PSYCHOACTIVE PHARMACEUTICALS AND NEW PSYCHOACTIVE SUBSTANCE RESIDUES IN WASTE- AND RECEIVING WATERS: EPIDEMIOLOGICAL AND ENVIRONMENTAL INVESTIGATION

Maria Laimou-Geraniou

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Prof. Dr. Ester Heath

Evaluation Board:
Prof. Dr. Milena Horvat, Chair, Jožef Stefan Institute and Dean of the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia
Prof. Dr. Helena Prosen, Member, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia
Prof. Dr. Richard Bade, Member, Queensland Alliance for Environmental Health Sciences, University of Queensland, Brisbane, Australia

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL



Maria Laimou-Geraniou

PSYCHOACTIVE PHARMACEUTICALS AND NEW PSYCHOACTIVE SUBSTANCE RESIDUES IN WASTE- AND RECEIVING WATERS: EPIDEMIOLOGICAL AND ENVIRONMENTAL INVESTIGATION

Doctoral Dissertation

OSTANKI PSIHOAKTIVNIH ZDRAVILNIH UČINKOVIN IN NOVIH PSIHOAKTIVNIH SNOVI V ODPADNIH IN POVRŠINSKIH VODAH: EPIDEMIOLOŠKI IN OKOLJSKI VIDIK

Doktorska disertacija

Supervisor: Prof. Dr. Ester Heath

Ljubljana, Slovenia, November 2025

To my parents, Lena and Markos
& my brother Konstantinos

"The mind, once stretched by a new idea,
never returns to its original dimensions."
Ralph Waldo Emerson

Acknowledgments

With this PhD thesis finally complete, one important chapter of my life comes to an end — a chapter full of personal growth, challenges, and countless lessons — and a new one in my professional journey begins. Words fall short when I try to express my gratitude to all the people who helped me reach this point, who stood by me through both struggles and happy moments, and who made Ljubljana feel like my second home. I could easily write a chapter for each of you, but for the sake of the trees sacrificed for this thesis, I will try to keep it short.

First of all, I would like to thank my supervisor, *prof. dr. Ester Heath*, for her consistent support, encouragement, and guidance. Thank you for choosing me, for believing in me from the start, for being both demanding and understanding, and for creating an environment in which I could grow both professionally and personally. I am grateful for the genuine care you have shown me, for giving me the push I needed at the right times, and for your honest feedback. Although our back-and-forth "ping-pong" manuscript revisions were sometimes challenging, I know how much they shaped my writing and thinking; thank you for never letting things slide, even when it might have been easier. Thank you for your patience, for always being there when I needed advice, for introducing me to so many wonderful people, and for opening doors to numerous exciting opportunities. Thank you for being my supervisor throughout this journey.

I would also like to thank *dr. David Heath*, for his thoughtful feedback and valuable recommendations on this thesis and my scientific publications. Beyond his scientific advice, I am deeply grateful for his encouragement, trust, and optimism, and for always being there with kind words, meaningful discussions, and genuine support — both academic and personal — whenever I needed it most.

My sincere thanks also go to the members of the evaluation board: *prof. dr. Milena Horvat*, *prof. dr. Helena Prosen*, and *dr. Richard Bade*, for taking the time to carefully read, review, and evaluate the work of my thesis.

An important part of this work was made possible through the collaboration with *prof. dr. Adrian Covaci*, *prof. dr. Alexander L. N. van Nuijs*, and *Maarten Quireyns* at the Toxicological Center, University of Antwerp. I am deeply grateful to *Adrian* and *Alexander* for hosting me twice during my secondments, where I had the opportunity to broaden my scientific knowledge and gain valuable research experience. *Adrian*, thank you also for sharing your amazing taste in music; it added a special note to my time in Antwerp. My heartfelt thanks go to my colleague and friend, *Maarten*, for his excellent collaboration, his selfless sharing of knowledge, and his constant support. Thank you for all your help, from assistance with lab work to help with R scripts, data analysis, and graphs. I am also very thankful to *Maarten*, *dr. Tim Boogaerts* and *Natan Van Wichelen*, the "WBE boys", for welcoming me into your team and for making our work together both productive and

enjoyable during my study visits. Many thanks as well to *Florence Maes* for her help with sample preparation and analysis during her stay; your contribution was greatly appreciated. To all of you, the "WBE team", thank you not only for the inspiring scientific collaboration but also for the wonderful moments we shared outside the lab, including our climbing adventures, which made my time in Antwerp truly special.

I would also like to thank *Allen Jun Anies* from ECOSPHERE (University of Antwerp) for our collaboration and for introducing me to the field of active-passive samplers. We worked together on related projects both during my secondments in Antwerp and later when he visited our lab at the Jožef Stefan Institute in Ljubljana. I appreciate the professional exchange and the experience gained through our joint work.

A big thank you also goes to all the colleagues at the Toxicological Centre, who, even though we did not collaborate directly, made my stay in Antwerp really unforgettable. *Giulia*, *Hugo*, *Steven*, *Yunsun*, *Tom*, *Catalina*, *Alicia*, *Fatima*, *Manuela*, thank you for your warm welcome to the office and for creating such a friendly and supportive atmosphere. *Yunsun*, my amazing flatmate and buddy, thank you for being one of the most unique people I know. I still remember that first night when you welcomed me with beers as soon as I stepped into our flat. Who could have imagined that one day, together with *Tom*, we would be brewing our own homemade ones? The Delirium beer will always remind me of you. Thank you for your nice energy at home, for the always interesting conversations, for sharing your stories, your struggles, your "jenever expertise", for the dinners and the crazy fun nights, even when we stayed at home. Thank you for being such a real friend. Special thanks to *Catalina*, *Alicia*, and *Fatima* for the sushi nights, the beers, the parties, the concerts, the gatherings, the chatting, and the joyful memories we created.

I would also like to express my gratitude to *prof. dr. Jochen Mueller*, *prof. dr. Kevin Thomas*, and *dr. Richard Bade* for accepting me for a secondment at QAEHS; it gave me the opportunity to work in a great environment and to visit a lot of exciting locations (from Brisbane to Lady Elliot Island). I am especially grateful to *Richard* and *Dhaya Nadarajan* for warmly welcoming me into your research group and for making me feel part of the "NPS team" during my stay. *Richard*, thank you for your trust, your guidance, your constant support, and for all your valuable advice throughout my research visit. *Dhaya,* thank you for your kindness and support from the very first day, for taking me out to lunch, showing me around the city, and helping me settle in. Thank you for all your guidance and help in the lab, for the inspiring discussions, your generosity in sharing your knowledge, and for always being so encouraging and supportive. You were not only the best lab partner I could have wished for, but also someone with whom I quickly felt a genuine connection. Thank you for being both a wonderful colleague and a dear friend.

I also thank *Andrew Chapell* for the nice collaboration and the enjoyable moments we shared during our overlapping time in the lab. Working together with *Richard*, *Dhaya*, and *Andrew* was an amazing experience, both scientifically and personally. My sincere thanks also go to *Jake* for the great conversations, for introducing me to trivia nights, and, together with *Richard*, *Andrew*, and *Dhaya*, for the dinners, the beers, the laughter, and the memorable moments we shared. Brisbane would not have been nearly as adventurous without *Gabriel*, *Emma*, and *Reena*; thank you for all the trips, hikes, and wonderful adventures exploring Queensland together. My time at QAEHS was truly unforgettable, not only for the professional growth it offered, but also for the wonderful people and friendships that made it so special.

Of course, I would like to thank my colleagues at Jožef Stefan Institute, *dr. Žiga Tkalec*, *dr. Taja Verovšek*, *dr. Ana Kovačič*, *dr. David Škufca*, *dr. Milka Ljoncheva*, *dr. Tjaša Gornik*, *Eirini Andreasidou*, *Helena Plešnik*, *Anja Vehar*, and *Žan Rekar*, for supporting me, for sharing experiences and ideas, for our coffee breaks, for our enthusiastic workouts, and for being such wonderful people to work and have fun with. *Žiga,* thank you for trusting me to take care of the plants, for all the funny moments, for our long discussions, for making our office such a nicer place to be, and for always being the sweetest. *Tjaša Kos*, thanks a lot for helping with sample preparation during your Master's studies in our group.

Many thanks to *dr. Tina Kosjek*, *prof. dr. Helena Prosen*, *assist. prof. dr. Tina Eleršek*, *Denisa Krevh,* and *Labtim d.o.o.* for fruitful cooperation in the research process. Special thanks go to the personnel of *Central wastewater treatment plants of Ljubljana* and *Domžale-Kamnik, Komunalno podjetje Velenje, Marjetica Koper, Komunala Novo Mesto, AquaSystems, and Komunala Kranj,* who enabled my studies by providing (waste)water samples. I would also like to thank João Matias from the European Union Drugs Agency for the nice collaboration and for welcoming me to the agency's offices during my short study visit in Lisbon. I would like to acknowledge the *Slovenian Research and Innovation Agency* (ARIS) for financially supporting my work through the Program group P1—0143, the ARIS—FWO Project N1-0143, and the national Project L1—9191. This work was also supported by the University of Ljubljana (interdisciplinary preparative project Nanostructurome 802-12/2024-5) and the TunTWIN project funded by the European Union (research project GA N° 952306).

I would also like to thank all the visitors who joined us over the years. It was wonderful meeting you, sharing ideas, and learning more about your diverse scientific backgrounds, experiences, and personal stories. *Ouns*, *Ines*, *Samira*, and *Wided*, I truly enjoyed working with you during your study visit. I am also deeply grateful for your kindness and hospitality during the conference in Hammamet, Tunisia, and for taking the time to show me around; it made the experience even more memorable.

Also, I would like to express my gratitude to all the people whom I have met at the *Jožef Stefan Institute* over the years. Special thanks to *dr. Margarita Antoniou*, *dr. Arman Korajac*, *dr. Lamprini Athanasopoulou*, *dr. Michele Tammaro*, and *dr. Leonardo B. Rizzuto*. I really don't know how my life in Ljubljana would be without all of you. Thank you for all the fun, the great discussions, the lunch breaks, the "kafedaki" times, the relaxing moments, the gatherings, the dinners, the beers, the parties, the concerts, and the excursions. Thank you for always being so supportive, for your friendship, and for the wonderful memories we have shared over the years. *Margarita*, my beloved friend, you truly became like family to me. Thank you for being by my side through everything — the laughter and the tears, the struggles and the small victories, for coming over during the pandemic, for our endless chats, long walks, and shared adventures, for listening to me vent for hours when things felt overwhelming, and for all the joy we created together. With you, everything felt easy, natural, and genuine. Thank you for your help with programming, for your delicious dinners and sweets, and mostly for being the person I could always count on — my calm in the chaos. You were always understanding, encouraging, and caring, and you supported me through every decision, big and small. You grounded me, reminded me what truly matters in life, and honestly, I don't think this thesis — or my sanity — would have made it to the end without you. I am endlessly grateful for your friendship, your kindness, and for being my safe space all these years in Ljubljana.

I was also fortunate to meet some truly amazing people outside of work. *Erika*, my sunshine, you were the first person I met in Ljubljana and have been a true friend ever since. I feel so lucky to have had you by my side through all these years. Thank you for all the laughter, for always believing in me, and for your endless kindness and support. Even though we did not see each other often, every time we met, it felt as if no time had passed; everything was as easy and joyful as always. You brightened my days in Ljubljana and even travelled to see me in Antwerp, bringing not only your positivity but also a large bottle of medica, this honey brandy, to keep me warm — a true act of friendship! I will always treasure the moments we shared, our constant laughter, and our perfectly synchronised lateness; somehow always late, but always together. Thank you for being such a wonderful friend.

Rapolas, or *Kumasi*, as I like to call you, we have known each other since our first Erasmus days in Ljubljana. When I started my PhD and found out you were back in the city too, it was the most wonderful surprise and a real gift, especially during the long and isolating days of the pandemic. Thank you for being such a great friend, always supportive, and always there to make me laugh, or to cheer me up with wine and good company ☺. You were there for me in all my hard moments, and I'll never forget your kindness and steady presence. Thank you for the great chats, the excursions, the hikes, the parties, the spritzers, the festivals, the concerts, the dinners at your place, the gardening sessions in your amazing garden, the lake swims, and even our mushroom-picking adventures. You are one of the most loyal, funny, smart, and caring people I know. I am deeply grateful for your friendship and for your constant support in every aspect of my life.

I would also like to thank *Mina* and *Lidija*, two wonderful friends with whom I shared countless moments in Ljubljana. From coffees and endless chats to concerts and parties, your friendship and support meant a lot to me. Thank you for being there for me through my struggles, for all the laughter, and for the amazing experiences, and yes, that secret bunker still awaits us to visit.

A big thank you to my *Rog-Fu Banda* team for all the Kung Fu training — those kicks were the best stress relief. I am also grateful to my *[A]Infoshop*, *PLAC*, and *Radio Študent* "families", each of which played a vital role in my experience and well-being while doing science. Thank you for the sense of community, inspiration, and good energy you brought into my life. *Božo*, thank you for all the lessons, the gym sessions, our exciting and reflective discussions, your steady support, and your friendship. *Andrej*, thank you for all your support, the long walks, the dinners at your place, those "pelinkovec nights", and your 3D printings; this little 3D-printed cat you made for me has been sitting on my desk ever since. *Marko*, my friend, thank you for the berry, apple, walnut, and chestnut picking, and for all the laughter along the way. Special thanks also to *Julio*, *Hannah*, *Andreja*, *Nejc*, *Mazinga*, *Cooky*, *Tine*, *Vaiva*, *Aneda*, *Maša*, *Aja*, *Urša*, *Sašo*, *Urška*, *Makis*, *Špela*, *Slobodan*, *Petra*, *Blaž*, *Lenča*, *Katja*, *Anja*, *Mača*, and *Jerry*. Thank you for the great discussions, the book fair, the amazing homemade pizza and wine nights, for all the carnivals we celebrated, for all the fun, the concerts, the festivals, the trips, the cooking sessions, the vegan dinners, and for all the wonderful moments we shared. Most of all, thank you for listening, for your support, and for making my life in Ljubljana brighter and happier, each of you in your own unique way.

Since Ljubljana is not exactly the most convenient destination, I would like to thank all my amazing friends who still managed to visit me over the years, bringing a bit of home with them each time: *Eirini*, *Nikoleta*, *Marina* "*μωράκι τ' ουρανού*", *Paraskevoula*, *Vasso*,

Nancy, *Lara*, *Bill*, *Fey*, *Lina*, and *Natalia*. To those who were farther away, *Spyro*, *Naya*, *Aspa*, *Miro*, and *Lavrenti*, thank you for the countless virtual sessions that kept us connected. *Nikoleta*, *Naya (κουκουνάκι μου)*, *Aspa (μπιζελάκι)*, *Miro*, *Paras*, *Marina*, and *Anastasia*, we have known each other since our very first university days. Thank you for being my constants, my true friends who have stood by me through everything! Thank you for the support, the endless discussions, the trips, the patience, the laughter, and all the beautiful and crazy moments we have shared. Thank you for always being there for me, no matter what. *Spyro* (*Σπυράκο μου*), thank you for your friendship, for our laughter, for our artistic photo-shootings, for our fun adventures, for the loud car singing, the crazy car dancing, and for our holidays together; I honestly don't know how I would have survived all those winters in Ljubljana without our amazing summers. Thank you for always being there for me, even from miles away. To all my beloved friends, *Eirini* (*κουμπάκι μου*), *Evi*, *Niko K.*, *Vasso*, *Niko M.*, *Deborah, Niko A., Xristina*, *Michali* (*σεφ μου*), Niko M., *Eirini & Korina* (and your fur boy *Sotiri Sotiropoulo*, his cuddling towards the end, was priceless), *Lara & Bill*, *Foivo*, and *Stella*, thank you for your love and your support throughout this journey. I hope you don't need this text to know how incredibly thankful I am for having you in my life. My amazing friends, I don't know what I would do without you. Life, both in Ljubljana and beyond, would not have been the same without your care, laughter, and friendship. I am endlessly grateful for your patience through these years, for all the time we spent together, for your unwavering support, for your trust, and for always believing in me. I would also like to express my heartfelt thanks to my friends *Eleni M.*, *Eugenia*, *Gogo*, *Aris*, *Pakos*, *Alekos*, *Vasia*, and *Panos*, who entered my life during the final years of my PhD — a period that proved to be among the most demanding and challenging of this journey. Even though our paths crossed later on, your solidarity, understanding, kindness, and patience meant more to me than words can express. I am deeply grateful to each of you for your genuine support and for standing by me during this journey.

George (*Rote*), my dearest friend, I truly don't know where to begin. Thank you for the beautiful gift you gave me the night before I left for Ljubljana, that unique hand-painted tattoo flash sheet you made for me, which found its place in my new home immediately. It has been with me ever since, through every moment there, giving me courage and strength, as if you were right beside me all along. This thesis would never have been completed without your presence and friendship. Thank you for your constant support, for being a pillar of companionship in my life, and for always being my safe space. Thank you for the endless discussions, the midnight walks, the motorcycle rides, our wonderful adventures, the trips, the drinks, the parties, your music; for being my therapist when everything felt like a mess, and for being there when everything seemed to be falling apart. You were always there for me, even during your own difficult moments, and I will never forget that. Thank you for your patience with all my complaining and for always understanding me without the need for many words. Thank you for helping me think clearly when I couldn't, for grounding me, and for reminding me to keep going when I wanted to give up. I always carry your care, your words, and your advice with me everywhere I go. Thank you for believing in me, for making me stronger, and for being by my side, in your own way; through your words, your actions, your art, and even in the quiet moments that spoke louder than anything else. You are a truly special friend to me!

This thesis would not be complete without a heartfelt thank you to my family. I am privileged beyond words to have such an incredible family to acknowledge. First, to my brother *Konstantinos* (*Ντινούλη μου*), thank you for your constant support, for the amazing trips we've taken together, for all our funny moments, and for always being there for me. Thank you for the concerts, for your energy, and for coming to Athens every single

time I was back from Ljubljana. You always made my visits to Greece feel as if I had never left. A huge thank you to my beloved grandmother *Maria*, who spent many years wishing for me to become a scientist, and whom I sadly lost during my PhD. *Γιαγιά Μάρω*, you didn't get to see the end of this journey, but I carry you with me in every step. I am deeply grateful for everything I learned from you, and I hope you will be watching proudly from above, together with the other grandparents, during my PhD defence. Many thanks also to my cousins, *Kostas* and *Giorgos*, and to their families, for the support and for all the joyful moments we've shared over the years. Thank you for always having my back, for helping me whenever I needed it, and for making me feel supported and cared for, no matter the distance. Of course, I should also acknowledge my fur babies, *Troufa*, *Koukou*, and *Roubis*, for their companionship, cuddles, and unconditional love. Thank you for making the long hours in front of the computer so much more bearable and fun. You were, and are, the best stress relief life could offer.

Finally, a very special thank you to my parents, *Lena* and *Markos*. I'm writing this in English so that everyone will know how amazing you both are. *Μαμά και μπαμπά*, thank you for everything you have taught me, for your example, for your endless patience, for all the moving houses, for your tireless help, for the music, the dancing, the humour, the imagination, and, above all, for making me feel that I am never alone. Thank you for always coming to visit me wherever life or work took me, and for the beautiful travels we shared together, memories I will always treasure. The love and the values you filled me with are the same ones I put into my work and that allow me to connect with all the wonderful people I have met along this journey. Thank you for your unwavering support, motivation, and understanding, even when I stumble. I should say it more often than I do, but I love you and admire you both immensely. Σας αγαπώ πολύ και σας ευχαριστώ για όλα!

One last thank you goes to all the artists for their music, pictures, colours, photos, words, and stories. I could never have survived this PhD without their art.

To all of you, thank you for shaping this journey and for making it truly unforgettable.

And always remember, wastewater never lies!

Abstract

The analysis of psychoactive substance residues in wastewater (influent and effluent) and receiving surface waters offers valuable insight into the epidemiological and environmental implications of psychoactive substance use. This thesis describes the use of wastewater-based epidemiology (WBE) to study spatiotemporal trends in the consumption of psychoactive pharmaceuticals and new psychoactive substances (NPS) among the general Slovenian population. It also examines the influence of the COVID-19 pandemic on psychoactive pharmaceuticals and NPS use. Furthermore, this thesis provided an opportunity to assess the removal efficiency of wastewater treatment and the occurrence of psychoactive pharmaceutical residues in the environment. Additionally, the thesis explores the application of novel active-passive sampling strategies in wastewater and compares the results with those obtained from conventional 24-hour composite sampling. Finally, the thesis addresses a significant knowledge gap regarding the impact of psychoactive pharmaceutical residues on aquatic organisms.

First, robust bioanalytical methods were established for the quantification of psychoactive pharmaceutical residues in multiple aquatic matrices. These include the development of target analytical methods based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the simultaneous detection of trace levels of psychoactive pharmaceutical biomarkers, including antidepressants, antipsychotics, and benzodiazepines, in influent wastewater. Additionally, to enhance throughput and reduce sample volume requirements, a novel 96-well solid phase extraction (SPE) protocol was optimised for the quantification of 28 biomarkers of antidepressants, opioids, and drugs of abuse in influent wastewater. This method, which requires only 2 mL of influent wastewater, proved effective for large-scale, long-term monitoring campaigns while maintaining sufficiently low limits of detection (LODs) and adequate analytical sensitivity.

In parallel, analytical protocols based on LC-MS/MS (QTrap-based targeted analysis and QTOF-based "suspect-screening") were applied to investigate the presence of NPS, highlighting their dynamic emergence and widespread distribution. Targeted LC-MS/MS methods were developed to determine the presence of psychoactive pharmaceutical residues in wastewater (influent and effluent) samples and receiving surface water. These methods assess the removal efficiency of these residues during wastewater treatment processes and determine their occurrence in effluent-receiving surface waters. Furthermore, the acute and chronic toxicity of psychoactive pharmaceutical residues to aquatic organisms was explored using *in silico* methods (Ecological Structure-Activity Relationships Software – ECOSAR).

Given the uncertainties regarding biomarker stability in wastewater, systematic experiments were conducted to evaluate the stability of 11 antipsychotic biomarkers under various storage and sampling conditions. Results demonstrated compound-specific degradation patterns: several compounds rapidly degraded under acidification or repeated freeze–thaw cycles, while only quetiapine and risperidone remained highly stable across most tested conditions. Storage of extracted samples on SPE cartridges in a freezer (−20 °C) was highly effective, maintaining high stability for up to three months for almost all antipsychotic biomarkers, making them suitable for sample transport and batch

analysis. Additionally, the stability of selected NPS on loaded SPE cartridges was evaluated under different temperatures and time intervals to mimic various storage conditions. The findings indicated that keeping extracts at −20 °C maintained recoveries within the acceptable range (80–120%) for most compounds for up to four months.

The major research findings on the use of psychoactive pharmaceuticals and NPS in the general population, based on wastewater (influent) analysis, reveal distinct trends with numerous temporal and spatial variations. Most psychoactive pharmaceuticals showed stable usage throughout the week; however, the few observed differences between weekends and weekdays could not be directly attributed to the pandemic. Moreover, although for most antidepressants the consumption levels increased during the first wave of the pandemic, no clear pandemic-related spatial patterns were found across locations. In contrast, antipsychotics and benzodiazepines consumption decreased during the pandemic, especially during the first wave of COVID-19. Notably, these results align with available annual prescription data for psychoactive pharmaceuticals, supporting the reliability of WBE results. Among the detected NPS, 3-methylmethcathinone (3-MMC) had the highest detection frequency, appearing in almost all sites and sampling periods, followed by mitragynine, which had the highest mass loads. The findings also align with other international wastewater studies, which report that 3-MMC is one of the predominant stimulant NPS in Europe. It is worth noting that mass loads of 3-MMC increased significantly during the pandemic. Suspect screening revealed the presence of additional NPS across at least one site during the sampling campaigns.

Analysis of wastewater treatment plant (WWTP) effluent revealed the incomplete removal of psychoactive pharmaceutical residues during different conventional biological wastewater treatments (activated sludge, sequential batch reactor–SBR, SBR with UV disinfection, membrane bioreactor–MBR, and moving bed biofilm reactor–MBBR), making it possible to detect their presence in effluent-receiving surface waters within the ng/L range. In addition, a good comparison between direct surface water analyses and predicted environmental concentrations indicates that predictive approaches, as described in this thesis, may serve as a practical substitute for conventional water analysis. The ECOSAR model further indicated potential effects on aquatic organisms, especially when exposed to venlafaxine, *O*-desmethylvenlafaxine, citalopram, and sertraline. These results emphasise the importance of upgrading treatment technologies and the need for further *in vivo* studies.

Finally, the thesis presents an innovative sampling strategy that utilised active–passive sampling (APS) in real wastewater. APS devices were deployed at the inflow and outflow of a Slovenian wastewater treatment plant to compare the occurrence of psychoactive substances, including antidepressants, illicit drugs, and opioids. The results from APS were compared with those obtained from conventional 24-hour composite sampling followed by SPE. While APS was ineffective for influent samples due to excessive particulate content, a comparison of APS with conventional 24-hour composite sampling in effluent during the same sampling period showed good agreement for most studied compounds. The results highlight the potential of APS for monitoring campaigns.

To summarise, this thesis successfully addressed several gaps related to developing and validating advanced analytical methods for the determination of psychoactive pharmaceuticals and NPS, evaluating their stability, estimating their use through WBE, and understanding the environmental impact of psychoactive pharmaceutical residues. At the same time, it highlighted new research challenges in WBE, such as integrating non-target screening into the early warning system. In addition, investigations are needed into their occurrence in drinking water supplies and the associated risks to human health, as well as further ecotoxicological studies assessing risks to non-target organisms, especially in sensitive environments.

Povzetek

Analiza ostankov psihoaktivnih snovi v komunalnih odpadnih vodah (neprečiščeni vtoki in iztoki iz čistilnih naprav) ter v površinskih vodah, v katere se iztekajo iztoki iz čistilnih naprav, ponuja dragocen vpogled v epidemiološke in okoljske vplive uporabe psihoaktivnih snovi. Doktorska disertacija opisuje uporabo epidemiologije na osnovi analize neprečiščene odpadne vode (ang. Wastewater-Based Epidemiology – WBE) za preučevanje prostorsko-časovnih trendov porabe psihoaktivnih zdravilnih učinkovin in novih psihoaktivnih snovi (NPS) v splošni populaciji v Sloveniji. Poleg tega obravnava vpliv pandemije COVID-19 na uporabo psihoaktivnih zdravilnih učinkovin in NPS, preučuje uspešnost odstranitve teh spojin iz odpadnih vod ter posledično njihovo pojavnost v okolju. Disertacija prav tako raziskuje uporabo novih strategij aktivno-pasivnega vzorčenja v odpadnih vodah ter primerja rezultate s konvencionalnim 24-urnim kompozitnim vzorčenjem. Nazadnje se osredotoča na pomembno vrzel v poznavanju vplivov ostankov psihoaktivnih zdravilnih učinkovin na vodne organizme.

V okviru doktorske naloge smo sprva vzpostavili robustne bioanalitske metode za kvantifikacijo ostankov psihoaktivnih zdravilnih učinkovin v različnih vodnih matrikah. Med te metode sodi razvoj analiznih postopkov na osnovi tekočinske kromatografije sklopljene s tandemsko masno spektrometrijo (LC-MS/MS) za analizo sledov biomarkerjev psihoaktivnih zdravilnih učinkovin, vključno z antidepresivi, antipsihotiki in benzodiazepini v neprečiščenih odpadnih vodah (vtoki) komunalnih čistilnih naprav. Za povečanje pretočnosti analiz in zmanjšanje potrebnega volumna vzorca smo optimizirali nov analizni protokol na osnovi ekstrakcije na trdo fazo (SPE) s "96-well plate" za kvantifikacijo 28 biomarkerjev antidepresivov, opioidov in zdravil, ki se zlorabljajov vtokih čistilnih naprav. Ta metoda, ki zahteva le 2 mL vzorca (vtok), je predvsem uporabna za obsežne in dolgotrajne nadzorne meritve, pri čemer ohranja zadostne meje zaznave.

Vzporedno smo uporabili analizne protokole na osnovi LC-MS/MS (QTrap, tarčna analiza in QTOF, "suspect screening" protokoli) za preučevanje prisotnosti NPS in spremljanje njihove dinamične pojavnosti in široke razširjenosti. V ta namen smo razvili tarčne LC-MS/MS protokole za določanje ostankov psihoaktivnih zdravilnih učinkovin v odpadnih in površinskih vodah. Te metode omogočajo oceno učinkovitosti odstranjevanja ostankov preučevanih snovi v čistilnih napravah ter določanje njihove prisotnosti v površinskih vodah, ki prejemajo iztoke iz čistilnih naprav. Poleg tega smo z uporabo "*in silico*" metod (programska oprema ECOSAR –Ecological Structure-Activity Relationships) preučevali tudi akutno in kronično toksičnost ostankov psihoaktivnih zdravilnih učinkovin na vodne organizme.

Glede na negotovosti, povezane s stabilnostjo biomarkerjev v odpadnih vodah, smo izvedli sistematične poskuse za oceno stabilnosti 11 biomarkerjev antipsihotikov pri različnih pogojih shranjevanja in vzorčenja. Rezultati so pokazali specifične vzorce razgradnje posameznih spojin – več spojin se je razgradilo ob nakisanju vzorcev in pri ponavljajočih se ciklih zamrzovanja in odtaljevanja, medtem ko sta kvetiapin in risperidon ostala stabilna pri večini testiranih pogojev. Shranjevanje ekstraktov na SPE kartušah v zamrzovalniku (−20 °C) omogoča za večino biomarkerjev antipsihotikov visoko stabilnost

do treh mesecev, kar nakazuje, da je tovrstna hramba vzorcev primerna tudi za daljši transport in obdelavo večje serije vzorcev. S simuliranjem različnih scenarijev shranjevanja vzorcev smo dodatno ocenili stabilnost izbranih NPS na SPE kartušah pri petih različnih temperaturah in v petih časovnih intervalih. Rezultati so pokazali, da shranjevanje ekstraktov pri −20 °C ohranja izkoristke ekstrakcije znotraj sprejemljivega območja (80−120%) za večino spojin v časovnem razponu do štirih mesecev.

Glavne ugotovitve raziskav o uporabi psihoaktivnih zdravilnih učinkovin in NPS v splošni populaciji na osnovi analize neprečiščene odpadne vode razkrivajo izrazite trende s številnimi časovnimi in prostorskimi variacijami. Večina psihoaktivnih zdravilnih učinkovin je imela stabilno porabo med tednom, opaženih razlik med vikendi in delovnimi dnevi pa ni bilo mogoče neposredno povezati s pandemijo. Poleg tega se je pri večini antidepresivov poraba povečala med prvim valom pandemije, pri čemer nismo zaznali jasnih prostorskih trendov. Nasprotno pa se je poraba antipsihotikov in benzodiazepinov med pandemijo zmanjšala, zlasti med prvim valom COVID-19. Pomembno je tudi, da so pridobljeni rezultati skladni z razpoložljivimi letnimi podatki predpisovanja psihoaktivnih zdravilnih učinkovin, kar dodatno potrjuje zanesljivost rezultatov, pridobljenih z WBE. Med zaznanimi NPS je imel 3-metilmetkatinon (3-MMC) najvišjo frekvenco zaznave, saj je bil prisoten na skoraj vseh vzorčnih mestih in v vseh obdobjih vzorčenja, sledil pa mu je mitraginin, ki je imel najvišje masne obremenitve. Ugotovitve so skladne tudi z drugimi mednarodnimi WBE študijami, ki poročajo, da je 3-MMC ena izmed prevladujočih stimulativnih NPS v Evropi. Omeniti velja, da so se masne obremenitve 3-MMC med pandemijo opazno povečale. Aplikacija "suspect screening" protokolov pa je razkrila dodatne NPS na vsaj eni lokaciji med različnimi vzorčenji.

Zaradi nepopolnega odstranjevanja ostankov psihoaktivnih zdravilnih učinkovin pri različnih konvencionalnih bioloških postopkih čiščenja odpadnih vod (aktivno blato, šaržni biološki reaktor–SBR, SBR z UV–dezinfekcijo, membranski bioreaktor–MBR, reaktor s plavajočimi nosilci biomase–MBBR) smo določili njihovo prisotnost v površinskih vodah, ki sprejemajo iztoke iz čistilnih naprav (ng/L). Poleg tega smo s primerjavo rezultatov analiz površinske vode in napovedanimi okoljskimi koncentracijami pokazali, da lahko napovedni pristopi, kot je opisano v tej disertaciji, služijo kot alternativa konvencionalnim analizam vode. Model ECOSAR pa je dodatno nakazal možne učinke na vodne organizme, zlasti ob njihovi izpostavitvi venlafaksinu, *O*-desmetilvenlafaksinu, citalopramu in sertralinu. Ti rezultati poudarjajo pomen nadgradnje tehnologij čiščenja in potrebo po nadaljnjih *in vivo* raziskavah.

Nazadnje disertacija obravnava uporabo inovativnega aktivno-pasivnega vzorčenja (APS) v realnih odpadnih vodah. APS vzorčevalnike smo namestili v vtok in iztok iz komunalne čistilne naprave z namenom primerjave prisotnosti ostankov psihoaktivnih snovi (antidepresivov, prepovedanih drog in opioidov) določenih z APS in konvencionalnim 24 urnim kompozitnim vzorčenjem s sledečo SPE ekstrakcijo. Čeprav APS v neprečiščenih vzorcih vtoka zaradi prevelike vsebnosti trdnih delcev ni delovalo, je primerjava učinkovitosti APS s konvencionalnim vzorčenjem v iztoku pokazala dobro ujemanje za večino preučevanih spojin in potencial tega vzorčenja za nadzorne meritve.

V predloženi disertaciji smo uspešno naslovili več vrzeli, povezanih z razvojem in validacijo naprednih analitskih metod za določanje psihoaktivnih zdravilnih učinkovin in NPS. Obravnavali smo tudi oceno njihove stabilnosti, uporabo pristopa WBE ter razumevanje okoljskih vplivov ostankov teh snovi. Raziskava je nakazala tudi nove izzive na področju WBE, med katere spadajo vključitev netarčnih analiznih protokolov v sistem zgodnjega opozarjanja (EWS) ter raziskave prisotnosti teh snovi v pitni vodi in s tem povezanih tveganj za zdravje ljudi. Poleg tega so potrebne dodatne ekotoksikološke študije za oceno tveganj za netarčne organizme, zlasti v občutljivih okoljih.

Contents

List of Figures **xxi**

List of Tables **xxiii**

Abbreviations **xxv**

Symbols **xxxiii**

Glossary **xxxv**

1 Introduction **1**
1.1 Psychoactive Substances ... 1
1.1.1 Antidepressants ... 4
1.1.2 Antipsychotics ... 6
1.1.3 Benzodiazepines ... 8
1.1.4 New psychoactive substances ... 10
1.2 Monitoring the Prevalence of Psychoactive Pharmaceutical and NPS Use ... 12
1.2.1 Psychoactive pharmaceuticals: Consumption trends and prescription data ... 13
1.2.1.1 Antidepressants ... 13
1.2.1.2 Antipsychotics ... 14
1.2.1.3 Benzodiazepines ... 15
1.2.2 Current dynamics of the NPS market ... 15
1.3 Wastewater-Based Epidemiology ... 19
1.3.1 WBE methodology ... 20
1.3.1.1 Sampling ... 20
1.3.1.2 Biomarker selection ... 24
1.3.1.3 Sample preparation ... 25
1.3.1.4 Target analysis ... 26
1.3.1.5 Suspect screening ... 27
1.3.1.6 Back-estimation of consumption ... 28
1.3.2 Advantages and limitations ... 29
1.3.2.1 Advantages and WBE potential as a complementary data source ... 29
1.3.2.2 Limitations and uncertainties ... 30
1.3.3 WBE as a complementary information source during the COVID-19 pandemic ... 32
1.4 Environmental Aspect ... 33
1.4.1 Analytical methods for the determination of psychoactive pharmaceutical residues in the aqueous environment ... 35
1.4.2 Occurrence and removal of psychoactive pharmaceutical residues in wastewater ... 36

1.4.3 Occurrence of psychoactive pharmaceutical residues in receiving surface waters 37
1.4.4 Toxicity of psychoactive pharmaceutical residues to aquatic organisms. 39

2 Aims and Hypotheses 41

3 Publications 43
3.1 Analytical Methods for the Determination of Psychoactive Substances in Influent Wastewater 45
3.1.1 Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology 45
3.1.2 Optimisation, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater 71
3.2 WBE as a Complementary Data Source: Application in the Slovenian Population 79
3.2.1 Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters 79
3.2.2 Three years of wastewater surveillance for new psychoactive substances from 16 countries 91
3.2.3 Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis 103
3.3 Reducing WBE Uncertainties: Stability of Psychoactive Pharmaceuticals and NPS 115
3.3.1 Stability of antipsychotic biomarkers for wastewater-based epidemiology 115
3.3.2 Wastewater analysis as a global toxicovigilance tool for new psychoactive substances monitoring 124
3.4 Psychoactive Pharmaceutical Residues in the Aqueous Environment: Removal, Occurrence, and Ecotoxicity 136
3.4.1 Psychoactive pharmaceuticals: Consumption trends, removal during wastewater treatment and occurrence in receiving waters with associated environmental risks 136
3.5 Novel Sampling Approaches for Psychoactive Substances in Wastewater 151
3.5.1 In-situ evaluation of an active-passive sampling (APS) technique for monitoring psychoactive compounds in wastewater 151
3.5.1.1 Introduction 152
3.5.1.2 Materials and methods 153
3.5.1.2.1 Chemicals and sorbents 153
3.5.1.2.2 Compounds of interest 153
3.5.1.2.3 Active-passive sampling (APS) device 156
3.5.1.2.4 Organic diffusive gradients in thin films (o-DGT) ... 156
3.5.1.2.5 Field experiment setup 157
3.5.1.2.6 Sample preparation 158
3.5.1.2.6.1 Conventional 24-hour composite samples 158
3.5.1.2.6.2 APS and o-DGT receiving phases 158
3.5.1.2.7 Gas chromatography-tandem mass spectrometry 159
3.5.1.2.8 Method validation 160
3.5.1.2.9 Calculation and Data analysis/Statistical analysis .. 161
3.5.1.3 Results and Discussion 161

3.5.1.3.1 Derivatisation procedure 161
3.5.1.3.2 Method performance 162
3.5.1.3.3 Application to real samples 164
3.5.1.3.4 Conventional 24-hour composite sampling vs APS 165
3.5.1.4 Conclusions 168

4 Overall Conclusions **169**

5 Future Perspectives **177**

References **181**

Bibliography **205**

Biography **211**

List of Figures

Figure 1: Schematic overview of the main steps in WBE. 19
Figure 2: Deploying a passive sampler (POCIS) at municipal WWTP (Photo: Maria Laimou-Geraniou). 21
Figure 3: (A) Schematic and (B) cross-section (not to scale) of the APS diffusion cell [2], [114]. 22
Figure 4: Deployment of APS in (A) influent and (B) effluent wastewater, at municipal WWTP (Photo (A): David Heath and photo (B): Maria Laimou-Geraniou). 23
Figure 5: Scheme of top-down and bottom-up approach (adapted from [103]). 24
Figure 6: SPE manifold with extraction cartridges and wastewater samples (Photo: Maria Laimou-Geraniou). 26
Figure 7: Fishbone presentation of uncertainty sources in drug consumption estimates obtained by WBE. 30
Figure 8: Conceptual overview of the psychoactive pharmaceutical transfer pathway, from production and consumption to the environment. 34
Figure 9: Photo showing surface water grab sampling (Photo: Eirini Andreasidou). 36
Figure 10: Physicochemical properties, chemical structures and speciation (*cationic, neutral, zwitterionic, anionic*), at pH 7.0 – 8.0, of the target psychoactive compounds. *Data obtained from ChemAxon, 2024.* 154
Figure 11: The active-passive sampling (APS) device. 156
Figure 12: (A) Commercially available o-DGT samplers with HLB sorbent topped with 0.8 mm agarose diffusive gel and PTFE filter membrane and (B) lab-assembled o-DGT samplers using APS sorbent phases (HLB and MCX) topped with agarose diffusive gel and a PES filter membrane. 157
Figure 13: Deployment of APS in (A) influent and (B) effluent wastewater, in Ljubljana WWTP (Photo (A): David Heath and photo (B): Maria Laimou-Geraniou). 157
Figure 14: Comparison between microwave-assisted derivatisation and derivatisation with conventional heating. 162
Figure 15: Scatterplot and boxplots showing the average concentrations of target psychoactive pharmaceuticals with all sampling methods tested during all sampling periods. 166
Figure 16: Correlation plots comparing sampling methods. Obtained concentrations are log-transformed, and the diagonal line represents a 1:1 correlation. 167

List of Tables

Table 1: International and Slovenian scheduling of controlled psychoactive substances (adapted by [14], [15], [17]). For full compound names corresponding to the abbreviations used in this table, please refer to the Abbreviations section. 3
Table 2: Classification of prescribed antidepressants in Slovenia. 5
Table 3: Classification of prescribed antipsychotics in Slovenia. 7
Table 4: Classification of prescribed benzodiazepines in Slovenia. 9
Table 5: NPS classification based on their origin, chemical structure, and pharmacological effect, including representative examples [32]. 11
Table 6: Identified NPS in Slovenia from 2015 to 2025 (acute emergency cases, anonymous testing of street drugs and drug seizures), adapted by [79], [80]. 17
Table 7: Compounds of interest (human metabolic residues). 154
Table 8: GC-MS/MS parameters. 160
Table 9: Validation results. 163
Table 10: Average concentrations of psychoactive compounds measured with conventional 24-hour composite sampling (SPE), APS, and o-DGT during a 3-, 6-, 9-, 12-, and 14-day sampling period. All concentrations are reported in ng/L. 164

Abbreviations

1cP-LSD	1-Cyclopropionyl-LSD
1P-LSD	1-Propionyl-LSD
25B-NBOH	[[2-(4-Bromo-2,5-dimethoxyphenyl)ethylamino]methyl]phenol
25E-NBOH	2-({[2-(4-Ethyl-2,5-dimethoxyphenyl)ethyl]amino}methyl)phenol.
25I-NBF	*N*-[(2-Fluorophenyl)methyl]-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine.
25I-NBOMe	2-(4-Iodo-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine
2-AI	2-Aminoindane
2C-B	4-Bromo-2,5-dimethoxyphenethylamine
2C-TMF	4-Trifluoromethyl-2,5-dimethoxyphenethylamine
2-FDCK	2-Fluoro deschloroketamine
2-FMA	2-Fluoromethamphetamine
2-MAPB	1-(Benzofuran-2-yl)-*N*-methylpropan-2-amine
2-MMC	2-(Methylamino)-1-(4-methylphenyl)propan-1-one
2-Oxo-PCE	*N*-Ethyldeschloroketamine
3,4-DMMC	2-(Methylamino)-1-(3,4-dimethylphenyl)propan-1-one
3Cl-N,N-DMC	2-(Dimethylamino)-1-(3-chlorophenyl)propan-1-one
3-CMC	3-Chloromethcathinone
3F-alpha-PVP	3-Fluoro-alpha-pyrrolidinovalerophenone
3F-NEB	3-Fluoro-*N*-ethylbuphedrone
3-HO-PCE	3-Hydroxyeticyclidine
3-MeOMC	2-(Methylamino)-1-(3-methoxyphenyl)propan-1-one
3-MeO-PCE	3-Methoxieticyclidine
3-MeO-PCP	3-Methoxyphencyclidine
3-MMC	3-Methylmethcathinone
4,4-F-CUMYL-PIPETINACA (SGT-235)	*N*-(1-Methyl-1-phenylethyl)-1-(4,4-difluoropiperidin-1-ylmethyl)-1H-indazole-3-carboxamide
4-AcO-DMT	4-Acetoxy-*N,N*-dimethyltryptamine
4-ANBP	Benzylfentanyl
4Cl-PVP	4-Chloro-alphapyrrolidinovalerophenone
4-CMC	4-Chloromethcatinone
4-CN-CUMYL-BUTCZCA	*N*-(1-Methyl-1-phenylethyl)-1-(4-cyanobutyl)-1H-indazole-3-carboxamide
4-CDC	4-Chloro-*N,N*-dimethylcathinone
4Cl-alpha-PHP	4-Chloro-alpha-pyrrolidinohexiophenone

4Cl-Cyclopentyl-F ... 4-Chloro-cyclopentanoyl fentanyl
4Cl-iBF ... 4-Chloroisobutyrfentanyl
4-FA ... 4-Fluoroamphetamine
4F-BF ... 4-Fluoro-butyryl-fentanyl
4F-EMB-BUTICA ... Ethyl-*N*-[(1*S*)-1-(4-fluorobenzyl)-1H-indole-3-carbonyl]-*L*-tert-leucinate
4-FMA ... 4-Fluoromethamphetamine
4F-CUMYL-5F-PICA ... *N*-(1-Methyl-1-phenylethyl)-1-(5-fluoropentyl)-1-(4-fluorobenzyl)-1H-indole-3-carboxamide
4F-MPH ... 4-Fluoromethylphenidate
4-HO-MET ... 4-Hydroxy-*N*-methyl-*N*-ethyltryptamine
4-HO-MiPT ... 4-Hydroxy-*N*-methyl-*N*-isopropyltryptamine
4-MeO-BF ... 4-Methoxybutyrylfentanyl
4-MeO-PCE ... 4-Methoxyeticyclidine
4-MMC ... 4-Methylmethcathinone
5-BPDi ... 1-(2,3-Dihydro-1*H*-inden-5-yl)-2-(pyrrolidin-1-yl)hexan-1-one
5C-AKB48 ... *N*-(1-Adamantyl)-1-(cyclopentylmethyl)-1H-indazole-3-carboxamide
5Cl-AB-PINACA ... *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(5-chloropentyl)-1H-indazole-3-carboxamide
5F-3,5-AB-PFUPPYCA ... *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide
5F-AKB48 ... *N*-(1-Adamantyl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
5F-AMBICA ... *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-1*H*-indole-3-carboxamide
5F-APICA ... *N*-(1-Adamantyl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
5F-APP-PICA_(PX1) ... *N*-(1-Adamantyl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
5F-CYPPICA ... *N*-(Cyclopropyl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
5F-JWH-018-adamantyl analogue ... 1-(5-Fluoropentyl)-3-(adamantan-1-yl-carbonyl)-1H-indole
5-MAPDI ... Indanylmethylaminopropane
5F-MDMB-P4AICA ... Methyl-*N*-[(1*S*)-1-(5-fluoropentyl)-1H-indole-3-carbonyl]-*L*-tert-leucinate
5F-MDMB-PINACA ... Methyl-*N*-[(1*S*)-1-(5-fluoropentyl)-1H-indazole-3-carbonyl]-*L*-tert-leucinate
5F-MN-18 ... 1-(5-Fluoropentyl)-*N*-(naphthalen-1-yl)-1H-indazole-3-carboxamide
5F-PB-22 ... Quinolin-8-yl-1-(5-fluoropentyl)-1H-indole-3-carboxylate
5F-PY-PICA ... *N*-(Pyrrolidin-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
5F-SDB-005 ... (1-(5-Fluoropentyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone
5-IAI ... 5-Iodo-2-aminoindane
5-MAPB ... 1-(Benzofuran-5-yl)-*N*-methylpropan-2-amine
5-MeO-DMT ... 5-Methoxy-*N,N*-dimethyltryptamine

6-APB . . . 6-(2-Aminopropyl)benzofuran
6-APDB . . . 6-(2Aminopropyl)-2,3-dihydrobenzofuran
AB-CHMINACA . . . *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide
AB-FUBINACA . . . *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
AB-PINACA . . . *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide
ACN . . . Acetonitrile
Acryloyl-F . . . Acryloylfentanyl
ADAMANTYL-THPINACA . . . *N*-(1-Adamantyl)-1-(tetrahydropyran-4-ylmethyl)-1*H*-indazole-3-carboxamide
ADB-5Br-4-en-PINACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-5-bromo-1-(pent-4-en-1-yl)-1H-indazole-3-carboxamide
ADB-5Br-BUTINACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-5-bromo-1-butyl-1H-indazole-3-carboxamide
ADB-5Br-PINACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-5-bromo-1-pentyl-1H-indazole-3-carboxamide
ADB-BUTINACA . . . *N*-(1-AAmino-3,3-dimethyl-1-oxobutan-2-yl)-1-butyl-1H-indazole-3-carboxamide
ADB-CHMICA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indole-3-carboxamide
ADB-D-5Br-INACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-5-bromo-1-decyl-1H-indazole-3-carboxamide
ADB-FUBICA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indole-3-carboxamide
ADB-HEXINACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-hexyl-1H-indazole-3-carboxamide
ADB-INACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1H-indazole-3-carboxamide
ADB-P-5Br-INACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-5-bromo-1-propyl-1H-indazole-3-carboxamide
ADB-PHETINACA . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-phenethyl-1H-indazole-3-carboxamide
ADB-PINACA_isomer 2 . . . *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide
ADHD . . . Attention-Deficit/Hyperactivity Disorder
AKB48 . . . *N*-(1-Adamantyl)-1-pentyl-1*H*-indazole-3-carboxamide
ALD-52 . . . 1-Acetyl-LSD (1A-LSD)
Alpha-PHP . . . Alpha-pyrrolidinohexanophenone
Alpha-PHiP . . . 4-Methyl-alpha-pyrrolidinopentaphenone
Alpha-PVP . . . 1-Phenyl-2-(1-pyrrolidinyl)-1-pentanone
AM-2201 . . . 1-(5-Fluoropentyl)-3-(1-naphthoyl)indole
AMB-CHMICA . . . Methyl-*N*-[(1*S*)-1-(cyclohexylmethyl)-1H-indole-3-carbonyl]-*L*-valinate
AMB-FUBICA . . . Methyl-*N*-[(1*S*)-1-(4-fluorobenzyl)-1H-indole-3-carbonyl]-*L*-valinate
AMT . . . 2-(1H-Indol-3-yl)-1-methyl-ethylamine
AMP . . . Amphetamine
APAAN . . . *alpha*-Phenylacetoacetonitrile

APP-BUTINACA ... *N*-(1-Adamantyl)-1-butyl-1H-indazole-3-carboxamide
APS ... Active–Passive Sampling
ARIS ... Slovenian Research and Innovation Agency
ATHPINACA_isomer 1 ... *N*-(1-Adamantyl)-1-(tetrahydrofuran-3-ylmethyl)-1H-indazole-3-carboxamide
ATHPINACA_isomer 2 ... *N*-(1-Adamantyl)-1-(tetrahydrofuran-2-ylmethyl)-1H-indazole-3-carboxamide
Benzyl-4-CN_BUTINACA ... *N*-(4-Cyanobenzyl)-1-butyl-1H-indazole-3-carboxamide
BK-2C-B ... 2-Amino-1-(4-bromo-2,5-dimethoxyphenyl)ethanone hydrochloride
BMDP ... Benzylone
BOD ... Biological Oxygen Demand
BSTFA ... *N,O*-Bis-(trimethylsilyl)trifluoroacetamide
BZE ... Benzoylecgonine
BZP ... Benzylpiperazine
CEC ... Contaminants of Emerging Concern
CF ... Correction Factor
COD ... Chemical Oxygen Demand
COVID-19 ... Coronavirus Disease 2019 (SARS-CoV-2)
CUMYL-4CN-B7AICA ... *N*-(1-Methyl-1-phenylethyl)-1-(4-cyanobutyl)-1H-indole-3-carboxamide
CUMYL-5F-P7AICA ... *N*-(1-Methyl-1-phenylethyl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
CUMYL-5F-PICA ... *N*-(1-Methyl-1-phenylethyl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
CUMYL-5F-PINACA ... *N*-(1-Methyl-1-phenylethyl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
CUMYL-BICA ... 1-Butyl-*N*-(2-phenylpropan-2-yl)-1H-indole-3-carboxamide
CUMYL-CBMICA ... *N*-(1-Methyl-1-phenylethyl)-1-(cyclobutylmethyl)-1H-indole-3-carboxamide
CUMYL-CBMINACA ... *N*-(1-Methyl-1-phenylethyl)-1-(cyclobutylmethyl)-1H-indazole-3-carboxamide
CUMYL-CHSINACA ... *N*-(1-methyl-1-phenylethyl)-1-(cyclohexylsulfinylmethyl)-1H-indazole-3-carboxamide
CUMYL-PeGaClone ... *N*-(1-Methyl-1-phenylethyl)-1-pentyl-1H-indazole-3-carboxamide
CUMYL-PICA ... *N*-(1-Methyl-1-phenylethyl)-1H-indole-3-carboxamide
CUMYL-PINACA ... (*S*)-1-(1-Methyl-1H-indazole-3-carbonyl)-3,3-dimethylbutan-2-yl (1-pentyl-1H-indole-3-carboxylate)
CUMYL-PIPETINACA ... *N*-(1-Methyl-1-phenylethyl)-1-(piperidin-1-ylmethyl)-1H-indazole-3-carboxamide
CUMYL-THPINACA ... *N*-(1-Methyl-1-phenylethyl)-1-((tetrahydro-2H-pyran-4-yl)methyl)-1H-indazole-3-carboxamide
DB-MDBP ... Difluoromethylenedioxybenzylpiperazine
DDA ... Data-Dependent Acquisition
DDD ... Defined Daily Dose

DIA ... Data-Independent Acquisition
DMAA ... 1,3-Dimethylamylamine
DMT ... Dimethyltryptamine
DOF ... 2,5-Dimethoxy-4-fluoroamphetamine
DOM ... 2,5-Dimethoxy-4-methylamphetamine
DRIs ... Dopamine Reuptake Inhibitors
DUR ... Drug Utilisation Research
ECOSAR ... Ecological Structure-Activity Relationships (software)
EDDP ... 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EDMB-PINACA ... Ethyl-*N*-[(1*S*)-1-pentyl-1H-indazole-3-carbonyl]-*L*-tert-leucinate
EF ... Excretion Factor
ER ... Extraction Recovery
ESI ... Electrospray Ionization
Ethylbenzyl-CYBINACA ... *N*-(1-Ethylbenzyl)-1H-indazole-3-carboxamide
EU ... European Union
EUDA ... European Union Drugs Agency
EWA ... Early Warning Advisory on new psychoactive substances
EWS ... Early Warning System
F-AMB-PICA ... Methyl-N-[(1*S*)-1-(5-fluoropentyl)-1H-indole-3-carbonyl]-*L*-valinate
Furanyl UF-17 ... *N*-[2-(Dimethylamino)cyclohexyl]-*N*-phenylfuran-2-carboxamide
FUB-144 ... *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
FUB-AKB48 ... *N*-(1-Adamantyl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
FUBIMINA ... *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
FUB-NPB-22 ... Quinolin-8-yl-1-(4-fluorobenzyl)-1H-indole-3-carboxylate
FU-F ... Furanylfentanyl
GABA ... Gamma-aminobutyric acid
GAD ... Generalised Anxiety
GBL ... Gamma butyrolactone
GC ... Gas Chromatography
GC-MS/MS ... Gas Chromatography coupled with Tandem Mass Spectrometry
GHB ... Gamma-hydroxybutyric acid
HBM ... Human Biomonitoring
HBP ... Hydroxybupropion
HE ... High Energy mode
HLB ... Hydrophilic–Lipophilic Balanced (sorbent)
HPLC ... High-Performance Liquid Chromatography
HRMS ... High-Resolution Mass Spectrometry
ISTD ... Internal Standard
JWH-018 ... 1-Pentyl-3-(1-naphthoyl)indole
JWH-020 ... 1-Pentyl-3-(1-naphthoyl)indole
JWH-072 ... 1-Pentyl-3-(2-methoxybenzoyl)-1H-indole
JWH-072-N-carboxy ... (1-Pentyl-1H-indol-3-yl)oxoacetic acid

JWH-147 ... 1-Pentyl-3-(2-iodobenzoyl)-1H-indole
JWH-166 ... 1-Pentyl-3-(2-chlorobenzoyl)-1H-indole
JWH-308 ... 1-Pentyl-3-(4-methoxybenzoyl)-1H-indole
JWH-368 ... 1-Pentyl-3-(4-chlorobenzoyl)-1H-indole
JWH-369 ... 1-Pentyl-3-(3-methoxybenzoyl)-1H-indole
LC ... Liquid Chromatography
LC-MS/MS ... Liquid Chromatography coupled with Tandem Mass Spectrometry
LC-HRMS ... Liquid Chromatography coupled with High-Resolution Mass Spectrometry
LC-QTOF-MS ... Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry
LE ... Low Energy mode
LLOQ ... Lower Limit of Quantification
LOD ... Limit of Detection
LOQ ... Limit of Quantification
LSD ... Lysergic acid diethylamide
LSZ ... Lysergic acid 2,4-dimethylazetidide
MAOIs ... Monoamine Oxidase Inhibitors
MAPA ... Methyl-alpha-phenylacetoacetate
MBBR ... Moving Bed Biofilm Reactor
MBR ... Membrane Bioreactor
MCX ... Mixed-Mode Cation Exchange (sorbent)
MCX 96-well ... 96-well solid-phase extraction plate using MCX sorbent
MDA-19 ... *N*-(Adamantan-1-yl)-1-pentyl-1H-indole-3-carboxamide
MDAI ... 5,6-Methylenedioxy-2-aminoindane
MDD ... Major Depressive Disorder
MDMA ... 3,4-Methylenedioxymethamphetamin
MDMB-4en-PINACA ... Methyl-*N*-[(1*S*)-1-(pent-4-en-1-yl)-1H-indazole-3-carbonyl]-*L*-tert-leucinate
MDMB-CHM7AICA ... Methyl-*N*-[(1*S*)-1-(cycloheptylmethyl)-1H-indole-3-carbonyl]-*L*-tert-leucinate
MDMB-CHMCZCA ... Methyl-(2S)-2-(9-(cyclohexylmethyl)-9H-carbazole-3-carboxamido)-3,3-dimethylbutanoate
MDMB-CHMICA ... *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1H-indole-3-carboxamide
MDMB-CHMINACA ... Methyl-*N*-[(1*S*)-1-(cyclohexylmethyl)-1H-indazole-3-carbonyl]-*L*-tert-leucinate
MDMB-INACA ... Methyl-*N*-[(1*S*)-1H-indazole-3-carbonyl]-*L*-tert-leucinate
MeOH ... Methanol
MDMB-FUB7AICA ... Methyl-*N*-[(1*S*)-1-(4-fluorobenzyl)-1H-indole-3-carbonyl]-*L*-tert-leucinate
MEP-FUBICA ... Ethyl-*N*-[(1*S*)-1-(4-fluorobenzyl)-1H-indole-3-carbonyl]-*L*-valinate
MMB-022 ... Methyl-*N*-[(1*S*)-1-(pent-4-en-1-yl)-1H-indole-3-carbonyl]-*L*-tert-leucinate
MO-CHMINACA ... *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(methoxybenzyl)-1H-indazole-3-carboxamide
MOR ... Morphine

MPA	. . . Methiopropamine
MPPP	. . . 4-Methyl-*alpha*-pyrrolidinopropiophenone
MPT	. . . Microporous Polyethylene Tubes
MRM	. . . Multiple Reaction Monitoring
MS	. . . Mass Spectrometry
MS/MS	. . . Tandem Mass Spectrometry
MSTFA	. . . *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
MXE	. . . Methoxetamine
MXiPr	. . . Methoxisopropamin
MXPr	. . . Methoxpropamine
NaSSAs	. . . Noradrenergic and Specific Serotonergic Antidepressants
NBOMe	. . . *N*-Methoxybenzyl-substituted phenethylamines
NM-2201	. . . *N*-(1-Adamantyl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
N-Methyl-bk-MMDA-2	. . . 1-(6-Methoxy-1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one
Noopept	. . . *N*-Phenylacetyl-*L*-prolylglycine ethyl ester
NPS	. . . New Psychoactive Substances
OCD	. . . Obsessive-Compulsive Disorder
o-DGT	. . . Organic Diffusive Gradients in Thin films
ODV	. . . *O*-desmethylvenlafaxine
OECD	. . . Organisation for Economic Co-operation and Development
P2NP	. . . 1-Phenyl-2-nitropropene
PARC	. . . European Partnership for the Assessment of Risks from Chemicals (H2020 project)
PB-22	. . . Quinolin-8-yl-1-pentyl-1H-indole-3-carboxylate
PC:M	. . . Parent Compound-Metabolite ratio
PCP	. . . Phencyclidine
PDA	. . . Partial Dopamine Agonist
Phenibut	. . . A
PNML	. . . Population-Normalised Mass Load
POCIS	. . . Polar Organic Chemical Integrative Sampler
PTSD	. . . Post-Traumatic Stress Disorder
PV8	. . . Alpha-pyrrolidinoheptiophenone
PV9	. . . 1-Phenyl-2-(pyrrolidin-1-yl)octan-1-one
QC	. . . Quality Control
QqQ	. . . Triple Quadrupole mass analyser
QTOF	. . . Quadrupole Time-of-Flight mass analyser
QTRAP	. . . Hybrid Triple Quadrupole/Linear Ion Trap mass analyser
RCS-4-C4 homologue	. . . 1-(Butyl)-3-(1-pentyl-1H-indol-3-yl)propan-1-one
RCS-8	. . . 1-(Adamantan-1-yl)-3-(1-pentyl-1H-indol-3-yl)propan-1-one
RP	. . . Reverse Phase
RSD	. . . Relative Standard Deviation
SAD	. . . Seasonal Affective Disorder
SARIs	. . . Serotonin Antagonist and Reuptake Inhibitors
SBR	. . . Sequential Batch Reactor
SCORE	. . . The Sewage analysis CORe group Europe network
SDB-006-N-phenyl analogue	. . . (1-Pentyl-1H-indol-3-yl)(phenyl)methanone

SPE	... Solid-Phase Extraction
SPM	... Suspended Particulate Matter
SNRIs	... Serotonin-Norepinephrine Reuptake Inhibitors
SSREs	... Selective Serotonin Reuptake Enhancers
SSRIs	... Selective Serotonin Reuptake Inhibitors
STS-135	... *N*-(Adamantan-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
TBZD	... Triazolobenzodiazepine
TCAs	... Tricyclic Antidepressants
TFA	... Trifluoroacetic anhydride
TFMPP	... 3-Trifluoromethylphenylpiperazine
THC	... Tetrahydrocannabinol
THJ-2201	... 1-(5-Fluoropentyl)-1H-indazole-3-carboxamide
TIANEPTIN	... 7-[(3-Chloro-6-methyl-2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methylamino]heptanoic acid
TMCS	... Trimethylchlorosilane
TRA	... Tramadol
TWA	... Time-Weighted Average
U-47700	... 3,4-Dichloro-*N*-((1*S*,2*S*)-2-(dimethylamino)cyclohexyl)-*N*-methylbenzamide
UN61	... Single Convention on Narcotic Drugs 1961
UN71	... Convention on Psychoactive Substances 1971
UN88	... 1988 United Nations Convention against the illicit traffic in narcotic drugs and psychoactive substances
UNODC	... United Nations Office on Drugs and Crime
UPLC	... Ultra-Performance Liquid Chromatography
UPLC-MS/MS	... Ultra-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry
US	... United States
UWWTD	... Urban Wastewater Treatment Directive
WBE	... Wastewater-Based Epidemiology
WHO	... World Health Organization
WL	... Watch List
WWTP	... Wastewater Treatment Plant
X-FEA	... X-fluoroethamphetamine

Symbols

Δg	...	Thickness of the diffusive layer
φ	...	Daily wastewater flow
A	...	Exposure surface area
c	...	Measured concentration of the target biomarker
CE	...	Collision energy
D	...	Diffusion coefficient of the target analyte in the diffusive layer
K_{OW}	...	Octanol-water partition coefficient
K_{sw}	...	Sorbent-water partition coefficient
k_x	...	Accumulation rate constant
M	...	Mass of the target analyte in the sorbent phase
N	...	Number of people in the catchment area
pK_a	...	The negative logarithm of the acid dissociation constant
Sw	...	Water solubility
t	...	Deployment time
t_R	...	Retention time

Glossary

Active-Passive sampling (APS) – a hybrid sampling device combining active pumping and passive diffusion mechanisms to collect samples under controlled hydrodynamic conditions.

Biofouling – the accumulation of microorganisms, plants, algae, or small animals on sampler surfaces, potentially affecting analyte uptake.

Biomarker – the excreted metabolic residue of the substance under investigation. Its levels in raw wastewater are used to estimate drug consumption.

Composite sampling – a conventional sampling approach that collects multiple wastewater aliquots over a set period (e.g., 24 hours) to obtain a time-averaged sample.

Contaminants of Emerging Concern (CEC) – defined by EU NORMAN as "Substances that have been detected in the environment, but which are currently not included in routine monitoring programs at EU level and whose fate, behaviour and (eco)toxicological effects are not well understood".

Defined Daily Dose (DDD) – "the assumed average maintenance dose per day for a drug used for its main indication in adults" and "does not necessarily reflect the recommended or prescribed daily dose", defined by the World Health Organization (WHO).

Drug Utilisation Research (DUR) – a discipline which combines aspects of pharmacotherapy, epidemiology, and health services research into an interdisciplinary set of methods for analysing and assessing the prescribing, dispensing and consumption of medicines.

Ecotoxicity – is defined according to the United Nations Office on Drugs and Crime (UNODC) as "The potential for biological, chemical or physical agents to affect ecosystems and thereby, indirectly, biodiversity".

Enantiomers - molecules that are a pair of mirror images of each other.

Grab sample – a single, discrete sample collected at a specific time and location to provide a "snapshot" of the conditions at that moment.

Limit of detection (LOD) – the lowest concentration of an analyte that can be detected with a stated probability.

Limit of quantification (LOQ) – the lowest concentration of an analyte that can be quantified with an acceptable method performance (accuracy and repeatability).

Matrix effect – the effect (enhancement or suppression) of co-eluting residual matrix components of the sample on the ionisation of the target analyte in mass spectrometry.

Passive sampling – a sampling method where analytes accumulate on a sorbent or membrane through natural diffusion over extended periods.

Substitutional therapy – a medical treatment where a legal drug, which is pharmacologically similar to a drug of addiction, is prescribed to replace illicit drugs to manage dependence and withdrawal symptoms.

Wastewater influent – the raw or untreated wastewater entering a wastewater treatment plant.

Wastewater effluent – the treated wastewater emitted to the environmental waters from the wastewater treatment plant.

Wastewater Treatment Plant (WWTP) – a facility that uses mechanical, physical, chemical, and biological processes to remove pollutants from wastewater before it is released to the environment or reused.

Chapter 1

Introduction

Over the past decade, the global use of pharmaceuticals (measured in defined daily doses) has increased by 36%, reflecting advances in medical science, increased access and a growing dependence of societies on pharmacological treatments for both physical and mental health [1]. The increasing prevalence of mental health disorders, chronic conditions, and lifestyle pressures has resulted in unprecedented levels of psychoactive pharmaceutical consumption, particularly in developed countries [2], [3]. This trend has been further accelerated by recent global challenges, including the COVID-19 pandemic, which disrupted daily life, heightened stress levels, and profoundly impacted mental health worldwide, amplifying the demand for psychoactive medications [4], [5].

Although psychoactive pharmaceuticals play a critical role in improving quality of life and managing neuropsychiatric disorders, their increasing use is accompanied by challenges of misuse, dependence, and diversion to non-medical purposes [6]. Alongside these prescribed psychoactive medications, the rise of new psychoactive substances (NPS), defined as synthetic or natural compounds designed to mimic the effects of controlled drugs while often evading regulation, has added complexity to drug monitoring and control, since their rapid emergence often outpaces regulation [7]. The expanding use of psychoactive pharmaceuticals and NPS creates significant burdens for individuals, healthcare systems, and law enforcement, while also raising concerns for the environment. Alongside risks of addiction and diversion, residues of psychoactive pharmaceuticals are increasingly detected in wastewater and receiving surface waters, where their ecological impacts are poorly understood [8]. This thesis applies wastewater-based epidemiology (WBE) to investigate psychoactive pharmaceuticals and NPS in Slovenia, combining public health and environmental perspectives to address a growing societal concern.

1.1 Psychoactive Substances

Psychoactive substances are chemicals that, when administered, influence the functioning of the central nervous system (brain and spinal cord) and alter an individual's perception, cognition, mood, consciousness, behaviour and emotions [1]. These substances can be categorised based on their pharmacological effects, origin, and legal status. Certain psychoactive substances are prescribed for therapeutic purposes, such as alleviating pain and psychological suffering, whereas others are used recreationally for their mood- or perception-altering effects.

According to the United Nations Office on Drugs and Crime (UNODC), psychoactive substances can be classified as stimulants, hallucinogens, and depressants based on their pharmacological effect [9]. Among these, psychoactive pharmaceuticals represent a

significant subclass of medications prescribed for the management of various neuropsychiatric and somatic disorders such as depression, anxiety, schizophrenia, bipolar disorder, insomnia, and chronic pain and include antidepressants, antipsychotics, benzodiazepines, mood stabilisers, anxiolytics, and hypnotics [10].

Psychoactive substances can be broadly categorised according to their primary effects on the central nervous system. Stimulants, such as amphetamines, are known to heighten alertness, increase arousal and elicit behavioural excitement, while some have established therapeutic uses in conditions like attention-deficit/hyperactivity disorder (ADHD) [11]. In contrast, hallucinogens (or "psychedelics") can alter consciousness, perception, thought processes, and emotions, often inducing synaesthesia or varying levels of auditory or visual hallucinations [12]. Depressants, including opioids, sedatives, tranquillisers, and hypnotics, mainly suppress, inhibit or decrease brain activity [13].

According to their origin, psychoactive substances are classified into three categories: natural, semi-synthetic, and synthetic. Natural or plant-based substances are directly derived from plants or fungi. In contrast, semi-synthetic substances are produced by modifying natural precursors through substitution or addition reactions, yielding compounds with a similar chemical structure. Alternatively, synthetic substances are synthesised through a series of chemical reactions using chemical precursors [7]. Notably, antidepressants, antipsychotics, and benzodiazepines are entirely synthetic compounds developed through medicinal chemistry to target specific neurotransmitter pathways. These drug classes have no known direct natural equivalents but are structurally inspired by earlier central nervous system depressants and modulators [6].

The legal regulation of psychoactive substances operates on both national and international levels, classifying substances as licit (legal or unregulated) or illicit (controlled). Globally, the control of psychoactive substances is governed by three key United Nations conventions: the Single Convention on Narcotic Drugs in 1961 (UN61) [14], the Convention on Psychoactive Substances in 1971 (UN71) [15], and the Convention Against Illicit Traffic in Narcotic Drugs and Psychoactive Substances in 1988 (UN88) [16]. These conventions collectively govern the production, distribution, and use of over 300 psychoactive substances, which, based on their potential for abuse, associated health risks, and therapeutic value, can be classified as shown in **Table 1**. National authorities implement these conventions through domestic legislation, which may differ in how psychoactive substances are classified and enforced. Slovenia, as a signatory to all three UN conventions (UN61, UN71, and UN88), has incorporated these controls into its national legislation framework [17], classifying psychoactive substances into three levels (**Table 1**).

In contrast, psychoactive substances that are not restricted by law in terms of consumption and trade are considered legal. These include prescription pharmaceuticals (i.e., antidepressants, antipsychotics, and benzodiazepines), some NPS, alcohol and nicotine. Although legal, their abuse, misuse, unauthorised production, possession, or trafficking is considered illicit and may constitute a criminal offence under both Slovenian and international law. Furthermore, in some cases, such as with substitutional therapies involving synthetic opioids, their use is further restricted under international control (UN61) due to their high misuse potential [14].

Table 1: International and Slovenian scheduling of controlled psychoactive substances (adapted by [14], [15], [17]). For full compound names corresponding to the abbreviations used in this table, please refer to the Abbreviations section.

Single Convention on Narcotic Drugs (1961)			
Schedule	**Classification criteria**	**Degree of control**	**Examples**
I	Highly addictive substances High risk of abuse	Very strict: subject to all measures of control under the Convention of 1961	Cannabis, cocaine, heroin, morphine, methadone, opium
II	Substances used for medical purposes Lower risk of abuse than substances in Schedule I	Less strict	Codeine
III	Preparations of substances listed in Schedule II and cocaine	Lenient	<2.5% codeine <0.1% cocaine
IV	The most dangerous substances already listed in Schedule I Particularly harmful and of extremely limited or no accepted medical/therapeutic value	Very strict: complete ban on their production, manufacture, export/import, trade, possession and use Exceptions: medical and scientific research	Heroin
Convention on Psychoactive Substances (1971)			
Schedule	**Classification criteria**	**Degree of control**	**Examples**
I	Substances with a high risk of abuse that pose a serious threat to public health Very little/ no therapeutic value	Very strict: prohibited use Exceptions: scientific or limited medical purposes	LSD, THC, MDMA (ecstasy), mescaline, psilocybin
II	Substances with a risk of abuse that pose a serious threat to public health Low/ moderate therapeutic value	Less strict	Amphetamines
III	Substances with a risk of abuse that pose a serious threat to public health Moderate/ high therapeutic value	Available for medical purposes	Barbiturates, buprenorphine
IV	Substances with a risk of abuse that pose a minor threat to public health High therapeutic value	Available for medical purposes	Tranquilisers, analgesics, narcotics, benzodiazepines

Regulation on the classification of illicit drugs in Slovenia No. 69/19, 157/20, 162/21, 8/23, and 113/23			
Group	**Classification criteria**	**Degree of control**	**Examples**
I	Substances (and plants) with a high risk of abuse that pose a serious threat to public health No therapeutic value	Very strict: prohibited use Exceptions: scientific or educational purposes	Heroin, LSD, MDMA, cocaine
II	Substances (and plants) with a moderate to high risk of abuse that pose a serious threat to public health Known therapeutic value	May be produced, possessed, traded and used for medical, veterinary, educational, or scientific purposes	Cannabis, methadone, codeine
III	Substances (and plants) with a risk of abuse that pose a medium threat to public health Known therapeutic value	May be produced, possessed, traded and used for medical, veterinary, educational, or scientific purposes	Antipsychotics, benzodiazepines, morphine, barbiturates

1.1.1 Antidepressants

Antidepressants are psychoactive medications primarily prescribed for the treatment of major depressive disorder (MDD). They are also utilised in managing generalised anxiety disorder (GAD), obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), social anxiety disorder (SAD), and certain chronic pain conditions. They function in various ways by modulating neurotransmitters such as serotonin, norepinephrine, and dopamine, which help regulate mood and alleviate depressive symptoms [18]. Antidepressants are categorised into different classes, according to their chemical structure and working mechanism (**Table 2**), including selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), noradrenergic and specific serotonergic antidepressants (NaSSA), serotonin antagonist and reuptake inhibitors (SARIs), dopamine reuptake inhibitors (DRIs), and selective serotonin reuptake enhancers (SSREs) [18]. Although closely related and structurally similar to tricyclic antidepressants, carbamazepine and its active metabolite (carbamazepine-10,11-epoxide) are primarily classified as mood stabilisers a class of medications used to treat mood disorders characterised by intense mood fluctuations, and are best known for the treatment of bipolar disorder and mania [19].

In the European Union (EU) and the United States (US), antidepressants are classified as prescription-only medications. Some antidepressants, such as dibenzepin, melitracen, moclobemide, mianserin, and lorpiprazole, are not approved or marketed in the US but are available in the EU and Slovenia. In Slovenia, antidepressants are not listed under the Decree on the Classification of Illicit Drugs (Official Gazette RS, No. 45/2020) and therefore are not classified as controlled substances, as they generally have a low potential for abuse and dependence [20]. However, their prescription and dispensation are regulated to prevent misuse.

Table 2: Classification of prescribed antidepressants in Slovenia.

Class	**Trade name**	**Parent compound (PC)/ main active metabolite (M)**	**Primary therapeutic uses**	**Legal status**
SSRIs	Cipramil, Celexa, Citalon	Citalopram (PC) Desmethylcitalopram or Norcitalopram (M)	MDD, GAD	Prescription-only, not scheduled
	Prozac, Sarafem, Adofen	Fluoxetine (PC) Norfluoxetine (M)	MDD, OCD, bulimia nervosa	Prescription-only, not scheduled
	Fevarin, Luvox	Fluvoxamine (PC)	MDD, OCD, anxiety disorders	Prescription-only, not scheduled
	Seroxat, Paxil, Loxamine	Paroxetine (PC)	MDD, OCD, panic disorder, SAD, PTSD	Prescription-only, not scheduled
	Zoloft, Lustral, Asentra	Sertraline (PC) Desmethylsertraline or Norsertraline (M)	MDD, OCD, panic disorder, PTSD, SAD	Prescription-only, not scheduled
SNRIs	Cymbalta, Drizalma, Ariclaim, Yentreve	Duloxetine (PC)	MDD, GAD, fibromyalgia, neuropathic pain	Prescription-only, not scheduled
	Efectin	Venlafaxine (PC) *O*-desmethylvenlafaxine (M)	MDD, GAD, panic disorder, SAD	Prescription-only, not scheduled
TCAs	Elavil	Amitriptyline (PC) Nortriptyline (M)	MDD, chronic pain, migraine prophylaxis	Prescription-only, not scheduled
	Norpramin, Pertofrane	Desipramine (PC) 2-Hydroxydesipramine (M)	MDD, ADHD, neuropathic pain	Prescription-only, not scheduled
	Sinequan, Quitaxon, Aponal	Doxepin (PC) *N*-Desmethyldoxepin or Nordoxepin (M)	Depression, anxiety disorders, insomnia, chronic hives	Prescription-only, not scheduled
	Noveril	Dibenzepin (PC)	MDD	Prescription-only, not scheduled
	Tofranil, Melipramine	Imipramine (PC) Desipramine (M)	Depression, enuresis (bedwetting), panic disorder	Prescription-only, not scheduled
	Adaptol, Dixeran, Melixeran, Thymeol, Trausabun	Melitracen (PC)	Depression, anxiety disorders	Prescription-only, not scheduled
	Surmontil	Trimipramine (PC) Desmethyltrimipramine (M) Trimipramine *N*-oxide (M)	Depression, insomnia	Prescription-only, not scheduled

	Anafranil	Clomipramine (PC) Desmethylclomipramine or Norclomipramine (M)	OCD, depression, panic disorder	Prescription-only, not scheduled
MAOIs	Aurorix, Manerix	Moclobemide (PC)	Depression, social anxiety disorder	Prescription-only, not scheduled
	Nardil	Phenelzine (PC) β-phenylethylidenehydrazine (M)	Depression, anxiety disorders	Prescription-only, not scheduled
NaSSAs	Tolvon, Athymil	Mianserin (PC) Desmethylmianserin or Normianserin (M)	Depression, insomnia	Prescription-only, not scheduled
	Remeron, Mirataz, Avanza, Mirzaten	Mirtazapine (PC) *N*-Desmethylmirtazapine or Normirtazapine (M)	MDD, GAD, insomnia	Prescription-only, not scheduled
SARIs	Trittico, Desyrel, Molipaxin, Oleptro, Trazorel	Trazodone (PC) *m*-chlorophenylpiperazine, mCPP (M)	MDD, insomnia	Prescription-only, not scheduled
DRIs	Wellbutrin, Zyban	Bupropion (PC) Hydroxybupropion (M)	MDD, SAD, smoking cessation aid	Prescription-only, not scheduled
Mood stabilizers	Tegretol, Carbatrol, Epitol, Equetro	Carbamazepine (PC) Carbamazepine 10,11-epoxide (M)	Trigeminal neuralgia, epilepsy, bipolar disorder	Prescription-only, not scheduled

MDD: major depressive disorder, GAD: generalised anxiety disorder, OCD: obsessive-compulsive disorder, SAD: social anxiety disorder, PTSD: post-traumatic stress disorder, ADHD: attention-deficit/hyperactivity disorder

1.1.2 Antipsychotics

Antipsychotics, also known as neuroleptics, are a class of psychoactive medications primarily used to manage psychosis, including symptoms such as delusions, hallucinations, and disordered thought processes. They are essential in the treatment of schizophrenia, schizoaffective disorder, and bipolar disorder, and are sometimes used adjunctively in major depressive disorder. There are three categories of antipsychotics: first-generation, also known as typical antipsychotics, which are primarily dopamine D_2 receptor antagonists, second-generation, also known as atypical antipsychotics, which are traditionally described as serotonin-dopamine (5-HT_2A/D_2) antagonists, and third-generation, which act mainly as partial dopamine agonists (PDAs) (**Table 3**) [21], [22]. However, the serotonin–dopamine antagonism model for atypical antipsychotics is now considered oversimplified, as agents such as clozapine and risperidone display variable D_2 receptor occupancy and diverse receptor-binding profiles. Their clinical effects are thought to arise from broader multi-receptor activity and kinetic properties rather than solely 5-HT_2A/D_2 antagonism [23], [24].

Antipsychotics are prescription-only medications in both the USA and the EU. In Slovenia, they are primarily classified under Group III of the Decree on the Classification

of Illicit Drugs (Official Gazette RS, No. 45/2020), indicating that they have therapeutic value but still pose some public health risks when misused [20]. Exceptions are haloperidol and clozapine, which are classified under Group II, indicating their higher potential for abuse. Consequently, although haloperidol and clozapine are legally available for medical use, their production, distribution, and prescription are subject to stringent regulatory controls [20].

Table 3: Classification of prescribed antipsychotics in Slovenia.

Class	**Trade name**	**Parent compound (PC)/ main active metabolite (M)**	**Primary therapeutic uses**	**Legal status**
Typical (first generation)	Thorazine, Largactil	Chlorpromazine (PC) 7-hydroxychlorpromazine (M)	Schizophrenia, the manic phase of bipolar disorder	Prescription-only, Group III
	Prolixin	Fluphenazine (PC) 7-Hydroxyfluphenazine (M)	Schizophrenia	Prescription-only, Group III
	Haldol	Haloperidol (PC)	Schizophrenia, acute psychosis, Tourette syndrome	Prescription-only, Group II
	Trilafon	Perphenazine (PC) 7-Hydroxyperphenazine (M)	Schizophrenia, nausea	Prescription-only, Group III
Atypical (second generation)	Saphris	Asenapine (PC) *N*-Desmethyl-asenapine (M)	Schizophrenia, bipolar disorder	Prescription-only, Group III
	Clozaril Leponex, Versacloz	Clozapine (PC) *N*-Desmethylclozapine or Norclozapine (M)	Treatment-resistant schizophrenia	Prescription-only, Group II
	Zyprexa	Olanzapine (PC) *N*-desmethyl-olanzapine (M)	Schizophrenia, bipolar disorder	Prescription-only, Group III
	Invega, Xeplion, Trevicta	Paliperidone (PC)	Schizophrenia, schizoaffective disorder	Prescription-only, Group III
	Seroquel, Kventiax	Quetiapine (PC) *N*-Desalkylquetiapine or Norquetiapine (M)	Schizophrenia, bipolar disorder, major depression	Prescription-only, Group III
	Risperdal, Rispolux, Torendo	Risperidone (PC) 9-hydroxyrisperidone (M)	Schizophrenia, bipolar disorder, autism	Prescription-only, Group III
Third generation	Abilify, Aryzalera	Aripiprazole (PC) Dehydroaripiprazole (M)	Schizophrenia, bipolar disorder, depression	Prescription-only, Group III
	Reagila, Vraylar	Cariprazine (PC) Desmethylcariprazine (M) Didesmethylcariprazine (M)	Schizophrenia, bipolar disorder	Prescription-only, Group III

1.1.3 Benzodiazepines

Benzodiazepines are a class of psychoactive drugs known for their sedative, anxiolytic, muscle relaxant, and anticonvulsant properties. They are commonly prescribed for the short-term treatment of anxiety disorders, insomnia, seizures, and as premedication for medical procedures [25], [26]. They are also known as 'minor tranquillisers' and sedatives (or sleeping pills). Benzodiazepines enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the GABA-A receptor, producing a calming effect. There are three types of benzodiazepines (**Table 4**), including long-, intermediate-, and short-acting, with the latter being more addictive and having a stronger withdrawal and 'come down' effect [25].

In addition to benzodiazepines, a group of medications known as non-benzodiazepines has emerged, which exert similar hypnotic and sedative effects by targeting the same GABA-A receptors. Although structurally distinct, these agents—commonly referred to as "Z-drugs" (e.g., zolpidem, zaleplon, and zopiclone)—promote GABAergic activity, which contributes to their effectiveness in managing sleep-related disorders such as insomnia. Due to their rapid onset and short half-life, they are primarily used for short-term sleep induction [26].

Benzodiazepines can be highly addictive, whether they are taken under medical supervision or used illegally for recreational purposes, to feel a sense of euphoria (or high) or after taking stimulants (cocaine, amphetamines, ecstasy) to help with the 'come down' [27]. They can cause an overdose, particularly when used with alcohol or other drugs, and should not be used long-term. For instance, more than 50% of drug-induced deaths in Denmark, Austria, Portugal, and Finland in 2021 involved benzodiazepines [28], while during the same year (2021), there were approximately 12,500 deaths involving benzodiazepine overdoses in the US [29]. Benzodiazepines are also implicated in cases of drug-facilitated sexual assault due to their sedative effects and ability to impair memory. Flunitrazepam (Rohypnol) is among the most recognised, as it can induce amnesia and drowsiness, making it a tool of misuse in such crimes. Other benzodiazepines, including clonazepam and diazepam, have also been associated with similar misuse [27]. Additionally, non-benzodiazepine drugs like zolpidem have been identified in related contexts due to their comparable effects [27].

Although benzodiazepines are prescription-only medications, in the US, they are regulated under Schedule IV of the Controlled Substances ACT [30], while in the EU, their legal status varies slightly among countries, falling under national drug control laws. Particularly in Slovenia, benzodiazepines are classified under the Decree on the Classification of Illicit Drugs (Official Gazette RS, No. 45/2020) as Group III substances [20]. This classification indicates that they have recognised medical uses but also have the potential for misuse and dependence. Their prescription and dispensation are strictly regulated to prevent abuse. One exception is temazepam, which is regulated as a Group II substance, indicating stricter prescribing regulations and controls due to its higher potential for abuse [20].

Table 4: Classification of prescribed benzodiazepines in Slovenia.

Class	**Trade name**	**Parent compound (PC)/ main active metabolite (M)**	**Primary therapeutic uses**	**Legal status**
Short acting	Xanax, Xanor, Niravam, Aripsiq	Alprazolam (PC) α-hydroxy-alprazolam (M)	Anxiety, panic disorders	Prescription-only, Group III
	Noctamid, Loramet	Lormetazepam (PC) *N*-Demethylated lormetazepam (M)	Insomnia	Prescription-only, Group III
	Versed	Midazolam (PC) α-Hydroxy midazolam (M)	Sedation, Anaesthesia	Prescription-only, Group III
	Serax, Alepam	Oxazepam (PC)	Anxiety, Alcohol Withdrawal	Prescription-only, Group III
	Halcion	Triazolam (PC) α-Hydroxytriazolam (M) 4-Hydroxytriazolam (M)	Insomnia	Prescription-only, Group III
Intermediate acting	Prosom, Esilgan, Eurodin	Estazolam (PC) 4-Hydroxyestazolam (M)	Insomnia	Prescription-only, Group III
	Rohypnol	Flunitrazepam (PC) 7-Aminoflunitrazepam (M)	Severe Insomnia	Not approved
	Ativan, Tavor, Temesta	Lorazepam (PC)	Anxiety, Insomnia, Seizures	Prescription-only, Group III
	Mogadon, Alodorm, Nitrazadon	Nitrazepam (PC) 7-Aminonitrazepam (M)	Insomnia	Prescription-only, Group III
	Restoril, Normison, Nortem	Temazepam (PC) Oxazepam (M)	Insomnia	Prescription-only, Group II
Long acting	Lexotan, Lexotanil, Lekotam, Lexaurin	Bromazepam (PC) 3-Hydroxybromazepam (M)	Anxiety	Prescription-only, Group III
	Frisium	Clobazam (PC) *N*-Desmethylclobazam (M)	Epilepsy	Prescription-only, Group III
	Klonopin, Rivotril	Clonazepam (PC) 7-Amino-clonazepam (M)	Seizures, panic disorders	Prescription-only, Group III
	Valium, Apaurin	Diazepam (PC) Nordiazepam (M) Temazepam (M) Oxazepam (M)	Anxiety, muscle spasms, seizures	Prescription-only, Group III

Z-drugs	Sonata, Starnoc, Andante	Zaleplon (PC) 5-Oxo-zaleplon (M)	Short-term treatment of insomnia	Prescription-only, Group III
	Ambien, Sanval, Zolsana	Zolpidem (PC) Zolpidem phenyl-4-carboxylic acid (M)	Short-term treatment of insomnia	Prescription-only, Group III
	Imovane, Zimovane, Dopareel	Zopiclone (PC) N-Desmethylzopiclone (M)	Short-term treatment of insomnia	Prescription-only, Group III

1.1.4 New psychoactive substances

New psychoactive substances are a diverse and continuously evolving group of synthetic plant-based substances, either in a pure form or a preparation, that are structurally similar to internationally controlled illicit drugs but not regulated under UN61 or UN71 [31]. The term "new" primarily refers to substances that have recently emerged on the drug market, rather than to new inventions [7]. According to the UNODC, NPS are classified into 15 primary groups [32], based on their origin, chemical structure, and pharmacological effects (**Table 5**). These substances appear on the drug market under various names, including "legal highs", "herbal highs", "research chemicals ", "bath salts", and "designer drugs". NPS are often designed to mimic the effects of conventional illicit drugs by making minor modifications (addition/substitution of functional chemical groups) to the molecular structure of controlled substances, while bypassing legal restrictions [32].

By the end of 2024, over 1,300 NPS had been identified across 151 countries worldwide, with 47 new NPS reported in the European market [33], [34]. However, only 83 NPS had been scheduled under international control by October 2024 [32]. This fact highlights a significant delay between detection and regulation. For example, 3-methylmethcathinone (3-MMC), one of the most frequently detected NPS in the EU, was only classified as Schedule II Drug under the Convention of Psychotropic Substances of 1971 and placed under international control in March 2023 [35], [36] – one year after the EU regulated it through Commission Delegated Directive (EU) 2022/1326 [37], and following earlier action by most Member States at the national level.

The legal status of NPS varies widely between countries. To date, more than 65 countries, including Slovenia, have implemented legal responses to control NPS [31]. Some countries have adopted broader legal measures, such as blanket bans or comprehensive prohibitions on all NPS, while others use more targeted approaches, regulating these substances based on their chemical similarity (analogue legislation) or through individual listing [31], [38]. Additionally, NPS may fall under various legal frameworks, including customs regulations, pharmaceutical laws, or consumer protection acts, depending on each country's legal context [38]. Considering the large and very dynamic NPS market, coupled with the lack of comprehensive toxicological data, long-term effects, potential carcinogenicity, and compound purity pose significant public health risks, increasing the chance of acute intoxication in users, and necessitate timely regulatory responses.

Table 5: NPS classification based on their origin, chemical structure, and pharmacological effect, including representative examples [32]. For full compound names corresponding to the abbreviations used in this table, please refer to the Abbreviations section.

NPS class	**Origin / Chemical group**	**Pharmacological effect**	**Examples**
Aminoindanes	Synthetic / Indane derivatives	Stimulant, Entactogen	MDAI, 5-IAI, 2-AI
(New) benzodiazepines	Synthetic / Benzodiazepine-related	Sedative, anxiolytic	Flubromazolam, diclazepam, etizolam
Fentanyl analogues	Synthetic / Fentanyl-related	Potent μ-opioid agonists; depressant	Carfentanil, acetylfentanyl
Lysergamides	Synthetic / Lysergic acid derivatives (LSD-like)	Hallucinogen, psychedelic (5-HT2A agonism)	1P-LSD, LSZ
Nitazenes	Synthetic / Benzimidazole opioids	Potent synthetic opioids	Isotonitazene, metonitazene
Phencyclidine-type substances	Synthetic / Phencyclidine-related arylcyclohexylamines	Stimulant, Dissociative (NMDA antagonism)	3-MeO-PCP, 3-MeO-PCE
Phenethylamines	Synthetic / Amphetamine derivatives	Stimulant, hallucinogen	2C-B, DOM,
Phenidates	Synthetic / derivatives of methylphenidate	Stimulant	Ethylphenidate, isopropylphenidate, 4F-MPH
Phenmetrazines	Synthetic / Phenmetrazine derivatives	Stimulant	3-fluorophenmetrazine, phendimetrazine
Piperazines	Synthetic / Piperazine ring	Stimulant, entactogenic	BZP, TFMPP
Plant-based substances	Natural / Diverse origins	Various	Kratom (mitragynine), *Salvia divinorum* (salvinorin A)
Synthetic cannabinoids	Synthetic / Cannabinoid receptor agonists	Cannabinoid-like, Psychoactive	JWH-018, AM-2201, 5F-MDMB-PINACA (5F-ADB)
Synthetic cathinones	Synthetic / β-keto amphetamines	Stimulant	Mephedrone, 3-MMC, alpha-PVP
Tryptamines	Synthetic or natural / Indolealkylamines	Hallucinogenic	4-HO-MET, psilocybin, DMT, 5-MeO-DMT
Other substances	Synthetic / Structurally diverse	Hallucinogen, stimulant, opioid, sedative/hypnotic	n.a

n.a.: not applicable

1.2 Monitoring the Prevalence of Psychoactive Pharmaceutical and NPS Use

Increasing mental disorders and drug abuse create a significant social, economic, and public health burden [3]. For instance, mental health disorders globally contribute to 32% of years lived with disability and account for 13% of the disability-adjusted life-years [39], while illicit drugs are responsible for 5% of all substance-use-related deaths and 9% of all substance-use-related lost healthy years [40]. Additionally, the COVID-19 pandemic profoundly impacted the social and psychological well-being of populations worldwide [41]. Measures such as lockdowns, reduced social interactions, and the temporal closure of public spaces led to sudden changes to the lifestyle and health-related behaviours among the general population, contributing to increased levels of stress, anxiety, and depression [42].

Accurate monitoring of psychoactive substance use is essential for developing effective prevention strategies and comprehensive mental health policies and plans [43]. The monitoring of psychoactive pharmaceuticals typically uses conventional socio-epidemiological methods, including hospital records, general population surveys, and prescription and sales data [44]. Although such data provide valuable information about consumption patterns in the general population, they are also subject to inherent limitations and challenges, including bias, ethical concerns, time constraints, significant time lags in the reported data, and a lack of geographical resolution [45], [46]. Prescription and sales data are also subject to multiple sources of uncertainty, such as non-prescribed sales, improper or unauthorised use, and household disposal of medication [47], [48].

Traditional drug utilisation research (DUR) also often fails to capture information on the clandestine production or illegal distribution of psychoactive pharmaceuticals, leading to potential underestimation of their consumption [49], [50]. Furthermore, incomplete healthcare system datasets may lead to discrepancies between prescribed and administered medication data. For example, prescriptions may not be filled, taken, or consumed by someone other than the intended patient, or the patient may not follow the recommended dosage. Additionally, the site of prescription fulfilment may not align with the location of consumption, and economic factors or adverse effects may prevent patients from obtaining or adhering to their medications [51].

Estimating the NPS use is essential from a public health and policy perspective, yet it poses several distinct challenges due to the dynamic and short-lived nature of these substances on the drug market[52], [53], [54]. Again, traditional indicators, such as seizure quantities or reported consumption, are often inaccurate for NPS, as data on typical doses and usage patterns remain limited [55]. Furthermore, NPS can quickly emerge and disappear, making consistent tracking difficult. As a result, monitoring NPS use trends often relies on identifying and counting the number of new substances entering the market. However, drug users are often unaware of the specific NPS they consume, as new products containing these substances typically provide minimal or no details about their composition [7], [56]. Another issue is the use of NPS in combination with other substances (i.e., traditional illicit drugs and/or NPS), either intentionally or unintentionally, as "adulterants" in controlled drugs [7], [57]. Because of these factors, at the European level, the Early Warning System (EWS) was introduced in 1997 to collect, evaluate, and disseminate information related to NPS, while on a global scale, the UNODC established the Early Warning Advisory (EWA) in 2013 to track, assess, and report emerging trends in NPS use worldwide.

1.2.1 Psychoactive pharmaceuticals: Consumption trends and prescription data

1.2.1.1 Antidepressants

According to the World Health Organization (WHO), approximately 300 million people worldwide suffer from depression and anxiety disorders [3]. Depression affects individuals of all ages, genders, and backgrounds, with a notable increase in cases due to factors such as economic instability, the COVID-19 pandemic, and social isolation. Since 2010, the global consumption of antidepressants has been steadily increasing, leading to further research on their usage patterns [58]. This growth is also reflected in the global antidepressant market, which was valued at USD 11.67 billion in 2019, rising to USD 15.84 billion in 2021, USD 19.60 billion in 2024, and is projected to reach USD 37.9 billion by 2034 [59], [60].

Selective serotonin reuptake inhibitors (SSRIs), in particular, remain the most commonly prescribed class of antidepressants, accounting for a significant market share due to their efficacy and favourable safety profiles [60]. Data from the Organisation for Economic Co-operation and Development (OECD) indicate that between 2011 and 2021, antidepressant use in OECD countries increased by nearly 50%, with countries like Chile, Korea, Latvia, and Estonia reporting a doubling or tripling of consumption rates [58].

The COVID-19 pandemic further exacerbated mental health issues, leading to a significant rise in anxiety and depression prevalence in several countries, which likely contributed to increased antidepressant use [4]. According to the latest OECD data, in 2022, Iceland continues to have the highest level of antidepressant use, with an average consumption of 157 defined daily doses (DDD) per day per 1,000 inhabitants [61]. Several other countries, including Canada, the United Kingdom, and Sweden, were also among the biggest consumers, with more than 110 DDD/day/1,000 inhabitants reported [61]. However, data for the USA were not reported.

In the European Union, antidepressant consumption patterns vary significantly across countries, with an overall trend of substantial growth. Southern European countries such as Portugal and Spain have traditionally reported higher usage rates (151 and 98.4 DDD/day/1,000 inhabitants, respectively; 2022 data), whereas some Eastern European countries, such as Romania and Bulgaria, have shown more rapid relative increases in recent years [61].

In Slovenia, antidepressant use has followed the broader EU trend, with a steady rise observed from 2010 onwards. According to OECD data for 2023, Slovenia ranked in the middle range in global antidepressant consumption, with 70.2 DDD/day/1,000 inhabitants [58], [62]. Recent data from the national prescription records showed an average increase of 33% in antidepressant prescriptions between 2010 and 2023, with SSRIs, particularly citalopram and sertraline, being the most commonly prescribed antidepressants, accounting for approximately 70% of all prescriptions [62]. The COVID-19 pandemic further amplified antidepressant usage, particularly during lockdown periods, with a notable increase of 10% in prescriptions among the younger population, especially during the first wave of the pandemic [63], [64]. These trends highlight the growing dependence on pharmacological treatments for mental health care across general and specific populations, emphasising the need for comprehensive monitoring strategies.

1.2.1.2 Antipsychotics

Consumption of antipsychotics has also increased substantially over the past decade. The global antipsychotic drug market was valued at USD 12.42 billion in 2020, increasing to USD 16.88 billion in 2023, and is estimated to reach USD 26.48 billion by 2032 [65]. From a market perspective, North America holds the largest share, accounting for over 50% of global antipsychotic drug sales, driven by high diagnosis rates, access to healthcare, and adoption of long-acting injectable formulations. Europe accounts for roughly one-third of the global market, with growth driven by an ageing population, mental health reforms, and technological advancements. Meanwhile, the Asia-Pacific region is experiencing the fastest growth, supported by expanding healthcare access and increased mental health awareness [66]. Overall, the antipsychotic drug market is driven by an increase in the prevalence of mental health disorders, progress in drug development, and growing public awareness regarding mental health issues [66]. According to recent WHO data, one in every eight people lives with mental disorders, with schizophrenia and bipolar disorder affecting approximately 24 and 40 million people globally [3].

The use of antipsychotics, especially second-generation (atypical) agents like risperidone, olanzapine, and quetiapine, has expanded substantially in both clinical and off-label settings [65], [66]. High-income countries generally led global trends [66]. In Europe, the average antipsychotic consumption was around 14–15 DDD/day/1,000 inhabitants, while countries like Greece have reported significantly higher usage rates, exceeding 25 DDD/day/1,000 inhabitants (2019 data) [66]. In contrast, Asian countries reported much lower consumption rates, typically around 2 DDD/day/1,000 inhabitants [66]. Between 2010 and 2020, OECD data showed a moderate but consistent increase in antipsychotic use, i.e., Germany's rates rose slightly, while several countries, already at high levels (e.g., Spain and the UK), also climbed [67]. In the U.S., antipsychotic use followed similar growth patterns, although country-level data (DDD/day/1,000 inhabitants) were less often compared internationally. Still, U.S. pharmaceutical sales indicate that antipsychotic consumption is rising, driven not just by schizophrenia and bipolar treatment but also for off-label uses, such as behavioural symptoms in dementia and mood disorders [65].

The pandemic also increased antipsychotic demand globally, largely through expanded telepsychiatry and rising mental health concerns. While DDD-level data are still emerging, the overall market and prescription volumes grew during 2020–2022. Specific vulnerable groups impacted included older adults, who have high baseline antipsychotic usage, and adolescents, who experienced increased prescriptions amid pandemic stressors [68]. Many defining drug markets, such as France, Italy, the UK, South Korea, and the USA, reported notable increases in antipsychotic prescriptions among dementia patients during the early COVID-19 months, with usage levels that remained elevated into 2021 [69]. The increase, particularly among dementia and long-term care patients, is believed to stem from pandemic-induced disruptions such as restricted family visits and mounting neuropsychiatric symptoms in care settings [69].

In Slovenia, the number of antipsychotic prescriptions increased by 45% between 2010 and 2023, indicating that antipsychotic use has mirrored broader European trends, with atypical antipsychotics dominating the Slovenian market due to their improved safety and efficacy profiles [62]. Moreover, the COVID-19 pandemic likely contributed to these trends, as a 12% increase in prescriptions was observed in Slovenia during the lockdown periods (2020-2022) [62]. Compared to other EU countries, Slovenia's consumption rates are moderate, similar to those of countries reporting approximately 5–10 DDD/day/1,000 inhabitants [62]. According to national prescription records, the most commonly prescribed antipsychotic was quetiapine, followed by olanzapine and risperidone [62].

1.2.1.3 Benzodiazepines

Regarding benzodiazepines, the general trend differed from antidepressants and antipsychotics, which, on average, demonstrated increasing rates of use globally. Benzodiazepine consumption, however, has decreased, mainly in high-income countries that historically have shown the highest consumption rates [70]. Specifically, a cross-sectional study encompassing 64 countries found that between 2014 and 2019, benzodiazepine use decreased by 13% in high-income countries, increased by 4% in middle-income countries, and increased by 5% in low-income countries [70]. Across Europe and globally, traditional benzodiazepine usage has gradually declined pre-2020, with a parallel increase in prescriptions of Z-drugs and short-acting agents, such as diazepam and alprazolam [71]. Notably, from 2008 to 2019, the use of benzodiazepines showed a mean annual decline of 1.9% in DDD/day/1,000 inhabitants, while Z-drug usage increased by 3.3% per year, reflecting shifting prescribing practices across more than 60 countries worldwide [71].

This global trend is also mirrored in Slovenia. The analysis of national prescription data from 2006 to 2023 revealed a steady long-term decline of 25%, with the total number of prescriptions decreasing from approximately 1.77 million in 2006 to 1.19 million in 2023 [62]. This trend could potentially reflect changing clinical guidelines, increasing awareness of dependence risks, and the adoption of alternative therapies. Focusing on the last decade (2014-2023), prescriptions have continued to decline steadily. However, a notable sharp drop was observed in 2016, with a 48% decrease compared to the previous year. This decrease was primarily driven by reductions in prescriptions for bromazepam, diazepam, and lorazepam, possibly indicating changes in prescribing policy or supply-related availability [62].

In contrast, a moderate increase of approximately 8% in total benzodiazepine prescriptions was observed during 2020–2021, potentially linked to elevated anxiety and sleep disorders associated with the COVID-19 pandemic. Prescription volumes subsequently returned to levels similar to those seen before the pandemic [62]. During the COVID-19 pandemic, global benzodiazepine consumption patterns varied significantly worldwide. However, a similar trend was also observed in the US, Estonia, and Spain, where a significant increase in the number of benzodiazepine prescriptions, mainly among women, was recorded at the start of the pandemic, in spring 2020, while long-term trends remained largely unchanged.

In Slovenia, zolpidem, alprazolam, and bromazepam were the most frequently prescribed benzodiazepines over the past decade [62]. Their continued prevalence suggests a clinical preference for short-acting compounds with a rapid onset of action, consistent with international trends aimed at minimising long-term dependency.

1.2.2 Current dynamics of the NPS market

New psychoactive substances are rapidly emerging, quickly changing, and swiftly replaced by new variants, preventing the development of a well-established, long-standing market and long-term demand for any specific NPS. Despite international efforts, including continuous identification and scheduling processes by the UNODC and the European Union Drugs Agency (EUDA) (Chapter 1.1.4 New psychoactive substances), the NPS market remains resilient and highly dynamic. According to the UNODC Early Warning Advisory, up to 2024, approximately 1,300 NPS had been reported in 141 countries and territories worldwide [72], with EUDA monitoring over 1,000 NPS [73], reflecting the widespread global distribution and the rapidly changing nature of the NPS market, evidenced by the emergence of 47 new substances within Europe in 2024 alone [74].

The number of readily available NPS expanded globally after 2009 and stabilised at around 550 available NPS by 2018. Interestingly, the number of new NPS cases reported annually increased to 627 in 2023, setting a record for the highest number of unique NPS cases reported in a single year [75]. Synthetic cannabinoids, in particular, reached a peak with the unprecedented number of 191 unique substances reported in 2023 alone, the highest number reported since UNODC began NPS monitoring, highlighting the increasing complexity of the synthetic drug landscape [75]. This trend was further reinforced by increasing diversification within a subgroup known as "semi-synthetic" cannabinoids. While some of the early compounds in this category were derived from plant-based cannabinoids like CBD or THC, many later examples were entirely synthetic but still marketed under the same label [75].

The latest data from 2023 showed that stimulants continue to constitute the largest and most diverse group of NPS, with a percentage of 35%, followed by synthetic cannabinoids (32%), classic hallucinogens (15%) and synthetic opioids (12%) [75]. The latter showed a significant diversification from a single opioid NPS in 2009 to 87 by 2020, but there was a notable drop in the number of synthetic opioids, which fell from 82 unique substances in 2022 to just 41 in 2023. This reduction was mainly attributed to a sharp decline in fentanyl analogues, decreasing from 55 reported variants in 2022 to only 18 in 2023 [75]. Meanwhile, the nitazenes group, which also includes potent opioid agonists, grew significantly, with 16 distinct compounds identified in 2023, compared to 11 the previous year. This increase was coupled with wider geographic distribution, as 26 countries and territories reported the presence of nitazenes in their drug markets [75].

The NPS market dynamics differ significantly by region, with NPS use appearing to decrease in North America and most parts of Western Europe, whereas it is likely to rise in Eastern Europe, Asia, and Africa [40]. Two distinct markets have emerged – synthetic and plant-based. The synthetic NPS market has experienced rapid geographical expansion, with the number of countries reporting seizures increasing from 30 (2009-2010) to 57 (2019-2020), driven by reports from East and South-East Asia. Among these substances, ketamine has emerged as the most commonly seized synthetic NPS [40], [76]. On the contrary, the trafficking of plant-based NPS has expanded more gradually, with seizures reported in 28 countries between 2009 and 2010, increasing to 37 countries between 2019 and 2020. Plant-derived materials such as kratom, khat, and *Salvia divinorum* remain the most commonly reported plant-based NPS, with kratom leading in terms of global seizures and accounting for approximately 50% of all plant-based NPS seized [76].

Local-level data further illustrate NPS trends. In Slovenia, according to the national report on the drug situation in 2019, less than 1% of Slovenian adults reported the use of any NPS [77]. Nevertheless, data from alternative sources, such as online surveys, suggest that NPS use, either on its own or in combination with traditional illicit drugs, could be more prevalent among specific subgroups, particularly among young people in recreational environments [78]. Although in 2021, university students in Ljubljana reported their first use of NPS at the age of 21, data from broader studies suggested that the first NPS use was in even lower ages, including 2% of 17-year-olds in 2018 and 5.3% of 15–16-year-olds in 2019 [77], [78], [79], [80]. Among students at the University of Ljubljana, benzodiazepines and ketamine were the most recognised NPS (83%), followed by gamma-hydroxybutyric acid/gamma-butyrolactone (GHB/GBL) (52%) and DMT (33%) [80].

Based on the latest Slovenian data, the number of reported NPS cases was relatively low until 2020, but increased ninefold in 2021. Twenty different NPS were reported, mainly synthetic cathinones, including 3-MMC, MDPHP, and pentedrone, which were identified [81]. The identified NPS from street drug analysis in Slovenia from 2015 to 2025, grouped by their chemical class, are shown in **Table 6** [82], [83]. This comprehensive list provides a foundation for more targeted toxicological and epidemiological analyses.

Table 6: Identified NPS in Slovenia from 2015 to 2025 (acute emergency cases, anonymous testing of street drugs and drug seizures), adapted by [82], [83]. For full compound names corresponding to the abbreviations used in this table, please refer to the Abbreviations section.

Class	**Identified Substances**
Aminoindanes	5-IAI, 2-AI
Arylalkylamines	2-MAPB, Methiopropamine (MPA), BK-2C-B, 6-APDB, Beta-ketomethiopropamine
Arylcyclohexylamines	3-MeO-PCP, Methoxetamine (MXE), Ketamine, N-Ethyldeschloroketamine (2-oxo-PCE, O-PCE), 2-Methoxyketamine, 3-MeO-PCE, 2F-Ketamine, 3-HO-PCE, 4-MeO-PCE, MXPr, 2F-Deschloronorketamine, 3-Fluoro-PCP, 3-Chloro-PCP, MXiPr, N-Boc Norketamine, 3-Fluoro-deschloroketamine, 2-Fluoro-2-oxo-PCE, 2F-Deschloroketamine (2-FDCK)
Benzodiazepines	Nifoxipam, Phenazepam-4-oxide, Pivoxazepam, Metizolam, Nitrazolam, Ro5-4864 (4-chlorodiazepam), Fonazepam, Clonazolam, Etizolam, Flubromazepam, 3-Hydroxyflubromazepam, Bromazolam, Flualprazolam, Norflurazepam (Norfludiazepam), Desalkylgidazepam, Triazolobenzodiazepine (TBZD), Flubromazolam, Meclonazepam
Cannabinoids	MDMB-CHMCZCA, AB-FUBINACA, CUMYL-BICA, 5F-AMBICA, CUMYL-THPINACA, AB-CHMINACA, CUMYL-PINACA, ADB-CHMICA, FUB-AKB48, CUMYL-5F-P7AICA, CUMYL-PICA, AKB48, ADAMANTYL-THPINACA, PB-22, CUMYL-5F-PINACA, CUMYL-5F-PICA, 5C-AKB48, FUBIMINA, MDMB-CHMICA, STS-135, AMB-CHMICA, JWH-072-N-carboxy, RCS-8, 5F-APICA, JWH-147, AB-PINACA, JWH-020, JWH-072, JWH-018, 5F-MN-18, 5F-APP-PICA_(PX1), 5F-PY-PICA, MO-CHMINACA, 5-Fluoropentyl-3-pyridinoylindole, FUB-NPB-22, ADB-PINACA_isomer 2, 5F-AKB48, JWH-166, JWH-368, 5-Fluoro-SDB-005, JWH-308, SDB-006-N-phenyl analogue, 5-Fluoro-JWH-018-adamantyl analogue, 5F-MDMB-PINACA, JWH-369, AMB-FUBICA, 5F-PB-22, NM-2201, MDMB-CHMINACA, 5F-3,5-AB-PFUPPYCA, ADB-FUBICA, ATHPINACA_isomer 1, ATHPINACA_isomer 2, 5Cl-AB-PINACA, 5F-CYPPICA, MEP-FUBICA, CUMYL-PeGaClone, FUB-144, CUMYL-4CN-B7AICA, 4F-CUMYL-5F-PICA, MDMB-4en-PINACA, MMB-022 (MMB-4en-PICA), THJ-2201 (AM-2201 indazole analogue), RCS-4-C4 homologue, F-AMB-PICA, CUMYL-PIPETINACA, 4,4-F-CUMYL-PIPETINACA-(SGT-235), Ethylbenzyl-CYBINACA, Isobutyl-1-pentyl-1H-indazole-3-carboxylate, MDMB-INACA, APP-BUTINACA, 5F-MDMB-P4AICA, ADB-BUTINACA, CUMYL-CBMICA, MDMB-CHM7AICA, Benzyl-4-CN_BUTINACA, MDMB-FUB7AICA, 4-CN-CUMYL-BUTCZCA, 4F-EMB-BUTICA, EDMB-PINACA, ADB-HEXINACA, ADB-PHETINACA, MDA-19, CUMYL-CBMINACA, ADB-D-5Br-INACA, ADB-5Br-BUTINACA, ADB-5Br-PINACA, CUMYL-CHSINACA, ADB-5Br-4-en-PINACA, ADB-INACA, ADB-P-5Br-INACA

Synthetic cathinones	2-MMC, 3-MMC, 4-MMC, 4-Methyl-alpha-pyrrolidinopentiophenone (alpha-PHiP), Alpha-PVP, Pyrovalerone, Pentedrone, *N*-Ethylnorpentedrone, 3-CMC, 5-BPDi, *N*-methyl-bk-MMDA-2, alpha-PHP, *N*-ethylhexedrone, PV9, PV8, Naphyrone, 4-Ethylethcathinone, 4-Ethylmethcathinone, 3,4-DMMC, 4Cl-PVP, Eutylone, BMDP, 4-CDC, MPPP, 3-MeOMC, N-Ethylpentylone, 3Cl-*N,N*-DMC, 4-Ethylpentedrone, 4Cl-alpha-PHP, 2Cl-*N,N*-Dimethylcathinone, 4-Methyldiethcathinone, 3F-alpha-PVP, 4Cl-Buphedrone, 3-MeO-4-EtO-*N,N*-Diethylpentedrone, 3-MeO-4EtO-*N,N*-Diethylhexedrone, 3-EtO-4-MeO-*N,N*-Diethylpentedrone, 5-MeO-*N,N*-Diethyl hexylone, 3-EtO-4-MeO-*N,N*-Diethylhexedrone, 6-MeO-*N,N*-Diethylpentylone, 3F-NEB, 4-Me-*N*-Propylcathinone, Propylcathinone, 3-Chlorocathinone, *N*-Propylmethylone, 4-Chloromethcathinone (4-CMC)
Indolalkylamines (tryptamines)	4-HO-MET, 1P-LSD, 4-AcO-DMT, 4-HO-MiPT, AMT, ALD-52, 1cP-LSD
Opioids	4-MeO-BF, 4F-BF, FU-F, U-47700, Acryloyl-F, 4Cl-iBF, Methoxyacetyl-F, Methene-U-47700, Benzyl-carfentanil, Benzylfentanyl, Beta-methylfentanyl, 3-Methylbutyrylfentanyl, Furanylethyl fentanyl, Cyclohexyl fentanyl, Cyclopropyl fentanyl, 4Cl-Acrylfentanyl, 4Cl-Cyclopentyl-F, Despropionyl-methylfentanyl, Isophentanyl (cis), Propyl U-47700, 2,3-seco-fentanyl, para-Fluorobenzylfentanyl, ortho-fluoro 4-ANBP, Despropionyl-4-methoxyfentanyl, Despropionyl-4-bromofentanyl, TIANEPTIN, *N*-Me-4-Me phenyl fentanyl, *N*-Me-3-Me Phenyl fentanyl, Brorphine, Fluonitazene, Etazene, Furanyl UF-17, Etonitazepyne, *N*-Pyrrolidino etonitazene
Phenethylamines	4-Fluoroamphetamine (4-FA), X-fluoroethamphetamine (X-FEA), 1-(Benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB), 25I-NBF, DOF, Acetylamphetamine, 2C-TFM, 4-Chloromethamphetamine, 2-Bromo-4,5-MDMA, Allylescaline, 25I-NBOMe, 4-Bromo-2,5-dimethoxyphenethylamine (2C-B), 5-MAPDI, Hordenine, 25E-NBOH, 25B-NBOH, 2-Fluoromethamphetamine (2-FMA), 4-FLuoromethamphetamine (4-FMA), 6-(2-Aminopropyl) benzofuran (6-APB)
Piperazine derivatives	DB-MDBP, 1-(2,5-Dimethoxybenzyl)piperazine, Acetildenafil, Sunifiram, Methoxypiperamide, Kleferein
Plants & extracts	Mitragynine, Harmine
Others	2-MeO-Diphenidine, Dimethocaine, DMAA, Noopept, P2NP, Phenibut, Phenylpiracetam
Precursor and pre-precursor chemicals (associated with the synthesis of NPS)	4-Methylpropiophenone, APAAN, MAPA

1.3 Wastewater-Based Epidemiology

A wastewater treatment plant (WWTP) is typically the endpoint of a sewerage or wastewater collection system that gathers wastewater from households, businesses, and other facilities through a network of sewers and pumping stations. Therefore, the content of wastewater influent acts as a mirror of our society, reflecting a population's health and lifestyle. For example, everything we consume is excreted from the body in urine and faeces as parent compounds or related metabolites, and subsequently ends up in the sewer system, where it is typically transported to a local WWTP. Therefore, a single influent wastewater sample contains a wealth of information about the population served by a particular WWTP. From this perspective, collecting multiple samples enables the acquisition of epidemiological insights across defined population groups, in various locations, and at different periods [84]. Over the past decade, wastewater-based epidemiology (WBE) has become a well-established alternative to conventional epidemiological methods (Chapter 1.2 Monitoring the Prevalence of Psychoactive Pharmaceutical and NPS Use), providing objective, anonymous complementary information on spatiotemporal trends in licit and illicit drug use [2].

Wastewater-based epidemiology involves analysing human metabolic excretion products (biomarkers), including parent compounds and their relative metabolites, in influent wastewater to investigate consumption and exposure to different xenobiotics within defined populations [85]. Trace concentrations of these biomarkers can be measured using highly accurate and precise analytical methods and instrumentation. The measured concentrations of human biomarkers in untreated (raw) influent wastewater are then converted to population-normalised mass loads (PNMLs), expressed as mg biomarker/day/1,000 inhabitants, by multiplying by the corresponding daily wastewater flow rates (L/day) and dividing by the population equivalent contributing to the catchment area on a given day [85]. The PNMLs can serve as proxies for human consumption or exposure, allowing comparisons of usage or exposure patterns across different locations and time periods.

Since its first application in 2005 by Zuccato, to investigate cocaine consumption in Italy [86], WBE has been subjected to continuous development and improvement [87], [88], and most WBE studies have adopted the same approach, including the following steps: sampling of raw influent wastewater, targeted wastewater analysis for selected biomarkers, and back-calculation of their consumption (**Figure 1**).

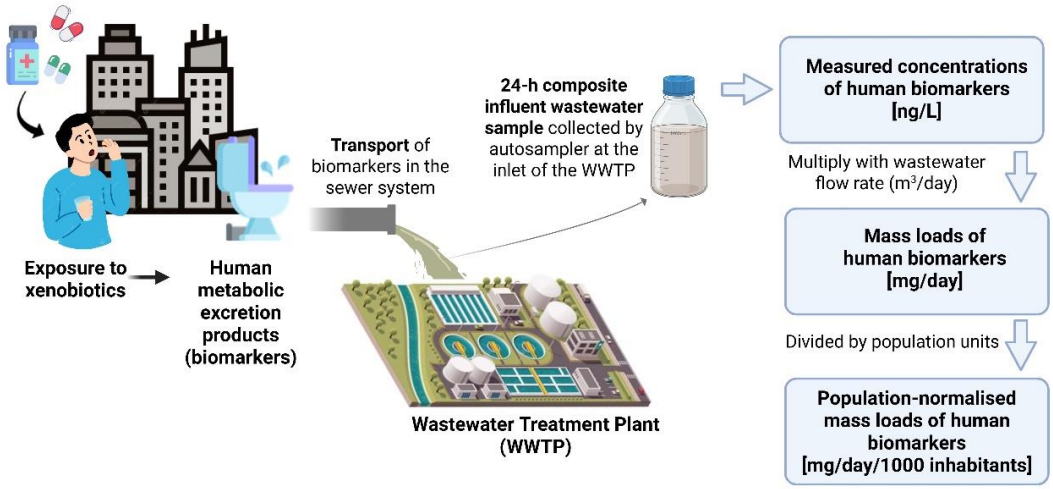


Figure 1: Schematic overview of the main steps in WBE.

In 2010, the WBE approach was harmonised through the Sewage Analysis CORe group Europe (SCORE) COST Action. This initiative has developed best-practice protocols for wastewater sampling, storage, and analysis and has led to the formation of the SCORE network to monitor illicit drug use. Since then, apart from the large number of studies extensively applied to monitor the consumption of illicit drugs, the application of WBE extended to monitoring the consumption of alcohol, tobacco [89], [90], caffeine [91], opioids [92], [93], food and artificial sweeteners [94], [95], personal care products [96], [97], pharmaceuticals [5], [98], [99], NPS [100], [101], [102], [103], and lately to track the spread of infectious diseases like SARS-CoV-2 in populations [104], [105]. Additionally, non-targeted wastewater analysis has been incorporated into the WBE protocol to detect both known substances (via suspect screening) and previously unidentified ones, potentially leading to the identification of new biomarkers. This approach is particularly beneficial for monitoring NPS, as it provides a more comprehensive overview of their consumption patterns [106].

1.3.1 WBE methodology

1.3.1.1 Sampling

Typically, wastewater influent is collected at the inlet of a WWTP to estimate psychoactive substance consumption in the general population, such as within a city or municipality. WBE best practices strongly recommend wastewater sampling during dry periods, as rainfall can dilute wastewater and make biomarker detection more challenging. Additionally, in the event of an overflow, untreated wastewater may be discharged, leading to inaccurate average consumption estimates. Sampling should also be avoided during special events at the WWTPs, including planned maintenance, public holidays or festivals, which may significantly affect the results, as population size (people migration) and psychoactive substance consumption may differ from typical, unless this is the study's objective, like determining NPS during the new year period [100].

The most reliable approach in the WBE is to use 24-hour composite sampling over multiple days (usually a whole week) to capture weekday and weekend variability. Typically, 24-hour composite wastewater samples are collected using an autosampler, which operates in flow-, time-, or flow-proportional mode. In terms of best sampling practice, Ort et al. [107] suggested using autosamplers that operate in flow-proportional mode, collecting samples of varying volumes proportional to the wastewater flow at constant time intervals. Alternatively, the autosampler should operate in volume-proportional mode, where fixed-volume samples are collected at varying time intervals adjusted according to the wastewater flow. In contrast, the time-proportional sampling mode, where both the sampling frequency and volume remain constant, is considered less preferable due to the higher level of uncertainty it introduces into the sampling process. Nevertheless, regardless of the chosen sampling mode, the sampling frequency should be tailored to the specific characteristics of the catchment area, such as exfiltration and the prevalence of psychoactive substances, ideally with a maximum interval of ten minutes [107].

According to recent reviews [2], [102], [106], 24-hour composite influent wastewater samples collected at the WWTPs are the most widely applied sampling method for assessing psychoactive pharmaceutical and NPS consumption in the general population. Although autosamplers typically operate in time-proportional mode at different time intervals (ranging from 10 minutes up to 6 hours), primarily for operational reasons, volume- and flow-proportional sampling modes have also been employed [2]. Only a few studies have reported using grab or passive sampling [45], [96], [108], [109], [110], [111],

[112]. Grab sampling is considered less suitable for WBE applications because it represents wastewater conditions at a specific time, failing to capture short- or long-term fluctuations in concentration, which can result in a misleading concentration profile for the system under investigation [2]. Although grab sampling is simple, low-cost, and practical for rapid screening, it lacks temporal representativeness. In contrast, 24-hour composite sampling integrates temporal variation, offering more accurate and reliable estimates of community-level substance use, albeit with higher equipment and maintenance requirements [2]. Overall, composite sampling remains the standard approach for quantitative WBE applications; however, alternative techniques such as passive sampling are increasingly being explored to overcome some of its operational limitations and enable longer-term monitoring.

The use of passive samplers, such as polar organic integrative samplers (POCIS), is an interesting approach because they enhance *in-situ* detection by accumulating biomarkers on a polymer-based sorbent that remains exposed to wastewater for extended periods, ranging from days to weeks [2]. Except for POCIS (**Figure 2**), the use of other passive samplers, including microporous polyethylene tubes (MPT) [96] and diffusive gradients in thin-film (o-DGT) [113], has also been reported. POCIS are particularly effective for monitoring a broad range of hydrophilic organic compounds, while o-DGT samplers rely on diffusion through a gel layer, thereby reducing boundary-layer effects and providing more stable time-weighted average concentrations [114]. In contrast, MPT devices employ a microporous polyethylene membrane that minimises flow dependence and have been extensively calibrated for polar organic contaminants, making them suitable for wastewater applications [115], [116].



Figure 2: Deploying a passive sampler (POCIS) at municipal WWTP (Photo: Maria Laimou-Geraniou).

Nevertheless, for a better understanding of human psychoactive substance abuse behaviour between weekdays and weekends, 24-hour composite sampling remains more practical, as it can capture fluctuations in consumption on specific days [2], [112]. Passive sampling devices, however, can be advantageous in wastewater analysis when autosamplers

are unavailable, as they provide a straightforward, low-cost sampling approach, *in-situ* enrichment of analytes, increased sensitivity, and time-weighted average (TWA) concentrations, thus offering a time-integrated representation of exposure [2], [102]. Passive samplers also simplify sample transport and storage, as analytes are pre-concentrated onto the sorbent material, requiring less refrigerated volume and reducing the risk of degradation compared with large-volume composite samples. Their low power demand, operational simplicity, and ability to be deployed simultaneously at multiple sites make them particularly attractive for long-term or remote monitoring campaigns [2], [45].

Despite the advantages of passive samplers — time-integrated results, reduced power requirements, and operational simplicity — their applicability remains limited. This limitation arises from the highly complex nature of influent wastewater matrices, which contain variable loads of suspended solids, organic matter, and interfering contaminants, as well as the need for site-specific in-field calibration. This limited application is also due to their uptake being affected by environmental conditions, biofouling, low robustness, and the limited number of extracted biomarkers. Another limitation of passive samplers is the potential for membranes to clog with solid particles that accumulate on the sampler surface during deployment, thereby substantially affecting the analytical results [2], [45].

An alternative, more recent development in this field is the active-passive sampling (APS) device, which combines the benefits of both active and passive sampling [117], [118]. The APS samplers are designed with a diffusion cell loaded with multiple sorbents and powered by a pump that maintains laminar flow. This design enables better control over sampling conditions and reduces dependency on ambient hydrodynamics (**Figure 3**). When first applied in seawater at the Port of Zeebrugge, Belgium, APS showed promising results in detecting a wide range of contaminants (including the psychoactive pharmaceuticals carbamazepine and temazepam) with greater accuracy [119]. The APS device also demonstrated the ability to measure concentrations of target biomarkers with diverse physicochemical properties, highlighting its potential as a promising tool for monitoring influent wastewater in WBE applications (**Figure 4**) [119].

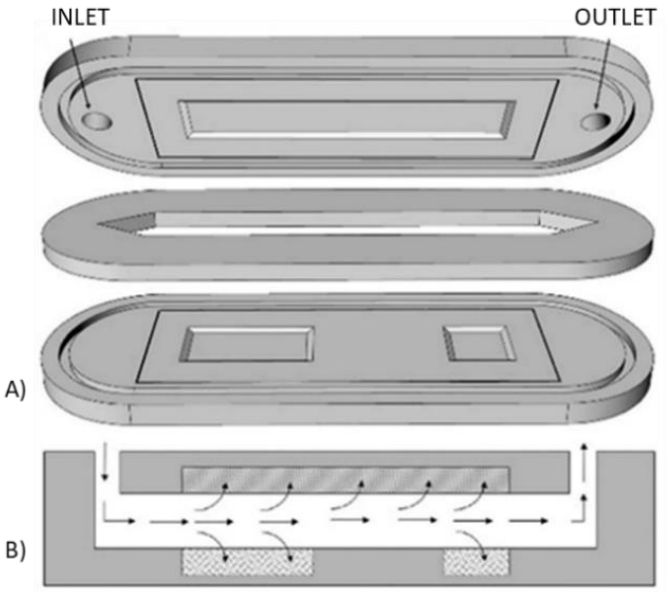


Figure 3: (A) Schematic and (B) cross-section (not to scale) of the APS diffusion cell [2], [117]. The top and bottom blocks can accommodate multiple sorbents, allowing for the sampling of a diverse range of compounds. The sample is actively pumped through the

device, as indicated by the arrows in (B). Water sealing is ensured by two O-rings placed in circular cavities machined in both top and bottom blocks.

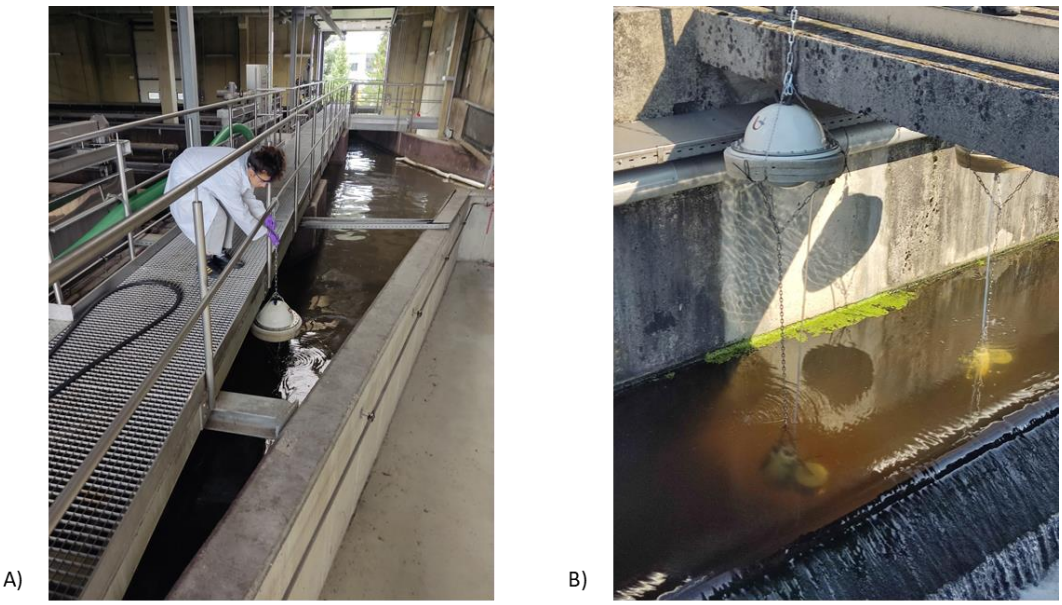


Figure 4: Deployment of APS in (A) influent and (B) effluent wastewater, at municipal WWTP (Photo (A): David Heath and photo (B): Maria Laimou-Geraniou).

Studies targeting specific sub-catchments, such as hospitals, prisons, educational institutions, or even events like football games and festivals, require adapted sampling strategies [2], [120], [121]. In such cases, samples are often collected directly from the sewage outlet before it enters the main municipal network (i.e., at prisons or educational institutions) or from the inlet of an on-site wastewater treatment facility (i.e., at hospitals). Sampling during special events, such as festivals, is commonly carried out at municipal WWTPs [2], [120]. However, in cases where the event location is not connected to a WWTP, sewage is transported to the corresponding WWTPs through local sewers or on-site sanitation systems, such as portable toilets. In the latter case, pooled sewage samples are collected directly and transported for further analysis [2], [120]. Specific sites or small sub-catchments (i.e., hospitals, prisons, educational institutions, and fitness centres) present additional challenges in obtaining representative wastewater samples due to the physical boundaries of the sewer system (i.e. depth of the sewer), limited power supply for autosamplers (in case of composite sampling), inconsistent or reduced wastewater flow, and fewer discharge events, such as toilet flushes containing the biomarkers of interest [120]. While flow-proportional sampling with short intervals (i.e., 1 min) is recommended [107], time-proportional sampling with modified sampling periods ranging from 2 to 72 hours is more frequently applied in specific catchments [2].

The appropriate transport and storage conditions for wastewater samples are determined by the matrix properties, which are influenced by factors such as temperature and source, as well as the stability and preservation requirements of the target analytes. Thus, before any sampling and analysis, the stability of target biomarkers in wastewater should be established under relevant environmental and storage conditions. Since the stability of many psychoactive pharmaceuticals and NPS in wastewater remains unknown, wastewater samples that cannot be processed immediately must be stored appropriately (−20 °C) to avoid degradation of target biomarkers [2]. Another acceptable practice is the extraction of biomarkers on solid-phase extraction (SPE) cartridges within 24 h, with the

addition of internal standard (ISTD), and storing the cartridges in the freezer (−20 °C) [2]. Loaded POCIS [45], [112], [122] and MPTs [96] samplers, stored at −20 °C, have also been reported.

1.3.1.2 Biomarker selection

Selection of biomarkers in WBE can follow either a top-down or bottom-up approach (**Figure 5**). The top-down approach involves selecting biomarkers from a set of human-derived substances identified in pharmacokinetic and human biomonitoring (HBM) studies, for which data on metabolism, excretion rates, and stability are well established. In contrast, the bottom-up approach relies on non-target analysis to identify human-related substances present in wastewater. After evaluating their stability, suitable biomarkers are proposed for further study. Although the bottom-up approach provides a wealth of information, including data for yet unknown substances that could reveal emerging psychoactive substance use patterns, it is less commonly applied than the top-down approach due primarily to the difficulty in identifying potential biomarkers, which are often present at very low concentrations, and the high levels of organic matter in wastewater [106].

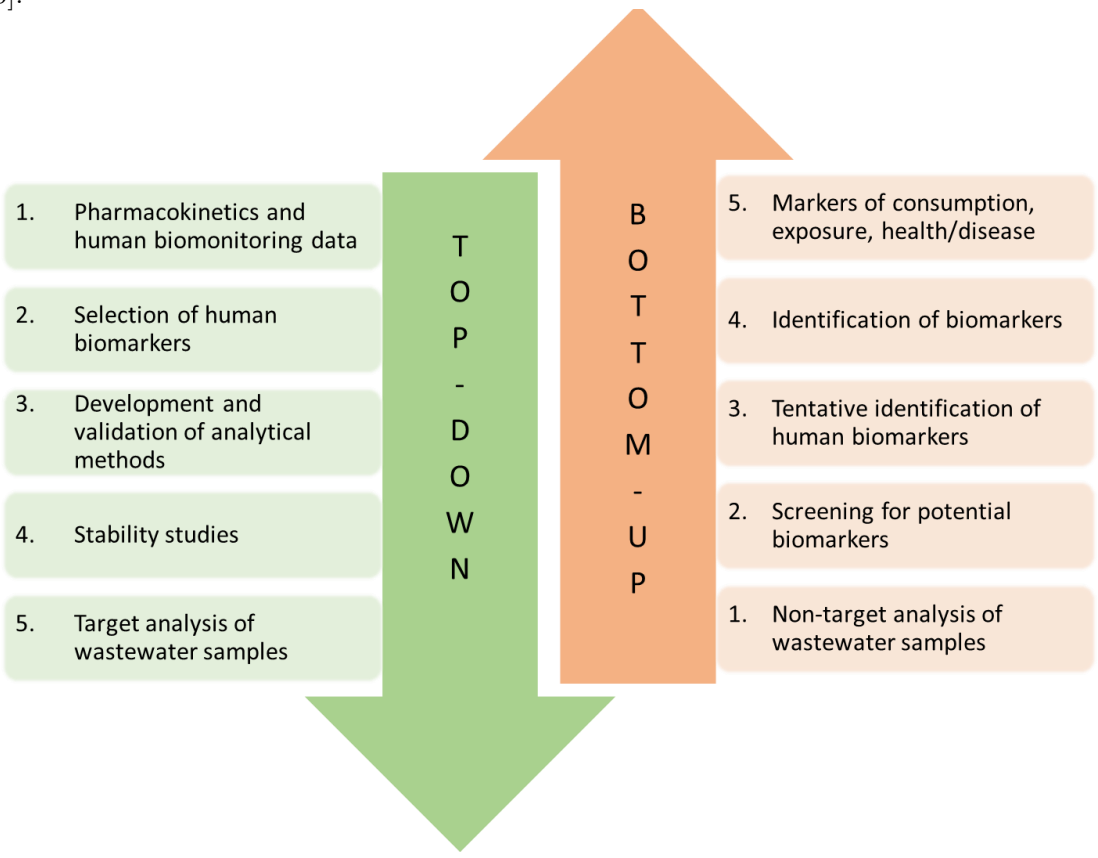


Figure 5: Scheme of top-down and bottom-up approach (adapted from [106]).

Regardless of the selected approach, suitable biomarkers for WBE purposes should fulfil the following requirements [123]: they have to (i) be specific to the substance under investigation, (ii) have well-defined metabolic pathways, (iii) be specific to human metabolism, as exogenous sources may lead to overestimation of substance use, (iv) have high urinary excretion to be detectable in raw wastewater, and (v) have high stability in influent wastewater and low tendency to adsorb (i.e., onto the particulate matter in the sewer). In WBE studies, human metabolites are generally preferred over parent compounds

as more suitable biomarkers, since measured parent compound levels can be affected by direct disposal into the sewage system [87]. However, metabolic biomarkers that fulfil all the desired criteria are not always available, leading to the common practice of using parent compounds in various WBE applications [43], [123].

1.3.1.3 Sample preparation

Analytical challenges in determining psychoactive pharmaceuticals and NPS biomarkers in wastewater stem from matrix complexity and high concentrations of interfering compounds [124]. Therefore, to ensure analytical accuracy and reliability, best practice guidelines recommend the use of isotopically labelled internal standards such as deuterated or ^{13}C isotopically labelled analyte analogues, specific to each target biomarker [87], which can compensate for signal variations caused by matrix effects (ion suppression or enhancement) or analyte losses during sample preparation (e.g., filtration) [125], [126], [127].

As an initial step in sample preparation, suspended solids are typically removed through centrifugation [5], [84], [96], [128], filtration [45], [101], [122], or centrifugation and filtration consecutively [129], [130], [131], [132]. However, a limited number of studies have employed direct injection of filtered wastewater for the determination of psychoactive pharmaceuticals [133], [134], [135] (mainly in hospital effluent wastewater) and NPS biomarkers [136]. For substances like nicotine and ethanol (alcohol), which are generally found in wastewater at relatively high concentrations (μg/L), direct analysis of filtered samples is more feasible [137]. However, bypassing sample cleanup via direct injection can lead to matrix-related issues, such as poor ionisation efficiency and increased limits of detection followed by limited detectability of the target analytes.

In general, the determination of psychoactive pharmaceuticals and NPS, which are usually present in wastewater at trace concentrations (ng/L), requires extraction and pre-concentration. The latter are most commonly achieved using solid-phase extraction (SPE) (**Figure 6**), which offers broad applicability due to the wide choice of available sorbents and solvents [2]. Acidification prior to extraction is an optional step depending on the physicochemical properties of the targeted psychoactive substances (e.g., polarity and pK_a) and the type of SPE cartridges used [102], [138]. Employing reversed-phase (RP) sorbents, such as Oasis HLB, or mixed-mode RP-cation-exchange sorbents, such as Oasis MCX, is among the most popular choices for extracting psychoactive substances [2]. However, the use of multiple other SPE sorbents, often used as mixtures or sequentially, allows the extraction of a wide range of compounds, including neutral, aromatic, acidic, and basic as well as polar analytes [49], [139], [140].



Figure 6: SPE manifold with extraction cartridges and wastewater samples (Photo: Maria Laimou-Geraniou).

1.3.1.4 Target analysis

In WBE, the analysis of psychoactive substances primarily relies on chromatographic separation (liquid or gas chromatography) coupled with mass spectrometry. This analytical combination provides the selectivity and sensitivity required to detect and quantify psychoactive pharmaceutical and NPS biomarkers in wastewater. Although GC-MS/MS is accepted as the gold standard in forensic drug analysis, its application in WBE studies has been limited to a few individual studies addressing the occurrence of antidepressants [141], including only fluoxetine, paroxetine, and sertraline, as well as NPS [111] in wastewater. For GC analysis, derivatisation agents such as trifluoroacetic anhydride (TFA), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), or *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with 1% TMCS are often required to increase analyte volatility [2], [141]. However, the majority of psychoactive pharmaceuticals and NPS are polar, thermally labile, and non-volatile, making them poorly amenable to GC analysis, even after derivatisation, regardless of the detector used. Consequently, liquid chromatography-mass spectrometry (LC-MS) has largely replaced GC-MS in WBE applications, as it enables analysis of psychoactive pharmaceutical and NPS in their native form without the need for derivatisation, detects compounds with a wide range of polarities, low volatilities, and thermal instabilities, and supports more generic sample preparation approaches [2].

Both high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are commonly used for the separation of psychoactive pharmaceuticals and NPS in wastewater [2]. Selecting UPLC over HPLC offers several benefits, such as improved separation efficiency and accordingly reduced analysis time (i.e., UPLC can be 3 to 9 times faster compared to HPLC). Currently in WBE studies, reversed-phase (RP) chromatography is the most commonly used for the separation of psychoactive pharmaceuticals and NPS biomarkers [2]. In RP, mobile phases consist of a biphasic system comprising water and an organic solvent (i.e., methanol or acetonitrile). Modifiers, such as ammonium acetate, ammonium formate, and formic or acetic acid, are also added to improve peak shape and compound ionisation [2]. Nevertheless, mobile phases without any additives have also been reported [85], [142].

LC coupled with tandem mass spectrometry (LC-MS/MS) has become the method of choice for target analysis in WBE, as it provides good robustness, reproducibility, high

selectivity, and sensitivity for the quantification of target compounds, often reaching detection limits in the low ng/L range [2]. Its selectivity arises from a two-stage mass selection process, in which specific precursor ions are isolated and fragmented to generate characteristic product ions, enabling precise identification even in complex matrices. Efficient ionisation, low background noise, and the monitoring of specific ion transitions enhance the signal-to-noise ratio, contributing to low method detection limits and enabling the quantification of trace analytes [143], [144]. The most frequently used detectors include triple quadrupole (QqQ) and hybrid triple quadrupole/linear ion trap (QTRAP) mass analysers, which usually operate in multiple reaction monitoring (MRM) mode.

Electrospray ionisation (ESI) is the most commonly used ionisation technique for the detector interface in wastewater analysis [2]. However, ESI is particularly prone to matrix effects, where co-eluting substances can either enhance or suppress the analyte signal, leading not only to reduced sensitivity (signal suppression) but also to potential errors in quantification [145]. To compensate for these effects, internal calibration is commonly employed, in which the analyte signal is normalised to that of its deuterated internal standard, which is assumed to exhibit behaviour similar to that of the analyte throughout the analytical process [145]. Additionally, most published methods are designed for multi-analyte detection rather than targeting individual compounds, resulting in a broad range of reported limits of detection (LODs) and limits of quantification (LOQs), typically ranging from low ng/L to low µg/L [2].

In recent years, high-resolution mass spectrometry (HRMS) instruments such as quadrupole time-of-flight (QTOF) and Orbitrap analysers have become increasingly important in WBE. HRMS provides accurate mass measurements and resolving powers that typically exceed 20,000-100,000 full width at half maximum (FWHM). Compared with low-resolution MS/MS (LRMS) systems, HRMS offers enhanced mass accuracy and mass resolution, enabling the precise determination of elemental compositions, differentiation of isobaric compounds and higher confidence in compound identification [146]. Also, other advantages of HRMS include scan speed, a wide dynamic range, and the acquisition of full-scan spectra, which allow retrospective data analysis of previously unconsidered analytes without the need to reanalyse samples [146]. Although HRMS instruments are generally less sensitive for trace-level quantification (potentially leading to analyte concentrations below detection) than triple-quadrupole systems, they are indispensable for qualitative and semi-quantitative suspect and non-target screening, providing complementary information to targeted LC–MS/MS measurements. The importance and specific applications of HRMS in suspect and non-target screening are discussed in Section 1.3.1.5.

1.3.1.5 Suspect screening

WBE studies primarily focus on analysing well-known biomarkers at detectable, quantifiable concentrations. However, the number of non-target and suspect screening WBE studies has increased, particularly concerning NPS, for which only a limited number of standards are available [2], [111]. Non-target analysis of wastewater is typically performed using liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) instruments [106]. Despite having a lower resolution than Orbitrap, the QqTOF is the most widely used HRMS analyser [106]. Mass spectral data are gathered using either data-independent (DIA) or data-dependent acquisition (DDA) mode. In DIA, mass spectral data are acquired simultaneously using multiple collision energies: the low energy (LE) mode generates (de)protonated molecular ions, while the high energy (HE) mode produces corresponding fragment ions. In contrast, DDA involves performing a full scan spectrum to identify the 5–20 most abundant precursor ions, which are then selected for fragmentation to generate their HRMS/MS spectra. However, because psychoactive

compound residues in wastewater typically occur at low concentrations, DIA is generally more suitable for WBE applications [106], [111].

Two workflows (suspect screening and non-target) can then be used to analyse the data. Suspect screening includes examining a predefined list of compounds expected to be present in the sample [106], [111]. Based on the criteria proposed by Schymanski et al. [147], compounds are assigned different confidence levels. At a minimum, tentative compound identification (Level 3) requires the accurate mass of the (de)protonated molecule and at least one characteristic fragment ion. In contrast, unequivocal identification (Level 1), which represents the highest level of confidence, can only be achieved when a corresponding reference standard is used.

In a non-target workflow, unknown compounds are identified without prior selection of specific target compounds [106], [111]. Software is employed for noise and background removal, peak picking, dataset comparison, and feature candidate extraction. Following this, retrospective data mining and grouping of the identified compounds are performed using multivariate statistical data analysis [106]. However, this approach requires an extensive and detailed examination of mass spectral data for each chromatographic peak, making it a complex and time-consuming task. As a result, its potential for identifying unknown and rapidly emerging NPS remains limited [111].

Overall, target analysis and suspect screening by UHPLC-QTOF are a broad analytical tool for analysing different illicit and prescribed drugs in wastewater. A noteworthy study by Bade et al. [148] examined the determination of psychoactive pharmaceuticals and NPS in influent wastewater using both qualitative screening and a quantitative approach. In their study, a subset of these compounds was included in a quantitative assessment, while qualitative data were obtained using a QqTOF instrument in Sequential Window Acquisition of All Theoretical Fragment-ion Spectra (SWATH mode). SWATH enables the fragmentation of every analyte in a sample, providing comprehensive MS and MS/MS data for all detectable compounds. This feature enables the differentiation of analytes with similar fragment ions but different $[M + H]^+$ masses. Importantly, a database of retention times and exact masses was created from standard solutions, which were assessed using the SWATH technique.

Although quantitative analysis and qualitative screening for psychoactive pharmaceuticals and NPS in wastewater have their purposes, with target analysis providing quantitative results for compounds with available reference standards, while suspect and non-target screening are primarily qualitative or semi-quantitative approaches, as they rely on accurate mass and fragmentation data rather than calibrated reference solutions, combining them can be valuable, as it provides a more comprehensive overview of their consumption patterns [2], [148]. In this direction, a newly developed comprehensive workflow using the InSpectra open-source, free platform, designed explicitly for suspect screening of NPS and related psychoactive drugs, was recently employed [149].

1.3.1.6 Back-estimation of consumption

The principle underlying the back-calculation of community drug consumption, first described by Zuccato et al. [86], assumes that wastewater losses within the sewer system are negligible and that the selected biomarkers remain sufficiently stable during transport, sampling, and analysis. The back-calculation is outlined in **Equations 1 and 2**:

$$\text{Consumption [mg/day/1,000 inhabitants]} = \frac{c \times \varphi \times CF}{N} \tag{1}$$

$$\mathrm{CF} = \frac{molar\ mass\ ratio\ between\ parent\ compound\ and\ metabolite}{biomarker\ excretion\ rate} \quad (2)$$

Here c represents the measured concentration of the target biomarker in influent wastewater (ng/L), φ the daily wastewater flow (m^3/day), CF a correction factor, and N the number of people in the catchment area.

In contrast to the WBE studies targeting illicit drugs, in which CFs are applied to convert measured biomarker loads into estimates of substance consumption, WBE studies focusing on NPS and pharmaceuticals such as antidepressants, antipsychotics, and benzodiazepines generally report results only as PNMLs in mg/day/1,000 inhabitants. The main reason for this difference lies in the limited and variable pharmacokinetic data available for these compounds. Excretion fractions are often poorly characterised and subject to considerable inter-individual variability due to differences in metabolism, route of administration, co-medication, and physiological conditions. Moreover, many pharmaceuticals undergo extensive biotransformation, leading to the formation of multiple metabolites and conjugates with uncertain excretion profiles [43], [85].

1.3.2 Advantages and limitations

1.3.2.1 Advantages and WBE potential as a complementary data source

WBE can serve as a valuable complementary tool, providing epidemiological information and helping address existing knowledge gaps related to public health across different regions. It enables continuous, near-real-time tracking of lifestyle and health-related indicators across various communities [2], [123]. One of WBE's key strengths is its ability to monitor consumption trends at high spatiotemporal resolution. By monitoring at high spatial resolution, WBE enables assessment of intra- and inter-country differences in the PNML of various biomarkers. Unlike some conventional epidemiological data sources, which may lack detailed geospatial resolution, WBE can capture consumption patterns at different locations, including within countries at the city/village level or monitor consumption trends at the scale of regions and suburbs within a metropolitan area [5]. Normalising data to PNMLs further supports comparisons across cities on a global scale.

The high temporal resolution of WBE also enables the gathering of information on the long-term consumption of psychoactive substances, indicating whether their use is stable, increasing, decreasing, or fluctuating [2]. Moreover, seasonal variations can highlight temporal changes within specific months of the year or recurring annual patterns. WBE's strong spatiotemporal capabilities make it a powerful early-warning tool for identifying rapidly emerging shifts in consumption. This real-time insight equips policymakers with essential data to support prompt decision-making and to assess the impact of newly implemented policies or local and national public health initiatives.

In addition, WBE can provide consumption estimates for substances or pharmaceuticals that are not recorded by other methods, either because they are used illegally [150] or because they are absent from existing data collections [151]. For example, WBE can complement traditional datasets by indicating the proportion of pharmaceuticals consumed by the population, rather than sold, prescribed, or dispensed. Since WBE measures human metabolic excretion products in influent wastewater, it reflects real usage. This ability is particularly valuable given that prescribed medications are not always taken as directed or may be diverted to others, meaning dispensing data alone may not accurately represent actual consumption.

WBE biomarkers also have the potential to serve as a proxy for the spread and circulation of infectious diseases at the population level [152], [153]. Biomarkers of infectious agents, such as viral genomes, bacterial gene fragments, and antibiotic resistance genes, are excreted by infected individuals, then pooled and transported through the wastewater system. The concentration of these pathogens in influent wastewater reflects the spatial and temporal dynamics of disease transmission within a population, independent of individual participation in clinical testing [154], [155], [156], [157]. This ability makes WBE a valuable tool for the early detection and prevention of infectious disease outbreaks (e.g., SARS-CoV-2), the identification of potential hotspots, and the assessment of the effectiveness of large-scale public health interventions across communities [158], [159], [160].

1.3.2.2 Limitations and uncertainties

Although WBE provides high-resolution spatiotemporal information at the population level [2], [48], it also has limitations regarding individual use of substances, doses, and administration frequency. For example, suppose an increase in the biomarker's PNMLs was detected. In that case, WBE cannot distinguish whether it is due to (i) more individuals consuming at the same rate, (ii) the same number of individuals increasing their consumption, (iii) specific subgroups within the population consuming more than others, or (iv) a combination of these scenarios. Although individual socio-demographic features are not accessible through WBE, it does allow for spatial comparisons between communities with varying socio-economic backgrounds, offering valuable context at the population level [2], [48].

Despite significant advancements in research over the past decade, WBE still faces inherent uncertainties in wastewater flow sampling methods, biomarker stability, chemical analysis, the application of correction factors, and population size estimation, all of which require further attention. **Figure 7** outlines the key steps involved in implementing the WBE approach and highlights the various sources of uncertainty associated with each stage.

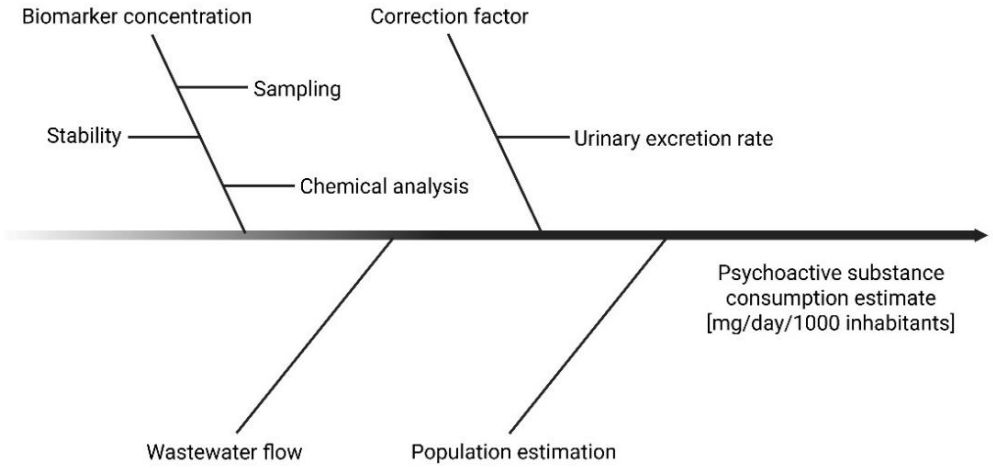


Figure 7: Fishbone presentation of uncertainty sources in drug consumption estimates obtained by WBE.

Efforts have been made to assess these uncertainties and enhance the accuracy of WBE estimations. As previously described (Chapter 1.3.1.1 Sampling), continuous 24-hour composite sampling using flow-proportional methods is recommended as the best practice for collecting influent wastewater. However, in practice, time-proportional sampling is often employed due to logistical constraints. To properly assess sampling biases, high temporal resolution is needed for wastewater flow and intra-day variations in biomarker concentrations. Sampling bias is strongly dependent on specific catchment characteristics, such as population size, the number of substance users, and the operation of pump stations in the sewer system [87]. Additionally, Lai et al. [161] found that the systematic error (approximately 20%) associated with flow measurements could be minimised through routine calibration of flowmeters.

A significant source of uncertainty arises from population size in the catchment area. The majority of studies investigating psychoactive substance consumption use fixed population numbers when calculating their PNMLs, as provided by the WWTPs. This approach does not consider population mobility and contributes to overall uncertainty levels as high as 55% [87], [128]. This uncertainty poses a challenge, especially when verifying whether observed fluctuations in PNMLs are due to changes in consumption patterns, differences in the number of consumers or variations in the actual catchment population. Therefore, it would be more suitable to use dynamic population size numbers, utilising either anthropogenic biomarkers [162] or alternative sources, such as mobile phone data [128], [162], [163].

Additionally, the excretion rate of human biomarkers is considered another important source of uncertainty, as it varies between individuals or even within the same individual under different health conditions. The urinary excretion rate depends strongly on factors such as the route of administration, dose, gender, age, physical condition, and even concurrent drug use [87], [164], [165], [166]. Consequently, the excretion factor (EF), which represents the fraction of a substance excreted as the measured biomarker, obtained from these processes, significantly contributes to overall uncertainty when back-calculating substance consumption in WBE. Since the correction factor used in these calculations incorporates the EF, variability in biomarker excretion propagates directly into uncertainty in the CF and, in turn, in the estimated consumption.

Additionally, EF values used in WBE are typically based on a small number of studies and may not accurately represent the average excretion profile in large populations [48]. Although efforts have been made to refine EF values for different compounds, these efforts have been limited primarily to illicit drugs [167]. Consequently, the application of CFs to back-calculate the absolute consumption of psychoactive pharmaceuticals and NPS should be approached with great care, as it would introduce substantial and unquantifiable uncertainty. However, the primary objective of WBE has been to serve as a complementary method for monitoring spatiotemporal trends in consumption patterns of psychoactive pharmaceuticals and NPS. To address the uncertainty arising from EFs, a practical approach is to use PNMLs and focus on trend analysis with relative comparisons [48].

Biomarker stability is also an important source of uncertainty in WBE. Many studies have been conducted to investigate degradation and transformation processes, aiming to evaluate and model in-sample and in-sewer stability of illicit drug biomarkers in order to understand the extent of this uncertainty better; however, only in the last few years there has been a growing number of publications assessing the stability of antidepressants and benzodiazepines, while stability of antipsychotics and NPS remain unexplored [2].

To limit chemical changes in biomarkers after collection, preservatives such as hydrochloric acid (HCl) and storage at low temperatures (below −20 °C) are commonly used. However, since preservatives can only be added at the point of sampling, they do not prevent degradation that occurs within the sewer network. For biological biomarkers,

degradation may still occur under these storage conditions, necessitating the use of alternative sample-handling methods [2].

In-sewer stability introduces even greater uncertainty due to limited control over the microbiota present in the sewer system, particularly the biofilms that form within it. Factors such as higher wastewater temperatures, a higher biofilm surface area relative to the bulk water volume, and extended hydraulic retention times can all enhance biomarker transformation in the sewer system [168]. Additionally, for specific compounds, adsorption to suspended particulate matter (SPM) or biofilm, particularly those with high $\log K_{ow}$ values, can further increase uncertainty [138].

Finally, further uncertainties may arise from the direct disposal of unused substances into the sewage system or the formation of other chemicals or conjugated forms of substances [169]. Direct disposal of unused drugs into the sewers systematically affects WBE estimations, particularly when the parent compound is used as a biomarker of substance use (overestimation) [170]. Consequently, there is a need for analytical methods capable of distinguishing between actual consumption and the direct disposal of psychoactive substances.

To date, investigations of dumping events have been limited and typically initiated only after wastewater analysis revealed unusually high mass loads of specific biomarkers [171]. According to Quireyns et al. [171], direct disposal is most commonly addressed using the parent compound-to-metabolite (PC:M) ratios. In this approach, the cut-off values representing the limit between disposal and human consumption are set based on the substance's excretion profile. Despite the simplicity of the approach, it requires a thorough understanding of the excretion profile and data on the concentrations of both parent compounds and metabolites, which are often not readily available. Other methods include enantiomeric profiling (only for chiral compounds) and the detection of chemical indicators (for illicit drug production) by non-target and suspect screening workflows.

Overall, the high spatial and temporal resolution offered by WBE makes it particularly well-suited for monitoring trends in psychoactive substance use over time (i.e., intra-week or long-term) and across different locations (i.e., inter-municipality and international comparison), rather than for determining absolute substance use within a specific population. As a result, certain sources of uncertainty, i.e., variation in excretion rates, become less critical. However, to ensure the reliability of spatial and temporal comparisons, sampling campaigns should be performed following standardised protocols, and, ideally, under comparable weather conditions. This principle is applied in the European SCORE network, which organises annual monitoring campaigns each spring to ensure consistent sampling conditions for assessing both temporal and spatial trends of illicit drugs. Nevertheless, methodological improvements remain important to minimise systematic over- and underestimation of substance use and to evaluate consumption patterns more thoroughly.

1.3.3 WBE as a complementary information source during the COVID-19 pandemic

Despite its potential to monitor public health interventions and track spatiotemporal trends in xenobiotic consumption and chemical or pathogen exposure, WBE remains poorly integrated into government strategies and policymaking. The COVID-19 pandemic, which led to widespread lockdowns and significant disruptions to daily life, offered a unique opportunity to assess WBE as a supplementary information tool. In this context, WBE can help evaluate the impact of socially disruptive measures on populations served by WWTPs and monitor changes in PNMLs of both chemical and biological biomarkers. Some governmental bodies, such as the EUDA, the Australian Crime Intelligence Commission,

and the New Zealand Police, already recognise WBE as a reliable complementary source for tracking a wide range of illicit and legal drug biomarkers [145], [172], [173]. However, its broader potential remains underutilised, and further research is essential to integrate WBE into policy development and evaluation.

WBE, due to its high temporal resolution, can also be effectively used to examine the short-term impacts of public health countermeasures on human consumption and exposure. Its flexible sampling temporal frequencies allow for the retrospective generation of aggregated consumption data with minimal delay in response to policy changes, offering a faster alternative to conventional information sources. Importantly, WBE also serves as a valuable complementary tool, especially during the COVID-19 pandemic, when existing data sources may not be fully in agreement. This inconsistency highlights the importance of developing additional epidemiological tools and triangulating data across multiple methodologies. Thomaidis et al. [174] also demonstrated WBE's ability to capture temporal trends during an economic crisis and the implementation of austerity measures, further highlighting its relevance in monitoring the societal impacts of major disruptions. Consequently, WBE could provide essential insights to public health authorities and institutions, supporting the development and refinement of harm-reduction, prevention, and treatment programs. However, while WBE effectively reflects population-level consumption patterns, it does not capture the socio-demographic characteristics of individuals using xenobiotics [175]. This limitation reinforces the need for integrating WBE with other epidemiological data sources to more comprehensively assess the effects of events like the COVID-19 pandemic on substance use [175], [176].

Finally, analysing influent wastewater can be used to monitor infectious disease biomarkers within different communities. Even asymptomatic individuals infected with SARS-CoV-2 shed the virus in their faeces, which enters the sewer system [177]. This ability allows WBE to assess the extent of viral transmission across various catchments and potentially forecast future outbreaks. Moreover, WBE also shows promise as an early warning system for detecting SARS-CoV-2 in the general population [178], [179], [180]. It is particularly effective for identifying isolated or sporadic cases, where mass testing of thousands of individuals would be impractical and inefficient.

1.4 Environmental Aspect

The harmful effects of chemicals, including aromatic compounds, polychlorinated biphenyls, heavy metals, and pesticides, on human and aquatic organisms are well established [181], [182], [183]. However, growing attention is now being directed toward “emerging contaminants,” with pharmaceuticals standing out as a major concern. The heightened focus stems from two key features of pharmaceutical substances: they can exert biological effects even at the low concentrations typically found in the environment, and they are intentionally designed to remain stable long enough to reach their target site before breaking down. This persistence contributes to environmental concerns, as these compounds often enter aquatic ecosystems without being fully degraded [184], [185].

Since the late 1990s, there has been an exponential increase in scientific publications on the presence of pharmaceutical compounds in the environment. This surge is largely due to advances in analytical methods that enable the detection of drugs at very low concentrations across various environmental media. Today, the volume of data on pharmaceutical contamination and its potential impacts is vast and continually expanding [185]. Currently, approximately 4000 different pharmacologically active substances are being administered worldwide, including prescription medications for humans, over-the-counter drugs, and veterinary drugs [186]. Global medicine consumption is on the rise, with

an estimated 14% increase over the last five years, and is projected to rise by another 12% by 2028, reaching an annual total of 3.8 trillion defined daily doses [187]. However, psychoactive pharmaceuticals only recently received particular attention compared to other therapeutic classes, due to their widespread consumption by modern society, which was further increased due to the pandemic, especially for antidepressants and antipsychotics, their persistence in water compartments, and their potential ecotoxicity [185], [188].

Each stage in a pharmaceutical's life cycle (**Figure 8**), including production, consumption, and disposal, can serve as a potential pathway for its entry into different environmental matrices, such as surface waters, groundwater, drinking water, soil, manure, biota, sediment and the food chain [189]. Although the relative impact of each source varies by region and substance type, the primary source of pharmaceuticals in the environment is considered to be treated effluent wastewater from municipal WWTPs and, to a lesser extent, untreated domestic discharges where sewerage systems are absent or inadequate [190]. Additionally, emissions from manufacturing plants, intensive agriculture, and aquaculture can be important pollution hotspots locally [185], [188].

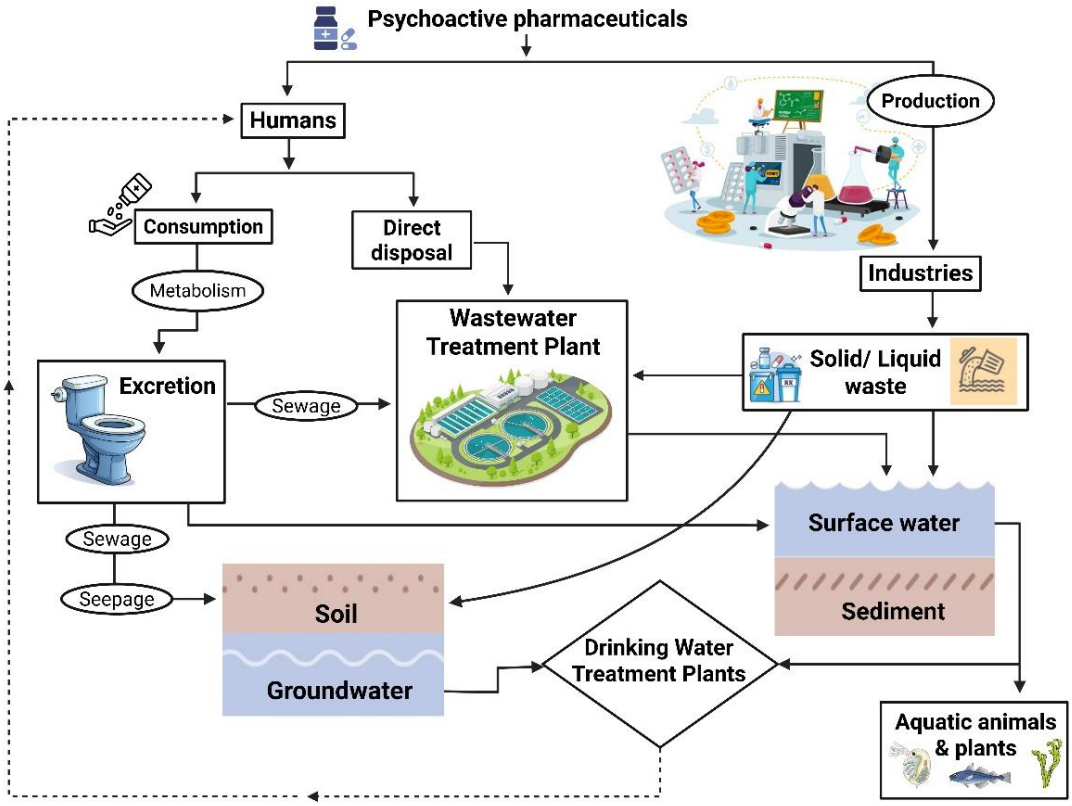


Figure 8: Conceptual overview of the psychoactive pharmaceutical transfer pathway, from production and consumption to the environment.

The ubiquitous presence of psychoactive pharmaceuticals, including both parent compounds and their corresponding metabolites, in both wastewater and environmental waters is of particular concern. Importantly, since psychoactive pharmaceuticals and their metabolites are inherently bioactive, they can have unintended, negative impacts on non-target organisms.

Harmful effects on aquatic organisms have been reported at the environmental level. For instance, the antidepressant fluoxetine can change fish behaviour, making them less risk-averse and more susceptible to predators [189]. Although psychoactive pharmaceuticals

and their metabolites are frequently detected in influent wastewater to monitor consumption trends in the general population through WBE, their occurrence in environmental waters and the effects they may have on ecosystems remain poorly understood and relatively unresearched [8]. In particular, most of the existing studies have examined mainly antidepressants [191], [192], [193], [194], while only a few have focused on a limited number of antipsychotics [195], [196] and benzodiazepines [197] and their associated ecological risk in aquatic ecosystems.

Most OECD countries have already implemented watch-lists and voluntary monitoring programs targeting specific pharmaceuticals in surface waters. However, the vast majority of active pharmaceutical ingredients (APIs), their metabolites, and transformation products are still largely unmonitored and lack ecotoxicological data. As a result, significant uncertainties remain in assessing the environmental risks of pharmaceuticals, stemming from limited knowledge about their behaviour in the environment, their effects on ecosystems and human health, and the potential impacts of mixtures with other chemicals [189]. Therefore, together with the global increase in psychoactive pharmaceutical use, the UNODC pointed to the need to develop effective strategies for environmental protection based on a deeper understanding of how drug residues impact the environment. This need includes thorough monitoring of both parent compounds and metabolites in wastewater and environmental waters, as well as conducting comprehensive hazard assessments.

1.4.1 Analytical methods for the determination of psychoactive pharmaceutical residues in the aqueous environment

As WBE serves as a link between environmental and social sciences [198], it is understandable that detecting psychoactive pharmaceutical residues in environmental waters largely relies on techniques that are only slightly adapted, such as increased sample volumes, initially designed to assess population psychoactive pharmaceutical use through wastewater analysis. Accurate determination of psychoactive pharmaceutical residues in environmental waters also requires a robust sample preparation process, which includes effective analyte pre-concentration, efficient removal of interfering substances, and the use of highly sensitive analytical methods [197]. Consequently, solid-phase extraction (SPE), commonly using Oasis MCX or HLB cartridges, is frequently employed to extract drug residues, which are then quantified by targeted LC-MS/MS analysis, as detailed in the recent literature [197], [199].

Sampling strategies vary, and in contrast to wastewater, where sample collection follows established best practice protocols, sampling of receiving surface water lacks standardisation. At present, it is commonly performed using grab samples (**Figure 9**), which can only provide a "snapshot" at the time of sampling and do not reflect temporal variations in contaminant concentrations [8]. To address this limitation, passive sampling using POCIS, low-density polyethylene film devices, solid-phase microextraction fibres, and Chemcatcher® devices has been employed [96], [200], [201].

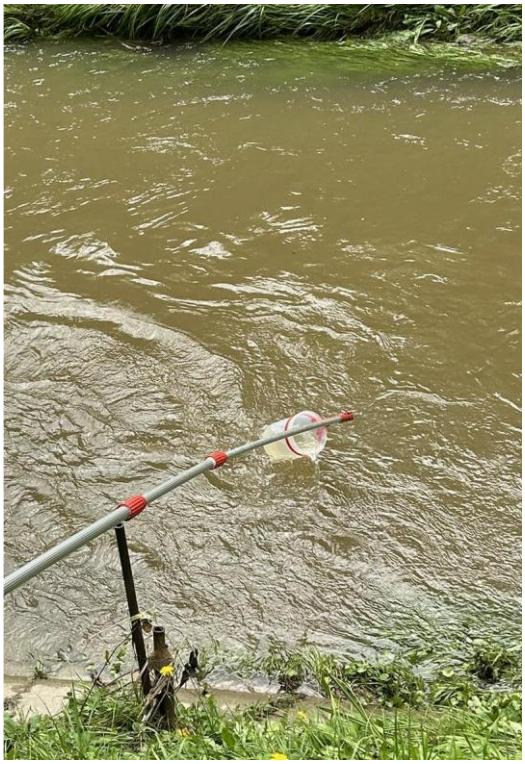


Figure 9: Photo showing surface water grab sampling (Photo: Eirini Andreasidou).

1.4.2 Occurrence and removal of psychoactive pharmaceutical residues in wastewater

Like many other drugs, psychoactive pharmaceuticals are excreted from the body through urine and faeces, either as unchanged compounds or as metabolites, and then enter the sewage system, where they eventually reach a local WWTP. Accordingly, raw wastewater is the primary destination for human biological fluids, such as urine, faeces, saliva, and sweat, which commonly contain psychoactive pharmaceutical residues in concentrations ranging from ng/L to μg/L. Since conventional WWTPs were not explicitly designed to remove persistent organic pollutants, such as certain CECs, including psychoactive pharmaceutical residues, these substances often persist in the wastewater effluent [8], [184], [191], [197], [202]. However, they are typically found at lower concentrations (ng/L) than those in untreated influent wastewater (ng/L to μg/L).

Various wastewater treatment technologies have been studied for their effectiveness in removing psychoactive pharmaceutical residues from raw influent wastewater, commonly including activated sludge, sequential batch reactors (SBR), trickling filters, and membrane bioreactors (MBR) [8]. Their removal efficiency was shown to depend on multiple factors, such as the treatment technology used, physicochemical properties, influent concentration, operational parameters at the WWTP, and in-sewer conditions [8], [203]. As a result, removal rates can vary significantly even for the same compound and treatment technology. Generally, higher removals were found for antipsychotics, followed by benzodiazepines and antidepressants [8], [204], [205], [206].

Removal efficiency was also found to be compound-dependent, with some psychoactive pharmaceutical residues (i.e., citalopram, venlafaxine, and oxazepam) exhibiting poor removal under different biological treatments (activated sludge, SBR, SBR with UV disinfection, MBR, MBBR). Additionally, negative removal has been observed, where

higher concentrations of bupropion, citalopram, venlafaxine, clozapine, oxazepam, and zolpidem are detected in the treated effluent compared to the influent [8], [207], [208], [209].

These cases attributed to factors such as desorption from sludge, deconjugation of glucuronide conjugates, mutual transformation of conjugated metabolites and parent compounds, transformation of parent compound/precursor during wastewater treatment and limitations in sampling accuracy due to an inadequate matching of influent and effluent wastewater samples, i.e., outside of the HRT range [8], [193], [203], [206]. Although several studies [8], [191], [192], [193], [202], [210] have attempted to investigate the presence of psychoactive pharmaceutical residues in wastewater and their removal with different wastewater treatment technologies, the variability of findings and limited compound selection [195], [196], [197] suggest further research in the area.

From a regulatory perspective, the removal of micropollutants, including psychoactive pharmaceutical residues from wastewater, has gained increasing attention within the framework of European Union environmental legislation. To protect the environment and public health from the harmful impacts of wastewater discharges originating from municipal and certain industrial sources, the Urban Wastewater Treatment Directive (UWWTD), initially established in 1991 to set the requirements for the collection, treatment, and discharge of urban wastewater, was recently revised and came into effect on January 1, 2025 [211], [212].

One of the key updates in the Directive is the regulation of micropollutant discharges from WWTP effluents into the environment, particularly into surface waters, for the first time. To monitor the removal efficiency of WWTPs, the revised UWWTD identifies 13 model micropollutants, whose treatment performance must be tracked. The Directive requires that at least 80% of six out of these 13 compounds be removed during treatment. If this criterion is not met, additional quaternary treatment must be implemented at each WWTP, with the installation timeline depending on the plant's size.

Through this revision, the European Commission has formally incorporated into legislation the need to protect the environment from potentially harmful chronic exposure to low concentrations of residues. Notably, among the 13 regulated micropollutants are three antidepressants: carbamazepine, citalopram, and venlafaxine, which are the focus of this thesis. Although the current directive primarily focuses on limiting the concentrations of pharmaceutical and personal care products in treated effluent wastewater [212], it is anticipated that this list will be expanded to include additional compounds that may pose environmental risks, particularly in regions affected by extensive pollution.

1.4.3 Occurrence of psychoactive pharmaceutical residues in receiving surface waters

Due to their continuous release, psychoactive pharmaceutical residues, which include both parent compounds and their corresponding metabolites, exhibit pseudo-persistence in aquatic ecosystems. Consequently, psychoactive pharmaceutical residues have been detected in various receiving surface waters such as rivers, lakes, and oceans [8], [195], [197], mainly in the ng/L range, with differences related to the sampling site, dilution factors, time (seasonal variations), and trends in drug consumption and production.

Once released into the environment, the fate and distribution of psychoactive pharmaceutical residues depend on their physicochemical properties and external environmental conditions, including pH, dilution, temperature, and exposure to sunlight [8]. Based on their physicochemical properties, most psychoactive pharmaceutical residues (except for certain antipsychotics and benzodiazepines, i.e., quetiapine, aripiprazole, zolpidem, flunitrazepam, etc.) are considered hydrophilic ($\log K_{OW}<4$) and exhibit relatively high water solubility (Sw$>$ 8.4 mg/L). As a result, they tend to remain in the aqueous

phase rather than bind to solid particles, making them highly mobile in aquatic systems and capable of leaching into groundwater [184], [185], [188].

Additionally, environmental factors, particularly pH, also affect the aqueous solubility and mobility of such residues, with ionised forms typically exhibiting greater solubility and mobility. At environmentally relevant pH levels (pH 5–9), most psychoactive pharmaceutical residues, with pK_a values ranging from 4.21 to 10.2, are expected to exist in neutral or protonated forms, thereby enhancing their persistence in the aqueous phase [184], [185], [188].

Once in the environment, parent compounds or metabolites of psychoactive pharmaceutical residues continue to undergo various transformations. These include complex metabolic processes by different organisms as well as physicochemical mechanisms such as photodegradation and adsorption to solids, resulting in the formation of so-called "transformation products" [185]. For instance, Trawiński et al. [213] conducted a review on the photodegradation of psychoactive pharmaceuticals, showing that certain compounds are persistent and may remain chemically stable in the environment for decades. For example, oxazepam has been found unchanged in the sediments of Swedish lakes for over 30 years [214].

Currently, there are no established regulatory limits for psychoactive pharmaceutical residues in surface waters. However, as our understanding of the environmental impact of psychoactive pharmaceuticals continues to grow, further research into their presence and behaviour in surface water systems is needed. The EU has established a Surface Water Watch List, under the Water Framework Directive (WFD), to identify and monitor substances that may pose a significant risk to aquatic environments but lack sufficient data for full regulatory action.

Together, the UWWTD and WFD form the legislative foundation for managing micropollutant emissions and improving water quality across the European Union. The UWWTD addresses removal at the treatment stage, while the WFD Watch List focuses on environmental monitoring of substances that escape treatment and enter surface waters. This integrated policy framework reflects the growing recognition of pharmaceuticals as contaminants of emerging concern and underscores the need for coordinated treatment, monitoring, and regulatory measures at the European level.

Venlafaxine and its primary active metabolite, *O*-desmethylvenlafaxine, were among the first antidepressants added to the EU Watch List (WL) in 2020. This inclusion was formalised through Commission Implementing Decision (EU) 2020/1161 of 4 August 2020, following growing evidence of their frequent detection in European surface waters and concerns over their bioactive effects on non-target aquatic organisms [215].

In 2022, the European Commission considered 61 compounds, including the antidepressant fluoxetine, for which data on their environmental occurrence and ecotoxicity indicators were considered candidates for the 4th Watch List under the Water Framework Directive. Subsequently, fluoxetine, a commonly prescribed selective serotonin reuptake inhibitor (SSRI), was added to the Watch List in 2022 as per Commission Implementing Decision (EU) 2022/1307 of 22 July 2022. Its inclusion was driven by similar environmental concerns, particularly its persistence in aquatic systems and potential to disrupt aquatic life even at low concentrations [216]. To date, no antipsychotic or benzodiazepine compounds have been included in the EU Surface Water Watch List, highlighting a potential gap in regulatory monitoring despite their known environmental occurrence and pharmacological activity.

1.4.4 Toxicity of psychoactive pharmaceutical residues to aquatic organisms

Because of the pseudo-persistent nature of psychoactive pharmaceutical residues in aquatic environments, aquatic organisms are continuously exposed to low concentrations of these substances [8], [184]. The detection of psychoactive pharmaceutical residues or other chemical substances in the environment does not necessarily indicate a risk or harmful effect. However, existing research has shown that antidepressants, antipsychotics, and benzodiazepines can impact bacteria, algae, vertebrates, and invertebrates. The reported effects include disruptions in reproductive, physiological, and morphological development, as well as changes in swimming behaviour and reduced survival rates [184], [185]. Psychoactive pharmaceutical residues may also affect aquatic organisms at trace levels, i.e., through chronic exposure.

Overall, exposure to psychoactive pharmaceuticals has been shown to impact non-target organisms in multiple ways. For instance, SSRIs have been linked to changes in behaviour, reproduction, morphology, antioxidant activity, gene expression and development in both vertebrates and invertebrates. SNRIs affect gene expression, behaviour, reproduction, and circadian rhythms, and may reduce survival in fish. TCA exposure can lead to developmental and morphological abnormalities, and even affect the survival of organisms in early life stages [184].

Exposure to the antipsychotic clozapine has been linked to constipation in fish [217], while benzodiazepines can accumulate in plants, potentially impacting their GABAergic systems [218]. In addition, enantiomer-selective toxicity is predicted for chiral psychoactive pharmaceutical residues (e.g., fluoxetine*),* indicating that individual enantiomers can have different adverse effects on aquatic organisms. Castillo-Zacarías et al. [219] and Moreira et al. [184] have recently reviewed the available literature on the occurrence and effects of antidepressants in the environment. Despite what is known about the behaviour of antipsychotic and benzodiazepine residues, as environmental pollutants, they have been underrated so far [8], [185].

To date, studies have primarily focused on parent compounds and a limited range of organisms, mainly from the groups of microorganisms, vertebrates, and invertebrates. The ecotoxicity of antidepressant, antipsychotic, and benzodiazepine residues has been confirmed across a range of organisms and at various concentrations, including those found in the environment. However, there is a lack of research specifically examining their impact on algae, which are key primary producers in aquatic ecosystems [220].

Furthermore, most studies do not focus on the effects of individual enantiomers of chiral psychoactive pharmaceuticals, even though these may differ in toxicity. For example, in one of the rare studies addressing the stereoselective toxicity of the antidepressant fluoxetine for *Pimephales promelas* and *Tetrahymena thermophila*, the authors confirmed that (S)-fluoxetine was found to be more toxic than its respective enantiomer [221], while (R)-fluoxetine was considered more harmful to *Pseudokirchneriella subcapitata.*

Accordingly, to establish action thresholds and accurately assess the environmental risks associated with psychoactive pharmaceutical residues, further ecotoxicological research is required. These studies should encompass a broad range of organisms, including primary producers like algae, and also consider the stereoselective toxicity of chiral compounds.

Chapter 2

Aims and Hypotheses

A review of the current literature emphasises the importance and usefulness of wastewater analysis in monitoring consumption trends of psychoactive substances such as drugs of abuse, illicit drugs, and prescribed pharmaceuticals from a public health perspective. However, despite the global increase in psychological disorders exaggerated by lifestyle-related stressors and, more recently, the COVID-19 pandemic, the use of WBE to monitor consumption trends in psychoactive pharmaceuticals is still developing. Consequently, there remain shortcomings in applied methodologies (e.g., a broader range of psychoactive pharmaceuticals, the stability of antipsychotics, and suitable sampling strategies) and a lack of WBE studies in many countries, including Slovenia. Moreover, a lack of data on their removal in WWTPs and their occurrence in environmental waters raises concerns about their environmental impact.

This thesis aimed to address these important gaps by estimating trends in psychoactive pharmaceutical consumption among the general Slovenian population and evaluating the potential risks they may pose to aquatic organisms. Particularly, it aimed to:

i. Determine the presence of a broad range of representative antidepressant, antipsychotic, and benzodiazepine biomarkers in different aquatic matrices (influent and effluent wastewater, receiving surface water). The goal is to develop and validate analytical methods for the trace analysis of selected biomarkers using sample preparation (SPE and 96-well SPE) and LC-MS/MS.
ii. Evaluate the stability of antipsychotic biomarkers in influent wastewater under various experimental conditions (e.g., storage and sampling).
iii. Investigate spatiotemporal trends in the use of psychoactive pharmaceuticals in general populations in Slovenia.
iv. Explore the presence of NPS in Slovenia using targeted (LC-MS/MS) and suspect screening (LC-QTOF-MS).
v. Evaluate the influence of the pandemic on the use of psychoactive pharmaceuticals and NPS in Slovenia.
vi. Assess the removal efficiency of psychoactive pharmaceutical residues by WWTPs (different sizes and configurations) and evaluate the occurrence of these residues (n=42) in waste- and receiving surface waters.
vii. Investigate active-passive sampling strategies for wastewater and compare them with conventional 24-hour composite sampling.
viii. Estimate the acute and chronic toxicity of psychoactive pharmaceutical residues to aquatic organisms (using the ECOSAR predictive model).

More specifically, this work set out to test the following five hypotheses (H):

H1: Measured levels of psychoactive pharmaceutical biomarkers in influent wastewater accurately reflect available prescription data on their consumption trends within Slovenian communities.

H2: WBE serves as a complementary data source to evaluate the impact of the COVID-19 pandemic on the use of psychoactive pharmaceuticals and NPS in Slovenia.

H3: Conventional biological wastewater treatment technologies are not able to entirely remove psychoactive pharmaceutical residues to <LOQ.

H4: APS provides a more convenient and efficient sampling strategy compared to conventional 24-hour composite sampling.

H5: Measured levels of psychoactive pharmaceutical residues in surface waters impacted by WWTP effluent do not pose a risk to aquatic organisms based on the ECOSAR predictive model.

Chapter 3

Publications

The work presented in this dissertation comprises nine scientific papers, eight of which are published in peer-reviewed journals, and another is in preparation for submission to Environmental Pollution (December 2025).

The publications are divided into four sections:

1. Analytical methods for the determination of psychoactive substances in influent wastewater

The publications (*3.1.1 Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology* and *3.1.2 Optimisation, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater) i*ncluded in the first section address **hypotheses H1, H2, and H4**. Analytical methods for the simultaneous determination of a broad range of psychoactive substances in influent wastewater within the scope of WBE are described. Additionally, this section provides an overview of novel advancements in sampling techniques, sample preparation and analysis, as well as the stability and consumption of psychoactive substances at high spatiotemporal resolution worldwide.

2. WBE as a complementary data source: Application to the Slovenian population

The work presented here (*3.2.1 Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters*, *3.2.2 Three years of wastewater surveillance for new psychoactive substances from 16 countries*, and *3.2.3 Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis*) addresses **hypotheses H1 and H2**. It explores the potential of WBE to estimate psychoactive substance use in the general population across different Slovenian municipalities (WWTP catchments). Data obtained on psychoactive pharmaceutical use are compared with the available prescription data **(H1)**. Additionally, consumption trends on psychoactive pharmaceuticals and NPS in Slovenia are compared with findings from other international epidemiological studies, while the impact of COVID-19 on their use is evaluated **(H2)**.

3. Reducing WBE uncertainties: Stability of psychoactive pharmaceuticals and NPS

Here, the publications (*3.3.1 Stability of antipsychotic biomarkers for wastewater-based epidemiology* and *3.3.2 Wastewater analysis as a global toxicovigilance tool for new psychoactive substances monitoring*) also address **H2** by further supporting WBE as a complementary data source to evaluate consumption trends of psychoactive pharmaceuticals and NPS. In WBE, accurate estimations depend on the stability of drug biomarkers in influent wastewater; however, data on the stability of certain psychoactive substances remain insufficient. Accordingly, the work performed (3.3.1 and 3.3.2) focuses on evaluating the stability of antipsychotic and NPS biomarkers under various conditions to improve our understanding of the degree of this uncertainty.

4. Psychoactive pharmaceutical residues in the aqueous environment: removal, occurrence, and ecotoxicity

This section (*3.4.1 Psychoactive pharmaceuticals: Consumption trends, removal during wastewater treatment and occurrence in receiving waters with associated environmental risks)* addresses hypotheses H2, H3 and H5. The first part of this publication focuses on evaluating temporal trends in the use of psychoactive pharmaceuticals in Slovenia, covering the periods before, during, and after the pandemic **(H2)**. The second part explores the environmental impact of psychoactive pharmaceutical usage. The efficiency of conventional biological wastewater treatment technologies in eliminating psychoactive pharmaceutical residues **(H3)** is discussed, along with their occurrence in receiving surface waters. Notably, this is the first study to report on the performance of the moving bed biofilm reactor (MBBR) for removing psychoactive pharmaceutical residues. Furthermore, potential ecological risks to aquatic organisms are assessed based on measured concentrations of psychoactive pharmaceutical residues in receiving surface waters and predicted toxicity thresholds using the ECOSAR software **(H5)**.

5. Novel sampling approaches for psychoactive substances in wastewater

This final part (*3.5 Novel Sampling Approaches for Psychoactive Substances in Wastewater*) addresses **hypothesis H4** and evaluates the performance of the APS device compared to conventional 24-hour composite sampling. The aim is to determine their relative efficiency in monitoring psychoactive substances in wastewater, providing valuable insights for improved environmental surveillance and wastewater-based epidemiological applications. To our knowledge, this is the first such deployment of an APS device for monitoring wastewater and is a part of a publication in preparation (Allen Jun Anies, Maria Laimou-Geraniou, Maarten Quireyns, Tim Boogaerts, Taja Verovšek, David Heath, Ester Heath, Alexander L. N. van Nuijs, Adrian Covaci, Ronny Blust, Raewyn Town, "In-situ evaluation of an active-passive sampling (APS) technique for monitoring psychoactive compounds in wastewater").

3.1 Analytical Methods for the Determination of Psychoactive Substances in Influent Wastewater

3.1.1 Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology

Published: M. Laimou-Geraniou, D. Heath, E. Heath, 2023, *Trends in Environmental Analytical Chemistry*, vol. 37, pp. e00192.

Regarding the publication, I was solely responsible for the conceptualisation, methodology, investigation, visualisation, and drafting of the manuscript.

The wastewater-based epidemiology (WBE) approach is a powerful and versatile tool for estimating illicit drug use and has been used to evaluate lifestyle-related substances such as alcohol, tobacco, and caffeine; health-related biomarkers including pharmaceuticals, plasticisers, and flame retardants; and, more recently, to track SARS-CoV-2 prevalence at the population level (Chapter 1.3 Wastewater-Based Epidemiology). Accordingly, several reviews have been published summarising established WBE biomarkers for licit and illicit drugs, pharmaceuticals, personal care products, and other xenobiotics, discussing analytical approaches, spatiotemporal trends, and highlighting the risks to society [43], [48], [102], [106], [222]. However, a gap remains in the literature regarding a comprehensive review of sampling and sample preparation methods, analytical methodologies, and the stability of biomarkers, with a focus on psychoactive pharmaceuticals such as antidepressants, antipsychotics, and benzodiazepines (Chapter 1.3 Wastewater-Based Epidemiology).

This comprehensive review focuses on sample collection, sample preparation, and analytical methodologies developed to determine antidepressants, antipsychotics, benzodiazepines, and their metabolites in influent wastewater, in the scope of WBE. It also provides information on recent advancements in sampling techniques (e.g., active-passive sampling), as well as on the stability and consumption of psychoactive pharmaceuticals at high spatiotemporal resolution worldwide. Additionally, limitations, uncertainties, challenges and future recommendations are also presented. Seventy-four peer-reviewed WBE studies monitoring the consumption of psychoactive pharmaceuticals published since 2010 are included.

Notably, SPE for sample preparation and target analysis by either LC-QqQ-MS or QTrap-MS was the most commonly used technique in WBE due to its high sensitivity and reliability for detecting low concentrations of pharmaceuticals and their metabolites. HRMS has also gained popularity in wastewater analysis, particularly for suspect- and non-targeted screening of NPS, enabling early detection of emerging compounds in the community. When applied globally, WBE revealed widespread and varied consumption of antidepressants, antipsychotics, and benzodiazepines across countries, regions, and timeframes.

The literature also shows how WBE complements traditional surveillance methods and can serve as a valuable early warning system for public health monitoring and tracking the emergence of new substances. However, the WBE is not without its limitations. These are primarily related to sampling strategies, excretion rates, compound stability (both in-sewer and in-sample), flow dynamics, and the accurate estimation of population catchment sizes (Chapter 1.3.2 Advantages and limitations). Given the many advantages of WBE, which provides continuous, near-real-time monitoring of psychoactive pharmaceutical

consumption trends at high spatiotemporal resolution, further studies on methodology and applicability are warranted.

Trends in Environmental Analytical Chemistry 37 (2023) e00192

Contents lists available at ScienceDirect

Trends in Environmental Analytical Chemistry

journal homepage: www.elsevier.com/locate/treac

Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology

Maria Laimou-Geraniou [a,b], David Heath [a], Ester Heath [a,b,*]

[a] Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia

ARTICLE INFO

Keywords:
Psychoactive pharmaceuticals
Influent
Wastewater
Sampling
Sample preparation
Extraction
Chromatography
Mass spectrometry
Stability
Spatiotemporal trends

ABSTRACT

The wastewater-based epidemiology approach, popular for estimating illicit drug use, has been used to evaluate lifestyle habits such as alcohol, tobacco and caffeine consumption, health biomarkers including pharmaceuticals, plasticizers and flame retardants, and recently to track SARS-CoV-2 at the population level. Equally, the number of WBE studies investigating psychoactive pharmaceuticals such as antidepressants, antipsychotics and benzodiazepines has also increased, which can be connected to the overall growth of psychological disorders worldwide. This review aims to discuss novelties in sampling techniques and analytical methodologies, including sample preparation and analysis, developed for estimating the consumption of psychoactive pharmaceuticals in defined populations. Seventy-four peer-reviewed studies monitoring psychoactive pharmaceutical consumption published since 2010 have been systematically reviewed. Its findings show that a broad range of bioanalytical methodologies is used to simultaneously measure several antidepressants, antipsychotics, benzodiazepines, and their metabolites from influent wastewater samples in low concentrations and different time periods. The application of WBE commenting on the temporal and spatial variations worldwide, showing widespread consumption, is also discussed. Despite much progress and excellent studies, there remains a need for research, and deeper knowledge is needed to reduce method uncertainty, especially since excretion rates, their transformation, and in-sewer and in-sample stability for many psychoactive pharmaceutical biomarkers are not available.

Abbreviations: 1-OH-MDZ, 1-hydroxymidazolam; 10-OH-CBZ, 10-hydroxycarbamazepine; 2-ACB, 2-amino-5-chlorobenzophenone; 2-OH-CBZ, 2-hydroxycarbamazepine; 3-OH-CBZ, 3-hydroxycarbamazepine; 4CDP, 4'-chlorodiazepam; 5-CMB, 5-chloro-2-(methylamino)benzophenone; 6-CPQ, 6-chloro-4-phenyl-2(1H)-quinazolinone; 7-ACL, 7-aminoclonazepam; 7-AF, 7-aminoflunitrazepam; 7-ANIZ, 7-aminonitrazepam; 8-OH-MIRT, 8-hydroxymirtazapine; ALP, alprazolam; AMIT, amitriptyline; AMS, Amisulpride; AMT, alpha-methyltryptamine; APS, active-passive sampling; APZ, Aripiprazole; BUP, bupropion; BUS, buspirone; BZP, bromazepam; CBH, cellobiohydrolase column; CBZ, carbamazepine; CBZ-E, carbamazepine-10,11-epoxide; CDD, chlorodesmethyldiazepam; CDP, chlordiazepoxide; CIT, citalopram; CLB, clobazam; CLOZ, clozapine; CMI, clomipramine; CPZ, chlorpromazine; CZP, clonazepam; DARI, dehydroaripiprazole; DDD, defined daily doses; DMI, desimipramine; DiOH-CBZ, 10,11-dihydro-10,11-dihydroxycarbamazepine; DMO, N-desmethylolanzapine; DOX, doxepin; DSP, dosulepin; DUR, Drug Utilization Research; DUX, duloxetine; DZP, diazepam; EMCDDA, European Monitoring Centre for Drugs and Drug Addiction; ESCIT, escitalopram; EST, estazolam; ETZ, etizolam; FA, formic acid; FLV, fluvoxamine; FNZ, flunitrazepam; FPX, flupentixol; FXT, fluoxetine; FZP, flurazepam; GC, gas chromatography; HAL, haloperidol; HLB, Hydrophilic-Lipophilic Balanced reversed-phase sorbent; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; IMP, Imipramine; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; LOR, lorazepam; LORM, lormetazepam; MAP, maprotiline; MCLZ, meclonazepam; mCPP, m-chlorophenylpiperazine; MCX, mixed-mode cation exchange sorbent; MDMA, 3,4-methylenedioxymethamphetamine; MDZ, midazolam; MEL, melitracen; MeOH, methanol; MES, Mesoridazine; MET, methylphenidate; MIAN, mianserin; MIRT, mirtazapine; MOC, moclobemide; MPA, mobile device-based population activity; MPT, Microporous Polyethylene Tube; MS/MS, tandem mass spectrometry; ND, not detected; nor-CIT, norcitalopram; nor-CLO, norclozapine; nor-CMI, norclomipramine; nor-DZP, nordiazepam; nor-FXT, norfluoxetine; nor-MIAN, normianserin; nor-MIRT, nor-Mirtazapine; nor-MAP, normaprotiline; nor-QTP, norquetiapine; nor-SER, norsertraline; NORT, nortriptyline; NTZ, nitrazepam; ODVEN, O-desmethylvenlafaxine; OH-BUP, hydroxybupropion; OH-BZP, hydroxybromazepam; OLZ, olanzapine; OXA, oxazepam; PAL, paliperidone; PAR, paroxetine; PEM, pemoline; PLZ, phenelzine; POCIS, polar organic integrative samplers; PYR, pyrazolam; PZP, prazepam; QqQ, quantitative triple quadrupole; QTP, quetiapine; RIS, risperidone; SCORE, Sewage analysis CORe group Europe; SER, sertraline; SPE, solid-phase extraction; SUL, sulpiride; TEM, temazepam; TZD, trazodone; TRMP, trimipramine; UPLC, ultra-performance liquid chromatography; VEN, venlafaxine; WBE, wastewater-based epidemiology; WRRF, water resource and recovery facility; ZOL, zolpidem; ZPC, zopiclone; ZPCA, zolpidem phenyl-4-carboxylic acid; α-OH-ALP, alpha -hydroxy-alprazolam.

* Correspondence to: Jamova 39, 1000 Ljubljana, Slovenia.
E-mail address: ester.heath@ijs.si (E. Heath).

https://doi.org/10.1016/j.teac.2022.e00192
Received 7 September 2022; Received in revised form 14 December 2022; Accepted 19 December 2022
Available online 23 December 2022
2214-1588/© 2022 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Increasing mental disorders, including depression, anxiety, bipolar disorder, schizophrenia, and other psychoses, create a significant social, health, and economic burden [1,2]. According to the World Health Organization (WHO), an estimated 280 million patients suffer from depression worldwide [2]. Since 2010, the growth in antidepressant usage globally has been steadily increasing (Fig. 1), leading to further research on their consumption and use patterns [2,3]. This growth is also reflected in the global market for antidepressants, i.e., in 2019, the market was estimated to be worth USD 11.67 billion, rising to 15.84 billion in 2021, and is projected to grow to 21.28 billion by 2025 [4]. In the case of psychoactive pharmaceuticals, antipsychotics are mainly used for treating schizophrenia, the second most common mental disease affecting more than 21 million people worldwide. Similarly, the global antipsychotic drug market, valued at USD 12.42 billion in 2020, increased to USD 14.54 billion in 2021 and is estimated to reach USD 26.04 billion by 2030. The global psychoactive drug market was valued at a staggering USD 3.8 billion in 2020 [5].

Benzodiazepines are another important class of psychoactive pharmaceuticals. They are among the most widely prescribed medications to treat various conditions such as anxiety, insomnia, epilepsy, and muscle spasms and manage withdrawal symptoms from alcohol. In most countries, benzodiazepines are considered prescription-only medications with restrictions on their supply, use and possession under drug control laws [6]. However, increased usage of benzodiazepines has been observed among prisoners, high-risk drug users and some groups of recreational drug users due to them being readily available and low cost and among the general population due to pandemic-related mental health issues [7]. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the misuse of benzodiazepines, diverted either from therapeutic medical or illegal use in Europe, is also becoming an issue [8]. New benzodiazepines, which are not controlled under the international drug control system, but are monitored as new psychoactive substances (NPS) by the European Union Early Warning System, have been sold as 'legal' versions for authorised benzodiazepines on the drug market in Europe [7]. Currently, the EMCDDA is monitoring 30 new benzodiazepines through the EU Early Warning System, 21 of which were first detected in Europe in 2015. Some of these are sold as fake versions of commonly prescribed medication such as alprazolam (Xanax) and diazepam, making use of existing distribution networks in the illicit drug market, while others are sold online, sometimes under their own brand names.

The ability to accurately monitor psychoactive pharmaceutical use is of high importance. Typically, such data have been painstakingly collected using conventional socio-epidemiological methods such as general population surveys, hospital records, and prescription and sales data within a population [9]. The limitation of these traditional estimation methods is that they can be biased, ethically questionable, have high uncertainty, be time-consuming, and suffer a significant time lag in the reported data [10,11]. For these reasons, wastewater-based epidemiology (WBE), also called sewage epidemiology, is becoming a popular alternative to epidemiological surveys since it offers objective and anonymous information on licit and illicit drug use or abuse in defined populations by analysing human metabolic excretion products (biomarkers) in wastewater. Non-invasiveness, cost-effectiveness, the capability to assess trends in pharmaceutical use over time and the ability to gather data in near real-time are just a few of the advantages of WBE [1]. WBE has also turned into a vital tool for monitoring spatio-temporal consumption patterns and trends both within and between countries and time points, in illicit drugs and psychoactive pharmaceuticals use that allow the tracking of human habits and lifestyle and the associated outcomes on health, education and crime [12–14]. Since its first application in 2005, investigating the consumption of cocaine in Italy [15], WBE has been subjected to constant improvement and development [16], [17]. At present, apart from a large number of studies for illicit drugs, the application of WBE extends to monitoring the consumption of tobacco [18–22], caffeine [23], alcohol [19,22,24], opioids [22,25], artificial sweeteners [26,27], pharmaceuticals [28–33], new psychoactive substances [34–38], personal care products [29,39,40] and lately to track the spread of the Sars-CoV-2 in populations [41,42].

To date, a large number of studies have been published related to sample collection, extraction, stability, validation and back-calculating antidepressant, antipsychotic and benzodiazepine consumption by wastewater analysis, taking into account the flow rate and the population units, mainly in Europe [43–51], USA [52–54], Australia [29,55] and China [32,56,57], but also in Mexico [58] South Korea [59], South Africa [60], Maldives [61] India [62,63] and Brazil [64]. However,

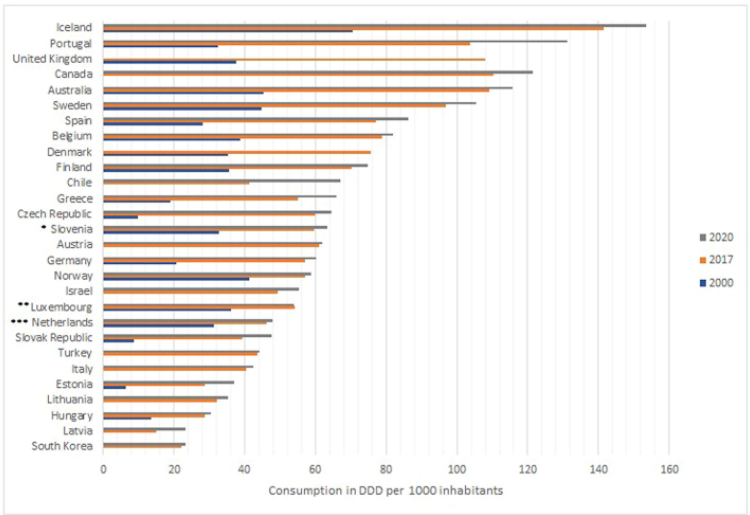


Fig. 1. Consumption of antidepressants in defined daily doses (DDD) per 1000 inhabitants, 2000, 2007 and 2020. *Data for 2006 (not 2000). * *Data for 2003 (not 2000). * ** Data for 2001 (not 2000).
Source: OECD Health Statistics 2019 and 2021.

several studies have also highlighted the methodological uncertainties associated with WBE [16,65–67]. These uncertainties include the stability of biomarkers in the sewage system and during storage, sampling, sample pre-treatment, extraction, and quantification. Furthermore, many uncertainties arise when back-calculating psychoactive pharmaceutical consumption. These uncertainties include, for example, the specific characteristics of Water Resource and Recovery Facilities (WRRFs, formerly WasteWater Treatment Plants, WWTPs), the population size of the contributing wastewater catchment, wastewater flow, time a biomarker takes to arrive at the WRRF and associated stability, biomarker pharmacokinetics, excretion rate, and dose estimation [16, 52,68].

Many excellent reviews on WBE can be found in the recent literature. For example, Choi et al. [69] summarized the established wastewater-based epidemiology (WBE) biomarkers for licit and illicit drugs, pharmaceuticals and personal care products, industrial chemicals, and biochemical markers of stress, food and diet. Their paper also describes the current capabilities and challenges of WBE. Similarly, Picó and Barceló [70] classify the different systematic approaches used to identify and measure a broad range of biomarkers in wastewater while discussing analytical trends in WBE. A comprehensive review of analytical approaches and global trends that only focused on detecting NPS in wastewater was also recently published by Gent and Paul [34]. Alternatively, Boogaerts et al. [71] focused on current WBE applications relating to pharmaceutical consumption, spatiotemporal trends and the relationship between pharmaceutical use and socio-demographic parameters of the specific catchment. They also show how triangulating WBE data with other drug utilization research (DUR) can help understand better how WBE can further contribute to DUR. In the same year, Melchor-Martínez et al. [72] also published a review paper on common antidepressants in wastewater and sludge, highlighting the risks to society, the environment and their increasing use during the SARS-CoV-2 pandemic. Despite such excellent reviews, there remains a gap in the literature regarding sampling methods, sample pre-treatment, analytical methodologies, and stability of biomarkers focusing only on psychoactive pharmaceuticals such as antidepressants, antipsychotics and benzodiazepines.

This review paper focuses on sample collection, sample preparation, extraction and quantification methods in wastewater influent specific to antidepressants, antipsychotics, benzodiazepines, and their metabolites in the scope of WBE. It also provides information on novel advancements in sampling techniques, stability and consumption of psychoactive pharmaceuticals in high spatiotemporal resolution worldwide. In total, 74 WBE publications were selected. The referenced papers were found by searching Science Direct, SCOPUS, Springer and Google Scholar for the following keywords: wastewater, influent, wastewater analysis, wastewater-based epidemiology, pharmaceuticals, psychoactive pharmaceuticals, prescribed pharmaceuticals, antidepressants, antipsychotics and benzodiazepines. Only peer-reviewed studies published since 2010 were included. Table 1 shows the different keyword combinations applied to find eligible studies for this review. We included WBE studies that estimated population-normalised mass loads (mg/day/1000 inhabitants) of excreted human metabolic biomarkers of antidepressants, antipsychotics and benzodiazepines. Wastewater studies that focused on special events, evaluation of removal efficiencies during wastewater treatment and investigation of their ecotoxicity in aquatic environments were excluded.

2. Determination of psychoactive pharmaceuticals in wastewater

Sample collection, pre-treatment, preparation, and instrumental analysis are the first steps in the WBE approach. The following sections discuss the state-of-the-art in each of the steps involved in analysing antidepressants, antipsychotics, benzodiazepines, and their metabolites in influent wastewater.

Table 1
Applied search combinations during the advanced literature search.

Search terms	Hits
(wastewater OR "wastewater-based epidemiology") AND ("psychoactive pharmaceuticals")	7274
(wastewater OR "wastewater-based epidemiology") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)	608
(wastewater OR "wastewater-based epidemiology") AND ("psychoactive pharmaceuticals") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)	374
((wastewater OR "wastewater-based epidemiology") AND ("psychoactive pharmaceuticals") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)) AND (influent)	368
((wastewater OR "wastewater-based epidemiology") AND ("psychoactive pharmaceuticals") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)) AND (influent) AND (consumption)	355
(((wastewater OR "wastewater-based epidemiology") AND ("psychoactive pharmaceuticals") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)) AND (influent)) NOT (effluent) NOT (removal)	238
("wastewater-based epidemiology") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)	40
((("wastewater-based epidemiology") AND (antidepressants) AND (antipsychotics) AND (benzodiazepines)) AND ("influent wastewater")) AND (consumption)	25

2.1. Sampling

Practitioners of the WBE approach strongly recommend that sampling is performed during dry periods since rain dilutes wastewater and makes detecting biomarkers in wastewater more difficult. In rare cases of wastewater overflow, it may also lead to incorrect average consumption estimations if part of the wastewater is discharged untreated and not included in the flow measurement. In general, sampling should also be avoided during special events at WRRFs, such as scheduled maintenance or, for example, public holidays and festivals that may affect the results, unless this is the subject of research, like determining new psychoactive substance use over the new year period [73]. During special events, population size (people migration) and pharmaceutical consumption may differ from the average, leading to incorrect consumption estimations in the catchment population [74]. Developing a sampling strategy can be influenced by several factors, including the characteristics of the WRRF catchment area or less explored sub-catchments such as wastewater from hospitals [40,43,75], aged care facilities [76], prisons [14,77,78] educational institutions [14,22,79], football games [80] and festivals [14], [81]. Here it is important to clarify that when monitoring the previously mentioned sub-catchments, other sampling points have been used than the usually sampled influent wastewater at the WRRFs. In the case of site-specific studies, including educational institutions, and prisons, raw wastewater samples are typically collected directly from the sewage outlet before reaching the main municipal sewage network or at the inlet of a wastewater plant installed at the studied site (e.g. hospitals) [14,43]. In addition, sampling during special events (e.g. festivals) has been chiefly performed at municipal WRRFs [14]. If the festival area is not connected to any WRRF or portable toilets are used, the sewage is transported to the corresponding WRRFs via local sewers. The approach regarding sampling frequency can differ based on the number of possible users per population, pharmacokinetics (metabolic/ transformation pathways) and characteristics of the sewage system (matrix complexity) [16,34].

Different sampling techniques can be used for sampling wastewater. Grab sampling of wastewater [58,62,82–84] is not the most suitable sampling method since it represents only the wastewater conditions at a specific point in time without capturing the short or long-term variation of concentration, providing a potential false concentration profile in the studied system [85]. In WBE, it is crucial to create composite samples to ensure the collection of representative samples. Typically, 24-hour composite samples for multiple days (usually seven consecutive days) are collected to account for variations between weekdays and weekends

(Table 2). However, in some cases, composite samples of 72-hour [86] and 12-hour, from 10 am to 10 pm [61], have been reported. Composite samples are commonly collected with an autosampler, working in one of three modes: (1) volume-proportional mode, i.e., taking samples of equal volume at varying time intervals, proportional to wastewater flow); (2) time-proportional mode, i.e., taking equal sampling volumes at constant time intervals or (3) flow-proportional, i.e., varying sampling volumes, proportional to the wastewater flow in constant time intervals [16]. Auto-samplers allow researchers to collect as much sample (and consequently data) as possible within a predefined time frame and volume [34]. Although flow-proportional sampling seems to be more suitable for smaller populations [16], it remains, that is according to Ort et al. [87], the best sampling practice for WBE estimations generally, which agrees with the number of publications that use this sampling technique [32,37,38,48,57,79,83,86,88–93]. Volume-proportional mode is acceptable for short sampling intervals, while time-proportional sampling can be used for slight variations in flow or pollutant concentrations. For operational reasons, time-proportional sampling is also widely applied at different time intervals: 10 min [39,40,45,67,94], 15 min [44,52,54,95,96], 20 min [31], 30 min [46,47,58], 60 min [53,56,59], 2 h [64,75] or 6 h [43].

An alternative approach is to use passive samplers, e.g., polar organic integrative samplers (POCIS), that can overcome the limitations of active samplers, like capturing low contaminant levels, potential changes in flow, and power requirements [11,53]. Passive samplers, consisting of polymeric-based sorbent material, are commonly deployed for extended periods and can adsorb trace analytes on the sorbent during the sampling period [97]. Criquet et al. [98], who studied pesticides and pharmaceuticals, including carbamazepine, in effluent wastewater, suggested that POCIS have certain advantages over grab sampling due to its ability to obtain time-integrated results. Regarding the analysis of psychoactive pharmaceuticals and illicit drugs, a comparison between POCIS and 24 h auto-sampling has been reported, showing similar concentrations of the collected influent wastewater samples [46,53]. However, concentrations obtained by POCIS were lower in the case of more complex and untreated wastewater [99]. Baz-Lomba et al. [46] and Bishop et al. [53] applied POCIS when investigating antidepressants, benzodiazepines and illicit drugs in sewage alongside 24 h composite auto-sampling. In both cases, their data were in better agreement with concentrations that may have been expected between both sampling methods. Although, for some compounds, including temazepam, Bishop et al. [53] reported that POCIS underestimated the influent concentrations (3 times lower) compared to the median 24 h composite values. However, the differences were lower when considering the 24 h composite values range [53]. Another limitation of POCIS is the potential occlusion of the membrane due to solid particles on the sampler surface during the deployment period, which could significantly affect the results [53].

Using a similar approach, McKay et al. [29] developed, calibrated, and validated a Microporous Polyethylene Tube (MPT) passive sampler with a multi-mode sorbent (Strata-X in agarose) as a tool for detecting and quantifying licit and illicit drugs, including carbamazepine and temazepam in WRRF influent. For carbamazepine and temazepam, along with methamphetamine, MDMA, nicotine and morphine, the authors found that the uptake was linear over a 29-day calibration period [29]. In another variation, Liu et al. [108] evaluated the use of diffusive gradients in thin-film (o-DGT) passive samplers, using three different types of resin gels (HLB, XAD 18, and XDA-1) to sample pharmaceuticals and illicit drugs for WBE. From the WBE perspective, passive samplers have also been used for the long-term monitoring of community-level licit and illicit drug consumption [11,29,53]. Nevertheless, for a better understanding of the human drug abuse behaviour between weekends and daily use, a 24 h composite sampling is more practical since it can show the fluctuations in consumption on a specific day [53]. Passive sampling devices can be advantageous in wastewater analysis when auto-sampling is not used, as they provide a more straightforward sampling process, in-situ enrichment of chemicals, increased sensitivity and a time-integrated sample [34,109]. At present, this sampling mode is still under development, and the influence of environmental factors (flow rate, temperature, pH, biofouling) on the sampling rate and the mechanisms controlling uptake (sorption and desorption of chemicals due to flow) are still to be explored for psychoactive pharmaceuticals [11].

More recently, an alternative, novel in-situ sampling technique that combines active and passive sampling (APS) has been reported [110,111]. For instance, Amato et al. [110–112] developed such a device to simultaneously measure a wide range of contaminants (metals, polar and non-polar compounds). This device uses a diffusion cell (loaded with three different sorbents: Chelex, HLB and silicone rubber) supplied with an exposure medium using a pump. At the same time, a flow meter establishes accurate measurements of flow rates. Fig. 2 illustrates the schematic and cross-section of the APS diffusion cell comprised of two blocks separated by a 0.50 cm thick spacer. In such a device, a controlled laminar flow is created within the cell, allowing the obtained measurements to be independent of the hydrodynamic conditions in the bulk exposure medium and, in doing so, overcomes one of the limitations of passive samplers (e.g. POCIS) [111]. In the first field application conducted in the Port of Zeebrugge, Belgium, Amato et al. [112] showed that the measurements of specific polar organic compounds, namely carbamazepine, using the APS were in good agreement (difference <15%) with conventional passive sampling techniques such as organic diffusive gradients in thin films (o-DGT), but were both consistently higher (~30 and ~50% for APS and o-DGT, respectively) than those obtained using grab samples. The APS device was also capable of establishing the concentrations of target biomarkers of contaminants in seawater with a broad range of different physico-chemical properties, thereby making it a promising technique for monitoring wastewater influent in WBE applications [112].

2.1.1. *Sample storage*

Standard procedures for transporting and storing wastewater samples depend on the matrix (e.g. wastewater is a complex matrix influenced by temperature and source), target compound stability and preservation requirements. Since the stability of many psychoactive pharmaceuticals in wastewater is largely unknown, especially for antipsychotics, the choice of temperature during transportation and storage may influence their stability and affect the beginning of sample preparation, e.g., by adding acidification and adding preservative agents [34]. In the case of low stability, the samples must be preserved. Transporting samples usually involves careful packaging and transport on ice, followed by either immediate analysis [96] or chilled storage until analysis. All the details on sample storage conditions for determining psychoactive drugs are summarised in Table 2 and include refrigeration in the dark at 4 °C when processed within six to 72 h [38,43,52,53,57,64,83,84,94], or more commonly at − 20 °C [1,29,31,37,39,40,44,45,47,51,54,58,61,63,67,79,83,86,88–91,93,101–105] or even − 80 °C [30], if the samples are to be kept for longer periods (e.g. from two weeks up to three months). Hydrochloric acid or sulphuric acid [32,56,59,82,106] are also used to adjust sample pH (pH 2–3) to prevent bacterial activity and maintain analyte stability. The latter is compound dependent and can, in some cases, lower the stability of certain benzodiazepines, including clonazepam, flubromazepam nitrazepam [55] and the metabolites 7-aminoclonazepam and 7-aminoflunitrazepam [113]. Similarly, sodium metabisulphite has been used as a preservative agent when analysing benzodiazepines [55] and traditional illicit drugs [34,65,114]. Rice et al. [95] took a slightly different approach. The samples were extracted within 24 h of sampling and stored on the SPE cartridges in the freezer until the final elution step was performed. No stability issues were reported. In the case of loaded POCIS samplers [11,46,53] and MPTs [29], these were stored at − 20 °C.

Table 2
Literature review on sample type, storage, sampling period, sampling location and population equivalent.

Sample	Sample type	Additional sampling information	Sample storage	Sampling period	Sampling location	Population	Ref.
Wastewater (influent)	24-hour composite sample (weekdays) 72-hour composite sample (weekend, Friday-Monday)	Autosampler (flow-proportional A total of 30 samples)	-20 °C	10th June-21th July (2016)	1 WRRF, Vestfjorden Avløpselskap (VEAS), Oslo, Norway	VEAS 600,000	[86]
Wastewater (influent)	24-hour composite sample	Autosampler (100 mL every 60 min)	-20 °C, pH 2 adjustment with 2 M HCl (processed within 2 weeks)	19–22 April 2018	3 WRRFs, Busan, South Korea	2235,245	[59]
Wastewater (influent)	24-hour composite sample	Autosampler (every 15 min)	4 °C (processed within 6 h)	7 consecutive days in late summer 2018	2 WRRFs, eastern Kentucky, USA	$WRRF_A$ (industrial, metropolitan areas) ~190,000 $WRRF_B$ (suburban) ~150,000	[52]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	4 °C pH 2–3 adjustment with 4 M H_2SO_4 (processed within 24 h)	WRRFc: once a month for six consecutive months from November 2018 to April 2019	11 WRRFs Guangzhou, Huizhou and Dongguan of Guangdong province, China	LB: 300,000 TS-1: 428,000 DTS-2: 570,000 HY: 425,000 MH: 380,000 $WRRF_A$: 410,000 LJ:540,000 JX:130,000 HJ:130,000 $WRRF_B$: 200,000 $WRRF_C$:390,000	[32]
Wastewater (influent)	24-hour composite sample	Autosampler (100 mL every 60 min)	4 °C (processed within 24–48 h)	April, May, June 2019	2 WRRFs, Western USA	$WRRF_A$: < 45,000 $WRRF_B$: < 4000	[53]
	Sample gathered by passive sampling (POCIS)	30 days of exposure	POCIS stored at − 20 °C until extraction				
Wastewater (influent)	24-hour composite sample	Autosampler (10 mL sample every 15 min)	extracted within 24 h and stored on SPE (freezer)	2014–2018	1 WRRF, South-West UK	886,650	[95]
Wastewater (influent)	24-hour composite sample	Autosampler (volume- or time-proportional)	-80 °C	March, April, May 2017	6 WRRFs, Italy	Milan: 1080,000 Bologna: 530,000 Palermo: 360,000 Bari: 459,650 Gorizia: 34,844 Potenza: 90,000	[30]
Wastewater (influent)	24-hour composite sample	Autosampler (time or flow-proportional)	-20 °C	October 2017	4 WRRFs, Belgium	Ostend: 160,000 Boom: 30,000 Brussels: 954,200 Turnhout: 43,200	[1]
Wastewater (influent)	24-hour composite sample	Autosampler (every 15 min)	-20 °C	2017	1 WRRF, Kentucky, USA	~20,000	[54]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	-20 °C	2012 and 2014	1 WRRF, Amsterdam, Netherlands	769,000	[37]
Wastewater (influent)	24-hour composite sample	Grab sample	-20 °C	17,23,31 January and 6th of February 2017	1 WRRF, South East Queensland, Australia	~200,000	[29]
	Analytes gathered by passive sampling (MPTs)	30 days of exposure	MPTs stored at − 20 °C until extraction	12th of January-10th of February (2017)			
Wastewater (influent)	24-hour composite sample	Autosampler (100 mL every 60 min)	-20 °C, pH 2	August-September 2014, May-September 2015	36 WRRFs, (STPs) 18 major cities, China	Total 26.29 million	[56]
Wastewater (influent)	24-hour composite sample	Autosampler (time or flow-proportional)	-20 °C, $Na_2S_2O_5$ (0.5 g/L)	27 11 – 3.12 2019 and 25.12.2019–3.01.2020	4WRRFs, South Australia	$WRRF_A$: 728,759 $WRRF_B$: 75,225	[55], [100]

(continued on next page)

Table 2 (*continued*)

Sample	Sample type	Additional sampling information	Sample storage	Sampling period	Sampling location	Population	Ref.
						$WRRF_C$: 212,309 $WRRF_D$: 155,604	
Wastewater (influent	24-hour composite sample	Autosampler (flow-proportional)	4 °C	April-May 2010	6 WRRFs, Germany	NR	[83]
	Grab sample	-	-20 °C	April-May 2010	2 WRRFs, Aveiro, Portugal	NR	[83]
Wastewater (influent)	24-hour composite sample	Autosampler (every 30 min)	-20 °C	March 2011	1WRRF, London, UK	3400,000	[47]
Wastewater (influent)	Grab sample	-	4 °C (processed within 18 h)	13th–19th April 2011	1 WRRF, Czech Republic	> 300,000	[84]
Wastewater (influent)	24-hour composite sample	Autosampler (50 mL every 30 min)	-20 °C	Oslo: February 2014 Trondheim: Spring 2014	2 WRRFs Oslo and Trondheim Norway	WRRF, Oslo: 600,000 WRRF, Trondheim: 180,000	[46]
	Sample gathered by passive sampling (POCIS)	2 weeks of exposure	POCIS stored at − 20 °C until extraction	Oslo: February 2014	1 WRRF Oslo, Norway	WRRF, Oslo: 600,000	
Wastewater (influent)	Sample gathered by passive sampling (POCIS)	2 weeks of exposure	POCIS stored at − 20 °C until extraction	29.12.2011 – 03.01.2014	1 WRRF, Oslo, Norway	WRRF, Oslo: 600,000	[11]
Wastewater (influent)	24-hour composite sample	Autosampler (10 mL sample every 15 min)	4 °C (processed immediately)	NR	1 WRRF, UK	NR	[96]
Wastewater (influent)	24-hour composite sample	Autosampler (flow- or time-proportional)	-20 °C	August 2016	22 WRRFs, 6 states and territories, Australia	1000–2.5 million	[101]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	4 °C	one week of March in years 2015–2018	1 WRRF Psyttalia, Athens, Greece	~3700,000	[38]
Wastewater (influent)	24-hour composite sample	Autosampler (300 mL every 20 min)	-20 °C	16–20 October 2019	2 WRRFs, Barcelona, Girona, Spain	BCN: 1619,602 GRN: 151,076	[31]
Wastewater (influent)	24-hour composite sample	Autosampler (time-proportional)	-20 °C	4 sampling periods: spring, summer and autumn 2013, winter 2014	15 WRRFs, 5 regions (North, Centre, Lisbon and Tagus Valley, Alentejo and Algarve), Portugal	From 6850 to 756,000	[102], [103]
Wastewater (influent, hospital effluent)	24-hour composite sample	Autosampler (time-proportional, 2 h intervals)	4 °C (analysed within 7 days)	July-November 2013	4 WRRFs 6 hospitals New York, USA	NR	[75]
Wastewater (influent)	24-hour composite sample	Autosampler (time-proportional, 15 min intervals)	-20 °C	January- December 2014	8 WRRFs, 7 major cities (Bratislava-Central and Bratislava-Petržalka, Košice, Prešov, Banská Bystrica, Zvolen, Trenčín, and Piešťany), Slovakia	Bratislava: 350,000 Petržalka: 110,000 Košice: 175,000 Prešov: 90,000 B. Bystrica: 75,000 Zvolen: 42,000 Trenčín: 47,000 Piešťany: 34,000	[44]
Wastewater (influent)	Grab sample	-	4 °C (processed within 48 h), pH 2 adjustment with H_2SO_4 (95–97%)	NR	Ponte da Moreira's WRRF, Maia region, Porto, Portugal	25,000	[82]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	-20 °C	One-week period per month from August to December 2017	University campus, South-western USA	15,000–60,000	[79]
Wastewater (influent)	24-hour composite sample	Autosampler (time-proportional, every 10 min)	4 °C (processed within 12 h)	5 consecutive days in July 2013	1WRRF, Sant Pere Pescador, north-east coastal region of Catalonia, Spain	Estimated population in the sampling period: 7500	[94]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional	-20 °C	7 consecutive days, Wednesday to Tuesday between June and October 2015	5 WRRFs, South-West UK	A: 37,714 B: 68,453 C: 109,543 D: 18,274 E: 867,244	[88]
Wastewater (influent)	24-hour composite sample	Autosampler (every 6 h)	4 °C (processed within 48 h)	7 consecutive days, two sampling periods; October 2016 and December 2016	1 municipal WRRF, Ioannina city, Greece	100,000	[43]

(*continued on next page*)

Table 2 (*continued*)

Sample	Sample type	Additional sampling information	Sample storage	Sampling period	Sampling location	Population	Ref.
Wastewater (influent)	24-hour composite sample	Autosampler (50 mL every 10 min)	-20 °C (analysis within 2 months)	5 consecutive days during one week in mid-March from 2011 to 2015	1 municipal WRRF, Barcelona, Spain	1159,071–1149,722	[45]
Wastewater (influent)	24-hour composite sample	Autosampler (100 mL every 10 min)	Processed immediately	7 consecutive days during February 2018/2019 and July 2018/2019	2 WRRFs, Western Cape Province, South Africa	WRRF1: 248,000 WRRF2: 1102,000	[60]
Wastewater (influent)	12-hour composite sample (10 am to 10 pm)	Autosampler (time proportional)	-20 °C (processed within 2 months)	9 consecutive days. (12.03.2015–20.03.2015)	9 pumping stations, Malé , Republic of Maldives	153,940	[61]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional	-20 °C	7 consecutive days during 13–19.03.2019 and 25.03–08.4.2020	1 WRRF, Athens, Greece	2019: 3995,020 2020: 4009,346	[89], [90]
Wastewater (influent)	24-hour composite sample	Autosampler	-20 °C (processed within 7 days)	September 2018, May and July 2019	2 WRRFs, Thessaloniki, Greece	WRRF-T: 1100,000 WRRF-A: 90,000	[104]
Wastewater (influent)	24-hour composite sample	Autosampler (every 30 min)	-20 °C	UK: 5 – 7 April 2019 USA: 9–15 September 2019	WRRF, London, UK WRRF, city in the south-west USA	UK: 3400,000 USA: 60,888	[58]
	Grab sample	-	NR	Mexico: 19–25 February 2019	WRRF, Monterrey, Mexico	Mexico: 1708,190	[58]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional	NR	January 2012	33 WRRFs across Sweden	>10,000 each	[48]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional	-20 °C (processed within 7 days)	9 sampling periods: October 2013-June 2014	2 WRRFs, Leiria, Portugal	WRRF 1: 21,726 WRRF 2: 110,131	[91]
Wastewater (influent)	24-hour composite sample	Autosampler (100 mL every 10 min)	-20 °C	4 sampling periods 2013–2014 (winter, spring, summer, autumn)	1 WRRF, Volos, Greece	~145,000	[39]
Wastewater (influent)	24-hour composite sample	Autosampler (100 mL every 10 min)	-20 °C	3 consecutive days, sampling campaigns from 2015 to 2017	WRRF-S, WRRF-A Thessaloniki, WRRF-L Larissa, WRRF-V, Volos, Greece	WRRF-S: 1333,000 WRRF-A: 50,264 WRRF-L: 284,325 WRRF-VI.: 45,000	[40]
Wastewater (influent, effluent)	24-hour composite sample	Autosampler (1 L every 2 h)	4 °C (processed within 27 h)	February to May 2019	5 WRRFs, Campinas (São Paulo State), Brazil	WRRF-A: ~203,000 WRRF-B: ~44,000 WRRF-C: ~55,000 WRRF-D: ~288,000 WRRF-E: ~182,000	[64]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	NR	Before lockdown, 7–22 consecutive days (2016–2020) During lockdown, daily 12.03–15.04/2020	Innsbruck, Austria	174,000	[92]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	-20 °C	February 2011 - June 2012	WRRF, Southeast Queensland, Australia	230,000	[93]
Wastewater (influent)	Grab sample	-	-20 °C	July and August 2012	5 STPs, Saidpur, Beur, Coimbatore, Udupi, Manipal, India	STP_{SP}: 350,000 STP_{BU}: 275,000 STP_{CO}: 350,000 STP_{UP}: 10,000 STP_{MP}: 12,000	[62]
Wastewater (influent)	24-hour composite sample	NR	-20 °C	7 consecutive days, 19–25 May 2013	2 STPs, Udupi, Mangalore, India	STP_L: ~150,000 STP_M: ~450,000	[63]
Wastewater (influent)	24-hour composite sample	NR	NR	7 consecutive days, 12–18 July 2013	2 WRRFs, Albany, New York, USA	WRRFA: ~15,000 WRRFB: ~100,000	[105]
Wastewater (influent)	24-hour composite sample	Autosampler (volume-proportional, 50 mL every 30 m^3)	NR	86 consecutive days 21 March-13 June 2016	1 WRRF, Region Centre-Val de Loire, France	70,000	[50]
Wastewater (influent)	24-hour composite sample	Autosampler (time-proportional)	-20 °C	17 April-1 May 2012	3 WRRFs, Valencia, Spain	Pinedo-I: 351,198 Pinedo-II: 942,774 Quart-Benager: 166,942	[51]
Wastewater (influent)	24-hour composite sample	Autosampler (volume-proportional)	-20 °C, pH 2.5 adjustment with 1 M HCl	5 sampling campaigns 5–11 December 2010 3–10 April 2011 2–9 April 2012 6–16 March 2013 11–18 March 2014	1 WRRF, Athens, Greece	~4562,500	[106]

(*continued on next page*)

Table 2 (*continued*)

Sample	Sample type	Additional sampling information	Sample storage	Sampling period	Sampling location	Population	Ref.
Wastewater (influent)	24-hour composite sample	Autosampler (time-proportional, every 10 min)	-20 °C	2007	5 WRRFs, Belgium	STP 1: 59,400 STP 2: 63,000 STP 3: 58,500 STP 4: 43,200 STP 5: 54,900	[67]
Wastewater (influent)	24-hour composite sample	Autosampler (flow-proportional)	4 °C (processed within 72 h)	2–3 sampling campaigns: September-October 2018 3 sampling campaigns: December 2018 -March 2019	6 WRRFs, Hong Kong, China	WRRF A: 685,500 WRRF B: 5700,000 WRRF C: 318,400 WRRF D: NA WRRF E: 27,000 WRRF F: 200,000	[57]
Wastewater (influent)	24-hour composite sample	Autosampler (time-proportional, every 15 min)	-20 °C	March to October 2013	6 WRRFs, Slovakia	Bratislava: 350,000 Petržalka: 110,000 Košice: 175,000 Zvolen: 42,000 Banská Bystrica: 75,000 Trenčín: 47,000 Prešov: 90,000 Piešťany: 34,000	[49]
Wastewater (influent)	24-hour composite sample	Autosampler	-20 °C	March 2014	1 WRRF, Thessaloniki, Greece	~908,593	[107]

NR: not reported

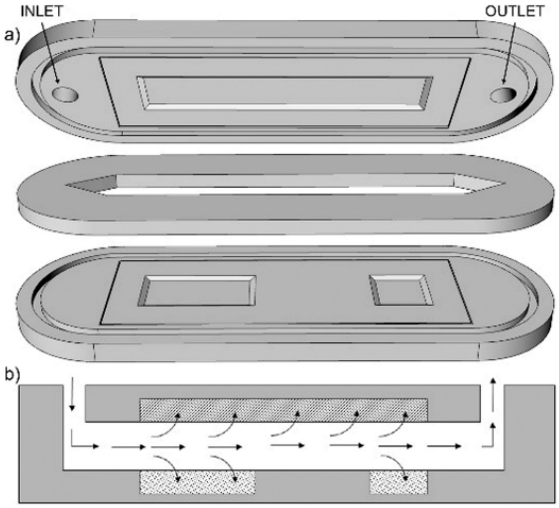


Fig. 2. a) Schematic and b) cross-section (not to scale) of the APS diffusion cell [110]. Top and bottom blocks can accommodate sorbents (topped with agarose gel) for sampling a diverse range of compounds. The sample is actively pumped through the device, as indicated by the arrows in (b). Water sealing is ensured by two o-rings placed in circular cavities machined in both top and bottom blocks.

2.2. Sample preparation

Pre-treatment protocols (Table 3) for determining organic contaminants, like pharmaceutical residues in wastewater, are similar to standard analytical protocols. Principally, filtration and extraction are used before injection into the appropriate analytical system. Acidification, before extraction, is an optional step that may be applied depending on the physico-chemical properties of the psychoactive pharmaceuticals (e.g., polarity and pKa) and the type of SPE cartridges used [34,115]. Matrix complexity and low concentrations of target analytes represent a significant analytical challenge. A pre-treatment step is essential since particulate matter and other compounds (interferences) may clog the adsorbent bed during the preparation step. In order to remove these solid particles from the matrix, samples are centrifuged [1,29,86], filtered [11,46,67], or centrifuged and filtered consecutively [52,54,105,107]. Vacuum filtration is commonly applied using mainly glass fiber filters. However, the use of nylon membrane [45,53,91,94] and polyvinylidene difluoride (PVDF) membrane filters [31,64] or, in some cases, cellulose ester filters [30,44,61] and PTFE filters [61] have been reported. Also, possible analyte loss due to filtration should be considered, especially when compounds are in low concentrations or are hydrophobic [33,34]. González-Mariño et al. [33] researched the sorption of nine benzodiazepines and 13 antidepressants on different filter types, including glass microfiber, mixed cellulose membranes, hydrophilic nylon membranes and hydrophilic PVDF membranes. Independently of the filter types applied, González-Mariño et al. [33] reported analyte losses below 30% in the case of all nine benzodiazepines. However, the majority of tested antidepressants gave different results. An ANOVA statistical test at the 95% confidence level showed that for the antidepressants: citalopram, N-desmethylcitalopram, fluoxetine, norfluoxetine, sertraline, mirtazapine, duloxetine, paroxetine and trazodone, the differences between mean losses observed with the four types of filters were statistically significant. Their study also showed that glass fibre and hydrophilic nylon filters adsorbed the least amount of analytes among the studied benzodiazepines and antidepressants, regarded as the best filtering materials. At the same time, the highest analyte losses were observed with cellulose membranes (80–95%), followed by PVDF filters (60–90%) [33]. However, the addition of internal standard (IS) prior to filtration allows to compensate for the losses during filtration, and also avoids a potential underestimation of the concentration detected in wastewater.

2.2.1. Sample extraction

Two classical approaches, direct injection (after filtration, centrifugation or any other mechanical or physical operation) and solid-phase extraction (SPE), are the most common. The chosen method depends on matrix complexity, biomarker concentration and its retention on the sorbent. For instance, antidepressants, antipsychotics, benzodiazepines and illicit drugs are mainly extracted using SPE. In contrast, biomarkers of licit drugs, including tobacco and alcohol (cotinine and ethyl sulphate), are commonly determined by direct injection because of their higher consumption and, accordingly, higher concentrations in

Table 3
Literature review on sample pre-treatment, sample preparation and analysis.

Analyte	Sample pre-treatment	Sample preparation	Analysis		LOQ (ng/L)	Year publ.	Ref
			Separation	**Detection**			
CIT, MET, CBZ	Centrifugation (16,200 g for 5 min)	SPE: HLB 96-well plate Sample volume: 1 mL Conditioning: 1 mL MeOH, 1 mL H_2O Washing: 1 mL H_2O Elution: 50 μL MeOH, 50 μL 1% NH_4OH in MeOH, 50 μL 1% FA in MeOH	UPLC: Acquity BEH C8 column (2.1 × 100 mm, 1.7 μm) Mobile phase: A: 0.1% NH4OH (aq.)B: ACN	QqQ MS; ionization mode: ESI(+); detection mode: MRM	CIT: 1.1 MET: 0.2 CBZ: 1.0	2017	[86]
ZOL [ZPCA]	Filtration (glass microfiber filter - 0,45 μm)	SPE: Oasis-HLB, pH 6 Sample volume 250 mL Conditioning: 3 mL MeOH, 3 mL Milli-Q Washing: 5% MeOH in H_2O Elution: 2 × 5% NH_4OH in MeOH	HPLC: ZORBAX Eclipse XDB-C18 column (4.6 ×150 mm, 3.5 μm) Mobile phase: A: ACN/H_2O (1:99, v/v) with 0.1% FAB: H_2O/ACN (5:95, v/v) with 0.1% FA	QqQ MS; ionization mode: ESI (+); detection mode: MRM	ZOL: 0.84 ZOL-COOH: 1.98	2020	[59]
CIT, FXT, SER, VEN, QTP, ALP, DZP, LOR, OXA, TEM	Centrifugation (4500 rpm for 5 min), Filtration (glass fiber filters - 1.2 μm)	SPE: Oasis-HLB Sample volume 100 mL Conditioning: 3 mL MeOH, 3 mL H_2O Elution: 4 mL MeOH Reconstitution: MeOH	UPLC: Force Biphenyl column (2.1 × 100 mm, 1.8 μm) Mobile phase: A: MeOH B: 0.1% FA in H_2O	QqQ MS; ionization mode: ESI (+); detection mode: SRM	NR	2020	[52]
DZP [nor-DZP], LOR, PZP, OXA, ALP, BZP, TEM, CDP, CZP, FZP, NTZ, FNZ, CLB, MDZ, CLOZ, EST, 6-CPQ, 2-ACB, 5-CMB	Filtration (glass fiber filters - 0.7 μm)	SPE: OASIS HLB Sample volume 1000 mL Conditioning: 10 mL MeOH, 10 mL Milli-Q Washing: 2 × 25 mL of 5% MeOH in H_2O Elution: 5 mL MeOH, 4 mL EtAc, 3 mL DCM Reconstitution: 1 mL MeOH	HPLC: Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) Mobile phase: A: citric acid buffer (5 mM CH_3COONH_3 + 0.05% HCOOH) B: ACN	QqQ MS; ionization mode: ESI(+); detection mode: MRM	2-ACB: 0.330 5-CMB: 0.038 6-CPQ: 0.121 ALP: 0.063 BZP: 0.290 CDP: 0.313 CLB: 0.270 CZP: 0.088 CLOZ: 0.053 DZP: 0.083 EST: 0.109 FNZ: 0.289 FZP: 0.104 LOR: 0.404 MDZ: 0.054 NTZ: 0.916 nor-DZP: 0.224 OXA: 0.487 PZP: 0.040 TEM: 0.763	2021	[32]
AMIT, NORT, FXT [nor-FXT] ALP [α-OH-ALP], CZP [7-ACL], DZP [nor-DZP], TEM, OXA, ZOL	Filtration (nylon membrane filters - 0.2 μm)	SPE: Oasis MCX (150 mg) Sample volume 100 mL Conditioning: 5 mL MeOH, 5 mL H_2O, 5 mL H_2O (pH 2) Washing: 5 mL MeOH/1% FA Elution: 5 mL MeOH/6% NH_4OH Reconstitution: 100 μL 95% H_2O/5% MeOH (0.1% FA)	HPLC: Kinetex Phenyl-Hexyl column (4.5 ×50 mm, 2.6 μm) Mobile phase: A: 0.1% NH_4HCO_2/H2OB: 0.1% FA in MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	10 ng/L	2020	[53]
AMIT, FXT [nor-FXT], NORT, VEN [ODVEN] DZP [nor-DZP], LOR, NTZ [7ANIZ], OXA, TEM, ZOL, ZPC	Filtration (glass microfiber filters - GF/F)	SPE, Oasis HLB Sample volume 50 mL Conditioning: 2 mL MeOH, 2 mL H_2O Washing: 3 mL H_2O Elution: 4 mL MeOH Reconstitution: 85:15% v/v 1 mM NH_4OAc(aq.)/MeOH	UPLC, ChiralPak CBH column (1.0 × 100 mm, 1.7 μm) Isocratic mobile phase: 1 mM NH_4OAc(aq.)/MeOH (85:15% v/v)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	NR	2020	[95]
CIT [nor-CIT]	Filtration (GF/A 1.6 μm, mixed cellulose ester filters ME 0.45 μm)	SPE, Oasis MCX Sample volume 25 mL Conditioning: 6 mL MeOH, 3 mL H_2O, 3 mL H_2O (pH 2.5) Elution: 2 mL MeOH, 2 mL of 2% NH_3/MeOH	HPLC, Kinetex C18 column (2.1 × 150 mm, 5 μm) Mobile phase: A: H_2O (0.1% FA)B: ACN	QqQ MS; ionization mode: ESI (+); detection mode: MRM	CIT: 5.17 ng/L Nor-CIT: 1.97 ng/L	2020	[30]
MOC, MIRT [nor-MIRT], BUP [OH-BUP], VEN [ODVEN], TZD [mCPP], MIAN [nor-MIAN], CIT [nor-CIT], DOX [nor-DOX], FLV, IMP [DMI], MAP [nor-MAP], AMIT [NORT],	Centrifugation (2465 g for 30 min)	SPE, Oasis HLB Sample volume 50 mLConditioning: 4 mL MeOH, 4 mL H_2OWashing: 5% v/v MeOH/ H_2OElution: MeOH (2% v/v FA) SPE, Oasis MCX Sample volume 50 mL Conditioning: 4 mL MeOH,	UPLC, Kinetex XB-C18 column (2.1 × 150 mm, 2.6 μm) Mobile phase: A: H_2O (0.1% v/v FA)B: 10/90 H_2O /ACN (0.1% FA)	QqQ MS; ionization mode: ESI (+); detection mode: dMRM	1–25 ng/L	2019	[1]

(continued on next page)

Table 3 (*continued*)

Analyte	Sample pre-treatment	Sample preparation	Analysis		LOQ (ng/L)	Year publ.	Ref
TRMP, FXT, SER, MEL, CMI [nor-CMI]		4 mL H_2O (pH 2) Washing: MeOH (2% v/v FA) Elution: MeOH (5% v/v NH_3)					
CIT, FXT, SER, VEN, APZ, QTP, ALP, DZP, LOR, OXA, TEM, CBZ	Centrifugation (4500 rpm for 5 min), Filtration (glass fibre filters - 1.2 μm)	SPE, Oasis HLBSample volume 100 mL Conditioning: 3 mL MeOH and 3 mL H_2OElution: 4 mL MeOH, 3 mL 5% ammonia in MeOHReconstitution: MeOH	HPLC: Force Biphenyl column (2.1 × 100 mm, 1.8 μm) Mobile phase:A: H_2O (0.1% v/v FA)B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	0.5–16 ng/mL	2018	[54]
VEN, CLOZ, DZP [nor-DZP], OXA, TEM, mCPP	Filtration (GF/A - 1.6 μm)	SPE, Oasis HLB Sample volume 50 mLConditioning: 8 mL of MeOH, 8 mL H_2OWashing: 4 mL of ultra-pure water Elution: 8 mL MeOHReconstitution: H_2O/MeOH 90:10, v/v	UPLC: Phenomenex Biphenyl column (2.1 × 100 mm, 2.6 μm) Mobile phase: A: H_2OB: ACN /H_2O (80:20, v/v)(both with 0.04% FA)	1. QTOF-MS; ionization mode: ESI (+) 2. LTQ Orbitrap MS; ionization mode: ESI (+)	NR	2017	[37]
CIT [nor-CIT], FXT, VEN, DZP [nor-DZP], TEM	Centrifugation (2500 rpm for 10 min)	SPE, Strata-X Sample volume 100 mL Conditioning: 4 mL of MeOH, 4 mL H_2OElution: 2 × 4 mL of MeOH	HPLC: Phenomenex Biphenyl column (2.1 × 50 mm, 2.6 μm) Mobile phase: A: MeOH (0.1% FA) B: H_2O (0.1% FA)	QqLIT; ionization mode: ESI (+) (-); detection mode: MRM	10–150 ng/L	2020	[29]
mCPP	Filtration (glass fibre filters)	SPE, Oasis MCX Sample volume 50 mL Conditioning: 6 mL MeOH, 4 mL H_2O, 4 mL H_2O (pH = 2) Washing: 3 mL H_2O, 2 mL H_2O (pH = 2), 3 mL MeOH Elution: 4 mL MeOH, 4 mL 5% NH_3/MeOH Reconstitution: 200 μL MeOH/H_2O (1/1, v/v)	UPLC: ACQUITY HSS T3 (2.1 × 50 mm, 1.8 μm) Mobile phase: A: H_2O (0.1% FA) B: ACN (0.1% FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	2.0 ng/L	2017	[56]
ALP [α-OH-ALP], CZP [7-ACL], 4CDP, CDD, ETZ, FNZ, LOR, LORM, MCLZ, MDZ, NTZ, OXA, PYR, QTP, TEM	Filtration (glass microfibre filter GF/A - 1.6 μm	SPE, CleanScreen XCEL II Sample volume 100 mL Conditioning: 6 mL MeOH, 6 mL sodium acetate buffer (100 mM pH 6 acidified with 10% acetic acid) Washing: 6 mL sodium acetate buffer Elution: 4 mL EtAc containing 2% ammonia Reconstitution: 40 μL FA/ACN (0.1/100, v/v) and 160 μL FA/ H_2O (0.1/100, v/v)	UPLC: Kinetex Biphenyl column (2.1 × 150 mm, 1.7 μm) Mobile phase: A: 95% H_2O (0.1% FA/5% ACN) B: 95% ACN (0.1% FA/5% H_2O)	QTrap MS; ionization mode: ESI(+); detection mode: MRM	< 10 ng/L LORM: 18 ng/L OXA: 300 ng/L TEM: 75 ng/L QTP: 75 ng/L	2020	[55]
CBZ [DiOH-CBZ, 10-OH-CBZ, CBZ-E, 2-OH-CBZ, 3-OH-CBZ]	NR	SPE, Strata-X (200 mg) Sample volume 100 mL Preconditioning: 5 mL acetone/EtAc (50:50) Conditioning: 5 mL MeOH, 15 mL H_2O Elution: 9 mL acetone/EtAc (50:50) Reconstitution: 10 mL 30:70 MeOH/H_2O	HPLC: RP: UltraSep ES Phen C18 column (2.1 ×250 mm, 5 μm) Mobile phase: A: H_2O B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	CBZ: 0.03 μg/L DiOH-CBZ: 0.2 μg/L 2-OH-CBZ: 0.05 μg/L 10-OH-CBZ: 0.13 μg/L 3-OH-CBZ: 0.05 μg/L	2014	[83]
DZP [nor-DZP], OXA, TEM AMIT [NORT], FXT [nor-FXT], VEN, NTZ [7-ANIZ], CDP, DSP	Filtration (glass fibre filter GF/D - 2.7 μm and GF/F - 0.7 μm)	SPE, Oasis MCX Sample volume 100 mL Conditioning: 2 mL MeOH, 2 mL 2% HCOOH/H_2O (pH 2) Washing: 2 mL 0.6%HCOOH/MeOH (pH 2) Elution: 3 mL 7% NH_4OH/MeOH Reconstitution: 500 μL 0.3% CH_3COOH/5% MeOH/H_2O	UPLC: ACQUITY BEH C18 column (1.0 ×150 mm, 1.7 μm) Mobile phase: A: 79.7% H_2O/20% MeOH/ 0.3% CH_3COOHB: 99.7% MeOH/0.3% CH_3COOH	QqQ MS; ionization mode: ESI (+); detection mode: SRM	NR	2012, 2014	[47], [84]
CIT, CBZ, CZP, LOR, OXA, ALP, DZP	Filtration	SPE, HLB disks (47 mm) Conditioning: 10 mL MeOH, 10 mL H_2O Washing: 4 × 5% MeOH/H_2O Elution: 6 mL 5% NH_4OH/MeOH, 6 mL 5% CH_3COOH/MeOH Reconstitution: 400 μL 25% MeOH/H_2O	UPLC: HSS C18 column (2.1 ×150 mm, 1.8 μm) Mobile phase: A (pH 3): 5 mM NH_4HCO_2 (aq.) B: ACN (0.1% v/v FA)	QTOF-MS; ionization mode: ESI (+); detection mode: SRM	0.09–2.31 ng/mL	2016, 2017	[11], [46]
AMIT, FXT [nor-FXT], VEN [ODVEN], DZP [nor-DZP] OXA, TEM,	Filtration (GF/F 0.7 μm)	SPE, Oasis HLB Sample volume 100 mLConditioning: 2 mL MeOH, 2 mL H_2OWashing:	UPLC: CHIRALPAK CBH column (2.0 mm × 10 cm, 5 μm) Isocratic mobile phase:	QqQ MS; ionization mode: ESI (+);	1.3–58.8 ng/L	2016	[96]

(*continued on next page*)

Table 3 (*continued*)

Analyte	Sample pre-treatment	Sample preparation	Analysis		LOQ (ng/L)	Year publ.	Ref
LOR, NTZ [7-ANIZ], ZOL, ZPC		3 mL H_2OElution: 4 mL MeOHReconstitution: 85:15% v/v 1 mM NH4OAc(aq.)/MeOH	1 mM NH_4OAc (aq.)/MeOH (85:15, v/v)	detection mode: MRM			
BUP, VEN, QTP, ALP, CZP, DZP [nor-DZP], FNZ, FZP, LOR, MDZ, NTZ, OXA, TEM	Filtration (GF/F - 0.7 μm)	SPE, in-house cartridges: 200 mg Strata-X, 100 mg Strata-X-AW, 100 mg Strata-X-CW and 150 mg IsoluteENV+ Sample volume 100 mL Conditioning: 3 mL MeOH, 3 mL H_2O Elution: 4 mL of EtAc/MeOH (50/50 v/v) containing 2% ammonia hydroxide (v/v), 2 mL of EtAc/MeOH (50/50 v/v) containing FA 1.7% v/v Reconstitution: 500 μL MeOH/H_2O (50/50 v/v)	UPLC: Acclaim RSLC 120 C18 (2.1 × 100 mm, 2.2 μm) Mobile phase: A: 90% H_2O, 10% MeOH, 5 mM $HCOONH_4$, 0.01% HCOOH B: MeOH, 5 mM $HCOONH_4$, 0.01% HCOOH	QTOF-MS; ionization mode: ESI (+); HRMS/MS	LOD: 0.5–305 ng/L	2019	[38]
CBZ [2-OH-CBZ], CIT, FXT [nor-FXT], VEN [ODVEN], LOR	Filtration (glass fibre filters – 1 μm, PVDF membrane filters - 0.45 μm)	SPE, Oasis HLB Sample volume: 50 mL Reconstitution: 1 mL MeOH:H_2O 15:85 v/v)	HPLC	QTrap MS; ionization mode: ESI(+) (-); detection mode: SRM	2-OH-CBZ: 0.88 CBZ: 2.67 CIT: 2.29 Codeine: 1.40 FXT: 3.75 nor-FXT; 7.56 LOR: 3.65 VEN: 0.88 ODVEN: 1.26	2021	[31]
ALP, LOR, ZOL	Filtration (glass fibre filters – 0.45 μm, polyamide membrane filters – 0.2 μm	SPE, Oasis MAX Sample volume: 50 mL Conditioning: 6 mL MeOH, 3 mL H_2O (pH 2) Washing: 3 mL H_2O Elution: 2 × 3 mL MeOH Reconstitution: 200 μL MeOH: H_2O (35:65 v/v)	HPLC: Pursuit UPS C18 (2.1 × 50 mm, 2.4 μm) Mobile phase: A: MeOH B: H_2O (10 mM FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	ALP: 4.0 LOR: 5.6 ZOL: 5.0	2015, 2016	[102], [103]
CBZ, CBZ-E, CZP, AMIT, BUP [OH-BUP], nor-CIT, DUX, ESCIT, FXT [nor-FXT], PAR, SER, TZD, VEN [ODVEN], ALP, APZ [DARI], CLB, DZP, FNZ, FZP, LOR, NTZ, QTP [nor-QTP], OXA, PZP, TEM, ZOL [ZPCA]	Filtration (PTFE filter)	Direct injection Sample volume: 50 μL	UPLC: Acquity BEH C18 (2.1 ×100 mm, 1.7 μm) Mobile phase: A: H_2O (5 mM Ammonium Acetate)	QqQ MS; ionization mode: ESI (+); detection mode: dMRM	NR	2015	[75]
CBZ, RIS, BUP, FPX	Filtration (cellulose syringe filter - 0.45 μm)	on-line-SPE sample volume: 1 mL Hypersil Gold C18 (2.1 × 20 mm, 12 μm) A: H_2O (0.1% FA) B: MeOH (0.1% FA)	HPLC: Hypersil Gold C18 (2.1 × 50 mm, 3 μm) Mobile phase: A: H_2O (0.1% FA) B: ACN (0.1% FA)	HRMS: LTQ Orbitrap MS; ionization mode: HESI-II (+);detection mode: SRM	NR	2017	[44]
FXT [nor-FXT], PAR, SER	Filtration (glass fibre filters - 0.7 μm)	SPE, Oasis MCX (150 mg, 6 cc) Sample volume: 500 mL Washing: 4 mL H_2O (2% FA) Elution: 4 mL of 5% NH_4OH/EtOH Reconstitution: 250 μL MeOH Derivatisation: MSTFA with 1% TMCS (70 °C for 1 h)	GC: Restek 5% diphenyl, 95% dimethyl column (30 m × 0.25 mm, 0.25 μm) GC oven: 140 °C for 0.5 min 11 °C/min to 215 °C, 29 °C/min to 283 °C (held for 6 min), increased to 300 °C (held for 7.7 min), total run time 24 min	Q MS/EI at 70 eV/SIM mode	0.15–104.2 ng/L	2019	[82]
ALP [α-OH-ALP]	NR	SPE, Oasis HLB (150 mg) Sample volume: 200 mL Washing: H_2O Elution: 4 mL Acetone/MeOH (50:50 v/v) with 0.5% FA	HPLC: Symmetry C18 (6.4 × 75 mm, 3.5 μm) Mobile phase: A: MeOH (0.2% FA) B: H_2O (0.2% FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	ALP: 0.5 ng/L α-OH-ALP: 0.2 ng	2018	[79]
CBZ, CIT, VEN, LOR	Filtration (glass fibre filters - 1 μm, nylon membrane filter – 0,45 μm)	SPE, OASIS HLB, Sample volume: 50 mL Elution: 6 mL MeOH Reconstitution: 1 mL MeOH/H_2O (10:90, v/v)	UPLC: Acquity HSS T3 (2.1 × 50 mm ×, 1.8 μm) Mobile phase: A: MeOH B: 10 mM FA/ NH_4HCO_2 (pH 3.2)	QqLIT, ionization mode: ESI (+); detection mode: SRM	10–14 ng/L	2015	[94]
AMIT [NORT] CBZ [CBZ-E], CIT [nor-CIT], FXT [Nor-FXT], MIRT, nor-SER, VEN	Filtration (GF/F - 0.7 μm)	SPE, Oasis HLB (60 mg) Sample volume: 50 mL Conditioning: 2 mL MeOH, 2 mL H_2OElution: 4 mL	UPLC: ACQUITY BEH C18 (1.0 x 150 mm, 1.7 μm) Mobile phase: A: 80/20 H_2O/MeOH 1 mM NH_4F	QqQ MS; ionization mode: ESI (+) (-); detection mode: MRM	0.66–12.4 ng/L	2021	[88]

(*continued on next page*)

Table 3 (*continued*)

Analyte	Sample pre-treatment	Sample preparation	Analysis		LOQ (ng/L)	Year publ.	Ref
[ODVEN], LOR, OXA, TEM		MeOHReconstitution: 500 μL 80:20 H_2O/MeOH	B: 5/95 H_2O/MeOH 1 mM NH_4F				
BUP, FXT [nor-FXT], MIRT, PAR, SER [nor-SER], VEN [ODVEN] CLOZ, OLZ [DMO], QTP	Filtration (GF/B - 1 mm)	SPE, Oasis HLB (200 mg, 6 cc) Sample volume: 150 mL Conditioning: 5 mL MeOH, 5 mL H_2OWashing: 5 mL 20% MeOH in 2% $NH_4CH_3CO_2$Elution: 2 × 5 mL 2% CH_3COOH in MeOHReconstitution: 0.5 mL MeOH: H_2O, 10:90 (v/v)	UPLC: RP: SpeedCore C18 (2.1 × 50 mm, 2.6 μm) Mobile phase: A: H_2O (0.1% v/v FA)B: MeOH (0.1% v/v FA)	HRMS: LTQ-FT Orbitrap MS; ionization mode: ESI (+)	1.5–41.3 ng/L	2019	[43]
ALP, DZP	Filtration (glass fibre filters – 1 μm, nylon membrane filters - 0.45 μm)	On-line SPE, Sample volume: 5 mL PLRPs cartridges Elution: ACN/H_2O with 20 mM FA/ammonium formate buffer (pH 3.8) constant flow rate 0.3 mL/min	ion-pair LC: Purospher Star RP-18 end-capped (2.0 × 125 mm, 5 μm) Mobile phase: A: ACNB: H_2O	QqLIT; ionization mode: ESI (+); SRM mode	LOD ALP: 1.5 ng/L DZP: 1.4: ng/L	2017	[45]
CBZ, DiOH-CBZ	Centrifugation (5000 rpm for 15 min, 4 °C) Filtration (GF/F – 0.7 μm)	SPE, Oasis HLB (3 cc, 60 mg) Sample volume: 50 mL Conditioning: 2 mL MeOH, 2 mL H_2OElution: 4 mL MeOHReconstitution: 1 mL MeOH	UPLC, RP: Waters BEH C18 (2.1 × 100 mm, 1.7 μm) Mobile phase: A: H_2O (0.1% v/v FA)B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	CBZ: 0.25 μg/L DiOH-CBZ: 1.0 μg/L	2021	[60]
CBZ	Filtration (GF/A - 1.6 μm, mixed cellulose ester microfiber filter ME2–0.45 μm)	SPE, OASIS MCX (60 mg) Conditioning: 5 mL MeOH, 3 mL MilliQ, 3 mL MilliQ (pH 2–2.5) Elution: 2 mL MeOH, 2 mL 2% NH_3/MeOH	HPLC, Luna C8 (2.0 ×50 mm, 3 μm) Mobile phase: A: H_2O (0.1% v/v FA) B: ACN	QqQ MS; ionization mode: ESI (+); detection mode: SRM	NR	2020	[61]
FXT [Nor-FXT], VEN [ODVEN], PAR, SER, CBZ [CBZ-E], AMS	Filtration (glass fibre filters)	SPE, Strata-X (200 mg) Conditioning: 3 mL MeOH, 3 mL H_2O Washing: 3 mL of 5% MeOH (aq. solution) Elution: 2 × 3 mL MeOH Reconstitution: 200 μL of H_2O/MeOH (80/20, v/v)	1D-LC, Kinetex C18 (3.0 × 50 mm, 1.7 μm) 2D-LC, RP: Synergy Max-RP (3.0 × 150 mm, 4 μm) Mobile phase: A: H_2O (0.01% CH_3COOH)B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: SRM	0.5–25 ng/L	2021	[116]
CBZ	Filtration (GF/F – 0.7 μm)	SPE, mixed bed multilayer cartridges (Oasis HLB, Isolute ENV+, Strata-X-AW and Strata-X-CV) Sample volume: 100 mL Conditioning: 3 mL MeOH, 3 mL H_2O Elution: 4 mL of MeOH/EtAc (50:50, v/v) containing 2% ammonia, 2 mL of MeOH/EtAc (50:50, v/v) containing 1.7% FA Reconstitution: 0.5 mL MeOH/H_2O (50:50, v/v)	UPLC, Atlantis T3 C18 (2.1 ×100 mm, 3 μm) Mobile phase: A: H_2O (0.0 1% v/v FA) B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	LOD 1.1 ng/L	2021	[89]
LOR, OXA, ALP, BZP, 7AF, DZP, MDZ [1-OH-MDZ], TEM, MIRT [8-OH MIRT, CIT [nor-CIT], PAR, VEN, FXT, SER [nor-SER], AMIT, NORT, CMI, DOX, AMT, PLZ, CLOZ [nor-CLOZ], OLZ, AMS, BUS, HAL, MFS, PEM, QTP, SUL	Filtration (GF/F – 0.7 μm)	SPE, mixed bed multilayer cartridges (Strata-X, Strata-X-CW, Strata-X-AW, Isolute ENV+) Sample volume: 100 mL Conditioning: 3 mL MeOH, 3 mL H_2O Elution: 4 mL of MeOH/EtAc (50:50, v/v) containing 2% ammonia, 2 mL of MeOH/EtAc (50:50, v/v) containing 1.7% FA Reconstitution: 0.5 mL MeOH/H_2O (50:50, v/v)	UPLC, Atlantis T3 C18 (2.1 ×100 mm, 3 μm) Mobile phase: A: H_2O (0.0 1% v/v FA) B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	NR	2021	[90]
ALP, BZP, BUS, CLB, DZP, FNZ [7AF], LOR, MDZ, OXA, AMIT, CBZ, BUP, CIT, DOX, DUX, FLV, SER, VEN, AMS, OLZ, QTP, RIS	Filtration (glass fibre filter GF/B – 1 μm)	SPE, Oasis HLB (200 mg) Sample volume: 250 mL Conditioning: 5 mL MeOH, 5 mL H_2O Washing: 5 mL H_2OElution: 7 mL MeOHReconstitution: 1 mL MeOH	UPLC, Thermo Hypersil GOLD aQ (2.1 ×50 mm, 1.9 μm) Mobile phase: A: H_2O (0.1% v/v FA)B: MeOH (0.1% v/v FA)	Orbitrap MS/MS; ionization mode: HESI (+), detection mode: full scan	0.1–10.0 ng/L	2022	[104]
ALP, DZP [nor-DZP], OXA, TEMAMIT, NORT, BUP, CBZ, CBZ-E, CIT, FXT, SER, VEN, CLOZ, RIS	Filtration (GF/D 2.7 μm)	Direct injection injection volume 10 μL sample volume 1 mL	UPLC, Raptor biphenyl (5.0 × 3.0 mm, 2.7 μm) Mobile phase: A: H_2O (0.1% v/v FA) B: ACN/MeOH, 1:1, v/v (0.1% FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	1–185 ng/L	2020	[58]

(*continued on next page*)

Table 3 (*continued*)

Analyte	Sample pre-treatment	Sample preparation	Analysis		LOQ (ng/L)	Year publ.	Ref
ALP, OXA, FNZ, MDZ, ZOL	Filtration (syringe filter - 0.45 μm)	On-line SPE, Oasis HLB column (2.1 × 20, 15 μm) load sample from 1 mL loop A: MeOH (0.1% FA) B: H_2O (0.1% FA)	HPLC, Thermo Hypersil Gold AQ (2.1 ×50 mm, 5 μm)Mobile phase: A: MeOH (0.1% v/v FA)B: H_2O (0.1% v/v FA)	QqQ MS; ionization mode: ESI (+); detection mode: SRM	1–10 ng/L	2014	[48]
CBZ [CBZ-E], CIT, FXT, [Nor-FXT], PAR, SER, [nor-SER], TZD, VENDZP	Filtration (nylon membrane filters-0.22 μm)	SPE, Strata-X (200 mg) Sample volume: 50 mL Conditioning: 5 mL MeOH, 5 mL H_2O, 5 mL H_2O (pH 2) Washing: 5 mL H_2O Elution: 10 mL MeOH Reconstitution: 500 μL ACN: H_2O (30:70, v/v)	UPLC, Cortecs C18 + (2.1 × 100 mm, 1.6 μm) Mobile phase: A: H_2O (0.1% v/v FA)B: ACN	QqQ MS; ionization mode: ESI (+); detection mode: MRM	0.12–13.1 ng/L	2016	[91]
OLZ, RIS, BUP, CBZ, DUX, SER, VEN, FLV	Filtration (GF/B - 1 μm)	SPE, Oasis HLB (samples: 0,1% Na_2EDTA) Sample volume: 150 mL Conditioning: 5 mL H_2O, 5 mL MeOH Elution: 2 × 5 mL MeOH Reconstitution: 0,5 mL MeOH	HPLC, C18 Hypersil Gold (2.1 × 100 mm, 1.9 μm) Mobile phase: A: ACN (0.02% FA) B: NH_4OH aq. (5 μM) (0.02% FA)	QqQ MS; ionization mode: ESI (+); detection mode: SRM	4.9–532 ng/L	2016	[39]
BZP, DZP, OLZ, QTP, RIS BUP, CBZ, CIT, DUX, SER, VEN, BUS, DOX, FLV	Filtration (GF/B - 1 μm)	SPE, Oasis HLB (samples: 0,1% Na_2EDTA) Sample volume: 150 mL Conditioning: 5 mL H_2O, 5 mL MeOH Elution: 2 × 5 mL MeOH Reconstitution: 0,5 mL MeOH	HPLC, C18 Hypersil Gold (2.1 × 100 mm, 1.9 μm) Mobile phase: A: 5 mM NH_4HCO_2/ H_2O B: 5 mM NH_4HCO_2/ MeOH	QqQ MS; ionization mode: ESI (+); detection mode: SRM	0.3–19.5 ng/L	2019	[40]
AMIT, BUP, CBZ FXT, NORT, SER, ESCIT, TZD, ALP, CZP	Filtration (PVDF membranes - 0.22 μm)	On-line SPE, OASIS HLB (2.1 × 30 mm, 10 μm) H_2O/ACN (80:20 v/v)	UPLC, Acquity CSH C18 (2.1 ×100 mm, 1.7 μm) A: H_2O (0.1%FA)B: ACN	QqQ MS; ionization mode: ESI (+); detection mode: SRM	50 ng/L	2020	[64]
OXA, CBZ, VEN	NR	SPE, Strata X Sample volume: 5 mL Conditioning: 2 mL MeOH, 2 mL H_2O Washing: 2 mL H_2O Elution: 2 × 750 μL ACN (2% FA) Reconstitution: 50 μL 0.01% HFBA (aq.) with 5% MeOH (v/v)	HPLC, Kinetex Biphenyl (2.1 × 100 mm, 2.6 μm) Mobile phase: A: MeOHB: 0.1% CH_3COOH /H_2O	QTrap MS; ionization mode: ESI (+); detection mode MRM	NR	2021	[92]
CBZ	NR	Direct injection	UPLC, Kinetics C18 (3 × 100 mm, 2.6 μm) Mobile phase: A: H_2O (0.1%FA) B: 95% ACN (0.1%FA)	QTrap MS; ionization mode: ESI (+); detection mode MRM	50 ng/L	2017	[93]
APZ [DARI], BUP, CBZ, CIT [nor-CIT], SER [nor-SER], VEN, QTP [nor-QTP], ALP [α-OH-ALP], DZP [nor-DZP], LOR, OXA	NR	SPE, Oasis HLB (200 mg) Sample volume: 50 mL Conditioning: 5 mL of MeOH, 5 mL H_2O Elution: 6 mL MeOH, 3 mL acetone: methanol: ethyl acetate (2:2:1 v/v/v), 3 mL MeOH (5% ammonia) Reconstitution: 1 mL MeOH	HPLC, Ultra-Biphenyl (2.1 × 100 mm, 5 μm) Mobile phase: A: MeOH B: H_2O (0.1% v/v FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	0.1–20 ng/L	2015	[62]
QTP [nor-QTP], APZ [DARI], LOR, ALP, DZP [nor-DZP], OXA, CBZ, VEN, BUP, SER [nor-SER]	NR	SPE, Oasis HLB (200 mg) Sample volume: 100 mL Conditioning: 5 mL of MeOH, 5 mL H_2O Elution: 6 mL MeOH, 4 mL acetone: methanol: ethyl acetate (2:2:1 v/v/v), 4 mL MeOH (5% ammonia) Reconstitution: 1 mL MeOH	HPLC, Ultra-Biphenyl (2.1 × 100 mm, 5 μm) Mobile phase: A: MeOH B: H_2O (0.1% v/v FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	0.1–50 ng/L	2017	[63]
BUP, CBZ, CIT [nor-CIT], SER [nor-SER], VEN, APZ [DARI], QTP [nor-QTP], ALP [α-OH-ALP], DZP [nor-DZP], LOR, OXA	Centrifugation (5000g for 10 min), Filtration (glass fibre filters - 1 μm)	SPE, Oasis HLB (200 mg) Sample volume: 100 mL Conditioning: 5 mL MeOH, 5 mL H_2O Elution: 6 mL MeOH, 3 mL acetone: MeOH: EtAc (2:2:1 v/v/v), 3 mL MeOH (5% NH_3) Reconstitution: 1 mL MeOH	HPLC, Hypersil Gold column (2.1 × 150 mm, 3 μm) Mobile phase: A: H_2O (0.1% v/v FA) B: MeOH (0.1% v/v FA)	QqQ MS; ionization mode: ESI (+); detection mode: MRM	0.1–20 ng/L	2015	[105]
CBZ, OXA	Filtration (glass-fiber filters, GF/F - 0.7 μm)	SPE, Chromabond HR-X (500 mg) Sample volume: 250 mL Conditioning: 6 mL MeOH,	HPLC, Nucleodur C18 Gravity (4.3 × 150 mm, 3 μm)	QqQ MS; ionization mode: H-ESI	OXA: 6.4 ng/L CBZ: 4.6 ng/L	2017	[50]

(*continued on next page*)

Table 3 (*continued*)

Analyte	Sample pre-treatment	Sample preparation	Analysis		LOQ (ng/L)	Year publ.	Ref
		6 mL H_2O Washing: 6 mL H_2O Elution: 6 mL MeOH Reconstitution: H_2O (0.1% FA)	Mobile phase: A: H_2O (0.1% v/v FA) B: ACN (0.1% v/v FA)	(+); detection mode: MRM			
CBZ, CPZ, CLOZ, [nor-CLOZ], OLZ, RIS, PAL, ALP, BZP, CDP, CLB, DZP [nor-DZP], FNZ [7AF], LOR, NTZ, MDZ, OXA, TEM, ZPC, VEN, FXT, PAR, SER [nor-SER], MIRT [8-OH-MIRT], AMIT, NORT, CMI, IMP, DOX	Filtration (glass-fiber filters GF/F - 0.7 μm)	SPE, Strata-X cartridges (200 mg) Sample volume: 50 mL (0.1% w/v EDTA) Conditioning: 6 mL MeOH, 6 mL H_2O Washing: 3 mL H_2O Elution: 6 mL MeOH Reconstitution: 500 μL H_2O (FA 0.05%):MeOH (75:25 v/v)	UPLC, Atlantis T3 C18 (2.1 × 100 mm, 3 μm) Mobile phase: A: H_2O (0.01% v/v FA) B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: SRM	1.0–23.1 ng/L 80.8 ng/L for FNZ	2016	[106]
VEN	Filtration (glass microfiber filters – 0.45 μm)	SPE, Oasis HLB Sample volume: 50 mL Washing: H_2O Elution: 6 mL MeOH Reconstitution: 500 μL MeOH/ H_2O (25:75, v/v)	HPLC, Chiral CBH (2.0 ×100 mm, 5 μm) Isocratic mobile phase (pH–5.0): 90% H_2O, 10% 2-propanol and 1 mM ammonium acetate	QqQ MS; ionization mode: ESI (+); detection mode: SRM	5.7 ng/L	2014	[51]
CIT, FXT, VEN, CBZ	Filtration	SPE, Oasis HLB or MCX Sample volume: 50 mL	HPLC	QqQ MS; ionization mode: ESI (+); detection mode: MRM	1–25 ng/L	2015	[67]
AMIT, CIT, CMI, DZP, FXT, HAL, MIAN, MIRT, RIS, SER	Filtration (glass fibre membrane filters- 0.5 μm) Acidification (pH 4 with FA)	SPE, Oasis MCX (500 mg) Conditioning: 10 mL MeOH, 5 mL H_2O, 5 mL H_2O (pH 4) Washing: 5 mL H_2O (pH 4) Elution: 5 mL MeOH (F1, for acidic compounds) and 5 mL MeOH (0,5% NH_3) (F2, for basic compounds) F1 was further diluted to 50 mL H_2O (pH 11) SPE, Oasis MAX (500 mg) Conditioning: 10 mL MeOH, 5 mL H_2O, 5 mL H_2O (pH 11) Washing: 5 mL H_2O (pH 11) Elution: 5 mL MeOH, 5 mL MeOH (2% FA) Reconstitution: 1 mL MeOH/ H_2O (1:1, v/v with 10 mM ammonium formate and 0.005% FA)	HPLC, CHIROBIOTIC V (2.1 ×150 mm, 5 μm)Mobile phase: A: H_2O B: MeOH Both with 0.005% FA and 10 mM ammonium formate	QTrap MS; ionization mode: ESI (+); detection mode MRM	0.02–2.9 ng/L	2021	[57]
MDZ, VEN, RIS, OXA, CIT	Filtration (cellulose syringe filter-0.45-μm)	on-line-SPE sample volume: 1 mL Hypersil Gold C18 (2.1 × 20 mm, 12 μm) A: H_2O (0.1% FA) B: MeOH (0.1% FA)	HPLC, Cogent bidentate C18 (2.1 ×50 mm, 3 μm) Mobile phase: A: H_2O (0.1% FA) B: ACN (0.1% FA)	Orbitrap Q Exactive HRPS	NR	2016	[49]
FXT, 7AF, RIS, ALP, OXA, BZP, DUX, QTP, DZP, HAL, SER, CPZ, CLOZ	Centrifugation (4200 rpm for 30 min), Filtration	SPE, Oasis HLB (200 mg) Sample volume:400 mL Conditioning: 6 mL MeOH, 6 mL H_2O Washing: H_2O (0.5% FA) Elution: 10 mL MeOH (0.5% FA), 6 mL H_2O (3% NH_4OH) Reconstitution: 1 mL H_2O (FA 0.5%):MeOH (95:5 v/v)	UPLC, BEH C18 (100 × 2.1 mm, 1.7 μm) Mobile phase: A: H_2O (0.1% FA) B: MeOH	QqQ MS; ionization mode: ESI (+); detection mode: MRM	0.8–4.7 ng/L	2021	[107]

NR: not reported.

wastewater [117]. Direct injection has also been reported for psychoactive pharmaceuticals [58,75,93]. Prior to filtration and extraction, an internal standard is added to the sample to correct for the losses in response (ion suppression) or an increase in response (ion enhancement) due to matrix effect or losses derived from sample preparation (e.g., filtration) [33,74,118]. To a certain extent, the application of isotopically labelled internal standards accounts for these losses, and this practice is commonly applied.

Solid-phase extraction offers broad application due to the wide choice of available stationery phases (sorbents) and solvents. SPE has become the extraction technique of choice for WBE, enabling trace-level pharmaceutical biomarkers (low ng/L range) to be extracted and concentrated from an aqueous matrix while removing many interferences present in sewage samples [119]. Reversed phase (RP), hydrophilic-lipophilic-balanced, mixed-mode, mix-mode RP-cation-exchange or cation exchange and hydrophilic are popular choices for SPE sorbents when analysing antidepressants, antipsychotics and benzodiazepines. Depending on the SPE sorbent and the target compounds' physiochemical properties (e.g. pKa), the pH of the samples must be adjusted. For example, basic compounds, like most psychoactive pharmaceuticals (pKa 6–10), should be loaded onto the MCX (mixed-mode cation exchange) sorbent in an acidic aqueous solution. In this case, the

compounds will be ionised (positively charged), ensuring ion exchange and reverse-phase retention. In classical SPE, the influent wastewater sample volume is usually 50 or 100 mL [29,37,52,54,56,95], but volumes ranging from five to 1000 mL have also been assessed [32,45,59,82].

In WBE, a multi-residue analysis is usually applied to determine different compounds in a single analytical method. Accordingly, several researchers have used similar hydrophilic and high-capacity polymeric sorbents, such as Oasis Hydrophilic-Lipophilic Balanced reversed-phase sorbent (HLB) [1,31,32,37,39,40,43,51,52,54,59,60,62,63,79,88,94–96,104,105,107] or Strata X [29,83,91,92,106,116] for the extraction of antidepressants, antipsychotics and benzodiazepines. Oasis HLB is widely used to extract polar and nonpolar compounds with acidic, basic, and neutral properties (Table 3). Pugajeva et al. [116] compared the extraction of pharmaceuticals including the antidepressants fluoxetine, venlafaxine, sertraline, paroxetine, carbamazepine and the antipsychotic amisulpride, using Oasis HLB (60 mg/ 3 mL and 500 mg/ 6 mL) and Strata-X (60 mg/ 3 mL, 200 mg/ 3 mL and 500 mg/ 6 mL) cartridges. Overall recoveries were similar, except for Strata-X (60 mg/ 3 mL), which showed a decrease in signal intensity, especially when the eluting solvent was acidified [116] The Strata-X (200 mg) cartridges were chosen for further optimisation due to a higher percentage of acceptable recoveries (80–100%) and economic reasons [116]. Ribeiro et al. [120] developed a green SPE-based method to extract and quantify micropollutants, including venlafaxine, fluoxetine and its metabolite norfluoxetine, from Portuguese wastewater influent. Using OASIS HLB cartridges, the authors successfully applied ethanol, an eco-friendly solvent, as the conditioning and eluting solvent [120]. The use of ethanol in the SPE process produced similar recoveries to those obtained with methanol, with venlafaxine having the highest recovery (97.2–104%), followed by fluoxetine (42.8–53.2%), and then norfluoxetine (11.9–15.3%) [120]. To reduce solvent volume and sample preparation time, Thomas et al. [86] used an HLB 96-well plate to extract illicit drugs and antidepressants from influent wastewater samples (VEAS, Oslo). The authors used a small volume of sample (1 mL sample volume) and solvent for conditioning (1 mL MeOH) and μElution (50 μL MeOH, 50 μL 1% NH_4OH/MeOH and 50 μL 1%FA/MeOH). For citalopram, methylphenidate and carbamazepine, the LOQs were 1.1, 0.2 and 1.0 ng/L, respectively, while the method gave recoveries of $>$ 80%. Additionally, the application of HLB disks has also been reported for antidepressants (citalopram and carbamazepine) and benzodiazepines (midazolam, nitrazepam, alprazolam, flunitrazepam and etizolam) [11,46]. Baz-Lomba et al. [46] loaded high-volume samples onto the HLB disk at a flow rate of 100 mL/min. Designed to handle large sample volumes ($>$ 1 L), any sample matrix, regardless of particulate level, and eliminate the filtration step, are a few advantages of SPE disks. Compared with SPE cartridges, when handling the extraction of larger sample volumes, using SPE disks is more straightforward, cost-effective, and can maintain fast flow rates, while maintaining similar high recoveries. Additionally, LOQs should be lower when higher volumes are extracted with SPE disks.

Mixed-mode ion-exchange sorbents, such as Oasis MCX [1,30,47,53,56,57,61,82,84], are also used in WBE studies due to their ability to increase analyte specificity, sensitivity and selectivity, which is advantageous considering the range of chemical and physical properties of psychoactive pharmaceuticals. Principally, Oasis MCX is based on the Oasis HLB copolymer but chemically modified with sulfonic groups (pKa$<$1), so a part of the reversed-phase interactions (unspecific) is capable of creating cation-exchange interactions (specific) with analytes with basic characteristics. Oasis MCX has higher selectivity for basic compounds (i.e., most psychoactive pharmaceuticals) and retains fewer interfering compounds. When using this type of sorbent, the sample is loaded under acidic conditions (pH 2–3) because it promotes ionic interactions, while elution is performed in basic conditions, typically using MeOH containing 2–7% ammonium hydroxide or ammonia. Pugajeva et al. [116] evaluated several different cartridges, including Strata C18 E (silica-based sorbent), Strata-X (surface-modified styrene divinylbenzene polymer-based sorbent), Strata Screen A (mixed mode C8 and strong anion exchange sorbent), Strata Screen C (mixed mode C8 and strong cation exchange sorbent) and Strata ABW (silica-based cation/anion mixed-mode exchange sorbent). The best recoveries were achieved with polymeric reversed-phase sorbents, except for fluoxetine and paroxetine, where Strata Screen C cartridges performed better. After considering all the data, the authors chose Strata-X cartridges due to their broader applicability, including antidepressants, antipsychotics, other pharmaceuticals, opioids, alcohol, tobacco, and veterinary medication [116]. Overall, both mechanisms can effectively be used for simultaneous extraction, showing relatively high analyte recovery when using either Oasis HLB or MCX cartridges, covering the range of physical and chemical properties of targeted biomarkers. A good example is a study by Boogaerts et al. [1], where multiple SPE procedures (one using Oasis HLB and the other Oasis MCX cartridges) were developed and validated for the extraction of 27 antidepressants due to their wide range of physicochemical properties. In their study, LOQs ranged between 1 and 25 ng/L for both SPE procedures [1].

The use of UCT CleanScreen XCEL II [55] and Chromabond HR-X [50] solid phase extraction columns have also been reported in the literature. Clean Screen Xcel columns reduce sample preparation since the conditioning step is eliminated and the sample size and solvent required are minimized, showing improved sensitivity and recoveries. As for the Chromabond HR-X, the spherical structure of the hydrophobic polystyrene-divinylbenzene copolymer provides a very homogenous surface and optimised pore structure that enables high recovery rates for the enrichment of pharmaceuticals. Both these sorbents can extract a broad range of basic compounds, including benzodiazepines and opioids. Bade et al. [55] and Thiebault et al. [50] used UCT CleanScreen XCEL II and Chromabond HR-X, respectively, to extract 20 prescribed and designer benzodiazepines and their metabolites, the antipsychotic quetiapine and the antidepressant carbamazepine among other pharmaceuticals, illicit drugs and opioids. The authors obtained acceptable recoveries (80–120%) and LOQs ($<$10 ng/L) for the majority of the studied psychoactive pharmaceuticals. The exceptions were 7-aminoclonazepam, 7-aminonimetazepam, midazolam and quetiapine. Diamanti et al. [38] and Alygizakis et al. [90] used in-house prepared SPE cartridges made from four different sorbent types (Strata-X, Strata-X-CW, Strata-X-AW and Isolute ENV+), while Galani et al. [89] used mixed bed multilayer cartridges comprising Oasis HLB, Isolute ENV+, Strata-X-AW and Strata-X-CV sorbents to extract a broad array of compound types including antidepressants, antipsychotics and benzodiazepines. This combination of sorbents and elution with acidic and basic solutions allowed the extraction of an extensive compound range from neutral, aromatic and acidic to basic and polar analytes [38,89,90].

Solid phase extraction is typically performed offline, but it can also form part of an automated system coupled online to an LC system [49]. Online SPE reduces laboratory time, simplifies sample preparation and improves reproducibility while decreasing the risk of analyte loss due to evaporation steps [121]. Online SPE only requires a small sample volume (1–5 mL) and makes use of an HLB column [48,64] or a C18 column [44] to extract and concentrate different psychoactive pharmaceuticals, including antidepressants, antipsychotics and benzodiazepines. So far, online SPE, with either HLB or C18 column, has been used to determine carbamazepine, risperidone, bupropion, flupentixol, alprazolam, diazepam, oxazepam, flunitrazepam, midazolam, zolpidem, amitriptyline, nortriptyline, fluoxetine, sertraline, escitalopram, trazodone and clonazepam in influent wastewater. The method gave recoveries and LOQs similar to offline SPE, ranging from 1 to 50 ng/L. Mastroianni et al. [45] also used online SPE with PLRPs cartridges to determine alprazolam and diazepam having LOQs 1.5 and 1.4 ng/L, respectively. In their study, the samples were diluted with HPLC water (1:10, v/v) before loading the cartridges, possibly to reduce matrix effects. However, compared to offline SPE, limited portability, expensive equipment and higher matrix effects are a few drawbacks of the online SPE [121].

Dispersive liquid-liquid microextraction (DLLME) of olanzapine, clozapine, chlorpromazine, risperidone, haloperidol and pimozide from hospital wastewater has also been reported with LOQs < 1.4 μg/L [122]. Also, a method combining gel electro membrane extraction (GEL-EME) and switchable hydrophilicity solvent-based homogeneous liquid-liquid microextraction (SHS-HLLME) was developed by Behpour et al. [122] to extract desipramine, clozapine and citalopram from wastewater. The method gave LODs and LOQs ≤ 1.0 and ≤ 5.0 ng/mL, respectively [123]. Although not used in WBE, both reported methods have been used to extract antidepressants and antipsychotics from complex matrices, such as wastewater, breast milk and human serum, with 70–90% recoveries, albeit with higher LODs and LOQs when compared with SPE. Even though these extraction methods are not so common in wastewater analysis, they can be regarded as "green" choices. In the DLLME, ethanol was selected as the disperser solvent. In the case of GEL-EME, an agarose-based membrane was used to substitute the supported liquid membrane, which is usually used in common electromembrane extraction, to avoid toxic organic solvents.

2.3. Instrumental analysis

In WBE, the predominant technique for separating psychoactive pharmaceuticals involves chromatographic separation *(liquid or gas chromatography)* coupled with mass spectrometry. It enables a high level of separation and the sensitivity required for qualitative and quantitative analysis of antidepressants, antipsychotics, benzodiazepines and their metabolites in wastewater. Gas chromatography-mass spectrometry (GC-MS) has been overtaken by liquid chromatography-mass spectrometry (LC-MS) because it allows the determination of compounds with a broad range of polarity, low volatility and thermolability and the application of a more generic sample treatment strategy [97]. However, for the determination of fluoxetine, its metabolite norfluoxetine, paroxetine and sertraline, Gonçalves et al. [82] used GC-MS. In this case, the target analytes required derivatisation with N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with the addition of 1% TMCS (2,2,2-Trifluoro-N-methyl-N-(trimethylsilyl)-acetamide, Chlorotrimethylsilane), to increase their volatility. In general, the detection, identification and quantification of psychoactive pharmaceutical biomarkers are typically made using LC hyphenated to a highly sensitive and quantitative triple quadrupole (QqQ) mass analyser.

2.3.1. Chromatography

Good chromatographic separation is essential for the required selectivity, sensitivity and identification power to monitor psychoactive pharmaceuticals in wastewater [97]. Both high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are equally applied to the analysis of prescribed pharmaceuticals in wastewater (Table 3). Selecting UPLC over HPLC has several advantages, including more efficient separation and shorter analysis time. Regarding the mobile phases, most methods use a biphasic system comprising water and an organic solvent (methanol or acetonitrile). Volatile salts, like ammonium acetate or ammonium formate (1–10 mM), can be added in the aqueous mobile phase to improve peak shape. Modifiers designed to improve ionization (positive or negative) can also be added to the aqueous or organic mobile phase. In contrast, formic or acetic acid (0.01–0.1%) is mostly added to improve the ionisation in positive ionisation mode. When working in positive ionization mode, a combination of methanol-water or acetonitrile-water with formic acid as a mobile phase are the most popular choices among the WBE studies, giving excellent chromatographic performance [1,30,43,48,50,54–56,59,62,63,79,89,91,106–110]. Nevertheless, mobile phases without additives were reported [47,80].

Currently, most published methods for the quantification of psychoactive pharmaceuticals and their metabolites in wastewater focus on analysing the compounds after extraction using reverse-phase LC columns packed with different stationary phases such as C18 [32,40,56,59,60,75,79,88,90,104,107,115] or biphenyl [29,52,54,55,62,63,92]. In the Bade et al. [55] paper, the authors compared three chromatographic columns: biphenyl, C18 and Pentafluorophenyl F5, for the analysis of 22 prescribed and designer benzodiazepines and their metabolites, including 7-amino clonazepam, 7-amino nimetazepam, alpha-hydroxy alprazolam, alprazolam, clonazepam, delorazepam, deschloroetizolam, diazepam, diclazepam, etizolam, flubromazepam, flunitrazepam, lorazepam, lormetazepam, meclonazepam, midazolam, nimetazepam, nitrazepam, oxazepam, pyrazolam, quetiapine and temazepam. They found that Pentafluorophenyl F5 gave poor separation for all tested compounds, while except for quetiapine, it was possible to separate all of the remaining analytes using the biphenyl and C18 columns. The best peak shapes were obtained using the biphenyl column [55]. Others, such as Mastroianni [45] and Östman [48], used a polar endcapped C18 column to enhance analyte retention and resolution to analyse benzodiazepines (alprazolam, diazepam, flunitrazepam, midazolam and oxazepam) and zolpidem. Overall, reverse phase columns seem the most suitable chromatographic technique for obtaining a generic but satisfactory separation of a wide range of psychoactive pharmaceuticals, including antidepressants, benzodiazepines and antipsychotics.

To reduce analysis time, Ng et al. [58] developed an ultra-fast high-throughput direct injection LC-MS/MS method (total run time of 5.0 min) to quantify 135 contaminants of emerging concern (CECs), including five benzodiazepines (alprazolam, diazepam, nordiazepam, oxazepam, temazepam) and ten antidepressants (amitriptyline, nortriptyline, bupropion, carbamazepine, citalopram, fluoxetine, sertraline, venlafaxine, clozapine and risperidone, using only 10 μL of filtered sample per injection on a short biphenyl column. For the psychoactive pharmaceuticals included, the detection limits ranged from 3 ng/L (diazepam and fluoxetine) to 56 ng/L (nordiazepam), and a matrix effect was < 11%. Moreover, Pugajeva et al. [116] introduced a two-dimensional liquid chromatography-mass spectrometry (2D-LC-MS/MS) as an effective way of assessing a broader range of pharmaceuticals with significantly different polarities. The authors' optimised and validated heart-cutting 2D-LC–MS/MS method, which relies on reversed-phase C18 (1D) and reversed-phase C12 (2D) stationary phases, simultaneously quantified 62 biomarkers, including eight antidepressants and the antipsychotic amisulpride, with LOQ from 0.1 to 50 ng/L.

2.3.2. Enantiomeric separation

Enantiomeric analysis has also been used to study psychoactive pharmaceuticals in wastewater. Many psychoactive pharmaceuticals have chiral centres and are produced either as racemic mixtures such as citalopram (a mixture of the two stereo-isomers R-citalopram and S-citalopram) or as a single enantiomer, as in the case of escitalopram (S-citalopram). Thus, the stereochemistry cannot be ignored when estimating their consumption in WBE studies. Chiral analysis can also be a helpful tool in WBE because it helps to understand (i) the origin of the pharmaceutical residue, i.e., discriminate between consumption and direct disposal of unused drugs in the sewage, since human metabolism is stereoselective, (ii) the different synthesis routes, (iii) the distinction between legal and illicit use (especially for drugs of abuse) and (iv) the potency of abused pharmaceuticals. Unfortunately, the chiral analysis still has limited WBE application [124]. In the literature, two ways of chiral analysis have been reported, either using a chiral column and LC-MS analysis or using a chiral derivatisation agent and GC-MS analysis.

Chiral LC-MS methods are primarily applied to a limited number of chiral pharmaceuticals, mainly focusing on illicit drugs and having a complementary role alongside non-chiral LC-MS methods. For instance, Vazquez-Roig et al. [51] used a chiral CBH (cellobiohydrolase) column with an isocratic mobile phase (90% H_2O, 10% 2-propanol and 1 mM ammonium acetate) for the enantiomeric separation of chiral illicit drugs and pharmaceuticals, including venlafaxine from WRRFs in Valencia, Spain. However, venlafaxine enantiomers were not fully

separated, probably because of the matrix effect [51]. Rice et al. [95] used a ChiralPak CBH column but only for the enantiomeric separation of illicit drugs, even though antidepressants, benzodiazepines and Z-drugs (zolpidem and zopiclone) were included in the study. Castrignanò et al. [96] proposed for the first time a multi-residue method based on a chiral CBH column with an isocratic mobile phase (1 mM NH_4OAc (aq.):MeOH, 85:15, v/v) for the analysis of 56 drug biomarkers at the enantiomeric level. These included five antidepressants and eight benzodiazepines from a WRRF in the UK. For the analytes injected as racemates, including illicit drugs and pharmaceuticals: venlafaxine, O-desmethyl venlafaxine, fluoxetine, norfluoxetine, enantiomeric fractions (EF) were on average 0.49 and were reproducible among different concentrations tested [96]. For most analytes, the enantiomeric resolution was acceptable ($Rs \geq 1.0$) and allowed the quantification of individual enantiomers, except for fluoxetine and O-desmethylvenlafaxine, which showed lower enantiomeric resolution. Consequently, the results for their single enantiomers should be considered semi-quantitative. The authors also tested two more columns, Chirobiotic V and T, having macrocyclic antibiotics as chiral selectors. The Chirobiotic T column showed higher enantioselectivity ($Rs \geq 2.2$) only for benzodiazepines, which enantiomerically were unresolved using the CBH column. Notably, for most chiral benzodiazepines, polar organic mobile phases containing only methanol and 99% methanol/0.005% FA/1 mM ammonium acetate provide the best chiral recognition. However, Castrignanò et al. [96] showed that mobile phases containing comparable quantities of an acid as a mobile phase additive showed better chiral recognition at lower concentrations of ammonium acetate. Based on a comparison between Chiral CBH and Chirobiotic V columns published by Bagnall et al. [125], it can be concluded that, at least in the case of antidepressants venlafaxine and fluoxetine, the Chirobiotic V column was more selective with $Rs > 4.3$ and > 2.2 respectively.

Other methods for separating enantiomers are also possible. For instance, Gonçalves et al. [82] used derivatisation, developing an indirect multi-residue analytical GC–MS method for the enantiomeric quantification of three different classes of compounds: synthetic psychoactive stimulants, antidepressants and β-blockers. Although other enantiomerically pure chiral derivatising reagents (EPDRs) were investigated, (R)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (R-MTPA-Cl) proved the best. However, from the 24 diastereomers formed, they could not separate the two pairs of sertraline and paroxetine enantiomers. Verovšek et al. [126] also used the chiral derivatising agent R-MTPA-Cl and GC-MS/MS for enantiomeric profiling of amphetamines in wastewater. Their study concluded that the unusually high MDMA loads in Ljubljana likely resulted from the disposal of the unused drug, while typically, MDMA loads enriched in the R-enantiomer indicate consumption [126]. Chiral derivatisation can also be cost-effective compared to chiral LC chromatographic columns, e.g., when analysing one-off samples [126]. Although enantiomeric profiling gives a new dimension to WBE, it can also provide more accurate information regarding evidence of the occurrence of chiral pharmaceuticals in the environment for a more precise environmental risk assessment. A good example is fluoxetine, which is often used as a model compound for assessing the antidepressant impact on aquatic organisms. The toxic effects of fluoxetine are enantiomer-dependent, with the toxicity of the S-enantiomer being almost an order of magnitude higher to the fathead minnow (*Pimephales promelas*) than the R-enantiomer [51].

2.3.3. *Mass spectrometry*

In WBE studies, antidepressants, antipsychotics, benzodiazepines and their metabolites have been determined by various mass spectrometers using electrospray ionization (ESI), most frequently in positive mode (Table 3). ESI is known for its susceptibility to the matrix effect that enhances or suppresses the analyte ionization signal. Most reported methodologies include deuterated internal standards to compensate for matrix effects by using them for internal calibration since deuterated standards are expected to behave similarly to non-deuterated substances during ionization [123]. Mass spectrometers include low resolution (LR), such as triple quadrupole mass spectrometer (QqQ) and quadrupole linear ion-trap (QTRAP), and high resolution (HR) instruments, such as quadrupole Orbitrap, QTRAP-Orbitrap and quadrupole time-of-flight (QqTOF) that can capture a wide m/z value range at a good sensitivity without a predetermined target list.

The most widely used analyser for reliable determination and quantification of psychoactive pharmaceuticals in wastewater is triple quadrupole (QqQ) because of its good sensitivity with low ng/L LODs [1,32,40,48,53,56,58,60,61,67,75,79,83,86,88–91,95,102,103,107], followed by QTRAP [29,31,45,55,57,92–94] with similar LODs. Targeted LC-MS/MS or UHPLC-MS/MS methods using either QqQ or QTrap mass analysers are operated in Multiple Reaction Monitoring (MRM) or Selected Reaction Monitoring (SRM) acquisition mode (Table 3). Most MS/MS instruments can simultaneously scan many m/z transitions at high frequency (up to 0.02 s/scan) and have the option to program several segments or monitor different m/z transitions for a short interval before and after the target analytes retention time. However, dynamic Multiple Reaction Monitoring (dMRM) was also used [1,75] since this approach addresses the limitations of classical MRM methods for a large number of analytes by replacing group segmentation with individual time windows for every compound transition and by minimising the amount of individual MRM transitions that are monitored during each cycle. For each analyte, the most abundant transition concerning absolute abundance and signal-to-noise ratio (S/N) was used for quantification (quantifier), while the second transition was used for confirmation (qualifier) [1]. Boogaerts et al. [1] used a sensitive UHPLC-MS/MS using ESI in positive ionization and dMRM modes to determine 27 antidepressants in Belgian WRRFs. The total run time was 20.0 min with lower limits of quantification (LLOQ) for all 27 compounds ranging from 1 to 25 ng/L, with 18 out of the 27 studied biomarkers being confirmed in the wastewater samples.

The use of two HRMS instruments, namely QTOF-MS [11,37,38,46] and LTQ Orbitrap MS [37,43,44,104], for broad-scope screening of pharmaceuticals have been reported in the literature having LOQs from 0.1 to 50 ng/L. Mackuľak et al. [49] used a hybrid Q-Exactive Orbitrap mass analyser to investigate and monitor prescribed psychoactive pharmaceuticals. Although quantitative target monitoring can be performed using HRMS instruments, their application in the WBE studies is limited because of their generally lower sensitivity (potential for a low concentration of analytes to remain undetected) than low-resolution MS/MS instruments. Compared with QqTOF, Orbitrap has a higher resolution at low m/z but a slower data acquisition rate. Commonly, WBE studies focus on target analysis of well-known biomarkers in concentrations that can be detected and quantified. However, the number of non-target and suspect screening WBE studies has increased but focused on new psychoactive substances (NPS). Causanilles et al. [37] presented a suspect screening approach with LC coupled to HRMS, where the data were processed using an algorithm that involves the extraction of accurate masses (calculated based on the molecular formula) of expected m/z from an in-house database containing about 2000 entries, including the antidepressant venlafaxine, the antipsychotic clozapine, benzodiazepines (diazepam, oxazepam and temazepam), NPS and transformation products. The findings highlight the presence of benzodiazepines among other pharmaceuticals and NPS, while mCPP was identified for the first time.

Target and suspect screening methodologies by UHPLC-QTOF have been introduced to establish a broad analytical tool for analysing different illicit and prescribed drugs in sewage-based samples, such as wastewater and passive sample extracts [11,46]. Similar suspect screening has been reported by Diamanti et al. [38], Bijlsma et al. [36] and Bade et al. [73], but only focused on the occurrence of NPS in influent wastewater. Nevertheless, it is worth mentioning the approach of Bade et al. [100] for determining psychoactive pharmaceuticals and

NPS in influent wastewater using a qualitative screening and a quantitative approach during a detailed investigation of licit and illicit drugs in Australian sewage. In their study, a subset of these compounds was included in a quantitative assessment, while qualitative data was captured using a QqTOF Sequential Window Acquisition of all Theoretical fragment-ion spectra (SWATH mode). SWATH allows fragmentation of every available analyte in a sample providing comprehensive MS and MS/MS data for everything detectable in the sample. This feature allows analytes with similar fragment ions but different $[M + H]^+$ to be differentiated [100]. A database recording retention time and exact mass were created from standard solutions assessed using the SWATH technique. As for the quantitative approach, the number of recovered data is reduced compared to the screening approach. The authors used a QqQ that targeted 22 drugs but with higher sensitivity. Overall, Bade et al. [100] confirmed or detected 100 analytes, including the antidepressants: amitriptyline, dothiepin and doxepin, and the antipsychotics: amisulpride and clozapine, in all the samples. As we can see, qualitative screening and quantitative analysis for psychoactive pharmaceuticals and NPS in wastewater have their purpose, but their combined use can be valuable because it gives a more comprehensive overview of their consumption patterns.

2.4. Stability

Several uncertainties are associated with WBE, especially the stability of biomarkers in wastewater. For example, stability is important in 24-h composite sampling and during sample storage since certain compounds may degrade significantly with time [127]. In the WBE literature, many studies evaluate the stability of illicit drug biomarkers in sewage and the uncertainties related to back-calculation of their use; however, only in the last few years has the number of publications increased evaluating the in-sample and in-sewer stability of antidepressants and benzodiazepines [1,16,33,46,55,113,128–132]. The stability of a pharmaceutical in wastewater depends on the matrix complexity (including microorganisms and potential biodegradation), the residence time, the pH, the temperature, and the influence of suspended solids [52]. In order to assess stability, the residual percentage is used for each compound separately and based on McCall et al. [132], compounds are classified into four groups, e.g. those with high stability (< 20% transformation), medium stability (20–60% transformation), low stability (60–100% transformation) and variable stability during a 24 h-period. Boogaerts et al. [1] suggest biomarkers showing medium to low stability should be replaced, if possible, with more suitable ones (stable). Otherwise, they must be excluded from the analytical assays as they will not be suitable for performing back-calculations in WBE.

In reported stability studies, the experimental conditions included different temperatures (−20 to 20 °C), sampling times (e.g. 2, 5, 6, 10, 12, 24, 48, 72 h) and pH (2–9). In-sample stability of antidepressants at standard room temperature and at different time points during up to 48 h has been investigated for mirtazapine, normirtazapine, venlafaxine, O-desmethylvenlafaxine, sertraline, norsertraline, fluoxetine, norfluoxetine, duloxetine, citalopram, desmethylcitalopram and trazodone, with no significant degradation in any case and relative responses > 80% [33]. Boogaerts et al. [1] also showed similar results for 27 antidepressants, including the previously mentioned ones, with all targeted biomarkers having high in-sample stability, except for mianserin which showed only medium stability (> 20% decrease). However, they showed that its metabolite normianserin, showing higher stability, could be considered a more suitable biomarker. Long-term storage stability on either Oasis HLB or MCX cartridges (storage at −20 °C for six weeks) was confirmed for the antidepressants [1,33,128], making this storage method for multi-class compounds more suitable than freezing large volumes of wastewater samples. In-sample stability of benzodiazepines has also been investigated by Bade et al. [55], Racamonde et al. [113], and Baker et al. [127] at different wastewater matrices (natural pH and acidified to pH 2) at three temperatures (4 °C, −20 °C and room temperature: 20 °C) with similar results and in agreement that at − 20 °C most of the analytes were stable under all experimental conditions (<20% transformation) for up to one week. All compounds were stable at room temperature for up to 72 h in raw wastewater (natural pH), except for clonazepam and flunitrazepam, where a 50% decrease was observed already during the first 24 h. At the same time, after a week, > 80% of diazepam and nordiazepam had degraded and approximately 50% of the chlordiazepoxide, lorazepam, lormetazepam, midazolam, oxazepam, temazepam and zolpidem. Furthermore, although refrigeration at 4 °C improved the stability of certain benzodiazepines, clonazepam and flunitrazepam were still 50% degraded within 24 h [55,113,128]. Acidification (pH 2) improved compound stability, especially for pyrazolam, clonazepam, flunitrazepam, diazepam and nordiazepam [113]. However, the benzodiazepine metabolites: 7-aminoclonazepam and 7-aminoflunitrazepam, which have 7-amino functionalities, completely disappeared immediately when the sample was adjusted to pH = 2 [113]. Alternatively, Bade et al. [55] reported that acid preservation was worse than wastewater without prior acidification for the compounds clonazepam, flubromazepam and nitrazepam, which exhibited low stability (>60% degradation) after one week. These results agree with previous studies [113,128] indicating that acidification can reduce the stability of certain benzodiazepines. Additionally, Bade et al. [55] also examined stability with sodium metabisulphite, which is used as a disinfectant, antioxidant, and preservative. They recommend it as a good preservative for wastewater samples since analytes were found to be stable at all tested temperatures (−20, 4 and 20 °C) for up to one week.

Storing samples at low temperatures (−20, −80 °C) and using preservatives are suitable ways of minimising biomarker transformation. However, they can only be applied at the sampling point and cannot prevent any in-sewer degradation. Uncertainties for in-sewer stability could be much higher because of sewer biofilms since there is a lack of control over the microbiota in the sewer system [71]. For some biomarkers with higher log Kow, i.e. fluoxetine, sorption to biofilm or particulate matter can also contribute to the overall uncertainty of the WBE approach [71]. Different methods have been applied to explore the in-sewer transformation since WBE biomarkers degradation in the sewer is a significant knowledge gap in the literature with high uncertainties [132]. Baz-Lomba et al. [46] estimated biomarker degradation, including benzodiazepines and antidepressants, during the in-pipe transit time for a single wastewater sample. Results included 120 h monitoring of compound stability, which showed degradation of many target analytes, such as nitrazepam (−79%) and phenazepam (−70%), agreeing with other studies [16]. Gao et al. [128] used a pilot sewer system to evaluate the stability of biomarkers, including antidepressants, under realistic and variable sewer conditions and observed that carbamazepine, citalopram, venlafaxine and trazodone remain stable. Additionally, Choi et al. [130] compared the stability of 33 licit drug and pharmaceutical biomarkers, including carbamazepine, venlafaxine, O-desmethylvenlafaxine, citalopram, norcitalopram, moclobemide, mirtazapine and amisulpride, in in-sewer biofilm reactors and in biofilm-free (BFF) conditions showing most of the analytes transformed in the presence of biofilm, while being stable under biofilm-free conditions. Carbamazepine, a known bio-recalcitrant pharmaceutical, was stable under both conditions [130]. It is worth mentioning that stability under biofilm-free conditions contradicted degradation under sewer conditions, indicating that biofilm-free conditions do not reflect sewer conditions in the same manner, which is expected since biofilm-free conditions cannot support biochemical activities. [130]. These results agree with Lin et al. [129], who investigated the biodegradation of antidepressants, among others, in wastewater and biofilm sewers under different temperatures, pH and biofilm conditions, confirming that the in-sewer biodegradation with either anaerobic or aerobic biofilms is higher than in BFF conditions, with some compounds such as paroxetine and sertraline having low stability (80% transformation within 6 h). In addition, despite that the number of papers investigating the stability of

pharmaceutical biomarkers in WBE studies has increased, in the case of antidepressants and benzodiazepines, the stability of antipsychotics remains unexplored and more studies of the in-sample and in-sewer fate of target pharmaceuticals are needed to reduce the uncertainty in the estimation of population consumption and, therefore, provide a more accurate and reliable application in WBE.

3. WBE applications on psychoactive pharmaceuticals

3.1. Population size estimation

Most reported studies investigating psychoactive pharmaceuticals use population numbers provided by the WRRFs, which do not consider population mobility and contribute to overall uncertainty levels as high as 55% [16,86]. This uncertainty is problematic, especially when distinguishing whether the fluctuations in population-normalised mass loads were due to changes in consumption patterns, differing numbers of consumers, or variations in the catchment population. Thus, it would be more appropriate to use dynamic population size numbers using either an anthropogenic biomarker [133] or other data such as mobile phone data [86,109]. Been et al. [133] showed that fluctuations in the size of the population over long periods or during major events could be monitored by back-calculating the number of inhabitants (using population equivalents of NH_4–N) or using raw NH_4–N loads as an indirect marker of the population if the objective is to monitor consumption in a specific area.

O'Brien et al. [109] collected wastewater samples on a Census day as a basis for a model to estimate the number of people contributing to a given wastewater sample. The de facto population was then estimated through Bayesian inference, incorporating chemical markers representing population size into a population estimation model and validating this model using cross-validation with measured chemical mass loads. Thomas et al. [86] proposed using mobile device-based population activity (MPA) patterns to calculate real-time population within a specific community, which could be helpful in WBE studies. The authors concluded that mobile device analytics might be promising since it addresses the uncertainties associated with short- and long-term changes in population. As evidenced by Thomas et al. [86] and Baz Lomba et al. [134], using MPA data is the most accurate option for estimating the *de facto* population contributing to a specific catchment area and the back calculation of the population-normalised mass loads. Very recently, Boogaerts et al. [135] published their study regarding monitoring temporal trends in stimulants' use during the COVID-19 pandemic in Belgium, using mobile network data as a dynamic population marker for the normalisation of mass loads. However, these mobile device data resources are not always available and may be costly.

In addition, MPA data provide WBE scientists with population data that can be used to investigate and validate new (or improved) (hydro) chemical and hydrodynamic population markers in cases where using mobile device data is not an option [86]. In this light, Baz-Lomba et al. [134] assessed and compared for the first time the performance of three different population size proxies, including drinking water production, electricity consumption, and online measurements of ammonium in wastewater for the population normalisation of WBE data from a wastewater catchment in Oslo (Norway) using MPA data. The ammonium model showed the best correlation ($R^2 = 0.88$) with the estimated mean annual population to be 4% higher than the "de jure" population reported by the WRRF. Drinking water production and electricity consumption showed more discrepancies due to changing conditions and seasonality, underestimating the population by 27% and overestimating the population by 59%, respectively [134]. Nevertheless, drinking water production and electricity consumption are two promising complementary proxies that need further evaluation with supplementary MPA data.

3.2. Spatial and temporal analysis

The increasing number of publications shows the ability of WBE to investigate spatial and temporal differences in the consumption of antidepressants, antipsychotics and benzodiazepines [1], [11], [30]–[32], [39], [40], [43]–[50], [53]–[55], [57]–[64], [67], [79], [84], [89], [90], [92], [93], [95], [96], [102], [103], [105], [106], [116]. However, measured concentrations must be normalized for varying flow rates and population units to allow reliable comparisons between locations and periods. Population-normalized mass loads in mg/day/1000 inhabitants can compare intra- and inter-country patterns and gather information on the long-term consumption of pharmaceuticals, indicating whether their use is stable or fluctuating. Fig. 3 illustrates the global data on consumption in mg/day/1000 inhabitants for some of the most frequently detected psychoactive pharmaceuticals, namely carbamazepine and venlafaxine for antidepressants, quetiapine for antipsychotics and oxazepam for benzodiazepines. Between 2010 and 2014, a period of a severe economic crisis in Greece, Thomaidis et al. [106] reported a high increase in the per capita consumption of antipsychotics (35-fold), benzodiazepines (19-fold) and antidepressants (11-fold) in Athens. Later, other studies performed in Athens, during 2019–2020 (before and during the COVID-19 pandemic), by Galani et al. [89] and Alygizakis et al. [90] revealed a 20% increase for benzodiazepines, 3% for antidepressants (with a notable increase for venlafaxine, i.e., from 383 g/day in 2019 to 1059 g/day in 2020) and no increase for antipsychotics. A remarkable increase of 60% and 77% was observed in the mass loads of carbamazepine and lorazepam, respectively. However, the detected mass loads for some benzodiazepines did not follow the same trend, such as oxazepam, which showed a slight decrease of 16%. Reinstadler et al. [92] report similar observations in Austria in 2016–2020 (before and during the COVID-19 pandemic lockdown). Their data show no statistically significant difference between 2016 and 2020, except for venlafaxine, for which a temporal trend was observed (an increase in annual mass loads of 5–10%). Kosma et al. [43] estimated pharmaceutical mass loads ranging from 0.2 to 129 mg/day/1000 inhabitants, with the highest mass loads observed for venlafaxine and its metabolite O-desmethylvenlafaxine, followed by fluoxetine, sertraline and mirtazapine. The results were similar or lower than those reported in other studies in Greece, Belgium, India and the USA [1,39,54,62,63,109].

Papageorgiou et al. [39] showed that the mass loads for psychiatric drugs were stable during all seasons compared to non-steroidal anti-inflammatory drugs (NSAIDs), which recorded higher mass loads in winter and autumn. In contrast, Mackul'ak et al. [49] found higher mass loads for venlafaxine and citalopram during winter, while oxazepam did not show any fluctuations during the long-term monitoring. In their study in Hong Kong, Wu et al. [57] reported no apparent seasonal variation for most of the studied pharmaceuticals except for mirtazapine, fluoxetine, citalopram, and sertraline at higher levels during the wet season compared to the dry season. Boogaerts et al. [1] observed spatial differences for all the studied antidepressants, with venlafaxine, citalopram, trazodone and their metabolites showing the highest population-normalized mass loads. Regarding temporal variations, only trazodone followed a monotonic trend, according to the Mann-Kendall trend analysis that had been applied. Baker et al. [84] in the Czech Republic showed no clear weekly pattern for antidepressants, although an increase of 23% for amitriptyline during the weekend could indicate a possible temporal variation. Archer et al. [60] did not observe any notable trend in temporal variation for carbamazepine and its metabolite 10,11-dihydro-11-hydroxycarbamazepine during a two-year study in South Africa, trying to investigate any correlation between weekdays and seasons. Subedi and Kannan [105] reported for the first-time concentrations of norquetiapine and α-hydroxy alprazolam, respectively, metabolites of quetiapine and alprazolam in wastewater influents, emphasizing the importance of monitoring metabolites as more appropriate biomarkers for the calculation of mass loads. According to a study

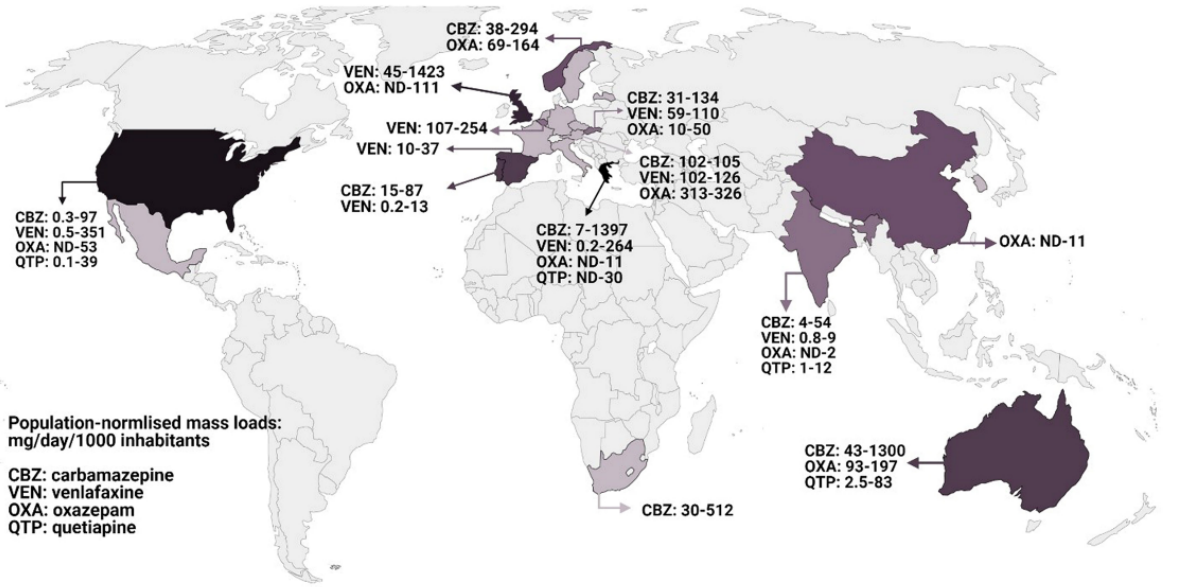


Fig. 3. Population-normalised mass loads in mg/day/1000 inhabitants globally for some of the most frequently detected psychoactive pharmaceuticals, namely carbamazepine, venlafaxine, quetiapine and oxazepam. The coloured countries provided data on population-normalised mass loads: the darker the country's colour, the larger number of published studies.

in Spain by Gushgari et al. [79], mass loads for alprazolam were higher during the week than at weekends, but this was not the case for its metabolite, α-hydroxy alprazolam, which may be explained due to in-sewer transformation and degradation.

Lei et al. [32] monitored the consumption of ten benzodiazepines for six consecutive months in Guangzhou, China. The total estimated consumption was 1012 mg/day/1000 inhabitants, with clozapine, diazepam, and estazolam the most widely used and higher than a study in London, by Baker et al. [47], showing an average consumption estimation of 760, 121 and 95.3 mg/day/1000 inhabitants, respectively. From the measured benzodiazepines and their metabolites in South Australia during two sampling periods, Bade et al. [55] found no significant differences, with oxazepam showing the highest mass loads (100–180 mg/day/1000 inhabitants), followed by temazepam (35–120 mg/day/1000 inhabitants) and quetiapine (5–80 mg/day/1000 inhabitants), which was expected since oxazepam has multiple contributing parent compounds. Even though the mass loads for alprazolam and etizolam were below 1 mg/day/1000 inhabitants, etizolam is concerning since it is an illicit alternative to alprazolam in Australia and USA, while it is legal in Italy, India and Japan [55]. In the UK, from 2014 to 2018, Rice et al. [95] showed a significant increase in mass loads for oxazepam, temazepam, and venlafaxine. Temazepam is an excellent example of the most typical trend, showing a notable decrease in daily intake from 2015 to 2016, followed by significant increases between 2016 and 2018 without any weekend effect. The zolpidem use rate was relatively high in South Korea, according to Kim and Oh [59], where zolpidem and its metabolite mCPP were detected in all studied WRRFs with a mean estimated consumption rate to be 2.3 times higher than the maximum mean concentration rate found by Oliveira et al. in their study in the USA [75]. Among the measured pharmaceuticals by Ng et al. [58] in WRRFs from the UK, USA and Mexico, the mass loads for clozapine, carbamazepine and its metabolite carbamazepine-10,11-epoxide were higher in Monterrey than in London. At the same time, fewer psychoactive pharmaceutical residues were detected in Monterrey compared to London. In the same study, oxazepam showed noticeably high mass loads in the USA, whereas carbamazepine was rarely measured. The different profiles of psychoactive pharmaceuticals and their metabolites measured within the same country and in other countries indicate differences in consumption patterns and trends (Table S1).

4. Conclusions and future trends

Influent wastewater is a treasure of information reflecting population health and lifestyle, and though WBE is a relatively new approach, many studies have shown its potential to provide complementary, accurate, independent and timely epidemiologic information on the consumption of xenobiotics at high spatial and temporal levels. Efforts have been made to develop a broader range of bioanalytical methods that simultaneously measure several antidepressants, antipsychotics, benzodiazepines, and their metabolites from influent wastewater samples in low concentrations and different periods. However, WBE also has its limitations regarding the individual use of pharmaceuticals, the dose or the frequency of administration since it cannot distinguish between one user using large amounts versus many users using small amounts. A better understanding of methodological and compound-specific uncertainties such as sampling and storage conditions, metabolism, biomarker excretion, in-sewer and in-sample stability, flow rates, and population catchment size will be needed to convert concentrations of human biomarkers to population-normalized mass loads and measure the pharmaceutical consumption. When applying WBE in site-specific studies, the major uncertainty that derives from the sampling stage (commonly used time proportional) can be overcome by using flow-proportional sampling or passive sampling. Although passive samplers are still somewhat limited and need to be improved and further explored, a novel active-passive sampling approach for simultaneously measuring a wide range of pollutants at different concentration levels seems promising.

From an analytical point of view, SPE for the sample preparation and conventional target analysis by liquid chromatography coupled to mass spectrometry, either LC-QqQ-MS or QTrap MS, were the most widely used techniques in WBE for the identification and quantification of human biomarkers due to their high sensitivity. Recently, high-resolution mass spectrometry (HRMS) methods have gained popularity

in wastewater analysis, especially for identifying NPS through suspect and non-target screening. WBE is one of the few data sources that can objectively measure pharmaceutical ab(use) and the extent of illegal pharmaceutical consumption in the population with a better understanding of the biomarkers. At the same time, it can also be used as an early warning system to monitor new substances in communities at a high spatiotemporal resolution.

In the future, more research needs to be done regarding the in-sample and in-sewer stability of psychoactive pharmaceuticals, especially for benzodiazepines and antipsychotics, since, for the latter, this field remains almost unexplored. Additionally, it is important to make efforts towards replacing fixed population units with a dynamic population proxy, using mobile network data or measurements of ammonium in wastewater, when normalising mass loads in order to decrease the uncertainty in the estimation of population consumption and, therefore, have a more precise, accurate and reliable application in WBE.

CRediT authorship contribution statement

Maria Laimou-Geraniou: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **David Heath:** Conceptualization, Writing – review & editing, Visualization. **Ester Heath:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Financial support: This work was supported by the Slovenian Research Agency (ARRS Program group P1–0143 and Project N1–0143).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.teac.2022.e00192.

References

[1] T. Boogaerts, M. Degreef, A. Covaci, A.L.N. van Nuijs, 'Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach', Talanta vol. 200 (2019) 340–349, https://doi.org/10.1016/j.talanta.2019.03.052.
[2] 'Mental disorders'. {https://www.who.int/news-room/fact-sheets/detail/mental-disorders} (accessed Mar. 24, 2022).
[3] 'Antidepressant consumption in selected countries 2020', *Statista*. {https://www.statista.com/statistics/283072/antidepressant-consumption-in-selected-countries/} (accessed Jun. 07, 2022).
[4] 'Antidepressants Market Size, Growth | Global Report, 2020–2027'. {https://www.fortunebusinessinsights.com/antidepressants-market-105017} (accessed Jun. 07, 2022).
[5] 'Antipsychotic Drugs Market Size & Share | Global Report [2029]'. {https://www.fortunebusinessinsights.com/industry-reports/antipsychotic-drugs-market-101390} (accessed Jun. 07, 2022).
[6] 'New Benzodiazepines In Europe – A Review PDF Documents', *123doku.com*. {https://123doku.com/document/1604c_new-benzodiazepines-in-europe-a-review.html} (accessed Jun. 07, 2022).
[7] 'European drug report 2021: trends and developments', p. 60.
[8] Observatoire européen des drogues et des toxicomanies, European Drug Report: Trends and Developments, Publications office of the European Union,, Luxembourg, 2021.
[9] D. Vitale, M.M. Suárez-Varela, Y. Picó, 'Wastewater-based epidemiology (WBE), a tool to bridge biomarkers of exposure, contaminants and human health', Curr. Opin. Environ. Sci. Health (2021), 100229 https://doi.org/10.1016/j.coesh.2021.100229.
[10] B. Subedi, Estimation of community usage of drugs utilizing sewage epidemiology, in: R.A. Musah (Ed.), Analysis of Drugs of Abuse, Springer, New York, NY, 2018, pp. 141–147, doi: 10.1007/978-1-4939-8579-1_14.
[11] J.A. Baz-Lomba, C. Harman, M. Reid, K.V. Thomas, 'Passive sampling of wastewater as a tool for the long-term monitoring of community exposure: Illicit and prescription drug trends as a proof of concept', Water Res. vol. 121 (2017) 221–230, https://doi.org/10.1016/j.watres.2017.05.041.
[12] M.J. Andrés-Costa, V. Andreu, Y. Picó, 'Liquid chromatography–mass spectrometry as a tool for wastewater-based epidemiology: Assessing new psychoactive substances and other human biomarkers', TrAC Trends Anal. Chem. vol. 94 (2017) 21–38, https://doi.org/10.1016/j.trac.2017.06.012.
[13] A.L.N. van Nuijs, et al., Illicit drug consumption estimations derived from wastewater analysis: A critical review', Sci. Total Environ. vol. 409 (19) (2011) 3564–3577, https://doi.org/10.1016/j.scitotenv.2010.05.030.
[14] T. Verovšek, I. Krizman-Matasic, D. Heath, E. Heath, 'Site- and event-specific wastewater-based epidemiology: current status and future perspectives', Trends Environ. Anal. Chem. (2020), e00105 https://doi.org/10.1016/j.teac.2020.e00105.
[15] E. Zuccato, et al., Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse', Environ. Health vol. 4 (1) (2005) 14, https://doi.org/10.1186/1476-069X-4-14.
[16] S. Castiglioni, et al., Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers', Environ. Sci. Technol. vol. 47 (3) (2013) 1452–1460, https://doi.org/10.1021/es302722f.
[17] A.L.N. van Nuijs, et al., Multi-year inter-laboratory exercises for the analysis of illicit drugs and metabolites in wastewater: Development of a quality control system', TrAC Trends Anal. Chem. vol. 103 (2018) 34–43, https://doi.org/10.1016/j.trac.2018.03.009.
[18] E. Gracia-Lor, N.I. Rousis, E. Zuccato, S. Castiglioni, 'Monitoring caffeine and nicotine use in a nationwide study in Italy using wastewater-based epidemiology', Sci. Total Environ. vol. 747 (2020), 141331 https://doi.org/10.1016/j.scitotenv.2020.141331.
[19] J. Gao, et al., Using wastewater-based epidemiology to estimate consumption of alcohol and nicotine in major cities of China in 2014 and 2016', Environ. Int. vol. 136 (2020), 105492 https://doi.org/10.1016/j.envint.2020.105492.
[20] R. Montes, et al., First nation-wide estimation of tobacco consumption in Spain using wastewater-based epidemiology', Sci. Total Environ. vol. 741 (2020), 140384 https://doi.org/10.1016/j.scitotenv.2020.140384.
[21] Q. Zheng, et al., Long-term trends in tobacco use assessed by wastewater-based epidemiology and its relationship with consumption of nicotine containing products', Environ. Int. vol. 145 (2020), 106088 https://doi.org/10.1016/j.envint.2020.106088.
[22] T. Verovšek, I. Krizman-Matasic, D. Heath, E. Heath, 'Investigation of drugs of abuse in educational institutions using wastewater analysis', Sci. Total Environ. vol. 799 (2021), 150013 https://doi.org/10.1016/j.scitotenv.2021.150013.
[23] E. Gracia-Lor, N.I. Rousis, E. Zuccato, S. Castiglioni, 'Monitoring caffeine and nicotine use in a nationwide study in Italy using wastewater-based epidemiology', Sci. Total Environ. vol. 747 (2020), 141331 https://doi.org/10.1016/j.scitotenv.2020.141331.
[24] E. López-García, et al., Assessing alcohol consumption through wastewater-based epidemiology: Spain as a case study', Drug Alcohol Depend. vol. 215 (2020), 108241 https://doi.org/10.1016/j.drugalcdep.2020.108241.
[25] T. Boogaerts, M. Quireyns, A. Covaci, H. De Loof, A.L.N. van Nuijs, 'Analytical method for the simultaneous determination of a broad range of opioids in influent wastewater: Optimization, validation and applicability to monitor consumption patterns', Talanta vol. 232 (2021), 122443 https://doi.org/10.1016/j.talanta.2021.122443.
[26] D. Li, et al., Trends in artificial sweetener consumption: A 7-year wastewater-based epidemiology study in Queensland, Australia', Sci. Total Environ. vol. 754 (2021), 142438 https://doi.org/10.1016/j.scitotenv.2020.142438.
[27] D.R. Van Stempvoort, S.J. Brown, J. Spoelstra, D. Garda, W.D. Robertson, S. A. Smyth, 'Variable persistence of artificial sweeteners during wastewater treatment: Implications for future use as tracers', Water Res. vol. 184 (2020), 116124 https://doi.org/10.1016/j.watres.2020.116124.
[28] Y. Zhang, et al., Efficient multiresidue determination method for 168 pharmaceuticals and metabolites: Optimization and application to raw wastewater, wastewater effluent, and surface water in Beijing, China', Environ. Pollut. vol. 261 (2020), 114113 https://doi.org/10.1016/j.envpol.2020.114113.
[29] S. McKay, et al., Calibration and validation of a microporous polyethylene passive sampler for quantitative estimation of illicit drug and pharmaceutical and personal care product (PPCP) concentrations in wastewater influent', Sci. Total Environ. vol. 704 (2020), 135891 https://doi.org/10.1016/j.scitotenv.2019.135891.
[30] F. Riva, S. Castiglioni, C. Pacciani, E. Zuccato, 'Testing urban wastewater to assess compliance with prescription data through wastewater-based epidemiology: First case study in Italy', Sci. Total Environ. vol. 739 (2020), 139741 https://doi.org/10.1016/j.scitotenv.2020.139741.
[31] M. Escolà Casas, et al., Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution', Environ. Int. vol. 150 (2021), 106404 https://doi.org/10.1016/j.envint.2021.106404.
[32] H.-J. Lei, et al., Occurrence, fate and mass loading of benzodiazepines and their transformation products in eleven wastewater treatment plants in Guangdong

province, China', Sci. Total Environ. vol. 755 (2021), 142648 https://doi.org/10.1016/j.scitotenv.2020.142648.
[33] I. González-Mariño, et al., Multi-residue determination of psychoactive pharmaceuticals, illicit drugs and related metabolites in wastewater by ultra-high performance liquid chromatography-tandem mass spectrometry', J. Chromatogr. A vol. 1569 (2018) 91–100, https://doi.org/10.1016/j.chroma.2018.07.045.
[34] L. Gent, R. Paul, 'The detection of new psychoactive substances in wastewater. A comprehensive review of analytical approaches and global trends', Sci. Total Environ. vol. 776 (2021), 146028 https://doi.org/10.1016/j.scitotenv.2021.146028.
[35] R. Bade, J.M. White, C. Gerber, 'How the recreational stimulant market has changed: Case study in Adelaide, Australia 2016–2019', Sci. Total Environ. vol. 757 (2021), 143728 https://doi.org/10.1016/j.scitotenv.2020.143728.
[36] L. Bijlsma, A. Celma, F.J. López, F. Hernández, 'Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations', Curr. Opin. Environ. Sci. Health vol. 9 (2019) 1–12, https://doi.org/10.1016/j.coesh.2019.03.002.
[37] A. Causanilles, et al., Qualitative screening for new psychoactive substances in wastewater collected during a city festival using liquid chromatography coupled to high-resolution mass spectrometry', Chemosphere vol. 184 (2017) 1186–1193, https://doi.org/10.1016/j.chemosphere.2017.06.101.
[38] K. Diamanti, R. Aalizadeh, N. Alygizakis, A. Galani, M. Mardal, N.S. Thomaidis, 'Wide-scope target and suspect screening methodologies to investigate the occurrence of new psychoactive substances in influent wastewater from Athens', Sci. Total Environ. vol. 685 (2019) 1058–1065, https://doi.org/10.1016/j.scitotenv.2019.06.173.
[39] M. Papageorgiou, C. Kosma, D. Lambropoulou, 'Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece', Sci. Total Environ. vol. 543 (2016) 547–569, https://doi.org/10.1016/j.scitotenv.2015.11.047.
[40] M. Papageorgiou, I. Zioris, T. Danis, D. Bikiaris, D. Lambropoulou, 'Comprehensive investigation of a wide range of pharmaceuticals and personal care products in urban and hospital wastewaters in Greece', Sci. Total Environ. vol. 694 (2019), 133565 https://doi.org/10.1016/j.scitotenv.2019.07.371.
[41] D. Barceló, 'Wastewater-Based Epidemiology to monitor COVID-19 outbreak: Present and future diagnostic methods to be in your radar', Case Stud. Chem. Environ. Eng. vol. 2 (2020) https://doi.org/10.1016/j.cscee.2020.100042.
[42] P. Foladori, et al., SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review', Sci. Total Environ. vol. 743 (2020) https://doi.org/10.1016/j.scitotenv.2020.140444.
[43] C.I. Kosma, C.I. Nannou, V.I. Boti, T.A. Albanis, 'Psychiatrics and selected metabolites in hospital and urban wastewaters: Occurrence, removal, mass loading, seasonal influence and risk assessment', Sci. Total Environ. vol. 659 (2019) 1473–1483, https://doi.org/10.1016/j.scitotenv.2018.12.421.
[44] M. Fáberová, I. Bodík, L. Ivanová, R. Grabic, T. Mackuľak, 'Frequency and use of pharmaceuticals in selected Slovakian town via wastewater analysis', Mon. Chem. vol. 148 (3) (2017) 441–448, https://doi.org/10.1007/s00706-016-1853-0.
[45] N. Mastroianni, E. López-García, C. Postigo, D. Barceló, M. López de Alda, Five-year monitoring of 19 illicit and legal substances of abuse at the inlet of a wastewater treatment plant in Barcelona (NE Spain) and estimation of drug consumption patterns and trends', Sci. Total Environ. vol. 609 (2017) 916–926, https://doi.org/10.1016/j.scitotenv.2017.07.126.
[46] J.A. Baz-Lomba, M.J. Reid, K.V. Thomas, 'Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF', Anal. Chim. Acta vol. 914 (2016) 81–90, https://doi.org/10.1016/j.aca.2016.01.056.
[47] D.R. Baker, L. Barron, B. Kasprzyk-Hordern, 'Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates', Sci. Total Environ. vol. 487 (2014) 629–641, https://doi.org/10.1016/j.scitotenv.2013.11.107.
[48] M. Östman, J. Fick, E. Näsström, R.H. Lindberg, 'A snapshot of illicit drug use in Sweden acquired through sewage water analysis', Sci. Total Environ. vol. 472 (2014) 862–871, https://doi.org/10.1016/j.scitotenv.2013.11.081.
[49] T. Mackuľak, et al., Wastewater analysis: the mean of the monitoring of frequently prescribed pharmaceuticals in Slovakia', Environ. Monit. Assess. vol. 188 (1) (2016) 18, https://doi.org/10.1007/s10661-015-5011-7.
[50] T. Thiebault, L. Fougère, E. Destandau, M. Réty, J. Jacob, 'Temporal dynamics of human-excreted pollutants in wastewater treatment plant influents: Toward a better knowledge of mass load fluctuations', Sci. Total Environ. vol. 596–597 (2017) 246–255, https://doi.org/10.1016/j.scitotenv.2017.04.130.
[51] P. Vazquez-Roig, B. Kasprzyk-Hordern, C. Blasco, Y. Picó, 'Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters of Valencia (Spain, Sci. Total Environ. vol. 494–495 (2014) 49–57, https://doi.org/10.1016/j.scitotenv.2014.06.098.
[52] T.L. Croft, R.A. Huffines, M. Pathak, B. Subedi, 'Prevalence of illicit and prescribed neuropsychiatric drugs in three communities in Kentucky using wastewater-based epidemiology and Monte Carlo simulation for the estimation of associated uncertainties', J. Hazard. Mater. vol. 384 (2020), 121306 https://doi.org/10.1016/j.jhazmat.2019.121306.
[53] N. Bishop, T. Jones-Lepp, M. Margetts, J. Sykes, D. Alvarez, D.E. Keil, 'Wastewater-based epidemiology pilot study to examine drug use in the Western United States', Sci. Total Environ. vol. 745 (2020), 140697 https://doi.org/10.1016/j.scitotenv.2020.140697.
[54] A.J. Skees, K.S. Foppe, B. Loganathan, B. Subedi, 'Contamination profiles, mass loadings, and sewage epidemiology of neuropsychiatric and illicit drugs in wastewater and river waters from a community in the Midwestern United States', Sci. Total Environ. vol. 631–632 (2018) 1457–1464, https://doi.org/10.1016/j.scitotenv.2018.03.060.
[55] R. Bade, M. Ghetia, J.M. White, C. Gerber, 'Determination of prescribed and designer benzodiazepines and metabolites in influent wastewater', Anal. Methods vol. 12 (28) (2020) 3637–3644, https://doi.org/10.1039/D0AY00560F.
[56] T. Gao, P. Du, Z. Xu, X. Li, 'Occurrence of new psychoactive substances in wastewater of major Chinese cities', Sci. Total Environ. vol. 575 (2017) 963–969, https://doi.org/10.1016/j.scitotenv.2016.09.152.
[57] R. Wu, Y. Ruan, H. Lin, C.N.T. Yuen, H. Feng, P.K.S. Lam, 'Occurrence and Fate of Psychiatric Pharmaceuticals in Wastewater Treatment Plants in Hong Kong: Enantiomeric Profiling and Preliminary Risk Assessment', ACS EST Water vol. 1 (3) (2021) 542–552, https://doi.org/10.1021/acsestwater.0c00081.
[58] K.T. Ng, et al., High-throughput multi-residue quantification of contaminants of emerging concern in wastewaters enabled using direct injection liquid chromatography-tandem mass spectrometry', J. Hazard. Mater. vol. 398 (2020), 122933 https://doi.org/10.1016/j.jhazmat.2020.122933.
[59] K.Y. Kim, J.-E. Oh, 'Evaluation of pharmaceutical abuse and illicit drug use in South Korea by wastewater-based epidemiology', J. Hazard. Mater. vol. 396 (2020), 122622 https://doi.org/10.1016/j.jhazmat.2020.122622.
[60] E. Archer, M. Volschenk, L. Brocker, G.M. Wolfaardt, 'A two-year study of emerging micro-pollutants and drugs of abuse in two Western Cape wastewater treatment works (South Africa, Chemosphere vol. 285 (2021), 131460, https://doi.org/10.1016/j.chemosphere.2021.131460.
[61] L. Fallati, et al., Use of legal and illegal substances in Malé (Republic of Maldives) assessed by wastewater analysis', Sci. Total Environ. vol. 698 (2020), 134207 https://doi.org/10.1016/j.scitotenv.2019.134207.
[62] B. Subedi, K. Balakrishna, R.K. Sinha, N. Yamashita, V.G. Balasubramanian, K. Kannan, 'Mass loading and removal of pharmaceuticals and personal care products, including psychoactive and illicit drugs and artificial sweeteners, in five sewage treatment plants in India', J. Environ. Chem. Eng. vol. 3 (4) (2015) 2882–2891, https://doi.org/10.1016/j.jece.2015.09.031.
[63] B. Subedi, K. Balakrishna, D.I. Joshua, K. Kannan, 'Mass loading and removal of pharmaceuticals and personal care products including psychoactives, antihypertensives, and antibiotics in two sewage treatment plants in southern India', Chemosphere vol. 167 (2017) 429–437, https://doi.org/10.1016/j.chemosphere.2016.10.026.
[64] R.C. Pivetta, C. Rodrigues-Silva, A.R. Ribeiro, S. Rath, 'Tracking the occurrence of psychotropic pharmaceuticals in Brazilian wastewater treatment plants and surface water, with assessment of environmental risks', Sci. Total Environ. vol. 727 (2020), 138661 https://doi.org/10.1016/j.scitotenv.2020.138661.
[65] C. Chen, C. Kostakis, R.J. Irvine, P.D. Felgate, J.M. White, 'Evaluation of pre-analysis loss of dependent drugs in wastewater: stability and binding assessments', Drug Test. Anal. vol. 5 (8) (2013) 716–721, https://doi.org/10.1002/dta.1428.
[66] A.-K. McCall, R. Palmitessa, F. Blumensaat, E. Morgenroth, C. Ort, 'Modeling in-sewer transformations at catchment scale – implications on drug consumption estimates in wastewater-based epidemiology', Water Res. vol. 122 (2017) 655–668, https://doi.org/10.1016/j.watres.2017.05.034.
[67] A.L.N. van Nuijs, et al., Do concentrations of pharmaceuticals in sewage reflect prescription figures, Environ. Sci. Pollut. Res vol. 22 (12) (2015) 9110–9118, https://doi.org/10.1007/s11356-014-4066-2.
[68] M. Lorenzo, Y. Picó, 'Wastewater-based epidemiology: current status and future prospects', Curr. Opin. Environ. Sci. Health vol. 9 (2019) 77–84, https://doi.org/10.1016/j.coesh.2019.05.007.
[69] P.M. Choi, et al., Wastewater-based epidemiology biomarkers: Past, present and future', TrAC Trends Anal. Chem. vol. 105 (2018) 453–469, https://doi.org/10.1016/j.trac.2018.06.004.
[70] Y. Picó, D. Barceló, 'Identification of biomarkers in wastewater-based epidemiology: Main approaches and analytical methods', TrAC Trends Anal. Chem. vol. 145 (2021), 116465 https://doi.org/10.1016/j.trac.2021.116465.
[71] T. Boogaerts, et al., Current and future perspectives for wastewater-based epidemiology as a monitoring tool for pharmaceutical use', Sci. Total Environ. vol. 789 (2021), 148047 https://doi.org/10.1016/j.scitotenv.2021.148047.
[72] E.M. Melchor-Martínez, et al., Antidepressants surveillance in wastewater: Overview extraction and detection', Case Stud. Chem. Environ. Eng. vol. 3 (2021), 100074 https://doi.org/10.1016/j.cscee.2020.100074.
[73] R. Bade, et al., International snapshot of new psychoactive substance use: Case study of eight countries over the 2019/2020 new year period', Water Res. vol. 193 (2021), 116891 https://doi.org/10.1016/j.watres.2021.116891.
[74] C. Ort, et al., 'Wastewater Analysis for Community-Wide Drugs Use Assessment', in: H.H. Maurer, S.D. Brandt (Eds.), New Psychoactive Substances: Pharmacology, Clinical, Forensic and Analytical Toxicology, Springer International Publishing, Cham, 2018, pp. 543–566, https://doi.org/10.1007/164_2018_111.
[75] T.S. Oliveira, M. Murphy, N. Mendola, V. Wong, D. Carlson, L. Waring, 'Characterization of Pharmaceuticals and Personal Care products in hospital effluent and waste water influent/effluent by direct-injection LC-MS-MS', Sci. Total Environ. vol. 518–519 (2015) 459–478, https://doi.org/10.1016/j.scitotenv.2015.02.104.
[76] C. Gómez-Canela, T. Sala-Comorera, V. Pueyo, C. Barata, S. Lacorte, 'Analysis of 44 pharmaceuticals consumed by elderly using liquid chromatography coupled to tandem mass spectrometry', J. Pharm. Biomed. Anal. vol. 168 (2019) 55–63, https://doi.org/10.1016/j.jpba.2019.02.016.
[77] T. Néfau, O. Sannier, C. Hubert, S. Karolak, and Y. Lévi, 'Analysis of drugs in sewage: an approach to assess substance use, applied to a prison setting', p. 21.

[78] C. Postigo, M.L. de Alda, D. Barceló, 'Evaluation of drugs of abuse use and trends in a prison through wastewater analysis', Environ. Int. vol. 37 (1) (2011) 49–55, https://doi.org/10.1016/j.envint.2010.06.012.

[79] A.J. Gushgari, E.M. Driver, J.C. Steele, R.U. Halden, 'Tracking narcotics consumption at a Southwestern U.S. university campus by wastewater-based epidemiology', J. Hazard. Mater. vol. 359 (2018) 437–444, https://doi.org/10.1016/j.jhazmat.2018.07.073.

[80] W. Gul, S.W. Gul, B. Stamper, M. Godfrey, M.A. ElSohly, 'LC-MS-MS Method Development and Analysis of Stimulants, Opiates, Synthetic Opiates, PCP, and Benzodiazepines in Wastewater. Preponderance of these Drugs During Football Games', in: R.A. Musah (Ed.), Analysis of Drugs of Abuse, Springer, New York, NY, 2018, pp. 149–182, doi: 10.1007/978-1-4939-8579-1_15.

[81] T. Mackul'ak, et al., Music festivals and drugs: Wastewater analysis', Sci. Total Environ. vol. 659 (2019) 326–334, https://doi.org/10.1016/j.scitotenv.2018.12.275.

[82] R. Gonçalves, et al., Multi-residue method for enantioseparation of psychoactive substances and beta blockers by gas chromatography–mass spectrometry', J. Chromatogr. B vol. 1125 (2019), 121731 https://doi.org/10.1016/j.jchromb.2019.121731.

[83] A. Bahlmann, W. Brack, R.J. Schneider, M. Krauss, 'Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal', Water Res. vol. 57 (2014) 104–114, https://doi.org/10.1016/j.watres.2014.03.022.

[84] D.R. Baker, V. Očenášková, M. Kvicalova, B. Kasprzyk-Hordern, 'Drugs of abuse in wastewater and suspended particulate matter — Further developments in sewage epidemiology', Environ. Int. vol. 48 (2012) 28–38, https://doi.org/10.1016/j.envint.2012.06.014.

[85] A. Rodayan, M. Majewsky, V. Yargeau, 'Impact of approach used to determine removal levels of drugs of abuse during wastewater treatment', Sci. Total Environ. vol. 487 (2014) 731–739, https://doi.org/10.1016/j.scitotenv.2014.03.080.

[86] K.V. Thomas, A. Amador, J.A. Baz-Lomba, M. Reid, 'Use of mobile device data to better estimate dynamic population size for wastewater-based epidemiology', Environ. Sci. Technol. vol. 51 (19) (2017) 11363–11370, https://doi.org/10.1021/acs.est.7b02538.

[87] C. Ort, M.G. Lawrence, J. Reungoat, J.F. Mueller, 'Sampling for PPCPs in wastewater systems: comparison of different sampling modes and optimization strategies', Environ. Sci. Technol. vol. 44 (16) (2010) 6289–6296, https://doi.org/10.1021/es100778d.

[88] B. Kasprzyk-Hordern, et al., Estimation of community-wide multi-chemical exposure via water-based chemical mining: Key research gaps drawn from a comprehensive multi-biomarker multi-city dataset', Environ. Int. vol. 147 (2021), 106331 https://doi.org/10.1016/j.envint.2020.106331.

[89] A. Galani, N. Alygizakis, R. Aalizadeh, E. Kastritis, M.-A. Dimopoulos, N. S. Thomaidis, 'Patterns of pharmaceuticals use during the first wave of COVID-19 pandemic in Athens, Greece as revealed by wastewater-based epidemiology', Sci. Total Environ. vol. 798 (2021), 149014 https://doi.org/10.1016/j.scitotenv.2021.149014.

[90] N. Alygizakis, A. Galani, N.I. Rousis, R. Aalizadeh, M.-A. Dimopoulos, N. S. Thomaidis, 'Change in the chemical content of untreated wastewater of Athens, Greece under COVID-19 pandemic', Sci. Total Environ. vol. 799 (2021), 149230 https://doi.org/10.1016/j.scitotenv.2021.149230.

[91] P. Paíga, L.H.M.L.M. Santos, S. Ramos, S. Jorge, J.G. Silva, C. Delerue-Matos, 'Presence of pharmaceuticals in the Lis river (Portugal): Sources, fate and seasonal variation', Sci. Total Environ. vol. 573 (2016) 164–177, https://doi.org/10.1016/j.scitotenv.2016.08.089.

[92] V. Reinstadler, et al., Monitoring drug consumption in Innsbruck during coronavirus disease 2019 (COVID-19) lockdown by wastewater analysis', Sci. Total Environ. vol. 757 (2021), 144006 https://doi.org/10.1016/j.scitotenv.2020.144006.

[93] D. Phung, et al., Can wastewater-based epidemiology be used to evaluate the health impact of temperature? – An exploratory study in an Australian population', Environ. Res. vol. 156 (2017) 113–119, https://doi.org/10.1016/j.envres.2017.03.023.

[94] A. Jelic, S. Rodriguez-Mozaz, D. Barceló, O. Gutierrez, 'Impact of in-sewer transformation on 43 pharmaceuticals in a pressurized sewer under anaerobic conditions', Water Res. vol. 68 (2015) 98–108, https://doi.org/10.1016/j.watres.2014.09.033.

[95] J. Rice, A.M. Kannan, E. Castrignanò, K. Jagadeesan, B. Kasprzyk-Hordern, 'Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - A UK perspective', Sci. Total Environ. vol. 735 (2020), 139433 https://doi.org/10.1016/j.scitotenv.2020.139433.

[96] E. Castrignanò, A. Lubben, B. Kasprzyk-Hordern, 'Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry', J. Chromatogr. A vol. 1438 (2016) 84–99, https://doi.org/10.1016/j.chroma.2016.02.015.

[97] L. Bijlsma, R. Bade, F. Been, A. Celma, S. Castiglioni, 'Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial', Anal. Chim. Acta (2020) https://doi.org/10.1016/j.aca.2020.08.058.

[98] J. Criquet, et al., 'Comparison of POCIS passive samplers vs. composite water sampling: A case study', Sci. Total Environ. vol. 609 (2017) 982–991, https://doi.org/10.1016/j.scitotenv.2017.07.227.

[99] V. Yargeau, B. Taylor, H. Li, A. Rodayan, C.D. Metcalfe, 'Analysis of drugs of abuse in wastewater from two Canadian cities', Sci. Total Environ. vol. 487 (2014) 722–730, https://doi.org/10.1016/j.scitotenv.2013.11.094.

[100] R. Bade, J.M. White, C. Gerber, 'Qualitative and quantitative temporal analysis of licit and illicit drugs in wastewater in Australia using liquid chromatography coupled to mass spectrometry', Anal. Bioanal. Chem. vol. 410 (2) (2018) 529–542, https://doi.org/10.1007/s00216-017-0747-2.

[101] P.M. Choi, et al., 'Social, demographic, and economic correlates of food and chemical consumption measured by wastewater-based epidemiology', PNAS vol. 116 (43) (2019) 21864–21873 (Oct.).

[102] A.M.P.T. Pereira, L.J.G. Silva, C.M. Lino, L.M. Meisel, A. Pena, 'Assessing environmental risk of pharmaceuticals in Portugal: An approach for the selection of the Portuguese monitoring stations in line with Directive 2013/39/EU', Chemosphere vol. 144 (2016) 2507–2515, https://doi.org/10.1016/j.chemosphere.2015.10.100.

[103] A.M.P.T. Pereira, L.J.G. Silva, L.M. Meisel, C.M. Lino, A. Pena, 'Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment', Environ. Res. vol. 136 (2015) 108–119, https://doi.org/10.1016/j.envres.2014.09.041.

[104] A. Ofrydopoulou, C. Nannou, E. Evgenidou, A. Christodoulou, D. Lambropoulou, 'Assessment of a wide array of organic micropollutants of emerging concern in wastewater treatment plants in Greece: Occurrence, removals, mass loading and potential risks', Sci. Total Environ. vol. 802 (2022), 149860 https://doi.org/10.1016/j.scitotenv.2021.149860.

[105] B. Subedi, K. Kannan, 'Occurrence and fate of select psychoactive pharmaceuticals and antihypertensives in two wastewater treatment plants in New York State, USA', Sci. Total Environ. vol. 514 (2015) 273–280, https://doi.org/10.1016/j.scitotenv.2015.01.098.

[106] N.S. Thomaidis, et al., Reflection of Socioeconomic Changes in Wastewater: Licit and Illicit Drug Use Patterns', Environ. Sci. Technol. vol. 50 (18) (2016) 10065–10072, https://doi.org/10.1021/acs.est.6b02417.

[107] C. Christophoridis, et al., Determination of illicit drugs and psychoactive pharmaceuticals in wastewater from the area of Thessaloniki (Greece) using LC–MS/MS: estimation of drug consumption', Environ. Monit. Assess. vol. 193 (5) (2021) 249, https://doi.org/10.1007/s10661-021-09035-9.

[108] X. Liu, et al., Field evaluation of diffusive gradients in thin-film passive samplers for wastewater-based epidemiology', Sci. Total Environ. vol. 773 (2021), 145480 https://doi.org/10.1016/j.scitotenv.2021.145480.

[109] J.W. O'Brien, et al., A model to estimate the population contributing to the wastewater using samples collected on census day', Environ. Sci. Technol. vol. 48 (1) (2014) 517–525, https://doi.org/10.1021/es403251g.

[110] E.D. Amato, et al., A novel active-passive sampling approach for measuring time-averaged concentrations of pollutants in water', Chemosphere vol. 209 (2018) 363–372, https://doi.org/10.1016/j.chemosphere.2018.06.079.

[111] E.D. Amato, et al., Characterization of the accumulation of metals and organic contaminants on a novel active-passive sampling device under controlled water flow conditions', Chemosphere vol. 236 (2019), 124400 https://doi.org/10.1016/j.chemosphere.2019.124400.

[112] E.D. Amato, et al., Field application of a novel active-passive sampling technique for the simultaneous measurement of a wide range of contaminants in water', Chemosphere vol. 279 (2021), 130598 https://doi.org/10.1016/j.chemosphere.2021.130598.

[113] I. Racamonde, R. Rodil, J.B. Quintana, E. Villaverde-de-Sáa, R. Cela, 'Determination of benzodiazepines, related pharmaceuticals and metabolites in water by solid-phase extraction and liquid-chromatography–tandem mass spectrometry', J. Chromatogr. A vol. 1352 (2014) 69–79, https://doi.org/10.1016/j.chroma.2014.05.064.

[114] B.J. Tscharke, C. Chen, J.P. Gerber, J.M. White, 'Temporal trends in drug use in Adelaide, South Australia by wastewater analysis', Sci. Total Environ. vol. 565 (2016) 384–391, https://doi.org/10.1016/j.scitotenv.2016.04.183.

[115] D.R. Baker, B. Kasprzyk-Hordern, 'Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography–positive electrospray ionisation tandem mass spectrometry', J. Chromatogr. A vol. 1218 (12) (2011) 1620–1631, https://doi.org/10.1016/j.chroma.2011.01.060.

[116] I. Pugajeva, L.E. Ikkere, M. Jansons, I. Perkons, V. Sukajeva, V. Bartkevics, 'Two-dimensional liquid chromatography - mass spectrometry as an effective tool for assessing a wide range of pharmaceuticals and biomarkers in wastewater-based epidemiology studies', J. Pharm. Biomed. Anal. vol. 205 (2021), 114295 https://doi.org/10.1016/j.jpba.2021.114295.

[117] T. Verovšek, D. Heath, E. Heath, 'Occurrence, fate and determination of tobacco (nicotine) and alcohol (ethanol) residues in waste- and environmental waters', Trends Environ. Anal. Chem. vol. 34 (2022), e00164 https://doi.org/10.1016/j.teac.2022.e00164.

[118] E. López-García, C. Postigo, B. Zonja, D. Barceló, M. López de Alda, Chapter Two - Analysis of Psychoactive Pharmaceuticals in Wastewater and Surface Water Using LC-MS, in: A. Cappiello, P. Palma (Eds.), Comprehensive Analytical Chemistry, vol. 79, Elsevier, 2018, pp. 29–52, doi: 10.1016/bs.coac.2017.08.009.

[119] N. Fontanals, E. Pocurull, F. Borrull, R.M. Marcé, 'Role of solid-phase extraction in wastewater-based epidemiology', Curr. Opin. Environ. Sci. Health vol. 9 (2019) 26–33, https://doi.org/10.1016/j.coesh.2019.03.006.

[120] A.R. Ribeiro, M. Pedrosa, N.F.F. Moreira, M.F.R. Pereira, A.M.T. Silva, 'Environmental friendly method for urban wastewater monitoring of micropollutants defined in the Directive 2013/39/EU and Decision 2015/495/EU', J. Chromatogr. A vol. 1418 (2015) 140–149, https://doi.org/10.1016/j.chroma.2015.09.057.

[121] S. Rodríguez-Mozaz, M. Lopez de Alda, D. Barcelo, 'Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography-mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water', J. Chromatogr. A vol. 1152 (2007) 97–115, https://doi.org/10.1016/j.chroma.2007.01.046.

[122] J.F. Reichert, D.M. Souza, A.F. Martins, 'Antipsychotic drugs in hospital wastewater and a preliminary risk assessment', Ecotoxicol. Environ. Saf. vol. 170 (2019) 559–567, https://doi.org/10.1016/j.ecoenv.2018.12.021.

[123] M. Behpour, S. Nojavan, S. Asadi, A. Shokri, 'Combination of gel-electromembrane extraction with switchable hydrophilicity solvent-based homogeneous liquid-liquid microextraction followed by gas chromatography for the extraction and determination of antidepressants in human serum, breast milk and wastewater', J. Chromatogr. A vol. 1621 (2020), 461041 https://doi.org/10.1016/j.chroma.2020.461041.

[124] B. Kasprzyk-Hordern, D.R. Baker, 'Estimation of community-wide drugs use via stereoselective profiling of sewage', Sci. Total Environ. vol. 423 (2012) 142–150, https://doi.org/10.1016/j.scitotenv.2012.02.019.

[125] J.P. Bagnall, S.E. Evans, M.T. Wort, A.T. Lubben, B. Kasprzyk-Hordern, 'Using chiral liquid chromatography quadrupole time-of-flight mass spectrometry for the analysis of pharmaceuticals and illicit drugs in surface and wastewater at the enantiomeric level', J. Chromatogr. A vol. 1249 (2012) 115–129, https://doi.org/10.1016/j.chroma.2012.06.012.

[126] T. Verovšek, D. Heath, E. Heath, 'Enantiomeric profiling of amphetamines in wastewater using chiral derivatisation with gas chromatographic-tandem mass spectrometric detection', Sci. Total Environ. vol. 835 (2022), 155594 https://doi.org/10.1016/j.scitotenv.2022.155594.

[127] D.R. Baker, B. Kasprzyk-Hordern, 'Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry', J. Chromatogr. A vol. 1218 (44) (2011) 8036–8059, https://doi.org/10.1016/j.chroma.2011.09.012.

[128] J. Gao, et al., Systematic evaluation of biomarker stability in pilot scale sewer pipes', Water Res. vol. 151 (2019) 447–455, https://doi.org/10.1016/j.watres.2018.12.032.

[129] W. Lin, et al., Evaluating the stability of prescription drugs in municipal wastewater and sewers based on wastewater-based epidemiology', Sci. Total Environ. vol. 754 (2021), 142414 https://doi.org/10.1016/j.scitotenv.2020.142414.

[130] P.M. Choi, et al., Considerations for assessing stability of wastewater-based epidemiology biomarkers using biofilm-free and sewer reactor tests', Sci. Total Environ. vol. 709 (2020), 136228 https://doi.org/10.1016/j.scitotenv.2019.136228.

[131] E. Gracia-Lor, et al., Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives', Environ. Int. vol. 99 (2017) 131–150, https://doi.org/10.1016/j.envint.2016.12.016.

[132] A.-K. McCall, et al., Critical review on the stability of illicit drugs in sewers and wastewater samples', Water Res. vol. 88 (2016) 933–947, https://doi.org/10.1016/j.watres.2015.10.040.

[133] F. Been, L. Rossi, C. Ort, S. Rudaz, O. Delémont, P. Esseiva, 'Population normalization with ammonium in wastewater-based epidemiology: application to illicit drug monitoring', Environ. Sci. Technol. vol. 48 (14) (2014) 8162–8169, https://doi.org/10.1021/es5008388.

[134] J.A. Baz-Lomba, F. Di Ruscio, A. Amador, M. Reid, K.V. Thomas, 'Assessing alternative population size proxies in a wastewater catchment area using mobile device data', Environ. Sci. Technol. vol. 53 (4) (2019) 1994–2001, https://doi.org/10.1021/acs.est.8b05389.

[135] T. Boogaerts, et al., Temporal monitoring of stimulants during the COVID-19 pandemic in Belgium through the analysis of influent wastewater', Int. J. Drug Policy vol. 104 (2022), 103679 https://doi.org/10.1016/j.drugpo.2022.103679.

3.1.2 Optimisation, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater

Published: T. Boogaerts, M. Quireyns, F. Maes, M. Laimou-Geraniou, N. Van Wichelen, E. Heath, B. Pussig, B. Aertgeerts, A. Covaci, A. L. N. van Nuijs, 2022, *Drug testing and analysis,* vol. 15 (2), pp. 240-246.

This study is a collaboration between the Jožef Stefan Institute in Ljubljana (Slovenia) and the Ecotoxicological Centre at the University of Antwerp (Belgium). In this work, I contributed to method optimisation and validation, sample preparation, instrumental analysis, data analysis, and manuscript preparation.

Due to the complexity of the wastewater matrix and the low concentrations of human biomarkers within sewage systems, it is essential to have highly sensitive analytical methods available [2]. Furthermore, to enhance the role of WBE as a complementary epidemiological tool, there is a growing demand to analyse a broader range of compounds across more sampling sites with a higher number of samples. To address this, a high-throughput miniaturised SPE protocol was developed using an Oasis MCX 96-well plate with only 2 mL of influent wastewater, followed by LC-MS/MS analysis to quantify 28 psychoactive drug biomarkers, including parent compounds and metabolites of antidepressants, opioids, and drugs of abuse.

The method was validated for linearity, limits of quantification, extraction recovery, selectivity, process efficiency, intra- and inter-day accuracy, and precision, following the guidelines of the European Medicines Agency (EMA), with minor adjustments [223]. LOQs were mostly ≤10 ng/L, making the method sufficiently sensitive for WBE applications despite the reduced pre-concentration compared to conventional SPE protocols. Following the successful application of the developed method to 24-hour composite samples obtained from an urban WWTP in Leuven, Belgium, it was also applied to 24-hour influent wastewater samples from six WWTPs across Slovenia (see 3.2.1 Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters).

The 96-well SPE protocol significantly reduced sample and solvent volume requirements and processing time, making it highly suitable for large-scale or high-frequency WBE campaigns. Overall, this study presented a robust, efficient, precise, and sensitive high-throughput method for monitoring psychoactive substances in wastewater, supporting its integration into national WBE networks for public health surveillance and drug trend monitoring.

The developed method was presented at the 6th International Conference on Testing the Waters (TTW6): The International Conference on Wastewater Surveillance, held in Oxford, UK, June 26-27, 2023 (poster presentation).

Check for updates

Received: 14 September 2022 | Revised: 6 October 2022 | Accepted: 17 October 2022
DOI: 10.1002/dta.3392

SHORT COMMUNICATION

WILEY

Optimization, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater

Tim Boogaerts[1] | Maarten Quireyns[1] | Florence Maes[1] | Maria Laimou-Geraniou[2,3] | Natan Van Wichelen[1] | Ester Heath[2,3] | Bram Pussig[4] | Bert Aertgeerts[4] | Adrian Covaci[1] | Alexander L. N. van Nuijs[1]

[1]Toxicological Centre, University of Antwerp, Wilrijk, Belgium

[2]Department of Environmental Sciences, Jožef Stefan Institute, Ljubljana, Slovenia

[3]Jožef Stefan International Postgraduate School, Ljubljana, Slovenia

[4]Academic Centre for General Practice, KU Leuven, Leuven, Belgium

Correspondence
Tim Boogaerts, Toxicological Centre, University of Antwerp, Wilrijk, Belgium.
Email: tim.boogaerts@uantwerpen.be

Funding information
European Union's Justice Programme-Drugs Policy Initiatives, Grant/Award Number: 861602; Research Agency of Republic of Slovenia (ARRS), Grant/Award Numbers: P1-0143, N1-0143; Research Scientific Foundation Flanders, Grant/Award Number: G060920N

Abstract

Wastewater-based epidemiology (WBE) is based on the analysis of human metabolic excretion products (biomarkers) of xenobiotics in wastewater, to gain information about various lifestyles and health aspects of a population in an evidence-based manner. Due to the complex wastewater matrix and trace level occurrence of human biomarkers in the sewage network, it is crucial to have sensitive analytical procedures available. Additionally, to improve the value of WBE as a complementary epidemiological source, there is increasing pressure on the analysis of more compounds, more locations and more samples. A high-throughput method based on 96-well Oasis MCX solid-phase extraction (SPE), requiring less influent wastewater (2 mL), was developed in accordance with the European Medicines Agency guidelines. Validation was successful for 28 parent drugs and metabolites of antidepressants, opioids and drugs of abuse. The selection of biomarkers and quantification limit was chosen to be relevant for WBE and was predominantly 10 ng/L or below. The final method was successfully applied to 24-h composite samples of October 2019 ($n = 27$), obtained from an urban wastewater treatment plant in Leuven (Belgium).

KEYWORDS
96-well solid-phase extraction, high-throughput analysis, psychoactive substances, wastewater-based epidemiology

1 | INTRODUCTION

Wastewater-based epidemiology (WBE) is based on the chemical analysis of human metabolic excretion products in influent wastewater (IWW) samples. Concentrations of these biomarkers are measured to gain information about various health and lifestyle aspects of the catchment population.[1–3] To improve the value of WBE as a complementary epidemiological data source, there is increasing pressure on the analysis of more compounds, locations and samples. However, sample preparation is a clear limitation, as it is often a time-consuming procedure requiring solid-phase extraction (SPE) and relatively large volumes (≥50 mL) of IWW. Although there have been efforts to investigate longitudinal patterns in drugs of abuse (DOA) use through extensive daily sampling, to date, most community efforts to analyse DOA consumption using WBE consist in monitoring a few cities per country for 1 week per year.[4] However, many compounds have weekly and/or seasonal consumption trends (e.g., 3,4-methylenedioxymethamphetamine (MDMA)) that may not

Tim Boogaerts and Maarten Quireyns joint first author.

 © 2022 John Wiley & Sons Ltd.

19427611, 2023, 2, Downloaded from https://analysisandsensing.onlinelibrary.wiley.com/doi/10.1002/ansa.3982 by Jozef Stefan Institute, Wiley Online Library on [01/12/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

be properly captured when analysing only 1 week per year.[5,6] Additionally, the chosen, often urban, locations may not be representative for consumption at country level.

Long-term spatiotemporal data require therefore the analysis of a large set of samples. Even when using a stratified random sampling approach to reduce the number of samples, a large set of samples has to be analysed each year (>54, depending on the analysed compound and allowed uncertainty), which quickly increases if more locations are included.[5,6] Within WBE, Baz-Lomba et al.[7] published a high-throughput method for 13 drugs and metabolites (e.g., amphetamine, atenolol and carbamazepine). The method consists of HLB μElution 96-well plates for sample preparation, liquid chromatography (LC) using high-volume injection and post-loop mixing for separation and detection using tandem mass spectrometry (MS/MS). Although this is the first study that applied 96-well SPE for the extraction of different chemicals in IWW using small sample volumes (1 mL), it requires physical alteration of the LC system.

The objective of this work was to create a rapid and sensitive method based on 96-well SPE for the high-throughput analysis of 28 parent compounds and metabolites of antidepressants, opioids and DOA in IWW. The method coupled to liquid chromatography with tandem mass spectrometry (LC–MS/MS) should be sensitive enough to detect the low concentration levels (ng/L) of the biomarkers using low volumes of IWW (2 mL) and avoiding physical alteration (e.g., post-loop mixing) of the LC instrument.

2 | MATERIALS AND METHODS

2.1 | Chemicals, materials and samples

Stock solutions of reference and internal standard (IS) ranged from 95 to 5 μg/μL. Working solutions, ranging from 1 to 200 ng/μL, were prepared from the stock solutions in methanol. All stock and working solutions were stored at −18°C. Sample preparation was performed on a 96-well extraction plate manifold with Oasis MCX 96-well plates (30 μm, 10 mg) from Waters (Massachusetts, USA). More details on the standards, materials and suppliers can be found in the Section S1 in the Supporting Information.

Daily 24-h composite IWW samples (250 mL) were collected in October 2019 from a wastewater treatment plant (WWTP) in Leuven, Belgium ($n = 27$). Samples were collected during a normal period (i.e., no special events occurring), in a time-proportional manner (frequency < 10 min). Samples were stored at −18°C after sample collection until analysis.

2.2 | Biomarker choice

Based on a preliminary screening with existing offline SPE methods, a selection of 30 biomarkers of psychoactive pharmaceuticals and DOA was made for method development and validation.[8–10] The selected compounds were found previously in sufficiently high concentrations (>50 ng/L in Belgian IWW), which make them relevant for WBE purposes. Furthermore, these biomarkers were specific for the target compound and were sufficiently stable in wastewater (Section S7 in the Supporting Information, Biomarker stability).

2.3 | Method development

Instrumental analysis was performed on an Agilent 1290 Infinity II Ultra High-Performance LC system coupled to an Agilent 6495 Triple Quadrupole MS/MS with a Jet Stream Electrospray Ionisation interface operating in positive ionisation mode. Chromatographic separation was based on earlier optimised methods and was carried out on a Waters Atlantis T3 Column (150 × 2.1 mm, 3 μm) maintained at 30°C.[9] Mobile Phase A consisted of 0.1% v/v formic acid in ultrapure water, and Mobile Phase B consisted of 0.1% v/v formic acid in methanol. The gradient that achieved the best separation was 0–0.5 min: 5% B; 0.5–5 min: increase to 25% B; 5–16 min: increase to 30% B; 16–21 min: increase to 95% B; 21–21.1 min decrease to 5% B; and equilibration at 5% B up to 25 min. An injection volume of 4 μL was used based on sensitivity and peak shape of the analytes of interest. The flow rate was 0.275 mL/min. All 30 compounds were eluted within 21 min.

Source- and compound-dependent MS/MS parameters can be found in Section S2 in the Supporting Information. Identification of the compounds was based on the quantifier/qualifier ratios with a tolerance level of 30% relative standard deviation (RSD). The relative retention time should be within ±2.5% RSD of that of the calibrators.[11]

2.4 | Sample preparation

The final sample preparation procedure can be found in Figure S1. In the first step, 2 mL of IWW is acidified with 6 μL of formic acid and subsequently spiked with 20 μL of an IS mixture, resulting in a final concentration of 100 ng/L (except for tramadol-D_8, 500 ng/L). This was the maximum loading volume of the used 96-well collection plate, and this resulted in highest sensitivity for analytes of interest. The samples are centrifuged (5 min, 10,000×*g*) to remove solid particles. An Oasis MCX 96-well plate (30 μm, 10 mg) is conditioned under vacuum with 500 μL of methanol and 500 μL ultrapure water, both with 0.3% v/v formic acid. Then, 1.8 mL of the supernatant is loaded on the 96-well plate under vacuum. After loading the samples, the sorbent is washed with (i) 500 μL ultrapure water with 0.3% v/v formic acid, (ii) 500 μL methanol with 0.3% v/v formic acid and (iii) 500 μL 30/70 (v/v) methanol in water. Next, the 96-well sorbent is dried for 5 min under vacuum (5 mmHg). Elution is done with 200 μL of 5% v/v ammonia in methanol into a collection plate. The eluent is evaporated in a Biotage SPE Dry 96 device at 37°C until dry, using nitrogen (25 L/min bottom, 50 L/min top). Afterwards, the samples are reconstituted in 120 μL of 5/95 methanol/ultrapure water with 0.1% v/v formic acid. The reconstituted samples are vortexed (2.5 min,

TABLE 1 Method validation criteria for the 4 QC levels: intra- and inter-run accuracy and precision

	Accuracy (%)								Precision (%RSD)							
	Intra-run ($n = 5$)				Inter-run ($n = 15$)				Intra-run ($n = 5$)				Inter-run ($n = 15$)			
Compound	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH	LLOQ	QCL	QCM	QCH
Amitriptyline	108	107	105	101	102	104	104	97	4	13	6	8	6	9	4	6
Amphetamine	89	98	108	99	90	98	107	98	7	7	4	4	8	7	2	5
Benzoylecgonine	93	101	112	109	91	99	107	106	2	3	3	3	5	2	2	4
Bupropion	101	101	101	104	101	100	97	96	5	6	4	5	5	5	5	7
Bupropion-OH	104	99	99	109	102	96	96	100	5	6	5	6	6	5	4	8
Citalopram	101	101	98	108	99	99	99	102	7	5	6	3	8	4	4	5
Codeine	106	99	106	112	103	97	107	110	8	9	7	3	9	8	5	3
Dihydrocodeine	103	104	105	113	97	981	105	109	9	10	12	4	8	8	7	4
EDDP	102	96	101	107	92	95	100	101	12	6	4	6	13	7	3	7
Hydromorphone	113	92	102	106	105	91	103	100	5	3	5	1	8	5	4	5
mCPP	112	103	93	101	104	101	99	97	5	4	6	4	7	6	6	5
MDMA	100	106	105	98	104	109	103	100	4	4	5	3	4	6	3	5
Melitracen	93	102	102	102	98	105	100	98	7	4	3	11	10	5	6	8
Methadone[a]	114	103	107	109	101	98	105	106	5	8	6	5	13	8	5	5
Methamphetamine	85	96	110	104	94	97	104	96	4	6	3	5	9	4	5	7
Mirtazapine	97	96	107	108	76	90	104	103	4	8	5	3	35	12	4	6
Moclobemide[a]	106	102	100	105	101	98	100	99	8	8	5	5	12	7	3	7
Morphine	103	98	105	96	100	97	106	97	8	5	5	8	6	5	3	6
Norcitalopram	106	113	102	106	95	109	101	102	6	8	4	6	11	7	3	6
Normirtazapine	109	101	108	111	97	94	109	107	4	6	3	4	11	7	3	4
Normorphine	114	102	105	108	108	100	107	107	13	6	6	6	10	5	4	4
Noroxycodone	105	103	103	107	98	103	101	106	9	5	7	7	15	7	5	6
Nortilidine	115	112	107	108	113	105	106	99	3	12	4	6	11	9	3	8
O-DMV[b]	99	81	113	116	133	88	106	105	5	6	5	6	36	13	7	9
O-DMV[c]	101	93[c]	109	110	101	93[c]	104	101	1	1	5	6	2	2	5	8
ODT	96	103	108	106	93	99	106	99	14	6	3	5	10	7	3	6
Oxycodone	107	107	108	103	102	96	104	97	8	5	4	7	12	9	7	7
Tilidine	112	110	110	105	101	104	108	103	3	6	4	5	10	7	3	5
Tramadol	103	101	110	106	102	99	109	102	3	5	2	5	3	4	5	5
Trazodone	93	99	99	106	92	97	101	103	3	5	5	7	12	5	4	5
Venlafaxine	106	104	102	100	97	100	100	96	2	5	6	4	9	5	4	5

Note: Intra-run results are from the validation first day. In general, 5 replicates were used per QC level and repeated on 3 days.
Abbreviations: EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; LLOQ, lower limit of quantification; mCPP, m-chlorophenylpiperazine; O-DMV, O-desmethylvenlafaxine; ODT, O-desmethyltramadol; QC, quality control; RSD, relative standard deviation.
[a]Only four replicates were included on a specific day (not used for intraday comparison).
[b]Intra- and interday accuracy and precision of O-DMV with a seven-level calibration range (10–1500 ng/L) that failed the initial method validation.
[c]O-DMV with a six-level range (50–1500 ng/L) that passed method validation had only two replicates for intraday assessment of precision and accuracy of QCL and a total of nine replicates for the assessment of interday accuracy and precision of QCL.

19427611, 2023, 2, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/dta.3392 by Jozef Stefan Institute, Wiley Online Library on [01/12/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

1500 rpm) and are transferred to a filter plate (0.2 μm). Finally, the samples are filtered under vacuum into an autosampler plate (0.5 mL, 96-well plate) to be analysed with LC–MS/MS.

2.5 | Method validation

Method validation was performed according to the bioanalytical method validation guideline by the European Medicines Agency (EMA) with minor adjustments.[12] Because it is not possible to obtain blank IWW, method validation was performed with tap water as an alternative matrix. Selectivity was assessed by analysing three zero blank samples (i.e., containing no analyte and IS) for interferences. The method is sufficiently selective if the response of the quantifier transition is less than 20% of the lower limit of quantification (LLOQ) peak area and 5% for the IS peak area. The lowest calibration point corresponded with the LLOQ.

For each compound, a seven-level calibration curve was constructed (Table S2). The analyte signal at the LLOQ level was at least five times the noise signal present in the blank sample. Carry-over was evaluated in a blank sample after injection of the highest calibration level. The peak area in the blank sample should be less than 20% of the peak area of the LLOQ and 5% for the IS. Intra- and interday accuracy and precision were evaluated by analysing four quality con-

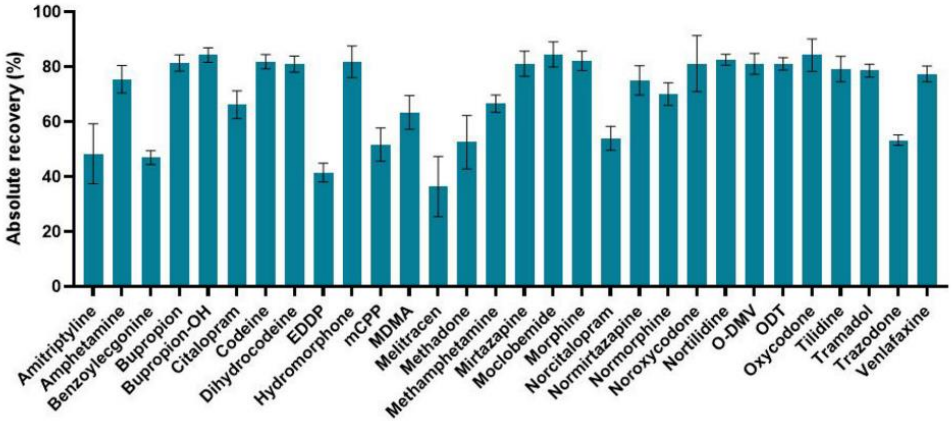


FIGURE 1 Mean absolute recovery ± SD ($n = 8$) in tap water for all compounds via the 96-well elution protocol. EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; mCPP, m-chlorophenylpiperazine; O-DMV, O-desmethylvenlafaxine; ODT, O-desmethyltramadol [Colour figure can be viewed at wileyonlinelibrary.com]

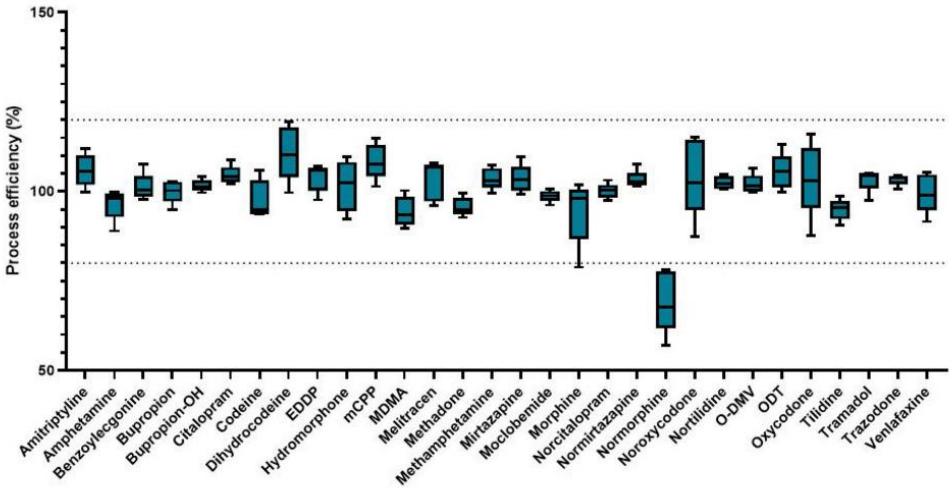


FIGURE 2 Process efficiency of each compound. Standard addition experiments in influent wastewater were performed on samples from five different locations. Dotted lines represent the acceptable 80%–120% range. EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; mCPP, m-chlorophenylpiperazine; O-DMV, O-desmethylvenlafaxine; ODT, O-desmethyltramadol [Colour figure can be viewed at wileyonlinelibrary.com]

19427611, 2023, 2, Downloaded from https://analysisandsensing.onlinelibrary.wiley.com/doi/10.1002/ansa.3892 by Jozef Stefan Institute, Wiley Online Library on [01/12/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

trol (QC) samples at three different days: LLOQ, QC Low (within three times LLOQ concentration), QC Medium (within 30%–50% of the calibration curve) and QC High (at least 75% of calibration curve). Results were compared with the acceptable criteria of ≤15% bias and ≤15% RSD, except for the LLOQ, which should be within 20% bias and ≤20% RSD. Absolute recovery (RE) experiments were executed on multiple days in tap water at QC Medium concentrations. Process efficiency (PE) experiments according to Boogaerts et al.[9] performed during method validation to verify if the IS adequately compensates for possible interferences and loss during sample preparation. An overview of the experiment is given in Section S5 in the Supporting Information.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

In accordance with EMA, performance criteria were met for 28 out of 30 biomarkers, except for mirtazapine (i.e., due to poor interday accuracy and precision) and normorphine (i.e., due to an inacceptable PE). A seven-level linear calibration curve was constructed for most compounds with a weighing factor of 1/x or $1/x^2$, as illustrated in Table S2. Only O-desmethylvenlafaxine (O-DMV) had six calibration levels. The LLOQ ranged between 1 and 100 ng/L and were based on actual concentrations found in IWW from previous research.[8,9] Even though the preconcentration step with the 96-well elution (±20-fold) is less than offline SPE (±250-fold), the LC–MS/MS proved to be sensitive enough to measure the analytes of interest at the LLOQ levels. This could potentially also be explained by differences in matrix effects between the 96-well elution and the classical SPE procedure. No significant carry-over was observed (<5% for the analytes and <1% for the IS). The method proved to be selective as no interferences were noted in the zero samples.

Intra- and interday accuracy and precision, measured at the different QC levels, fulfilled the EMA requirements for these 28 analytes (Table 1). For mirtazapine, it was not possible to obtain the required interday accuracy (76% at LLOQ level) and interday precision (36%). Increasing the LLOQ did not result in better performance during method validation. Mirtazapine was therefore excluded from the method. However, consumption of mirtazapine can still be measured through its metabolite normirtazapine, making this not an issue.

For most compounds, reproducible and high RE (>70%) were obtained (Figure 1). For benzoylecgonine, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), m-chlorophenylpiperazine (mCPP), MDMA, methamphetamine, methadone, norcitalopram and trazodone, the recoveries were lower but reproducible (RSD < 20%). The high RSD obtained for amitriptyline (22.5%) and melitracen (30.0%) suggests a variable recovery. Instrumental sensitivity was sufficiently high over all samples and days during validation to cover this variability in RE (i.e., not corrected using IS).

All analytes had acceptable PE, except for normorphine with a mean PE of 69.3% ± 7.1% (Figure 2). Amitriptyline and melitracen had acceptable PE, with only minor variations between the different locations. The RSD of the RE was below the threshold of 20% and across different days. This indicates that the relative recovery is acceptable and that the IS robustly compensates for losses during sample preparation and matrix effects. For this reason, it can be concluded that amitriptyline and melitracen could be included in method validation. Normorphine does not have a deuterated analogue as IS and morphine-D_3 were used as an IS. No further efforts were made to improve the PE of normorphine at this point, but it is recommended to test an isotope-labelled analogue in future research. Additionally, normorphine has only a medium in-sample stability (Section S7 in the

TABLE 2 Quantification frequency (number of samples >LLOQ) and average PNML loads of validated compounds during 1 month of sampling in October 2019, Leuven, Belgium ($n = 27$)

Compound	Quantification frequency (%)	Mean PNML ± SD (mg/day/1000p)
Amitriptyline	89	16.8 ± 7.2
Amphetamine	100	126.8 ± 55.9
Benzoylecgonine	100	292.4 ± 175.5
Bupropion	67	4.6 ± 1.5
Bupropion-OH	100	44.8 ± 16.4
Citalopram	100	49 ± 19.4
Codeine	100	51.3 ± 20.9
Dihydrocodeine	96	6.4 ± 2
EDDP	100	6.5 ± 2.5
Hydromorphone	0	
mCPP	0	
MDMA	100	52.7 ± 36.9
Melitracen	0	
Methadone	96	4.1 ± 1.4
Methamphetamine	74	2.7 ± 1.3
Moclobemide	96	3.7 ± 1.9
Morphine	100	57.6 ± 28.1
Norcitalopram	100	26.3 ± 9.9
Normirtazapine	93	8.2 ± 2.9
Noroxycodone	4	[a]
Nortilidine	100	3.6 ± 1.4
O-DMV	100	367.1 ± 130.3
ODT	100	359.2 ± 138.6
Oxycodone	0	
Tilidine	11	[a]
Tramadol	100	493.3 ± 198
Trazodone	100	28.6 ± 12.8
Venlafaxine	100	145.2 ± 54

Abbreviations: EDDP, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; LLOQ, lower limit of quantification; mCPP, m-chlorophenylpiperazine; O-DMV, O-desmethylvenlafaxine; ODT, O-desmethyltramadol; PNML, population normalised mass load.
[a]Not calculated given low quantification frequency.

19427611, 2023, 2, Downloaded from https://analysisandsensing.onlinelibrary.wiley.com/doi/10.1002/ttc.3392 by Ateneo Sistan Instanic, Wiley Online Library on [01/11/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Supporting Information, Biomarker stability). For these reasons, this compound was excluded from the assay at this point. The PE of normorphine was not reproducible enough to allow the use of a correction factor. Using the classical SPE protocol, the PE of normorphine was acceptable.[9] This could potentially indicate that matrix effects can be different when a different preconcentration step is applied.

3.2 | Method application

The applicability of the method was evaluated on samples collected in October 2019 from Leuven ($n = 27$, Section 2.1). Measured analyte concentrations (ng/L) were back-calculated to population normalised mass load (PNML) by considering the flow rate (L/day) and de facto population on each day (Section S6 in the Supporting Information).[3] As illustrated in Table 2, most validated compounds were quantified at least once, except for hydromorphone, melitracen, oxycodone and mCPP. Furthermore, the highest PNMLs were obtained for O-DMV (367.1 ± 130.3 mg/day/1000p). No discernible trend was noted over the full-sampled month (Figure 3), but a week–weekend change in PNML was present, potentially due to changing demographics in the catchment population. Furthermore, the application of the 96-well SPE proved to be less time-consuming and required less elution solvent volume and less sample volume compared to traditional SPE (2 mL vs > 50 mL).

ACKNOWLEDGEMENTS

This study was funded by the European Union's Justice Programme—Drugs Policy Initiatives (EuSeME) (project number: 861602) and Research Scientific Foundation Flanders (FWO, project number: G060920N). Tim Boogaerts and Natan Van Wichelen acknowledge PhD fellowships from the University of Antwerp. Additionally, the

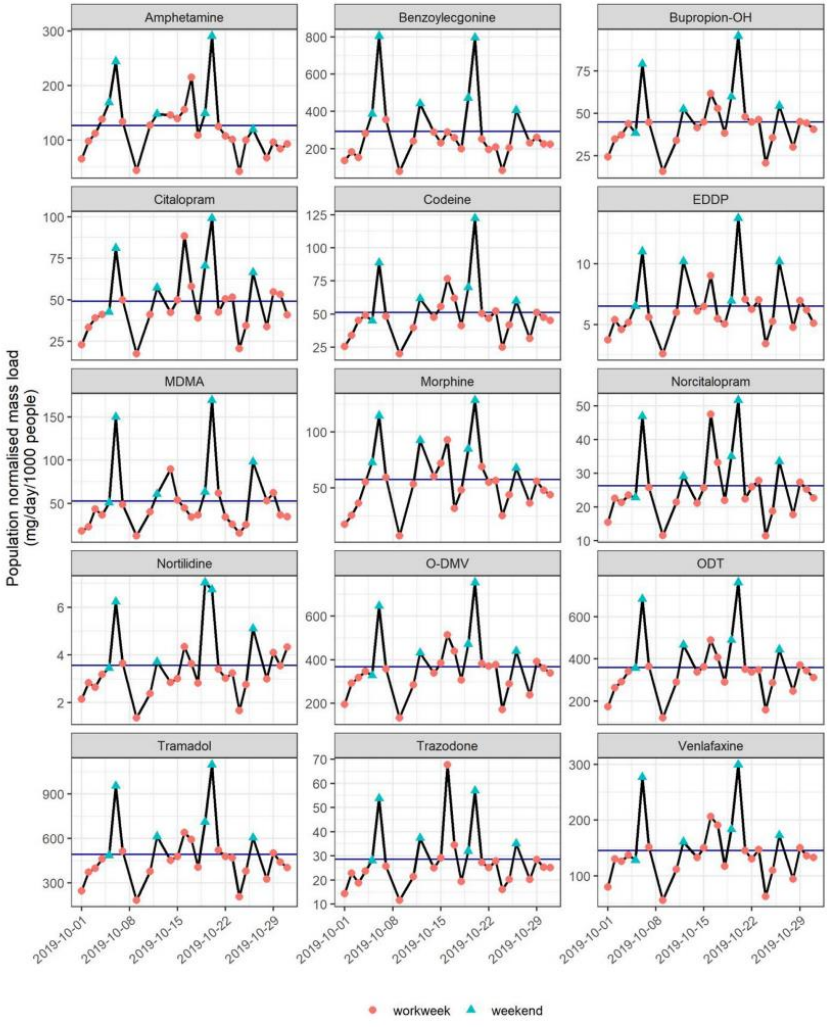


FIGURE 3 Temporal population normalised mass load (PNML) of compounds during 1 month of sampling in October 2019, Leuven, Belgium ($n = 27$). Only compounds with a quantification frequency of 100% are shown. The mean PNML level for each compound is visualised as a horizontal blue line. [Colour figure can be viewed at wileyonlinelibrary.com]

19427611, 2023, 2, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/bmc.5392 by Inzet Stefan Inzianc, Wiley Online Library on [01/12/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

authors wish to acknowledge the Research Agency of Republic of Slovenia (ARRS) for funding the programme group P1-0143 and the ARRS-FWO Project N1-0143.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

ORCID

Tim Boogaerts https://orcid.org/0000-0002-3802-3174
Maarten Quireyns https://orcid.org/0000-0003-3397-3186
Alexander L. N. van Nuijs https://orcid.org/0000-0002-9377-6160

REFERENCES

1. Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R. Estimating community drug abuse by wastewater analysis. *Environ Health Perspect*. 2008;116(8):1027-1032. doi:10.1289/ehp.11022
2. Choi PM, Tscharke BJ, Donner E, et al. Wastewater-based epidemiology biomarkers: past, present and future. *TrAC - Trends Anal Chem*. 2018;105:453-469. doi:10.1016/j.trac.2018.06.004
3. Gracia-Lor E, Castiglioni S, Bade R, et al. Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives. *Environ Int*. 2017;1(99):131-150. doi:10.1016/j.envint.2016.12.016
4. González-Mariño I, Baz-Lomba JA, Alygizakis NA, et al. Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. *Addiction*. 2020;115(1):109-120. doi:10.1111/add.14767
5. Humphries MA, Bruno R, Lai FY, et al. Evaluation of monitoring schemes for wastewater-based epidemiology to identify drug use trends using cocaine, methamphetamine, MDMA and methadone. *Environ Sci Technol*. 2016;50(9):4760-4768. doi:10.1021/acs.est.5b06126
6. Ort C, Eppler JM, Scheidegger A, Rieckermann J, Kinzig M, Sörgel F. Challenges of surveying wastewater drug loads of small populations and generalizable aspects on optimizing monitoring design. *Addiction*. 2014;109(3):472-481. doi:10.1111/add.12405
7. Baz-Lomba JA, Löve ASC, Reid MJ, Ólafsdóttir K, Thomas KV. A high-throughput solid-phase microextraction and post-loop mixing large volume injection method for water samples. *J Chromatogr a*. 2018;1531:32-38. doi:10.1016/j.chroma.2017.11.051
8. Boogaerts T, Degreef M, Covaci A, van Nuijs ALN. Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach. *Talanta*. 2019;200:340-349. doi:10.1016/j.talanta.2019.03.052
9. Boogaerts T, Quireyns M, Covaci A, De Loof H, van Nuijs ALN. Analytical method for the simultaneous determination of a broad range of opioids in influent wastewater: optimization, validation and applicability to monitor consumption patterns. *Talanta*. 2021;232:122443. doi:10.1016/j.talanta.2021.122443
10. van Nuijs ALN, Tarcomnicu I, Bervoets L, et al. Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2009;395(3):819-828. doi:10.1007/s00216-009-3017-0
11. Eurpean Commission. Commission decision of 12 august 2002 implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *J Eur Commun*. 2002;221:8-36.
12. Guideline on bioanalytical method validation [Internet]. European Medicines Agency; [cited 2021 Aug 26]. Report No.: EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2**. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Boogaerts T, Quireyns M, Maes F, et al. Optimization, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater. *Drug Test Anal*. 2023;15(2):240-246. doi:10.1002/dta.3392

19427611, 2023, 2, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/dta.3392 by Inzet Sudan insaanic, Wiley Online Library on [01/11/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

3.2 WBE as a Complementary Data Source: Application in the Slovenian Population

3.2.1 Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters

Published: M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, N. Van Wichelen, D. Heath, A. L.N. van Nuijs, A. Covaci, E. Heath, 2023, *Science of The Total Environment*, vol. 903, pp. 166586.

With the assistance of M. Quireyns and T. Boogaerts, I performed the experimental work, including sample preparation, instrumental analysis, and method validation. I was also responsible for data analysis and evaluation, as well as writing the manuscript. This study is a collaboration between the Jožef Stefan Institute in Ljubljana (Slovenia) and the Ecotoxicological Centre at the University of Antwerp (Belgium).

Antidepressant use has been increasing worldwide, reflecting shifts in mental health care and prescribing practices. This trend became even more pronounced with the onset of the COVID-19 pandemic, which coincided with a further rise in antidepressant consumption (1.2.1 Psychoactive pharmaceuticals: Consumption trends and prescription data). Previous work has shown that WBE provides valuable information on the consumption patterns of psychoactive pharmaceuticals in near real time, with high spatiotemporal resolution [2]. Despite growing interest in monitoring the effects of the COVID-19 pandemic on spatiotemporal trends in antidepressant consumption patterns, only a limited number of studies include countries in Central Europe.

In response, for the first time, a comprehensive WBE study was conducted to evaluate temporal and spatial trends in the consumption of commonly prescribed antidepressants in Slovenia, before and during the COVID-19 pandemic. Influent wastewater samples (24-hour composite) were collected from six WWTPs serving approximately 30% of the Slovenian population during five sampling periods between summer 2019 (before the COVID-19 pandemic) and spring 2021 (the third wave of the pandemic).

A previously developed analytical method [224], which is based on 96-well SPE (Oasis MCX) and target analysis (LC-MS/MS), was applied for the determination of antidepressant biomarkers. The measured concentrations of target biomarkers were converted to PNMLs to facilitate direct comparisons across sites and time frames. Ten out of twelve antidepressant biomarkers, including amitriptyline, bupropion, bupropion-OH, citalopram, norcitalopram, normirtazapine, venlafaxine, *O*-desmethylvenlafaxine, trazodone, and moclobemide, were above the lower limit of quantification (LLOQ) and were included in the spatiotemporal assessment.

The highest PNMLs were recorded for *O*-desmethylvenlafaxine (mean ± SD: 82.1 ± 21.2 mg/day/1,000 inhabitants) and venlafaxine (38.0 ± 10.6 mg/day/1,000 inhabitants), followed by citalopram (27.0 ± 10.7 mg/day/1,000 inhabitants). In addition, the mean metabolite-to-parent compound ratios were consistent with those of other WBE studies, indicating that the detected substances largely originated from human consumption rather than direct disposal.

Most antidepressant biomarkers showed stable usage throughout the week; however, the few observed differences between weekdays and weekends could not be directly linked to the pandemic. Numerous temporal and spatial variations were revealed. Although antidepressant levels increased during the sampling campaigns, and particularly during the

first wave of the pandemic (spring 2020), no clear spatial patterns were observed across locations. Some seasonal variation was noted, with higher PNMLs generally observed in winter, potentially linked to increased antidepressant use during months when symptoms of seasonal affective disorder (SAD) are more common. Notably, the results align with available national annual prescription data, supporting the reliability of WBE for assessing psychoactive pharmaceutical use. These findings highlight the value of WBE as a complementary tool for public health monitoring.

Study outcomes were presented at three scientific conferences, i.e., 28th Annual Meeting of the Slovenian Chemical Society, September 21-23, 2022, Portorož, Slovenia; Chem2Change, Environmental Chemistry towards Global Change, 2nd Online ACE Seminar on Chemistry and the Environment Led by Early-Career Scientists, March 15-16, 2022; and Throughout Knowledge Towards a Green New World: 13th Jožef Stefan International Postgraduate School Students' Conference and 15th Young Researchers' Day of Chemistry, material science, biochemistry and environment, (CMBE day), May 27-28, 2021.

Science of the Total Environment 903 (2023) 166586

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters

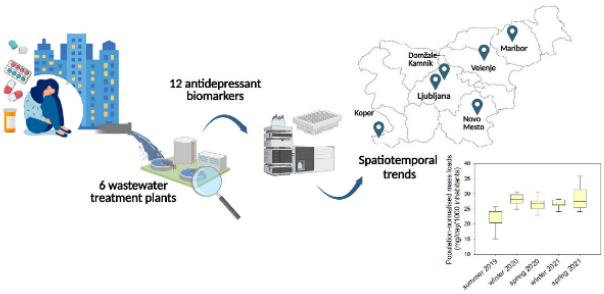
Maria Laimou-Geraniou [a,b], Maarten Quireyns [c], Tim Boogaerts [c], Natan Van Wichelen [c], David Heath [a], Alexander L.N. van Nuijs [c], Adrian Covaci [c], Ester Heath [a,b,*]

[a] Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia
[c] Toxicological Centre, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

HIGHLIGHTS

- Evaluation of spatiotemporal trends in antidepressant use during the COVID-19 pandemic
- *O*-desmethylvenlafaxine had the highest population-normalised mass loads.
- Antidepressants use increased during the pandemic.
- No clear pandemic-related spatial patterns found.
- The WBE results align with the annual prescription data in Slovenia.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Dimitra A Lambropoulou

Keywords:
Antidepressants
Wastewater-based epidemiology
Spatiotemporal trends
Slovenia
COVID-19

ABSTRACT

This study utilizes wastewater-based epidemiology (WBE) to evaluate spatiotemporal changes in the consumption of antidepressants before and during the COVID-19 pandemic in Slovenia. Composite 24-h influent wastewater samples (n = 210) were collected from six wastewater treatment plants between summer 2019 and spring 2021. The samples were extracted using 96-well solid-phase extraction and analysed by liquid chromatography-tandem mass spectrometry. The measured concentrations of target antidepressant biomarkers were then converted to population-normalised mass loads (PNMLs), taking into account flow rate and catchment population. Ten biomarkers, including amitriptyline, bupropion, bupropion-OH, citalopram, norcitalopram, normirtazapine, venlafaxine, *O*-desmethylvenlafaxine, trazodone, and moclobemide, were above the lower limit of quantification and were included in the spatiotemporal temporal assessment. The highest PNMLs were detected for *O*-desmethylvenlafaxine (mean ± SD: 82.1 ± 21.2 mg/day/1000 inhabitants) and venlafaxine (38.0 ± 10.6 mg/day/1000 inhabitants), followed by citalopram (27.0 ± 10.7 mg/day/1000 inhabitants). In addition, the mean metabolite/parent compound ratios were comparable with other WBE

* Corresponding author at: Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia
E-mail address: ester.heath@ijs.si (E. Heath).

https://doi.org/10.1016/j.scitotenv.2023.166586
Received 12 July 2023; Received in revised form 23 August 2023; Accepted 24 August 2023
Available online 26 August 2023
0048-9697/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

studies indicating consumption rather than direct disposal. Overall, the results indicated significant spatiotemporal variations depending on the location, and the PNMLs of most biomarkers increased during the first wave of the COVID-19 pandemic (spring of 2020). However, no clear spatial patterns were revealed related to the pandemic.

1. Introduction

Since the outbreak of the COVID-19 pandemic, there has been a growing interest in examining the pandemic's impact on people's mental health. Several epidemiological studies worldwide have reported an increase in the consumption of psychoactive pharmaceuticals, such as antidepressants, anxiolytics, sedatives, and hypnotics, during the pandemic due to depression, higher rates of acute psychological distress, insomnia, anxiety, post-traumatic stress disorder, and addiction problems that originated in the general population (Diaz-Camal et al., 2022; Melchor-Martínez et al., 2021; Pazzagli et al., 2022; Tiger et al., 2023; Winkler et al., 2021; Xiong et al., 2020). For these reasons, understanding the mental well-being of the population is vital since mental illness creates a substantial health, social, and economic burden (WHO, 2022). In Slovenia, approximately 85,000 people (4.3 % of the population) suffered from depression in 2019 (GBDS, 2019). In fact, according to OECD data for 2021, Slovenia ranked 13th (63.6 defined daily doses/1000 people) worldwide in antidepressant consumption (Statista, 2021). More recent studies also suggest that the COVID-19 pandemic has been responsible for increased stress, anxiety, and depression, especially among the younger population (Kozina et al., 2022; Rus Prelog et al., 2022), which is reflected in a 10 % increase in antidepressant prescriptions between 2019 and 2020, i.e., when the COVID-19 pandemic began (ZZZS, 2022).

Wastewater-based epidemiology (WBE) is a well-established approach that offers objective, complementary, and anonymous information on consumption and exposure to different xenobiotics in defined populations by analysing human metabolic excretion products (biomarkers) in influent wastewater (Baker et al., 2014). At present, besides the numerous WBE studies on illicit drugs, alcohol, tobacco, and new psychoactive substance consumption (Bade et al., 2021; Estévez-Danta et al., 2022; Gent and Paul, 2021; Gracia-Lor et al., 2020; Huizer et al., 2021; López-García et al., 2020), the application of WBE also extends to a small number of studies addressing psychoactive pharmaceutical consumption (Boogaerts et al., 2021, 2019; Escolà Casas et al., 2021; Laimou-Geraniou et al., 2023; Quireyns et al., 2022; Rice et al., 2020; Riva et al., 2020). Studies have shown that WBE provides valuable information on the consumption patterns of psychoactive pharmaceuticals over time and has the benefit of high temporal resolution. This knowledge holds significance as weekly patterns can shed light on potential recreational use. Additionally, seasonal patterns can highlight temporal changes within months of the year or between the same months in different years. Long-term trends can also provide insights into the stability or fluctuations in the use of antidepressants over time (Tscharke et al., 2016). The WBE approach also provides good spatial resolution sufficient for identifying variations in the usage of psychoactive pharmaceuticals at different locations, including within countries at the city/village level or monitor consumption trends at the scale of regions and suburbs within a metropolitan area (Laimou-Geraniou et al., 2023). This advanced spatial resolution offers the potential to conduct area-based evaluations that would otherwise be unattainable solely through nationally compiled prescription data.

Several studies have already utilized the WBE approach to identify the effects of the COVID-19 pandemic on spatiotemporal trends in psychoactive pharmaceutical consumption patterns (Alygizakis et al., 2021; Boogaerts et al., 2023a; Galani et al., 2021; Reinstadler et al., 2021; Tomsone et al., 2022; Yavuz-Guzel et al., 2022). However, only a limited number of WBE studies include countries in Central Europe, i.e. Austria (Reinstadler et al., 2021), Slovakia (Fáberová et al., 2017; Mackuľak et al., 2016) and the Czech Republic (Baker et al., 2014). Accordingly, this study aims to fill this gap by monitoring the spatiotemporal use of antidepressants at a higher degree of granularity in Slovenia using WBE before and during the pandemic. Fortunately, a comprehensive sampling campaign conducted from the summer of 2019 to the spring of 2021 (Verovšek et al., 2023) provided a valuable opportunity to retrospectively evaluate the influence of the pandemic on the usage patterns of specific antidepressant medications in Slovenia.

2. Materials and methods

2.1. Sampling

Six wastewater treatment plants (WWTPs) from across Slovenia (Ljubljana, Maribor, Domžale-Kamnik, Velenje, Koper and Novo Mesto), covering approximately 30 % of the Slovenian population, were included in the study (Table S1). Influent wastewater samples (24-h composites) were collected for seven consecutive days, representing "normal" conditions at the WWTPs; these sampling periods excluded any special events such as festivals, maintenance activities, or extreme weather events. The samples ($n = 210$) were collected during the summer of 2019, winter of 2020, spring of 2020, winter of 2021 and spring of 2021. All samples were stored at −20 °C until analysis. It is noteworthy that the first pandemic wave hit Slovenia in the spring of 2020 (March 2020), the second wave started in the autumn of 2020 (October 2020), and the third in the spring of 2021 (March 2021).

2.2. Target biomarkers

Target biomarkers of antidepressants were selected based on sales, prescription frequency, preliminary WBE concentrations (Laimou-Geraniou et al., 2022) in Slovenia and available validated analytical methods (Boogaerts et al., 2023b). In addition, all the investigated compounds have high in-sample stability (Boogaerts et al., 2019; Choi et al., 2020). The final list of biomarkers (Table S2) included amitriptyline (AMIT), bupropion (BUP), bupropion-OH (BUP-OH), citalopram (CIT), norcitalopram (nor-CIT), melitracen (MEL), moclobemide (MOC), normirtazapine (nor-MIRT), trazodone (TZD), *m*-chlorophenylpiperazine (mCPP), venlafaxine (VEN), and *O*-desmethylvenlafaxine (ODVEN). Even though sertraline is among the most prescribed antidepressants in Slovenia, it was excluded from the bioanalytical assay because it did not pass the requirements for method validation. All details regarding reagents, standards, materials and suppliers can be found in the Supplementary material (SM: Materials and methods).

2.3. Sample preparation and analysis

A previously validated method, as described by Boogaerts et al. (2023b), was employed for sample preparation and analysis. The procedure involved the following steps. Initially, 2 mL of influent wastewater was spiked with 6 μL of formic acid and 20 μL of an internal standard mixture. Centrifugation (10,000 ×*g*, 5 min) was performed to separate the solid particles. Subsequently, extraction was carried out using 96-well Oasis MCX solid phase extraction plates (SPE, Oasis MCX 10 mg). The sorbents were first conditioned under vacuum using 500 μL of methanol and 500 μL ultrapure water, with 0.3 % *v*/v formic acid. Then, the supernatant (1.8 mL) was loaded under vacuum, followed by a series of washing steps using (i) 500 μL ultrapure water +0.3 % formic acid, (ii) 500 μL methanol +0.3 % formic acid, and finally, (iii) 500 μL

30/70 (v/v) methanol/water. The sorbent was dried under vacuum for 5 min, after which the analytes of interest were eluted with 200 μL of 5 % (v/v) ammonia in a methanol solution. The eluent was then evaporated to dryness on a Biotage SPE Dry 96 device with heated nitrogen (37 °C, 25 L/min bottom, 50 L/min top). The resulting residues were reconstituted by adding 120 μL of 95/5 (v/v) ultrapure water/methanol containing 0.1 % formic acid. The samples were then vortexed (2.5 min at 1500 ×g) and filtered (Agilent 0.2 μm polypropylene, 96 well, 1 mL). Finally, the samples were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Chromatographic separation was carried out on a Waters Atlantis T3 Column (150 × 2.1 mm, 3 μm) maintained at 30 °C with Mobile Phase A consisted of 0.1 % v/v formic acid in ultrapure water, and Mobile Phase B consisted of 0.1 % v/v formic acid in methanol. Compounds were eluted using gradient elution (total run time 25 min). The gradient was as follows: 0–0.5 min: 5 % B; 0.5–5 min: increase to 25 % B; 5–16 min: increase to 30 % B; 16–21 min: increase to 95 % B; 21–21.1 min decrease to 5 % B; and equilibration at 5 % B up to 25 min. The flow rate was 0.275 mL/min. The injection volume of 4 μL was used based on the sensitivity and peak shape of the analytes of interest.

Complete details can be found in the supplementary information (see SM: Materials and methods, Measured compounds, and Table S3).

2.4. Back-calculations

In the present study, daily mass loads of antidepressant biomarkers were calculated by multiplying their measured values with the corresponding daily wastewater flow rates. Mass loads were then normalised to the number of people contributing to the WWTPs to back-calculate population-normalised mass loads (PNMLs) expressed as mg biomarker/day/1000 inhabitants. The WWTPs provided data for the daily flow rates (L/day) and population equivalents. The equation for the estimation of the PNMLs is given in Eq. (1):

Back-calculation to population-normalised mass loads

$$\text{PNMLs (mg/day/1000 inhabitants)} = \frac{\textit{concentration}\left(\frac{ng}{L}\right) \times \textit{wastewater flow rate}\left(\frac{L}{day}\right)}{\textit{population equivalent}} \quad (1)$$

2.5. Statistical analysis

A statistical evaluation was conducted using SigmaPlot 14.0 and R Studio. The normality and equality of variance of the data were tested using the Shapiro-Wilk test and Brown-Forsythe test, respectively, at a significance level of 5 %. To compare differences between two groups, we employed either a Student *t*-test (data normality and equality of variance were assumed), a Welch t-test (data normality but equality of variance was not assumed), or a Mann-Whitney Rank Sum Test (data normality was not assumed) at the 5 % significance level. To assess spatiotemporal differences in the use of psychoactive pharmaceuticals, either One-way ANOVA or Kruskal-Wallis test (for non-parametric analysis), followed by Tukey's post-hoc test, was used. In addition, to evaluate temporal variations in psychoactive pharmaceutical use, the calculated PNMLs (mg/day/1000 inhabitants) were scaled to daily proportions by dividing the mass loads by the sum of the mass loads within seven days of sampling. In this manner, locations could be combined to examine overall temporal patterns in psychoactive pharmaceutical use while facilitating comparisons between different periods within a single location. A Mann-Kendall trend analysis was applied to detect any monotonic increase (positive S value) or decrease (negative S value) in psychoactive pharmaceutical use across the 7-day sampling periods at all sampling locations.

3. Results and discussion

3.1. Method application

Out of the twelve biomarkers investigated, ten were quantified at least once (Table 1) and were subsequently selected for spatiotemporal analysis. However, melitracen and mCPP were below the lower limit of quantification (LLOQ), which is the minimum concentration of an analyte that can be accurately and precisely measured. Consequently, these two compounds were excluded from the analysis.

The measured PNMLs in all locations during the sampling periods can be found in Table S4. On average, the highest PNMLs were found for the antidepressants *O*-desmethylvenlafaxine (mean ± SD: 82.1 ± 21.2 mg/day/1000 inhabitants), venlafaxine (38.0 ± 10.6 mg/day/1000 inhabitants), citalopram (27.7 ± 11.4 mg/day/1000 inhabitants), and norcitalopram (12.3 ± 7.2 mg/day/1000 inhabitants) across all samples. This observation is consistent with the annual number of prescriptions obtained from the database of the Health Insurance Institute

Table 1
Quantification frequency (percentage of samples >LLOQ) of target compounds during all sampling periods (summer 2019, winter/spring 2020 and winter/spring 2021) in Ljubljana, Maribor, Novo Mesto, Velenje, Domžale-Kamnik and Koper (n = 35 per location).

Compound	LLOQ (ng/L)	Quantification frequency (%)					
		Ljubljana (LJ)	Maribor (MB)	Novo Mesto (NM)	Velenje (VE)	Domžale-Kamnik (DK)	Koper (KP)
Amitriptyline	25	0	0	66	0	0	0
Bupropion	10	6	6	0	0	0	21
Bupropion-OH	10	100	100	100	91	100	100
Citalopram	10	100	100	100	100	100	100
Norcitalopram	20	100	100	100	85.7	100	100
Melitracen	25	0	0	0	0	0	0
Moclobemide	2.5	100	100	100	97	100	94
Normirtazapine	10	91	94	91	6	66	89
Trazodone	5	100	100	100	94	100	100
mCPP	30	0	0	0	0	0	0
Venlafaxine	5	100	100	100	97	100	100
O-desmethylvenlafaxine	50	100	100	100	97	100	100

of Slovenia (ZZZS, 2022). Venlafaxine, citalopram, trazodone, and mirtazapine were the most frequently prescribed antidepressants. Similar findings have been reported in other studies conducted in Belgium, Greece, India, Turkey, and the USA, where the highest PNMLs of antidepressants were observed for venlafaxine/*O*-desmethylvenlafaxine (Boogaerts et al., 2019; Kosma et al., 2019; Papageorgiou et al., 2016; Quireyns et al., 2022; Skees et al., 2018; Subedi et al., 2017; Yavuz-Guzel et al., 2022).

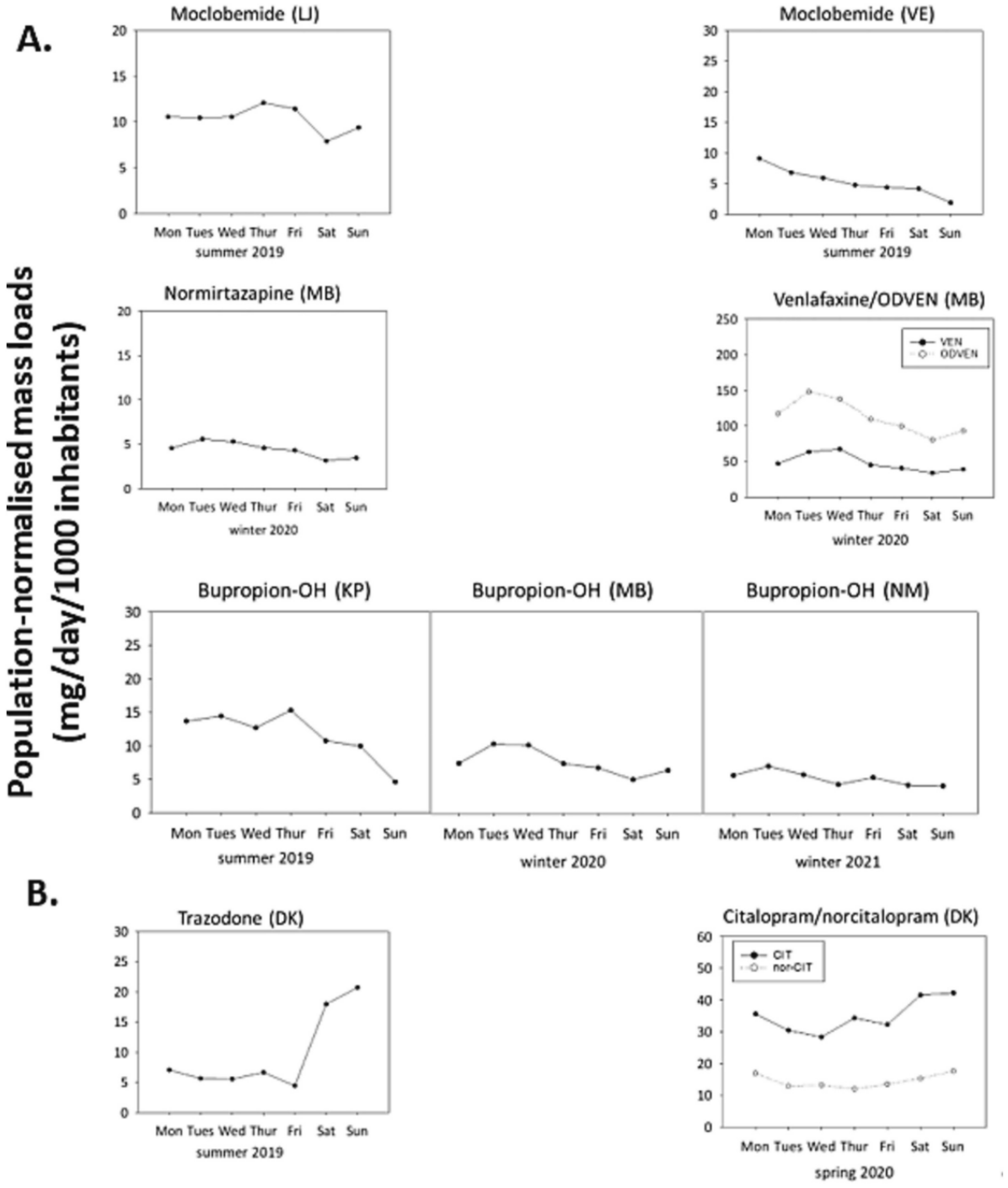


Fig. 1. Plots showing population-normalised mass loads (PNMLs) in mg/day/1000 inhabitants of biomarkers of antidepressants. Data shows statistically significant lower PNMLs and a decreasing trend during weekends (A) and higher PNMLs and an increasing trend at weekends (B).

3.2. Temporal differences in antidepressant use

3.2.1. Weekly trends (weekdays vs. weekends)

In general, the consumption of antidepressants is expected to be stable since patients are required to take their medication continuously and at strict time intervals. Initiating or discontinuing treatment is usually done by gradually adjusting the dosage. When a trend analysis was conducted to examine weekday-weekend (Mon-Fri vs Sat-Sun) antidepressant use, a few minor trends were identified; however, no clear overall pattern in the PNMLs emerged. Most statistically significant differences were observed prior to the pandemic, with a decrease ($p < 0.05$) in the PNMLs of moclobemide in Ljubljana (summer 2019), normirtazapine in Maribor (winter 2020), and bupropion-OH in Koper (summer 2019) during the weekend (Fig. 1A). Conversely, a statistically significant increase ($p < 0.05$) in the PNMLs of trazodone in Domžale-Kamnik (summer 2019) was observed during the weekend (Fig. 1B). Furthermore, the negative S-value of the Mann-Kendall trend indicated a decreasing trend over the sampling week (Fig. 1A) for moclobemide ($S = -21$) in Velenje (summer 2019), venlafaxine/*O*-desmethylvenlafaxine ($S = -13/-15$), and bupropion-OH ($S = -15$) in Maribor (winter 2020).

The only discernible effect of the pandemic, as indicated by a comparison between weekdays and weekends, was observed for citalopram/norcitalopram in Domžale-Kamnik during the first wave in spring 2020 and for bupropion-OH in Novo Mesto during the second wave in winter 2021. The data demonstrate a statistically significant increase in the PNMLs of citalopram and norcitalopram during the weekend ($p < 0.05$), whereas the PNMLs of bupropion-OH decreased ($p < 0.05$). The Mann-Kendall trend analysis revealed similar outcomes, indicating a monotonic decrease in the PNMLs of bupropion-OH in Novo Mesto ($S = -15$) during the sampling week of winter 2021. Conversely, the PNMLs of the other antidepressants exhibited independent distributions throughout the sampling periods, suggesting consistent use throughout the week (Fig. S1-S6). A trend in the PNMLs of antidepressants between weekdays and weekends was also observed in Belgium during the pre-pandemic period and second lockdown (October 2020–May 2021), with the highest PNMLs mainly observed during the weekends (Boogaerts et al., 2023a). However, in contrast to the findings of Boogaerts et al. (2023a) and this study, other studies by Yavuz-Guzel et al. (2022) and Reinstadler et al. (2021) did not reveal a statistically significant difference in antidepressant consumption between weekdays and weekends. Consequently, it is challenging to draw definite conclusions regarding whether the differences observed in our data are related to the pandemic or influenced by other factors, such as population movement.

During the pandemic and the associated social restrictions, including lockdown measures and remote working, the reduced disparity in the PNMLs between workdays and weekends could be attributed to changes in the socio-demographic characteristics of the population within the catchment area. To test this, Boogaerts et al. (2023a) employed a dynamic population proxy, specifically mobile phone data, to attribute the observed weekly pattern to demographic shifts induced by the pandemic measures within the catchment population. These shifts included reduced weekday commuting, increased weekend visitors, and the impact of reduced tourism. However, in our study, we relied on a fixed population size provided by the WWTPs, as accessing mobile phone data was not feasible.

3.2.2. Long-term trends (2019–2021)

Diverse temporal patterns in psychoactive pharmaceutical use were identified across the six locations in Slovenia (Fig. 2), with Domžale-Kamnik exhibiting the highest degree of variation while Velenje and Koper showed the least variability. In Domžale-Kamnik, we observed a significant increase ($p < 0.05$) in the PNMLs of bupropion-OH, citalopram, norcitalopram, moclobemide and normirtazapine in both winter and spring 2020 compared to winter and spring 2021 (Fig. 2 and Fig. S7). Conversely, in Ljubljana, there was relatively less temporal variation in the usage of antidepressants, suggesting a consistent pattern in the PNMLs of these medications, except for moclobemide which exhibited a 50 % decrease in consumption from 10.3 ± 1.4 mg/day/1000 inhabitants in summer 2019 to 5.1 ± 1.7 mg/day/1000 inhabitants during lockdown in spring 2021 (Fig. 2 and Fig. S8).

A similar trend for moclobemide was also observed in Novo Mesto, with a 36 % decrease in PNMLs from summer 2019 to spring 2021 (Fig. 2 and Fig. S9). This decline was also reflected in the number of annual prescriptions for moclobemide, which decreased by 16 % between 2019 and 2021. Moclobemide belongs to a class of antidepressants that, despite being among the most effective for treating unipolar major depression, is not commonly prescribed due to dietary restrictions, side effects and safety concerns (Sub Laban and Saadabadi, 2023). It is worth noting that there were no significant fluctuations in the mass loads of trazodone, except during winter 2021 when the PNMLs reached 27.9 mg/day/1000 inhabitants on Wednesday, compared to the average of 7.4 ± 1.1 mg/day/1000 inhabitants during the rest of the sampling periods. Additionally, in Novo Mesto, only a few temporal variations were observed. Except for moclobemide, there was a general increase in the PNMLs of several antidepressants during the COVID-19 pandemic (Fig. 2 and Fig. S9). For instance, the PNMLs of trazodone increased by 35 % between the pre-pandemic and lockdown periods. Moreover, an increase in the PNMLs of amitriptyline, normirtazapine, venlafaxine, and *O*-desmethylvenlafaxine, was also observed, especially during the spring 2021 lockdown (7.9, 5.2, 35.7, and 67.6 mg/day/1000 inhabitants, respectively), compared to winter 2020 (3.1, 2.8, 22.2, 44.2 mg/day/ 1000 inhabitants, respectively).

The variations in PNMLs may be attributed to changes in the population dynamics during the pandemic, such as the implementation of work-from-home policies, restrictions on movement between municipalities, and the absence of tourism. According to the available Mobility Report for Slovenia, the percentage of time spent in the municipality of residence and working from home increased to 25 % and 55 %, respectively, during the spring 2020 and winter 2021 lockdown periods compared to the pre-pandemic period (Mathieu et al., 2020). Another possible explanation could be the sharp decline in international tourist arrivals in Slovenia, mainly in Ljubljana, during the pandemic. Annual reports indicate an average 60–70 % decrease in tourist numbers between 2019 and 2021 (Tourism in numbers/Turizem v številkah, 2020,2021). Boogaerts et al. (2023a) also observed increased PNMLs of antidepressants during the initial lockdown phase (March–June 2020), likely resulting from the demographic shifts occurring during the COVID-19 pandemic rather than consumption habits.

In Velenje, there was an increasing trend in the consumption of trazodone during both winter and spring 2021, with a 60 % increase in the PNMLs observed in spring 2021 (18.8 ± 10.5 mg/day/1000 inhabitants) compared to spring 2020 (7.5 ± 0.9 mg/day/1000 inhabitants). The data also indicated slightly increased PNMLs for citalopram, norcitalopram, venlafaxine, and *O*-desmethylvenlafaxine from summer 2019 to spring 2021 (Fig. 2 and Fig. S10). However, it is important to note that the PNMLs for citalopram during one day (Tuesday) in the sampling week of spring 2020 were exceptionally high (351.3 mg/day/1000 inhabitants) compared to the rest of the week (average 30.1 ± 13.4 mg/day/1000 inhabitants). This fluctuation was not observed in the PNMLs of its metabolite, norcitalopram (11.6 mg/day/1000 inhabitants on that day and 11.0 ± 1.7 mg/day/1000 inhabitants on average). This discrepancy suggests direct disposal of citalopram rather than consumption (Fig. 2 and Fig. S10). In Koper (Fig. 2 and Fig. S11), there were no significant variations observed in the consumption of antidepressants, except for bupropion, which showed an increase in consumption between 2020 (<LLOQ) and 2021 (4 ± 1.7 mg/day/1000 inhabitants). Similar studies have reported different findings; for instance, some show a significant increase in PNMLs (Boogaerts et al., 2023a; Yavuz-Guzel et al., 2022), while others found no statistically significant difference or a slight increase, except for venlafaxine, which exhibited a notable increase (Alygizakis et al., 2021; Galani et al., 2021; Reinstadler et al., 2021).

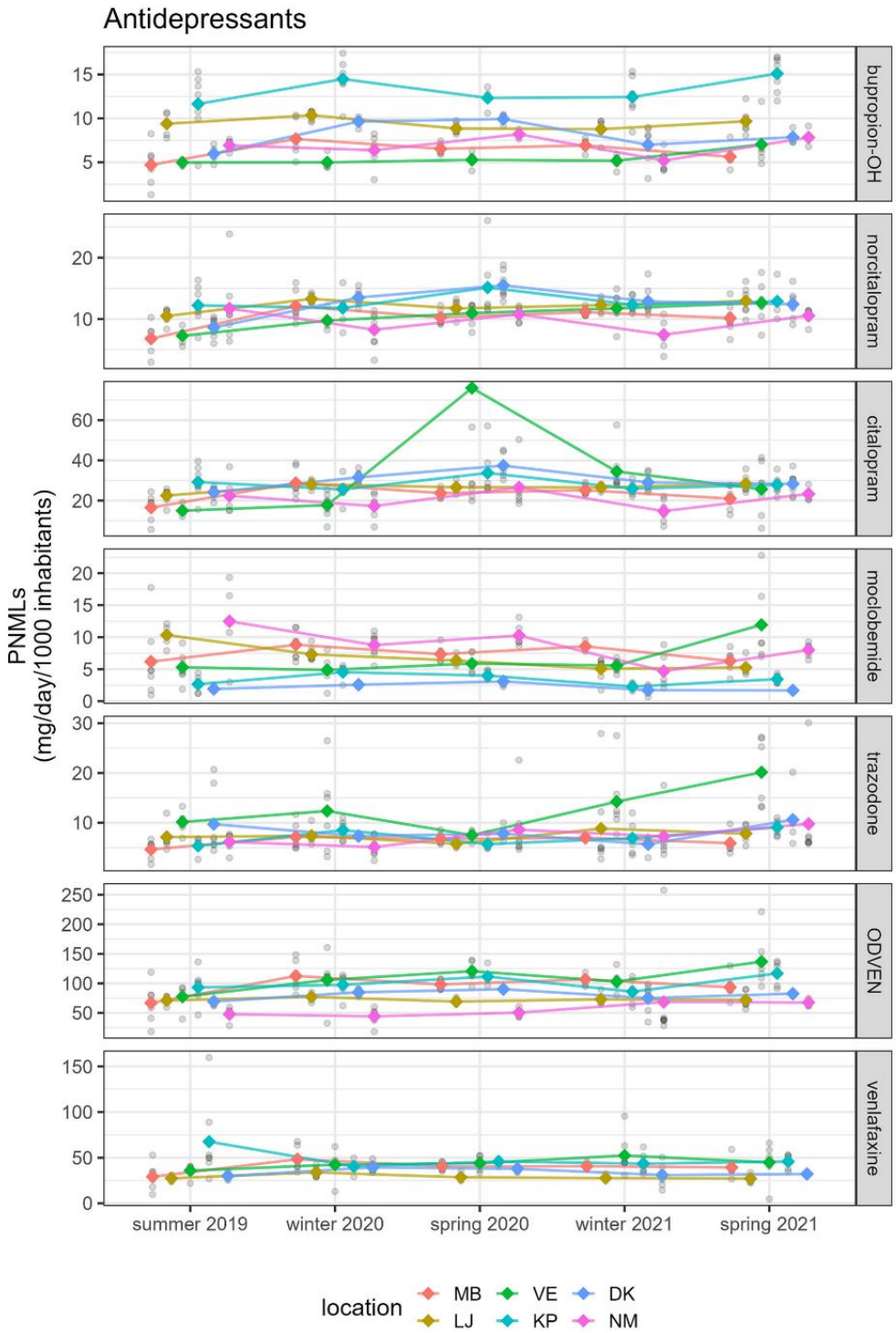


Fig. 2. Graphs showing the long-term trends in the population-normalised mass loads (PNMLs) of the different antidepressant biomarkers in all WWTPs. Only compounds with detection frequency > 85 % in each location are shown. Coloured dots represent the average of 7-day values of PNMLs which are shown in grey. For citalopram, the outlier due to dumping has been removed from the figure (but not the mean calculation).

The overall increase in the PNMLs of venlafaxine, normirtazapine, and trazodone from summer 2019 to winter 2021 aligns with the rise in their annual number of prescriptions, specifically a 4 % increase in venlafaxine, a 5 % increase for the parent drug mirtazapine, and a 10 % increase for trazodone ("ZZZS", 2022). However, there was a 7 % increase in mirtazapine annual prescriptions in 2020, followed by a slight 2 % decrease compared to 2021. Similarly, bupropion and citalopram exhibited a comparable trend, with a 5 % and 7 % increase in the annual number of prescriptions between 2019 and 2020, respectively, returning to similar levels as 2019 in 2021. It is important to note that sales and prescription data are compiled nationally, while WBE operates at different spatial resolutions, such as city-level or national-level assessments, and this may account for the discrepancies between the number of prescriptions and WBE data. Furthermore, such differences could arise from (i) prescriptions may not be filled; (ii) filled prescriptions may not be consumed; (iii) pharmaceuticals may not be taken at the recommended dose and (iv) specific insurance modalities are not included.

3.2.3. Seasonal trends

This study compared seasons across different years, which means that longitudinal trends could have influenced the observed variations. However, verifying intra-year seasonal patterns is challenging due to the limited coverage of each season in the sample collection. Also, since the samples were collected as a part of a previous study (Verovšek et al., 2023), i.e., not specifically for studying the effects of the pandemic, the sample size was limited in terms of the seasons and periods within each season. Nevertheless, longitudinal trends across all locations, resulting from ANOVA and Kruskal-Wallis test with Tukey's post-hoc test, are visualised in Fig. S13-S18. The results demonstrate comparable patterns in Ljubljana, Maribor, and Domžale-Kamnik. In winter 2020, the PNMLs for four (citalopram, norcitalopram, venlafaxine, and *O*-desmethylvenlafaxine), five (citalopram, norcitalopram, venlafaxine, *O*-desmethylvenlafaxine, and normirtazapine) and six (bupropion-OH, citalopram, norcitalopram, venlafaxine, *O*-desmethylvenlafaxine, and moclobemide) antidepressant biomarkers, respectively, were statistically higher compared to summer 2019. This observation could be attributed to seasonal variation, as both sampling campaigns occurred before the pandemic. Similar findings of higher mass loads during winter have been reported in long-term monitoring studies for antidepressants (Golovko et al., 2014), such as citalopram, venlafaxine and tramadol (Mackuľak et al., 2016).

This seasonal increase could be associated with seasonal affective disorder (SAD), a mood disorder where individuals with normal mental health throughout most of the year exhibit depressive symptoms at the same time each year, commonly but not always in winter months when sunlight is reduced. In Maribor, seasonal variation is more apparent, as the PNMLs of citalopram, norcitalopram, *O*-desmethylvenlafaxine and normirtazapine in both winter 2020 and winter 2021 were higher compared to summer 2019 ($p < 0.05$) (Fig. 2 and Fig. S12).

3.3. Spatial differences

Spatial differences, determined by ANOVA or Kruskal-Wallis test with Tukey's post-hoc test, are visualised in Fig. S19. The data reveals spatial trends for all compounds in almost all sampling periods (Fig. 3). These differences could be attributed to variations in the demographic characteristics of the catchment areas, such as average age, the proportion of university students, and the tourist numbers. On average, the highest PNMLs for *O*-desmethylvenlafaxine were obtained in Velenje (106.9 ± 19.6 mg/day/1000 inhabitants), displaying a statistically significant difference ($p < 0.05$) compared to Novo Mesto, which had the lowest PNMLs (55.7 ± 11.5 mg/day/1000 inhabitants). Both venlafaxine and *O*-desmethylvenlafaxine exhibited similar spatial variations across all sampling periods, with the highest variation observed in spring 2020 during the first wave of the pandemic. Notably, they show identical spatial variations in winter and spring 2020 (Fig. 3. and Fig. S20).

Spatial variations were observed for citalopram and its metabolite norcitalopram in winter 2020, while no spatial variations were observed in spring 2021. The highest PNMLs (Fig. 3 and Fig. S20) for trazodone (12.3 ± 4.4 mg/day/1000 inhabitants) were found in Velenje (12.3 ± 4.4 mg/day/1000 inhabitants). Variations in PNMLs of trazodone were mainly observed during the winter and spring of 2021. Amitriptyline was only detected above LLOQ in Novo Mesto. On the other hand, bupropion was detected and quantified primarily in Koper (sampling periods summer 2019 and winter and spring 2021), as well as in Ljubljana and Maribor, while it was <LLOQ in Velenje, Domžale-Kamnik and Novo Mesto (Fig. 3 and Fig. S20). Koper exhibited the highest PNMLs for bupropion-OH (13.2 mg/day/1000 inhabitants), consistent with the higher PNMLs of bupropion as the parent compound in the same location. Moclobemide had significantly lower PNMLs in Domžale-Kamnik than the other WWTPs, with most spatial variations observed in spring 2020 during the first lockdown. However, for normirtazapine, which was measured in all WWTPs except for Velenje (only in spring 2021 was >LLOQ), the greatest spatial variation was in spring 2021 (Fig. 3 and Fig. S20). Overall, the results show that spatial variations were mainly evident for bupropion, venlafaxine, moclobemide and tramadol during spring 2020, coinciding with the first wave of the pandemic in Slovenia.

3.4. Parent compound/metabolite ratios

In the present study, both the parent compound and the metabolite were included for the following substances: bupropion, citalopram, trazodone, and venlafaxine. The metabolite/parent compound ratio (M/PC ratio) was not calculated for trazodone/mCPP since the concentration of mCPP was below the LLOQ. The M/PC ratios for citalopram/norcitalopram and venlafaxine/*O*-desmethylvenlafaxine remained relatively stable across all the sampling periods and locations, with a relative standard deviation (% RSD) of the measured M/PC ratio below 25 %. The measured M/PC ratios in influent wastewater are consistent with other studies (Table 2). However, the average M/PC ratios of bupropion/bupropion-OH were slightly lower than in other WBE studies (Boogaerts et al., 2019). Notably, the bupropion/bupropion-OH ratios were only above the LLOQ in a limited number of samples. The relatively stable spatiotemporal pattern in the M/PC ratios suggests that the mass loads of the biomarkers likely derive from consumption rather than direct disposal of the parent compound. However, for citalopram, there was a notable change in the M/PC (0.03 compared to the average of 0.44) on a specific sampling day during spring 2020 in Velenje, indicating potential dumping (Section 3.2.2).

3.5. Limitations of the study

The sampling campaign involved six WWTPs in urban and rural areas, accounting for just 30 % of the Slovenian population; however, they cover the main geographic regions, and are used in the SCORE monitoring. Due to logistical considerations and the involvement of multiple sampling locations and periods, the start of sampling collection varied by location, resulting in different orders of data points across sampling periods and locations. Therefore, the data was arranged from Monday to Sunday to facilitate statistical comparisons and evaluate spatiotemporal trends. Another limitation is the inclusion of only one week of sampling within each period (summer 2019, winter 2020, spring 2020, winter 2021 and spring 2021) for the spatiotemporal comparisons. Consequently, consumption rates may not fully represent the rest of the year. However, since all sampling periods were conducted in a "typical" week, which should reflect baseline consumption, we assume that weekdays and weekends represent baseline use within a regular working week. Furthermore, the fact that we did not consistently sample in the same season adds complexity to the interpretation of any seasonal trends.

Another limitation is the use of fixed population data provided by the

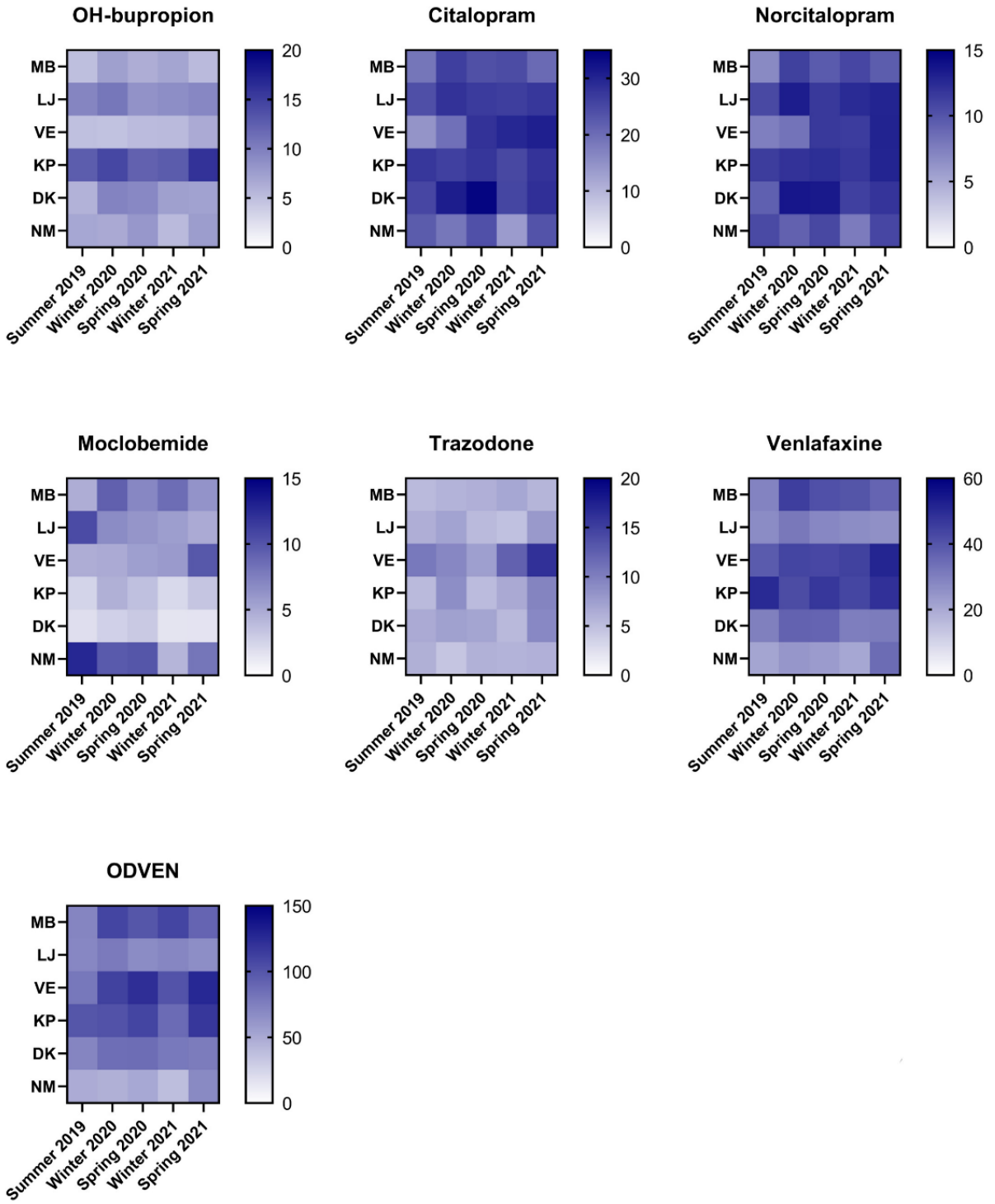


Fig. 3. Heatmaps of the spatiotemporal variations, visualised by heatmaps, in the population-normalised mass loads of the different biomarkers of antidepressant use. The darker the colour, the higher the PNMLs. Only compounds with detection frequency > 85 % in each location are shown.

Table 2
Comparison of the measured metabolite/parent compound ratios with other WBE studies.

Parent compound/ metabolite	Measured M/ PC ratio in this study (average ± SD)	Measured M/ PC ratio in other studies	Ref.
Bupropion/Bupropion-OH	3.98 + 1.36	4.5	(Boogaerts et al., 2019)
Citalopram/ Norcitalopram	0.44 ± 0.06	0.5–0.8	(Boogaerts et al., 2023a, 2019)
Venlafaxine/*O*-desmethylvenlafaxine	2.33 ± 0.25	2.0–5.8	(Boogaerts et al., 2023a, 2019; Rúa-Gómez and Püttmann, 2012)

WWTPs, in the back-calculations of psychoactive pharmaceutical consumption. It is important to note that variations in population could occur due to pandemic measures resulting in factors such as changes in commuting habits and tourism. An increase in the mass loads could be attributed to a greater number of people consuming psychoactive pharmaceuticals, higher quantities, or a combination of both. To address this limitation, the use of dynamic population markers is recommended. Previous studies have utilised markers such as mobile phone data (Boogaerts et al., 2023a), chemical oxygen demand, biological oxygen demand, phosphorus, nitrogen, and ammonium loads (Di Marcantonio et al., 2022; Reinstadler et al., 2021; Tomsone et al., 2022) to account for variations in the socio-demographics of the catchment population. However, in this study, we attempted to use mobile phone tracking data, but permission to access this data was denied. Additionally, using other dynamic population markers, such as nitrogen, is not feasible due to the inclusion of wastewater from agricultural activities.

4. Conclusions

This study highlights the potential of WBE as a valuable tool for monitoring trends in psychoactive pharmaceutical consumption with a high level of temporal and spatial resolution, surpassing other data sources such as general population surveys, hospital records, and prescription and sales data. The findings of the study revealed numerous temporal and spatial variations. However, no clear patterns related to the pandemic were observed in the analysis of weekdays vs weekends and spatial trends. Incorporating dynamic population numbers in the calculation of PNMLs would be beneficial in elucidating changes in the socio-demographic characteristics of the population within the catchment area. Among the compounds detected, *O*-desmethylvenlafaxine, venlafaxine, citalopram and norcitalopram exhibited the highest PNMLs, while the M/PC ratios of the different biomarkers were consistent with findings from other WBE studies, demonstrating relative stability throughout the entire sampling campaign. This finding suggests that measured PNMLs likely derive from human consumption rather than dumping. Overall, the PNMLs of most psychoactive pharmaceuticals showed an increasing trend during the sampling campaigns, particularly during the first wave of the pandemic in the spring of 2020. These findings align with available data on annual prescriptions, further supporting the validity of the results obtained through WBE.

CRediT authorship contribution statement

Maria Laimou-Geraniou: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Maarten Quireyns:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – review & editing. **Tim Boogaerts:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – review & editing. **Natan Van Wichelen:** Methodology, Investigation, Writing – review & editing. **David Heath:** Conceptualization, Writing – review & editing. **Alexander L.N. van Nuijs:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Adrian Covaci:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Ester Heath:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Financial support: This work was supported by the Slovenian Research Agency (ARRS Program group P1-0143 and Projects N1-0143, J2-4427, and L7-4422) and Research Scientific Foundation Flanders (FWO, project number: G060920N).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166586.

References

Alygizakis, N., Galani, A., Rousis, N.I., Aalizadeh, R., Dimopoulos, M.-A., Thomaidis, N. S., 2021. Change in the chemical content of untreated wastewater of Athens, Greece under COVID-19 pandemic. Sci. Total Environ. 799, 149230 https://doi.org/10.1016/j.scitotenv.2021.149230.

Antidepressant consumption in selected countries 2021 [WWW Document], n.d. Statista. URL https://www.statista.com/statistics/283072/antidepressant-consumption-in-selected-countries/ (accessed 4.20.23).

Bade, R., White, J.M., Chen, J., Baz-Lomba, J.A., Been, F., Bijlsma, L., Burgard, D.A., Castiglioni, S., Salgueiro-Gonzalez, N., Celma, A., Chappell, A., Emke, E., Steenbeek, R., Wang, D., Zuccato, E., Gerber, C., 2021. International snapshot of new psychoactive substance use: case study of eight countries over the 2019/2020 new year period. Water Res. 193, 116891 https://doi.org/10.1016/j.watres.2021.116891.

Baker, D.R., Barron, L., Kasprzyk-Hordern, B., 2014. Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: chemical analysis and drug use estimates. Sci. Total Environ. 487, 629–641. https://doi.org/10.1016/j.scitotenv.2013.11.107.

Boogaerts, T., Degreef, M., Covaci, A., van Nuijs, A.L.N., 2019. Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach. Talanta 200, 340–349. https://doi.org/10.1016/j.talanta.2019.03.052.

Boogaerts, T., Ahmed, F., Choi, Phil.M., Tscharke, B., O'Brien, J., De Loof, H., Gao, J., Thai, P., Thomas, K., Mueller, J.F., Hall, W., Covaci, A., van Nuijs, A.L.N., 2021. Current and future perspectives for wastewater-based epidemiology as a monitoring tool for pharmaceutical use. Sci. Total Environ. 789, 148047 https://doi.org/10.1016/j.scitotenv.2021.148047.

Boogaerts, T., Quireyns, M., De Loof, H., Bertels, X., Van Wichelen, N., Pussig, B., Saevels, J., Lahousse, L., Bonmariage, P., Hamelinck, W., Aertgeerts, B., Covaci, A., van Nuijs, A.L.N., 2023. Do the lockdown-imposed changes in a wastewater treatment plant catchment's socio-demographics impact longitudinal temporal trends in psychoactive pharmaceutical use? Sci. Total Environ. 876, 162342 https://doi.org/10.1016/j.scitotenv.2023.162342.

Boogaerts, T., Quireyns, M., Maes, F., Laimou-Geraniou, M., Van Wichelen, N., Heath, E., Pussig, B., Aertgeerts, B., Covaci, A., van Nuijs, A.L.N., 2023. Optimization, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater. Drug Test. Anal. 15, 240–246. https://doi.org/10.1002/dta.3392.

Choi, P.M., Li, J., Gao, J., O'Brien, J.W., Thomas, K.V., Thai, P.K., Jiang, G., Mueller, J.F., 2020. Considerations for assessing stability of wastewater-based epidemiology biomarkers using biofilm-free and sewer reactor tests. Sci. Total Environ. 709, 136228 https://doi.org/10.1016/j.scitotenv.2019.136228.

Di Marcantonio, C., Chiavola, A., Gioia, V., Frugis, A., Cecchini, G., Ceci, C., Spizzirri, M., Boni, M.R., 2022. Impact of COVID19 restrictions on organic micropollutants in

wastewater treatment plants and human consumption rates. Sci. Total Environ. 811, 152327 https://doi.org/10.1016/j.scitotenv.2021.152327.
Diaz-Camal, N., Cardoso-Vera, J.D., Islas-Flores, H., Gómez-Oliván, L.M., Mejía-García, A., 2022. Consumption and ocurrence of antidepressants (SSRIs) in pre- and post-COVID-19 pandemic, their environmental impact and innovative removal methods: a review. Sci. Total Environ. 829, 154656 https://doi.org/10.1016/j.scitotenv.2022.154656.
Escolà Casas, M., Schröter, N.S., Zammit, I., Castaño-Trias, M., Rodriguez-Mozaz, S., Gago-Ferrero, P., Corominas, Ll, 2021. Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: comparison against prescription data collected at fine spatial resolution. Environ. Int. 150, 106404 https://doi.org/10.1016/j.envint.2021.106404.
Estévez-Danta, A., Bijlsma, L., Capela, R., Cela, R., Celma, A., Hernández, F., Lertxundi, U., Matias, J., Montes, R., Orive, G., Prieto, A., Santos, M.M., Rodil, R., Quintana, J.B., 2022. Use of illicit drugs, alcohol and tobacco in Spain and Portugal during the COVID-19 crisis in 2020 as measured by wastewater-based epidemiology. Sci. Total Environ. 836, 155697 https://doi.org/10.1016/j.scitotenv.2022.155697.
Fáberová, M., Bodík, I., Ivanová, L., Grabic, R., Mackuľak, T., 2017. Frequency and use of pharmaceuticals in selected Slovakian town via wastewater analysis. Monatsh. Chem. 148, 441–448. https://doi.org/10.1007/s00706-016-1853-0.
Galani, A., Alygizakis, N., Aalizadeh, R., Kastritis, F., Dimopoulos, M.-A., Thomaidis, N. S., 2021. Patterns of pharmaceuticals use during the first wave of COVID-19 pandemic in Athens, Greece as revealed by wastewater-based epidemiology. Sci. Total Environ. 798, 149014 https://doi.org/10.1016/j.scitotenv.2021.149014.
Gent, L., Paul, R., 2021. The detection of new psychoactive substances in wastewater. A comprehensive review of analytical approaches and global trends. Sci. Total Environ. 776, 146028 https://doi.org/10.1016/j.scitotenv.2021.146028.
Global Burden of Disease Study 2019 (GBD 2019) Data Resources | GHDx [WWW Document], n.d. URL https://ghdx.healthdata.org/gbd-2019 (accessed 4.17.23).
Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant. Chemosphere 111, 418–426. https://doi.org/10.1016/j.chemosphere.2014.03.132.
Gracia-Lor, E., Rousis, N.I., Zuccato, E., Castiglioni, S., 2020. Monitoring caffeine and nicotine use in a nationwide study in Italy using wastewater-based epidemiology. Sci. Total Environ. 747, 141331 https://doi.org/10.1016/j.scitotenv.2020.141331.
Huizer, M., ter Laak, T.L., de Voogt, P., van Wezel, A.P., 2021. Wastewater-based epidemiology for illicit drugs: a critical review on global data. Water Res. 207, 117789 https://doi.org/10.1016/j.watres.2021.117789.
Kosma, C.I., Nannou, C.I., Boti, V.I., Albanis, T.A., 2019. Psychiatrics and selected metabolites in hospital and urban wastewaters: occurrence, removal, mass loading, seasonal influence and risk assessment. Sci. Total Environ. 659, 1473–1483. https://doi.org/10.1016/j.scitotenv.2018.12.421.
Kozina, A., Peras, I., Veldin, M., Pivec, T., 2022. The psychological response and perception of stress during the COVID-19 pandemic in Slovenia: three-wave repeated cross-sectional study. Stress. Health 38, 950–960. https://doi.org/10.1002/smi.3147.
Laimou-Geraniou, M., Heath, D., Heath, E., 2023. Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology. Trends Environ. Anal. Chem. 37, e00192 https://doi.org/10.1016/j.teac.2022.e00192.
López-García, E., Pérez-López, C., Postigo, C., Andreu, V., Bijlsma, L., González-Mariño, I., Hernández, F., Marcé, R.M., Montes, R., Picó, Y., Pocurull, E., Rico, A., Rodil, R., Rosende, M., Valcárcel, Y., Zuloaga, O., Quintana, J.B., López de Alda, M., 2020. Assessing alcohol consumption through wastewater-based epidemiology: Spain as a case study. Drug Alcohol Depend. 215, 108241 https://doi.org/10.1016/j.drugalcdep.2020.108241.
Mackuľak, T., Birošová, L., Gál, M., Bodík, I., Grabic, R., Ryba, J., Škubák, J., 2016. Wastewater analysis: the mean of the monitoring of frequently prescribed pharmaceuticals in Slovakia. Environ. Monit. Assess. 188, 18. https://doi.org/10.1007/s10661-015-5011-7.
Mathieu, E., Ritchie, H., Rodés-Guirao, L., Appel, C., Giattino, C., Hasell, J., Macdonald, B., Dattani, S., Beltekian, D., Ortiz-Ospina, E., Roser, M., 2020. Coronavirus Pandemic (COVID-19). Our World in Data.
Melchor-Martínez, E.M., Jiménez-Rodríguez, M.G., Martínez-Ruiz, M., Peña-Benavides, S.A., Iqbal, H.M.N., Parra-Saldívar, R., Sosa- Hernández, J.E., 2021. Antidepressants surveillance in wastewater: overview extraction and detection. Case Stud. Chem. Environ. Eng. 3, 100074 https://doi.org/10.1016/j.cscee.2020.100074.
Papageorgiou, M., Kosma, C., Lambropoulou, D., 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. Sci. Total Environ. 543, 547–569. https://doi.org/10.1016/j.scitotenv.2015.11.047.
Pazzagli, L., Reutfors, J., Lucian, E., Zerial, G., Perulli, A., Castelpietra, G., 2022. Increased antidepressant use during the COVID-19 pandemic: findings from the Friuli Venezia Giulia region, Italy, 2015–2020. Psychiatry Res. 315, 114704 https://doi.org/10.1016/j.psychres.2022.114704.
Quireyns, M., Boogaerts, T., Van Wichelen, N., Pussig, B., De Loof, H., Covaci, A., Van Nuijs, A., 2022. Temporal monitoring of pharmaceutical consumption using a wastewater-based epidemiologic approach. In: Toxicologie Analytique et Clinique, 30th Meeting of SFTA- 59th Meeting of TIAFT - September 2022 34, S72. https://doi.org/10.1016/j.toxac.2022.06.099.
Reinstadler, V., Ausweger, V., Grabher, A.-L., Kreidl, M., Huber, S., Grander, J., Haslacher, S., Singer, K., Schlapp-Hackl, M., Sorg, M., Erber, H., Oberacher, H., 2021. Monitoring drug consumption in Innsbruck during coronavirus disease 2019 (COVID-19) lockdown by wastewater analysis. Sci. Total Environ. 757, 144006 https://doi.org/10.1016/j.scitotenv.2020.144006.
Rice, J., Kannan, A.M., Castrignanò, E., Jagadeesan, K., Kasprzyk-Hordern, B., 2020. Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - a UK perspective. Sci. Total Environ. 735, 139433 https://doi.org/10.1016/j.scitotenv.2020.139433.
Riva, F., Castiglioni, S., Pacciani, C., Zuccato, E., 2020. Testing urban wastewater to assess compliance with prescription data through wastewater-based epidemiology: first case study in Italy. Sci. Total Environ. 739, 139741 https://doi.org/10.1016/j.scitotenv.2020.139741.
Rúa-Gómez, P.C., Püttmann, W., 2012. Occurrence and removal of lidocaine, tramadol, venlafaxine, and their metabolites in German wastewater treatment plants. Environ. Sci. Pollut. Res. 19, 689–699. https://doi.org/10.1007/s11356-011-0614-1.
Rus Prelog, P., Matić, T., Pregelj, P., Sadikov, A., 2022. Risk of depression, anxiety, and stress during the second wave of COVID-19 in Slovenia. Frontiers. Psychiatry 12.
Skees, A.J., Foppe, K.S., Loganathan, B., Subedi, B., 2018. Contamination profiles, mass loadings, and sewage epidemiology of neuropsychiatric and illicit drugs in wastewater and river waters from a community in the Midwestern United States. Sci. Total Environ. 631–632, 1457–1464. https://doi.org/10.1016/j.scitotenv.2018.03.060.
Sub Laban, T., Saadabadi, A., 2023. Monoamine Oxidase Inhibitors (MAOI). StatPearls. StatPearls Publishing, Treasure Island (FL), in.
Subedi, B., Balakrishna, K., Joshua, D.I., Kannan, K., 2017. Mass loading and removal of pharmaceuticals and personal care products including psychoactives, antihypertensives, and antibiotics in two sewage treatment plants in southern India. Chemosphere 167, 429–437. https://doi.org/10.1016/j.chemosphere.2016.10.026.
Tiger, M., Wesselhoeft, R., Karlsson, P., Handal, M., Bliddal, M., Cesta, C.E., Skurtveit, S., Reutfors, J., 2023. Utilization of antidepressants, anxiolytics, and hypnotics during the COVID-19 pandemic in Scandinavia. J. Affect. Disord. 323, 292–298. https://doi.org/10.1016/j.jad.2022.11.068.
Tourism in numbers/ Turizem v številkah, 2020, pdf. URL:https://www.slovenia.info/uploads/dokumenti/tvs/2020/2021_03_STO_TVS_2020_SLO.pdf (accessed 4.20.23).
Tourism in numbers/ Turizem v številkah, 2021, pdf. URL:https://www.slovenia.info/uploads/dokumenti/tvs/2021/2021_Turizem_v_%C5%A1tevilkah.pdf (accessed 4.20.23).
Tomsone, L.E., Perkons, I., Sukajeva, V., Neilands, R., Kokina, K., Bartkevics, V., Pugajeva, I., 2022. Consumption trends of pharmaceuticals and psychoactive drugs in Latvia determined by the analysis of wastewater. Water Res. 221, 118800 https://doi.org/10.1016/j.watres.2022.118800.
Tscharke, B.J., Chen, C., Gerber, J.P., White, J.M., 2016. Temporal trends in drug use in Adelaide, South Australia by wastewater analysis. Sci. Total Environ. 565, 384–391. https://doi.org/10.1016/j.scitotenv.2016.04.183.
Verovšek, T., Šuštarič, A., Laimou-Geraniou, M., Krizman-Matasic, I., Prosen, H., Eleršek, T., Kramarič Zidar, V., Mislej, V., Mišmaš, B., Stražar, M., Levstek, M., Cimrmančič, B., Lukšič, S., Uranjek, N., Kozlovič-Bobič, T., Kosjek, T., Kocman, D., Heath, D., Heath, E., 2023. Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment. Sci. Total Environ. 866, 161257 https://doi.org/10.1016/j.scitotenv.2022.161257.
Winkler, P., Mohrova, Z., Mlada, K., Kuklova, M., Kagstrom, A., Mohr, P., Formanek, T., 2021. Prevalence of current mental disorders before and during the second wave of COVID-19 pandemic: an analysis of repeated nationwide cross-sectional surveys. J. Psychiatr. Res. 139, 167–171. https://doi.org/10.1016/j.jpsychires.2021.05.032.
World Health Organization, 2022. Mental disorders: fact sheets. https://www.who.int/en/news-room/fact-sheets/detail/mental-disorders. (Accessed 20 March 2023).
Xiong, J., Lipsitz, O., Nasri, F., Lui, L.M.W., Gill, H., Phan, L., Chen-Li, D., Iacobucci, M., Ho, R., Majeed, A., McIntyre, R.S., 2020. Impact of COVID-19 pandemic on mental health in the general population: a systematic review. J. Affect. Disord. 277, 55–64. https://doi.org/10.1016/j.jad.2020.08.001.
Yavuz-Guzel, E., Atasoy, A., Gören, İ.E., Daglioglu, N., 2022. Impact of COVID- 19 pandemic on antidepressants consumptions by wastewater analysis in Turkey. Sci. Total Environ. 838, 155916 https://doi.org/10.1016/j.scitotenv.2022.155916.
ZZZS Podatki o porabi zdravil [WWW Document], n.d. URL https://partner.zzzs.si/wps/portal/portali/aizv/zdravila_in_zivila_za_posebne_zdravstvene_namene/podatki_o_porabi_zdravil/!ut/p/z1/04_Sj9CPykssy0xPLMnMz0vMAfljo8ziTQxdPd2N_Q08LSyCDQ0cjZzMzXz8XQ0sTAz0C7IdFQGdnpEx/ (accessed 3.31.23).

3.2.2 Three years of wastewater surveillance for new psychoactive substances from 16 countries

Published: R. Bade, N. Rousis, S. Adhikarib, C. Baduel, L. Bijlsma, E. Bizani, T. Boogaerts, D. Burgard, S. Castiglioni, A. Chappell, A. Covaci, E. M. Driverc, F. Fabriz Sodre, D. Fatta-Kassinos, A. Galani, C. Gerber, E. Gracia Lor, E. Gracia Marin, R. U. Halden, E. Heath, E. Jaunay, F. Yin Lai, H.-Jun Lee, M. Laimou-Geraniou, J.-E. Oh, K. Olafsdottir, K. Phung, M. Pineda Castro, M. Psichoudaki, X. Shao, N. Salgueiro-Gonzalez, R. Silva Feitosa, C. Silvino Gomes, B. Subedi, A. Sue Ching Löve, N. Thomaidis, D. Tran, A. van Nuijs, T. Verovšek, D. Wang, J. M. White, V. Yargeau, E. Zuccato, J. Mueller, 2023, *Water Research* X, vol. 19, pp. 100179.

My role in this international work included performing experimental work (sample preparation of Slovenian wastewater samples) and reviewing and editing the manuscript.

NPS represent a significant public health issue on a global scale and require systematic and comprehensive information on their availability and usage trends. Due to the ambiguous legal status of NPS, their rapidly evolving market, and the lack of information on their identity and related safety and health risks, this issue warrants attention. However, data on the worldwide prevalence of NPS remains scarce (1.1.4 New psychoactive substances and 1.2.2 Current dynamics of the NPS market). To address this gap, the present international study aimed to examine spatial and temporal trends in NPS use by applying a harmonised wastewater analysis protocol and comparing these findings with other available data sources.

A total of 546 municipal influent wastewater samples were collected over three consecutive New Year periods—2019/2020, 2020/2021, and 2021/2022. Over this time, the number of participating countries (sites) increased from eight (12 sites) to ten (25 sites) and, in the last year of the study, to 16 (47 sites). Notably, Slovenia participated in the sampling campaign (the 2021/2022 New Year period), contributing data from Ljubljana, Maribor, Novo Mesto, and Kranj.

To ensure consistency and comparability of the results across all locations, a standardised sampling and analysis protocol was followed. Each participating country was required to collect a maximum of seven consecutive 24-hour composite samples of raw influent wastewater at each sampling site during a predefined period (e.g., December 29, 2021, to January 4, 2022) and to extract the analytes using the same SPE extraction protocol with UCT XtracT DAU cartridges. All extracts were analysed at the University of Queensland, Australia.

Eighteen NPS, primarily synthetic cathinones, followed by phenethylamines and designer benzodiazepines, were detected within the three years of monitoring, which aligns with UNODC reports [1]. Their occurrence varied across all participating countries, following distinctive regional trends. Particularly, 3-MMC and *N*-ethylhexedrone were dominant in Europe, while eutylone and mephedrone were more prevalent in New Zealand, 2F-DCK was more frequently found in China, and the US showed the highest mass loads of mitragynine.

Temporal shifts in NPS usage were also observed, including the impact of the COVID-19 pandemic. However, 3-MMC was consistently detected across all campaigns, with its presence spreading from Europe to Oceania and its consumption increasing significantly from 5 mg/day/1,000 inhabitants in 2019/2020 to >100 mg/day/1,000 inhabitants by 2021/2022. The highest mass loads were found in Spain and Slovenia. Eutylone and mitragynine were also detected in samples from Slovenia.

This study highlights the utility and effectiveness of wastewater analysis for monitoring NPS use worldwide. It also establishes a foundation for further systematic surveillance of specific NPS use through wastewater analysis, following the example of the existing initiatives such as the SCORE network, and encourages the development of advanced workflows, including non-target approaches.

Study outcomes were presented at two scientific conferences: the Annual Conference of the International Association of Forensic Toxicologists (TIAFT 2024), St. Gallen, Switzerland, September 2–6, 2024, and Testing the Waters 6 (TTW6), Oxford, United Kingdom, June 26–27, 2023.

Water Research X 19 (2023) 100179

Contents lists available at ScienceDirect

Water Research X

journal homepage: www.sciencedirect.com/journal/water-research-x

Three years of wastewater surveillance for new psychoactive substances from 16 countries

Richard Bade [a,*], Nikolaos Rousis [a], Sangeet Adhikari [b,c], Christine Baduel [d], Lubertus Bijlsma [e], Erasmia Bizani [f], Tim Boogaerts [g], Daniel A. Burgard [h], Sara Castiglioni [i], Andrew Chappell [j], Adrian Covaci [g], Erin M. Driver [c,k], Fernando Fabriz Sodre [l], Despo Fatta-Kassinos [m], Aikaterini Galani [f], Cobus Gerber [n], Emma Gracia-Lor [o], Elisa Gracia-Marín [e], Rolf U. Halden [b,c,k,p], Ester Heath [q], Felix Hernandez [e], Emma Jaunay [n], Foon Yin Lai [r], Heon-Jun Lee [s], Maria Laimou-Geraniou [q], Jeong-Eun Oh [s], Kristin Olafsdottir [t], Kaitlyn Phung [j], Marco Pineda Castro [u], Magda Psichoudaki [m], Xueting Shao [v], Noelia Salgueiro-Gonzalez [i], Rafael Silva Feitosa [l], Cezar Silvino Gomes [w], Bikram Subedi [x], Arndís Sue Ching Löve [t], Nikolaos Thomaidis [f], Diana Tran [h], Alexander van Nuijs [g], Taja Verovšek [q], Degao Wang [v], Jason M. White [n], Viviane Yargeau [u], Ettore Zuccato [i], Jochen F. Mueller [a]

[a] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Cornwall Street, Woolloongabba, QLD 4102, Australia
[b] School of Sustainable Engineering and Built Environment, Arizona State University, Tempe, AZ, 85281, United States
[c] Biodesign Center for Environmental Health Engineering, Biodesign Institute, Arizona State University, 1001 S. McAllister Ave., Tempe, AZ 85281, United States
[d] Université Grenoble Alpes, CNRS, IRD, Grenoble INP, IGE, Grenoble, France
[e] Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Avda, Sos Baynat s/n, E-12071 Castellón, Spain
[f] Laboratory of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece
[g] Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp, 2610 Wilrijk, Belgium
[h] Department of Chemistry and Biochemistry, University of Puget Sound, Tacoma, WA 98416, United States
[i] Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Department of Environmental Health Sciences, Via Mario Negri 2, 20156, Milan, Italy
[j] Institute of Environmental Science and Research Limited (ESR), Christchurch Science Centre: 27 Creyke Road, Ilam, Christchurch 8041, New Zealand
[k] AquaVitas, LLC, Scottsdale, Arizona, 85251, United States
[l] Institute of Chemistry, University of Brasília, Brasília, DF, 70910-000, Brazil
[m] Nireas-International Water Research Centre and Department of Civil and Environmental Engineering, University of Cyprus, P.O. Box 20537, 1678, Nicosia, Cyprus
[n] Health and Biomedical Innovation, UniSA: Clinical and Health Sciences, University of South Australia, Adelaide 5001, South Australia, Australia
[o] Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, Avenida Complutense s/n, 28040 Madrid, Spain
[p] OneWaterOneHealth, Arizona State University Foundation, 1001 S. McAllister Avenue, Tempe, AZ 85287-8101, United States
[q] Jožef Stefan Institute and International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia
[r] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences (SLU), SE-75007 Uppsala, Sweden
[s] Department of Civil and Environmental Engineering, Pusan National University, Jangjeon-dong, Geumjeong-gu, Busan 46241, Republic of Korea
[t] University of Iceland, Department of Pharmacology and Toxicology, Hofsvallagata 53, 107 Reykjavik, Iceland
[u] Department of Chemical Engineering, McGill University, Montreal, QC
[v] College of Environmental Science and Engineering, Dalian Maritime University, No. 1 Linghai Road, Dalian, 116026, PR China
[w] Laboratory of Forensic Chemistry, Brazilian Federal Police, PB, Brazil
[x] Department of Chemistry, Murray State University, Murray, Kentucky 42071-3300, United States

ARTICLE INFO

Keywords:
Illicit drugs
Public health
Monitoring
3-methylmethcathinone
Wastewater analysis

ABSTRACT

The proliferation of new psychoactive substances (NPS) over recent years has made their surveillance complex. The analysis of raw municipal influent wastewater can allow a broader insight into community consumption patterns of NPS. This study examines data from an international wastewater surveillance program that collected and analysed influent wastewater samples from up to 47 sites in 16 countries between 2019 and 2022. Influent wastewater samples were collected over the New Year period and analysed using validated liquid chromatography – mass spectrometry methods. Over the three years, a total of 18 NPS were found in at least one site.

* Corresponding author.
E-mail address: r.bade@uq.edu.au (R. Bade).

https://doi.org/10.1016/j.wroa.2023.100179
Received 20 December 2022; Received in revised form 13 March 2023; Accepted 5 April 2023
Available online 6 April 2023
2589-9147/© 2023 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Synthetic cathinones were the most found class followed by phenethylamines and designer benzodiazepines. Furthermore, two ketamine analogues, one plant based NPS (mitragynine) and methiopropamine were also quantified across the three years. This work demonstrates that NPS are used across different continents and countries with the use of some more evident in particular regions. For example, mitragynine has highest mass loads in sites in the United States, while eutylone and 3-methylmethcathinone increased considerably in New Zealand and in several European countries, respectively. Moreover, 2F-deschloroketamine, an analogue of ketamine, has emerged more recently and could be quantified in several sites, including one in China, where it is considered as one of the drugs of most concern. Finally, some NPS were detected in specific regions during the initial sampling campaigns and spread to additional sites by the third campaign. Hence, wastewater surveillance can provide an insight into temporal and spatial trends of NPS use.

1. Introduction

New psychoactive substances (NPS) are compounds that have been designed to mimic the effect of conventional illicit drugs, while evading legal restrictions and are thus of international public health concern. From fentanyl-laced heroin (Jannetto et al., 2019) to counterfeit Xanax (Blakey et al., 2022) and adulterated MDMA (Krotulski et al., 2021; HighAlert, 2021), NPS may pose a risk to illicit users. The United Nations Office of Drugs and Crime (UNODC) runs an Early Warning Advisory (EWA) program on NPS, which is updated with information from international drug enforcement and intelligence agencies, health authorities and toxicologists. As of mid-2022, 136 countries and territories, covering all continents had reported more than 1100 NPS to the EWA.

The consumption of NPS is influenced by their specific effects, availability, price, potential undetectability in routine drug tests and their use as adulterants (Peacock et al., 2019; United Nations Office on Drugs and Crime 2022). It is imperative from a public health viewpoint to monitor the use of these drugs given our limited knowledge of their specific effects, their interactions with other drugs, and the harms that they cause. It is difficult for law enforcement to control the circulation of these compounds because they are generally manufactured in much smaller quantities than traditional illicit drugs such as cocaine and methamphetamine and many have a 'grey' legal status.

Information on the prevalence of NPS use is collected from forensic analyses, surveys, and pill testing as well as social media and dark web monitoring (Peacock et al., 2019; Pascoe et al., 2022; Barenholtz et al., 2021). Each of these serves as a complementary tool for identification, monitoring, surveillance, control and ultimately evaluation of public health impacts of NPS use. Each has its distinct advantages such as rapid identification of substances, early warning capabilities and outlining demographics and profiles of users. However, not all jurisdictions have access to these data sources and thus some communities could be misinformed. There is increased concern around music festivals, where NPS-adulterated drugs can be mistakenly consumed. This can increase the risk of overdoses, resulting in more emergency department presentations. Wastewater analysis can help to fill this gap and has been used in many countries to assess licit and illicit drug use (Gracia-Lor et al., 2017).

The global prevalence of NPS use remains unknown and data are limited to a few countries with appropriate resources (UNODC 2022; Khaled et al., 2016). The current work presents data from wastewater sampling across the New Year period in three consecutive years (2019–20, 2020–21 and 2021–22). The use of NPS typically increases during festivals such as those of the New Year period that are associated with parties and festivals. The number of countries (and sites) has increased from 8 (12) in the first iteration to 10 (25) and finally to 16 (47). Throughout this project, the number of targeted analytes changed to include the most relevant NPS, based on findings from the UNODC EWA on NPS, international forensic findings, and published scientific literature. The aims of this work are: i) to study international spatial trends in NPS use; ii) to examine if preferences for NPS drugs change from year to year; iii) to evaluate the impact of the COVID-19 pandemic on NPS use and iv) to establish whether data on NPS obtained from wastewater analysis is comparable to that from other data sources.

2. Results

Across the three years, the number of countries (and sites) investigated increased from 8 (12) to 10 (25) and finally 16 (47). Across all three campaigns, a total of 546 individual samples were analysed, between 115 (2019/20) and 287 (2021/22). A total of 18 NPS were found across the three sampling campaigns (Table 1, structures in Table S1), with yearly totals from nine (2019–20 and 2021–22) to ten (2020–21) individual NPS. Each year, the number of analytes included in the analytical method has changed (Table S2) based on information from the UNODC EWA, forensic agencies, peer reviewed publications and availability of reference analytical standards. For example, 3-methylmethcathinone (3-MMC), mephedrone and methylone were analysed across all years, while 2F-deschloroketamine (2F-DCK), mitragynine, clonazolam and etizolam were only included in the third campaign. 4-Methylethcathinone (4-MEC), methylenedioxypyrovalerone (MDPV), methiopropamine, methoxetamine and *para*-methoxyamphetamine (PMA) were not included in the 2021–22 campaign because there was limited identification in international forensic analyses and in the early warning systems of the UNODC and EMCDDA.

A variety of classes of NPS were found during this campaign. Synthetic cathinones were the most common (Table 1). Phenethylamines (4-fluoroamphetamine and PMA), designer benzodiazepines (clonazolam and etizolam), ketamine analogues (2F-DCK and methoxetamine) as well as the plant-based NPS mitragynine and methiopropamine were also quantified at least once across the three years.

2.1. Spatial trends

Over the three years of these data, some spatial patterns emerged. The synthetic cathinone 3-MMC was found consistently in all years, primarily in Europe (Fig. 1). However, in 2020–21 and 2021–22, it was found in at least one site in New Zealand. In the latter sampling period, it was also found on selected days in the United States. Mephedrone and methylone were also found across the three campaigns. They were primarily located in Oceania and North America, with highest levels in New Zealand. Across the European sites, they were only found in one site in Spain in the 2021–22 collection (Table 1). Like mephedrone, eutylone was also primarily seen in sites in New Zealand (up to 55 mg/day/1000 people) in both 2020–21 and 2021–22 (Fig. 2A).

N-ethylpentylone, pentylone and ethylone had the lowest levels of synthetic cathinones found across the three sampling campaigns. N-ethylpentylone and pentylone had highest levels in the United States. Several compounds were only seen once: 4-fluoroamphetamine, 4-MEC and MDPV in the Netherlands, methiopropamine and methoxetamine in Australia and PMA in New Zealand (Table 1).

Some substances were only quantified in the most recent surveillance campaign in 2021-22. Mitragynine (Fig. 3) was found at the highest levels in the United States, mostly between 1000 and 5000 mg/day/1000 people. Sites in Sweden had the next highest levels. Most other sites where the compound was found had levels below 50 mg/day/1000 people. There was generally no increase in levels over the New Year in

Table 1
Table of all NPS found over 3 years (Country with sites of the highest levels are highlighted in bold).

	2F-Deschloroketamine (2F-DCK)	3-Methylmethcathinone (3-MMC)	4-Fluoroamphetamine	4-Methylethcathinone (4-MEC)	Clonazolam	Etizolam	Ethylone	Eutylone	Methylenedioxypyrovalerone (MDPV)	Mephedrone	Methiopropamine	Methoxetamine	Methylone	Mitragynine	N-ethylhexedrone	N-ethylpentylone	Pentylone	*para*-Methoxyamphetamine (PMA)
2019-20	N.D.	ES IT **NL**	NL	NL	N.A.	N.A.	AU	N.A.	NL	AU **NZ**	N.D.	N.D.	NL **NZ**	N.A.	N.A.	AU NZ **US**	US	N.D.
2020-21	N.D.	**BE** ES IT NZ	N.D.	N.D.	N.A.	N.A.	AU **CA** NZ	AU CA **NZ** US	N.D.	AU CA **NZ** US	AU	AU	AU **NZ**	N.A.	N.A.	AU **CA** KR NZ	US	NZ
2021-22	CA **CN** ES FR IS IT US	BE **ES** FR GR IS IT NZ SE SI US	N.D.	N.A.	**IS**	CA **IS** IT KR NZ SE US	N.A.	AU BE BR CA ES GR IT KR **NZ** SI US	N.A.	NZ ES	N.A.	N.A.	**ES**	AU BE BR CA CY ES FR GR IS IT KR NZ SE SI **US**	BE ES SE	N.D.	N.A.	N.A.

Abbreviations: N.D.: Not Detected; N.A.: Not Analysed.

AU: Australia; BE: Belgium; BR: Brazil; CA: Canada; CN: China; CY: Cyprus; ES: Spain; FR: France; GR: Greece; IS: Iceland; IT: Italy; KR: Republic of Korea; NL: the Netherlands; NZ: New Zealand; SE: Sweden; SI: Slovenia; US: United States

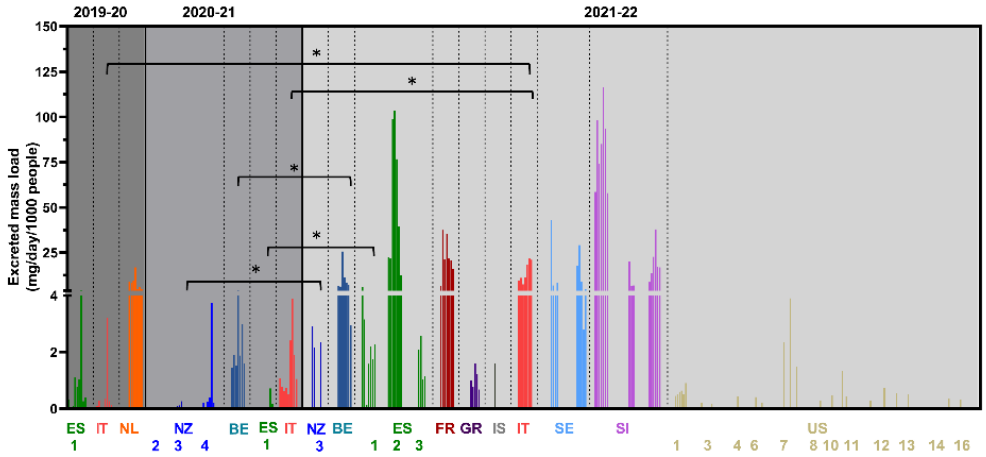


Fig. 1. Estimated excreted mass loads of 3-MMC from all sites over the three-year sampling period. Note: 2019–20 and 2020–21 values are taken from previous publications (Bade et al., 2021; Bade et al., 2022). Sites are ordered by continent. Countries with multiple sites are numbered according to Table S3. Only sites where the compound was found are included in the figure. *: Statistically significant difference ($p < 0.05$) following independent t-tests (for sites with two years of data) or a one-way ANOVA, followed by a pairwise *t*-test with Bonferroni correction (for sites with three years of data). ES: Spain; IT: Italy; NL: the Netherlands' BE: Belgium; NZ: New Zealand; FR: France; GR: Greece; IS: Iceland; SE: Sweden; SI: Slovenia; US: United States.

any site except for specific sites in Slovenia and Sweden. N-ethylhexedrone was only quantifiable in two sites in Sweden and Spain, with particularly high levels in one site from Sweden on New Year's Day. The designer benzodiazepines clonazolam and etizolam were also found, with highest loads at a site in Iceland, while levels in wastewater at other sites were below the limit of quantification of our method.

2F-DCK was found in samples from nine sites across Canada, China, Spain, France, Iceland, Italy, and the United States (Fig. 4). Increases over New Year's Eve were evident in Canada and Spain (Table S4), while highest levels were seen across the entire sampling week in China. It was only quantifiable on certain days in Iceland, Italy, and the United States.

2.2. Temporal trends

For sites and compounds that were monitored over multiple years, statistical tests were performed to determine significance. 3-MMC was monitored across all three years and the measured excreted mass loads increased from 5 to 10 mg/day/1000 people in the first two sampling campaigns to more than 100 mg/day/1000 people in 2021–22. The highest levels were found in the 2021–22 sampling campaign at sites in Spain and Slovenia. In sites monitored over multiple years, there was also an increase (i.e. Belgium (BE), Spain (ES 1), New Zealand (NZ 3), and Italy (IT)). A statistically significant increase was seen in sites in Belgium, Italy and Spain between 2020-21 and 2021–22 ($p < 0.05$). There was also a statistically significant increase in the site in Italy between 2019-20 and 2021–22 ($p < 0.05$). In the New Zealand site where 3-MMC was found in both 2020–21 and 2021–22, there was also a statistically significant increase ($p < 0.05$). In most sites, there was an increase in mass loads over New Year's Eve and New Year's Day.

Eutylone was analysed over the two most recent campaigns. The three sites in New Zealand that were monitored over 2020–21 were also included in 2021–22. NZ 2 and NZ 3 showed similar trends for the different periods, with statistically significant increases found ($p < 0.05$), while eutylone at NZ 4 had no significant difference between the two sampling periods . Eutylone was also found at much lower levels in sites in Australia, North America, Brazil, Republic of Korea and specific sites in Europe (Fig. 2**B**). These differences for both 3-MMC and eutylone from 2020 to 21 to 2021–22 could reflect the impact of COVID-19 restrictions.

3. Discussion

The societal burden attributable to illicit drug use is increasing. For example, in Australia, the societal burden due to illicit drug use increased by 35% from 2003 to 2018 and was responsible for 72% of all burden due to poisonings and almost all burden related to drug use disorders (excluding alcohol) (Australian Institute of Health and Welfare 2021). However, the burden specifically related to NPS use is not easily quantified. This wastewater-based study provides a broader insight into NPS use and establishes which NPS are most used across sites in 16 countries and may potentially contribute to disease burden. Highest consumption was recorded for most NPS around the New Year period, indicating that consumption increases at festivals and parties. This may be due to higher consumption of regular consumers and/or "new" users who consume NPS intentionally or unintentionally. It must be noted that in countries where Lunar New Year is celebrated (i.e. China and the Republic of Korea), the December-January collection does not coincide with a specific holiday season. Nevertheless, this allows this work to also show the use of NPS in sites during a 'normal' time of the year.

3.1. Impact of the COVID-19 pandemic

The novelty of this work is emphasised by comparing the sampling campaigns covering sites before the COVID-19 pandemic (2019–20), when most severe COVID-19 related restrictions were in place (2020–21) and when most restrictions had eased (2021–22). Despite these three distinct periods and the impacts on the population, there was little difference in the total number of NPS detections. In fact, for a few compounds, high levels were seen during the 2020–21 period, such as eutylone in sites in New Zealand. Increases between 2020-21 and 2021–22 were observed in two sites in New Zealand, while a third had no significant change. These results are not unexpected, as pill testing over both periods carried out in New Zealand found that eutylone was the most commonly found cathinone (KnowYourStuffNZ 2023). Most compounds were at lower levels during the 2020–21 period particularly for 3-MMC in the European sites. It is acknowledged that the restriction of movement, as well as the cancellation of festivals and social gatherings likely resulted at these lower levels.

Moreover, the use of synthetic cathinones such as 3-MMC and eutylone is linked with that of MDMA (Pascoe et al., 2022). For example,

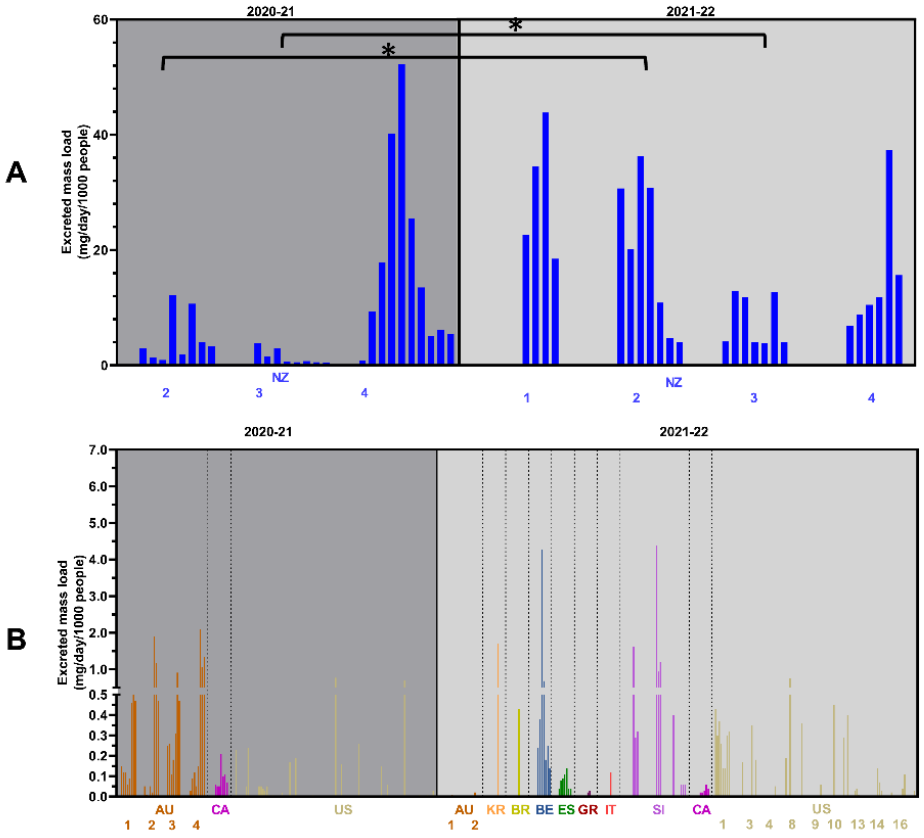


Fig. 2. Estimated excreted mass loads of eutylone from all sites. Figure is separated to show the sites with highest levels (A) and the lower levels (B). Note: 2020–21 values are taken from a previous publications (Bade et al., 2022). Sites are ordered by continent. Countries with multiple sites are numbered according to Table S3. Only sites where the compound was found are included in the figure. *: Statistically significant difference ($p < 0.05$) following independent t-tests (for sites with two years of data).
NZ: New Zealand; AU: Australia; CA: Canada; US: United States; BE: Belgium; BR: Brazil; ES: Spain; GR: Greece; IT: Italy; KR: Republic of Korea; SI: Slovenia.

the increase in eutylone in the summer of 2020–21 was hypothesised to be due to a reduction of MDMA in the country (Radio New Zealand 2022; Radio New Zealand 2021). MDMA was reported to have declined during 2020 in Europe, Australia and Canada, which could have resulted in the increased levels of 3-MMC and/or eutylone in this work (Bade et al., 2021; Been et al., 2021; Alygizakis et al., 2021; Statistics Canada 2023).

With the same time periods being monitored over the three years, this study is able to provide a 'snapshot' of NPS consumption. As such, the primary difference between the 2020–21 collection and those before and after is the COVID-19 pandemic. We therefore hypothesise that this was the driving factor for the differences observed.

3.2. Spatial and temporal trends

With samples collected at the same time every year, it is possible to see spatial and temporal changes in consumption trends (Supporting Information, Fig. S1). Many NPS seemingly follow regional trends. For example, mitragynine has highest loads in sites in the United States while eutylone and mephedrone were most common in New Zealand, 3-MMC and N-ethylhexedrone in Europe and 2F-DCK in China. Some NPS that were initially detected in specific regions spread to additional parts of the world in the third sampling campaign. For instance, 3-MMC was initially found in Europe, then spread to Oceania in 2020–21 and North America in 2021–22. Eutylone that was only detected in Oceania and North America in the second campaign but was detected in Europe in the last campaign, 2021–22. This study has identified the potential origin of NPS use around the world and could be used to monitor rapid changes in global consumers habits.

These results suggest that reducing NPS use and protecting public health should not only be done at the national level; it requires an organised global campaign. They also show the global spread of NPS in spite of current legislation. There are blanket bans on any NPS in countries such as China, New Zealand and Australia, while compound-specific bans also exist internationally. For example, the Convention on Psychotropic Substances 1971 from the United Nations is a treaty designed to control psychoactive drugs. The World Health Organisation (WHO) Expert Committee on Drug Dependence meets regularly to discuss the possible addition of new substances to the list of controlled drugs. In recent years, the NPS eutylone, N-ethylhexedrone and N-ethylpentylone have been added to the convention, while 3-MMC is currently under review and mitragynine is under surveillance

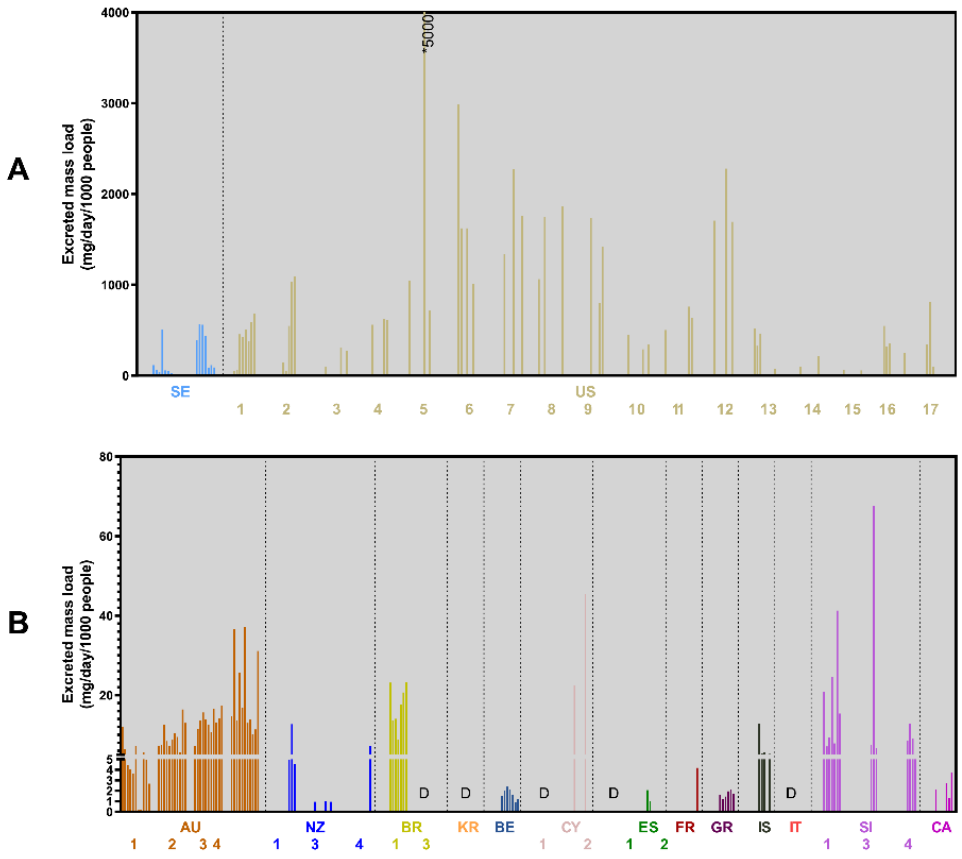


Fig. 3. Estimated excreted mass loads of mitragynine from all sites from the 2021–22 collection. Sites are ordered by continent. Figure is separated to show the sites with highest levels (A) and the lower levels (B). *D* = detected at levels below LOQ. Countries with multiple sites are numbered according to Table S3. Only sites where the compound was found are included in the figure.
AU: Australia; BE: Belgium; BR: Brazil; CA: Canada; CY: Cyprus; ES: Spain; FR: France; GR: Greece; IS: Iceland; IT: Italy; KR: Republic of Korea; NZ: New Zealand; SE: Sweden; SI: Slovenia US: United States.

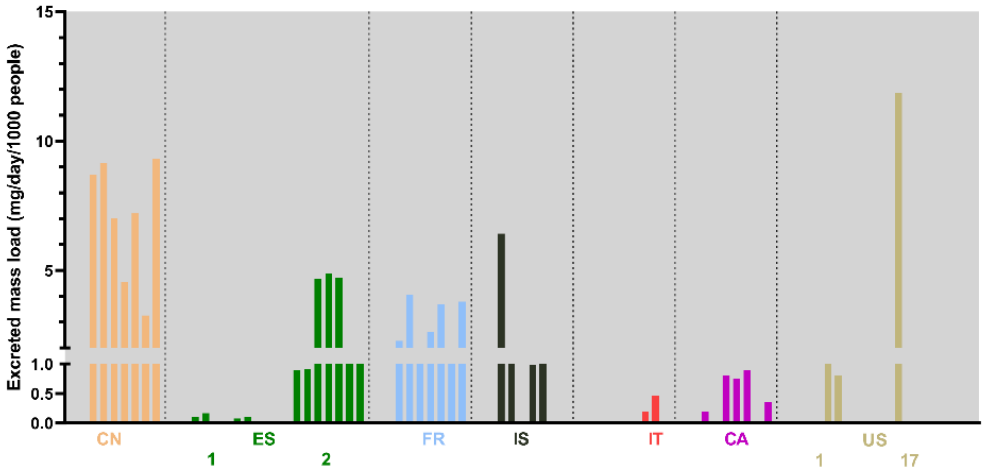


Fig. 4. Estimated excreted mass loads of 2F-DCK from all sites from the 2021–22 collection. Sites are ordered by continent. Countries with multiple sites are numbered according to Table S3. Only sites where the compound was found are included in the figure.
CA: Canada; CN: China; ES: Spain; FR: France; IS: Iceland; IT: Italy; US: United States.

(International Narcotics Control Board 2021; World Health Organization 2021; World Health Organization 2022).

3.2.1. Synthetic cathinones

The synthetic cathinones are stimulants that constitute the largest group of NPS reported to the UNODC EWA (United Nations Office on Drugs and Crime 2021) and 3-MMC, eutylone, N-ethylhexedrone and mephedrone were all found in this work. 3-MMC is of particular concern in Europe. Since it was first reported in 2012 in Sweden, it has gained in popularity, particularly since 2020 (European Monitoring Centre for Drugs and Drug Addiction 2022). This is reflected in acute poisonings in the Netherlands (Nugteren-van Lonkhuyzen et al., 2022), while it was one of the most common NPS seized in Italy between May and October 2020 (Vincenti et al., 2021), and was also the NPS with the highest mass loads found in a recent national study conducted in Italy (Salgueiro-González et al., 2022). This European prevalence is reflected in Fig. 1. In the sites with multiple years of data, an increase in measured mass loads is evident. This increase in use has unfortunately resulted in at least 291 acute poisonings and 27 deaths in Europe (European Monitoring Centre for Drugs and Drug Addiction 2022). In early 2022, after a risk assessment report of 3-MMC commissioned by the EMCDDA, the European Commission recommended control measures and member states were given six months to introduce national legislation (European Monitoring Centre for Drugs and Drug Addiction 2022). The WHO has also decided to include 3-MMC amongst nine NPS for critical review by its Expert Committee on Drug Dependence because of its dependence-producing properties and potential harms (World Health Organization 2022).

In this study, the only sites outside of Europe where 3-MMC was found were in New Zealand and the United States, albeit at much lower levels than in Europe. There is no current literature around the use of 3-MMC in the United States or New Zealand but the New Zealand drug information website, High Alert, did release an article about 3-MMC in mid-2021 (HighAlert 2021). In New Zealand in late 2020, it was reported that there was also a decline in MDMA, which was maintained through 2021 (New Zealand Police 2021; New Zealand Police 2022). With some of the sites in the New Zealand locations incorporating music festivals, it is possible that the 3-MMC measured may have been from the consumption of adulterated MDMA. This hypothesis is supported by a study from the United Kingdom on drug use at festivals, which found that as MDMA detection decreased, cathinone detections increased and 3-MMC represented more than 20% of all cathinones found (Pascoe et al., 2022).

Mephedrone (4-methylmethcathinone) is an isomer of 3-MMC and was one of the most popular NPS in the early 2010s but has maintained its popularity despite legislation restrictions (Hutton, 2020). In the three years of this work, it has been found at relatively low levels, particularly in New Zealand and Australia. However, in 2021–22, it was also found in a site in Spain.

In this work, eutylone had highest levels in New Zealand. In late 2020, the New Zealand Drug Information website put out an alert for eutylone (HighAlert, 2021). Over the 2020–21 summer festival season in New Zealand, testing of party drugs across the country found that up to 50% of samples thought to be of MDMA contained eutylone. This reduced to around 10% the following year (Radio New Zealand 2022). However, in Fig. 2, similar levels were obtained across both years while site NZ 2 showed a statistically significant increase in 2021–22. In other sites where eutylone was monitored over 2020–21 and 2021–22, there was a decrease in sites in Australia and Canada, while eutylone was found for the first time in samples from sites in Italy, Belgium, and the Republic of Korea. According to seizure data, the most common NPS stimulants in Europe were 3-MMC and N-ethylhexedrone (European Monitoring Centre for Drugs and Drug Addiction. 2022), so it is interesting that some eutylone was found in several European sites. N-ethylhexedrone was only found in a couple of European sites (in Spain, Belgium and Sweden), with quite high levels found in a site Sweden.

3.2.2. Mitragynine

Mitragynine is the primary alkaloid derived from a plant (kratom) found in South-East Asia, where it is traditionally used to combat fatigue and improve work productivity (Cinosi et al., 2015; Suwanlert, 1975). In recent years, it has become particularly popular in the United States as a 'legal high' for its stimulant and/or opioid-like effects (Tobacyk et al., 2022). The U.S. Food and Drug Administration has repeatedly warned of the dangers of kratom, including addiction, abuse and dependence (United States Food and Drug Administration 2022). It is not currently federally regulated in the United States, but some states have banned the substance, while others have imposed age restrictions. Use of the compound is legal in all the sites analysed in this study in the United States, but age restrictions may be in place to limit consumption by minors. Estimates of how many people use kratom in the United States vary, but the National Institute on Drug Abuse estimate that 0.6% (i.e. 1.7 million people) of the population aged 12 or older in 2021 reported using kratom in the past 12 months (Substance Abuse and Mental Health Services Administration 2022). Amongst the other sites in this project, mitragynine was only legal in Brazil, Belgium, Canada, Spain, and Greece. The legal status of mitragynine was not necessarily reflected in the measured mass loads calculated in this work. While sites in the United States had by far the highest levels of mitragynine, the next highest were in Sweden, Slovenia, and Australia – where it is illegal.

3.2.3. 2F-deschloroketamine

2F-deschloroketamine (2F-DCK) is an analogue of ketamine that has emerged over the past few years. Ketamine has been one of the drugs of most concern in China but major interventions have resulted in a large decline in registered ketamine users (United Nations Office on Drugs and Crime 2020). In recent years, ketamine analogues have emerged and become particularly prevalent in South-East Asia, including China. The highest levels of 2F-DCK were found in samples from one site in China and were similar to those previously reported (Shao et al., 2021; Li et al., 2022). It was also evident that there was no weekend (New Year) peak in use in the Chinese site, in contrast to other sites where 2F-DCK was found (e.g. Spain and Canada). It was also found infrequently in sites in the United States, Italy, and Iceland, at relatively low levels. According to the UNODC EWA, 2F-DCK is the second-most prevalent dissociative – behind ketamine – in all reporting countries since 2020 (United Nations Office on Drugs and Crime 2022).

3.2.3. Designer benzodiazepines

Etizolam and clonazolam are designer benzodiazepines, a class associated with the greatest fatalities (United Nations Office on Drugs and Crime 2021) and hence of greatest public health concern. Etizolam was found in eight countries in this work, with most at or below our limit of quantification. However, a site in Iceland had the highest mass loads. Clonazolam was only found in this same site in Iceland. According to the Nordic Health and Welfare Statistics, Iceland has the highest prescription sales rate of benzodiazepines of all Nordic countries (Nordic Health and Welfare Statistics 2022). Both etizolam and clonazolam have been sold as counterfeit Xanax (Blakey et al., 2022) so it is possible that the high etizolam levels in Iceland were due to illicit or unwitting use of Xanax/alprazolam.

3.3. Future perspectives

The SPE method used for this work has previously been validated for synthetic cathinones, phenethylamines and opioids (Bade et al., 2020). However, recoveries for synthetic cannabinoids, benzodiazepines and plant-based NPS range between 10 and 50% (data not shown). Additionally, studies have shown that acidified conditions are unsuitable for the optimal quantification of cannabinoids and benzodiazepines (Bade et al., 2021; Pandopulos et al., 2020). While internal standards were included in this work to help cater for losses during extraction, it is possible that the limited detection frequency of the designer

benzodiazepines and synthetic cannabinoids could be due to their instability in acidic conditions. To make a single method suitable for such a wide range of classes is challenging, but several methods have been developed to detect multiple classes of NPS in wastewater (Bade et al., 2020; O'Rourke and Subedi, 2020). As the variety and number of NPS continue to rise, it is important to continue developing new methods suitable to cover a wide number of classes in a single extraction.

The results of this study highlighted the need for a global campaign, as the issue of NPS use affects all countries to a different level and degree. Therefore, organisations, such as the UNODC and the EMCDDA, could adopt a similar wastewater analysis approach as a surveillance tool for NPS, since it allows the assessment of larger populations while minimising costs, provides data in an objective way, reducing the impact of self-reported data and presents no ethical implications, as the samples are anonymous. Annual and targeted (e.g., New Year period, music festivals and other special events) campaigns could act as a warning system for the prevalence of NPS.

4. Conclusion

Monitoring and surveillance of NPS internationally is an ongoing and complex problem. This wastewater-based study provides an insight into the NPS market internationally over the past three years, including before, during and after the COVID-19 pandemic. The use of NPS was mainly lower during the pandemic with a few exceptions, such as eutylone in specific sites. Higher NPS consumption around the world was found during the New Year holiday period and there were specific regional trends in which NPS was detected. For example, mitragynine had highest loads in sites in the United States, eutylone and mephedrone in New Zealand, 3-MMC and N-ethylhexedrone in Europe and 2F-DCK in China. These data indicate the promise of more systematic wastewater analyses to identify and monitor trends in the use of specific NPS in different populations and identify temporal and spatial patterns in their global spread of use.

5. Materials and methods

5.1. Compounds

A total of 52 compounds were analysed across the three years of this project, with between 26 and 34 analysed each year (Table S2).

5.2. Sampling campaigns

All information related to the wastewater collection and sites (collection dates, flow rates and population) can be found in the Supplemental Files (Table S3). As part of an ongoing global surveillance program, the number of countries (and sites) has increased from 8 (12) in the first iteration to 10 (25) and finally 16 (47). Data pertaining to the first two campaigns have been previously published (2019–20) (Bade et al., 2021) and 2020–21 (Bade et al., 2022). The population covered has increased from five to more than 17 million inhabitants. All samples were collected at the end of December – early January, coinciding with the New Year period. The first sampling campaign was performed before the COVID-19 pandemic, the second during the pandemic and the third when restrictions were ended/eased or presented only in a few countries.

5.3. Sampling, instrumentation and quality control

The best practice for WBE protocols were followed to minimise uncertainties relating to sample collection, storage and analytical methodology (Castiglioni et al., 2013). Briefly, 24-h composite influent wastewater was collected for between one and nine consecutive days. The wastewater was acidified upon collection and stored at -20 °C until sample treatment.

All samples were analysed using validated targeted LC-MS/MS methods (Bade et al., 2020; Bade et al., 2023). Procedural blanks were run throughout the analysis, after every 10 injections with the aim to identify any contamination originating from solvents and laboratory conditions. A quality control (blank spiked with 100 ng/L standards) was analysed after every 20 injections. Instruments were cleaned before the analysis according to vendors' recommendations.

For quantification purposes, both transitions needed to be present, while the ion ratio (within 20%) as well as retention time had to compare with the standard (within 2%). If only one transition was present, the compound was deemed at above the limit of detection (LOD) but below the limit of quantification (LOQ). For calculation purposes, this was given as the midpoint between the LOD and LOQ. As no analyte-specific internal standards were used for this method, quantification was based on the peak area ratios between native and surrogate internal standards compared to an external calibration curve. All data were acquired and processed with SCIEX OS. Further details about the analytical methodology are provided in the Supplemental Files.

5.4. Calculations

For each compound, a calibration curve of up to 11 points was constructed from 0.1 to 10,000 ng/L. Concentrations were calculated using the isotope dilution method and processed using SCIEX OS or Multiquant 3.0.2. As labelled internal standards were not available for the NPS in this work, a surrogate internal standard was utilised (Table S4). The flow rates and population data provided by each collaborating laboratory (Table S3) were then used to calculate excreted mass loads (Table S4 for 2022–23; (2019–20 (Bade et al., 2021) and 2020–21 (Bade et al., 2022)).

5.5. Statistical analysis

Statistical tests were performed on all sites and compounds where more than one year of data was available. For sites with two years of data, independent t-tests were performed. For sites with three years of data, a one-way ANOVA was performed, followed by a pairwise *t*-test with Bonferroni correction. Differences were deemed statistically significant with $p < 0.05$. All statistical analyses were performed using R, version 4.2.1.

CRediT authorship contribution statement

Richard Bade: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Funding acquisition. **Nikolaos Rousis:** Investigation, Formal analysis, Writing – review & editing. **Sangeet Adhikari:** Resources, Writing – review & editing. **Christine Baduel:** Resources, Writing – review & editing. **Lubertus Bijlsma:** Resources, Writing – review & editing. **Erasmia Bizani:** Resources, Writing – review & editing. **Tim Boogaerts:** Resources, Writing – review & editing. **Daniel A. Burgard:** Resources, Writing – review & editing. **Sara Castiglioni:** Resources, Writing – review & editing. **Andrew Chappell:** Resources, Writing – review & editing. **Adrian Covaci:** Resources, Writing – review & editing. **Erin M. Driver:** Resources, Writing – review & editing. **Fernando Fabriz Sodre:** Resources, Writing – review & editing. **Despo Fatta-Kassinos:** Resources, Writing – review & editing. **Aikaterini Galani:** Resources, Writing – review & editing. **Cobus Gerber:** Resources, Supervision, Writing – review & editing. **Emma Gracia-Lor:** Resources, Writing – review & editing. **Elisa Gracia-Marín:** Resources, Writing – review & editing. **Rolf U. Halden:** Resources, Writing – review & editing. **Ester Heath:** Resources, Writing – review & editing. **Felix Hernandez:** Resources, Writing – review & editing. **Emma Jaunay:** Resources, Formal analysis, Writing – review & editing. **Foon Yin Lai:** Resources, Writing – review & editing. **Heon-Jun Lee:**

Resources, Writing – review & editing. **Maria Laimou-Geraniou:** Resources, Writing – review & editing. **Jeong-Eun Oh:** Resources, Writing – review & editing. **Kristin Olafsdottir:** Resources, Writing – review & editing. **Kaitlyn Phung:** Resources, Writing – review & editing. **Marco Pineda Castro:** Resources, Writing – review & editing. **Magda Psichoudaki:** Resources, Writing – review & editing. **Xueting Shao:** Resources, Writing – review & editing. **Noelia Salgueiro-Gonzalez:** Resources, Writing – review & editing. **Rafael Silva Feitosa:** Resources, Writing – review & editing. **Cezar Silvino Gomes:** Resources, Writing – review & editing. **Bikram Subedi:** Resources, Writing – review & editing. **Arndís Sue Ching Löve:** Resources, Writing – review & editing. **Nikolaos Thomaidis:** Resources, Writing – review & editing. **Diana Tran:** Resources, Writing – review & editing. **Alexander van Nuijs:** Resources, Writing – review & editing. **Taja Verovšek:** Resources, Writing – review & editing. **Degao Wang:** Resources, Writing – review & editing. **Jason M. White:** Resources, Writing – review & editing. **Viviane Yargeau:** Resources, Writing – review & editing. **Ettore Zuccato:** Resources, Writing – review & editing. **Jochen F. Mueller:** Supervision, Resources, Funding acquisition, Writing – review & editing.

Declaration of competing interest

RUH and EMD are cofounders of AquaVitas, LLC, Phoenix, Arizona, United States, an Arizona State University startup company providing commercial services in wastewater-based epidemiology. RUH also is the founder of OneWaterOneHealth, a nonprofit project of the Arizona State University Foundation.

Data availability

Data will be made available on request

Acknowledgements

The authors sincerely thank the wastewater treatment plant operators for their help in collecting samples and the support provided by the lab staff who performed the extraction of the samples. The authors also thank SCIEX for the use of their instrument and Liam O'Brien for his statistical advice. The University of Iceland wants to particularly acknowledge Efla hf. and Veitur ohf. for their assistance with sample collection. SLU acknowledges the support of Inga Haalck, Oscar Götlind and Gabriel Persson for sample collection. IGE acknowledges the support of Nelly Laforet and Myriam Millet-Perrin for sample collection. The Queensland Alliance for Environmental Health Sciences, The University of Queensland, gratefully acknowledges the financial support of the Queensland Department of Health. R. B. is the recipient of an Australian Research Council Discovery Early Career Award (project number DE220100381), and J. M. is the recipient of an Australian Research Council Australian Laureate Fellowship (project number FL200100028) funded by the Australian Government. N.R acknowledges funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska–Curie Grant Agreement No. 896141. L. B. acknowledges the grant RYC2020–028936-I funded by MCIN/AEI/ 10.13039/501100011033 and by "ESF Investing in your future". E.G-L. acknowledges the financial support of the Madrid Government (Comunidad de Madrid- Spain) under the Multiannual Agreement with Complutense University in the line Program to Stimulate Research for Young Doctors in the context of the V PRICIT (Regional Programme of Research and Technological Innovation) (project number PR65/19–22432). T.B, A.C. and A. v. N. would like to thank European Union's Justice Programme—Drugs Policy Initiatives, EuSeME (project number: 861602) and the University of Antwerp and Research Scientific Foundation Flanders (FWO, project number: G060920N) for the funding of their research. The Slovenian Research Agency (ARRS) Programme group P1–0143 and ARRS-Flanders Research Foundation (project: N1–0143) is also acknowledged. The Italian group thank the Dipartimento Politiche Antidroga (Presidenza del Consiglio dei Ministri, Rome; "Aque reflue" project) for partial funding of this research, and Metropolitana Milanese for sampling collection. Samples from Arizona were acquired with partial support from an award by the J.M. Kaplan Fund to RUH: OneWaterOneHealth nonprofit project 30009070 of the Arizona State University Foundation. The views expressed in this article are those of the author(s) and not necessarily those of the funding agencies.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2023.100179.

References

Alygizakis, N, Galani, A, Rousis, NI, Aalizadeh, R, Dimopoulos, M-A, Thomaidis, NS., 2021. Change in the chemical content of untreated wastewater of Athens, Greece under COVID-19 pandemic. Sci. Total Environ. [Internet], 149230. https://doi.org/10.1016/j.ribaf.2020.101309.

Australian Institute of Health and Welfare. Australian Burden of Disease Study 2018: interactive data on risk factor burden [Internet]. 2021 [cited 2022 Sep 28]. Available from: https://www.aihw.gov.au/reports/burden-of-disease/abds-2018-interactive-data-risk-factors/contents/illicit-drug-use.

Bade, R., Abdelaziz, A.E., Nguyen, L., Pandopulos, A.J., White, J.M., Gerber, C., 2020. Determination of 21 synthetic cathinones, phenethylamines, amphetamines and opioids in influent wastewater using liquid chromatography coupled to tandem mass spectrometry. Talanta 208, 120479. https://doi.org/10.1016/j.talanta.2019.120479.

Bade, R, Eaglesham, G, Shimko, KM, Mueller, J, 2023. Quantification of new psychoactive substances in Australian wastewater utilising direct injection liquid chromatography coupled to tandem mass spectrometry. Talanta 251, 123767. https://doi.org/10.1016/j.talanta.2022.123767.

Bade, R, Tscharke, BJ, O'Brien, JW, Magsarjav, S, Humphries, M, Ghetia, M, et al., 2021 Sep 14. Impact of COVID-19 controls on the use of illicit drugs and alcohol in Australia. Environ. Sci. Technol. Lett. 8 (9), 799–804. https://doi.org/10.1039/D0AY00560F.

Bade, R, White, JM, Chen, J, Baz-Lomba, JA, Been, F, Bijlsma, L, et al., 2021. International snapshot of new psychoactive substance use: case study of eight countries over the 2019/2020 new year period. Water Res. [Internet] 193, 116891. https://doi.org/10.1016/j.watres.2021.116891.

Bade, R, White, JM, Ghetia, M, Adiraju, S, Adhikari, S, Bijlsma, L, et al., 2022. A taste for new psychoactive substances: wastewater analysis study of 10 countries. Environ. Sci. Technol. Lett. [Internet] 9 (1), 57–63. https://doi.org/10.1021/acs.estlett.1c00807.

Barenholtz, E, Krotulski, AJ, Morris, P, Fitzgerald, ND, Le, A, Papsun, DM, et al., 2021. Online surveillance of novel psychoactive substances (NPS): monitoring Reddit discussions as a predictor of increased NPS-related exposures. Int. J. Drug Pol. [Internet] 98, 103393. https://doi.org/10.1016/j.drugpo.2021.103393.

Been, F, Emke, E, Matias, J, Baz-lomba, JA, Castiglioni, S, Campos-Mañas, M, et al., 2021. Changes in drug use in European cities during early COVID-19 lockdowns - A snapshot from wastewater analysis. Environ. Int. [Internet] 153, 106540. https://doi.org/10.1016/j.envint.2021.106540.

Blakey, K, Thompson, A, Matheson, A, Griffiths, A., 2022. What's in fake 'Xanax'?: a dosage survey of designer benzodiazepines in counterfeit pharmaceutical tablets. Drug Test. Anal. 14 (3), 525–530.

Castiglioni, S, Bijlsma, L, Covaci, A, Emke, E, Hernández, F, Reid, M, Ort, C, Thomas, KV, van Nuijs, AL, de Voogt, P, Zuccato, E, 2013. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers. Environ. Sci. Technol. 47 (3), 1452–1460. https://doi.org/10.1021/es302722f.

Cinosi, E, Martinotti, G, Simonato, P, Singh, D, Demetrovics, Z, Roman-Urrestarazu, A, et al., 2015. Following "the Roots" of Kratom (Mitragyna speciosa): the evolution of an enhancer from a traditional use to increase work and productivity in southeast asia to a recreational psychoactive drug in western countries. Biomed. Res. Int. 2015.

European Monitoring Centre for Drugs and Drug Addiction. European Commission adopts measures to control two harmful new drugs amidst health concerns and surge in supply [Internet]. 2022 [cited 2022 Sep 8]. Available from: https://www.emcdda.europa.eu/news/2022/3/european-commission-adopts-measures-control-two-harmful-new-drugs_en.

European Monitoring Centre for Drugs and Drug Addiction. Risk assessment report on the new psychoactive substance 2-(methylamino)-1-(3-methylphenyl)propan-1-one (3- methylmethcathinone, 3-MMC) in accordance with Article 5c of Regulation (EC) No 1920/2006 (as amended. Vol. 2006. Luxembourg; 2022.

European Monitoring Centre for Drugs and Drug Addiction. European drug report 2022: trends and developments [Internet]. 2022. Available from: https://www.emcdda.europa.eu/system/files/publications/14644/EDR_2022_18-ONLINE.pdf.

Gracia-Lor, E, Castiglioni, S, Bade, R, Been, F, Castrignanò, E, Covaci, A, et al., 2017. Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives. Environ. Int. [Internet] 99,

131–150. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0160412016306936.
HighAlert. What is 3-MMC? [Internet]. 2021 [cited 2022 Sep 8]. Available from: https://www.highalert.org.nz/articles/what-is-3-mmc/.
HighAlert. Be aware of dangerous cathinones this summer [Internet]. [cited 2021 May 17]. Available from: https://highalert.org.nz/articles/be-aware-of-dangerous-cathinones-this-summer/.
Hutton, F., 2020. Cultures of Intoxication: `New' Psychoactive Substances. In: Hutton, F (Ed.), Cultures of Intoxication: Key Issues and Debates [Internet]. Springer International Publishing, Cham, pp. 87–110. https://doi.org/10.1007/978-3-030-35284-4_5.
International Narcotics Control Board. Green List - List of Psychotropic Substances Under International Control [Internet]. 2021 [cited 2022 Sep 14]. Available from: https://www.incb.org/incb/en/psychotropics/green-list.html.
Jannetto, PJ, Helander, A, Garg, U, Janis, GC, Goldberger, B, Ketha, H., 2019. The fentanyl epidemic and evolution of fentanyl analogs in the United States and the European Union. Clin. Chem. 65 (2), 242–253.
Khaled, SM, Hughes, E, Bressington, D, Zolezzi, M, Radwan, A, Badnapurkar, A, et al., 2016. The prevalence of novel psychoactive substances (NPS) use in non-clinical populations: a systematic review protocol. Syst. Rev. [Internet] 5 (1), 1–7. https://doi.org/10.1186/s13643-016-0375-5.
KnowYourStuffNZ. Testing reports [Internet]. 2023 [cited 2023 Mar 7]. Available from: https://knowyourstuff.nz/our-results-2/testing-results/testing-reports/.
Krotulski, AJ, Papsun, DM, Chronister, CW, Homan, J, Crosby, MM, Hoyer, J, et al., 2021. Eutylone intoxications—an emerging synthetic stimulant in forensic investigations. J. Anal. Toxicol. 45 (1), 8–20.
Li, X, Feng, L, Fan, X, Huang, J, Du, Y., 2022. Wastewater-based monitoring of 2-fluorodeschloroketamine abuse from 2019 to 2021 in a southern Chinese province. Sci. Total Environ. [Internet] 826, 154191. https://doi.org/10.1016/j.scitotenv.2022.154191.
New Zealand Police. Wastewater drug testing in New Zealand: national overview quarter one 2021 [Internet]. 2021. Available from: https://www.police.govt.nz/sites/default/files/publications/wastewater-results-quarter-1-2021.pdf.
New Zealand Police. Wastewater drug testing in New Zealand: national overview quarter one 2022. 2022;(November 2018):21–23. Available from: https://www.police.govt.nz/sites/default/files/publications/wastewater-results-quarter-1-2021.pdf.
Nordic Health & Welfare Statistics. Use of addictive medicine [Internet]. 2022 [cited 2022 Sep 23]. Available from: https://nhwstat.org/health/thematic-articles-health/use-addictive-medicine.
Nugteren-van Lonkhuyzen, JJ, Essink, S, Rietjens, SJ, Ohana, D, de Lange, DW, van Riel, AJHP, et al., 2022. 3-Methylmethcathinone (3-MMC) poisonings: acute clinical toxicity and time trend between 2013 and 2021 in the Netherlands. Ann. Emerg. Med. [Internet] 80 (3), 203–212. https://doi.org/10.1016/j.annemergmed.2022.04.022.
O'Rourke, CE, Subedi, B, 2020. Occurrence and Mass Loading of Synthetic Opioids, Synthetic Cathinones, and Synthetic Cannabinoids in wastewater treatment plants in four U.S. communities. Environ. Sci. Technol. 54 (11), 6661–6670. https://doi.org/10.1021/acs.est.0c00250.
Pandopulos, AJ, Bade, R, O'Brien, J, Tscharke, Ben, Mueller, J, Thomas, K, White, J, Gerber, C, 2020. Towards an efficient method for the extraction and analysis of cannabinoids in wastewater. Talanta 217, 121034. https://doi.org/10.1016/j.talanta.2020.121034.
Pascoe, MJ, Radley, S, Simmons, HTD, Measham, F., 2022. The cathinone hydra: increased cathinone and caffeine adulteration in the English MDMA market after Brexit and COVID-19 lockdowns. Drug Sci. Pol. Law [Internet] 8, 205032452210992. http://journals.sagepub.com/doi/10.1177/20503245221099209.
Peacock, A, Bruno, R, Gisev, N, Degenhardt, L, Hall, W, Sedefov, R, et al., 2019. New psychoactive substances: challenges for drug surveillance, control, and public health responses. Lancet [Internet] 394 (10209), 1668–1684. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673619322317.
Radio New Zealand. Serious side effects reported after fake MDMA circulates [Internet]. 2021 [cited 2021 Nov 18]. Available from: https://www.rnz.co.nz/news/national/434255/serious-side-effects-reported-after-fake-mdma-circulates.
Radio New Zealand. Drug testing: dramatic drop in bath salts being sold as MDMA [Internet]. 2022 [cited 2022 Sep 13]. Available from: https://www.rnz.co.nz/news/national/459181/drug-testing-dramatic-drop-in-bath-salts-being-sold-as-mdma.
Salgueiro-González, N, Zuccato, E, Castiglioni, S, 2022. Science of the Total Environment Nationwide investigation on the use of new psychoactive substances in Italy through urban wastewater analysis. Sci. Total Environ. [Internet] 843 (March), 156982. https://doi.org/10.1016/j.scitotenv.2022.156982.
Shao, XT, Yu, H, Lin, JG, Kong, XP, Wang, Z, Wang, DG., 2021. Presence of the ketamine analog of 2-fluorodeschloroketamine residues in wastewater. Drug Test. Anal. (February), 1–8.
Statistics Canada. Drug metabolites in wastewater in select Canadian cities, by month. 2023; Available from: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=1310082001.
Substance Abuse and Mental Health Services Administration. Key substance use and mental health indicators in the United States: results from the 2021 National survey on drug use and health. HHS Publication No. PEP19-5068, NSDUH Series H-54. 2022.
Suwanlert S. A study of kratom eaters in Thailand [Internet]. 1975. Available from: https://www.unodc.org/unodc/en/data-and-analysis/bulletin/bulletin_1975-01-01_3_page003.html.
Tobacyk, J, Parks, BJ, Lovelady, N, Brents, LK., 2022. Qualitative content analysis of public responses to an FDA inquiry on the impact of scheduling changes to kratom. Int. J. Drug Pol. [Internet] 108, 103817. https://doi.org/10.1016/j.drugpo.2022.103817.
United Nations Office on Drugs and Crime. Synthetic Drugs in East and Southeast Asia. 2020;(May):1–91. Available from: http://www.unodc.org/southeastasiaandpacific.
United Nations Office on Drugs and Crime. Current NPS Threats: volume IV. 2021.
United Nations Office on Drugs and Crime. Early warning advisory on new psychoactive substances [Internet]. [cited 2022 May 25]. Available from: https://www.unodc.org/unodc/en/scientists/ewa/data.html.
United Nations Office on Drugs and Crime. World Drug Report 2022: drug Market Trends - cocaine, amphetamine-type substances, new psychoactive substances [Internet]. 2022. Available from: https://www.unodc.org/res/wdr2022/MS/WDR22_Booklet_4.pdf.
United States Food and Drug Administration. FDA and Kratom [Internet]. 2022 [cited 2022 Sep 13]. Available from: https://www.fda.gov/news-events/public-health-focus/fda-and-kratom.
UNODC. Current NPS Threats. Vol. 4. 2022.
Vincenti, F, Gregori, A, Flammini, M, Di Rosa, F, Salomone, A, 2021. Seizures of New Psychoactive Substances on the Italian territory during the COVID-19 pandemic. Forensic Sci. Int. [Internet] 326, 110904. https://doi.org/10.1016/j.forsciint.2021.110904.
World Health Organization. Summary of assessments, findings and recommendations of the 44th WHO ECDD, 11-15 October 2021. 2021.
World Health Organization. 45th expert committee on drug dependence documents [Internet]. 2022 [cited 2022 Sep 8]. Available from: https://www.who.int/groups/who-expert-committee-on-drug-dependence/45th-ecdd-documents.

3.2.3 Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis

Published: M. Laimou-Geraniou, D. Nadarajan, R. Bade, E. Heath, 2025, *Journal of Hazardous Materials Advances*, vol. 19, pp. 100781.

This study is a collaboration between the Jožef Stefan Institute in Ljubljana (Slovenia) and the Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland (Australia). My role in this joint work included sample preparation, instrumental analysis, data analysis and evaluation, visualisation, and writing the manuscript.

Since 2021, Slovenia has participated in both European [103], [225] and global [100] campaigns monitoring NPS use during the Christmas and New Year period, revealing interesting findings regarding NPS consumption in specific sites. Concerns have also been raised about the presence of NPS within educational institutions [226]. Accordingly, this study applied WBE to gain additional information on the spatiotemporal use of NPS across Slovenia over four years of monitoring (2019–2022) during "normal" non-holiday periods. Furthermore, the COVID-19 pandemic in Slovenia (March 2020) provided a valuable opportunity to assess its impact on NPS consumption trends.

Influent wastewater samples were collected from seven WWTPs in Slovenia during six sampling periods: pre-pandemic (summer 2019 and winter 2020), during-pandemic (spring 2020 and winter/spring 2021), and post-pandemic (spring 2022). Following SPE, pooled weekly influent composite wastewater samples (n=37) were analysed using a combination of targeted LC-MS/MS method and suspect screening via LC-QTOF-MS in combination with the HighResNPS database of >2000 NPS and other compounds of toxicological relevance. Although the combination of qualitative methods (SWATH acquisition on QTOF) and quantitative approaches (QqQ) provides a more comprehensive overview of NPS consumption trends, it remains limited in application [100], [103], [227].

Five NPS, including 3-methylmethcathinone (3-MMC), eutylone, mephedrone, 4-fluoroamphetamine (4-FA), and mitragynine, were semi-quantified at least once across all sites. 3-MMC was the most frequently detected compound, appearing in nearly all sites and all sampling periods. It was followed by mitragynine, which showed the highest PNMLs (up to 40 mg/day/1,000 inhabitants), especially in Novo Mesto and Ljubljana. This finding aligns with other international wastewater studies that reported 3-MMC as one of the predominant stimulant NPS in Europe, showing an increase in its consumption between 2018 and 2022.

Temporal monitoring revealed a noticeable increase in mass loads of 3-MMC during the pandemic. Spatial differences were also evident: Koper had the highest number of NPS, likely due to its status as a key port city, while Velenje showed no detectable NPS levels. Following suspect screening, 17 additional psychoactive compounds, including NPS pharmaceuticals, opioids, and illicit drugs, were identified across at least one site during the sampling campaigns. It is also worth noting that some NPS were detected at only one site: phentermine and *N*-methyl-2AI in Kranj, and 4-FA, 4-OH-MET, mephedrone, and levamisole in Koper, possibly indicating that supply meets demand or reflects regional preference. This finding is believed to be the first report of phentermine and 4-HO-MET in influent wastewater.

These findings demonstrate the utility of WBE for monitoring emerging drug trends and can serve as an early warning system for potential public health concerns. Continued monitoring will reveal changes in NPS consumption trends and is essential for informing

public health and drug policy, particularly in rapidly evolving markets like NPS, thereby contributing to beneficial outcomes for society.

The study's outcome was presented at two scientific conferences: the SETAC Europe 34th Annual Meeting in Seville, Spain, May 5-9, 2024, and the SPECTROMED Mediterranean Congress on Mass Spectrometry and Its Applications in Hammamet, Tunisia, April 21-25, 2024.

Journal of Hazardous Materials Advances 19 (2025) 100781

Contents lists available at ScienceDirect

Journal of Hazardous Materials Advances

journal homepage: www.elsevier.com/locate/hazadv

Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis

Maria Laimou-Geraniou [a,b,1], Dhayaalini Nadarajan [c,1], Richard Bade [c,*], Ester Heath [a,b,*]

[a] Jožef Stefan Institute, Jamova 39 1000 Ljubljana, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova 39 1000 Ljubljana, Slovenia
[c] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Cornwall Street, Woolloongabba, QLD 4102, Australia

ARTICLE INFO

Keywords:
Designer drugs
Wastewater-based epidemiology
High-resolution mass spectrometry
COVID-19 pandemic

ABSTRACT

New psychoactive substances (NPS) are a continuously evolving class of psychoactive compounds, marketed initially as legal alternatives to conventional illicit drugs while bypassing legal regulations and, as such, are of international public health concern. Due to their continuous evolution, with new derivatives continually appearing on the drug market, monitoring and surveillance of NPS pose a challenge when employing traditional socio-epidemiological methods. In this study, we applied wastewater-based epidemiology (WBE) to gain additional information on the spatiotemporal consumption patterns of NPS. Samples of influent wastewater were collected from seven wastewater treatment plants (WWTPs) in Slovenia throughout six sampling periods, covering the pre- and during-pandemic periods (summer 2019, winter/spring 2020 and winter/spring 2021), and when most of the restrictions had been eased (spring 2022) due to the COVID-19 pandemic. Following solid-phase extraction, pooled samples were analysed using two separate methods: a targeted LC-MS/MS method for NPS analysis and suspect screening using LC-QTOF-MS in combination with the HighResNPS database of >2000 NPS and other compounds of toxicological relevance. Five NPS (3-methylmethcathinone (3-MMC), 4-fluoroamphetamine (4-FA), eutylone, mephedrone, and mitragynine) were semi-quantified across all sites. 3-MMC and mitragynine were the most prevalent NPS detected, with the latter having the highest population-normalised mass loads (up to 40 mg/day/1000 inhabitants). A noticeable increase in 3-MMC mass loads was observed during the pandemic. Following suspect screening, 17 additional compounds were detected at least once across all sites. This study highlights the potential for WBE to provide insight into Slovenia's NPS market.

1. Introduction

In recent years, the proliferation of new psychoactive substances (NPS) has emerged as a global public health concern, challenging traditional drug monitoring and regulatory frameworks. These substances, often designed to mimic the effects of conventional illicit drugs such as ecstasy (MDMA), cocaine, and cannabis while bypassing legal restrictions, pose significant risks to individual health and societal well-being (Peacock et al., 2019). According to the Early Warning Advisory (EWA) of the United Nations Office on Drugs and Crime (UNODC), >1200 NPS have been reported in 141 countries and territories worldwide up to 2024, including synthetic cathinones, synthetic cannabinoids, synthetic stimulants, synthetic opioids, benzodiazepines, dissociatives, hallucinogens, phenethylamines, and plant-based substances (UNODC, 2023a). The consumption of NPS is influenced by various factors such as their specific effects, price, availability, use as adulterants, and potential difficulty in detection through routine drug testing (UNODC, 2022). From a public health perspective, it is crucial to monitor the usage of these substances due to the limited knowledge of their identity and toxicity, interactions with other drugs, and potential side effects. Unlike conventional illicit drugs, which undergo stringent regulatory processes, NPS rapidly enter the market, exploiting legal loopholes and evading detection by traditional socio-epidemiological methods, including self-reported surveys, seizure data, forensic and toxicological analysis, as well as social media and dark web monitoring (Barenholtz et al., 2021; Peacock et al., 2019). Therefore, innovative approaches are needed to monitor drug consumption patterns and prevalence effectively. Wastewater-based epidemiology (WBE) has

* Corresponding authors.
E-mail addresses: r.bade@uq.edu.au (R. Bade), ester.heath@ijs.si (E. Heath).
[1] Joint first author

https://doi.org/10.1016/j.hazadv.2025.100781
Received 9 January 2025; Received in revised form 16 May 2025; Accepted 7 June 2025
Available online 9 June 2025
2772-4166/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

emerged as one such complementary tool for public health surveillance.

Wastewater-based epidemiology is a well-established approach that measures trace concentrations of metabolic drug residues (biomarkers) in influent wastewater. These measurements are then converted into *per capita* mass load estimates of drugs (mg/day/1000 inhabitants) using a pharmacokinetic approach, daily flow rates of wastewater, and population in the catchment area (Laimou-Geraniou et al., 2023a). At present, besides the numerous WBE studies on illicit drugs (Estévez-Danta et al., 2022; Huizer et al., 2021; Perkons et al., 2022), pharmaceuticals (Boogaerts et al., 2023; Laimou-Geraniou et al., 2023b; Quireyns et al., 2022), and lifestyle substances like alcohol, tobacco, and caffeine (Gracia-Lor et al., 2020; López-García et al., 2020; Rousis et al., 2023a; T. Verovšek et al., 2022), WBE has been applied to address NPS consumption at the national (Bade et al., 2023a; Brandeburová et al., 2020; Diamanti et al., 2019; Gao et al., 2017; Jaunay et al., 2024; Lee and Oh, 2023; Moslah et al., 2023; O'Rourke and Subedi, 2020; Perkons et al., 2022; Rice et al., 2020; Salgueiro-González et al., 2022) and international level (Bade et al., 2023b; R. 2022, 2021; Castiglioni et al., 2021; Gatica-Bahamonde et al., 2023; Gent and Paul, 2021; González-Mariño et al., 2016; Salgueiro-Gonzalez et al., 2024) in which spatiotemporal trends in NPS use were evaluated. New Psychoactive Substance use was also investigated in specific small populations such as educational institutions (Verovšek et al., 2023a) and during social events, like music festivals (Bijlsma et al., 2020b; Causanilles et al., 2017; Rousis et al., 2023b; Senta et al., 2023) and over the New Year period (Bade et al., 2023b; R. 2022, 2021).

Monitoring of NPS by WBE has many challenges because of the diverse and constantly evolving nature of these substances, their expected low concentration levels, the lack of pharmacokinetic studies, and limited stability data (Bijlsma et al., 2020a; Reid et al., 2014). To address these challenges, targeted (liquid chromatography-tandem mass spectrometry, LC-MS/MS) and suspect screening (high-resolution mass spectrometry, HRMS) methodologies have been developed to enhance the detection of NPS in wastewater. However, the combination of both techniques remains limited in application (Bade et al., 2023b; Rousis et al., 2023b; Salgueiro-Gonzalez et al., 2024), although it provides a more comprehensive overview of trends in NPS usage.

Slovenia has been included in European campaigns (Castiglioni et al., 2021; Salgueiro-Gonzalez et al., 2024) and in a global campaign on surveillance of NPS during the Christmas and New period since 2021, with some interesting findings regarding the consumption of NPS in some specific sites (Bade et al., 2023b). Additionally, the presence of NPS in educational institutions was of great concern (Verovšek et al., 2023a). This study aims to investigate the spatiotemporal use of NPS at a higher degree of granularity in Slovenia over four years of monitoring (2019–2022) during regular non-holiday periods, applying both quantitative and qualitative analysis. Moreover, the outbreak of the COVID-19 pandemic in Slovenia in March 2020 also provided a valuable opportunity to evaluate the influence of the pandemic on NPS use.

2. Materials and methods

2.1. Chemicals and materials

The NPS selected for targeted analysis were based on the European Union Drugs Agency, EUDA (formerly the European Monitoring Centre for Drugs and Drug Addiction, EMCDDA) and the United Nations Office on Drugs and Crime (UNODC) list. Full details concerning the chemicals used are given in the Supporting Information (see SM: S2. Materials and methods).

2.2. Sample collection

Seven wastewater treatment plants (WWTPs) in urban and rural areas across Slovenia (Ljubljana, Maribor, Kranj, Domžale-Kamnik, Novo Mesto, Koper, and Velenje) were included in the study (Table S1). The selected WWTPs, which account for 31 % of the Slovenian population, cover the main geographic regions and form part of the Sewage analysis CORe group – Europe (SCORE) monitoring. Influent wastewater samples (24-h composites) were collected for seven consecutive days, representing "normal" conditions at the WWTPs; these sampling periods excluded any special events such as maintenance activities, festivals, or extreme weather events. The samples ($n = 259$) were collected during the summer of 2019, winter of 2020, spring of 2020, winter of 2021, spring of 2021, and spring of 2022 (Table S1). These sampling periods also coincided with the COVID-19 pandemic and the related social restrictions in Slovenia. Particularly, the first wave hit Slovenia in the spring of 2020 (March 2020), the second wave started in the autumn of 2020 (October 2020), and the third in the spring of 2021 (March 2021), while most restrictions had eased during the final sampling period of spring 2022) The WWTP of Kranj was only included in the last sampling period of spring 2022. This work aimed to investigate long-term trends rather than weekly trends. For this, 37 pooled weekly samples were prepared by mixing aliquots (20 mL) of daily influent wastewater collected for seven consecutive days from each WWTP during each sampling period. All samples were stored at −20° C until analysis.

2.3. Sample preparation

Pooled influent wastewater samples were extracted using previously published protocols (Bade et al., 2020). Briefly, samples (100 mL) were filtered under vacuum using glass microfibre filter paper (GF/A 1.6 µm, Whatman, Kent, U.K.). Before extraction, samples were spiked with deuterated internal standards (illicit drug reference standard mixture, spiked at 150 ng/L), and their pH was adjusted to 4.5–5 using hydrochloric acid (37 %). Subsequently, solid phase extraction (SPE) cartridges (UCT XtracT DAU, 500 mg/6 mL; UCT Inc., Bristol, PA, USA) were conditioned with methanol (6 mL) and sodium acetate buffer (20 mM, 6 mL). The samples were then loaded on the cartridges under gravity, and the cartridges were successively washed with sodium acetate buffer (20 mM, 6 mL), 0.1 M acetic acid (2 mL), and methanol (6 mL). The sorbent was dried under vacuum for 15 min. The dried cartridges were stored at −20 °C before shipping to the University of Queensland (UQ) for analysis. On arrival at UQ, cartridges were again stored at −20 °C for less than one week before elution. The cartridges were then thawed to room temperature, and a mixture of dichloromethane/isopropanol/aqueous ammonia (80:16:4 v/v/v, 6 mL) was used to elute the analytes of interest from the cartridges. The eluent was then evaporated to approximately 200 µL under nitrogen at 40 °C, after which 1 % HCl in methanol (20 µL) was added, and the sample evaporated to dryness. The dry residue was reconstituted with 0.1 % formic acid in methanol (20 µL) and 0.1 % formic acid in ultrapure water (80 µL), giving a final volume of 100 µL (i.e., a 1000 times concentration factor).

2.4. Instrumental analysis

Quantitative analysis was performed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method utilising a Shimadzu Nexera LC-40 (Kyoto, Japan) coupled to a SCIEX Triple Quad 7500 system (Framingham, MA, USA). Chromatographic separation was achieved using a Phenomenex Kinetex Biphenyl column (50 mm x 2.1 mm, 2.6 µm) fitted with a Phenomenex security guard ultra cartridges HPLC Biphenyl 2.1 mm ID columns at a flow rate of 0.45 mL/min and an injection volume of 2 µL. Mobile phase A consisted of 95 % ultrapure water with 5 % MeOH and 0.1 % formic acid, while mobile phase B consisted of 95 % MeOH with 5 % ultrapure water and 0.1 % formic acid. The chromatographic gradient was as follows: 5 % B at 0 min and held for 1 min, linear increase to 100 % B at 7 min and held for 2 min, then decreased to 5 % B at 9.1 min and kept steady for the final 1.9 min to equilibrate the system. The total runtime was 11 min. The mass spectrometer was run in scheduled multiple reaction monitoring

(sMRM) mode in positive ionisation mode, with a 30 s retention time window around the expected retention time of each analyte. Optimised sMRM conditions and retention times for all NPS can be found in the supplementary information (S2.1 Acquisition parameters, Table S2).

Qualitative analysis was performed using LC—HRMS utilising a Shimadzu UHPLC system (Nexera X2) coupled to a SCIEX TripleTOF 5600+ mass spectrometer (Bade et al., 2024). The qualitative analysis employed a previously published suspect screening workflow, with data processing incorporating the open-source InSpectra platform (Bade et al., 2024).

2.5. Quantification, back-calculations, and quality control

The extracts were semi-quantified using a targeted LC-MS/MS method. The analytical MS method was based on a previously published direct injection method (Nadarajan et al., 2024), adapted to the SPE method. As additional compounds have been included in the analytical method, the SPE method was revalidated, with 54 compounds passing. To confirm a compound in the wastewater samples, both transitions had to be present, with an ion ratio within 30 % and a retention time within 0.1 min of the reference standard (SANTE/11312/2021v2, 2021). As analyte-specific internal standards were unavailable for all analytes, the concentration of NPS compounds were determined based on the peak area ratios between native and surrogate internal standards compared to an external calibration curve. For the analytes detected in this method, MDMA-d5 was utilised as a surrogate to enable semi-quantification.

Linearity, limit of detection (LOD), limit of quantification (LOQ), repetability, extraction recovery, and matrix effect can be found in the supplementary information (Table S3). Both LOD and LOQ were evaluated by analysing duplicate wastewater samples that had been spiked at in-sample concentrations of 1, 5, 10 and 50 ng/L. The LOD was determined as the lowest level in which both analyte transitions could be seen with a signal-to-noise ratio of 3, with LOQ at a signal-to-noise ratio of 10. To provide a more accurate estimation, where the signal-to-noise level at the lowest level was above 3 (or 10), the LOD (or LOQ) was back-calculated to determine the level at which a signal-to-noise ratio of 3 (or 10) could be achieved. Intra-day repeatability was assessed as the relative standard deviation (% RSD) of three replicate analyses of spiked samples at 10 and 100 ng/L, to evaluate precision. Extraction recovery was evaluated at 10 and 100 ng/L in pre- and post-extracted samples, as previously described (Matuszewski et al., 2003). The matrix effect was assessed at 10 and 100 ng/L, comparing the post-extracts and the neat solvent. Extraction recovery and matrix effect were both determined in triplicates. A linear response ($R^2 > 0.99$) was observed between LOQ–1000 ng/L (nine-point calibration curves) for all NPS, with LOD: 0.10–3.88 ng/L and LOQ: 0.29–11.8 ng/L.

In the present study, NPS mass loads were calculated by multiplying their concentrations (ng/L) of pooled samples by the average influent wastewater daily flow rates (m^3/day) reported in Table S1. Mass loads were then normalised to the population contributing to the WWTPs (Table S1) to back calculate population-normalised mass loads (PNMLs) expressed as mg/day/1000 inhabitants. The WWTPs provided data for the daily flow rates and population equivalents. The NPS consumption was estimated according to the PNMLs as shown in Eq. (1)

$$PNMLs\ (mg/day/\ 1000\ inhabitants) = \frac{concentration\ \left(\frac{ng}{L}\right) \times wastewater\ flow\ rate\ \left(\frac{m^3}{day}\right)}{population\ equivalent} \quad (1)$$

Most WBE approaches estimate substance consumption (e.g. illicit drugs) by back-calculating using correction factors that account for metabolism (excretion rates). However, the lack of comprehensive data on NPS metabolism complicates this step. Consequently, PNMLs of parent compounds (or metabolites) were solely considered to assess NPS use.

Quality control for the quantitative method includes control at 100 ng/L and 250 ng/L for every 10 samples. As part of quality control assurance, a double-blank injection without internal standards was injected for every five samples. All standards were freshly made on the day of analysis.

Regarding the HRMS qualitative method QC procedure, all samples were spiked with internal standards ($n = 15$), which were used to monitor instrument performance such as sensitivity, sample variation and retention time drift. For identification purposes, a mixture of NPS and illicit drug standards (and internal standards) were injected at the beginning and end of each run and after regular intervals to monitor chromatographic and mass spectrometric performance. Instrumental blanks of Milli-Q water were analysed within the sample batch after every 10 samples. The instrument was calibrated before each batch and after every 15 samples using the SCIEX ESI Calibration Solution to ensure mass accuracy.

3. Results and discussion

3.1. Quantification of NPS

A total of five NPS from three different classes: synthetic cathinones (eutylone, mephedrone, and 3-methylmethcathinone 3-MMC), phenethylamines (4-fluoroamphetamine 4-FA), and the plant-based NPS mitragynine, were semi-quantified across all sites at least once and were subsequently selected for descriptive spatiotemporal analysis (Table 1). However, Velenje WWTP was the only site where NPS were below the LOQ (Table S3) during all sampling periods. Structures of all detected compounds are given in the supporting information (Table S4).

The highest average population-normalised mass loads (PNMLs) were found for mitragynine (3.7 mg/day/1000 inhabitants (Koper) to 39.7 mg/day/1000 inhabitants (Novo Mesto), while for the remaining NPS, the PNMLs were lower than 2 mg/day/1000 inhabitants. Measured PNMLs were relatively low for most of the compounds, especially for 3-MMC and eutylone, compared with findings from previous international wastewater studies, including Slovenia (Bade et al., 2023b; Salgueiro-Gonzalez et al., 2024). However, this is probably expected since pooled samples were analysed, and stimulants, such as those found in this study, are expected to have higher use on the weekends. In addition, previous studies reported significantly higher use of NPS during festivals (Brandeburová et al., 2020; Rousis et al., 2023b), special events such as during the New Year period (Bade et al., 2023b; R. 2022), and weekends (González-Mariño et al., 2016; Salgueiro-Gonzalez et al., 2024; Salgueiro-González et al., 2022; Tscharke et al., 2016), indicating their use in a recreational context. The measured PNMLs in all locations during the sampling periods can be found in Table S5.

3.1.1. Spatial trends

The data reveals several spatial patterns over the four-year moni-

Table 1
Quantification frequency of target compounds during all sampling periods (summer 2019, winter/spring 2020 and winter/spring 2021, spring 2022) in Ljubljana, Maribor, Novo Mesto, Velenje, Domžale-Kamnik, and Koper. Kranj was included only in the sampling period of spring 2022.

Compound	Quantification frequency (%)						
	Ljubljana (LJ) ($n = 6$)	Maribor (MB) ($n = 6$)	Domžale-Kamnik (DK) ($n = 6$)	Velenje (VE) ($n = 6$)	Novo Mesto (NM) ($n = 6$)	Koper (KP) ($n = 6$)	Kranj (KR) ($n = 1$)
3-MMC	100 (6/6)	33.3 (2/6)	83.3 (5/6)	0	33.3 (2/6)	50 (3/6)	100 (1/1)
4-FA	0 (0/6)	0 (0/6)	0 (0/6)	0	0	50 (3/6)	0
Eutylone	16.7 (1/6)	16.7 (1/6)	16.7 (1/6)	0	0	16.7 (1/6)	0
Mephedrone	0	0	16.7 (1/6)	0	0	83.3 (5/6)	0
Mitragynine	33.3 (2/6)	50 (3/6)	50 (3/6)	0	16.7 (1/6)	16.7 (1/6)	0

Abbreviations: 3-MMC, 3-methylcathinone; 4-FA, 4-Fluoroamphetamine.

toring period (Fig. 1). These variations could be attributed to differences in the demographic characteristics of the regions being studied, including factors like the proportion of university students, the average age, and the number of tourists (Laimou-Geraniou et al., 2023b).

The highest number of NPS detections ($n = 5$) was in the WWTP from Koper, a major port servicing central Europe. Although no specific NPS was found in all WWTPs, the synthetic cathinone 3-MMC was the most abundant, identified in all catchment areas except for Velenje (Fig. 1A). The highest PNMLs for 3-MMC (Table S5), ranging from 0.1 to 1.3 mg/day/1000 inhabitants were found in Ljubljana and Domžale-Kamnik, followed by Koper (0.1–0.8 mg/day/1000 inhabitants). According to UNODC and EUDA, 3-MMC is one of the predominant stimulant NPS in Europe (EUDA, 2022a; UNODC, 2023b). This finding is in line with previous international wastewater studies, which also reported that 3-MMC had the highest levels in multiple European countries (Bade et al., 2023b; R. 2022, 2021; Rousis et al., 2023b; Salgueiro-Gonzalez et al., 2024; Salgueiro-González et al., 2022). In particular, from 2018 to 2022, according to WBE studies, the use of 3-MMC increased in Europe (Bade et al., 2023b; Salgueiro-Gonzalez et al., 2024).

In 2021, the EUDA added 3-MMC to its monitoring list (EUDA, 2022b), and recently, in March 2023, the Commission on Narcotic Drugs (CND) decided to subject 3-MMC under international regulation, following the World Health Organization's (WHO) suggestion (WHO, 2022). The use of 3-MMC in Slovenia was reported for the first time in 2012 (Sande, 2016) and recently demonstrated in forensic (NFL, 2022) and wastewater analysis (Bade et al., 2023b; Salgueiro-Gonzalez et al., 2024; Verovšek et al., 2023a). After the ban on the sales of mephedrone, and until 2014, 3-MMC was one of the most sought-after drugs in the country, even though it was already legally controlled in Slovenia (Sande, 2016). Its popularity has since decreased, but it is still among the most frequently used and well-known NPS among users, including young people (primary, secondary, and tertiary educational institutions) (Ferreira et al., 2019; Verovšek et al., 2023a), and has been associated with drug intoxication (NIJZ, 2019). The prevalence of 3-MMC in Slovenia could be partly attributed to the rapid growth of the NPS market, the geographical positioning of Slovenia, and its legal status remaining intact for an extended period in neighbouring countries.

In our study, the second most widespread NPS was mitragynine, measured in five out of seven wastewater treatment plants at least once (Fig. 1B). Mitragynine showed the highest PNMLs in Novo Mesto ($\leq$ 40 mg/day/1000 inhabitants) even though it was only present in the sampling period of spring 2021. More frequently, it was detected in Maribor, Domžale-Kamnik, and Ljubljana with measured PNMLS up to 32.0, 12.4, and 32.7 mg/day/1000 inhabitants respectively. Mitragynine, the principal alkaloid extracted from the kratom plant, native to Southeast Asia, has traditionally been used in the region to enhance work efficiency and productivity (Zhang et al., 2022). Although mitragynine stands out as one of the most frequently reported plant-based NPS, especially in the USA (UNODC, 2023b), it is primarily used as an opioid-like pain relief rather than a stimulant. Mitragynine is considered a legal drug in some European countries (e.g. Belgium, Greece, and Spain); however, this was not necessarily reflected in the measured PNMLS (Bade et al., 2023b; Rousis et al., 2023b). Surprisingly, Sweden and Slovenia, where mitragynine is illegal, have the highest consumption in Europe (Bade et al., 2023b).

Eutylone was sporadically detected in Ljubljana, Maribor, Domžale-Kamnik, and Koper, having the lowest PNMLS (0.02 - 0.06 mg/day/1000 inhabitants) among measured NPS (Fig. 1C). 4-FA was exclusively detected in Koper (on average 0.3 mg/day/1000 inhabitants), while mephedrone showed the highest PNMLs (0.77 mg/day/1000 inhabitants) in Domžale-Kamnik, even though it was only measured there once during the sampling campaign (Fig. 1D and Fig. 1E). Interestingly, in this work, mephedrone was mainly found in Koper, which is located close to the border with Italy, where mephedrone was also identified in 2020 only in Trieste and Gorizia (Salgueiro-González et al., 2022). Mephedrone (4-methylmethcathinone) is an isomer of 3-MMC, and in the early 2010s, it was one of the most popular NPS reported in Europe (Castiglioni et al., 2015; Mwenesongole et al., 2013; Sande and Purkart, 2011). In Slovenia, mephedrone was categorised as a Class I controlled substance in 2011 (Decree RS 157/20, 2020; Sande, 2016), as the Slovenian government updated its regulations regarding the classification of illegal drugs. This change was prompted by the European Council's decision, which mandated EU Member States to regulate the new psychoactive substance in the preceding year (Sande, 2016). Despite legal restrictions, mephedrone maintained its popularity among the most reported NPS in Europe (UNODC, 2023b), particularly in the UK (Hutton, 2020).

3.1.2. *Temporal trends*

By monitoring the same period from 2019 to 2022, this study can provide a "snapshot" of NPS consumption in Slovenia and evaluate temporal trends on the six sites investigated. The WWTP of Kranj was only included in the last sampling period; therefore, temporal evaluation could not be performed. Diverse temporal patterns in NPS use were identified in this study (Fig. 2).

A common observation for all the detected NPS is their disappearance during the sampling period of spring 2020, except for 3-MMC, which was measured in Ljubljana at 0.25 mg/day/1000 inhabitants. During that sampling period, Slovenia was in a strict lockdown with severe restrictions on movement between municipalities and limited social interactions due to the COVID-19 pandemic. 3-MMC was the only substance found in all sampling periods, with its measured PNMLs showing a progressive increase with time, indicating rising use in most catchments (Fig. 2A). In Ljubljana and Domžale-Kamnik, PNMLs of 3-MMC increased from 0.11 mg/day/1000 inhabitants (summer 2019) to respectively 1.27 and 1.32 mg/day/1000 inhabitants (spring 2021). In the following sampling period (spring 2022), where almost all restrictions had been eased, PNMLs of 3-MMC showed a decrease of 84 % and 36 % in Ljubljana and Domžale-Kamnik, respectively. A similar trend was observed in Koper. In particular, 3-MMC was first measured in Koper during the winter 2021 sampling period, with PNMLs corresponding to 0.25 mg/day/1000 inhabitants, then increased to 0.77 mg/day/1000 inhabitants (spring 2021) and finally decreased to 0.12 mg/day/1000 inhabitants (84 % decrease) in spring 2022. In Maribor, 3-

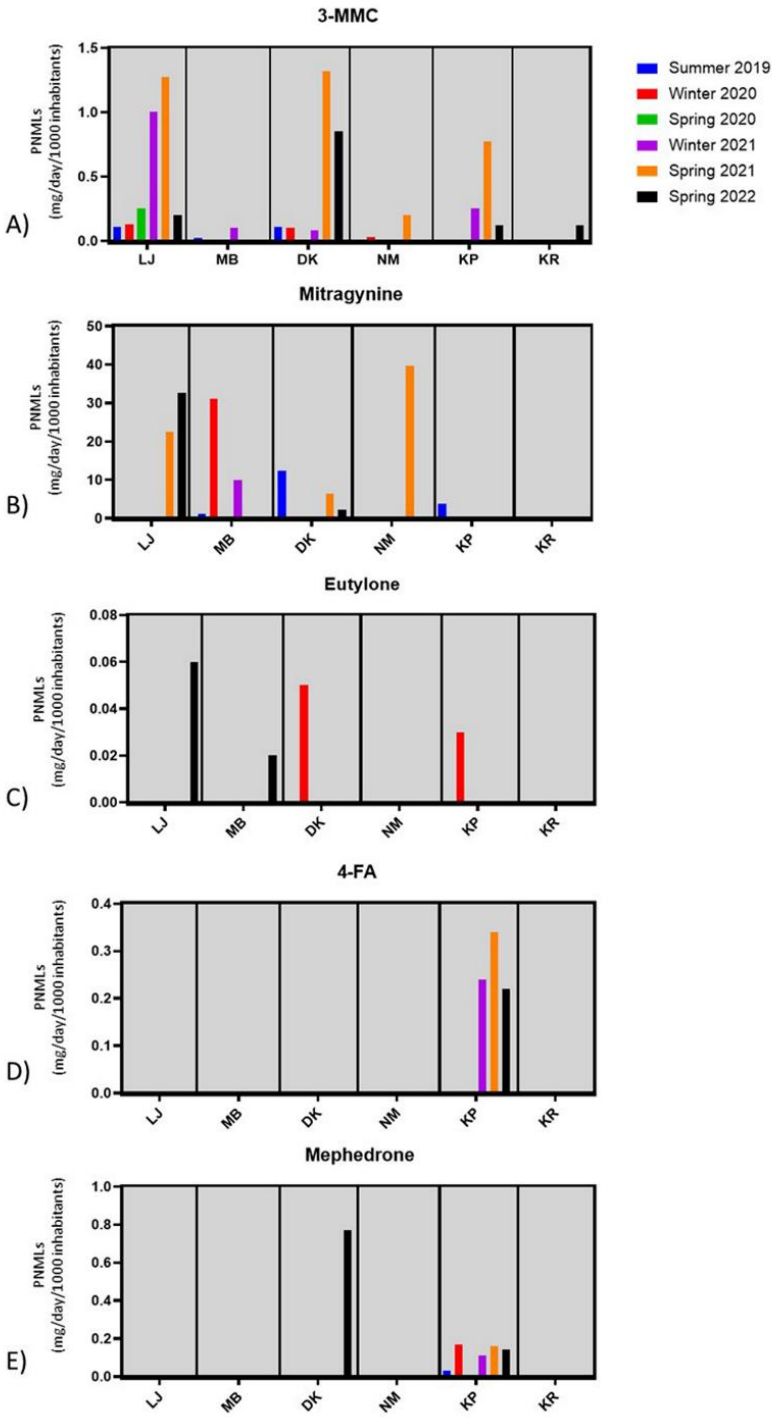


Fig. 1. Graphs showing the spatial variations in the PNMLs of NPS in all WWTPs. No NPS were detected in Velenje and is not included in the graphs.

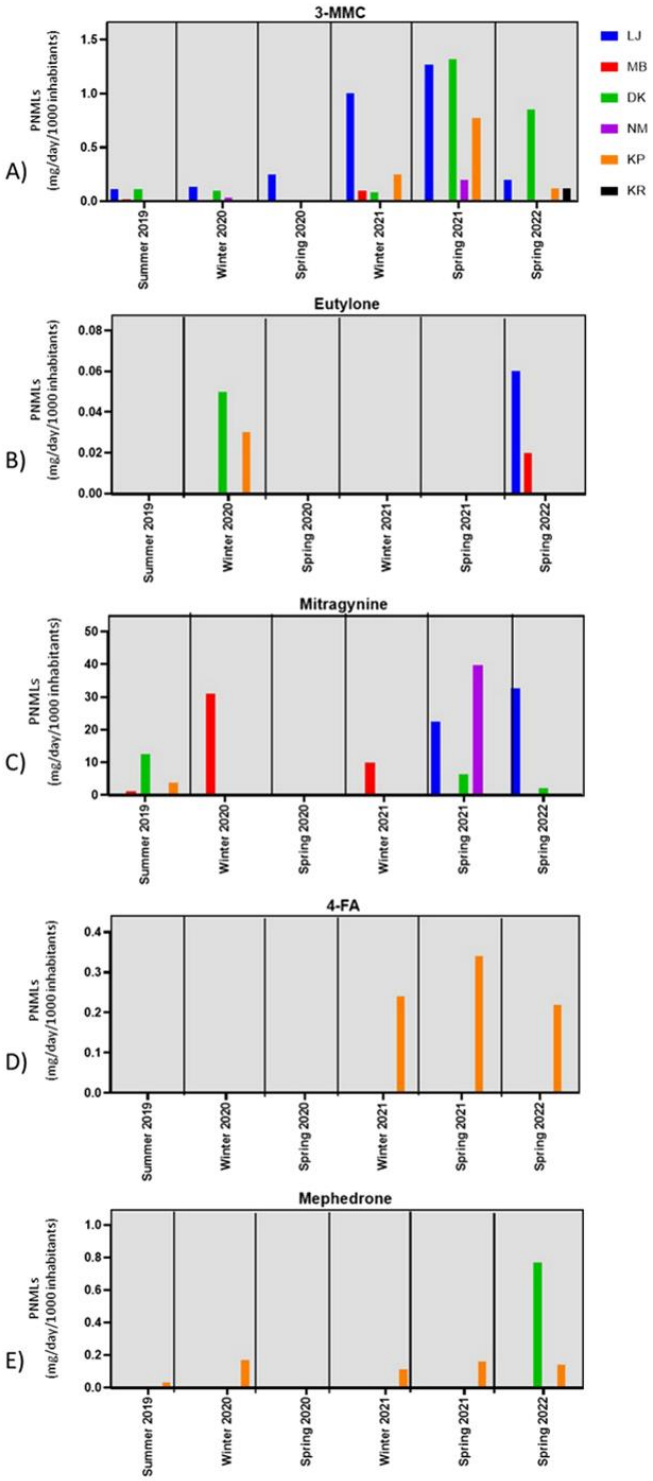


Fig. 2. Graphs showing the temporal variations in the PNMLs of NPS found in the present study. No NPS were detected in Velenje; thus, it is not included in the graphs.

MMC was only measured twice: in summer 2019 (0.02 mg/day/1000 inhabitants) and winter 2021 (0.10 mg/day/1000 inhabitants). Similarly, in Novo Mesto, 3-MMC was measured in winter 2020 (0.03 mg/day/1000 inhabitants) and spring 2021 (0.20 mg/day/1000 inhabitants), while it remained below LOQ for the rest of the sampling periods in both catchments (Fig. 2A). These results are in line with an international study (Bade et al., 2023b), according to which, increased levels of 3-MMC and eutylone between 2020–21 and 2021–22 could be linked with the decreased levels of MDMA reported in Europe, Australia, and Canada during the COVID-19 pandemic (EUDA, 2024; Price et al., 2022).

In the current study, eutylone was detected infrequently (four times) with very low PNMLs (Fig. 2B). Eutylone appeared before the pandemic (winter 2020) in Domžale-Kamnik (0.05 mg/day/1000 inhabitants) and Koper (0.03 mg/day/1000 inhabitants) and also during the spring 2022 sampling period in Ljubljana (0.06 mg/day/1000 inhabitants) and Maribor (0.02 mg/day/1000 inhabitants). In Ljubljana, mitragynine appeared in the spring of 2021, and its PNMLs increased from 22.5 to 32.7 mg/day/1000 inhabitants during spring 2022. In contrast, mitragynine PNMLs decreased from 12.4 mg/day/1000 inhabitants (summer 2019) to 2.14 mg/day/1000 inhabitants (spring 2022) in Domžale-Kamnik (Fig. 2C). An opposite trend was found in Maribor, i.e., mitragynine significantly increased from 1.23 mg/day/1000 inhabitants (summer 2019) to 32.0 mg/day/1000 inhabitants (winter 2020), then dramatically decreased by 69 % (winter 2021) until it was below LOD during the remaining sampling periods. 4-FA, detected only in Koper (Fig. 2D), increased by 29 % between winter 2021, when it was first measured, and spring 2021, followed by a decrease to the previous levels (0.22 mg/day/1000 inhabitants).

Lastly, mephedrone, which was also mainly detected in Koper, increased from 0.03 to 0.17 mg/day/1000 inhabitants between summer 2019 and winter 2020, while the PNMLs remained roughly stable afterwards (Fig. 2E). According to a study conducted in 2016 and 2017 (Castiglioni et al., 2021) mephedrone mass loads in Ljubljana, decreased by 40 %, while in the present study, it was not detected in Ljubljana during any sampling period. The absence of mephedrone in Ljubljana, its detection in Koper, and the variations in temporal trends indicate a potential change in the market or the use of NPS over the investigated period.

3.2. Qualitative screening of NPS

A qualitative screening method was applied to all pooled wastewater samples, using a database of >2000 NPS, pharmaceuticals of potential abuse, and illicit drugs (Bade et al., 2024). Five to 11 compounds were identified at levels 1 and 2 for each WWTP. After the final reprocessing, a total of 17 psychoactive compounds, including NPS (Table 2), pharmaceuticals, opioids, and illicit drugs (Table S7), were found across at least one site during the sampling campaigns. Nine were identified at confidence level 1, with a reference standard available for confirmation, while the remaining eight were identified at confidence level 2, based on actual retention time within the set threshold of 3 min and comparative fragmentation (Table S6) to that reported in HighResNPS database (Mardal et al., 2019). All identified NPS (levels 1 and 2) are listed in Table 2, while the remaining detected psychoactive compounds can be found in the Supplementary Information (Table S7).

Due to strict identification criteria regarding intensity and the presence of fragment ions, alongside the typically lower usage rates compared to conventional illicit drugs and pharmaceuticals. Although it was anticipated that NPS would not be detected in high numbers, six NPS were identified: 4-hydroxy-N-methyl-N-ethyltryptamine (4-HO-MET), levamisole, ephedrine, phentermine, N-methyl-2AI, and amantadine (Table 2). This workflow offers a complementary insight into the Slovenian NPS market and the spatiotemporal trends in NPS use. Levamisole, an adulterant illicit street drug (e.g. cocaine, heroin, and fentanyl), and 4-HO-MET, a lesser-known psychedelic drug that is a functional and structural analogue of psilocin, were only identified in Koper during all sampling periods. Similarly, the stimulant phentermine and the designer drug N-methyl-2AI (rigid analogue of methamphetamine) were only identified in Kranj. Their detection in only one location possibly indicates supply meeting demand or regional preference. To the authors' knowledge, this is the first time that 4-HO-MET and phentermine have been identified in influent wastewater. Levamisole and N-methyl-2AI have been previously reported in Europe, Brazil, and the US (Bade et al., 2024; Salgueiro-González et al., 2019).

Among the unequivocally identified stimulants (Table 2), ephedrine is the only one that can be of natural or synthetic origin. Apart from its use as a medication in traditional and Eastern medicine, ephedrine has several other uses, such as a precursor in the clandestine synthesis of methamphetamine and as a drug of abuse (mainly by athletes or drug users). It is expected to be detected in wastewater since it is a common ingredient in preparations for treating asthma, colds, and narcolepsy

Table 2
NPS identified at confidence levels 1 and 2 (Schymanski et al., 2014) in pooled influent wastewater samples across all sites and during all sampling periods by qualitative analysis, ordered alphabetically.

Psychoactive compound	Confidence level	Type	WWTP found	Sampling period					
				Summer 2019	Winter 2020	Spring 2020	Winter 2021	Spring 2021	Spring 2022
4-HO-MET	Level 2	NPS - stimulant	KP	✓	✓	✓	✓	✓	✓
Amantadine	Level 2	NPS - other	DK	✓	✓	×	✓	✓	✓
			VE	×	✓	✓	NA	NA	✓
Ephedrine	Level 1	NPS - stimulant	DK	✓	✓	×	✓	✓	✓
			VE	×	✓	✓	NA	NA	✓
			MB	✓	✓	×	✓	×	✓
			KR	NA	NA	NA	NA	NA	✓
			NM	✓	✓	×	✓	✓	✓
Levamisole	Level 2	NPS/ cocaine adulterant	KP	✓	✓	✓	✓	✓	✓
N-methyl-2AI	Level 2	NPS - other	KR	NA	NA	NA	NA	NA	✓
Phentermine	Level 2	NPS - stimulant	KR	NA	NA	NA	NA	NA	✓

Velenje (VE) winter/spring 2021 samples are missing due to an instrumental issue.
Abbreviations: 4-HO-MET, 4-hydroxy-N-methyl-N-ethyltryptamine; N-methyl-2AI, N-methyl-2-aminoindane; NA: not analysed.

(Gad et al., 2021). However, in Slovenia, medical ephedrine is only available in the injectable form of ephedrine hydrochloride, used to treat hypotension during local and general anaesthesia (CBZ, 2023). Therefore, there have been reports of recreational use of ephedrine in Slovenia (NIJZ, 2019; Verovšek et al., 2023a), and while this work cannot confirm this, it is noteworthy that ephedrine was identified (mainly in Maribor, Novo Mesto, Domžale-Kamnik, and Velenje) during all sampling periods except for the spring 2020 when strict lockdown was enforced due to the first wave of COVID-19 pandemic.

Morphine, tramadol, and codeine were the most detected opioid analgesics, found unequivocally during all sampling periods in almost all sampled Slovenian wastewater (Table S7). Two additional opioids, tapentadol and levorphanol, met level 1 and 2 identification criteria, respectively (Table S7). Tapentadol, a relatively new opioid, at the beginning of the sampling campaign, was found sporadically in six out of seven sites across Slovenia. In contrast, since the sampling period of winter 2021, it has been detected in all WWTPs, indicating its increasing popularity. It has previously been reported in Australia (Ahmed et al., 2023; Bade et al., 2024) and Greece (Alygizakis et al., 2021), with the latter study showing that its use declined during the pandemic. Levorphanol was also found sporadically, mainly in Koper, Maribor, Ljubljana, and Domžale-Kamnik during all sampling periods, with the difference disappearing in the spring of 2020 (first lockdown). Even though opioid-like psychoactive substances are frequently used as easily accessible alternatives to prescription medications under medical supervision (Andersson and Kjellgren, 2016), there is limited available metadata regarding their presence in Slovenia to confirm their use as street drugs. Finally, the illicit drugs cocaine and MDMA, the antidepressant venlafaxine, and its metabolite *O-desmethylvenlafaxine* were identified and confirmed in almost all WWTPs and sampling periods (Table S7). Their presence in Slovenia has already been reported in other studies (Laimou-Geraniou et al., 2023b; Verovšek et al., 2023b; T. 2022) and also in the frame of SCORE international monitoring on stimulant consumption (SCORE, 2023).

3.3. Limitations

As the study was focussed on long-term temporal trends, rather than weekly patterns, all analysed samples were weekly pools. It is thus possible that the numbers of NPS reported in this work is an underrepresentation as the continuous use of NPS is not expected throughout the week. Moreover, with pooled weekly samples, the mass loads are not necessarily comparable to previous studies.

4. Conclusions

This study provides an insight into the NPS market in Slovenia, including before, during and after the COVID-19 pandemic, highlighting the potential of WBE as a valuable tool for monitoring trends in NPS consumption by providing primary spatiotemporal information that is complementary to seized data. The findings of the study revealed several temporal and spatial variations. Five substances were semi-quantified across all sites at least once, with 3-MMC being the most prevalent, followed by mitragynine, which showed the highest population-normalised mass loads. This finding is in line with other international wastewater studies reporting 3-MMC as one of the predominant stimulant NPS in Europe, showing an increase in its use from 2018 to 2022. It is also worth noting that some NPS were only detected in one specific site, such as phentermine and N-methyl-2AI in Kranj and 4-FA, 4-OH-MET, mephedrone, and levamisole in Koper. Temporal monitoring revealed an increasing trend of 3-MMC use during the pandemic. Following suspect screening, 17 additional compounds were detected across all sites at least once. Due to the dynamic nature of the market, capturing all NPS in targeted analysis poses challenges. However, employing qualitative methods (SWATH acquisition on QTOF) and quantitative approaches (QqQ) provides a more comprehensive overview of trends in NPS usage. Continued monitoring will reveal other NPS consumption trend changes and help to identify and control emerging trends in NPS use, thereby contributing to beneficial outcomes for society.

Funding information

This study was supported by the TunTWIN project funded by the European Union (research project GA N° 952306), by the Slovenian Research and Innovation Agency (ARIS Program group P1-0143 and Project N1-0143), and by University of Ljubljana (interdisciplinary preparative project Nanostructurome 802-12/2024-5). R. B. is the recipient of an Australian Research Council Discovery Early Career Award (project number DE220100381), funded by the Australian Government. The Queensland Alliance for Environmental Health Sciences, The University of Queensland, gratefully acknowledges the financial support of the Queensland Department of Health.

CRediT authorship contribution statement

Maria Laimou-Geraniou: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Dhayaalini Nadarajan:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Richard Bade:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ester Heath:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hazadv.2025.100781.

Data availability

Data will be made available on request.

References

Ahmed, F., Tscharke, B., O'Brien, J.W., Hall, W.D., Cabot, P.J., Sowa, P.M., Samanipour, S., Thomas, K.V., 2023. National wastewater reconnaissance of analgesic consumption in Australia. Env. Sci. Technol. 57, 1712–1720. https://doi.org/10.1021/acs.est.2c06691.

Alygizakis, N., Galani, A., Rousis, N.I., Aalizadeh, R., Dimopoulos, M.-A., Thomaidis, N. S., 2021. Change in the chemical content of untreated wastewater of Athens, Greece under COVID-19 pandemic. Sci. Total. Environ. 799, 149230. https://doi.org/10.1016/j.scitotenv.2021.149230.

Andersson, M., Kjellgren, A., 2016. Aspects of substance displacement - from illicit drugs to novel psychoactive substance. J Addict Res Ther. 7.

Bade, R., Abdelaziz, A., Nguyen, L., Pandopulos, A.J., White, J.M., Gerber, C., 2020. Determination of 21 synthetic cathinones, phenethylamines, amphetamines and opioids in influent wastewater using liquid chromatography coupled to tandem mass spectrometry. Talanta 208, 120479. https://doi.org/10.1016/j.talanta.2019.120479.

Bade, R., Eaglesham, G., Shimko, K.M., Mueller, J., 2023a. Quantification of new psychoactive substances in Australian wastewater utilising direct injection liquid chromatography coupled to tandem mass spectrometry. Talanta 251, 123767. https://doi.org/10.1016/j.talanta.2022.123767.

Bade, R., Rousis, N., Adhikari, S., Baduel, C., Bijlsma, L., Bizani, E., Boogaerts, T., Burgard, D.A., Castiglioni, S., Chappell, A., Covaci, A., Driver, E.M., Sodre, F.F., Fatta-Kassinos, D., Galani, A., Gerber, C., Gracia-Lor, E., Gracia-Marín, E., Halden, R. U., Heath, E., Hernandez, F., Jaunay, E., Lai, F.Y., Lee, H.-J., Laimou-Geraniou, M.,

Oh, J.-E., Olafsdottir, K., Phung, K., Castro, M.P., Psichoudaki, M., Shao, X., Salgueiro-Gonzalez, N., Feitosa, R.S., Gomes, C.S., Subedi, B., Löve, A.S.C., Thomaidis, N., Tran, D., van Nuijs, A., Verovšek, T., Wang, D., White, J.M., Yargeau, V., Zuccato, E., Mueller, J.F., 2023b. Three years of wastewater surveillance for new psychoactive substances from 16 countries. Water. Res. X. 19, 100179. https://doi.org/10.1016/j.wroa.2023.100179.

Bade, R., van Herwerden, D., Rousis, N., Adhikari, S., Allen, D., Baduel, C., Bijlsma, L., Boogaerts, T., Burgard, D., Chappell, A., Driver, E.M., Sodre, F.F., Fatta-Kassinos, D., Gracia-Lor, E., Gracia-Marín, E., Halden, R.U., Heath, E., Jaunay, E., Krotulski, A., Lai, F.Y., Löve, A.S.C., O'Brien, J.W., Oh, J.-E., Pasin, D., Castro, M.P., Psichoudaki, M., Salgueiro-Gonzalez, N., Gomes, C.S., Subedi, B., Thomas, K.V., Thomaidis, N., Wang, D., Yargeau, V., Samanipour, S., Mueller, J., 2024. Workflow to facilitate the detection of new psychoactive substances and drugs of abuse in influent urban wastewater. J. Hazard. Mater. 469, 133955. https://doi.org/10.1016/j.jhazmat.2024.133955.

Bade, R., White, J.M., Chen, J., Baz-Lomba, J.A., Been, F., Bijlsma, L., Burgard, D.A., Castiglioni, S., Salgueiro-Gonzalez, N., Celma, A., Chappell, A., Emke, E., Steenbeek, R., Wang, D., Zuccato, E., Gerber, C., 2021. International snapshot of new psychoactive substance use: case study of eight countries over the 2019/2020 new year period. Water. Res. 193, 116891. https://doi.org/10.1016/j.watres.2021.116891.

Bade, R., White, J.M., Ghetia, M., Adiraju, S., Adhikari, S., Bijlsma, L., Boogaerts, T., Burgard, D.A., Castiglioni, S., Celma, A., Chappell, A., Covaci, A., Driver, E.M., Halden, R.U., Hernandez, F., Lee, H.-J., van Nuijs, A.L.N., Oh, J.-E., Pineda Castro, M.A., Salgueiro-Gonzalez, N., Subedi, B., Shao, X.-T., Yargeau, V., Zuccato, E., Gerber, C., 2022. A taste for new psychoactive substances: wastewater analysis study of 10 countries. Env. Sci. Technol. Lett. 9, 57–63. https://doi.org/10.1021/acs.estlett.1c00807.

Barenholtz, E., Krotulski, A.J., Morris, P., Fitzgerald, N.D., Le, A., Papsun, D.M., Logan, B. K., Hahn, W.E., Goldberger, B.A., Cottler, L.B., Palamar, J.J., 2021. Online surveillance of novel psychoactive substances (NPS): monitoring Reddit discussions as a predictor of increased NPS-related exposures. Int. J. Drug Policy. 98, 103393. https://doi.org/10.1016/j.drugpo.2021.103393.

Bijlsma, L., Bade, R., Been, F., Celma, A., Castiglioni, S., 2020a. Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – a tutorial. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2020.08.058.

Bijlsma, L., Celma, A., Castiglioni, S., Salgueiro-González, N., Bou-Iserte, L., Baz-Lomba, J.A., Reid, M.J., Dias, M.J., Lopes, A., Matias, J., Pastor-Alcañiz, L., Radonić, J., Turk Sekulic, M., Shine, T., van Nuijs, A.L.N., Hernandez, F., Zuccato, E., 2020b. Monitoring psychoactive substance use at six European festivals through wastewater and pooled urine analysis. Sci. Total. Environ. 725, 138376. https://doi.org/10.1016/j.scitotenv.2020.138376.

Boogaerts, T., Quireyns, M., Maes, F., Laimou-Geraniou, M., Van Wichelen, N., Heath, E., Pussig, B., Aertgeerts, B., Covaci, A., van Nuijs, A.L.N., 2023. Optimization, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater. Drug Test. Anal. 15, 240–246. https://doi.org/10.1002/dta.3392.

Brandeburová, P., Bodík, I., Horáková, I., Žabka, D., Castiglioni, S., Salgueiro-González, N., Zuccato, E., Špalková, V., Mackuľak, T., 2020. Wastewater-based epidemiology to assess the occurrence of new psychoactive substances and alcohol consumption in Slovakia. Ecotoxicol. Env. Saf. 200, 110762. https://doi.org/10.1016/j.ecoenv.2020.110762.

Castiglioni, S., Borsotti, A., Senta, I., Zuccato, E., 2015. Wastewater analysis to monitor spatial and temporal patterns of use of two synthetic recreational drugs, ketamine and mephedrone, in Italy. Env. Sci. Technol. 49, 5563–5570. https://doi.org/10.1021/es5060429.

Castiglioni, S., Salgueiro-González, N., Bijlsma, L., Celma, A., Gracia-Lor, E., Beldean-Galea, M.S., Mackuľak, T., Emke, E., Heath, E., Kasprzyk-Hordern, B., Petkovic, A., Poretti, F., Rangelov, J., Santos, M.M., Sremački, M., Styszko, K., Hernández, F., Zuccato, E., 2021. New psychoactive substances in several European populations assessed by wastewater-based epidemiology. Water. Res. 195, 116983. https://doi.org/10.1016/j.watres.2021.116983.

Causanilles, A., Kinyua, J., Ruttkies, C., van Nuijs, A.L.N., Emke, E., Covaci, A., de Voogt, P., 2017. Qualitative screening for new psychoactive substances in wastewater collected during a city festival using liquid chromatography coupled to high-resolution mass spectrometry. Chemosphere 184, 1186–1193. https://doi.org/10.1016/j.chemosphere.2017.06.101.

CBZ, 2023. Central database of pharmaceuticals 2 = Centralna Baza Zdravil 2 (2023) [WWW Document]. URL. http://www.cbz.si/cbz/bazazdr2.nsf/Search/$searchForm?SearchView (accessed 3.19.24).

Decree RS 157/20, 2020. Decree R.S. Decree amending the Decree on the classification of illicit drugs (Uredba o dopolnitvah Uredbe o razvrstitvi prepovedanih drog) Uradni list RS, št, 157 (20) (2020) 157/20 [WWW Document]. URL https://pisrs.si/pregledPredpisa?id=URED8133 (accessed 6.13.24).

Diamanti, K., Aalizadeh, R., Alygizakis, N., Galani, A., Mardal, M., Thomaidis, N.S., 2019. Wide-scope target and suspect screening methodologies to investigate the occurrence of new psychoactive substances in influent wastewater from Athens. Sci. Total. Environ. 685, 1058–1065. https://doi.org/10.1016/j.scitotenv.2019.06.173.

Estévez-Danta, A., Bijlsma, L., Capela, R., Cela, R., Celma, A., Hernández, F., Lertxundi, U., Matias, J., Montes, R., Orive, G., Prieto, A., Santos, M.M., Rodil, R., Quintana, J.B., 2022. Use of illicit drugs, alcohol and tobacco in Spain and Portugal during the COVID-19 crisis in 2020 as measured by wastewater-based epidemiology. Sci. Total. Environ. 836, 155697. https://doi.org/10.1016/j.scitotenv.2022.155697.

EUDA, 2024. EUDA (European union drugs agency). MDMA – the current situation in Europe (European drug report 2024) | [Document]. URL. www.euda.europa.eu. https://www.euda.europa.eu/publications/european-drug-report/2024/mdma_en [Document]. URL(accessed 6.26.24).

EUDA, 2022a. EUDA (European Union Drugs Agency), 2022, European Drug Report 2022: Trends and Developments. Publications Office of the European Union, Luxembourg. https://doi.org/10.2810/75644. Publications Office, LU.

EUDA, 2022b. EUDA (European Union Drugs Agency). New psychoactive substances: 25 Years of Early Warning and Response in Europe: an Update from the EU Early Warning System. Publications Office, LU.

Ferreira, B., Dias da Silva, D., Carvalho, F., de Lourdes Bastos, M., Carmo, H., 2019. The novel psychoactive substance 3-methylmethcathinone (3-MMC or metaphedrone): a review. Forensic Sci. Int. 295, 54–63. https://doi.org/10.1016/j.forsciint.2018.11.024.

Gad, M.Z., Azab, S.S., Khattab, A.R., Farag, M.A., 2021. Over a century since ephedrine discovery: an updated revisit to its pharmacological aspects, functionality and toxicity in comparison to its herbal extracts. Food Funct. 12, 9563–9582. https://doi.org/10.1039/D1FO02093F.

Gao, T., Du, P., Xu, Z., Li, X., 2017. Occurrence of new psychoactive substances in wastewater of major Chinese cities. Sci. Total. Environ. 575, 963–969. https://doi.org/10.1016/j.scitotenv.2016.09.152.

Gatica-Bahamonde, G., Godynyuk, E.A., Neicun, J., Roberts, E., Tangerli, M.M., van Kessel, R., Czabanowska, K., Humphreys, K., Roman-Urrestarazu, A., 2023. Analysing the use trends of new psychoactive substances using wastewater-based epidemiology in Europe: a systematic review. Emerg. Trends. Drugs Addict. Health 3, 100053. https://doi.org/10.1016/j.etdah.2023.100053.

Gent, L., Paul, R., 2021. The detection of new psychoactive substances in wastewater. A comprehensive review of analytical approaches and global trends. Sci. Total. Environ. 776, 146028. https://doi.org/10.1016/j.scitotenv.2021.146028.

González-Mariño, I., Gracia-Lor, E., Rousis, N.I., Castrignanò, E., Thomas, K.V., Quintana, J.B., Kasprzyk-Hordern, B., Zuccato, E., Castiglioni, S., 2016. Wastewater-based epidemiology to monitor synthetic cathinones use in different European countries. Env. Sci, Technol. 50, 10089–10096. https://doi.org/10.1021/acs.est.6b02644.

Gracia-Lor, E., Rousis, N.I., Zuccato, E., Castiglioni, S., 2020. Monitoring caffeine and nicotine use in a nationwide study in Italy using wastewater-based epidemiology. Sci. Total. Environ. 747, 141331. https://doi.org/10.1016/j.scitotenv.2020.141331.

Huizer, M., ter Laak, T.L., de Voogt, P., van Wezel, A.P., 2021. Wastewater-based epidemiology for illicit drugs: a critical review on global data. Water. Res. 207, 117789. https://doi.org/10.1016/j.watres.2021.117789.

Hutton, F., 2020. Cultures of intoxication: 'new' Psychoactive substances. In: Hutton, F. (Ed.), Cultures of Intoxication: Key Issues and Debates. Springer International Publishing, Cham, pp. 87–110. https://doi.org/10.1007/978-3-030-35284-4_5.

Jaunay, E.L., Bade, R., Paxton, K.R., Nadarajan, D., Barry, D.C., Zhai, Y., Tscharke, B.J., O'Brien, J.W., Mueller, J., White, J.M., Simpson, B.S., Gerber, C., 2024. Monitoring the use of novel psychoactive substances in Australia by wastewater-based epidemiology. Sci. Total. Environ. 919, 170473. https://doi.org/10.1016/j.scitotenv.2024.170473.

Laimou-Geraniou, M., Heath, D., Heath, E., 2023a. Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology. Trends Environ. Anal. Chem. 37, e00192. https://doi.org/10.1016/j.teac.2022.e00192.

Laimou-Geraniou, M., Quireyns, M., Boogaerts, T., Van Wichelen, N., Heath, D., van Nuijs, A.L.N., Covaci, A., Heath, E., 2023b. Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters. Sci. Total. Environ. 903, 166586. https://doi.org/10.1016/j.scitotenv.2023.166586.

Lee, H.-J., Oh, J.-E., 2023. Target and suspect screening of (new) psychoactive substances in South Korean wastewater by LC-HRMS. Sci. Total. Environ. 875, 162613. https://doi.org/10.1016/j.scitotenv.2023.162613.

López-García, E., Pérez-López, C., Postigo, C., Andreu, V., Bijlsma, L., González-Mariño, I., Hernández, F., Marcé, R.M., Montes, R., Picó, Y., Pocurull, E., Rico, A., Rodil, R., Rosende, M., Valcárcel, Y., Zuloaga, O., Quintana, J.B., López de Alda, M., 2020. Assessing alcohol consumption through wastewater-based epidemiology: spain as a case study. Drug Alcohol Depend. 215, 108241. https://doi.org/10.1016/j.drugalcdep.2020.108241.

Mardal, M., Andreasen, M.F., Mollerup, C.B., Stockham, P., Telving, R., Thomaidis, N.S., Diamanti, K.S., Linnet, K., Dalsgaard, P.W., 2019. HighResNPS.Com: an online crowd-sourced HR-MS database for suspect and non-targeted screening of new psychoactive substances. J. Anal. Toxicol. 43, 520–527. https://doi.org/10.1093/jat/bkz030.

Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M., 2003. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. Anal. Chem. 75, 3019–3030. https://doi.org/10.1021/ac020361s.

Moslah, B., Smaoui, O., Nouioui, M.A., Araoud, M., Chaouali, N., Laribi, M., Amira, D., Ben Salah, N., Hedhili, A., 2023. Sewage analysis as an alternative tool for assessing drug of abuse and new psychoactive substances in Tunisia. Forensic Sci. Int. 347, 111672. https://doi.org/10.1016/j.forsciint.2023.111672.

Mwenesongole, E.M., Gautam, I., Hall, S.W., Waterhouse, J.W., Cole, M.D., 2013. Simultaneous detection of controlled substances in waste water. Anal. Methods 5, 3248–3254. https://doi.org/10.1039/C3AY40655E.

Nadarajan, D., O'Brien, J., Cresswell, S., Kele, B., Mueller, J., Bade, R., 2024. Application of design of experiment for quantification of 71 new psychoactive substances in influent wastewater. Anal. Chim. Acta 1321, 343036. https://doi.org/10.1016/j.aca.2024.343036.

NFL, 2022. NFL,2022 NFL National forensic laboratory, chemical examination section [WWW Document]. URL. https://www.policija.si/o-slovenski-policiji/organizirano st/generalna-policijska-uprava/nacionalni-forenzicni-laboratorij/nfl-oddelek-za-kemijske-preiskave (accessed 3.4.24).

NIJZ, 2019. Report on the drug situation 2019 of the republic of Slovenia National institute of public health, Ljubljana (2019). Nijz. URL. https://nijz.si/publikacije/report-on-the-drug-situation-2019-of-the-republic-of-slovenia-2/ (accessed 3.4.24).

O'Rourke, C.E., Subedi, B., 2020. Occurrence and mass loading of synthetic opioids, synthetic cathinones, and synthetic cannabinoids in wastewater treatment plants in four U.S. Communities. Env. Sci. Technol. 54, 6661–6670. https://doi.org/10.1021/acs.est.0c00250.

Peacock, A., Bruno, R., Gisev, N., Degenhardt, L., Hall, W., Sedefov, R., White, J., Thomas, K.V., Farrell, M., Griffiths, P., 2019. New psychoactive substances: challenges for drug surveillance, control, and public health responses. Lancet 394, 1668–1684. https://doi.org/10.1016/S0140-6736(19)32231-7.

Perkons, I., Tomsone, L.E., Sukajeva, V., Neilands, R., Kokina, K., Pugajeva, I., 2022. Qualitative fingerprinting of psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater: a year-long study from Riga, Latvia. J. Environ. Chem. Eng. 10, 108110. https://doi.org/10.1016/j.jece.2022.108110.

Price, O., Man, N., Bruno, R., Dietze, P., Salom, C., Lenton, S., Grigg, J., Gibbs, D., Wilson, T., Degenhardt, L., Chan, R., Thomas, N., Peacock, A., 2022. Changes in illicit drug use and markets with the COVID-19 pandemic and associated restrictions: findings from the EcSTASY AND RELATED DRUGS REPORTING SYstem, 2016–20. Addiction 117, 182–194. https://doi.org/10.1111/add.15620.

Quireyns, M., Boogaerts, T., Van Wichelen, N., Pussig, B., De Loof, H., Covaci, A., Van Nuijs, A., 2022. Temporal monitoring of pharmaceutical consumption using a wastewater-based epidemiologic approach. In: Toxicologie Analytique et Clinique, 30th meeting of SFTA- 59th meeting of TIAFT - September 2022 34, S72. https://doi.org/10.1016/j.toxac.2022.06.099.

Reid, M.J., Baz-Lomba, J.A., Ryu, Y., Thomas, K.V., 2014. Using biomarkers in wastewater to monitor community drug use: a conceptual approach for dealing with new psychoactive substances. Sci. Total. Environ. 487, 651–658. https://doi.org/10.1016/j.scitotenv.2013.12.057.

Rice, J., Kannan, A.M., Castrignanò, E., Jagadeesan, K., Kasprzyk-Hordern, B., 2020. Wastewater-based epidemiology combined with local prescription analysis as a tool for temporal monitoring of drugs trends - A UK perspective. Sci. Total. Environ. 735, 139433. https://doi.org/10.1016/j.scitotenv.2020.139433.

Rousis, N., Bade, R., Gracia-Lor, E., 2023a. Wastewater-based epidemiology as a surveillance tool to assess human consumption of psychotropic substances: alcohol, nicotine and caffeine as case studies. TrAC Trends Anal. Chem. 167, 117230. https://doi.org/10.1016/j.trac.2023.117230.

Rousis, N., Bade, R., Romero-Sánchez, I., Mueller, J.F., Thomaidis, N.S., Thomas, K.V., Gracia-Lor, E., 2023b. Festivals following the easing of COVID-19 restrictions: prevalence of new psychoactive substances and illicit drugs. Env. Int. 178, 108075. https://doi.org/10.1016/j.envint.2023.108075.

Salgueiro-Gonzalez, N., Béen, F., Bijlsma, L., Boogaerts, T., Covaci, A., Baz-Lomba, J.A., Kasprzyk-Hordern, B., Matias, J., Ort, C., Bodík, I., Heath, E., Styszko, K., Emke, E., Hernández, F., van Nuijs, A.L.N., Castiglioni, S., 2024. Influent wastewater analysis to investigate emerging trends of new psychoactive substances use in Europe. Water. Res., 121390 https://doi.org/10.1016/j.watres.2024.121390.

Salgueiro-González, N., Castiglioni, S., Gracia-Lor, E., Bijlsma, L., Celma, A., Bagnati, R., Hernández, F., Zuccato, E., 2019. Flexible high resolution-mass spectrometry approach for screening new psychoactive substances in urban wastewater. Sci. Total. Environ. 689, 679–690. https://doi.org/10.1016/j.scitotenv.2019.06.336.

Salgueiro-González, N., Zuccato, E., Castiglioni, S., 2022. Nationwide investigation on the use of new psychoactive substances in Italy through urban wastewater analysis. Sci. Total. Environ. 843, 156982. https://doi.org/10.1016/j.scitotenv.2022.156982.

Sande, M., 2016. Characteristics of the use of 3-MMC and other new psychoactive drugs in Slovenia, and the perceived problems experienced by users. Int. J. Drug Policy. 27, 65–73. https://doi.org/10.1016/j.drugpo.2015.03.005.

Sande, Purkart, 2011. Cocaine use in nightlife in Slovenia and Italy. In: Sande, M. (Ed.), Nightlife Reconsidered. The Etnoblog Intercultural Association, Trieste, pp. 73–85 (2011). http://pefprints.pef.uni-lj.si/688/1/Nightlife_reconsidered.pdf.

SANTE/11312/2021v2, 2021. Analytical quality control and method validation procedures for pesticide residues analysis in food and feed.

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying small molecules via high resolution mass spectrometry: communicating confidence. Env. Sci. Technol. 48, 2097–2098. https://doi.org/10.1021/es5002105.

SCORE, 2023. SCORE, Sewage analysis CORe group — Europe, homepage (2023). URL. https://score-network.eu/monitoring/ (accessed 3.18.24).

Senta, I., Krizman-Matasic, I., Kostanjevecki, P., Gonzalez-Mariño, I., Rodil, R., Quintana, J.B., Mikac, I., Terzic, S., Ahel, M., 2023. Assessing the impact of a major electronic music festival on the consumption patterns of illicit and licit psychoactive substances in a Mediterranean city using wastewater analysis. Sci. Total. Environ. 892, 164547. https://doi.org/10.1016/j.scitotenv.2023.164547.

Tscharke, B.J., Chen, C., Gerber, J.P., White, J.M., 2016. Temporal trends in drug use in Adelaide, South Australia by wastewater analysis. Sci. Total. Environ. 565, 384–391. https://doi.org/10.1016/j.scitotenv.2016.04.183.

UNODC, 2023a. UNODC (United nations office on drugs and crime), 2023. Current NPS trends VI August 2023 [WWW Document]. URL. https://www.unodc.org/res/scientists/ewa/Current_NPS_Threats_VI.pdf (accessed 6.26.24).

UNODC, 2023b. UNODC (United nations office on drugs and crime), 2023. Data from the UNODC early warning advisory on new psychoactive substances [WWW Document]. URL. https://www.unodc.org/LSS/Page/NPS/DataVisualisations (accessed 3.4.24).

UNODC, 2022. United Nations office on drugs and crime. World drug report 2022: drug Market trends - cocaine, amphetamine-type substances, new psychoactive substances [Internet]. 2024. Available from. https://www.unodc.org/res/wdr2022/MS/WDR22_Booklet_4.pdf.

Verovšek, T., Celma, A., Heath, D., Heath, E., Hernández, F., Bijlsma, L., 2023a. Screening for new psychoactive substances in wastewater from educational institutions. Env. Res. 237, 117061. https://doi.org/10.1016/j.envres.2023.117061.

Verovšek, T., Heath, D., Heath, E., 2022. Occurrence, fate and determination of tobacco (nicotine) and alcohol (ethanol) residues in waste- and environmental waters. Trends Environ. Anal. Chem. 34, e00164. https://doi.org/10.1016/j.teac.2022.e00164.

Verovšek, T., Janža, M., Heath, D., Šuštarič, A., Prosen, H., Heath, E., 2023b. Occurrence and sources of residues of drugs of abuse in an urban aquifer: chemical analysis and solute transport modelling. Sci. Total. Environ. 892, 164364. https://doi.org/10.1016/j.scitotenv.2023.164364.

WHO, 2022. World health organisation, W.H.O., 2022. 45th expert committee on drug dependence documents. [WWW Document]. URL. https://www.who.int/groups/ecdd/45th-ecdd-documents (accessed 3.4.24).

Zhang, M., Sharma, A., León, F., Avery, B., Kjelgren, R., McCurdy, C.R., Pearson, B.J., 2022. Plant growth and phytoactive alkaloid synthesis in kratom [Mitragyna speciosa (Korth.)] in response to varying radiance. PLoS. One 17, e0259326. https://doi.org/10.1371/journal.pone.0259326.

3.3 Reducing WBE Uncertainties: Stability of Psychoactive Pharmaceuticals and NPS

3.3.1 Stability of antipsychotic biomarkers for wastewater-based epidemiology

Published: M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, D. Heath, A. Covaci, A. L.N. van Nuijs, E. Heath, 2025, *Journal of Pharmaceutical and Biomedical Analysis*, vol. 257, pp. 116702.

This study is a collaboration between the Jožef Stefan Institute in Ljubljana (Slovenia) and the Ecotoxicological Centre at the University of Antwerp (Belgium). All aspects of the work – from experimental design, data analysis and interpretation to manuscript writing were shared equally between me and M. Quireyns (co-author).

WBE has emerged as a powerful tool for assessing community-wide pharmaceutical consumption. However, accurate estimations depend on the in-sewer and in-sample stability of drug biomarkers. Degradation or transformation of target biomarkers may occur at various stages, including excretion, in-sewer transport, sampling, storage, and analysis, leading to under- or overestimation of actual consumption levels.

The number of publications evaluating the in-sample and in-sewer stability of antidepressants and benzodiazepines has increased, but despite this, information on the stability of antipsychotics in influent wastewater is lacking. To the date, this is the first comprehensive study of the stability of 11 antipsychotic biomarkers, including parent compounds and metabolites, in influent wastewater, using a series of experiments mimicking in-sewer transport (in-sewer setup and deconjugation) and in-sample stability (benchtop, frozen cartridge, WWTP autosampler, long-term sample storage, and freeze-thaw) under various conditions (i.e., filtration, acidification) commonly used for storage. All experiments used spiked influent wastewater samples processed by SPE and analysed by UPLC–MS/MS.

The results reveal that biomarker stability is highly compound-specific, with quetiapine, *N*-desalkylquetiapine, and risperidone showing notably high stability across most tested conditions. In contrast, *N*-desmethylolanzapine and dehydroaripiprazole, metabolites of olanzapine and aripiprazole, respectively, degraded rapidly. Acidification before storage resulted in significant degradation of several compounds, thereby decreasing the accuracy of WBE consumption estimates and highlighting its unsuitability as a sample storage method. Freeze–thaw testing revealed that a single cycle was generally acceptable, but repeated cycles reduced stability for specific compounds, especially when acidified. Storage on frozen SPE cartridges was highly effective, maintaining high stability for up to three months, except for *N*-desalkylquetiapine (up to 6 weeks), making them suitable for sample transport and batch analysis.

These findings are essential for enhancing the accuracy, reliability, and comparability of WBE studies focused on antipsychotic drug surveillance. Overall, this study suggests that spatiotemporal comparisons should be approached cautiously and tailored to specific biomarkers to ensure accurate conclusions.

Journal of Pharmaceutical and Biomedical Analysis 257 (2025) 116702

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Stability of antipsychotic biomarkers for wastewater-based epidemiology

Maria Laimou-Geraniou [a,b,1], Maarten Quireyns [c,1], Tim Boogaerts [c], David Heath [a], Adrian Covaci [c], Alexander L.N. van Nuijs [c], Ester Heath [a,b,*]

[a] Jožef Stefan Institute, Jamova 39, Ljubljana 1000, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova 39, Ljubljana 1000, Slovenia
[c] Toxicological Centre, University of Antwerp, Universiteitsplein 1, Wilrijk 2610, Belgium

ARTICLE INFO

Keywords:
Antipsychotics
Influent wastewater
In-sewer stability
In-sample stability
Storage
Wastewater-based epidemiology

ABSTRACT

Wastewater-based epidemiology (WBE) offers a promising approach by providing objective data on antipsychotic drug consumption within a population that can be used to monitor abuse, misuse, and regional prescription patterns. However, accurate estimations depend on knowing the stability of drug biomarkers. This study provides a comprehensive analysis of the stability of 11 antipsychotic biomarkers (parent drugs and their metabolites) in influent wastewater, using a series of experiments mimicking in-sewer transport (in-sewer setup and deconjugation) and in-sample stability (benchtop, frozen cartridge, long-term sample storage, and freeze-thaw). In-sample stability experiments are exposed to various conditions (i.e., filtration, acidification) commonly used for storage. The results reveal compound-specific stability profiles, with quetiapine showing notable high stability across most experiments. In contrast, N-desmethylolanzapine and dehydroaripiprazole demonstrated rapid degradation. Acidification before storage led to significant degradation of some compounds, decreasing the accuracy of WBE consumption estimates and rendering it unsuitable as a sample storage method. Overall, the study found that compound-specific degradation patterns underscore the necessity for tailored analytical approaches when using WBE to monitor antipsychotic drug use. Future research should focus on bioreactor studies mimicking in-sewer conditions and the application of correction factors to enhance the accuracy of consumption estimates, facilitating more reliable public health surveillance through WBE.

1. Introduction

Antipsychotics are used to treat a variety of disorders, including acute antipsychotic symptoms, and as a maintenance therapy to prevent relapse. They are commonly prescribed for schizophrenia, which is associated with increased social and physical burden, such as increased risk of cardio-cerebrovascular disease, obesity, and significantly reduced life expectancy [1,2]. At the population level, aggregated data provide an overview that helps identify trends and patterns in drug use. From a public health perspective, it would be interesting to know how antipsychotic use changes due to various factors such as rising mental health issues, increased diagnosis, or a change in prescription patterns. While antipsychotics are mainly used for the treatment of severe mental conditions, there is scientific evidence that certain antipsychotic medications, such as quetiapine and olanzapine, have been misused or even abused [3–5]. Wastewater-based epidemiology (WBE), a technique based on the analysis of biomarkers (i.e., human metabolic excretion products of xenobiotics) in wastewater, can provide insights into population-wide drug consumption patterns and public health trends. Untreated wastewater, typically composite 24-h influent wastewater samples, is collected at the wastewater treatment plant (WWTP). Knowing the concentration of the biomarker in the wastewater, the flow rate and the size of the population in the treatment plant catchment area, it is possible to calculate the population-normalised mass load of a biomarker (PNML, mg/day/1000 people), which acts as a proxy for consumption [6]. Tracking antipsychotic consumption using WBE has significant public health benefits. It offers valuable, cost-effective, objective, spatiotemporal information on the consumption of antipsychotics in the general population that may be difficult to measure using traditional drug research, e.g., surveys, number of prescriptions, sales, and dispensing data. Over time and across locations, it enables the identification of overall trends and usage patterns that may not be

* Corresponding author at: Jožef Stefan Institute, Jamova 39, Ljubljana 1000, Slovenia.
E-mail address: ester.heath@ijs.si (E. Heath).
[1] Joint first author

https://doi.org/10.1016/j.jpba.2025.116702
Received 11 September 2024; Received in revised form 23 January 2025; Accepted 23 January 2025
Available online 25 January 2025
0731-7085/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

captured through surveys or prescription records.

In WBE, accurate estimations depend on the stability of drug biomarkers in influent wastewater, but unfortunately, current information on the stability of antipsychotics in influent wastewater during sampling, storage, and sample preparation is lacking. At different stages, from excretion, transport, sampling, sample storage and final analysis, degradation and formation of the target biomarkers may occur, leading to an under- or overestimation of their actual consumption. It is essential, therefore, that the chosen biomarker is stable or that a suitable correction factor is applied to make correct assumptions regarding consumption [7]. To date, the publication by Logarinho *et al.* is the only one that examined the stability of six antipsychotic drugs: chlorpromazine, clozapine, cyamemazine, haloperidol, levomepromazine, and quetiapine. It is limited to the short-term stability of spiked influent wastewater left at room temperature for 24 hours [8]. This study is the first comprehensive report on the stability of antipsychotic drugs and their metabolites in wastewater. More precisely, we evaluate the stability of 11 antipsychotic biomarkers (parent drugs and their metabolites) using a series of experiments mimicking in-sewer transport (in-sewer setup, deconjugation) and in-sample stability (benchtop, frozen cartridge, long-term sample storage, WWTP autosampler, and freeze-thaw). In-sample stability experiments are exposed to various conditions (i.e., filtration, acidification) commonly used for storage.

2. Materials and methods

2.1. Materials

Eleven biomarkers of the most popular antipsychotic drugs were selected based on sales and prescription data in Slovenia and Belgium. These included the parent compounds and their main metabolites: aripiprazole and dehydroaripiprazole, clozapine and N-desmethylclozapine, olanzapine and N-desmethylolanzapine, risperidone and paliperidone, quetiapine and N-desalkylquetiapine, and haloperidol. Further details regarding reagents and materials can be found in the Supplementary material (S1. Materials and Methods).

2.2. Sample collection and treatment

Composite 24-h influent wastewater samples were collected from the wastewater treatment plant (WWTP) servicing Domžale-Kamnik (population 77,981), Slovenia. Samples were collected in a flow-proportional manner (1 L of sample is collected every hour according to flow) on the same day as the experimental analysis during a "normal" period, i.e., no special events occurred. The samples were collected in 1 L high-density polyethylene (HDPE) bottles, transported directly to the laboratory and processed immediately.

Pre-concentration of target compounds was achieved using Oasis Prime MCX solid-phase extraction (SPE) cartridges (60 mg/3 mL, Waters, Milford, MA, USA), followed by analysis using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). Any addition of standards and modifiers such as HCl (when needed) was done after filtration as it was not intended to assess filtration recovery. Prior to SPE, 50 mL aliquots were spiked with 6 µL of working internal standard (ISTD) mixture (final concentration 240 ng/L), acidified (HCl 37 %) to pH 2, and centrifuged for 25 min at 10,000 rpm to sediment any solid particles. The aliquot was loaded on the cartridges and washed with 5 mL of ultrapure water. The sorbent was dried under vacuum for 30 min, and the analytes were eluted using 6 mL of 0.5 % ammonium solution in methanol. The samples were evaporated to dryness at 35 °C and reconstituted in 200 µL of the starting mobile phase (ultrapure water: methanol (3:1 v/v) + 0.1 % of formic acid). Before injection, the extracts were centrifugally filtered (0.45 µm) for 3 min at 14,000 rpm, transferred to an autosampler vial and analysed using UPLC-MS/MS.

2.3. Liquid chromatography-tandem mass spectrometry

All samples were analysed using UPLC (Shimadzu, Kyoto, Japan) coupled to MS/MS (AB Sciex 4500 QTRAP, Sciex, Framingham, MA, USA). Chromatographic separation was achieved using a Phenomenex Kinetex Phenyl-Hexyl (150 × 2.1 mm, 2.6 µm) reversed-phase column (Phenomenex Inc. Torrance, CA, USA). A flow rate of 0.3 mL/min and an injection volume of 1 µL was used. Ultrapure water + 0.1 % formic acid (A) and methanol + 0.1 % formic acid (B) were used as the mobile phases. The chromatographic gradient was as follows: 25 % B at 0 min and held for 0.5 min, increase to 80 % B at 13 min, 87 % B at 15 min, 100 % B at 16 min and held for 1 min, then decreased to 25 % B at 17.5 min and kept steady for the final 9.5 min to equilibrate the system. The total run time was 27.1 min. The mass spectrometer was run in scheduled multiple reaction monitoring (sMRM) mode in positive ionisation mode, with a 60 s retention time window around the expected retention time of each analyte. For compound identification, retention times, two transitions and peak area ratios between the transitions were used (European Union Decision 2002/657/EC, 2002), while relative response factors of the analyte to the ISTD were used for quantification [9]. Optimised sMRM conditions and retention times for all analytes can be found in the supplementary information (S1.1 Acquisition parameters, Table S1).

2.4. Method validation

The method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), extraction recovery (ER), matrix effect (ME), accuracy, and repeatability, following the SANTE protocol, with minor adjustments, at two concentration levels (S1.2 Method validation, Table S2) [9]. Tap water was used as a matrix, and a deuterated ISTD mix, structurally similar to the compounds under investigation, was used to correct for matrix effects. A linear response ($R^2 > 0.99$) was observed between LOQ–1000 ng/mL (final extract concentration) for all biomarkers, with LOD: 0.5–3.6 ng/L and LOQ: 1.5–11.9 ng/L (Table S2).

2.5. Stability experiments

In each experiment, the samples were spiked in separate pools to ensure that any transformation of the parent to metabolite (and *vice versa)* did not affect the results (Table 1). Spiking of each pool, with either parent or metabolite mixture, was performed at a final concentration of 1000 ng/L and the internal standard (ISTD) mixture (final concentration 240 ng/L) after filtration (when applicable). This spiked concentration was well above native concentrations, ranging from

Table 1
Included antipsychotic biomarkers, internal standards, and the wastewater pool.

Compound	ISTD	Spiked pool
Aripiprazole	Aripiprazole-D_8	Parent
Aripiprazole, Dehydro	Aripiprazole-D_8	Metabolite
Clozapine	Olanzapine-D_8	Parent
Clozapine, N-desmethyl	Olanzapine-D_8	Metabolite
Haloperidol	Haloperidol-D_4	Parent
Olanzapine	Olanzapine-D_8	Parent
Olanzapine, N-desmethyl	Olanzapine-D_8	Metabolite
Paliperidone	Risperidone-D_4	Metabolite (parent drug and metabolite of risperidone)
Quetiapine	Olanzapine-D_8	Parent
Quetiapine, N-desalkyl	Olanzapine-D_8	Metabolite
Risperidone	Risperidone-D_4	Parent

below LOQ to 80 ng/L of influent wastewater, according to preliminary analysis. The substantially higher concentration ensures that the spiked biomarkers would be detectable above any background noise and reduce analytical uncertainty in the wastewater matrix. Individual samples were prepared according to the Section 2.2 Sample collection and treatment. Concentrations of biomarkers of interest were obtained using an external calibration curve. When needed, samples were collected and extracted at different sampling times, and the SPE cartridges containing the adsorbed analytes were dried and frozen. Subsequently, all cartridges were eluted and analysed simultaneously.

The final stability classification at each sampling time followed the criteria outlined by McCall et al.: categorised as *high* if the transformation was < 20 %, *medium* if between 20 % and 60 %, *low* if > 60 %, and results deviating from previously published literature are categorised as *variable* [7]. The 95 % confidence interval was calculated based on a student t-distribution with n-1 degrees of freedom (n = analysed number of replicates). In all cases, triplicates (n = 3) were taken except where otherwise indicated.

2.5.1. In-sewer transport

2.5.1.1. In-sewer stability. Aerobic in-sewer stability was evaluated at 4 °C and 20 °C representing a broad range of sewer wastewater temperatures [10]. In winter, the average wastewater temperature in Slovenian WWTPs (including Domžale-Kamnik) is around 8 °C. According to the literature, snowmelt inflow significantly impacts sewer system temperatures, lowering the average daily sewage temperature by 2–3 degrees. [11]. Considering Slovenia's Alpine geography, in our study 4 °C were selected as an extreme scenario (worst-case condition) rather than the typical 10 °C winter temperature. For each temperature, two 1200 mL raw wastewater samples were spiked with either metabolites or parent compounds. During the experiment, each wastewater sample was maintained at the desired temperature using a water bath and continuously stirred to simulate sewer flow. This setup does not fully mimic the real-life sewer conditions, as discussed later (3.1.1.1 In-sewer stability). At each designated sampling time: immediate (0 h), 2 h, 6 h, 10 h, 24 h, and 48 h; three 50 mL aliquots were collected and processed (including the addition of ISTD mixture). After the extraction, the cartridges were thoroughly dried and stored in the freezer (-20 °C). All samples were eluted immediately after collecting the final sample at t = 48 h. The mean concentrations were then normalised against the initial time point (t = 0 h) to evaluate stability based on the specified criteria. The 48-hour mark was chosen as the final sampling time based on the average residence time (<12 h, exceptionally <24 h) for most WWTPs [7].

2.5.1.2. Deconjugation. Urinary excretion of antipsychotic drugs and metabolites is typically heavily conjugated [12,13]. To test for remaining levels of glucuronidated biomarkers in wastewater, enzymatic deconjugation by β-glucuronidase from Helix pomatia (Type H-2) was used. No sulphated metabolites were reported in the literature for the included compounds [12,14]. The experimental setup was similar to that of Ryu et al. [15] and used nine 50 mL aliquots of composite influent wastewater. These were divided into three groups: (i) *initial*, extracted without undergoing any additional treatment; (ii) *treated*, each aliquot was spiked with 0.25 mL β-glucuronidase; and (iii) control, *untreated* but underwent the same sample procedure as the *treated* group. The *treated* and *untreated* groups were deconjugated by incubating them in the dark on a rotary shaker (80 rpm) for 2 h at 37 °C. The pH of the influent wastewater was pH 6.5 (unadjusted). The mean concentrations of the *treated* and *untreated* groups were then normalised against the *initial* concentrations.

2.5.2. In-sample stability

In-sample stability was assessed under different storage conditions using a combination of filtration (filtered or unfiltered) and modifiers (raw wastewater or acidified to pH 2). Again, each pool was spiked with either parent or metabolite mixture at a final concentration of 1000 ng/L and the internal standard (ISTD) mixture at a final concentration of 240 ng/L.

2.5.2.1. Frozen cartridge stability. Each raw wastewater sample was divided into two portions of 1200 mL, and each portion was spiked, as mentioned above. These spiked pools were subdivided into 50 mL aliquots and processed using the standard sample preparation procedure described in Section 2.2, including the SPE drying step. Subsequently, the cartridges were stored at 20 °C in an air-tight freezer bag for the different sampling times: immediate (0 h), 1 week, 6 weeks, and 3 months. Cartridges were prepared in triplicate for each sampling time and immediately analysed after elution. The mean concentrations were then normalised against time zero (0 h) to evaluate the stability based on the specified criteria.

2.5.2.2. Benchtop stability. Benchtop stability or short-term stability at room temperature indicates the analyte stability under sample preparation conditions. The benchtop stability experiment was exposed to indirect sunlight. Two different wastewater pools were created and spiked for each experimental combination (filtered, unfiltered, raw, and acidified) with 1000 ng/L final concentration of either parent or metabolite. All sampling was performed in triplicate. For benchtop stability, 20 °C was chosen and sampled at t = 0 h, 1 h, 3 h, and 6 h. At each designated sampling time, three 50 mL aliquots were collected and processed, including the addition of the ISTD. After the extraction, the cartridges were dried and stored in the freezer (-20 °C). All samples were eluted immediately after collecting the final sample and analysed at the end of the experiment. Measured concentrations were normalised against the initial time point (0 h).

2.5.2.3. WWTP autosampler stability. During composite sampling at the WWTP, multiple samples are collected at the WWTP composite sampling device over the required time (e.g. 24 hours). Stability is an important factor, given that compounds can already degrade during the sample collection, especially during the extended exposure time. Therefore, during collection, the sample is commonly stored at the WWTP composite sampling devices exposed at 4 °C to reduce degradation processes. The WWTP autosampler stability, referring to the stability at the WWTP composite sampling devices, was assessed at 4 °C in influent raw wastewater (not stirred). Again, two raw wastewater pools were spiked with either metabolites or parent compound and ISTD mixture and sampled at t = 0 h, 6 h, 24 h, and 48 h. The experiment ran up to 48 hours as an extreme case. At each designated sampling time, three 50 mL aliquots were collected and processed, including the addition of the ISTD. After the extraction, the cartridges were dried and stored in the freezer (-20 °C). All samples were eluted immediately after collecting the final sample and analysed at the end of the experiment. Measured concentrations were normalised against the initial time point (0 h).

2.5.2.4. Long-term sample storage stability. Long-term sample storage stability at −20 °C was tested under different conditions (filtered, unfiltered, raw, and acidified). Two different wastewater pools were created and spiked for each experimental combination with 1000 ng/L final concentration of either parent or metabolite and the ISTD mixture. Each spiked pool was sampled at t = 0 h, 1 week, 6 weeks, and 3 months. At each designated sampling time, three 50 mL aliquots were collected and processed, including the addition of the ISTD. After the extraction, the cartridges were dried and stored in the freezer (-20 °C). All samples were eluted immediately after collecting the final sample and analysed at the end of the experiment. Measured concentrations were normalised against the initial time point (0 h).

2.5.2.5. Freeze-thaw stability. To test the effect of multiple freeze-thaw

cycles on stability, each sample was frozen for at least 12 hours before being thawed to room temperature. The sample then underwent sample treatment or was subjected to another freeze-thaw cycle. The influence of different storage conditions was evaluated in this experiment using a combination of filtration (filtered or unfiltered) and acidification (raw wastewater or acidified to pH 2). Two different wastewater pools were created, and after filtration and acidification, depending on the experiment, they were spiked with either a parent or metabolite mixture. Each freeze-thaw cycle experimental setup was prepared in triplicate.

Freeze-thaw experiments were conducted on consecutive days, with time points initial (fresh sample, no freeze-thaw cycle), 1, 2, and 3 freeze-thaw cycles. Again, intermediate time points were processed, including the addition of the ISTD. After the extraction, the cartridges were dried and stored in the freezer (-20 °C). All samples were eluted immediately after the last freeze-thaw cycle and analysed at the end of the experiment. Measured concentrations at each freeze-thaw cycle are normalised against the initial time point (unfrozen).

3. Results and discussion

3.1. In-sewer transport

3.1.1. In-sewer stability

In-sewer stability was assessed at 4 °C and 20 °C over 48 h. The results (Fig. 1, Table S3 and Table S4) show that only quetiapine and risperidone were stable at both temperatures for 48 h, with most other compounds being stable for at least 10 h. For most WWTPs, this is not an issue since the mean residence time is < 12 h (exceptionally <24 h), but this finding emphasises the importance of considering individual WWTP transit times and temperature variations for accurate uncertainty assessments [7].

We hypothesise that the observed increase in clozapine (at 20 °C) could be caused by an unknown compound that is transformed to clozapine or deconjugation occurs (see 3.1.1.2). Similarly, the increase in paliperidone might be due to the transformation of an unknown compound in the influent wastewater [7,16]. However, in the case of clozapine this increase was not observed at 4 °C. Considering that influent wastewater is such a complex matrix, we could only hypothesise that this transformation could be affected by higher temperatures, as slower formation at lower temperatures could be possible. These differences enhance our results revealing compound-specific stability profiles. Olanzapine and its metabolite N-desmethylolanzapine show rapid degradation over time in influent wastewater. In other biological matrices, results on the stability of the parent compound olanzapine were mixed [17–19]. The transit time, which is dependent on the specific WWTP, must be taken into account.

A correction factor could be used if the degradation is shown to be constant over different WWTPs with varying characteristics. However,

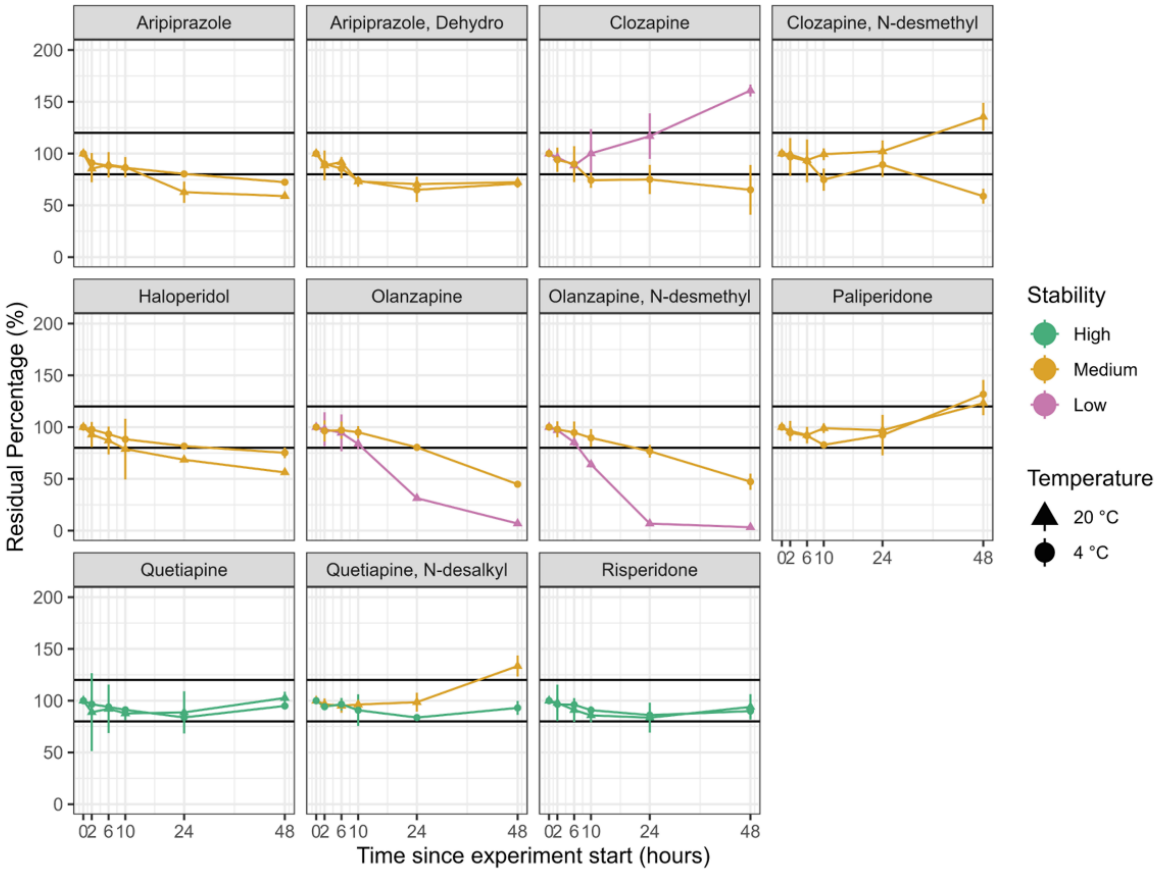


Fig. 1. Plots showing the stability of antipsychotics up to 48 hours, measured under biofilm-free aerobic in-sewer conditions at temperatures of 4 °C and 20 °C. The concentration of each time point is normalised against the initial time point.

the in-sewer stability experiment, though rudimentary, served only as an initial exploration of antipsychotic stability within gravity sewer systems. A more comprehensive evaluation should be performed using bioreactors, which better mimic the sewer system by considering biofilm, suspended solids, and other parameters [20]. However, our study provides an initial and conservative estimate of stability, which is important since previous studies on pharmaceuticals and illicit drugs were consistently overestimated using lab-scale sewer reactors [21].

3.1.2. Deconjugation

Previous research has shown that the absence of conjugated compounds is attributed to the presence of β-glucuronidase enzymes produced by faecal bacteria that end up in the sewer [22,23]. Since urinary excretion of antipsychotic drugs and metabolites is typically heavily conjugated, this experiment was performed to confirm the complete deglucuronidation of biomarkers before analysis [12,13]. An obvious limitation of this experiment is that, since no spiking is performed of conjugated standards, it is only relevant for compounds consumed by the serviced WWTP catchment area on the sampling day of the experiment. The *untreated* group is used as a reference for residual deconjugation in the native influent wastewater sample, as bacteria and enzymes may still be present, and to control for the effect of temperature degradation. If the *treated* and *untreated* groups are lower than the *initial* group, this indicates loss of the compound over time. Compared to the *initial* group, an increase in the residual percentage of the *treated* group, but not the *untreated*, would indicate deconjugation. However, if both increase, it indicates the transformation of other compounds or deconjugation in both samples (i.e., residual enzyme in the *untreated* sample). An overview of the experimental outcome can be found in the supplementary information (Table S5). The Kruskal-Wallis test (significance level $\alpha = 0.05$) was used to determine if all groups (*initial*, *treated*, and *untreated*) originated from the same distribution. There was no significant significance for olanzapine (p value 0.094), borderline significance for quetiapine (p value 0.050), and significant for clozapine (p value 0.027) and N-desalkylquetiapine (p value 0.027).

Clozapine increased by 38 % in the *treated* (95 % confidence interval, 95CI: 6 %-71 %) and 98 % *untreated* (95CI: 60–135 %) groups, suggesting that transformation (incl. deconjugation) processes are involved. Hydroxylation can also occur in-sewer; however, the transformation of N-desmethylclozapine as a significant metabolic pathway is minimal, given the structure and the fact that it was <LOQ across all experimental groups [24]. The decrease in N-desalkylquetiapine in both the *treated* (-41 %, 95CI -49 % to -33 %) and *untreated* (-24 %, 95CI -26 % to -21 %) groups illustrates the impact of temperature on degradation, i.e., during the in-sewer experiment at 20 °C, N-desalkylquetiapine was not degraded to the same extent over 48 hours (Fig. 1, section 3.1.1.1 In-sewer stability). For quetiapine, more research would be required on why it could not be quantified in the *treated* group, despite the only difference being the addition of the β-glucuronidase enzyme. The concentration in the *initial* and *untreated* group was >LOQ. Matrix effects are a possible explanation since the composition of wastewater differs daily, and during validation, matrix effects were only assessed in tap water.

3.2. In-sample stability

In-sample stability, including frozen cartridge (Table S6), benchtop (Fig. S1), WWTP autosampler (Fig. S2), long-term sample storage (Fig. S3), and freeze-thaw stability (Fig. S4) was assessed since in-sample conditions during storage and analysis can lead to biomarker transformation and subsequently, the under- or overestimation of antipsychotic mass loads when applying WBE.

3.2.1. Frozen cartridge stability

Except for the metabolite N-desalkylquetiapine, which was stable for at least six weeks, all the remaining antipsychotics and metabolites were stable when stored frozen on the SPE cartridge for at least three months (Table S6). The results show how, with the exception of N-desalkylquetiapine (6 weeks), the analytes of interest can be stored on the cartridges for up to three months. Such a storage method could be more practical than freezing substantial volumes of wastewater samples or transporting samples to (other) laboratories for analysis. Additionally, processing many samples can be more efficiently achieved by eluting more cartridges overall and reducing potential instrumental variability.

3.2.2. Benchtop stability

Most targeted compounds showed high (>85 %) benchtop stability under all conditions at 20 °C up to 6 h when exposed to standard laboratory conditions. Exceptions were dehydroaripiprazole, risperidone, and N-desalkylquetiapine, which showed medium (63–78 %) stability when unfiltered and acidified. Metabolite N-desalkylquetiapine was only medium stable when filtered and acidified (Table S7). In the study of Andreassen *et al.*, the stability of olanzapine in serum remained undegraded after 24 h under intense light (3000 lux) but was degraded by 50 % after 48 h. Therefore, it is still recommended that samples should be stored in the dark [25].

3.2.3. WWTP autosampler stability

Regarding WWTP autosampler stability at 4 °C in influent raw wastewater without stirring, almost half of the included antipsychotics, including haloperidol, olanzapine, N-desmethylolanzapine, quetiapine, N-desalkylquetiapine, and risperidone, showed high (84–97 %) stability up to 48 hours (Table S8). Ceolotto et al. showed similar results (high stability of 85–100 %) for quetiapine, the only antipsychotic included in their study [26]. Aripiprazole, dehydroaripiprazole, clozapine, and N-desmethylclozapine showed medium (69–76 %) stability after 6 or 24 h (Table S8). What is noticeable, is the increase (125 %) of paliperidone after 24 h, similar to the in-sewer results (3.1.1.1) after 24 h in both 4 °C (132 %) and 20 °C (123 %).

3.2.4. Long-term sample storage stability

Additionally, long-term sample storage at − 20 °C in all conditions (filtered, unfiltered, raw, and acidified) was assessed. The results showed high (82–103 %) stability for most of the compounds up to 3 months in all conditions, except for N-desalkylquetiapine, paliperidone, and N-desmethylclozapine which showed medium (86–69 %) stability after six weeks when unfiltered and acidified. Only risperidone showed medium (76–54 %) stability already in the first week of storage when unfiltered and acidified (Table S9).

3.2.5. Freeze-thaw stability

Samples were frozen for at least 12 h (approx. 18 h each time) before being thawed to room temperature on consecutive days. Only clozapine and haloperidol (Fig. S4 and Table S10) showed high stability in all conditions during three freeze-thaw cycles. In contrast, olanzapine and its metabolite N-desmethylolanzapine showed medium stability after the second freeze-thaw cycle under all conditions. The remaining compounds showed high stability when filtered and raw (not acidified) during all freeze-thaw cycles, while N-desmethylclozapine showed high stability when filtered and acidified. Finally, the compounds risperidone, paliperidone, and N-desalkylquetiapine showed medium stability only when unfiltered and acidified. In conclusion, one freeze-thaw cycle is acceptable for all compounds under all storage conditions, but multiple may influence consumption estimates.

3.3. Stability overview

This study presents comprehensive data on the stability of antipsychotics in influent wastewater under various conditions to date. Fig. 2 provides a comparative overview of the different storage/sampling conditions and associated stability, including the time the compound is highly stable under the given conditions. The results showed that in-sewer degradation is compound-specific and is influenced by the

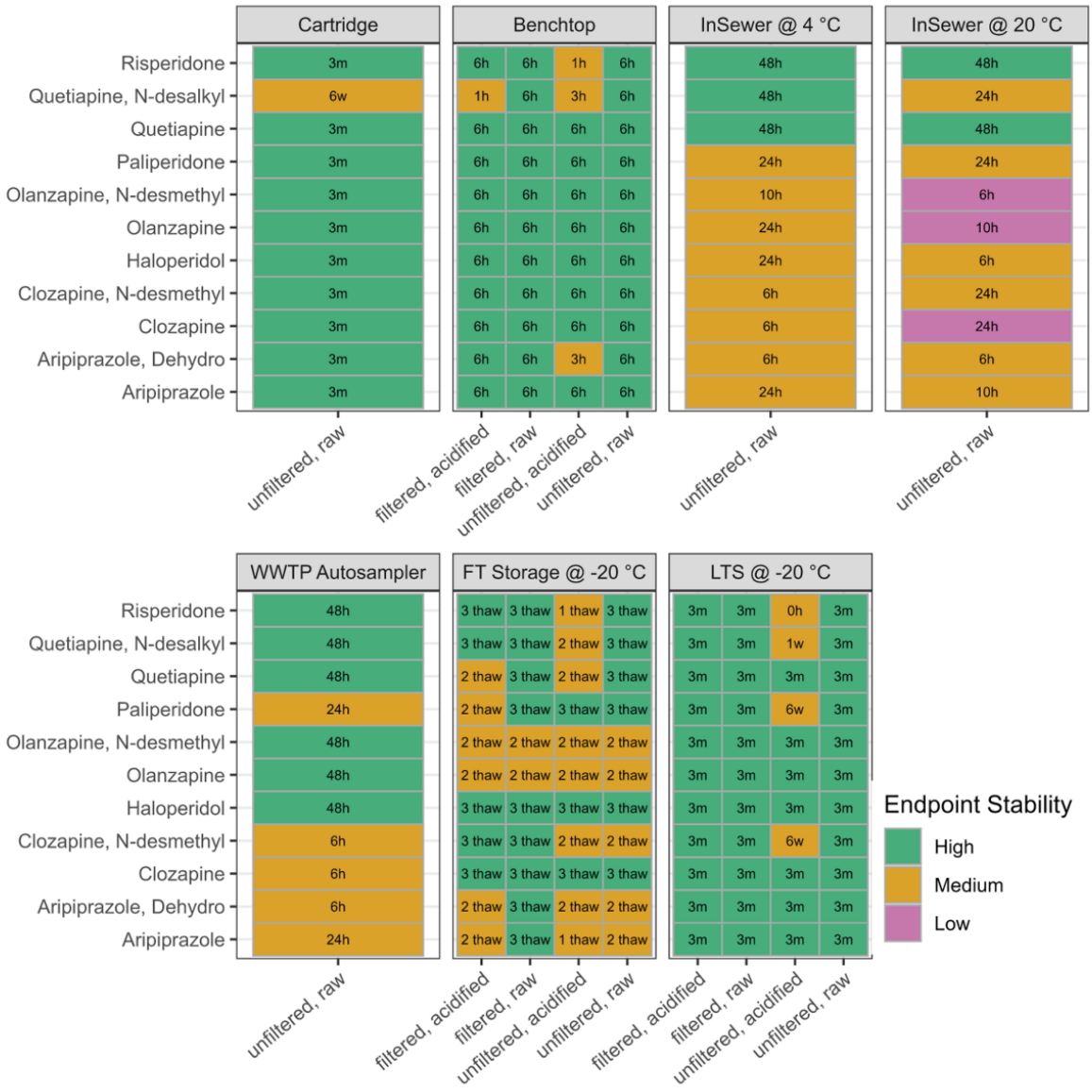


Fig. 2. Overview of stability of included antipsychotic biomarkers showing the final stability over the entire experiment duration (coloured) and the minimum time the compound was highly stable during the experiment (text label)—abbreviations: FT, freeze-thaw; LTS, long-term storage; WWTP, wastewater treatment plant.

environmental conditions present in the sewers, such as temperature and residence time. For most WWTPs, the residence time ranges between 30 min and 12 h, and therefore, it might not be relevant for many compounds [7]. In addition, in-sewer results show that some compounds, such as clozapine and aripiprazole, are unstable, a fact that should be taken into account. Acidification before storage is, in most use cases, not recommended as some analytes already degrade at low pH within the first week of storage. Also, when acidified, some biomarkers showed only medium stability after the second freeze-thaw cycle. However, SPE cartridges could be used for sample storage since all compounds except quetiapine were stable for at least three months. Overall, among the tested compounds, quetiapine, N-desalkylquetiapine, and risperidone seem to be the most stable under all tested conditions.

Given these results, spatiotemporal comparisons should be cautiously approached and tailored to specific biomarkers to ensure correct conclusions are made. Future WBE research to refine antipsychotic consumption estimates should focus on in-sewer bioreactor degradation studies and the potential of applying a correction factor.

3.4. Limitations

The inclusion of lab-scale sewer reactors experiments would further provide insights into the current in-sewer degradation findings.

However, this requires specialised equipment optimized for stability studies which is only available in a limited number of laboratories. However, previous research indicates that lab-scale sewer reactors may overestimate degradation [21]. Therefore, the experiments in this study provide an initial and more conservative estimate.

The experimental setup of WWTP autosampler stability could be further optimized by mimicking the addition of a specific portion of the wastewater to the collection pool. However, the exact volume and the frequency of the added wastewater sample also depend on the autosampler collection method (e.g., time-, flow-proportional). According to recent studies evaluating the stability of new psychoactive substances (NPS), stimulants, opioids, personal care products, and other pharmaceuticals, a similar setup of refrigerated influent wastewater pool at 4 °C was used to mimic sampling conditions related to storage within an autosampler at a WWTP [26,27]. In our study, refrigerated storage of influent wastewater at 4 °C for 48 hours, as an extreme case, has been explored mainly as an indication of WWTP autosampler stability considering worst-case scenarios.

CRediT authorship contribution statement

Laimou-Geraniou Maria: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Quireyns Maarten:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Boogaerts Tim:** Writing – review & editing, Supervision. **Heath David John:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Covaci Adrian:** Writing – review & editing, Supervision, Conceptualization. **L.N. van Nuijs Alexander:** Writing – review & editing, Supervision, Conceptualization. **Heath Ester:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the Slovenian Research and Innovation Agency (ARIS Program group P1–0143 and Projects N1–0143, J2–4427, and L7–4422) and the Research Foundation - Flanders (FWO), under grant number G060920N (Maarten Quireyns) and 1225924 N (Tim Boogaerts).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.116702.

References

[1] C.U. Correll, M. Solmi, G. Croatto, L.K. Schneider, S.C. Rohani-Montez, L. Fairley, N. Smith, I. Bitter, P. Gorwood, H. Taipale, J. Tiihonen, Mortality in people with schizophrenia: a systematic review and meta-analysis of relative risk and aggravating or attenuating factors, World Psychiatry 21 (2022) 248–271, https://doi.org/10.1002/wps.20994.

[2] J. Lally, J.H. MacCabe, Antipsychotic medication in schizophrenia: a review, Br. Med. Bull. 114 (2015) 169–179, https://doi.org/10.1093/bmb/ldv017.

[3] K.E. Evoy, C. Teng, V.G. Encarnacion, B. Frescas, J. Hakim, S. Saklad, C.R. Frei, Comparison of quetiapine abuse and misuse reports to the FDA adverse event reporting system with other second-generation antipsychotics, Subst. Use 13 (2019) 1178221819844205, https://doi.org/10.1177/1178221819844205.

[4] M.E. Montebello, J. Brett, Misuse and associated harms of quetiapine and other atypical antipsychotics, in: S. Nielsen, R. Bruno, S. Schenk (Eds.), Non-Medical and Illicit Use of Psychoactive Drugs, Springer International Publishing, Cham, 2017, pp. 125–139, https://doi.org/10.1007/7854_2015_424.

[5] S. Roy, R. Charreteur, M. Peries, F. Kheloufi, C. Eiden, N. Nagot, H. Donnadieu-Rigole, J. Micallef, H. Peyrière, Abuse and misuse of second-generation antipsychotics: an analysis using VigiBase, the World Health Organisation pharmacovigilance database, Br. J. Clin. Pharm. 88 (2022) 4646–4653, https://doi.org/10.1111/bcp.15420.

[6] H.E. Jones, M. Hickman, B. Kasprzyk-Hordern, N.J. Welton, D.R. Baker, A.E. Ades, Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part B: placing back-calculations in a formal statistical framework, Sci. Total Environ. 487 (2014) 642–650, https://doi.org/10/ghhtmt.

[7] A.-K. McCall, R. Bade, J. Kinyua, F.Y. Lai, P.K. Thai, A. Covaci, L. Bijlsma, A.L. N. van Nuijs, C. Ort, Critical review on the stability of illicit drugs in sewers and wastewater samples, Water Res 88 (2016) 933–947, https://doi.org/10.1016/j.watres.2015.10.040.

[8] F. Logarinho, T. Rosado, C. Lourenço, M. Barroso, A.R.T.S. Araujo, E. Gallardo, Determination of antipsychotic drugs in hospital and wastewater treatment plant samples by gas chromatography/tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1038 (2016) 127–135, https://doi.org/10.1016/j.jchromb.2016.10.031.

[9] T. Pihlström, A.R. Fernández-Alba, C.F. Amate, M.E. Poulsen, B. Hardebusch, M. Anastassiades, R. Lippold, L.C. Cabrera, A. de Kok, F. ORegan, et al., Analytical quality control and method validation procedures for pesticide residues analysis in food and feed, Sante 11312 (2021) v2.

[10] I. Senta, I. Krizman, M. Ahel, S. Terzic, Assessment of stability of drug biomarkers in municipal wastewater as a factor influencing the estimation of drug consumption using sewage epidemiology, Sci. Total Environ. 487 (2014) 659–665, https://doi.org/10.1016/j.scitotenv.2013.12.054.

[11] Impact of Snowmelt Inflow on Temperature of Sewage Discharged to Treatment Plants, Pol. J. Environ. Stud. 21 (n.d.) 381–386.

[12] R.C. Baselt. Disposition of Toxic Drugs and Chemicals in Man, ninth ed., Biomedical Publications, Seal Beach (Cal.), 2011.

[13] M.C. Mauri, S. Paletta, M. Maffini, A. Colasanti, F. Dragogna, C. Di Pace, A. C. Altamura, Clinical pharmacology of atypical antipsychotics: an update, EXCLI J. 13 (2014) 1163–1191.

[14] C. Knox, M. Wilson, C.M. Klinger, M. Franklin, E. Oler, A. Wilson, A. Pon, J. Cox, N. E.L. Chin, S.A. Strawbridge, M. Garcia-Patino, R. Kruger, A. Sivakumaran, S. Sanford, R. Doshi, N. Khetarpal, O. Fatokun, D. Doucet, A. Zubkowski, D. Y. Rayat, H. Jackson, K. Harford, A. Anjum, M. Zakir, F. Wang, S. Tian, B. Lee, J. Liigand, H. Peters, R.Q.R. Wang, T. Nguyen, D. So, M. Sharp, R. da Silva, C. Gabriel, J. Scantlebury, M. Jasinski, D. Ackerman, T. Jewison, T. Sajed, V. Gautam, D.S. Wishart, DrugBank 6.0: the DrugBank Knowledgebase for 2024, Nucleic Acids Res 52 (2024) D1265–D1275, https://doi.org/10.1093/nar/gkad976.

[15] Y. Ryu, M.J. Reid, K.V. Thomas, Liquid chromatography–high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater, J. Chromatogr. A 1409 (2015) 146–151, https://doi.org/10.1016/j.chroma.2015.07.060.

[16] N.V. Heuett, S.R. Batchu, P.R. Gardinali, Understanding the magnitude of emergent contaminant releases through target screening and metabolite identification using high resolution mass spectrometry: illicit drugs in raw sewage influents, J. Hazard Mater. 282 (2015) 41–50, https://doi.org/10.1016/j.jhazmat.2014.08.009.

[17] D.S. Fisher, S.J. Partridge, S.A. Handley, R.J. Flanagan, Stability of some atypical antipsychotics in human plasma, haemolysed whole blood, oral fluid, human serum and calf serum, Forensic Sci. Int 229 (2013) 151–156, https://doi.org/10.1016/j.forsciint.2013.03.021.

[18] L. Patteet, K.E. Maudens, B. Sabbe, M. Morrens, M. De Doncker, H. Neels, High throughput identification and quantification of 16 antipsychotics and 8 major metabolites in serum using ultra-high performance liquid chromatography–tandem mass spectrometry, Clin. Chim. Acta 429 (2014) 51–58, https://doi.org/10.1016/j.cca.2013.11.024.

[19] E. Saar, D. Gerostamoulos, O.H. Drummer, J. Beyer, Assessment of the stability of 30 antipsychotic drugs in stored blood specimens, Forensic Sci. Int 215 (2012) 152–158, https://doi.org/10.1016/j.forsciint.2011.02.022.

[20] J. Gao, J. Li, G. Jiang, A.H. Shypanski, L.M. Nieradzik, Z. Yuan, J.F. Mueller, C. Ort, P.K. Thai, Systematic evaluation of biomarker stability in pilot scale sewer pipes, Water Res 151 (2019) 447–455, https://doi.org/10.1016/j.watres.2018.12.032.

[21] P.M. Choi, J. Li, J. Gao, J.W. O'Brien, K.V. Thomas, P.K. Thai, G. Jiang, J. F. Mueller, Considerations for assessing stability of wastewater-based epidemiology biomarkers using biofilm-free and sewer reactor tests, Sci. Total Environ. 709 (2020) 136228, https://doi.org/10.1016/j.scitotenv.2019.136228.

[22] I. González-Mariño, R. Rodil, I. Barrio, R. Cela, J.B. Quintana, Wastewater-based epidemiology as a new tool for estimating population exposure to phthalate plasticizers, Environ. Sci. Technol. 51 (2017) 3902–3910, https://doi.org/10.1021/acs.est.6b05612.

[23] I. Senta, S. Rodríguez-Mozaz, L. Corominas, M. Petrovic, Wastewater-based epidemiology to assess human exposure to personal care and household products – a review of biomarkers, analytical methods, and applications, Trends Environ. Anal. Chem. 28 (2020) e00103, https://doi.org/10.1016/j.teac.2020.e00103.

[24] J. Kinyua, N. Negreira, A.-K. McCall, T. Boogaerts, C. Ort, A. Covaci, A.L.N. van Nuijs, Investigating in-sewer transformation products formed from synthetic cathinones and phenethylamines using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, Sci. Total Environ. 634 (2018) 331–340, https://doi.org/10.1016/j.scitotenv.2018.03.253.

[25] T.N. Andreassen, B.M.H. Falch, O. Spigset, A UPLC–MSMS method for the analysis of olanzapine in serum—with particular emphasis on drug stability testing,

J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1006 (2015) 112–120, https://doi.org/10.1016/j.jchromb.2015.10.029.

[26] N. Ceolotto, P. Dollamore, A. Hold, B. Balne, K.K. Jagadeesan, R. Standerwick, M. Robertson, R. Barden, B. Kasprzyk-Hordern, A new Wastewater-Based Epidemiology workflow to estimate community wide non-communicable disease prevalence using pharmaceutical proxy data, J. Hazard Mater. 461 (2024) 132645, https://doi.org/10.1016/j.jhazmat.2023.132645.

[27] N. Salgueiro-Gonzalez, F. Béen, L. Bijlsma, T. Boogaerts, A. Covaci, J.A. Baz-Lomba, B. Kasprzyk-Hordern, J. Matias, C. Ort, I. Bodík, E. Heath, K. Styszko, E. Emke, F. Hernández, A.L.N. van Nuijs, S. Castiglioni, Influent wastewater analysis to investigate emerging trends of new psychoactive substances use in Europe, Water Res. 254 (2024) 121390, https://doi.org/10.1016/j.watres.2024.121390.

3.3.2 Wastewater analysis as a global toxicovigilance tool for new psychoactive substances monitoring

Published: D. Nadarajan, M. Laimou-Geraniou, A. Chappell, C. Baduel, L. Bijlsma, T. Boogaerts, D. A. Burgard, S. Castiglioni, N. Ceolotto, E. M. Driver, F. Fabriz Sodre, D. Fatta Kassinos, H. Flores Quintana, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R. U. Halden, E. Heath, J. Huchthausen, B. Kasprzyk-Hordern, E. L. Keller, F. Yin Lai, A. Sue-Ching Löve, J. Matias, V. Ocenaskova, J. E. Oh, T. Oluseyi, K. Phung, M. Pineda-Castro, M. Psichoudaki, N. Salgueiro-Gonzalez, J. M. Santos, C. Silvino-Gomes, B. Subedi, N. Thomaidis, D. Wang, N. Van Wichelen, V. Yargeau, S. Cresswell, J. Mueller, R. Bade, 2026, *Water Research*, vol. 289 (A), pp. 124847.

In this international work, I contributed to experimental work, instrumental analysis, and reviewing and editing the manuscript.

NPS represent an ongoing global health and policy challenge due to their rapid emergence, often unregulated status, and potential health risks. NPS regulations vary globally, with some countries adopting broad definitions and implementing blanket bans or total prohibition of all NPS. In contrast, others employ more targeted approaches, scheduling NPS based on their structural scaffolds (analogue control) or through individual listings (1.1.4 New psychoactive substances). This study provides recent data on the international snapshot of NPS use via WBE, analysing influent wastewater from 52 sites across 20 countries during the 2022/2023 New Year period. Using SPE followed by targeted LC-MS/MS, 21 of 54 monitored NPS were detected, with mitragynine, 3-methylmethcathinone (3-MMC), and eutylone being the most prevalent. Findings confirm that synthetic cathinones remain the dominant class, particularly in Europe, while designer benzodiazepines and ketamine analogues were geographically specific.

A significant feature of this study, crucial for accurate WBE estimations, was the investigation of the stability of 61 selected NPS, including seven ISTDs, on loaded SPE cartridges, under five temperatures (−80 °C, −20 °C, 4 °C, 22 °C, and 37 °C) across five time points (3 days to 4 months), simulating differing storage conditions, as well as the winter and summer conditions over the New Year period around the world.

Results showed that storage at −20 °C maintained acceptable recovery (80–120%) for most analytes for up to four months, making it an ideal condition for international shipment and potential delayed analysis. Only two compounds, including phenibut and acetoxymethylketobemidone, exhibited lower stability (<80%), while others (e.g., six nitazenes) showed elevated recoveries (>120%). These findings provide critical insight for future global WBE campaigns, where consistent, long-term storage and transportation conditions are necessary. Overall, this study highlights the value of WBE not only for global monitoring of emerging NPS threats but also for policy evaluation and assessment.

In addition to the spatiotemporal investigation of NPS occurrence, this study retrospectively examined the longitudinal trends of 3-MMC as a case study to understand the general decline in its mass loads. The decrease observed in European cities could be related to the strengthening of existing local regulations following the COVID-19 pandemic, along with ongoing scheduling of 3-MMC at both the European Union and international levels.

The study's outcome was presented at two scientific conferences: The 61st Annual Conference of the International Association of Forensic Toxicologists (TIAFT 2025), Aotearoa New Zealand, November 23-28, 2025, and in Testing the Waters 8 (TTW8): International conference on wastewater-based epidemiology/surveillance, Tacoma, WA, United States, June 2-4, 2025.

Water Research 289 (2026) 124847

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Wastewater analysis as a global toxicovigilance tool for the monitoring of new psychoactive substances

Dhayaalini Nadarajan [a], Maria Laimou-Geraniou [b], Andrew Chappell [c], Christine Baduel [d], Lubertus Bijlsma [e], Tim Boogaerts [f], Daniel A. Burgard [g], Sara Castiglioni [h], Nicola Ceolotto [i], Erin M. Driver [j], Fernando Fabriz Sodre [k], Despo Fatta Kassinos [l], Harold Flores Quintana [m], Cobus Gerber [n], Emma Gracia-Lor [o], Elisa Gracia-Marín [e], Rolf U. Halden [j], Ester Heath [b], Julia Huchthausen [p], Barbara Kasprzyk-Hordern [i], Emma L. Keller [n], Foon Yin Lai [m], Arndís Sue-Ching Löve [q], João Matias [r], Vera Ocenaskova [s], Jeong-Eun Oh [t], Temilola Oluseyi [u], Kaitlyn Phung [c], Marco Pineda-Castro [v], Magda Psichoudaki [l], Noelia Salgueiro-Gonzalez [h], Cezar Silvino-Gomes [w], Fábio Marley Sousa Nascimento [k], Bikram Subedi [x], Nikolaos Thomaidis [y], Natan Van Wichelen [f], Degao Wang [z], Viviane Yargeau [v], Sarah Cresswell [aa], Jochen F. Mueller [a], Richard Bade [a,*]

[a] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, Woolloongabba, QLD, Australia
[b] Jožef Stefan Institute and International Postgraduate School, Slovenia
[c] Institute of Environmental Science and Research Limited (ESR), New Zealand
[d] Université Grenoble Alpes, France
[e] Research Institute for Pesticides and Water, University Jaume I, Spain
[f] Toxicological Centre, University of Antwerp, Universiteitsplein 1, Antwerp, 2610, Belgium
[g] University of Puget Sound, United States
[h] Istituto di Ricerche Farmacologiche Mario Negri IRCCS, via Mario Negri 2, Milan, 20156, Italy
[i] Centre of Excellence in Water Based Early Warning Systems for Health Protection, University of Bath, Claverton Road, Bath, BA2 7AY, United Kingdom
[j] Arizona State University, United States
[k] University of Brasília, Brazil
[l] University of Cyprus, P.O. Box 20537, Nicosia, 1678, Cyprus
[m] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences (SLU), Uppsala, SE 75007, Sweden
[n] Health and Biomedical Innovation, Clinical and Health Sciences, University of South Australia, Australia
[o] Complutense University of Madrid, Spain
[p] Helmholtz Centre for Environmental Research, Germany
[q] University of Iceland, Iceland
[r] European Union Drug Agency, Lisbon, Portugal
[s] TG. Masaryk Water Research Institute, Czech Republic
[t] Pusan National University, Republic of Korea
[u] University of Lagos, Nigeria
[v] McGill University, Canada
[w] Brazilian Federal Police, Brazil
[x] Department of Environmental Sciences, Louisiana State University, Baton Rouge, LA, USA
[y] National and Kapodistrian University of Athens, Greece
[z] Dalian Maritime University, China
[aa] School of Environment and Science, Griffith University, QLD, Australia

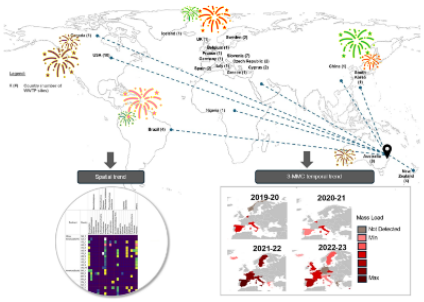
* Corresponding author.
E-mail address: r.bade@uq.edu.au (R. Bade).

https://doi.org/10.1016/j.watres.2025.124847
Received 29 June 2025; Received in revised form 30 September 2025; Accepted 21 October 2025
Available online 21 October 2025
0043-1354/© 2025 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

HIGHLIGHTS

- 21 new psychoactive substances (NPS) were found across 52 sites in 20 countries.
- The most frequently detected NPS was mitragynine, 3- MMC, and eutylone.
- 3,4-methylenedioxy PV8 was detected for the first time in sites in Australia and the US.
- A decline of 3-MMC was seen in European sites from 2021–2022 to 2022–2023.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Designer drugs
Wastewater-based epidemiology
3-methylmethcathinone
Legislation
Stability

ABSTRACT

New psychoactive substances are a group of synthetic or naturally occurring drugs that mimic the effects of controlled illicit drugs. With limited information around potency, effects and health risks, there is international concern around their use and thus surveillance efforts are needed for public health. This study presents a global assessment of new psychoactive substances in influent wastewater from 52 sites across 20 countries during the 2022/2023 New Year period. Using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry, 21 new psychoactive substances were detected with mitragynine, 3-methylmethcathinone, and eutylone being the most prevalent. Notably, 3,4-methylenedioxy-PV8 was identified for the first time in sites in Australia and the United States. A retrospective analysis of population normalised mass loads for 3-methylmethcathinone at European sites revealed downward trends in 2022/2023 sampling period compared to the previous years, suggesting a possible impact of regional scheduling measures. Additionally, this work demonstrates the stability of new psychoactive substances in loaded cartridges for up to four months when stored at -20 °C. These findings highlight the value of wastewater-based epidemiology for global monitoring of emerging new psychoactive substances threats and policy evaluation.

Introduction

New psychoactive substances (NPS) are a group of synthetic or naturally occurring chemicals that mimic the effects of controlled illicit drugs but are not listed in the United Nations International Drug Convention 1961–1971 (United Nations Office on Drugs and Crime, 2024). These substances are not necessarily 'new'. Some, such as benzimidazole synthetic opioids, were synthesized in the 1950s and never intended for human consumption (Ujváry et al., 2021). New chemicals have continued to circumvent the early warning systems replacing illicit drugs and scheduled NPS in the drug market (European Monitoring Centre for Drugs and Drug 2021). As of 2024, the United Nations Office on Drugs and Crime (UNODC) have identified 1300 NPS (United Nations Office on Drugs and Crime, 2023), while the European Union Drug Agency (EUDA) (European Union Drug Agency, 2024) were monitoring over 1000 NPS, with 47 new substances emerging in European market during 2024 (European Union Drugs Agency 2024). In October 2024, 83 NPS were placed under international control (United Nations Office on Drugs and Crime, 2023), underscoring the continuing global need for regulation informed by toxicological risk assessments related to human consumption.

The fatalities due to toxicity and increased recreational use of NPS has been well documented (Kronstrand et al., 2018; Andreasen et al., 2015; Nugteren-van Lonkhuyzen et al., 2022; Montanari et al., 2022). Given the dynamic nature of the drug market and the sporadic emergence of new compounds, a range of complementary tools are employed to monitor NPS occurrence. These include drug seizure records (Vincenti et al., 2021), clinical toxicology reports, forensic post-mortems, population surveys (12), dark-web surveillance (Catalani et al., 2021), and wastewater-based epidemiology (WBE) (Borova et al., 2015; Castiglioni et al., 2021). Some countries have also implemented early warning systems (EWS) to rapidly detect and respond to emerging NPS threats, enhancing coordination across forensic and public health sectors (Evans-Brown and Sedefov, 2018). Each method serves a distinct role and offers insight at different stages with its uncertainty, ranging from early detection and tracking of consumption patterns to assessing health outcomes and regulatory action.

WBE is recognised for its near real-time reporting of population-level sewage biomarkers through anonymised, aggregate samples collected from wastewater treatment plants (Daughton, 2018). Following ingestion, exogenous compounds are excreted in urine and faeces either unchanged as parent compounds or as metabolites. The concentration of these biomarkers in raw wastewater can be used to estimate population-level consumption by back calculating based on the catchment population size and flow rate data (Daughton, 2001; Daughton, 2011). However, with limited pharmacokinetic data available for NPS, accurate back calculation requires cautious interpretation. Nevertheless, WBE has provided valuable insight for public health, which was demonstrated during the Covid-19 outbreak, where population-level monitoring was achieved in real time without recruiting individuals and thus avoided human ethics and logistic procedures (Bivins et al., 2020).

In 2019, an international NPS wastewater monitoring was initiated to provide insight into NPS use during the New Year's period (Bade et al., 2021). A similar study was also conducted in Europe, comparing pooled weekdays and weekends for weekly use patterns (Castiglioni

et al., 2021). Other NPS studies have offered country-specific insights (Salgueiro-González et al., 2022; Jaunay et al., 2024; O'Rourke and Subedi, 2020; Gao et al., 2017; Senta et al., 2015; Bade et al., 2020) or focus on specific events (Bijlsma et al., 2020; Nadarajan et al., 2024), collectively demonstrating the feasibility of WBE as the complementary tool for near real time analysis.

The regulation of NPS varies globally. Some countries have broad definitions, implementing blanket bans or total prohibition of all NPS, while others use more targeted approaches, scheduling NPS based on their structural scaffolds (analogue control), or through individual listing (Evans-Brown et al., 2022). NPS may also be regulated under customs acts, medicinal legislation, and/or through consumer law, depending on national context. Among the NPS, 3-methylmethcathinone (3-MMC) has consistently been one of the most frequently detected NPS in all iterations of the international wastewater project since its inception in 2019/2020 (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). It has also been one of the most frequently reported NPS to the European Union Early Warning System, with notable rises in customs seizures recorded in 2020 (European Union Drug Monitoring, EU Drug Market, 2024).

Due to the potential for problematic patterns of use, efforts were made to include 3-MMC under international control (UNODC 2024). Although it was not scheduled following the initial UNODC critical review in 2016, growing evidence from customs seizures and toxicology cases prompted a second review in 2022 (World Health Organisation 2022). Eventually, in March 2023, 3-MMC was formally placed under international control, alongside six other NPS, following the recommendation by the World Health Organisation (WHO) expert committee (UNODC 2022). It is now classified as Schedule II Drug under the Convention of Psychotropic Substances of 1971 and entered into force in November 2023.

While the list of NPS from EUDA is similar to UNODC, regulatory actions within European Union may precede the international scheduling. For example, 3-MMC was added to the EU's list of controlled drugs on 18 March 2022, a year before the UNODC, through Commission Delegated Directive (EU) 2022/1326 (Commission, 2022). The member states were given a year until 18 February 2023 to adopt the directives into their national legislation. Before this directive, 15 Member States controlled 3-MMC under national drug control legislation, six under NPS legislation, and one under other legislation.

The New Year period is recognised as a time of the year of potentially increased recreational drug use (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). The overarching aim of this study is to investigate global NPS use during 2022/2023 New Year's period. The specific objectives were: (i) to determine the geographical prevalence of NPS use during this period; and (ii) to assess the potential impact of 3-MMC legislation by analysing longitudinal wastewater data in the context of national and international scheduling. In addition to the specific objectives, the study also included an assessment of stability of NPS on loaded solid phase extraction (SPE) cartridges to support reliable sample shipment and analysis. Materials and Methods

2.1. Selection of NPS

This work investigates 54 NPS (*Table S1–1, Electronic Supplementary Information 1)*. They were selected based on data published by UNODC Early Warning Advisory (United Nations Office on Drugs and Crime, 2023), EUDA Early Warning System (European Union Drug Agency, 2024), and in verbal consultation with local and international forensic agencies and are thus all of public health relevance.

2.2. Chemicals and reagents

Analytical reference standards used for quantification at The University of Queensland were kindly donated by Forensic Science Queensland, Australia. Deuterated internal standards (ISTD) benzoylecgonine-d3, ketamine-d4, 3,4-methylenedioxymethamphetamine (MDMA)-d5, mephedrone-d3, methylone-d3, and *O*-desmethyl-cis-tramadol-d6 were purchased from Cerilliant (Round Rock, TX, USA) and morphine-d3 monohydrate from Lipomed GmbH (Germany). Formic acid (Optima LC/MS grade, purity ≥99.0 %), isopropanol, dichloromethane, glacial acetic acid, and ammonia (28 %) were from Fisher Scientific (Tingalpa, QLD, Australia). LC/MS hypergrade methanol (MeOH, purity ≥99.9 %) and hydrochloric acid (HCl fuming 37 %) were obtained from Merck (Highway Bayswater, Australia). Working standards consisting of 54 analytes at 0.05 ng/mL, 5 ng/mL, and 50 ng/mL in MeOH were prepared along with mixed ISTD at 12.5 ng/mL in MeOH. All standards were stored in amber vials at −20 °C until use. Filtered ultrapure water (resistivity 18.2 MΩ cm at 25 °C) was from a Milli-Q system (Millipore, Bedford, USA).

2.3. Sample collection

Influent wastewater samples were collected from three to 11 days from the 25th of December 2022 to the 5th of January 2023. For the majority of sites, they were collected for between 7 and 11 consecutive days (Table SI-2). However, in one site in the United Kingdom, samples were collected on seven non-consecutive days between 11th to 25th December 2022 while for eight sites in the United States, samples were collected for three non-consecutive days between 27th December 2022 to 4th Jan 2023. In total, samples were collected from 52 wastewater treatment plants in 20 countries: Australia (two-letter ISO code: AU, n = 8 sites), Belgium (BE; 1), Brazil (BR; 4), Canada (CA; 1), China (CN; 1), Cyprus (CY; 2), Czechia (CZ; 2), France (FR; 1), Germany (DE; 1), Greece (GR; 1), Iceland (IS; 1), Italy (IT; 1), Republic of Korea (KR; 1), New Zealand (NZ; 4), Nigeria (NG; 1), Slovenia (SI; 7), Spain (ES; 2), Sweden (SE; 2), United Kingdom (UK; 1), and the United States (US; 10). These sites covered about 20 million inhabitants from both small and large cities as well as holiday destinations. The sampling date, flow rate, and population size are reported in *Table S1–2, Electronic Supplementary Information 1*. A standard protocol for the collection procedure was provided to all collaborators to minimise variability. Briefly, 24-hour composite samples were collected by flow or time proportion. The collected samples were acidified immediately to pH 2 using 2 M hydrochloric acid to suppress microbial growth, then stored at −20 °C until SPE sample extraction.

2.4. SPE extraction and validation

The sample treatment followed an SPE protocol (Bade et al., 2020) distributed to all participants using UCT XtracT DAU, 500 mg/ 6 mL cartridges (UCT Inc, Bristol, PA, USA). Steps 1–7 (*Text S1. Supplementary Methodology - 1. SPE Extraction, Electronic Supplementary Information 1*) were carried out in the country of collection. The ISTD were added after filtration and pH adjustment in Step 3 at the country of origin except for samples from Germany, Greece, and United States Site 2. After washing, the cartridges were dried, stored at −20 °C (Step 7), and shipped to The University of Queensland for elution and LC-MS/MS analysis (Steps 8 to 12).

The SPE method in this work was an adapted method with the addition of new compounds (*Table S1–3*) and has been validated previously (Laimou-Geraniou et al., 2025). The method validation includes selectivity, recovery, matrix effect, limits of detection (LOD), and limits of quantification (LOQ) in wastewater. The linearity and *R*-squared values were assessed using a 10 % MeOH solution. In addition to the validation, loaded cartridges were examined for NPS stability under various temperatures and storage conditions. The details for the validation procedure and cartridge stability are included in *Electronic Supplementary Information 1* in Text S1: Supplementary Methodology, Section 2 (SPE validation) and 3 (Stability of loaded cartridges), respectively.

2.5. HPLC-MS/MS

The instrument was an LC (Shimadzu Nexera LC-40, Kyoto, Japan) coupled to a tandem mass spectrometry instrument (SCIEX Triple Quad 7500 system, Framingham, MA, USA) (Nadarajan et al., 2024). *Table S1–4* details the operating conditions for liquid chromatography and mass spectrometry. All data were acquired in positive ionisation with scheduled multiple reaction monitoring (sMRM) mode as shown in *Table S1–1*.

2.6. Quantification and quality control

The identification and confirmation of NPS compounds followed N° SANTE/11,312/2021 (40) guidelines based on the two product ion transitions with an ion ratio of ± 30 % and a retention time of ± 0.1 min of a reference standard. The concentration of NPS compounds was determined using a nine-point external calibration curve (0.5 – 1000 ng/L) prepared in 10 % MeOH containing ISTD. Quantification of unknown was based on the peak area ratio between known analyte in the calibration curve and ISTD. Sites without ISTD were reported qualitatively as 'Detected'. Procedural blanks were analysed at the beginning of each sample site. To confirm the identity of NPS detected for the first time in wastewater, reference standards were spiked into SPE extracts and compared with their respective unspiked counterparts. Instrumental blanks and quality control standards were sequenced after every ten samples to monitor the batch performance. All reported concentrations were procedural blank-corrected, whenever needed.

The population normalised mass load (PNML) was estimated based on Eq. (1). The concentration (ng/L) obtained from the sample was multiplied by the daily influent wastewater flow rate (Megalitre, ML) for the population served in the catchment, normalised to 1000 people. In this work, the specific correction factor on parent/metabolite ratio was not included in the back-calculation due to limited information on NPS pharmacokinetic studies.

$$\text{Population normalised mass load (PNML, mg / day / 1000 people)} = \text{concentration}\left(\frac{\text{ng}}{\text{L}}\right) \times \text{flow rate (ML)} \times \frac{1000}{\text{population}} \quad (1)$$

2.7. Statistical method and data analysis

For the 3-MMC trend analysis, additional statistics were performed. The normality of the distribution for temporal data was tested using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. Due to the non-normal distribution of the data, Kruskal-Wallis's test was applied to check the significance of the temporal trend of the 3-MMC PNML. The mean rank contribution of results within the groups was statistically compared pairwise using post-hoc Dunn's test. For the number of sites $n = 2$, the Mann-Whitney U test was applied to check the null hypothesis. For all results, the null hypothesis was not rejected when the p-value > 0.05 at two-tailed. Sites with $n \leq 2$ were compared visually using descriptive statistics.

Peak integration was done using SCIEX OS. Statistical analysis and visualisation were performed using Microsoft Excel Version 2410 and GraphPad Prism Version 10.1.2 for Windows (Boston, MA, USA).

Results and discussion

3.1. SPE method validation

The SPE method was validated and reported previously for selectivity, limit of detection (LOD), limit of quantification (LOQ), recovery, and matrix effects (Laimou-Geraniou et al., 2025). The analytical figures of merit are provided in *Table S1–5 in Electronic Supplementary Information 1*. The LOQ ranged between 0.29 ng/L and 12 ng/L across all compounds. Most NPS exhibited SPE recoveries between 80 % and 120 %. Acetoxymethylketobemidone, clonazolam, flutoprazepam, phenibut, and SL-164 had recoveries below 50 % at 10 ng/L and 100 ng/L. Meanwhile, flubromazolam recovered at 121 % ± 3.2 % at 10 ng/L and 194 % ± 3.8 % at 100 ng/L. The matrix effect was substantial, with ion suppression predominating in the SPE matrix. Thirty compounds at 10 ng/L and 19 compounds at 100 ng/L exhibited a matrix effect of ± 51 %. Due to the unavailability of isotopically labelled ISTD for most target NPS, MDMA-d5 was employed as a surrogate ISTD for quantification.

3.2. Stability of analytes in loaded SPE cartridges

One critical aspect of this study was assessing the stability of NPS on SPE cartridges, particularly to ensure the analytes remain stable during international shipping and potential delays. As such, an experiment was conducted to test the stability of all analytes spiked at 500 ng/L in influent wastewater and loaded onto SPE cartridges. As the standard protocol involves shipping dried SPE cartridges to Australia, the stability study commenced after loading and drying steps. A total of 61 analytes, including the seven ISTD, were evaluated for stability at five temperatures (−80 °C, −20 °C, 4 °C, 22 °C, and 37 °C) across five time points (three days to four months), simulating differing storage conditions, as well as the winter and summer conditions over the New Year period around the world. Stability was considered acceptable with recoveries remaining between 80 % and 120 %. Out of 54 compounds, two NPS (phenibut, and acetoxymethylketobemidone) had recovery below 80 % and six (isotonitazene, etonitazepyne, 25iP-NBOMe, protonitazene, N-desethyl isotonitazene, butonitazene, and desoxymethoxetamine) had recovery above 120 % at −20 °C up to four months storage. Data were unavailable for the three-day and one-month time points at −20 °C and day three at 22 °C due to sample loss. Individual stability profiles for each analyte are presented *in Fig. S1–1 in Electronic Supplementary Information 1*.

These findings provide a foundation for future monitoring studies of current and emerging NPS and their stability on cartridge. For example, the nitazene compounds exhibited slightly more variation in their stability over time. Nevertheless, based on our study, storage at −20 °C is ideal, along with prompt shipment of cartridges within four months and immediate analysis of extracts after extraction to minimise the degradation of sensitive NPS.

3.3. 2022/2023 NPS snapshot

A summary of the 2022/2023 New Year campaign conducted across 52 sites from 20 countries using 343 samples is presented in Figs. 1 and 2. Based on Fig. 2, the most prevalent NPS classes in this cohort were synthetic cathinones (42 %), followed by benzodiazepines (23 %), phencyclidine-type substances (14 %) and phenmetrazines, nitazenes,

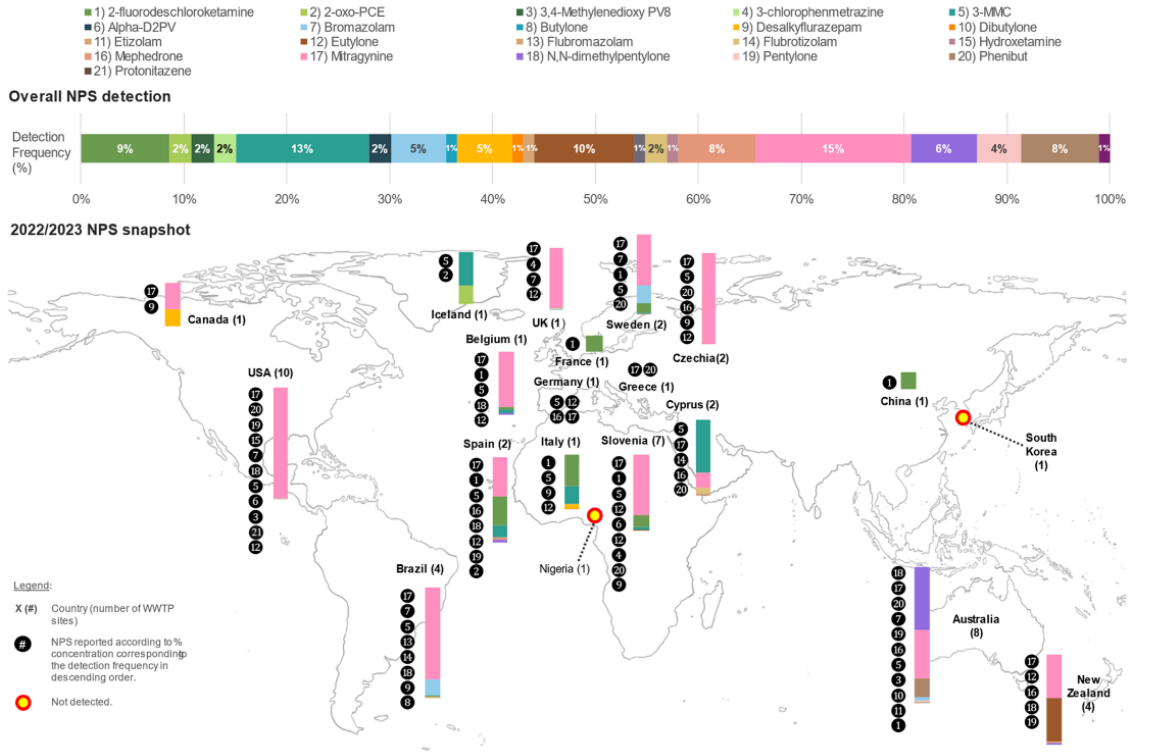


Fig. 1. Summary of detected NPS across sampling sites, including their respective detection frequencies. Note: PNML estimates are based on individual WWTP catchments and should not be extrapolated to entire cities or countries.

plant-based and others, each at 4.8 %. Of the 54 NPS in the method, 21 were detected: 2-fluorodeschloroketamine (2F-DCK), 2-oxo-PCE, 3,4-methylenedioxy PV8, 3-chlorophenmetrazine, 3-methylmethcathinone, alpha-D2PV, bromazolam, butylone, desalkylflurazepam, dibutylone, etizolam, eutylone, flubromazolam, flubrotizolam, hydroxetamine, mephedrone, mitragynine, *N,N*-dimethylpentylone, pentylone, phenibut, and protonitazene. The chemical and structural information is provided in *Table S1–6*. The most frequently detected NPS was mitragynine (38/55 sites), followed by 3-MMC (28/55) and eutylone (25/55), which is consistent with previous work (Bade et al., 2023). Compounds such as flubromazolam and butylone were only found in sites in Brazil, whereas protonitazene and hydroxetamine were only detected in sites in the United States. Although methcathinone and *O*-desmethyltramadol were detected at high concentrations in all sites in this work, they were excluded from the discussion, due to the uncertainty around their consumption. Methcathinone is potentially an in-sewer oxidation product of ephedrine or pseudoephedrine (Simpson et al., 2022), while *O*-desmethyltramadol is a metabolite of tramadol. However, it has also been sold and used as an NPS on its own (Castro et al., 2022). Therefore, this study could not distinguish between the licit and illicit use of tramadol and its metabolite, *O*-desmethyltramadol. No NPS were detected in samples from Nigeria and the Republic of Korea. However, previous studies in Korea have reported the presence of *N*-ethyl pentylone and methcathinone, phenmetrazine, 25E-NBOMe, 25D-NBOMe, N-methyl-2-AI, *N*-ethylpentylone, etizolam, and eutylone (Bade et al., 2022; Bade et al., 2023; Kim et al., 2023; Lee and Oh, 2023). Although both local and international media have reported the use of plant-based and non-NPS substances in Nigeria, a 2021 review emphasised that empirical research on NPS in the country remain scarce (Dumbili et al., 2021). The current method includes limited number of plant-based target analytes, which may restrict the detection of such compounds. The PNML and the detection frequency for each NPS and site are provided in *Electronic Supplementary Information 2*.

3.3.1. *Synthetic cathinones*

As shown in *Fig. 2*, synthetic cathinones were the most detected class of NPS in this work. Among the cathinones, 3-MMC was the most frequently found, particularly in Europe, where it was detected in all but two sites. It was also present in samples from the United States and Australia. Mephedrone and eutylone were the next most prevalent cathinones, with the highest PNML levels observed in sites in New Zealand. Both compounds were also detected at lower levels in sites across Europe and the United States. Mephedrone is an isomer of 3-MMC, the current chromatographic method was able to differentiate between the two compounds (*Fig. S1–2*).

Although mephedrone has been prohibited in Europe since 2010, it was consistently detected between 2019 and 2022 (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). In the present study, mephedrone was detected in five European sites: Cyprus (CY1), Czechia(CZ1), Germany, Slovenia (SI4), Spain (ES1), and the United Kingdom with the highest PNML of 4.3 mg/day/1000 people observed in the United Kingdom on 18th December 2022. It is also detected at two sites in Australia and one in New Zealand.

As shown in *Table S1–6,* eutylone, a structural isomer of pentylone, was detected in Belgium, Czechia, Germany, Italy, New Zealand, Slovenia, Spain, the United Kingdom and the United States. The highest PNML recorded in this study was in a site in New Zealand (653 mg/day/1000 people), marking the highest level detected since monitoring began in this international study (Bade et al., 2022; Bade et al., 2023).

N,N-dimethylpentylone and pentylone (also a metabolite of *N,N*-

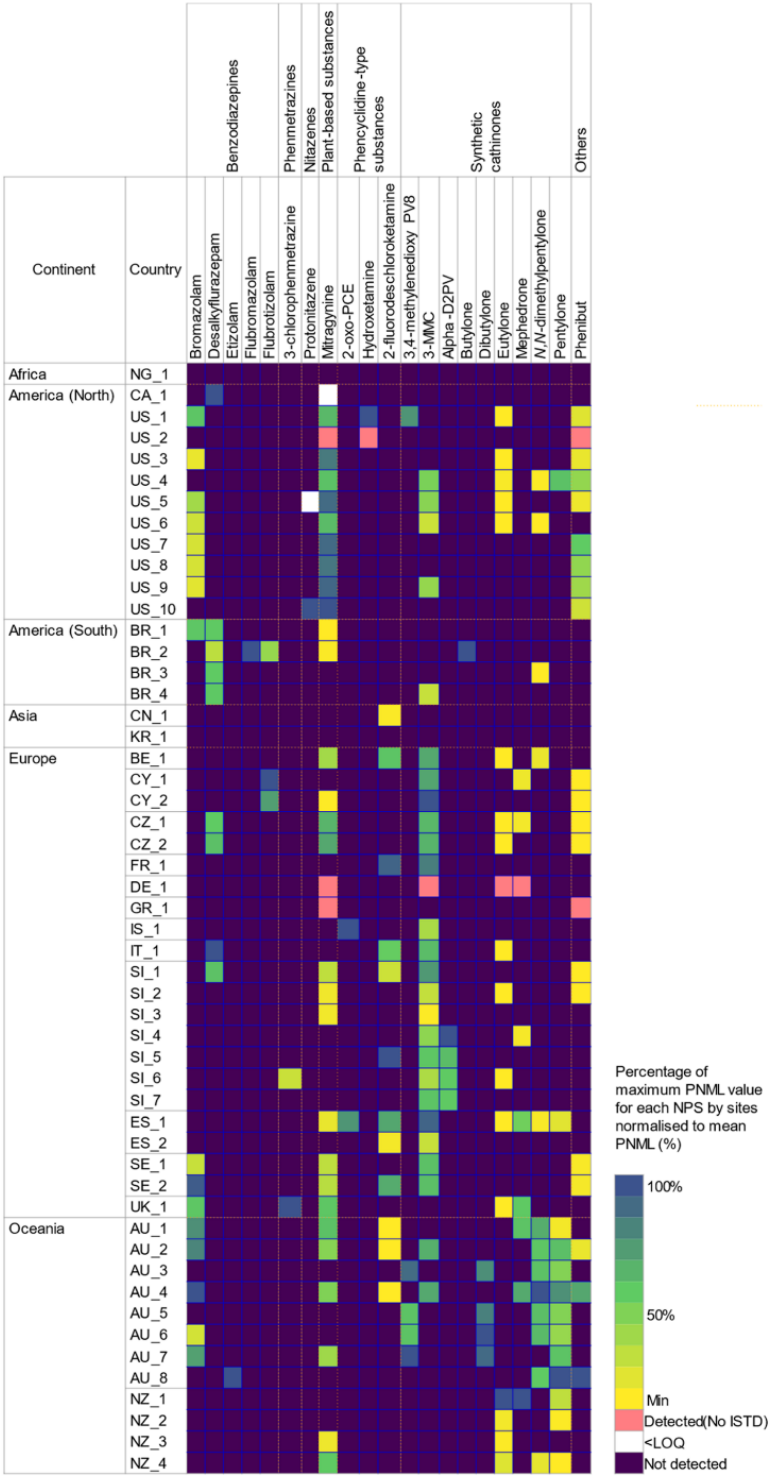


Fig. 2. Heat map showing percentage of maximum PNML for NPS detected at 52 sites.

dimethylpentylone) (Jaunay et al., 2024), were found almost exclusively in Oceania, appearing across sites in Australia and New Zealand. The highest PNML for *N,N*-dimethylpentylone, 63 mg/day/1000 people, was observed at site AU7 on 3rd January 2023. The highest for pentylone was 15 mg/day/1000 people at site AU8 on 2nd of January 2023.

In this work, one synthetic cathinone, 3,4-methylenedioxy PV8, was detected for the first time at sites in Australia and the United States. To confirm its identification via LC-MS/MS, a secondary experiment was conducted, in which the extract was spiked with a reference standard (*Fig. S1–3*), confirming the presence of the compound at a retention time of 5.1 min. 3,4-Methylenedioxy PV8 was detected at four sites in Australia (AU3, AU5, AU6 and AU7) throughout the entire sampling week, with increased levels observed over the New Year period, reaching a maximum PNML of 0.98 mg/day/1000 people. In the United States, 3,4-methylenedioxy PV8 was detected at one site, albeit at much lower levels. The first 3,4-methylenedioxy PV8 clinical case was reported in Sweden in 2019 through a retrospective analysis of oral fluid samples (Axelsson et al., 2022). In September of the same year, the compound was formally notified to the EMCDDA (now the EUDA). Subsequently, in May 2020, a surveillance tool based on web crawling known as NPSfinder® reported the online discussion of the same compound on social media (Catalani et al., 2021).

3.3.2. *Designer benzodiazepines*

Three different classes of designer benzodiazepines were detected in the 2022/2023 New Year period: 1,4-benzodiazepin-2-one (desalkylflurazepam), thienotriazolobenzodiazepine (flubrotizolam), and triazolobenzodiazepine (bromazolam and flubromazolam). The highest PNML values were observed for bromazolam (48 mg/day/1000 people) in Sweden, desalkylflurazepam (4.2 mg/day/1000 people) in Canada, flubrotizolam (2.3 mg/day/1000 people) in Cyprus, and etizolam (0.33 mg/day/1000 people) in Australia.

Bromazolam was detected at five sites in Australia, one in Brazil, two in Sweden, one in the United Kingdom, and six in the United States. First reported in Sweden in 2016 (WHO 2023), bromazolam continues to be detected in the wastewater samples. Desalkylflurazepam (known as norflurazepam) (Manchester et al., 2018) was detected in sites in Canada, Brazil (BR1- BR4), Czechia (CZ1 - CZ2), Italy, and Slovenia (SL1). It is both an active metabolite and structural analogue of several benzodiazepines, including flurazepam, flutoprazepam, fludiazepam, midazolam, flutazolam, quazepam, and ethyl loflazepate (Manchester et al., 2018; Miyaguchi et al., 2006). Flubrotizolam was detected in Brazil (BR2) and Cyprus (CY1 and CY2), whereas flubromazolam was detected only at site BR2 in Brazil. Due to the high recovery of flubromazolam during method validation, the result should be interpreted cautiously.

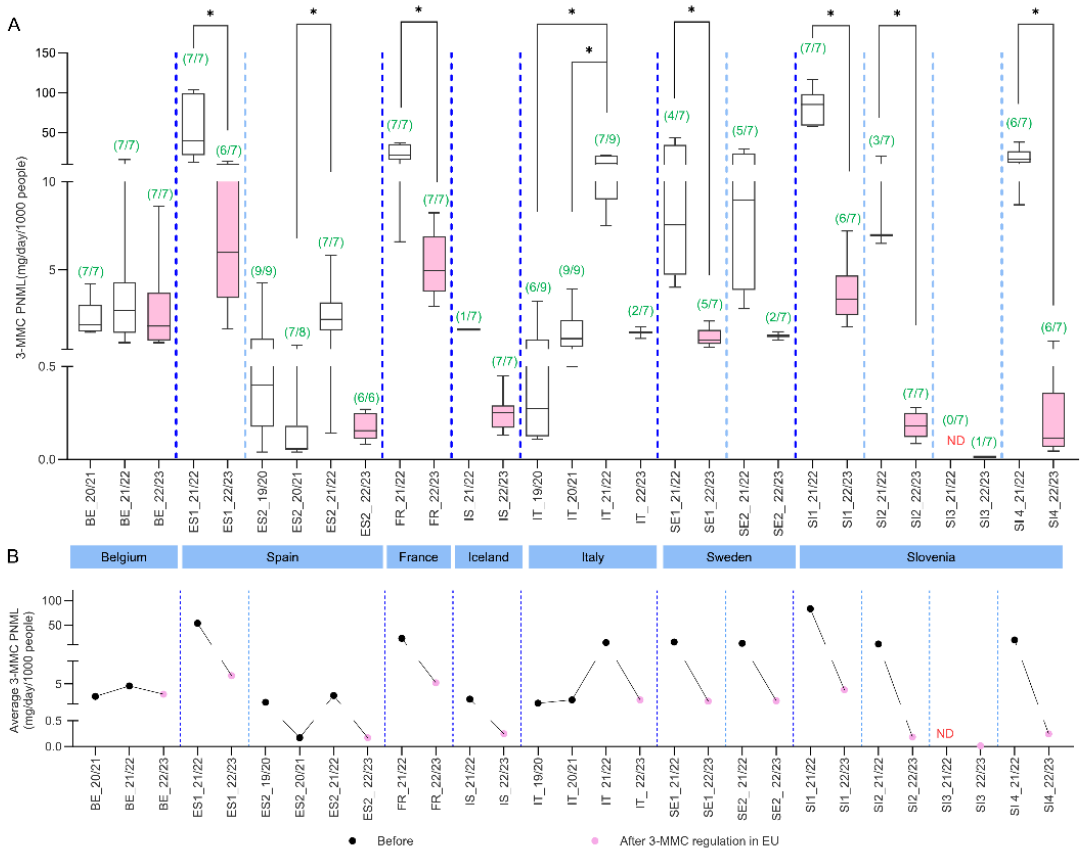


Fig. 3. A) Population normalised mass loads (PNML) for 3-MMC at 12 European sites in this study from 2019/20 to 2022/23. Statistically significant differences ($p < 0.05$) are indicated with a star (*). Numbers in green with brackets represent the detection frequency (number of days 3-MMC detected / total sampling days). 'ND' in red denotes 'not detected'. Pink bars represent PNML calculated in this study (i.e. 2022/2023) during the introduction of 3-MMC legislation in EU. The dark and light blue dash lines differentiate between countries and the sites within country, respectively.
B) Summary of trend before and after the implementation of Commission Delegated Directive (EU) 2022/1326 in March 2022. Black circles (pre-implementation); pink circle: after implementation.

3.3.3. Other compounds

Mitragynine was the most frequently detected NPS in this study and also exhibited the highest PNML among all the NPS in this work. However, as it remains legal in some countries, including all states in the United States where samples were collected, distinguishing between licit and illicit use remains challenging. The synthetic opioid protonitazene was detected in two sites in the United States, as previously published (Bade et al., 2024). 3-Chlorophenmetrazine was found at two sites in Europe (Slovenia and United Kingdom). Although phenibut is not classified as an NPS under the UNODC definition, it was detected across sites in the United States, Europe and Oceania. Three ketamine analogues were also identified: hydroxetamine, 2-oxo PCE and 2F-DCK, with 2F-DCK being the most frequently seen in sites in Australia, Belgium, China, France, Italy, Spain, Sweden and Slovenia. The highest PNML for 2F-DCK was recorded in Australia at the site AU4 with PNML of 72 mg/day/1000 people.

3.4. Temporal trend of 3-MMC 2019/2020 – 2022/2023

A temporal trend analysis for 3-MMC was conducted across multiple years with a focus on European sites located in Belgium, Spain, France, Iceland, Italy, Sweden, and Slovenia. This region was selected based on the EUDA 3-MMC scheduling decision in 2022 and the availability of longitudinal wastewater data from 2019/2020 and 2021/2022 (Bade et al., 2021; Bade et al., 2022; Bade et al., 2023). As shown in *Fig. 3*, significant trends in 3-MMC were observed in Europe. Belgium (BE) was the only site that showed no changes in PNML across the three sampling years from 2020/2021 to 2022/2023.

Sites in Spain (ES1 and ES2), France, Iceland, Italy, Sweden (SE1 and SE2), and Slovenia (SI1- SI4) had high 3-MMC PNML during 2021/2022 compared to the preceding and/or subsequent years. A Kruskal-Wallis's test showed significant differences across sampling years at the Spanish site ES2 (H (3) = 13.89, p = 0.003). Post hoc Dunn's test indicated significant increase from 2020/2021 and 2021/2022 (p = 0.002) at the same site. Similarly, in Italy, an increase was observed in 2021/2022 compared to preceding years. Both sites showed a statistically significant increase of 3-MMC PNML during 2021/2022 in conjunction with Covid-19 pandemic. A subsequent decline was observed at all sites in the following year (present study) except in Belgium, Spain (ES2), and Slovenia (SI3). Although 3-MMC appears to persist in some sites, none of the mass loads have reached the heights of 2021–2022.

The mass loads in this study were consistent with the wastewater data reported for 3-MMC in Europe from 2019 to 2021. For example, multiday pooled wastewater samples collected on weekdays and weekends in March and April 2021 ranged from 0.91 to 8.50 mg/day/1000 people in two Belgian cities (Salgueiro-Gonzalez et al., 2024). The three years New Year results showed a similar mass load range, between 0.87 to 16.15 mg/day/1000 people, indicating relatively stable use of 3-MMC in Belgium, which may not be influenced by festival activities. Additionally, studies conducted in Italy show similar trends to our work. The multiday pooled weekends mass loads increased from 0.12 – 3.80 mg/day/1000 people in 2020 (Salgueiro-González et al., 2022) to 3.64 to 6.99 mg/day/1000 people in 2021 (Salgueiro-Gonzalez et al., 2024).

Other data sources corroborate these increases in 3-MMC observed in sites in Europe. Postal seizures in Italy between May and October 2020 show that 3-MMC was the most seized NPS (Vincenti et al., 2021). A sharp increase was also observed in 3-MMC poisonings reported by healthcare professionals to the Dutch Poisons Information Centre in the Netherlands from 2019 to the first half of 2021 (Nugteren-van Lonkhuyzen et al., 2022).

Official reports from the European Union have identified synthetic cathinones, including 3-MMC, being sold as "legal" alternatives of MDMA or other stimulants (Commission, 2022). This is supported by drug-checking services across 13 European sites, which reported synthetic cathinones as the most common adulterants found in MDMA samples (EUDA 2024). However, adulteration with 3-MMC was not specifically mentioned in their report. Since the scheduling of mephedrone, 3-MMC has replaced other cathinones in the European market (Ferreira et al., 2019; Bäckberg et al., 2015). To further understand the relationship between 3-MMC and MDMA, wastewater data from 2019 to 2023 was analysed for spatial trends for Belgium, Italy, Spain, Slovenia, and Sweden using the EUDA/ SCORE dashboard data (EUDA 2024). A lower consumption of MDMA was observed at all sites for the year 2021/2022 compared to 2020/2021 and 2022/2023 (*Fig. S1–4*). While wastewater data alone cannot definitively show compound substitution and/or adulteration, it is worth noting that the observed trends align with the findings of the EUDA.

Overall, based on the four years data, a decline population-normalised mass loads were observed at sites in Spain and Italy in 2022/2023 compared to the previous years, except for Belgium. A general declining trend was observed at sites in Iceland, France, Slovenia, and Sweden. This coincides with legislation being introduced in Europe and internationally to control 3-MMC. It is potentially too early to confirm this trend is directly associated with the legislation, as there could be multiple factors at play. Hence, long term continuous monitoring is recommended to understand the trend and effectiveness of these control measures.

3.5. Challenges in the present work

The present work demonstrates the application of a wastewater analysis workflow on a global scale to understand the spatial-temporal trend of NPS, covering a population of over 20 million people across 20 countries for the 2022/2023 New Year period. However, it must be noted that the sites within this study cannot reflect the entire country. The method targeted 54 NPS, representing, <5 % of the over 1300 known NPS reported to date. Therefore, the observed prevalence is inherently method-dependent and does not imply the absence of other NPS in the sites and countries monitored. High-resolution mass spectrometry may serve as a complementary approach, provided the instrument's sensitivity is sufficient to detect trace levels of NPS (Bijlsma et al., 2021). Moreover, non-detections of compounds within this study do not mean that these compounds were not used, considering the different dose sizes of the NPS and the potential small user pool.

This study focused exclusively on samples collected during the New Year period, a time specifically selected as there will be typically increased drug use associated with festive occasions. However, this means that the levels of NPS observed in the current work may be higher than those normally seen in 'normal' weeks, and care should be taken when comparing studies. Additionally, it is important to recognise that data from a single site does not represent the entire country. Nevertheless, detection of an NPS at a single location may still indicate localised use, which could have public health implications. For example, the detection of protonitazene, a potent synthetic opioid, at even one site may warrant serious attention.

Finally, a recognised uncertainty in wastewater-based epidemiology is the estimation of the catchment population. The New Year period typically involves population movement due to holidays and travel (Boogaerts et al., 2024). This could skew the data towards potentially higher consumption trends in the regions where the occasion is celebrated. As such, there may be a larger than normal uncertainty associated with the population used due to holiday travel. In this study, many sites used static population estimates based on census data across sampling weeks, which may lead to over- or underestimation of PNML. At present, there is no universally accepted population biomarker to account for population movement. This remains an active area of research, and the integration of multiple biomarkers could represent an important future direction which could refine population estimates and improve the robustness of cross-national WBE assessments.

4. Conclusions

The present study provides a snapshot of the international prevalence of NPS in influent wastewater from 52 sites across 20 countries during the 2022/2023 New Year. A total of 21 NPS were detected in this work. These findings were based on an updated analytical method, applied to the emerging compounds relevant at the time of study. The most commonly identified NPS were synthetic cathinones, benzodiazepines, and phencyclidine-type substances, with mitragynine, 3-MMC, and eutylone being the most frequently reported compounds. In addition to the spatiotemporal investigation of NPS occurrence, the study examined temporal trends of 3-MMC to understand the general declining trend of 3-MMC PNML mass loads. The decline in European cities could be associated with the enhancement of existing local legislation after Covid-19, in addition to ongoing scheduling of 3-MMC in the European Union and at the international level. Furthermore, the evaluation of the loaded SPE cartridge provides a body of knowledge on the stability of NPS compounds at various storage conditions, with −20 °C being the ideal temperature up to four months. As new NPS emerge onto the drug market, spatiotemporal wastewater analysis in combination with scheduling information of controlled NPS can offer insights into dynamic drug markets. The present work demonstrated the possible application of wastewater as an auxiliary assessment tool to understand the effectiveness and impact of scheduling of NPS through long-term surveillance.

Declaration of generative AI and AI-assisted technologies in the writing process

Declaration of generative AI and AI-assisted technologies in the writing process. During the preparation of this work the author(s) used ChatGPT-4o to check grammar, spelling, and improve the readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Dhayaalini Nadarajan: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Laimou-Geraniou:** Writing – review & editing, Resources, Formal analysis. **Andrew Chappell:** Resources, Formal analysis. **Christine Baduel:** Resources, Formal analysis. **Lubertus Bijlsma:** Writing – review & editing, Resources, Formal analysis. **Tim Boogaerts:** Writing – review & editing, Resources, Formal analysis. **Daniel A. Burgard:** Resources, Formal analysis. **Sara Castiglioni:** Writing – review & editing, Resources, Formal analysis. **Nicola Ceolotto:** Resources, Formal analysis. **Erin M. Driver:** Resources, Formal analysis. **Fernando Fabriz Sodre:** Writing – review & editing, Resources, Formal analysis. **Despo Fatta Kassinos:** Writing – review & editing, Resources, Formal analysis. **Harold Flores Quintana:** Writing – review & editing, Resources, Formal analysis. **Cobus Gerber:** Writing – review & editing, Resources, Formal analysis. **Emma Gracia-Lor:** Resources, Formal analysis. **Elisa Gracia-Marín:** Writing – review & editing, Resources, Formal analysis. **Rolf U. Halden:** Writing – review & editing, Methodology, Formal analysis. **Ester Heath:** Writing – review & editing, Resources, Formal analysis. **Julia Huchthausen:** Resources, Formal analysis. **Barbara Kasprzyk-Hordern:** Writing – review & editing, Resources, Formal analysis. **Emma L. Keller:** Writing – review & editing, Resources, Formal analysis. **Foon Yin Lai:** Writing – review & editing, Resources, Formal analysis. **Arndís Sue-Ching Löve:** Writing – review & editing, Resources, Formal analysis. **João Matias:** Writing – review & editing. **Vera Ocenaskova:** Resources, Formal analysis. **Jeong-Eun Oh:** Resources, Formal analysis. **Temilola Oluseyi:** Resources, Formal analysis. **Kaitlyn Phung:** Resources, Formal analysis. **Marco Pineda-Castro:** Resources, Formal analysis. **Magda Psichoudaki:** Writing – review & editing, Resources, Formal analysis. **Noelia Salgueiro-Gonzalez:** Writing – review & editing, Resources, Formal analysis. **Cezar Silvino-Gomes:** Writing – review & editing, Resources, Formal analysis. **Fábio Marley Sousa Nascimento:** Resources, Formal analysis. **Bikram Subedi:** Resources, Formal analysis. **Nikolaos Thomaidis:** Resources, Formal analysis. **Natan Van Wichelen:** Writing – review & editing, Resources, Formal analysis. **Degao Wang:** Resources, Formal analysis. **Viviane Yargeau:** Resources, Formal analysis. **Sarah Cresswell:** Writing – review & editing, Supervision. **Jochen F. Mueller:** Writing – review & editing, Visualization, Supervision, Resources. **Richard Bade:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Despo Fatta-Kassinos, an author of this submission, serves as an editor of Water Research. The manuscript was handled and reviewed independently by other editors, and Despo Fatta-Kassinos is recusing herself from any decisions related to the manuscript evaluation and acceptance. The other authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

Acknowledgements

The authors thank all participating wastewater treatment plant technicians and collaborators for their invaluable contributions for sample collection, extraction, and shipment. The Queensland Alliance for Environmental Health Sciences (QAEHS) gratefully acknowledges the financial support of Queensland Health, Australia. The authors also thank SCIEX for providing access to the 7500 Triple Quad mass spectrometer. D. Nadarajan is the recipient of The University of Queensland Research Training Program Scholarship. R. Bade is the recipient of the Australian Research Council Discovery Early Career Award (project number DE220100381), and J. Mueller is the recipient of the Australian Research Council Australian Laureate Fellowship (project number FL200100028) funded by the Australian Government. T. Boogaerts was funded by the Research Foundation Flanders for his WBE-related research (1225924 N). SLU wants to particularly acknowledge Uppsala Vatten (Oscar Götlind) and Stockholm Vatten och Avfall (Gabriel Persson) for their assistance with sample collection in Sweden. E. Heath and M. Laimou-Geraniou would like to acknowledge financial support of Slovene Research Agency (P1–0143, N1–0047, L7–4422). M. Psichoudaki and D. Fatta-Kassinos wish to express their gratitude to the National Addictions Authority of Cyprus (NAAC) for their generous funding and support. L. Bijlsma acknowledges grant RYC2020–028936-I funded by MCIN/AEI/ 10.13039/501100011033 and "ESF Investing in your future". The views expressed in this article are those of the author (s) and not necessarily those of the funding agencies. B. Kasprzyk-Hordern and N. Ceolotto would like to acknowledge Wessex Water and support by Research England under the Expanding Excellence in England (E3) funding stream'. S. Castiglioni and N. Salgueiro- Gonzalez would like to acknowledge Metropolitana Milanese for samples collection. E. Gracia-Lor acknowledges the support of the Government Delegation for the National Drugs Plan, with funds from the Recovery, Transformation, and Resilience Mechanism of the European Union–NextGenerationEU (project number EXP2022/008817). The views expressed in this article are those of the author(s) and not necessarily those of the funding agencies.

Supplementary materials

Supplementary material associated with this article can be found, in

the online version, at doi:10.1016/j.watres.2025.124847.

Data availability

Data will be made available on request.

References

Andreasen, M.F., Telving, R., Rosendal, I., Eg, M.B., Hasselstrøm, J.B., Andersen, L.V., 2015. A fatal poisoning involving 25C-NBOMe. Forensic Sci. Int. 251, e1–e8. https://doi.org/10.1016/j.forsciint.2015.03.012.

Axelsson, M.A.B., Lövgren, H., Kronstrand, R., Green, H., Bergström, M.A., 2022. Retrospective identification of new psychoactive substances in patient samples submitted for clinical drug analysis. Basic Clin. Pharmacol. Toxicol. 131, 420–434. https://doi.org/10.1111/bcpt.13786.

Bäckberg, M., Lindeman, E., Beck, O., Helander, A., 2015. Characteristics of analytically confirmed 3-MMC-related intoxications from the Swedish STRIDA project. Clin. Toxicol. 53, 46–53. https://doi.org/10.3109/15563650.2014.981823.

Bade, R., White, J.M., Nguyen, L., Tscharke, B.J., Mueller, J.F., O'Brien, J.W., Thomas, K. V., Gerber, C., 2020a. Determining changes in new psychoactive substance use in Australia by wastewater analysis. Sci. Total Environ. 731. https://doi.org/10.1016/j.scitotenv.2020.139209.

Bade, R., Abdelaziz, A., Nguyen, L., Pandopulos, A.J., White, J.M., Gerber, C., 2020b. Determination of 21 synthetic cathinones, phenethylamines, amphetamines and opioids in influent wastewater using liquid chromatography coupled to tandem mass spectrometry. Talanta 208, 120479. https://doi.org/10.1016/j.talanta.2019.120479.

Bade, R., White, J.M., Chen, J., Baz-Lomba, J.A., Been, F., Bijlsma, L., Burgard, D.A., Castiglioni, S., Salgueiro-Gonzalez, N., Celma, A., Chappell, A., Emke, E., Steenbeek, R., Wang, D., Zuccato, E., Gerber, C., 2021. International snapshot of new psychoactive substance use: case study of eight countries over the 2019/2020 new year period. Water. Res. 193, 116891. https://doi.org/10.1016/j.watres.2021.116891.

Bade, R., White, J.M., Ghetia, M., Adiraju, S., Adhikari, S., Bijlsma, L., Boogaerts, T., Burgard, D.A., Castiglioni, S., Celma, A., Chappell, A., Covaci, A., Driver, E.M., Halden, R.U., Hernandez, F., Lee, H.J., Van Nuijs, A.L.N., Oh, J.E., Pineda Castro, M. A., Salgueiro-Gonzalez, N., Subedi, B., Shao, X.T., Yargeau, V., Zuccato, E., Gerber, C., 2022. A taste for new psychoactive substances: wastewater analysis study of 10 countries. Environ. Sci. Technol. Lett. 9, 57–63. https://doi.org/10.1021/acs.estlett.1c00807.

Bade, R., Rousis, N., Adhikari, S., Baduel, C., Bijlsma, L., Bizani, E., Boogaerts, T., Burgard, D.A., Castiglioni, S., Chappell, A., Covaci, A., Driver, E.M., Sodre, F.F., Fatta-Kassinos, D., Galani, A., Gerber, C., Gracia-Lor, E., Gracia-Marín, E., Halden, R. U., Heath, E., Hernandez, F., Jaunay, E., Lai, F.Y., Lee, H.J., Laimou-Geraniou, M., Oh, J.E., Olafsdottir, K., Phung, K., Castro, M.P., Psichoudaki, M., Shao, X., Salgueiro-Gonzalez, N., Feitosa, R.S., Gomes, C.S., Subedi, B., Löve, A.S.C., Thomaidis, N., Tran, D., van Nuijs, A., Verovšek, T., Wang, D., White, J.M., Yargeau, V., Zuccato, E., Mueller, J.F., 2023. Three years of wastewater surveillance for new psychoactive substances from 16 countries. Water. Res. X. 19. https://doi.org/10.1016/j.wroa.2023.100179.

Bade, R., Nadarajan, D., Driver, E.M., Halden, R.U., Gerber, C., Krotulski, A., Hall, W., Mueller, J.F., 2024. Wastewater-based monitoring of the nitazene analogues: first detection of protonitazene in wastewater. Sci. Total Environ. 920. https://doi.org/10.1016/j.scitotenv.2024.170781.

Bijlsma, L., Celma, A., Castiglioni, S., Salgueiro-González, N., Bou-Iserte, L., Baz-Lomba, J.A., Reid, M.J., Dias, M.J., Lopes, A., Matias, J., Pastor-Alcañiz, L., Radonić, J., Turk Sekulic, M., Shine, T., van Nuijs, A.L.N., Hernandez, F., Zuccato, E., 2020. Monitoring psychoactive substance use at six European festivals through wastewater and pooled urine analysis. Sci. Total Environ. 725, 138376. https://doi.org/10.1016/j.scitotenv.2020.138376.

Bijlsma, L., Bade, R., Been, F., Celma, A., Castiglioni, S., 2021. Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – a tutorial. Anal. Chim. Acta 1145, 132–147. https://doi.org/10.1016/j.aca.2020.08.058.

Bivins, A., North, D., Ahmad, A., Ahmed, W., Alm, E., Been, F., Bhattacharya, P., Bijlsma, L., Boehm, A.B., Brown, J., Buttiglieri, G., Calabro, V., Carducci, A., Castiglioni, S., Cetecioglu Gurol, Z., Chakraborty, S., Costa, F., Curcio, S., de los Reyes, F.L., Delgado Vela, J., Farkas, K., Fernandez-Casi, X., Gerba, C., Gerrity, D., Girones, R., Gonzalez, R., Haramoto, E., Harris, A., Holden, P.A., Islam, Md.T., Jones, D.L., Kasprzyk-Hordern, B., Kitajima, M., Kotlarz, N., Kumar, M., Kuroda, K., La Rosa, G., Malpei, F., Mautus, M., McLellan, S.L., Medema, G., Meschke, J.S., Mueller, J., Newton, R.J., Nilsson, D., Noble, R.T., van Nuijs, A., Peccia, J., Perkins, T.A., Pickering, A.J., Rose, J., Sanchez, G., Smith, A., Stadler, L., Stauber, C., Thomas, K., van der Voorn, T., Wigginton, K., Zhu, K., Bibby, K., 2020. Wastewater-based epidemiology: global collaborative to maximize contributions in the fight against COVID-19. Environ. Sci. Technol. 54, 7754–7757. https://doi.org/10.1021/acs.est.0c02388.

Boogaerts, T., Van Wichelen, N., Quireyns, M., Burgard, D., Bijlsma, L., Delputte, P., Gys, C., Covaci, A., van Nuijs, A.L.N., 2024. Current state and future perspectives on de facto population markers for normalization in wastewater-based epidemiology: a systematic literature review. Sci. Total Environ. 935, 173223. https://doi.org/10.1016/j.scitotenv.2024.173223.

Borova, V.L., Gago-Ferrero, P., Pistos, C., Thomaidis, N.S., 2015. Multi-residue determination of 10 selected new psychoactive substances in wastewater samples by liquid chromatography–tandem mass spectrometry. Talanta 144, 592–603. https://doi.org/10.1016/j.talanta.2015.06.080.

Castiglioni, S., Salgueiro-González, N., Bijlsma, L., Celma, A., Gracia-Lor, E., Beldean-Galea, M.S., Mackuľak, T., Emke, E., Heath, E., Kasprzyk-Hordern, B., Petkovic, A., Poretti, F., Rangelov, J., Santos, M.M., Sremački, M., Styszko, K., Hernández, F., Zuccato, E., 2021. New psychoactive substances in several European populations assessed by wastewater-based epidemiology. Water. Res. 195, 116983. https://doi.org/10.1016/j.watres.2021.116983.

Castro, A.L., da Costa, M.J.C.S.P., Tarelho, S., Sousa, L., Russo, F., Franco, J.M., 2022. Intoxication by 3-MeO-PCP and O-desmethyltramadol: an unusual NPS mix. Int. J. Legal. Med. 136, 1297–1301. https://doi.org/10.1007/s00414-022-02818-w.

Catalani, V., Arillotta, D., Corkery, J.M., Guirguis, A., Vento, A., Schifano, F., 2021. Identifying new/emerging psychoactive substances at the time of COVID-19; A web-based approach. Front. Psychiatry 11. https://doi.org/10.3389/fpsyt.2020.632405.

Commission, European, 2022. Commission delegated Directive (EU) 2022/1326 of 18 March 2022 amending the Annex to Council Framework Decision 2004/757/JHA as regards the inclusion of new psychoactive substances in the definition of 'drug'. Off. J. Eur. Union 1–3. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32022L1326 (accessed April 13, 2025).

Daughton, C.G., Illicit drugs in municipal sewage, in: 2001: pp. 348–364. https://doi.org/10.1021/bk-2001-0791.ch020.

Daughton, C.G., 2011. Illicit drugs: contaminants in the environment and utility in Forensic epidemiology, in: D. Whitacre (Ed.). Rev. Environ. Contam. Toxicol. 59–110. https://doi.org/10.1007/978-1-4419-7615-4_3.

Daughton, C.G., 2018. Monitoring wastewater for assessing community health: sewage chemical-information mining (SCIM). Sci. Total Environ. 748–764. https://doi.org/10.1016/j.scitotenv.2017.11.102, 619–620.

Dumbili, E.W., Ebuenyi, I.D., Ugoeze, K.C., 2021. New psychoactive substances in Nigeria: a call for more research in Africa, emerging trends in drugs. Addictions Health 1, 100008. https://doi.org/10.1016/j.etdah.2021.100008.

EUDA, 2024. MDMA – the current situation in Europe (European Drug Report 2024). https://www.euda.europa.eu/publications/european-drug-report/2024/mdma_en (accessed December 10, 2024).

European Monitoring Centre for Drugs and Drug, 2021. Addiction (EMCDDA), European Drug Report. Trends Dev. 2021. www.emcdda.europa.eu.

European Union Drug Monitoring, EU Drug Market, 2024. New psychoactive substances — distribution and supply in Europe: synthetic cathinones. https://www.euda.europa.eu/publications/eu-drug-markets/new-psychoactive-substances/distribution-and-supply/synthetic-cathinones_en.

European Union Drug Agency, 2024. Early Warning System on NPS. https://www.euda.europa.eu/publications/topic-overviews/eu-early-warning-system_en.

European Union Drugs Agency, 2024. The EU Early Warning System monitors 1000 NPS. Lisbon.

Evans-Brown, M., Sedefov, R., Responding to new psychoactive substances in the European Union: early warning, risk assessment, and control measures, in: 2018: pp. 3–49. https://doi.org/10.1007/164_2018_160.

Evans-Brown, M., Hughes, B., Sedefov, R., 2022. Legal classification and international systems for monitoring and responding to novel psychoactive substances. Novel Psychoactive Substances. Elsevier, pp. 3–56. https://doi.org/10.1016/B978-0-12-818788-3.00016-4.

Ferreira, B., Dias da Silva, D., Carvalho, F., de Lourdes Bastos, M., Carmo, H., 2019. The novel psychoactive substance 3-methylmethcathinone (3-MMC or metaphedrone): a review. Forensic Sci. Int. 295, 54–63. https://doi.org/10.1016/j.forsciint.2018.11.024.

Gao, T., Du, P., Xu, Z., Li, X., 2017. Occurrence of new psychoactive substances in wastewater of major Chinese cities. Sci. Total Environ. 575, 963–969. https://doi.org/10.1016/j.scitotenv.2016.09.152.

Jaunay, E.L., Bade, R., Paxton, K.R., Nadarajan, D., Barry, D.C., Zhai, Y., Tscharke, B.J., O'Brien, J.W., Mueller, J., White, J.M., Simpson, B.S., Gerber, C., 2024. Monitoring the use of novel psychoactive substances in Australia by wastewater-based epidemiology. Sci. Total Environ., 170473 https://doi.org/10.1016/j.scitotenv.2024.170473.

Kim, U., Sim, W., Cha, H.J., Oh, J.-E., 2023. Development of a simultaneous multi component analytical method for 37 new psychoactive substances in wastewater using LC MS/MS. Environ. Eng. Res. 29. https://doi.org/10.4491/eer.2023.491, 230491–0.

Kronstrand, R., Guerrieri, D., Vikingsson, S., Wohlfarth, A., Gréen, H., Fatal poisonings associated with new psychoactive substances, in: 2018: pp. 495–541. https://doi.org/10.1007/164_2018_110.

Laimou-Geraniou, M., Nadarajan, D., Bade, R., Heath, E., 2025. Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis. J. Hazardous Mater. Adv., 100781 https://doi.org/10.1016/j.hazadv.2025.100781.

Lee, H.-J., Oh, J.-E., 2023. Target and suspect screening of (new) psychoactive substances in South Korean wastewater by LC-HRMS. Sci. Total Environ. 875, 162613. https://doi.org/10.1016/j.scitotenv.2023.162613.

Manchester, K.R., Lomas, E.C., Waters, L., Dempsey, F.C., Maskell, P.D., 2018. The emergence of new psychoactive substance (NPS) benzodiazepines: a review. Drug Test. Anal. 10, 37–53. https://doi.org/10.1002/dta.2211.

Miyaguchi, H., Kuwayama, K., Tsujikawa, K., Kanamori, T., Iwata, Y.T., Inoue, H., Kishi, T., 2006. A method for screening for various sedative-hypnotics in serum by liquid chromatography/single quadrupole mass spectrometry. Forensic Sci. Int. 157, 57–70. https://doi.org/10.1016/j.forsciint.2005.03.011.

Montanari, E., Madeo, G., Pichini, S., Busardò, F.P., Carlier, J., 2022. Acute intoxications and fatalities associated with Benzimidazole opioid (Nitazene Analog) use. Systematic Rev. http://journals.lww.com/drug-monitoring.

Nadarajan, D., O'Brien, J., Cresswell, S., Kele, B., Mueller, J., Bade, R., 2024. Application of design of experiment for quantification of 71 new psychoactive substances in influent wastewater. Anal. Chim. Acta 1321, 343036. https://doi.org/10.1016/j.aca.2024.343036.

No SANTE/11312/2021v2, 2024. Analytical quality control and method validation procedures for pesticide residues analysis in food and feed Sante, 11312/2021 v2. Supersedes Document No. SANTE/11312/2021. Implemented by 01/01/2024. https://food.ec.europa.eu/system/files/2023-11/pesticides_mrl_guidelines_wrkdoc_2021-11312.pdf (accessed April 14, 2025).

Nugteren-van Lonkhuyzen, J.J., Essink, S., Rietjens, S.J., Ohana, D., de Lange, D.W., van Riel, A.J.H.P., Hondebrink, L., 2022. 3-Methylmethcathinone (3-MMC) poisonings: acute clinical toxicity and time trend between 2013 and 2021 in the Netherlands. Ann. Emerg. Med. 80, 203–212. https://doi.org/10.1016/j.annemergmed.2022.04.022.

O'Rourke, C.E., Subedi, B., 2020. Occurrence and mass loading of synthetic opioids, synthetic cathinones, and synthetic cannabinoids in wastewater treatment plants in four U.S. Communities. Environ. Sci. Technol. 54, 6661–6670. https://doi.org/10.1021/acs.est.0c00250.

Salgueiro-González, N., Zuccato, E., Castiglioni, S., 2022. Nationwide investigation on the use of new psychoactive substances in Italy through urban wastewater analysis. Sci. Total Environ. 843, 156982. https://doi.org/10.1016/j.scitotenv.2022.156982.

Salgueiro-Gonzalez, N., Béen, F., Bijlsma, L., Boogaerts, T., Covaci, A., Baz-Lomba, J.A., Kasprzyk-Hordern, B., Matias, J., Ort, C., Bodík, I., Heath, E., Styszko, K., Emke, E., Hernández, F., van Nuijs, A.L.N., Castiglioni, S., 2024. Influent wastewater analysis to investigate emerging trends of new psychoactive substances use in Europe. Water. Res. 254, 121390. https://doi.org/10.1016/j.watres.2024.121390.

Senta, I., Krizman, I., Ahel, M., Terzic, S., 2015. Multiresidual analysis of emerging amphetamine-like psychoactive substances in wastewater and river water. J. Chromatogr. A 1425, 204–212. https://doi.org/10.1016/j.chroma.2015.11.043.

Simpson, B.S., Jaunay, E.L., Ghetia, M., Nguyen, L., Bade, R., White, J.M., Gerber, C., 2022. Methcathinone in wastewater: drug of choice, or artefact? Sci. Total Environ. 836. https://doi.org/10.1016/j.scitotenv.2022.155696.

Ujváry, I., Christie, R., Evans-Brown, M., Gallegos, A., Jorge, R., de Morais, J., Sedefov, R., 2021. DARK Classics in chemical neuroscience: etonitazene and related benzimidazoles. ACS. Chem. Neurosci. 12, 1072–1092. https://doi.org/10.1021/acschemneuro.1c00037.

United Nations Office on Drugs and Crime, 2024. What are NPS? https://www.unodc.org/LSS/Page/NPS.

United Nations Office on Drugs and Crime, 2023. UNODC early warning advisory on new psychoactive substances. https://www.unodc.org/LSS/Country/List.

UNODC, 2022. WHO: world Health Organization recommends seven NPS for scheduling. https://www.unodc.org/LSS/Announcement/Details/dc9d7f3c-1fd3-4d61-9770-2d72b198252e.

UNODC, 2024. The challenge of new psychoactive substances - a technical update. https://www.unodc.org/documents/scientific/The_Challenge_of_NPS_A_technical_update_2024.pdf (accessed December 11, 2024).

Vincenti, F., Gregori, A., Flammini, M., Di Rosa, F., Salomone, A., 2021. Seizures of new psychoactive substances on the Italian territory during the COVID-19 pandemic. Forensic Sci. Int. 326, 110904. https://doi.org/10.1016/j.forsciint.2021.110904.

WHO, 2023. Expert Committee on Drug Dependence forty-sixth meeting: critical review report: bromazolam. Geneva. https://cdn.who.int/media/docs/default-source/46th-ecdd/bromazolam_46th-ecdd-critical-review_public-version.pdf?sfvrsn=4f1bccfa_1 (accessed December 11, 2024).

Winstock, A., Munksgaard, R., Davies, E., Ferris, J., Zhuparris, A., Barratt, M., Global Drug Survey A different way of looking at drugs 2022 Drug Trend Report: time, music, clubbing, age, and diet, n.d. https://www.globaldrugsurvey.com/gds-2021/.

World Health Organisation, 2022. Critical review report of 3-methylmethcathinone (3-MMC) for 45th ECDD Meeting. Geneva. Geneva, 2022. https://cdn.who.int/media/docs/default-source/controlled-substances/45th-ecdd/3-mmc_draft.pdf?sfvrsn=f046b91f_1 (accessed April 13, 2025).

3.4 Psychoactive Pharmaceutical Residues in the Aqueous Environment: Removal, Occurrence, and Ecotoxicity

3.4.1 Psychoactive pharmaceuticals: Consumption trends, removal during wastewater treatment and occurrence in receiving waters with associated environmental risks

Published: M. Laimou-Geraniou, T. Kos, D. Heath, E. Heath, 2025, *Journal of Hazardous Materials Advances*, vol. 19, pp. 100816.

I was fully responsible for the experimental work, method optimisation and validation, instrumental analysis, data analysis and evaluation, visualisation, and writing the manuscript.

Although recent studies have used WBE to examine the impact of the COVID-19 pandemic on temporal trends in psychoactive pharmaceutical consumption, they predominantly focus on antidepressants, with fewer looking at benzodiazepines or antipsychotics. Additionally, the widespread use of psychoactive pharmaceuticals has raised concerns about their residues in the environment, as they are excreted from the body (either parent compounds or metabolites) and subsequently enter the sewer system, where they are transported to the local WWTP. Due to incomplete removal during wastewater treatment, psychoactive pharmaceutical residues are continuously released into receiving environmental waters via wastewater effluent, potentially affecting the aquatic ecosystems.

Similar to WBE studies, several studies have already addressed the efficiency of certain wastewater treatment technologies for removing antidepressant residues and investigated their impact on the aquatic ecosystems; however, only a few have focused on a limited number of antipsychotics and benzodiazepines. Furthermore, additional studies are needed considering the recently revised UWWTD that came into effect on 1.1.2025 (UWWTD, 2024), which indicates the requirement of additional quaternary treatment in order to achieve >80% removal of at least 6 out of 13 listed organic micropollutants, including the antidepressants carbamazepine, citalopram, and venlafaxine.

To fill this knowledge gap, this study aimed to evaluate temporal trends in the use of psychoactive pharmaceuticals in Slovenia, including before, during, and after the pandemic and to determine the removal efficiency of seven Slovenian WWTPs varying in size and configuration (AS, SBR, SBR with UV, MBR, and MBBR), for removing antidepressant, antipsychotic, and benzodiazepine residues. For the first time, removal efficiencies of psychoactive pharmaceuticals were reported for a moving bed biofilm reactor (MBBR). Additionally, the occurrence of psychoactive pharmaceuticals in receiving surface waters was explored using developed analytical methods and a prediction approach based on measured concentrations in effluent wastewater combined with site-specific dilution factors. Finally, an environmental risk assessment was made for the studied receiving surface waters based on measured concentrations of psychoactive pharmaceutical residues and effect concentrations predicted using the ECOSAR software.

Regarding WBE, the antidepressants sertraline and carbamazepine, the antipsychotics clozapine and quetiapine, and the benzodiazepines zolpidem and oxazepam showed the highest PNMLs (50-110, 10-75, and 8-78 mg/day/1,000 inhabitants, respectively). The remaining compounds ranged from 1 to 30 mg/day/1,000 inhabitants, while others were detected but not quantified. Temporal patterns reveal variability in psychoactive pharmaceutical use, which likely reflects the influence of external factors such as

COVID-19-related public health measures, seasonal trends and changes in regional population dynamics. Overall, the PNMLs of most psychoactive pharmaceuticals selected for temporal analysis showed a decreasing trend during the pandemic, and particularly during the first wave in spring 2020.

Psychoactive pharmaceutical residues were detected in wastewater influents and effluents of seven Slovene WWTPs in the range of ng/L to μg/L. Results showed that treatment technologies exhibit varying removal efficiencies for psychoactive pharmaceuticals, with AS and MBR bioreactors achieving the highest (50-60%) and MBBR the lowest (20%). Removal efficiency was also compound-dependent, with citalopram, venlafaxine, and oxazepam exhibiting poor removal in all cases. Poor removal (<80%) of three compounds listed in the revised UWWTD (carbamazepine, citalopram, and venlafaxine) indicated the need for additional quaternary treatment at conventional WWTPs. Overall, the highest average REs (>80%) were observed only for the antipsychotics paliperidone (88%) and quetiapine (87%). On average, higher removals were found for antipsychotics (73%), followed by benzodiazepines (45%) and antidepressants (39%).

The removal of psychoactive pharmaceutical residues at various WWTPs influences their occurrence in receiving surface waters, which are detected at ng/L levels. Comparable results were obtained from both water analysis and predictions of environmental concentrations, suggesting that prediction methods could be used as a practical alternative to the cost- and labour-intensive water analysis. Environmental risk assessment using the ECOSAR predictive model indicated potential acute risks to aquatic organisms, particularly from venlafaxine, *O*-desmethylvenlafaxine, citalopram, and sertraline. These findings underscore the need to upgrade treatment processes, particularly in plants with low dilution factors or inefficient technologies, to mitigate ecological impacts.

The study results were presented at the 7th International Conference on Testing the Waters (TTW7): The International Conference on Wastewater Surveillance, held in Lisbon, Portugal, October 21-22, 2024.

Journal of Hazardous Materials Advances 19 (2025) 100816

Contents lists available at ScienceDirect

Journal of Hazardous Materials Advances

journal homepage: www.elsevier.com/locate/hazadv

Psychoactive pharmaceuticals: Consumption trends, removal during wastewater treatment and occurrence in receiving waters with associated environmental risks

Maria Laimou-Geraniou [a,b], Tjaša Kos [a], David Heath [a], Ester Heath [a,b,*]

[a] Department of Environmental Sciences, Jožef Stefan Institute, Jamova c. 39, Ljubljana 1000, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova c. 39, Ljubljana 1000, Slovenia

ARTICLE INFO

Keywords:
Antidepressants
Antipsychotics
Benzodiazepines
Temporal trends
COVID-19 pandemic
Surface water
ECOSAR

ABSTRACT

This study applied wastewater-based epidemiology to evaluate temporal changes in the consumption of psychoactive pharmaceuticals before, during, and after the COVID-19 pandemic in Slovenia. 24-hour influent composite wastewater samples were collected over seven consecutive days within seven distinct sampling periods (summer 2019, winter/spring 2020, winter/spring 2021, spring 2022, and spring 2023) from the Ljubljana wastewater treatment plant (WWTP). The antidepressants sertraline and carbamazepine, the antipsychotics clozapine and quetiapine, and the benzodiazepines zolpidem (Ambien) and oxazepam showed the highest population-normalised mass loads (50–110, 10–75, and 8–78 mg/day/1000 inhabitants, respectively). The remaining compounds ranged between 1–30 mg/day/1000 inhabitants, while others were detected but not quantified. Temporal patterns reveal variability in psychoactive pharmaceutical use, which likely reflects the influence of external factors such as, COVID-19 related public health measures, seasonal trends and changes in regional population dynamics. Forty-one psychoactive pharmaceutical residues were also determined in wastewaters (influent and effluent) of seven Slovene WWTPs differing in size and configuration and receiving surface waters. Results showed that treatment technologies have varying removal efficiencies for psychoactive pharmaceuticals, with activated sludge and membrane bioreactor having the highest (50–60 %) and moving bed biofilm reactor having the lowest efficiency (20 %). Removal efficiency was also compound-dependent, with citalopram, venlafaxine, and oxazepam exhibiting poor removal in all cases. Poor removal (<80 %) of three compounds listed in the revised Urban Waste Water Treatment Directive (carbamazepine, citalopram, and venlafaxine) indicates the need for additional quaternary treatment at common WWTPs. On average, higher removals were found for antipsychotics (73 %), followed by benzodiazepines (45 %) and antidepressants (39 %). Finally, environmental risk assessment revealed that levels of citalopram, sertraline, and venlafaxine could pose a risk to aquatic organisms.

1. Introduction

According to the World Health Organization (WHO), substance dependence and mental health disorders, such as anxiety, depression, post-traumatic stress disorder (PTSD), insomnia, bipolar disorder, and schizophrenia, affect >360 million people worldwide (WHO, 2022). The result is the increasing use of psychoactive pharmaceuticals, including antidepressants, antipsychotics, and benzodiazepines. Additionally, the COVID-19 pandemic profoundly impacted people's lives, which, together with the subsequent lockdowns, is known to have had a significant effect on people's mental health (Diaz-Camal et al., 2022; Laimou-Geraniou et al., 2023b; Pazzagli et al., 2022; Tiger et al., 2023).

In Slovenia, the first epidemic was declared on the 12th of March 2020, followed by the first lockdown (i.e., the closing of all educational institutions, restaurants, bars, and nightlife), prior to a full lockdown on the 20th of March 2020, including the closure of all non-essential activities, moving restrictions to the municipality of residence, limited public gatherings, and a de facto quarantine (Bâldea, 2021). Restrictions were gradually relaxed by May 2020, when Slovenia became the first European country to declare the epidemic officially over (Bâldea, 2021). However, a second wave in October 2020 prompted a new lockdown with the closure of schools, a night-time curfew, a strict ban on all

* Corresponding author.
E-mail address: ester.heath@ijs.si (E. Heath).

https://doi.org/10.1016/j.hazadv.2025.100816
Received 13 April 2025; Received in revised form 24 May 2025; Accepted 7 July 2025
Available online 9 July 2025
2772-4166/© 2025 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

unnecessary commercial activities, and the suspension of most public services and inter-municipality travel. A brief holiday relaxation was followed by a third wave and a partial lockdown in April 2021, during which schools and non-essential businesses and services were again closed. The epidemic officially ended in June 2021 (HSRM, 2021). From mid-June 2021 onwards, although the epidemic was officially declared over in Slovenia, many control measures remained enforced. Despite high case numbers during the upcoming autumn and winter months, a gradual easing of restrictions began in early 2022. By February 2022, most public health mandates were lifted, including travel-related quarantines, and by the end of May 2022, all remaining COVID-19 measures were officially disbanded, signaling a return to pre-pandemic conditions (HSRM, 2021). Finally, in May 2023, the WHO declared an end to the public health emergency of international concern.

Wastewater-based epidemiology (WBE) is a well-established approach for monitoring substance consumption or exposure to various xenobiotics within specific populations by analysing human metabolic excretion products (biomarkers) in influent wastewater (Gracia-Lor et al., 2018; Laimou-Geraniou et al., 2023a). Besides the numerous WBE studies focused on the consumption of alcohol, tobacco, illicit drugs, and new psychoactive substances (Bade et al., 2021; Castiglioni et al., 2021; Estévez-Danta et al., 2022; Gent and Paul, 2021; Gracia-Lor et al., 2020; Huizer et al., 2021; Verovšek et al., 2022, 2020; Zheng et al., 2023), it has been successfully applied to evaluate psychoactive pharmaceutical consumption (Boogaerts et al., 2021, 2019; Laimou-Geraniou et al., 2023a; Li et al., 2024; Quireyns et al., 2022; Rice et al., 2020). Wastewater-based epidemiology also provides valuable insights into the consumption trends of psychoactive pharmaceuticals over time, with high temporal resolution. This information is particularly relevant as weekly patterns can help identify potential recreational use. Moreover, recent studies have utilised WBE to examine the impact of the COVID-19 pandemic's effect on temporal trends in psychoactive pharmaceutical consumption (Boogaerts et al., 2023; Laimou-Geraniou et al., 2023b; Tiger et al., 2023; Tomsone et al., 2022; E. E. Yavuz-Guzel et al., 2022). However, these studies predominantly focus on antidepressants, with fewer looking at benzodiazepines (Adhikari et al., 2023; Bade et al., 2020) or antipsychotics (Kosma et al., 2019).

The widespread use of psychoactive pharmaceuticals has also raised concerns about the presence of their residues in the environment, as, like most drugs, they are excreted from the body in urine and faeces as either parent compounds or metabolites and subsequently enter the sewer system, where they are typically transported to a local wastewater treatment plant (WWTP) (Mohan et al., 2021; Pivetta et al., 2020). Their removal, however, depends on their physicochemical properties, influent concentration, treatment technology, and environmental parameters (Verovšek et al., 2023). In most cases, conventional WWTPs have limited removal efficiency in eliminating such compounds (Jiang et al., 2024; Pivetta et al., 2020; Singh et al., 2022; A. A. Zergui and Nkontcheu Kenko, 2024). Consequently, psychoactive pharmaceutical residues, including parent compounds and metabolites, due to their continuous release, exhibit pseudo persistence in the aquatic environment and are present in receiving waters such as rivers, lakes and oceans and have even been detected in drinking water (Escudero et al., 2021; A. A. Zergui and Nkontcheu Kenko, 2024).

The presence of psychoactive pharmaceutical residues in receiving surface waters, even at low concentrations (ng/L), potentially poses a risk to non-target aquatic organisms, and prolonged exposure can result in chronic ecological and evolutionary impacts (Grabicová et al., 2020; Jiang et al., 2024; Niu et al., 2024; Rathi et al., 2021). Furthermore, their presence may contribute to additive or synergistic effects (Farré et al., 2008). In order to protect the environment and public health from the adverse effects of wastewater discharges from municipal and specific industrial sources, the urban wastewater treatment directive (UWWTD) was recently revised (UWWTD, 1991; UWWTD, 2024), coming into effect on 1.1.2025. Among other adaptations, urban WWTPs will be required to include additional quaternary treatment (time on installation depends on WWTP size) in order to remove >80 % of at least 6 out of 13 listed organic micropollutants (Contaminants of Emerging Concern, CEC). Among these 13 CECs are the antidepressants carbamazepine, citalopram, and venlafaxine, which are also the subject of this study.

Unfortunately, comprehensive data on psychoactive pharmaceutical removal and occurrence in environmental waters remain limited due to the number of residues tested and the diversity of exposed aquatic organisms. Although several studies have investigated the removal of antidepressants and their impact on the aquatic environment (Jiang et al., 2024; Mohan et al., 2021; Niu et al., 2024; Pivetta et al., 2020; Schlüsener et al., 2015; Singh et al., 2022; Wang et al., 2023), few have addressed limited number of antipsychotics (Escudero et al., 2021; Reichert et al., 2019) or benzodiazepines (A. A. Zergui and Nkontcheu Kenko, 2024) and their risk assessment in aquatic ecosystems.

This study evaluates (i) temporal trends in the use of psychoactive pharmaceuticals in Slovenia, including before, during, and after the pandemic, (ii) the removal efficiency of seven Slovenian WWTPs varying in size and configuration, for removing antidepressant, antipsychotic, and benzodiazepine residues; (iii) their occurrence in receiving surface waters, and (iv) the ecological risk posed by measured psychoactive pharmaceutical residue concentrations in receiving surface waters using the Ecological Structure-Activity Relationships (ECOSAR) predictive model (US EPA, 2015).

2. Materials and methods

2.1. Compounds of interest

Forty-one biomarkers of the most widely prescribed psychoactive pharmaceuticals (antidepressants, antipsychotics, and benzodiazepines) in Slovenia, including the parent compounds and their primary metabolites in most cases, were selected for analysis. The selection was based on national prescription frequency and sales data and limited information, especially for antipsychotics and benzodiazepines, concerning their occurrence, removal, and potential impact on the aquatic environment. These included the parent compounds and their primary metabolites. More specifically, 17 residues of antidepressants *(amitriptyline- AMIT, nortriptyline- NORT, bupropion- BUP, hydroxybupropion- BUP-OH, carbamazepine-CBZ, Carbamazepine-10,11-epoxide- CBZ-E, citalopram- CIT, norcitalopram- norCIT, duloxetine- DUX; fluoxetine- FXT, normirtazapine-norMIRT, paroxetine- PAR, sertraline- SER, trazodone- TZD, m-chlorophenylpiperazine- mCPP, venlafaxine- VEN, and O-desmethylvenlafaxine-ODVEN)*, 11 residues of antipsychotics *(aripiprazole- APZ, dehydroaripiprazole- DARI, clozapine- CLOZ, norclozapine- norCLOZ, haloperidol-HAL, quetiapine- QTP, norquetiapine- norQTP, olanzapine- OLZ, desmethylolanzapine- DMO, risperidone- RIS, and paliperidone-PAL)*, and 13 residues of benzodiazepines *(alprazolam- ALP, a-hydroxyalprazolam- a-OH-ALP, bromazepam- BZP, hydroxybromazepam- BZP-OH, clonazepam-CZP, 7-aminoclonazepam- 7-ACL, diazepam- DZP, nordiazepam- norDZP, lorazepam- LOR, oxazepam- OXA, temazepam- TEM, zolpidem- ZOL, and zolpidem-phenyl-carboxylic acid- ZPCA)* were targeted in wastewater (influents and effluents) and receiving surface waters. Mirtazapine was excluded from the bioanalytical assay because it did not meet the method validation requirements due to poor accuracy and precision. However, its consumption can still be assessed through its metabolite, normirtazapine, which met all validation criteria. The stability of antidepressants (Boogaerts et al., 2019; Choi et al., 2020), antipsychotics (Laimou-Geraniou et al., 2025), and benzodiazepines (Bade et al., 2020; Racamonde et al., 2014) has been previously investigated. All details regarding standards, reagents, materials and suppliers are provided in the Supplementary Material (S1. Materials and methods).

2.2. Sampling

Influent wastewater samples were collected from the Ljubljana WWTP, Slovenia (covering approximately 13 % of the Slovenian population), in order to evaluate temporal trends in psychoactive pharmaceutical consumption during seven sampling periods, including summer 2019, winter 2020, spring 2020, winter 2021, spring 2021, spring 2022, and spring 2023 (Table S1). These sampling periods also coincided with the COVID-19 pandemic and the related social restrictions in Slovenia. The pandemic's first wave hit Slovenia in the spring of 2020 (March 2020), the second in the autumn of 2020 (October 2020), and the third in the spring of 2021 (April 2021), while most restrictions had eased during the sampling period of spring 2022, and the WHO declared an end to the public health emergency of international concern. The time-proportional samples (n = 49) were collected over 24 h for seven consecutive days, representing "normal" conditions at the WWTPs, i.e., no special events such as maintenance activities, festivals, or extreme weather events. After transport, all samples were stored at −20 °C until analysis. Details about the WWTP, including flow rates and population equivalents, are presented in Table S1.

In the spring of 2022, in addition to Ljubljana WWTP, psychoactive pharmaceutical residues were also determined in wastewaters (influent and effluent) of six Slovene WWTPs and receiving surface waters to evaluate their occurrence and removal and perform an environmental risk assessment. Seven WWTPs varying in catchment size (25,414–270,305 inhabitants) and type of wastewater treatment technology (activated sludge – AS, sequential batch reactor – SBR, SBR with UV disinfection, membrane bioreactor – MBR, and moving bed biofilm reactor – MBBR) were included in the study (Table S2). For seven consecutive days, 24-h composite wastewater influent (n = 49) and effluent (n = 49) samples were collected using time- or flow-proportional sampling, taking hydraulic-retention times (HRTs) into account. Surface waters (Table S2) varying in hydrological conditions, such as dilution factor (2.17–815), calculated as the ratio between the receiving surface water flow and wastewater effluent flow, were collected (for seven consecutive days) as grab samples (n = 42) approximately 100 m downstream of the WWTPs outflows. Grab sampling is not the most suitable method, as it only represents the conditions at a particular point in time, failing to account for short or long-term concentration variations, which could lead to an inaccurate concentration profile of the studied system (Laimou-Geraniou et al., 2023a). In the case of WWTP 4, the collection of surface water was not possible (Table S2). After transport, all samples were stored at −20 °C until analysis. Full details about the WWTPs and receiving surface waters are given in Table S2.

2.3. Sample preparation and analysis

Pre-concentration of antidepressant, antipsychotic, and benzodiazepine biomarker residues was achieved following the method of Laimou-Geraniou et al. (2025) with minor modifications. Prior to SPE, wastewater (influent: 50 mL, effluent: 250 mL) and receiving surface water (500 mL) samples were spiked with an isotopically labelled internal standard (ISTD) mixture (60 ng/mL in final extract) and acidified (HCl 37 %) to pH 2.

Influent wastewater samples were centrifuged for 25 min at 10,000 rpm to sediment any solid particles, while effluent wastewater and receiving surface water samples were sequentially filtered using GF/D (2.7 μm) and GF/C (1.2 μm) glass microfiber filters (Whatman, USA). Each sample was then loaded on the SPE cartridges (influent wastewater: Oasis Prime MCX, 60 mg/ 3 mL, effluent wastewater and receiving surface water: Oasis Prime MCX, 150 mg/ 6 mL, Waters Milford, MA, USA) and washed with 5 mL of Milli-Q water. After drying the sorbent under vacuum for 30 min, the analytes of interest were eluted with 6 mL of a 0.5 % ammonium solution in methanol. The samples were evaporated to dryness at 35 °C and reconstituted in 200 μL (influent wastewater), 400 μL (effluent wastewater), and 100 μL (surface water) of the starting mobile phase (ultrapure water: methanol (3:1 v/v) + 0.1 % of formic acid). Before injection, the extracts were centrifugally filtered (0.45 μm) for 3 min at 14,000 rpm and transferred to an LC autosampler vial.

All samples were analysed using reverse-phase UPLC (Shimadzu, Kyoto, Japan) coupled to MS/MS (AB Sciex 4500 QTRAP, Sciex, Framingham, MA, USA). Chromatographic separation was achieved using a Phenomenex Kinetex Phenyl-Hexyl (150 × 2.1 mm, 2.6 μm) column (Phenomenex Inc. Torrance, CA, USA) with Mobile Phase A consisted of ultrapure water + 0.1 % formic acid, and Mobile Phase B consisted of methanol + 0.1 % formic acid. A 0.3 mL/min flow rate and an injection volume of 1 μL were used. The chromatographic gradient (total run time 27.1 min) was as follows: 25 % B at 0 min and held for 0.5 min, increase to 80 % B at 13 min, 87 % B at 15 min, 100 % B at 16 min and held for 1 min, then decreased to 25 % B at 17.5 min and kept steady for the final 9.5 min to equilibrate the system. The total run time was 27.1 min.

The mass spectrometer was run in scheduled multiple reaction monitoring (sMRM) mode in positive electrospray ionisation mode (ESI+), with a 60 s retention time window around the expected retention time of each analyte. Retention times, two transitions and the peak area ratios between the transitions were used to identify the targeted psychoactive pharmaceutical residues (European Union Commission Decision 2002/657/EC, 2002), while relative response factors of the analyte to the ISTD were used for quantification (SANTE/11312/2021v2, 2021). Retention times and optimised sMRM conditions of all analytes are presented in the supplementary information (S1.2. Acquisition parameters, Table S3).

2.4. Method validation

The methods were validated by assessing the following parameters: extraction recovery (ER), accuracy, repeatability, matrix effect (ME) at two concentration levels, linearity, limits of detection (LOD), and limits of quantification (LOQ), following the SANTE protocol (SANTE/11312/2021v2, 2021), with minor adjustments (S1.3 Method validation). Deuterated ISTDs, structurally similar to the compounds under investigation, were used to correct for matrix effects. A linear response (R^2 > 0.99) was observed between LOQ–1000 ng/mL (final extract concentration) for all psychoactive pharmaceutical residues. Exceptions were: BUP/BUP-OH (LOQ-750 ng/mL), ZOL/ZPCA (LOQ-500 ng/mL), AMIT (LOQ-750 ng/mL), and 7-ACL (LOQ-500 ng/mL) for influent wastewater (Table S4), BUP/BUP-OH (LOQ-750 ng/mL), HAL (LOQ-750 ng/mL), and 7-ACL (LOQ-500 ng/mL) for effluent wastewater (Table S5), and CBZ-E (LOQ-500 ng/mL), norMIRT (LOQ-500 ng/mL), PAL (LOQ-750 ng/mL), and 7-ACL (LOQ-500 ng/mL) for receiving water (Table S6). For influent wastewater, the LOD ranged between 3.13–10.1 ng/L (except DUX: 27.0 ng/L) and the LOQ between 1.04–33.7 ng/L (except DUX:90.0 ng/L), for effluent wastewater, the LOD ranged between 0.406–9.73 ng/L (except DUX: 26.2 ng/L) and the LOQ between 0.703–16.6 ng/L (except BZP-OH: 32.4 ng/L, and DUX: 87.2 ng/L), while for receiving water, the LOD ranged between 0.0322–0.750 ng/L (except DUX: 7.32 ng/L, BZP-OH: 5.00 ng/L, and LOR: 6.43 ng/L) and the LOQ between 0.107–2.50 ng/L (except DUX: 24.4 ng/L, BZP-OH: 16.7 ng/L, and LOR: 21.4 ng/L). The higher LODs and LOQs in the case of DUX, BZP-OH, and LOR could be due to low ionization efficiency under the applied ESI+ conditions, which leads to weak MS signals and lower sensitivity, especially at low concentrations; as such, the ones used for the determination of LOD and LOQ. For influent wastewater, the relative and absolute extraction recoveries (Tables S4–S6) were 74–114 % and 81–109 %, respectively, and for effluent wastewater ranged between 81 and 115 % and 83–116 %, respectively. For receiving surface water, recoveries were 71–114 % and 74–117 %, respectively. Finally, signal enhancement or suppression (Tables S4–S6) was observed for influent wastewater (ME: −37–22 %), effluent wastewater (ME: −28–13 %), and receiving surface water (ME:

−26–18 %). Despite higher matrix effects in some cases (i.e., paliperidone, norclozapine), good accuracies were obtained for all targeted compounds (85–111 %). Additionally, for all targeted compounds, the RSDs of both relative and absolute extraction recoveries were below the threshold of 20 %, indicating that the ISTDs robustly compensate for matrix effects and losses during sample preparation. All validation results are presented in Tables S4-S6.

2.5. Back-calculations

In the present study, daily mass loads of psychoactive pharmaceutical (antidepressants, antipsychotics, and benzodiazepines) biomarkers were calculated by multiplying their measured concentrations (ng/L) by the corresponding daily influent wastewater flow rates (m^3/day) reported in Table S1. Mass loads were normalised to the population serviced by the Ljubljana WWTP to back-calculate population-normalised mass loads (PNMLs) expressed as mg/day/ 1000 inhabitants. Ljubljana WWTP provided daily flow rates and population equivalents. The psychoactive pharmaceutical consumption was estimated according to the PNMLs as shown in Eq. (1):

$$PNMLs\ (mg/day/\ 1000\ inhabitants) = \frac{concentration\ \left(\frac{mg}{L}\right) \times wastewater\ flow\ rate\ \left(\frac{m^3}{day}\right)}{population\ equivalent} \quad (1)$$

2.6. Determining removal efficiency (RE)

Removal efficiency (RE) was calculated according to Eq. (2):

$$RE(\%) = 100 - \left(\frac{Ceff}{Cinf} \times 100\right) \quad (2)$$

where C_{eff} is the concentration of the target biomarker (ng/L) in wastewater effluent, and C_{inf} is the concentration of the target biomarker (ng/L) in wastewater influent. When the C_{inf} was <LOQ in wastewater influent, the RE was not calculated, whereas when the C_{eff} was <LOQ in wastewater effluent, LOQ was used as a worst-case scenario.

2.7. Predicting environmental concentration of psychoactive pharmaceutical residues in receiving surface waters

An approach adapted from previously published studies (Keller et al., 2014; Verovšek et al., 2023) was employed to predict environmental concentrations of down-the-drain chemicals in surface waters by generating dilution factors. Psychoactive pharmaceutical residue concentrations in receiving surface waters were predicted using these calculated dilution factors (Table S2) alongside the measured concentrations in wastewater effluents (Table S7). The predicted concentrations were then compared with those measured directly in the receiving surface waters (Table S7).

2.8. Environmental risk assessment

An environmental risk assessment (ERA) addressed the ecological threat of psychoactive pharmaceutical residues in receiving surface waters. In the present study, for the characterisation of potential ERA, based on Directive 93/67/EEC on Risk assessment for newly notified substances, Regulation (EC) no. 1488/94 on Risk assessment for existing substances, Directive 98/8/EC concerning the placing of biocidal products on the market (Directive 93/67/EC, 1488/94 and 98/8/EC, 2003), and US Environmental Protection Agency (EPA) guidelines (US EPA, 2024), we used the risk quotient (RQ). The RQ of each psychoactive pharmaceutical residue is the ratio between measured environmental concentration (MEC) and predicted no-effect concentration (PNEC), as shown in Eq. (3):

$$RQ = \frac{MEC}{PNEC} \quad (3)$$

The MEC corresponds to the maximal and average amount of each psychoactive pharmaceutical residue in the receiving surface water. If the measured concentrations were <LOQ, the LOQ was used in the calculation as a worst-case scenario. The PNEC was calculated (i) for acute (short-term) toxicity by dividing the lowest values of the median lethal concentration (LC_{50}) or median effective concentration (EC_{50}) with an assessment factor (AF) and (ii) for chronic (long-term) toxicity, by dividing the values of the no observed effect concentration (NOEC) with the relative AF (Table S8). An AF=1000 was used to assess acute effects, while an AF=50 was used to address chronic risks (European Directive 93/67/EC, 1488/94 and 98/8/EC, 2003). Without experimental data for most of the target psychoactive pharmaceutical residues, the LC_{50} and EC_{50} were predicted (Table S8) using the ECOSAR software (version 2.2). This software is widely used (Singh et al., 2022; Verovšek et al., 2023; Wang et al., 2023) to predict the toxicity of new or untested chemicals based on their structural similarities to substances whose toxicity in the aquatic environment has been estimated. NOEC was calculated by dividing the chronic value (ChV) derived from ECOSAR software with $\sqrt{2}$ (European Directive 93/67/EC, 1488/94 and 98/8/EC, 2003).

Finally, the RQ values expressing risk to aquatic organisms (fish, daphnids, and algae) were compared to the levels of concern (LOC) determined by the US EPA (Table S9) (US EPA, 2024). When RQ>1, there is a potential acute risk to aquatic plants, while when RQ>0.05, there is a potential acute risk to aquatic animals. An RQ>1 indicates a chronic effect on aquatic animals, while no chronic risk assessment was made for aquatic plants.

2.9. Statistical analysis

Univariate and multivariate analysis revealed any differences in RE between the WWTPs (variation between WWTPs) and in the occurrence of psychoactive pharmaceutical residues in different receiving surface waters (variation between rivers). The data were assessed for normality and variance equality using the Shapiro-Wilk and Brown-Forsythe tests, respectively, at a confidence level of 95 % ($\alpha = 0.05$). To evaluate differences between the two groups, a Student *t*-test, where data normality and equality of variance were assumed; a Welch *t*-test, where data normality but equality of variance was not assumed; and a Mann-Whitney Rank Sum Test, where data normality was not assumed, was used. All three were tested at the 95 % confidence level ($\alpha = 0.05$).

To assess temporal differences in the use of psychoactive pharmaceuticals, we employed either One-way ANOVA or a Kruskal-Wallis test for non-parametric analysis, followed by Tukey's post-hoc test. The Mann-Kendall trend analysis was applied to identify monotonic trends in psychoactive pharmaceutical use, indicating an increase (positive S value) or a decrease (negative S value) within the 7-day sampling periods.

Principal component analysis – PCA (unsupervised) was used to

explore the variation within the data set, while orthogonal projection to latent squares-discriminant analysis – OPLS-DA (supervised) was employed to assess the importance of the variables (psychoactive pharmaceutical residues) in the projection (VIP) score at 95 % confidence interval. A permutation test ($n = 100$) was performed to prevent overfitting of the OPLS-DA model. For both PCA and OPLS-DA, data were scaled using Pareto (Par) or unit variance (UV), with logarithmic transformation applied where necessary. Statistical evaluations were conducted using GraphPad Prism 8.4.3, SigmaPlot 14.0, Simca 15.0, and R Studio.

3. Results

3.1. Temporal differences in psychoactive pharmaceutical use

A study on the spatiotemporal trends of 12 antidepressant biomarkers (amitriptyline, bupropion, bupropion-OH, citalopram, norcitalopram, melitracen, moclobemide, normirtazapine, trazodone, mCPP, venlafaxine, and O-desmethylvenlafaxine) in Slovenian wastewaters has been previously conducted (Laimou-Geraniou et al., 2023b). However, antidepressant biomarkers of carbamazepine and its metabolite CBZ-E, fluoxetine, nortriptyline, paroxetine, duloxetine, and sertraline have been additionally investigated in the present study.

Out of the 31 remaining psychoactive pharmaceutical biomarkers investigated, 16 biomarkers, including four antidepressants (CBZ, CBZ-E, SER, and FXT), seven antipsychotics (APZ, CLOZ, norCLOZ, QTP, norQTP, OLZ, and PAL) and five benzodiazepines (norDZP, OXA, TEM, ZOL, and ZPCA) were quantified at least once in Ljubljana WWTP during all sampling periods, and were subsequently selected for temporal analysis. The rest of the psychoactive pharmaceutical biomarkers were below the LOQ and were excluded from further temporal analysis.

The measured PNMLs at the Ljubljana WWTP during all sampling periods can be bound in Table S10. On average the highest PNMLs were found for the antidepressants sertraline (mean±SD: 57.5 ± 18.7 mg/day/1000 inhabitants) and carbamazepine (55.7 ± 16.3 mg/day/1000 inhabitants), for the antipsychotics clozapine (40.2 ± 15.2 mg/day/1000 inhabitants) and quetiapine (15.8 ± 8.2 mg/day/1000 inhabitants), and the benzodiazepines ZPCA (58.0 ± 20.3 mg/day/1000 inhabitants) a zolpidem metabolite, and oxazepam (7.3 ± 2.1 mg/day/1000 inhabitants). These findings align with the yearly prescription data for Slovenia (NIJZ, 2024a; 2024b; ZZZS, 2023). Sertraline, quetiapine, and zolpidem were among the most frequently prescribed psychoactive pharmaceuticals.

3.1.1. Weekly trends: weekends vs weekdays

Psychoactive pharmaceutical consumption is expected to remain stable, as patients need to take their medication consistently and at strictly prescribed intervals. Starting or stopping treatment is typically managed through gradual dosage adjustments. A trend analysis was performed to investigate differences in antidepressant, antipsychotic, and benzodiazepine use between weekdays (Mon-Fri) and weekends (Sat-Sun), revealing a few minor trends; however, no distinct general pattern in the PNMLs emerged. Most statistically significant differences were observed either prior or post to the pandemic. According to Mann-Kendall trend analysis, the negative S-values indicated a negative trend (monotonic decrease) in the PNMLs for clozapine ($S=-13$) (Fig. S1-a), norclozapine ($S=-15$) (Fig. S1-b), and sertraline ($S=-15$) (Fig. S2-d) within the sampling week of summer 2019, for temazepam ($S=-19$) within the sampling week of spring 2023 (Fig. S3-b), and ZPCA ($S=-21$ and $S=-14$) within the sampling week of winter 2020 and spring 2022, respectively (Fig. S3-d). Additionally, during the sampling period of spring 2022, when most restrictions had been eased, a statistically significant decrease ($p < 0.05$) in the PNMLs of quetiapine (Fig. S1-c) and its metabolite norquetiapine (Fig. S1-d) was observed during the weekend.

The only noticeable potential effect of the pandemic, as indicated by a monotonic increase in the PNMLs within the sampling week, was observed for the antidepressant fluoxetine ($S = 7$) and the benzodiazepine temazepam ($S=12$) during the sampling period of winter 2021 (second wave of the pandemic) (Fig. S2-c and Fig S3-b, respectively). On the contrary, a decreasing trend in the PNMLs of the antipsychotic clozapine ($S=-17$) was observed during the same sampling week (Fig. S1-a). Meanwhile, the PNMLs of the other psychoactive pharmaceuticals showed independent distributions across the sampling periods—such a finding suggests consistent daily usage throughout the week (Fig. S1-S3). However, determining if differences are from the pandemic or are a result of other factors, such as population movement, remains challenging.

Throughout the pandemic, social restrictions such as lockdowns, suspension of inter-municipality travel, and remote work likely influenced the population dynamics within the WWTP catchment area, leading to a decrease in PNMLs between weekdays and weekends. Only a few studies (Boogaerts et al., 2023, 2022) used mobile phone data as a dynamic population marker to account for fluctuations in population units during the COVID-19 pandemic. Previously published studies have shown that mobile network data can effectively capture and accurately account for variations in population movement (Baz-Lomba et al., 2019; Thomas et al., 2017). However, in this study, fixed population equivalents reported by the WWTP were used due to the unavailability of real-time mobile population data.

3.1.2. Long-term trends (2019–2023)

Temporal patterns were identified in the usage of psychoactive pharmaceuticals in Ljubljana WWTP (Fig. 1–3). A significant decrease ($p < 0.05$) in the PNMLs of the antipsychotics quetiapine and norquetiapine was observed during the first wave of the pandemic (spring 2020, Fig. 1A and Fig. 1B, respectively). Similar results were found for the benzodiazepine temazepam, which showed a significant decrease of 34 % in consumption during the sampling period of spring 2020 compared to the other sampling periods (Fig. 2A). On the contrary, a significant increase ($p < 0.05$) for the antipsychotics Fig. 1C) and paliperidone (Fig. 1D) was observed during the pandemic, in the winter 2021 sampling period. In particular, olanzapine exhibited a 72 % increase in the PNMLs from 3.2 mg/day/1000 inhabitants to 11.5 mg/day/1000 inhabitants during lockdown in winter 2021 (Fig. 1C).

The antipsychotic aripiprazole exhibited a gradual decrease and became undetectable after the first wave of the pandemic (Fig. 1E). Additionally, ZPCA, the major metabolite of zolpidem revealed a significant ($p < 0.05$) reduction (34 %) in PLMS during the pandemic followed by a notable increase (53 %) in spring 2022 and spring 2023 (Fig. 2C). The data also showed a significant reduction in PNMLs for the benzodiazepine oxazepam from winter 2020 to spring 2020, while it remained relatively stable for the remaining sampling periods (Fig. 2D). In contrast to studies reporting a significant increase antidepressants use during the pandemic (Boogaerts et al., 2023; Laimou-Geraniou et al., 2023b; E. E. Yavuz-Guzel et al., 2022), this study found a significant ($p < 0.05$) decrease in the PNMLs of carbamazepine (Fig. 3A) and its metabolite CBZ-E (Fig. 3B) from winter 2021, i.e., the second wave of pandemic onwards. Sertraline also decreased during the pandemic; however, its PNMLs increased by 50 % in spring 2021 (third pandemic wave) before returning to pre-pandemic levels (Fig. 3C).

The fluctuations in PNMLs could be linked to shifts in the population dynamics during the pandemic, including travel restrictions between municipalities, implementation of work-from-home policies, and decline in tourism activity. Based on the Mobility Report for Slovenia, there was a significant rise in the proportion of time individuals spent in their home municipalities and working remotely, reaching 25 % and 55 %, respectively, during the spring 2020 and winter 2021 lockdowns compared to pre-COVID-19 levels (Ritchie, 2020). Additionally, a notable drop in international tourism, particularly in Ljubljana, may have contributed to these trends, with official records showing a 60–70 % decrease in tourist arrivals between 2019 and 2021.

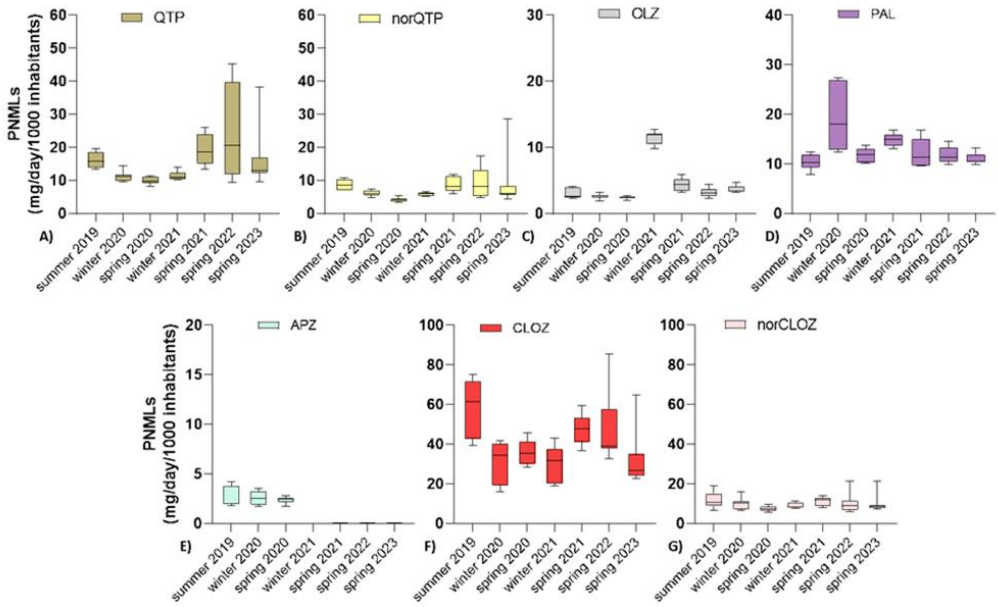


Fig. 1. Boxplots showing the long-term trends in population-normalised mass loads of the antipsychotic biomarkers quetiapine (A), norquetiapine (B), olanzapine (C), paliperidone (D), aripiprazole (E), clozapine (F), and norclozapine (G) in Ljubljana WWTP. Pre-pandemic: summer 2019 and winter 2020; during pandemic: spring 2020, winter 2021, and spring 2021; post pandemic: spring 2022 and spring 2023.

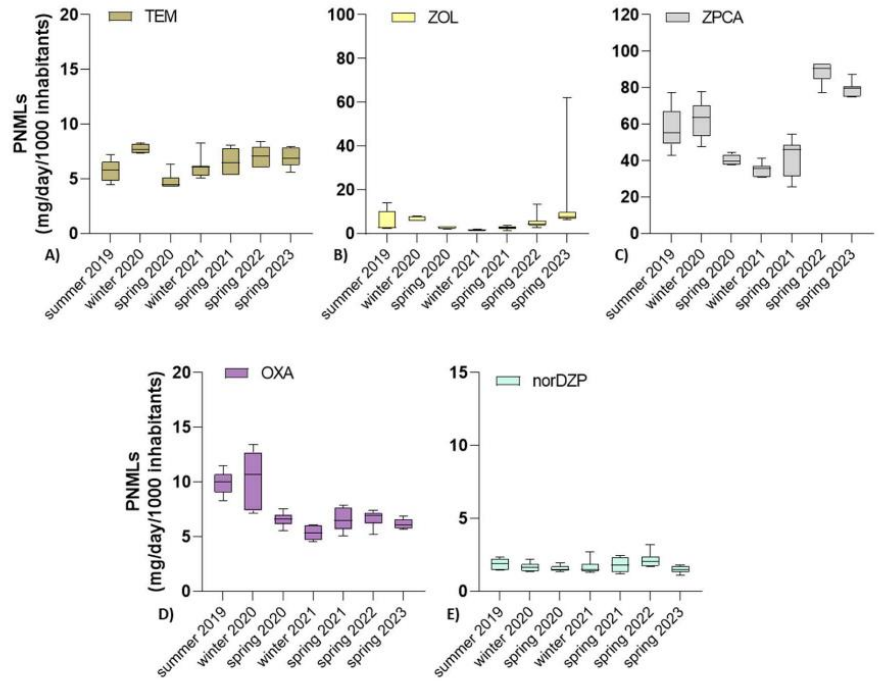


Fig. 2. Boxplots showing the long-term trends in population-normalised mass loads of the benzodiazepine biomarkers temazepam (A), zolpidem (B), ZPCA (C), oxazepam (D), and nordiazepam (E) in Ljubljana WWTP. Pre-pandemic: summer 2019 and winter 2020; during pandemic: spring 2020, winter 2021, and spring 2021; post pandemic: spring 2022 and spring 2023.

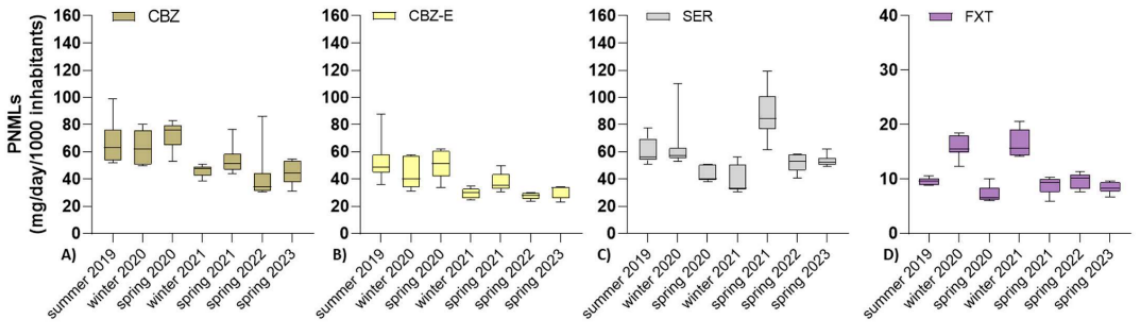


Fig. 3. Boxplots showing the long-term trends in population-normalised mass loads of the antidepressant biomarkers carbamazepine (A), CBZ-E (B), sertraline (C), and fluoxetine (D) in Ljubljana WWTP. Pre-pandemic: summer 2019 and winter 2020; during pandemic: spring 2020, winter 2021, and spring 2021; post pandemic: spring 2022 and spring 2023.

Another notable finding is a significant ($p < 0.0.5$) increase (50 %) in the PNMLs of fluoxetine during winter 2020 and winter 2021 compared to other sampling periods, suggesting possible seasonal variation (Fig. 3D). Seasonal variations have been reported for other antidepressants including venlafaxine, citalopram, and normirtazapine (Golovko et al., 2014; Laimou-Geraniou et al., 2023b; Mackuľak et al., 2016). This seasonal variation may be associated with seasonal affective disorder (SAD), a mood disorder characterised by recurring depressive symptoms in individuals with otherwise stable mental health, typically in winter months due to reduced sunlight exposure.

3.2. Occurrence in wastewaters

In the influent, 35 out of the 41 psychoactive pharmaceutical residues had high detection frequencies (DF ≥80 %), while low DF (≤20) were found for nortriptyline (14 %), aripiprazole (16 %), 7-aminoclonazepam (12 %), and bromazepam (12 %) had (Fig. S4). The amount of psychoactive pharmaceuticals is closely related to their consumption levels and other factors, such as excretion rates (Croft et al., 2020; Laimou-Geraniou et al., 2023a). The DF and the levels of psychoactive pharmaceutical residues in aqueous samples collected in spring 2022 are given in Table S7. The antidepressant residues in the highest concentrations were O-desmethylvenlafaxine (189–496 ng/L), the primary metabolite of venlafaxine, followed by carbamazepine (138–460 ng/L) and sertraline (74.8–313). Notably, significantly higher concentrations of O-desmethylvenlafaxine and carbamazepine compared to other antidepressants have been reported previously (Jiang et al., 2024; Kosma et al., 2020; Niu et al., 2024; Wang et al., 2023; A. A. Zergui and Nkontcheu Kenko, 2024). The most abundant antipsychotics were clozapine (28.5–528 ng/L) and quetiapine (17.2–174 ng/L). (Reichert et al., 2019) reported the highest concentration of haloperidol (1.43—2.73 μg/L), risperidone (0.92—0.98 μg/L), and clozapine (0.56—0.97 μg/L). However, in this study, risperidone (Risperdal) was <LOQ in all WWTPs. Among benzodiazepine residues, ZPCA, a zolpidem (Ambien) metabolite, was in the highest concentration (37.2–509 ng/L), followed by temazepam (Restoril) (16.4–69.2 ng/L) and oxazepam (9.88–46.0 ng/L).

In effluent, nineteen of the 41 psychoactive pharmaceutical residues had a DF >80 %, whereas the antipsychotics olanzapine (10 %) and paliperidone (10 %), and the antidepressants amitriptyline (10 %), fluoxetine (10 %), and paroxetine (10 %) had low DF ≤20 % (Fig S4). Also, thirteen psychoactive pharmaceutical residues, including nortriptyline, duloxetine, aripiprazole, dehydroaripiprazole, desmethylolanzapine, risperidone, alprazolam, a-OH-alprazolam, diazepam, lorazepam, clonazepam, 7-aminoclonazepam, and bromazepam were <LOD in all WWTP effluents. As expected, most psychoactive pharmaceutical residues had lower concentrations in the effluent than in the influent (Table S7). The effluent contained antidepressant residues up to 437 ng/L (O-desmethylvenlafaxine), antipsychotic residues up to 76.3 ng/L (clozapine), and benzodiazepine residues up to 200 ng/L (ZPCA).

3.3. Removal efficiencies

Removal efficiencies were evaluated only for the compounds (n = 28) detected in the influent at a concentration above the LOQ. The REs of the selected psychoactive pharmaceuticals in the WWTPs varied. On average, the REs of antipsychotic residues ranged from 62 % to 88 %, followed by 31 % to 53 % for benzodiazepine residues, while the average REs of antidepressant residues (9 % to 69 %) were the lowest (Table 1). RE has been considered as high (>70 %), moderate (25–70 %), low (<25 %), and negative (<0 %). The highest average REs (>80 %) were observed only for the antipsychotics paliperidone (88 %) and quetiapine (87 %), while the lowest average REs were found for the antidepressants citalopram (24 %) and venlafaxine (17 %), together with their corresponding metabolites norcitalopram (26 %) and O-desmethylvenlafaxine (9 %), respectively (Table 1). Most results agree with published albeit fragmented data (Table 1). However, any discrepancies could be attributed to factors such as different treatment technologies applied, specific operational and design parameters of each WWTP (i.e., hydraulic retention time, sludge retention time, characteristics or activated sludge, pH, and microbial activity during the sampling period), the presence or not of tertiary treatment, wastewater composition, and environmental conditions (Choi et al., 2018; Ofrydopoulou et al., 2022). Furthermore, comparing results across different WWTPs is challenging, not only because of these operational differences but also due to the variability in the efficiency of removal mechanisms such as biodegradation and sludge adsorption.

The negative REs of bupropion-OH, citalopram, normirtazapine, venlafaxine, O-desmethylvenlafaxine, clozapine, norquetiapine, oxazepam, and ZPCA, (Table 1) indicate that these psychoactive pharmaceutical residues were present in higher concentration in effluent wastewater than the influent. Notably, negative REs, especially for antidepressants, have been widely reported (Table 1). They are likely due to the mutual transformation of conjugated metabolites and parent compounds, deconjugation of glucuronide conjugates, and the transformation of parent compound/precursor during wastewater treatment, or by an inadequate matching of influent and effluent wastewater samples, i.e., sampling occurring outside of the HRT range (Gurke et al., 2015; Niu et al., 2024; Verlicchi et al., 2012; Verovšek et al., 2023).

The differences in the RE among the seven WWTPs for the studied psychoactive pharmaceuticals were explored using PCA (Fig. 4A). According to PCA visualisations, only WWTP_6, which employs MBBR biological treatment, formed a distinct cluster separate from the other WWTPs. This finding agrees with the average removal efficiencies that are significantly lower for most of the compounds in WWTP_6 compared to the other WWTPs (Fig. 4B), as shown by an ANOVA. For the

Table 1
Individual antidepressant, antipsychotic, and benzodiazepine residue removal efficiencies, RE (average and range) in the present study and related literature data.

Psychoactive pharmaceutical residue		Average RE (range) [%]	Other studies	Ref.
Antidepressants	Amitriptyline*	35 (28–44)	15, 5, 63	(Gurke et al., 2015; Lopez-Herguedas et al., 2023; Ofrydopoulou et al., 2022)
	Bupropion	42 (1–64)	–	–
	Bupropion-OH	32 (–12–59)	21	(Schlüsener et al., 2015)
	Carbamazepine	42 (8–65)	7, 32, 60, –12, –7, 2, –65, 35, 43, 50	(Afonso-Olivares et al., 2017; Čelić et al., 2019; Collado et al., 2014; Golovko et al., 2014; Gurke et al., 2015; Li et al., 2024; Lopez-Herguedas et al., 2023; Molnarova et al., 2024; Nieto-Juárez et al., 2021; Ofrydopoulou et al., 2022)
	CBZ-E	39 (7–54)	16	(Čelić et al., 2019)
	Citalopram	24 (–44–73)	27, 70, 18, 10, 26, 53, 35, 35	(Čelić et al., 2019; Collado et al., 2014; Golovko et al., 2014; Gurke et al., 2015; Li et al., 2024; Molnarova et al., 2024; Ofrydopoulou et al., 2022; Schlüsener et al., 2015)
	Norcitalopram	26 (3–56)	–13, 42	(Gurke et al., 2015; Schlüsener et al., 2015)
	Fluoxetine*	69 (58–77)	63, 53, 65	(Afonso-Olivares et al., 2017; Čelić et al., 2019; Schlüsener et al., 2015)
	Normirtazapine	47 (–3–79)	–	–
	Paroxetine*	31 (17–47)	20, 76	(Collado et al., 2014; Schlüsener et al., 2015)
	Sertraline	54 (15–74)	50, 81, 79, 82	(Čelić et al., 2019; Golovko et al., 2014; Gornik et al., 2020; Schlüsener et al., 2015)
	Trazodone	52 (23–82)	39, 40	(Čelić et al., 2019; Collado et al., 2014)
	mCPP	48 (2–78)	–	–
	Venlafaxine	17 (–6–48)	10, 25, 1, 8, –81, 39, 18	(Čelić et al., 2019; Collado et al., 2014; Golovko et al., 2014; Gurke et al., 2015; Li et al., 2024; Ofrydopoulou et al., 2022; Schlüsener et al., 2015)
	ODVEN	9 (–8–23)	–31	(Gurke et al., 2015)
Antipsychotics	Clozapine	64 (–31–95)	30	(Gurke et al., 2015)
	Norclozapine	71 (24–95)	–	–
	Haloperidol	62 (25–84)	–	–
	Quetiapine	87 (51–94)	67	(Gurke et al., 2015)
	Norquetiapine	68 (–19–90)	–	–
	Olanzapine*	70 (63–81)	87, 100	(Čelić et al., 2019; Ofrydopoulou et al., 2022)
	Paliperidone*	88 (83–91)	–	–
Benzodiazepines	Bromazepam-OH	42 (28–51)	–	–
	Nordiazepam	41 (5–67)	–	–
	Oxazepam	31 (–11–49)	–17, 12, 33	(Golovko et al., 2014; Molnarova et al., 2024; Ofrydopoulou et al., 2022)
	Temazepam	53 (16–75)	14	(Li et al., 2024)
	Zolpidem	52 (11–81)	47	(Molnarova et al., 2024)
	ZPCA	52 (–2–74)	–	–

* calculated only from one WWTP

Abbreviations: RE– removal efficiency, mCPP– m-chlorophenylpiperazine, ZPCA– zolpidem-phenyl-carboxylic acid.

projection, the difference in removal efficiencies of bupropion-OH, carbamazepine, CBZ-E, clozapine, norclozapine, norquetiapine, temazepam, sertraline, and ZPCA residues was important (OPLS-DA) with significantly lower removals obtained for WWTP_6 (bupropion-OH: $U_{statistic}$=14, $p \leq 0.001$, α=0.05; carbamazepine: $U_{statistic}$=16, $p \leq 0.001$, α=0.05; CBZ-E: $U_{statistic}$=7, $p \leq 0.001$, α=0.05; clozapine: $U_{statistic}$=0, $p \leq 0.001$, α=0.05; norclozapine: $U_{statistic}$=0, $p \leq 0.001$, α=0.05; norquetiapine: $U_{statistic}$=0, $p \leq 0.001$, α=0.05; temazepam: $U_{statistic}$=0, $p \leq 0.001$, α=0.05; sertraline: $U_{statistic}$=2, $p \leq 0.001$, α=0.05; ZPCA: $U_{statistic}$=4, $p \leq 0.001$, α=0.05). Other WWTPs, e.g., WWTP_3 and WWTP_4, form a tight cluster indicating similar treatment efficiencies. Considering that WWTP_3 employs SBR and WWTP_4 employs SBR with UV disinfection, the results indicate that UV treatment does not significantly enhance RE (Fig. 4B). Similar results are also reported elsewhere (Collado et al., 2014; Verovšek et al., 2023; Wang et al., 2023).

It is known that RE of various pollutants, except for the size and type of wastewater received, also depends on the commonly employed wastewater treatment technologies (Jiang et al., 2024; Yadav et al., 2017), and in terms of efficiency is ranked as follows: MBR > AS > trickling filters (Nguyen et al., 2024; Niu et al., 2024; Pérez-Bou et al., 2024; Wang et al., 2023; A. A. Zergui and Nkontcheu Kenko, 2024). The present study observed no difference (clustering) in REs between AS and MBR. Moreover, RE is compound specific (Gurke et al., 2015; Jiang et al., 2024), as confirmed by the results for citalopram, norcitalopram, venlafaxine, O-desmethylvenlafaxine, and oxazepam being poorly removed in all WWTPs (Table S11). It is important to emphasise that

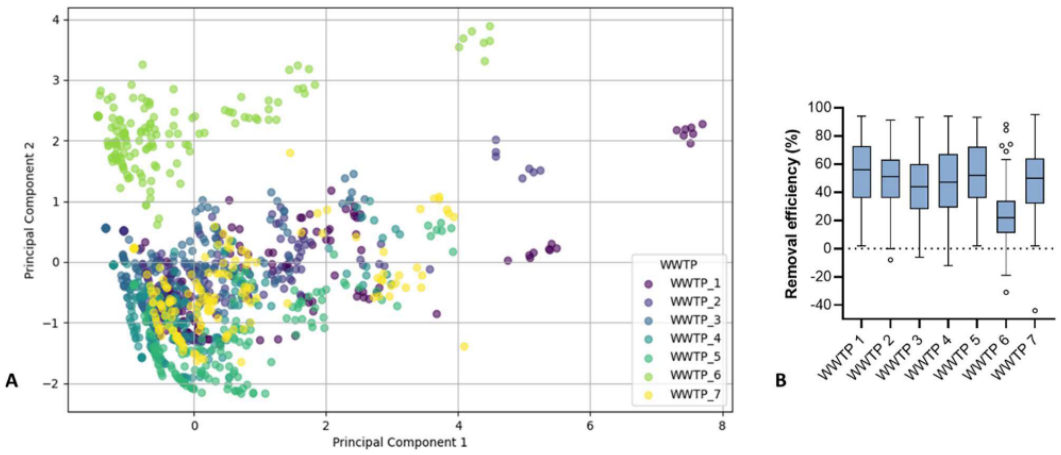


Fig. 4. PCA scatter plot (A) and boxplots (B) show variation in REs' dataset in the studied WWTPs (WWTP1 to WWTP7).

venlafaxine, citalopram, and carbamazepine are among the 13 CECs included in the revised UWWTD. The data on their removal can be found in the literature, but they are fragmented, making a proper comparison difficult (Table 1). In contrast, the present study offers a simultaneous sampling of wastewater from different WWTPs, including different wastewater compositions, sizes, and treatment technologies (AS, MBR, SBR, SBR with UV disinfection, and MBBR). Results showed that REs of carbamazepine, citalopram, and venlafaxine, together with their corresponding metabolites, were in all cases <80 %. Considering that the UWWTD mandates six out of the 13 CECs to have removal efficiencies >80 %, our comprehensive study (seven WWTPs with different sizes and configurations) shows that carbamazepine, citalopram, and venlafaxine do not reach this criterion for all involved WWTPs indicating the need for additional quaternary treatment of common urban WWTPs.

Finally, although MBBR treatment technology has been previously studied on drugs of abuse and illicit drugs (Verovšek et al., 2023), this is believed to be the first time MBBR was studied for the removal of psychoactive pharmaceuticals, indicating poor REs for three antidepressant residues (bupropion-OH, carbamazepine, CBZ-E) three antipsychotic residues (clozapine, norclozapine, norquetiapine) and three benzodiazepine residues (temazepam, sertraline, ZPCA).

3.4. Occurrence in receiving surface waters

The presence of psychoactive pharmaceutical residues in receiving surface waters (Table S7) is expected due to their occurrence in WWTP effluent. Sixteen out of 41 psychoactive pharmaceutical residues had high average DF >80 % (Fig S5), while low average DF (<20 %, Fig S5) was obtained for bupropion, fluoxetine, haloperidol, olanzapine, paliperidone, and bromazepam-OH. Thirteen psychoactive pharmaceutical residues, including two antidepressants (nortriptyline and fluoxetine), four antipsychotics (aripiprazole, dehydroaripiprazole, desmethylolanzapine, and risperidone), and seven benzodiazepines (alprazolam, a-OH-alprazolam, diazepam, lorazepam, clonazepam, 7-aminoclonazepam, and bromazepam) residues were < LOD in the tested receiving surface waters (Fig S5). As expected, lower levels of psychoactive pharmaceutical residues were detected in receiving surface waters than in the effluents (Table S7) due to dilution (Table S2). However, higher DFs in receiving surface waters (100 %) than in the effluent (71 %) were found for amitriptyline, fluoxetine, olanzapine, and paliperidone (Fig S5, Table S7). These results can be explained by the much lower LOD/LOQ (2–21 fold) obtained for surface waters (Table S6). In general, in receiving surface waters, antidepressant residues were detected in

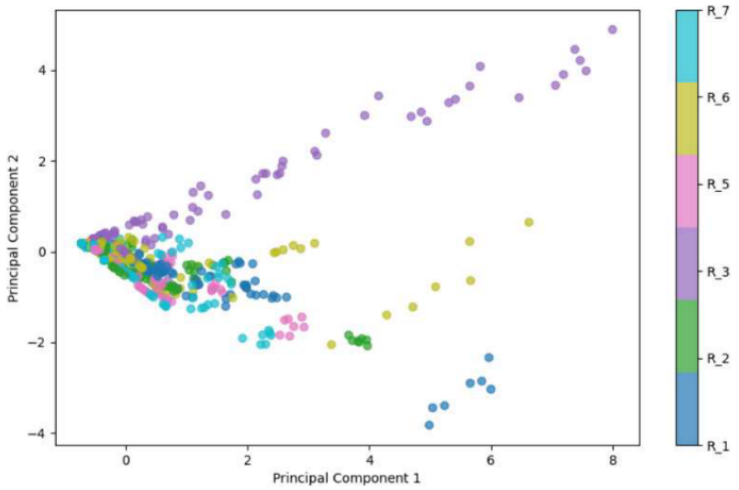


Fig. 5. Scatter plot (PCA) of samples with data on the occurrence of psychoactive pharmaceutical residues in the studied rivers (R_1 to R_7).

concentrations up to 102 ng/L (O-desmethylvenlafaxine), antipsychotic residues in concentrations up to 24.4 ng/L (clozapine), and residues of benzodiazepines in concentrations up to 12.7 ng/L (ZPCA) (Table S7). The presence of psychoactive pharmaceutical residues, mainly antidepressants, in river waters has also been reported by (Liang et al., 2021; Ma et al., 2020; Petrie and Moffat, 2022; Pivetta et al., 2020; Schlüsener et al., 2015; Singh et al., 2022; Wang et al., 2023; Wu et al., 2017).

The variations in the occurrence of psychoactive pharmaceutical residues were explored in six different rivers with different dilution factors using multi-variant analysis. The PCA visualisations (Fig. 5) show how R_3 (WWTP_3) and R_6 (WWTP_6) form separate clusters. For the projection, the difference in the occurrence of bupropion-OH, carbamazepine, citalopram, norcitalopram, venlafaxine, O-desmethylvenlafaxine, clozapine, norclozapine, quetiapine, and norquetiapine residues was important (OPLS-DA), with significantly higher concentrations measured in R_3 and R_6 (Table S12). These results are likely due to a lower dilution factor of R_3 (1.97–2.35) and R_6 (4.44–5.37) compared to other studied rivers (17.6–1004) along with the lower REs, enhancing the impact of effluent wastewater on the quality of receiving surface waters.

3.5. Measured vs predicted values of psychoactive pharmaceutical residues in surface waters

A comparison between measured vs estimated levels of psychoactive pharmaceutical residues in receiving surface waters showed that the variations fall within an order of magnitude, with an average ratio of 17.20 between the measured and estimated values. With a few exceptions, the measured values were higher than the predicted values (Fig S6-S11), likely due to sampling-related factors. Surface water sampling from the riverbank does not fully capture the complex hydrodynamics of the river section. Additionally, the surface water samples were collected as grab samples, which makes getting a representative sample more challenging (Verovšek et al., 2023).

This assumption is supported by the discrepancies between measured and estimated concentrations, which are more noticeable in rivers with higher flow rates. Other factors include the representativeness of the selected monitoring stations and uncertainties in the river discharge data. The most significant discrepancies were observed at WWTP_2 and WWTP_7, where dilution is highest (WWTP_2: 376–1004, WWTP_7: 335–882; other WWTPs: 1.97–236), and the closest accessible hydrological stations are situated approximately 20 and 10 km downstream, accordingly. At these two sites, the most significant differences between measured and estimated values were recorded, with a maximum measured-to-estimated ratio of 111 for R_2 and 65.3 for R_7. Despite a few variations, the findings suggest that predicting environmental concentrations can be a viable alternative to surface water analysis when reliable river flow data are available. This approach can be especially beneficial in locations where sampling is challenging or where analytical costs are a concern.

3.6. Environmental risk assessment

The concern regarding the increased use of psychoactive pharmaceuticals and their occurrence in surface waters is the uncertainty surrounding their potential toxicological impact on aquatic organisms. Although several studies have been published on the presence of psychoactive pharmaceuticals in aqueous matrices, only a limited number have conducted a risk assessment, focusing mainly on antidepressants (Moreira et al., 2022; Singh et al., 2022; Wang et al., 2023; Wu et al., 2017) and less on antipsychotics (Kosma et al., 2020; Reichert et al., 2019) or benzodiazepines (Ofrydopoulou et al., 2022). The acute and chronic toxicity of psychoactive pharmaceutical residues, including parent compounds and corresponding metabolites in most cases, was predicted using the ECOSAR software, similar to other studies (Singh et al., 2022; Verovšek et al., 2023; Wang et al., 2023), as experimental data were not always available for most of the targeted compounds, especially for antipsychotics and benzodiazepines. Risk quotients corresponding to maximum and mean concentrations of the target psychoactive pharmaceutical residues in the receiving surface waters are given in Tables S13-S19. While interactions among different psychoactive pharmaceuticals may lead to additive and synergistic effects (Farré et al., 2008), this study focused solely on predicting the effects of individual psychoactive pharmaceutical residues.

The results showed that none of the targeted compounds, at average or maximal measured concentrations, pose an acute risk for aquatic plants. However, among the antidepressant residues, acute effects to aquatic animals were predicted for O-desmethylvenlafaxine and sertraline at average (ODVEN: 36.8–90.3 ng/L; RQ average= 0.07–0.18 and SER: 3.86–6.11ng/L; RQ average= 0.06–0.08) and maximal (ODVEN: 31.6–102 ng/L; RQ max= 0.06–0.20 and SER: 3.62–6.67 ng/L; RQ max= 0.05–0.09) measured concentrations in the receiving rivers R_1, R_3 and R_6. Additionally, in R_3, acute risks for aquatic animals were predicted for venlafaxine at maximal (61.6 ng/L; RQ max= 0.06) measured concentration and for citalopram at average (69.1 ng/L; RQaverage=0.11) and maximal (79.2 ng/L; RQ max=0.12) measured concentrations. Other studies (Kosma et al., 2020; Ofrydopoulou et al., 2022; Papageorgiou et al., 2019) that used values of LC_{50}/EC_{50} found from available data reported in the literature also reported acute effects of venlafaxine, sertraline, and citalopram on aquatic organisms. Finally, among the antipsychotic and benzodiazepine residues, no acute effects on aquatic animals were predicted for the measured concentrations. In contrast, Reichert et al. (2019) reported high environmental risk (RQ>1) for the antipsychotics clozapine and risperidone, indicating high persistence, bioaccumulation and toxicity. Considering that venlafaxine, O-desmethylvenlafaxine, citalopram, and sertraline could affect aquatic animals at the levels detected in the studied rivers, monitoring and regulatory actions may be warranted.

3.7. Limitations of the study

A limitation of this study is that the temporal evaluation was based on only one week of sampling within each period (i.e., summer 2019, winter/spring 2020, winter/spring 2021, spring 2022, and spring 2023). As a result, these weekly snapshots may not fully capture broader annual consumption patterns. Nevertheless, since all sampling campaigns were conducted during a "typical" week, intended to reflect standard weekday and weekend consumption, the results are assumed to represent baseline consumption levels during a regular working week. Additionally, not sampling consistently during the same season each year complicated the identification and interpretation of seasonal variations.

Another limitation lies in using fixed population units, provided by the WWTPs, to back-calculate psychoactive pharmaceutical consumption. Population dynamics likely shifted during the pandemic due to changes in commuting behavior and tourism, which may have influenced observed mass loads. Increased loads could reflect a rise in the number of users, greater consumption quantities, or both. While using dynamic population markers is recommended to reduce such uncertainties (Boogaerts et al., 2023), we could not integrate mobile phone tracking data due to restrictions in data access. Similarly, alternative markers such as nitrogen or phosphorus were unsuitable for this study, as the wastewater included discharges from agricultural sources, which could bias population estimates.

4. Conclusion

The present study highlighted the potential of WBE as an effective tool for monitoring trends in antidepressant, antipsychotic, and benzodiazepine use with a high level of temporal resolution, surpassing other conventional socio-epidemiological sources. Although the study revealed several temporal variations, no clear patterns associated with the pandemic were observed between weekdays and weekends, as most

statistically significant differences observed were before or after the pandemic. Only the biomarkers fluoxetine, temazepam, and clozapine followed a monotonic trend (increase or decrease) within the 7-day sampling period (Monday to Sunday) of winter 2021, which coincided with the pandemic. Among the psychoactive pharmaceuticals detected, sertraline, clozapine, and ZPCA exhibited the highest PNMLs for antidepressants, antipsychotics, and benzodiazepines, respectively. Overall, the PNMLs of most psychoactive pharmaceuticals selected for temporal analysis showed a decreasing trend during the pandemic, particularly during the first wave in spring 2020.

Although the presence of psychoactive pharmaceutical residues in wastewater is well-known, comprehensive information on their removal efficiencies and related occurrence in surface waters, particularly concerning their ecotoxicity, remains limited. This study addressed these gaps by evaluating the efficiency of different biological treatments (AS, SBR, SBR with UV, MBR, and MBBR) in eliminating known psychoactive pharmaceutical residues from wastewater. It also assessed their occurrence in downstream surface waters, using LC-MS/MS analysis, and compared them to predicted values. The potential ecological risks of the detected psychoactive pharmaceutical residues were further estimated using ECOSAR prediction software. The study showed that the different treatment technologies remove psychoactive pharmaceuticals to differing degrees, with AS and MBR being the most efficient and MBBR the least efficient. It also revealed how removal is compound dependent, with, e.g., oxazepam, venlafaxine/O-desmethylvenlafaxine, and citalopram/norcitalopram being poorly removed (average RE< 40 %) in all cases. Overall, the highest average REs (>80 %) were observed only for the antipsychotics paliperidone (88 %) and quetiapine (87 %). On average, better removals were found for antipsychotics (REs=73 %), followed by benzodiazepines (REs=45 %), then antidepressants (REs=39 %). Furthermore, the study revealed that applied treatment technologies also impact the levels of psychoactive pharmaceutical residues in the receiving surface water. Comparable results were obtained through water analysis and the prediction of environmental concentrations. In addition, ECOSAR predicted EC_{50} values suggest that determined levels of venlafaxine, O-desmethylvenlafaxine, citalopram, and sertraline residues in the studied rivers R_1, R_3, and R_6 may pose an acute toxicity risk to aquatic animals in the receiving waters.

The findings highlighted the need for targeted monitoring and regulatory measures to address specific psychoactive pharmaceutical residues in receiving surface waters and associated environmental risks. The study also highlights the need for enhanced wastewater treatment, aligning with the revised Urban Wastewater Treatment Directive, which mandates quaternary treatment for WWTPs of a certain size when the removal efficiency for specific CECs, including carbamazepine, citalopram, and venlafaxine, analysed in this study, is below 80 %. Based on their proven efficiency in removing persistent psychoactive residues, quaternary technologies such as ozonation and activated carbon adsorption are recommended. Ozonation is particularly suitable for large-scale WWTPs, while PAC dosing offers a flexible and cost-effective solution for smaller plants. These technologies are aligned with the revised Urban Wastewater Treatment Directive and could be feasible for Slovenian WWTPs, particularly when adapted to WWTP size, infrastructure, and regional funding capabilities. Nevertheless, future research should expand the list of target compounds and incorporate suspect screening and non-targeted analysis to better understand the cycling and effects of psychoactive substances (including their metabolites and transformation products) in wastewater and receiving waters.

Funding

This study was supported by the Slovenian Research Agency (ARRS Program group P1–0143 and ARIS-FWO project N1–0143, L7–4422, J2–4427 and J2–50,047) and involved WWTPs.

CRediT authorship contribution statement

Maria Laimou-Geraniou: Writing – original draft, Software, Formal analysis, Validation, Investigation, Visualization, Methodology, Data curation. **Tjaša Kos:** Formal analysis. **David Heath:** Supervision, Writing – review & editing, Methodology. **Ester Heath:** Resources, Funding acquisition, Supervision, Investigation, Writing – review & editing, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Dr. Tina Kosjek for her valuable guidance on analytical method optimisation. This study was supported by the Slovenian Research Agency (ARRS Program group P1–0143 and ARIS-FWO project N1–0143, L7–4422, J2–4427 and J2–50047), University of Ljubljana interdisciplinary preparative (project Nanostructurome 802–12/2024–5), and involved WWTPs.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hazadv.2025.100816.

Data availability

Data will be made available on request.

References

Adhikari, S., Kumar, R., Driver, E.M., Bowes, D.A., Ng, K.T., Sosa-Hernandez, J.E., Oyervides-Muñoz, M.A., Melchor-Martínez, E.M., Martínez-Ruiz, M., Coronado-Apodaca, K.G., Smith, T., Bhatnagar, A., Piper, B.J., McCall, K.L., Parra-Saldivar, R., Barron, L.P., Halden, R.U., 2023. Occurrence of Z-drugs, benzodiazepines, and ketamine in wastewater in the United States and Mexico during the Covid-19 pandemic. Sci. Total Environ. 857, 159351. https://doi.org/10.1016/j.scitotenv.2022.159351.

Afonso-Olivares, C., Sosa-Ferrera, Z., Santana-Rodríguez, J.J., 2017. Occurrence and environmental impact of pharmaceutical residues from conventional and natural wastewater treatment plants in Gran Canaria (Spain). Sci. Total Environ. 599–600, 934–943. https://doi.org/10.1016/j.scitotenv.2017.05.058.

Bade, R., Ghetia, M., White, J.M., Gerber, C., 2020. Determination of prescribed and designer benzodiazepines and metabolites in influent wastewater. Anal. Methods 12, 3637–3644. https://doi.org/10.1039/D0AY00560F.

Bade, R., White, J.M., Chen, J., Baz-Lomba, J.A., Been, F., Bijlsma, L., Burgard, D.A., Castiglioni, S., Salgueiro-Gonzalez, N., Celma, A., Chappell, A., Emke, E., Steenbeek, R., Wang, D., Zuccato, E., Gerber, C., 2021. International snapshot of new psychoactive substance use: case study of eight countries over the 2019/2020 new year period. Water Res. 193, 116891. https://doi.org/10.1016/j.watres.2021.116891.

Bâldea, I., 2021. What Can We Learn from the Time Evolution of COVID-19 Epidemic in Slovenia? Adv. Theory Simul. 4, 2000225. https://doi.org/10.1002/adts.202000225.

Baz-Lomba, J.A., Di Ruscio, F., Amador, A., Reid, M., Thomas, K.V., 2019. Assessing Alternative Population Size Proxies in a Wastewater Catchment Area Using Mobile Device Data. Environ. Sci. Technol. 53, 1994–2001. https://doi.org/10.1021/acs.est.8b05389.

Boogaerts, T., Ahmed, F., Choi, Phil.M., Tscharke, B., O'Brien, J., De Loof, H., Gao, J., Thai, P., Thomas, K., Mueller, J.F., Hall, W., Covaci, A., van Nuijs, A.L.N., 2021. Current and future perspectives for wastewater-based epidemiology as a monitoring tool for pharmaceutical use. Sci. Total Environ. 789, 148047. https://doi.org/10.1016/j.scitotenv.2021.148047.

Boogaerts, T., Degreef, M., Covaci, A., van Nuijs, A.L.N., 2019. Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach. Talanta 200, 340–349. https://doi.org/10.1016/j.talanta.2019.03.052.

Boogaerts, T., Quireyns, M., De Loof, H., Bertels, X., Van Wichelen, N., Pussig, B., Saevels, J., Lahousse, L., Bonmariage, P., Hamelinck, W., Aertgeerts, B., Covaci, A., van Nuijs, A.L.N., 2023. Do the lockdown-imposed changes in a wastewater treatment plant catchment's socio-demographics impact longitudinal temporal

trends in psychoactive pharmaceutical use? Sci. Total Environ. 876, 162342. https://doi.org/10.1016/j.scitotenv.2023.162342.
Boogaerts, T., Quireyns, M., De prins, M., Pussig, B., De Loof, H., Matheï, C., Aertgeerts, B., Van Coppenolle, V., Fransen, E., Covaci, A., van Nuijs, A.L.N., 2022. Temporal monitoring of stimulants during the COVID-19 pandemic in Belgium through the analysis of influent wastewater. Int. J. Drug Policy 104, 103679. https://doi.org/10.1016/j.drugpo.2022.103679.
Castiglioni, S., Salgueiro-González, N., Bijlsma, L., Celma, A., Gracia-Lor, E., Beldean-Galea, M.S., Mackuľak, T., Emke, E., Heath, E., Kasprzyk-Hordern, B., Petkovic, A., Poretti, F., Rangelov, J., Santos, M.M., Sremački, M., Styszko, K., Hernández, F., Zuccato, E., 2021. New psychoactive substances in several European populations assessed by wastewater-based epidemiology. Water Res. 195, 116983. https://doi.org/10.1016/j.watres.2021.116983.
Čelić, M., Gros, M., Farré, M., Barceló, D., Petrović, M., 2019. Pharmaceuticals as chemical markers of wastewater contamination in the vulnerable area of the Ebro Delta (Spain). Sci. Total Environ. 652, 952–963. https://doi.org/10.1016/j.scitotenv.2018.10.290.
Choi, J.-W., Zhao, Y., Bediako, J.K., Cho, C.-W., Yun, Y.-S., 2018. Estimating environmental fate of tricyclic antidepressants in wastewater treatment plant. Sci. Total Environ. 634, 52–58. https://doi.org/10.1016/j.scitotenv.2018.03.278.
Choi, P.M., Li, J., Gao, J., O'Brien, J.W., Thomas, K.V., Thai, P.K., Jiang, G., Mueller, J.F., 2020. Considerations for assessing stability of wastewater-based epidemiology biomarkers using biofilm-free and sewer reactor tests. Sci. Total Environ. 709, 136228. https://doi.org/10.1016/j.scitotenv.2019.136228.
Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I., Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. Environ. Pollut. 185, 202–212. https://doi.org/10.1016/j.envpol.2013.10.040.
Croft, T.L., Huffines, R.A., Pathak, M., Subedi, B., 2020. Prevalence of illicit and prescribed neuropsychiatric drugs in three communities in Kentucky using wastewater-based epidemiology and Monte Carlo simulation for the estimation of associated uncertainties. J. Hazard. Mater. 384, 121306. https://doi.org/10.1016/j.jhazmat.2019.121306.
Diaz-Camal, N., Cardoso-Vera, J.D., Islas-Flores, H., Gómez-Oliván, L.M., Mejía-García, A., 2022. Consumption and ocurrence of antidepressants (SSRIs) in pre- and post-COVID-19 pandemic, their environmental impact and innovative removal methods: a review. Sci. Total Environ. 829, 154656. https://doi.org/10.1016/j.scitotenv.2022.154656.
Escudero, J., Muñoz, J.L., Morera-Herreras, T., Hernandez, R., Medrano, J., Domingo-Echaburu, S., Barceló, D., Orive, G., Lertxundi, U., 2021. Antipsychotics as environmental pollutants: an underrated threat? Sci. Total Environ. 769, 144634. https://doi.org/10.1016/j.scitotenv.2020.144634.
Estévez-Danta, A., Bijlsma, L., Capela, R., Cela, R., Celma, A., Hernández, F., Lertxundi, U., Matias, J., Montes, R., Orive, G., Prieto, A., Santos, M.M., Rodil, R., Quintana, J.B., 2022. Use of illicit drugs, alcohol and tobacco in Spain and Portugal during the COVID-19 crisis in 2020 as measured by wastewater-based epidemiology. Sci. Total Environ. 836, 155697. https://doi.org/10.1016/j.scitotenv.2022.155697.
European Directive 93/67/EC, 1488/94 and 98/8/EC, 2003. Technical Guidance Document on risk assessment in support of Commission Directive 93/67/EEC on risk assessment for new notified substances, Commission Regulation (EC) No 1488/94 on risk assessment for existing substances, Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Part III [WWW Document]. URL https://op.europa.eu/en/publication-detail/-/publication/212940b8-3e55-43f8-8448-ba258d0374bb (accessed 1.29.25).
European Union Commission Decision 2002/657/EC, 2002. European Union Commission Decision 2002/657/EC. Off. J. Eur. Commun. 45, 8–36.
Farré, M.la, Pérez, S., Kantiani, L., Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC Trends Analy. Chem., Adv. MS Analy. Metabolites and Degradation Prod. - II 27, 991–1007. https://doi.org/10.1016/j.trac.2008.09.010.
Gent, L., Paul, R., 2021. The detection of new psychoactive substances in wastewater. A comprehensive review of analytical approaches and global trends. Sci. Total Environ. 776, 146028. https://doi.org/10.1016/j.scitotenv.2021.146028.
Golovko, O., Kumar, V., Fedorova, G., Randak, T., Grabic, R., 2014. Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant. Chemosphere 111, 418–426. https://doi.org/10.1016/j.chemosphere.2014.03.132.
Gornik, T., Kovacic, A., Heath, E., Hollender, J., Kosjek, T., 2020. Biotransformation study of antidepressant sertraline and its removal during biological wastewater treatment. Water Res. 181, 115864. https://doi.org/10.1016/j.watres.2020.115864.
Grabicová, K., Grabic, R., Fedorova, G., Kolářová, J., Turek, J., Brooks, B.W., Randák, T., 2020. Psychoactive pharmaceuticals in aquatic systems: a comparative assessment of environmental monitoring approaches for water and fish. Environ. Pollut. 261, 114150. https://doi.org/10.1016/j.envpol.2020.114150.
Gracia-Lor, E., Rousis, N.I., Hernández, F., Zuccato, E., Castiglioni, S., 2018. Wastewater-Based Epidemiology as a Novel Biomonitoring Tool to Evaluate Human Exposure To Pollutants. Environ. Sci. Technol. 52, 10224–10226. https://doi.org/10.1021/acs.est.8b01403.
Gracia-Lor, E., Rousis, N.I., Zuccato, E., Castiglioni, S., 2020. Monitoring caffeine and nicotine use in a nationwide study in Italy using wastewater-based epidemiology. Sci. Total Environ. 747, 141331. https://doi.org/10.1016/j.scitotenv.2020.141331.
Gurke, R., Rößler, M., Marx, C., Diamond, S., Schubert, S., Oertel, R., Fauler, J., 2015. Occurrence and removal of frequently prescribed pharmaceuticals and corresponding metabolites in wastewater of a sewage treatment plant. Sci. Total Environ. 532, 762–770. https://doi.org/10.1016/j.scitotenv.2015.06.067.
HSRM, 2021. COVID-19 Health System Response Monitor: European Observatory On Health Systems and Policies: [WWW Document]. URL. https://eurohealthobservatory.who.int/monitors/hsrm/hsrm-countries. accessed 5.20.25.
Huizer, M., ter Laak, T.L., de Voogt, P., van Wezel, A.P., 2021. Wastewater-based epidemiology for illicit drugs: a critical review on global data. Water Res. 207, 117789. https://doi.org/10.1016/j.watres.2021.117789.
Jiang, T., Wu, W., Ma, M., Hu, Y., Li, R., 2024. Occurrence and distribution of emerging contaminants in wastewater treatment plants: a globally review over the past two decades. Sci. Total Environ. 951, 175664. https://doi.org/10.1016/j.scitotenv.2024.175664.
Keller, V.D.J., Williams, R.J., Lofthouse, C., Johnson, A.C., 2014. Worldwide estimation of river concentrations of any chemical originating from sewage-treatment plants using dilution factors. Environ. Toxicol. Chem. 33, 447–452. https://doi.org/10.1002/etc.2441.
Kosma, C.I., Kapsi, M.G., Konstas, P.-S.G., Trantopoulos, E.P., Boti, V.I., Konstantinou, I. K., Albanis, T.A., 2020. Assessment of multiclass pharmaceutical active compounds (PhACs) in hospital WWTP influent and effluent samples by UHPLC-Orbitrap MS: temporal variation, removals and environmental risk assessment. Environ. Res. 191, 110152. https://doi.org/10.1016/j.envres.2020.110152.
Kosma, C.I., Nannou, C.I., Boti, V.I., Albanis, T.A., 2019. Psychiatrics and selected metabolites in hospital and urban wastewaters: occurrence, removal, mass loading, seasonal influence and risk assessment. Sci. Total Environ. 659, 1473–1483. https://doi.org/10.1016/j.scitotenv.2018.12.421.
Laimou-Geraniou, M., Heath, D., Heath, E., 2023a. Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology. Trends Environ. Analy. Chem. 37, e00192. https://doi.org/10.1016/j.teac.2022.e00192.
Laimou-Geraniou, M., Quireyns, M., Boogaerts, T., Heath, D., Covaci, A., van Nuijs, A.L. N., Heath, E., 2025. Stability of antipsychotic biomarkers for wastewater-based epidemiology. J. Pharm. Biomed. Anal. 257, 116702. https://doi.org/10.1016/j.jpba.2025.116702.
Laimou-Geraniou, M., Quireyns, M., Boogaerts, T., Van Wichelen, N., Heath, D., van Nuijs, A.L.N., Covaci, A., Heath, E., 2023b. Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters. Sci. Total Environ. 903, 166586. https://doi.org/10.1016/j.scitotenv.2023.166586.
Li, Z., Li, J., Hu, Y., Yan, Y., Tang, S., Ma, R., Li, L., 2024. Evaluation of pharmaceutical consumption between urban and suburban catchments in China by wastewater-based epidemiology. Environ. Res. 250, 118544. https://doi.org/10.1016/j.envres.2024.118544.
Liang, J., Meng, H., Zhou, J., Yin, Y., Zhen, H., Zhao, Y., Zhang, K., 2021. Simultaneous Occurrence of Psychotropic Pharmaceuticals in Surface Water of the Megacity Shanghai and Implication for Their Ecotoxicological Risks. ACS EST Water 1, 825–836. https://doi.org/10.1021/acsestwater.0c00174.
Lopez-Herguedas, N., Irazola, M., Alvarez-Mora, I., Orive, G., Lertxundi, U., Olivares, M., Zuloaga, O., Prieto, A., 2023. Comprehensive micropollutant characterization of wastewater during Covid-19 crisis in 2020: suspect screening and environmental risk prioritization strategy. Sci. Total Environ. 873, 162281. https://doi.org/10.1016/j.scitotenv.2023.162281.
Ma, R., Qu, H., Wang, B., Wang, F., Yu, G., 2020. Widespread monitoring of chiral pharmaceuticals in urban rivers reveals stereospecific occurrence and transformation. Environ. Int. 138, 105657. https://doi.org/10.1016/j.envint.2020.105657.
Mackuľak, T., Birošová, L., Gál, M., Bodík, I., Grabic, R., Ryba, J., Škubák, J., 2016. Wastewater analysis: the mean of the monitoring of frequently prescribed pharmaceuticals in Slovakia. Environ. Monit. Assess. 188, 18. https://doi.org/10.1007/s10661-015-5011-7.
Mohan, H., Rajput, S.S., Jadhav, E.B., Sankhla, M.S., Sonone, S.S., Jadhav, S., Kumar, R., 2021. Ecotoxicity, occurrence, and removal of pharmaceuticals and illicit drugs from aquatic systems. Biointerface Res. Appl. Chem. 11, 12530–12546. https://doi.org/10.33263/BRIAC115.1253012546.
Molnarova, L., Halesova, T., Tomesova, D., Vaclavikova, M., Bosakova, Z., 2024. Monitoring Pharmaceuticals and Personal Care Products in Healthcare Effluent Wastewater Samples and the Effectiveness of Drug Removal in Wastewater Treatment Plants Using the UHPLC-MS/MS Method. Molecules 29, 1480. https://doi.org/10.3390/molecules29071480.
Moreira, D.G., Aires, A., de Lourdes Pereira, M., Oliveira, M., 2022. Levels and effects of antidepressant drugs to aquatic organisms. Comparative Biochem. Physiol. Part C: Toxicol. Pharmacol. 256, 109322. https://doi.org/10.1016/j.cbpc.2022.109322.
Nguyen, M.-K., Lin, C., Bui, X.-T., Rakib, Md.R.J., Nguyen, H.-L., Truong, Q.-M., Hoang, H.-G., Tran, H.-T., Malafaia, G., Idris, A.M., 2024. Occurrence and fate of pharmaceutical pollutants in wastewater: insights on ecotoxicity, health risk, and state–of–the-art removal. Chemosphere 354, 141678. https://doi.org/10.1016/j.chemosphere.2024.141678.
NIJZ, 2024a. National Institute of Health: Consumption of prescribed medicines in Slovenia in 2023 [WWW Document]. URL https://nijz.si/wp-content/uploads/2024/05/Zdravila-na-recept-2023-Final-9.5.2024.pdf.
NIJZ, 2024b. Data portal - Data on prescription drug dispensing, according to ATC classification (level 5), Slovenia, annually - "Podatki o izdajah zdravil na recept, po ATC klasifikaciji (5. nivo), Slovenija, letno" [WWW Document]. Podatkovni portal. URL https://podatki.nijz.si:443/pxweb/sl/NIJZ podatkovni portal/NIJZ podatkovni portal_6 Zdravila_1 Izdana zdravila na recept/60100005.px/ (accessed 7.12.25).
Nieto-Juárez, J.I., Torres-Palma, R.A., Botero-Coy, A.M., Hernández, F., 2021. Pharmaceuticals and environmental risk assessment in municipal wastewater treatment plants and rivers from Peru. Environ. Int. 155, 106674. https://doi.org/10.1016/j.envint.2021.106674.

Niu, J., Lu, Y., Wang, Haiyan, Qiao, X., Wang, Hui, Ma, C., Liu, Y., 2024. Occurrence, removal and environmental risk assessment of pharmaceutical active compounds (PhACs) and metabolites in hospital wastewater. J. Hazard. Mater. 480, 136348. https://doi.org/10.1016/j.jhazmat.2024.136348.

Ofrydopoulou, A., Nannou, C., Evgenidou, E., Christodoulou, A., Lambropoulou, D., 2022. Assessment of a wide array of organic micropollutants of emerging concern in wastewater treatment plants in Greece: occurrence, removals, mass loading and potential risks. Sci. Total Environ. 802, 149860. https://doi.org/10.1016/j.scitotenv.2021.149860.

Papageorgiou, M., Zioris, I., Danis, T., Bikiaris, D., Lambropoulou, D., 2019. Comprehensive investigation of a wide range of pharmaceuticals and personal care products in urban and hospital wastewaters in Greece. Sci. Total Environ. 694, 133565. https://doi.org/10.1016/j.scitotenv.2019.07.371.

Pazzagli, L., Reutfors, J., Lucian, E., Zerial, G., Perulli, A., Castelpietra, G., 2022. Increased antidepressant use during the COVID-19 pandemic: findings from the Friuli Venezia Giulia region, Italy, 2015–2020. Psychiatry Res. 315, 114704. https://doi.org/10.1016/j.psychres.2022.114704.

Pérez-Bou, L., Rosa-Masegosa, A., Vílchez-Vargas, R., Link, A., Gonzalez-Martinez, A., Gonzalez-Lopez, J., Muñoz-Palazon, B., 2024. Treatment of hospital wastewater using aerobic granular sludge technology: removal performance and microbial dynamics. J. Water Process. Eng. 60, 105206. https://doi.org/10.1016/j.jwpe.2024.105206.

Petrie, B., Moffat, C.F., 2022. Occurrence and fate of chiral and achiral drugs in estuarine water – a case study of the Clyde Estuary, Scotland. Environ. Sci.: Processes Impacts 24, 547–556. https://doi.org/10.1039/D1EM00500F.

Pivetta, R.C., Rodrigues-Silva, C., Ribeiro, A.R., Rath, S., 2020. Tracking the occurrence of psychotropic pharmaceuticals in Brazilian wastewater treatment plants and surface water, with assessment of environmental risks. Sci. Total Environ. 727, 138661. https://doi.org/10.1016/j.scitotenv.2020.138661.

Quireyns, M., Boogaerts, T., Van Wichelen, N., Pussig, B., De Loof, H., Covaci, A., Van Nuijs, A., 2022. Temporal monitoring of pharmaceutical consumption using a wastewater-based epidemiologic approach. In: Toxicologie Analytique et Clinique, 30th meeting of SFTA- 59th meeting of TIAFT - September 2022, 34, p. S72. https://doi.org/10.1016/j.toxac.2022.06.099.

Racamonde, I., Rodil, R., Quintana, J.B., Villaverde-de-Sáa, E., Cela, R., 2014. Determination of benzodiazepines, related pharmaceuticals and metabolites in water by solid-phase extraction and liquid-chromatography–tandem mass spectrometry. J. Chromatogr. A 1352, 69–79. https://doi.org/10.1016/j.chroma.2014.05.064.

Rathi, B.S., Kumar, P.S., Show, P.-L., 2021. A review on effective removal of emerging contaminants from aquatic systems: current trends and scope for further research. J. Hazard. Mater. 409, 124413. https://doi.org/10.1016/j.jhazmat.2020.124413.

Reichert, J.F., Souza, D.M., Martins, A.F., 2019. Antipsychotic drugs in hospital wastewater and a preliminary risk assessment. Ecotoxicol. Environ. Saf. 170, 559–567. https://doi.org/10.1016/j.ecoenv.2018.12.021.

Rice, J., Kannan, A.M., Castrignanò, E., Jagadeesan, K., Kasprzyk-Hordern, B., 2020. Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - A UK perspective. Sci. Total Environ. 735, 139433. https://doi.org/10.1016/j.scitotenv.2020.139433.

Ritchie, H., 2020. Google Mobility Trends: How has the Pandemic Changed the Movement of People Around the world? Our World in Data.

SANTE/11312/2021v2, 2021. Analytical Quality Control and Method Validation Procedures For Pesticide Residues Analysis in Food and Feed.

Schlüsener, M.P., Hardenbicker, P., Nilson, E., Schulz, M., Viergutz, C., Ternes, T.A., 2015. Occurrence of venlafaxine, other antidepressants and selected metabolites in the Rhine catchment in the face of climate change. Environ. Pollut. 196, 247–256. https://doi.org/10.1016/j.envpol.2014.09.019.

Singh, A., Saidulu, D., Gupta, A.K., Kubsad, V., 2022. Occurrence and fate of antidepressants in the aquatic environment: insights into toxicological effects on the aquatic life, analytical methods, and removal techniques. J. Environ. Chem. Eng. 10, 109012. https://doi.org/10.1016/j.jece.2022.109012.

Thomas, K.V., Amador, A., Baz-Lomba, J.A., Reid, M., 2017. Use of Mobile Device Data To Better Estimate Dynamic Population Size for Wastewater-Based Epidemiology. Environ. Sci. Technol. 51, 11363–11370. https://doi.org/10.1021/acs.est.7b02538.

Tiger, M., Wesselhoeft, R., Karlsson, P., Handal, M., Bliddal, M., Cesta, C.E., Skurtveit, S., Reutfors, J., 2023. Utilisation of antidepressants, anxiolytics, and hypnotics during the COVID-19 pandemic in Scandinavia. J. Affect. Disord. 323, 292–298. https://doi.org/10.1016/j.jad.2022.11.068.

Tomsone, L.E., Perkons, I., Sukajeva, V., Neilands, R., Kokina, K., Bartkevics, V., Pugajeva, I., 2022. Consumption trends of pharmaceuticals and psychoactive drugs in Latvia determined by the analysis of wastewater. Water Res. 221, 118800. https://doi.org/10.1016/j.watres.2022.118800.

US EPA, 2024. United States Environmental Protection Agency (US EPA), Technical overview of ecological risk assessment: problem formulation [WWW Document]. URL https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/technical-overview-ecological-risk-assessment (accessed 1.29.25).

US EPA, O., 2015. Ecological Structure Activity Relationships (ECOSAR) Predictive Model [WWW Document]. URL. https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar-predictive-model. accessed 3.9.25.

UWWTD, 2024. Directive (EU) 2024/3019 of the European Parliament and of the Council of 27 November 2024 concerning urban wastewater treatment (recast) [WWW Document]. URL https://eur-lex.europa.eu/eli/dir/2024/3019/oj/eng (accessed 3.9.25).

UWWTD, 1991. Council Directive 91/271/EEC of 21 May 1991 concerning urban wastewater treatment [WWW Document]. URL https://eur-lex.europa.eu/eli/dir/1991/271/oj/eng (accessed 3.9.25).

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment—A review. Sci. Total Environ., Special Section - Arsenic in Latin Am., An Unrevealed Continent: Occurr., Health Effects Mitigat. 429, 123–155. https://doi.org/10.1016/j.scitotenv.2012.04.028.

Verovšek, T., Heath, D., Heath, E., 2022. Occurrence, fate and determination of tobacco (nicotine) and alcohol (ethanol) residues in waste- and environmental waters. Trends in Environ. Analy. Chem. 34, e00164. https://doi.org/10.1016/j.teac.2022.e00164.

Verovšek, T., Krizman-Matasic, I., Heath, D., Heath, E., 2020. Site- and event-specific wastewater-based epidemiology: current status and future perspectives. Trends Environ. Analy. Chem., e00105 https://doi.org/10.1016/j.teac.2020.e00105.

Verovšek, T., Šuštarič, A., Laimou-Geraniou, M., Krizman-Matasic, I., Prosen, H., Fleršek, T., Kramarič Zidar, V., Mislej, V., Mišmaš, B., Stražar, M., Levstek, M., Cimrmančič, B., Lukšič, S., Uranjek, N., Kozlovič-Bobič, T., Kosjek, T., Kocman, D., Heath, D., Heath, E., 2023. Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment. Sci. Total Environ. 866, 161257. https://doi.org/10.1016/j.scitotenv.2022.161257.

Wang, W., Zhang, J., Hu, M., Liu, X., Sun, T., Zhang, H., 2023. Antidepressants in wastewater treatment plants: occurrence, transformation and acute toxicity evaluation. Sci. Total Environ. 903, 166120. https://doi.org/10.1016/j.scitotenv.2023.166120.

WHO, 2022. World Health Organization: Health topics – Drugs (psychoactive) [WWW Document]. URL. https://www.who.int/health-topics/drugs-psychoactive. accessed 2.21.25.

Wu, M., Xiang, J., Chen, F., Fu, C., Xu, G., 2017. Occurrence and risk assessment of antidepressants in Huangpu River of Shanghai. China Environ. Sci. Pollut. Res. 24, 20291–20299. https://doi.org/10.1007/s11356-017-9293-x.

Yadav, M.K., Short, M.D., Aryal, R., Gerber, C., van den Akker, B., Saint, C.P., 2017. Occurrence of illicit drugs in water and wastewater and their removal during wastewater treatment. Water Res. 124, 713–727. https://doi.org/10.1016/j.watres.2017.07.068.

Yavuz-Guzel, E., Atasoy, A., Gören, İ.E., Daglioglu, N., 2022a. Impact of COVID-19 pandemic on antidepressants consumption by wastewater analysis in Turkey. Sci. Total Environ. 838, 155916. https://doi.org/10.1016/j.scitotenv.2022.155916.

Zergui, A., Nkontcheu Kenko, D.B., 2024a. Drugs of abuse and psychoactive pharmaceuticals in wastewater as worldwide contaminants of emerging concern: a systematic review. Environ. Pollut. Manage. 1, 71–86. https://doi.org/10.1016/j.epm.2024.08.001.

Zheng, Q., Gerber, C., Steadman, K.J., Lin, C.-Y., Tscharke, B.J., O'Brien, J.W., Hobson, P., Toms, L.-M., Mueller, J.F., Thomas, K.V., Thai, P.K., 2023a. Improving Wastewater-Based Tobacco Use Estimates Using Anabasine. Environ. Sci. Technol. 57, 7958–7965. https://doi.org/10.1021/acs.est.3c01510.

ZZZS, 2023a. Data on drug consumption [WWW Document]. URL https://partner.zzzs.si/wps/portal/portali/aizv/zdravila_in_zivila_za_posebne_zdravstvene_namene/podatki_o_porabi_zdravil/!ut/p/z1/04_Sj9CPykssy0xPLMnMz0vMAfIjo8ziTQxdPd2N_Q08LSyCDQ0cjZzMzXz8XQ0sTAz0C7IdFQGdnpEx/ (accessed 11.26.23).

Yavuz-Guzel, E., Atasoy, A., Gören, İ.E., Daglioglu, N., 2022b. Impact of COVID-19 pandemic on antidepressants consumption by wastewater analysis in Turkey. Sci. Total Environ. 838, 155916. https://doi.org/10.1016/j.scitotenv.2022.155916.

Zergui, A., Nkontcheu Kenko, D.B., 2024b. Drugs of abuse and psychoactive pharmaceuticals in wastewater as worldwide contaminants of emerging concern: a systematic review. Environ. Pollut. Manage. 1, 71–86. https://doi.org/10.1016/j.epm.2024.08.001.

Further reading

Zheng, Q., Gerber, C., Steadman, K.J., Lin, C.-Y., Tscharke, B.J., O'Brien, J.W., Hobson, P., Toms, L.-M., Mueller, J.F., Thomas, K.V., Thai, P.K., 2023. Improving Wastewater-Based Tobacco Use Estimates Using Anabasine. Environ. Sci. Technol. 57, 7958–7965. https://doi.org/10.1021/acs.est.3c01510.

watres. 2017.07.068.

ZZZS, 2023b. Data on drug consumption [WWW Document]. URL https://partner.zzzs.si/wps/portal/portali/aizv/zdravila_in_zivila_za_posebne_zdravstvene_namene/podatki_o_porabi_zdravil/!ut/p/z1/04_Sj9CPykssy0xPLMnMz0vMAfIjo8ziTQxdPd2N_Q08LSyCDQ0cjZzMzXz8XQ0sTAz0C7IdFQGdnpEx/(accessed 11.26.23).

3.5 Novel Sampling Approaches for Psychoactive Substances in Wastewater

Based on the following publication:

3.5.1 In-situ evaluation of an active-passive sampling (APS) technique for monitoring psychoactive compounds in wastewater

To be submitted to Environmental Pollution (December 2025): A. Jun Anies[a*], M. Laimou-Geraniou[b, c], M. Quireyns[d], T. Boogaerts[d], T. Verovšek [b,c], D. Heath[b], E. Heath[b, c], A. L. N. van Nuijs[d], A. Covaci[d], R. Blust[a], R. Town[a].

[a] ECOSPHERE, Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium
[b] Department of Environmental Sciences, Jožef Stefan Institute, Jamova c. 39, 1000 Ljubljana, Slovenia,
[c] Jožef Stefan International Postgraduate School, Jamova c. 39, 1000, Ljubljana, Slovenia
[d] Toxicological Centre, University of Antwerp, Universiteitsplein 1, Wilrijk 2610, Antwerp, Belgium

This study is a collaboration between the Jožef Stefan Institute in Ljubljana (Slovenia), the Ecotoxicological Centre at the University of Antwerp (Belgium) and ECOSPHERE (Belgium). The development and optimisation of the APS devices, as well as the characterisation of the sorption dynamics of biomarkers to the APS sorbents, took place in ECOSPHERE (Belgium). My part included all aspects of analytical method development and validation for the quantification of target biomarkers in wastewater and sorbents using GC-MS/MS, the application and deployment of APS devices in a Slovenian WWTP, and data processing, which took place at the Jožef Stefan Institute in Ljubljana (Slovenia). In addition, I performed data evaluation and assisted in drafting the analytical part of the manuscript regarding the Slovenian samples.

An overall issue to be addressed in WBE is the sampling process. In this study, an innovative sampling strategy that exploits APS strategies developed at the University of Antwerp was employed in real wastewater. For the first time, APS devices were fully deployed in WWTPs in Slovenia and Belgium for the determination of illicit drug, opioid, and psychoactive pharmaceutical biomarkers in wastewater. The following subchapter is part of a broader common study to be published in Environmental Pollution (to be *submitted in December 2025).* This subchapter presents the method development and application of the APS device to Slovenian wastewater and compares it with conventional 24-hour composite sampling to assess the relative efficiency of APS for monitoring psychoactive compounds.

3.5.1.1 Introduction

The presence of psychoactive compounds, including prescription medications and illicit drugs, in the aquatic environment has raised significant environmental and public health concerns. These substances, excreted as parent compounds or metabolites, enter WWTPs and may persist in effluents, eventually reaching surface waters [8], [203], [210], [228]. Exposure to these contaminants has been associated with endocrine disruption, neurotoxicity, and behavioural alterations in aquatic organisms [228], [229]. Given the widespread consumption of psychoactive substances and their incomplete removal during wastewater treatment, it is crucial to monitor their presence and fate in the environment.

Wastewater-based epidemiology (WBE) has emerged as a valuable tool for tracking community-wide drug and pharmaceutical consumption by analysing wastewater samples and back-calculating their population-normalised mass loads (PNMLs) in mg/day/1,000 inhabitants. [5], [48], [198], [230]. Traditionally, 24-hour composite sampling followed by solid-phase extraction (SPE) and high-resolution analytical techniques, such as liquid or gas chromatography coupled with mass spectrometry, have been used to detect these substances [2], [148]. However, conventional methods, such as grab or composite sampling, have limitations in obtaining time-weighted average (TWA) concentrations, including the need to sample and store numerous samples and their costly analysis, as well as potential fluctuations in analyte concentrations due to degradation or interactions with suspended particulate matter (SPM) [231].

To overcome these challenges, passive sampling techniques have been developed, allowing for *in-situ* pre-concentration of contaminants and providing time-weighted average concentrations [110], [232]. Unlike composite sampling, which captures only a snapshot of contaminant levels during a specific period (e.g. 24 hours), passive samplers allow for TWA concentrations of compounds of interest over much more extended periods (typically 1-3 weeks), thereby providing a more representative and accurate measure of exposure that accounts for short-term fluctuations and enables the detection of contaminants that may occur intermittently or at low concentrations. Additionally, passive sampling devices can be deployed for specific periods without manual operation or external power sources, at a comparatively low cost.

Common passive sampling devices currently available for organic compounds include the polar organic chemical integrative sampler (POCIS) [213], microporous polyethylene membrane samplers [87], Chemcatcher [201], and organic diffusive gradients in thin films (o-DGT) [233]. Out of these, POCIS, o-DGT, and Chemcatcher® have been applied for the analysis of over 300 polar organic substances in aquatic samples, including pharmaceuticals and illicit drugs, as well as other polar contaminants such as pesticides [45], [96], [112], [113], [234], [235], [236]. These samplers are designed to monitor trace levels of organic contaminants in aquatic environments. They are particularly effective for tracking TWA concentrations of hydrophilic pharmaceuticals, such as antidepressants, antipsychotics, benzodiazepines, and other psychoactive substances, which are often present at low levels and may fluctuate due to varying usage patterns or discharges. Despite their advantages (TWA concentration, *in-situ* pre-concentration, low operational cost, no need for power systems, minimal human intervention, and suitability for monitoring a broad range of polar organic compounds), passive samplers rely on diffusion-driven uptake, making them susceptible to water turbulence, temperature, and flow dynamics.

An alternative is to use an APS device, designed to overcome the limitations of both composite and passive sampling approaches. An APS integrates active and passive sampling mechanisms, incorporating a controlled laminar flow system that enables the accumulation of target compounds independently of external hydrodynamic conditions

[117], [118], [119]. By actively pumping the aqueous sample through a diffusion cell containing a specific receiving phase, such as Hydrophilic-Lipophilic Balanced (HLB) or Mixed-mode Cation Exchange (MCX) sorbents, both of which are commonly used for extracting polar compounds (i.e., psychoactive substance residues) in SPE applications, APS combines the benefits of passive pre-concentration with improved control over sampling conditions. This hybrid approach has been proposed as a promising tool for WBE and environmental monitoring campaigns. So far, APS have been used for the simultaneous measurement of a wide range of contaminants (i.e., metal cations, polar and non-polar organic compounds) mainly in surface waters [119].

The primary aim of this study is to compare the APS device with o-DGT and conventional 24-hour composite sampling to assess their relative efficiency in monitoring psychoactive compounds in wastewater. This comparison provides valuable insights for improved environmental surveillance and wastewater-based epidemiological applications. To the best of our knowledge, this is the first such application of an APS device in real wastewater.

3.5.1.2 Materials and methods

3.5.1.2.1 Chemicals and sorbents

Reference standards and deuterated ISTDs for the analytes under investigation (purity > 98%) were obtained as solutions in methanol (MeOH) or acetonitrile (ACN) at concentrations of 1 mg/mL or 100 µg/mL from Cerilliant Corporation (Round Rock, Texas, USA). Working solutions (analytes: 10 mg/L, deuterated analogues: 2 mg/L) were prepared from the stock solutions in methanol. Standards and working solutions were stored in the dark at 20 °C. LC-MS grade MeOH was purchased from JT Baker (Philipsburg, USA), LC-MS grade hydrochloric acid (HCl, 37%) from VWR Chemicals (Pennsylvania, USA), formic acid (HCOOH) from Fluka (Switzerland) and aqueous ammonia (NH_3, 25%) from Merck (Darmstadt, Germany). The derivatisation agent MSTFA + 1% TMCS was purchased from Merck (Darmstadt, Germany). Milli-Q was obtained using the Millipore Direct-Q purifying system.

HLB- and MCX-based polymeric sorbents were purchased from Affinisep (FR) as custom-made AttractSPE™ pads, with 90% w/w sorbent material and a thickness of 0.5 mm. The sorbent pads were conditioned with MeOH and stored in deionised water (HLB) and in pH 2 deionised water (MCX) at 4 °C until further use.

3.5.1.2.2 Compounds of interest

Seven biomarker residues of psychoactive compounds such as illicit drugs, opioids, and antidepressants were selected based on different functionalities, prescription frequency and sales in Slovenia, high prevalence in wastewater, and ecological relevance. These included either parent compounds or their corresponding metabolites (**Table 7**). More specifically, two biomarkers of illicit drugs, three biomarkers of opioids, and two biomarkers of antidepressants were targeted in wastewater influents and effluents.

Table 7: Compounds of interest (human metabolic residues).

Psychoactive drug/ pharmaceutical		**Compound of interest (target compound)**	**Abbreviation**	**Internal standard**
Illicit drugs	Amphetamine	Amphetamine	AMP	AMP-d6
	Cocaine	Benzoylecgonine	BZE	BZE-d3
Opioids	Methadone	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP	EDDP-d3
	Morphine	Morphine	MOR	MOR-d3
	Tramadol	Tramadol	TRA	VEN-d6
Antidepressants	Bupropion	Hydroxybupropion	HBP	VEN-d6
	Venlafaxine	*O*-desmethylvenlafaxine	ODV	VEN-d6

Chemical structure, physicochemical properties, and chemical speciation (data obtained from ChemAxon 2024) of the target compounds at pH 7.8 (average pH of wastewater) are presented in **Figure 10**. Morphine, EDDP, tramadol, and *O*-desmethylvenlafaxine, all having tertiary ammonium ions, and amphetamine with a primary ammonium group predominantly exist as cationic (+1) species; hydroxybupropion primarily exists in neutral form (approx. 59%); however, the remaining fraction is present mainly as a monocationic species due to protonation of the amine group; while benzoylecgonine is mainly in a zwitterionic form (**Figure 10**). The stability of illicit drugs [237], opioids [92], [237], and antidepressants [84], [238] has been previously investigated.

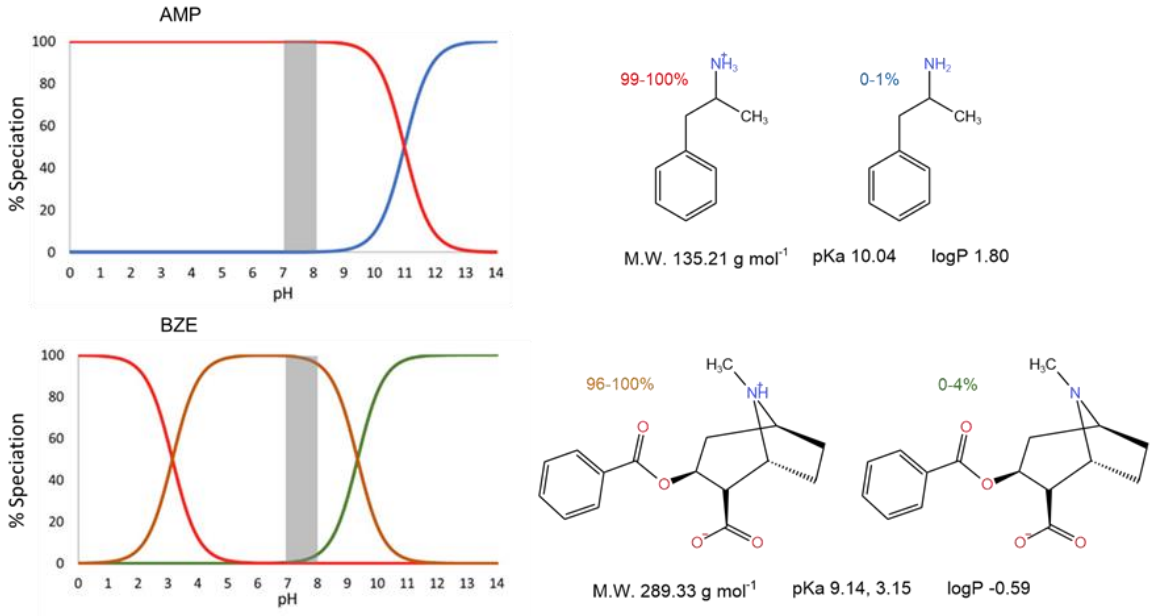


Figure 10: Physicochemical properties, chemical structures and speciation (*cationic*, *neutral*, *zwitterionic*, *anionic*), at pH 7.0 – 8.0, of the target psychoactive compounds. *Data obtained from ChemAxon, 2024. The logP (elsewhere referred to as log Kow) values shown in this figure represent the octanol–water partition coefficient of the neutral species, as provided by ChemAxon.*

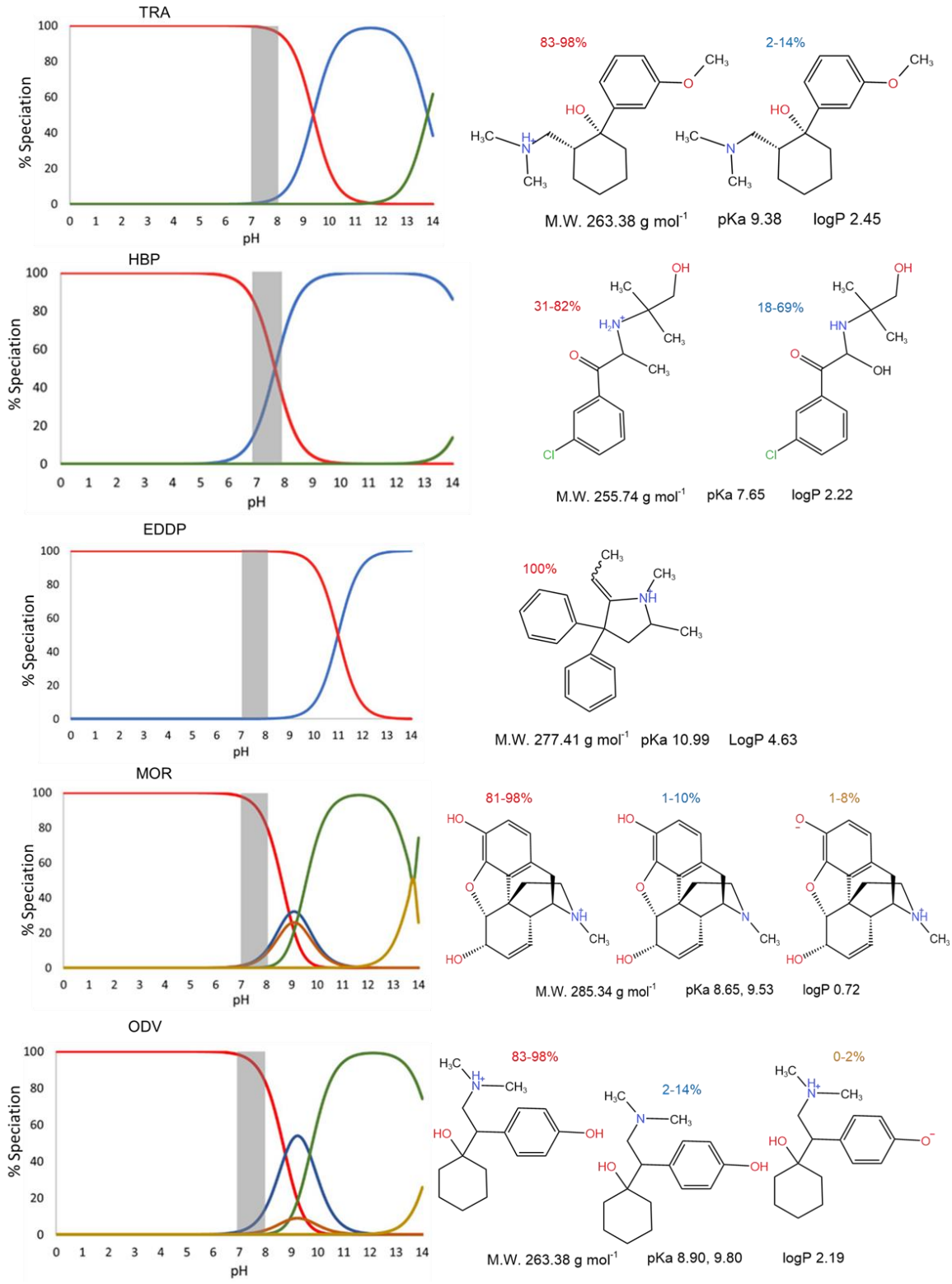


Figure 10 (continued): Physicochemical properties, chemical structures and speciation (*cationic*, *neutral*, *zwitterionic*, *anionic*), at pH 7.0 – 8.0, of the target psychoactive compounds. *Data obtained from ChemAxon, 2024. The logP (elsewhere referred to as log Kow) values shown in this figure represent the octanol–water partition coefficient of the neutral species, as provided by ChemAxon.*

3.5.1.2.3 Active-passive sampling (APS) device

The APS device (**Figure 11**) incorporates a diffusion cell constructed from polyether ether ketone (PEEK), a pump at the inlet, a flow meter at the outlet to maintain a constant flow rate, and temperature and humidity sensors [118]. Regular measurements of flow rate (set at 5.4 cm^3 s^{-1}), water temperature and air humidity were logged using the sampler's embedded online software. The diffusion cell consists of two blocks separated by a 5 mm thick spacer. Each block contains two pockets designed to house the receiving phases (HLB- and MCX-based) in 40 x 20 mm and 20 × 20 mm sizes, with two replicates per sorbent (**Figure 11**). Each receiving phase was topped with a filter membrane (polyethersulfone, PES, 0.2 µm pore size, 145 µm thickness). The APS device is coupled with an external 100 µm Nylon mesh filter to prevent the entry of large particles into the diffusion cell.

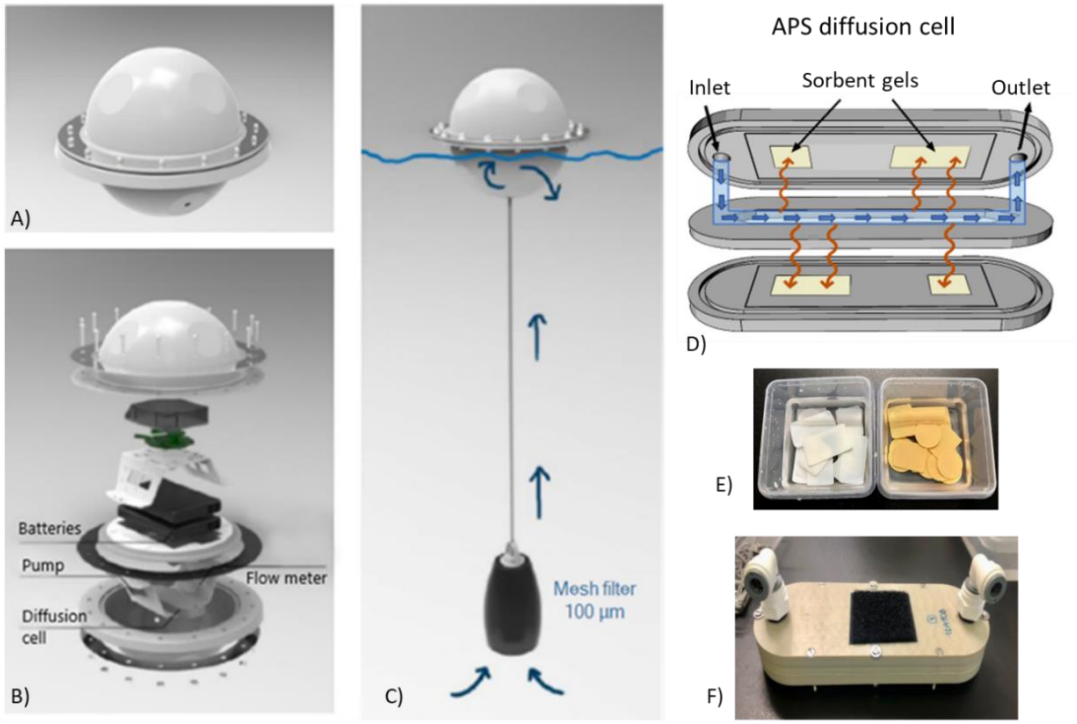


Figure 11: The active-passive sampling (APS) device. (A and B) The diffusion cell, pump, and flowmeter are placed inside the floating device, along with a set of batteries to provide power to the system. (C) A filter connected externally to the inlet of the device. (D) Schematic and cross-section (not to scale) of the APS diffusion cell. (E) Affinisep™ HLB and MCX (in PTFE matrix) SPE pads. (F) Picture illustrating the external appearance of the APS diffusion cell.

3.5.1.2.4 Organic diffusive gradients in thin films (o-DGT)

The o-DGT samplers (**Figure 12**) used in the sampling campaigns were constructed according to the standard DGT configuration, with the identical sorbents (HLB and MCX) as those used in the APS device. The HLB- and MCX-based binding layers were topped with an agarose diffusive gel (1.5% w/w agarose gel, 0.75 mm thickness) and a PES filter membrane (0.2 µm pore size, 145 µm thickness). For comparison, commercially available o-DGT samplers with an HLB binding layer, topped with a 0.8 mm agarose diffusive gel and a PTFE filter membrane, were purchased from DGT® Research (UK).

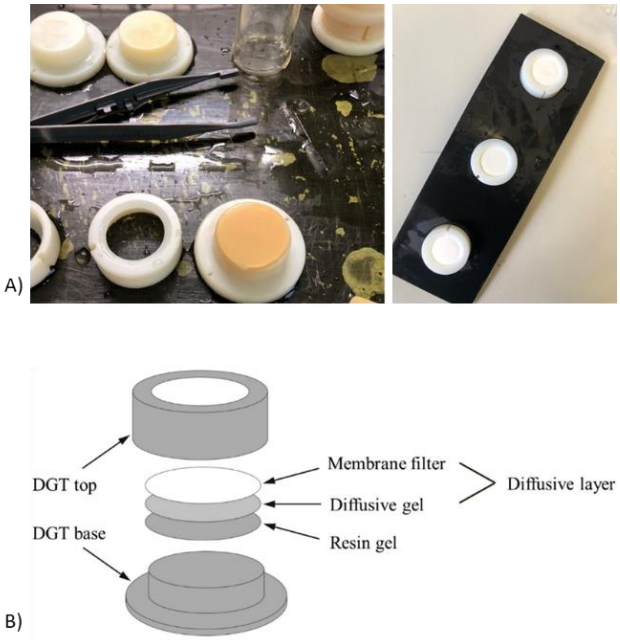


Figure 12: (A) Commercially available o-DGT samplers with HLB sorbent topped with 0.8 mm agarose diffusive gel and PTFE filter membrane and (B) lab-assembled o-DGT samplers using APS sorbent phases (HLB and MCX) topped with agarose diffusive gel and a PES filter membrane.

3.5.1.2.5 Field experiment setup

Field experiments were conducted at the Ljubljana WWTP in Slovenia, covering approximately 13% of the Slovenian population. Although designed to float, each sampler was suspended from a bridge with the mesh-filter intake submerged in the effluent at 0.3 m, while the o-DGT devices were placed on aluminium holders, securely attached to the APS mesh filter (**Figure 13**). The o-DGT samplers were included in the setup to assess the differences between the two sampling techniques.

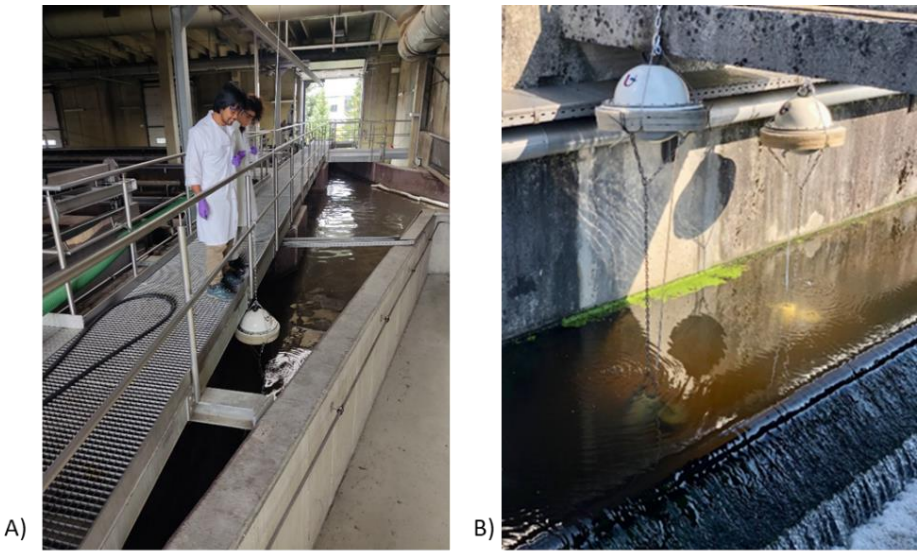


Figure 13: Deployment of APS in (A) influent and (B) effluent wastewater, in Ljubljana WWTP (Photo (A): David Heath and photo (B): Maria Laimou-Geraniou).

The APS and o-DGT samplers were simultaneously deployed at both the influent and effluent of the WWTP in Ljubljana (population: 270,305) to obtain relevant information on analyte concentrations and to assess treatment efficiency. For the influent sampling, the APS sampler was deployed after the clarifier tank to avoid contact with large debris and particulate matter. However, after only 18 hours of exposure, the filters became clogged, resulting in pump failure. Thus, the sampling campaign in the influent wastewater was cancelled. Consequently, APS and o-DGT sampling devices were deployed as planned only in the effluent of Ljubljana WWTP for five sampling periods: 3 days (26-29/09/2023), 6 days (03-09/09/2023), 9 days (27/09-06/10/2023), 12 days (11-23/10/2023), and 14 days (26/09-10/10/2023). Daily conventional 24-hour composite samples were collected and analysed separately during the same sampling periods to determine psychoactive compounds and for subsequent comparison with APS and o-DGT devices.

3.5.1.2.6 Sample preparation

Two different extraction procedures were employed: a classical solid-phase extraction (SPE) for the 24-hour composite effluent wastewater samples and a sonication-assisted extraction for the passive sample (APS and o-DGT) sorbent gels (MCX and HLB)

3.5.1.2.6.1 Conventional 24-hour composite samples

Pre-concentration of psychoactive compounds in composite effluent wastewater samples was achieved using SPE with OASIS MCX cartridges, following the method of Laimou-Geraniou et.al [8] with minor modifications. Prior to SPE, effluent wastewater samples (125 mL) were spiked with the isotopically labelled ISTD mixture (200 ng/mL in final extract) and subsequently filtered through GF/D (2.7 µm) and GF/C (1.2 µm) glass microfiber filters (Whatman, USA). After acidification to pH 2 with HCl (37 %), analytes were loaded onto Oasis Prime MCX cartridges (150 mg/6 mL, Waters, Milford, MA, USA) and washed with 5 mL of Milli-Q water. After drying the sorbent under vacuum for 30 minutes, the target analytes were eluted with 6 mL of a 0.5 % ammonium solution in methanol. The samples were evaporated to dryness at 35 °C and reconstituted in 100 µL of methanol. A 50 µL aliquot (for EDDP) was transferred into GC-MS vials and stored at −10 °C until further analysis. The remaining aliquot of 50 µL was then evaporated to dryness under a gentle stream of N_2, and the dry extract was derivatised using 50 µL of MSTFA + 1% TMCS in a microwave oven at 800 W for 3 min. After cooling to room temperature, the extracts were transferred to GC glass inserts and then to GC vials, where they were analysed using gas chromatography-tandem mass spectrometry (GC-MS/MS).

3.5.1.2.6.2 APS and o-DGT receiving phases

Extraction of psychoactive compounds from sorbent gels (APS and o-DGT) was achieved using sonication-assisted extraction, following the method of Anies et al. [239]. Prior to extraction, exposed sorbent gels were spiked with an isotopically labelled ISTD mixture (200 ng/mL in the final extract). For the HLB sorbent, 3 mL of 2% formic acid in methanol was added, followed by vortex mixing for 30 seconds and sonication for 10 minutes. This extraction process was then repeated twice (3 mL then 2 mL), and the supernatants were pooled and evaporated to dryness (35 °C, N_2). For the MCX sorbent, the same extraction procedure was performed, using 3 mL of methanol followed by 3 mL and 2 mL of 1.25% NH_3 in methanol. The supernatants were then pooled and evaporated to dryness (35 °C, N_2). Dried extracts were then reconstituted with 100 µL of methanol. Similar to the SPE

procedure, a 50 µL aliquot (for EDDP) was transferred into GC-MS vials and stored at −10 °C until further analysis. The remaining aliquot of 50 µL was then evaporated to dryness (N_2) and the dry extracts derivatised using 50 µL of MSTFA + 1% TMCS in a microwave oven at 800 W for 3 min. After cooling down to room temperature, the extracts were transferred to GC vials and analysed using GC-MS/MS.

3.5.1.2.7 Gas chromatography-tandem mass spectrometry

All samples were analysed using an 7890B gas chromatograph (GC) coupled with a 7000 GC/MS Triple quadrupole mass spectrometer (Agilent Technologies, US). The injection was performed in splitless mode (260 °C) with an injection volume of 1 µL. Chromatographic separation was achieved using a DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, US) and helium as the carrier gas (1 mL/min). The initial oven temperature was maintained at 100 °C for 1 minute, then increased to 320 °C at 15 °C/min (held for 5 minutes), resulting in a total run time of 20.5 minutes. The ionisation was achieved in electron impact (EI) at 70 eV. The MS was operated in selected reaction monitoring (SRM) mode. Retention times and the two most intense transitions were used for identification (**Table 8**). Quantification was performed based on the relative response factors of the analyte to the corresponding ISTD. The obtained data were processed using MassHunter software. GC-MS/MS parameters for analytes and ISTD were optimised as presented in **Table 8**.

Before analysis, the instrument's performance was checked using an air-water control (leakage check) and by analysing a diazepam solution at 1 µg/mL (intra-laboratory check of the instrument response). MS was auto-tuned. Experimental quality control was performed by analysing solvent (ethyl acetate) and procedural blanks (wastewater spiked only with the internal standard) at the beginning of the batch. QC samples (points of calibration curves or spiked solvents) were analysed after every 14th sample in the batch to control potential drift in the signal.

Table 8: GC-MS/MS parameters.

Analyte	**Retention time [min]**	**MRM Transitions [*m/z*]**	**CE [eV]**	**Dwell time [ms]**
AMP	6.06	116 → 73 (Q)	25	50
		117 → 74 (q)	30	50
BZE	13.19	240 → 82 (Q)	35	50
		82 → 67 (q)	45	50
EDDP	11.75	277 → 262 (Q)	30	50
		277 → 235 (q)	25	50
		262 → 105 (q)	35	50
MOR	14.44	236 → 146 (Q)	20	50
		236 → 220 (q)	25	50
TRA	11.20	58 → 43 (Q)	45	80
		245 → 58 (q)	30	80
		335 → 58 (q)	30	80
HBP	10.53	188 → 145 (Q)	15	50
		188 → 44 (q)	25	50
		224 → 166 (q)	15	50
ODV	12.30	58 → 43 (Q)	45	80
		171 → 73 (q)	30	80
		191 → 177 (q)	10	80
AMP-d6	6.03	120 → 73 (Q)	25	50
BZE-d3	13.18	243 → 85 (Q)	35	50
EDDP-d3	11.71	280→ 265 (Q)	35	50
MOR-d3	14.43	239 → 149 (Q)	25	50
VEN-d6	11.94	64 → 46 (Q)	45	50

3.5.1.2.8 Method validation

The method was validated for linearity, limit of detection, limit of quantification, repeatability, extraction recovery, and accuracy, following the SANTE protocol [240], with minor adjustments. Deuterated ISTDs, structurally similar to the psychoactive compounds under investigation, were used to correct for instrumental variations. Spiked tap water at two concentration levels (100 ng/mL and 500 ng/mL) of the final extracts was used as the matrix.

Linearity and linearity range were determined from seven- to twelve-point calibration curves by plotting the peak area ratios (analyte vs. ISTD) as a function of analyte concentration and assessed using the coefficient of determination (R^2).

The LOD and LOQ were determined by calculating signal-to-noise ratios (S/N = 3 and S/N = 10) in water matrices spiked with a low concentration in five replicates. Following the European Union Decision 2002/657/EC, two distinct transitions were required for positive compound identification. In this case, LOD refers to the S/N = 3 ratio for the transition with the lower intensity (qualifier), and LOQ refers to the S/N = 10 ratio for the transition used for quantification (quantifier).

Inter-day repeatability was assessed as the relative standard deviation (%RSD) of five replicate analyses of water samples spiked to evaluate accuracy. Instrumental repeatability

was determined as the %RSD of three consecutive injections of the same spiked water sample.

Extraction recovery (ER) was assessed by spiking water samples (in triplicate) with analytes prior to (sample spike) and after extraction (eluate spike). The extraction recovery was calculated based on analyte-to-ISTD peak areas ratios (A) as shown in **Equation 3**:

$$ER\ (\%) = \frac{A(sample\ spike)}{A(eluate\ spike)} \times 100 \tag{3}$$

Method accuracy was determined by comparing measured concentrations (c) of spiked water samples before sample preparation (sample spike) and spiked solvent (STD spike), as shown in **Equation 4**. All sample sets were prepared in five replicates.

$$Accuracy\ (\%) = \frac{C\ (sample\ spike)}{C\ (STD\ spike)} \times 100 \tag{4}$$

Quality control (QC) was performed by using procedural tap water (spiked only with ISTD) and processed using the described sample preparation procedure and instrument (solvent spiked only with ISTD) blanks. QC samples (points of calibration curves or spiked solvents) were analysed after every 14th sample in the batch to control potential drift in the signal.

3.5.1.2.9 Calculation and Data analysis/Statistical analysis

For APS, accumulation curves were constructed by plotting the calculated concentrations in the sorbent, c_s, over deployment time. Subsequent calculation of the concentrations in the water c_w was done using a nonlinear regression following the first-order kinetics equation for the passive samplers (**Equation 5**), where K_{sw} is the previously determined sorbent-water partition coefficient of the target analyte and k_x is the accumulation rate constant [239].

$$c_s = c_w K_{sw}(1 - exp^{-k_x t}) \tag{5}$$

For DGT, c_w was calculated following the DGT principle as described in previous studies [234], [241] and based on Fick's first law of diffusion (**Equation 6**).

$$c_w = \frac{M\Delta g}{DAt} \tag{6}$$

M is the mass of the target analyte in the sorbent phase, Δg is the thickness of the diffusive layer, A is the exposure surface area, and t is the deployment time. D is the diffusion coefficient of the target analyte in the diffusive layer, previously determined following the method reported by Amato et al. [118].

3.5.1.3 Results and Discussion

3.5.1.3.1 Derivatisation procedure

In this study, a comparison was made between conventional derivatisation (70 °C for 1 h) and a faster microwave-heating method at 800 W nominal power (3 min). For this purpose, two sample sets containing the target analytes (except for EDDP) at concentrations of 100 and 500 ng/mL (final extract) were prepared in triplicate. All samples were extracted using

the described SPE method, and derivatisation was performed using both heating techniques to assess their effectiveness. No statistically significant differences ($p>0.05$) were found (**Figure 14**).

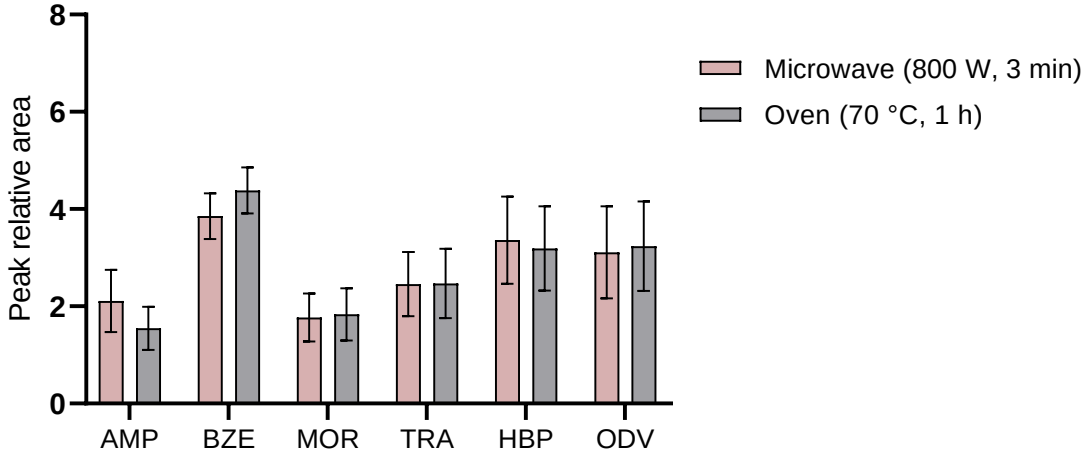


Figure 14: Comparison between microwave-assisted derivatisation and derivatisation with conventional heating.

3.5.1.3.2 Method performance

The analytical method demonstrated good performance (**Table 9**) when evaluated according to the SANTE/11312/2021_v2 Guidance. A linear response ($R^2 > 0.992$) was observed within the range LOQ–5000 ng/mL (final extract concentration) for all target psychoactive compounds, with LOD: 1.02-7.06 ng/L and LOQ: 2.19-18.50 ng/L (**Table 9**). Accuracy ranged from 91% to 110%. Extraction recoveries ranged from 77% to 101%. Inter-day and instrumental repeatability showed %RSD of 1–9%, all of which fall within the acceptable SANTE limits for both concentration levels (100 and 500 ng/mL, final extract). All validation results are presented in **Table 9**.

Table 9: Validation results.

Analyte	**Linearity range in ng/mL (R^2)**	**LOD [ng/L]**	**LOQ [ng/L]**	**Concentration level used for validation [ng/mL]**	**Accuracy [%]**	**Extraction recovery [%]**	**Inter-day repeatability [%RSD]**	**Instrumental repeatability [%RSD]**
AMP	LOQ-5,000 (0.998)	7.06	14.17	100	104	101	5	4
				500	103	93	1	7
BZE	LOQ-5,000 (0.997)	1.78	5.91	100	105	89	5	3
				500	103	81	7	1
EDDP	LOQ-5,000 (0.997)	5.56	18.50	100	98	79	7	5
				500	110	77	6	5
MOR	LOQ-5,000 (0.996)	4.07	13.55	100	96	90	6	9
				500	109	96	6	3
TRA	LOQ-5,000 (0.995)	1.02	3.39	100	109	98	7	7
				500	106	90	7	6
HBP	LOQ-5,000 (0.992)	1.62	2.19	100	91	93	7	9
				500	108	90	9	9
ODV	LOQ-5,000 (0.993)	2.87	11.58	100	99	91	9	5
				500	105	88	7	3

3.5.1.3.3 Application to real samples

Of the seven investigated biomarkers, six were quantified in wastewater effluent at least once and were subsequently selected for comparison among APS, o-DGTs and conventional 24-hour composite sampling. The measured average concentrations of target psychoactive compounds obtained using the three different sampling methods during all sampling periods are presented in **Table 10**. Only amphetamine was below LOQ in all cases (different sampling methods) during all sampling periods. This finding could be explained by its high average removal efficiency (97%) [203]. Consequently, amphetamine was excluded from the comparison between the conventional 24-hour composite sampling (classical SPE), the APS, and the o-DGT device. Based on SPE results, the highest average concentrations were found for *O*-desmethylvenlafaxine (546 ng/L) and tramadol (399 ng/L), followed by hydroxybupropion (85.1 ng/L) and EDDP (53.4 ng/L) across all samples.

Table 10: Average concentrations of psychoactive compounds measured with conventional 24-hour composite sampling (SPE), APS, and o-DGT during a 3-, 6-, 9-, 12-, and 14-day sampling period. All concentrations are reported in ng/L.

3 days of sampling (26-29/09/2023)						
Substance	**SPE**	**APS**		**o-DGT**		
	MCX	**HLB**	**MCX**	**HLB-1**	**HLB-2**	**MCX**
AMP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BZE	10.1	20.6	<LOQ	<LOQ	<LOQ	<LOQ
EDDP	50.2	31.6	21.4	<LOQ	92.8	110
MOR	22.9	29.3	45.1	<LOQ	<LOQ	<LOQ
TRA	355	*	324	700	559	598
HBP	81.4	*	137	<LOQ	<LOQ	113
ODV	542	296	441	323	408	*
6 days of sampling (03-09/10/2023)						
Substance	**SPE**	**APS**		**o-DGT**		
	MCX	**HLB**	**MCX**	**HLB-1**	**HLB-2**	**MCX**
AMP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BZE	18.4	29.2	<LOQ	18.4	31.6	*
EDDP	51.2	63.7	29.8	60.0	67.4	*
MOR	18.5	25.2	33.2	<LOQ	<LOQ	*
TRA	439	684	335	693	699	*
HBP	94.6	44.0	125	12.0	14.0	*
ODV	594	458	361	471	717	*
9 days of sampling (27/09-06/10/2023)						
Substance	**SPE**	**APS**		**o-DGT**		
	MCX	**HLB**	**MCX**	**HLB-1**	**HLB-2**	**MCX**
AMP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BZE	15.7	22.0	<LOQ	15.9	18.9	19.2
EDDP	50.7	60.3	20.7	46.3	63.7	120
MOR	20.5	27.3	39.5	22.0	25	40.7
TRA	429	597	403	333	473	681
HBP	93.8	47	131	48.9	<LOQ	99.7
ODV	593	453	431	404	413	818

12 days of sampling (11-23/10/2023)						
Substance	**SPE**	**APS**		**o-DGT**		
	MCX	**HLB**	**MCX**	**HLB-1**	**HLB-2**	**MCX**
AMP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BZE	23.8	20.3	<LOQ	13.1	16.9	10.8
EDDP	63.6	38.5	17.3	44.0	46.0	42.5
MOR	18.5	17.1	29.5	<LOQ	18.8	17.8
TRA	354	410	356	259	327	214
HBP	64.5	51.8	49.6	14.2	12.4	30.8
ODV	422	305	421	273	317	326
14 days of sampling (26/09-10/10/2023)						
Substance	**SPE**	**APS**		**o-DGT**		
	MCX	**HLB**	**MCX**	**HLB-1**	**HLB-2**	**MCX**
AMP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
BZE	16.7	24.8	<LOQ	11.9	12.1	12.7
EDDP	51.3	62.5	16.7	60.3	58.5	74.0
MOR	19.6	26.5	37.5	15.4	16.6	23.7
TRA	418	661	266	528	337	269
HBP	91.4	45.3	73.7	7.4	<LOQ	56.8
ODV	577	416	409	413	276	260

*values without replicates-outliers

3.5.1.3.4 Conventional 24-hour composite sampling vs APS

The measured concentrations of target psychoactive compounds obtained with SPE, APS and o-DGT are visualised in **Figure 15**. The x-axis represents the sampling method used, and the y-axis shows the obtained target biomarker concentrations (ng/L) in effluent wastewater during the five discrete sampling periods. SPE-MCX refers to the composite samples extracted using SPE with MCX cartridges. APS (HLB and MCX) refers to the gels in the APS device. DGT-HLB_1 is the purchased DGT, while DGT-HLB_2 and DGT-MCX are the lab-assembled DGTs. SPE values were represented as dots to indicate daily concentrations. The red bars represent the average concentration over the sampling period, with vertical lines indicating the standard deviation of the mean. APS and DGT values are presented as box plots to represent the time-weighted average concentrations over each sampling period. Thin bars or missing values for the DGT are due to the lack of replicates, due to the damage/loss of the gels in the field.

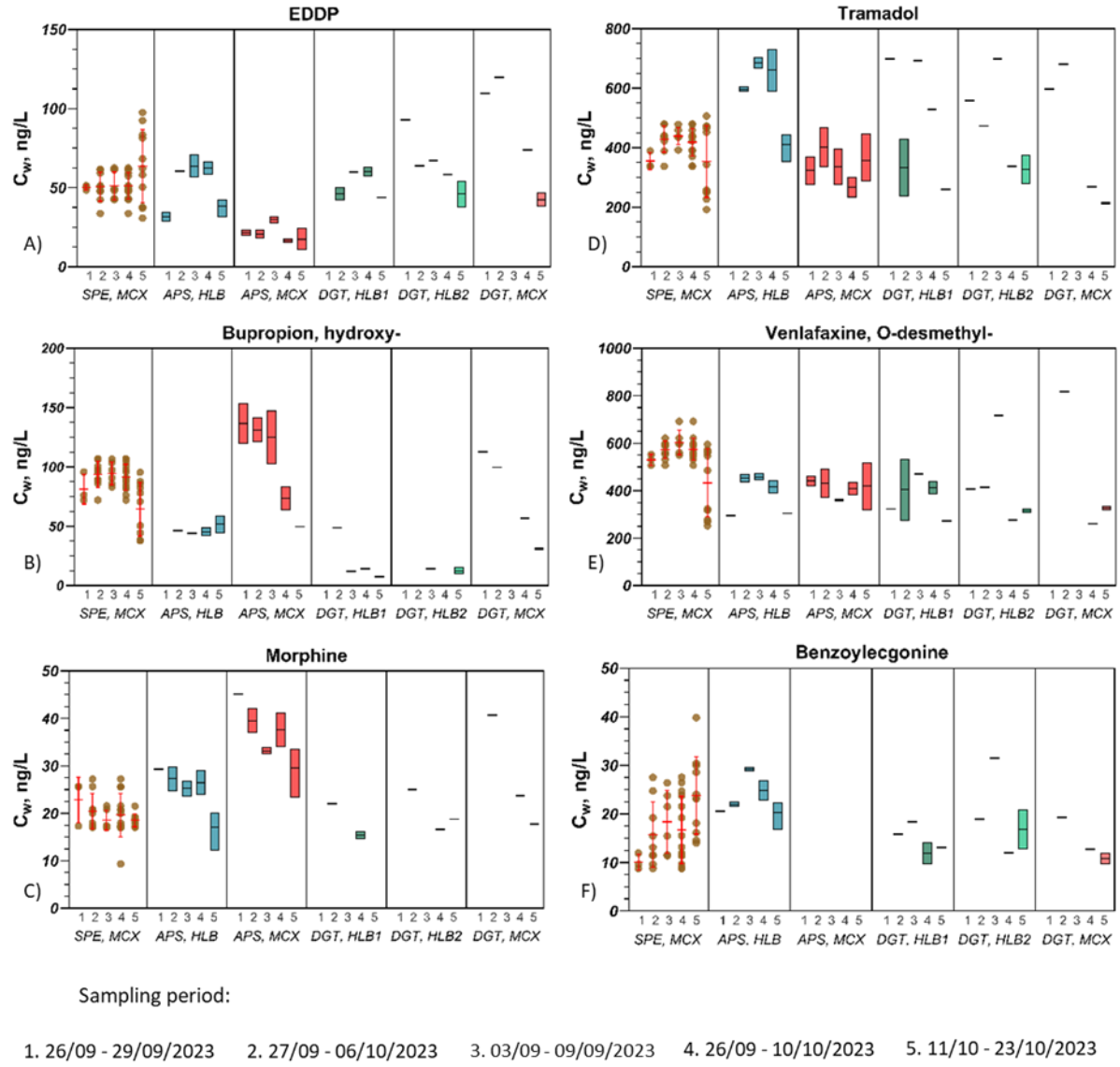


Figure 15: Scatterplot and boxplots showing the average concentrations of target psychoactive pharmaceuticals with all sampling methods tested during all sampling periods.

On average, concentrations of most psychoactive compounds measured using the APS (HLB and MCX) were in good agreement compared to both conventional 24-hour composite sampling (followed by SPE) and o-DGT devices (generally <40% difference), following deployment of 3, 6, 9, 12, and 14 days (**Figure 16**). One exception was EDDP, which, according to our results, showed a significantly ($p<0.05$) lower average concentration (60% difference) in the APS-MCX compared to the SPE (**Figure 15A** and **Figure 16A**), while no significant discrepancies were found when comparing the SPE, APS-HLB, and o-DGT-HLB concentrations.

Bäuerlein et al. [242] emphasised the influence of analyte functional groups on sorption to sorbents such as HLB and MCX. Generally, apolar groups, particularly aromatic rings, play a more significant role in sorption than hydrogen bonding, as both the sorbent and sorbate can interact with water molecules through H-bonding. Since MCX has fewer accessible apolar groups compared to HLB, sorption occurs more rapidly on HLB via π-π interactions. In contrast, MCX provides an additional sorption mechanism through cation

exchange for cationic species [242]. Our results align with a recent study [239] showing that EDDP exhibits the strongest sorption on HLB, likely due to its relatively high apolar surface area. In contrast, sorption of cationic compounds on MCX depends on the accessibility of their charged functional groups.

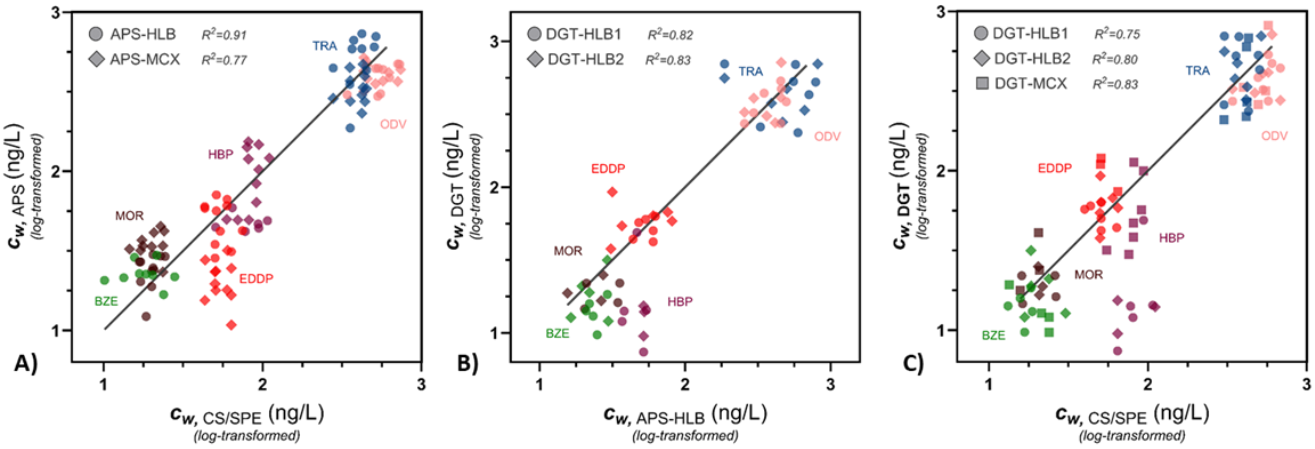


Figure 16: Correlation plots comparing sampling methods. Obtained concentrations are log-transformed, and the diagonal line represents a 1:1 correlation.

Similarly, hydroxybupropion showed a statistically lower average concentration (50% difference) with the APS, but this time using the HLB sorbent, compared to the SPE (**Figure 15B**). In general, there were no major discrepancies in the concentrations of targeted compounds. However, for morphine (**Figure 15C**) and tramadol (**Figure 15D**), the APS concentrations, when using MCX and HLB, respectively, were approximately two times higher than those obtained by SPE composite sampling. *O*-desmethylvenlafaxine was the only compound where measured SPE concentrations were slightly higher (<30% difference) compared to both APS-HLB and APS-MCX concentrations (**Figure 15E**). Variations in concentration could be linked to interactions between compound species and micro- and nanoparticles in wastewater, potentially influencing their sorption rate and extent onto the sorbent. What is also noteworthy is the case of BZE, whose concentration was <LOQ when in the APS-MCX sorbent (**Figure 15F**). This observation could be attributed to the cation-exchange properties of MCX, which enhance the sorption of cationic species but are less effective for zwitterionic compounds such as BZE. The average sorbed concentrations of BZE on APS-HLB sorbent were in good agreement with SPE (25% difference).

Moreover, as shown in **Figure 16B**, comparing APS-HLB and o-DGT-HLB revealed variations in hydroxybupropion concentrations. In particular, the concentration of hydroxybupropion was on average 60% lower for o-DGT-HLB (both HLB_1 and HLB_2) compared to APS-HLB. This difference could be due to the smaller exposed surface area of the o-DGT sorbent compared to that of the APS (3.14×10^{-4} and 8.0×10^{-4} m^2, respectively). For the remaining target compounds, no significant differences (<30%) were observed between APS-HLB and o-DGT-HLB. Additionally, the comparison between o-DGT-HLB_1 and o-DGT-HLB_2 revealed no significant differences, with differences <20% in all cases. Finally, when comparing the o-DGT (HLB_1, HLB_2, and MCX) with the SPE concentrations, results showed good agreement for most target compounds, with only a few variations, mainly for hydroxybupropion (**Figure 16C**). According to the results, hydroxybupropion showed a significantly ($p<0.05$) lower average concentration (75%

difference) with the o-DGT-HLB device compared to composite samples extracted using SPE (**Figure 16C** and **Figure 15B**).

3.5.1.4 Conclusions

This study demonstrated the first successful *in-situ* application of the APS for determining psychoactive compounds (antidepressants, illicit drugs, and opioids) in wastewater effluents. It compared the APS's performance with o-DGT (both commercial and lab-assembled) and conventional 24-hour composite sampling (followed by SPE) during the same sampling period. Across five deployment periods (3, 6, 9, 12, and 14 days), APS showed good agreement with both 24-hour composite sampling (followed by SPE) and o-DGT devices for most compounds, with concentration differences generally within 40%. APS was remarkably consistent with 24-hour SPE-based measurements for all compounds, except for EDDP and hydroxybupropion, where compound-specific deviations were observed, likely driven by differences in sorbent affinities, analyte speciation, or interactions with particulate matter. Observed variations in compound behaviour suggest the need for further investigation of chemical speciation, sorption, partitioning, and accumulation rates on the sorbents, to optimise analytical approaches and include a broader range of compounds in the future.

APS integrates the advantages of both passive and active sampling techniques, combining controlled uptake dynamics with broad analyte detection, while avoiding reliance on high-cost equipment or daily sample handling. Both APS and o-DGT can pre-concentrate analytes over time, enabling improved detection of low-level compounds without the complexity or expense of autosamplers. Additionally, APS is particularly advantageous where controlled flow rates, low detection limits, and integrated monitoring over multiple days are desired.

Although the results showed the potential of APS in monitoring TWA concentrations of psychoactive compounds in wastewater effluents, complementing conventional 24-hour composite sampling by providing continuous monitoring and capturing temporal variations, APS is not without its limitations. Despite promising results in effluent wastewater applications, the initial deployment of the APS device in influent wastewater failed after 18 hours due to filter clogging. Influent matrices are highly complex, containing large particulate matter, suspended solids, and biological debris, which obstruct flow and damage system components.

Future research should also include optimisation of the APS device for challenging matrices, such as influent wastewater monitoring. Such improvements could include physical modifications (e.g., a metal cage or protective mesh around the PES membrane to prevent clogging by large debris and solids) and alternative pumping mechanisms, such as more resilient micro-pump types (e.g., peristaltic or diaphragm pumps) that are less prone to fouling. Besides allowing for their exploitation in wastewater-based epidemiological studies, this could also enable the evaluation of WWTP treatment efficiency, which is in line with the upgraded UWWTD (2024, 1991) [211], [212].

Chapter 4

Overall Conclusions

Analytical methods for psychoactive substance biomarkers for WBE

Wastewater-based epidemiology (WBE) is a well-established approach that has quickly demonstrated its potential to provide objective, independent, cost-effective, and near real-time data, offering valuable insights into human exposure and consumption of xenobiotics with high spatial and temporal resolution. Since its first application in 2005 on cocaine consumption in Italy, WBE has undergone continuous improvements and advancements.

While WBE was initially focused on monitoring illicit drug use, its applications have since expanded significantly, as several proof-of-concept studies have demonstrated that WBE is also suitable for evaluating community health. For instance, WBE has been applied to monitor population-level consumption of alcohol, tobacco, caffeine, opioids, artificial sweeteners, various pharmaceuticals, new psychoactive substances, and personal care products, to estimate the exposure to emerging chemical contaminants, such as flame retardants and pesticides, and more recently to monitor the spread of SARS-CoV-2 within communities. However, compared to illicit drugs, the number of WBE studies focusing on psychoactive pharmaceuticals, including antidepressants, antipsychotics, and benzodiazepines, remains limited.

The initial stages of the WBE approach involve sample collection, pre-treatment, preparation, and instrumental analysis. This thesis highlighted the literature gap and provided a comprehensive overview of each step involved in analysing antidepressants, antipsychotics, benzodiazepines, and their corresponding metabolites in influent wastewater, within the scope of WBE. From an analytical perspective, SPE for sample preparation and targeted LC-MS/MS analysis (LC-QqQ-MS or QTrap MS) has been the most commonly used approach for WBE detection and quantification of psychoactive pharmaceutical biomarkers, largely due to its high sensitivity.

More recently, HRMS has gained popularity in wastewater analysis, particularly for the identification of new psychoactive substances (NPS) through both suspect and non-target screening. Efforts have been made to develop a broader range of bioanalytical methods that simultaneously measure several antidepressants, antipsychotics, benzodiazepines, and their metabolites from influent wastewater samples in low concentrations and over different periods. However, most available WBE studies include only a limited selection of psychoactive pharmaceutical biomarkers, focusing mainly on antidepressants. Therefore, more efforts are needed to detect and quantify additional psychoactive pharmaceutical biomarkers in wastewater.

Accordingly, within the thesis, analytical methods were developed and validated for determining a broad range of psychoactive pharmaceutical and NPS biomarkers in influent

wastewater. In particular, a sensitive analytical method based on SPE and LC-QTrap-MS was developed and validated for the simultaneous determination of 42 psychoactive pharmaceutical biomarkers, including 18 antidepressant, 11 antipsychotic, and 13 benzodiazepine biomarkers (parent compounds and metabolites) at low ng/L levels in influent wastewater. The performance criteria for 41 biomarkers (one antidepressant biomarker was excluded) met the acceptable criteria set by SANTE guidelines. Additionally, following SPE, a targeted LC-QqQ-MS method for analysing more than 60 NPS biomarkers in influent wastewater was developed and validated. Again, the performance criteria for 54 NPS biomarkers met the acceptable criteria set by SANTE guidelines.

Although SPE is the most widely used sample preparation method for the pre-concentration of psychoactive substance biomarkers in wastewater, it often requires relatively large sample volumes (≥50mL) of influent wastewater to achieve the required LODs. Most of these analytical methods are both labour-intensive and time-consuming.

Few studies have applied direct injection methods to analyse psychoactive substance biomarkers in influent wastewater; pre-concentration is typically required to detect and accurately quantify these biomarkers at trace levels. In this thesis, a high-throughput method based on a 96-well elution SPE protocol and LC-MS/MS was developed, optimised, and validated for the quantification of a broad range of psychoactive substances, including biomarkers for antidepressants, opioids, and illicit drugs in influent wastewater. Performance criteria were within the EMA-acceptable limits for 28 of 29 psychoactive substance biomarkers.

This innovative 96-well SPE sample preparation protocol, which requires only 2 mL of influent wastewater (instead of >50 mL), has been shown to be especially effective for handling large volumes of influent wastewater samples and for collecting long-term spatiotemporal data on the consumption of various psychoactive substances. Although the pre-concentration achieved using 96-well SPE (approximately 20-fold) is lower than that with traditional SPE methods (around 250-fold), LC-MS/MS still demonstrated sufficient sensitivity to detect the target analytes at low ng/L concentrations. This difference may be attributed to variations in matrix effects (ME) between the 96-well elution technique and conventional SPE protocols.

Currently, there are no reported applications in the literature of time-efficient, miniaturised SPE versions for analysing a broad spectrum of psychoactive substance biomarkers in influent wastewater. However, because this approach does not require any modifications to the LC instrumentation, it could be compatible with nearly all analytical laboratories. The implementation of this miniaturised sample preparation method significantly decreases sample processing time, overall analysis duration, and solvent consumption.

WBE as a complementary tool for monitoring spatiotemporal changes in psychoactive substance consumption: Application to the Slovenian population

The use of psychoactive substances (psychoactive pharmaceuticals and NPS) has a significant impact on public health. Especially since the outbreak of the COVID-19 pandemic, there has been a growing interest in exploring how it affected people's mental health. Additionally, NPS rapidly enter the market as legal alternatives to conventional illicit drugs, bypassing legal restrictions and thus becoming an international public health concern. Conventional socio-epidemiological methods, such as general population surveys, hospital records, and prescription and sales data, are used to obtain information on trends

in psychoactive pharmaceutical consumption. Self-reported surveys, seizure data, and forensic and toxicological analysis are also employed to collect data on NPS consumption trends in the population; however, these methods have many limitations. To overcome these challenges, complementary approaches such as WBE are necessary.

WBE offers the potential to serve as an indicator of lifestyle and public health characteristics within populations, providing data quickly and with high spatial and temporal resolution. Analysis of only a small number of influent wastewater samples can deliver anonymous, objective, complementary and real-time data about a community's health and lifestyle. Due to its high spatiotemporal resolution, WBE can also serve as an early warning system for detecting emerging trends in consumption and exposure to different xenobiotics in defined populations.

In the thesis, the developed and validated analytical methods were used to measure spatiotemporal changes in the PNMLs of psychoactive pharmaceutical and NPS biomarkers, and to evaluate their consumption trends in the Slovenian population. Short-term weekly monitoring (weekdays vs weekends) can reveal potential recreational use of psychoactive pharmaceuticals, while long-term trends can also provide insights into the stability or fluctuations in the use of both psychoactive pharmaceuticals and NPS over time. Additionally, the high spatial resolution enabled area-based evaluations that would not have been possible with only conventional epidemiological data.

To our knowledge, this is the first WBE study to investigate the spatiotemporal use of psychoactive pharmaceuticals and NPS at a higher degree of granularity in Slovenia, and to retrospectively evaluate the influence of the pandemic on their usage patterns. Specifically, seven WWTPs in urban and rural areas across Slovenia, which cover approximately 31% of the Slovenian population, were included in the study. In total, samples were collected during six sampling periods, spanning the pre- and post-pandemic periods, when most restrictions had been eased.

Our results revealed several temporal and spatial variations for both psychoactive pharmaceuticals and NPS. Regarding psychoactive pharmaceuticals, there was a general alignment between measured PNMLs and annual prescription data in Slovenia, further supporting the validity of the WBE results. However, some notable discrepancies emerged. For instance, an exceptionally high concentration of citalopram was observed in Velenje during spring 2020, without a corresponding increase in its metabolite, suggesting direct disposal rather than consumption. Some psychoactive pharmaceuticals exhibited temporary increases or decreases in wastewater levels that were not mirrored in prescription records, indicating potential changes in usage behaviour or population dynamics.

Furthermore, differences in spatial resolution between the localised WBE data and nationally aggregated prescription data may contribute to such discrepancies. Accordingly, hypothesis 1 **(H1): "Measured levels of psychoactive pharmaceutical biomarkers in influent wastewater accurately reflect available prescription data on their consumption trends within Slovenian communities"** was **partially confirmed**. Therefore, while WBE does not perfectly mirror prescription data, it serves as a valuable complementary tool to traditional monitoring methods, enhancing the understanding of pharmaceutical consumption trends in the Slovenian population and offering insights beyond what prescription data alone can provide.

In addition, the results of this thesis showed that the PNMLs of most antidepressant biomarkers increased during the sampling campaigns, particularly during the first wave of the pandemic (spring 2020). On the contrary, the PNMLs of most antipsychotic and benzodiazepine biomarkers selected for long-term temporal analysis showed a decreasing trend during the same sampling period and particularly during the first wave in spring 2020. However, no clear patterns related to the pandemic were observed in the short-term

analysis of weekdays vs weekends, as most statistically significant differences were observed either before or after the pandemic.

This thesis also demonstrated the ability of WBE to overcome limitations associated with conventional NPS data collection, including identifying which NPS are circulating on the market without concrete data on their prevalence or use. Through targeted analysis, it is possible to quantify NPS use within the target population. Incorporating suspect screening into the workflow enables the rapid identification of newly emerging NPS. Accordingly, wastewater monitoring of NPS trends was first implemented during the 2019/2020 New Year period as part of an international initiative, with the long-term aim of establishing a global surveillance NPS system similar to the SCORE model.

Slovenia was first included in the 3rd international NPS monitoring campaign (New Year period 2021/22), revealing some interesting findings regarding the consumption of NPS in some specific sites, such as the detection of eutylone, mitragynine, and 3-MMC, with the latter having one of the highest mass loads detected within the study. These results led to a broader investigation of spatiotemporal trends in NPS use in Slovenia over four years of monitoring (2019–2022) during regular non-holiday periods.

The outbreak of the COVID-19 pandemic in Slovenia in March 2020 also provided a valuable chance to evaluate the influence of the pandemic on NPS use. Five NPS were quantified across all sites at least once, with 3-MMC being the most prevalent, followed by mitragynine, which showed the highest PNMLs. One common observation for all detected NPS was their disappearance during the first wave of COVID-19 (spring 2020), except for 3-MMC, which, in addition to being measured during all sampling periods, also showed an increasing trend during the pandemic.

One main limitation regarding the spatiotemporal evaluation of both psychoactive pharmaceutical and NPS use was that only a single week of sampling was included within each sampling period. As a consequence, consumption rates may not fully capture the rest of the year. However, since all sampling campaigns were conducted in a "typical" week, it is assumed that the data reflect baseline use, with weekdays and weekends representing baseline consumption within a regular working week.

Overall, the results showed that WBE was capable of monitoring the temporal and spatial changes in psychoactive substance use during the COVID-19 pandemic, **confirming** hypothesis 2 **(H2): "WBE can serve as a complementary data source to evaluate the impact of the COVID-19 pandemic on the use of psychoactive pharmaceuticals and NPS in Slovenia"**.

WBE uncertainties: Stability of psychoactive pharmaceuticals and NPS

Although WBE is a well-established approach for monitoring (ab)use, misuse, and regional prescription patterns by providing objective information on psychoactive substance use within a population, there remains a need to reduce methodological uncertainties, such as stability. In WBE, accurate estimations rely on the stability of substance biomarkers in influent wastewater; however, current information on the stability of psychoactive pharmaceuticals, especially antipsychotics, during sampling, storage, and sample preparation is limited.

Throughout various stages, ranging from excretion and in-sewer transport to sampling, storage, and final analysis, target biomarkers may be degraded or transformed, potentially resulting in under- or overestimation of actual substance use. Therefore, it is crucial to ensure that the selected biomarker is stable or that an appropriate correction factor is applied to enable an accurate assessment of consumption. This study presents the first comprehensive analysis of the stability of 11 representative antipsychotic biomarkers, including parent compounds and related metabolites, in influent wastewater. It uses a series

of experiments that mimic in-sewer transport and in-sample stability under various conditions.

The results showed that in-sewer degradation is compound-specific and influenced by environmental conditions, such as temperature and residence time, although the latter is often too short to affect many biomarkers significantly. Acidification before storage should generally be discouraged because it degrades some analytes at low pH and reduces their stability after freeze-thaw cycles, making this approach unsuitable for preserving samples. In contrast, SPE cartridges offer a more reliable storage option, with all compounds except quetiapine remaining stable for up to three months. Given these results, WBE should be used cautiously to monitor the spatiotemporal use of antipsychotics and tailored to specific biomarkers to ensure accurate conclusions are made.

Additionally, within the framework of international NPS monitoring, another key focus of this thesis was evaluating the stability of NPS on SPE cartridges, particularly to ensure that NPS biomarkers remain stable during international shipping and potential delays. A total of 54 NPS biomarkers were evaluated for stability at five temperatures (−80 °C, −20 °C, 4 °C, 22 °C, and 37 °C) across five time points (three days to four months), simulating differing storage conditions, as well as the winter and summer conditions over the New Year period around the world. The findings showed that the majority of NPS biomarkers (n = 46) remained stable for up to three months when loaded onto SPE cartridges at −20 °C.

Overall, understanding the degradation processes and evaluating the in-sample and in-sewer biomarker stability further strengthens the accuracy of WBE, as a complementary data source for monitoring spatiotemporal trends in the consumption of psychoactive pharmaceuticals and NPS. Accordingly, the confirmation of hypothesis 2 **(H2): "WBE can serve as a complementary data source to evaluate the impact of the COVID-19 pandemic on the use of psychoactive pharmaceuticals and NPS in Slovenia"** is **further supported**.

The ecological impact of psychoactive pharmaceutical residues in the aquatic environment

There is growing recognition that the use of psychoactive pharmaceuticals, including antidepressants, antipsychotics, and benzodiazepines, not only affects human health and well-being but also has a significant impact on the environment. The increased consumption of psychoactive pharmaceuticals in modern society has driven higher production by the pharmaceutical industries, leading to a greater discharge of pharmaceutical and hospital waste, beyond municipal waste, into the aquatic environment. In response to the global rise in psychoactive pharmaceutical use, the UNODC recognised psychoactive pharmaceutical residues as an increasing environmental threat and advocated for expanded research into their environmental occurrence and toxicological effects.

Due to their physicochemical properties, particularly high hydrophilicity, psychoactive pharmaceutical residues tend to accumulate in aquatic environments, with treated wastewater being their primary source. Accordingly, several studies have assessed the removal efficiency of psychoactive pharmaceutical residues using conventional wastewater treatment methods, including activated sludge, membrane bioreactors (MBR), and sequential batch reactors (SBR).

Comprehensive data on the removal of psychoactive pharmaceuticals remains limited due to the number of residues and treatment methods (i.e., moving bed biofilm reactor, MBBR) tested. Additionally, most of the studies have been focused on the removal of antidepressants, while only a limited number of antipsychotics or benzodiazepines have

been addressed. Consequently, further research is essential, especially in light of the EU's recently revised urban wastewater treatment directive, which requires the inclusion of additional quaternary treatment to remove >80% of at least six out of 13 listed micropollutants, including three antidepressants (citalopram, carbamazepine, and venlafaxine), which are also the subject of this study.

A significant part of this thesis focused on the removal efficiency of antidepressant, antipsychotic, and benzodiazepine residues in seven Slovenian WWTPs, which varied in catchment size and type of wastewater treatment technology, including activated sludge, SBR, SBR with UV, MBR, and MBBR. Among the studied treatment technologies, the efficiency of MBBR in removing psychoactive pharmaceutical residues was tested for the first time. The results showed that the different treatment technologies remove psychoactive pharmaceuticals to differing degrees, with AS and MBR being the most efficient and MBBR the least efficient.

This thesis also revealed how removal is compound dependent, with, e.g., oxazepam, venlafaxine/*O*-desmethylvenlafaxine, and citalopram/norcitalopram residues being poorly removed (average RE<40%) in all cases. On average, better removals were found for antipsychotics (REs=73%), followed by benzodiazepines (REs=45%), then antidepressants (REs=39%). Although a few antipsychotics, including clozapine, quetiapine, and paliperidone, were effectively removed (<90%) with some treatment methods, complete mineralisation was not achieved, **confirming H3: "Conventional biological wastewater treatment technologies do not entirely remove psychoactive pharmaceutical residues to <LOQ"**. Furthermore, bupropion-OH, carbamazepine, CBZ-E, clozapine, norclozapine, norquetiapine, temazepam, sertraline, and ZPCA residues showed significantly lower removal rates in MBBR systems compared to other treatment technologies. Psychoactive pharmaceutical residues were also found in various amounts in effluent-receiving surface waters, reflecting their degree of removal during treatment.

As a result of their continuous release into wastewater, psychoactive pharmaceutical residues, including both parent compounds and their metabolites, demonstrate pseudo-persistence in the aquatic environment and have been detected in various receiving waters, such as rivers, lakes, and oceans, as well as in some cases, even in soil or drinking water supplies. Even at trace levels (ng/L), their presence in surface waters could pose a threat to non-target aquatic organisms, and long-term exposure leads to chronic ecological and evolutionary impacts. Unfortunately, data on their occurrence in environmental waters remain limited due to the number of residues tested and the diversity of aquatic organisms exposed. Consequently, more data are required on the environmental presence and toxic effects of psychoactive pharmaceutical residues to better assess ecological risks.

This thesis explored the occurrence of a broad range of antidepressants, antipsychotics, and benzodiazepine residues in Slovenian effluent receiving surface waters. Additionally, the ecological risk posed by the measured concentrations of psychoactive pharmaceutical residues in the tested receiving surface waters was evaluated using the ECOSAR predictive model. At the measured concentrations, the effect on aquatic organisms was predicted for venlafaxine, *O*-desmethylvenlafaxine, citalopram, and sertraline residues, which leads to the **partial rejection** of **H5: "Measured levels of psychoactive pharmaceutical residues in surface waters impacted by WWTP effluent do not pose a risk to aquatic organisms based on the ECOSAR predictive model"**, indicating that some psychoactive pharmaceutical residues may pose an acute toxicity risk to the aquatic organisms at the determined levels. Considering the *in silico* data, further *in vivo* studies would be beneficial.

Novel sampling strategies for the determination of psychoactive substances in wastewater

An overall issue to be addressed in WBE is the sampling process. This thesis employed an innovative sampling strategy that utilised APS devices developed at the University of Antwerp. In brief, the APS relies on generating a controlled flow across sorbents selective for several classes of compounds, thereby providing on-site, *in-situ* pre-concentration of target biomarkers in a well-defined manner. Such an approach yields average concentrations over the device's deployment time (e.g., a few days to a few weeks), reducing the required sampling frequency and aligning them with the WBE approach. This deployment was the first *in-situ* application of the APS device in wastewater for the determination of six psychoactive compound biomarkers (antidepressants, illicit drugs, and opioids), and compared its performance with o-DGT passive samplers (both purchased and lab-assembled) and conventional 24-hour composite sampling (followed by SPE) during the same sampling period.

Data from over five deployment periods (3, 6, 9, 12, and 14 days) revealed that APS results were in good agreement with both 24-hour composite sampling (followed by SPE) and o-DGT devices for most biomarkers, with concentration differences generally within 40%. APS results were also remarkably consistent with the 24-hour SPE-based measurements for all biomarkers, except for EDDP and hydroxybupropion, for which compound-specific deviations were observed, likely driven by chemical speciation, differences in sorbent binding or interactions with particulate matter.

The APS offers a hybrid approach that integrates the strengths of both passive and active sampling, providing controlled uptake rates and broad analyte coverage without the need for expensive equipment or daily maintenance. Like o-DGT, APS enables the time-integrated pre-concentration of analytes, thereby enhancing the detection of low-concentration substances while minimising the complexity and associated costs of autosamplers. Additionally, APS benefits are particularly evident in applications that require controlled flow rates, low detection limits, and extended monitoring periods. Although this thesis demonstrated APS's potential to complement traditional 24-hour composite sampling by enabling continuous measurement and capturing temporal fluctuations in psychoactive compound levels in wastewater effluents, APS does have certain limitations that should be considered. Despite the promising results in effluent wastewater applications, initial deployment of the APS device in influent wastewater failed due to filter clogging within 18 hours, which leads to a **partial rejection** of **H4: "APS provides a more convenient and efficient sampling strategy than conventional 24-hour composite sampling"**. Further improvements would provide valuable opportunities for WBE applications and environmental surveillance.

To summarise, determining psychoactive substance residues in waste and environmental waters provides significant information on the epidemiological and environmental aspects of psychoactive substance use. These aspects are closely related and require a multidisciplinary approach to be understood and addressed effectively. This thesis used a combination of different methods, including chemical analysis, ecotoxicity predictive model, and risk assessment, to (i) determine psychoactive substance biomarkers in aquatic matrices, (ii) monitor spatiotemporal use of psychoactive pharmaceuticals and NPS in the Slovenian population using WBE and compare the data with the available prescriptions (for psychoactive pharmaceuticals), (iii) evaluate the influence of the COVID-19 pandemic on psychoactive pharmaceutical and NPS consumption trends, (iv) assess the stability of antipsychotic and NPS biomarkers, (v) evaluate removal efficiency of psychoactive pharmaceutical residues during conventional biological wastewater treatments,

(vi) determine the occurrence of psychoactive pharmaceutical residues in receiving surface waters, (vii) estimate associated acute and chronic toxicity to aquatic organisms, and (viii) deploy and compare innovative (active-passive) and conventional (24-hour composite) wastewater sampling strategies.

Overall, the work has made significant contributions to both science and society. Importantly, it has enabled methodological advancements for determining psychoactive pharmaceuticals and NPS in wastewater within the WBE framework. In particular, classical SPE protocols and a high-throughput miniaturised SPE protocol using Oasis MCX 96-well plates were developed and validated for the quantification of a broad range of psychoactive substance biomarkers in waste and surface waters. Additionally, it has provided timely information on changes in the spatiotemporal patterns of psychoactive pharmaceutical and NPS use in Slovenia and evaluated the pandemic's influence on their use, thereby enhancing our understanding of these international trends. It also comprehensively assessed the stability of antipsychotic and NPS biomarkers, further supporting WBE in obtaining more accurate consumption data and strengthening its validity as a complementary approach.

The thesis also contributes additional data on removing psychoactive pharmaceutical residues during wastewater treatment, including, for the first time, their removal using an MBBR. Moreover, it has evaluated the prevalence of psychoactive pharmaceutical residues in receiving surface waters and assessed associated environmental risks by using the ECOSAR predictive model. Lastly, this thesis investigated, for the first time, innovative active-passive sampling strategies in wastewater and compared them to conventional 24-hour composite sampling.

These contributions are documented in eight peer-reviewed scientific publications and one publication to be submitted for revision in Environmental Pollution in December 2025. The published papers include renowned peer-reviewed publications with impact factors ranging from 2.6 to 12.4. Three publications were published in the journals with outstanding scientific performance (A” according to the Slovene Research and Innovation Agency) classification. The doctoral candidate is the first author in five of the publications. In addition, the research findings have been presented at eleven scientific conferences, including both oral presentations and poster sessions.

Despite the contributions this thesis has made, many questions remain unanswered, and there is considerable scope for further research. In this regard, this work serves as a foundation for future studies. Future directions (Chapter 5) include, among others, methodological advancements, applications to specific populations, and further investigation of the impact of psychoactive substance residues in aquatic ecosystems.

Chapter 5

Future Perspectives

Methodological improvements

This section highlights key insights and future opportunities for WBE to further contribute as a complementary epidemiological source, providing independent, accurate, and timely information on xenobiotic consumption trends at high spatiotemporal resolution. Although WBE has already been used for two decades to investigate licit and illicit drug consumption, and it has proven useful in monitoring trends in the use of psychoactive pharmaceuticals in the general population, additional research is needed on methodological issues.

New biomarkers (parent compounds or corresponding metabolites) for a selection of frequently prescribed pharmaceuticals, particularly for antipsychotics and benzodiazepines, should be included in the target analytical methods, such as the high-throughput 96-well SPE method. Considering that for some psychoactive pharmaceuticals of interest, such as fluoxetine, paroxetine, duloxetine, haloperidol, and lorazepam, only parent compounds have been investigated, their corresponding metabolites should be added to analytical methods.

The available Phase I and II metabolism studies in the scientific literature should be further reviewed to identify additional metabolites to be targeted in influent wastewater. Additionally, it is essential to assess biomarker suitability by evaluating their pharmacokinetics and stability to address associated uncertainties, particularly when using parent compounds as biomarkers. This data is also needed for the accurate quantification of NPS in influent wastewater (e.g., within the framework of international NPS monitoring). Currently, data on their excretion and metabolism are limited, making it more challenging to estimate their consumption.

In-sewer (i.e., temperature, pH, time, biofilm presence) and in-sample (i.e., storage, freeze-thaw, benchtop) conditions can result in the degradation and (bio)transformation of biomarkers. In-sewer and in-sample stability for several of the investigated biomarkers (mainly antidepressants and benzodiazepines) have been studied in various other publications, while there is only limited research for antipsychotics and NPS.

Finding consensus on a suitable experimental setup that fully mimics sewer conditions remains challenging. A variety of approaches, such as gravity sewers, biofilm-free systems, and rising main sewer reactors, along with different conditions (pH, temperature, duration, and biofilm content), have been reported, making it difficult to compare findings across studies. Similarly, various experiments (benchtop, frozen cartridge, long-term sample storage, WWTP autosampler, and freeze-thaw) have been used in different studies to evaluate in-sample stability under different conditions (filtration, temperature, and pH).

Consequently, the in-sewer and in-sample stability of several target psychoactive substance biomarkers should be further assessed.

The sampling schemes used in this thesis primarily consisted of daily 24-hour composite sampling for one week at different times between 2019 and 2023. The applicability of novel sampling approaches, such as active-passive sampling, should be further explored for spatiotemporal trend analysis within the WBE framework. Although APS was successfully applied to effluent wastewater, further optimisation is needed for more complex matrices such as influent wastewater.

Improvements may include physical adaptations such as installing a metal cage or protective mesh around the PES membrane to prevent blockages caused by large debris and suspended solids. Additionally, integrating more robust pumping systems—such as peristaltic or diaphragm micro-pumps—could help reduce fouling and improve reliability. Moreover, with the inclusion of a broader range of psychoactive substances in this approach, these advancements would not only expand the applicability of APS in WBE studies but also support the assessment of WWTP performance, aligning with the objectives of the revised Urban Wastewater Treatment Directive.

Further research is also required to reduce uncertainty in estimating the population size contributing to the WWTP. This omission is of great importance, as failing to account for population fluctuations may lead to incorrect conclusions about consumption trends or exposure levels. While various efforts have already been made to track changes in population sizes to reduce this uncertainty, proposed methods, including the use of mobile phone data and the determination of hydro-chemical parameters, remain inadequate. Mobile phone data, for instance, is not easily accessible or even available in all countries (including Slovenia), and hydro-chemical indicators like chemical oxygen demand (COD) and biological oxygen demand (BOD) are influenced not only by human activity but also by industrial and agricultural inputs. In the future, further research and alternative methods for applying dynamic population proxies are needed to address this methodological issue effectively.

WBE: application to the Slovenian population

International NPS monitoring is expected to continue, and interlaboratory studies and best-practice protocols will need to be developed. Slovenia's ongoing participation in NPS monitoring is beneficial at both national and international levels. Accordingly, during the New Year periods of 2023/2024 and 2024/2025, Slovenia contributed to international NPS monitoring by providing wastewater samples from seven municipalities. However, future participation also depends on funding.

Beyond methodological development, efforts should also focus on exploring the potential of applying wastewater analysis to specific populations as a non-invasive alternative to existing methods, such as individual drug testing. These could include educational institutions, prisons, and sports settings. Although studies have been made in Slovenian educational institutions to investigate the use of illicit drugs, monitoring the use of psychoactive pharmaceuticals would provide additional value. Educational institutions, mainly high schools and universities, are increasingly associated with the use of medications (i.e., antidepressants, anxiolytics) either for therapeutic purposes, cognitive enhancement, or non-medical use. WBE would provide valuable insight into students' mental health, which could inform campus health services, awareness campaigns, and policy interventions. However, ethical considerations, including informed consent and the potential for misinterpretation or stigmatisation of institutions, must be carefully addressed.

In correctional facilities, WBE could become a powerful tool to monitor the use of psychoactive pharmaceuticals and NPS in a comprehensive and real-time manner. Prisons

are high-risk settings for substance misuse, both through the smuggling of illicit drugs and the diversion of prescribed medications. WBE can serve as a tool for early detection of drug misuse trends, help evaluate the effectiveness of treatment and harm reduction programs, and guide health interventions tailored to the specific needs of the incarcerated population. Additionally, its non-invasive nature and ability to assess drug use at the population level provide a practical alternative to conventional methods such as random drug testing, which can be resource-intensive and limited in scope. However, implementing WBE in prisons must be handled with caution, ensuring strict adherence to ethical standards that protect privacy and prevent the misuse of data for punitive or discriminatory purposes.

Recreational and professional athletes represent another potential setting, where wastewater analysis could be applied to monitor psychoactive substance misuse. Current doping control methods rely primarily on random individual testing, which may not capture broader trends or detect the use of new or designer substances that fall outside conventional testing panels. Here, wastewater analysis could serve as an initial screening method, providing insight into group-level drug use and guiding more targeted anti-doping strategies. However, since such an application has not yet been implemented in practice in the sports domain, careful consideration must be given to ethical implications, such as the potential stigmatisation of entire teams or clubs, especially when interpreting group-level data. Beyond identifying drug misuse, the use of suspect screening or non-targeted analytical approaches in wastewater analysis could also help detect emerging trends in the use of psychoactive substances in sports. Transparent communication and regulatory frameworks are essential to ensure fair, responsible, and scientifically grounded applications in the athletic community.

Ultimately, wastewater analysis is expected to expand its applications beyond estimating population-level consumption of psychoactive substances. Another promising direction of WBE is its application to assess the spread of viruses and other diseases within populations, a role that proved valuable during the COVID-19 pandemic. Moreover, WBE can be applied to explore population-wide exposure to substances not intended for human consumption, such as pesticides, plasticisers, and various household or industrial chemicals, thereby serving as a complementary approach to human biomonitoring. Such an application is currently being explored through the ongoing European Partnership for the Assessment of Risks from Chemicals (PARC) project, co-funded by the European Union. The initiative focuses on evaluating the risks associated with known, emerging, and novel pollutants, aligning with the EU's objective of achieving a pollution-free environment.

Psychoactive pharmaceutical residues in receiving surface waters and associated risks

While this thesis has made important contributions, further research is necessary to deepen our understanding of the presence of psychoactive pharmaceuticals in environmental waters. The predominant use of grab sampling as a technique for sampling surface waters presents limitations because it captures only a snapshot of substance concentrations at the time of collection and fails to provide time-resolved data. Therefore, alternative methods, such as passive or active-passive sampling techniques, require further investigation. Additionally, the evaluation of psychoactive pharmaceutical residues should also be expanded to include groundwater. Consequently, there is a need for studies that include a broader spectrum of psychoactive pharmaceuticals and their metabolites to support future environmental risk assessments, including those linked to the EU Watch List and Groundwater Watch List under the Water Framework Directive.

Furthermore, suspect screening and non-target analysis should be considered to detect substances that may occur in the environment but cannot be detected by targeted analytical methods. Moreover, conducting studies on psychoactive pharmaceutical residues in environmental waters is especially critical in developing regions and countries with limited access to wastewater treatment infrastructure. In these areas, untreated or inadequately treated wastewater can lead to higher concentrations of psychoactive pharmaceutical residues in the environment, posing significantly greater risks to ecosystems and public health.

Exploring the potential impact of psychoactive pharmaceutical residues on other subsurface aquatic ecosystems (i.e., caves) would be of great interest, given their ecological sensitivity and fragility, particularly in light of Slovenia's well-known karst landscapes. However, to gain a comprehensive understanding of how pharmaceutical residues move, disperse, and persist in subterranean and cave aquatic systems, it is essential to investigate the stability of these compounds during their passage through soil and within water bodies.

Additionally, research into the effects of psychoactive pharmaceutical residues on wildlife is necessary, as their impact on aquatic organisms at environmental levels and no-effect concentrations remains largely unknown. Furthermore, ecotoxicological testing should be performed on subterranean species across different trophic levels, as such data are currently scarce or non-existent, although these organisms play vital roles in delicate karst ecosystems. While the environmental risk assessment in this thesis focused on psychoactive pharmaceuticals, future studies should also extend predictive ecotoxicological modelling approaches, such as ECOSAR, to NPS, once appropriate analytical methods, reference standards, and exposure data become available.

Lastly, more attention should be given to studies that determine the occurrence of psychoactive pharmaceutical residues in drinking water and evaluate their potential risks to human health. Many psychoactive pharmaceutical residues are chiral molecules, and their enantiomers can exhibit differing levels of toxicity. Investigating stereoselective toxicity is another area for advancing environmental risk assessments of psychoactive pharmaceutical residues.

References

[1] WHO, 'World Health Organization: Health topics – Drugs (psychoactive)'. Accessed: Feb. 21, 2025. [Online]. Available: https://www.who.int/health-topics/drugs-psychoactive

[2] M. Laimou-Geraniou, D. Heath, and E. Heath, 'Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology', *Trends in Environmental Analytical Chemistry*, vol. 37, p. e00192, Mar. 2023, doi: 10.1016/j.teac.2022.e00192.

[3] WHO, 'Mental disorders: fact sheets'. Accessed: Mar. 24, 2022. [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/mental-disorders

[4] WHO, 'Mental Health and COVID-19: Early evidence of the pandemic's impact: Scientific brief, 2 March 2022'. Accessed: Jun. 18, 2025. [Online]. Available: https://www.who.int/publications/i/item/WHO-2019-nCoV-Sci_Brief-Mental_health-2022.1

[5] M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, N. Van Wichelen, D. Heath, A. L. N. van Nuijs, A. Covaci, and E. Heath, 'Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters', *Science of The Total Environment*, vol. 903, p. 166586, Dec. 2023, doi: 10.1016/j.scitotenv.2023.166586.

[6] J. M. Witkin and J. E. Barrett, 'ANXIOLYTICS: Origins, drug discovery, and mechanisms', *Pharmacology Biochemistry and Behavior*, vol. 245, p. 173858, Dec. 2024, doi: 10.1016/j.pbb.2024.173858.

[7] F. Zapata, J. M. Matey, G. Montalvo, and C. García-Ruiz, 'Chemical classification of new psychoactive substances (NPS)', *Microchemical Journal*, vol. 163, p. 105877, Apr. 2021, doi: 10.1016/j.microc.2020.105877.

[8] M. Laimou-Geraniou, T. Kos, D. Heath, and E. Heath, 'Psychoactive pharmaceuticals: Consumption trends, removal during wastewater treatment and occurrence in receiving waters with associated environmental risks', *Journal of Hazardous Materials Advances*, p. 100816, Jul. 2025, doi: 10.1016/j.hazadv.2025.100816.

[9] UNODC, 'United Nations Office on Drugs and Crime: homepage', United Nations: Office on Drugs and Crime. Accessed: Apr. 05, 2025. [Online]. Available: //www.unodc.org/unodc/en/index.html

[10] S. M. Stahl, *Stahl's essential psychopharmacology: Neuroscientific basis and practical applications, 4th ed.* in Stahl's essential psychopharmacology: Neuroscientific basis and practical applications, 4th ed. New York, NY, US: Cambridge University Press, 2013, pp. xv, 608.

[11] UNODC, 'United Nations Office on Drugs and Crime: World Drug Report 2019 – Stimulants', United Nations: World Drug Report 2019. Accessed: Apr. 05, 2025. [Online]. Available: //wdr.unodc.org/wdr2019/en/stimulants.html

[12] UNODC, 'United Nations Office on Drugs and Crime: World Drug Report 2019 – Cannabis and hallucinogens', United Nations: World Drug Report 2019. Accessed: Apr. 05, 2025. [Online]. Available: //wdr.unodc.org/wdr2019/en/cannabis-and-hallucinogens.html

[13] UNODC, 'United Nations Office on Drugs and Crime: World Drug Report 2019 – Depressants', United Nations: World Drug Report 2019. Accessed: Apr. 05, 2025. [Online]. Available: //wdr.unodc.org/wdr2019/en/depressants.html

[14] 'Single Convention on Narcotic Drugs, 1961'. Accessed: Apr. 09, 2025. [Online]. Available: https://www.unodc.org/pdf/convention_1961_en.pdf

[15] 'Convention on Psychoactive Substances, 1971'. Accessed: Apr. 09, 2025. [Online]. Available: https://www.unodc.org/pdf/convention_1971_en.pdf

[16] 'United Nations Convention Against Illicit Traffic in Narcotic Drugs and Psychoactive Substances, 1988'. [Online]. Available: https://www.unodc.org/pdf/convention_1988_en.pdf

[17] 'Uradni list RS: Uredba o razvrstitvi prepovedanih drog (Eng., Official Gazette of the Republic of Slovenia: Regulation on the Classification of Illicit Drugs)'. Accessed: Apr. 09, 2025. [Online]. Available: https://pisrs.si/pregledPredpisa?id=URED7970

[18] S. M. R. Wille, S. G. Cooreman, H. M. Neels, and W. E. E. Lambert, 'Relevant Issues in the Monitoring and the Toxicology of Antidepressants', *Critical Reviews in Clinical Laboratory Sciences*, vol. 45, no. 1, pp. 25–89, Jan. 2008, doi: 10.1080/10408360701713112.

[19] R. Henry, 'Mood stabilisers', *Medicine*, vol. 52, no. 9, pp. 577–582, Sep. 2024, doi: 10.1016/j.mpmed.2024.07.003.

[20] 'JAZMP - Agency for Medicinal Products and Medical Devices of the Republic of Slovenia: Legislation'. Accessed: Apr. 16, 2025. [Online]. Available: https://www.jazmp.si/en/legislation/

[21] O. Vasiliu, 'Third-generation antipsychotics in patients with schizophrenia and non-responsivity or intolerance to clozapine regimen: What is the evidence?', *Front Psychiatry*, vol. 13, p. 1069432, Nov. 2022, doi: 10.3389/fpsyt.2022.1069432.

[22] K. Chokhawala and L. Stevens, 'Antipsychotic Medications', in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2025. Accessed: Apr. 30, 2025. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK519503/

[23] D. Grinchii and E. Dremencov, 'Mechanism of Action of Atypical Antipsychotic Drugs in Mood Disorders', *International Journal of Molecular Sciences*, vol. 21, no. 24, p. 9532, Jan. 2020, doi: 10.3390/ijms21249532.

[24] D. A. Sykes, J. Lochray, H. M. F. Comfort, P. Jain, and S. J. Charlton, 'Exploring the kinetic selectivity of antipsychotics for dopamine D2 and 5-HT2A receptors: implications for the prevalence of EPS and receptor occupancy', Nov. 14, 2021, *bioRxiv* [preprint]. doi: 10.1101/2021.11.14.468520.

[25] A. N. Edinoff, C. A. Nix, J. Hollier, C. E. Sagrera, B. M. Delacroix, T. Abubakar, E. M. Cornett, A. M. Kaye, and A. D. Kaye, 'Benzodiazepines: Uses, Dangers, and

Clinical Considerations', *Neurol Int*, vol. 13, no. 4, pp. 594–607, Nov. 2021, doi: 10.3390/neurolint13040059.

[26] A. Herrero Babiloni, G. Beetz, A. Bruneau, M. O. Martel, P. A. Cistulli, D. R. Nixdorf, J. M. Conway, and G. J. Lavigne, 'Multitargeting the sleep-pain interaction with pharmacological approaches: A narrative review with suggestions on new avenues of investigation', *Sleep Medicine Reviews*, vol. 59, p. 101459, Oct. 2021, doi: 10.1016/j.smrv.2021.101459.

[27] UNODC, ''United Nations Office on Drugs and Crime: Guidelines for the Forensic analysis of drugs facilitating sexual assault and other criminal acts'', United Nations: Office on Drugs and Crime. Accessed: May 01, 2025. [Online]. Available: //www.unodc.org/unodc/en/scientists/guidelines-for-the-forensic-analysis-of-drugs-facilitating-sexual-assault-and-other-criminal-acts_new.html

[28] European Monitoring Centre for Drugs and Drug Addiction, *European drug report 2023*. in European drug report ... (Online). LU: Publications Office, 2023. Accessed: Sep. 23, 2025. [Online]. Available: https://data.europa.eu/doi/10.2810/161905

[29] 'Benzodiazepines Statistics - 2024 Data on Benzo Use and Abuse', AddictionHelp.com. Accessed: Sep. 23, 2025. [Online]. Available: https://www.addictionhelp.com/benzodiazepines/statistics/

[30] DEA, 'United States Drug Enforcement Administration: Drug Scheduling'. Accessed: May 13, 2025. [Online]. Available: https://www.dea.gov/drug-information/drug-scheduling

[31] UNODC, 'United Nations Office on Drugs and Crime: What are NPS?' Accessed: May 05, 2025. [Online]. Available: https://www.unodc.org/LSS/Page/NPS

[32] UNODC, 'United Nations Office on Drugs and Crime: NPS Substance Groups'. Accessed: May 05, 2025. [Online]. Available: https://www.unodc.org/LSS/SubstanceGroup/GroupsDashboard?testType=NPS

[33] UNODC, 'United Nations Office on Drugs and Crime, Early warning advisory on new psychoactive substances: NPS Data Visualisation'. Accessed: May 06, 2025. [Online]. Available: https://www.unodc.org/LSS/Page/NPS/DataVisualisations

[34] EUDA, 'European Union Drug Agency. The EU Early Warning System on new psychoactive substances (NPS) | www.euda.europa.eu'. Accessed: May 06, 2025. [Online]. Available: https://www.euda.europa.eu/activities/eu-early-warning-system-on-nps_en

[35] 'EU Drug Market: New psychoactive substances — Distribution and supply in Europe: Synthetic cathinones | www.euda.europa.eu'. Accessed: Jun. 28, 2025. [Online]. Available: https://www.euda.europa.eu/publications/eu-drug-markets/new-psychoactive-substances/distribution-and-supply/synthetic-cathinones_en

[36] UNODC, 'UNODC, December 2022 – WHO: World Health Organization recommends seven NPS for scheduling'. Accessed: Jun. 28, 2025. [Online]. Available: https://www.unodc.org/LSS/Announcement/Details/dc9d7f3c-1fd3-4d61-9770-2d72b198252e

[37] European Commission, *Commission Delegated Directive (EU) 2022/1326 of 18 March 2022 amending the Annex to Council Framework Decision 2004/757/JHA*

as regards the inclusion of new psychoactive substances in the definition of 'drug'., Official Journal of the European Union (2022) 1–3, vol. 200. 2022. Accessed: Jun. 29, 2025. [Online]. Available: http://data.europa.eu/eli/dir_del/2022/1326/oj/eng

[38] M. Evans-Brown, B. Hughes, and R. Sedefov, 'Chapter1 - Legal classification and international systems for monitoring and responding to novel psychoactive substances', in *Novel Psychoactive Substances (Second Edition)*, P. Dargan and D. Wood, Eds, Boston: Academic Press, 2022, pp. 3–56. doi: 10.1016/B978-0-12-818788-3.00016-4.

[39] D. Vigo, G. Thornicroft, and R. Atun, 'Estimating the true global burden of mental illness', *The Lancet Psychiatry*, vol. 3, no. 2, pp. 171–178, Feb. 2016, doi: 10.1016/S2215-0366(15)00505-2.

[40] UNODC, 'United Nations Office on Drugs and Crime: World Drug Report 2022 – Booklet 1', United Nations: Office on Drugs and Crime. Accessed: May 07, 2025. [Online]. Available: //www.unodc.org/unodc/en/data-and-analysis/wdr-2022_booklet-1.html

[41] M. Tiger, G. Castelpietra, R. Wesselhoeft, J. Lundberg, and J. Reutfors, 'Utilization of antidepressants, anxiolytics, and hypnotics during the COVID-19 pandemic', *Transl Psychiatry*, vol. 14, no. 1, pp. 1–6, Apr. 2024, doi: 10.1038/s41398-024-02894-z.

[42] E. W. Flanagan, R. A. Beyl, S. N. Fearnbach, A. D. Altazan, C. K. Martin, and L. M. Redman, 'The Impact of COVID-19 Stay-At-Home Orders on Health Behaviors in Adults', *Obesity*, vol. 29, no. 2, pp. 438–445, 2021, doi: 10.1002/oby.23066.

[43] P. M. Choi, B. J. Tscharke, E. Donner, J. W. O'Brien, S. C. Grant, S. L. Kaserzon, R. Mackie, E. O'Malley, N. D. Crosbie, K. V. Thomas, and J. F. Mueller, 'Wastewater-based epidemiology biomarkers: Past, present and future', *TrAC Trends in Analytical Chemistry*, vol. 105, pp. 453–469, Aug. 2018, doi: 10.1016/j.trac.2018.06.004.

[44] D. Vitale, M. M. Suárez-Varela, and Y. Picó, 'Wastewater-based epidemiology (WBE), a tool to bridge biomarkers of exposure, contaminants and human health', *Current Opinion in Environmental Science & Health*, p. 100229, Jan. 2021, doi: 10.1016/j.coesh.2021.100229.

[45] J. A. Baz-Lomba, C. Harman, M. Reid, and K. V. Thomas, 'Passive sampling of wastewater as a tool for the long-term monitoring of community exposure: Illicit and prescription drug trends as a proof of concept', *Water Research*, vol. 121, pp. 221–230, Sep. 2017, doi: 10.1016/j.watres.2017.05.041.

[46] B. Subedi, 'Estimation of Community Usage of Drugs Utilizing Sewage Epidemiology', in *Analysis of Drugs of Abuse*, R. A. Musah, Ed., in Methods in Molecular Biology. , New York, NY: Springer, 2018, pp. 141–147. doi: 10.1007/978-1-4939-8579-1_14.

[47] J. A. Baz-Lomba, S. Salvatore, E. Gracia-Lor, R. Bade, S. Castiglioni, E. Castrignanò, A. Causanilles, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A.-K. McCall, A. van Nuijs, C. Ort, B. G. Plósz, P. Ramin, M. Reid, N. I. Rousis, Y. Ryu, P. de Voogt, J. Bramness, and K. Thomas, 'Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and

consumption data for 8 European cities', *BMC Public Health*, vol. 16, no. 1, p. 1035, Oct. 2016, doi: 10.1186/s12889-016-3686-5.

[48] T. Boogaerts, F. Ahmed, Phil. M. Choi, B. Tscharke, J. O'Brien, H. De Loof, J. Gao, P. Thai, K. Thomas, J. F. Mueller, W. Hall, A. Covaci, and A. L. N. van Nuijs, 'Current and future perspectives for wastewater-based epidemiology as a monitoring tool for pharmaceutical use', *Science of The Total Environment*, vol. 789, p. 148047, Oct. 2021, doi: 10.1016/j.scitotenv.2021.148047.

[49] K. Diamanti, R. Aalizadeh, N. Alygizakis, A. Galani, M. Mardal, and N. S. Thomaidis, 'Wide-scope target and suspect screening methodologies to investigate the occurrence of new psychoactive substances in influent wastewater from Athens', *Science of The Total Environment*, vol. 685, pp. 1058–1065, Oct. 2019, doi: 10.1016/j.scitotenv.2019.06.173.

[50] A. L. N. van Nuijs, A. Covaci, H. Beyers, L. Bervoets, R. Blust, G. Verpooten, H. Neels, and P. G. Jorens, 'Do concentrations of pharmaceuticals in sewage reflect prescription figures?', *Environ Sci Pollut Res*, vol. 22, no. 12, pp. 9110–9118, Jun. 2015, doi: 10.1007/s11356-014-4066-2.

[51] M. Elseviers, B. Wettermark, A. B. Almarsdóttir, M. Andersen, R. Benko, M. Bennie, I. Eriksson, B. Godman, J. Krska, E. Poluzzi, K. Taxis, V. Vlahovic-Palcevski, and R. vander Stichele, Eds, *Drug Utilization Research: Methods and Applications*. Wiley, 2016.

[52] K. Higgins, N. O'Neill, L. O'Hara, J.-A. Jordan, M. McCann, T. O'Neill, M. Clarke, T. O'Neill, and A. Campbell, *Evidence for public health on novel psychoactive substance use: a mixed-methods study*. in Public Health Research. Southampton (UK): NIHR Journals Library, 2019. Accessed: Jan. 27, 2026. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK544999/

[53] I. C. Santos, D. Maia, R. J. Dinis-Oliveira, and D. J. Barbosa, 'New Psychoactive Substances: Health and Legal Challenges', *Psychoactives*, vol. 3, no. 2, pp. 285–302, Jun. 2024, doi: 10.3390/psychoactives3020018.

[54] 'Health and social responses to drug problems: a European guide | www.euda.europa.eu'. Accessed: Jan. 27, 2026. [Online]. Available: https://www.euda.europa.eu/publications/health-and-social-responses-a-european-guide_en

[55] J. J. Palamar, A. Salomone, and M. J. Barratt, 'Drug checking to detect fentanyl and new psychoactive substances', *Current Opinion in Psychiatry*, vol. 33, no. 4, p. 301, Jul. 2020, doi: 10.1097/YCO.0000000000000607.

[56] J. B. Zawilska and D. Andrzejczak, 'Next generation of novel psychoactive substances on the horizon – A complex problem to face', *Drug and Alcohol Dependence*, vol. 157, pp. 1–17, Dec. 2015, doi: 10.1016/j.drugalcdep.2015.09.030.

[57] C. V. Giné, I. F. Espinosa, and M. V. Vilamala, 'New psychoactive substances as adulterants of controlled drugs. A worrying phenomenon?', *Drug Testing and Analysis*, vol. 6, no. 7–8, pp. 819–824, 2014, doi: 10.1002/dta.1610.

[58] 'Pharmaceutical consumption', OECD. Accessed: Jun. 17, 2025. [Online]. Available: https://www.oecd.org/en/publications/health-at-a-glance-2023_7a7afb35-en/full-report/pharmaceutical-consumption_4b6cb013.html

[59] ‘Antidepressants Market Size, Growth | Global Report, 2020-2027’. Accessed: Jun. 07, 2022. [Online]. Available: https://www.fortunebusinessinsights.com/antidepressants-market-105017

[60] ‘Antidepressant Drugs Market Size & Share Report, 2025 – 2034’, Global Market Insights Inc. Accessed: Jun. 17, 2025. [Online]. Available: https://www.gminsights.com/industry-analysis/antidepressant-drugs-market

[61] A. Fleck, ‘STATISTA: The Growing Global Reliance on Antidepressants’, Statista Daily Data. Accessed: Jun. 18, 2025. [Online]. Available: https://www.statista.com/chart/28358/antidepressant-consumption-daily-doses-per-100-population

[62] NIJZ, ‘National Institute of Health: Consumption of prescribed medicines in Slovenia in 2023’. [Online]. Available: https://nijz.si/wp-content/uploads/2024/05/Zdravila-na-recept-2023-Final-9.5.2024.pdf

[63] A. Kozina, I. Peras, M. Veldin, and T. Pivec, ‘The psychological response and perception of stress during the COVID-19 pandemic in Slovenia: Three-wave repeated cross-sectional study’, *Stress and Health*, vol. 38, no. 5, pp. 950–960, 2022, doi: 10.1002/smi.3147.

[64] P. Rus Prelog, T. Matić, P. Pregelj, and A. Sadikov, ‘Risk of Depression, Anxiety, and Stress During the Second Wave of COVID-19 in Slovenia’, *Frontiers in Psychiatry*, vol. 12, 2022, Accessed: Apr. 17, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.788898

[65] ‘Antipsychotic Drugs Market Size, Share, Trends & Growth, 2032’. Accessed: Jun. 19, 2025. [Online]. Available: https://www.fortunebusinessinsights.com/industry-reports/antipsychotic-drugs-market-101390

[66] ‘Antipsychotic Drugs Market Size and Share Report, 2030’. Accessed: Jun. 19, 2025. [Online]. Available: https://www.grandviewresearch.com/industry-analysis/antipsychotic-drugs-market

[67] ‘Health at a Glance: Europe 2022’, OECD. Accessed: Jun. 20, 2025. [Online]. Available: https://www.oecd.org/en/publications/health-at-a-glance-europe-2022_507433b0-en.html

[68] C. Leong, K. Kowalec, S. Eltonsy, J. M. Bolton, M. W. Enns, Q. Tan, M. Yogendran, D. Chateau, J. A. Delaney, J. Sareen, J. Falk, R. Spiwak, S. Logsetty, and S. Alessi-Severini, ‘Psychotropic Medication Use Before and During COVID-19: A Population-Wide Study’, *Front. Pharmacol.*, vol. 13, Apr. 2022, doi: 10.3389/fphar.2022.886652.

[69] H. Luo, W. C. Y. Lau, Y. Chai, C. O. Torre, R. Howard, K. Y. Liu, X. Lin, C. Yin, S. Fortin, D. M. Kern, D. Y. Lee, R. W. Park, J.-W. Jang, C. S. L. Chui, J. Li, C. Reich, K. K. C. Man, and I. C. K. Wong, ‘Rates of Antipsychotic Drug Prescribing Among People Living With Dementia During the COVID-19 Pandemic’, *JAMA Psychiatry*, vol. 80, no. 3, pp. 211–219, Mar. 2023, doi: 10.1001/jamapsychiatry.2022.4448.

[70] O. Alabaku, A. Yang, S. Tharmarajah, K. Suda, S. Vigod, and M. Tadrous, ‘Global trends in antidepressant, atypical antipsychotic, and benzodiazepine use: A cross-

sectional analysis of 64 countries', *PLoS One*, vol. 18, no. 4, p. e0284389, Apr. 2023, doi: 10.1371/journal.pone.0284389.

[71] T.-T. Ma, Z. Wang, X. Qin, C. Ju, W. C. Y. Lau, K. K. C. Man, D. Castle, W. Chung Chang, A. Y. L. Chan, E. C. L. Cheung, C. S. L. Chui, and I. C. K. Wong, 'Global trends in the consumption of benzodiazepines and Z-drugs in 67 countries and regions from 2008 to 2018: a sales data analysis', *Sleep*, vol. 46, no. 10, p. zsad124, Oct. 2023, doi: 10.1093/sleep/zsad124.

[72] 'United Nations Office on Drugs and Crime, UNODC Early warning advisory on new psychoactive substances'. Accessed: Jul. 01, 2025. [Online]. Available: https://www.unodc.org/LSS/Country/List

[73] EUDA, 'The EU Early Warning System on new psychoactive substances (NPS) | www.euda.europa.eu'. Accessed: Jul. 01, 2025. [Online]. Available: https://www.euda.europa.eu/activities/eu-early-warning-system-on-nps_en

[74] EUDA, 'The EU Early Warning System monitors 1000 NPS | www.euda.europa.eu'. Accessed: Jul. 01, 2025. [Online]. Available: https://www.euda.europa.eu/publications/poster/eu-early-warning-system-monitors-1000-nps_en

[75] 'News: October 2024 – UNODC EWA: Significant diversification among synthetic cannabinoids and opioids in 2023'. Accessed: Jul. 01, 2025. [Online]. Available: https://www.apaic.net/2024/10/18/unodc-ewa-significant-diversification-among-synthetic-cannabinoids-and-opioids-in-2023/

[76] UNODC, 'United Nations Office on Drugs and Crime: World Drug Report 2022 – Booklet 4', United Nations : Office on Drugs and Crime. Accessed: Jul. 01, 2025. [Online]. Available: //www.unodc.org/unodc/en/data-and-analysis/wdr-2022_booklet-4.html

[77] NIJZ, 'Report on the Drug Situation 2019 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2019', Nijz. Accessed: Mar. 04, 2024. [Online]. Available: https://nijz.si/publikacije/report-on-the-drug-situation-2019-of-the-republic-of-slovenia-2/

[78] H. J. Klanšček, M. Roškar, A. Drev, V. Pucelj, H. Koprivnikar, T. Zupanič, and A. Korošec, 'Z zdravjem povezana vedenja v šolskem obdobju med mladostniki v Sloveniji, izsledki mednarodne raziskave HBSC, 2018'. Accessed: Jul. 01, 2025. [Online]. Available: https://nijz.si/publikacije/z-zdravjem-povezana-vedenja-v-solskem-obdobju-med-mladostniki-v-sloveniji-izsledki-mednarodne-raziskave-hbsc-2018/

[79] NIJZ, 'Report on the Drug Situation 2021 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2021'. Accessed: Jul. 01, 2025. [Online]. Available: https://nijz.si/en/publications/report-on-the-drug-situation-2021-of-the-republic-of-slovenia/

[80] NIJZ, 'Report on the Drug Situation 2022 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2022'. Accessed: Jul. 01, 2025. [Online]. Available: https://nijz.si/en/publications/report-on-the-drug-situation-2022-of-the-republic-of-slovenia/

[81] NIJZ, 'Report on the Drug Situation 2023 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2023'. Accessed: Jul. 01, 2025. [Online]. Available: https://nijz.si/en/publications/report-on-the-drug-situation-2023-of-the-republic-of-slovenia/

[82] 'NPS AND RELATED COMPOUNDS DATABASE'. Accessed: Jul. 07, 2025. [Online]. Available: https://www.policija.si/apps/nfl_response_web/seznam.php

[83] 'General information about testing - "Splošno o testiranju"', DrogArt. Accessed: Jul. 07, 2025. [Online]. Available: https://www.drogart.org/splosne-informacije/

[84] T. Boogaerts, M. Degreef, A. Covaci, and A. L. N. van Nuijs, 'Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach', *Talanta*, vol. 200, pp. 340–349, Aug. 2019, doi: 10.1016/j.talanta.2019.03.052.

[85] D. R. Baker, L. Barron, and B. Kasprzyk-Hordern, 'Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates', *Science of The Total Environment*, vol. 487, pp. 629–641, Jul. 2014, doi: 10.1016/j.scitotenv.2013.11.107.

[86] E. Zuccato, C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea, and R. Fanelli, 'Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse', *Environ Health*, vol. 4, no. 1, p. 14, Aug. 2005, doi: 10.1186/1476-069X-4-14.

[87] S. Castiglioni, L. Bijlsma, A. Covaci, E. Emke, F. Hernández, M. Reid, C. Ort, K. V. Thomas, A. L. N. van Nuijs, P. de Voogt, and E. Zuccato, 'Evaluation of Uncertainties Associated with the Determination of Community Drug Use through the Measurement of Sewage Drug Biomarkers', *Environ. Sci. Technol.*, vol. 47, no. 3, pp. 1452–1460, Feb. 2013, doi: 10.1021/es302722f.

[88] A. L. N. van Nuijs, F. Y. Lai, F. Been, M. J. Andres-Costa, L. Barron, J. A. Baz-Lomba, J.-D. Berset, L. Benaglia, L. Bijlsma, D. Burgard, S. Castiglioni, C. Christophoridis, A. Covaci, P. de Voogt, E. Emke, D. Fatta-Kassinos, J. Fick, F. Hernandez, C. Gerber, I. González-Mariño, R. Grabic, T. Gunnar, K. Kannan, S. Karolak, B. Kasprzyk-Hordern, Z. Kokot, I. Krizman-Matasic, A. Li, X. Li, A. S. C. Löve, M. Lopez de Alda, A.-K. McCall, M. R. Meyer, H. Oberacher, J. O'Brien, J. B. Quintana, M. Reid, S. Schneider, S. S. Simoes, N. S. Thomaidis, K. Thomas, V. Yargeau, and C. Ort, 'Multi-year inter-laboratory exercises for the analysis of illicit drugs and metabolites in wastewater: Development of a quality control system', *TrAC Trends in Analytical Chemistry*, vol. 103, pp. 34–43, Jun. 2018, doi: 10.1016/j.trac.2018.03.009.

[89] R. Montes, R. Rodil, A. Rico, R. Cela, I. González-Mariño, F. Hernández, L. Bijlsma, A. Celma, Y. Picó, V. Andreu, M. L. de Alda, E. López-García, C. Postigo, E. Pocurull, R. M. Marcé, M. Rosende, M. Olivares, Y. Valcárcel, and J. B. Quintana, 'First nation-wide estimation of tobacco consumption in Spain using wastewater-based epidemiology', *Science of The Total Environment*, vol. 741, p. 140384, Nov. 2020, doi: 10.1016/j.scitotenv.2020.140384.

[90] Q. Zheng, C. Gartner, B. J. Tscharke, J. W. O'Brien, J. Gao, F. Ahmed, K. V. Thomas, J. F. Mueller, and P. K. Thai, 'Long-term trends in tobacco use assessed

by wastewater-based epidemiology and its relationship with consumption of nicotine containing products', *Environment International*, vol. 145, p. 106088, Dec. 2020, doi: 10.1016/j.envint.2020.106088.

[91] E. Gracia-Lor, N. I. Rousis, E. Zuccato, and S. Castiglioni, 'Monitoring caffeine and nicotine use in a nationwide study in Italy using wastewater-based epidemiology', *Science of The Total Environment*, vol. 747, p. 141331, Dec. 2020, doi: 10.1016/j.scitotenv.2020.141331.

[92] T. Boogaerts, M. Quireyns, A. Covaci, H. De Loof, and A. L. N. van Nuijs, 'Analytical method for the simultaneous determination of a broad range of opioids in influent wastewater: Optimization, validation and applicability to monitor consumption patterns', *Talanta*, vol. 232, p. 122443, Sep. 2021, doi: 10.1016/j.talanta.2021.122443.

[93] T. Verovšek, I. Krizman-Matasic, D. Heath, and E. Heath, 'Investigation of drugs of abuse in educational institutions using wastewater analysis', *Science of The Total Environment*, vol. 799, p. 150013, Dec. 2021, doi: 10.1016/j.scitotenv.2021.150013.

[94] D. Li, J. W. O'Brien, B. J. Tscharke, P. M. Choi, F. Ahmed, J. Thompson, J. F. Mueller, H. Sun, and K. V. Thomas, 'Trends in artificial sweetener consumption: A 7-year wastewater-based epidemiology study in Queensland, Australia', *Science of The Total Environment*, vol. 754, p. 142438, Feb. 2021, doi: 10.1016/j.scitotenv.2020.142438.

[95] D. R. Van Stempvoort, S. J. Brown, J. Spoelstra, D. Garda, W. D. Robertson, and S. A. Smyth, 'Variable persistence of artificial sweeteners during wastewater treatment: Implications for future use as tracers', *Water Research*, vol. 184, p. 116124, Oct. 2020, doi: 10.1016/j.watres.2020.116124.

[96] S. McKay, B. Tscharke, D. Hawker, K. Thompson, J. O'Brien, J. F. Mueller, and S. Kaserzon, 'Calibration and validation of a microporous polyethylene passive sampler for quantitative estimation of illicit drug and pharmaceutical and personal care product (PPCP) concentrations in wastewater influent', *Science of The Total Environment*, vol. 704, p. 135891, Feb. 2020, doi: 10.1016/j.scitotenv.2019.135891.

[97] M. Papageorgiou, I. Zioris, T. Danis, D. Bikiaris, and D. Lambropoulou, 'Comprehensive investigation of a wide range of pharmaceuticals and personal care products in urban and hospital wastewaters in Greece', *Science of The Total Environment*, vol. 694, p. 133565, Dec. 2019, doi: 10.1016/j.scitotenv.2019.07.371.

[98] M. Escolà Casas, N. S. Schröter, I. Zammit, M. Castaño-Trias, S. Rodriguez-Mozaz, P. Gago-Ferrero, and Ll. Corominas, 'Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution', *Environment International*, vol. 150, p. 106404, May 2021, doi: 10.1016/j.envint.2021.106404.

[99] H.-J. Lei, B. Yang, P. Ye, Y.-Y. Yang, J.-L. Zhao, Y.-S. Liu, L. Xie, and G.-G. Ying, 'Occurrence, fate and mass loading of benzodiazepines and their transformation products in eleven wastewater treatment plants in Guangdong province, China', *Science of The Total Environment*, vol. 755, p. 142648, Feb. 2021, doi: 10.1016/j.scitotenv.2020.142648.

[100] R. Bade, N. Rousis, S. Adhikari, C. Baduel, L. Bijlsma, E. Bizani, T. Boogaerts, D. A. Burgard, S. Castiglioni, A. Chappell, A. Covaci, E. M. Driver, F. F. Sodre, D. Fatta-Kassinos, A. Galani, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R. U. Halden, E. Heath, F. Hernandez, E. Jaunay, F. Y. Lai, H.-J. Lee, M. Laimou-Geraniou, J.-E. Oh, K. Olafsdottir, K. Phung, M. P. Castro, M. Psichoudaki, X. Shao, N. Salgueiro-Gonzalez, R. S. Feitosa, C. S. Gomes, B. Subedi, A. S. C. Löve, N. Thomaidis, D. Tran, A. van Nuijs, T. Verovšek, D. Wang, J. M. White, V. Yargeau, E. Zuccato, and J. F. Mueller, 'Three years of wastewater surveillance for new psychoactive substances from 16 countries', *Water Research X*, vol. 19, p. 100179, May 2023, doi: 10.1016/j.wroa.2023.100179.

[101] M. Laimou-Geraniou, D. Nadarajan, R. Bade, and E. Heath, 'Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis', *Journal of Hazardous Materials Advances*, vol. 19, p. 100781, Aug. 2025, doi: 10.1016/j.hazadv.2025.100781.

[102] L. Gent and R. Paul, 'The detection of new psychoactive substances in wastewater. A comprehensive review of analytical approaches and global trends', *Science of The Total Environment*, vol. 776, p. 146028, Jul. 2021, doi: 10.1016/j.scitotenv.2021.146028.

[103] N. Salgueiro-Gonzalez, F. Béen, L. Bijlsma, T. Boogaerts, A. Covaci, J. A. Baz-Lomba, B. Kasprzyk-Hordern, J. Matias, C. Ort, I. Bodík, E. Heath, K. Styszko, E. Emke, F. Hernández, A. L. N. van Nuijs, and S. Castiglioni, 'Influent wastewater analysis to investigate emerging trends of new psychoactive substances use in Europe', *Water Research*, vol. 254, p. 121390, May 2024, doi: 10.1016/j.watres.2024.121390.

[104] D. Barceló, 'Wastewater-Based Epidemiology to monitor COVID-19 outbreak: Present and future diagnostic methods to be in your radar', *Case Studies in Chemical and Environmental Engineering*, vol. 2, p. 100042, 2020, doi: 10.1016/j.cscee.2020.100042.

[105] P. Foladori, F. Cutrupi, N. Segata, S. Manara, F. Pinto, F. Malpei, L. Bruni, and G. La Rosa, 'SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review', *Science of the Total Environment*, vol. 743, p. 140444, 2020, doi: 10.1016/j.scitotenv.2020.140444.

[106] Y. Picó and D. Barceló, 'Identification of biomarkers in wastewater-based epidemiology: Main approaches and analytical methods', *TrAC Trends in Analytical Chemistry*, vol. 145, p. 116465, Dec. 2021, doi: 10.1016/j.trac.2021.116465.

[107] C. Ort, M. G. Lawrence, J. Reungoat, and J. F. Mueller, 'Sampling for PPCPs in Wastewater Systems: Comparison of Different Sampling Modes and Optimization Strategies', *Environ. Sci. Technol.*, vol. 44, no. 16, pp. 6289–6296, Aug. 2010, doi: 10.1021/es100778d.

[108] B. Subedi, K. Balakrishna, D. I. Joshua, and K. Kannan, 'Mass loading and removal of pharmaceuticals and personal care products including psychoactives, antihypertensives, and antibiotics in two sewage treatment plants in southern India', *Chemosphere*, vol. 167, pp. 429–437, Jan. 2017, doi: 10.1016/j.chemosphere.2016.10.026.

[109] A. Bahlmann, W. Brack, R. J. Schneider, and M. Krauss, 'Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal', *Water Research*, vol. 57, pp. 104–114, Jun. 2014, doi: 10.1016/j.watres.2014.03.022.

[110] J. Criquet, D. Dumoulin, M. Howsam, L. Mondamert, J.-F. Goossens, J. Prygiel, and G. Billon, 'Comparison of POCIS passive samplers vs. composite water sampling: A case study', *Science of The Total Environment*, vol. 609, pp. 982–991, Dec. 2017, doi: 10.1016/j.scitotenv.2017.07.227.

[111] L. Bijlsma, R. Bade, F. Been, A. Celma, and S. Castiglioni, 'Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial', *Analytica Chimica Acta*, vol. 1145, pp. 132–147, Sep. 2020, doi: 10.1016/j.aca.2020.08.058.

[112] N. Bishop, T. Jones-Lepp, M. Margetts, J. Sykes, D. Alvarez, and D. E. Keil, 'Wastewater-based epidemiology pilot study to examine drug use in the Western United States', *Science of The Total Environment*, vol. 745, p. 140697, Nov. 2020, doi: 10.1016/j.scitotenv.2020.140697.

[113] X. Liu, R. Zhang, H. Cheng, M. S. Khorram, S. Zhao, T. T. Tham, T. M. Tran, T. B. Minh, B. Jiang, B. Jin, and G. Zhang, 'Field evaluation of diffusive gradients in thin-film passive samplers for wastewater-based epidemiology', *Science of The Total Environment*, vol. 773, p. 145480, Jun. 2021, doi: 10.1016/j.scitotenv.2021.145480.

[114] J. K. Challis, X. O. Almirall, P. A. Helm, and C. S. Wong, 'Performance of the organic-diffusive gradients in thin-films passive sampler for measurement of target and suspect wastewater contaminants', *Environmental Pollution*, vol. 261, p. 114092, Jun. 2020, doi: 10.1016/j.envpol.2020.114092.

[115] R. Verhagen, B. J. Tscharke, J. Clokey, C. Gerber, M. Ghetia, S. L. Kaserzon, K. V. Thomas, and J. F. Mueller, 'Multisite Calibration of a Microporous Polyethylene Tube Passive Sampler for Quantifying Drugs in Wastewater', *Environ Sci Technol*, vol. 55, no. 19, pp. 12922–12929, Oct. 2021, doi: 10.1021/acs.est.1c02900.

[116] J. E. Clokey, D. W. Hawker, R. Verhagen, S. Ghorbani Gorji, E. R. Knight, K. V. Thomas, and S. L. Kaserzon, 'Calibration of a microporous polyethylene tube passive sampler for polar organic compounds in wastewater effluent', *Science of The Total Environment*, vol. 874, p. 162497, May 2023, doi: 10.1016/j.scitotenv.2023.162497.

[117] E. D. Amato, A. Covaci, R. M. Town, J. Hereijgers, B. Bellekens, V. Giacometti, T. Breugelmans, M. Weyn, F. Dardenne, L. Bervoets, and R. Blust, 'A novel active-passive sampling approach for measuring time-averaged concentrations of pollutants in water', *Chemosphere*, vol. 209, pp. 363–372, Oct. 2018, doi: 10.1016/j.chemosphere.2018.06.079.

[118] E. D. Amato, D. T. Nguyen, D. Subotic, J. Hereijgers, T. Breugelmans, M. Weyn, F. Dardenne, L. Bervoets, A. Covaci, R. M. Town, and R. Blust, 'Characterization of the accumulation of metals and organic contaminants on a novel active-passive sampling device under controlled water flow conditions', *Chemosphere*, vol. 236, p. 124400, Dec. 2019, doi: 10.1016/j.chemosphere.2019.124400.

[119] E. D. Amato, F. Pfeiffer, N. Estoppey, D. Subotic, L. Herweyers, T. Breugelmans, M. Weyn, E. Du Bois, F. Dardenne, A. Covaci, R. M. Town, and R. Blust, 'Field

application of a novel active-passive sampling technique for the simultaneous measurement of a wide range of contaminants in water', *Chemosphere*, vol. 279, p. 130598, Sep. 2021, doi: 10.1016/j.chemosphere.2021.130598.

[120] T. Verovšek, I. Krizman-Matasic, D. Heath, and E. Heath, 'Site- and event-specific wastewater-based epidemiology: current status and future perspectives', *Trends in Environmental Analytical Chemistry*, p. e00105, Oct. 2020, doi: 10.1016/j.teac.2020.e00105.

[121] C. I. Kosma, C. I. Nannou, V. I. Boti, and T. A. Albanis, 'Psychiatrics and selected metabolites in hospital and urban wastewaters: Occurrence, removal, mass loading, seasonal influence and risk assessment', *Science of The Total Environment*, vol. 659, pp. 1473–1483, Apr. 2019, doi: 10.1016/j.scitotenv.2018.12.421.

[122] J. A. Baz-Lomba, M. J. Reid, and K. V. Thomas, 'Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF', *Analytica Chimica Acta*, vol. 914, pp. 81–90, Mar. 2016, doi: 10.1016/j.aca.2016.01.056.

[123] E. Gracia-Lor, S. Castiglioni, R. Bade, F. Been, E. Castrignanò, A. Covaci, I. González-Mariño, E. Hapeshi, B. Kasprzyk-Hordern, J. Kinyua, F. Y. Lai, T. Letzel, L. Lopardo, M. R. Meyer, J. O'Brien, P. Ramin, N. I. Rousis, A. Rydevik, Y. Ryu, M. M. Santos, I. Senta, N. S. Thomaidis, S. Veloutsou, Z. Yang, E. Zuccato, and L. Bijlsma, 'Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives', *Environment International*, vol. 99, pp. 131–150, Feb. 2017, doi: 10.1016/j.envint.2016.12.016.

[124] M. Lorenzo and Y. Picó, 'Wastewater-based epidemiology: current status and future prospects', *Current Opinion in Environmental Science & Health*, vol. 9, pp. 77–84, Jun. 2019, doi: 10.1016/j.coesh.2019.05.007.

[125] I. González-Mariño, V. Castro, R. Montes, R. Rodil, A. Lores, R. Cela, and J. B. Quintana, 'Multi-residue determination of psychoactive pharmaceuticals, illicit drugs and related metabolites in wastewater by ultra-high performance liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*, vol. 1569, pp. 91–100, Sep. 2018, doi: 10.1016/j.chroma.2018.07.045.

[126] C. Ort, L. Bijlsma, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, F. Hernández, M. Reid, A. L. N. van Nuijs, K. V. Thomas, and B. Kasprzyk-Hordern, 'Wastewater Analysis for Community-Wide Drugs Use Assessment', in *New Psychoactive Substances : Pharmacology, Clinical, Forensic and Analytical Toxicology*, H. H. Maurer and S. D. Brandt, Eds, in Handbook of Experimental Pharmacology. , Cham: Springer International Publishing, 2018, pp. 543–566. doi: 10.1007/164_2018_111.

[127] E. López-García, C. Postigo, B. Zonja, D. Barceló, and M. López de Alda, 'Chapter Two - Analysis of Psychoactive Pharmaceuticals in Wastewater and Surface Water Using LC-MS', in *Comprehensive Analytical Chemistry*, vol. 79, A. Cappiello and P. Palma, Eds, in Advances in the Use of Liquid Chromatography Mass Spectrometry (LC-MS), vol. 79. , Elsevier, 2018, pp. 29–52. doi: 10.1016/bs.coac.2017.08.009.

[128] K. V. Thomas, A. Amador, J. A. Baz-Lomba, and M. Reid, 'Use of Mobile Device Data To Better Estimate Dynamic Population Size for Wastewater-Based

Epidemiology', *Environ. Sci. Technol.*, vol. 51, no. 19, pp. 11363–11370, Oct. 2017, doi: 10.1021/acs.est.7b02538.

[129] T. L. Croft, R. A. Huffines, M. Pathak, and B. Subedi, 'Prevalence of illicit and prescribed neuropsychiatric drugs in three communities in Kentucky using wastewater-based epidemiology and Monte Carlo simulation for the estimation of associated uncertainties', *Journal of Hazardous Materials*, vol. 384, p. 121306, Feb. 2020, doi: 10.1016/j.jhazmat.2019.121306.

[130] A. J. Skees, K. S. Foppe, B. Loganathan, and B. Subedi, 'Contamination profiles, mass loadings, and sewage epidemiology of neuropsychiatric and illicit drugs in wastewater and river waters from a community in the Midwestern United States', *Science of The Total Environment*, vol. 631–632, pp. 1457–1464, Aug. 2018, doi: 10.1016/j.scitotenv.2018.03.060.

[131] B. Subedi and K. Kannan, 'Occurrence and fate of select psychoactive pharmaceuticals and antihypertensives in two wastewater treatment plants in New York State, USA', *Science of The Total Environment*, vol. 514, pp. 273–280, May 2015, doi: 10.1016/j.scitotenv.2015.01.098.

[132] C. Christophoridis, S. Veloutsou, E. Mitsika, C. K. Zacharis, C. Christia, N. Raikos, and K. Fytianos, 'Determination of illicit drugs and psychoactive pharmaceuticals in wastewater from the area of Thessaloniki (Greece) using LC–MS/MS: estimation of drug consumption', *Environ Monit Assess*, vol. 193, no. 5, p. 249, Apr. 2021, doi: 10.1007/s10661-021-09035-9.

[133] K. T. Ng, H. Rapp-Wright, M. Egli, A. Hartmann, J. C. Steele, J. E. Sosa-Hernández, E. M. Melchor-Martínez, M. Jacobs, B. White, F. Regan, R. Parra-Saldivar, L. Couchman, R. U. Halden, and L. P. Barron, 'High-throughput multi-residue quantification of contaminants of emerging concern in wastewaters enabled using direct injection liquid chromatography-tandem mass spectrometry', *Journal of Hazardous Materials*, vol. 398, p. 122933, Nov. 2020, doi: 10.1016/j.jhazmat.2020.122933.

[134] T. S. Oliveira, M. Murphy, N. Mendola, V. Wong, D. Carlson, and L. Waring, 'Characterization of Pharmaceuticals and Personal Care products in hospital effluent and waste water influent/effluent by direct-injection LC-MS-MS', *Science of The Total Environment*, vol. 518–519, pp. 459–478, Jun. 2015, doi: 10.1016/j.scitotenv.2015.02.104.

[135] D. Phung, J. Mueller, F. Y. Lai, J. O'Brien, N. Dang, L. Morawska, and P. K. Thai, 'Can wastewater-based epidemiology be used to evaluate the health impact of temperature? – An exploratory study in an Australian population', *Environmental Research*, vol. 156, pp. 113–119, Jul. 2017, doi: 10.1016/j.envres.2017.03.023.

[136] D. Nadarajan, J. O'Brien, S. Cresswell, B. Kele, J. Mueller, and R. Bade, 'Application of design of experiment for quantification of 71 new psychoactive substances in influent wastewater', *Analytica Chimica Acta*, vol. 1321, p. 343036, Sep. 2024, doi: 10.1016/j.aca.2024.343036.

[137] T. Verovšek, D. Heath, and E. Heath, 'Occurrence, fate and determination of tobacco (nicotine) and alcohol (ethanol) residues in waste- and environmental

waters', *Trends in Environmental Analytical Chemistry*, vol. 34, p. e00164, Jun. 2022, doi: 10.1016/j.teac.2022.e00164.

[138] D. R. Baker and B. Kasprzyk-Hordern, 'Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography–positive electrospray ionisation tandem mass spectrometry', *Journal of Chromatography A*, vol. 1218, no. 12, pp. 1620–1631, Mar. 2011, doi: 10.1016/j.chroma.2011.01.060.

[139] N. Alygizakis, A. Galani, N. I. Rousis, R. Aalizadeh, M.-A. Dimopoulos, and N. S. Thomaidis, 'Change in the chemical content of untreated wastewater of Athens, Greece under COVID-19 pandemic', *Science of The Total Environment*, vol. 799, p. 149230, Dec. 2021, doi: 10.1016/j.scitotenv.2021.149230.

[140] A. Galani, N. Alygizakis, R. Aalizadeh, E. Kastritis, M.-A. Dimopoulos, and N. S. Thomaidis, 'Patterns of pharmaceuticals use during the first wave of COVID-19 pandemic in Athens, Greece as revealed by wastewater-based epidemiology', *Science of The Total Environment*, vol. 798, p. 149014, Dec. 2021, doi: 10.1016/j.scitotenv.2021.149014.

[141] R. Gonçalves, C. Ribeiro, S. Cravo, S. C. Cunha, J. A. Pereira, J. O. Fernandes, C. Afonso, and M. E. Tiritan, 'Multi-residue method for enantioseparation of psychoactive substances and beta blockers by gas chromatography–mass spectrometry', *Journal of Chromatography B*, vol. 1125, p. 121731, Sep. 2019, doi: 10.1016/j.jchromb.2019.121731.

[142] W. Gul, S. W. Gul, B. Stamper, M. Godfrey, and M. A. ElSohly, 'LC-MS-MS Method Development and Analysis of Stimulants, Opiates, Synthetic Opiates, PCP, and Benzodiazepines in Wastewater. Preponderance of these Drugs During Football Games', in *Analysis of Drugs of Abuse*, R. A. Musah, Ed., in Methods in Molecular Biology. , New York, NY: Springer, 2018, pp. 149–182. doi: 10.1007/978-1-4939-8579-1_15.

[143] C. Li, S. Chu, S. Tan, X. Yin, Y. Jiang, X. Dai, X. Gong, X. Fang, and D. Tian, 'Towards Higher Sensitivity of Mass Spectrometry: A Perspective From the Mass Analyzers', *Front. Chem.*, vol. 9, p. 813359, Dec. 2021, doi: 10.3389/fchem.2021.813359.

[144] G. Kaklamanos, E. Aprea, and G. Theodoridis, '11 - Mass spectrometry: principles and instrumentation', in *Chemical Analysis of Food (Second Edition)*, Y. Pico, Ed., Academic Press, 2020, pp. 525–552. doi: 10.1016/B978-0-12-813266-1.00011-5.

[145] A. L. N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M. L. de Alda, H. Neels, E. Zuccato, D. Barcelo, and A. Covaci, 'Illicit drug consumption estimations derived from wastewater analysis: A critical review', *Science of The Total Environment*, vol. 409, no. 19, pp. 3564–3577, Sep. 2011, doi: 10.1016/j.scitotenv.2010.05.030.

[146] S. S. Andra, C. Austin, D. Patel, G. Dolios, M. Awawda, and M. Arora, 'Trends in the application of high-resolution mass spectrometry for human biomonitoring: An analytical primer to studying the environmental chemical space of the human exposome', *Environ Int*, vol. 100, pp. 32–61, Mar. 2017, doi: 10.1016/j.envint.2016.11.026.

[147] E. L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H. P. Singer, and J. Hollender, 'Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence', *Environ. Sci. Technol.*, vol. 48, no. 4, pp. 2097–2098, Feb. 2014, doi: 10.1021/es5002105.

[148] R. Bade, J. M. White, and C. Gerber, 'Qualitative and quantitative temporal analysis of licit and illicit drugs in wastewater in Australia using liquid chromatography coupled to mass spectrometry', *Anal Bioanal Chem*, vol. 410, no. 2, pp. 529–542, Jan. 2018, doi: 10.1007/s00216-017-0747-2.

[149] R. Bade, D. van Herwerden, N. Rousis, S. Adhikari, D. Allen, C. Baduel, L. Bijlsma, T. Boogaerts, D. Burgard, A. Chappell, E. M. Driver, F. F. Sodre, D. Fatta-Kassinos, E. Gracia-Lor, E. Gracia-Marín, R. U. Halden, E. Heath, E. Jaunay, A. Krotulski, F. Y. Lai, A. S. C. Löve, J. W. O'Brien, J.-E. Oh, D. Pasin, M. P. Castro, M. Psichoudaki, N. Salgueiro-Gonzalez, C. S. Gomes, B. Subedi, K. V. Thomas, N. Thomaidis, D. Wang, V. Yargeau, S. Samanipour, and J. Mueller, 'Workflow to facilitate the detection of new psychoactive substances and drugs of abuse in influent urban wastewater', *Journal of Hazardous Materials*, vol. 469, p. 133955, May 2024, doi: 10.1016/j.jhazmat.2024.133955.

[150] I. González-Mariño, J. A. Baz-Lomba, N. A. Alygizakis, M. J. Andrés-Costa, R. Bade, A. Bannwarth, L. P. Barron, F. Been, L. Benaglia, J.-D. Berset, L. Bijlsma, I. Bodík, A. Brenner, A. L. Brock, D. A. Burgard, E. Castrignanò, A. Celma, C. E. Christophoridis, A. Covaci, O. Delémont, P. de Voogt, D. A. Devault, M. J. Dias, E. Emke, P. Esseiva, D. Fatta-Kassinos, G. Fedorova, K. Fytianos, C. Gerber, R. Grabic, E. Gracia-Lor, S. Grüner, T. Gunnar, E. Hapeshi, E. Heath, B. Helm, F. Hernández, A. Kankaanpaa, S. Karolak, B. Kasprzyk-Hordern, I. Krizman-Matasic, F. Y. Lai, W. Lechowicz, A. Lopes, M. López de Alda, E. López-García, A. S. C. Löve, N. Mastroianni, G. L. McEneff, R. Montes, K. Munro, T. Nefau, H. Oberacher, J. W. O'Brien, R. Oertel, K. Olafsdottir, Y. Picó, B. G. Plósz, F. Polesel, C. Postigo, J. B. Quintana, P. Ramin, M. J. Reid, J. Rice, R. Rodil, N. Salgueiro-González, S. Schubert, I. Senta, S. M. Simões, M. M. Sremacki, K. Styszko, S. Terzic, N. S. Thomaidis, K. V. Thomas, B. J. Tscharke, R. Udrisard, A. L. N. van Nuijs, V. Yargeau, E. Zuccato, S. Castiglioni, and C. Ort, 'Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring', *Addiction*, vol. 115, no. 1, pp. 109–120, 2020, doi: 10.1111/add.14767.

[151] L. Mellish, E. A. Karanges, M. J. Litchfield, A. L. Schaffer, B. Blanch, B. J. Daniels, A. Segrave, and S.-A. Pearson, 'The Australian Pharmaceutical Benefits Scheme data collection: a practical guide for researchers', *BMC Research Notes*, vol. 8, no. 1, p. 634, Nov. 2015, doi: 10.1186/s13104-015-1616-8.

[152] K. Mao, K. Zhang, W. Du, W. Ali, X. Feng, and H. Zhang, 'The potential of wastewater-based epidemiology as surveillance and early warning of infectious disease outbreaks', *Current Opinion in Environmental Science & Health*, vol. 17, pp. 1–7, Oct. 2020, doi: 10.1016/j.coesh.2020.04.006.

[153] N. Sims and B. Kasprzyk-Hordern, 'Future perspectives of wastewater-based epidemiology: Monitoring infectious disease spread and resistance to the community

level', *Environment International*, vol. 139, p. 105689, Jun. 2020, doi: 10.1016/j.envint.2020.105689.

[154] S. Diemert and T. Yan, 'Municipal Wastewater Surveillance Revealed a High Community Disease Burden of a Rarely Reported and Possibly Subclinical Salmonella enterica Serovar Derby Strain', *Applied and Environmental Microbiology*, vol. 86, no. 17, pp. e00814-20, Aug. 2020, doi: 10.1128/AEM.00814-20.

[155] J. Majlander, V.-J. Anttila, W. Nurmi, A. Seppälä, J. Tiedje, and W. Muziasari, 'Routine wastewater-based monitoring of antibiotic resistance in two Finnish hospitals: focus on carbapenem resistance genes and genes associated with bacteria causing hospital-acquired infections', *Journal of Hospital Infection*, vol. 117, pp. 157–164, Nov. 2021, doi: 10.1016/j.jhin.2021.09.008.

[156] M. V. A. Corpuz, A. Buonerba, G. Vigliotta, T. Zarra, F. Ballesteros, P. Campiglia, V. Belgiorno, G. Korshin, and V. Naddeo, 'Viruses in wastewater: occurrence, abundance and detection methods', *Science of The Total Environment*, vol. 745, p. 140910, Nov. 2020, doi: 10.1016/j.scitotenv.2020.140910.

[157] D. Schmiege, N. Zacharias, E. Sib, T. Falkenberg, S. Moebus, M. Evers, and T. Kistemann, 'Prevalence of multidrug-resistant and extended-spectrum beta-lactamase-producing *Escherichia coli* in urban community wastewater', *Science of The Total Environment*, vol. 785, p. 147269, Sep. 2021, doi: 10.1016/j.scitotenv.2021.147269.

[158] W. Ahmed, N. Angel, J. Edson, K. Bibby, A. Bivins, J. W. O'Brien, P. M. Choi, M. Kitajima, S. L. Simpson, J. Li, B. Tscharke, R. Verhagen, W. J. M. Smith, J. Zaugg, L. Dierens, P. Hugenholtz, K. V. Thomas, and J. F. Mueller, 'First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community', *Science of The Total Environment*, vol. 728, p. 138764, Aug. 2020, doi: 10.1016/j.scitotenv.2020.138764.

[159] M. Hamouda, F. Mustafa, M. Maraqa, T. Rizvi, and A. Aly Hassan, 'Wastewater surveillance for SARS-CoV-2: Lessons learnt from recent studies to define future applications', *Science of The Total Environment*, vol. 759, p. 143493, Mar. 2021, doi: 10.1016/j.scitotenv.2020.143493.

[160] G. Medema, L. Heijnen, G. Elsinga, R. Italiaander, and A. Brouwer, 'Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands', *Environ. Sci. Technol. Lett.*, vol. 7, no. 7, pp. 511–516, Jul. 2020, doi: 10.1021/acs.estlett.0c00357.

[161] F. Y. Lai, C. Ort, C. Gartner, S. Carter, J. Prichard, P. Kirkbride, R. Bruno, W. Hall, G. Eaglesham, and J. F. Mueller, 'Refining the estimation of illicit drug consumptions from wastewater analysis: Co-analysis of prescription pharmaceuticals and uncertainty assessment', *Water Research*, vol. 45, no. 15, pp. 4437–4448, Oct. 2011, doi: 10.1016/j.watres.2011.05.042.

[162] J. W. O'Brien, P. K. Thai, G. Eaglesham, C. Ort, A. Scheidegger, S. Carter, F. Y. Lai, and J. F. Mueller, 'A Model to Estimate the Population Contributing to the

Wastewater Using Samples Collected on Census Day', *Environ. Sci. Technol.*, vol. 48, no. 1, pp. 517–525, Jan. 2014, doi: 10.1021/es403251g.

[163] T. Boogaerts, M. Quireyns, M. De prins, B. Pussig, H. De Loof, C. Matheï, B. Aertgeerts, V. Van Coppenolle, E. Fransen, A. Covaci, and A. L. N. van Nuijs, 'Temporal monitoring of stimulants during the COVID-19 pandemic in Belgium through the analysis of influent wastewater', *International Journal of Drug Policy*, vol. 104, p. 103679, Jun. 2022, doi: 10.1016/j.drugpo.2022.103679.

[164] R. Kato, 'Sex-Related Differences in Drug Metabolism', *Drug Metabolism Reviews*, vol. 3, no. 1, pp. 1–32, Jan. 1975, doi: 10.3109/03602537408993737.

[165] S. Shi and U. Klotz, 'Age-Related Changes in Pharmacokinetics', *Current Drug Metabolism*, vol. 12, no. 7, pp. 601–610, 2011, doi: 10.2174/138920011796504527.

[166] S. Yasuda, L. Zhang, and S.-M. Huang, 'The Role of Ethnicity in Variability in Response to Drugs: Focus on Clinical Pharmacology Studies', *Clinical Pharmacology & Therapeutics*, vol. 84, no. 3, pp. 417–423, 2008, doi: 10.1038/clpt.2008.141.

[167] E. Gracia-Lor, E. Zuccato, and S. Castiglioni, 'Refining correction factors for back-calculation of illicit drug use', *Science of The Total Environment*, vol. 573, pp. 1648–1659, Dec. 2016, doi: 10.1016/j.scitotenv.2016.09.179.

[168] J. W. O'Brien, A. P. W. Banks, A. J. Novic, J. F. Mueller, G. Jiang, C. Ort, G. Eaglesham, Z. Yuan, and P. K. Thai, 'Impact of in-Sewer Degradation of Pharmaceutical and Personal Care Products (PPCPs) Population Markers on a Population Model', *Environ. Sci. Technol.*, vol. 51, no. 7, pp. 3816–3823, Apr. 2017, doi: 10.1021/acs.est.6b02755.

[169] E. Bettington, J. Spinks, F. Kelly, A. Gallardo-Godoy, S. Nghiem, and A. J. Wheeler, 'When is a medicine unwanted, how is it disposed, and how might safe disposal be promoted? Insights from the Australian population', *Aust. Health Review*, vol. 42, no. 6, pp. 709–717, Dec. 2018, doi: 10.1071/AH16296.

[170] B. Petrie, R. Barden, and B. Kasprzyk-Hordern, 'A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring', *Water Research*, vol. 72, pp. 3–27, Apr. 2015, doi: 10.1016/j.watres.2014.08.053.

[171] M. Quireyns, T. Boogaerts, N. Van Wichelen, A. Covaci, and A. L. N. van Nuijs, 'State-of-the-art analytical approaches and strategies to assess disposal of drugs for wastewater-based epidemiology', *WIREs Forensic Science*, vol. 5, no. 1, p. e1469, 2023, doi: 10.1002/wfs2.1469.

[172] EUDA, *EUDA (European Union Drugs Agency), 2022, European Drug Report 2022: Trends and Developments. Publications Office of the European Union, Luxembourg. doi: 10.2810/75644.* LU: Publications Office, 2022. Accessed: Mar. 04, 2024. [Online]. Available: https://www.euda.europa.eu/publications/edr/trends-developments/2022_en

[173] EUDA, *EUDA (European Union Drugs Agency). New psychoactive substances: 25 years of early warning and response in Europe: an update from the EU Early Warning System.* LU: Publications Office, 2022. Accessed: Mar. 04, 2024. [Online]. Available: https://data.europa.eu/doi/10.2810/396103

[174] N. S. Thomaidis, P. Gago-Ferrero, C. Ort, N. C. Maragou, N. A. Alygizakis, V. L. Borova, and M. E. Dasenaki, 'Reflection of Socioeconomic Changes in Wastewater: Licit and Illicit Drug Use Patterns', *Environ. Sci. Technol.*, vol. 50, no. 18, pp. 10065–10072, Sep. 2016, doi: 10.1021/acs.est.6b02417.

[175] T. Boogaerts, M. Quireyns, H. De Loof, X. Bertels, N. Van Wichelen, B. Pussig, J. Saevels, L. Lahousse, P. Bonmariage, W. Hamelinck, B. Aertgeerts, A. Covaci, and A. L. N. van Nuijs, 'Do the lockdown-imposed changes in a wastewater treatment plant catchment's socio-demographics impact longitudinal temporal trends in psychoactive pharmaceutical use?', *Science of The Total Environment*, vol. 876, p. 162342, Jun. 2023, doi: 10.1016/j.scitotenv.2023.162342.

[176] M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, N. Van Wichelen, D. Heath, A. L. N. van Nuijs, A. Covaci, and E. Heath, 'Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters', *Science of The Total Environment*, vol. 903, p. 166586, Dec. 2023, doi: 10.1016/j.scitotenv.2023.166586.

[177] T. Boogaerts, L. Jacobs, N. De Roeck, S. Van den Bogaert, B. Aertgeerts, L. Lahousse, A. L. N. van Nuijs, and P. Delputte, 'An alternative approach for bioanalytical assay optimization for wastewater-based epidemiology of SARS-CoV-2', *Science of The Total Environment*, vol. 789, p. 148043, Oct. 2021, doi: 10.1016/j.scitotenv.2021.148043.

[178] G. Medema, F. Been, L. Heijnen, and S. Petterson, 'Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges', *Current Opinion in Environmental Science & Health*, vol. 17, pp. 49–71, Oct. 2020, doi: 10.1016/j.coesh.2020.09.006.

[179] W. Ahmed, B. Tscharke, P. M. Bertsch, K. Bibby, A. Bivins, P. Choi, L. Clarke, J. Dwyer, J. Edson, T. M. H. Nguyen, J. W. O'Brien, S. L. Simpson, P. Sherman, K. V. Thomas, R. Verhagen, J. Zaugg, and J. F. Mueller, 'SARS-CoV-2 RNA monitoring in wastewater as a potential early warning system for COVID-19 transmission in the community: A temporal case study', *Science of The Total Environment*, vol. 761, p. 144216, Mar. 2021, doi: 10.1016/j.scitotenv.2020.144216.

[180] G. Orive, U. Lertxundi, and D. Barcelo, 'Early SARS-CoV-2 outbreak detection by sewage-based epidemiology', *Science of The Total Environment*, vol. 732, p. 139298, Aug. 2020, doi: 10.1016/j.scitotenv.2020.139298.

[181] P. S. K. Ngoubeyou, C. Wolkersdorfer, P. P. Ndibewu, and W. Augustyn, 'Toxicity of polychlorinated biphenyls in aquatic environments – A review', *Aquatic Toxicology*, vol. 251, p. 106284, Oct. 2022, doi: 10.1016/j.aquatox.2022.106284.

[182] L. Montano, G. M. Baldini, M. Piscopo, G. Liguori, R. Lombardi, M. Ricciardi, G. Esposito, G. Pinto, C. Fontanarosa, M. Spinelli, I. Palmieri, D. Sofia, C. Brogna, C. Carati, M. Esposito, P. Gallo, A. Amoresano, and O. Motta, 'Polycyclic Aromatic Hydrocarbons (PAHs) in the Environment: Occupational Exposure, Health Risks and Fertility Implications', *Toxics*, vol. 13, no. 3, p. 151, Mar. 2025, doi: 10.3390/toxics13030151.

[183] A. Alengebawy, S. T. Abdelkhalek, S. R. Qureshi, and M.-Q. Wang, 'Heavy Metals and Pesticides Toxicity in Agricultural Soil and Plants: Ecological Risks and Human

Health Implications', *Toxics*, vol. 9, no. 3, p. 42, Mar. 2021, doi: 10.3390/toxics9030042.

[184] D. G. Moreira, A. Aires, M. de Lourdes Pereira, and M. Oliveira, 'Levels and effects of antidepressant drugs to aquatic organisms', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 256, p. 109322, Jun. 2022, doi: 10.1016/j.cbpc.2022.109322.

[185] J. Argaluza, S. Domingo-Echaburu, G. Orive, J. Medrano, R. Hernandez, and U. Lertxundi, 'Environmental pollution with psychiatric drugs', *World J Psychiatry*, vol. 11, no. 10, pp. 791–804, Oct. 2021, doi: 10.5498/wjp.v11.i10.791.

[186] OECD, 'Organisation for Economic Co-operation and Development: Pharmaceutical Residues in Freshwater Hazards and Policy Responses.' Accessed: Jul. 13, 2025. [Online]. Available: https://www.oecd.org/en/publications/pharmaceutical-residues-in-freshwater_c936f42d-en.html

[187] 'The Global Use of Medicines 2024: Outlook to 2028'. Accessed: Jul. 14, 2025. [Online]. Available: https://www.iqvia.com/insights/the-iqvia-institute/reports-and-publications/reports/the-global-use-of-medicines-2024-outlook-to-2028

[188] D. L. Cunha, M. P. Mendes, and M. Marques, 'Environmental risk assessment of psychoactive drugs in the aquatic environment', *Environ Sci Pollut Res*, vol. 26, no. 1, pp. 78–90, Jan. 2019, doi: 10.1007/s11356-018-3556-z.

[189] 'Pharmaceutical Residues in Freshwater', OECD. Accessed: Jul. 14, 2025. [Online]. Available: https://www.oecd.org/en/publications/pharmaceutical-residues-in-freshwater_c936f42d-en.html

[190] M. Caban and P. Stepnowski, 'How to decrease pharmaceuticals in the environment? A review', *Environ Chem Lett*, vol. 19, no. 4, pp. 3115–3138, Aug. 2021, doi: 10.1007/s10311-021-01194-y.

[191] T. Jiang, W. Wu, M. Ma, Y. Hu, and R. Li, 'Occurrence and distribution of emerging contaminants in wastewater treatment plants: A globally review over the past two decades', *Science of The Total Environment*, vol. 951, p. 175664, Nov. 2024, doi: 10.1016/j.scitotenv.2024.175664.

[192] H. Mohan, S. S. Rajput, E. B. Jadhav, M. S. Sankhla, S. S. Sonone, S. Jadhav, and R. Kumar, 'Ecotoxicity, occurrence, and removal of pharmaceuticals and illicit drugs from aquatic systems', *Biointerface Research in Applied Chemistry*, vol. 11, no. 5, pp. 12530–12546, 2021, doi: 10.33263/BRIAC115.1253012546.

[193] J. Niu, Y. Lu, H. Wang, X. Qiao, H. Wang, C. Ma, and Y. Liu, 'Occurrence, removal and environmental risk assessment of pharmaceutical active compounds (PhACs) and metabolites in hospital wastewater', *Journal of Hazardous Materials*, vol. 480, p. 136348, Dec. 2024, doi: 10.1016/j.jhazmat.2024.136348.

[194] R. C. Pivetta, C. Rodrigues-Silva, A. R. Ribeiro, and S. Rath, 'Tracking the occurrence of psychotropic pharmaceuticals in Brazilian wastewater treatment plants and surface water, with assessment of environmental risks', *Science of The Total Environment*, vol. 727, p. 138661, Jul. 2020, doi: 10.1016/j.scitotenv.2020.138661.

[195] J. Escudero, J. L. Muñoz, T. Morera-Herreras, R. Hernandez, J. Medrano, S. Domingo-Echaburu, D. Barceló, G. Orive, and U. Lertxundi, 'Antipsychotics as

environmental pollutants: An underrated threat?', *Science of The Total Environment*, vol. 769, p. 144634, May 2021, doi: 10.1016/j.scitotenv.2020.144634.

[196] J. F. Reichert, D. M. Souza, and A. F. Martins, 'Antipsychotic drugs in hospital wastewater and a preliminary risk assessment', *Ecotoxicology and Environmental Safety*, vol. 170, pp. 559–567, Apr. 2019, doi: 10.1016/j.ecoenv.2018.12.021.

[197] A. Zergui and D. B. Nkontcheu Kenko, 'Drugs of abuse and psychoactive pharmaceuticals in wastewater as worldwide contaminants of emerging concern: a systematic review', *Environmental Pollution and Management*, vol. 1, pp. 71–86, Oct. 2024, doi: 10.1016/j.epm.2024.08.001.

[198] C. Daughton, 'Pharmaceuticals and Personal Care Products in the Environment: Overarching Issues and Overview', in *ACS Symposium Series*, vol. 791, 2001, pp. 2–38. doi: 10.1021/bk-2001-0791.ch001.

[199] N. Diaz-Camal, J. D. Cardoso-Vera, H. Islas-Flores, L. M. Gómez-Oliván, and A. Mejía-García, 'Consumption and ocurrence of antidepressants (SSRIs) in pre- and post-COVID-19 pandemic, their environmental impact and innovative removal methods: A review', *Science of The Total Environment*, vol. 829, p. 154656, Jul. 2022, doi: 10.1016/j.scitotenv.2022.154656.

[200] A. K. Richardson, M. Chadha, H. Rapp-Wright, G. A. Mills, G. R. Fones, A. Gravell, S. Stürzenbaum, D. A. Cowan, D. J. Neep, and L. P. Barron, 'Rapid direct analysis of river water and machine learning assisted suspect screening of emerging contaminants in passive sampler extracts', *Anal. Methods*, vol. 13, no. 5, pp. 595–606, Feb. 2021, doi: 10.1039/D0AY02013C.

[201] J. K. Kingston, R. Greenwood, G. A. Mills, G. M. Morrison, and L. B. Persson, 'Development of a novel passive sampling systemfor the time-averaged measurement of a range of organic pollutants in aquatic environments', *J. Environ. Monit.*, vol. 2, no. 5, pp. 487–495, Jan. 2000, doi: 10.1039/B003532G.

[202] A. Singh, D. Saidulu, A. K. Gupta, and V. Kubsad, 'Occurrence and fate of antidepressants in the aquatic environment: Insights into toxicological effects on the aquatic life, analytical methods, and removal techniques', *Journal of Environmental Chemical Engineering*, vol. 10, no. 6, p. 109012, Dec. 2022, doi: 10.1016/j.jece.2022.109012.

[203] T. Verovšek, A. Šuštarič, M. Laimou-Geraniou, I. Krizman-Matasic, H. Prosen, T. Eleršek, V. Kramarič Zidar, V. Mislej, B. Mišmaš, M. Stražar, M. Levstek, B. Cimrmančič, S. Lukšič, N. Uranjek, T. Kozlovič-Bobič, T. Kosjek, D. Kocman, D. Heath, and E. Heath, 'Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment', *Science of The Total Environment*, vol. 866, p. 161257, Mar. 2023, doi: 10.1016/j.scitotenv.2022.161257.

[204] A. Ofrydopoulou, C. Nannou, E. Evgenidou, A. Christodoulou, and D. Lambropoulou, 'Assessment of a wide array of organic micropollutants of emerging concern in wastewater treatment plants in Greece: Occurrence, removals, mass loading and potential risks', *Science of The Total Environment*, vol. 802, p. 149860, Jan. 2022, doi: 10.1016/j.scitotenv.2021.149860.

[205] M. Čelić, M. Gros, M. Farré, D. Barceló, and M. Petrović, 'Pharmaceuticals as chemical markers of wastewater contamination in the vulnerable area of the Ebro Delta (Spain)', *Science of The Total Environment*, vol. 652, pp. 952–963, Feb. 2019, doi: 10.1016/j.scitotenv.2018.10.290.

[206] R. Gurke, M. Rößler, C. Marx, S. Diamond, S. Schubert, R. Oertel, and J. Fauler, 'Occurrence and removal of frequently prescribed pharmaceuticals and corresponding metabolites in wastewater of a sewage treatment plant', *Science of The Total Environment*, vol. 532, pp. 762–770, Nov. 2015, doi: 10.1016/j.scitotenv.2015.06.067.

[207] L. Molnarova, T. Halesova, D. Tomesova, M. Vaclavikova, and Z. Bosakova, 'Monitoring Pharmaceuticals and Personal Care Products in Healthcare Effluent Wastewater Samples and the Effectiveness of Drug Removal in Wastewater Treatment Plants Using the UHPLC-MS/MS Method', *Molecules*, vol. 29, no. 7, p. 1480, Mar. 2024, doi: 10.3390/molecules29071480.

[208] Z. Li, J. Li, Y. Hu, Y. Yan, S. Tang, R. Ma, and L. Li, 'Evaluation of pharmaceutical consumption between urban and suburban catchments in China by wastewater-based epidemiology', *Environmental Research*, vol. 250, p. 118544, Jun. 2024, doi: 10.1016/j.envres.2024.118544.

[209] N. Lopez-Herguedas, M. Irazola, I. Alvarez-Mora, G. Orive, U. Lertxundi, M. Olivares, O. Zuloaga, and A. Prieto, 'Comprehensive micropollutant characterization of wastewater during Covid-19 crisis in 2020: Suspect screening and environmental risk prioritization strategy', *Science of The Total Environment*, vol. 873, p. 162281, May 2023, doi: 10.1016/j.scitotenv.2023.162281.

[210] W. Wang, J. Zhang, M. Hu, X. Liu, T. Sun, and H. Zhang, 'Antidepressants in wastewater treatment plants: Occurrence, transformation and acute toxicity evaluation', *Science of The Total Environment*, vol. 903, p. 166120, Dec. 2023, doi: 10.1016/j.scitotenv.2023.166120.

[211] UWWTD, 'Council Directive 91/271/EEC of 21 May 1991 concerning urban wastewater treatment'. Accessed: Mar. 09, 2025. [Online]. Available: https://eur-lex.europa.eu/eli/dir/1991/271/oj/eng

[212] UWWTD, 'Directive (EU) 2024/3019 of the European Parliament and of the Council of 27 November 2024 concerning urban wastewater treatment (recast)'. Accessed: Mar. 09, 2025. [Online]. Available: https://eur-lex.europa.eu/eli/dir/2024/3019/oj/eng

[213] J. Trawiński and R. Skibiński, 'Studies on photodegradation process of psychotropic drugs: a review', *Environ Sci Pollut Res*, vol. 24, no. 2, pp. 1152–1199, Jan. 2017, doi: 10.1007/s11356-016-7727-5.

[214] J. Klaminder, T. Brodin, A. Sundelin, N. J. Anderson, J. Fahlman, M. Jonsson, and J. Fick, 'Long-Term Persistence of an Anxiolytic Drug (Oxazepam) in a Large Freshwater Lake', *Environ. Sci. Technol.*, vol. 49, no. 17, pp. 10406–10412, Sep. 2015, doi: 10.1021/acs.est.5b01968.

[215] *Commission Implementing Decision (EU) 2020/1161 of 4 August 2020 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council*

(notified under document number C(2020) 5205) (Text with EEA relevance), vol. 257. 2020. Accessed: Jul. 16, 2025. [Online]. Available: http://data.europa.eu/eli/dec_impl/2020/1161/oj/eng

[216] *Commission Implementing Decision (EU) 2022/1307 of 22 July 2022 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council (notified under document C(2022) 5098) (Text with EEA relevance)*, vol. 197. 2022. Accessed: Jul. 16, 2025. [Online]. Available: http://data.europa.eu/eli/dec_impl/2022/1307/oj/eng

[217] K. a. F. de Alvarenga, E. K. Sacramento, D. V. Rosa, B. R. Souza, V. B. de Rezende, and M. A. Romano-Silva, 'Effects of antipsychotics on intestinal motility in zebrafish larvae', *Neurogastroenterology & Motility*, vol. 29, no. 5, p. e13006, 2017, doi: 10.1111/nmo.13006.

[218] L. J. Carter, M. Williams, S. Martin, S. P. B. Kamaludeen, and R. S. Kookana, 'Sorption, plant uptake and metabolism of benzodiazepines', *Science of The Total Environment*, vol. 628–629, pp. 18–25, Jul. 2018, doi: 10.1016/j.scitotenv.2018.01.337.

[219] C. Castillo-Zacarías, M. E. Barocio, E. Hidalgo-Vázquez, J. E. Sosa-Hernández, L. Parra-Arroyo, I. Y. López-Pacheco, D. Barceló, H. N. M. Iqbal, and R. Parra-Saldívar, 'Antidepressant drugs as emerging contaminants: Occurrence in urban and non-urban waters and analytical methods for their detection', *Science of The Total Environment*, vol. 757, p. 143722, Feb. 2021, doi: 10.1016/j.scitotenv.2020.143722.

[220] F. Zindler, S. Stoll, L. Baumann, S. Knoll, C. Huhn, and T. Braunbeck, 'Do environmentally relevant concentrations of fluoxetine and citalopram impair stress-related behavior in zebrafish (Danio rerio) embryos?', *Chemosphere*, vol. 261, p. 127753, Dec. 2020, doi: 10.1016/j.chemosphere.2020.127753.

[221] M. J. Andrés-Costa, K. Proctor, M. T. Sabatini, A. P. Gee, S. E. Lewis, Y. Pico, and B. Kasprzyk-Hordern, 'Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance', *Sci Rep*, vol. 7, no. 1, Art. no. 1, Nov. 2017, doi: 10.1038/s41598-017-15585-1.

[222] E. M. Melchor-Martínez, M. G. Jiménez-Rodríguez, M. Martínez-Ruiz, S. A. Peña-Benavides, H. M. N. Iqbal, R. Parra-Saldívar, and J. E. Sosa- Hernández, 'Antidepressants surveillance in wastewater: Overview extraction and detection', *Case Studies in Chemical and Environmental Engineering*, vol. 3, p. 100074, Jun. 2021, doi: 10.1016/j.cscee.2020.100074.

[223] 'Guideline on bioanalytical method validation. European Medicines Agency. Report No.: EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2**'. Accessed: Jun. 27, 2025. [Online]. Available: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

[224] T. Boogaerts, M. Quireyns, F. Maes, M. Laimou-Geraniou, N. Van Wichelen, E. Heath, B. Pussig, B. Aertgeerts, A. Covaci, and A. L. N. van Nuijs, 'Optimization, validation and application of a high-throughput 96-well elution protocol for the

quantification of psychoactive substances in influent wastewater', *Drug Testing and Analysis*, vol. 15, no. 2, pp. 240–246, 2023, doi: 10.1002/dta.3392.

[225] S. Castiglioni, N. Salgueiro-González, L. Bijlsma, A. Celma, E. Gracia-Lor, M. S. Beldean-Galea, T. Mackuľak, E. Emke, E. Heath, B. Kasprzyk-Hordern, A. Petkovic, F. Poretti, J. Rangelov, M. M. Santos, M. Sremački, K. Styszko, F. Hernández, and E. Zuccato, 'New psychoactive substances in several European populations assessed by wastewater-based epidemiology', *Water Research*, vol. 195, p. 116983, May 2021, doi: 10.1016/j.watres.2021.116983.

[226] T. Verovšek, A. Celma, D. Heath, E. Heath, F. Hernández, and L. Bijlsma, 'Screening for new psychoactive substances in wastewater from educational institutions', *Environmental Research*, vol. 237, p. 117061, Nov. 2023, doi: 10.1016/j.envres.2023.117061.

[227] N. Rousis, R. Bade, I. Romero-Sánchez, J. F. Mueller, N. S. Thomaidis, K. V. Thomas, and E. Gracia-Lor, 'Festivals following the easing of COVID-19 restrictions: Prevalence of new psychoactive substances and illicit drugs', *Environment International*, vol. 178, p. 108075, Aug. 2023, doi: 10.1016/j.envint.2023.108075.

[228] H. Jin, D. Yang, P. Wu, and M. Zhao, 'Environmental occurrence and ecological risks of psychoactive substances', *Environment International*, vol. 158, p. 106970, Jan. 2022, doi: 10.1016/j.envint.2021.106970.

[229] L. Wang, R. Liu, X. Liu, and H. Gao, 'Sampling Rate of Polar Organic Chemical Integrative Sampler (POCIS): Influence Factors and Calibration Methods', *Applied Sciences*, vol. 10, no. 16, Art. no. 16, Jan. 2020, doi: 10.3390/app10165548.

[230] L. E. Tomsone, I. Perkons, V. Sukajeva, R. Neilands, K. Kokina, V. Bartkevics, and I. Pugajeva, 'Consumption trends of pharmaceuticals and psychoactive drugs in Latvia determined by the analysis of wastewater', *Water Research*, vol. 221, p. 118800, Aug. 2022, doi: 10.1016/j.watres.2022.118800.

[231] I. B. Roll and R. U. Halden, 'Critical review of factors governing data quality of integrative samplers employed in environmental water monitoring', *Water Research*, vol. 94, pp. 200–207, May 2016, doi: 10.1016/j.watres.2016.02.048.

[232] B. Vrana, I. J. Allan, R. Greenwood, G. A. Mills, E. Dominiak, K. Svensson, J. Knutsson, and G. Morrison, 'Passive sampling techniques for monitoring pollutants in water', *TrAC Trends in Analytical Chemistry*, vol. 24, no. 10, pp. 845–868, Nov. 2005, doi: 10.1016/j.trac.2005.06.006.

[233] C.-E. Chen, H. Zhang, and K. C. Jones, 'A novel passive water sampler for in situ sampling of antibiotics', *Journal of Environmental Monitoring*, vol. 14, no. 6, pp. 1523–1530, 2012, doi: 10.1039/C2EM30091E.

[234] J. K. Challis, M. L. Hanson, and C. S. Wong, 'Development and Calibration of an Organic-Diffusive Gradients in Thin Films Aquatic Passive Sampler for a Diverse Suite of Polar Organic Contaminants', *Anal. Chem.*, vol. 88, no. 21, pp. 10583–10591, Nov. 2016, doi: 10.1021/acs.analchem.6b02749.

[235] R. Z. Hahn, M. F. Bastiani, L. de Lima Feltraco Lizot, I. C. da Silva Moreira, Y. F. Meireles, A. Schneider, C. A. do Nascimento, and R. Linden, 'Determination of a comprehensive set of drugs of abuse, metabolites and human biomarkers in

wastewater using passive sampling followed by UHPLC-MS/MS analysis', *Microchemical Journal*, vol. 172, p. 106960, Jan. 2022, doi: 10.1016/j.microc.2021.106960.

[236] S. L. MacLeod, E. L. McClure, and C. S. Wong, 'Laboratory calibration and field deployment of the Polar organic chemical integrative sampler for pharmaceuticals and personal care products in wastewater and surface water', *Environmental Toxicology and Chemistry*, vol. 26, no. 12, pp. 2517–2529, 2007, doi: https://doi.org/10.1897/07-238.1.

[237] X. Che, P. Liu, Y. Ding, W. Tao, X. Zheng, B. Di, and H. Qiao, 'In-sample stability and postsampling analysis of 21 illicit drugs, their metabolites and cotinine in wastewater', *Ecotoxicology and Environmental Safety*, vol. 270, p. 115900, Jan. 2024, doi: 10.1016/j.ecoenv.2023.115900.

[238] P. M. Choi, J. Li, J. Gao, J. W. O'Brien, K. V. Thomas, P. K. Thai, G. Jiang, and J. F. Mueller, 'Considerations for assessing stability of wastewater-based epidemiology biomarkers using biofilm-free and sewer reactor tests', *Science of The Total Environment*, vol. 709, p. 136228, Nov. 2020, doi: 10.1016/j.scitotenv.2019.136228.

[239] A. J. Anies, J. He, S. Lefever, T. Boogaerts, A. Covaci, R. Blust, and R. M. Town, 'Sorption of psychoactive compounds on passive sampling sorbents in the presence of wastewater suspended particulate matter', *Environmental Pollution*, vol. 385, p. 127104, Nov. 2025, doi: 10.1016/j.envpol.2025.127104.

[240] SANTE/11312/2021v2, 'Analytical quality control and method validation procedures for pesticide residues analysis in food and feed', 2021.

[241] Z. Fang, K. Li, Y. Li, H. Zhang, K. C. Jones, X. Liu, S. Liu, L. Q. Ma, and J. Luo, 'Development and Application of the Diffusive Gradients in Thin-Films Technique for Measuring Psychiatric Pharmaceuticals in Natural Waters', *Environ. Sci. Technol.*, vol. 53, no. 19, pp. 11223–11231, Oct. 2019, doi: 10.1021/acs.est.9b03166.

[242] P. S. Bäuerlein, J. E. Mansell, T. L. ter Laak, and P. de Voogt, 'Sorption Behavior of Charged and Neutral Polar Organic Compounds on Solid Phase Extraction Materials: Which Functional Group Governs Sorption?', *Environ. Sci. Technol.*, vol. 46, no. 2, pp. 954–961, Jan. 2012, doi: 10.1021/es203404x.

Bibliography

Publications Related to the Thesis

Journal Articles

M. Laimou-Geraniou, T. Kos, D. Heath, E. Heath, "Psychoactive pharmaceuticals: Consumption trends, removal during wastewater treatment and occurrence in receiving waters with associated environmental risks", *Journal of Hazardous Materials Advances*, vol. 19, pp. 100816, 2025. DOI: https://doi.org/10.1016/j.hazadv.2025.100816.

M. Laimou-Geraniou, D. Nadarajan, R. Bade, E. Heath, "Spatiotemporal trends of new psychoactive substances in Slovenia through influent wastewater analysis", *Journal of Hazardous Materials Advances*, vol. 19, pp. 100781, 2025. DOI: https://doi.org/10.1016/j.hazadv.2025.100781.

M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, D. Heath, A. Covaci, A. L.N. van Nuijs, E. Heath, "Stability of antipsychotic biomarkers for wastewater-based epidemiology", *Journal of Pharmaceutical and Biomedical Analysis*, vol. 257, pp. 116702, 2025. DOI: https://doi.org/10.1016/j.jpba.2025.116702.

D. Nadarajan, M. Laimou-Geraniou, A. Chappell, C. Baduel, L. Bijlsma, T. Boogaerts, D. A. Burgard, S. Castiglioni, N. Ceolotto, E. M. Driver, F. Fabriz Sodre, D. Fatta Kassinos, H. Flores Quintana, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R. U. Halden, E. Heath, J. Huchthausen, B. Kasprzyk-Hordern, E. L. Keller, F. Yin Lai, A. Sue-Ching Löve, J. Matias, V. Ocenaskova, J. E. Oh, T. Oluseyi, K. Phung, M. Pineda-Castro, M. Psichoudaki, N. Salgueiro-Gonzalez, J. M. Santos, C. Silvino-Gomes, B. Subedi, N. Thomaidis, D. Wang, N. Van Wichelen, V. Yargeau, S. Cresswell, J. Mueller, R. Bade, "Wastewater Analysis as a Global Toxicovigilance Tool for New Psychoactive Substances Monitoring", *Water Research*, vol. 289 (A), pp. 124847, 2026. DOI: https://doi.org/10.1016/j.watres.2025.124847.

M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, N. Van Wichelen, D. Heath, A. L.N. van Nuijs, A. Covaci, E. Heath, "Retrospective spatiotemporal study of antidepressants in Slovenian wastewaters", *Science of The Total Environment*, vol. 903, pp. 166586, 2023. DOI: https://doi.org/10.1016/j.scitotenv.2023.166586.

M. Laimou-Geraniou, D. Heath, E. Heath, "Analytical methods for the determination of antidepressants, antipsychotics, benzodiazepines and their metabolites through wastewater-based epidemiology", *Trends in Environmental Analytical Chemistry*, vol. 37, pp. e00192, 2023. DOI: https://doi.org/10.1016/j.teac.2022.e00192.

T. Boogaerts, M. Quireyns, F. Maes, M. Laimou-Geraniou, N. Van Wichelen, E. Heath, B. Pussig, B. Aertgeerts, A. Covaci, A. L.N. van Nuijs, "Optimization, validation and application of a high-throughput 96-well elution protocol for the quantification of psychoactive substances in influent wastewater" Drug testing and analysis, vol. 15 (2), pp. 240-246, 2023. DOI: https://doi.org/10.1002/dta.3392.

R. Bade, N. Rousis, S. Adhikari, C. Baduel, L. Bijlsma, E. Bizani, T. Boogaerts, D.A. Burgard, S. Castiglioni, A. Chappell, A. Covaci, E.M. Driver, F. Fabriz Sodre, D. Fatta-Kassinos, A. Galani, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R.U. Halde, E. Heath, F. Hernandez, E. Jaunay, F.Y. Lai, H.J. Lee, M. Laimou-Geraniou, J.E. Oh, K. Olafsdottir, K. Phung, M. Pineda Castro, M. Psichoudaki, X. Shao, N. Salgueiro-Gonzalez, R. Silva Feitosa, C. Silvino Gomes, B. Subedi, A.S. Ching Löve, N. Thomaidis, D. Tran, A. L.N van Nuijs, T. Verovšek, D. Wang, J.M. White, V. Yargeau, E. Zuccato, J.F. Mueller, "Three years of wastewater surveillance for new psychoactive substances from 16 countries", *Water Research X*, vol. 19, pp. 100179, 2023. DOI: https://doi.org/10.1016/j.wroa.2023.100179.

In-Preparation Articles

A. Jun Anies, M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, T. Verovšek, D. Heath, E. Heath, A. L.N. van Nuijs, A. Covaci, R. Blust, R. Town, "In-situ evaluation of an active-passive sampling (APS) technique for monitoring psychoactive compounds in wastewater", to be submitted to *Environmental Pollution* in December 2025.

Conference Contribution Abstracts

M D. Nadarajan, M. Laimou-Geraniou, A. Chappell, C. Baduel, L. Bijlsma, T. Boogaerts, D. A. Burgard, S. Castiglioni, N. Ceolotto, E. M. Driver, F. Fabriz Sodre, D. Fatta Kassinos, H. Flores Quintana, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R. U. Halden, E. Heath, J. Huchthausen, B. Kasprzyk-Hordern, E. L. Keller, F. Yin Lai, A. Sue-Ching Löve, J. Matias, V. Ocenaskova, J. E. Oh, T. Oluseyi, K. Phung, M. Pineda-Castro, M. Psichoudaki, N. Salgueiro-Gonzalez, J. M. Santos, C. Silvino-Gomes, B. Subedi, N. Thomaidis, D. Wang, N. Van Wichelen, V. Yargeau, S. Cresswell, J. Mueller, R. Bade, "3-Methylmethcathinone: A wastewater-based case study on the impact of control measures" in *TIAFT 2025–The 61st Annual Conference of the International Association of Forensic Toxicologists*, Aotearoa New Zealand, 2025.

D. Nadarajan, M. Laimou-Geraniou, A. Chappell, C. Baduel, L. Bijlsma, T. Boogaerts, D. A. Burgard, S. Castiglioni, N. Ceolotto, E. M. Driver, F. Fabriz Sodre, D. Fatta Kassinos, H. Flores Quintana, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R. U. Halden, E. Heath, J. Huchthausen, B. Kasprzyk-Hordern, E. L. Keller, F. Yin Lai, A. Sue-Ching Löve, J. Matias, V. Ocenaskova, J. E. Oh, T. Oluseyi, K. Phung, M. Pineda-Castro, M. Psichoudaki, N. Salgueiro-Gonzalez, J. M. Santos, C. Silvino-Gomes, B. Subedi, N. Thomaidis, D. Wang, N. Van Wichelen, V. Yargeau, S. Cresswell, J. Mueller, R. Bade, "Wastewater analysis to explore spatial and temporal trends of new psychoactive substances: case studies in early warning and the impact of legislation" in *Testing the Waters 8 (TWW8): International conference on wastewater-based epidemiology/surveillance*, Tacoma, WA United States, 2025.

M. Laimou-Geraniou, T. Kos, D. Heath, E. Heath, "Evaluating the Effect of the COVID-19 Pandemic on Consumption Trends of Psychoactive Pharmaceuticals in Slovenia and their Occurrence in the Aqueous Environment" in *Testing the Waters 7 Conference*, Lisbon, Portugal, 2024.

M. Laimou-Geraniou, D. Nadarajan, R. Bade, E. Heath, "Qualitative and quantitative spatiotemporal analysis of new psychoactive substances in Slovenia through the analysis of influent wastewater" in *SETAC Europe 34th Annual Meeting*, Seville, Spain, 2024.

M. Laimou-Geraniou, D. Nadarajan, R. Bade, E. Heath, "Monitoring the Use of New Psychoactive Substances in Slovenia Using Wastewater Analysis" in *Spectromed: Mediterranean Congress on Mass Spectrometry and Its Applications*, Hammamet, Tunisia, 2024.

R. Bade, N. Rousis, S. Adhikari, C. Baduel, L. Bijlsma, E. Bizani, T. Boogaerts, D.A. Burgard, S. Castiglioni, A. Chappell, A. Covaci, E.M. Driver, F. Fabriz Sodre, D. Fatta-Kassinos, A. Galani, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R.U. Halde, E. Heath, F. Hernandez, E. Jaunay, F.Y. Lai, H.J. Lee, M. Laimou-Geraniou, J.E. Oh, K. Olafsdottir, K. Phung, M. Pineda Castro, M. Psichoudaki, X. Shao, N. Salgueiro-Gonzalez, R. Silva Feitosa, C. Silvino Gomes, B. Subedi, A.S. Ching Löve, N. Thomaidis, D. Tran, A. L.N van Nuijs, T. Verovšek, D. Wang, J.M. White, V. Yargeau, E. Zuccato, J.F. Mueller, "Insights from four years of wastewater surveillance for novel psychoactive substances from up to 20 countries" in *TIAFT 2024–The 60th Annual Conference of the International Association of Forensic Toxicologists*, St. Gallen, Switzerland, 2024.

R. Bade, N. Rousis, S. Adhikari, C. Baduel, L. Bijlsma, E. Bizani, T. Boogaerts, D.A. Burgard, S. Castiglioni, A. Chappell, A. Covaci, E.M. Driver, F. Fabriz Sodre, D. Fatta-Kassinos, A. Galani, C. Gerber, E. Gracia-Lor, E. Gracia-Marín, R.U. Halde, E. Heath, F. Hernandez, E. Jaunay, F.Y. Lai, H.J. Lee, M. Laimou-Geraniou, J.E. Oh, K. Olafsdottir, K. Phung, M. Pineda Castro, M. Psichoudaki, X. Shao, N. Salgueiro-Gonzalez, R. Silva Feitosa, C. Silvino Gomes, B. Subedi, A.S. Ching Löve, N. Thomaidis, D. Tran, A. L.N van Nuijs, T. Verovšek, D. Wang, J.M. White, V. Yargeau, E. Zuccato, J.F. Mueller, "Trends from three years of wastewater monitoring for new psychoactive substances in 16 countries" in *Testing the Waters 6 Conference*, Oxford, United Kingdom, 2023.

N. Van Wichelen, M. Quireyns, T. Boogaerts, F. Maes, M. Laimou-Geraniou, E. Heath, B. Pussig, B. Aertgeerts, A. Covaci, A. L.N. van Nuijs, "A High-throughput 96-well Elution Protocol for the Quantification of Psychoactive Substances in influent Wastewater as an Alternative for Traditional Solid-Phase Extraction" in *Testing the Waters 6 Conference*, Oxford, United Kingdom, 2023.

M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, N. Van Wichelen, A. Covaci, A. L.N. van Nuijs, E. Heath, "Monitoring trends in consumption of antidepressants in Slovenia using wastewater analysis", in *Slovenian chemical days 2022 – 28th Annual Meeting of the Slovenian Chemical Society*, Portorož, Slovenia, 2022.

M. Laimou-Geraniou, M. Quireyns, T. Boogaerts, N. Van Wichelen, A. Covaci, A. L. N. van Nuijs, E. Heath, "Understanding temporal and spatial trends in antidepressant drug use in Slovenia using wastewater-based epidemiology" in *Chem2Change, Environmental Chemistry towards Global Change, the 2nd Online ACE Seminar on Chemistry and the Environment Led by Early-Career Scientists*, on-line, 2022.

T. Verovšek, A. Šuštarič, M. Laimou-Geraniou, I. Krizman-Matasic, H. Prosen, T. Eleršek, V. Kramarič Zidar, V. Mislej, B. Mišmaš, M. Stražar, M. Levstek, B. Cimrmančič, S. Lukšič, N. Uranjek, T. Kozlovič-Bobič, T. Kosjek, D. Kocman, D. Heath, E. Heath, "Residues of drugs of abuse: treatment efficiency, environmental occurrence and risk assessment" in *EMEC 22, the 22nd European Meeting on Environmental Chemistry*, Ljubljana, Slovenia, 2022.

T. Verovšek, I. Krizman-Matasic, M. Laimou-Geraniou, A. Šuštarič, H. Prosen, T. Eleršek, D. Heath, E. Heath, "Residues of drugs of abuse: removal, occurrence and environmental risk assessment" in *IASWS 2022, the 15th International Symposium on the Interactions Between Sediments and Water*, Piran, Slovenia, 2022;

T. Verovšek, I. Krizman-Matasic, M. Laimou-Geraniou, A. Šuštarič, H. Prosen, D. Heath, E. Heath, "Removal of residues of drugs of abuse during wastewater treatment and their occurrence in the aquatic environment" in *Chem2Change, Environmental Chemistry towards Global Change, the 2nd Online ACE Seminar on Chemistry and the Environment Led by Early-Career Scientists*, on-line, 2022.

M. Laimou-Geraniou, T. Verovšek, D. Heath, E. Heath, "Development and application of the method for determination of selected psychoactive pharmaceuticals in wastewater" in *the 13th Jožef Stefan International Postgraduate School Students' Conference and 15th Young Researchers' Day of Chemistry,* Ljubljana, Slovenia, 2021.

Other Publications

Journal Articles

T. Verovšek, A. Šuštarič, M. Laimou-Geraniou, I. Krizman-Matasic, H. Prosen, T. Eleršek, V. Kramarič Zidar, V. Mislej, N. Uranjek, T. Kozlovič-Bobič, T. Kosjek, D. Kocman, D. Heath, E. Heath, "Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment", *Science of The Total Environment, vol.* 866, pp. 161257, 2023. DOI: https://doi.org/10.1016/j.scitotenv.2022.161257.

National Reports

T. Verovšek, M. Laimou-Geraniou, U. Blaznik, A. Hočevar-Grom, D. Heath, E. Heath, "Wastewater-based epidemiology: drug consumption in seven Slovenian municipalities" in M. Jadl, A. Hočevar-Grom, A. Belščak Čolaković, A. Drev, N. Havaši, A. Tratar: Report on the drug situation 2024 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2024, pp. 43-87. ISSN 1855-8003. https://nijz.si/wp-content/uploads/2024/12/NP_2024_obl_eng_3_4_25.pdf.

T. Verovšek, U. Blaznik, A. Hočevar-Grom, D. Heath, M. Laimou-Geraniou, E. Heath, "Wastewater- based epidemiology: drug consumption in seven Slovenian municipalities" in M. Jadl, A. Hočevar-Grom, A. Drev, A. Belščak Čolaković, I. Kvaternik, N. Havaši: Report on the drug situation 2023 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2023, pp. 65-70. ISSN 1855-8003. https://nijz.si/wp-content/uploads/2023/12/NP_2023_obl_final.pdf.

T. Verovšek, U. Blaznik, A. Hočevar-Grom, D. Heath, M. Laimou-Geraniou, E. Heath, "Wastewater- based epidemiology: drug consumption in six Slovenian municipalities" in M. Jadl, A. Hočevar-Grom, A. Drev, A. Belščak Čolaković, I. Kvaternik: Report on the drug situation 2022 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2022, pp. 69-75. ISSN 1855-8003. https://nijz.si/wp-content/uploads/2022/12/NP_ang_2022_pop_obl.pdf.

T. Verovšek, U. Blaznik, A. Hočevar-Grom, D. Heath, M. Laimou-Geraniou, E. Heath, "Wastewater-based assessment of drug use in Slovenia" in M. Jadl, A. Hočevar-Grom, A. Drev, A. Belščak Čolaković: Report on the drug situation 2021 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2021, pp.65-71. ISSN 1855-8003. https://www.nijz.si/sites/www.nijz.si/files/publikacije-datoteke/nacionalno_porocilo_2021_ang.pdf.

Public Dissemination

Interview: Epidemija odvisnosti (Eng., Addiction epidemic): RTV Slovenija 1, Tednik (Eng., Weekly), 24 May 2021.

Biography

Education and Research Activities

- **2020-Present:** Jožef Stefan International Postgraduate School, Ljubljana: Doctoral study program Ecotechnologies;

- **2016-2020:** M.Sc. in "Chemical Analysis- Quality Control", Chemistry Department, National and Kapodistrian University of Athens, in collaboration with the Aristotle University of Thessaloniki, Greece. Thesis title: "Determination of pesticide residues in soil samples using QuEChERS technique in combination with liquid chromatography tandem mass spectrometry";

- **2010-2016:** Bachelor's Degree in Chemistry, Chemistry Department, Aristotle University of Thessaloniki, Greece. Thesis title: "Removal of Trimethoprim as organic micropollutant in aqueous matrices using modified chitosan super-absorbent beads".

Foreign Work and Study Experiences

- **2025** (1 month): Erasmus+ Internship at Democritus University of Thrace, Department of Agricultural Biotechnology and Oenology, Drama, Greece;

- **2024** (1 week): Erasmus+ Internship at the European Union Drugs Agency (EUDA), Lisbon, Portugal;

- **2023** (1 month): Internship as a visiting research student at Queensland Alliance for Environmental Health Sciences, University of Queensland, Brisbane, Australia;

- **2022** (2 weeks): Toxicological Centre, Department of Pharmaceutical Sciences, at the University of Antwerp, Belgium, Project N1-0143;

- **2021** (1 month): Toxicological Centre, Department of Pharmaceutical Sciences, at the University of Antwerp, Belgium, Project N1-0143;

- **2019-2020**: Quality Control Analyst, Quality Control Laboratory at KORRES Natural Products S.A., Athens, Greece;

- **2019**: Research and Development Analyst (R&D Analyst), Development and Validation Laboratory, DEMO S.A. Pharmaceutical Industry, Kryoneri, Greece;

- **2018** (6 months): Erasmus+ Traineeship at Jozef Stefan Institute, Department of Environmental Organic Analysis Department, Ljubljana, Slovenia;

- **2014-2015** (6 months): Erasmus+ Traineeship at Jozef Stefan Institute, Department of Environmental Organic Analysis Department, Ljubljana, Slovenia;

- **2014** (2 months): Practical training at the Thessaloniki Water Supply and Sewerage Company (EYATH S.A), Sewerage and Environment Quality Control Laboratory, Thessaloniki, Greece.

Grants

- **2024:** Meeting Registration Grant for SETAC Europe Students: SETAC Europe 34th Annual Meeting, 2024, Seville, Spain.

Research Projects

- Project **N1-0143**: Novel approaches for the estimation of the use of psychoactive pharmaceuticals and illicit drugs by wastewater analysis.

- Project **L1-9191**: Illicit drugs, alcohol and tobacco: wastewater-based epidemiology, treatment efficiency and vulnerability assessment of water catchments.