LICIT AND ILLICIT DRUGS IN WASTE- AND ENVIRONMENTAL WATERS: EPIDEMIOLOGICAL AND ENVIRONMENTAL APPLICATIONS

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Doctoral Dissertation
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Doctoral Dissertation

DOLOČANJE DOVOLJENIH IN NEDOVOLJENIH DROG V ODPADNIH IN OKOLJSKIH VODAH: EPIDEMIOLOŠKI IN OKOLJSKI VIDIK

Doktorska disertacija

Supervisor: Prof. Dr. Ester Heath

Ljubljana, Slovenia, September 2023

To my children, Tevž and Lenart

"If you're always trying to be normal, you will never know, how amazing you can be."
(Maya Angelou)

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Abstract

The analysis of drug residues in waste and environmental waters offers valuable insights into the epidemiological and environmental implications of drug use. This study employs a wastewater-based epidemiology (WBE) approach to estimate the use of licit and illicit drugs and new psychoactive substances (NPS) among both general and specific populations. In the latter case, the main focus is on evaluating drug use within educational institutions. Notably, this study comprehensively assessed the presence of illicit drugs in primary schools for the first time. Previously, except for cannabis, such data have not been obtained even through conventional surveys. Additionally, the study evaluates the use of WBE as part of an early warning system capable of detecting the presence of a wide range of psychoactive substances in educational institutions without exposing individuals, as is the case with drug testing. Lastly, this research aims to address a significant knowledge gap regarding the impact of drug use on aquatic ecosystems.

Herein are described the target analytical methods (LC-MS/MS) developed to determine both licit and illicit drug residues in raw wastewater. To study new psychoactive substances (NPS) in educational institutions, suspect screening (LC-IMS-HRMS) was applied. Enantiomeric profiling (chiral derivatization, GC-MS/MS) and determining isotopic composition (GC-C-IRMS) of carbon in biomarkers were explored as complementary approaches to the obtained WBE data. Targeted analytical methods (LC-MS/MS) were also developed to determine drug residues in treated wastewater, surface water, and groundwater. The aim was to assess the removal efficiency of drug residues using conventional biological wastewater treatment processes and determine their occurrence in effluent-receiving rivers and groundwater. In addition, the distribution of drug residues in groundwater was predicted using solute transport modelling.

The toxicity of drug residues towards green algae *Chlamydomonas reinhardtii*, which serves as a representative primary producer in the aquatic food web, was also explored. This was accomplished through *in vivo* testing, specifically the algal growth inhibition test. Furthermore, the environmental risks posed by drug residues in effluent-receiving rivers were assessed using *in silico* methods (ECOSAR).

The research findings on the use of licit, illicit drugs and NPS in the general population reveal distinct patterns. The use of stimulants shows similarities to Western and Southern European countries, with cocaine being the predominant drug detected, as observed through international SCORE monitoring. Among the NPS identified, the use of eutylone, 3-MMC, and mitragynine is confirmed based on international NPS monitoring, with 3-MMC having one of the highest detected mass loads within the study. In educational institutions, drugs are also detected, with nicotine, alcohol, and cannabis being the most prevalent, along with the unequivocal identification of four NPS (stimulants: 3-MMC, ephedrine, 4-chloro-α-PPP, and ethcathinone). It is worth noting that, despite methodological differences, the results obtained through WBE only partially align with data from other sources, regardless of the target population.

In addition to WBE data, a dumping event is confirmed solely through enantiomeric profiling of MDMA, a drug with a straightforward synthesis and excretion profile. Although

the effectiveness of determining the isotopic composition of light elements (C^{13}/C^{12}) in drug residues from raw wastewater for reducing the uncertainty of WBE consumption estimates has not been established, it could be used as a complementary method to drug profiling, potentially providing an early warning system for detecting changes in drug supply. However, further studies are required to substantiate this claim.

Due to the incomplete removal of drug residues during conventional biological wastewater treatment, their presence has been detected in effluent-receiving rivers within the ng/L range. In the algal growth inhibition test conducted on *Chlamydomonas reinhardtii*, a species of green algae, no adverse effects were observed, even at concentrations higher than those measured (1 mg/L). However, *in silico* predictions indicate potential effects on aquatic organisms for nicotine, methadone, EDDP, morphine, and MDMA, which warrant further *in vivo* studies. Furthermore, drug residues have also been found in groundwater within the low ng/L range, which raises concerns considering that groundwater is a primary source of drinking water globally. Contrary to expectations, it was found that the River Sava, which recharges the Ljubljansko polje aquifer and contains drug residues (ng/L range), is not their primary source. Instead, the source has been identified as Ljubljana's leaky sewer infrastructure, emphasizing the need to address raw wastewater as an important source of pollutants when studying urban aquifers.

To summarize, while the thesis successfully addressed several gaps related to estimating drug use through WBE and understanding the environmental impact of drug residues, it has also raised numerous new research questions in epidemiology and environmental and human risk evaluation. For instance, the application of determining the isotopic composition of drug residues in raw wastewater opens up opportunities for further investigation of its applicability as support for forensic intelligence. Additionally, there is a need for ecotoxicological studies to assess the potential harm caused by drug residues (*e.g.,* to subterranean organisms) and studies to examine the occurrence and impact of these residues in drinking water.

Povzetek

Analiza ostankov zdravil v odpadnih in okoljskih vodah omogoča vpogled v stanje na področju uporabe drog (epidemiologija) ter vpogled v vplive, ki jih uporaba drog ima na okolje. V okviru študije smo z epidemiologijo na osnovi odpadnih vod (WBE) ocenili uporabo dovoljenih in prepovedanih drog ter novih psihoaktivnih snovi (NPS) v splošni in specifični populaciji, s poudarkom na oceni uporabe drog v izobraževalnih ustanovah. Kot prvič je bila celovito ocenjena prisotnost prepovedanih drog v osnovnih šolah, saj se tovrstni podatki, z izjemo uporabe konoplje, ne zbirajo niti s klasičnimi raziskavami (vprašalniki). Ocenili smo tudi aplikativnost WBE za sistem zgodnjega opozarjanja kot postopek, ki je sposoben zaznati prisotnost širokega nabora psihoaktivnih snovi v izobraževalnih ustanovah, ne da bi pri tem izpostavil posameznike (kot je to v primeru testiranja na droge v urinu). Poleg tega smo v raziskavi odpravili veliko vrzel v poznavanju posledic uporabe drog na vodne ekosisteme.

Za dosego ciljev smo razvili tarčne analizne metode (LC-MS/MS), s katerimi smo določili ostanke dovoljenih in prepovedanih drog v neprečiščeni odpadni vodi. Za preučevanje pojavnosti novih psihoaktivnih snovi (NPS) v odpadni vodi izobraževalnih ustanov smo uporabili netarčno analizo (*Eng., suspect screening*; LC-IMS-HRMS). Dodatno smo raziskali uporabo enantiomernega profiliranja (derivatizacija s kiralnim reagentom, GC-MS/MS) in določanje izotopske sestave (GC-C-IRMS) ogljika biomarkerjev za identifikacijo neuporabljene droge, ki je bila odvržena v kanalizacijski sistem kot dopolnitev podatkov o uporabi drog, ki so bili pridobljeni s klasično analizo neprečiščene odpadne vode (WBE). Tarčne analizne metode (LC-MS/MS) smo razvili tudi za določanje ostankov drog v prečiščeni odpadni in površinski vodi ter podtalnici s ciljem, da bi ocenili učinkovitost odstranjevanja ostankov drog med običajnimi biološkimi postopki čiščenja odpadne vode, pridobili podatke o pojavljanju ostankov drog v rekah, v katere se prečiščena odpadna voda iz čistilnih naprav izliva, in ocenili pojavnost ostankov drog v podzemni vodi. Poleg tega smo z modeliranjem prenosa snovi predvideli porazdelitev ostankov drog v podzemni vodi. Toksičnost ostankov drog smo raziskali za zelene alge *Chlamydomonas reinhardtii*, ki predstavljajo primarnega proizvajalca prehranjevalne verige v vodnem okolju. Izvedli smo tudi *in vivo* test zaviranja rasti alg, tveganja, ki jih predstavljajo ostanki drog v rekah, v katera se izliva prečiščena odpadna voda, pa smo ocenili *in silico* (ECOSAR).

Rezultati raziskav o uporabi dovoljenih, prepovedanih drog in NPS v splošni populaciji so pokazali specifične vzorce uporabe drog v Sloveniji. Na primer, v mednarodnem SCORE monitoringu je bilo ugotovljeno, da se v Sloveniji, podobno kot v državah zahodne in južne Evrope, med stimulansi največ uporablja kokain, na podlagi NPS monitoringa pa je bila detektirana uporaba eutilona, 3-MMC in mitraginina, pri čemer so bile vrednosti masnih obremenitev 3-MMC med najvišjimi v študiji. V izobraževalnih ustanovah smo največkrat zaznali nikotin, alkohol in konopljo, od NPS pa smo identificirali štiri stimulanse: 3-MMC, efedrin, 4-kloro- α -PPP in etkatinon. Zaradi metodoloških razlik so se rezultati, pridobljeni z WBE, ne glede na tarčno populacijo, vedno le delno ujemali s podatki iz drugih virov.

Poleg pridobljenih WBE podatkov o uporabi drog smo zaznali tudi odvrženo neuporabljeno drogo v kanalizacijskem sistemu. In sicer smo z enantiomernim profiliranjem potrdili prisotnost neuporabljene MDMA kot droge z enostavnim enantiomernim profilom sinteze in izločanja. Čeprav z določanjem izotopske sestave lahkih elementov (ogljika) biomarkerjev, prisotnih v neprečiščeni odpadni vodi, nismo mogli zmanjšati negotovosti uporabe drog v populaciji, bi določanje izotopske sestave ostankov drog lahko uporabili kot sistem zgodnjega opozarjanja na spremembe v ponudbi drog na trgu in tako dopolnili podatke, ki se običajno pridobivajo s profiliranjem (zaseženih) drog. Vendar pa so za utemeljitev te trditve potrebne nadaljnje študije.

Zaradi nepopolne odstranitve ostankov drog med konvencionalnim biološkim čiščenjem odpadne vode smo ostanke drog (ng/L) določili tudi v rekah, v katere se iztoki iz čistilnih naprav izlivajo. Pri testu zaviranja rasti alg, opravljenem na zelenih algah *Chlamydomonas reinhardtii*, nismo opazili učinkov na njihovo rast, čeprav so bile testirane koncentracije (1 mg/L) precej višje od izmerjenih rečnih koncentracij. Ker pa smo predvideli učinke na vodne organizme pri teh koncentracijah *in silico* za nikotin, metadon, EDDP, morfij in MDMA, bi bilo za te ostanke drog smiselno opraviti nadaljnje *in vivo* ekotoksikološke študije. Ostanke drog smo detektirali tudi v podzemni vodi (spodnje območje ng/L), kar vzbuja zaskrbljenost tudi v svetovnem merilu, saj je podzemna voda običajno glavni vir pitne vode. V nasprotju s pričakovanji smo v študiji ugotovili, da v preiskovanem vodonosniku (Ljubljansko polje) reka Sava, ki vodonosnik napaja in vsebuje ostanke drog (ng/L), ne doprinese pomembno k pojavljanju ostankov drog v podzemni vodi. Kot njihov primarni vir smo identificirali puščanje kanalizacijskega sistema Ljubljane, kar poudarja potrebo po obravnavanju neprečiščene odpadne vode kot pomembnega vira onesnaževal pri preučevanju urbanih vodonosnikov.

Medtem ko smo v okviru doktorske disertacije uspešno premostili številne vrzeli, ki so povezane z ocenjevanjem uporabe drog z uporabo WBE in razumevanjem njenega vpliva na okolje, smo odprli tudi številna nova raziskovalna področja, ki so povezana z uporabo drog, tako v okviru epidemiologije kot tudi ocenjevanja okoljskega tveganja in tveganja za zdravje ljudi. Na primer, določanje izotopske sestave ostankov drog v neprečiščeni odpadni vodi odpira možnosti za nadaljnje raziskave njene uporabnosti kot podpore obveščevalnim službam. Po drugi strani pa so se kot potrebne izkazale dodatne ekotoksikološke študije ostankov drog na okoljske organizme (npr. na podzemne organizme), pa tudi študije, ki preučujejo pojav ostankov drog v pitni vodi in njihov morebitni vpliv na človeka.

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Abbreviations

2-AI	...	2-aminoindane
2C-B	...	4-bromo-2,5-dimethoxyphenethylamine
2-FDCK	...	2-fluoro deschloroketamine
2-FEA	...	2-fluoromethamphetamine
2-Oxo-PCE	...	*N*-ethyldeschloroketamine
3-FPM	...	3-fluorophenmethazine
3-MMC	...	3-methylmethcathinone
4Cl-PVP	...	4-chloro-alphapyrrolidinovalerophenone
4-CMC	...	4-chloromethcatinone
4-FA	...	4-fluoroamphetamine
4-FMA	...	4-fluoromethamphetamine
4F-MPH	...	4-fluoromethylphenidate
4-HO-MiPT	...	4-hydroxy-*N*-methyl-*N*-isopropyltryptamine
4-MMC	...	4-methylmethcathinone
5-MAPB	...	1-(benzofuran-5-yl)-*N*-methylpropan-2-amine
6-AM	...	6-acetylmorphine
6-APB	...	6-(2-aminopropyl)benzofuran
alpha-PHP	...	alpha-pyrrolidinohexanophenone
Alpha-PHiP	...	4-methyl-alpha-pyrrolidinopentaphenone
Alpha-PVP	...	1-phenyl-2-(1-pyrrolidinyl)-1-pentanone
AMP	...	Amphetamine
ARIS	...	Slovenian Research and Innovation Agency
ARRS	...	Slovenian Research Agency
BE	...	Benzoylecgonine
BOD	...	Biological oxygen demand
BSTFA	...	*N,O*-bis-(trimethylsilyl)trifluoroacetamide
BZP	...	1-benzylpiperazine
CBH	...	Cellobiohydrolase
CCS	...	Collision cross-section value
CEC	...	Contaminants of emerging concern
CF	...	Correction factor
COC	...	Cocaine
COD	...	Codeine
COE	...	Cocaethylene
COT	...	Cotinine
DDA	...	Data-dependent acquisition
DDD	...	Defined daily dose
DF	...	Detection frequency
DIA	...	Data-independent acquisition
DMT	...	Dimethyltryptamine
EC	...	European Commission

ECOSAR	...	Ecological structure-activity relationships (software)
EDDP	...	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EF	...	Enantiomeric fraction
EMCDDA	...	European Monitoring Centre for Drugs and Drug Addiction
ENDS	...	Electronic nicotine delivery systems
ENNDS	...	Electronic non-nicotine delivery systems
ESI	...	Electrospray ionization
ESPAD	...	European School Survey Project on Alcohol and Other Drugs
EtS	...	Ethyl sulfate
EU	...	European Union
EWA	...	Early Warning Advisory on New Psychoactive Substances
EWS	...	Early Warning System on NPS
FCTC	...	WHO Framework Convention on Tobacco Control
GBL	...	Gamma butyrolactone
GC	...	Gas chromatography
GC-C-IRMS	...	Gas chromatography-combustion-isotope ratio mass spectrometry
GC-MS/MS	...	Gas chromatography coupled with tandem mass spectrometry
GHB	...	Gamma-hydroxybutyric acid
GWWL	...	Groundwater Watch List
HBM	...	Human biomonitoring
HCOT	...	*Trans*-3′-hydroxycotinine
HE	...	High energy mode
HILIC	...	Hydrophilic interaction liquid chromatography
HRMS	...	High-resolution mass spectrometry
HTP	...	Heated tobacco products
IMS	...	Ion mobility separation
IRMS	...	Isotope ratio mass spectrometry
IS	...	Internal standard
KET	...	Ketamine
LC	...	Liquid chromatography
LC-MS/MS	...	Liquid chromatography coupled with tandem mass spectrometry
LC-HRMS	...	Liquid chromatography coupled with high resolution mass spectrometry
LE	...	Low energy mode
LOD	...	Limit of detection
LOQ	...	Limit of quantification
LSD	...	Lysergic acid diethylamide
MAMP	...	Methamphetamine
MBBR	...	Moving bed biofilm reactor
MBR	...	Membrane bioreactor
MDA	...	3,4-methylenedioxyamphetamine
MDEA	...	3,4- methylenedioxymethamphetamine
MDMA	...	3,4-methylenedioxymethamphetamine
MDPV	...	3,4-methylenedioxypyrovalerone
MOR	...	Morphine
MRM	...	Multiple reaction monitoring
MS	...	Mass spectrometry
MS/MS	...	Tandem mass spectrometry
MTHD	...	Methadone
NBOMe	...	*N*-methoxybenzyl-substituted phenethylamines

NIC	...	Nicotine
NIJZ	...	National Institute of Public Health (*Slovene: Nacionalni inštitut za javno zdravje*)
NPS	...	New psychoactive substance
P:M	...	Parent compound-metabolite ratio
PARC	...	European Partnership for the Assessment of Risks from Chemicals (H2020 project)
PCP	...	Phencyclidine
PFAS	...	Poly- and per-fluoroalkyl substances
POCIS	...	Polar organic chemical integrative sampler
QqQ	...	Triple quadrupole mass analyzer
QTOF	...	Quadrupole time-of-flight mass analyzer
QTRAP	...	Hybrid triple quadrupole/linear ion trap mass analyzer
R-MTPCl	...	(−)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride
RP	...	Reverse phase
RSD	...	Relative standard deviation
SBR	...	Sequential batch reactor
SCORE	...	The Sewage analysis CORe group Europe network
S-HFBPrCl	...	S-(−)-*N*-(heptafluorobutyryl)propyl chloride
SPE	...	Solid-phase extraction
S-TPC	...	S-(−)-*N*-(trifluoroacetyl)propyl chloride
TBZD	...	Triazolobenzodiazepine
TFA	...	Trifluoroacetic anhydride
THC	...	Tetrahydrocannabinol
THC-COOH	...	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
TMCS	...	Trimethylchlorosilane
UN61	...	Single Convention on Narcotic Drugs 1961
UN71	...	Convention on Psychoactive Substances 1971
UN88	...	1988 United Nations Convention against illicit traffic in narcotic drugs and psychoactive substances
UNODC	...	United Nations Office on Drugs and Crime
US	...	United States
VPDB	...	Vienna-pee dee belemnite
VSMOW	...	Vienna-standard mean ocean water
WBE	...	Wastewater-based epidemiology
WHO	...	World Health Organization
WL	...	Watch List
WWTP	...	Wastewater treatment plant

Symbols

φ	...	Daily wastewater flow
$\delta^{i}E$	...	Isotopic ratio of an element in the sample
CF	...	Correction factor
E_1	...	R-enantiomer
E_2	...	S-enantiomer
K_H	...	Henry's law constant
K_{OW}	...	Octanol-water partition coefficient
M_R	...	Accurate mass
N	...	Number of people in the catchment area
pK_a	...	The negative logarithm of the acid dissociation constant
R_{Ref}	...	The ratio between the heavier and the lighter isotope in reference material
R_S	...	The ratio between the heavier and the lighter isotopes in the sample
S_w	...	Water solubility
t_R	...	Retention time

Glossary

Alcohol – ethanol (if not specified otherwise).

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

Chiral molecules – molecules that contain an asymmetric (chiral) carbon atom, which results in two enantiomeric forms: R(−) and S(+).

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, behavior and (eco)toxicological effects are not well understood".

Dark web – also known as the darknet, the Dark web is an internet network that can be accessed only with specific software, configuration, or authorization.

Decriminalization – the process in which a criminal offense is reclassified as non-criminal through legislation. The behavior remains an offense but is addressed by other means than criminal law. Decriminalization is within the provisions of international drug control conventions.

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).

Depenalization – when behavior remains a criminal offense but with reduced criminal sanctions. A change in the legal framework is not needed.

Diastereomers – are molecules of the same chemical composition that differ in 3D configuration at one or more stereocenters, but are not mirroring images of one another.

(Current) drinkers – are defined by the WHO as "those who have consumed a drink containing alcohol in the last 12 months".

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.

FAIR data – are the data that meet four foundational principles, namely findability, accessibility, interoperability, and reusability.

Heavy drinkers – drinkers, that consume more alcohol than the established limit of reduced-risk drinking, *i.e.,* more than four standard drinks per day (14 standard drinks per week) for men and three drinks per day (seven drinks per week) for women.

Isotopes – atoms of the same element with the same number of protons and electrons (same atomic number) but differing numbers of neutrons, *i.e.,* atomic mass. Isotopes can be stable (non-radioactive) or unstable (radioactive).

Legalization – the commercialization of controlled substances for non-medical and non-scientific purposes, including production, export/import, and distribution, entailing no penalty. Legalization is not within the provisions of international drug control conventions.

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 ionization of the target analyte in mass spectrometry.

Recorded alcohol – alcohol beverages, recorded in official statistics on sales and taxation.

Second-hand smoke – smoke emitted from lighted tobacco products, namely cigarettes, bidis, and water pipes, and exhaled smoke of the smoker.

(Standard) daily dose – defined by WHO as "the assumed average maintenance dose per day for a drug used for its main indication in adults".

Substitutional drugs – pharmaceutical opioids used to treat people with opioid use disorder.

Unrecorded alcohol – alcohol, such as homemade alcohol (legal and illegal), smuggled alcohol, alcohol intended for medical and industrial use, alcohol obtained from cross-border marketing and surrogate alcohol (e.g., mouthwash and medical tinctures), which is produced outside of government control and is not accounted for in official statistics on sales and taxation.

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.

Chapter 1

Introduction

1.1 Psychoactive Substances

Psychoactive substances are chemicals that, after administration, act upon the central nervous system and change a person's perception, consciousness, cognition, mood, and emotions [1]. They can be classified based on their pharmaceutical effect, origin, and legal status (**Table 1**). The United Nations Office on Drugs and Crime (UNODC) [2] classifies psychoactive substances as hallucinogens, stimulants and depressants based on their pharmaceutical effect. Hallucinogens, sometimes called "psychedelics", induce synesthesia and alter a person's perception of reality. In contrast, dissociative anesthetics, another type of hallucinogen, create feelings of detachment and separation from self and environment [3], while stimulants increase alertness, heighten arousal and cause behavioral excitement. Some stimulants, *e.g.,* amphetamines or amphetamine-type stimulants, have established therapeutic uses [4]. In contrast, depressants, namely opioids, sedatives, tranquilizers and hypnotics, suppress brain activity. The term "opioids" refers to several groups of substances, including natural opium poppy alkaloids known as opiates, their semi-synthetic derivatives, and synthetic opioids, primarily synthesized by the pharmaceutical industry to enhance the effectiveness of painkillers [5].

Based on their origin, psychoactive substances are divided into natural, semi-synthetic and synthetic. Natural or plant-based substances are directly extracted from plants or fungi, while semi-synthetic substances are synthesized from these natural precursors using addition or substitution reactions and share a similar chemical structure. In contrast, synthetic substances are synthesized from chemical precursors using multistep chemical reactions [5].

Table 1: Classification of psychoactive drugs with most prominent examples [2].

Pharmaceutical effect	**Origin**	**International legal status**
Hallucinogens: - **Classic hallucinogens (psychedelics)** - **Dissociative anesthetics** - **Cannabinoids**	**Classic hallucinogens (psychedelics):** - natural: mescaline, psilocybin, naturally-occurring tryptamines - semi-synthetic: lysergic acid diethylamide (LSD) - synthetic: dimethyltryptamine (DMT) analogs, NBOMe series **Dissociative anesthetics:** - synthetic: phencyclidine (PCP), ketamine **Cannabinoids:** - natural: tetrahydrocannabinol (THC) - synthetic: synthetic cannabinoids (*e.g.,* HU-210, CP-47,497, JWH-018)	**Most of the common hallucinogens:** Regulated under UN71 **Some synthetic hallucinogens:** Unregulated – labeled as NPS (e.g., ketamine)
Stimulants	- natural: cocaine, "crack" cocaine, nicotine, caffeine - synthetic: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA)*, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDEA), and synthetic cathinones (*e.g.,* mephedrone)	**Legal stimulants:** Nicotine, caffeine **Regulated stimulants:** Regulated under UN61, UN71 (e.g., cocaine, amphetamines, MDMA) **Newly emerged stimulants:** Unregulated – labeled as NPS (e.g., synthetic cathinones)

Table 1: Continued.

Depressants: - **Opioids** - **Sedatives, tranquilizers and hypnotics**	**Opioids:** - natural: opiates, namely morphine and codeine - semi-synthetic: opiates, namely heroin - synthetic: fentanyl **Sedatives, tranquilizers and hypnotics:** - natural: alcohol (ethanol) - synthetic: benzodiazepines, barbiturates, gabapentinoids, methaqualone, gamma-hydroxybutyric acid (GHB)	**Legal depressants:** Alcohol (ethanol) **Regulated depressants:** Regulated under UN61 (most opioids), UN71 (most sedatives, tranquilizers and hypnotics) **Newly emerged depressants:** Unregulated – labeled as NPS (e.g., synthetic opioids)

*Since 2002, MDMA has been classified as a hallucinogen and a stimulant.

NBOMe – *N*-methoxybenzyl-substituted phenethylamines, NPS – new psychoactive substances, UN61 – the Single Convention on Narcotic Drugs 1961, UN71 – the Convention on Psychoactive Substances 1971, UN88 – 1988 United Nations Convention Against Illicit Traffic in Narcotic Drugs and Psychoactive Substances

The legal status of psychoactive substances is regulated under international and national laws, which classify substances as legal (licit or unregulated) and illicit (controlled). The prohibition of the recreational use of selected psychoactive substances (called illicit or controlled substances) began in the late 19th and early 20th century, considering factors such as health and social risks of use, colonial interests and culture [6]. Alcohol and tobacco, for example, which are socially accepted drugs in European countries, have never been considered subject to international laws, even though they are known to contribute more overall harm to society than the misuse of some controlled drugs, such as ecstasy [6].

Coherent international law controlling illicit psychoactive drug consumption, possession and trade was established after World War II. More than 300 psychoactive substances were classified under the United Nations Convention on Narcotic Drugs in 1961 (UN61) [7] and the Convention on Psychoactive Substances in 1971 (UN71) [8] based on their therapeutic value, risk of abuse, and health risks (**Table 2**). In addition, the United Nations Convention Against Illicit Traffic in Narcotic Drugs and Psychoactive Substances (UN88) [9] was written in 1988 to control the trade of drug precursors and reagents. Parties to the conventions can also prohibit specific substances as part of their national legal frameworks. Accordingly, different concepts of legalization, namely depenalization, decriminalization and legalization, are used when discussing the status of, for example, cannabis (*Cannabis sativa* L.). Slovenia has signed the UN61, UN71, and UN88 conventions and classifies illicit psychoactive drugs into three levels (**Table 2**); cannabis is classified under level II [10].

Substances for which trade and consumption are not prohibited by law are marked as legal. These can be divided into prescription pharmaceuticals, recreational or culturally used substances (*e.g.,* nicotine and alcohol), and new psychoactive substances (NPS). Despite being licit, misuse, abuse, unauthorized synthesis, and trade of prescribed pharmaceuticals are considered illicit. In some cases, when misuse is of particular concern, *i.e.,* in the case of substitutional drugs (pharmaceutical opioids used to treat people with opioid use disorder), their use is internationally controlled (UN61) [7].

The numerous proven adverse effects of tobacco and alcohol use have resulted in legislation controlling their prevalence. Internationally, the Framework Convention on Tobacco Control (FCTC) [11] and The Tobacco Products Directive (2014/40/EU) [12] regulate the production, distribution, taxation, sale, packaging, advertisement, and use of tobacco products. In 2018, heated tobacco products (HTP) became subject to FCTC control, while other novel tobacco and nicotine products, namely electronic nicotine delivery systems (ENDS) and electronic non-nicotine delivery systems (ENNDS), remain unregulated [13], [14]. In the case of alcohol, guidelines for reducing its harmful use were published by the World Health Organization (WHO) in 2010 [15]. In Slovenia, the first tobacco restrictions were introduced in 1996. It was later adopted to implement the European directive 2014/40/EU [16], while restrictions on alcohol sales and advertisement were made in 2003 [17]. Among others, tobacco, tobacco-related products and alcohol were prohibited from being sold to persons under 18 years of age. In addition, their use and sale were banned within educational institutions [17], [18].

Table 2: International and national substance scheduling (adapted by [7], [8], [10]).

Single Convention on Narcotic Drugs (1961)				
Classification	Schedule I	Schedule II	Schedule III	Schedule IV
Type of substances *(examples)*	Highly addictive substances with a high risk of abuse *(cannabis, cocaine, heroin, methadone, morphine, coca leaf, opium)*	Substances used for medical purposes, with a lower risk of abuse than substances in Schedule I *(codeine)*	Preparations containing a low number of substances in Schedule II and cocaine *(<2.5 % codeine, <0.1 % cocaine)*	The most dangerous substances, already listed in Schedule I, with limited therapeutic value *(heroin)*
Degree of control	Very strict: taking all measures of control under the 1961 convention	Less strict	Lenient	Very strict: a complete ban on the production, export/import, trade, possession, and use. Exceptions: medical and scientific purposes
Convention on Psychoactive Substances (1971)				
Classification	Schedule I	Schedule II	Schedule III	Schedule IV
Type of substances *(examples)*	Substances with a high risk of abuse and little/no therapeutic value, which pose a serious threat to public health *(LSD, MDMA, cathinone, THC)*	Substances with a risk of abuse and low/moderate therapeutic value which pose a serious threat to public health *(amphetamines)*	Substances with a risk of abuse and moderate/high therapeutic value that pose a serious threat to public health *(barbiturates, buprenorphine)*	Substances with a risk of abuse and high therapeutic value, which pose a minor threat to public health *(tranquilizers, analgesics, and narcotics)*
Degree of control	Very strict: use limited to medical and scientific purposes	Less strict	Available for medical purposes	Available for medical purposes

Table 2: Continued.

Decree on the classification of illicit drugs in Slovenia, no. 69/19, 157/20,162/21			
Classification	Group I	Group II	Group III
Type of substances *(examples)*	Substances (and plants) with no therapeutic value, which pose a serious threat to public health if abused *(heroin, MDMA, coca leaves)*	Substances (and plants) with a known therapeutic value that pose a serious threat to public health if abused *(Cannabis sativa L. – herb, extracts and resins, cocaine, codeine, methadone)*	Substances (and plants) with known therapeutic value, which pose a medium threat to public health if abused *(GHB)*
Degree of control	Very strict: production, trade, and use limited to educational and scientific purposes	They may be produced, traded, possessed, and used for medical, veterinary, educational, or scientific purposes.	

GHB – gamma-hydroxybutyric acid, LSD – lysergic acid diethylamide, MDMA – 3,4-methylenedioxymethamphetamine, THC – tetrahydrocannabinol

1.1.1 New psychoactive substances

New psychoactive substances are substances that have recently appeared on the drug market and are not controlled under UN61 or UN71 [19], [20]. According to UNODC [20], they are classified into 15 main groups based on their pharmacological effect, origin and chemical structure (**Table 3**).

Table 3: UNODC NPS classification (June 2023) [20].

NPS class	Pharmaceutical effect	Substituted illicit drug
Aminoindanes	Stimulant	2-aminoindane (2-AI)
(New) benzodiazepines	Sedative/hypnotics	Benzodiazepines
Fentanyl analogs	Depressant	Fentanyl
Lysergamides	Hallucinogen	Lysergic acid diethylamide (LSD)
Nitazenes	Depressant	n.a.
Phencyclidine-type substances	Stimulant, dissociative	Phencyclidine (PCP), ketamine
Phenethylamines	Stimulant, hallucinogen	Amphetamine, methamphetamine, and MDMA
Phenidates	Stimulant	Methylphenidate
Phenmetrazines	Stimulant	n.a.
Piperazines	Antidepressant, opioid (rarely)	MDMA, 1-benzylpiperazine (BZP)
Plant-based substances	n.a.	n.a.
Synthetic cannabinoids	Stimulants, hallucinogens, dissociative	Cannabinoids (THC)
Synthetic cathinones	Stimulant	Natural cathinone (khat plant)
Tryptamines	Hallucinogen	Dimethyltryptamine (DMT)
Other substances	Hallucinogen, stimulant, opioid, sedative/hypnotics	n.a.

n.a. – not applicable;
MDMA – 3,4-methylenedioxymethamphetamine, THC – tetrahydrocannabinol

New psychoactive substances are intended to replicate the effects of controlled substances, as outlined in **Table 3**. Although they may also be present as adulterants in controlled substances, they are primarily accessible as legal alternatives [21], [22]. With time, individual NPS and their classes may come under control primarily within national laws established in more than 60 countries, including Slovenia [20], [23]. However, the regulation of NPS at the international level is slow, considering the rapidly growing number of newly produced and identified NPS. For example, in the 15 years up to December 2021, 1,127 NPS were newly identified, while only 300 psychoactive substances have been scheduled under international drug conventions since 1961 [19].

The ease of NPS synthesis through minor modification (substitution/addition of functional groups; **Figure 1**) also makes NPS regulation problematic, and their market is large and dynamic. For example, in 2021 alone, 52 new NPS were reported in Europe and 50 in the United States [20], [24]. Moreover, the lack of safety data on toxicity, carcinogenic

potential, long-term effects, purity, and compound identity presents a challenge for public health and puts users at risk of intoxication [20], [21], [24].

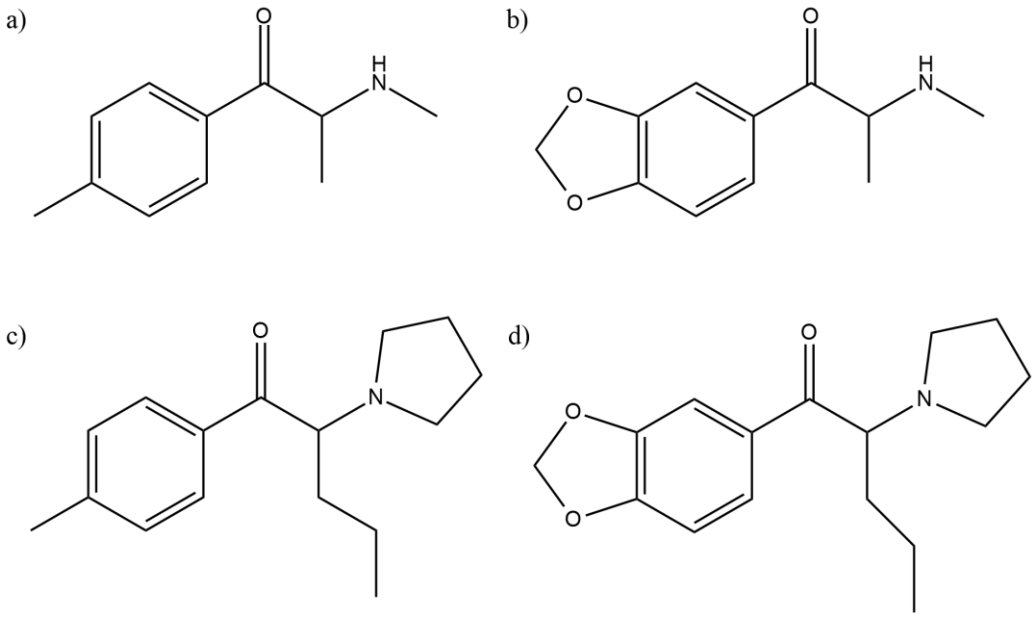


Figure 1: Examples of synthetic cathinones: (a) mephedrone, (b) methylone, (c) pyrovalerone and (d) 3,4-methylenedioxypyrovalerone (MDPV).

1.2 Estimating Drug Use Prevalence

Drug abuse imposes a significant burden on society, the economy, and public health. For instance, in 2019, licit drugs such as tobacco and alcohol, as well as illicit drugs, accounted for 5 % of all substance use-related deaths and 9 % of all substance use-related healthy years of life lost [22]. Therefore, to establish effective prevention programs, accurately monitoring the prevalence of drug use is crucial [25], [26]. The prevalence of legal drug use is usually estimated using sales data and self-reported population surveys. In contrast, the use of illicit drugs is usually evaluated using self-reported population surveys, medical and toxicological reports, drug-related crime statistics, police seizures, and data from treatment programs [27], [28].

Estimating the use of NPS is equally essential, but it faces many unique challenges due to, for example, the short-lived nature of these drugs on the market. Also, estimating NPS use by reporting quantities of consumed or seized NPS remains inaccurate, as little is known about their typical dose. Accordingly, NPS trends are mainly monitored by counting their number on the drug market and acquiring their identity. At the European Union (EU) level, the European Union Early Warning System (EWS) was established in 1997 to collect, analyze, assess, and communicate data on NPS [29], while the Early Warning Advisory (EWA), established by the UNODC in 2013 [20], monitors, analyzes, and reports trends in the use of NPS at the global level.

1.2.1 Licit drugs: production, trafficking and consumption trends

1.2.1.1 Pharmaceutical opioids

When expressed in standard daily doses, the most widely used pharmaceutical opioid globally in 2020 was codeine (and its preparations), followed by hydrocodone, fentanyl,

methadone, buprenorphine, oxycodone and morphine. However, codeine use declined by 30 % between 2016-2020, while the use of substitutional drugs (opioids, used in the treatment of opioid use disorder) has an increase over a more extensive period (1999–2019), *i.e.,* the amount of methadone and buprenorphine, the most frequently prescribed substitutional drugs, increased six-fold [22], [30]. In general, the availability of substitutional drugs depends on a country's income, the prevalence of their use as analgesics, the number of people with opioid use disorder and policies related to opioid agonist treatment. For example, opioids are extensively available in North America, Western and Central Europe, and the most developed parts of Oceania, while their availability is limited in Africa and Asia [30]. The COVID-19 pandemic has significantly impacted the availability of substitutional treatment. In countries with limited resources, there has been a reduced availability of substitutional drugs, while in others, there has been a decrease in supervised services; for instance, users in the United States were provided with up to 28 days of take-home medicines [31].

Substitution treatment in Slovenia is fully financed by national health insurance and is available under supervision at centers for the prevention and treatment of illicit drug addiction, as well as in prison clinics. In 2020, users included in the substitution treatment (approximately 3,100) were mainly prescribed methadone (59 %), buprenorphine (31 %), and morphine (10 %). Most users included in the substitution treatment program (62 %) were over 40 years of age [32].

1.2.1.2 Tobacco and related products

Tobacco is grown on approximately 4 million ha of land across 125 low-and-middle-income countries, mainly in southern Africa, the Middle East, south and east Asia, Latin America, and the Caribbean [33], [34]. Within those countries, cigarettes are produced in approximately 500 factories [34]. In 2020, tobacco was used by 1.3 billion people (22 % of the global population), among which 10 % were children (age 13–15), 14 % were adolescents (age 15–24), and 22 % were young adults (age 25–34). As a result of international and national tobacco controls, the trend in tobacco use is declining, with 20 % of the global population predicted to use tobacco in 2025. Regardless, the so-called tobacco epidemic is considered the biggest health threat and is responsible for more than 8 million deaths annually, among which 1.2 million are related to exposure to second-hand smoking [13].

Since 2014, a new health threat has arisen from the increasing popularity of novel tobacco and nicotine products, whose use, in contrast to traditional tobacco use, is expected to continue to grow, mainly at the expense of new users. These products were launched on the tobacco market by companies independent of the tobacco industry. Still, their increasing popularity attracted the tobacco industry's attention, which purchased shares in those companies and started to develop new brands [13]. Currently, novel tobacco and nicotine products are available in more than 40 countries, with Western Europe and North America being the largest markets. Nevertheless, they still represent only a small share of the total tobacco market, *e.g.,* 2 % of the global market are ENDS, while cigarettes represent 91 % [13].

According to the latest data, every fifth adult (18-74 years of age) and almost every 10th youth (15 years of age) smokes tobacco in Slovenia, while the primary users of novel tobacco and nicotine products have been young people (<25 years of age). For example, e-cigarettes were used by 13 % of 13-, 28 % of 15-, 31 % of 17-year-olds, and only 4 % of 25–74-year-olds in 2020. Following global trends, tobacco use declined in Slovenia between 2000–2020, while the use of novel tobacco and nicotine products has been increasing since they became available in 2017. Regardless, tobacco smoking still prevails; 20 % of tobacco

and related product users report tobacco smoking, 1.9 % report using smokeless tobacco products, and 2.2 % of electronic cigarettes [35].

1.2.1.3 Alcohol (ethanol)

Since ancient times, alcoholic beverages have been produced in tribes and villages in batches by fermentation or distilling various local vegetables and fruits. With the industrial revolution, alcohol (ethanol) production became industrialized. Nowadays, the alcohol market is centralized, with ten transnational alcohol corporations dominating the production and branding. On the retail level, alcohol sales are mainly organized separately from its producers (recorded alcohol), considering national legislation. However, traditional production of alcohol (unrecorded alcohol) still represents approximately one-quarter of all consumed alcohol [36].

According to the latest data on global consumption of alcohol (2016), 2.3 billion people use alcohol (are current drinkers). While, on average, 57 % of the world's adult population (aged 15 and above) reported not drinking alcohol (being abstinent) in the last 12 months, three WHO regions - Europe (60 % drinkers), the Americas (54 % drinkers), and the West Pacific Region (54 % drinkers) - reported alcohol consumption by more than half of their population [37]. Adults generally drink 6.4 L of pure alcohol per year, while the highest consumption of alcohol (10 L of pure alcohol/adult/year) was reported for the WHO European Region [37]. According to the European School Survey Project on Alcohol and Other Drugs (ESPAD), alcohol is the psychoactive substance that is most available to young people [38]. The ratio between abstinent and current drinkers among the young population (15–19 years of age) reflects drinking habits in the general population, *i.e.,* the highest number of young people reported drinking was in the WHO European Region (44 %), followed by Region of America (38 %) and Western Pacific Region (approx. 38 %) [37].

In 2020, only 21 % of Slovene adults (18-74 years of age) were alcohol abstinent, and 55 % were heavy drinkers [39]. Adolescents (11-, 13-, 15- and 17-year-olds) also reported drinking alcohol, with the percentage of those who had tried alcohol at least once in their lifetime increasing with age (11-year-olds: 15 %, 17-years old: 86 %) [40]. Compared to the WHO average global consumption of registered alcohol (6.4 L of pure alcohol/adult/year [37]), Slovenia, with 10.4 L of pure alcohol/adult/year, is way above the average. Additionally, it is estimated that 1.8 L/adult/year of unrecorded alcohol was consumed [41].

1.2.2 Illicit drugs: production and trafficking

Cannabis cultivation is a global phenomenon, as it is known to be cultivated both indoors and outdoors in at least 190 countries worldwide [42]. In contrast to cultivating plants to produce other illicit drugs (*e.g.,* cocaine and opiates), cannabis is mainly produced in the countries where it is consumed [31]. Due to the lack of systematic monitoring of cannabis cultivation, the global area of cannabis cultivation cannot be estimated. However, its trafficking can be evaluated through seizure data, which suggests cannabis trafficking has increased in the last decades and during the COVID-19 pandemic [42], [43].

In contrast to other illicit drugs, hallucinogens are mainly concentrated and trafficked in America, accounting for 88 % of the hallucinogens seized between 2015 and 2017, while European seizures accounted for 10 % during the same period. The trafficking of hallucinogens has increased over the past two decades, with LSD dominating [3].

The total area under coca bush cultivation is estimated at 234,200 ha and is mainly located in Columbia (61 %), followed by Peru and Bolivia. Cocaine is typically trafficked

by sea (89 % of cocaine in 2021) to North America and Europe via well-known routes, with Columbia and Brazil as the leading countries of departure. Globally, cocaine trafficking is increasing and expanding from its major markets to Africa, Asia and Indonesia [31]. Trafficking of other conventional stimulants, namely amphetamine, methamphetamine and ecstasy, is also increasing, with four-, five, and three-fold increases in their seizure in the past decade, respectively. Methamphetamine is the most sized (2016–2020: 72 % of seizures were related to methamphetamine), with its trafficking showing a worldwide spread, *i.e.,* 117 countries reported seizures in 2016–2020 compared with 84 countries in 2006–2010 [19]. In comparison to cocaine, where production and trafficking were not disrupted during the COVID-19 pandemic, production and trafficking of other stimulants (especially ecstasy) were affected by the unavailability of precursors and applied measures [22], [43].

Globally, Afghanistan, followed by Myanmar (South-East Asia) and Mexico, accounted for 95 % of cultivated opium poppy (total cultivation area: 246,800 ha) in 2021 and for 97 % of illicit opium produced between 2017–2021 [31], [42]. The trafficking of opiates continues to increase, with seized amounts doubling in the period 2000-2020 [42]. No effect of COVID-19 was observed on opium and heroin production in 2020 [43]. Similarly, trafficking with pharmaceutical opioids increased in the last decade, with tramadol being the most seized in quantity and fentanyl in the number of defined daily doses [42].

1.2.2.1 Drug production and trafficking in Slovenia

Slovenia is known to be an important logistic point for organized criminal groups across Europe and beyond. It lies on the main route for the illicit trafficking of heroin (produced in Turkey) and cannabis (produced in Western Balkan countries), known as the Balkan route. Recently, it has become an important European entry point for cocaine. Illicit drugs entering Slovenia are typically not intended for the Slovene market but are transported through Slovenia to other countries by heavy goods vehicles. The Slovenian illicit drug market is self-sufficient with locally grown cannabis, which is even exported to neighboring countries, namely Austria, Italy, Croatia, and Germany [44]. Stimulants, such as amphetamine, MDMA, and cocaine, intended for the Slovenian market are mainly smuggled to Slovenia from the Netherlands, while clandestine amphetamine laboratories have also been reported to exist in Slovenia [16].

1.2.3 Illicit drugs: Consumption trends

From 2000 to 2020, the number of drug users (15–74-year-olds) grew by 26 % to 284 million, partly due to an increasing global population. In 2020, cannabis was the drug of choice (209 million users), followed by opioids (61 million users, out of which 31 million are using opiates, mainly heroin), amphetamines (34 million users), cocaine (21 million users) and ecstasy (20 million users). The number of cannabis users has increased by 23 % over the past decade and remains the highest in North America [22], [30].

Opioids remain a significant health risk, *i.e.,* accounting for two-thirds of drug use-related deaths, primarily due to overdose. There are two epidemics of non-medical use of opioids, namely fentanyl (North America) and tramadol (North and West Africa, the Near and Middle East and South-West Asia). The use of cocaine continues to grow in North America and Europe and is spreading in Africa and Asia. Similarly, the prevalence of amphetamines has increased, with the highest use reported in North America and the highest number of users in East and South-East Asia [22], [30].

The latest data indicate that 284,600 (21 %) Slovenians (15–64 years of age) have used illicit drugs at least once. Cannabis is the most commonly used illicit drug (the use reported by 21 % of users), followed by ecstasy (2.9 %), cocaine (2.6 %), amphetamine (2.3 %) and

LSD (2.2 %). Between young adults (15–34 years of age), the lifetime prevalence of illicit drug use was 34 %. Again, cannabis was the most commonly used (21 % of 15- and 42 % of 17-year-olds), while a lower percentage of young people used other drugs. For example, 4.5 % of 17-year-olds reported using ecstasy, 4.2 % non-medical use of pharmaceuticals (stimulants, tranquilizers and analgesics), 4.1 % magic mushrooms, 3.8 % cocaine, 3.8 % amphetamine or methamphetamine, 3.6 % solvents, 2.8 % LSD and <1 % heroin [16], [40], [45].

1.2.4 NPS market

New psychoactive substances have no established, large or long-standing market; NPS are rapidly changing and quickly replaced, making it impossible to generate long-term demand for a specific NPS. Regarding the number of NPS available, the market expanded globally after 2009 and stabilized in 2018 at approximately 550 readily available NPS. The most diverse among them are stimulants, followed by synthetic cannabinoids and opioids. The latter has diversified in the past decade, from one known opioid NPS in 2009 to 87 in 2020 [19], [22].

In general, NPS use is assumed to be decreasing in North America and most parts of Europe, while it is likely to increase in Eastern Europe, Asia, and Africa [22]. Two important NPS markets have formed: synthetic and plant-based. The synthetic NPS market has expanded geographically, from 30 countries reporting their seizure between 2009 and 2010 to 57 between 2019 and 2020 (based mainly on reports from East and South-East Asia) [19], [22]. Among synthetic NPS, ketamine was the most commonly seized [19], [22]. In contrast, the trafficking of plant-based NPS expanded more slowly, with 28 countries reporting their seizure between 2009 and 2010 and 37 between 2019 and 2020. Among plant-based NPS, kratom was the most seized in 2020, followed by khat [19].

According to the latest data, 0.3 % of inhabitants of Slovenia (15–64-year-olds) reported using NPS [32], [40]. Although students of the University of Ljubljana reported their first NPS use at the age of 21 in 2021 [45], data from other studies suggest that the age of first NPS use is even lower. For example, 2 % of 17-year-olds reported using NPS in 2018, and 5 % of 15–16-year-olds reported their use in 2019 [16], [32], [40]. The most commonly recognized NPS by the students of the University of Ljubljana were benzodiazepines (83 %), followed by ketamine (83 %), gamma-hydroxybutyric acid/gamma butyrolactone GHB/GBL (52 %), and DMT (33 %). Identified NPS from street drug analysis for Slovenia in 2021 are shown in **Table 4** [45].

Table 4: Identified NPS in Slovenia in 2021 (acute emergency cases, anonymous testing of street drugs and drug seizures, adapted by [45]).

NPS class	NPS
(New) benzodiazepines	- Triazolobenzodiazepine (TBZD) - Clonazolam - Bromazolam - Etizolam - Flualprazolam - Flubromazepam - Flubromazolam - Meclonazepam
Fentanyl analogs	- Brorphine
Lysergamides	- 1cP-LSD - 1P-LSD
Nitazenes	- Etazene - Etonitazepyne - Metonitazene
Phencyclidine-type substances	- *N*-ethyldeschloroketamine (2-Oxo-PCE) - 2-fluoro deschloroketamine (2-FDCK)
Phenethylamines	- X-fluoroethamphetamine (X-FEA) - 2-fluoromethamphetamine (2-FMA) - 4-fluoromethamphetamine (4-FMA) - 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB) - 6-(2-aminopropyl)benzofuran (6-APB) - 4-bromo-2,5-dimethoxyphenethylamine (2C-B) - 4-fluoroamphetamine (4-FA)
Phenidates	- 4-fluoromethylphenidate (4F-MPH)
Phenmetrazines	- 3-fluorophenmetrazine (3-FPM)
Synthetic cannabinoids	- MDMB-4EN-PINACA - ADB-BUTINACA
Synthetic cathinones	- 3-methylmethcathinone (3-MMC) - 4-methylmethcathinone (4-MMC) - 4-methyl-alpha-pyrrolidinopentiophenone (alpha-PHiP) - 4-chloro-alpha-pyrrolidinovalerophenone (4Cl-PVP) - 4-chloromethcatinone (4-CMC) - alpha-pyrrolidinohexanophenone (alpha-PHP) - 1-phenyl-2-(1-pyrrolidinyl)-1-pentanone (alpha-PVP) - Eutylone - *N*-Ethylhexedrone
Tryptamines	- 4-hydroxy-*N*-methyl-*N*-isopropyltryptamine (4-HO-MiPT)

1.2.5 World Wide Web – an alternative market for licit and illicit drugs

The internet provides new opportunities for tobacco (and related products) and alcohol marketing. Websites (including video games) and applications provide platforms for cross-border advertisement through banners, pop-up advertisements, and videos. At the same time, social media present new ways of engaging consumers, *e.g.,* via shared media. Taking personalized advertisements and the power of social influence into account, marketing on the internet is highly effective and may (non-)intentionally target users whom it should not reach, *i.e.,* youth [36], [46]. In the case of tobacco or novel tobacco and nicotine products, internet marketing is especially prominent and raises additional concerns due to deceptive health claims [46].

With the high levels of anonymity, increased interconnectivity, and continuing evolution of online platforms, internet sales using social media, messaging, and dating applications means the internet has become an alternative way of illicit drug distribution. Transactions are done in person, by an online payment system, or by cryptocurrency. The latter is used especially on the dark web, which quadrupled between 2017–2020 compared to 2011–2017. In 2021, cannabis was the most sold drug on the dark web (49 % of the sales on investigated markets), followed by amphetamines (16 %), cocaine (12 %), benzodiazepines (6 %), opioids and pharmaceuticals (5 %). Between 2019 and 2020, shipments of the drugs bought on the dark web were mainly sent to Eastern Europe, with notable geographic expansion to marketplaces in Asia and South America. Despite the growth in illicit drug sales on 19 monitored dark web markets, darknet sales present only 0.2 % of total illicit drug sales in the United States (US) and the EU (2017–2020). Dark web sales remained stable during the COVID-19 pandemic, *i.e.,* no pronounced increase or decrease was observed. However, the smuggling trend shifted towards remote and contactless modes, such as mail and food delivery services [31].

1.3 Wastewater-Based Epidemiology – A Complementary Approach to Conventional Epidemiological Approaches

Estimating illicit drug consumption is essential from many aspects, *e.g.,* public health and crime control, and is typically done using socio-epidemiological methods (Chapter 1.2 Estimating Drug Use Prevalence. These methods pose ethical challenges and are susceptible to recall bias, sampling limitations, and time lag in reported data [19], [47], suggesting the need for a different approach not subject to such limitations. Accordingly, in 2001, Daughton *et al.* [48] proposed a complementary method for the estimation of illicit drug consumption using targeted chemical analysis of raw (untreated) wastewater for pre-selected drug metabolic residues (biomarkers). The proposed approach, known as wastewater-based epidemiology (WBE), is non-invasive, cost-effective, and can gather spatial and temporal consumption data in near real-time [49]. It was first implemented in 2005 to address the consumption of cocaine in Milan, Italy [50]. Since then, most WBE studies have adopted the same approach, which consists of the following steps (**Figure 2**): raw wastewater collection, targeted wastewater analysis for selected biomarkers, and back-calculation of drug consumption [51], [52]. In 2010, the approach was synchronized in the frame of the Sewage Analysis CORe group Europe (SCORE) COST Action [53], which developed a best practice protocol considering wastewater sampling, sample storage, and wastewater analysis [51] and established the SCORE network.

From estimating illicit drug consumption, the use of WBE has expanded to include consumption estimation of licit drugs (e.g., tobacco and alcohol), food and artificial sweeteners; use of pharmaceuticals and personal care products; determining human

exposure to hazardous substances (e.g., to pesticides, mycotoxins, and bisphenols); and population health assessment (e.g., stress, and COVID-19) [52]. Moreover, non-target wastewater analysis was introduced into the WBE protocol to identify known (suspect screening) and unknown substances in wastewater, which may reveal possible new biomarkers. The approach is mainly used when addressing NPS [52].

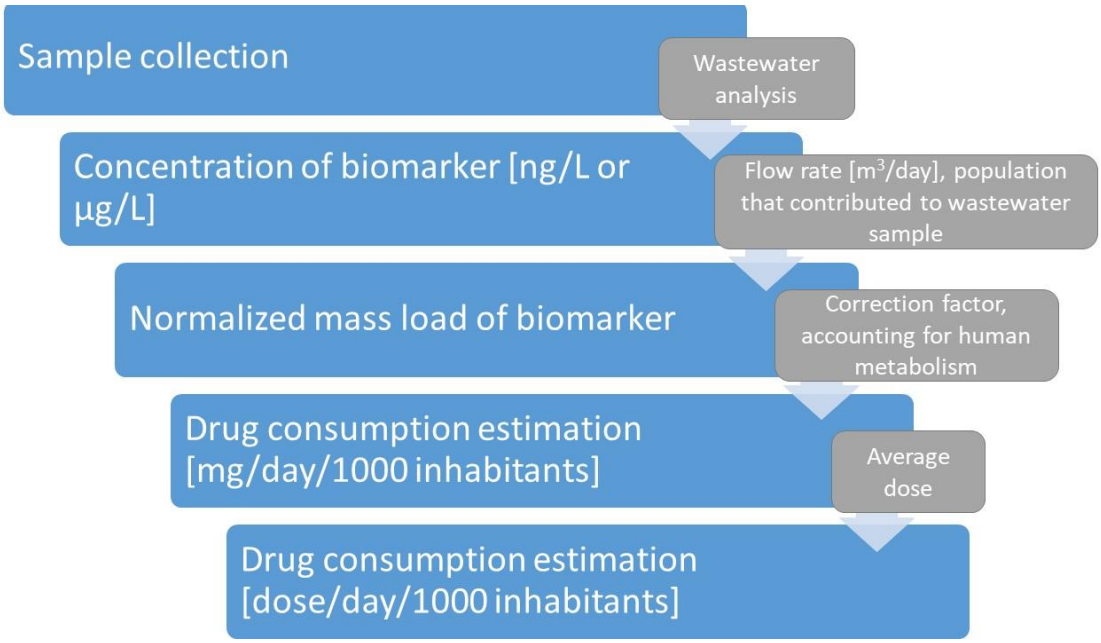


Figure 2: Schematic showing the main steps (blue squares) and required data (grey squares) in the WBE approach (adapted from [51], [52]).

1.3.1 Methodology

1.3.1.1 Wastewater sampling

To estimate drug consumption in the general population, *e.g.,* city and municipality level, wastewater influent is sampled at the wastewater treatment plant's (WWTP) inlet [54]. Following best practices, sampling is advised to be carried out during dry periods on a typical day (without WWTP maintenance). Rain dilutes wastewater and thus obstructs the detection of biomarkers. Moreover, some wastewater is commonly discharged untreated during overflow, leading to incorrect consumption estimates. Special events such as public holidays and festivals should also be avoided when targeting average drug consumption in the population since during such events, population size (migration) and drug consumption may vary from typical. Also, wastewater samples should be collected as 24 h composites using an autosampler that operates in flow-proportional mode, where subsamples are collected in proportion to wastewater flow at constant time intervals. Alternatively, the sampler should operate in volume-proportional mode, where the sampling frequency is adjusted according to wastewater flow while the sampling volume is kept constant. Due to the increase in sampling uncertainty, time-proportional sampling (sampling frequency and volume are constant) is less favorable. However, regardless of the sampling mode, the sampling frequency should be adapted to the catchment characteristics, *e.g.,* exfiltration and expected drug prevalence, but ideally, it should not exceed ten-minute intervals [54].

According to reviews by Huizer *et al.* [55] and Verovšek *et al.* [56], 24-h composite wastewater samples are most commonly collected at the WWTP to address drug

consumption in the general population. Autosamplers typically work in time-proportional mode; however, volume- or flow-proportional modes have also been applied. Few studies reported using grab or passive sampling (**Figure 3**) [55]–[58]. The latter is interesting, given the possibility of increasing substance detection by accumulating analytes on a polymeric-based sorbent, which is exposed to wastewater over an extended period (days or weeks) [57]. However, a need for in-field calibration (uptake is affected by environmental conditions), biofouling, limitation in the number of extracted compounds and low robustness currently limit the applicability of passive samplers [56]. To evaluate the use of NPS on a city scale as well as during specific night-time settings (music festivals), pooled urine from portable urinals set across the city was also used [57].

Figure 3: Setting passive sampler (POCIS) at municipal WWTP (Photo: Taja Verovšek).

Obtaining representative wastewater samples from specific sites or small sub-catchments, i.e., educational institutions and prisons, is challenging due to the physical boundaries of the sewer system (depth of the sewer), power availability (in case of composite sampling), inconsistent wastewater flow and low number of pulses (*e.g.,* toilet flushes) containing drug residues [59]. Although flow-proportional sampling with short sampling intervals (1 min) is proposed [54], time-proportional sampling with an adjusted sampling period (2–72 h) is most commonly used in specific catchments (**Figure 4**). The sampling frequency was optimized in specific instances using a dye tracer test or dynamic flow analysis [59].

Figure 4: Example of sampling wastewater in specific sub-catchment using autosampler (Photo: Taja Verovšek).

Wastewater samples that cannot be processed immediately should be appropriately stored to prevent compound degradation. According to best practice, one of the following storage conditions should be used in the preferred order: extraction on solid-phase extraction (SPE) cartridges within 12 h with the addition of internal standard (IS) or kept frozen (–18 °C) after the addition of the IS (–18 °C) [51], [53].

1.3.1.2 Selection of biomarkers

Biomarker selection can be performed using a top-down or bottom-up approach (**Figure 5**). In the top-down approach, biomarkers are selected from a set of human-related substances obtained from pharmacokinetic and human biomonitoring (HBM) studies for which metabolism, excretion, and wastewater stability are well known. In a bottom-up approach, with non-target analysis, human-related substances present in wastewater are identified, and after studying their stability, biomarkers are proposed for studying compounds. Although this approach provides extensive data, including occurrence data for yet-unknown substances, which may contain important information about drug consumption trends, it is less used than the top-down approach. The reason is the problematic identification of potential biomarkers due to their low concentration and the large amount of organic matter in wastewater [52].

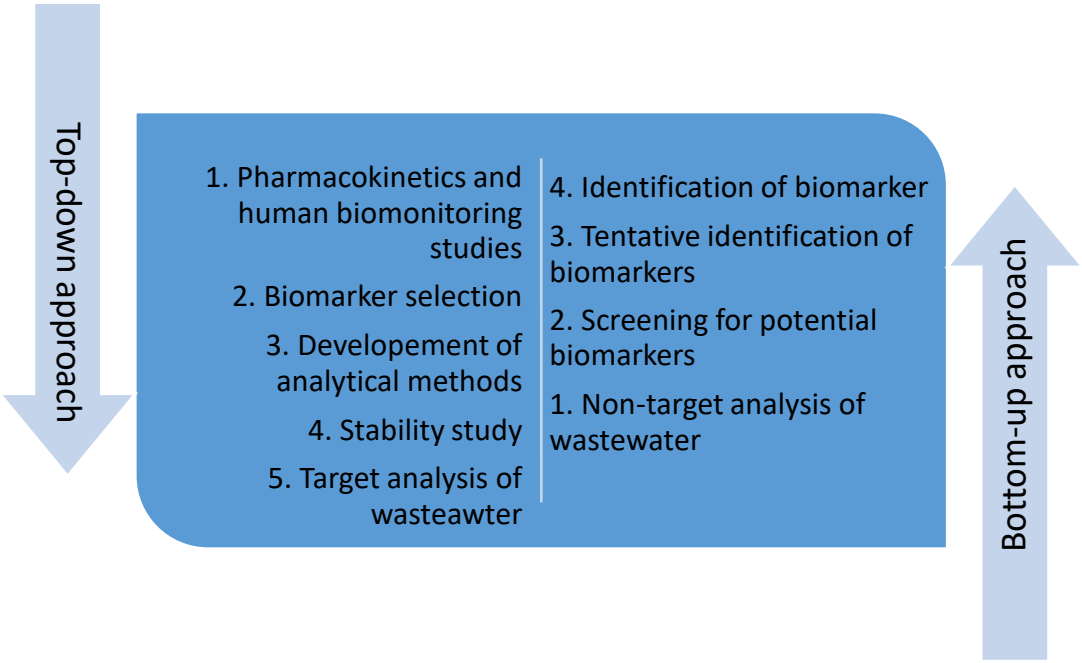


Figure 5: Scheme of top-down and bottom-up approach (adapted from [52]).

Regardless of the approach, selected biomarkers should ideally have a well-known metabolic pathway, high urinary excretion, high wastewater stability and a low tendency to adsorb, *e.g.,* on particulate matter in wastewater. Also, they should be unique to human metabolism and specific to the investigated substance [25]. However, meeting such criteria can be challenging in reality, and therefore, the closest approximations (**Table 5**) are selected as biomarkers [60]. For example, tobacco use is often estimated by measuring cotinine or the sum of cotinine and *trans*-3′-hydroxycotinine (HCOT), which are non-specific for tobacco. Both metabolites are also produced due to nicotine replacement therapies and the use of novel nicotine-related products, which result in the overestimation of tobacco use. Specific tobacco metabolites anabasine and anatabine have been proposed as alternatives, but they are not used routinely due to insufficient information on their excretion rates from the human body [60]. Since selected biomarkers may also originate from pharmaceutical use, so-called non-specific biomarkers are used when dealing with the use of amphetamine (amphetamine), methamphetamine (methamphetamine) and heroin (morphine) [60], [61]. In the case of heroin, the specific biomarker 6-acetylmorphine is often used, but it may underestimate the consumption of heroin, as it tends to degrade in the sewer [60].

Selecting parent compounds as biomarkers may also result in overestimating consumption since, in addition to consumption, these compounds can also originate from their direct disposal unused into the sewer. Typical examples are amphetamines: amphetamine, methamphetamine, and MDMA [61]. In the case of methamphetamine, its metabolite pholedrine has recently been proposed as an alternative biomarker, albeit studies proving its applicability are needed [62]. In some cases, the co-consumption of drugs can be estimated using a specific biomarker. For example, by determining levels of cocaethylene, simultaneous consumption of cocaine and alcohol can be evaluated [63].

Table 5: In-sewer stability and excretion data of biomarkers of selected drugs applied in WBE studies [25], [60], [63]–[65].

Drug	Metabolic residue	Information on metabolic residue and its specificity	In-sewer stability	Excretion rate (correction factor, CF)
		Licit drugs		
Tobacco	Cotinine	Non-specific metabolite	High	30 % (3.13)
	Cotinine + HCOT	Non-specific metabolite	High	74 % (1.35)
Alcohol (ethanol)	EtS	Human-specific metabolite	Medium-high	0.012 % (3047)
Codeine	Codeine	Parent compound	High	30 % (3.33)
Methadone	EDDP	Specific metabolite	High	55% (2.04)
		Illicit drugs		
Cannabis (THC)	THC-COOH	Specific metabolite	High	0.5 % (182)
Cocaine	Benzoylecgonine	Specific metabolite	High	29.2 % (3.59)
Amphetamine	Amphetamine	Parent compound (also a metabolite of methamphetamine and prescription drugs)	High	36.3 % (2.77)
Methamphetamine	Methamphetamine	Parent compound (also a metabolite of prescription drugs)	High	22.7 % (4.4)
Ecstasy	MDMA	Parent compound	High	22.5 % (4.4)

Table 5: Continued.

Heroin	**Morphine**	**Metabolite (also the parent compound of morphine and metabolite of other opioids, such as codeine)**	**High**	**42 % (3.1)**
	6-acetylmorphine	Specific metabolite	Low	1.3 % (86.9)
		NPS		
Ketamine	Ketamine	Parent compound	Medium	20 % (50)

EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EtS – ethyl sulfate, HCOT – *trans*-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol;
Stability classification: high (decay <20 %), medium (decay 20–60 %), low (decay >60 %).

1.3.1.3 Sample treatment

Analytical challenges in determining drug residues in wastewater are linked mainly to matrix complexity and the relatively high levels of compounds that may interfere with the analytes [66]. Best practice protocols suggest using isotopically labelled internal standards to ensure analytical quality, *e.g.,* deuterated or ^{13}C isotopically labelled analyte analogs, for each target analyte [51].

As a first step in sample preparation, suspended solids are removed using filtration, centrifugation, or successive filtration and centrifugation [66]. For nicotine and alcohol residues, typically present in wastewater in high concentrations (μg/L), direct analysis of filtered wastewater is possible [56]. However, using direct injection, a lack of sample clean-up may result in poor ionization and the loss of sensitivity [56]. In contrast to alcohol residues, where direct injection is the only reported method, SPE (Oasis HLB) appears to be the method of choice for extracting nicotine residues [67], [68].

The determination of medications of abuse (*e.g.,* methadone and codeine) and illicit drugs, which are present in wastewater in trace levels (ng/L), require extraction and pre-concentration. Usually, this involves SPE (**Figure 6**) using RP (*e.g.,* Oasis HLB) or mix-mode RP-cation-exchange sorbents (*e.g.,* Oasis MCX) [52], [54], [67], [69]–[73]. To reduce the matrix effect, Senta *et al.* [71] proposed an additional purification step for cannabinoids using a weak anion exchange sorbent (Strata NH_2). Extraction and pre-concentration are also needed when screening for NPS to extract the broadest range of compounds possible. Multiple SPE sorbents are often used sequentially or in a mixture [52], [73].

Figure 6: SPE manifold with extraction cartridges and wastewater samples (Photo: Blaž Samec).

1.3.1.4 Target analysis

The polar nature of the analytes, along with robustness, good reproducibility, selectivity, and sensitivity, makes liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) the analytical technique of choice for quantifying drug residues in wastewater [55], [56]. Although the polar nature of drug residues enables the utilization of hydrophilic interaction liquid chromatography (HILIC), reversed-phase (RP) chromatography is typically used for their separation [55], [56]. In RP, mobile phases consist of a mixture of water and organic solvent (*e.g.,* methanol or acetonitrile), in which modifiers, namely

ammonium acetate, formate or fluoride, formic or acetic acid, and trimethylamine, are added to improve peak shape and compound ionization [56], [74]–[77].

An ion-pair reagent can improve the interactions between highly polar drug residues and the hydrophobic stationary phase. Here, the reagent adsorbs onto the surface of the stationary phase, which retains the analytes through electrostatic interactions. For example, alcohol metabolites (*e.g.,* ethyl sulfate), which are anionic by nature, can be retained on RP columns by using positively charged ion-pair reagents, namely dibutylammonium acetate, dihexylammonium acetate, tetrabutylammonium bromide. Ion-pair reagents can be added to the mobile phase or the samples before analysis. Since higher amounts of the non-volatile ion-pair reagent can suppress the MS signal due to its build-up on the ion source, reducing the amount of the iron pair reagent is advisable by adding it to the sample, not to the mobile phase. Avoiding using it entirely is also an option. In the latter case, it is necessary to use an RP column with a ligand distributed within the stationary phase to promote the retention of polar compounds [56].

High selectivity and sensitivity with LODs in the low ng/L range make tandem mass spectrometry (MS/MS) the detection technique of choice when performing targeted analysis. The most commonly used detectors are triple quadrupole (QqQ) and hybrid triple quadrupole/linear ion trap (QTRAP) mass analyzers operating in multiple reaction monitoring (MRM) mode. Sufficient sensitivity can also be achieved using a high-resolution mass spectrometer (HRMS), such as an Orbitrap or quadrupole time-of-flight (QTOF) instrument [55], [56].

For the detector interface, electrospray ionization (ESI) is most commonly used [55], [56]. ESI is known to be susceptible to the matrix effect (enhancement or suppression of the analyte signal by co-eluting substances), which can result not only in reduced sensitivity (signal suppression) but also in quantification errors [77]. Internal calibration is used to compensate for this effect, *i.e.,* the intensity of the analyte is normalized to the intensity of its deuterated analogs (internal standard), which is expected to behave similarly to the analytes during the analysis [77]. Also, the methods reported in the literature are mostly multi-analyte (*i.e.,* not optimized to a single compound) and consequently a wide range of LODs and LOQs (low ng/L to low µg/L) have been reported [56], [78].

Although widely accepted as the gold standard in forensic drug analysis, gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) was used only in individual studies addressing the occurrence of nicotine, cotinine, cocaine, benzoylecgonine, morphine, and NPS in wastewater [56], [57], [79]–[81]. For GC analysis, derivatizing agents such as trifluoroacetic anhydride (TFA) and *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) are often required to enhance analyte volatility. However, derivatization is not needed when targeting nicotine and cotinine.

1.3.1.5 Non-target analysis

Non-target wastewater analysis is performed on liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) instruments [52] and is most commonly used to identify NPS, for which only a limited number of standards are available [57]. Despite having a lower resolution than an Orbitrap, the QTOF is the most used HRMS analyzer [52]. Mass spectral data are gathered in data-independent (DIA) or data-dependent (DDA) acquisition mode. DIA simultaneously acquires mass spectral data using different collision energies. Low energy mode (LE) is used to obtain a (de)protonated molecular ion, and high energy mode (HE) is used to obtain fragment ions.

In contrast, DDA provides a full scan spectrum (MS), from which 5–20 most abundant precursor ions are isolated and fragmented to obtain their HRMS/MS spectra. Since

residues of psychoactive compounds are mainly present in low concentrations in wastewater, DIA is more applicable in WBE [52], [57]. To provide clearer mass spectra and increase selectivity, LC-HRMS is coupled with advanced separation techniques, such as ion mobility separation (IMS) [57], [82]. IMS separates ions based on their size, shape and charge in the gas phase and the presence of an electric field. Drift time, *i.e.,* the time an ion travels through the mobility cell, is measured, and the collision cross-section (CCS) values are calculated. The CCS values obtained, which represent the surface of the sphere created by the ion, are robust, deviating by <2 %, independent of the instrument, *i.e.,* chromatographic conditions and sample matrix, and can be therefore used in addition to retention time (t_R) and mass spectral data for compound identification [57].

1.3.1.5.1 Compound identification workflows

Two workflows can be used to analyze obtained data: suspect screening and non-target. The suspect screening workflow includes searching a suspect list of compounds expected to be found in the sample [52], [57]. Based on different criteria proposed by Schymanski *et al.* [83] (LC-HRMS analysis) and upgraded by Celma *et al.* [82] (LC-IMS-HRMS analysis), compounds are identified with different levels of confidence. The accurate mass of (de)protonated molecule and one significant fragment ion are considered minimal requirements for tentative identification of the compound (Level 3). Alternatively, unequivocal identification (Level 1 – the highest level of confidence) can only be achieved using a reference standard (**Table 6**). In cases where reference standards are unavailable (*e.g.,* NPS), *in silico* tools can predict parameters, namely CCS values, $t_{R,}$ and MS fragmentation, for increased confidence in identification [82].

Table 6: Levels of confidence as proposed by Celma *et al.* [82].

Level	Name	Data available
Level 1	Unequivocally identified compound - Level 1*: due to the matrix effect of wastewater, higher deviation in Δt_R or accurate mass is permitted (but not simultaneously)	- Empirical data fully agree with a reference standard (are within criteria) - Criteria: o LC: $\Delta t_R \leq 0.1$ min o IMS: ΔCCS ≤ 2 % o MS: $\Delta M_R \leq 5$ ppm; precursor and one diagnostic fragment are visible
Level 2	Probable identification: - Level 2a: probable identification by library search - Level 2b: probable identification by diagnostic evidence (*in silico* prediction tools)	- LC, IMS: predicted - MS: experimental and library data - An exact structure can be proposed based on experimental data
Level 3	Probable candidate(s)	- MS: experimental data - Different chemical structures are compatible with experimental data
Level 4	Unequivocal molecular formula	- MS: molecular formula can be assigned
Level 5	The exact mass of interest	- MS: exact mass

CCS – collision cross-section value, IMS – ion mobility separation, LC – liquid chromatography, MS – mass spectrometry, M_R – accurate mass, t_R – retention time

When using a non-target workflow, unknown compounds are identified without pre-selecting targeted compounds [52], [57]. Software is used for peak picking, removal of noise and background, comparison between datasets, and extraction of feature candidates. Subsequently, retrospective data mining and grouping of identified compounds are performed using multivariate data analysis [52]. However, the process requires extensive examination of mass spectral data for each chromatographic peak, which is a difficult and time-consuming task; therefore, it has limited potential for identifying unknown and rapidly emerging NPS [57].

1.3.1.6 Back-calculation of consumption

The principle of back-calculation of drug consumption is based on Zuccato *et al.* [50], who assumed negligible losses of wastewater and stability of selected biomarkers. Back-calculation is performed as presented in the following equations (**Equations 1 and 2**):

$$Consumptionm\,[mg/day/1000\,inhabitants] = \frac{c \times \varphi \times CF}{N} \quad (1)$$

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

The c represents the measured concentration of the biomarker in wastewater (ng/L), φ the daily wastewater flow (m^3/day), CF a correction factor (**Table 7**), and N the number of

people in the catchment area. In most WBE studies, the static population is used in the calculation, often derived from census data (*de jure* population). Several studies consider population fluctuations by calculating *de facto* population from measured hydro-chemical parameters, such as chemical oxygen demand – COD, biological oxygen demand – BOD, total nitrogen, phosphorus and ammonium, mobile phone trace data and population biomarkers (*e.g.,* cotinine, cortisol, homovanillic acid, creatinine) [69], [84].

Taking into account additional data such as average dose, nicotine uptake per cigarette, amount of pure alcohol per standard drink (**Table 7**), the consumption of illicit drugs in doses/day/1000 inhabitants, tobacco in cigarettes/day/1000 inhabitants, and alcohol in standard drinks[1]/day/1000 inhabitants can be calculated and compared with socio-epidemiological data [63], [85], [86].

Considering uncertainties related to CF (Chapter 1.3.1.7 Uncertainty) and variability of average doses, the content of alcohol and nicotine in the products and drug uptake [51], [80], normalized mass loads, *i.e.,* measured concentrations normalized to wastewater flow and the number of people in the catchment, are usually reported and used in comparison studies such as SCORE monitoring [53], [87].

[1]One standard drink = 10 g of pure ethanol = 1 dcL of wine = 2.5 dcL of beer = 0,3 dcL of spirit [39]

Table 7: Data used to back-calculate drug consumption from measured biomarker concentrations [25], [39], [63], [65], [86], [88]–[90].

Drug	Biomarker	Correction factor (CF)	Average dose
Methadone	EDDP	1.63	100 mg*
Codeine	Codeine	3.33	15-25 mg*
Ketamine	Ketamine	5.00	8 mg*
Tobacco (nicotine)	Cotinine+HCOT	1.35	1.25 mg†
Alcohol	Ethyl sulfate	3,047	10 g‡
Cannabis (THC)	THC-COOH	182	83 mg
Cocaine	Benzoylecgonine	3.59	45 mg
Amphetamine	Amphetamine	2.77	47.5 mg
Methamphetamine	Methamphetamine	4.4	20 mg
Ecstasy	MDMA	4.4	95 mg
Heroin	6-acetylmorphine	86.9	10 mg

*Defined daily dose (DDD), †uptake per cigarette, ‡one standard drink;
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – *trans*-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC – tetrahydrocannabinol, THC-COOH – 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

1.3.1.7 Uncertainty

Uncertainty sources in drug consumption estimates using wastewater analysis were first evaluated for illicit drugs within the SCORE [51], [91], while Zheng *et al.* [92] evaluated uncertainties related to alcohol and tobacco consumption estimates. The measurement of wastewater flow, biomarker concentration, correction factor application, and population estimation are all recognized as potential sources of uncertainty (**Figure 7**).

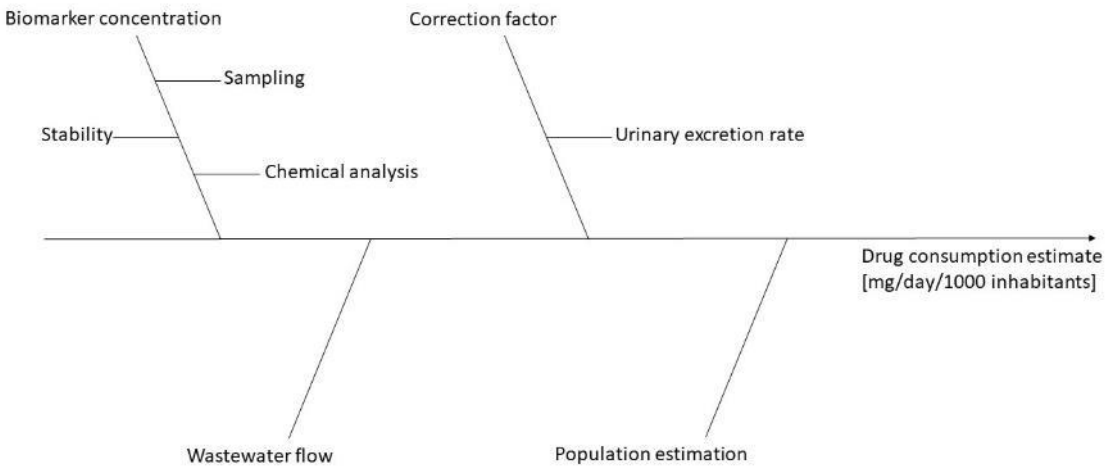


Figure 7: Fishbone presentation of uncertainty sources in drug consumption estimates obtained by WBE.

In order to properly evaluate sampling biases, a high temporal resolution is required for both wastewater flow and intra-day biomarker concentration profiles. Therefore, errors arising from the sampling strategy are only roughly estimated. Sampling bias strongly depends on the catchment characteristic, *i.e.,* population size, number of drug users, and operation of the pump station in the sewer system, and was estimated to range from negligible to 100 % [51], [55], [93].

Urinary excretion rate is considered an important source of uncertainty since it varies greatly depending on the route of administration, dose, gender, age, physical condition and even drug co-consumption. Moreover, it is derived from limited pharmacokinetic studies of a small cohort of typically healthy men [51], [92], [94]. For example, the variability in the excretion rate of benzoylecgonine was 26 % (RSD) [51]. Uncertainty contribution of the excretion rate is especially pronounced in small catchments with few users, and excretion cannot be averaged out [59].

Another dominant source of uncertainty is the estimated number of people in the catchment area. According to an inter-laboratory study, population variability, taking into account different population estimates (census data and data from hydro-chemical parameters were between 7 and 55 % (RSD) [51].

Analytical variability (up to 34 %, depending on the analyte) was estimated within the SCORE inter-laboratory study [51] and by Zheng *et al.* [92] and is considered a minor source of uncertainty. Also, biomarker in-sewer and in-sample stability are considered negligible (<10 % RSD) [51], [55], [84]. In addition, Lai *et al.* [93] showed that the systematic error (20 %) associated with flow measurements could be significantly reduced by regularly calibrating the flowmeters.

The high temporal and spatial resolution of WBE approaches makes it more suitable for tracking changes in drug use over time (*e.g.,* within-week or long-term) and across space (e.g., inter-municipality and international comparisons) than for evaluating absolute drug use in a particular population. Accordingly, individual sources of uncertainty, *e.g.,* variability of excretion rates, become less critical [95]. However, improvements in methods would still be beneficial to reduce systematic over- and under-estimation of drug use and critically evaluate obtained consumption estimates. For example, additional methods to differentiate between drug use and disposal are needed when parent compounds are selected as biomarkers, *e.g.,* amphetamines.

1.3.2 Addressing uncertainties in evaluating drug consumption

Currently, sewage disposal of waste from the large-scale clandestine production of illicit drugs is a growing concern in Europe, particularly in Belgium and the Netherlands [96], [97]. Such events and the disposal of unused drugs systematically affect WBE estimates when parent compounds are selected as biomarkers of drug use (overestimation). Therefore, additional analytical methods that distinguish between consumption and direct disposal are urgently needed [98], [99]. So far, investigation of dumping events has been rarely performed and always followed the analysis of wastewater, during which unusually high biomarker mass loads were observed [98]. As reviewed by Quireyns *et al.* [98], drug disposal is most commonly addressed using the parent compound-metabolite (P:M) ratios approach (72 % of 29 reviewed studies), enantiomeric profiling (28 %) or searching for markers of chemical waste from the illicit drug production by non-target and suspect screening workflows (10 %).

Another approach capable of providing data on the origin of organic compounds is through studying the isotopic composition of light elements (carbon, nitrogen, hydrogen and oxygen) using isotope ratio mass spectrometry (IRMS). The method is frequently used to determine food authenticity and traceability (*e.g.,* confirmation of the geographic origin

and distinguishing between natural and synthetic aromas), but also in forensic science to discriminate between batches of explosives and illicit drugs and to trace their sources [100], [101], [102]. Accordingly, determining the isotopic composition of light elements of biomarkers in wastewater may also provide data on their origin. However, such an approach is yet to be studied.

1.3.2.1 Parent compound-metabolite ratio approach

In the P:M approach, the ratio between wastewater concentrations of the parent compound and metabolite is calculated, and cut-off values, representing the limit between human consumption and disposal, are set based on the excretion profile of the drug. Despite the simplicity of the approach, its use requires a good knowledge of the drug excretion profile and data on the concentration of parent compounds and metabolites, which are only sometimes available [98].

1.3.2.2 Enantiomeric profiling

Enantiomeric profiling can only be used for chiral drugs, *i.e.,* molecules that contain an asymmetric (chiral) carbon atom, for which only parent compounds are being determined in wastewater, *e.g.,* amphetamines [98]. Enantiomeric profiling is based on the fact that enantiomers (molecules that are a pair of mirror images of each other; **Figure 8**) of the same chiral substance interact differently with other chiral molecules despite identical physical and chemical (except optical activity) properties [103]. Accordingly, synthesis, distribution, receptor binding, metabolism, and excretion processes are enantioselective, resulting in specific chiral signatures (ratio between enantiomers). By measuring the ratio between enantiomers, we can differentiate between consumption and direct disposal and also determine drug origin (illicit or licit pharmaceuticals), synthesis route (ratio of enantiomers differ based on synthesis route), and the potency of the drug in the illicit drug market (potency, as well as biological activity, differ between enantiomers) [61].

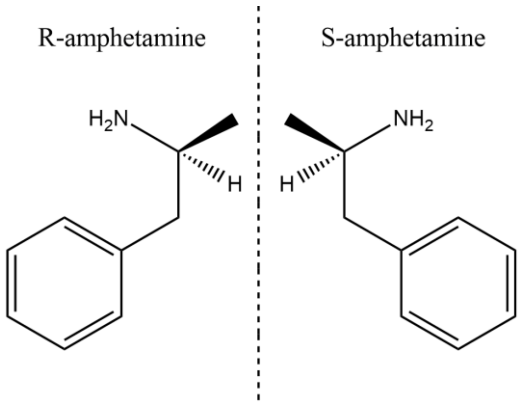


Figure 8: Enantiomers of amphetamine.

In wastewater analysis studies, enantiomers are determined using chiral LC-MS/MS. Chiral separation is achieved on a cellobiohydrolase (CBH) column, with the chiral selector being the enzyme CBH immobilized on the spherical silica particles [103]. Enantiomeric fraction (EF) is calculated using the following formula (**Equation 3**):

$$EF = \frac{E_1}{E_1 + E_2} \tag{3}$$

Here, E_1 and E_2 represent R- and S- enantiomer concentrations. The mixture is racemic when EF equals 0.5 [98]. However, the method does have its limitations. Namely, the enantiomeric profile of administered and excreted drugs must be known [98]. Also, there is the need for *ad hoc* sample preparation, which is time- and cost-intensive and requires collecting higher sample volumes [103].

As an alternative to chiral LC-MS/MS, chiral derivatization can be used when identifying enantiomers of optically active amines and alcohols. The approach is based on the reaction between enantiomers of a chiral drug and optically pure derivatizing agent, whereby diastereomers, *i.e.,* non-mirroring compounds, with differences in the 3D configuration on at least one stereocenter (chiral carbon), are formed (**Figure 9**) [104], [105]. In contrast to enantiomers, diastereomers have different physicochemical properties and can, therefore, be separated by conventional chromatography, *e.g.,* GC [104], [106].

Various chiral derivatizing agents are available for this task, namely S-(−)-*N*-(trifluoroacetyl)propyl chloride (S-TPC), R-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (R-MTPCl) and S-(−)-*N*-(heptafluorobutyryl)propyl chloride (S-HFBPrCl) [107]. However, chiral reagents containing α-proton on the chiral carbon, such as S-TPC and S-HFBPrCl, can undergo racemization due to keto-enol tautomerization of the α-proton on the chiral carbon with the neighboring carbonyl group and therefore derivatizing agents without the α-proton are preferred, *e.g.,* R-MTPCl [108]. Chiral derivatization followed by GC-MS analysis is widely used in the pharmaceutical industry, *e.g.,* in the process of discovering new pharmaceuticals, clinical (toxicological) and forensic studies, *e.g.,* to distinguish between the use of licit and illicit drugs, and linking clandestine drug production, traffickers and consumers [105], [107]–[111]. However, its applicability in wastewater analysis to complement WBE consumption data has not yet been investigated.

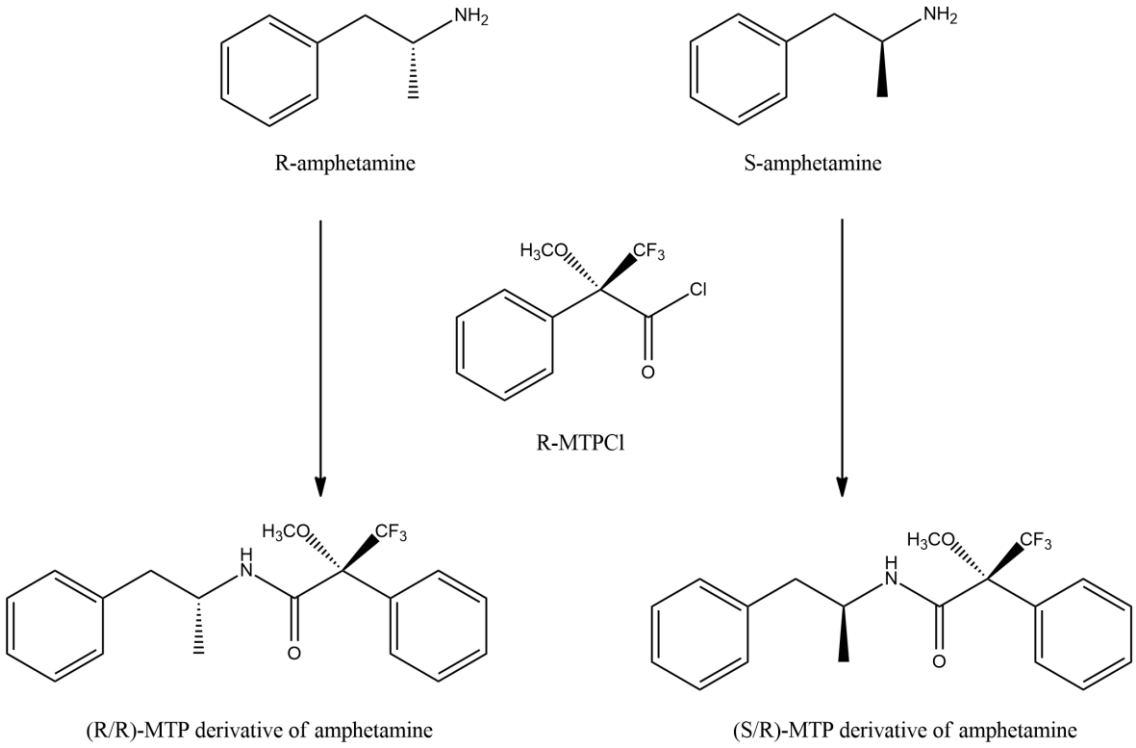


Figure 9: Acylation reaction between amphetamine enantiomers and R-MTPCl (adapted by [106]).

1.3.2.3 Non-target analysis supporting WBE data

Non-target analysis can be used as an alternative approach to detect dumping events by utilizing non-target and suspect screening workflows. The method identifies chemicals used in illicit drug production, *i.e.,* precursors (*e.g.,* amphetamine precursor 1-phenylpropan-2-one [112]), impurities and intermediates, and parent compounds in wastewater. The method provides comprehensive data on the incidence of drug production and the type of synthesis, making it a potential early warning system for identifying spatiotemporal variations in illicit drug production. A significant disadvantage of this approach is that it is laborious and cannot trace the clandestine production of pharmaceuticals [98].

To standardize the handling of dumping events, *i.e.,* when unusually high mass loads in wastewater are observed, Quireyns *et al.* [98] proposed a scoring system that requires the simultaneous use of more than one of the aforementioned approaches to confirm drug disposal. However, not all methods can be used in all cases, as they are specific to the compound and require specific analytical equipment (*e.g.,* chiral column and HRMS). Accordingly, further studies of analytical techniques and standardization of the approach are still needed, *e.g.,* establishing criteria for exclusion of WBE data in case of drug disposal evidence from total (average) consumption estimates [98].

1.3.2.4 Stable isotopic composition of light elements

Organic compounds with the same molecular formula and chemical structure (*e.g.,* biomarkers) can be distinguished based on the composition of stable isotopes, *i.e.,* atoms that differ in the number of neutrons, which result in a different atomic mass [113], [114]. This difference means that stable isotopes of the same element have different physicochemical properties and behave differently during physical and chemical processes, *e.g.,* breaking of bonds involving heavier atoms requires higher activation energies, which

causes enrichment or depletion of one of the stable isotopes relative to the other and hence result in isotopic fractionation [115]. Therefore, the determination of the most commonly used isotopic ratios of light elements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$) of organic compounds is widely used to study chemical and biological processes to obtain data on compound origin or history as well as source. In the case of organic compounds, the isotopic composition is usually determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), where the compounds are evaporated, separated and converted by combustion to gases, such as H_2, CO_2, N_2, and CO, for hydrogen, carbon, nitrogen and oxygen measurements, respectively. The analyte gas is then ionized (electron impact ionization, EI) and introduced into IRMS, where produced ions are separated by a magnetic sector and determined according to *m/z* ratios in dedicated Faraday cups explicitly positioned to collect the masses of interest [116].

In nature, the lighter isotope is much more abundant, *e.g.* approximately 98.9 % of all carbon is ^{12}C and 1.1 % is ^{13}C [117]. At this natural abundance, isotopic variations are tiny, making absolute isotopic abundances less important than the changes in isotopic abundance. For this reason, changes in natural abundance are expressed compared to reference material as δ-notation in *parts per thousand* or *per mil*, ‰. δ-notation is calculated as presented in **Equation 4**:

$$\delta^{i}E\ [‰] = \left(\frac{R_S}{R_{Ref}} - 1\right) \times 1000 \tag{4}$$

where $\delta^{i}E$ represents the isotopic composition of an element in the sample (*e.g.,* $\delta^{13}C$), and R_S and R_{Ref} indicate the ratio between the heavier and the lighter isotope (*e.g.,* $^{13}C/^{12}C$) in the sample and reference material, respectively [117], [118]. Typically, $\delta^{2}H$ and $\delta^{18}O$ values are reported relative to the Vienna-standard mean ocean water (VSMOW), $\delta^{13}C$ to the Vienna-pee dee belemnite (VPDB) and $\delta^{15}N$ to AIR [116], [119].

Stable isotope analysis is often used to detect adulterated food and flavors and determine their authenticity [102]. It is also used in forensic science where, among others, stable isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) have been used for illicit drug profiling [120]–[122]. The purpose of illicit drug profiling is to provide basic data for law enforcement agencies on illicit drug production and trafficking networks by linking seized drugs to the route of synthesis (synthetic and semi-synthetic illicit drugs) or geographic origin (semi-synthetic and natural illicit drugs). This task is usually achieved through physical and chemical characterization of seized drugs, including determining the isotopic composition of light elements of active substances using IRMS. For example, in the study of Thompson *et al.* [123], opium samples from Mexico were distinguished from South American samples based on the $\delta^{15}N$ values determined in extracted morphine, while Southwest Asian samples were isolated based on $\delta^{13}C$ values. Similarly, data on the isotopic composition of carbon and nitrogen were used to link seized synthetic drugs (*e.g.,* amphetamine, methamphetamine and MDMA) and NPS to a production batch and a common source [100], [101], [123], [124].

Given its wide use in forensics and its discriminating ability, analysis of the isotopic composition of light elements has excellent potential to complement WBE data by providing information on the origin of drug biomarkers present in wastewater. Nevertheless, the utility of determining the composition of stable isotopes in supplementing wastewater analysis data has yet to be investigated.

1.3.3 WBE: general population

Wastewater-based epidemiology is an established and synchronized approach for estimating consumption of conventional illicit drugs in the general population (e.g., cities and municipalities) by the SCORE network [53], which annually organizes an inter-laboratory study (to guarantee comparability of results) and international monitoring to track spatiotemporal trends in cocaine, amphetamines, cannabis (THC), and from 2022 also ketamine consumption. Since its first monitoring campaign in 2011, the number of participants has grown from 12 laboratories, 21 WWTPs, and 19 European municipalities participating; it stretched in 2022 to 41 laboratories, 132 WWTPs, and 118 worldwide municipalities participating.

SCORE data provide spatiotemporal trends in drug use and accordingly reveal city- and even country-specific characteristics of drug use. For example, data show that cocaine prevails in western and southern European cities (particularly in Belgium, the Netherlands, Portugal and Spain) and amphetamine in the north and east Europe (Sweden, Belgium, Germany, the Netherlands and Finland). Historically, methamphetamine is concentrated in Czechia and Slovakia, but the data suggests its use is increasing in Belgium, East Germany, Spain, Turkey and northern Europe (**Figure 10**). Ecstasy is the most prevalent in Belgium, Czechia, the Netherlands, Spain and Portugal, and cannabis in Czechia, Spain, the Netherlands and Portugal. Data from 2022 show ketamine was the most prevalent in Denmark, Italy, Spain and Portugal [87].

The SCORE monitoring system, which is capable of providing timely data on changes in drug use trends across various European cities simultaneously, has been recognized and supported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). The EMCDDA publishes monitoring data and utilizes them as an early warning of temporal and spatial changes in drug use throughout Europe [87], [125], [126].

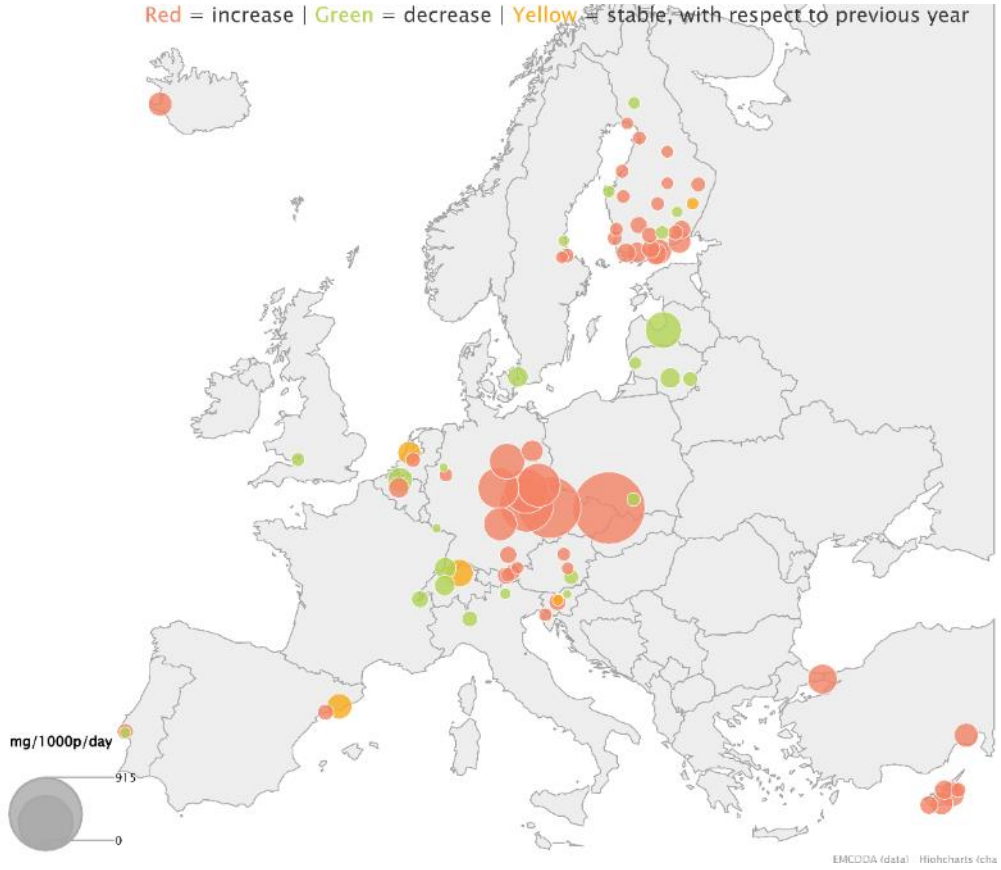


Figure 10: "Changes in the mean weekly methamphetamine metabolites from wastewater analyses in selected European cities between 2021 and 2022" (Source: © EMCDDA [126]).

1.3.3.1 Slovenia in SCORE monitoring

Slovenia became part of the SCORE monitoring in 2017 when the Group of Organic Analysis (Department of Environmental Science, Jožef Stefan Institute) joined the SCORE monitoring and provided the data on mass loads for the capital city, Ljubljana. In 2018, Maribor and Domžale-Kamnik were also included in the monitoring campaign [87]. Initial data (2017-2018) indicated that cocaine use prevailed in Slovenian municipalities, which placed them aside western and southern European cities.

The importance of Slovenia's participation in SCORE monitoring was also recognized by the Slovenian National Institute of Public Health (*Slovene: Nacionalni inštitut za javno zdravje,* NIJZ). NIJZ, as the Slovenian focal point, has a long history of collecting data on drug situation (*e.g.,* national drug policy, trends in drug use, prevention, treatment and harm reduction strategies and best practice protocols) from different sources, namely the ministries responsible for the national strategy in the field of drugs, government institutions, non-governmental organizations, universities and research institutions, and reporting the data to EMCDDA. Accordingly, annual reports on the drug situation of the Republic of Slovenia are published by the NIJZ, where WBE (SCORE monitoring) data are also included since 2018 [32], [127]. Initially (2017 and 2018 monitoring campaigns), SCORE partners analyzed Slovene wastewater samples in 2018 Slovenian Research and Innovation Agency (ARIS, former Slovenian Research Agency – ARRS) project "Illicit drugs, alcohol and tobacco: wastewater-based epidemiology, treatment efficiency and vulnerability assessment of water catchments" was approved, and in-house analytical methods were developed for integrating Slovenian WWTPs into the SCORE annual

monitoring campaigns. Aside from Ljubljana, Maribor and Domžale-Kamnik municipalities, namely Novo mesto, Koper and Velenje, were additionally included in the monitoring and are described in Chapter 3.1.1 National reports to the EMCDDA: Reports on the drug situation of the Republic of Slovenia (2019–2022).

1.3.4 WBE: Specific populations

Due to its non-invasiveness, WBE has a high potential to explore drug consumption trends in small, specific populations susceptible to drug use by sampling and analyzing wastewater of small sub-catchments upstream from WWTP (*e.g.,* an outlet of a particular institution). However, only a few WBE studies have been performed in educational institutions, prisons and other specific catchments, namely fitness centers and airports [59], [128], [129].

Monitoring drug use in educational institutions aims to track the spread of drugs among young people and is commonly done through surveys and drug testing [59], [130]. Since surveys are susceptible to biased reporting and have a time lag in reported data, and because drug testing is highly intrusive and can detect only a limited number of drugs, a few studies have explored the potential of wastewater analysis [59]. However, these studies have focused on only one type of institution, *i.e.,* high education institutions. Moreover, only the consumption of conventional illicit drugs and medications of abuse has been addressed, confirming the use of cannabis, cocaine, amphetamines, ecstasy, heroin, methadone, and codeine [59]. To date, only Gatido *et al.* [131] addressed the consumption of licit drugs (alcohol), while NPS have been addressed in the literature twice using targeted analysis (LC-MS/MS) [59], [131], [132].

Drug use in prisons impacts prison security and prisoners' reintegration into society [59]. Usually, drug use in prisons is investigated through mandatory drug testing and surveys, which requires additional security measures [59]. As an alternative approach, WBE was introduced. Studies have mainly assessed illicit drug and opioid use, while NPS were studied to a lesser extent. The use of substitutional drugs was detected daily, while the use of illicit drugs and NPS was detected occasionally. In such cases, WBE could provide an objective and comprehensive picture of drug use in prisons and be a non-invasive alternative to currently applied approaches [59].

Despite the many advantages of WBE, its application in specific populations also has drawbacks. For example, calculated consumption estimates may be biased, as irregular wastewater pulses in small sub-catchments prevent obtaining a representative sample. Moreover, they can be significantly affected by the application of correction factor taking into account the average drug excretion rate as discussed in Chapter 1.3.1.6 Back-calculation of consumption; the small number of drug users (*e.g.,* compared to the number of users in the whole municipality) means that the individual profile of drug excretion is more pronounced than for larger populations.

Also, sampling wastewater from the whole institution means drug prevalence can only be obtained for the whole population present at the time of sampling, and no extrapolation to a particular group of people, *e.g.,* pupils, prisoners, staff or visitors, can be made [59]. Finally, although WBE poses a low ethical risk when applied to the general population (individuals cannot be identified and traced), its application to small populations may raise specific ethical concerns. For example, obtained results may increase stigmatization, provoke sensationalized media reporting and inadvertently cause harm to the participants or the participating institution's reputation [133]. Accordingly, detailed planning, anonymity, and proper communication of the results are essential in such settings [133].

1.3.4.1 WBE: Special events

In addition to assessing drug use in specific populations, the WBE has also been applied to track drug use during special events, namely holidays, sporting events, and festivals, where the results can be used as an early warning for changes in licit (including NPS) and illicit drug consumption trends [59]. Wastewater for the determination of drug use during special events is usually collected from municipal WWTP [59], [134]–[138]. Only two studies have used pooled urine collected from portable urinals and toilets set up at festival venues to estimate drug prevalence [73], [139]. Trends in drug use showed to be event-specific; however, in general, a significant increase in the use of licit (*e.g.,* alcohol, tobacco, and NPS) and illicit drugs (*e.g.,* stimulants) was observed during various special events in comparison to "normal days", *i.e.,* weekdays without special events [59], [134]–[137], [140]–[143].

1.4 Environmental Perspective

Awareness of the impact that licit (e.g., nicotine-tobacco) and illicit psychoactive substances have on the environment began in the 1980s with studies that showed a connection between the cultivation of plants (*e.g.,* tobacco plant and coca bush) and deforestation [34], [144]. Later, in the 2000s, studies focused on the broader environmental and health aspects of the production (cultivation and synthesis) and consumption of psychoactive drugs (**Figure 11**).

In addition to deforestation, other adverse effects were associated solely with licit and illicit drug production: air, soil and water pollution (with the end product and production waste), soil and water depletion, biodiversity loss, and energy use [34], [144]. Although the impact of drug production on the environment on a global scale has been estimated to be small compared to the total impact of agriculture and pharmaceutical production, studies have shown its importance on a local scale, *i.e.,* where drug production is centralized. This fact is especially true in the case of illicit drugs, in which clandestine production takes place outside regulatory frameworks in remote and often even protected areas (e.g., national parks), where it has a high potential for harmful environmental effects [144]. The extent of the impact of drug production on the environment was reduced by the implementation of alternative development projects within the framework of already existing drug policy responses. The projects promote reforestation, agroforestry, shifting agriculture to alternative crops and reduction of the environmental footprint (*e.g.,* tobacco manufacturing carbon footprint) by the introduction of payment schemes [11], [145].

Compared to the amounts of contaminants released into the environment through drug production, the amounts of drug metabolites and unused drugs released into the environment due to drug consumption are estimated to be larger and contribute significantly to the overall environmental impact [144]. Of particular concern is their ubiquitous presence in waste- and environmental waters. Significantly, since drugs and individual metabolites are bioactive by nature (psychotropic), they have a high potential to affect non-target organisms negatively [56], [146]. Indeed, many adverse effects on aquatic organisms have already been demonstrated at the environmental level (Chapter 1.4.4 Effects on aquatic organisms). However, while drugs and their metabolites are commonly determined in wastewater in order to assess drug use in the population (WBE), their occurrence in the environmental waters, together with the impact they have on the ecosystem, remain understudied [56], [144], [146], [147].

Because of this and the global increase in drug use, the UNODC [144] pointed to the need to develop appropriate strategies for environmental protection, which require a better

understanding of the extent of the impact of drug residues on the environment, *i.e.,* comprehensive monitoring of drug residues (starting compounds and metabolites) in waste and environmental waters and evaluation of hazard assessment.

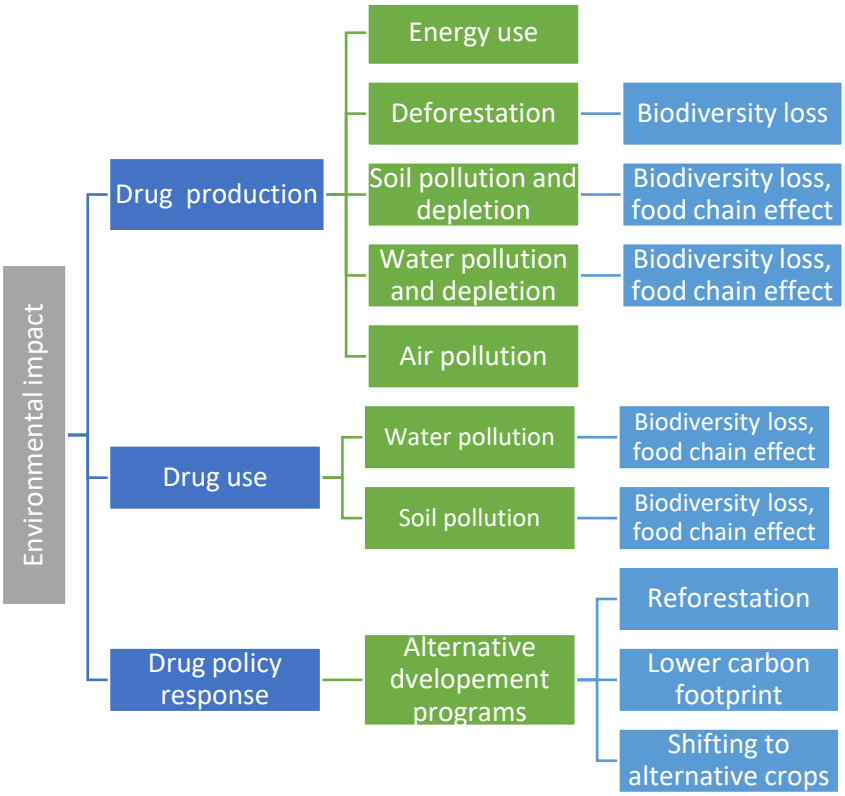


Figure 11: Routes of environmental impact (adapted from [144]).

1.4.1 Analytical methods used to determine drug residues in the aqueous environment

Since WBE presents a "bridge between the environmental and social sciences" [48], it is not surprising that the determination of drug residues in environmental waters is mainly carried out using slightly modified methods (*e.g.,* higher sample volume) primarily developed to estimate drug consumption in the population through wastewater analysis (Chapter 1.3.1 Methodology). Determining drug residues in environmental waters requires an efficient sample preparation step, including high pre-concentration of analytes, efficient removal of interferences and sensitive instrumental methods [78]. Accordingly, drug residues are most often extracted from environmental waters using SPE (Oasis MCX or HLB) and determined (target analysis) by LC-MS/MS, as presented in the latest reviews [56], [78].

However, the sampling strategies differ. Unlike wastewater, where sampling is done according to best practice protocol, surface water sampling strategies are not synchronized. Typically, samples are obtained as grab samples (**Figure 12**), which do not consider time-dependent fluctuations in concentrations and can only provide a "snapshot" at the time of sampling [56], [146], [148]. As an alternative, passive sampling using polar organic chemical integrative samplers (POCIS), low-density polyethylene film devices, solid-phase microextraction fiber and Chemcatcher® device were used [149]–[151]. In the case of

groundwater, sampling strategies are either not described [152], [153] or involve obtaining representative grab samples using existing protocols [154]–[156].

Figure 12: Photo showing grab sampling of surface water (Photo: Eirini Andreasidou).

1.4.2 Wastewater

As the primary recipient of human biological fluids such as urine, saliva, sweat, and feces, raw wastewater typically contains drug residues ranging from ng/L to µg/L (**Table 8**). As conventional WWTPs are not originally designed to eliminate recalcitrant organic contaminants (*e.g.,* selected contaminants of emerging concern, CEC) such as drug residues [56], [146], [157], they remain present in treated wastewater where they were detected, mostly in lower concentration range (ng/L-µg/L) than in wastewater influents (**Table 8**).

Most commonly activated sludge, sequential batch reactor (SBR), Bardenpho[2], trickling filters and membrane bioreactors (MBR) have been researched to eliminate drug residues from raw wastewater (**Table 9**). The removal efficiency depends on numerous factors, such as the treatment process, WWTP operational parameters, the compound's physicochemical properties and concentration, and in-sewer conditions [56], [148], [158]. Accordingly, reported removal efficiencies vary widely even for the same treatment technology and compound. In general, nicotine residues and amphetamines (except MDMA) are considered to be efficiently removed during biological treatment, followed by cannabinoids, opioids and cocaine-related compounds [56], [148], [158]. In some cases, negative removals, *i.e.,* drug residues present in higher concentration in treated than in raw wastewater, were

[2] "conventional activated sludge coupled with nutrient removal" [166]

reported for codeine, methadone, EDDP and MDMA and explained by desorption from sludge, deconjugation or poor sampling strategy (difficulty in obtaining appropriate influent/effluent pairs) [144], [159].

Although many studies have already addressed the occurrence of drug residues in wastewater and their removal during various wastewater treatment processes, inconsistent data indicate that the behavior of drug residues during wastewater treatment is still only partially understood and requires further studies [160]. Moreover, the recently proposed EU Directive on urban wastewater treatment [161] includes provisions for establishing limit values for CEC, which will be regulated by a minimal percentage of removal (80 %). Consequently, this would necessitate implementing an additional (quaternary) treatment step capable of effectively removing a broad range of organic contaminants. Although the directive currently focuses on limiting the concentration of pharmaceuticals and personal care products in treated wastewater [161], it is envisaged that this list will be extended to cover other compounds that pose an environmental risk, *e.g.,* in localized areas with extensive pollution.

Table 8: Determined concentrations (ng/L) of selected drug residues in waste- and environmental water [56], [146], [153]–[155], [157], [162]–[164].

Drug residue	Wastewater		Surface water			Groundwater
	Influent	Effluent	River	Lake	Sea	
NIC	<424,000	15–32,000	<9,340	n.a.	15–1,770	<8,070
COT	<42,300	<18,000	<6,580	<15	4–1,070	<130
HCOT	300–53,000	15-1,550	14-777	<77	n.a.	n.a.
EtS	500–33,000	<LOD	n.a.	n.a.	n.a.	n.a.
MOR	<2,400	<1,270	<631	n.a.	n.a.	n.a.
COD	35-6,460	25-3,970	0.8-342	2.1-4.4	n.a.	<2,440
MTHD	2.6–1,530	1.4–742	<40	1.1-2.5	n.a.	7.4
EDDP	3.7–1,030	2.6–1,150	0.1–71	1.9-8.7	n.a.	0.4
KET	0.78–447	0.19–278	0.05–205	n.a.	n.a.	n.a.
THC-COOH	<1,640	<170	<80	n.a.	n.a.	<LOD
COC	0.2-4,700	<496	<5,900	n.a.	<537	<3.8
BE	1.3-9,720	0.8-3.280	<3,580	0.3-2.40	<142	<1.5
COE	0.4–60	0.2-7.9	<5.2	n.a.	n.a.	0.05
AMP	<12,020	<755	<343	n.a.	n.a.	<LOD [153]
MAMP	<3,640	<675	<277	0.2-96	n.a.	<LOD
MDMA	<27,000	<1,270	<57	n.a.	n.a.	3.9 [153]
6-AM	<224	<96	<16	n.a.	n.a.	<LOD [153]

n.a. – not available; 6-AM – 6-acetylmorphine, AMP – amphetamine, BE – benzoylecgonine, COC – cocaine, COD – codeine, COE – cocaethylene, COT – cotinine, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EtS – ethyl sulfate, HCOT – *trans*-3'-hydroxycotinine, KET – ketamine, MAMP – methamphetamine, MDMA – 3,4-methylenedioxymethamphetamine, MOR – morphine, MTHD – methadone, NIC – nicotine, THC-COOH – 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

Table 9: Removal efficiency for selected drug residues using various treatment technologies [56], [158], [165], [166]–[171].

Compound	Activated sludge	Sequential batch reactor (SBR)	Bardenpho	Trickling filters	Membrane bioreactors (MBR)
NIC	>57 %	>21 %	>99 %	85-98 %	>99 %
COT	>46 %	>75 %	96-98 %	>82 %	>93 %
HCOT	66-99 %	n.a.	>99 %	n.a.	>99 %
EtS	80-99 %	n.a.	>99 %	n.a.	>99 %
MOR	73-89 %	60-95 %	>99 %	n.a.	>99 %
COD	<95 %	>10 %	82-92 %	50-75 %	78-93 %
MTHD	-120-39 %	>90 %	1-23 %	25 %	44-63 %
EDDP	-40-61 %	n.a.	13-30 %	46 %	20-40 %
KET	n.a.	>75 %	n.a.	n.a.	n.a.
THC-COOH	18-98 %	n.a.	n.a.	n.a.	n.a.
COC	>72 %	35-45 %	>-21 %	25-37 %	>99 %
BE	>33 %	>10 %	> 99 %	<27 %	>23 %
COE	>68 %	>25 %	n.a.	25 %	
AMP	>33 %	2-70 %	>99 %	89-95 %	>99 %
MAMP	>-130%	61-91 %	>99 %	60 %	> 63 %
MDMA	19-95 %	-40 %	>11 %	13 %	>99 %
6-AM	72 %	n.a.	n.a.	n.a.	n.a.

n.a. – not available;
6-AM – 6-acetylmorphine, AMP – amphetamine, BE – benzoylecgonine, COC – cocaine, COD – codeine, COE – cocaethylene, COT – cotinine, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EtS – ethyl sulfate, HCOT – *trans*-3'-hydroxycotinine, KET – ketamine, MAMP – methamphetamine, MDMA – 3,4-methylenedioxymethamphetamine, MOR – morphine, MTHD – methadone, NIC – nicotine, THC-COOH – 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

1.4.3 Environmental waters

Although drug residues enter environmental waters mainly through wastewater effluent, the clandestine landfilling of illicit drugs and their precursors, the leaching of nicotine from cigarette butts, the leaking sewer infrastructure and fertilization with biosolids from WWTPs also contribute to their presence in the environment [146], [148], [172], [173]. Once in the environment, the distribution and fate of drug residues depend on their physicochemical properties and environmental conditions, namely dilution, pH, temperature, and sunlight [56]. According to their physicochemical properties (**Table 10**), drug residues (except methadone, EDDP and THC-COOH) are hydrophilic compounds (logarithm of octanol-water partition coefficient, $\log K_{OW}<4$), which tend to remain in the aqueous phase (water solubility: $S_w>8.4$ mg/L) rather than be adsorbed on particulate matter. Accordingly, most drug residues are highly mobile in the aquatic environment and can reach groundwater [56], [146].

In addition, environmental conditions, such as pH, also influence their aqueous mobility, which is promoted when compounds are ionized [56]. At environmentally relevant pHs (pH 5-9), most drug residues (pK_a 4.21–10.2) will be neutral or protonated, while ethyl sulfate will be negatively charged. Biotransformation and indirect photodegradation, *i.e.,* reactions with substances that are produced during photodegradation of humic substances and NO_3^- ($\cdot OH$, $^1O_2$, H_2O_2, and e_{aq}^-), are the most probable processes for removing drug residues from environmental waters. At the same time, removal through adsorption and volatilization (lowK_{ow} and Henry's law constant, K_H) is unlikely. Accordingly, their tendency to bioaccumulate is considered low, except for THC-COOH ($\log K_{ow}$=5.14–5.24) [56], [146].

Table 10: Physicochemical properties of selected drug residues obtained by Ecological Structure-Activity Relationships software (ECOSAR) 2.0.

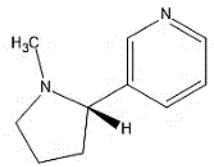
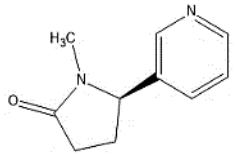
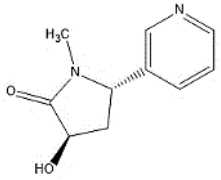
Drug	Drug residue (CAS)	Structural formula	M_R	pK_a	$logK_{OW}$	S_w [mg/L]	K_H (atm × m^3/mol, at 25 °C)
Nicotine	**Nicotine** (54-11-5)		162	8.58 [174]	1.17	1×10^6	8.1×10^{-9}
	Cotinine (486-56-6)		176	4,7–8.8 [56]	0.07	1×10^6	3.33×10^{-12}
	HCOT (34834-67-8)		192	4,5 [175]	-1.45	1×10^6	5.20×10^{-13}

Table 10: Continued.

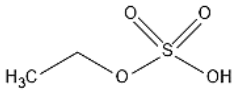
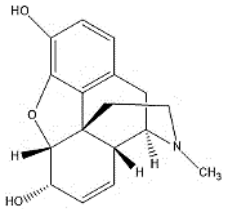
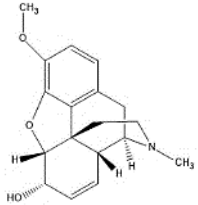
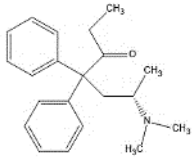
Alcohol	**Ethyl sulfate** (540-82-9)		126	-2.1 [174]	-2.49	1×10^{6}	1.08×10^{-8}
Morphine	**Morphine** (57-27-2)		285	8.21	0.89	149	1.33×10^{-16}
Codeine	**Codeine** (76-57-3)		299	8.21	1.19	9000	7.58×10^{-14}
Methadone	**Methadone** (76-99-3)		309	8.94	3.93	48.5	4.97×10^{-10}

Table 10: Continued.

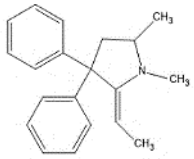
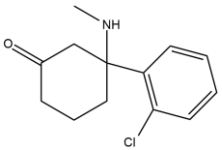
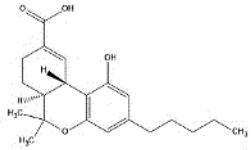
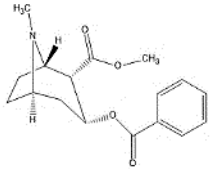
	EDDP (30223-73-5)		278	9.64 [174]	4.94	10.2	Not available
Ketamine	**Ketamine** (6740-88-1)		238	7.5 [176]	3.12	2,800	1.38×10^{-8}
Cannabis	**11-nor-9-carboxy-Δ^9-THC** (not available)		344	4.21 [174]	5.14–5.24 [174]	8.4 [174]	Not available
Cocaine	**Cocaine** (50-36-2)		303	8.61	2.30	1,800	4.24×10^{-11}

Table 10: Continued.

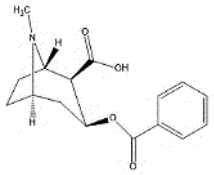
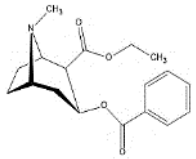
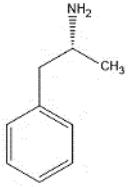
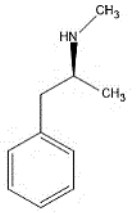
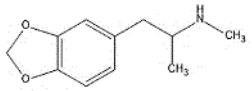
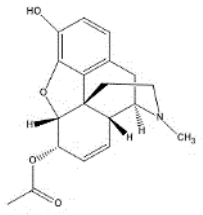
	Benzoylecgonine (519-09-5)		289	3.15 and 9.54 [174]	-1.32	88,300	1.03×10^{-13}
	Cocaethylene (529-38-4)		317	8.77 [174]	2.66	528	Not available
Amphetamine	**Amphetamine** (300-62-9)		135	10.1	1.76	28,000	1.08×10^{-6}

Table 10: Continued.

Methamphetamine	**Methamphetamine** (537-46-2)		179	9.87	2.07	13,300	2.37×10^{-6}
Ecstasy	**MDMA** (42542-10-9)		193	10.1 [174]	2.15	7,034 [176]	2.75×10^{-9} [176]
Heroin	**6-acetylmorphine** (2784-73-8)		327	10.2 [174]	1.55	4,093	Not available

EDDP - 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – *trans*-3'-hydroxycotinine, H_K - Henry's law constant, K_{ow} – octanol-water partition coefficient, MDMA – 3,4-methylenedioxymethamphetamine Mr – relative molecular mass, pK_a – negative logarithm of the acid dissociation constant, S_w – water solubility

1.4.3.1 Surface waters

Drug residues are detected in surface waters mainly in the ng/L range (**Table 8**), with differences related to the sampling site, time (seasonal variations), dilution factors, and trends in drug consumption and production [56], [148]. For example, high nicotine concentrations (9,340 ng/L) observed in Nigeria surface waters probably derived from agricultural activities [163], while high concentrations of cocaine (5,900 ng/L) and benzoylecgonine (3,580 ng/L) in urban streams in Brazilian reflect the use of cocaine, as untreated wastewater is directly discharged into streams in the area of the study [177].

Currently, limit values for drug residues in surface waters are not established. However, their pseudo-persistency in the environment [56], [146], together with already proven harmful effects of individual drug residues on aquatic organisms (Chapter 1.4.4 Effects on aquatic organisms), warrants further studies on their occurrence in surface waters. In 2022, the European Commission (EC) considered 61 compounds, including six illicit drugs and their metabolites: cannabinol, cocaine, benzoylecgonine, ephedrine, methamphetamine, MDMA and THC, for which there is data on their occurrence in the environment and ecotoxicity indicators, as candidates for the 4th Watch List (WL) under the Water Framework Directive [178]. However, in the EC's final evaluation, they were classified in the priority 2 category, *i.e.,* as "almost suitable candidates for the 4th WL, but for which there is lack of information on predicted no-effect contestation (PNEC) value or analytical methods". These substances will be re-evaluated in the next WL update in 2024 to allow time for the development of sensitive analytical methods that will provide more data on their occurrence in surface water (considering PNEC) and the performance of additional ecotoxicity studies [178].

1.4.3.2 Groundwater

On a global scale, groundwater represents an important source of freshwater, *i.e.,* it covers 40 % of irrigation demands and a quarter of industrial supplies and provides drinking water to one-third of the global population [179]. Accordingly, its quality is maintained through several legislations, *e.g.,* following the EU's Water Framework Directive 2000/60/EC [180] and the Groundwater Directive 2006/118/EC [181]. Among others, the directives also regulate the chemical status of groundwater, which includes monitoring only a limited number of chemicals (*e.g.,* nitrate and pesticides) and parameters (salinity). A group of compounds whose presence in groundwater is of increasing concern but are not covered by the directives are CEC, including licit and illicit drug residues [182].

To increase data available on such substances in groundwater, evaluate the risks, and identify substances for which limit values should be set within Groundwater Directive 2006/118/EC, Groundwater Watch List (GWWL), a voluntary mechanism developed in 2019 [183]. Based on the data on their leaching, occurrence, and hazard, 12 substances from classes of pharmaceuticals and polyfluoroalkyl substances (PFAS) have been included in the pilot version of GWWL, and more are expected to follow since the data gathered within GWWL showed 62 compounds, including cocaine, that are despite limited data availability already considered as commonly present in European groundwater.

Generally, drug residues in groundwater are poorly studied due to limited access to samples and demanding sampling, and more data are needed to evaluate the risks [56], [183]. So far, the occurrence of drug residues was mainly determined in the groundwater of EU member states and the USA [56], [183], where they were typically present in the low ng/L range (**Table 8**). However, significantly higher groundwater concentrations of nicotine and codeine (range μg/L) were also detected in the UK (nicotine: 8,070 ng/L)

[162] and Nigeria (nicotine: 3,530 ng/L, codeine: 2,440 ng/L) [163], which further emphasize the importance of studying drug residues in groundwater.

1.4.4 Effects on aquatic organisms

Due to the pseudo-persistence of drug residues in the aquatic environment, aquatic organisms are constantly exposed to (low levels) drug residues [144], [184]. Aquatic organisms, including algae, bacteria, invertebrates and vertebrates, have receptors to which drug residues can bind and thus affect their functioning [173]. The extent to which drug residues can affect the environment was clearly expressed in several local incidents in the province of Limburg in the Netherlands, where there was a mass die-off of amphibians, invertebrates and fish due to the release of large amounts of waste into the environment from the clandestine production of MDMA [144].

Drug residues may affect aquatic organisms also at trace levels, *e.g.,* through chronic exposure [144]. For example, amphetamines are generally known to disrupt catecholamine production in mammals and potentially affect catecholamine production in algae (primary producers in the aquatic food web), which would alter the algal defense against herbivores [173]. General findings also suggest that plant-derived alkaloids, such as nicotine, opioids, and cocaine, have antimicrobial properties and affect fungal and bacterial pathogens [185], [186]. Moreover, they may also influence the functioning of invertebrates since plant-derived alkaloids evolved to protect plants from herbivorous insects. On the other hand, vertebrates are expected to be affected by amphetamines through disruption of dopamine activity [173]. In addition, enantiomer-selective toxicity is predicted for chiral drug residues (*e.g.,* amphetamines), which means that individual drug enantiomers will cause different adverse effects on aquatic organisms in general [103].

Despite indications that drug residues may affect aquatic organisms, little research has been performed on their ecotoxicity [144]. Studies have focused mainly on parent compounds (drugs) and a limited number of organisms from the group of microorganisms, invertebrates and vertebrates (**Table 11**). The ecotoxicity of drug residues, namely nicotine, morphine, cocaine, benzoylecgonine, amphetamine, methamphetamine and MDMA, has been confirmed for various organisms at different concentration levels, including environmental ones. However, none of the studies addresses their effect on algae, an important primary producer in the aquatic food web. Similarly, studies mainly do not explicitly look at the effect of individual enantiomers of chiral drugs, which could also affect toxicity [103], [173]. For example, in one of the rare studies addressing stereoselective toxicity of illicit drugs, the authors confirmed that amphetamine enantioselectively interferes with invertebrate (*Daphnia magna*), *i.e.,* a sharper decrease in body size and number of eggs per daphnia caused by (R)-amphetamine in comparison to (S)- and racemic amphetamine [187]. Accordingly, to set action limits and evaluate the risks that drug residues pose in the environment, more ecotoxicological studies are needed that look at various organisms, including primary producers (algae and cyanobacteria) as well as stereoselective toxicity [103], [173].

Table 11: Summary of reported biological effects on aquatic organisms [146], [157], [173], [188]–[195].

Group of organisms	Tested compound	Tested organisms (exposure)	Reported acute biological effect
Microorganisms	Nicotine	**Bacteria:** *Streptococcus mutans*: 0–32 mg/mL	Repressed growth, increased biofilm formation
	Amphetamine and cannabinoids	**Bacteria:** *Pseudomonas fluorescens*: 5–1,000 µg/mL	Chemotactic effect: influencing respiration, nutrition transformation
Invertebrates	Nicotine	**Planaria:** *Dugesia dorotocephala*: 5 mM	Induce C-shape movement
	Morphine (alone and in mixture with other illicit drugs)	**Mussel:** - *Elliptio complanata:* 30–750 ng/g wet weight - *Dreissena polymorpha:* *100 ng/L MOR in a mixture* - *Zebra mussel: 100 ng/L MOR in a mixture* - *Bivalves: 50–5,000 ng/L*	Physiological changes, damage in macromolecules, increased antioxidant defense, triggering apoptotic process
		Crayfish: - *Orconectes rusticus*: 2000 ng/g	Increased mobility
	Tetrahydrocannabinol (THC)	**Mussel:** Zebra mussels: 500 ng/L	Oxidative stress, DNA damage
		Larvae: *Aedes albopictus*: 400 and 500 µg/L	High mortality rate
		Snail: *Physella acuta*: 100 µg/L	High mortality rate

Table 11: Continued.

	Cocaine	**Mussel**: - *Dreissena polymorpha:* 40, 200 and 10,000 ng/L - *Perna perna*: 500–500,000 ng/L - *Daphnia magna*: 50 and 500 ng/L	Cytotoxicity, oxidative stress, DNA damage, altered swimming behaviour and reproductive success
		Crustaceans: *Orconectes rusticus*: 2,000 and 10,000 ng/L	Increased mobility, behaviour changes
		Crayfish: 2.5-10 µg/g (species not specified)	Alter locomotion
		Planaria: *Girardia tigrina*: 1 mM	Induce C-shape movement
	Benzoylecgonine	**Mussel:** - *Dreissena polymorpha:* 500 and 1,000 ng/L - Freshwater mussels: 500 and 1,000 ng/L	Damage in macromolecules, oxidative stress
	Amphetamine (alone and in mixture with other illicit drugs)	**Mussel:** - Zebra mussels: 300 ng/L AMP in a mixture - Zebra mussels: 5,000 ng/L	Damage in macromolecules, increased antioxidant defense, oxidative and genotoxic damage

Table 11: Continued.

	Methamphetamine	**Crayfish** [146], [157]**:** *Orconectes rusticus:* 2,000 and 10,000 ng/L	Increased mobility
		Snail: *Lymnaea stagnalis*: 3.3 µM	Strengthen the long-term memory
Vertebrates	Nicotine	**Fish embryos**: - Zebrafish: 6.8 µM - Zebrafish: 1.3–1.7 mM	Dose-dependent effect: lower doses – tachycardia, higher doses – bradycardia
		Fish: Zebrafish: ≥0.49 µg/L	Neurotoxicity, altered predator escape response, 25-100 % reduction in survival
	Tetrahydrocannabinol, THC (alone or as an extract of *C. sativa*)	**a) Fish:** - *Cyprinus carpio*: 2,000–30,000 ng/L; - *Oreochromis niloticus*: 10.7 g/kg extract	Decreased alkaline phosphate activity in the gills and liver, high levels of transaminases and lactate dehydrogenase in blood, low growth rate, increased metabolism
	Cocaine	**Fish embryos:** Zebrafish: 0.3 and 1.0 µg/L	Tachycardia, decreased cell viability
		Fish: *Anguilla anguilla*: 20 ng/L	Affect the endocrine system and physiology. COC can accumulate in the tissue (brain, muscle, and liver)
	Amphetamine	**Fish:** 5,000 and 10,000 ng/L (species not specified)	Altered levels of brain monoamines, behavior changes
	3,4-methylenedioxymethamphetamine (MDMA)	**Fish:** Zebrafish: 40,000–120,000 ng/L	Reduce bottom swimming and immobility

Chapter 2

Aims and Hypotheses

Review of the existing literature highlights the significance and utility of wastewater analysis in tracking drug consumption from both health and environmental perspectives. Although the approach is well established for drug consumption estimation in the general population (SCORE monitoring), it still has methodological shortcomings (*e.g.,* differentiation between consumption and disposal of unused drugs) and a lack of applicability studies in some countries, including Slovenia, and in vulnerable populations, *e.g.,* educational institutions. Also, there is limited data on drug consumption's environmental impact. Accordingly, the thesis aimed to estimate drug use in general and specific populations (wastewater-based epidemiology, WBE) and to evaluate the impact that drug use has on aquatic ecosystems. Specifically, it aimed to:

- develop target analytical methods (LC-MS/MS) for the determination of 17 drug residues in aquatic matrices (wastewater influent and effluent, surface water, groundwater)
- explore analytical approaches to differentiate drug use and disposal, namely enantiomeric profiling (chiral derivatization, GC-MS/MS) and determination of the isotopic composition of light elements (GC-C-IRMS) of biomarkers present in raw wastewater;
- investigate the applicability of wastewater analysis using targeted analysis (LC-MS/MS) to explore the prevalence of licit and illicit drugs in specific populations, namely educational institutions;
- investigate the applicability of wastewater analysis using suspect screening (LC-IMS-HRMS) to explore the possible presence of NPS in educational institutions;
- assess the removal efficiency of drug residues by conventional biological wastewater treatment technologies
- determine the occurrence of drug residues in effluent receiving surface waters (rivers) and groundwater;
- evaluate the toxicity of selected drug residues toward green algae (*Chlamydomonas reinhardtii*) using the algal growth inhibition test and
- perform a risk assessment using an *in silico* methodology (Ecological structure-activity relationships software, ECOSAR);

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

H1: Determining drug biomarkers in wastewater influent can confirm reported socio-epidemiological data on trends in drug consumption in Slovenian communities.

H2: Enantiomeric profiling and stable isotope-ratio analysis can differentiate illicit drug consumption and direct disposal.

H3: Conventional wastewater treatment technologies do not fully mineralize drug residues.

H4: Levels of drug residues in studied receiving river waters do not pose a risk to the aquatic organisms.

Chapter 3

Publications

The work presented in this dissertation comprises three contributions to the National Reports on the Drug Situation in the Republic of Slovenia and ten scientific papers. Nine papers have already been published in peer-reviewed journals. In addition, one paper is in preparation and will be submitted to Environmental Science & Technology (October 2023).

The publications are divided into four sections:

1) WBE applications in Slovenian municipalities
The publications in the first section address **hypothesis 1 (H1)** and focus on the applicability of WBE to estimate drug consumption in the general Slovenian population, *i.e.,* six municipalities (WWTPs). Analytical methods and procedures used in international monitoring (SCORE and NPS) are described, and data obtained on illicit drugs and NPS use are compared with the data from other epidemiological studies.

2) Applicability of WBE to assess drug use in specific populations
The work presented here also addresses **H1** but focuses specifically on the applicability of WBE in specific populations. Analytical methods and approaches used to address drug use in various populations, namely prisons, educational institutions, and nightlife settings, are summarized, along with reported estimates of drug use. In particular, the work addresses the applicability of wastewater analysis to assess the prevalence of licit, illicit drugs and NPS in (Slovenian) educational institutions. For the first time, licit, illicit drugs and NPS in primary school wastewater were reported and compared between educational institutions of different types, *i.e.,* primary and secondary schools and high education institutions.

3) Supplementing WBE data: Reducing uncertainties in evaluating drug consumption
Here, developed analytical methods, *i.e.,* enantiomeric profiling of amphetamines (chiral derivatization, GC-MS/MS) and, to the best of our knowledge, for the first time, determining the isotopic composition of morphine (GC-C-IRMS) are presented and their applicability for complementing WBE data are discussed (**H2**).

4) Drug residues in the aqueous environment: occurrence and ecotoxicity
This last part addresses **H3** and **H4** and includes scientific publications on the environmental impact resulting from drug use. The efficiency of the removal of drug residues by various conventional biological wastewater treatment processes and the occurrence of drug residues in receiving rivers and groundwater are presented. To our knowledge, this is the first time the efficiency in removing drug residues by moving bed biofilm reactor (MBBR) has been addressed. Also, the impact on aquatic organisms is

evaluated based on the measured concentrations of drug residues in receiving rivers and predicted effect concentration using ECOSAR. Finally, the ecotoxicity of drug residues towards green algae (*Chlamydomonas reinhardtii*) is explored using an algal growth inhibition test.

3.1 WBE Applications in Slovenian Municipalities

3.1.1 National reports to the EMCDDA: Reports on the drug situation of the Republic of Slovenia (2019–2022)

1) *Published in 2022: Verovšek, T., Blaznik, U., Hočevar Grom, A., Heath, D., Laimou-Geraniou, M., Heath, E., 2022, National Institute of Public Health, Ljubljana, p. 69-75.*

2) *Published in 2021: Verovšek, T., Blaznik, U., Hočevar Grom, A., Heath, D., Laimou-Geraniou, M., Heath, E., 2021, National Institute of Public Health, Ljubljana, p. 65-71.*

3) *Published in 2020: Verovšek, T., Krizman-Matasic, I., Blaznik, U., Hočevar Grom, A., Kosjek, T., Heath, E., 2020, National Institute of Public Health, Ljubljana, p. 64-69.*

Following the best practice protocol [51], consumption of illicit drugs, namely cocaine, amphetamine, methamphetamine, ecstasy, and cannabis (THC), was estimated in the Slovenian general population (municipalities) within the SCORE monitoring 2019-2022. For the continuous inclusion of Slovene data, analytical methods had to be developed in a Slovene laboratory (Chapter 1.3.3 WBE: general population). Accordingly, in 2019, analytical methods were implemented to analyze Slovene WW influent. Their adequacy and comparability of results with the rest of the laboratories participating in SCORE were confirmed by an inter-laboratory study. Furthermore, a greater number of Slovenian municipalities were included in the monitoring, *i.e.,* in addition to Ljubljana (2017), Maribor, and Domžale-Kamnik (both 2017 and 2018), which previously participated in the monitoring, Novo mesto, Koper and Velenje were also included in 2019 (Chapter 1.3.3.1 Slovenia in SCORE monitoring).

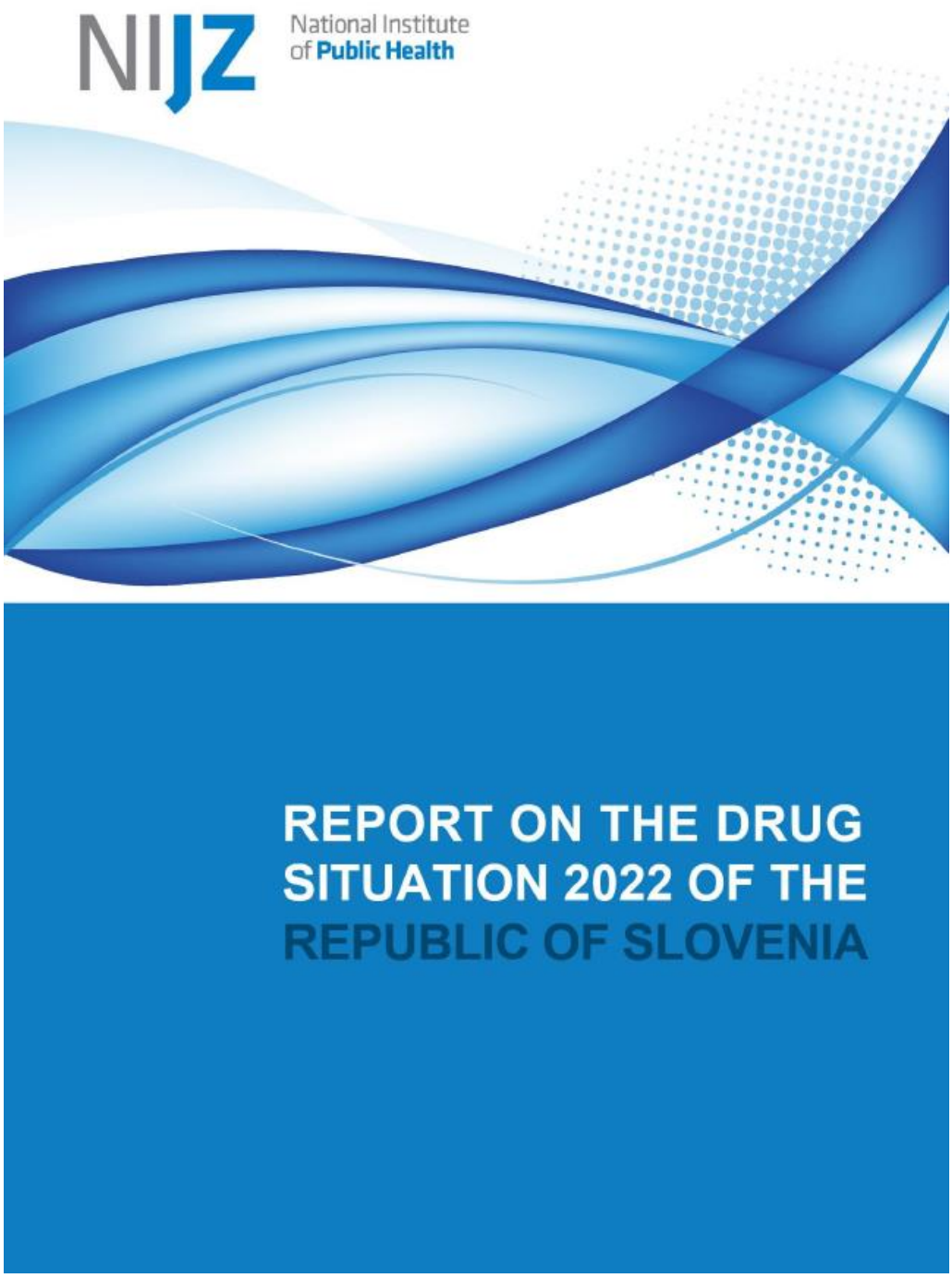
This work revealed that the most widespread drug in Slovenian municipalities was cannabis (THC), while contrary to survey results [32], cocaine was shown to be by far the most used stimulant. The consumption of individual drugs was usually the highest in Ljubljana, except for amphetamine, which was always the highest in Velenje. Stimulants showed a distinctive weekly pattern, with peak consumption observed over the weekend. Interestingly, we showed how the pattern changed during COVID-19 lockdowns by showing that the consumption of simulants (except cocaine) was relatively constant over the whole week. The findings supported other observations in other European cities [179], [180]. In 2020, an extremely high MDMA mass load was observed in Ljubljana and was speculated to be related to a dumping event.

When placing Slovenian municipalities in the broader European context, *i.e.,* comparing them with the other participating municipalities within SCORE monitoring, they ranked in the upper half of the range (municipalities with the highest consumption) for cocaine and cannabis (THC). The exception was Velenje, which in the investigated period ranked in the upper half of the range in terms of amphetamine use.

Knowledge of the spatiotemporal trends of drug use in Slovenia has supplemented that of drug use trends at the European level and provided timely data on changes in drug use within Slovenia. They were published as part of SCORE monitoring results by the EMCDDA [87] and as part of the presented National reports on the drug situation of the Republic of Slovenia by the national focal point, *i.e.,* the National Institute of Public Health [16], [32], [45]. In addition, the results of the studies were summarized within nine

working reports to final users, *i.e.,* Slovenian WWTPs, which showed that the data was also important for tracking changes in WWTP's catchment, which can affect the composition of wastewater and the efficiency of wastewater treatment. Finally, the results were of great interest to the Slovenian public, to which they were presented on more than ten occasions in the form of interviews, articles and popular science lectures, *e.g.,* Znanost na cesti, *Eng.:* Science on the street (**Figure 13**).

Figure 13: Science on the street, 30th September 2022 (Photo: Radojko Jaćimović).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2022 NATIONAL REPORT (2021 DATA) TO THE EMCDDA

by the Reitox National Focal Point

SLOVENIA

REITOX

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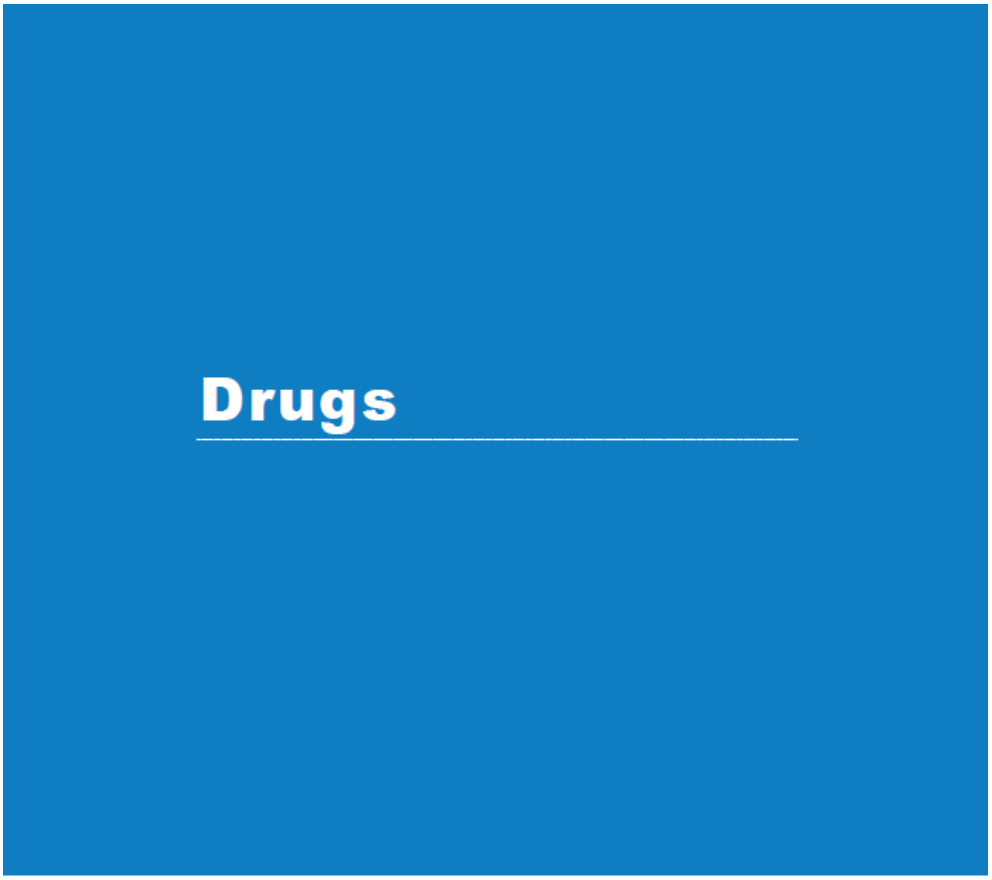
Drugs

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Experience assessment & side effects

When questioned about their experience with these drugs, 40.0% of respondents reported positive effects, 40.0% reported mixed both positive and negative effects, and 20.0% of respondents reported only negative effects. Some of the experiences included: burning sensation on nasal mucosa, feeling really bad, having fun, feeling good.

1.2.5 Injecting and other Routes of Administration

Among harm reduction programme participants who used cocaine in 2021, 68.4% reported injecting it, while 23.5% of amphetamine and methamphetamine users and 6.1% of ecstasy users reported injecting the drugs.

Cocaine is the stimulant drug which is most commonly injected by harm reduction programme participants. There was no significant change in the proportion of those injecting cocaine between 2020 and 2021.

0.5% of ESPAD respondents aged between 15 and 16 confirmed injecting a drug with a syringe, although this question referred to drugs that could be specifically used in this way, e.g. heroin.

2. New developments

2.1 New Developments in the Use of Stimulants

See 1.2.2., 1.2.3.,1.2.4., 4.1.

3. Additional information

3.1 Additional Sources of Information

Wastewater- based epidemiology: drug consumption in six Slovenian municipalities

Taja Verovšek, Urška Blaznik, Ada Hočevar Grom, David Heath, Maria Laimou-Geraniou, and Ester Heath

Wastewater-based epidemiology (WBE) was used to investigate the use of stimulants, namely amphetamine, methamphetamine, ecstasy or 3,4-methylenedioxymethamphetamine (MDMA) and cocaine, and cannabis (Δ-9-tetrahydrocannabinol, THC) in wastewater (obtained over 7 consecutive days) of six Slovenian municipalities: Ljubljana, Maribor, Domžale-Kamnik, Koper, Novo mesto and Velenje. Results on drug consumption in Slovenian municipalities were compared with the data obtained for other major cities within an international monitoring campaign organised by the Sewage Analysis CORe group Europe (SCORE) in 2021 [1,2]. Finally, timely trends in drug use in Slovenian municipalities were explored.

Results:

A) Biomarkers mass load

Mass loads (g/day) of biomarkers for cocaine (benzoylecgonine), amphetamine (amphetamine), methamphetamine (methamphetamine), ecstasy (MDMA), and cannabis (11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, THC-COOH) in wastewater were used to explore daily patterns in drug use within individual municipality. A distinctive weekly pattern, i.e., higher mass loads of biomarkers during weekends in connection with increased consumption of stimulants [3-5], was typically observed when analysing wastewater before COVID-19 pandemics (SCORE monitoring campaigns 2017–2019)[5,7]. In contrast, a distinctive weekly pattern was observed mainly for benzoylecgonine during the COVID-19 lockdown in 2020[8], confirming the effect of COVID-19 lockdowns on drug consumption patterns[9,10]. Similarly, in 2021 (short lockdown within the sampling period: 1.4.–11.4.2020), higher mass loads of benzoylecgonine were observed over the weekend in comparison to weekdays in all participating Slovenian municipalities (Figure 2), while mass loads of MDMA increased in Ljubljana, Domžale-Kamnik, and Koper. No distinctive weekly pattern was observed for THC-COOH, which is typical for drugs used regularly throughout week[3].

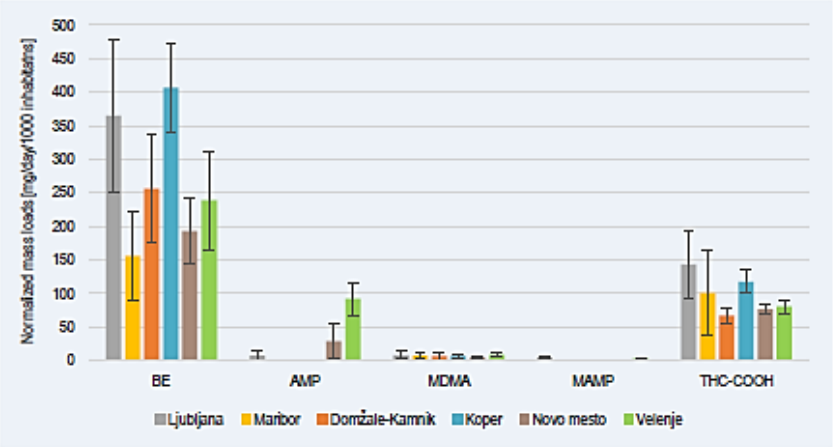
Figure 2. Daily variations in drug mass loads in 2021

AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol

All biomarker mass loads were normalised to the population served by wastewater treatment plant to compare the data between different-sized municipalities. The highest average mass loads of THC-COOH (143 mg/day/1000 inhabitants) and methamphetamine (3.75 mg/day/1000 inhabitants) were observed in Ljubljana, amphetamine (91.7 mg/day/1000 inhabitants) and MDMA (8.21 mg/day/1000 inhabitants) in Velenje and benzoylecgonine (407 mg/day/1000 inhabitants) in Koper (Figure 3).

Figure 3. Average mass loads and standard deviations (7 monitoring days) of selected drug biomarkers in six Slovenian municipalities in 2021

AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol



Slovenian municipalities mainly scored below the 2021 SCORE average for the monitored biomarkers of stimulants (Table 3). Only amphetamine in Velenje and benzoylecgonine in Koper were above 2021 SCORE average. Benzoylecgonine in Ljubljana was right below the 2021 SCORE average. In contrast, THC-COOH loads were above the 2021 SCORE average in all Slovenian municipalities.

Table 3. Average mass loads (7 monitoring days) and the estimated 2021 SCORE averages

AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH – 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol

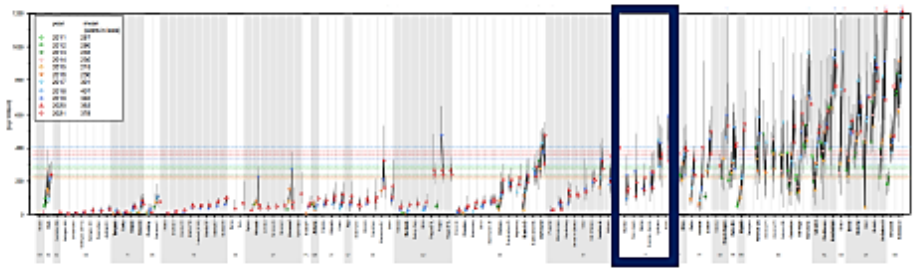
Biomarker of illicit drugs	Ljubljana	Maribor	Domžale-Kamnik	Koper	Novo mesto	Velenje	2021 SCORE average [1]
	Normalized mass loads [mg/day/1000 inhabitants]						
BE	365	156	256	407	193	239	378
AMP	6.78	n.a.	n.a.	n.a.	28.7	91.7	81
MDMA	7.49	6.86	6.33	6.42	4.59	8.21	23
MAMP	3.75	n.a.	0.642	1.14	n.a.	2.70	28
TCH-COOH	143	101	66.7	117	76.0	80.6	42

n.a. – not applicable (measured concentrations of biomarker were under the limit of quantification in all obtained wastewater samples)

In the SCORE, participating municipalities (2021: n=90) are ranked according to normalized mass loads of biomarkers (the 1st having the highest normalized mass load of biomarker). Slovenian municipalities (Figure 4. a–e)[2] ranked in the upper half regarding benzoylecgonine: Ljubljana (25th), Maribor (41st), Domžale-Kamnik (29th), Koper (20th), Novo mesto (38th), and Velenje (31st), while with mass loads of the biomarkers of other stimulants, they mainly ranked in the lower half: amphetamine (<37th), MDMA (<49th) and methamphetamine (<51st). The exception was Velenje, which ranked 15th for amphetamine. Regarding THC-COOH, Slovenian municipalities ranked 3rd–26th.

Figure 4. Ranking of Slovenian municipalities based on benzoylecgonine (a), amphetamine (b), MDMA (c), methamphetamine (d) and TCH-COOH (e) normalised mass loads in SCORE 2021 (adapted from SCORE graphical representation of results 2021[1]); y-axe: biomarker mass loads (mg/day/1000 inhabitants), x-axe: participating cities and countries.

(a)



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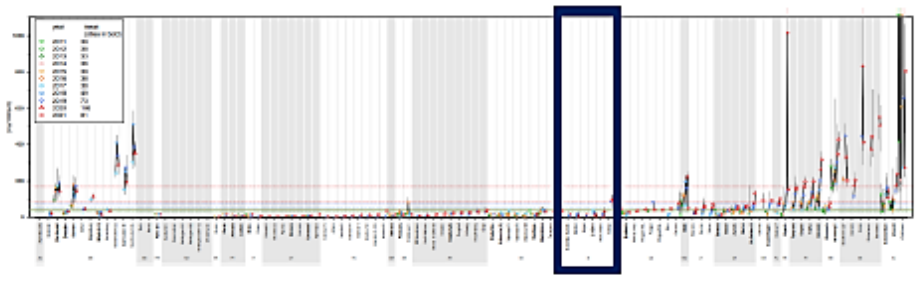

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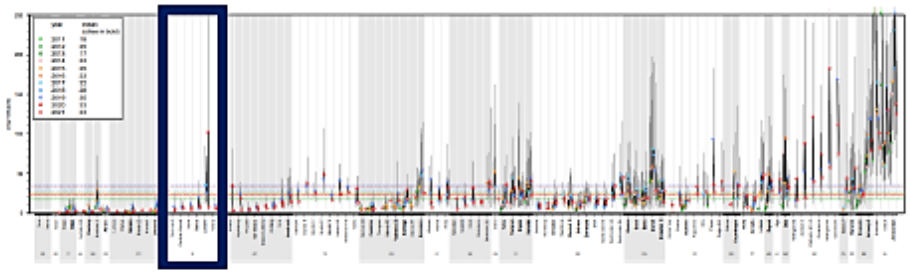

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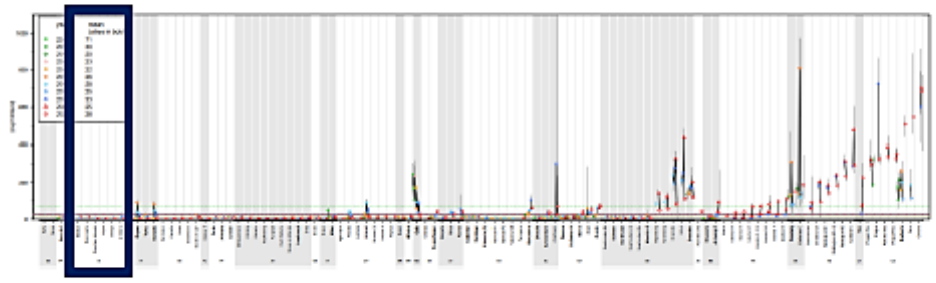

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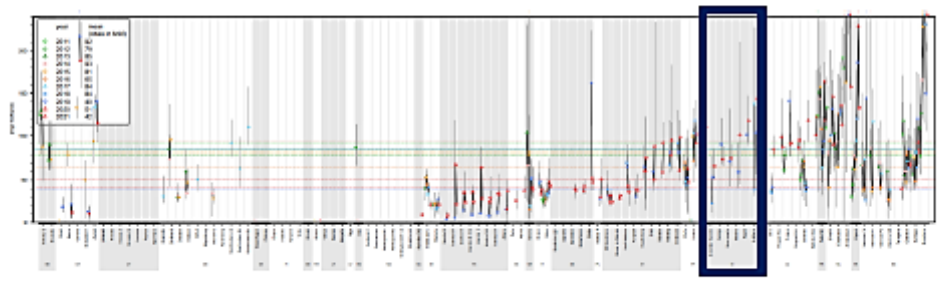
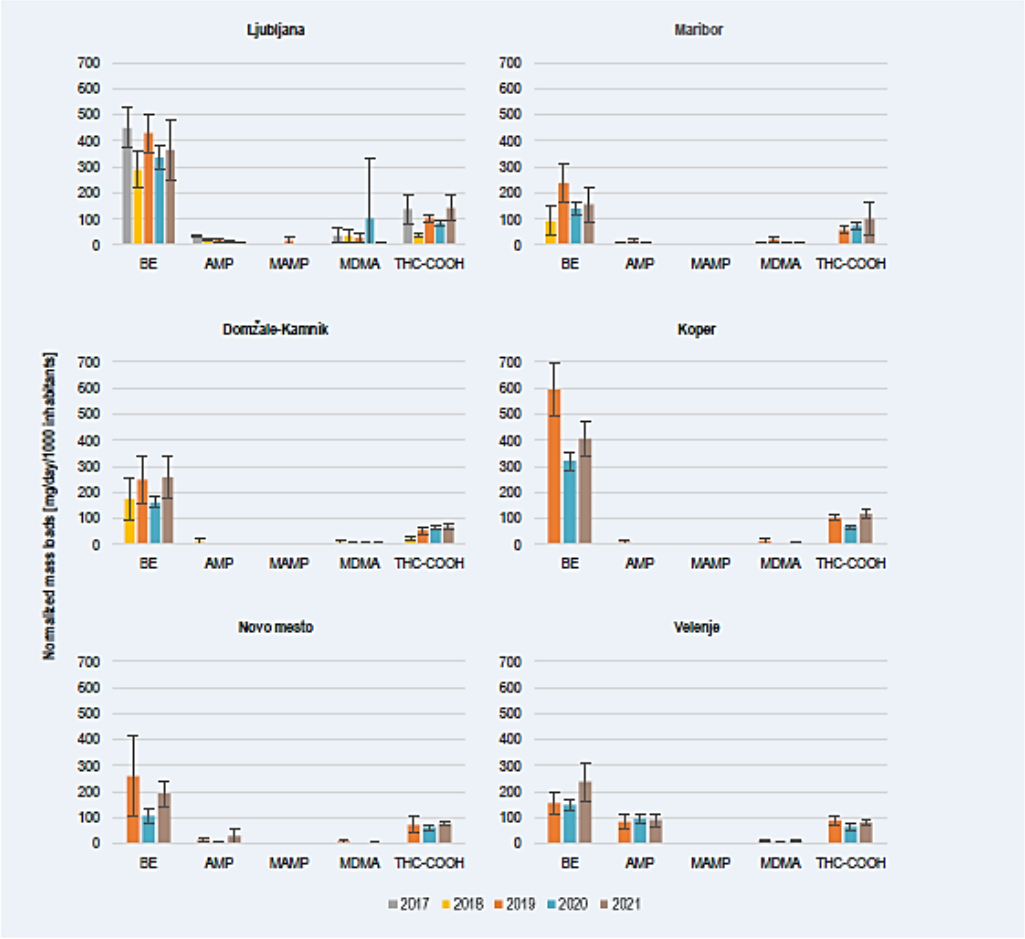


According to SCORE[1], at least five annual measurements are needed to predict temporal trends in drug use. In 2021, only Ljubljana was involved in SCORE for five consecutive years. However, specific trends can be observed in Maribor and Domžale-Kamnik, which were included in SCORE monitoring for four years (since 2018). During the five-year monitoring campaign, cocaine, amphetamine and MDMA consumption in Ljubljana (included in SCORE monitoring since 2017) generally decreased (Figure 5). The exception was the high average MDMA load in 2020 (a consequence of unusually high MDMA loads in one of seven wastewater samples), which most likely originated from unused MDMA disposed in the sewer system as it was indicated using enantiomeric profiling[11]. Consumption of methamphetamine and cannabis has been relatively constant. In Maribor (included in SCORE monitoring since 2018), the only trend observed is the increasing consumption of cannabis (Figure 5). In addition to cannabis, cocaine use has increased in Domžale-Kamnik (included in SCORE monitoring since 2018), while consumption of amphetamine continues to decline (Figure 5). Due to fluctuations in drug use in Koper, Novo mesto and Velenje (included in SCORE monitoring since 2019), no clear drug use patterns were observed (Figure 5). The exception is cocaine consumption in Velenje, which was higher in 2021.

Figure 5. Average mass loads and standard deviations (7 monitoring days) of selected drug biomarkers for Slovenian municipalities, participating in SCORE monitoring for three or more consecutive years
AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH – 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol



B) Drug consumption estimates

Drug consumption estimates (mg of drug/day/1000 inhabitants or doses/day/1000 inhabitants) are back-calculated from normalised mass loads of biomarkers (mg of biomarker/day/1000 inhabitants) taking into account correction factor that considers the percentage of parent drug metabolite excreted and the parent drug-to-metabolite molar mass ratio. Cannabis (Table 4) was the most consumed drug (average consumption: 167–314 doses/day/1000 inhabitants), while cocaine was the most used stimulant (average consumption: 12.4–32.4 doses/day/1000 inhabitants). The same consumption trend was also observed in 2020[8].

Table 4. Average illicit stimulant use

	Ljubljana	Maribor	Domžale-Kamnik	Koper	Novo mesto	Velenje
	Drug use [mg/day/1000 inhabitants]					
Cocaine	1310	560	920	1459	692	857
Amphetamine	18.8	n.a.	n.a.	n.a.	79.5	254
Methamphetamine	16.8	n.a.	2.77	4.88	n.a.	11.8
Ecstasy (MDMA)	32.8	30.1	27.7	28.2	20.2	36.0
Cannabis (THC)	26031	18319	12148	21352	13839	14671
	Drug use [doses/day/1000 inhabitants]					
Cocaine	29.1	12.4	20.4	32.4	15.3	19.0
Amphetamine	0.395	n.a.	n.a.	n.a.	1.67	5.343
Methamphetamine	0.838	n.a.	0.138	0.244	n.a.	0.588
Ecstasy (MDMA)	0.345	n.a.	0.291	0.297	0.213	0.379
Cannabis (THC)	314	221	146	257	167	177

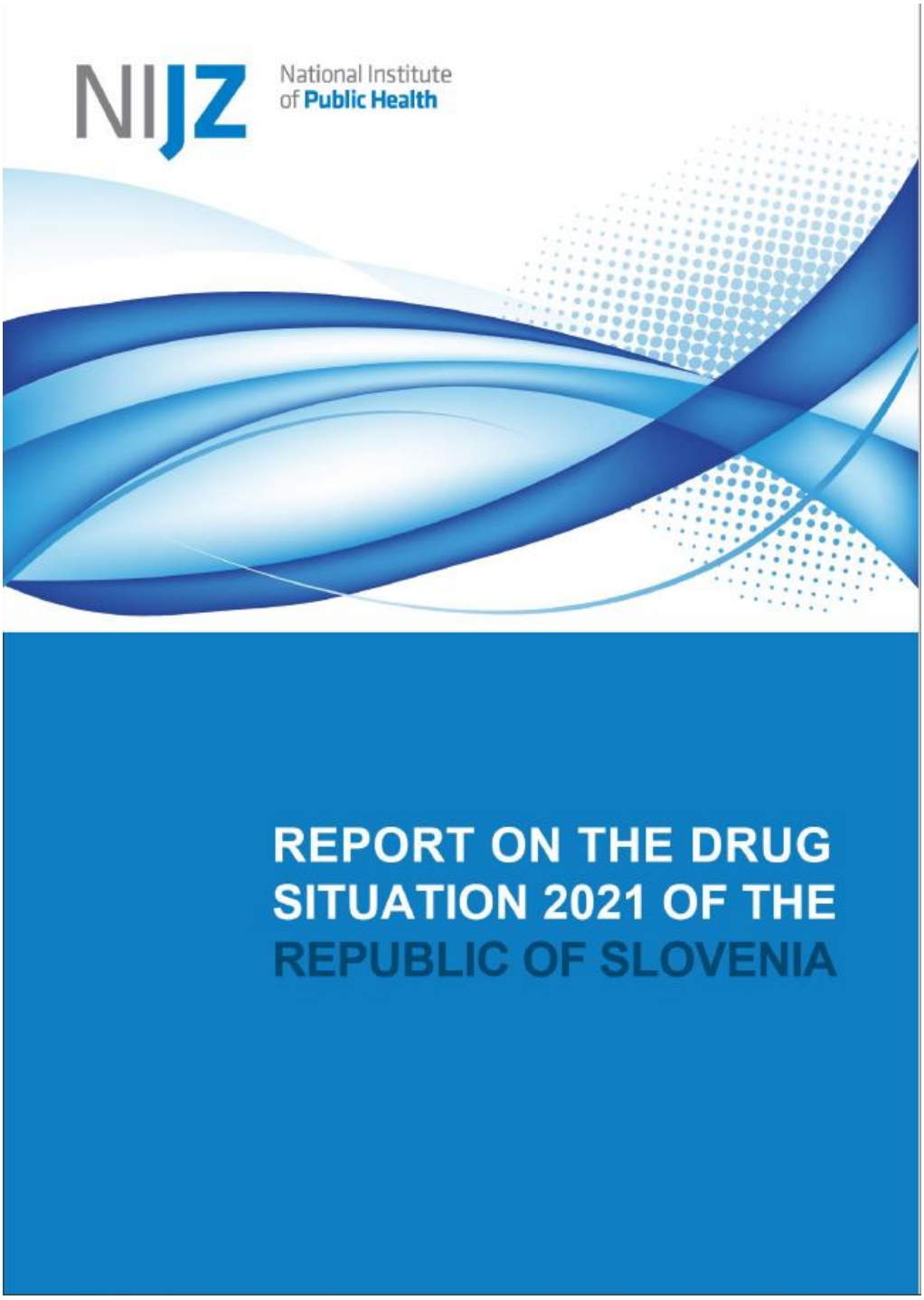
n.a. – not applicable (measured concentrations of the biomarker in all raw wastewater samples were under the limit of quantification)
THC – Δ-9-tetrahydrocannabinol

Conclusions

Six Slovenian municipalities (Ljubljana, Maribor, Domžale-Kamnik, Koper, Novo mesto and Velenje) participated in the 2021 SCORE monitoring. Among them, cannabis and methamphetamine use was highest in Ljubljana, while amphetamine and MDMA use was highest in Velenje. Koper had the highest cocaine use. Generally, normalized mass loads of stimulants' biomarkers were below the 2021 SCORE average in Slovenian municipalities, except for Koper (benzoylecgonine) and Velenje (amphetamine), while the average loads of THC-COOH were consistently above the SCORE average. The data from 3–5 years monitoring shows, cocaine, amphetamine and MDMA use has declined in Ljubljana, while the use of cannabis in Maribor and cannabis and cocaine in Domžale-Kamnik increased.

3.2 Further Aspects of Stimulant Use

Within the EWS, we investigated the reasons for the high levels of amphetamine found in waste water in the Municipality of Velenje. According to the data we collected, amphetamine use is present among drug users and construction workers in the Velenje area. This latter group are assumed to use amphetamine to help them handle their daily workload.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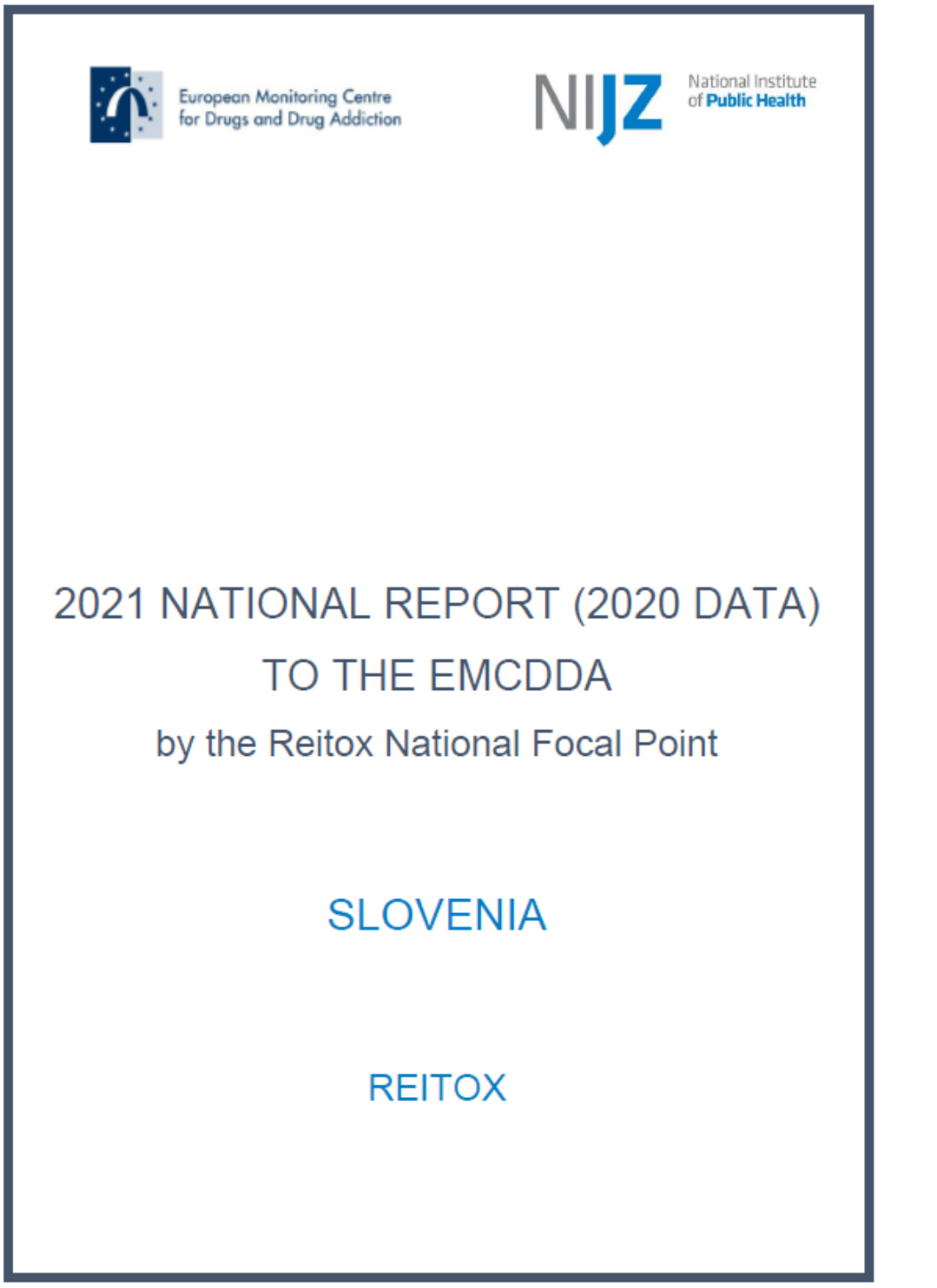
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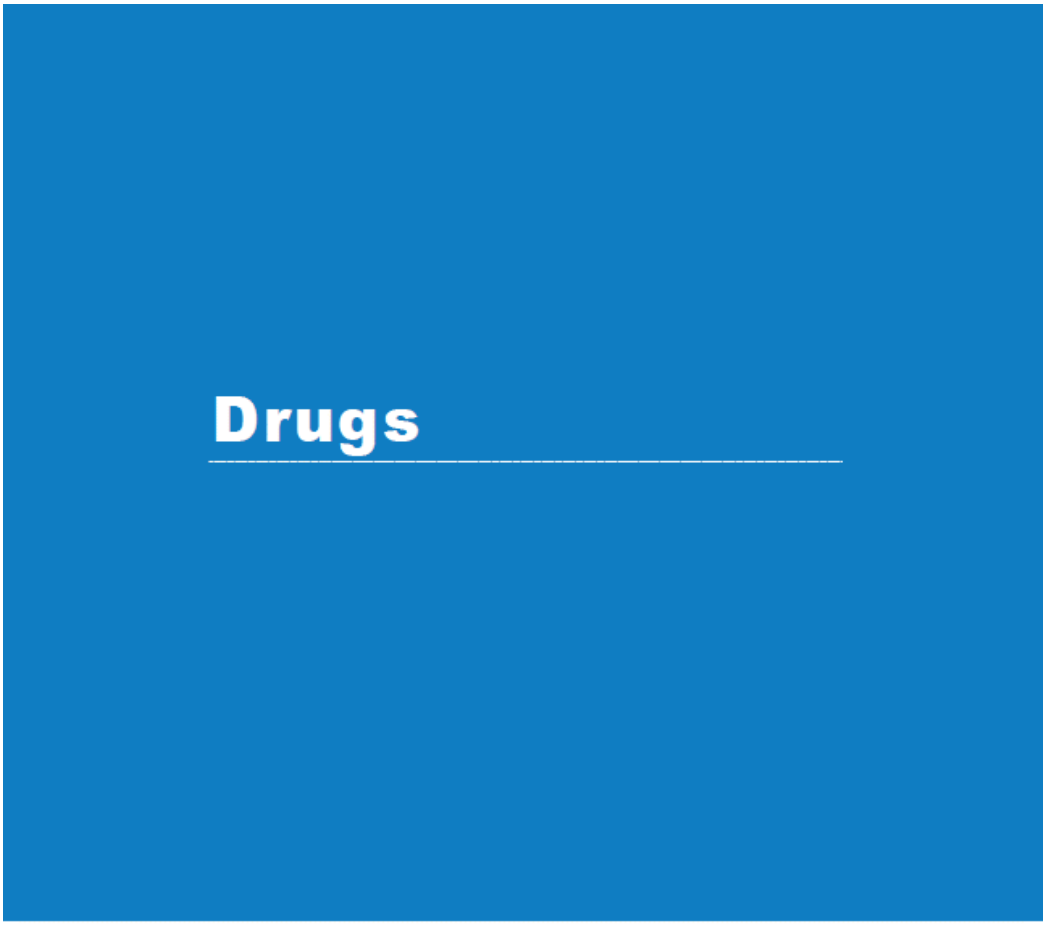
Drugs

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64.7% of respondents (out of all 51 who confirmed the use of at least one of the substances) reported having used cathinones for less than a month, 9.8% reported having used it for 2 years or more, while 13.7% reported that they still used the drug.

When questioned about the effects of the drugs, 52.9% of respondents (out of all 51 who confirmed the use of at least one of the substances) reported positive effects, 37.3% reported mixed effects, i.e. both positive and negative, and 9.8% of respondents reported only negative effects. Negative effects were described primarily as depression, paranoia, headache, feeling unwell, etc.

On a scale of 1 to 5 (1 representing lack of information), respondents also assessed their knowledge on the dangers of using synthetic cathinones, with 56.0% assessing their knowledge with 1 and 3.9% believing that they were well informed. The average amounted to 1.8% and we concluded that there is an even bigger lack of knowledge concerning synthetic cathinones than with synthetic cannabinoids.

Injecting and other Routes of Administration

Among harm reduction programme users who used cocaine in 2020, 67.8% reported they injected it, while 28.3% of amphetamine and methamphetamine users and 12.8% of ecstasy users reported they injected the drug.

Cocaine is the prevalent stimulant drug, injected by harm reduction programme users. There was no significant change in the injection of cocaine between 2019 and 2020.

Half of one per cent of ESPAD respondents aged between 15 and 16 replied that they had injected a drug using a syringe, although this question related to drugs that could be specifically used in this way, e.g. heroin.

2. Additional information

Wastewater-based assessment of drug use in Slovenia

Taja Verovšek, Urška Blaznik, Ada Hočevar Grom, David Heath, Maria Laimou-Geraniou, and Ester Heath

Wastewater-based epidemiology (WBE) was used to investigate the use of four illicit stimulants: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") and cocaine and cannabis in six Slovenian municipalities, including Ljubljana, Maribor, Domžale-Kamnik, Koper, Novo mesto and Velenje. Obtained results were compared with the data obtained for European cities and world capitals within an international monitoring campaign organized by Sewage Analysis CORe group Europe (SCORE) in 2020[1, 2]. Finally, this study examines timely changes in drug use patterns in Slovenian municipalities.

Results:

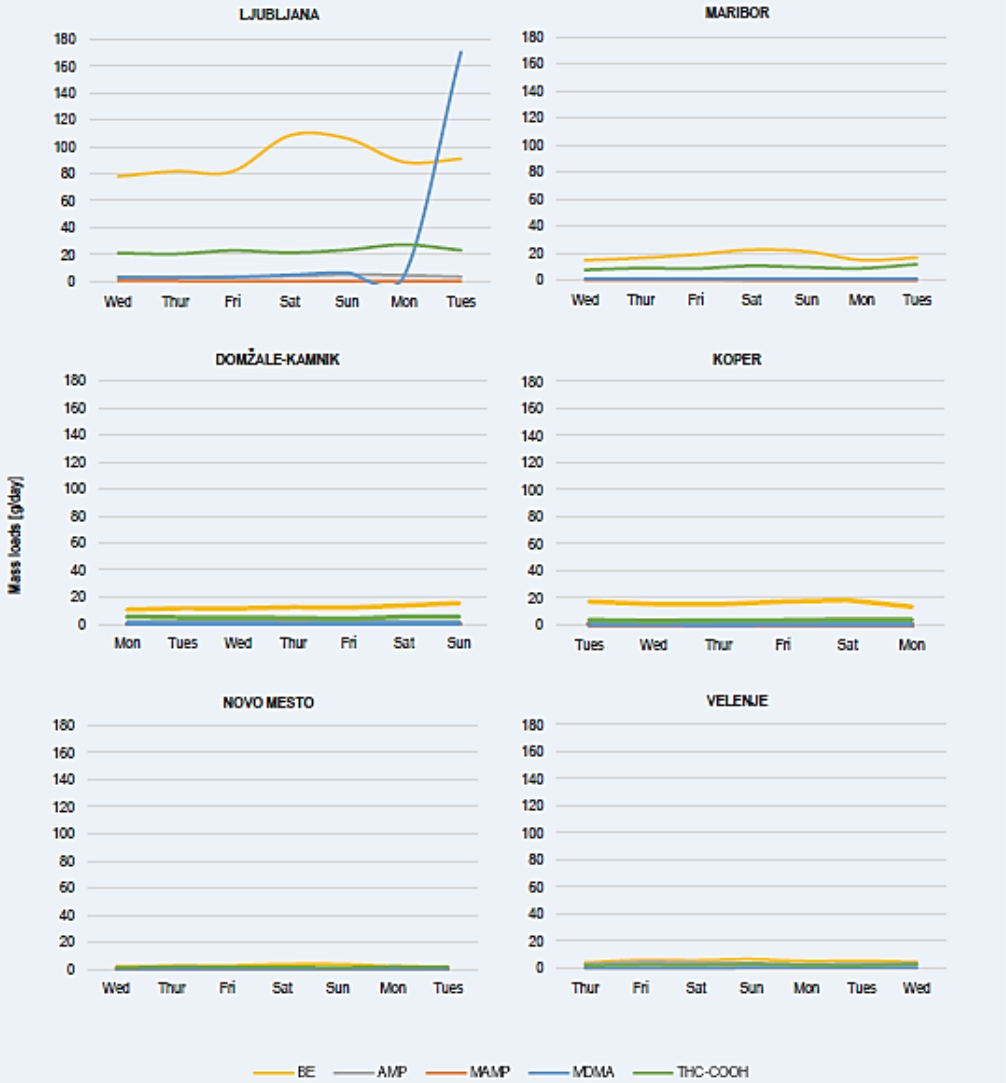
A) Mass loads of selected drug urinary biomarkers

Mass loads of illicit drug biomarkers are used to explore drug use patterns. When analyzing wastewater, stimulant residues show a distinctive weekly pattern [3-5], i.e., reflecting their higher consumption over the weekend than during weekdays, which was also observed in our previous studies (SCORE monitoring campaigns 2017–2019)[6,7]. In contrast, no distinctive weekly patterns were observed for amphetamine, methamphetamine and MDMA in this study, while the levels of benzoylecgonine (cocaine biomarker) did increase over the weekend in Ljubljana, Maribor, Domžale-Kamnik, Koper and Novo mesto. Weekly drug use patterns were also shown to be affected by the COVID-19 lockdowns [8, 9]. Extremely high MDMA mass load was observed in Ljubljana on sampling day seven (Tuesday) and may be related to either excessive consumption, deliberate disposal into the sewer or a combination of both. Although

additional studies are needed to explore the event further, excessive consumption seems the least possible explanation since Slovenia was in lockdown (12.3. – 31.5.2020) over the period when the samples were collected (30.3. – 21.4.2020). As expected, for those drugs used regularly throughout week 3, e.g., 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), a biomarker of cannabis no distinctive weekly patterns were observed.

Figure 1. Plots of daily variations in stimulant biomarkers in 2019

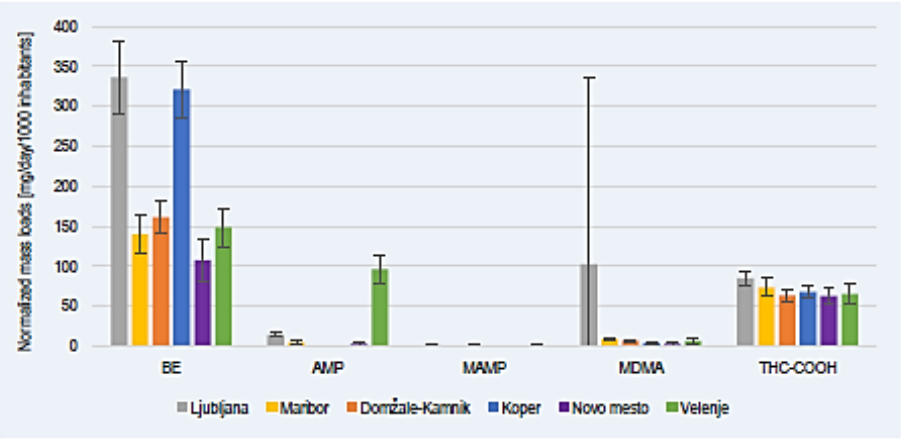
MDMA – 3,4-methylendioxymethamphetamine, MAMP – methamphetamine, AMP – amphetamine, BE – benzoylecgonine



In order to compare data from different-sized cities, all biomarker mass loads were normalized to the population served by each wastewater treatment plant. The highest average mass loads of THC-COOH (84.6 mg/day/1000 inhabitants), MDMA (102.5 mg/day/1000 inhabitants), methamphetamine (1.1 mg/day/1000 inhabitants) and benzoylecgonine (336 mg/day/1000 inhabitants) were observed in Ljubljana, while the average mass load of benzoylecgonine in Koper was right under Ljubljana (321 mg/day/1000 inhabitants). The average mass load of amphetamine (95.8 mg/day/1000 inhabitants) was highest in Velenje (Figure 2).

Figure 2. Histogram showing average mass loads of selected biomarkers in six Slovenian municipalities in 2020

AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol



All the municipalities except Ljubljana scored below the SCORE 2020 averages for the monitored biomarkers (Table 1). In Ljubljana, MDMA was above the average when taking into account all sampling days. However, if day seven is omitted (mass load of 14.6 mg/day/1000 inhabitants), the levels of MDMA match those reported in SCORE 2019 (26.3 mg/day/1000 inhabitants).

Table 1. Average mass loads and the estimated 2020 study averages (AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH - 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol)

Biomarker of illicit drugs	Ljubljana	Velenje	Koper	Maribor	Novo mesto	Domžale-Kamnik	Estimated 2020 study average [1,7]
	Normalized mass loads [mg/day/1000 inhabitants]						
BE	336	148	321	139	107	161	367
MDMA	103 (14.6*)	6.1	3.8	8.7	2.7	7.2	33
AMP	14.1	95.8	n.a.	4.4	2.7	n.a.	171
MAMP	1.1	1.0	0.2	n.a.	n.a.	0.7	61
TCH-COOH	84.6	64.9	67.7	73.6	62.5	63.0	97

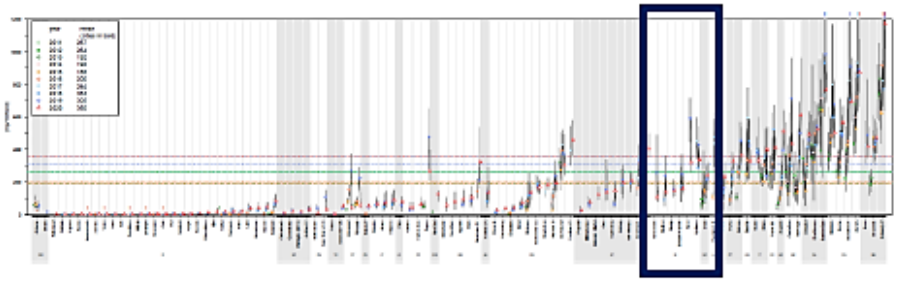
n.a. – not applicable (measured concentrations of biomarker were under the limit of quantification (AMP: 29.6 ng/L) in all obtained raw wastewater samples)

* – MDMA mass load calculated without the day with extreme value

When comparing Slovene municipalities with European cities and other world capitals (n=101) participating in SCORE 2020 (Figure 3. a–d), Slovenian municipalities ranked in the upper half or in the middle regarding benzoylecgonine (Ljubljana: 19 place, Maribor: 38 place, Domžale-Kamnik: 35 place, Koper: 23 place, Novo mesto: 43 place, Velenje: 36 place), while most cities ranked in the bottom with mass loads of the other monitored biomarkers (amphetamine: lower that 71 place, methamphetamine: lower than 69 place and MDMA: lower than 71 place). The exceptions were Velenje and Ljubljana, which ranked in the upper part of the scales based on amphetamine (Velenje: 36 place) and MDMA mass loads (Ljubljana: 3 place). THC-COOH was due to COVID-19 not submitted on time to be included in the SCORE graphical presentation

Figure 3. Ranking of Slovenian municipalities based on benzoylecgonine (a), MDMA (b), amphetamine (c) and methamphetamine (d) mass loads in SCORE 2019 (adapted from SCORE COST webpage – graphical representation of results 2019[1]): y-axe: biomarker mass loads (mg/day/1000 inhabitants), x-axe: participating cities and countries.

(a)



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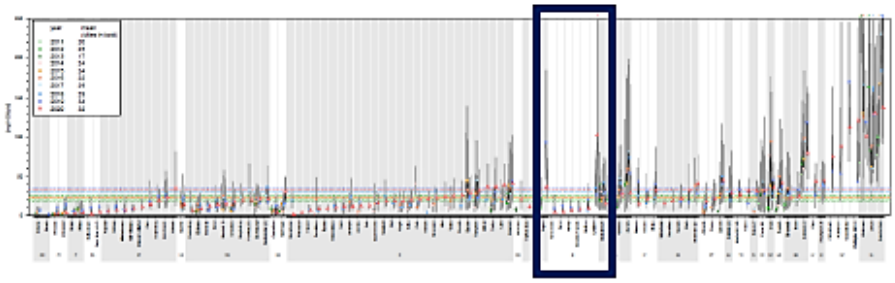

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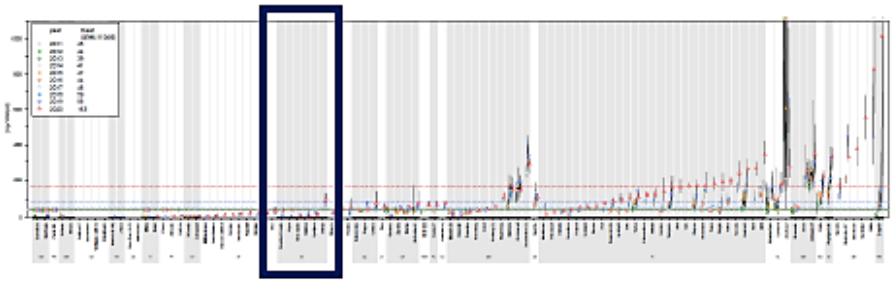
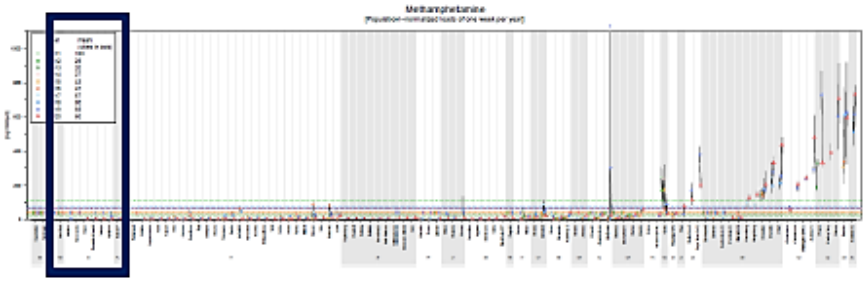


(d)



As all Slovenian municipalities are included in SCORE monitoring for at least two years (Ljubljana: four years; Maribor and Domžale-Kamnik: three years; Koper, Novo mesto and Velenje: two years), timely trends in drug consumption can be explored for all of them. Although some trends can be observed, according to SCORE, at least five annual measurements are needed to predict the timely trends.

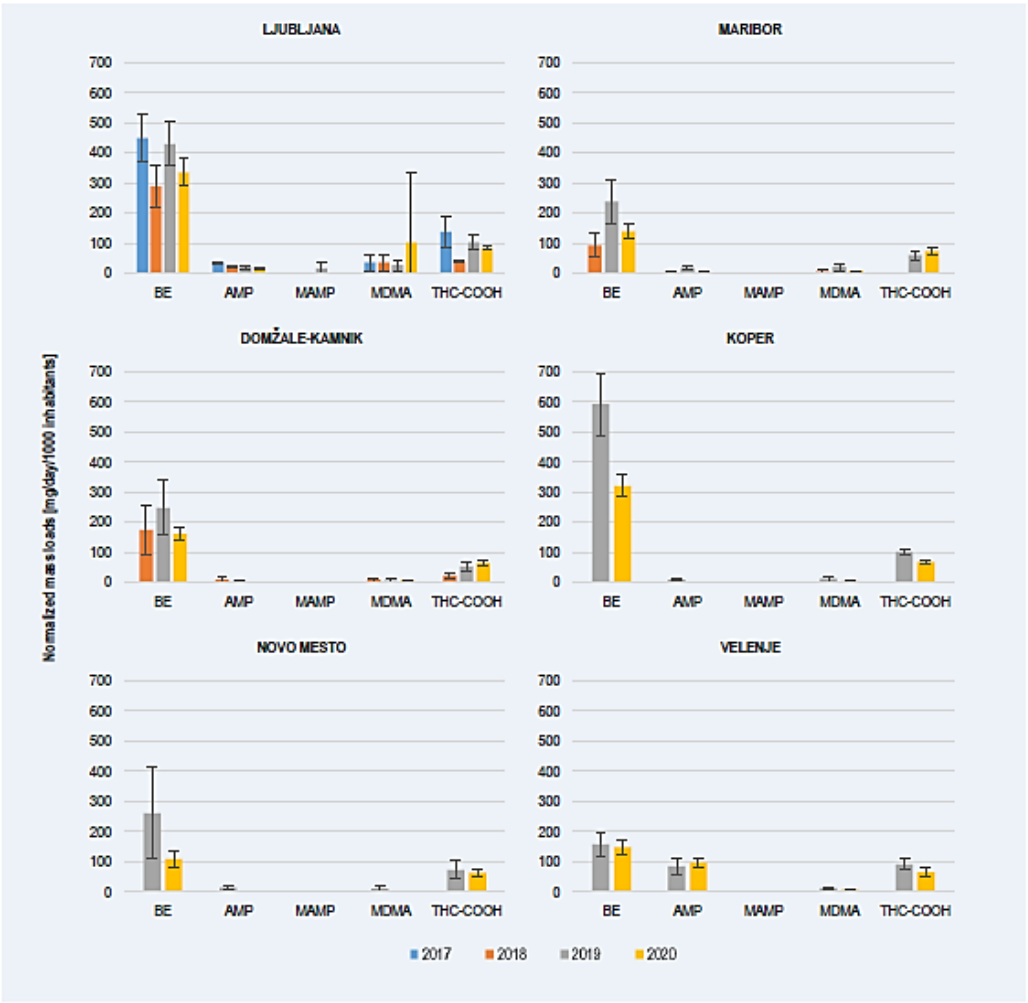
Lower consumption of monitored drugs can be observed in Ljubljana in 2020 in comparison to 2019, except for MDMA use, which was higher (Figure 4). Ljubljana is the only city that has been included in campaign for 4 years and throughout the monitoring program (monitored from 2017), cocaine and THC showed similar consumption trends (highest consumption in 2017 and lowest in 2018), while overall, their consumption is decreased. Similarly, consumption of amphetamine declined. The consumption of methamphetamine was up until 2019 but showed a decrease in 2020.

In Maribor (included in SCORE monitoring since 2018), a decline in stimulant use was observed between 2019 and 2020, while consumption of THC increased. In all three years of monitoring, the highest amounts of cocaine, MDMA and amphetamine residues were detected in 2019.

Compared to 2019, lower consumption of cocaine and amphetamine, unchanged consumption of MDMA and higher consumption of THC was observed in Domžale-Kamnik in 2020. The data also suggests that between three years of monitoring (2018–2020), amphetamine use declined while THC use increased. However, 5 successive year measurements are needed in order to add confidence in trends observed.

For the second time, Koper, Novo mesto and Velenje were included in SCORE (2019 and 2020). Except for methamphetamine, a decline in the consumption of all monitored drugs was observed in Koper while methamphetamine remained comparable. In Novo mesto, consumption of stimulants was lower in 2020, while Velenje stimulant use remained the same.

Figure 4. Histogram of average mass loads of selected illicit stimulant biomarkers for Slovenian municipalities, participating in SCORE monitoring for two or more consecutive years AMP – amphetamine, BE – benzoylecgonine, MAMP – methamphetamine, MDMA – 3, 4-methylendioxymethamphetamine, THC-COOH - 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol



B) Estimation of illicit stimulant use

When biomarkers mass loads are back-calculated to drug consumption (mg of drug/day/1000 inhabitants or doses/day/1000 inhabitants; Table 2), cannabis (THC) is the drug most consumed (average consumption: 137–185 doses/day/1000 inhabitants), while cocaine is the most commonly consumed stimulant (average consumption: 8.6–16.8 doses/day/1000 inhabitants)..

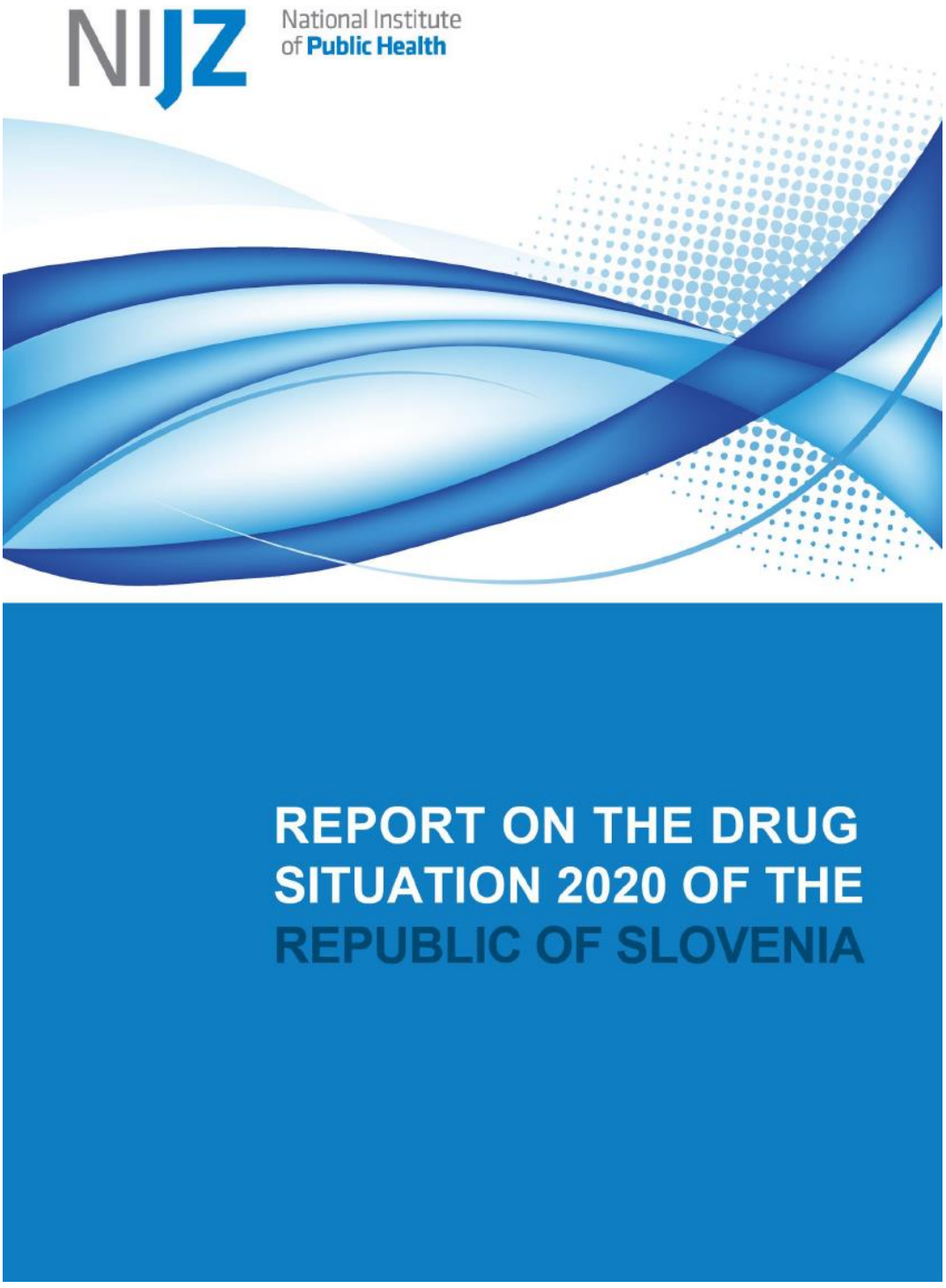
Table 2. Average illicit stimulant use

	Ljubljana	Velenje	Koper	Maribor	Novo mesto	Domžale-Kamnik
	Drug use [mg/day/1000 inhabitants]					
Cocaine	1210	532	1150	501	386	579
Amphetamine	39.0	266	n.a.	24.1	7.37	n.a.
Methamphetamine	4.92	4.28	1.80	n.a.	n.a.	3.21
Ecstasy (MDMA)	4.52	27.0	16.7	38.4	12.0	31.8
Cannabis (THC)	15400	11800	12300	13400	11400	11500
	Drug use [doses/day/1000 inhabitants]					
Cocaine	26.8	11.8	25.6	11.1	8.60	12.9
Amphetamine	0.82	5.59	n.a.	0.51	0.16	n.a.
Methamphetamine	0.25	0.21	0.09	n.a.	n.a.	0.16
Ecstasy (MDMA)	4.75	0.28	0.18	0.40	0.13	0.34
Cannabis (THC)	185	142	148	162	137	138

n.a. – not applicable (measured concentrations of the biomarker in all raw wastewater samples were under the limit of quantification)
THC – Δ-9-tetrahydrocannabinol

Conclusions

In 2020, six Slovenian municipalities (Ljubljana, Maribor, Domžale-Kamnik, Koper, Novo mesto and Velenje) participated in the SCORE. The data show cocaine, MDMA, methamphetamine and cannabis/THC use was the highest in Ljubljana, while Velenje had the highest amphetamine use. Except for MDMA in Ljubljana (when all sampling days are accounted for), all Slovenian municipalities were below the SCORE (2021) average for all monitored biomarkers. Cocaine use ranked in the upper half or middle of participating municipalities, while other drugs (except amphetamine in Velenje and MDMA in Ljubljana) ranked in the bottom half. Timely trends in drug use were specific for each Slovenian municipality; however, at least five consecutive annual measurements are needed to gain more confidence in trends observed.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SITUATION 2020 OF THE
REPUBLIC OF SLOVENIA

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NIJZ National Institute of Public Health

2020 NATIONAL REPORT (2019 DATA) TO THE EMCDDA

by the Reitox National Focal Point

SLOVENIA

REITOX

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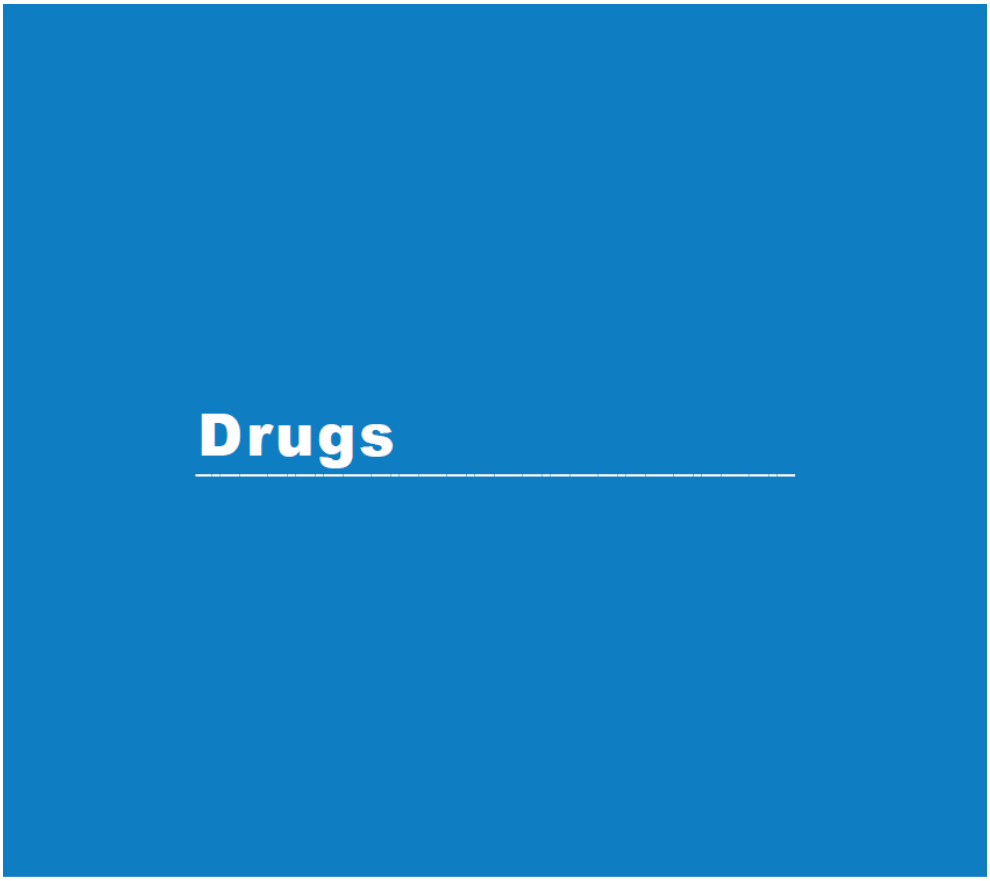
Drugs

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On a scale of 1 to 5 (1 representing lack of information), respondents also assessed their knowledge on the dangers of using synthetic cathinones, with 56.0% assessing their knowledge with 1 and 3.9% believing that they were well informed. The average amounted to 1.8% and we concluded that there is an even bigger lack of knowledge concerning synthetic cathinones than with synthetic cannabinoids.

1.2.5 Injecting and other Routes of Administration

Among harm reduction programme users who used cocaine in 2019, 66.5%reported they injected it, while 34.5% of amphetamine and methamphetamine users and 8%of ecstasy users reported they injected the drug.

Cocaine is the prevalent stimulant drug, injected by harm reduction programme users. In the period from 2015 (71.9%) to 2017 (77.4%), injecting cocaine as a route of administration increased, since 2018, it has been decreasing.

Half of one per cent of ESPAD respondents aged between 15 and 16 replied that they had injected a drug using a syringe, although this question related to drugs that could be specifically used in this way, e.g. heroin.

2. Additional information

2.1 Additional Sources of Information

Taja Verovšek, Ivona Krizman-Matasić, Urška Blaznik, Ada Hočevar Grom, Tina Kosjek in Ester Heath

Wastewater-based assessment of drug use in Slovenia

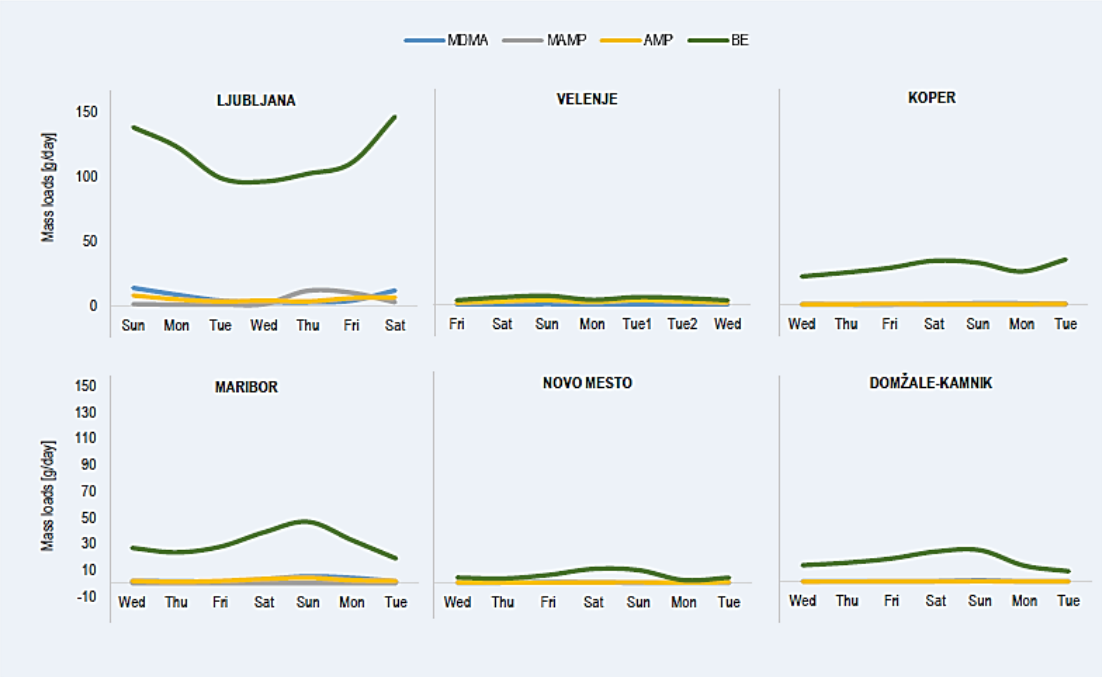
This study used Wastewater-based epidemiology (WBE) to investigate the use of four illicit stimulants: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") and cocaine in six Slovenian municipalities, including Ljubljana, Velenje, Koper, Maribor, Novo mesto and Domžale-Kamnik. The latest data (2019) were compared with WBE data obtained for European cities and world capitals, which participated in an international monitoring campaign, organized by Sewage Analysis CORe group Europe (SCORE) in 2019 [1, 2]. The data were also compared with the results obtained from the latest general population survey conducted among the Slovenian population in 2018. Finally, this study examines drug use patterns in Slovenian municipalities: Ljubljana, Maribor, and Domžale-Kamnik, that have participated in the SCORE program for two or more consecutive years.

Results:

A) Mass loads of selected drug urinary biomarkers

Illicit drug use patterns can be studied based on mass loads of illicit drug biomarkers. In this study, higher mass loads of all analyzed urinary biomarkers (benzoylecgonine, amphetamine, methamphetamine, MDMA) were observed during the weekends, suggesting higher use of illicit stimulants over this period in all six municipalities (Figure 8). Increased use of stimulant drugs has already been observed in different cities and municipalities over the weekend [3-5], including Ljubljana, Maribor, and Domžale-Kamnik (SCORE monitoring 2018[6]). However, 2019 saw an increase in mass loads of benzoylecgonine and amphetamine in Velenje midweek, although the reason for this remains unknown.

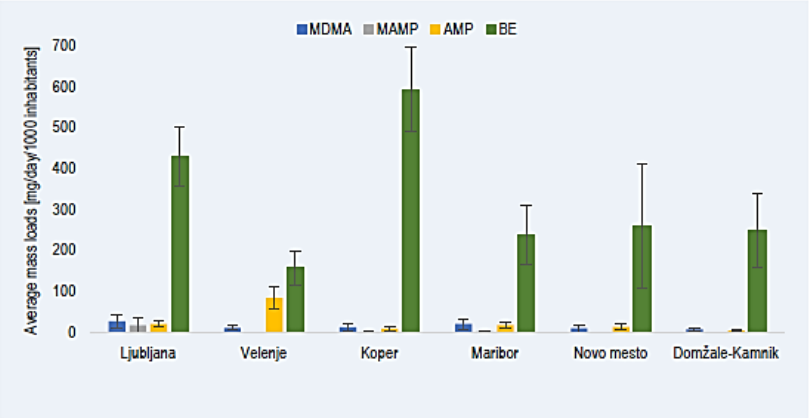
Figure 8. Plots of daily variations in stimulant biomarkers in 2019, MDMA – 3,4-methylendioxymethamphetamine, MAMP – methamphetamine, AMP – amphetamine, BE – benzoylecgonine



Source: Jožef Stefan Institute

Biomarker mass loads were normalized to the population served by each wastewater treatment plant to compare data from different-sized cities. Similar to 2018, the highest mass loads in wastewater from all Slovenian municipalities were observed for cocaine biomarker, benzoylecgonine (93–717 mg/day/1000 inhabitants) while the mass loads of other drug biomarkers were lower (up to 120 mg/day/1000 inhabitants). The highest average mass load of benzoylecgonine was observed in Koper (593 mg/day/1000 inhabitants), average mass loads of MDMA (26 mg/day/1000 inhabitants) and methamphetamine (17 mg/day/1000 inhabitants) in Ljubljana and amphetamine (84 mg/day/1000 inhabitants) in Velenje (Figure 9).

Figure 9. Histogram showing average mass loads of selected illicit stimulant biomarkers in six Slovenian municipalities in 2019, MDMA – 3,4-methylendioxymethamphetamine, MAMP – methamphetamine, AMP – amphetamine, BE – benzoylecgonine



Source: Jožef Stefan Institute

Table 12 presents the average mass loads calculated for six Slovenian municipalities, together with the SCORE 2019 averages (average mass loads of cities that participated in the monitoring campaign organized by the SCORE group for more than five years). Nearly all of the Slovenian municipalities scored below the study average for the majority of the monitored biomarkers. The exceptions were the average mass loads of benzoylecgonine in Koper and Ljubljana, and the mass loads of amphetamine in Velenje, which was slightly above the study average.

Table 12. Average mass loads and the estimated 2019 study averages, expressed in mg/day/1000 inhabitants (BE – benzoylecgonine, MDMA – 3,4-methylendioxymethamphetamine, AMP – amphetamine, MAMP – methamphetamine)

Biomarker of illicit drugs	Ljubljana	Velenje	Koper	Maribor	Novo mesto	Domžale-Kamnik	Estimated 2019 study average [1,7]
BE	430	157	593	237	260	248	380
MDMA	26	10	12	20	8.8	6.6	38
AMP	20	84	8.2	18	13	3.9	81
MAMP	17	n.a.	0.5	0.8	n.a.	0.4	70

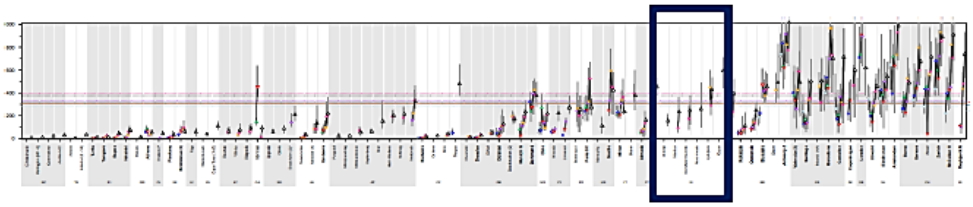
n.a. – not applicable (measured concentrations of biomarker were under the limit of quantification (2.3 ng/L) in all obtained raw wastewater samples)

Source: Jožef Stefan Institute

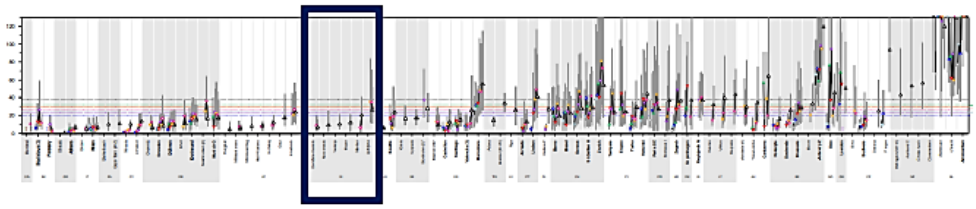
Among all SCORE 2019 participating countries (n=29) and cities (n=86), Slovenian municipalities ranked in the upper half or in the middle in the case of benzoylecgonine, while majority of the Slovenian cities ranked in the lower part for the remaining monitored biomarkers. The exceptions were Velenje and Ljubljana, which ranked in the middle of the scales based on amphetamine and MDMA mass loads (Figure 10. (a) – (d)).

Figure 10. Ranking of Slovenian municipalities based on benzoylecgonine (a), MDMA (b), amphetamine (c) and methamphetamine (d) mass loads in SCORE 2019 (adapted from SCORE COST webpage – graphical representation of results 2019[1]): y-axe: biomarker mass loads (mg/day/1000 inhabitants), x-axe: participating cities and countries

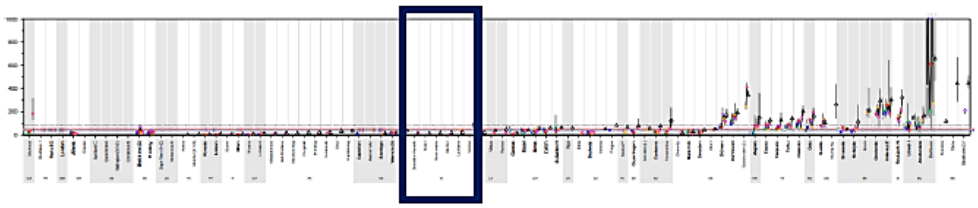
(a)



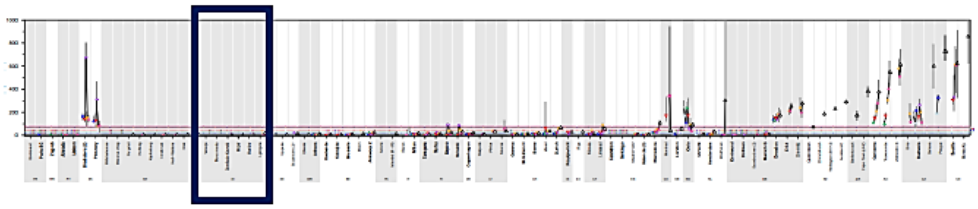
(b)



(c)



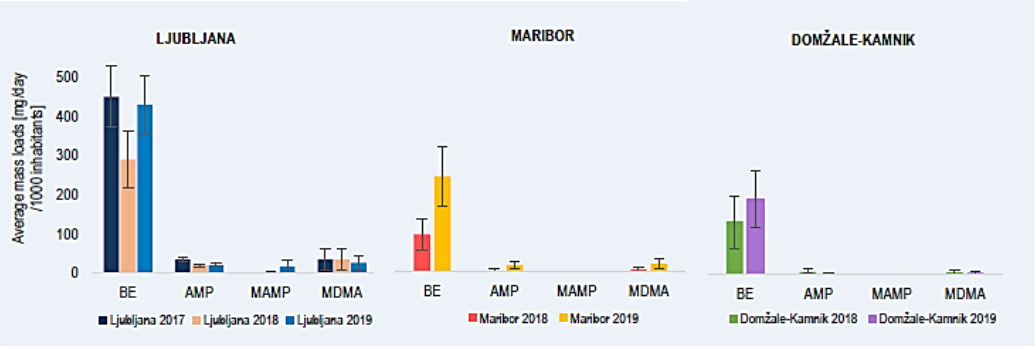
(d)



Source: Jožef Stefan Institute

As can be seen from Figure 11, in comparison to 2018, higher mass loads of benzoylecgonine can be observed in Slovenian municipalities participating in SCORE 2019 and 2018 (e.g., Ljubljana, Maribor, Domžale-Kamnik), suggesting increased cocaine consumption. However, the data for 2019 for Ljubljana is comparable to the 2017 and as suggested by SCORE, monitoring of at least 5 consecutive years is needed to make firm conclusions on drug use patterns. The data also show an increase in the consumption of methamphetamine, while the amphetamine and MDMA use was similar or slightly lower than in 2018. In Maribor, mass loads of all biomarkers increased in 2019. In Domžale-Kamnik, stimulant drug use (except cocaine) derived from the biomarker mass loads is comparable to use in 2018.

Figure 11. Histogram of average mass loads of selected illicit stimulant biomarkers for Slovenian municipalities, participating in SCORE monitoring for two or more consecutive years
BE – benzoylecgonine, AMP – amphetamine, MAMP – methamphetamine, MDMA – 3,4-methylendioxymethamphetamine



Source: Jožef Stefan Institute

B) Estimation of illicit stimulant use

Table 13 summarizes the average illicit stimulant use estimates in Slovenian municipalities. Cocaine use was highest in all six municipalities (334–2574 mg/day/1000 inhabitants), while the consumption of MDMA (8.5–229 mg/day/1000 inhabitants), amphetamine (6.0–331 mg/day/1000 inhabitants) and methamphetamine were lower (up to 194 mg/day/1000 inhabitants).

Table 13. Average illicit stimulant use

	Ljubljana	Velenje	Koper	Maribor	Novo mesto	Domžale-Kamnik
	Drug use [mg/day/1000 inhabitants]					
Cocaine	1543	562	2128	853	932	890
Amphetamine	56	232	23	49	36	11
Methamphetamine	73	n.a.	2.0	3.7	n.a.	1.1
Ecstasy (MDMA)	116	45	52	88	39	29
	Drug use [doses/day/1000 inhabitants]					
Cocaine	34	12	47	19	21	20
Amphetamine	1.2	4.9	0.48	1.0	0.76	0.23
Methamphetamine	3.6	n.a.	0.10	0.18	n.a.	0.06
Ecstasy (MDMA)	1.2	0.47	0.54	0.93	0.41	0.31

n.a. – not applicable (measured concentrations of the biomarker in all raw wastewater samples were under the limit of quantification)

Source: Jožef Stefan Institute

Comparison of the WBE results and results, obtained from survey 2018

A comparison of the results on illicit stimulant use obtained by SCORE 2019 and those reported in the 2018 National Survey on the Use of Tobacco, Alcohol and other Drugs in Slovenia [6] was made. According to the survey, cocaine (lifetime use reported by 2.6% of participants), ecstasy (lifetime use reported by 2.9% of participants) and amphetamine (lifetime use reported by 2.3% of participants) were the most commonly used stimulants among Slovenian inhabitants, aged 15–64. However, analysis of wastewater collected from the six Slovenian municipalities revealed that cocaine use (334–2574 mg/day/1000 inhabitants) is the highest among the illicit stimulants (MDMA: 8.5–229 mg/day/1000 inhabitants, amphetamine: 6.0–331 mg/day/1000 inhabitants, methamphetamine: up to 194 mg/day/1000 inhabitants). This discrepancy in the results may be a result of differences in the methodology used in each approach (questionnaire *vs* chemical analysis) and in the targeted populations. For example, 9161 Slovenes participated in the general population survey, while the SCORE project particular municipalities were targeted (in total 582761 people – based on census data). Also, the studies were conducted in different years (survey in 2018, WBE in 2019).

Conclusions

In 2019, six Slovenian municipalities (Ljubljana, Velenje, Koper, Maribor, Novo mesto, Domžale-Kamnik) participated in an international WBE study looking at the use of illicit stimulants. The data showed that Slovenian municipalities ranked in the upper half for cocaine use (the highest consumption in Koper), and the lower half regarding the other illicit stimulants. The exceptions are amphetamine and MDMA use where Velenje and Ljubljana ranked in the middle. Also, cocaine use was highest in Slovenian municipalities. The results are only partially in agreement with the 2018 survey, where along with cocaine, MDMA and amphetamine were reported as the most commonly used illicit stimulants. When comparing yearly WBE results (e.g., results obtained in 2018 and 2019), higher mass loads of cocaine biomarker were observed in Ljubljana, Maribor and Domžale-Kamnik. However, a more extended monitoring period is necessary to make firm conclusions about multiannual trends.

2.2 Further Aspects of Stimulant Use

Simon Kovačič

As part of the Kemseks programme, which involved three organisations (Društvo ŠKUC, Društvo informacijski center Legebitra and DrogArt), a snapshot of the situation was taken in 2019 involving 114 men who engage in sexual activity with other men and use psychoactive substances during sex. The main reasons why they used drugs during sex ('chemsex') were: to increase pleasure, to increase the duration and intensity of sex, to avoid discomfort during sex and to forget personal problems.

The drugs most often used in chemsex in Slovenia are GHB/GBL, ecstasy (MDMA), amphetamines, cocaine and 3-MMC (also known in Slovenia as 'sladoled' or 'ice cream'), followed by mephedrone (4-MMC), methamphetamine, methylone and ketamine. The use of poppers is also common. Just over a third of users of GHB/GBL had overdosed at some point in the last 12 months.

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

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Due to the legality of use, the dynamic market and the lack of data on identity and safety, NPS represent a global public health problem that requires comprehensive information on NPS availability and trends in their use. However, only limited data on the global prevalence of NPS is available (Chapters 1.1.1 New psychoactive substances and 1.2.4 NPS market). To fill the gap, this international study aimed to obtain spatiotemporal trends on NPS using synchronized wastewater analysis protocol and compare the data with the data available from other sources.

A total of 546 municipal wastewater samples were obtained in three New Year periods, namely 2019/2020, 2021/2022, and 2021/2022, whereby the number of participating countries (sites) increased from 8 (12) to 10 (25) and in the last year of the study at 16 (47). Notably, in the New Year period 2021/2022, Slovenia joined the study with Ljubljana, Maribor, Novo mesto and Kranj. The comparability of the results was ensured by applying a synchronized protocol. Each participating country was required to obtain a maximum of seven consecutive 24-h composite samples of raw wastewater per sampling site during a predefined period (*e.g.,* 29.12. 2021–4.1.2022) and extract analytes using the same extraction protocol (SPE: UCT XtracT DAU cartridges), while the extracts were analyzed at the University of Queensland, Australia.

In line with UNODC reports [2], NPS (n=18) detected within three years mainly were synthetic cathinones, followed by phenethylamines and benzodiazepines. They were used across all participating countries following distinctive regional trends, *i.e.,* 3-MMC and *N*-ethylhexedrone were most common in Europe, eutylone and mephedrone in New Zealand, 2F-DCK in China, while the highest mass loads of mitragynine were obtained in the US. Changes in timely trends were also observed, including the impact of the COVID-19 pandemic. However, 3-MMC was consistently found, with its use spreading (from Europe to Oceania) and increasing (from 5 in 2019/2020 to >100 mg/day/1000 people in 2021/2022, with the highest mass loads observed in Spain and Slovenia). Aside from 3-MMC, eutylone and mitragynine were also detected in Slovenia.

The results demonstrate the utility of wastewater analysis for monitoring NPS use in the general population globally. The study also provides the basis for further systematic monitoring of the use of specific NPS through wastewater analysis, following the example of the already established SCORE monitoring and developing new workflows, including non-target analysis.

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

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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.

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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 analyzed, 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 analyzed 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	NL	NL	N.A.	N.A.	AU	N.A.	NL	AU	N.D.	N.D.	NL	N.A.	N.A.	AU	US	N.D.
		IT								NZ			NZ			NZ		
		NL														US		
2020-21	N.D.	BE	N.D.	N.D.	N.A.	N.A.	AU	AU	N.D.	AU	AU	AU	AU	N.A.	N.A.	AU	US	NZ
		ES					CA	CA		CA			NZ			CA		
		IT					NZ	NZ		NZ						KR		
		NZ						US		US						NZ		
2021-22	CA	BE	N.D.	N.A.	IS	CA	N.A.	AU	N.A.	NZ	N.A.	N.A.	ES	AU	BE	N.D.	N.A.	N.A.
	CN	ES				IS		BE		ES				BE	ES			
	ES	FR				IT		BR						BR	SE			
	FR	GR				KR		CA						CA				
	IS	IS				NZ		ES						CY				
	IT	IT				SE		GR						ES				
	US	NZ				US		IT						FR				
		SE						KR						GR				
		SI						NZ						IS				
		US						SI						IT				
								US						KR				
														NZ				
														SE				
														SI				
														US				

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

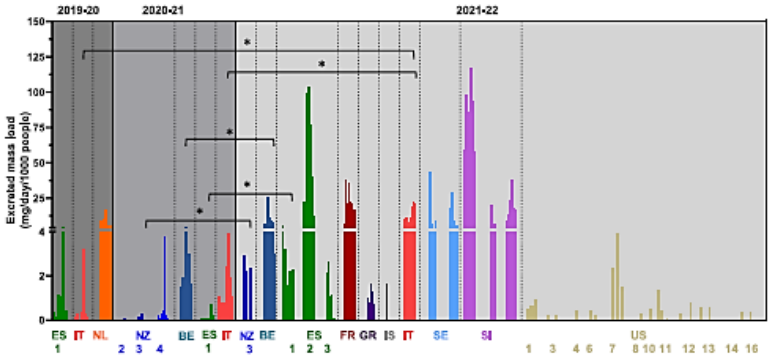

Fig. 1. Estimated excreted mass loads of 3-MMC from all sites over the three-year sampling period. Note: 2019–20 and 2020–21 values are taken from previous publications (Bade et al., 2021; Bade et al., 2022). Sites are ordered by continent. Countries with multiple sites are numbered according to Table 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 analyzed 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. 2B). 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 emphasized 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,

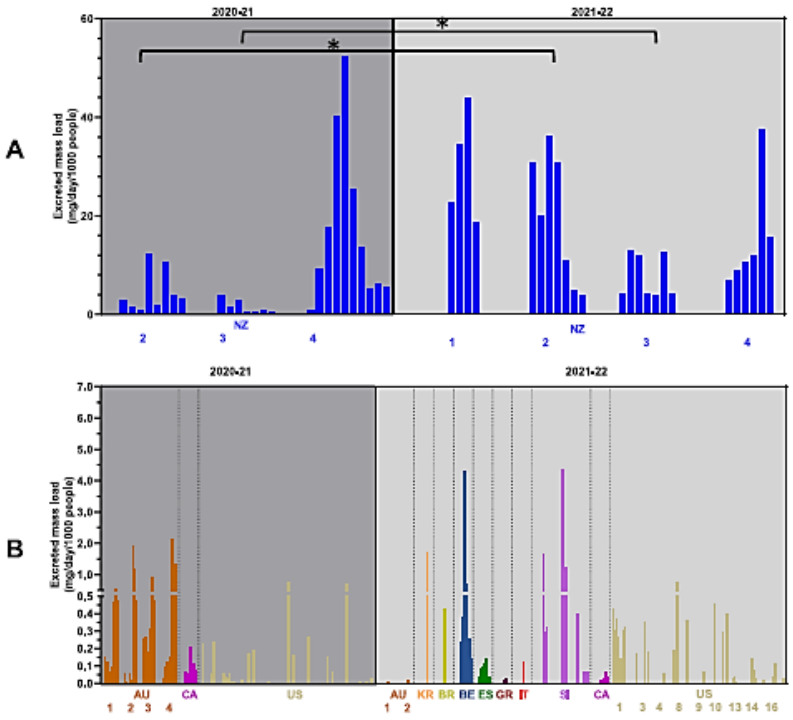

Fig. 2. Estimated excreted mass loads of eutylone from all sites. Figure is separated to show the sites with highest levels (A) and the lower levels (B). Note: 2020–21 values are taken from a previous publications (Bade et al., 2022). Sites are ordered by continent. Countries with multiple sites are 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 hypothesized 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 hypothesize 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 Organization (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

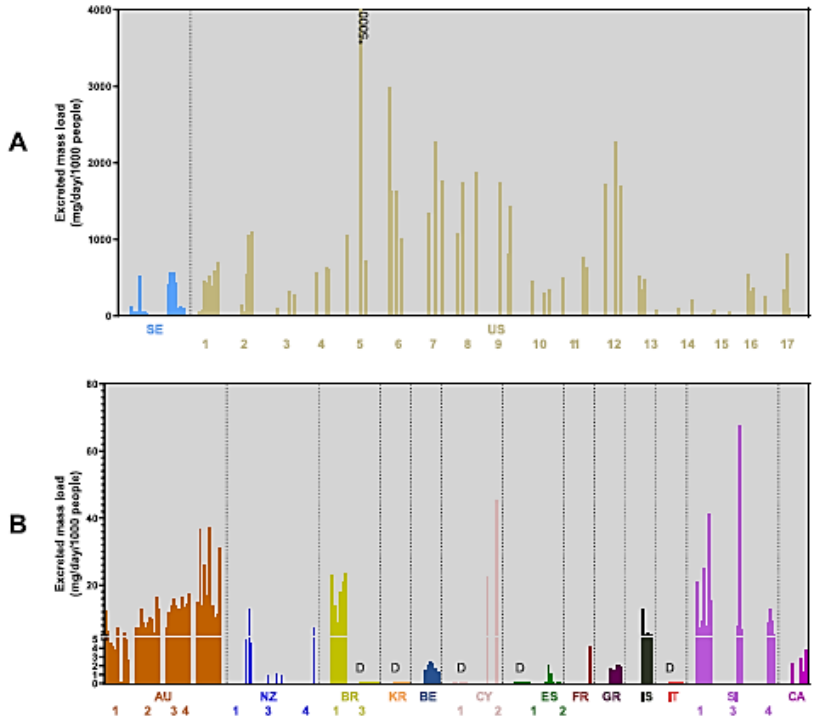

Fig. 3. Estimated excreted mass loads of mitragynine from all sites from the 2021–22 collection. Sites are ordered by continent. Figure is separated to show the sites with highest levels (A) and the lower levels (B). *D* = detected at levels below LOQ. Countries with multiple sites are numbered according 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.

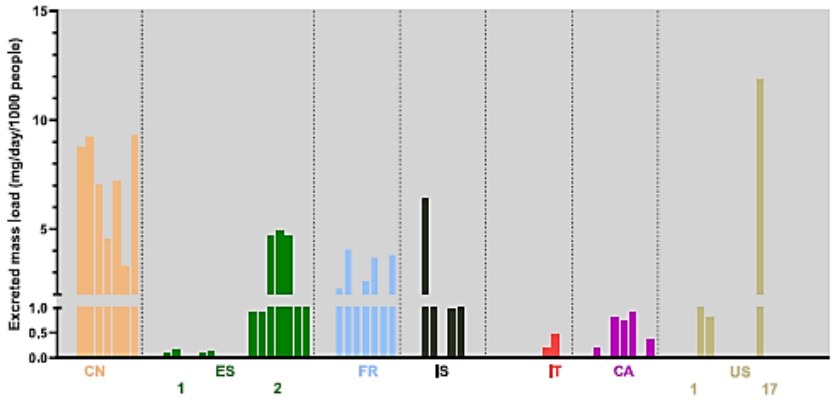

Fig. 4. Estimated excreted mass loads of 2F-DCK from all sites from the 2021–22 collection. Sites are ordered by continent. Countries with multiple sites are numbered according to Table S3. Only sites where the compound was found are included in the figure.
CA: Canada; CN: China; ES: Spain; FR: France;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

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Declaration of Competing Interest

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Data availability

Data will be made available on request

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Supplementary materials

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3.2 Applicability of WBE to Assess Drug Use in Specific Populations

3.2.1 Site- and event-specific wastewater-based epidemiology: Current status and future perspectives

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Due to its non-invasiveness, objectivity and capability of providing near-real-time data, WBE is suitable for monitoring drug use trends on the level of small, specific communities. Accordingly, it has already been used to assess licit (including NPS) and illicit drug use in specific populations, namely educational institutions, prisons, fitness centers and an airport (Chapter 1.3.4 WBE: Specific populations) and during special events, such as public holidays, sporting events, and festivals (Chapter 1.3.4.1 WBE: Special events). Although the number of sites- and event-specific WBE studies is increasing, such studies have not yet been included in any comprehensive literature review. In order to fill this gap, the methods and results of the published site- and event-specific studies were summarized, and current challenges and future perspectives were elaborated.

The literature has shown that WBE can be a valuable source of information on drug use in specific populations and can act as an early warning of changes in drug use trends when applied during special events. However, its application in specific sites is not without its limitations. Currently, the main limitations are related to difficulties in obtaining representative wastewater samples (in the case of sampling wastewater in small populations, where wastewater flow is inconsistent), pronounced variability in the drug excretion profile of individuals, the inability to extrapolate results to a particular group of people who contributed to wastewater sample, and ethical considerations (Chapters 1.3.4 WBE: Specific populations and 1.3.4.1 WBE: Special events). Given the many advantages of site- and event-specific WBE, further studies on methodology and applicability are relevant.

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Site- and event-specific wastewater-based epidemiology: Current status and future perspectives

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ABSTRACT

Wastewater-based epidemiology (WBE) can provide objective and reliable data to monitor spatio-temporal patterns of licit and illicit drug use. Numerous studies have been published relating to sampling, sample stability, validation of analytical protocols and the back-calculation of drug consumption. The majority of these studies focus on sampling from municipal sewage treatment plants, but an increasing number of studies have used WBE to monitor community-specific substance use and use during special events. This paper presents a systematic review of published WBE studies of drug use trends in educational institutions and prisons, as well as during music festivals, sporting events, and holidays. A discussion on the application and benefits of using wastewater-based epidemiology in these specific cases is presented together with an examination of current challenges and future perspectives.

1. Introduction

Licit and illicit drug abuse is a global issue and represents a social, economic and health burden for the abuser and on society [1]. According to World Health Organization (WHO) data, 2.3 billion people drink alcohol worldwide, and about 1.1 billion people smoke tobacco (15 years and older), making them the most popular licit drugs [2]. The European Drug Report (EDR) states that 29 % of adults (15–64 years) and 16 % of young adults (15–34 years) have tried illicit drugs [3]. Over the last decade, a large variety of new psychoactive substances (NPSs) have emerged that can be easily bought either on the drug market or in physical and online shops and on the darknet [3]. In this review, the consumption of the most common licit drugs (alcohol and nicotine/tobacco), and illicit drugs (cocaine, amphetamine, methamphetamine, ecstasy, lysergic acid diethylamide – LSD, heroin and cannabis), together

Abbreviations: 2CB, 2,5-dimethoxy-4-bromophenethylamine; 4-FA, 4-fluoroamphetamine; 4-MEC, 4-methylethcathinone; 6-AM, 6-acetylmorphine; ADHD, attention deficit hyperactivity disorder; AE, anhydroecgonine; AEME, anhydroecgonine methyl ester; AMP, amphetamine; BE, benzoylecgonine; BUP, buprenorphine; COC, cocaine; COD, codeine; COE, cocaethylene; COST, the European Cooperation in Science and Technology; COT, cotinine; DHNK, dehydronorketamine; ECG, ecgonine; EDDP, 2-ethylidene-15-dimethyl-3,3-diphenylpyrrolidine; EDR, the European Drug Report; EMCDDA, the European Monitoring Centre for Drugs and Drug Addiction; EME, ecgonine methyl ester; EPH, ephedrine; ESPAD, European School Survey Project on Alcohol and Other Drugs; EtS, ethyl sulfate; GHB, gamma-hydroxybutyrate; HBSC, Health Behavior in School-aged Children survey; HER, heroin; KET, ketamine; LC, liquid chromatography; LOQ, limit of quantification; LSD, lysergic acid diethylamide; LSD-OH, 2-oxo-3-hydroxy-lysergic acid diethylamide; M3G, morphine-3-glucuronide; MAMP, methamphetamine; MBDB, 3,4-methylenedioxy-N-methyl-butanphenamine; mCPP, meta-chlorophenylpiperazine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDPV, methylenedioxypyrovalerone; MDT, mandatory drug testing; MEPH, mephedrone; MOR, morphine; MPA, methiopropamine; MS/MS, tandem mass spectrometry; MTHD, methadone; MXE, methoxetamine; ND, not detected; NIC, nicotine; nor-BE, nor-benzoylecgonine; nor-COC, nor-cocaine; nor-KET, nor-ketamine; nor-LSD, N-demethyl-lysergic acid diethylamide; NPS, new psychoactive substance; PCP, phencyclidine; PEPH, pseudoephedrine; PMA, 4-methoxyamphetamine; PMMA, 4-methoxymethamphetamine; SCORE, Sewage analysis CORe group Europe; SPE, solid-phase extraction; THC, tetrahydrocannabinol; THC-COOH, 11-nor-Δ9-carboxy-Δ9-tetrahydrocannabinol; THC-OH, 11-hydroxy-Δ9-tetrahydrocannabinol; US, the United States; USA, the United States of America; WBE, Wastewater-based epidemiology; WHO, World Health Organization; WWTP, wastewater treatment plant.

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Table 1
Overview of WBE application in site-specific studies.

Population	Country	Sampling period	Type of raw wastewater sample	Sampling location and type of sampling	Target drugs	Reference
EDUCATIONAL INSTITUTIONS						
Educational institution *(less than 1 000 students)*	USA	Different days during regular class sessions, final exams and summer break *(sampling between 10 a.m.–12 a.m. and 12 a.m.–2 p.m.)*	2 -h composite samples	Sewer pipe output from the building Manual collection *(up to 500 mL every 20 min)*	Illicit drugs (cannabis, MDMA, AMP, COC, HER) NPSs (MDA) Opioids (COD, MOR)	[25]
Main university campus and dorms *(9 456 people in the university campus and 560 people in dorms)*	USA	a) Dorms (2012–2013): - Beginning of the semester (12 days at the beginning of September) - End of the semester (9 days during the second and third week in December) - Middle of the semester (17 days between February and March) b) Main university campus (sampling over 3 weeks in April 2013)	24 -h composite samples	Pump station Time-proportional sampling *(200 mL every4 h)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER, LSD) NPSs (MDA, MDEA) Opioids (COD, MOR, MTHD)	[32]
Private college of art *(4 dorms, 476 students)*	USA	a) First semester (4 samples): the first week, midterms, post-midterms, finals week (August–December 2011) b) Second semester (5 samples): the first week, midterms, post-midterms, last week, finals week *(January–July 2012)*	72 -h composite samples	Sanitary sewer line Time-proportional samples *(125 mL every hour)* integrated into a total volume over 72 -h period regarding the measured flow	Illicit drugs (AMP)	[22]
College campus (4 residences halls, 476 undergraduate students)	USA	The first week of school, midterms and shortly before final exams *Additionally: Web-based surveys (400–627 respondents) was conducted in the same time frames*	72 -h composite samples	On-campus sampling location Time-proportional samples *(125 mL every hour)* integrated into the total volume over 72 -h period regarding the measured flow	Illicit drugs (AMP)	[28]
University campus *(in total 15 000–60 000 persons)*	USA	7 consecutive samples, collected once per month over 5 months *(August–December 2017)*	24 -h composite samples	Two sampling locations at the campus sewer system Flow-proportional sampling	Illicit drugs (AMP, MDMA, COC, HER) Opioids (MOR, COD, MTHD, BUP, fentanyl)	[33]
8 secondary schools *(3 classic, scientific or artistic education and 5 professional or vocational schools)*, located in different cities *(Bologna, Florence, Milan, Naples, Palermo, Rome, Turin, Verona)* *(in total: <6 000 students)*	Italy	a) 5 or 6 consecutive daily samples *(May 2010)* b) Repeated sampling in schools from Rome, Turin, Verona *(October 2011, March and October 2012, November 2013)*	8 -h composite samples *(samples collected during lessons period)*	Main sewage pipe Time-proportional sampling *(1 L every hour)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER) NPSs (KET, MEPH) Opioids (MOR)	[24]
University campus without dormitories *(1 600 ± 130 people)* *Additional sampling sites: Mytilene (26 000 people), the island capital, and two small villages (1 250 people)*	Greece	a) University 5 consecutive days over the week b) Additional sampling sites 7 consecutive days *All samples were collected between 10th February to 10th March 2015.*	24 -h composite samples	3 WWTP, each serves a particular population (university, Mytilene or villages) Time-proportional sampling *(6 mL per minute)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER) NPSs (KET, butylone, ethylone, methylone, MPA, PMMA, PMA, MEPH, MXE, MDPV) Opioids (MTHD) Alcohol	[34]
PRISONS						

Two prisons *(covering three buildings)*	France	Not specified	24 -h composite samples	Sewer pipe output of the participated buildings Flow-proportional sampling	Illicit drugs (cannabis, MDMA, COC, HER) NPSs (MEPH,4-MEC) Opioids (MOR, MTHD, BUP)	[35]
Prison *(around 3 500 people in total)*	Spain	a) 10 or 11 consecutive days in the middle of each month *(June 2008–January 2009)* b) 1 sample every Monday *(during the rest of the month)*	24 -h composite samples	WWTP serving exclusively the penalty complex Type of sampling was not reported	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER, LSD) NPSs (EPH) Opioids (MOR, MTHD)	[36]
Prison *(no data on target population)*	USA	a) Sampling over 28 days (30 July–2 August 2011) b) Hourly samples collected over 3 days *(13–15 August 2011)* *Additionally: During the sampling period, 243 urinalysis tests were carried out.*	a) 24 -h composite samples b) Hourly samples	Details on sampling spot were not reported Constraint collection with a peristaltic pump *(flow rate of 8 ml/min)*	Illicit drugs (MAMP, COC)	[26]
Prison *(467–523 people in total)*	Australia	7 consecutive days *(January 2013)*	24 -h composite samples	Sewer pipe output outside the prison grounds Volume-proportional sampling *(median sampling interval: 2 min or 20 mL every 2.50 L of flow)*	Illicit drugs (cannabis, AMP, MAMP) NPSs (KET) Opioids (COD, MOR, MTHD)	[37]
Prison *(437 people in total)*	Australia	Two periods of 12 consecutive days *(May–July 2013)* *Additionally: During sampling periods, 40 urinalysis tests were carried out.*	24 -h composite samples	Sewer pipe output of the building Volume-proportional sampling *(median sampling interval: 2 min or 20 mL every 2.50 L of flow)*	Illicit drugs (cannabis, COC, MDMA, MAMP) NPSs (MDA, MDEA, KET, MEPH, methylone) Opioids (COD, MTHD, BUP)	[38]
OTHER SPECIFIC SITES						
3 fitness centres *(no data on targeted population)*	Germany	2 days *(12 samples per day)*	2 -h composite samples	Sewer pipe output from the building Time-proportional sampling	Illicit drugs (AMP, MAMP, MDMA) NPSs (EPH)	[23]
National airport of Amsterdam - Schiphol *(40 000 people)* *Additional sampling sites: Utrecht (529 000 people), Eindhoven (544 030 people), Apeldoorn (351 500 people), Amsterdam (913 435 people)*	Netherlands	a) Airport Sampling 3 days of the week and all weekend b) Additional sampling sites Sampling over one week. *All samples were collected between the third and fourth week of February 2010.*	a) Airport Weekdays: 24 -h composite samples Weekend: 72 -h composite sample b) Additional sampling sites: 24 -h composite samples	a) WWTP serving the airport b) WWTP serving each city All samples were collected in flow-proportional mode.	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER) NPSs (KET) Opioids (COD, MOR, MTHD)	[27]

4-MEC – 4-methylethcathinone, AMP – amphetamine, BUP – buprenorphine, COC – cocaine, COD – codeine, EPH – ephedrine, HER – heroin, KET – ketamine, LSD – lysergic acid diethylamide, MAMP – methamphetamine, MDA – 3,4-methylenedioxyamphetamine, MDEA – 3,4-methylenedioxy-N-ethylamphetamine, MDMA – 3,4-methylenedioxymethamphetamine, MDPV – methylenedioxypyrovalerone, MEPH – mephedrone, MOR – morphine, MPA – methiopropamine, MTHD – methadone, MXE – methoxetamine,
PMA – 4-methoxyamphetamine, PMMA – 4-methoxymethamphetamine.

with new psychoactive substances (NPSs) and opioids, such as morphine, codeine, methadone, buprenorphine and fentanyl, are discussed.

Drug consumption in the population is usually assessed by traditional epidemiological methods (e.g., surveys), while other information, such as crime statistics (e.g., seizure data) and medical records (e.g., overdoses) are also a valuable source of information [3–5]. The problem with surveys is that they are subject to errors arising from reporting biases, have low response rates, raise ethical issues and are slow to detect the use of NPSs [4,5]. This review is focused on studies utilizing an alternative approach referred to as wastewater-based epidemiology (WBE), which is becoming an increasingly popular approach for providing additional information on substance use and misuse due to its objectivity, cost-effectiveness and its ability to provide data in near-real-time. It is based on the accurate determination of human metabolic excretion products (biomarkers) of licit and illicit drugs in wastewater. From the measured levels of biomarkers, consumption in a target population can be back-calculated by taking in account additional information such as wastewater flow and by applying a correction factor that takes into account human metabolism [4,5]. Since its first application in 2005 [4], WBE has undergone continuous development and improvement [6,7]. For example, in 2012, a group of scientists supported by the European Cooperation in Science and Technology (COST) established the Sewage analysis CORe group Europe (SCORE) to gather experts to discuss, develop and standardize the WBE approach [8]. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has also recognized and supported the SCORE group and promoted WBE as an additional tool for estimating drug use [9]. To date, numerous studies relating to sample collection [10], sample stability [11], validation of analytical protocol [7,12] and the back-calculation of drug consumption [5,6] have been published, and are evidence of WBE's potential for studying spatio-temporal consumption patterns in the general population [13–16].

Obtaining drug use trends is not only of interest concerning the general population but also for specific populations that are highly susceptible to drug use (e.g., adolescents, prisoners). For example, in comparison to the general population, illicit drug use is more frequently reported in nightlife settings (clubs and bars) and during music festivals, where they are often co-consumed with other psychoactive substances, including alcohol and tobacco [3]. Moreover, according to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) "*prisoners report higher lifetime rates of drug use and more harmful patterns of use, including injecting, than the general population*" [3]. Several WBE studies exploring the impact and extent of substance abuse in specific catchments, e.g. educational institutions, prisons, fitness centers and at an airport (site-specific WBE), and during special events such as music festivals, sporting events and holidays (event-specific WBE), have emerged and are summarized and discussed in this review.

This review presents a discussion on the application and benefits of using WBE for assessing licit and illicit drug consumption in specific catchments. The results of published studies are presented together with an examination of current challenges and future perspectives. Referenced studies were found by searching the following keywords: wastewater analysis, wastewater-based epidemiology, illicit drug, alcohol, tobacco, school, prison, small population, event, festival, holiday.

2. Methodological challenges relating to sampling

Sample collection, filtration, sample preparation and instrumental analysis are the first steps in the WBE approach. Sample preparation mainly involves extraction of analytes by solid-phase extraction (SPE) followed by liquid chromatographic (LC) separation coupled to tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HRMS). When determining alcohol and tobacco biomarkers, direct sample injection without extraction is possible because of their high concentrations in the samples. Analytical protocols are well established and regulated by "best practice protocols" developed in the frame of COST SCORE actions [6,7] and summarized elsewhere [12]. In site- and event-specific studies, similar analytical protocols are applied. In this section, the focus is on sampling strategies since obtaining a representative wastewater sample represents a significant challenge in WBE when applied to specific catchments.

In the case of site-specific studies (studies conducted in small sub-catchments, e.g. educational institutions, prisons, fitness centers, airport), raw wastewater samples are typically collected directly from the sewage outlet or at the inlet of a wastewater treatment plant (WWTP) installed at the studied site (Table 1). At the same time, sampling during special events has been mostly performed at municipal WWTPs (Table 2). Site-specific studies face unique challenges, notably when sampling upstream from the WWTP in small sub-catchments or at specific sites [17,18]. Here, good knowledge of the sewer system is essential, since the physical boundary of the sewer system (e.g., depth of the sewer) and the availability of a power source can disable a sampling campaign (e.g., when an autosampler is used over an extended period) [19,20]. Also, obtaining an adequate composite wastewater sample using an autosampler becomes an issue, since a small and inconsistent wastewater flow may prevent subdivisions of the composite samples from being obtained, such as during times of no flow [21].

Moreover, low and inconsistant wastewater flow makes it difficult to measure wastewater flow, which is needed to calculate drug consumption estimates. In such cases, the flow rate can be estimated from the monthly water bill obtained for a specific site. If no leakage is assumed, the water used that month is drained as wastewater from the sampling site. The average daily wastewater flow can be calculated by dividing used water (read from the water bill) with the number of days in the month. However, calculating the flow this way introduces uncertainty into the consumption estimates, since wastewater flow can vary from day to day. When sampling at source in a site-specific situation, a higher content of solids, namely sanitary tissue, feces, and solid waste creates additional problems such as clogging of the autosampler [22]. Solutions to this problem include installing a solids separator and adapting the autosampler to operate with compressed air [23].

Typically, 24-h composite samples are collected in studies assessing drug consumption in the general population [13,14]. However, when sampling wastewater at specific sites, sampling intervals have to be frequently adjusted (Table 1). For instance, Zuccato et al. [24] collected samples during an 8-h school day, while others collected 2-h composite samples [23,25], hourly samples to study time-dependent use patterns [26], and samples collected over an extended period (72-h composite samples) at sites with limited access [22,27,28]. For estimating drug consumption during special events, 24-h composite samples are commonly collected (Table 2) while grab sampling, which is not recommended because of its inability to capture high intraday variability of drug excretion, has rarely been applied [29–31]. Tables 1 and 2, list the three most common autosampling modes: flow-, volume- and time-proportional, which were used to obtain composite wastewater samples in specific catchments and during special events. Flow-proportional sampling is used to overcome flow rate variability [27] and thus is recommended as the most suitable sampling mode for collection of wastewater samples in small catchments. Several site- and event-specific studies have used volume-proportional sampling, even though it does not provide

Table 2
Overview of WBE applications for monitoring drug consumption trends during special events.

Special event	Country	Population	Sampling period	Type of raw wastewater sample	Sampling location and type of sampling	Target drugs	Reference
MUSIC FESTIVALS							
Largest music festivals in Slovakia	Slovakia	a) Pohoda Festival – multicultural festival (near Trenčín) 30 000 attendants (47 000 inhabitants in Trenčín) b) Lodenica Festival – folk and country festival (near Piešťany) 10 000 attendants (35 000 inhabitants Piešťany)	a) 12–13 July 2013 b) 30–31 August 2013 *(plus control samples one week later)*	24-h composite sample	a) Trenčín WWTP including contents of special reservoirs Time proportional sampling b) Piešťany WWTP Time proportional sampling *(15-min intervals)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC)	[39]
Annual music festival *(includes a wide range of arts and attendants of different ages 6–60+)*	Australia	a) *festival 2010* In total 88 600 attendants	Music festival 2010 and 2011 One week in summer *Additionally: In 2010, samples were collected in a nearby urban area (~350 000 inhabitants)*	24-h composite sample	One-site WWTP *(received wastewater only from the festival)* a) festival 2010: Continuous sampling side stream Flow-proportional sampling	Illicit drugs (cannabis, COC, AMP, MAMP, MDMA) NPSs (MEPH, methylone, benzylpiperazine)	[40]
7 music festivals with different music genre preferences	The Czech Republic and Slovakia	a) Guláš Fest (Valašské Meziříčí) – country/folk music b) VanDaal fest (Zubří) – metal music c) Grape Festival (Piešťany) – dance music d) Topfest (Piešťany) – pop/rock music e) Gypsy Fest (Bratislava) f) Skalické dni (Skalica) – multi-genre music g) Pohoda Festival (Trenčín) – pop/rock music 8 000–20 000 festival attendants 14 000–45 000 inhabitants	several days before, during and after festivals	24-h composite sample	Wastewater from WWTPs of cities where festivals took place (including portable toilet Contents) Time-proportional sampling *(15-min intervals)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER, LSD) NPSs (MDEA, MBDB, KET, cathinone, mephedrone) Opioids (COD, MTHD, BUP)	[41]
Music day event	France	2 WWTPs about 500 000 inhabitants	9 consecutive days a) 15–23 June 2017 (including music event on 21 June) b)17–22 May 2018	24-h composite sample	2 WWTPs in Bordeaux Flow-weighted sampling *(6 times per hour)*	Illicit drugs (COC, MDMA, cannabis)	[42]
Two Amsterdam street festivals	Netherlands	300 000 attendants 769 000 inhabitants (Amsterdam)	Samples collected before and during the festival *(Thursday to Sunday in Summer 2012 and 2014)*	24-h composite sample	Amsterdam WWTP Flow-proportional sampling	NPSs (560 different NPSs)	[43]
The youth festival – Spring Scream	Taiwan	600 000 attendants	During the week of the youth festival *(1–7 April 2011)*	Grab sample	Nanwan and Kenting WWTPs	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER) NPSs (KET, PEPH, GHB) Tobacco	[30]
Music festivals with different music genre preferences	The Czech Republic and Slovakia	a) Guláš Fest (Valašské Meziříčí) – country/folk music b) VanDaal fest (Zubří) – metal music c) Grape Festival (Piešťany) – dance music d) Topfest (Piešťany) – rock/metal music e) Gypsy Fest (Bratislava) f) Skalické dni (Skalica) – multi-genre music	a) 18–20 July 2014 b) 16–18 August 2014 c) 15–16 August 2014 d) 26–28 June 2014 e) 8–9 August 2014 f) 20–21 September 2014	24-h composite sample	WWTPSs of cities where the festival took place Time-proportional sampling		[44]
Music festival *(Fallas festivity)*	Spain	Total of 1 500 000 inhabitants (Valencia city)	4–20 March 2014 (Fallas festivity:	24-h composite sample	3 WWTPs in Valencia city No data about sampling type	Alcohol	[45]
Largest Spanish and European annual pop, rock and	Spain	a) Music event Approximately 40 000 attendants b) Benicasim (city) 15 564 inhabitants	a) Music event *One week in July 2008 (the event took place on 17–20 July)* b) Periods without a music festival	24-h composite sample	Benicasim WWTP including contents of portable toilets Time-proportional sampling *(1 Levery hour)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC) NPSs (MDA, MDEA)	[46]

Table 2 (*Continued*)

Special event	Country	Population	Sampling period	Type of raw wastewater sample	Sampling location and type of sampling	Target drugs	Reference
electronic festivals			*(one week in June and January 2008)*				
The graduates' celebration – Russ	Norway	50 000 high school graduates 500 000 inhabitants (Oslo)	Year-long sampling in 2010 *(sampling period during the festival: 12)*	Passive sampling (Polar organic chemical integrative samplers, POCIS)	From the large gravity tunnel tube system before WWTP of Oslo POCIS were replaced every two weeks	Illicit drugs (AMP, MAMP, MDMA, COC)	[47]
Street parade	Switzerland	600 000 attendants	a) Street parade [illegible] (Wednesday after the event (Sunday)	24-h composite sample	Central WWTP Zurich Flow-proportional sampling	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER) Opioids (COD, MOR, MTHD)	[48]
			HOLIDAYS				
Christmas holiday season	Croatia	Zagreb *(688 163 inhabitants)*	a) holidays 2012–2013 *(21 December 2012–4 January 2013)* b) holidays 2013–2014 *(20 December 2013–3 January 2014)*	24-h composite sample	Central WWTP Zagreb Time-proportional sampling *(15-min intervals)*	Illicit drugs (AMP, MDMA, COC, HER) Opioids (MOR, MTHD)	[16]
Christmas holiday season	Australia	a) urban area *(350 000 inhabitants)* b) semi-rural area *(120 000 inhabitants)* c) vacation area *(1 100–2 400 inhabitants)*	*(23 December 2010–3 January 2011)* normal day *(26 February–3 March 2011)*	24-h composite sample	Urban area and vacation area: Flow-proportional sampling Semi-rural area: Volume-proportional sampling	Illicit drugs (Cannabis, MAMP, MDMA, COC)	[49]
Independence Day 2017 The 2017 solar eclipse The first week of an academic semester, 2017	USA	2 communities A: 20 000 inhabitants B: 25 000 inhabitants *(40 %of the B community are professors and students)*	a) Independence Day celebration *(30 June–6 July)* b) Total solar eclipse observation day *(19–22 August)* c) the first week of an academic year *(11–17 August)* d) typical week *(26 July–1 August 2017)*	24-h composite sample	2 WWTPs in Western Kentucky Time-proportional sampling *(15-min intervals)*	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC) NPSs (MDA, MDEA) Opioids (MOR, MTHD)	[50]
a) Memorial Day b) 4th of July c) Labor Day d) New Year	USA – New York	a) North River WWTP: 588 772 inhabitants b) Newton Creek WWTP: 1 068 012 inhabitants c) Hunts Point WWTP: 684 569 inhabitants d) Tallman Island WWTP: 410 812 inhabitants e) Jamaica WWTP: 728 123 inhabitants	Sampling on days before and after major holidays in 2016: Memorial Day *(27 and 31 May 2016)* 4th of July *(1 and 5 July 2016)* Labor Day *(2 and 6 September 2016)* New Year *(30 December, 3 January 2017)*	One-time grab samples between 8:00 am and 11:00 am	5 WWTPs No data about sampling type	Illicit drugs (AMP, MAMP, MDMA, COC) NPSs (MDA) Opioids (COD, MOR, MTHD, fentanyl) Tobacco	[31]
a) Chinese Spring Festival b) National Day	China	WWTP in Guangzhou No data about the population	8 weeks across 3 months 2017: 1–10 January 23 January–5 February (including Chinese Spring Festival) 9–22 May 27 September–12 October (including National Day)	24-h composite sample	WWTP in Guangzhou No data about sampling type	Illicit drugs (AMP, MAMP, MDMA, COC) NPSs (KET) Opioids (COD, MTHD)	[51]
Carnival	Brazil	A North-Wing WWTP: 145 000 inhabitants A South-Wing WWTP: 525 000 inhabitants	8 consecutive days *(30 May 2017–6 June 2017)* Carnival day *(13 February 2017)*	24-h composite sample	2 WWTPs Flow-proportional a sampling	Illicit drugs (COC)	[52]
Easter holiday (including the examination period at university and end of university year)	France	70 000 inhabitants *(students presented ~20 %of the total population)*	84 consecutive days *(21 March–11 June 2016; except on 31 March)*	24-h composite sample	WWTP Flow-proportional sampling *(50 mLevery 30 m^3 of Influent)*	Illicit drugs (AMP, HER, COC, cannabis, MDMA) Opioids (COD, MOR, MTHD, BUP)	[53]
	Croatia	a) Zadar	a Zadar			Illicit drugs (AMP, MDMA,	[54]

Table 2 (*Continued*)

Special event	Country	Population	Sampling period	Type of raw wastewater sample	Sampling location and type of sampling	Target drugs	Reference
Summer tourist Season		*(64 324 inhabitants + 16 % increase of population during summer season)* b) Zagreb *(688 163 inhabitants)*	- *off-season* - *tourist season (21 July–1 August 2013)* a) Zagreb	24-h composite sample	WWTP Zadar Time-proportional sampling *(15-min intervals)*	COC, HER) Opioids (MOR, MTHD)	
Summer holiday season	Korea	a) WWTP 1: semi-rural area b) WWTP2: residential area c) WWTP 3: vacation area No data about the population	*(24–31 July 2013)* 8–10 July 2013 (before the main holiday period) 1–3 August 2013 (in the peak Holiday period) 12–14 September 2013 (after the main holiday period)	24-h composite sample	3 WWTPs Time-proportional sampling	Illicit drugs (MAMP, MDMA, HER) NPSs (MDA, MDEA, meperidine, KET) Opioids (COD, MOR, MTHD, BUP, fentanyl)	[55]
Summer holiday season (coinciding with school holidays)	Italy	a) WWTP 1 Fondo Verde: 45 000 inhabitants b) WWTP 2 Acqua dei Corsari 340 000 inhabitants	March–November 2015 *(with the exception of May and August)*	24-h composite sample	2 WWTPs in Palermo No data about sampling type	Illicit drugs (cannabis, MAMP, MDMA COC) NPSs (MDA, MDEA)	[56]
Christmas holiday season	Australia	a) WWTP 1: b) WWTP 2: c) WWTP 3: d) WWTP 4:	19 December 2018–1 January 2019	24-h composite sample	4 WWTPs Flow-proportional sampling	NPSs (21 NPSs in total)	[57]
a) Christmas holiday season b) Australian Day	Australia	South-East Queensland 2015: 105 532–106 788 inhabitants 2016: 107 037–108 292 inhabitants 2017: 108 542–109 294 inhabitants	7 consecutive days in every second month *(February, April, June, August, October, December) 2012–2017* a) Christmas–New Year holiday *(21 December–31 January) 2015/2016 2016/2017* b) Australian Day *(26 January)*	24-h composite sample	WWTP located in South-East Queensland Flow-proportional sampling	Alcohol	[58]
			SPORTING EVENTS				
FIFA Soccer World Cup	Brazil	68 000 people per game only inside the stadium 700 000 inhabitants *(served by 2 WWTPs from Brazilian Federal District)*	a) Argentina vs Belgium [illegible] playoff [illegible] reference week *(21–22 April 2012)*	24-h composite sample	2 central WWTPs Flow-proportional	Illicit drugs (AMP, MAMP, MDMA, COC) NPSs (MDA, MDEA, MBDB)	[59]
National Football League's – Super Bowl	USA	Not reported	a) Super Bowl b) Reference week [illegible] *(7–8 March 2010)*	12-h composite sample	WWTP Time-proportional	Illicit drugs (cannabis, AMP, MAMP, MDMA, COC, HER) NPSs (MDA) Opioids (MOR)	[60]
Football game at the university	USA	a) University - *Fall semester: 18 800 students* - *During football games: >60 000 attendants (depends on the game held)* b) City of Oxford (20 800 inhabitants)	During weekends on which football home games were held: - Friday (before the game) - Saturday (during the game) - Sunday (after the game) Non-game days (Two Wednesdays, before the games)	Grab sample	a) University WWTP Samples manually collected from the influent reservoir b) Oxford WWTP Samples collected using a pump	Illicit drugs (AMP, MAMP, MDMA, COC, HER) NPSs (MDA, MDEA, PCP) Opioids (COD, MOR, MTHD, fentanyl)	[29]

AMP – amphetamine, BUP – buprenorphine, COC – cocaine, COD – codeine, GHB – gamma-hydroxybutyrate, HER – heroin, KET – ketamine, LSD – lysergic acid diethylamide, MAMP – methamphetamine, MBDB – 3,4-methylenedioxy-N-methyl-butanphenamine, MDA – 3,4-methylenedioxyamphetamine, MDEA – 3,4-methylenedioxy-N-ethylamphetamine, MDMA – 3,4 methylendioximethamphetamine, MEPH – mephedrone, MOR – morphine, MTHD – methadone, PCP – phencyclidine, PEPH – pseudoephedrine.

accurate averages of analyte concentrations since individual samples are not corrected for wastewater flow [10,19]. In this respect, volume-proportional sampling is similar to time-proportional sampling (constraint frequency and sampling volume), which is frequently used in site-specific studies [19].

A critical factor, when time-proportional sampling is used, is sampling frequency [19,20], and the main parameter dictating sampling frequency is the variability in the number of pulses, e.g., toilet flushes [10,19]. At specific sites, such as prisons and schools, a high sampling frequency is essential because of the low number of

Table 3
Overview of the reported biomarker concentrations and estimated drug consumptions in site-specific settings.

Concentration (ng/L)			Consumption (mg/day/1 000 inhabitants or mg/day)	Reference
EDUCATIONAL INSTITUTIONS				
Educational institution (range)	**Finals Week**	**Summer break**	**mg/day**	[25]
Regular class session	THC-COOH: 4.4–372.9	THC-COOH: ND–40.3	**Educational institution (all samplings average):**	
THC-COOH: ND–177.1	AMP: ND–153.6	AMP, MDA, MDMA: ND	COC: <100 mg	
AMP: ND–140.4	BE: 1.2–34.7	BE: 1.3–6.2	AMP: max 3.7 mg	
MDA: ND–173.9	COC: 2.5–9.6	COC:1.2–4.2		
MDMA: ND–3 266.0	COD: ND–25.6	COD: 6.1*		
BE: 1.9–10	M3G: ND–565.5	M3G: 63.6*		
COC: 2.4–13.8	MOR: ND–43.1	MOR: 95.4*		
COD: ND–71.4		6-AM: 7.7*		
M3G: ND–41.9		* one occasion		
6-AM, MOR: ND				
Dorms (range)	**College campus (range)**		**mg/day/1 000 people**	[32]
THC-COOH: 30–2 413	THC-COOH: 152–1 373		**Dorms (average)**	
THC: 22–2 070	AMP: 195–3 017		Cannabis: 29 125 ± 24 875	
AMP: 30–5 956	MAMP: 20–783		AMP: 660 ± 930	
MAMP: 26–56	MDMA: 79–108		COC: max 280	
MDA: 348*	BE: 13–1 214		HER: 69	
MDMA: 30*	COC: 40*		**Campus (range)**	
BE: 4–350	COD: 43–575		Cannabis: 1 587.5–5 750	
COC: 24–184	MOR: 23–491		AMP: 81–294	
COE: 77*	6-AM: 44*		COC: 10–20	
EDDP: 30*	THC, COE, EDDP, HER, LSD, MDEA, MDA, MTHD: *ND*		HER: 5.4–19.2	
COD: 14–981	*one occasion			
MOR: 21–217				
6-AM, HER, LSD, MDEA, MTHD: ND				
Private college (AMP average)	**Spring semester**		Not applicable	[22]
Fall semester)	1st week: 810 ± 6.0			
1st week: 330 ± 4.1	*Midterms:* 700 ± 5.8			
Midterms: 480 ± 3.6	*Post-midterms:* 650 ± 2.5			
Post-midterms: 310 ± 3.6	*Last week classes:* 810 ± 6.3			
Finals: 545 ± 0.8	*Finals:* 2 100 ± 8.9			
Additional information:	1 st week: 75 ± 51			
Average AMP concentration normalized for creatinine (*ng AMP/mg creatinine*)	Midterms: 120 ± 51			
1 st week: 74 ± 51	Post-midterms: 110 ± 50			
Midterms: 240 ± 55	Last week classes: 190 ± 50			
Post-midterms: 65 ± 51	Finals: 570 ± 51			
Finals: 110 ± 50				
College campus			Not applicable	[28]
No data on AMP concentration				
Additional information:				
Average AMP concentration normalized for creatinine - ng AMP/mg creatinine:				
1st week: 74 ± 7				
Midterms: 240 ± 60				
Finals: 111 ± 6				
Not applicable			**mg/day/1 000 people (average)**	[33]
			AMP: 256 ± 12	
			MDMA: 88 ± 35	
			COC: 551 ± 49	
			HER: 474 ± 32	
			COD: 50 ± 4	
			MOR: 18 ± 3	
			MTHD: 72 ± 8	
Cities where schools were located (range)			**mg/day/1 000 people**	[24]
Turin	***Verona***	**Naples**	**Classic, scientific or artistic education (range and median)**	
THC-COOH: 148–294	THC-COOH: 121–255	THC-COOH: 40–109	Cannabis: 663–2 893 (1 201)	
BE: 22–147	BE: 3–74	BE: 12–1 398	COC: 5.2–187 (45)	
COC:34–241	COC:13–2.2		**Professional or vocational**	
MOR: 58–382	MOR: 14–216			
6-AM: 39*	***Rome***			

Table 3 (Continued)

Concentration (ng/L)			Consumption (mg/day/1 000 inhabitants or mg/day)	Reference
*one occasion **Milan** THC-COOH: 37–147 BE: 57–3 516 COC: 23–421 ***Palermo*** BE: 4–205 THC-COOH: 7–40 *AMP, MAMP, MDMA, MEPH, KET: never detected in any of the school samples*	THC-COOH: 14–46 BE: 2–6.1 COC: ND–2.5 ***Florence*** THC-COOH: 0–16.5 BE: ND–3.4 COC: ND–3.8	COC:7–145 ***Bologna*** THC-COOH: ND–19.7	**education (range and median)** Cannabis: 49–1 062 (106) COC: 0–7.1 (0.6)	
University (range and average) THC-COOH: <20–329 (169) MDMA, BE, COC: <1 EtS: <1 700 *EME, AMP, MAMP, MTHD, EDDP, 6-AM, MXE, butylone, ethylone, methylone, MPA, PMMA, PMA, MEPH, MDPV, KET, DHNK and nor-KET were not detected in any of the samples.*	**The capital, Mytilene (range and average)** THC-COOH: 49.5–90.2 (70.7) MDMA: <1–6.2 (3.2) BE: <1–37.5 (9.4) COC: <1–7.5 (3.9) EtS: 2 190–12 243 (5 283)	**Villages (range and average)** THC-COOH: 22.5–50.0 (34.9) MDMA, BE, COC: <1 EtS: 2 316–7 814 (4 023)	**Illicit drugs mg/day/1 000 people, (range and average)** **University:** cannabis: 50–220 (100) **Mytilene:** cannabis: 2 400–3 400 (2 800) MDMA: 0.2–2.2 (1.2) COC: 0.5–33 (9.5) **Village:** cannabis: 600–1 700 (1 200) **Alcohol mL/day/1 000 people (range and average)** University: average 3.0 Mytilene: 2.2–11.2 (5.4) Villages: Alcohol: 1.7–7.2 (3.4)	[34]
PRISONS **Prison 1 (range or average)** THC-COOH: 347–3 152 BE: 970 EDDP: 51–353 MOR: <LOQ BUP: <LOQ MDMA, MEPH, 4-MEC: ND	**Prison 2 – building A (range or average)** THC-COOH: 1 021–8 900 BE: 1 083 MDMA: 21–226 MOR, MEPH, 4-MEC: ND EDDP: 313–8 507	**Prison 2 – building B (range or average)** THC-COOH: 640–6 240 BE: 492 MDMA, BUP: <LOQ MOR, MEPH, 4-MEC: ND EDDP: 51–605	**mg/day/1 000 people All sampling sites (range):** Cannabis: 24 000–94 000 COC: 90–282 MTHD: 255–1 707	[35]
Prison (average) THC-COOH:116 ± 52 THC: 67 ± 50 AMP: 97 MAMP:87 ± 66 MDMA: 61 ± 44 BE: 556 ± 291 COC:128 ± 94 6-AM: 63 ± 37 HER: 165 ± 186 MTHD:4 704 ± 1 424 EDDP: 9 262 ± 2 288 MOR: 1 346 ± 583 LSD-OH: 22 ± 9 LSD: 56 ± 62 EPH: 3 745 ± 2 789 THC-OH-,nor-LSD: ND			**mg/day/1 000 people (range and average)** Cannabis:375–2 193 (990) COC: 100–910 (300) MTHD: 1 677.5–7 060 (3 900) EPH: 87.5–3 187.5 (1 150) HER, MDMA, AMP, MAMP: *sporadic use*	[36]
Prison BE: <40 COC: <10			**mg/day/1 000 people (range)** MAMP: 180–960	[26]
Prison (range) THC-COOH: ND–0.04 AMP: 0.011–0.027 MAMP: 0.05–0.25 COD: 2.98–4.40 MOR: 0.19–0.28 MTHD: 0.35–1.79 EDDP: 0.57–1.24 KET: 0.017–0.043 nor-KET: 0.001–0.004			**mg/day (range)** Cannabis: <638–1 206 MAMP: 25–120 COD: 5 110–9 045	[37]
Prison THC-COOH, THC, MDMA, BE, COC, MDA, MDEA, MEPH: <LOQ			**mg/day (range)** MAMP: 2–319 COD: 1 077–12 015 KET: ND–107	[38]

Table 3 (Continued)

Concentration (ng/L)		Consumption (mg/day/1 000 inhabitants or mg/day)	Reference
		MTHD: 50–3 852 BUP: 331–6 243 Methylone: 537* *one occasion	
OTHER SPECIFIC SITES			
Fitness centres (range)		Not applicable	[23]
AMP: <2–2 340 MAMP: <2 MDMA: <2–93 EPH: 97–5 873			
Airport (range and mean)	**4 Dutch cities (range)**	Not applicable	[27]
THC-COOH: <33 AMP: 51–115 (81) MAMP: 16–17 (17) MDMA: 16–85 (58) BE: 659–2 933 (1 472) COC: 171–957 (559) 6-AM: <19 MOR: <360 MTHD: <45 COD: 336–894 (536) KET: <10	THC-COOH: 73–489 AMP: 40–1 779 MAMP: <15 MDMA: <12–241 BE: 260–3 701 COC: 87–673 6-AM: <19–73 MOR: <360 MTHD: <45 COD: 73–495 KET: <10–34		

ND – not detected, 4-MEC – 4-methylethcathinone, 6-AM – 6-acetylmorphine, AMP – amphetamine, BE – benzoylecgonine, BUP – buprenorphine, COC – cocaine, COD – codeine, CDE – cocaethylene, DHNK – dehydronorketamine, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EME – ecgonine methyl ester, EPH – ephedrine, EtS – ethyl sulfate, HER – heroin, KET – ketamine, LSD – lysergic acid diethylamide, LSD–OH – 2-oxo-3-hydroxy-lysergic acid diethylamide, M3G – morphine-3-glucuronide, MAMP – methamphetamine, MDA – 3,4-methylenedioxyamphetamine, MDEA – 3,4-methylenedioxy-N-ethylamphetamine, MDMA – 3,4-methylenedioxymethamphetamine, MDPV – methylenedioxypyrovalerone, MEPH – mephedrone, MOR – morphine, MPA – methiopropamine, MTHD – methadone, MXE – methoxetamine, nor-KET – nor-ketamine, nor-LSD – N-demethyl-lysergic acid diethylamide, PMA – 4-methoxyamphetamine, PMMA – 4-methoxymethamphetamine THC – tetrahydrocannabinol, THC–COOH – 11-nor-Δ9-carboxy-Δ9-tetrahydrocannabinol, THC–OH – 11-hydroxy-Δ9-tetrahydrocannabinol.

pulses. In such cases, the optimal sampling frequency should be determined using a preliminary dye tracer test [22,37] or by analyzing flow dynamics [24].

The primary source of uncertainty in sampling, which can exceed 100 %, depends on catchment characteristics, mode of sampling, and sampling frequency [10,29]. Burgard et al. [22] are the only group so far to determine sampling uncertainty connected with at source sampling (e.g., dorms) by measuring mass loads of creatinine and a human urine marker in wastewater. The authors surmise that since excretion, and consequently the mass load of creatinine, is assumed constant, the calculated deviation in measured loads is attributable to sampling uncertainty, which was 31 %. Others have also determined creatinine levels [22,28,31] but in order to account for population variations and dilutions.

Passive sampling can be used to overcome autosampling issues (e.g., varying wastewater flow, missing pulses – toilet flushes, power requirements) [19]. Passive samplers, such as polar organic chemical integrative sampler (POCIS), have been applied in WBE studies of the general population [47]. Their application is, however, limited due to practical challenges, e.g., clogging and difficulties in determining uptake rates [19]. To the author s' knowledge, passive samplers have not been used in site-specific WBE studies, although POCIS was used to monitor illicit drugs and their metabolites in wastewater during the Norwegian "russefeiring" or "the russ" celebrations for high school graduates [47].

To summarize, in site-specific studies flow-proportional sampling mode should be used to overcome variations in flow, while for time-proportional sampling, the sampling frequency should be optimized (using dye trace test or similar) to minimize the chance of missing wastewater pulses (e.g., toilet flushes). As an alternative to active sampling, passive sampling can be used, but its possible application in specific sites still needs to be explored. In the case of event-specific studies, wastewater is commonly collected at WWTP using standardized sampling procedures performed by the WWTPs' staff. As such, the sampling procedure is appropriate to address drug consumption patterns during special events, such as music festivals, holidays and sporting events and needs no adjustment.

3. Applicability of WBE for studying drug consumption patterns in site-specific studies

WBE has been utilized to gain insight into licit and illicit drug consumption patterns in susceptible populations, namely adolescents, prisoners, travelers and fitness users. Details of the sampling strategies used and the data obtained are presented in Tables 1 and 3, respectively. The data are hard to compare since the data are presented differently in different studies (e.g., as concentration, normalized concentrations, mass loads, consumption estimates), while consumption estimates are made based on different biomarkers (e.g., heroin consumption estimated from morphine or 6-acetylmorphine).

3.1. Educational institutions

Monitoring drug use is especially important for preventing the spread of drugs among young people, including schoolchildren and students. In Europe, data on drug use among teenagers derive mainly from the European School Survey Project on Alcohol and Other Drugs – ESPAD (15- to 16-year olds) [61] and the Health Behavior in School-aged Children – HBSC survey (11-, 13-, 15- and 17-year olds) [62]. In the US, several public secondary schools have considered mandatory drug testing (MDT) of their students, but such demands have so far been met with resistance by the public because of the personal invasiveness of the tests [25].

To date, several WBE studies in educational institutions, including universities [22,25,28,32–34] and secondary schools [24], have been completed. The focus was on opioid abuse and

consumption of NPSs, while only one study investigated ethyl sulfate, a biomarker of alcohol consumption (Table 1). Biomarkers of conventional drugs (cannabis, amphetamines, cocaine, and heroin) were present in wastewater from universities, as well as in wastewater from secondary schools (Table 3). The data show that cannabis was the most commonly used drug being used almost exclusively in secondary schools (cannabis consumption: up to 2 893 mg/day/1 000 people vs cocaine consumption: up to 187 mg/day/1 000 people). In contrast, biomarkers of opioids, such as codeine, morphine, methadone, and fentanyl, were detected sporadically although, in a US university, opioid biomarkers, such as codeine [32] and morphine-3-glucuronide [32] were present in 100 % and 98 % of the wastewater samples, respectively. Also, Gushgari et al. [33] observed a high average consumption of cocaine (551 ± 49 mg/day/1 000 people) and heroin (474 ± 32 mg/day/1 000 people, based on 6-acetylmorphine) at a US college campus (cannabis consumption was not examined) compared to other studies conducted in educational institutions, where up to 280 mg of cocaine/day/1 000 people and up to 69 mg of heroin/day/1 000 people (based on morphine) were estimated to be consumed. Among the NPSs, two US studies [25,32] report the presence of 3,4-methylenedioxyamphetamine (MDA). Ethyl sulfate (alcohol biomarker), although detected, was below the limit of quantification (LOQ) in wastewater from a university campus in Greece [34].

Several groups also used WBE to correlate drug use patterns with varying levels of stress during different school periods [22,25,28,32]. Panawennage et al. [25], found that drug use was higher during the final exam period compared to regular class time, with cannabis consumption doubling during this period. Heuett et al. [32] also observed an increase in cannabis consumption at the end of the semester, while amphetamine consumption remained relatively constant. In contrast, Burgard et al. [22], who explored the non-prescribed use of attention deficit hyperactivity disorder (ADHD) medications such as Adderall (mixed amphetamine salts, excreted in the urine as 30–40 % intact amphetamine) during periods of low and high stress, found an increase in amphetamine concentration during stressful times. Although sampling uncertainty was high because of the low sampling frequency, the results revealed an eight-fold increase in amphetamine concentration, normalized for creatinine, during times of high academic stress (final exams). Moore et al. [28] also reported that the use of Adderall increased during high-stress periods. In their study, up to a 3-times higher concentration of amphetamine (normalized for creatinine) was found in wastewater samples during high-stress periods compared to the first week of class. The results were in good agreement with results obtained by a self-reporting survey, conducted in parallel, where the number of people reporting the non-prescriptive use of Adderall increased by up to 4-times during high-stress periods.

Weekly consumption trends at a university campus were explored by Gushgari et al. [33] but found no statistically significant variation in the mass loads of opioids and 3,4-methylendioximethamphetamine (MDMA). In contrast, higher loads of amphetamine and benzoylecgonine (the main cocaine metabolite) were detected during weekdays and the weekend, respectively. Also, Heuett et al. [32] observed a higher consumption of cannabis and amphetamine during weekdays on the main campus, which is probably a likely consequence of the higher number of students present during weekdays. Annual variations in drug consumption in Italian secondary schools were explored by Zuccato et al. [24]. They found up to a 20-fold increase in cannabis consumption over a four-year monitoring period (2010–2013), although morphine and cocaine use varied such that no time consumption trends were observable.

Differences in drug consumption between institutions offering different educational programs, such as classic, scientific, artistic, vocational, and professional secondary schools, was also explored by Zuccato et al. [24]. The data show that illicit drug consumption is higher in secondary schools that offer classic, scientific or artistic education. For example, 11- and 75-times higher consumption of cannabis and cocaine was observed in the former compared to vocational or professional schools.

A good agreement between a national survey and WBE was reported by Panawennage et al. [25], both studies found that cannabis was the drug of choice among adolescents in the USA. In comparison to the general population, cannabis (schools in Milan, Turin, Verona and Naples) and morphine use (a school in Verona) in Italian secondary schools was in the same order of magnitude, while the consumption of morphine (except in a school in Verona) and cocaine was 40 % and 20 % lower [24]. Lower consumption of cannabis (100 mg/day/people) was observed on the university campus on the island of Lesvos, Greece compared to two small nearby villages (1 200 mg/day/1 000 inhabitants) and the capital, Mytilene (2 800 mg/day/1 000 inhabitants) [34].

Despite the usefulness of WBE, its application to educational institutions is not without its limitations, i.e., students spend only limited time in school and may excrete drug biomarkers elsewhere [24,33]. Also, an individual's excretion profile may have a significant effect on drug consumption trends and, therefore, sampling for an extended period at institutions with a larger number of students is desirable. In order to avoid bias, students should be unaware of the research being undertaken, and even if all of the purposed measures are taken into account, the data may not necessarily reflect drug use only among the students, since it is not possible to distinguish between students and staff.

3.2. Prisons

Another site-specific application, where WBE can be of benefit, is in evaluating drug use in prisons, where consumption has a significant impact on inmate detoxification, treatment, reintegration and prison security [37]. In Europe, the prevalence of drug consumption among prisoners is traditionally assessed by surveys and MDT, with urinalysis as the method of choice [63]. Accurate data is difficult to obtain from surveys, while MDT is expensive and may force prisoners to switch from less harmful drugs that persist longer in the body, such as cannabis, to "harder" drugs with shorter half-lives like heroin, methamphetamine, cocaine, or opioids like morphine [37,63]. In contrast to the methods commonly used to estimate drug consumption in prisons, WBE approach provides the possibility of obtaining more objective data [25]. Also, it can be performed without the prisoners' knowledge and does not require extra security measures [35]. Similar to an educational institution, an individual's excretion profile may affect drug consumption estimates, while the contribution of employees and visitors to overall drug consumption cannot be accurately determined [25].

So far, most WBE studies of prisons focus on illicit drugs (e.g., cannabis, amphetamines, cocaine) and opioids (e.g., substitutional drugs, such as morphine, methadone, buprenorphine), while fewer studies have looked at NPSs (MDA, 3,4-methylenedioxy-N-ethylamphetamine – MDEA, ketamine – KET, mephedrone – MEPH, ephedrine – EPH, methylone and 4-methylethcathinone – 4-MEC). The results suggest that WBE is suitable for detecting the use of new substances in such settings (Tables 1 and 3). Substitutional drugs were daily found in all of the prisons studied, while illicit drugs and NPSs were detected only sporadically or not at all (Table 3). The occurrence of illicit drugs in prisons (e.g., cannabis, cocaine, and methamphetamine), reflected that of a city or countrywide demand. For example, there was widespread use of cannabis in French prisons and the general population [35]. The consumption of cocaine and cannabis was also regularly detected in a Spanish prison and the city of Barcelona [36], and as a result of

its widespread usage in the general population, methamphetamine was frequently detected in Australian and US prison facilities [26,37,38]. Despite society perceptions, Postigo et al. [36] found that illicit drugs (e.g., cocaine, heroin, and amphetamines), except for cannabis, were consumed less in prison in comparison to the nearest major population (Barcelona).

Monitoring prescription drug use to prevent abuse is also of concern, and several authors [35,37,38] compared methadone loads in wastewater with the amounts administered to the inmates. In all three studies, there was an agreement between the estimated and the administrated amounts. The results suggested little if any additional non-prescribed usage of methadone over the monitoring periods. One reason could be the difficulties in dividing liquid methadone since its distribution in the prisons is strictly supervised or difficulties in smuggling a large number of methadone tablets into the prison [37]. Also, caution is needed when making such observations, since methadone consumption was estimated based on levels of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine – EDDP (methadone metabolite), the excretion rates of which can vary considerably [37,38,64]. Thus, consumption can be underestimated (using higher excretion rate – 55 %) or overestimated (using lower excretion rate – 3%). In contrast, up to 5-times higher consumption of buprenorphine was observed in comparison to administrated dose in the study by van Dyken et al. [38], suggesting greater buprenorphine misuse in a small regional prison in Australia. Buprenorphine is consumed mostly in tablet form or as a sublingual film, which makes its concealment easier. Other reasons include the availability of buprenorphine on the prison drug market, its increased use in the wider population, and the exclusion of buprenorphine from traditional prison screening processes. The levels of buprenorphine detected in French prisons is consistent with the proportions dispensed by the prison's pharmacy healthcare unit [35].

Weekly trends in drug use in prison were explored by Postigo et al. [36], who observed an increase in ephedrine (900 to 1 425 mg/day/1 000 people from Tuesday to Sunday) and cocaine (average working days: 280 mg/day/1 000 people, the weekend average: 350 mg/day/1 000 people) consumption through the week. In contrast, methadone consumption decreased over the week (Monday, Tuesday: 4 350 mg/day/1 000 people, Sunday: 3 025 mg/day/1 000 people), possibly due to weekend permits of the inmates.

A WBE approach is also a cost-effective alternative to MDT, providing a more comprehensive picture of substance use in prisons [26,38]. For example, in the study of van Dyken et al. [38], only a few of the drugs (cannabis, buprenorphine) were detected by both, WBE and MDTs, while drugs, such as methamphetamine, ketamine and methylone, were detected only by wastewater analysis. Alternatively, Brewer et al. [26] found good agreement between MDT and WBE data for methamphetamine and cocaine use. Methamphetamine consumption was confirmed, and cocaine consumption was disproven by both methods.

3.3. Other specific sites

The WBE approach has also been used to monitor drug consumption in public places, such as fitness centers and an airport (Tables 1 and 3). In the study of Schröder et al. [23] stimulants, e.g. ephedrine, amphetamine and MDMA were detected in the wastewater of three fitness centers (Aachen, Germany), where the highest concentration observed was for ephedrine (5 873 ng/L). Another case is Schiphol airport (Amsterdam, The Netherlands), where higher cocaine (1.3-times) and codeine (1.4-times) concentrations were measured at the airport in comparison to measured concentrations in the city of Amsterdam [27]. At the same time, methamphetamine was detected exclusively in airport wastewater, suggesting a relationship between its occurrence and international passengers. When the authors compared the populations of Schiphol airport and the city of Amsterdam and mass loads of drug biomarkers, a similar consumption pattern was observed. Also, weekly consumption patterns for cocaine (consumption mainly at the weekend) and codeine (continuous consumption over the week) was similar between the airport and the other four Dutch cities under investigation (Utrecht, Eindhoven, Apeldoorn, and Amsterdam). By utilizing WBE, the possible presence of drug traffickers can be shown at the airport based on the ratio of cocaine biomarkers (cocaine/benzoylecgonine). For example, the cocaine/benzoylecgonine ratio (0.85) in one wastewater sample from Schiphol airport exceeded the proposed cut-off cocaine/benzoylecgonine ratio value (0.75) from the literature [65,66], which indicates the disposal of cocaine directly into the sewage system. One reason could be drug traffickers disposing of the cocaine due to anxiety before passing customs control, but this hypothesis needs to be backed up by additional information [27].

4. Special events

Monitoring of drug use during special events may act as an early warning system concerning drug consumption trends. Generally, drug consumption during the event and control period (e.g., wastewater sampling during non-festival days, normal weeks or in places, where no event took place) are compared to assess changes in drug consumption (Table 4). Sampling in both periods should be carefully optimized regarding sampling protocol to make adequate conclusions (e.g., obtaining representative samples) and time (e.g., covering normal days and days during a special event) or location (e.g., places affected by special event vs places where no event takes place). In some cases, such as festivals and sporting events, WBE approach can underestimate levels of drug use since it does not take into account people who urinate elsewhere (outside the sewer system/portable toilets), but their percentage is probably low and should not significantly affect estimates. An overview of the available data for wastewater analysis performed during special events is presented in Tables 2 and 4.

4.1. Music festivals

Several authors have studied drug consumption patterns during music festivals [30,39–41,43–48], and show increased consumption in comparison to non-festival days. Among illicit drugs, increased consumption of cocaine [39,41,46,44–48], cannabis [41,46] and in some cases also amphetamine and methamphetamine [47,48] were observed during various festivals, but the most extreme case was reported for MDMA [30,39–41,46,44–48]. For example, during the Pohoda Festival (Trenčín, Slovakia), there was a ten-fold increase in MDMA consumption [39]. In Australia in 2010, during an annual music festival that attracts people of all ages, a higher consumption rate of MDMA was observed during the festival (320–4 600 mg/day/1 000 people) in comparison to that in the nearby urban area (80–560 mg/day/1 000 people) [40]. Also, an increase in MDMA mass loads was observed during the duration of the festival (14-times higher mass loads on the final day in comparison to the first day of the festival), suggesting an increase in MDMA consumption over the festival period. At the same festival held in 2011, the increase in MDMA mass loads was even more pronounced (final day: 510 mg/day/1 000 people, first day: 15 mg/day/1 000 people) [40]. No difference in MDMA consumption was observed during the Lodenica Festival (Piešťany, Slovakia), which can be explained by the higher average age of attendees in comparison to the Pohoda Festival in Trenčín, Slovakia (average age: 30 years), where a 10-fold increase in ecstasy (MDMA)

Table 4
Overview of the reported biomarker concentrations, mass loads and estimated drug consumptions during special events.

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg/day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
MUSIC FESTIVALS			
Pohoda Festival – Trenčín	**mg/day/1 000 inhabitants**	**mg/day/1 000 inhabitants**	[39]
MDMA: average 239	**Pohoda Festival – Trenčín (average)**	**Pohoda Festival - Trenčín (average)**	
BE: max 88	MAMP: 53	COC: 29	
COC: max 40	MDMA: 29	**Lodenica Festival - Piešťany (average)**	
Lodenica Festival – Piešťany	**Lodenica Festival – Piešťany (average)**	COC: 39	
BE: average 24	MAMP: 163	**Normal days - 8 WWTPs (average)**	
MDMA: <LOQ	**Normal days - 8 WWTPs (range)**	COC: max 141	
Non-festival days - 8 WWTPs (range)	AMP: 2–26		
THC-COOH: 42–140	MAMP: 16–169		
MAMP: 79–658	MDMA: <LOQ–5.8		
MDMA: <9.4–22			
BE: <4.6–124			
COC: <6.1–63			
Not reported	**mg/day/1 000 inhabitants**	**mg/day/1 000 inhabitants**	[40]
	Annual music festival 2010 (range and average)	**Consumption of both festivals (average)**	
	THC-COOH:5.8–12 (16)	Cannabis: 1 400	
	AMP:4.6–37 (19)	MAMP: 366 (in 2010), 91.5 (in 2011)	
	MAMP:61–270 (140)	MDMA: 1 440	
	MDMA:49–690 (204)	COC: 145	
	BE: 28–93 (45)	**Annual music festival 2010 (range)**	
	COC:1.3–5.9 (3.5)	Cannabis: 1 000–2 000	
	MEPH: <0.001–1.9 (1.3)	MAMP: 152.5–701.5	
	Methylone: 1.2–4.4 (2.7)	MDMA: 320–4 640	
	Annual music festival 2011 (range)	COC: 72.5–290	
	THC-COOH: 2.9–12 (7.8)	**Nearby urban areas 2010 (range)**	
	AMP: 1.8–18 (8.8)	Cannabis: 2 400–3 200	
	MAMP:5.1–67 (34)	MAMP: 213.5–457.5	
	MDMA:15–510 (190)	MDMA: 80–560	
	BE: 6.2–100 (37)	COC: 145–580	
	COC: 0.3–12 (3.5)		
	MEPH: <0.001–0.9 (0.7)		
	Methylone: <0.001–16 (5.4)		
Days around the festival / festival days	**mg/day/1 000 inhabitants**	Not reported	[41]
***Piešťany / Topfest*:**	**Days around the festival / festival days**		
THC-COOH: 22–56 / 65–142	***Piešťany / Topfest*:**		
AMP: <6.1–29 / 45–75	THC-COOH:14–21 / 15–43		
MAMP: 209–255 / 411–648	AMP: <7.0–11 / 10–23		
MDMA: <9 / <7.6–159	MAMP: 80–165 / 93–198		
COC: <5–12 / <7–52	MDMA: <2.3–5.8 / <2.9–49		
COD: 34–110 / 53–166	COC: <5–22 / <7–44		
***Piešťany / Grape Festival*:**	COD: 13–41 / 12–51		
THC-COOH: 29, 39 / 38, 109	***Piešťany / Grape Festival*:**		
AMP: 24, 26 / 32, 52	THC-COOH:16–31 / 12, 36		
MAMP: 235, 249 / 286, 455	AMP: 6–14 / 10, 17		
MDMA: <6.4 / 16, 330	MAMP: 44–136 / 89, 151		
COC: <6 / <6, 159	MDMA: <1.6–3.5 / 5, 109		
COD: 10, 20 / 35, 62	COC: <8–29 / <7, 146		
***Skalica / Skalické dni*:**	COD: 5–98 / 12, 19		
THC-COOH: 82, 169 / 62–100	***Skalica / Skalické dni*:**		
AMP: 22, 32 / 22–44	THC-COOH: 18, 31 / 9–17		
MAMP: 202, 243 / 200–427	AMP: 5, 6 / 3–7		
MDMA: <9.1/ <8.2–78	MAMP: 44, 45 / 28–71		
COC: <6.3, 58 / <5.4–25	MDMA: <15, <1.6 / <1.2–13		
COD: 171, 541 / 106–251	COC: <4, 29 / 2–12		
***Trenčín / Pohoda Festival*:**	COD: 38, 98 / 15–37		
THC-COOH: 55, 114 / 159–412	***Trenčín / Pohoda Festival*:**		
AMP: <9, 32 / 33–43	THC-COOH: 14,15 / 20–52		
MAMP: 86, 227 / 244–165	AMP: <2.5, 4.1 / 4.2–5.5		
MDMA: 10, 173 / <5.7–207	MAMP: 24, 29 / 31–34		
COC: <4.3, 24 / 12–66	MDMA: 2.8, 2.2 / <0.7–26		
COD: 59, 117 / 81–241	COC: <3.3, 8.4 / 4.2–23		
***Zubrí / VanDaal fest*:**	COD: 15, 16 / 10–31		
THC-COOH: 34–61 / 54–98	***Zubrí / VanDaal fest*:**		
AMP: <16–25/ 34–52	THC-COOH: 20–29 / 22–40		
MAMP: 267–411 / 457–616	AMP: <9–12 / 14–21		
MDMA: <6.4 / <6.4	MAMP: 158–193 / 189–247		
COC: <6.3 / <6.6–12	MDMA: <2.8–3.3 / <2.2–2.6		
COD: 9–43 / 16–45	COC: <3 / <2–5		
***Valašské Meziříčí / Guláš Fest*:**	COD: 5–20 / 7–15		
THC-COOH: 72–108 / 74–124	***Valašské Meziříčí / Guláš Fest*:**		
AMP: 42–68 / 69–90	THC-COOH: 21–35 / 19–25		
MAMP: 439–681 / 614–889	AMP: 12–17 / 17–19		
MDMA: <7.0/ <6.4–13.0	MAMP: 125–167 / 154–184		

Table 4 (Continued)

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg/day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
COC: <8.6 / <7.2 COD: 12–69 / 45–126 ***Gypsy Fest (Bratislava)*****: THC-COOH: 94–123 AMP: 44–64 MAMP: 584–738 MDMA: 13–46 COC: 146–193 COD: 89–175 **All sites:** HER, 6-AM, LDS, LSD-OH, MTHD, BUP, MDEA, KET, MBDB, cathinone, mephedrone: <LOQ **non-festival days were not investigated, due to failure in obtaining the wastewater samples (flushing of the sewer pipes)	MDMA: <1.5–2.1 / <1.4–2.6 COC: <2 / <1–2 COD: 4–17 / 11–26 ***Gypsy Fest (Bratislava):*** THC-COOH: 18–24 AMP: 7–12 MAMP: 101–138 MDMA: 2.4–7.8 COC: 76–91 COD: 17–34		
2017 (mean) **WWTP 1:** THC-COOH: 319 ± 93 BE: 647 ± 131 COC: 191 ± 70 **WWTP 2:** THC-COOH: 256 ± 59 BE: 968 ± 537 COC: 160 ± 72 **2018 (mean)** **WWTP 1:** THC-COOH: 451 ± 48 BE: 882 ± 169 COC: 233 ± 43 **WWTP 2:** THC-COOH: 362 ± 134 BE: 473 ± 341 COC: 127 ± 106	Not reported	**mg/day/1 000 inhabitants** **2017 (average all / Music day event)** **WWTP 1:** Cannabis: 4 614 ± 1 368 / 6 252 MDMA: 50 ± 76 / 0 COC: 552 ± 194 / 572 **WWTP 2:** Cannabis: 4 384 ± 1 081 / 5 019 MDMA: 51 ± 102 / 0 COC: 978 ± 516 / 855 **2018 (average all)** **WWTP 1:** Cannabis: 8 209 ± 1 530 MDMA: 199 ± 62 COC: 933 ± 101 **WWTP 2:** Cannabis: 10 005 ± 4 409 MDMA: 124 ± 170 COC: 725 ± 453	[42]
During Youth festival (average): MDMA: 940 **Non-festival days (average):** MDMA: 89.1 **2 WWTPs (range and average)** AMP: 52–84 (41)MAMP: 90–557 (164)MDMA: <2–413 (207)COC: <1–52 (14) HER: <1 COD: 2 207–3 967 (3 180) KET: 8 033–138 000 (18 633) PEPH: 12 133–44 667 (22 300) GHB: <2–5.5 (3.9)	Not applicable	Not reported	[30]
COT – festival (mean) *Gypsy Fest (Bratislava):* 2 495 *Grape Festival (Piešťany):* 2 600 Topfest (Piešťany):4 327 *Skalické dni (Skalica):* 4 657 *VanDaal fest (Zubří):* 3 927 *Guláš Fest (Valašské Meziříčí):* 3 540 **COT – normal days (mean)** *Bratislava:* 2 016 *Piešťany:* 1 093 *Skalica:* 5 152 *Zubří:* 3 283 *Valašské Meziříčí:* 2 485	**g/day** **COT – festival (mean)** *Gypsy Fest (Bratislava):* 182 *Grape Festival (Piešťany):* 31 *Topfest (Piešťany):* 54 *Skalické dni (Skalica):* 15 *VanDaal fest (Zubří):* 35 *Guláš Fest (Valašské Meziříčí):* 16 **COT – normal days (mean)** *Bratislava:* 175 *Piešťany:* 16 *Skalica:* 16 *Zubří:* 31 *Valašské Meziříčí:* 15	**NIC g/day/1 000 inhabitant** ***(additionall information - cigarettes/day/1 000 inhabitants)*** **Festival (mean)** *Gypsy Fest (Bratislava):* 4 (5 044) Grape festival (Piešťany): 6 (7 535) Topfest (Piešťany): 8 (10 442) Skalické dni (Skalica): 5 (6 363) VanDaal fest (Zubří): 11 (13 366) Guláš Fest (Valašské Meziříčí): 6 (6 826) **Non-festival days (mean)** *Bratislava:* 4 *(4 863)* *Piešťany:* 4 *(5 025)* *Skalica:* 9 *(10 678)* *Zubří:* 10 *(11 929)* *Valaské Medziříčí:* 5 *(6 170)*	[44]
EtS – Fallas festivity (range) *Pinedo I:* 9 150–19 850 *WWTP of Pinedo II:* 4 000–9 890 *WWTP of Quart-Benager:* 7 160–10 710 **EtS – normal days (range)** *Pinedo I:* 1 460–14 900 *WWTP of Pinedo II:* 1 580–5 920 *Quart-Benager:* 2 000–7 170	Not applicable	**mL/day/inhabitant** **Alcohol - Fallas festivity (range)** *Pinedo I:* 13.36–23.81 *Pinedo II:* 4.35–9.07 *Quart-Benager:* 26.99–56.11 **Alcohol – non-festival days (range)** *Pinedo I:* 1.11–18.31 *Pinedo II:* 1.07–6.44 *Quart-Benager:* 3.31–12.38	[45]

Table 4 (Continued)

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg/day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
PUBLIC HOLIDAYS			
Not reported	**g/day** **1st of January (New Year) 2013 and 2014** BE: 224 / 197 MDMA: 62 / 67 AMP: 42 / 60 **25th of December (Christmas) 2012 and 2013** BE: 166 / 130	**mg/day/1 000 inhabitants** **normal days 2012 and 2013** HER: 73 ± 71 /140 ± 51 COC: 213 ± 34 / 261 ± 27 AMP: 24 ± 7.1 / 37 ± 11 MDMA: 36 ± 17 / 37 ± 13 Cannabis: 7 717 ± 1 580 / 10 660 ± 2 116 MTHD: 267 ± 41 / 218 ± 31	[16]
Not reported	Not reported	**mg/day/1 000 inhabitants** **holidays / control period (maximum)** **Semi-rural area:** Cannabis: 1 220 / 1 480 MAMP: 192 / 168 COC: 17.4/ 4.64 MDMA: 11.2/ 1.2 **Urban area:** Cannabis: 3 400 / 1 400 MAMP: 427/ 244 COC: 537/ 96 MDMA: 800/ 26 **Vacation area:** Cannabis: 1 720 / 1 260 MAMP: 793 / 6.1 COC: 798 / 97 MDMA: 1 440 / 6	[49]
WWTPA (mean) **typical week / Independence day observation week (mean) / *1st week of the semester*:** THC: 22.1 / 21.7 / ND THC-COOH: 499 / 275 / 435 THC-OH: 1 130 / 1 620 / 622 AMP: 243 / 184 / 182 MAMP: 690 / 603 / 577 MDMA: 19.7 / 47.9 / 8.36 BE: 276 / 254 / 228 COC: 105 / 88.8 / 79.6 nor-COC: 25.7 / 48.9 / 9.22 COE: 20.0 / 43.4 / 8.57 HER: 226 / 859 / ND 6-AM: 22.4 / 554 / 11.2 MOR: 161 / 138 / 110 MTHD: 43.4 / 55.9 / 27.9 EDDP: 166 / 166 / 97.9 MDEA: 19.2 / 42.4 / 6.85 MDA: 26.8 / 56.2 / 6.67 **$WWTP_B$ (mean)** **typical week / Independence day observation week / Solar eclipse observation:** THC: 38.1 / 8.33 / 65.2 THC-COOH: 505 / 562 / 767 THC-OH: 176 / 98 / 263 AMP: 333 / 248 / 517 MAMP: 1 350 / 1 200 / 1 560 MDMA: <LOQ / <LOQ / 6.53 BE: 987 / 959 / 1 200 COC: 160 / 197 / 201 nor-COC: 9.73 / 4.93 / 6.54 COE: 9.39 / 14.5 / 6.87 HER: ND / 385 / ND 6-AM: 5.42 / 63.6 / 2.06 MOR: 141 / 107 / 193 MTHD: 42 / 28.9 / 55.4 EDDP: 177 / 137 / 204 MDEA: ND / ND / ND MDA: <LOQ / 4.96 / ND	**mg/day** **WWTPA (mean)** **typical week / Independence day observation week (mean) / *1st week of the semester*:** THC: 312 / 379 / Not applicable THC-COOH: 6 860 / 2 540 / 7 675 THC-OH: 16 000 / 27 600 / 11 000 AMP: 3 160 / 3 320 / 3 060 MAMP: 10 600 / 13 600 / 11 400 MDMA: 306 / 874 / 154 BE: 3 720 / 4 880 / 3 930 COC: 1 580 / 1 720 / 1 480 nor-COC: 448 / 1 000 / 192 COE: 308 / 797 / 159 HER: 8 990 / 41 200 / Not applicable 6-AM: 4 640 / 11 100 / 268 MOR: 1 550 / 1 800 / 1 340 MTHD: 647 / 1 150 / 527 EDDP: 2 640 / 3 790 / 2 040 MDEA: 274 / 706 / 117 MDA: 397 / 1 020 / 119 **WWTPB (mean)** **typical week / Independence day observation week / Solar eclipse observation:** THC: 799 / 190 / 1 330 THC-COOH: 11 200 / 14 700 / 14 500 THC-OH: 3 860 / 2 500 / 4 980 AMP: 6 900 / 5 920 / 8 780 MAMP: 33 200 / 34 800 / 31 500 MDMA: Not applicable / Not applicable / 126 BE: 21 100 / 23 500 / 21 000 COC: 3 680 / 5 120 / 3 730 nor-COC: 255 / 94.9 / 142 COE: 209 / 258 / 128 HER: Not applicable / 10 300 / Not applicable 6-AM: 78.2 / 1 120 / 47.4 MOR: 2 120 / 1 910 / 2 400 MTHD: 987 / 809 / 1 070 EDDP: 4 580 / 4 230 / 4 330 MDA: Not applicable / 126 / Not applicable	**mg/day/1 000 inhabitants** **WWTPA (mean)** **typical week / Independence day observation week (range) / *1st week of the semester*:** Cannabis: 62 400 / 35 200–51 900 / 69 800 AMP: 526 / 350–706 / 510 MAMP: 1240 / 1 060–2 500 / 1 330 MDMA: 59 / 43.9–83.4 / 29.7 COC: 434 / 287–773 / 458 MOR: 2380 / 1 310–3 340 / 2 060 MTHD: 1 100 / 793–1 720 / 844 MDEA: 72 / 36.3–94.5 / 30.7 **WWTPB (mean)** **typical week / Independence day observation week (range) / Solar eclipse observation:** Cannabis: 81500 / 129 000–172 000 / 169000AMP: 919 / 592–1 200 / 1 450 MAMP: 3 090 / 2 420–5 600 / 3 400 MDMA: Not applicable / Not applicable / 27.6 COC: 1 970 / 1 290–3 240 / 2 280 MOR: 2 610/ 1 630–4 610 / 3 470 MTHD: 1 520 / 1 260–2 200 / 1 750	[50]
All WWTPs (range) **Memorial day:** THC: ND–32.8 THC-COOH: 134.2–1 213.9 AMP: 37–237.5 MAMP: 51.6–342.1 MDMA: ND–81.7	Not reported	Not reported	[31]

Table 4 (Continued)

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg/day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
BE: 459.4–2 811.1 CDC: 141.7–1 140.7 CDE: 4.6–40.3 6-AM: ND–17 MOR: 177.4–1 373.5 MTHD: 12.8–270.3 EDDP: 32–532.3 CDD: 21.9–208.3 MDA: ND–14.8 nor-fentanyl: ND Fentanyl: ND CDT: 276–1 467.1 **4th of July:** THC: ND–23.2 THC-COOH: 68–626.2 AMP: 15.5–285.9 MAMP: 27.3–272.3 MDMA: ND–69 BE: 266.4–2 487.8 CDC: 76.8–1 546.4 CDE: 3.1–46.3 6-AM: ND–12 MOR: 148.3–1 096.0 MTHD: 9.2–250.4 EDDP: 23.6–481 CDD: 20.8–171.7 MDA: ND–35.3 nor-fentanyl: ND Fentanyl: ND–5.9 CDT: 306.8–1 373.1 **Labor Day:** THC: ND–24.1 THC-COOH: 85.6–1 451.9 AMP: 21–203.7 MAMP: 43.9–367.8 MDMA: ND–116.8 BE: 517.2–2 456 CDC: 221.7–787.1 CDE: 5.6–36.3 6-AM: ND–6.4 MOR: 145.8–918.6 MTHD: 13.6–205.3 EDDP: 23.6–442.6 CDD: 27.8–219.6 MDA: ND–25.6 nor-fentanyl: ND Fentanyl: ND CDT: 344.9–1 350.9 **New Year's Day:** THC: ND–33.3 THC-COOH: 140–898 AMP: 15–167.2 MAMP: 21.6–372.2 MDMA: ND–187 BE: 473.5–2 014.3 CDC: 184.1–788.6 CDE: 4.7–42.6 6-AM: ND–18.8 MOR: 149.8–578.5 MTHD: 12.1–157.4 EDDP: 26.8–267.2 CDD: 29.3–151.7 MDA: ND–51.1 nor-fentanyl: ND Fentanyl: ND CDT: 336.4–776.1 **Additional information:** **Concentration normalized for creatinine (ng/mg creatinine)** **– all WWTPs;** **Memorial Day / 4th July / Labor Day / New Year's (range):** THC: 3.2–30.4 / 5.3–24.2 / 9.1–19.9 / 3–17.3 THC-COOH: 176.7–1 354.8 / 172.6–681.2 / 177.5–2 29.0 / 200–630.8			

Table 4 (Continued)

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg/day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
AMP: 25.7–265.1 / 32.8–260.9 / 27.8–167.1 / 12.9–125.7 MAMP: 17.4–309.9 / 34.1–283.6 / 27.3–318.6 / 15.7–340.5 MDMA: 5.9–125.1 / 11.1–62.2 / 7.3–223.8 / 4.7–167.6 BE: 389.6–3 947.2 / 604.9–2 886.1 / 644.8–3 346.8 / 481.4–2 132.9 COC: 92.4–1 613.4 / 184.2–1 814.8 / 198.1–1 118.1 / 155.3–829.3 CDE: 6.6–57 / 7.9–53.7 / 9.1–45.6 / 5.8–22.1 6-AM: ND, 18.6 / 8, 25.4 / 4.8–9.4 / 5.2, 16.1 MOR: 184.7–1 942.7 / 151.9–1 271.5 / 178.2–1 436.9 / 82.7–674.8 MTHD: 19.6–361.2 / 23.4–290.5 / 28.2–323.8 / 17.3–135.1 EDDP: 49–655.9 / 59.9–558 / 49–698.1 / 36.4–229.4 COD: 31.5–294.6 / 43.9–199.2 / 41.6–321.1 / 34.2–132 MDA: 2.7–13.4 / 4.2–24.8 / 10–37.4 / 5.8–26.6 nor-fentanyl: ND / ND / ND / ND Fentanyl: ND / ND, 5.3 / ND / ND COT: 200.1–2 075.1 / 262.3–1 593 / 26.7 / 1 627.4 / 187.6–509.8			
Guangzhou (average) AMP: 17.0 ± 10.3 MAMP: 145.6 ± 100.3 MDMA: 3.2 ± 1.9 BE: 1.98 ± 1.38 COD: 4.8 ± 2.6 KET: 28.6 ± 15.6 nor-KET: 8.9 ± 5.9	Not reported	**mg/day/1 000 inhabitants** **Guangzhou (range)** MAMP: 14.7–470.7 MDMA: 1.7–18.4 COC: 0.9–9.5 MTHD: 0.6–2.6 KET: 64.9–673.7 COD: 1.8–18	[51]
North Wing (range) BE: 1 598 ± 127–5 920 ± 208 COC: 835 ± 58–1 502 ± 144 CDE: 59 ± 13–188 ± 34 **South Wing (range)** BE: 1 599 ± 106–8 559 ± 141 COC: 635 ± 79–1 983 ± 40 CDE: < 14(LOD)–155 ± 14	Not reported	**COC** **mg/day/1 000 inhabitants** **North Wing (average)** Weekdays: 2 296 ± 353 Weekend: 3 100 ± 233 Carnival Day: 6 229 ± 219 **South Wing (average)** Weekdays: 1 707 ± 250 Sunday: 7 385 ± 121	[52]
Semi-rural area (range) AMP: 2.35–4.83 MAMP: 37.53–53.32 COD: 7.33–25.27 Meperidine: 0.48–0.73 MOR: <LOQ–4.01 **Residential area (range)** AMP: 2.32–5.64 MAMP: 25.80–40.60 COD: 3.72–14.23 Meperidine: 0.41–0.74 MOR: 3.25–5.11 **Vacation area (range)** AMP: 1.81–4.65 MAMP: 18.01–47.08 COD: 2.64–11.97 Meperidine: 0.37–1.84 MOR: ND–5.4	**g/day** **July–September 2013 (range and average)** **Semi-rural area:** AMP: 0.454–0.0909 (0.0688) MAMP: 0.725–1.142 (0.895) COD: 0.142–0.467 (0.266) Meperidine: 0.009–0.015 (0.013) MOR: <LOQ–0.075 (0.024) Fentanyl: ND– <LOQ **Residential area:** AMP: 0.766–1.743 (1.431) MAMP: 8.531–12.755 (11.097) COD: 1.233–4.400 (2.527) Meperidine: 0.129–0.233 (0.181) MOR: <LOQ–1.619 (0.1138) Fentanyl: ND– <0.014 **Vacation area:** AMP: 0.128–0.330 (0.195) MAMP: 1.279–3.343 (2.192) COD: 0.187–0.850 (0.389) Meperidine: 0.026–0.130 (0.060) MOR: ND–0.384 (0.096) Fentanyl: ND–<LOQ	**mg/day/1 000 inhabitants** **Semi-rural area (range)** MAMP: 29.10–45.81 COD: 0.567–3.920 Meperidine: 2.273–3.686 **Residential area (range)** MAMP: 23.01–34.39 COD: 0.837–1.646 Meperidine: 2.136–3.865 **Vacation area (range)** MAMP: 13.80–36.07 COD: 0.384–1.104 Meperidine: 1.745–8.640	[55]
Palermo – all study period (range and average): THC-COOH: 38.15–62.75 (59.05 ± 16.3) BE: 160.69–330.19 (205.69 ± 60.82) COC: 38.45–116.09 MAMP, MDMA, MDA, MDEA < LOQ **Palermo – during summer holidays / other months (average):** BE: 154.33 ± 55.49 / 235.5 ± 30.7	Not reported	**mg/day/1 000 inhabitants** **Palermo – all study period (range and average)** Cannabis: 1 680–3 220 (2 929) COC: 120–250 (160)	[56]
Not reported	**mg/day/1 000 inhabitants** Butylone: ND–0.02 Butyryl fentanyl: ND–< LOQ	Not reported	[57]

Table 4 (Continued)

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg/day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
	Furanyl fentanyl: ND–< LOQ Methoxetamine: ND–1.27 N-ethylpentylone: ND–36.35 Pentylone: ND–0.08 Valeryl fentanyl: ND–<LOQ		
EtS **Yearly average (range 2012–2017):** 16 100–19 600 **Average 2015:** 19 500 ± 5 100 **Average 2016:** 19 600 ± 4 600 **Average 2017:** 16 200 ± 3 400	Not reported	**Alcohol (range)** **ml/person/day** **Yearly average (range and median 2012–2017):** 8.6–50.5 (19.5) **Average 2015:** 17.8–21.5 **Average 2016:** 19.7–23.9 **Average 2017:** 15.7–19.0 **4 days prior Christmas / Christmas 2015, 2016 / 3 days following Christmas (average):** 19.1 ± 4.5 / 21.7 ± 2.2 / 17.6 ± 3.2 **Christmas, New Years', Australia Day / normal weekdays (average 2015–2017):** 21.2 ± 4 / 16.9 ± 5.3	[58]
SPORTING EVENTS **North-Wing** ***Typical weekend/ 5th place playoff / 3rd place playoff:*** BE: 2.3, 2.9 / 2.9, 4.2 / 2.5, 3.1 COC: 0.69, 1 / 0.75, 0.8 / 0.6, 0.65 AE: Not analysed / 2.5, 2.6 / 1.9, 2.0 nor-BE, nor-COC, EME: <LOQ ECG, AMP, MDBD: ND or < LOQ AEME, MAMP, MDA, MDMA, MDEA: ND **South-Wing** ***5th place playoff / 3rd place playoff:*** BE: 2.4, 3.4 / 1.9, 2.5 COC: 0.58, 0.75 / 0.46, 0.49 nor-COC: <LOQ nor-BE, AEME, EME: ND or < LOQ ECG, AMP, MAMP, MDA, MDMA, MDEA, MBDB: ND	Not reported	**COC** **mg/day/inhabitant** **North-Wing** *Typical weekend*: 1.6, 2.1 *5th place playoff*: 1.8, 2.7 *3rd place playoff*: 2.4, 2.9 **South-Wing** *5th place playoff*: 0.9, 1.4 *3rd place playoff*: 0.7, 0.71	[59]
THC <100 THC-OH: <100 HER <25 6-AM <25	**g/day** **Super Bowl weekend (average)** AMP: 114 ± 17 MAMP: 806 ± 132 MDMA: 106 ± 30 BE: 718 ± 142 COC: 294 ± 70 nor-COC: 7 ± 2 ECG: 271 ± 52 EME: 161 ± 27 MOR: 231 ± 112 MDA: 17 ± 3 **Normal weekend (average)** AMP: 125 ± 23 MAMP: 930 ± 154 MDMA: 97 ± 43 BE: 494 ± 82 COC: 295 ± 47 nor-COC: 7 ± 2 ECG: 266 ± 40 EME: 135 ± 23 MOR: 269 ± 61 MDA: 18 ± 5	Not reported	[60]
University of Mississippi (range) AMP: 900–13 700 MAMP: ND–600 MDMA: ND–100 BE: <625–7 400 COC: ND–900 COD: 55–390 MOR: <44–250 MTHD < LOQ EDDP: <LOQ MDA: <LOQ	Not reported	Not reported	[29]

Table 4 (*Continued*)

Concentration (ng/L)	Mass loads (g/day) or normalized mass loads (mg /day/1 000 inhabitants)	Consumption* (g/day or mg/day/1 000 inhabitants)	Reference
MDEA, 6-AM, COD, PCP, fentanyl, nor-fentanyl: ND			
City of Oxford (range)			
AMP: 600–1 200			
MAMP: ND–400			
MDMA: ND–0.50			
BE: 300–2 400			
COC: ND–1 200			
COD:0.73–310			
MOR: <LOQ–170			
MTHD: <LOQ			
EDDP: <LOQ			
MDA: <LOQ			
MDEA, 6-AM, COD, fentanyl, PCP, nor-fentanyl: ND			

* Only for alcohol: ml/day/1 000 inhabitants or ml/person/day.
ND – not detected, 6-AM – 6-acetylmorphine, AE – anhydroecgonine, AEME – anhydroecgonine methyl ester, AMP – amphetamine, BE – benzoylecgonine, BUP – buprenorphine, COC – cocaine, COD – codeine, COE – cocaethylene, CDT – cotinine, ECG – ecgonine, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EME – ecgonine methyl ester, EtS – ethyl sulfate, GHB – gamma- hydroxybutyrate, HER- heroin, KET – ketamine, LDS – lysergic acid diethylamide, LSD–OH – 2-oxo-3-hydroxy-lysergic acid diethylamide, MAMP – methamphetamine, MBDB – 3,4-methylenedioxy-N-methyl-butanphenamine, MDA – 3,4-methylenedioxyamphetamine, MDEA – 3,4-methylenedioxy-N-ethylamphetamine, MDMA – 3,4- methylenedioxymethamphetamine, MEPH – mephedrone, MOR – morphine, MTHD – methadone, NIC – nicotine, nor-BE – nor-benzoylecgonine, nor–COC – nor–COCaine, nor-KET – nor-ketamine, PCP – phencyclidine, PEPH – pseudoephedrine, THC – tetrahydrocannabinol, THC–COOH – 11-nor-Δ9-carboxy-Δ9-tetrahydrocannabinol, THC–OH – 11-hydroxy-Δ9- tetrahydrocannabinol.

consumption was observed in comparison to consumption during the control week [39].

The connection between the consumption of a particular type of illicit drugs and the type of music festival has also been investigated by Mackulak et al. [41]. The authors found that MDMA and cocaine were the main drugs consumed at a dance (Grape Festival) and multi-genre (Skalické dni) themed festivals, while cannabis was the drug of choice at a pop/rock festival (Topfest, Pohoda Festival). No specific consumption patterns were related to folk/country (Guláš Fest) and metal (VanDaal fest) festivals. Devault et al. [42] explored the impact that a large outdoor event, the so-called "Music day" event, which allows amateur and professional musicians of various musical tastes to perform in the street, has on illicit drug use (e.g., amphetamine, MDMA, cocaine, and cannabis) and methadone consumption in Bordeaux, France. The authors found no correlation between a music festival and drug consumption which is surprising. The authors suggest that the results reflect the influence of a non-peer audience at the street festival, although further research is needed to support this hypothesis.

Exploring drug consumption during special events such as festivals is also important for monitoring NPSs [43], which otherwise presents a challenge due to the dynamic nature of the NPSs market, and because they are readily procured from the internet or smart shops. Several studies observed sporadic consumption of NPSs, such as benzylpiperazine [40], methylone [40], mephedrone [40], MDA [46], ketamine and pseudoephedrine [30] during various music festivals. Causanilles et al. [43] applied a screening approach (almost 2 000 analytes, 560 NPSs) to samples collected at the Amsterdam WWTP during the Amsterdam street festivals in 2012 and 2014. In this case, NPSs from several groups such as synthetic cathinone, phenethylamine and synthetic cannabinoids were detected during the festival. Meta-chlorophenylpiperazine (mCPP), 2,5-dimethoxy-4-bromophenethylamine (2CB) and 4-fluoroamphetamine (4-FA) were detected for the first time.

Few WBE studies have explored licit drug consumption, such as alcohol and tobacco (nicotine), during festivals [44,45]. Mackulak et al. [44] found a significant increase in cotinine (nicotine metabolite) concentrations during festivals in comparison to non-festival days in the Czech Republic and Slovakia. The highest usage was observed during the rock/metal festival – Topfest (4-fold increase) and dance festival – Grape Festival (2.5-fold increase) in Piešťany [44]. Also, Andrés-Costa and co-workers [45] observed a significant increase in alcohol consumption during the "Fallas" festival (4.35–56.11 mL/day/1 000 inhabitants; >15 years) in comparison to non-festival days (1.07–18.31 mL/day/1 000 inhabitants; >15 years) at all three investigated WWTPs in Spain. In contrast, compared to an increase in alcohol consumption observed during regular weekends [45,67,68], on average there were no differences in alcohol consumption between the weekend (20.88 mL/day/inhabitant) and weekdays (19.98 mL/day/inhabitant) during the "Fallas" festival [45].

4.2. Public holidays

Impact of various public holidays has also been the subject of WBE studies (Tables 2 and 4). The results reveal the influence of different holidays on drug consumption patterns. Table 4, shows increased cocaine consumption during Christmas and New Year [16,40] as well as during Carnival [52] and the Easter holiday [53] and specific US holidays: Independence Day and Labor Day [30,49], and the Chinese Spring Festival [51]. Also, an increase in methamphetamine consumption was observed during the US Independence Day, a Solar eclipse [31] and the summer holiday season in Italy [56]. For other drugs, such as amphetamine, MDMA, and certain opioids, the impact of holidays on consumption trends was lower. In a study performed in Zagreb (Croatia) by Krizman-Matasic et al. [16], a 2 to 3-fold increase in the consumption of cocaine and MDMA following New Year's Eve celebrations was observed, while over Christmas only cocaine consumption increased (2- to 3-fold increase in benzoylecgonine mass loads). The use of amphetamine-type drugs did not significantly change during Christmas, and the data most likely reflects the life-style differences between cocaine and amphetamine-type drug users [16]. Lai et al. [49] performed a study of drug consumption during the Christmas holidays at specific locations in a coastal urban area, an inland semi-rural area and an island vacation area in Australia. Their results indicated that specific consumption patterns observed during holidays were related to the different study

areas. Similar consumption patterns were observed in the vacation area (island) and coastal urban area, which is also a popular vacation destination with numerous nightclubs and festivals. Compared to the urban area, that showed an increase in the consumption of most of the drugs analyzed (MDMA: 31-fold, cocaine: 5.6-fold, cannabis: 2.4-fold, methamphetamine: 1.8-fold), levels of cannabis and methamphetamine use in the semi-rural area during the holiday period did not differ significantly from those in the control period. However, there was a 4- and 9-fold increase in cocaine and MDMA consumption.

Foppe et al. [50] observed an increase in the consumption of illicit drugs and opioids during Independence Day, a solar eclipse, and during the first week of the academic semester in the USA. Significantly, 1.3- to 2-fold increase in consumption of amphetamine, methamphetamine, cocaine, morphine, and methadone was observed on Independence Day compared to an average week in two similar-size communities. Drug consumption (including cannabis) was also significantly higher during the 2017 solar eclipse than that for a typical Monday, particularly in communities with a dynamic population (highway routes and airport). A US study performed by Centazzo et al. [31] investigated consumption trends during four public holidays, including Memorial Day, 4th of July, Labor Day and New Year. Their results found that there was no effect on the consumption of amphetamines, nicotine and cannabis, while only opioids and cocaine use varied significantly between particular holidays. For example, opioid use was higher during Memorial Day (184.7–1 942.7 ng of morphine/mg creatinine) and Labor Day (178.2–1 436.9 ng of morphine/mg creatinine) compared to New Year (82.7–674.8 ng of morphine/mg creatinine), while cocaine use was higher during Labor Day (644.8–3 346.8 ng of benzoylecgonine/mg creatinine) than during New Year eve celebration (481.4–2 132.9 ng of benzoylecgonine/mg creatinine). These results should be interpreted with caution since only grab samples were collected. The impact of public holidays on drug consumption was also explored during the Chinese Spring festival and National Day [51]. Consumption of methamphetamine and cocaine was significantly higher during National Day: 47 % higher for cocaine, compared to the control period while during Spring festival week, methamphetamine consumption also increased. Increased cocaine consumption was observed as well in Brazil during Carnival (6 229 ± 219 mg/day/1 000 inhabitants) in comparison to consumption on weekdays (2 296 ± 353 mg/day/1 000 inhabitants) and weekends (3 100 ± 233 mg/day/1 000 inhabitants) in the North-Wing, while in the South-Wing cocaine use was higher on a Sunday (7 385 ± 121 mg/day/1 000 inhabitants) than on the day of the Carnival (Monday) [52]. A significant increase in cocaine loadings was also observed during the Easter holiday [53] in France. Other biomarkers analyzed in this study, such as biomarkers of cannabis, codeine, morphine and heroin, also correlate with the Easter holidays. However, the focus of their study was on the impact of drug groups, e.g., opioids and stimulants rather than individual drugs.

In Croatia, Krizman et al. [54] observed the impact of the summer tourist season on the coastal touristic city of Zadar and the Croatian capital Zagreb. During the control period (spring), consumption of heroin, MDMA, cocaine, and cannabis was significantly higher in Zagreb compared to Zadar. In contrast, during the summer season, intercity differences in drug consumption were less pronounced, and the consumption of MDMA in Zadar was higher than in the capital. These differences in consumption are most likely a result of pronounced changes in population since the population of Zadar increases by 16 % in the summer bringing with it changes in lifestyle. Surprisingly, cannabis consumption in Zadar decreased during the summer season, which agrees with data obtained by Lai et al. [49]. The impact of the summer holiday season was also explored by Kim et al. [55], who investigated drug consumption in semi-rural, residential and vacation locations in Korea. Increased consumption of methamphetamine and mephedrone was observed, but only in the vacation area. Unlike previous research, a decrease in cocaine consumption during holidays, particularly during the summer (2nd week of June to the 1st week of September) compared to November through to March was observed in Palermo, Italy (140 vs 210 mg/day/inhabitants) [56]. Since the summer period coincides with the school holidays, lower cocaine use could be related to the reduced population size since many people move to their summer residence.

Except for the trends in classical illicit drug consumption, Bade et al. [57] has explored the impact of the Christmas–New Year period on NPSs consumption since recreational drug use in this period is also higher. Their study included a broad range of different NPSs including phenethylamines, synthetic cathinones, opioids and amphetamines (22 NPSs in total). Seven NPSs were detected and confirmed such as butylone, butyryl fentanyl, furanyl fentanyl, methoxetamine, N-ethylpentylone, pentylone and valeryl fentanyl. The highest mass loads were found for N-ethylpentylone (36.35 mg/day/1000 people) on 20 December, while mass loadings were much lower on subsequent days (e.g., 2.94 mg/day/1 000 people on 23 December, and 1.72 mg/day/1 000 people on 1 January). Despite this, caution should be taken in interpreting consumption, since only parent compounds were included in order to determine mass loadings, which could potentially arise from the direct disposal of the target drug along with or instead of drug consumption.

The impact of holidays, including the Christmas holiday season and Australia Day, on alcohol consumption, was explored by Zheng et al. [58]. Their results show a higher consumption of alcohol during all investigated holidays, e.g., Christmas, New Years, Australia Day, (21.2 ± 4 mL/person/day) in comparison to average weekdays (16.9 ± 5.3 mL/person/day) in years 2015–2017.

4.3. Sporting events

Wastewater-based epidemiology was also used to study drug consumption trends during various sporting events. For example, Sodre et al. [59] analyzed cocaine alkaloids, metabolites and adulterants as well as amphetamine-type substances in wastewater collected during the FIFA Soccer World Cup in 2014. A comparison of the data with that of an average weekend revealed a 25 % increase in cocaine consumption. Also, Gerrity et al. [60] observed increased benzoylecgonine loads during the 2010 US National Football League's Super Bowl (718 g/day) when compared to a typical week (494 g/day). Gul et al. [29] also analyzed wastewater collected from the Oxford Wastewater Treatment Plant in Oxford, (Mississippi, USA), and the University of Mississippi Wastewater Treatment Plant (Mississippi, USA) during weekends when the home football team was playing. The authors analyzed a broad range of stimulants and other drugs. The use of cocaine and amphetamine was observed at both the university and in the city. A significant increase in amphetamine (18-times) and benzoylecgonine (up to 8-times) concentrations during the two highest attended games was observed only at the University of Mississippi. Since the concentrations of amphetamine and benzoylecgonine in the City remained the same, the observed increase in the levels at the university is likely related to visitors watching the game.

5. Ethical issue related to WBE approach

Usually, WBE is applied to general populations, where the contribution of individuals to wastewater is non-identifiable, and where no information on individual drug use can be obtained [12,13,69]. According to Human Research Ethics Committees, WBE involves little ethical risk and typically does not require an ethical review of the studies. Although the WBE approach cannot be used

to target an individual *per se*, site-specific WBE studies can raise specific ethical concerns [70], and a series of ethical WBE guidelines have been developed by Prichard et al. [71] covering general and site-specific studies. Careful research planning is especially important when dealing with specific communities. To avoid ethical risks, researchers must obtain information on countries' regulations (regulations in some countries might still require their ethics committees' approval of the research conducted in specific catchments) and obtain approval from the relevant institutional authority (e.g., school principals and prison authorities). Also, gathering information on the reputation of institutions and stakeholders' ethical practices should be considered. For example, in prisons, WBE data could be used by authorities as a base for collective punishment of the prisoners, such as reducing family visits [37,69,70]. To avoid these problems, a careful discussion between researchers and prison authorities regarding a particular study, the results and ethics are essential [69]. During research planning, appropriate media communication protocols (e.g., determining who is the media contact person and specifying the information to be classified) and preservation of the anonymity of the studied sites needs to be considered, since sensationalizing of the study results and media attention can negatively affect the target population and institution [69,71]. In the case of prisons, negative media reports may harm opinion about the reintegration of ex-prisoners back into society or in the case of schools may harm a schools' reputation [69,70].

6. Practical challenges and future recommendation

The WBE approach enables the rapid non-invasive and cost-effective collection of objective data relating to drug consumption patterns and delivers this data in near-real-time, which is necessary for accurately tracking drug use. However, WBE is not without its disadvantages, for example, it cannot provide information on the type of users, multi-substance use or if any of the observed changes are related to an increasing number of estimated doses or an increase in the number of users.

The main uncertainty, when applying WBE in site-specific studies, derives from the sampling stage. A common practice is to collect samples with time-proportional sampling with reported sampling frequency from 1 min to 1 h (Table 1). From this, arise the question of representativeness of samples since irregular pulses (e.g., toilet flushes) might be missed. It is advised that preliminary analysis are performed, possibly using a tracer dye, prior to determining the sampling rate. Another way to overcome this difficulty is to use flow-proportional sampling or passive sampling; however, the use of passive sampling at site-specific studies still needs to be explored. To estimate drug consumption also requires knowing the flow rate, but since low and inconsistant wastewater flow makes it difficult to measure the flow rate in site-specific studies, as an alternative, the flow rate can be estimated from the monthly water bill (e.g., by dividing used water with the number of days in the month of sampling). However, the result should be treated with caution, since additional uncertainty is introduced into the result.

In specific populations, individual's excretion profiles may also have a significant effect on the overall results. Sampling for an extended period (e.g., repeated one-week samplings) is desirable as well as sampling larger populations to reveal, more precisely, the trends in drug consumption. Also, participants in a specific community should not be aware of any research being undertaken in order to avoid bias. In studies related to special events like music festivals, holidays and sporting events, sampling is not as problematic, since most commonly wastewater samples are obtained at WWTP using established procedures. However, the sampling period should be optimized to have adequate control samples, along with the samples collected during special events, which can be compared to obtain satisfactory conclusions.

Wastewater based epidemiology when applied to the general population typically does not have any significant ethical concerns, but issues may arise when applied to specific sites, especially in institutions like schools and prisons (site-specific studies). Careful research planning and compliance of developed ethical WBE guidelines should be considered to avoid ethical risks. For example, during research planning information on countrywide regulations should be obtained, good cooperation between the researcher and target facility must be established, and it is important to preserve the anonymity of the study site and use caution when reporting the results to the media.

Despite the uncertainties mentioned above, WBE is a useful tool for monitoring time and spatial trends in licit and illicit drug consumption in specific circumstances. Also, timely information on trends in new psychoactive substance use can be obtained. However, additional guidance on sampling is needed and the way data is reported standardized to enable an appropriate comparison of the results.

Declaration of Competing Interest

The authors report no declarations of interest.

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3.2.2 Investigation of drugs of abuse in educational institutions using wastewater analysis

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Both licit and illicit drug use harm the health and future of young people, with a higher likelihood of developing harmful patterns of drug use (*e.g.,* injection) and addiction closely related to a younger age of first use [182]. In order to establish efficient prevention programs, it is crucial to have good knowledge of trends in drug use among young people, which is usually obtained in the environment of educational institutions using surveys, such as ESPAD (targeting 15-16-year olds) and the study on Health Behavior in School-aged Children (HBSC, targeting 11-, 13-, 15-, and 17-year olds), and even drug testing [38], [114]. Also, WBE studies have been conducted in US, Italian and Greek educational institutions, although they mainly assessed drug use in individual high education institutions (Chapter 1.3.4 WBE: Specific populations)

In response, for the first time, a comprehensive WBE study was conducted to assess the prevalence of licit and illicit drugs in a large number (n=44) of educational institutions in Slovenia. Institutions were of different types, namely primary schools (6-15 yrs.), secondary schools (15-19 yrs.), mixed secondary and high education institutions (15+ yrs.) and high education institutions (19+ yrs.), and located in urban and non-urban areas of six municipalities. Analytical methods were developed to determine drug residues of licit (nicotine, alcohol, morphine, codeine and methadone) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy, and heroin) using SPE (Oasis MCX) and target analysis (LC-MS/MS).

Drug residues were present in all obtained wastewater samples, with residues of nicotine (detection frequency, DF>98 %), cannabis (DF=93 %) and alcohol (DF=80 %) being the most frequently detected. Moreover, cocaine residues were detected in >50 % of the samples, while other residues were less common (DF<40 %). Although differences in drug prevalence were found between educational institutions in different municipalities and urban and non-urban areas, the type of educational institution influenced the consumption patterns the most. Notably, a difference between primary schools and other institutions was observed. Also, the data only partially agreed with available data from ESPAD and HBSC but agreed with other WBE studies, *i.e.,* SCORE data and limited WBE studies performed in educational institutions abroad.

Although the study could not distinguish between pupils, staff and visitors, important information was obtained about which and when drugs enter young people's environment. It also demonstrated that as a non-invasive approach, WBE could be complementary in assessing drug use in specific populations. Its application is especially valuable when assessing the presence of drugs in vulnerable populations.

The developed method and study outcomes were presented at five scientific conferences, *i.e.,* the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day; Lisbon Addictions 2019: the 3rd European Conference on Addictive Behaviors and Dependencies; the 21st European Meeting on Environmental Chemistry; Testing the Waters 5 Conference 2021; and the 18th International Conference on Chemistry and the Environment. At the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, the presentation was awarded as "the best presentation of research achievements in terms of scientific quality and their applicability, the best oral presentation by a young researcher". In addition, the results were presented to the Slovenian public on four occasions through interviews in

national journals and radio/TV stations and popular science lectures (Science on the street, Slovene: *Znanost na cesti*). So far, the study globally presents the most extensive wastewater analysis study conducted in a specific population regarding the number of sites studied.

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Investigation of drugs of abuse in educational institutions using wastewater analysis

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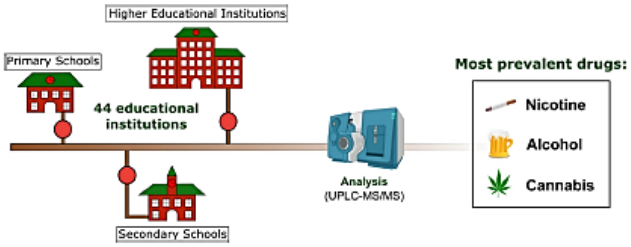
HIGHLIGHTS

- Drug prevalence in educational institutions was studied using wastewater analysis.
- Residues of drugs of abuse were present in all samples.
- Residues of nicotine, alcohol and cannabis were the most frequently detected.
- Educational level of the institute influenced consumption patterns the most.
- Drugs are present in young people's life in early stages of their education.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater analysis was used to investigate drug prevalence in primary and secondary schools and institutes of higher education located in urban and non-urban areas of six municipalities in Slovenia. Seven-hour composite raw wastewater samples from 44 educational institutions, including 19 primary schools (6–15 yrs.), ten secondary schools (15–19 yrs.), nine higher education institutions (19+ yrs.) and six mixed secondary and higher education institutions (15+ yrs.), were collected at the end of the 2018/2019 academic year. Metabolic residues of licit drugs (nicotine and alcohol), medications of abuse (morphine, codeine and methadone) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin) were targeted in the study. The analysis was carried out using solid-phase extraction and direct injection combined ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Biomarkers of nicotine, alcohol and cannabis intake were the most frequently detected, indicating a high prevalence of these drugs. Morphine and codeine were also detected, while among the stimulants, benzoylecgonine had the highest detection frequency. Drug differences were found between different levels of educational institution, geographic location (inter-municipality comparison) and degree of urbanization. However, t-distributed stochastic neighbour embedding (t-SNE) revealed that the level of educational institution was the main factor influencing the differences in drug prevalence. Although a good agreement between data from this study and other studies implementing wastewater analysis was observed, there was a discrepancy with Slovenian epidemiological survey data. Finally, despite certain drawbacks of the method, its application to detect drug residues in educational institutions provides a non-invasive insight into drug use trends.

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1. Introduction

Licit and illicit drug use can negatively impact a young person's health, such as cognitive functioning, psychological well-being, and future socio-economical position and success (Koprivnikar et al., 2018). The earlier a person begins using psychoactive substances, the greater the possibility that he or she will develop harmful patterns of drug use, which can lead to addiction and behavioural problems later in life; hence in Slovenia, many prevention programmes are targeted towards adolescents and young adults (Koprivnikar et al., 2018). For example, in 2017 and 2018, Slovenian non-governmental organizations organised various drug prevention and harm reduction programmes, workshops, and lectures aimed at young people, parents and school workers at the community level to aid young people in making healthy life choices (NIJZ, 2019b). However, proper implementation and evaluation of prevention programmes such as these require accurate knowledge about the extent of substance abuse (EMCDDA, 2019; López-García et al., 2019).

In Slovenia, data on drug use among 15-16-year-olds have been gathered through the European School Survey Project On Alcohol And Other Drugs (ESPAD Group, 2020) and from the Cross-National Survey On Health Behaviour In School-Aged Children (HBSC) study (Inchley et al., 2020), which surveyed 11-, 13-, 15- and 17-year-olds. Unfortunately, recall bias, the time lag in reported data and the respondents' subjectivity prevent surveys from gathering objective and timely information (Zuccato et al., 2008b). A more objective alternative is to determine selected metabolic excretion products (biomarkers) of consumed drugs in wastewater from which drug use in a community can be estimated (Gracia-Lor et al., 2017). This approach, called wastewater-based epidemiology (WBE), can provide evidence-based, objective, non-invasive, and near real-time estimates of community drug use at the regional, national and international level (Castiglioni et al., 2011; González-Mariño et al., 2019; Krizman-Matasic et al., 2019; Mastroianni et al., 2017; Zheng et al., 2017).

To date, few researchers have performed WBE studies of educational institutions (Burgard et al., 2013; Gatidou et al., 2016; Gushgari et al., 2018; Heuett et al., 2015; Panawennage et al., 2011; Verovšek et al., 2020; Zuccato et al., 2017). There have also been no studies exploring the prevalence of licit drugs, including medicines of abuse and illicit drugs, in primary and secondary schools and institutes of higher education, and knowledge of drug consumption patterns among educational institutions in areas with varying degrees of urbanization is lacking. With this in mind, paper seeks to address this knowledge gap by using wastewater analysis to investigate drug prevalence in Slovenian educational institutions in different locations classed as urban and non-urban by the Statistical Office of the Republic of Slovenia and compare the findings with available epidemiological data. In order to achieve our aim, wastewater samples from primary, secondary schools, higher education institutions (HEIs) and mixed secondary and higher education institutions (SHEIs) were analysed for metabolic residues of licit drugs (nicotine and alcohol), medications of abuse (morphine, codeine, methadone) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin).

2. Methods

2.1. Materials

Standard solutions (1 mg/mL) of 16 metabolic residues of licit drugs: nicotine (nicotine, cotinine, trans-3′-hydroxycotinine – HCOT) and alcohol (ethyl sulphate), medications of abuse: morphine (morphine), codeine (codeine) and methadone (methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine – EDDP), and illicit drugs: cannabis (11-nor-9-carboxy-Δ9-tetrahydrocannabinol – THC-COOH), cocaine (cocaine, benzoylecgonine and cocaethylene – a cocaine and alcohol co-consumption biomarker), amphetamine (amphetamine), methamphetamine (methamphetamine), ecstasy (3,4-methylenedioxymethamphetamine, MDMA) and heroin (6-acetylmorphine) and their labelled analogues (1 or 0.1 mg/mL) were purchased from Ceriliant Corp. (Round Rock, Texas, USA). Labelled analogues for each compound were used except for nicotine metabolites, where (±)-cotinine-d3 was used for all of them (Table S1). Working standards were prepared with final concentrations of 10 mg/L (analytes), 2 mg/L (labelled analogues of residues of medications of abuse and illicit drugs), and 0.5 mg/L (alcohol and nicotine residues labelled analogues). Methanol was purchased from JT Baker (Philipsburg, USA), LC-MS grade formic acid (HCOOH) and phosphoric acid (H_3PO_4) from Fluka (Switzerland), aqueous ammonia solution (NH3, 25%) from Merck (Darmstadt, Germany) and ammonium formate and tetrabutylammonium bromide (ion-pair reagent) from Sigma Aldrich (Missouri, USA). Millipore Direct-Q purifying system was used to obtain Milli-Q water.

2.2. Wastewater sampling

Forty-four educational institutions participated in the study. These consisted of 19 primary schools (6–15 yrs.), ten secondary schools (15–19 yrs.) comprising four gymnasiums (general education), three vocational, technical and other professional schools and three multi-programme schools, nine HEIs (19+ yrs.) comprising two institutions of social and seven of natural sciences, and six SHEIs (15+ yrs.). The location of each institution was defined as either urban or non-urban according to the Statistical Office of the Republic of Slovenia's definition. This definition considers the number of inhabitants, a surplus of jobs over the number of working people, the built-up area with urban character and number of workplaces and share of farms (Pavlin et al., 2004; SURS website). In our case, institutes were located in 37 urban and seven non-urban areas within seven municipalities (M1–7) from six different statistical regions (Fig. 1).

Sampling times, sampling location and methodology were based on a one-week preliminary study conducted at a secondary school in April 2019. Briefly, the samples obtained were analysed for 15 drug residues (ethyl sulphate was not included in the preliminary study) and results (see 3.2. Preliminary study results) were used to set appropriate days for sampling. For the full study, 40 composite raw wastewater samples (one sample per sampling site: 100 mL every 5 min over 7 h) were obtained mid-week, i.e., on either Tuesday, Wednesday or Thursday. Sampling took place at the end of the academic year 2018/2019 (May and June) except for two samples taken in March (Table S2). In specific cases, the sewer layout meant that some samples contained wastewater from more than one educational institution.

2.3. Sample preparation

For the determination of nicotine, cotinine, HCOT and ethyl sulphate, the samples were filtered through GF/D (2.7 μm, Whatman, USA), GF/C (1.2 μm, Whatman, USA) and cellulose membrane filters (0.45 μm, Sartorius, Gottingen, Germany) and spiked with labelled internal standards (final concentration: 10 ng/mL). For the determination of ethyl sulphate, the ion-pair agent (tetrabutylammonium bromide, TBA) was added to the samples (final concentration: 50 mM) to improve compound retention, peak shape and signal response (Rodríguez-Álvarez et al., 2014).

For basic drug residues, i.e., morphine, codeine, methadone, EDDP, cocaine, benzoylecgonine, cocaethylene, amphetamine, methamphetamine, MDMA and 6-acetylmorphine, and cannabinoid (THC-COOH) we used a modified method based on that of Senta et al. (2013). Briefly, each sample (125 mL) was spiked with labelled internal standards (final concentration: 60 ng/mL) and filtered through GF/D and GF/C filters (Whatman, USA). Sample pH was adjusted to pH 2 using concentrated H_3PO_4. Drug residues were extracted using solid phase extraction

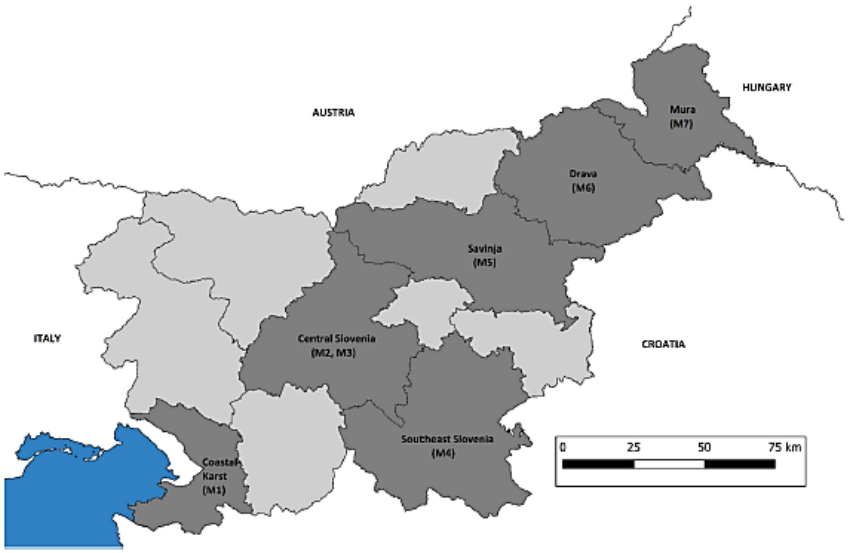

Fig. 1. Map of Slovenia showing sampling regions (dark grey) (Eurostat, GISCO; MOP, Geodesy Office, e-data; QGIS Geographic Information System).

(Oasis MCX, 150 mg/6 mL cartridges, Waters, Milford, MA, USA). After conditioning with methanol (5 mL), Milli-Q water (5 mL) and 25 mM H_3PO_4 (5 mL) and sample loading, a two-step elution was performed. Cannabinoids were eluted in the first fraction (methanol, 6 mL) and basic drug residues in the second (0.5% ammonium solution in methanol, 6 mL). Further purification of the acidified cannabinoid fraction (concentrated HCOOH, 60 μL) was performed using Strata NH_2 cartridges (200 mg/3 mL, Phenomenex, Torrance, California, USA). The analytes were eluted using 1% HCOOH in methanol (4 mL). All extracts were dried (40 °C, N_2) and reconstituted in 500 μL (250-times concentration) of either Milli-Q and methanol, 80:20, v/v with 0.1% formic acid (basic drug residues extract) or Milli-Q water and methanol, 30:70, v/v (cannabinoids-containing extract).

2.4. Analysis

Analysis was performed using a Shimadzu ultra-performance liquid chromatograph hyphenated to an AB Sciex 4500 QTRAP detector mass spectrometer (UPLC-MS/MS) with electrospray ionization (positive and negative) and operated in the multiple reaction monitoring (MRM) mode. Retention times, both transitions and the ratio between the transition peak areas were used to identify the targeted drug residues (European Union Commission Decision 2002/657/EC).

For alcohol residue analysis, 10 μL of the sample was injected on an Ascentis® Express C18 column (2 μm, 50 mm × 2.1 mm, Supelco, Pennsylvania, USA) at 40 °C. The mobile phase (flow rate: 0.3 mL/min) consisted of Milli-Q water (A) and methanol (B), containing 0.1% formic acid (elution gradient: 2% B, increase to 15% B at 10 min, 95% B at 11 min [hold: 1 min], then 2% B at 13 min). The ESI was in negative ionization mode (ESI−). For nicotine and basic drug residues, the method of Senta et al. (2013) was used. Briefly, 10 μL of the extract was injected onto a Synergi Polar-RP column (2.5 μm, 30 mm × 2 mm, Phenomenex, Torrance, California, USA) at 40 °C. In this case, the mobile phase (flow rate: 0.3 mL/min) was Milli-Q water (A) and Methanol (B) containing 5 mM ammonium formate and 0.1% formic acid (elution gradient: 2% B to 50% B at 6.9 min, 55% B at 7.3 min, 85% B at 8.7 min, 88% B at 10.7 min, and 100% B at 11 min [hold: 1.4 min]). The ESI was in the positive ion mode (ESI+). The MRM™ algorithm (MRM detection window: 120 s) was applied during acquisition. Separation of cannabinoids was achieved by injecting 10 μL of the extract on a Supelco Ascentis® Express C18 column (2 μm, 50 mm × 2.1 mm, Supelco, Pennsylvania, USA) at 40 °C using Milli-Q water (A) and methanol (B) as the mobile phase at a flow rate: 0.3 mL/min (elution gradient: 10% B to 50% B at 1.5 min, 60% B at 3.0 min [hold 4 min], increase to 85% B at 12.5 min, 10% B at 13 min [hold 2 min]). Here the ESI was operated in the negative ion mode (ESI-).

2.5. Method validation

The method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), relative extraction recovery (for residues of medications of abuse and illicit drugs), matrix effect (ME), accuracy and repeatability. Linearity was determined from the calibration curve (peak area ratio of the analyte vs its labelled analogue as a function of analyte concentration) and described using the coefficient of determination (R^2). The LOD/LOQ were determined by spiking the wastewater with labelled analyte analogues at low concentrations and calculated by averaging the signal-to-noise ratios (S/N = 3 or S/N = 10) of five replicates. Relative extraction recoveries, ME, accuracy and repeatability were also calculated using spiked samples.

2.6. Statistical analysis

A statistical evaluation was performed using Excel (Microsoft, USA), SigmaPlot (version: 14.0) and R (version: 4.0.3) (Eftimov et al., 2017). The data were analysed using the non-parametric Chi-Squared test for two or more samples (95% confidence level, $\alpha = 0.05$). When differences among multiple groups were observed, a Chi-Squared post-hoc test using adjusted residuals and Bonferroni adjustment of the significance level (adjusted α) was applied. Additionally, singular value

decomposition (SVD) was used to obtain a new representation of data instances, visualized using the dimensional reduction technique t-distributed stochastic neighbour embedding (t-SNE, R library: Rtsne). Perplexity was set to ten.

2.7. Ethics and consent

Although the benefit of WBE is that it avoids many of the ethical issues associated with surveys and drug testing, privacy concerns may arise when applied to smaller specific sites, such as educational institutions (Prichard et al., 2015). Guidelines for the researchers using wastewater analysis were developed by Prichard et al. (2015) and are hosted on the SCORE (Sewage Analysis CORe group Europe) website (SCORE website, Ethical guidelines for WBE). According to the guidelines, consent must be obtained when sampling from a specific site. In the study, informed consent was obtained from the Head of each institution. In return, they received an outline of the study aims and sampling procedure. Notably, an anonymity agreement was also signed to avoid identifying an individual institution and prevent possible stigmatization, i.e., by sensationalised media reporting.

3. Results and discussion

3.1. Method validation

In general, a linear response ($R2 > 0.99$) was observed between LOQ–1000 ng/mL for the drug residues. The exceptions were ethyl sulphate, EDDP, methamphetamine (LOQ–500 ng/mL) and methadone (LOQ–200 ng/mL). The LOD and LOQ for medications of abuse and illicit drugs were in the ng/L range (LOD: 0.31–3 ng/L, LOQ: 1–9.6 ng/L), while for licit drugs, they were 19–305 ng/L and 64–1020 ng/L. The matrix effect ranged from −2% to −115% for most drug residues. Nicotine was the only compound whose signal was enhanced, i.e., by 77%, at the lower spiking concentration. Relative extraction recoveries for medications of abuse and illicit drugs were between 71 and 110%, except for EDDP (23%). Accuracy was in the 84–136% range, while repeatability was below 10% (RSD). The only exception was ethyl sulphate at low concentrations (14% RSD).

3.2. Preliminary study results

The number of detected residues did not differ statistically between sampled days ($\chi^2 = 0.220$, $p = 0.974$, $\alpha = 0.05$), but differences in the detection frequency of particular metabolites, namely MDMA and benzoylecgonine, were observed. Stimulants are known to have a distinctive weekly consumption pattern, i.e., their higher consumption at weekends (Krizman et al., 2016; Thomas et al., 2012; Zuccato et al., 2008b) may explain the presence of MDMA and benzoylecgonine only on Mondays and Fridays. Therefore, to avoid the influence of weekend use, we chose Tuesday, Wednesday and Thursday (mid-week) as the most appropriate sampling days.

3.3. General findings

Despite certain limitations (e.g., the influence of the dose, excretion rate, sample preparation and LOD on the detection of individual analytes), drug prevalence was evaluated based on detection frequency (DF = percentage of samples containing drug residues above LOD) rather than consumption estimates (Verovšek et al., 2020; Zuccato et al., 2008b). The reason being that sampling in time-proportional mode meant that, although the sampling frequency was high (100 mL/5 min), small and inconsistent wastewater flows, i.e., episodes without wastewater in the sewer, prevented the collection of some composite subdivisions. Moreover, dilution with kitchen wastewater occurred at some sampling sites, making it difficult to compare all institutions quantitatively. The latter could be overcome by normalising the data (concentrations) to mass loads using flow data. However, the data on flow was impossible to obtain for all sampled sites and accordingly, mass loads could not be calculated. Aside from accurate flow measurements, flow-proportional sampling and the use of passive samplers (e.g., Polar Organic Chemical Integrative Sampler – POCIS and passive-active samplers) are also possible solutions (Amato et al., 2021; Verovšek et al., 2020). However, the application of passive samplers is only feasible under optimum conditions (i.e., passive samplers should not dry out during sampling), and further studies on the efficacy of passive samplers are needed (Verovšek et al., 2020).

The results show that between four to ten residues were detected in the samples (see Supplementary material: Fig. S1), with six being the most frequent (25% of the samples). High DFs of nicotine biomarkers (cotinine and HCOT; Table 1) suggest a high prevalence of nicotine use in Slovenian educational institutions. Since nicotine and alcohol metabolic residues were the most commonly detected in the study, we compared their detection frequencies. Compared to nicotine biomarkers, fewer samples contain ethyl sulphate ($\chi^2 = 13.514$, $p = 0.00024$, $\alpha = 0.05$), reflecting possible differences in consumption patterns, i.e., daily use of nicotine versus recreational use of alcohol, with peak consumption occurring over the weekend (Lai et al., 2018; Reid et al., 2011; Ryu et al., 2016).

Among the opioids, morphine had the highest DF (Table 1). Morphine is the second most commonly prescribed opioid (6500 prescriptions in 2019) in Slovenia after oxycodone (NIJZ, 2019a). Besides medical morphine, morphine may also originate from the metabolism of codeine and heroin (Baselt, 2000; Zuccato et al., 2008a). It is also produced in-sewer from the degradation of 6-acetylmorphine and glucuronide conjugates (Gracia-Lor et al., 2017; Senta et al., 2014; Zuccato et al., 2008b). Accordingly, we would expect to be able to detect its presence. Also present was codeine, which is a drug prescribed to treat mild to moderate pain or as a codeine-based cough syrup, and in some instances, people can purchase it as a codeine-based over-the-counter medication (CBZ website). It is less regulated than some opiates, but users risk developing tolerance and eventually dependence (Thai et al., 2016; van Dyken et al., 2014). Heroin use is unlikely to be the primary source of morphine in the samples since 6-acetylmorphine was <LOD. Also, because only 1.3% of the heroin dose is excreted as 6-acetylmorphine (Gracia-Lor et al., 2016; Postigo et al., 2008), the amounts of 6-acetylmorphine in wastewater is expected to be small (dilution) and in-sample transformation (degradation) could result in it being below the LOD.

Table 1
Detection frequencies of targeted drug residues in all of the obtained samples (n = 40).

Drug	Drug residue	DF [%]
Tobacco (nicotine)	HCOT[a]	98
	Cotinine[a]	100
	Nicotine	100
Alcohol	Ethyl sulphate[a]	80
Morphine	Morphine[a]	40
Codeine	Codeine[a]	23
Methadone	Methadone	n.d.
	EDDP[a]	n.d.
Cannabis	THC-COOH[a]	93
Cocaine	Cocaine	75
	Benzoylecgonine[a]	50
	Cocaethylene	8
Amphetamine	Amphetamine[a]	5
Methamphetamine	Methamphetamine[a]	13
Ecstasy	MDMA[a]	15
Heroin	6-Acetylmorphine[a]	n.d.

n.d. – not detected.
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.
[a] Drug biomarker used for consumption estimation.

Aside from licit drugs (nicotine and alcohol), cannabis was the most prevalent substance in Slovenian schools (Table 1). In fact, the DF of THC-COOH was higher than ethyl sulphate. A statistical evaluation of differences between their DFs reveals that cannabis is as prevalent as alcohol, i.e., the difference in the DFs of THC-COOH and ethyl sulphate was not statistically significant ($\chi^2 = 2.635$, $p = 0.1045$, $\alpha = 0.05$). Although cannabis use is widespread among young people in Slovenia (NIJZ, 2019b), it can only be obtained legally for medical purposes (Group II: illegal drugs regulated only for medical application), whereas alcohol can be legally purchased at 18 years of age (NIJZ, 2019b).

Compared to its metabolite benzoylecgonine, cocaine was detected in a significantly higher percentage of samples ($\chi^2 = 5.333$, $p = 0.0209$, $\alpha = 0.05$; Table 1). A possible explanation is the disposal of cocaine into the sewer, either from direct disposal or handwashing or wiping residual cocaine from the surfaces into the toilet/sink after its' use. This finding may be explained by the shorter time to peak concentration (Tmax) of cocaine (2–5 h) in urine in comparison to benzoylecgonine (4–8 h) (Cone et al., 1998; Huestis et al., 2007; Jufer et al., 2000), suggesting that cocaine use is taking place in the institutions. Other stimulants such as amphetamine, methamphetamine and MDMA were only detected sporadically, which agrees with their typical consumption patterns, i.e., peak consumption occurring over the weekends (Krizman et al., 2016; Thomas et al., 2012; Zuccato et al., 2008b), or it may indicate their overall low prevalence in educational institutions in general. Notably, although drug use in Slovenian educational institutions is discussed, except for the cocaine results, the presence of drug residues in samples may not necessarily indicate drug use directly in the institutions since the majority of drug residues under investigation have long excretion times (Table S3) and may be consumed elsewhere and only excreted in the institutions, e.g., at home.

3.4. Drug prevalence vs the level of educational institution

On average, six drug residues (min = 4, max = 9) were detected in primary school samples (Table S4). A significant difference ($\chi^2 = 14.843$, $p = 0.0006$, $\alpha = 0.05$) was observed in the number of samples containing nicotine, alcohol and cannabis biomarkers (Table 2). The results also reveal a significantly higher number of primary school samples containing nicotine biomarkers (Chi-squared post hoc test: $\chi 2 = 9.896$, $p = 0.0035$, adjusted $\alpha = 0.0083$) and a significantly lower number of primary school samples containing ethyl sulphate (Chi-squared post hoc test: $\chi^2 = 13.194$, $p = 0.0007$, adjusted $\alpha = 0.0083$). The order of prevalence of drugs in primary schools was nicotine>cannabis>alcohol. Among medications of abuse, the use of morphine and codeine was observed (Table 2). The results also reveal the use of cocaine (DF of benzoylecgonine = 37%) and methamphetamine and that cocaine and alcohol were co-consumed (detection of cocaethylene). Unfortunately, since, to our knowledge, this is the first application of WBE to investigate residues of licit drugs, medications of abuse and illicit drugs in primary schools, it is not possible to compare the results with other primary schools in other countries.

Table 2
Detection frequencies (%) of targeted drug residues in samples from educational institutions of different level.

Drug residue	Primary schools (n = 19)	Secondary schools (n = 8)	HEIs (n = 6)	SHEIs (n = 7)
HCOT	95	100	100	100
Cotinine	100	100	100	100
Nicotine	100	100	100	100
Ethyl sulphate	58	100	100	100
Morphine	37	25	83	29
Codeine	21	38	17	14
THC-COOH	84	100	100	100
Cocaine	68	100	67	71
Benzoylecgonine	37	63	67	57
Cocaethylene	11	n.d.	n.d.	14
Amphetamine	n.d.	n.d.	33	n.d.
Methamphetamine	16	13	n.d.	14
MDMA	n.d.	13	50	29

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.d. – not detected (<LOD).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, HEI – higher education institution, MDMA – 3,4-methylenedioxymethamphetamine, SHEI – mixed secondary and higher education institution, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

Secondary school samples contained an average of eight drug residues (min = 6, max = 10, Table S4). Also, nicotine, alcohol, and cannabis were equally prevalent, and medications of abuse (e.g., morphine and codeine) were detected (Table 2). Amphetamine was the only stimulant not detected in secondary schools (Table 2). The findings agree with Zuccato et al. (2017), who studied drug consumption in eight Italian secondary schools (age 15–19). Contrary to our study, methamphetamine and MDMA were not detected. On average, a different number of drug residues was observed in samples from secondary schools implementing different educational programmes (Table S5), although the difference was not significant ($\chi^2 = 0.711$, $p = 0.7008$, $\alpha = 0.05$). There were, however, differences in the type of drugs detected (Table 3). For example, only in vocational and technical schools were morphine, codeine, and MDMA found together, while methamphetamine was only identified in multi-programme schools. Although ethyl sulphate, cocaine, and benzoylecgonine were found in all samples from vocational and technical schools (Table 3), cocaethylene indicating alcohol and cocaine co-consumption was not detected. The lack of cocaethylene suggest that these substances were consumed separately (by different persons); however, their low concentration in wastewater, possibly due to low excretion in urine (0.7% of cocaine dose excreted in 24 h (Gracia-Lor et al., 2017)) and sampling difficulties may explain why they were not detected. Further studies, including a higher number of participating secondary schools, are needed to obtain more accurate data.

On average, eight drug residues (min = 5, max = 10) were detected in HEI (Table S4). Nicotine, alcohol and cannabis were also the most common suggesting their equal prevalence (Table 2). Among the medications of abuse, morphine showed a high prevalence (DF = 83%). Overall, the results agree with WBE studies previously conducted on different university campuses. For example, alcohol consumption was observed in a university campus in Greece with ethyl sulphate detected in all samples (Gatidou et al., 2016), a high DF of morphine (79%) was obtained on a US university campus (Heuett et al., 2015), and cannabis was reported to be one of the most frequently used drugs in two studies conducted in the US (Heuett et al., 2015; Panawennage et al., 2011). In these studies, cocaine and amphetamine were frequently reported. In the present study, the use of stimulants, such as cocaine (DF of benzoylecgonine = 67%), amphetamine (DF = 33%) and MDMA (DF = 50%) was observed, and although amphetamine was not as prevalent as cocaine, it was only specific to HEIs (Table 2). Also, a difference in the number of drug residues detected in HEIs offering different higher educational programmes (Table S5) is observed but is not statistically significant ($\chi^2 = 2.522$, $p = 0.1123$, $\alpha = 0.05$). However, there is an observable difference in the DF of drug residues, namely all residues had DF = 100% in HEIs offering social sciences, while different DFs were obtained in HEIs offering natural sciences (Table 3). Despite this, only amphetamine was observed in a statistically higher number of HEIs offering social sciences ($\chi^2 = 6.0$, $p = 0.0143$, $\alpha = 0.05$), suggesting its higher prevalence in those HEIs, although a higher number of samples from each type of HEIs would be needed to confirm this finding. Similar to vocational and technical secondary schools, alcohol and cocaine can be assumed to be consumed separately (ethyl sulphate, cocaine and benzoylecgonine were detected in all samples, while cocaethylene was <LOD; Table 3). However, non-detection due to the low concentration of cocaethylene in wastewater cannot be excluded. The SHEI samples contained an average of seven drug residues (min: 5, max: 10, Table S3). This finding is similar to that for secondary schools

Table 3
Detection frequencies (%) of targeted drug residues in samples of educational institutions regarding educational programme classification.

Drug residue	Gymnasiums (n = 3)	Vocational and technical schools (n = 2)	Multi-programme schools (n = 3)	HEIs for natural sciences (n = 4)	HEIs for social sciences (n = 2)
HCOT	100	100	100	100	100
Cotinine	100	100	100	100	100
Nicotine	100	100	100	100	100
Ethyl sulphate	100	100	100	100	100
Morphine	n.d.	50	33	75	100
Codeine	67	50	n.d.	25	n.d.
THC-COOH	100	100	100	100	100
Cocaine	100	100	100	50	100
Benzoylecgonine	67	100	33	50	100
Cocaethylene	n.d.	n.d.	n.d.	n.d.	n.d.
Amphetamine	n.d.	n.d.	n.d.	n.d.	100
Methamphetamine	n.d.	n.d.	33	n.d.	n.d.
MDMA	n.d.	50	n.d.	25	100

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.d. – not detected (<LOD).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, HEI – higher education institution, MDMA – 3,4-methylenedioxymethamphetamine, SHEI – mixed secondary and higher education institution, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

regarding the type of drugs present (Table 2), although cocaine and alcohol co-consumption was detected in SHEIs (Table 2).

Overall, no statistical difference in the number of detected residues was observed between primary, secondary schools, HEIs and SHEIs ($\chi^2 = 5.345$, $p = 0.1482$, $\alpha = 0.05$) although different types of drugs prevailed. Additionally, the data was visualized, and similarities between samples were explored using a dimensional reduction technique (t-SNE). In this case, two groups are observed when the data are arranged (in 2D space) based on the level of educational institution (Fig. 2). Group1 (lower left) mainly consisted of primary school samples and Group2 (upper right) of institutions offering secondary and higher education, suggesting a difference in drug prevalence observed is influenced by the level of the educational institution. Mostly, there is a difference between primary schools and the others.

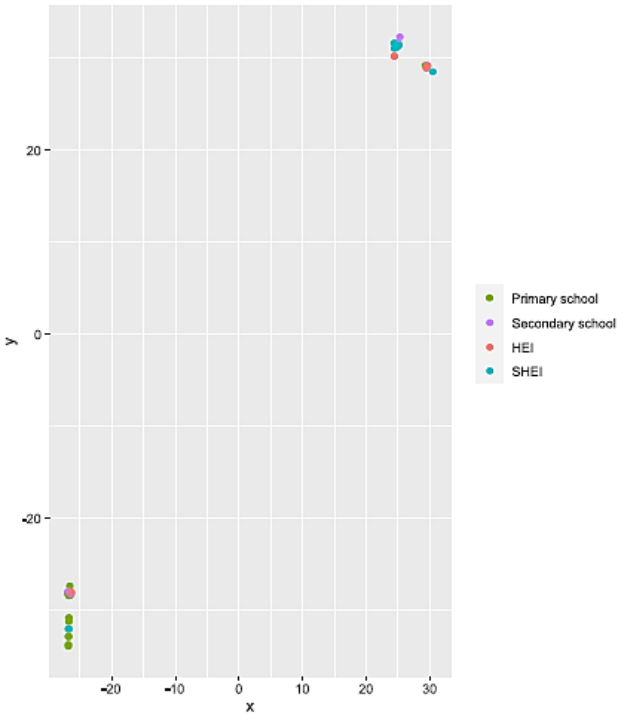

Fig. 2. SVD followed by t-SNE visualization of the data in 2D space (HEI – higher educational institution, SHEI – secondary and higher educational institution).

3.5. Geographic location vs drug prevalence

On average, a different number of drug residues were detected in samples from primary, secondary schools, HEIs and SHEIs from different municipalities. However, the differences were not statistically significant (Table S6). In addition, regardless of educational institute level, similar numbers of drugs were present in each municipality ($\chi^2 = 1.981$, $p = 0.9214$, $\alpha = 0.05$). Except for tobacco, alcohol, cannabis and cocaine, all other drug types varied. All investigated drugs were detected (biomarkers >LOD) in M2, i.e., Ljubljana, Slovenia's capital (Table 4). At least one prescription drug (morphine or codeine) was used in educational institutions in each municipality, except for M4 (primary schools and SHEIs), where neither were detected. Cocaine and alcohol co-consumption was observed in three municipalities: M2 (SHEIs), M5 (primary schools) and M6 (primary schools). Although ethyl sulphate and cocaine metabolites (cocaine and benzoylecgonine) were detected in all secondary school samples from M2 and M5 and SHEI samples from M4, cocaethylene was not detected. As already discussed, its absence suggests that alcohol was not co-consumed with cocaine suggesting consumption by different persons, but there is the possibility that cocaethylene is below the LOD. Amphetamine was detected only in M2, which is expected since it is specific to HEIs, seven of which are located in this municipality. Methamphetamine was detected in a higher number of municipalities than MDMA from

Table 4
Detection frequencies (%) of drug residues in educational institutions.

Statistical region		Costal-Karst	Central Slovenia		Southeast Slovenia	Savinja	Drava	Mura
Municipality (number of obtained samples)		M1 (n = 2)	M2 – Ljubljana (n = 16)	M3 (n = 6)	M4 (n = 4)	M5 (n = 6)	M6 (n = 3)	M7 (n = 3)
Drug residue	Samples	Detection frequency (%)						
HCOT	Primary school samples	100	100	100	100	100	67	100
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Cotinine	Primary school samples	100	100	100	100	100	100	100
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Nicotine	Primary school samples	100	100	100	100	100	100	100
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Ethyl sulphate	Primary school samples	100	60	33	100	67	33	50
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Morphine	Primary school samples	n.d.	20	67	n.d.	n.d.	100	50
	Secondary school samples	100	25	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	80	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	n.d.	n.d.	n.a.	100
Codeine	Primary school samples	n.d.	n.d.	33	n.d.	33	67	n.d.
	Secondary school samples	n.d.	75	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	20	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	n.d.	n.d.	n.a.	n.d.
THC-COOH	Primary school samples	100	100	100	50	100	67	50
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	100	100	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	100	n.a.	100	100	n.a.	100
Cocaine	Primary school samples	100	80	67	100	33	100	n.d.
	Secondary school samples	100	100	100	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	80	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	100	50	n.a.	100
Benzoylecgonine	Primary school samples	n.d.	40	33	50	33	67	n.d.
	Secondary school samples	100	75	n.d.	n.a.	100	n.a.	n.a.
	HEI samples	n.a.	80	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	100	50	n.a.	n.d.
Cocaethylene	Primary school samples	n.d.	n.d.	n.d.	n.d.	33	33	n.d.
	Secondary school samples	n.d.	n.d.	n.d.	n.a.	n.d.	n.a.	n.d.
	HEI samples	n.a.	n.d.	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	50	n.a.	n.d.	n.d.	n.a.	n.d.
Amphetamine	Primary school samples	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Secondary school samples	n.d.	n.d.	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	40	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.	n.d.
Methamphetamine	Primary school samples	n.d.	20	33	n.d.	n.d.	n.d.	50
	Secondary school samples	n.d.	n.d.	50	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	n.d.	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	n.d.	n.a.	50	n.d.	n.a.	n.d.
MDMA	Primary school samples	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Secondary school samples	n.d.	25	n.d.	n.a.	n.d.	n.a.	n.a.
	HEI samples	n.a.	60	n.d.	n.a.	n.a.	n.a.	n.a.
	SHEI samples	n.a.	n.d.	n.a.	100	n.d.	n.a.	n.d.

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.a. – not applicable (no samples were obtained), n.d. – no detected (<LOD).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, HEI – higher education institution, MDMA – 3,4-methylenedioxymethamphetamine, SHEI – mixed secondary and higher education institution, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

different statistical regions of Slovenia (four vs two). Both stimulants were detected in M2 (primary, secondary schools and HEIs) and M4 (SHEIs), while none were detected in M1, M5 and M6. No grouping of the results based on geographic location was observed in the t-SNE visualization (Fig. S2), suggesting other variables than municipality play a role in drug use trends.

3.6. Urban vs non-urban areas

An insufficient number of samples meant that only primary schools in urban (n = 13) and non-urban (n = 6) areas were used to explore differences due to urbanization. On average, six drug residues were detected in both urban (min = 5, max = 9) and non-urban (min = 4 max = 8) locations (Table S7), with no significant difference in the number of drugs residues detected ($\chi^2 = 0.425$, $p = 0.5144$, $\alpha = 0.05$). However, there was a difference in DFs of drug residues (Table 5). Although results obtained in other studies conducted in different sized cities (Krizman et al., 2016) and population type, i.e., capital and villages (Gatidou et al., 2016) suggest a higher drug consumption in urban areas. No such conclusion can be drawn in our study since individual drug consumption estimates were not calculated. When DFs of THC-COOH and ethyl sulphate are compared, the prevalence of cannabis was high and similar to that of alcohol (urban samples: $\chi^2 = 3.467$, $p = 0.0626$, $\alpha = 0.05$, non-urban samples: $\chi^2 = 0.343$, $p = 0.5582$, $\alpha = 0.05$). Urban samples also contained significantly more cocaine than its metabolite, benzoylecgonine ($\chi^2 = 3.939$, $p = 0.0472$, $\alpha = 0.05$), suggesting a connection between cocaine availability or its use in institutions and urbanization (see 3.3. General findings). According to the "Report on the drug situation in 2019 of the Republic of Slovenia" ("NIJZ, 2019b"), illicit drugs are more readily obtained in larger urban areas, which explain their availability in educational institutions. However, additional information is needed to support this claim. Interestingly, cocaine co-consumption with alcohol was only observed in urban areas (cocaethylene >LOD; Table 5). Also, there was no statistical difference ($\chi^2 = 2.030$, $p = 0.1542$, $\alpha = 0.05$) between the DF of methamphetamine in urban and non-urban samples. Despite this, no urban–non-urban grouping was observed in the t-SNE visualization (Fig. S3). However, there are only seven non-urban samples, so a higher number of samples from educational institutions located in non-urban areas are needed to confirm this finding.

3.7. Comparison with available epidemiological data

The results were compared with survey data from ESPAD 2015 (NIJZ, 2017) and HBSC 2018 (NIJZ, 2019b) and WBE data from SCORE 2019 covering six Slovenian municipalities (EMCDDA, Wastewater-based epidemiology and drugs topic page; SCORE-ES1307 COST Action). Despite our results, caution should be taken when extrapolating the results to schoolchildren and students since residues may also originate from members of staff and visitors since wastewater analysis only provides drug consumption of the whole population. There is also no socio-epidemiological data on drug consumption by specific groups (e.g., teachers, staff and visitors) associated with education in Slovenia, making it impossible to estimate the contribution from individual groups in educational institutions.

Differences in the DF of nicotine and alcohol biomarkers suggest a higher prevalence of nicotine than alcohol in Slovenian educational institutions ($\chi^2 = 13.514$, $p = 0.00024$, $\alpha = 0.05$). This finding contradicts the survey data, which suggests that alcohol use is more significant than tobacco among adolescents (Table S8). Differences in nicotine and alcohol use may explain this discrepancy, i.e., daily vs recreational use (Lai et al., 2018; Reid et al., 2011; Ryu et al., 2016).

The presence of codeine and morphine could indicate their therapeutic use, although codeine misuse cannot be ruled out. Codeine, for example, can be easily purchased over-the-counter in Slovenia (CBZ website), and it is reported in the ESPAD 2015 (NIJZ, 2017) that painkillers were used to get high by 2% of 15–16-year olds (Table S8). However, the low percentage of students using painkillers to get high is likely to mean that higher DFs (Tables 2 and 3) originate from (medical) usage by other groups of people present, although additional data is needed to support such a claim. In contrast, neither methadone nor EDDP was detected. Their absence could be explained by the fact that 65% of problem opioid users are between the ages 31 and 40 according to the Opioid Substitution Treatment program data (OST) (NIJZ, 2019b), whereas 41.8% of people ending higher education were <25 of years of age (SURS website).

Cannabis was the most common illicit drug in Slovenian educational institutions. This finding agrees with the survey data (Table S8), although the observed equal prevalence of cannabis and alcohol ($\chi^2 = 2.635$, $p = 0.1045$, $\alpha = 0.05$) does not, i.e., the survey data report alcohol as the most common drug (Table S8). Another discrepancy is in the use of stimulants. In our study, cocaine was the most prevalent, while amphetamine, methamphetamine and MDMA were detected only occasionally, whereas the survey data (ESPAD 2015; 15-16-years-olds and HBSC 2018; 17-year-olds) show the lifetime use of stimulants to be much more prevalent (Table S8). However, the results agree with the SCORE 2019 data (the year the study was performed), which show a higher prevalence of cocaine over other stimulants in the general population (EMCDDA, Wastewater-based epidemiology and drugs topic page; SCORE-ES1307 COST Action). Heroin was not detected, even though 1% of 15–16-year olds and 0.8% of 17-year olds reported using the drug (Table S8).

Overall, the results are inconsistent with those obtained by epidemiological surveys conducted in Slovenia. However, the discrepancy may result from differences in the methodology used (wastewater analysis vs questionnaires), time and mode of sampling/surveying, and reporting of results. Moreover, the number of samples containing individual drug biomarker(s) was used to predict drug prevalence, which means that no information can be obtained on the actual number of drug users at individual sites, while survey results offer direct insight into the number of users.

Table 5
Detection frequencies (%) of drug residues in primary schools located in urban and non-urban areas.

Drug residue	Urban	Non-urban
HCOT	92	100
Cotinine	100	100
Nicotine	100	100
Ethyl sulphate	62	50
Morphine	38	33
Codeine	23	17
THC-COOH	92	67
Cocaine	77	50
Benzoylecgonine	38	33
Cocaethylene	15	n.d.
Amphetamine	n.d.	n.d.
Methamphetamine	8	33
MDMA	n.d.	n.d.

Methadone, EDDP, 6-acetylmorphine were not detected in any of the samples.
n.d. – not detected.
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3'-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

4. Conclusions

The prevalence of licit drugs, medicines of abuse and illicit drugs was investigated in Slovenian educational institutions. In general, nicotine, alcohol and cannabis had the highest DFs. The most common medications of abuse were morphine and codeine, while cocaine was the most commonly detected stimulant. The number of detected residues did not vary between educational institutions regarding the level of education offered, geographic location and urbanization, but there were

differences in DFs and the type of drugs present. Amphetamine, for example, was detected only in HEIs. Ljubljana (M2) produced the greatest variety of drug residues, and urban areas were related to higher cocaine availability (evidenced by unused cocaine entering the sewer) and alcohol co-consumption. The level of educational institutions mainly influenced drug use patterns, i.e., differences were observed mainly between primary schools and other institutions. The observed DFs also agreed with other WBE studies conducted in educational institutions and the SCORE 2019 WBE study. Other variables that influence drug consumption patterns, however, require further exploration.

Overall, wastewater analysis is useful for investigating drug use in site-specific settings such as educational institutions since it is non-invasive and produces objective data in near-real-time. Importantly, the result obtained in this study may deepen our understanding of when drugs enter young peoples' lives and which drugs are the most common in different stages of education. Despite its many advantages, applying wastewater analysis to educational institutions is not without limitations. Inconsistent wastewater flows (e.g. periods with no flow) and different dilution factors (e.g. institutions with kitchens and those without), and, in this case, a lack of accurate flow data meant that it was impossible to quantify drug use, and such issue will need to be addressed in future site-specific studies. However, it did allow a comparison of drug types used. Also, maintaining an institution's anonymity in small catchments can be problematic, and disclosure could result in negative attention. Finally, caution is needed when interpreting the results since it is difficult to distinguish between students, staff and visitors.

CRediT authorship contribution statement

Taja Verovšek: Conceptualisation, Methodology, Validation, Investigation, Formal analysis, Data Curation, Writing – Original Draft, **Ivona Krizman-Matasic:** Conceptualisation, Methodology, Writing – Review & Editing, **David Heath:** Conceptualisation, Writing – Review & Editing, **Ester Heath:** Conceptualisation, Writing – Review & Editing, Supervision, Project administration.

All authors reviewed the manuscript and contributed to improving the quality of this paper.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150013.

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3.2.3 Data in brief: Dataset of residues of drugs of abuse in wastewater from Educational Institutions

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To produce FAIR data obtained in the framework of the WBE study carried out in Slovenian educational institutions (Chapter 3.2.2 Investigation of drugs of abuse in educational institutions using wastewater analysis, the data has been published in full in the peer-reviewed journal Data in Brief.

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Data Article

Data in brief: Dataset of residues of drugs of abuse in wastewaters from Educational Institutions

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ABSTRACT

Metabolic residue concentration data for two licit drugs (nicotine and alcohol), three medications of abuse (morphine, methadone and codeine) and six illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin) were obtained from raw wastewater samples collected from 44 Slovenian educational institutions are presented. Also, concentrations obtained at one secondary school during a preliminary study is provided. The wastewater samples were collected at the end of the 2018/2019 academic year using time proportional sampling and analysed for 16 drug residues, extracted using solid-phase extraction and analysed using ultra-performance liquid chromatography hyphenated to tandem mass spectrometry (UPLC-MS/MS). Residues of nicotine and alcohol were determined by direct injection of filtered wastewater onto the UPLC. Concentrations data were studied based on educational level (primary, secondary and tertiary) and institution type (secondary schools: gymnasiums, vocational and technical schools, multi-programme schools; higher education institutions: natural sciences and social sciences), geographic location (municipalities) and degree of urbanisation (urban and non-urban areas). Due to the

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large number of different educational institutions included in the study, provided datasets are valuable for further studies on drug consumption patterns among young people. Drug presence and prevalence data for primary schools (6–15 years) offer an objective insight into drugs present in the early stage of a young person's development and help establish effective prevention programs. More details on the study can be found in [1].

Specifications Table

Subject	Health and medical sciences
Specific subject area	Determination of licit drugs, medications of abuse and illicit drugs in educational institutions
Type of data	Table
How data were acquired	Instruments: Shimadzu ultra-performance liquid chromatograph hyphenated to AB Sciex 4500 QTRAP detector mass spectrometer (UPLC-MS/MS)
Data format	Raw, anonymised
Parameters for data collection	The data was obtained using UPLC-MS/MS analysis of seven-hour composite raw wastewater samples (n=40) obtained from different educational institutions (primary, secondary, and tertiary) in urban and non-urban areas in seven Slovenian municipalities. Also, data on chemical analysis conducted during the preliminary study (one secondary school sampled over one week) is provided.
Description of data collection	In total, 16 metabolic residues of licit drugs (nicotine, alcohol), medications of abuse (morphine, codeine, methadone) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin) were determined in wastewater samples using UPLC-MS/MS. Residues of medications of abuse and illicit drugs were enriched using solid phase extraction, while residues of licit drugs were filtered and analysed directly. Ion-pair reagent (tetrabutylammonium bromide) was added to the samples when analysing alcohol residues.
Data source location	Institutions: 44 educational institutions; 19 primary schools (6–15 years.), ten secondary schools (15–19 years), nine higher education institutions (19+ years.) and six mixed secondary and higher education institutions (15+ years.). City/ Town/Region: seven municipalities (including the capital) from five statistical regions (Coastal-Karst, Central Slovenia, Southeast Slovenia, Savinja, Drava and Mura) Country: Slovenia
Data accessibility	With the article
Related research article	T. Verovšek, I. Krizman-Matasic, D. Heath, E. Heath., Investigation of drugs of abuse in educational institutions using wastewater analysis, Science of The Total Environment, 799 (2021) 150013. https://doi.org/10.1016/j.scitotenv.2021.150013

Value of the Data

- Compared to similar datasets, this dataset provides concentrations of residues of licit drugs, medications of abuse and illicit drugs obtained from educational institutions of different educational levels (n=44), located in urban and non-urban areas within different municipalities across Slovenia.
- The data can be valuable for researchers studying drug use patterns among young people. Also, it may help with establishing prevention programmes and intervention strategies for young people since the data covers ages from 6–19+ years.

- Collected data can be used in comparison studies or as a base for (additional) experiments to study the variables influencing drug consumption trends in educational institutions, i.e. among young people.
- For the first time, wastewater analysis data on drugs present in primary schools (6–15 years) was obtained.

1. Data Description

Here the data for the wastewater samples, applied analytical methods, validation parameters, and concentrations (raw data) of residues of licit drugs (nicotine and alcohol), medications of abuse (morphine, methadone and codeine) and illicit drugs (cannabis, cocaine, amphetamine, methamphetamine, ecstasy and heroin) in wastewater samples are presented. No further calculation from concentration, i.e., to mass loads and consumption estimations [2], was possible due to a lack of data on wastewater flows.

In total, 40 wastewater samples were obtained from educational institutions offering different levels and types of education (Table 1) from urban and non-urban areas in six statistical regions in Slovenia (Table 2).

Standards solutions of targeted analytes (16 drug residues) and their labelled analogues used for identification and quantification by UPLC-MS/MS are listed in Table 3. Retention times and ionisation mode utilised are presented in Table 4 along with optimised UPLC-MS/MS parameters, namely declustering potentials (DP), collision energies (CE 1 and CE 2) and collision cell exit potential (CXP 1 and CXP 2), for each precursor–product ion pair.

Linearity, the limit of detection (LOD), the limit of quantification (LOQ), extraction recovery, matrix effect (ME), accuracy and repeatability were addressed during method validation. Signal suppression was observed (Table 5) for licit drug residues (2-115%) except for nicotine, for which signal enhancement (77%) was observed at low concentration (5 ng/mL). Accuracy values were in the 84-136% range. Repeatability was below 10% (RSD) for all compounds, except for ethyl sulphate (14% RSD). A linear response for nicotine residues was obtained between the LOQ and 1000 ng/mL, while for ethyl sulphate, linearity was achieved in LOQ–500 ng/mL range. LODs were in the range 19 to 305 ng/L, and LOQs were between 64 and 1020 ng/L.

Table 6 shows validation parameters for residues of medications of abuse and illicit drugs. Extraction recoveries were in 71–110% range (exception: 23% for 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EDDP) and signal suppression was observed (between 15% and 70%). Accu-

Table 1
Wastewater samples obtained in educational institutions of different level.

Educational institution (age of attendants)	Number of obtained samples
Primary schools (age 6–15)	19
Secondary schools (age 15–19)	8; - Three from gymnasiums (general upper secondary education); - Two from vocational and technical schools (vocational and technical education); - Three from multi-programme schools (general upper secondary education, vocational and technical education).
HEIs (age 19+)	6; - Four from institutions offering natural sciences; - Two from institutions offering social sciences.
SHEIs (age 15+)	7

HEIs – higher education institutions, SHEIs – mixed secondary and higher education institutions.

Table 2
Wastewater samples from each municipality and urban and non-urban areas divided based on the level of education offered by the institutions.

Geographic location/area	Statistical region of Slovenia	Total number of obtained samples	Number of primary school samples	Number of secondary school samples	Number of HEI samples	Number of SHEI samples
Inter-municipality comparison						
Municipality 1 (M1)	Costal-Karst	2	1	1	n.a.	n.a.
Municipality 2 (M2) – Ljubljana (Slovenian capital)	Central Slovenia	16	5	4	5	2
Municipality 3 (M3)	Central Slovenia	6	3	2	1	n.a.
Municipality 4 (M4)	Southeast Slovenia	4	2	n.a.	n.a.	2
Municipality 5 (M5)	Savinja	6	3	1	n.a.	2
Municipality 6 (M6)	Drava	3	3	n.a.	n.a.	n.a.
Municipality 7 (M7)	Mura	3	2	n.a.	n.a.	1
Urban *vs* non-urban areas						
Urban areas	Central Slovenia, Drava, Mura, Costal-Karst, Savinja, Southeast Slovenia	33	13	8	6	6
Non-urban areas	Central Slovenia, Drava, Mura, Savinja, Southeast Slovenia	7	6	n.a.	n.a.	1

n.a. – not applicable (no obtained samples).
HEI – higher education institution, SHEI – mixed secondary and higher education institution.

Table 3
Standard solutions of analytes and their deuterated analogues.

Analyte	[mg/mL] *(solvent)*	Labelled analogues (internal standards)	[mg/mL] *(solvent)*
Licit drug standard solutions			
HCOT	1 *(methanol)*	(±)-Cotinine-d3	1 *(methanol)*
(-)-Cotinine	1 *(methanol)*		
(S)-(-)-Nicotine	1 *(methanol)*		
Ethyl sulphate sodium salt	1 *(methanol)*	Ethyl-d5-sulphate sodium salt	1 *(methanol)*
Basic drug standard solutions			
Morphine	1 *(methanol)*	Morphine-d3	1 *(methanol)*
Codeine	1 *(methanol)*	Codeine-d3	1 *(methanol)*
(±)-Methadone	1 *(methanol)*	(±)-Methadone-d3	1 *(methanol)*
EDDP perchlorate	1 *(methanol)*	EDDP-d3 perchlorate	1 *(methanol)*
Cocaine	1 *(acetonitrile)*	Cocaine-d3	1 *(acetonitrile)*
Benzoylecgonine	1 *(methanol)*	Benzoylecgonine-d3	1 *(methanol)*
Cocaethylene	1 *(acetonitrile)*	Cocaethylene-d8	0.1 *(acetonitrile)*
(±)-Amphetamine	1 *(methanol)*	(±)-Amphetamine-d6	1 *(methanol)*
(±)-Methamphetamine	1 *(methanol)*	(±)-Methamphetamine-d5	1 *(methanol)*
(±)-MDMA	1 *(methanol)*	(±)-MDMA-d5	1 *(methanol)*
6-Acetylmorphine	1 *(acetonitrile)*	6-Acetylmorphine-d3	1 *(acetonitrile)*
Cannabinoid standard solutions			
(±)-THC-COOH	1 *(methanol)*	(±)-THC-COOH-d3	1 *(methanol)*

EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3'-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9- tetrahydrocannabinol.

racy values were in the 90-112% range. Repeatability was below 10% (RSD). For the majority of residues of medications of abuse and illicit drugs, a linear response was observed between LOQ and 1000 ng/mL, except for methamphetamine, EDDP (LOQ-500 ng/mL) and methadone (LOQ-200 ng/mL). LODs were between 0.31 and 3 ng/L, and LOQs were between 1 and 9.60 ng/L.

During a preliminary study, four daily wastewater samples obtained in one secondary school were analysed for nicotine residues, residues of medications of abuse and illicit drug residues. Out of 15 biomarkers (Table 7), on average, ten were detected in individual samples. 3,4-methylenedioxymethamphetamine – MDMA (Monday sample: 6.24 ng/L), benzoylecgonine (Monday sample: 42.0 ng/L and Friday sample: <LOQ but above LOD) and 6-acetylmorphine (<LOD only in Monday sample) were detected in different daily samples. Nicotine residues (nicotine: 4400–7500 ng/L, cotinine: 3000–5600 ng/L and trans-3'-hydroxycotinine – HCOT: 6700–9900 ng/L), 11-nor-9-carboxy-Δ9-tetrahydrocannabinolcocaine – THC-COOH (158–3232 ng/L), amphetamine (1.96–7.60 ng/L), morphine (up to 9.72 ng/L) and codeine (up to 29.84 ng/L) were detected in all samples and methamphetamine, EDDP, and cocaethylene were detected in none of them. Methadone was under LOD in three samples, while on a Wednesday (midweek), it was slightly higher (0.680 ng/L).

Concentrations of 16 drug residues obtained in 40 wastewater samples are presented in Table 8. Samples are grouped based on geographic location (municipalities: M1–7). Additional properties, such as level (PS – primary school, SS – secondary school, SHEI – mix secondary and higher education institution, HEI – higher education institution), type (G- gymnasiums, VTS – vocational and technical schools, MPS – multi-programme schools, IN- institutions offering natural science, IS – institutions offering social science) of educational institution and urbanisation (U – urban, NU – non-urban area) are also stated. As can be seen from Table 8, methadone (methadone, EDDP) and heroin (6-acetylmorphine, 6-AM) residues were always under LOD, while the highest concentrations were obtained for licit drug and cannabis residues.

Table 4
UPLC-MS/MS parameters.

Analyte	Retention time (min)	Ionisation mode	Precursor ion (m/z)	DP	Product ion 1 (m/z)	CE 1	CXP 1	Product ion 2 (m/z)	CE 2	CXP 2
Analytes										
HCOT	0.7	ESI+	**193**	61	**80**	47	6	**134**	27	4
Cotinine	1.2	ESI+	**177**	16	**80**	39	6	**136**	11	10
Nicotine	0.8	ESI+	**163**	41	**117**	32	8	**130**	27	10
Ethyl sulphate	3.3	ESI-	**125**	-5	**97**	-22	-15	**80**	-41	-7
Morphine	1.6	ESI+	**286**	96	**152**	79	10	**165**	51	8
Codeine	3.1	ESI+	**300**	81	**152**	83	14	**165**	55	12
Methadone	8.4	ESI+	**310**	16	**265**	21	10	**105**	35	10
EDDP	7.7	ESI+	**278**	66	**234**	40	10	**219**	57	14
THC-COOH	11.6	ESI-	**343**	-100	**299**	-30	-9	**245**	-36	-9
Cocaine	5.5	ESI+	**304**	51	**182**	27	6	**82**	39	8
Benzoylecgonine	4.3	ESI+	**290**	81	**168**	26	8	**77**	77	10
Cocaethylene	6.3	ESI+	**318**	101	**196**	25	10	**82**	41	8
Amphetamine	1.8	ESI+	**136**	51	**91**	23	8	**119**	11	8
Methamphetamine	2.4	ESI+	**150**	11	**91**	24	8	**119**	15	6
MDMA	3.1	ESI+	**194**	46	**163**	17	8	**105**	33	8
6-acetylmorphine	3.6	ESI+	**328**	101	**165**	51	6	**211**	35	8
Labelled internal standards										
Cotinine-d3	1.2	ESI+	**180**	76	**80**	37	6	**101**	29	8
Ethyl-d5-sulphate	3.3	ESI-	**130**	-45	**98**	-22	-13	**80**	-42	-11
Morphine-d3	1.6	ESI+	**289**	106	**152**	79	10	**165**	54	6
Codeine-d3	3.1	ESI+	**303**	81	**152**	85	8	**165**	55	6
Methadone-d3	8.4	ESI+	**313**	26	**268**	21	10	**105**	36	8
EDDP-d3	7.7	ESI+	**281**	56	**234**	42	10	**249**	32	8
THC-COOH-d3	11.6	ESI-	**346**	-90	**302**	-28	-11	**248**	-38	-7
Cocaine-d3	5.5	ESI+	**307**	86	**185**	27	8	**85**	43	6
Benzoylecgonine-d3	4.3	ESI+	**293**	81	**171**	27	8	**77**	75	6
Cocaethylene-d8	6.3	ESI+	**326**	96	**204**	27	8	**85**	43	8
Amphetamine-d6	1.8	ESI+	**142**	41	**93**	19	6	**125**	12	4
Methamphetamine-d5	2.4	ESI+	**155**	36	**92**	27	8	**91**	27	8
MDMA-d5	3.1	ESI+	**199**	56	**165**	17	6	**107**	32	8
6-Acetilmorphine-d3	3.6	ESI+	**331**	106	**165**	48	12	**211**	37	8

CE – collision energy, CXP – collision cell exit potential, DP – declustering potential, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3'-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9- tetrahydrocannabinol

2. Experimental Design, Materials and Methods

2.1. Preliminary study design, participants and wastewater sampling

A preliminary study was conducted at one of the participating secondary schools (15th–19th April 2019). Wastewater was obtained using an autosampler operating in time-proportional mode (100 mL in five minutes). Seven-hour composite raw wastewater samples were collected from Monday to Friday during lesson time (7:30–14:30). A technical error meant that Tuesday's sample was lost, and only four daily samples were obtained.

Forty-four educational institutions were included in the study; 19 primary schools (6–15 years.), ten secondary schools (15–19 years.), nine HEIs (19+ years.) and six SHEIs (15+ years.). Thirty-seven institutes were located in urban and seven in non-urban areas [3,4] of seven municipalities (M1–7) from six statistical regions of Slovenia. The intention was to collect one sample per participating institution (n=44) at the end of the 2018/2019 academic year. The sewer layout meant it was impossible to collect wastewater from just the institution at specific sam-

Table 5
Validation results for licit drug residues.

Analyte	Linearity - range in ng/mL (R^2)	LOD [ng/L]	LOQ [ng/L]	Concentration level used for validation [ng/mL]	Matrix effect [%]	Repeatability [%RSD]	Accuracy [%]
HCOT	LOQ–1000 (0.9991)	224.5	747.6	5	−28	2	86
				10	−20	3	87
				50	−26	3	84
Cotinine	LOQ–1000 (0.9967)	19.1	63.6	5	−22	3	90
				10	−20	5	91
				50	−19	3	94
Nicotine	LOQ–1000 (0.9993)	78.6	261.7	5	77	5	136
				10	−7.0	7	124
				50	−27	1	93
Ethyl sulphate	LOQ–500 (0.9996)	305.3	1016.8	5	−114.9	14	105
				20	−2.0	5	91
				50	−6.9	2	91
				100	−2.3	2	93

HCOT – trans-3'-hydroxycotinine.

pling sites. For this reason, forty composite raw wastewater samples covering all educational institutions were obtained (Tables 1 and 2). Wastewater samples were collected mid-week on either a Tuesday, Wednesday or Thursday using an autosampler (100 mL every five minutes). The samples were stored at −20°C until analysis.

2.2. Chemicals and materials

Standard solutions of targeted analytes (1 mg/mL) and labelled analogues (1 or 0.1 mg/mL) were purchased from Cerilliant (Round Rock, Texas, USA) and stored in the dark at −20°C (Table 3). Working standards were prepared by diluting the stock standards with methanol to give final concentrations of 10 mg/L for analytes, 2 mg/L for basic drug and cannabinoid standards, and 0.5 mg/L for alcohol and nicotine residues standards. All solutions were stored in the dark at −20°C. All HPLC solvents were purchased from JT Baker (Philipsburg, USA), while LC-MS grade formic (HCOOH) and phosphoric acid (H_3PO_4) were purchased from Fluka (Switzerland). Aqueous ammonia solution (NH_3, 25%) was purchased from Merck (Darmstadt, Germany) and ammonium formate and tetrabutylammonium bromide (ion-pair reagent) from Sigma Aldrich (Missouri, USA). Milli-Q water was obtained by Millipore Direct-Q purifying system.

2.3. Sample preparation

For nicotine and alcohol residues determination, the samples were filtered through three different-pore-size filters (2.7 µm – GF/D, 1.2 µm – GF/C, Whatman, USA, and 0.45 µm cellulose membrane filters, Sartorius, Gottingen, Germany) and spiked with labelled internal standards (final concentration of 10 ng/mL). To determine alcohol residue, tetrabutylammonium bromide (TBA) as an ion-pair reagent was added to the sample (final concentration of 50 mM) [5].

The method used for basic drugs and cannabinoids determination is based on Senta et al. [6]. Briefly, 125 mL of sample was spiked with labelled internal standards (60 ng/mL in final extracts) and filtered through two different-pore-size glass microfiber filters (GF/D and GF/C, Whatman, USA). The samples were then acidified to pH 2 using concentrated H_3PO_4. Drug residues were extracted and pre-concentrated on Oasis MCX (150 mg/6 mL, Waters, Milford, MA, USA) solid-phase extraction cartridges conditioned with 5 mL methanol, 5 mL Milli-Q water and 5 mL 25 mM H_3PO_4. A two-step elution followed sample loading. In the first fraction (6 mL of methanol), cannabinoids were eluted, while in the second fraction (6 mL of 0.5% ammonium solution in

Table 6
Validation results for residues of medications of abuse and illicit drugs.

Analyte	Extraction recovery [%]	Matrix effect [%]	Repeatability [% RSD]	Accuracy [%]	Linearity - range in ng/mL (R^2)	LOD [ng/L]	LOQ [ng/L]
Codeine	91	−41	3	98	LOQ–1000 (0.9997)	1.98	6.58
Methadone	88	−28	3	112	LOQ–200 (0.9958)	0.63	2.09
EDDP	23	−15	7	105	LOQ–500 (0.9944)	1.81	6.04
Morphine	110	−69	5	96	LOQ–1000 (0.9998)	1.39	4.61
THC-COOH	71	−70	5	104	LOQ–1000 (0.9985)	0.83	2.77
Cocaine	90	−30	2	97	LOQ–1000 (0.9952)	0.48	1.61
Benzoylecgonine	80	−28	5	90	LOQ–1000 (0.9936)	2.88	9.60
Cocaethylene	89	−23	4	100	LOQ–1000 (0.9941)	0.48	1.59
Amphetamine	101	−55	5	108	LOQ–1000 (0.9915)	0.31	1.03
Methamphetamine	81	−69	4	102	LOQ–500 (0.9955)	1.00	3.33
MDMA	72	−49	5	106	LOQ–1000 (0.9914)	0.83	2.78
6-acetylmorphine	76	−47	7	98	LOQ–1000 (0.9985)	1.44	4.80

EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, LOQ – limit of quantification, MDMA – 3,4-methylenedioxymethamphetamine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

Table 7
Concentrations (ng/L) of drug residues obtained during the preliminary study.

Day	HCOT	COT	NIC	MOR	COD	MTHD	EDDP	THC-COOH	COC	BE	COE	AMP	MAMP	MDMA	6-AM
Monday	6700	3000	4400	<4.61	29.84	<0.63	<1.81	158	10.52	42.0	<0.48	7.60	<1.00	6.24	<1.44
Wednesday	7300	4400	5300	5.68	<6.58	<2.09	<1.81	3232	2.40	<2.88	<0.48	2.00	<1.00	<0.83	<4.80
Thursday	9900	4900	5400	9.88	<6.58	<0.63	<1.81	728	2.40	<2.88	<0.48	4.64	<1.00	<0.83	5.16
Friday	8900	5600	7500	9.72	<6.58	<0.63	<1.81	248	2.16	<9.60	<0.48	1.96	<1.00	<0.83	<4.80

6-AM – 6-acetylmorphine, AMP – amphetamine, BE – benzoylecgonine, COC – cocaine, COD – codeine, COE – cocaethylene, COT – cotinine, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3'-hydroxycotinine, MAMP – methamphetamine, MDMA – 3,4-methylenedioxymethamphetamine, MOR – morphine, MTHD – methadone, NIC – nicotine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

Table 8
Concentrations (ng/L) of drug residues obtained in 40 wastewater samples.

No.	Properties of the sample	COT	HCOT	NIC	EtS	MOR	COD	MTHD	EDDP	THC-COOH	COC	BE	COE	AMP	MAMP	MDMA	6-AM
								M1									
1	PS, U	1040	2870	1630	2240	<1.39	<1.98	<0.63	<1.81	504	5.24	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
2	SS (MPS), U	3700	6770	3680	11200	12.4	<1.98	<0.63	<1.81	381	3.50	<9.60	<0.48	<0.31	<1.00	<0.83	<1.44
								M2 – Ljubljana									
3	PS, U	263	<747.6	1070	<305.3	<1.39	<1.98	<0.63	<1.81	<2.77	<1.61	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
4	PS, U	1860	3840	2290	<305.3	14.0	<1.98	<0.63	<1.81	1460	3.16	28.8	<0.48	<0.31	<1.00	<0.83	<1.44
5	PS, U	1100	1970	1710	8240	<1.39	<1.98	<0.63	<1.81	76	48.8	69.2	<0.48	<0.31	<1.00	<0.83	<1.44
6	PS, U	289	<747.6	926	<1016.8	<1.39	<1.98	<0.63	<1.81	5.84	<1.61	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
7	PS, NU	878	1730	1300	1110	<1.39	<1.98	<0.63	<1.81	9.24	<0.48	<2.88	<0.48	<0.31	<3.33	<0.83	<1.44
8	SS (G), U	964	1480	1670	228000	<1.39	16.2	<0.63	<1.81	163	<1.61	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
9	SS (G), U	674	1560	1650	1920	<1.39	24.6	<0.63	<1.81	128	3.57	<9.60	<0.48	<0.31	<1.00	<0.83	<1.44
10	SS (G), U	1000	2050	1350	10500	<1.39	<1.98	<0.63	<1.81	244	66.0	74.4	<0.48	<0.31	<1.00	<0.83	<1.44
11	SS (VTS), U	3640	6950	3260	4390	37.4	18.8	<0.63	<1.81	1330	63.6	1340	<0.48	<0.31	<1.00	10.8	<1.44
12	HEI (IN), U	1290	2840	1300	40000	<1.39	<1.98	<0.63	<1.81	333	<0.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
13	HEI (IN), U	1820	3010	1970	6290	12.0	<1.98	<0.63	<1.81	1140	10.2	196	<0.48	<0.31	<1.00	<2.78	<1.44
14	HEI (IS), U	1640	2950	3010	2420	11.2	<1.98	<0.63	<1.81	1460	12.6	25.7	<0.48	40.4	<1.00	7.56	<1.44
15	HEI (IS), U	3610	6090	3450	4250	19.1	<1.98	<0.63	<1.81	512	15.6	21.6	<0.48	180	<1.00	4.84	<1.44
16	HEI (IN), U	1880	5170	2550	4900	14.9	9.80	<0.63	<1.81	856	5.04	<9.60	<0.48	<0.31	<1.00	<0.83	<1.44
17*	SHEI, U	4730	8945	3035	5415	<1.39	<1.98	<0.63	<1.81	1890	<0.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
18*	SHEI, U	3945	7500	3755	12550	58.0	19.2	<0.63	<1.81	454	9.12	270	2.42	<0.31	<1.00	<0.83	<1.44
								M3									
19	PS, U	721	1590	1080	<305.3	36.4	111	<0.63	<1.81	31.4	2.26	62.8	<0.48	<0.31	<1.00	<0.83	<1.44
20	PS, U	1470	6260	2520	2910	<1.39	<1.98	<0.63	<1.81	21	<0.48	<2.88	<0.48	<0.31	<3.33	<0.83	<1.44
21	PS, NU	<63.6	<747.6	829	<305.3	8.20	<1.98	<0.63	<1.81	74	2.06	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
22	SS (MPS), U	6200	9120	5260	7790	<1.39	<1.98	<0.63	<1.81	672	10.8	<2.88	<0.48	<0.31	<3.33	<0.83	<1.44
23	SS (MPS), U	1400	3690	1590	3860	<1.39	<1.98	<0.63	<1.81	1130	4.52	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44

(*continued on next page*)

Table 8 (*continued*)

No.	Properties of the sample	COT	HCOT	NIC	EtS	MOR	COD	MTHD	EDDP	THC-COOH	COC	BE	COE	AMP	MAMP	MDMA	6-AM
24	HEI (IN), U	3850	6560	5520	22600	14.9	<1.98	<0.63	<1.81	67.6	<0.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
								M4									
25	PS, U	1260	2000	2580	3680	<1.39	<1.98	<0.63	<1.81	8.12	2.83	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
26	PS, NU	364	<747.6	1450	7150	<1.39	<1.98	<0.63	<1.81	<0.83	2.44	<9.60	<0.48	<0.31	<1.00	<0.83	<1.44
27	SHEI, U	3850	7160	3510	54000	<1.39	<1.98	<0.63	<1.81	524	5.08	<9.60	<0.48	<0.31	4.20	5.60	<1.44
28	SHEI, NU	3760	5460	3680	27100	<1.39	<1.98	<0.63	<1.81	130	3.68	18.7	<0.48	<0.31	<1.00	39.6	<1.44
								M5									
29	PS, U	1380	2950	8320	2210	<1.39	<1.98	<0.63	<1.81	35.6	177	1640	2.40	<0.31	<1.00	<0.83	<1.44
30	PS, U	516	1360	1130	4060	<1.39	<1.98	<0.63	<1.81	153.2	<0.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
31	PS, NU	529	772	1530	<305.3	<1.39	6.96	<0.63	<1.81	27.9	<0.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
32	SS (VTS), U	2140	3460	1830	4350	<1.39	<1.98	<0.63	<1.81	106	36.5	476	<0.48	<0.31	<1.00	<0.83	<1.44
33	SHEI, U	5860	10400	4340	9110	<1.39	<1.98	<0.63	<1.81	235	2.60	<9.60	<0.48	<0.31	<1.00	<0.83	<1.44
34	SHEI, U	3270	4870	2150	4970	<1.39	<1.98	<0.63	<1.81	14600†	<0.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
								M6									
35	PS, U	101	<224.5	1750	<305.3	9.72	8.96	<0.63	<1.81	<0.83	5.48	<2.88	<0.48	<0.31	<1.00	<0.83	<1.44
36	PS, U	701	968	32800	<305.3	12.0	47.6	<0.63	<1.81	4.44	3110	1530	<1.59	<0.31	<1.00	<0.83	<1.44
37	PS, NU	1630	3160	2070	2790	<4.61	<1.98	<0.63	<1.81	16.0	33.6	19.8	<0.48	<0.31	<1.00	<0.83	<1.44
								M7									
38	PS, U	209	554	1040	4440	113.2	<1.98	<0.63	<1.81	4.48	<0.48	<2.88	<0.48	<0.31	<1	<0.83	<1.44
39	PS, NU	1710	2650	1680	<305.3	<1.39	<1.98	<0.63	<1.81	<0.83	<0.48	<2.88	<0.48	<0.31	<3.33	<0.83	<1.44
40	SHEI, U	9390	20000	3730	72600	10.44	<1.98	<0.63	<1.81	304.4	2.10	<2.88	<0.48	<0.31	<1	<0.83	<1.44

* – average of two sampling days/samples,

† – estimated from the extrapolation of the calibration curve, G – gymnasiums, HEI – higher education institution, IN- institutions offering natural science, IS – institutions offering social science, MPS – multi-programme schools, NU – non-urban, PS – primary school, SHEI – mix secondary and higher education institution, SS – secondary school, U – urban, VTS – vocational and technical schools;6-AM – 6-acetylmorphine, AMP – amphetamine, BE – benzoylecgonine, COC – cocaine, COD – codeine, COE – cocaethylene, COT – cotinine, EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, EtS – ethyl sulphate, HCOT – trans-3'-hydroxycotinine, MAMP – methamphetamine, MDMA – 3,4-methylenedioxymethamphetamine, MOR – morphine, MTHD – methadone, NIC – nicotine, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

methanol), basic drugs were eluted. The cannabinoid fraction was further purified by acidification with concentrated HCOOH (60 µL) and passed through a Strata NH_2 cartridges (200 mg/3 mL, Phenomenex, Torrance, California, USA). The analyte was eluted using 2 × 2 mL of 1% HCOOH in methanol. The fractions were combined and reduced to dryness (40°C, N_2) and reconstituted in either 500 µL of Milli-Q and methanol, 80:20, v/v with 0.1% formic acid (basic drug residues extract) or Milli-Q water and methanol, 30:70, v/v (cannabinoids-containing extract).

2.4. Sample analysis

Samples were analysed using a Shimadzu ultra-performance liquid chromatograph hyphenated to an AB Sciex 4500 QTRAP detector mass spectrometer (UPLC-MS/MS). Ionisation was achieved with an electron ionisation (ESI) interface. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Retention times, both transitions and the ratio between the transition peak areas were used for identification [7]. Quantification was performed based on the relative response factors of the analyte to its isotopically labelled standard. Optimised LC-MS/MS parameters for analytes and labelled internal standards are presented in Table 4.

Alcohol residues were separated by injecting 10 µL of sample on Ascentis® Express C18 (2 µm, 50 mm × 2.1 mm, Supelco, Pennsylvania, USA) column at 40°C. Milli-Q water (A) and methanol (B), containing 0.1% formic acid, were used as eluents at a flow rate of 0.3 mL/min. The gradient elution was performed as follows: 2% B at 0 min, increase to 15% B at 10 min, 95% B at 11 min and hold the conditions for 1 min, then decreased to 2% B at 13 min. The ionisation of the compounds was achieved using electrospray ionisation in negative ionisation mode (ESI–).

For nicotine and basic drug residues, the analysis was based on Senta et al. [6]. Briefly, 10 µL of the extracted sample was injected onto the UPLC-MS/MS system. Analytes were separated on Synergi Polar-RP column (2.5 μm, 30 mm × 2 mm, Phenomenex, Torrance, California, USA) temperate at 40°C. Milli-Q water (A) and Methanol (B) containing 5 mM ammonium formate and 0.1% formic acid were used as eluents at a flow rate of 0.3 mL/min. The gradient elution was performed as follows: 2% B at 0 min, increase to 50% B at 6.9 min, 55% B at 7.3 min, 85% B at 8.7 min, 88% B at 10.7 min, and 100% B at 11 min, hold the condition till 11.4 min, then decrease to 2% B at 11.7 min and hold that percentage till 15.3 min. The ionisation of the compounds was conducted in positive ionisation mode (ESI+). During acquisition Scheduled MRM™ algorithm (MRM detection window: 120 s) was applied.

For cannabinoids, 10 µL of the sample was injected onto UPLC-MS/MS system, where the separation was performed on Supelco Ascentis® Express C18 (2 µm, 50 mm × 2.1 mm, Supelco, Pennsylvania, USA) column temperate at 40°C. Gradient elution using Milli-Q water (A) and methanol (B) at a flow rate of 0.3 mL/min was used as follows: 10% B at 0 min, increase to 50% B at 1.5 min, 60% B at 3.0 min and hold the conditions for 4 min and a half, increase to 85% B at 12.5min, then decrease to 10% B at 13 min and hold the condition for two minutes. The ionisation of the compounds was conducted in negative ionisation mode (ESI–).

2.5. Method validation

The method validation included parameters such as linearity, limits of detection (LOD), limits of quantification (LOQ), extraction recovery (for basic drug resides and cannabinoid), matrix effect (ME), accuracy and repeatability (Tables 5 and 6). The method performance was assessed in raw wastewater collected from a wastewater treatment plant (WWTP), while linearity was tested in a solvent. Matrix effect, accuracy and repeatability for alcohol residues were tested on four (5, 20, 50 and 100 ng/mL) and nicotine residues on three concentration levels (5, 10 and 50 ng/mL). In comparison, the functionality of transferred methods for basic drug residues and cannabinoids was confirmed at one concentration level (250 ng/mL).

The response's linearity was determined from a seven-to-twelve-point calibration curve and described using the linearity range and coefficient of determination (R^2). A calibration curve was obtained from plotting the peak area ratio of the analyte and its deuterated analogue as a function of analyte concentration.

Limits of detection (LOD) and quantification (LOQ) were determined by calculating the signal-to-noise ratio (S/N=3 and S/N=10) in real wastewater, spiked with deuterated analyte analogues at low concentration (2 ng/mL for nicotine, basic drug residues and cannabinoid, and 5 ng/mL for alcohol resides). The LOD and LOQ were calculated as an average S/N ratio obtained from five replicates.

The extraction recovery was assessed using two sets of spiked RW samples (four replicates). One set was spiked prior (RW spike) and one after the extraction (eluate spike). An additional set of samples (four replicates) was prepared only with labelled internal standards (RW original) and used to correct drug metabolites concentration already present in the wastewater. The extraction recovery was calculated based on obtained analyte peak areas as shown in Eq. (1), where A represents peak areas of analytes:

$$Extraction\ recovery\ (\%) = \frac{A(RW\ spiked) - A(RW\ original)}{A(eluate\ spiked) - A(RW\ original)} \times 100 \quad (1)$$

The matrix effect (ME) was evaluated by preparing two sets of spiked samples (each in four replicates). One set was spiked with analytes after the sample preparation procedure (final extract spiked), while the second was spiked only with labelled internal standards (RW original). Additionally, four replicates of Milli-Q water spiked with analytes were prepared (STD spiked). Matrix effects were evaluated based on a comparison between analytical response for biomarkers in the reconstructed sample (final spiked) and response for standard solutions as is shown in Eq. (2), where A represents the average peak areas of the analytes:

$$ME\ (\%) = \frac{A(final\ extract\ spiked) - A(RW\ original) - A(STD\ spiked)}{A(STD\ spiked)} \times 100 \quad (2)$$

Method accuracy was assessed by spiking a set of raw wastewater samples with analytes labelled internal standards at the beginning of the sample preparation procedure (RW spiked). One set of samples was spiked only with deuterated internal standards (RW original). All sample sets were prepared in four replicates and undergone the whole sampling preparation procedure. Additionally, four replicates of Milli-Q water spiked with analytes and deuterated standards were prepared (STD spiked). Method accuracy was evaluated by comparing the measured concentration of biomarkers in spiked wastewater influent and measured concentration in the standard solution as shown in Eq. (3), where c represents the average measured concentration of analytes:

$$Accuracy\ (\%) = \frac{c(RW\ spiked) - c(RW\ original)}{c(STD\ spiked)} \times 100 \quad (3)$$

Repeatability was assessed as relative standard deviation (RSD) of four replicate analyses of the spiked raw wastewater samples.

Quality control was performed by preparing and analysing procedural blanks (Milli-Q), analysing instrument blanks (Milli-Q water spiked only with deuterated internal standards), and quality control samples (points of calibration curve: 20 ng/mL for nicotine and alcohol residues, 30 ng/mL for basic drug residues and cannabinoid) after every 14th sample per batch.

Ethics Statement

Wastewater analysis requires no ethical approval for its application since individuals cannot be identified, it poses little risk of harming the participants. Accordingly, no approval from the ethics committee was needed prior to the study. However, following "The Ethical research guidelines for wastewater-based epidemiology and related fields", an informed consent form and an anonymity agreement were both signed by the Heads of participating institutions [8].

CRediT authorship contribution statement

Taja Verovšek: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing – original draft. **Ivona Krizman-Matasic:** Conceptualization, Methodology, Writing – review & editing. **David Heath:** Conceptualization, Writing – review & editing. **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 which have or could be perceived to have influenced the work reported in this article.

CRediT Author Statement

Taja Verovšek: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing – original draft; **Ivona Krizman-Matasic:** Conceptualization, Methodology, Writing – review & editing; **David Heath:** Conceptualization, Writing – review & editing; **Ester Heath:** Conceptualization, Writing – review & editing, Supervision, Project administration.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.dib.2021.107614.

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3.2.4 Screening for new psychoactive substances in wastewater from educational institutions

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The (mis)use of drugs among young people is a serious problem, which is further complicated by using readily available NPS, as their users are exposed to a high risk of intoxication (Chapter 1.1.1 New psychoactive substances). Accordingly, evaluating the prevalence of NPS among young people is essential and is commonly done by surveys and drug testing, while WBE studies were also performed to explore NPS use. However, in those WBE studies, only a limited number of NPS have been investigated using target analysis (Chapter 1.3.4 WBE: Specific populations).

As an upgrade of the research described in Chapter 3.2.2 Investigation of drugs of abuse in educational institutions using wastewater analysis, selected wastewater samples from Slovenian educational institutions (n=23) were screened for over 5600 NPS using liquid chromatography-ion mobility-high-resolution mass spectrometry (LC-IMS-HRMS). Educational institutions of all types, *i.e.,* from primary schools to high education institutions and of different geographic locations (two municipalities), were included in this study. The study aimed to examine the presence of NPS in educational institutions and assess WBE's usefulness for this purpose.

Results showed that NPS were present in all wastewater samples. In line with the study conducted in municipal wastewater (Chapter 3.1.2 Three years of wastewater surveillance for new psychoactive substances from 16 countries), most identified NPS in wastewater of educational institutions were synthetic cathinones, with 3-MMC, ephedrine, 4-chloro-α-PPP, and ethcathinone being identified unequivocally. No inter-institutional trends in the occurrence of NPS were observed, while there was a clear difference in their distribution regarding the geographic location of the institutions, *i.e.,* higher prevalence of NPS in the capital city.

The study indicates the presence of NPS in educational institutions and confirms the potential of wastewater analysis for their identification, even though NPS consumption cannot be linked to a specific group of people within an institution. Even though educational institutions should be drug-free environments, wastewater analysis, even without additional adaptations, can be used as a non-invasive approach for drug detection (including NPS) in such settings. In addition, WBE can complement otherwise invasive drug testing performed in various countries worldwide.

The study outcome was presented at two scientific conferences*, i.e.,* Testing the Waters 5 Conference 2021 and the 18th International Conference on Chemistry and the Environment.

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Screening for new psychoactive substances in wastewater from educational institutions

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ABSTRACT

Drug (ab)use among young people is a serious issue, negatively impacting their well-being and prospects. The emergence of new psychoactive substances (NPS) further complicates the situation as they are easily accessible (e.g., online), but users are at high risk of intoxication as their chemical identity is often unknown and toxicity poorly understood. While surveys and drug testing are traditionally used in educational institutions to comprehend drug use trends and establish effective prevention programs, they are not without their limitations. Accordingly, we investigated the occurrence of NPS in educational institutions through wastewater analysis and critically evaluated the viability of the approach. The study included eight wastewater samples from primary schools (ages 6–15 years), six from secondary schools (ages 15–19 years), three from institutions for both secondary and higher education (ages 15+), and six from higher educational institutions (ages 19+). Samples were obtained mid-week and evaluated in two Slovenian municipalities; the capital Ljubljana and a smaller one (M1). Samples were screened using liquid chromatography-ion mobility-high-resolution mass spectrometry (LC-IMS-HRMS), and NPS identified at three levels of confidence (Level 1: unequivocal, Level 2: probable, Level 3: tentative) from a suspect list containing over 5600 entries. NPS were identified in all types of educational institutions. Most were synthetic stimulants, with 3-MMC, ephedrine, 4-chloro-α-PPP, and ethcathinone being unequivocally identified. Also, NPS were present in wastewater from all educational institution types revealing potential spatial but no inter-institutional trends. Although specific groups cannot be targeted, the study, as a proof-of-concept, demonstrates that a suspect screening of wastewater employing LC-IMS-HRMS can be used as a radar for NPS in educational institutions and potentially replace invasive drug testing.

1. Introduction

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the term New Psychoactive Substances (NPS) refers to "narcotic or psychotropic drugs, in pure form or in preparation, that are not controlled by the United Nations drug conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions" (EMCDDA, 2022a). NPS are not necessarily newly synthesized substances, as the term suggests, but rather substances that have recently emerged on the drug market (UNODC, 2023). They are a broad and diverse category of drugs that are usually designed to mimic the effects of illegal drugs such as cocaine, cannabis and ecstasy and can be classified as synthetic cannabinoids, synthetic hallucinogens, dissociative drugs, synthetic stimulants, synthetic depressants and natural psychoactive compounds. (EMCDDA, 2022b). The NPS market is large and rapidly evolving due to the relative ease of synthesising NPS through minor modifications in their chemical structure. For example, the EU's Early Warning System reported the emergence of 52 NPS in 2021 alone (EMCDDA, 2022a; EMCDDA, 2022b). Furthermore, a wide range of NPS can be easily obtained online with little to no information about their identity and toxicity, increasing the risk of intoxication and drug-related death, as evidenced by

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drug-induced fatalities associated with the emergence of new benzodiazepines (EMCDDA, 2021; EMCDDA, 2022b).

Experimenting with NPS use among young people is of particular concern since, according to European School Survey Project on Alcohol and Other Drugs (ESPAD), 3.4% of 15-16-year-olds have reported using any NPS at least once (ESPAD, 2019). Studying NPS among young people is also essential to understand the motivations and risks associated with their use and to successfully implement prevention, education, and harm reduction programs, especially since the onset of risky drug consumption patterns has been linked to early drug use (NIJZ, 2019). Additionally, it can provide valuable insights into the changing drug landscape and inform public health strategies and law enforcement authorities (EMCDDA, 2021).

Educational institutions have been identified as an important setting for studying trends in drug use among young people (Sznitman et al., 2012), where surveys are commonly used to address substance and NPS use among schoolchildren and students, such as ESPAD. However, in addition to biased reporting and time lag in reported data, surveys raise ethical considerations and may require parental or respondent consent to be conducted (ESPAD, 2019). In another approach, known as "drug testing", pupils can be tested for drug use by either targeted or random urine analysis. Drug testing can be intrusive and seen as an invasion of privacy. Accordingly, it may create a hostile atmosphere, discouraging pupils from engaging in healthy behaviour (Sznitman et al., 2012). Moreover, due to the targeted nature of drug testing, substances can go undetected, especially for NPS, which are constantly changing (Sznitman et al., 2012; Vaccaro et al., 2022). Equally, there is no evidence to suggest that drug testing effectively deters drug use among young people (Sznitman et al., 2012).

Wastewater-based epidemiology (WBE) has become an increasingly popular approach for estimating drug consumption by analysing wastewater for metabolic drug residues (biomarkers) and has proven effective in providing complementary and near real-time objective data (Bade et al., 2023; Bijlsma et al., 2019; Salgueiro-González et al., 2022). Its non-invasive nature also makes it suitable for investigating drug prevalence in vulnerable populations, given that the data obtained cannot be traced to individuals, thereby ensuring anonymity (Verovšek et al., 2020). However, studies looking into NPS in educational institutions have focused on the targeted analysis of a few selected biomarkers, thus limiting the number of NPS that can be covered (Heuett et al., 2015; Panawennage et al., 2011; Zuccato et al., 2017). A potential solution is to use high-resolution mass spectrometry (HRMS), which provides full-spectrum accurate-mass data and allows non-targeted analysis and suspect screening for thousands of compounds (Klingberg et al., 2022). In addition, coupling HRMS with advanced separation techniques, such as ion mobility (D'Atri et al., 2017), can provide additional dimensions to the screening process, including collision cross-section values (CCS), thereby increasing confidence in compound identification (Bade et al., 2020; Celma et al., 2020). This extra information is especially important when monitoring NPS, as it is typically impossible to have all reference standards available in the laboratory for unequivocal identification (Bijlsma et al., 2021). Moreover, when acquiring in data independent acquisition mode, ion mobility allows to obtain cleaner mass spectra which facilitates data interpretation (Bijlsma et al., 2021).

In this study, we conducted a comprehensive screening strategy to assess the presence of NPS in wastewater samples from primary schools, secondary schools, institutions for secondary and higher education (SHEIs), and higher educational institutions (HEIs) within two different-sized municipalities in Slovenia. We included an extensive dataset of over 5600 entries in the screening workflow and compared the obtained data with available socio-epidemiological information. The goal of this proof-of-concept study was twofold: first, to evaluate the viability of using a complementary target and suspect screening for the detection and identification of NPS in wastewater, and second to get more insight on the presence of NPS in educational institutions by employing the WBE approach.

2. Methods

2.1. Wastewater samples and participating educational institutions

Raw wastewater samples (n = 23), consisting of 7-h composites, collected mid-week at the end of the 2018/2019 academic year in March, May and June, were taken directly from the main sewer outlet of an individual institution using an autosampler working in the time-proportional mode, i.e., every 5 min, 100 mL of wastewater was sampled (Verovšek et al., 2021a, 2021b). Although the sewer layout was examined and sampling conducted by trained personnel from municipal WTTPs, the construction of sewers in two cases made it impossible to obtain samples from the two institutions individually. Nevertheless, the results were included since, in all cases, the samples cover educational institutions of the same type.

Samples collected included eight samples from primary schools (6–15 yrs), six from secondary schools (15–19 yrs), three from SHEIs (15+ yrs) and six from HEIs (19+ yrs) located within two municipalities; Ljubljana and smaller municipality (M1). Both municipalities offer all levels of education but differ in character and size, with Ljubljana being the capital city and M1 being more provincial and a third the size. Seventeen of the 23 samples were from Ljubljana, and six were from M1. After collection, samples were stored at −20 °C. Samples were shortly defrosted once for the analysis of illicit drugs, prior to the analysis of NPS.

2.2. Chemicals and materials

Methanol (HPLC and LC-MS grade) was purchased from J.T. Baker (Philipsburg, USA), orthophosphoric (H_3PO_4, ≥85%, LC-MS grade) and formic (HCOOH) acid from Fluka (Switzerland) and aqueous ammonium (25%) from Merck (Darmstadt, Germany). Filters, namely glass microfiber filters (GF/D and GF/C) and nylon centrifugal filters, were obtained from Whatman (USA) and VWR (USA), respectively. Oasis (HLB and prime MCX) extraction cartridges were purchased from Waters (Milford, MA, USA) and Strata NH_2 extraction cartridges from Phenomenex (Torrance, California, USA). CORTECS chromatographic column was from Waters (Milford, MA, USA).

2.3. Sample preparation

All samples were processed using previously developed protocols (A and B). In order to ensure the extraction of the broadest possible range of psychoactive substances, these methods involved solid-phase extraction (SPE) using two stationary phases: Oasis HLB in protocol A (Celma et al., 2019) and MCX in protocol B (Verovšek et al., 2023).

2.3.1. Protocol A

Briefly, 25 mL of centrifuged wastewater (6000 rpm, 5 min; Domel, Centric CF 48, Slovenia) was loaded on an Oasis HLB (60 mg) SPE cartridge preconditioned with 6 mL of methanol and 6 mL of Milli-Q (Millipore Direct-Q purifying system). After sample loading, the cartridge was washed with 50 mL of Milli-Q and left under vacuum to dry. The retained compounds were eluted using 1 mL of methanol, and the resulting eluent was then dried using N_2 gas at 40 °C. Samples were reconstituted in 250 μL of methanol: Milli-Q (10:90, v/v) and filter-centrifuged (0.2 μm modified nylon centrifugal filter) at 14,000 RCF for 3 min before the analysis. All samples were stored at −20 °C prior to analysis.

2.3.2. Protocol B

The sample (125 mL) of filtered wastewater (glass microfiber filters: GF/D – 2.7 μm and GF/C – 1.2 μm) was first acidified (pH 2, ≥85% H_3PO_4) before being loaded on Oasis prime MCX (150 mg) SPE cartridge

and dried using a vacuum manifold. Retained compounds were first eluted with 6 mL of methanol (fraction B1) and then with 6 mL of 0.5% ammonia in methanol (fraction B2). After elution, fraction B2 was dried (40 °C, N_2), reconstituted in 500 μL of 0.1% HCOOH and filter-centrifuged (14,000 RCF, 3 min, modified nylon, 0.2 μm), while fraction B1 was acidified (60 μL of HCOOH) and purified using a Strata NH_2 (200 mg) SPE cartridge. After preconditioning with 4 mL of 1% HCOOH in methanol, the eluate (acidified B1) was passed through the cartridge and recollected, allowing interfering compounds to be retained. After drying (40 °C, N_2), the recollected fraction B1 was reconstituted in 500 μL of methanol: Milli-Q (70:30, v/v) and filter-centrifuged (14,000 RCF, 3 min, modified nylon, 0.2 μm). Both fractions B1 and B2 of all samples were stored at −20 °C prior to analysis.

2.4. Sample analysis

Sample analysis is based on Celma et al. (2020). The compounds were separated on a CORTECS C18 fused core column (2.1 × 100 mm, 2.7 μm) maintained at a temperature of 40 °C. Mobile phases consisting of Milli-Q (A) and methanol (B), both containing 0.01% HCOOH, were used, and the flow rate was set to 0.3 mL/min. After sample injection (5 μL), gradient elution was as follows: 10% B at 0 min, linear increase to 90% B over 14 min, hold the condition for 2 min, decrease to 10% B at 16.1 min and hold the condition for 2 min for the column to equilibrate.

Analysis was performed on an Acquity I-Class UPLC system (Waters, Milford, MA, USA) coupled to a VION IMS-QTOF, with an electrospray ionization interface operating in the positive mode. Mass spectral data were acquired in HDMSe mode over the range *m/z* 50–1000, with leucine encephalin used for mass correction. Low energy (LE, collision energy of 6 eV) and high energy (HE, collision energy ram of 28–56 eV) scans were done independently, with 0.3 s scan time. Data were examined using in-house built accurate mass screening workflow using the UNIFI platform (version 1.9.4). Additional data on VION parameters can be found in Table S1.

2.5. Suspect screening workflow

Empirical data for each chromatographic peak (feature) with an intensity >1000 cps was compared with library data. Comprehensive screening of NPS from various classes was conducted using two databases simultaneously: (1) the HighResNPS database (April 2021 version) – a extensive (5600 entries) centralised collection of NPS mass spectra provided by laboratories around the world (HighResNPS database, 2021), and (2) an in-house database including empirical data (LC, IMS and HRMS data) for 128 psychoactive substances (Celma et al., 2020), which were obtained by analysing reference standards under the same LC, IMS and HRMS conditions to the ones applied to wastewater samples within this study. Since no guidelines on compound identification in wastewater exist, threshold values, i.e., the acceptable deviation between empirical and library data was selected (Table 1) based on the SANTE/11312/2021 (2021) guidelines, and our previous experience in wastewater sample analysis, i.e., the higher deviation in retention times as proposed in SANTE was taken into account due to the influence of the wastewater matrix composition (Celma et al., 2020).

Table 1
Threshold values used for compound identification.

Analytical technique	Identification feature	Threshold value/criteria
HRMS	Mass accuracy	<5 ppm
	Number of fragment ions	Protonated molecule ion and the presence of (at least) one fragment ion
	Peak intensity	>1000 counts
LC	t_R	±0.2 min
IMS	CCS	±2%

CCS – collision cross-section values, HRMS – high-resolution mass spectrometry, IMS – ion mobility separation, LC – liquid chromatography, t_R – retention time.

2.6. Levels of confidence

Compounds were identified according to different confidence levels (Celma et al., 2020) depending on the information available: the availability of reference standards and the accuracy of the acquired chromatographic and mass spectral data. Briefly, Level 3 – tentative identification: this is the lowest level of confidence reported in this study. It is used when identified compounds meet the minimum requirements for tentative identification, i.e., an accurate mass and conformational mass fragment, but more than one possible candidate can be assigned to a single feature. Level 2 – probable identification: represents compounds that meet the requirements for tentative identification, with the difference that an exact structure of a substance could be proposed based on empirical data, leading to a single candidate. Within Level 2, Level 2a represents a probable identification by LC-HRMS library match, and Level 2b represents probable identification by *in silico* or GC-HRMS library data. Level 1 – unequivocal identification: this is the highest level of confidence and is used when data from reference standards support compound identity in all dimensions: chromatographic, IMS and mass spectral data. However, numerous spectral interferences in wastewater can affect mass accuracy and retention times, and accordingly, deviations beyond the established threshold (Table 1) for the HRMS or LC metrics (but not both simultaneously) were deemed acceptable and labelled as Level 1*.

2.7. Ethics

Although WBE studies raise limited ethical issues, as individuals cannot be identified, caution must be taken in specific sites, such as educational institutions, where there is a risk of stigmatisation and sensationalisation of the results. Therefore, we followed the ethical research guidelines developed by Prichard et al. (2014), where consent was obtained from the Heads of each institution, and an anonymity agreement was signed to protect the participants' identities. Additionally, the smaller municipality was anonymised to prevent tracking. All institutions willingly agreed to participate in the study and to the publication of the anonymised data. It should be noted that with the current study design, only data on the presence of psychoactive substances at the institution could be obtained, and the data cannot be inferred to a particular group, e.g., students, staff or visitors (see 4.1. Study strength, limitations and future perspectives).

3. Results

NPS were present in wastewater from all institutional types (Table 2). Four compounds, namely 3-MMC, ephedrine, 4-chloro-α-PPP and ethcathinone, were identified unequivocally (Level 1), with 4-chloro-α-PPP present in at least one sample from each institution type. Three compounds met Level 2 criteria, namely levorphanol, embutramide, and kavain and two features were related to 15 tentative (Level 3) candidates. More details to support the obtained results, i.e., relevant data on observed mass (m/z), mass error (ppm), retention time (min), CCS, and fragment ions, can be found in Table S2, while more data on (tentatively) identified compounds can be found in Table S3.

Out of nine features, six belonged to synthetic stimulants, two synthetic opioids and one to the natural plant-based psychoactive substance kavain, which was identified in 18 of the 23 wastewater samples. Overall, a similar number of features relating to NPS candidates were identified in samples from each type of institution (primary: n = 6, secondary: n = 6, SHEIs: = 6 and HEIs: n = 7), with Ljubljana having all of the features present and two being detected in M1.

Table 2
Summary of NPS identified for each relevant feature regardless of extraction method, organised according to NPS classification and level of confidence. Their legal status in Slovenia is also provided (Decree RS 157/20, 2020).

Feature	Exact mass[a] [g/mol]	NPS *(legality status in Slovenia)*	Confidence Level[c]	NPS class	Number of samples (n = 23)					
					Educational institution				Municipalities	
					Primary school (n = 8)	Secondary school (n = 6)	SHEI (n = 3)	HEI (n = 6)	Ljubljana (n = 17)	M1 (n = 6)
1	177.1154	3-MMC *(illicit)*	Level 1	Synthetic stimulants	1	0	0	0	1	0
2	165.1154	1R-2S-(−)-Ephedrine[b] *(licit)*	Level 1		0	1	0	3	4	0
3	237.0920	4-Chloro-α-PPP *(licit)*	Level 1*		1	1	1	2	5	0
4	177.1154	Ethcathinone *(licit)*	Level 1*		1	0	1	0	2	0
5	174.1157	5-IT, AMT *(illicit)* N-methyltryptamine, 6-IT *(licit)*	Level 3		6	1	3	1	9	2
6	191.1310	3-MBC, Pentedrone, 2-MBC *(illicit)* 2,3-DMMC, 2,4-DMMC, 4-MDMC, Isopentedrone, N- Ethylbuphedrone, 4-MPH, 2-NMC, N-Acetylmethamphetamine *(licit)*	Level 3		1	2	2	2	7	0
7	257.1780	Levorphanol *(illicit)*	Level 2a	Synthetic depressants (opioids)	0	1	1	1	3	0
8	293.1991	Embutramide *(licit)*	Level 2a		0	0	0	1	1	0
9	230.0943	Kavain *(licit)*	Level 2a	Natural (plant-based) psychoactive compounds	5	5	3	5	15	3

[a] (PubChem, 2023).
[b] Ephedrine can also be of natural origin (herb Ma-huang: *Ephedra sinica*).
[c] Level 1 = unequivocal, Level 1* = identification features align with reference standards, with a slight deviation in mass accuracy or retention times (but not both simultaneously; see 2.6. Levels of confidence), Level 2a = probable, Level 3 = tentative (Celma et al., 2020).

4. Discussion

4.1. NPS findings

In this study, four NPS were unequivocally identified, three met Level 2 criteria with high confidence, and 15 were tentatively identified as possible candidates (Level 3). The latter does not necessarily indicate the presence of all 15 different NPS in the wastewater samples. At Level 3 identification, multiple NPS could be assigned to an individual feature based on structural similarities but insufficient evidence to differentiate between candidates. Moreover, reference standards are required for final confirmation of the identity of NPS. Despite this, most (tentatively) identified NPS were synthetic stimulants, which agrees with their higher relative prevalence in Slovenia as reported by other socio-epidemiological data, namely from drug analysis, toxicological reports and questionnaires (NLZOH, 2021; NIJZ, 2019). Interestingly, synthetic cannabinoids, also common in Slovenia, were not detected. A possible reason could be their rapid metabolism in the human body (leading to lower concentrations) and hydrophobic properties, removing them from the aqueous phase of the wastewater (Bijlsma et al., 2019, 2021).

Among the unequivocally identified stimulants, i.e., 3-MMC, ephedrine, 4-chloro-α-PPP and ethcathinone, ephedrine is the only one that can be of synthetic or natural origin. It has many known uses, i.e., as medication in traditional and Eastern medicine, as a drug of abuse (by athletes and drug users) and as a precursor in the clandestine synthesis of methamphetamine (Gad et al., 2021). As a common ingredient in preparations to treat colds, asthma and narcolepsy (Gad et al., 2021), ephedrine is expected to be found in wastewater. However, in Slovenia, medical ephedrine is only available as an injection of ephedrine hydrochloride, used to treat hypotension during general and local anaesthesia (CBZ, 2023). Therefore, given the sample set (educational institutions), the presence of ephedrine is most likely related to its recreational use. This assertion gains further support from the reported instances of recreational ephedrine use in Slovenia (NIJZ, 2019).

Notably, 4-chloro-α-PPP was the only NPS unambiguously identified in at least one sample from all institution types. While this drug has been previously detected in street drug samples (NFL, 2022), it is not as commonly used or as well-known as 3-MMC, a controlled substance in Slovenia (NIJZ, 2019; Decree RS 157/20, 2020). 3-MMC, widely referred to as "ice cream" on the streets, gained popularity during the period of ecstasy and cocaine shortages, which coincided with economic and migrant crises. However, its popularity has since waned over time. Regardless, it is still among the most used and well-known NPS among drug users, including young users, and has been associated with drug intoxication (NIJZ, 2019). Although it has been reported as one of the most commonly used NPS among students at the University of Ljubljana (NIJZ, 2019, 2021), our study only detected its presence in a sample obtained from a primary school in Ljubljana.

Among probable candidates for synthetic stimulants, AMT and 6-IT (feature five), and MEC-type NPS, pentedrone, 2,3-DMMC, 4-MDMC and 4-MPH (feature six) are known to have been present on the Slovenian drug market (NIJZ, 2019, 2021). However, according to reports, only illicit MEC-type NPS were used by students of the University of Ljubljana in 2017/2018 and 2019/2020 (NIJZ, 2019, 2021), providing greater confidence in their identification of the samples analysed. Among all probable candidates, the most unusual and least expected is N-acetylmethamphetamine, as there are no indications (reports) that this substance is consumed as the parent compound (Barnes et al., 2019). N-acetylmethamphetamine is a by-product of methamphetamine synthesis and thus can be found in methamphetamine as an impurity. However, no methamphetamine was found in samples where N-acetylmethamphetamine was identified (Verovšek et al., 2021a, 2021b). Interestingly, except for 3-MMC, no other synthetic cathinones detected in Slovenian municipal wastewaters in 2016 (mephedrone, methylone and α-PVP), 2017 (mephedrone), and during the Christmas-New Year period in 2021/2022 (eutylone) (Castiglioni et al., 2021; Bade et al., 2023) were identified in wastewater from educational institutions analysed in this study. Their absence could be attributed to the dynamic nature of the NPS market or, alternatively, may be influenced by catchment specificities or their low concentration levels, which may fall below the detection limit of our method (Bade et al., 2020; NIJZ, 2021).

Although opioids were the least expected psychoactive substances, since unlike the stimulants, they do not increase alertness, morphine and codeine were found in wastewater from educational institutions in

Slovenia (Verovšek et al., 2021a). In this study, opioids levorphanol and embutramide met Level 2 identification criteria. Although opioid-type psychoactive substances are often used as a readily accessible substitute for prescription medications subject to medical supervision (Andersson and Kjellgren, 2016), we are unaware of any metadata regarding their occurrence in Slovenia to confirm their street use.

Kavain was the only naturally occurring (plant-based) psychoactive substance identified (Level 2) in 18 samples. Its high prevalence can be supported by easy accessibility, i.e., it can be readily obtained online in Slovenia (Google search by authors). It is one of the most abundant kavalactones in kava (*Piper methysticum*) and is responsible for the plant's psychoactive effect. Beyond its use in religious rituals in Melanesian societies, kava is known for its calming effect and is used in Western countries as a prescription-free alternative to benzodiazepines and recreationally as an alcoholic substitute (Chua et al., 2016). Despite being marketed as an alternative medicine in Slovenia, its safety was called into question in Europe between 1999 and 2000 due to a surge in reports highlighting its potential liver toxicity and lack of clinical evidence supporting its health benefits. (Kuchta et al., 2015). Accordingly, kavain (and the kava plant) was banned in several European countries, but its legal use was restored due to a lack of proper clinical research (Kuchta et al., 2015).

Regarding inter-institutional trends, we expected a higher number and diversity of NPS in HEIs, as NPS can be easily purchased online (Bijlsma et al., 2019), and the first instance of illicit drug use in Slovenia typically occurs between the ages of 19 and 23 (NIJZ, 2022). Moreover, NPS are often used as legal substitutes for illicit drugs, making their presence more likely among this age group and older. However, our study revealed a similar number of NPS features (six and seven) across all educational institutions, suggesting that the occurrence of NPS is comparable regardless of the institution type. Also, it remains unclear whether this reflects their prevalence in the general population, as no comparable data on wastewater-based epidemiology (WBE) exist for Slovenia.

Regarding potential spatial trends, all NPS corresponding features (n = 9) were found in Ljubljana, the capital of Slovenia, while in M1, there were only two features, none of which correspond to unequivocally identified NPS. Also, in M1, none of the likely candidates corresponds to synthetic opioids. Given the difference in the number of detected features, the results suggest that NPS were more prevalent in Ljubljana, which is a larger, more diverse municipality between the two addressed in the study, despite NPS being easily obtained online. The difference in the number of samples obtained from each municipality (17 *vs* 6) could also be a contributing factor to the results, as it highlights a substantially larger target population in Ljubljana. Nevertheless, a city-characteristical use of NPS was also confirmed by Brandeburová et al. (2020).

4.2. Study strengths, limitations and future perspectives

The non-invasiveness of WBE makes it an attractive technique for studying vulnerable populations, and it has already been applied successfully to prisons, festivals and educational institutions (Verovšek et al., 2020). Also, the integration of non-target data acquisition further enhances its effectiveness as a surveillance tool (radar) for NPS detection, which makes it particularly valuable (Bijlsma et al., 2019). However, the method is not without its limitations. For example, sampling wastewater in specific sub-catchments, such as single facilities, is challenging since it requires good knowledge of the sewer system, power availability when using an autosampler, and optimised sampling frequency to adjust to inconsistent wastewater flow (Verovšek et al., 2020). While extending the sampling period in this study was not feasible, it is recommended that future research considers sampling over multiple days; otherwise, it provides only a snapshot of drug use at a given time (Ort et al., 2010; Verovšek et al., 2020).

Another limitation specific to NPS is the need for reference standards for unequivocal identification. For this reason, having pre-knowledge of NPS used in countries from other socio-epidemiological sources is essential since it adds confidence to identification when no reference standards are available. Likewise, despite having an extensive database available, it can take time for NPS to be identified and included and may not appear in the study. One advantage of non-target data acquisition is that it allows for retrospective analysis, which can be performed at any future time to uncover additional information from the acquired data (Bijlsma et al., 2021). Moreover, due to limitations in method sensitivity and extraction specificity, certain compounds may remain undetected, despite utilising two different SPE cartridges to broaden the range of extracted NPS.

The main limitation of using wastewater analysis to investigate NPS use among younger individuals by sampling from educational institutions is that, at the institution level, it is challenging to distinguish between the target population (pupils and students) and other groups such as staff members or visitors (Verovšek et al., 2021a). Therefore, caution should be taken when extrapolating results to a particular group. Also, this makes comparison with socio-epidemiological study data problematic given that such data always relate to a particular group, e.g., the general population (15-64-year olds), students, or drug users. However, as stated above, such a comparison is essential in screening for NPS to gain confidence in the identification. A possible solution would be to leverage existing sewer infrastructure to target areas where pupils are known to congregate, such as bathrooms and locker rooms similar to that used in prison settings (van Dyken et al., 2014).

Despite the limitations identified, the combination of WBE and suspect analysis conducted in educational institutions holds significant promise. This approach provides valuable insight into the presence of psychoactive substances and serves as an effective tool (radar) for detecting NPS in environments where they should not be present, regardless of whether they are being consumed by pupils or staff. In that way, valuable data on when and with which NPS children and students come in contact are gathered. In addition, wastewater analysis can serve as a practical and non-invasive substitute for traditional drug testing methods to detect the presence of drugs, particularly in educational institutions, where drug testing may carry a social stigma and create a sense of shame for those who test positive. It is known that the association with drug use can also cause long-term psychological and social harm and encourage punitive actions rather than early prevention and education (Dupont et al., 2013). However, further research on replacing drug testing with wastewater analysis and evaluating its impact on drug use among young people is needed to support such a claim.

5. Conclusions

This study is the first to identify the presence of NPS in wastewater samples obtained from all levels of educational institutions, including primary schools, where data on the prevalence of psychoactive substances, including NPS, is scarce even at a global level. Also, no trends in NPS prevalence were observed when different types of institutions were compared, while more diverse substances were present in the larger of the two studied municipalities. Importantly, our proof of concept confirms that wastewater analysis has the potential to provide insight into the presence of NPS in educational institutions. It is worth noting that with specific adaptation (e.g., leveraging parts of existing sewer infrastructure covering parts of the institutions where pupils are congregating), this analysis may be able to attribute NPS consumption to a particular group of individuals within the institution (e.g., students). However, even in its current form, this analysis can still provide data on the presence of drugs in educational institutions, which is needed since these institutions are expected to maintain a drug-free environment. Accordingly, wastewater analysis could serve as a valuable non-invasive substitute for drug testing, which is currently carried out in various countries worldwide.

Credit authors statement

Taja Verovšek: Writing – original draft, Methodology, Formal analysis, Data curation, Visualization; Alberto Celma: Writing – review & editing; Investigation, Formal analysis; David Heath: Conceptualization, Data Curation, Writing – review & editing; Ester Heath: Project administration, Funding acquisition, Conceptualization, Supervision, Writing – review & editing; Félix Hernández: Resources, Writing – review & editing; Lubertus Bijlsma: Funding acquisition, Conceptualization, Supervision, Data curation, Writing – review & editing.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117061.

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3.3 Supplementing WBE Data: Reducing Uncertainty in Evaluating Drug Consumption

3.3.1 Enantiomeric profiling of amphetamines in wastewater using chiral derivatization with gas chromatographic-tandem mass spectrometric detection

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Enantiomeric profiling can distinguish between used and disposed or unused drugs and is commonly used to investigate so-called "dumping events" when high biomarker mass loads are observed in wastewater (Chapter 1.3.2 Addressing uncertainties in evaluating drug consumption). Until now, enantiomeric profiling has only been performed by chiral LC-MS/MS, which is impractical and requires an expensive chiral column purchased to analyze a small number of samples. In order to overcome these disadvantages, a cost-effective alternative method for enantiomeric profiling of amphetamines (amphetamine, methamphetamine and MDMA) in wastewater was developed. The method is based on chiral derivatization with (-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (R-MTPCl) and GC-MS/MS analysis and was applied to municipal wastewater samples obtained within SCORE monitoring (see 3.1.1 National reports to the EMCDDA: Reports on the drug situation of the Republic of Slovenia (2019–2022)). In particular, the work focused on samples with high MDMA (Ljubljana) and amphetamine (Velenje) mass loads, while a more representative sample in terms of amphetamine loads (Ljubljana) was used as a baseline.

Comparable method performance to chiral LC-MS/MS was observed, with a wide linearity range (LOQ-1000 ng/mL) and extraction recoveries of 81-99 %, an accuracy of 99-111 % and repeatability of 1-8 %RSD within the SANTE/12682/2019 guidelines [181]. Although the obtained LOD (120 ng/L) and LOQ (400 ng/L) were approximately 10-times higher compared to chiral LC-MS/MS methods (LOQ <20 ng/L), LOD/LOQ of the method was still sufficiently low for investigating higher concentrations of biomarkers (*e.g.,* amphetamine in Velenje) and potential dumping events (*e.g.,* MDMA in Ljubljana). Indeed, according to the enantiomeric profile, the high MDMA mass load in Ljubljana (sampled on Tuesday, 21. 4. 2020) was related to the disposal of unused drugs, while MDMA in the representative sample was related to drug consumption. Unfortunately, the complicated excretion of enantiomeric profiles of amphetamine and methamphetamine prevented investigating dumping events. However, it provided valuable information about the possible route of synthesis and potency of those drugs on the Slovenian illicit drug market (no amphetamine/methamphetamine-based prescription medications were prescribed in Slovenia at the time of sampling). The latter also highlights the importance of further developing analytical methods that complement WBE data by providing insight into spatiotemporal trends in drug production (*e.g.,* synthesis routes) and availability (drug potency).

The developed method and results were presented at one scientific conference, *i.e.,* the 26th International Symposium on Separation Sciences, and the presenter was awarded "Best oral presentation by a young researcher".

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Enantiomeric profiling of amphetamines in wastewater using chiral derivatisation with gas chromatographic-tandem mass spectrometric detection

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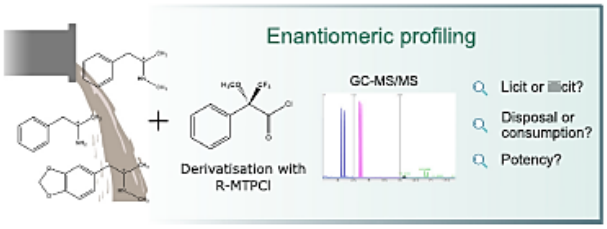
HIGHLIGHTS

- A chiral derivatisation GC–MS/MS method validated for amphetamines in wastewater.
- High MDMA loads in Ljubljana likely resulted from disposal.
- More potent S-methamphetamine is present on illicit drug market in Ljubljana.
- The method is a cost-effective alternative for enantiomeric profiling by chiral LC-MS/MS.

GRAPHICAL ABSTRACT



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ABSTRACT

Enantiomeric profiling can supplement wastewater-based epidemiology (WBE) by providing additional information on drug origin (licit or illicit), improving consumption estimates, i.e., differentiating between disposal and consumption, and offering an insight into the potency of drugs available on the illicit drug market. We report on the enantiomeric profiling of amphetamines in wastewater using R(-)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (R-MTPCl), a chiral derivatising agent and GC–MS/MS. The method performed well when evaluated against the SANTE/12682/2019 guidelines in terms of recovery (81–99%), accuracy (99–111%), repeatability (1–8%RSD) and linearity (LOQ–1000 ng/mL). The LOD and LOQ were 120 ng/L and 400 ng/L, respectively. The method was applied to samples of raw wastewater from two Slovene municipalities with unusual levels of amphetamines: Ljubljana (LJ1) and Velenje (VE1). LJ1 had an anomalously high mass load of MDMA (3,4-methylenedioxymethamphetamine) identified during SCORE 2020, and VE1 is a representative sample of the consistently high mass load of amphetamine. A second Ljubljana sample (LJ2) was chosen as a representative sample. The presence of racemic MDMA (EF = 0.511) in LJ1 indicated the disposal of the unused drug into the sewer, while the enrichment of R-MDMA (EF = 0.666) in the combined extract sample from Ljubljana (LJ2) indicated consumption. In the case of Velenje and Ljubljana, it is impossible to distinguish between the direct disposal and consumption of amphetamine and methamphetamine. Also, since amphetamine/methamphetamine-based prescription medications are unavailable in Slovenia, racemic amphetamine in VE1 (EF = 0.514) and LJ2 (EF = 0.459) indicate racemic and the more potent S-amphetamine are sold on the illicit drug market. Only S-methamphetamine was detected in wastewater (LJ2: EF = 0), indicating the presence of only the more potent S-methamphetamine on the illicit drug market. Overall, enantiomeric profiling provided useful information on amphetamine residues. In addition, chiral derivatisation can be a cost-effective alternative to using chiral chromatographic columns for the enantiomeric profiling of amphetamines in wastewater.

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1. Introduction

Drug use data are typically obtained through population surveys, crime statistics, production and seizure data and medical reports (WDR, 2021; Zuccato et al., 2008). However, the importance of acquiring objective, temporal and spatial data is leading to an increase in the use of wastewater-based epidemiology – WBE (EMCDDA, 2021), with the most well-known application being the SCORE (Sewage Analysis CORe group Europe) Europe-wide network (SCORE, 2022). The WBE approach estimates drug use in the community by determining drug biomarkers (parent compound or metabolite) in raw wastewater and adjusting their concentrations for wastewater flow and population in the targeted community (Gracia-Lor et al., 2016). The presence of the parent compound in wastewater may also originate from the disposal of the unused drug into the sewer, which can lead to biased consumption estimates when using it as a marker (Gracia-Lor et al., 2017; Cody, 2002). Progress towards finding appropriate metabolites for biomarkers is ongoing. For example, pholedrine—a methamphetamine metabolite, has recently been suggested by Bade et al. (2021), although further studies are still needed to prove its applicability. Accordingly, the parent compounds (amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine – MDMA) are still commonly used as biomarkers of use. Another problem is that amphetamine and methamphetamine are also metabolites of certain prescription drugs (e.g., amphetaminil, deprenyl, and fenethylline used to treat depression, Parkinson's disease and attention deficit hyperactivity disorder – ADHD), adding to the difficulty in interpreting their illicit use (Gracia-Lor et al., 2017; Cody, 2002).

Amphetamines, like many drugs, are chiral molecules that contain an asymmetric (chiral) carbon atom bonded to four different atoms or groups, resulting in two enantiomeric forms, R(−) and S(+) (Fig. S1). Different synthesis routes, metabolism in the human body and excretion represent enantioselective processes that result in a specific chiral signature (Kasprzyk-Hordern et al., 2010). Accordingly, knowing the enantiomeric profile of a drug residue (Eq. (1)) can offer valuable information on its origin (licit or illicit) as well as complement WBE estimates by discriminating between disposal of the unused drug into the sewer and actual consumption (Castrignanò et al., 2018). Moreover, since biological activity discriminates between enantiomers (S-enantiomers are more potent than R-enantiomers), the potency of amphetamines can also be evaluated (Kasprzyk-Hordern et al., 2010). However, despite its potential, so far, only a few studies have applied enantiomeric profiling to study amphetamines in wastewater (Castrignanò et al., 2016, 2018; Emke et al., 2014; Gao et al., 2018). Common to all of these studies is the use of chiral liquid chromatographic columns with mass spectrometric detection – LC-MS/MS (Castrignanò et al., 2016, 2018; Estévez-Danta et al., 2021; Gao et al., 2018; Kasprzyk-Hordern et al., 2010; Xu et al., 2017).

This study aimed to explore an alternative method to chiral LC-MS/MS for enantiomeric profiling of amphetamines in wastewater samples, focusing on samples with anomalously high mass loads to complement our WBE data. A novel approach using chiral derivatisation with R(-)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (R-MTPCl) followed by gas chromatography-tandem mass spectrometry (GC–MS/MS) detection was adopted. The method was applied for enantiomeric profiling of amphetamine in Slovenian wastewaters, particularly to investigate unusually high MDMA mass load observed in Ljubljana (SCORE 2020 compared to years 2017–2019) and consistently high amphetamine loads in Velenje as highlighted during the SCORE (2019–2020) monitoring campaigns (EMCDDA, 2021).

2. Experimental

2.1. Wastewater sampling and sample selection

Three raw wastewater samples were collected at the inflow of two Slovenian wastewater treatment plants (WWTPs) servicing Ljubljana (LJ1 and LJ2) and Velenje (VE1) and stored in the dark at −20 °C (Table 1). All samples were time-proportional 24-h composites, collected with 20 (Ljubljana) or 10 (Velenje) minute sampling intervals. Briefly, LJ1 had an unusually high MDMA mass load identified during the SCORE 2020 (EMCDDA, 2021), while LJ2 (16. 1. 2022; Table 1) is representative of Ljubljana in terms of levels of amphetamines commonly measured in this municipality (SCORE monitoring; EMCDDA, 2021). VE1 is representative of Velenje, the municipality where consistently higher mass loads of amphetamine were found during SCORE monitoring compared to other Slovenian municipalities participating in SCORE (SCORE, 2022; EMCDDA, 2021). For example, in 2019, the average amphetamine mass load in Velenje was 84 mg/day/1000 inhabitants, while it was more than 4-times lower in other Slovenian municipalities (<20 mg/day/1000 inhabitants; see Table S1).

2.2. Chemical analysis

The Supplementary Material (SM) provides full details on sample preparation and analysis (see SM section MATERIALS AND METHODS: Chemicals, Sample preparation and Sample analysis). Before treatment, a deuterated internal standard (IS) mixture was added to each sample. After filtering the raw wastewater (25 mL), the analytes were extracted using solid-phase extraction (SPE) with Oasis MCX cartridges. The dry extracts were reconstituted in 1-chlorobutanol. A solution of R-MTPCl in dry acetonitrile (50 μL/1 mL) was then used for derivatisation (25 μL, 80 °C, 2 h). Once cooled down, anhydrous ethanol (100 μL) was added to the samples to decompose any excess R-MTPCl. The samples were then re-heated (70 °C, 15 min), evaporated to dryness (N2), reconstituted in ethyl acetate (100 μL), centrifuged and analysed using GC–MS/MS (DB-5MS capillary column, 30 m × 0.25 mm × 0.25 μm). Since amphetamines in wastewater from Ljubljana (SCORE, 2022; EMCDDA, 2021) are typically <LOQ for enantiomeric profiling, extraction of a higher volume of wastewater was performed, i.e., ten separate 125 mL aliquots of LJ2 were extracted, and the extracts combined (concentration factor: 12500-times).

A detailed description of the validation procedure is described in the SM (section: Method validation). The method was validated in terms of linearity, LOD, LOQ, extraction recoveries, accuracy, and repeatability following the SANTE protocol using spiked wastewater influent at two concentration levels: 200 ng/mL and 800 ng/mL (final extracts). Since samples with unusually high concentrations of amphetamines were targeted, validation concentration levels were selected to bracket that of MDMA in LJ1. Enantiomeric resolution (R_s) was determined as described in the SM (section: Method validation).

Table 1
Sample information.

Sample ID	Sampling location (municipality)	Population covered by WWTP	Sampling date (day of sampling)	Other
LJ1	WWTP Ljubljana (Ljubljana)	270,305	21. 04. 2020 (Thursday)	- sample obtained during SCORE 2020 (SCORE, 2022) - high MDMA mass loads were observed: 102.5 mg/day/1000 inhabitants (EMCDDA, 2021)
LJ2	WWTP Ljubljana (Ljubljana)	270,305	16. 01. 2022 (Sunday)	- combined extracts (10 × 125 ml)
VE1	WWTP Šaleška Valley (Velenje)	35,280	30. 01. 2021 (Saturday)	/

AMP = amphetamine, WWTP = wastewater treatment plant, MDMA = 3,4- methylenedioxymethamphetamine, SCORE = Sewage Analysis Core group Europe.

Before analysis, the instrument's performance was checked by water-air control (leakage check) and analysis of diazepam solution—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 IS) at the beginning of the batch. IS were used to correct for instrumental variations.

2.3. Calculation and data analysis

The relative concentrations of R- and S- enantiomers were used to calculate the enantiomeric fraction (EF), as shown in Eq. (1):

$$EF = \frac{A(R)_a / A(R)_{IS}}{A(R)_a / A(R)_{IS} + A(S)_a / A(S)_{IS}} \quad (1)$$

Here $A(R)_a$ and $A(S)_a$ are peak areas of the R- and S-enantiomers of the analyte, and $A(R)_{IS}$ and $A(S)_{IS}$ are the peak areas of the R- and S-enantiomers of their corresponding ISs. The EF is 0.5 when the analyte present in the sample is racemic, while it equals 1 (R-enantiomer) or 0 (S-enantiomer) when there is a single enantiomeric form (Castrignanò et al., 2018; Kasprzyk-Hordern et al., 2010). Since racemic drug standards were used in the study, the EFs obtained in LJ1 and VE1 were evaluated against the EFs of the compounds in the spiked wastewater samples used for the calibration curve (Tables S4–S7). The normality of the data was tested using a Shapiro-Wilk test (95% confidence interval, $\alpha = 0.05$) and the equality of variance using the Brown-Forsythe test (95% confidence interval, $\alpha = 0.05$). The difference in the EFs of amphetamine (VE1) was evaluated using Welch's *t*-test (95% confidence interval, $\alpha = 0.05$) and the difference in the EFs of MDMA (LJ1) using a two-tailed Students' t-test (95% confidence interval, $\alpha = 0.05$). As there were no replicates of the LJ2 sample, the EF of each analyte was compared with the range of EFs obtained in the spiked wastewater samples. All statistical evaluation was performed using SigmaPlot (version: 14.0).

3. Results and discussion

3.1. Method performance

Good performance of the method (Table S7) was observed when evaluated against SANTE/12682/2019 Guidance. A linear response in the range LOQ–1000 ng/mL (sum of enantiomers) was observed for all compounds of interest ($R^2 > 0.99$). Extraction recoveries (81–99%), accuracies (99–111%) as well as inter-day recoveries (1–8% RSD) were within SANTE limits for both concentration levels. Also, an acceptable resolution of $R > 1$ was achieved (<2% overlap) for all enantiomers (Castrignanò et al., 2016; Kasprzyk-Hordern et al., 2010). The LOQ (sum of enantiomers) was determined as the lowest concentration that can be detected with sufficient accuracy (100 ± 20%) and repeatability (RSD < 20%), while the LODs were calculated as the LOQs divided by 3.33 and were 400 ng/L and 120 ng/L, respectively. When compared to chiral LC-MS/MS, the methods are comparable except in terms of LOQ, which is lower in the case of chiral LC-MS/MS, i.e., <20 ng/L based on S/N (Emke et al., 2014; Estévez-Danta et al., 2021; Kasprzyk-Hordern et al., 2010). However, determined LOQ were sufficiently low for compounds of interest in LJ1 and VE1.

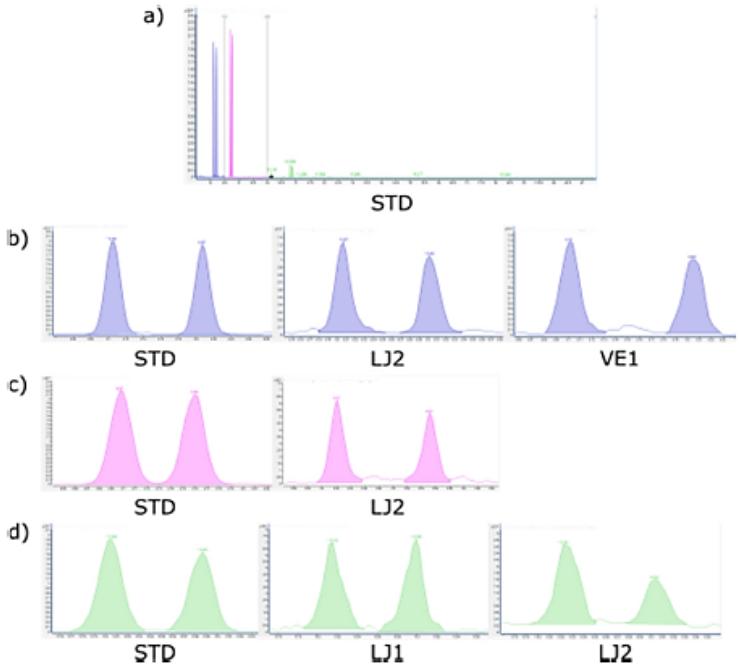

Fig. 1. Chromatograms (counts vs time in min) showing enantiomeric separation (MRM transition) of all studied amphetamines (a), amphetamine (b), methamphetamine (c) and 3,4-methylenedioxymethamphetamine – MDMA (d) in spiked wastewater (STD: 800 pg/μL) and samples (LJ1, LJ2 and VE1).

Also, the additional derivatisation step extends the analysis time. Despite these limitations, the method represents a cost-effective alternative to chiral LC-MS/MS when analysing a limited number of samples.

3.2. Enantiomeric profiling of amphetamines

Amphetamine in VE1 was racemic (Fig. 1), i.e., there was no statistically significant difference ($t = -1.453$, $p = 0.381$, $\alpha = 0.05$) between average EFs of amphetamine in VE1 and wastewater spiked with a racemic amphetamine standard (Table 2). In addition, the same can be assumed for LJ2 (Table 2). We know from the literature that when racemic amphetamine is administered, its' stereoselective metabolism yields higher amounts of the R-enantiomer since the S-enantiomer metabolises more rapidly (Kasprzyk-Hordern and Baker, 2012). Since racemic amphetamine is commonly synthesised in clandestine laboratories (Leuckart method), enrichment of R-amphetamine in wastewater is expected when consumption of illicit amphetamine is assumed (Emke et al., 2014; Kasprzyk-Hordern et al., 2010). As already mentioned, amphetamine may originate from the metabolism of certain medications (Cody and Schwarzhoff, 1993; Cody, 2002) and methamphetamine, 4–7% of which is excreted as amphetamine (Cody, 2002; Xu et al., 2017). However, given that such medications are no longer prescribed (since 2018) in Slovenia (CBZ, 2022; ZZZS, 2022), the amphetamine detected is from illicit use. Also, the presence of methamphetamine in Slovenian wastewater is always low compared to amphetamine (Table S1), suggesting that methamphetamine does not contribute significantly to amphetamine loads. It is also impossible to differentiate between disposal and consumption. As Emke et al. (2014) point out, there are two reasons for the presence of racemic amphetamine in wastewater (i) direct disposal of unused amphetamine and (ii) illicit use of both racemic and pure S-amphetamine. In our case, it is more reasonable to assume consumption since the samples contained no high mass loads of amphetamine attributable to direct disposal and that racemic amphetamine was found in the wastewater of both municipalities.

Since only low amounts of methamphetamine are detected in Slovenian wastewaters (EMCDDA, 2021), its enantiomeric profiling was only possible in composite samples (e.g. LJ2). The results show that methamphetamine was the pure S-enantiomeric form (Fig. 1 and Table 2). This finding shows that S-methamphetamine is clandestinely produced and abused (Kasprzyk-Hordern and Baker, 2012), which is consistent with other European countries (Castrignanò et al., 2018; Estévez-Danta et al., 2021; Kasprzyk-Hordern et al., 2010).

The drug MDMA is also typically synthesised using the Leuckart method (or other reductive aminations), resulting in a racemic product (Emke et al., 2014; Kasprzyk-Hordern and Baker, 2012), which is then excreted from the human body enriched in the R-enantiomer (Moore et al., 1996). In our study, enrichment of R-MDMA was observed in LJ2 (Fig. 1 and Table 2), indicating MDMA consumption. However, given there is no significant difference between the EF of LJ1 and the spiked wastewater ($t = 0.453$, $p = 0.656$, $\alpha = 0.05$), racemic MDMA present in LJ1 could result from the disposal of the unused drug, which is supported by the unusual high MDMA load in LJ1 (Fig. 1 and Table S1). Similarly, high mass loads were attributed to the disposal of unused MDMA in the Netherlands, associated with a police raid (Emke et al., 2014). Unfortunately, no police data could be obtained to support the argument for drug disposal in our case.

Table 2
Enantiomeric fractions (EFs, expressed as average, range or single value) obtained in standards (spike wastewater samples were used for calibration) and samples.

Sample	EF(AMP)	EF(MAMP)	EF(MDMA)
Standards	Average: 0.469 Range: 0.444–0.485	Average: 0.476 Range: 0.460–0.488	Average: 0.504 Range: 0.0471–0.537
LJ1	<LOD	<LOD	Average: 0.511
LJ2	0.459	0.000	0.666
VE1	Average: 0.514	<LOD	<LOD

AMP = amphetamine, MAMP = methamphetamine, MDMA = 3,4-methylenedioxymethamphetamine.
Ljubljana – LJ1: sample with unusually high MDMA levels.
Ljubljana – LJ2: representative sample of Ljubljana.
Velenje – VE1: representative sample of Velenje.

4. Conclusions

This study developed and validated a novel approach for WBE enantiomeric profiling using chiral derivatisation with GC–MS/MS to study amphetamines in wastewater. The method performed well when evaluated against SANTE/12682/2019 Guidance, and despite higher LOQs and longer sample preparation time, it is comparable to chiral LC-MS/MS methods. In this study, enantiomeric profiling of wastewater suggests that the sample with an unusually high MDMA load observed in Ljubljana with its racemic fraction likely originated from the disposal of the unused drug. In contrast, typical MDMA loads enriched in the R-enantiomer suggest MDMA consumption, while no assumptions about the disposal of unused amphetamine or methamphetamine could be made based on their enantiomeric profile. However, since no amphetamine/methamphetamine-based medications are prescribed in Slovenia, an insight into the potency of drugs present on the illicit drug market was gathered. Racemic amphetamine (Ljubljana and Velenje) probably indicates the availability of racemic and S-amphetamine on the market, and the presence of S-methamphetamine in wastewater indicated the availability of a more potent S-methamphetamine in Slovenia. Overall, chiral derivatisation can be a cost-effective alternative to chiral chromatography for the enantiomeric profiling of drug residues in WBE, especially for a limited number of samples with unusually high mass loads.

CRediT authorship contribution statement

Taja Verovšek: Conceptualisation, Methodology, Formal analysis, Investigation, Writing – Original Draft.
David Heath: Conceptualisation, Methodology, Writing – Original Draft.
Ester Heath: Conceptualisation, Writing – Review & Editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.155594.

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3.3.2 Combining a Stable Isotope Analysis with a Wastewater-Based Epidemiological Approach to Complement Illicit Drug Profiling

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Determining the composition of stable isotopes of organic molecules using isotope-ratio mass spectrometry (IRMS) is widely used to determine food authenticity, namely confirming geographic origin and detecting aroma adulterations, but also in forensic science to discriminate and track sources between batches of explosives and illicit drugs (Chapter 1.3.2.4 Stable isotopic composition of light elements). Accordingly, it was hypothesized that the isotopic composition of drug residues in raw wastewater may also provide data on their origin, e.g. used or dumped drug (Chapter 1.3.2 Addressing uncertainties in evaluating drug consumption). However, to our knowledge, this is the first attempt of a kind, *e.g.,* determining the isotopic composition of drug residues in wastewater and exploring potential applicability. In this study, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was used to determine the $\delta^{13}C$ value of morphine in raw wastewater. In order to complement forensic data [120] – [122], the $\delta^{13}C$ value of drugs, namely amphetamine, morphine, codeine and heroin in street drugs (amphetamine powder, opium resin and heroin powder) and laboratory analytical standards were also obtained.

For determining the $\delta^{13}C$ value of morphine in raw wastewater, it was extracted from a large volume of wastewater (51.5 L) using SPE-disks (AttractSPE™ Disks HLB), purified using SPE (Strata® C18-E) and isolated by flash chromatography (Biotage® Sfär C18 D). The morphine-containing fractions were then dried, derivatized (*N,O*-bis(trimethylsilyl)trifluoroacetamide, BSTFA) and analyzed using GC-C-IRMS. To eliminate fractionation that may occur during sample preparation and analysis, blank samples (1.5 L of spiked tap and raw wastewater) were prepared and analyzed following the same procedure. Before derivatization and analysis, street drugs were dissolved in the corresponding solvent and dried, while laboratory analytical standards already dissolved in methanol were only dried. Before GC-C-IRMS, the presence of analytes was confirmed in all samples using GC-MS/MS.

As expected due to its synthetic nature, highly negative $\delta^{13}C$ values were obtained for amphetamine (-42.6 to -40.0 ‰). Surprisingly, a highly negative value was also obtained for one opium resin ($\delta^{13}C$ values of morphine: -35.8 ‰), suggesting morphine in this drug is of synthetic origin. The $\delta^{13}C$ value of morphine isolated from raw wastewater was -33.7 ‰, which falls in the interval of $\delta^{13}C$ values of morphine in street drugs. Since morphine in wastewater may also originate from its medical use, further studies analyzing medical morphine are needed to evaluate the results more fully.

Based on the $\delta^{13}C$ value of morphine in wastewater alone, it was impossible to discern a possible dumping event, as no changes in carbon bonds were made during the metabolism. However, monitoring changes in $\delta^{13}C$ value over time may be used as an early warning detecting changes in drug supply in the illicit drug market and can be used to support drug profiling [123]. For example, in the case of natural and semi-synthetic drugs (*e.g.,* cannabis, cocaine, morphine/opium and heroin), GC-C-IRMS analysis of drug residues in wastewater may be used to detect the geographic origin of plant material used for their production. Of course, such applicability requires further studies that will, aside from determining the isotopic composition of carbon, consider the isotopic composition of other light elements, *i.e.,* nitrogen, oxygen and hydrogen. More importantly, to include GC-C-IRMS analysis in the routine analysis of drug residues in wastewater, investigating alternative pre-

concentration methods is needed, replacing the tedious extraction of high volumes (approx. 50+ L) of wastewater, *e.g.,* passive or active-passive sampling.

Combining a Stable Isotope Analysis with a Wastewater-Based Epidemiological Approach to Complement Illicit Drug Profiling

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KEYWORDS

Wastewater-based epidemiology, raw wastewater, drug profiling, illicit drug, drug origin, stable isotopes, CSIA, forensics

ABSTRACT

While wastewater analysis has gained recognition as an important tool in assessing drug usage in the population, the potential to trace the source of illicit drugs by combining WBE with the stable isotope approach is yet to be fully harnessed. In this study, the $\delta^{13}C$ values of drug residue (morphine) in wastewater were obtained for the first time. Moreover, the potential to complement forensic intelligence was assessed by analyzing authentic analytical standards and samples of street drugs, including amphetamine, opium, and heroin. Municipal wastewater was extracted using a combination of HLB disks and Strata C18-E and isolated using flash chromatography. The $\delta^{13}C$ values were obtained using gas chromatography-isotope ratio mass spectrometry (GC-C-IRMS). In the case of street drugs, highly negative $\delta^{13}C$ values for amphetamine (-42.6 to -40.0 ‰) and, surprisingly, for one opium resin sample ($\delta^{13}C$ value of morphine: -35.8 ‰) were observed, possibly relating to their synthetic origin. Despite the complexity of the wastewater matrix and trace levels of residues, a $\delta^{13}C$ value for morphine (-33.7 ‰) was determined, falling in the mid-range of the values observed for street drugs (-35.8 to -29.7 ‰). This study indicates that isotopic analysis of carbon in morphine residue from wastewater holds promise as a novel approach for tracking changes in sources of illicit drugs, offering potential applications in forensic intelligence and law enforcement.

SYNOPSIS

For the first time, this study combines stable isotope analysis with wastewater-based epidemiology to complement drug profiling. $\delta^{13}C$ values were obtained using compound-specific isotope analysis (CSIA).

1. INTRODUCTION

The production, trafficking, and use of illicit drugs profoundly affect public health and pose significant challenges to the rule of law, but assessing these risks requires sufficient high-quality data on their use and supply[1]. Typically, demand is assessed through population surveys, which are inherently subjective and have a time lag in reporting data[2,3]. As a response, an objective approach capable of providing timely data on trends in illicit drug use through analyzing municipal sewage was proposed by Daughton[4]. Wastewater-based epidemiology (WBE) relies on the chemical analysis of raw sewage for human metabolic drug residues, including parent compounds and their metabolites, and knowledge of drug excretion profile, wastewater flow and catchment size in order to estimate drug use in a population[5]. Since its first utilization in 2005[6], WBE has reached international recognition within Sewage analysis CORe group — Europe (SCORE) monitoring[7] and has become an established approach [2,3].

Since wastewater reflects the population's drug use activity, it represents an abundant data source. In addition, beyond its role in assessing drug use, it has the potential to serve as a valuable source of forensic information regarding illicit drug supply. Indeed, wastewater analysis using enantiomeric profiling or non-target analysis has already been used to detect drug manufacture and evaluate the potency of synthetic drugs [8,9]. However, such studies are rare and do not capture the full potential of WBE, for example, by combining WBE with stable isotope analysis.

Stable isotope analysis is already used to analyze seized drugs and obtain forensic intelligence on illicit drug production and trafficking due to its ability to discriminate origin, history and source with high precision [10,11]. This ability stems from the fact that the stable isotopes of the same element change their isotopic composition during physical, chemical and biological processes, i.e., isotopic fractionation. Determining the isotopic composition of light elements (*e.g.,* $^{13}C/^{12}C$ and $^{15}N/^{14}N$) has proven successful in differentiating between batches of synthetic drugs, such as amphetamines. It has also been effective in identifying the cultivation area (geographic origin) of raw materials, such as those used to produce natural and semi-synthetic drugs like cocaine and heroin, respectively[10,12]. For example, morphine is a common raw material used for clandestine synthesis of narcotics, such as heroin. It is typically extracted from opium, usually derived from the poppy plant *Papaver somniferum* using the "lime method" [13,14]. Once formed, morphine retains its natural isotopic composition, specific to where it grew, and will be influenced by environmental conditions, such as humidity, temperature and isotopic composition of CO_2[13]. Such differences in the isotopic composition can be used to determine the geographic origin of the poppy plant from which the morphine was extracted[13]. Nowadays, isotopic data on the origin of seized heroin (morphine) are obtained routinely by the United States Drug Enforcement Administration and others[11,15].

This study combines stable isotope analysis with wastewater-based epidemiology to complement drug profiling. This study determined the isotopic composition of carbon ($\delta^{13}C$ value) of drug residue extracted from raw wastewater using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Furthermore, $\delta^{13}C$ values were measured in street drugs, amphetamine powder, opium resin and heroin powder, and authentic analytical standards to assess the potential of combining both approaches.

2. MATERIALS AND METHODS

2.1. **Chemicals and materials.** Analytical standards of amphetamine, morphine, and codeine (1 mg/mL) were obtained from Cerilliant Corp (Round Rock, Texas, USA). All solvents, including HPLC grade methanol, acetone and ethyl acetate, were purchased from J. T. Baker (Philipsburg, USA). Formic acid (HCOOH) was obtained from Fluka (Switzerland), and N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma Aldrich (Missouri, USA). Ultrapure water (18.2 MΩ•cm resistivity at 25 °C) was obtained using the Millipore Direct-Q purifying system. Glass microfiber filters (GF/D – 2.7 µm and GF/C – 1.2 µm) were purchased from Whatman (USA) and modified nylon centrifugal filters (0.2 µm) from VWR (Vienna, Italy). Extraction disks (AttractSPE™ Disks HLB) were from Affinisep (Normandy, France) and solid phase extraction (SPE) cartridges (Strata C18-E; 55 µm, 6 mL, 70 Å) from Phenomenex (California, USA). Materials for flash chromatography, namely Sfär C18 samplets (25 g) and Sfär C18 Duo column (30 µm, 45 mL, 100 Å), were purchased from Biotage (Sweden). The accuracy of the $\delta^{13}C$ determination was checked using the following international reference materials: caffeine USGS61 obtained from the US Geology Survey (USGS, Virginia, USA) and caffeine IAEA-600 obtained from the International Atomic Energy Agency (IAEA, Austria).

2.2. **Selection of target compounds.** Targeted compounds included: amphetamine, morphine, codeine and heroin. Selection was based on the fact that data on amphetamines and opioid-based illicit drugs are collected within forensic studies to study their production and trafficking [11].

2.3. **Sample selection and preparation.** Three types of samples were prepared and analyzed: (i) street drug samples: two amphetamine samples (A1 and A2), two samples of opium resin (OR1 and OR2), and one heroin sample provided by the National Laboratory of Health, Environment and Food; (ii) authentic analytical standards of amphetamine, morphine, and codeine, and (iii) raw wastewater collected at the inlet of a municipal wastewater treatment plant (WWTP) serving 49,843 people, which had been previously analyzed for the presence of licit and illicit drug resideus[16].

2.3.1. Street drugs. Amphetamine samples (white powder) and heroin (brown powder) were dissolved in methanol, and opium (resin) in 60 % acetone in Milli-Q. Two hundred microliters of the prepared solutions were dried (40 °C, N_2), derivatized (section 2.3.4.) and analyzed using GC-MS/MS and GC-C-IRMS.

2.3.2. Laboratory analytical standards. An aliquot (50 µL) of each analytical standard (1 mg/mL) was dried and derivatized (section 2.3.4.) for GC-C-IRMS analysis. For GC-MS/MS analysis, the initial standard concentration was adjusted to 0.01 ng/mL with methanol.

2.3.3. Raw wastewater. After filtration (glass microfiber filters: GF/D and GF/C), the wastewater (51.5 L) was divided into approximately 1.5 L aliquots. Each aliquot was loaded onto a pre-conditioned (50 mL of methanol and 50 mL of Milli-Q water) AttractSPE™ HLB Disks. Each disk was washed with 50 mL of 5 % methanol in Milli-Q water and dried under vacuum. The morphine was then eluted with 50 mL of methanol. The eluate was acidified (pH 4) with HCOOH and purified by passing the eluant through a pre-conditioned (4 mL of methanol followed by 4 mL of 1 % HCOOH in methanol) Strata C18-E cartridge. Finally, the cartridge was washed with 4 mL of 1 % HCOOH in methanol. All extracts

were combined, dried (N_2, 40 °C) to a constant mass (final extract: 1.09 g) and stored at -20 °C.

Purification of the final extract was performed using flash chromatography (Biotage® Select). The extract was dissolved in 1 mL of Milli-Q and introduced into a Sfär C18 Duo flash chromatography column using a Sfär C18 samplet. Flash chromatography was achieved using Milli-Q water (A) and methanol (B) at a flow rate of 25 mL/min. The gradient elution was as follows: 5 % B hold for 0.5 column volumes (CV), increase to 100 % B in 8 CV and hold the condition for 1.2 CV. Fractions (14 mL) were collected throughout the chromatographic run (9.7 CV; **Figure S1**). To identify the morphine fractions, 10 µL of each fraction was dried (40 °C, N_2), derivatized (section 2.3.4.), filter-centrifuged (14,000 RCF, 3 min; Domel, Centric CF 48, Slovenia) using 0.2 µm modified nylon centrifugal filters and analyzed by GC-MS/MS. The fractions containing morphine were then combined, derivatized (section 2.3.4), centrifuged (3,000 RPM, 10 min) and analyzed using GC-C-IRMS.

2.3.4. *Derivatization.* Dried samples were reconstituted in 50 µL of ethyl acetate and derivatized at 70°C for 1h, using 50 µL BSTFA. Only in case GC-C-IRMS analysis of raw wastewater, final extract was reconstituted in 100 µL of ethyl acetate and derivatized (70°C, 1h) using 100 µL BSTFA.

2.3.5. GC-MS/MS analysis. Derivatized samples were analyzed using an Agilent Technologies (USA) gas chromatography system (AT 7890B) coupled with a triple quadrupole mass spectrometry detector (AT 7000 GC/MS). Samples (1 µL) were injected in spitless mode with an inlet temperature of 260 °C. Separation was achieved on an Agilent Technologies (Waltbronn, Germany) DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm) using

helium as the carrier gas (1.2 mL/min). The temperature program was as follows: 70 °C to 120 °C at 5 °C/min, 210 °C to 240 °C at 10 °C/min, 240 °C to 270 °C at 5 °C/min and hold 270 °C for 3 min, 270 °C to 300 °C at 3 °C/min with a hold time of 3 min. Ionization was achieved using electron impact (EI) at 70 eV, and the MS was operated in Scan and multiple reaction monitoring (MRM) mode (**Table 1**). Data analysis was performed using MassHunter software (Agilent Technologies, US).

Table 1. Selected transitions and optimized GC-MS/MS parameters.

Analyte	Retention time [min]	Precursor ion	Product ion	CE 1 [eV]	Dwell time [ms]
Amphetamine	14.3	116	73	20	26.7
		117	74	25	26.7
Morphine	29.2	236	146	20	8.9
		236	220	20	8.9

2.3.6. Stable carbon isotope ratio measurements. The $^{13}C/^{12}C$ ratios are reported in δ – notation in per mil (‰) and reported relative to the Vienna-Pee Dee Belemnite (V-PDB) standard following **Eq. 1**:

$$\delta\left({}^{i/j}E\right) = \delta^{i/j}E = \frac{{}^{i/j}R_P - {}^{i/j}R_{Ref}}{{}^{i/j}R_{Ref}} \quad (1)$$

Where superscripts i and j denote the highest and the lowest atomic mass number of elements E (which is C) and R_P and R_{Ref} indicate the ratio between the heavier and the lighter isotopes ($^{13}C/^{12}C$) in the sample (P) and reference material[17].

The isotopic composition was determined using an Agilent 6890N GC-C system coupled to an IsoPrime GV IRMS (GV Instruments, Manchester, UK). Separation was

achieved using a DB-5MS (30 m × 0.25 mm i.d., 0.25 µm) capillary column (Agilent Technologies) following the same temperature program reported previously (section 2.3.5). The carrier gas was helium in constant flow mode (1.2 mL/min). The injector temperature was 260 °C, and the oxidation reactor (Cu/O) in the 6890N GC/C system was 900 °C. Peak identification was made by comparing retention times of the unknown with an authentic morphine standard, which was analyzed at each measurement sequence. Stability was verified before each measurement sequence, ensuring it met the accepted criterion of ≤0.08‰. For data normalization, a two-point normalization method was performed using the international reference materials USGS61 ($\delta^{13}C$ = -35.05 ± 0.04 ‰) and IAEA-600 ($\delta^{13}C$ = -27.73 ± 0.04 ‰). The reproducibility was 0.5 to 0.8 ‰ (n=3), while the expanded uncertainty (U, k = 2) was ±1 ‰.

The isotopic composition of BSTFA was determined by elemental analysis IRMS (EA-IRMS) according to a standard procedure described in SDN-O2-ORG(01) using an IsoPrime 100 – Vario PYRO Cube combined with a Vario LS sampler (Liquid sampler for "cube" analyzer line; OH/CNS Pyrolyzer/Elemental Analyzer; IsoPrime, Cheadle, Hulme, UK) according to a standard procedure described in SDN-O2-ORG(01), 4th edition. Briefly, 1 µL of the sample was automatically injected into the elemental analyzer. To assure accuracy internal laboratory materials: absolute ethanol (LRM-2, $\delta^{13}C$ = -27.37 ± 0.11 ‰), a rum distillate (LRM-RUM-2, $\delta^{13}C$ = -13.45 ± 0.07 ‰) and control material distillate of wine (KM-1, $\delta^{13}C$ = -27.77 ± 0.08 ‰) were used. All laboratory reference materials were calibrated against international reference material BCR-656 ($\delta^{13}C$ = -26.91 ± 0.07 ‰). The expanded uncertainty (U, k = 2) was ± 0.5 ‰.

The $\delta^{13}C$ values of BSTFA based on the two lot numbers used were -30.12 ± 0.10 ‰ and -40.37 ± 0.40 ‰.

2.3.7. *Determination of $\delta^{13}C$ value of targeted compounds:* The isotopic shift due to the exogenous carbon introduced in the individual compound though derivatization was corrected using **Eq. 2**:

$$\delta^{13}C_{compound} = \frac{(N_{BSTFA(compound)}\delta^{13}C_{BSTFA(compound)} - (N_{BSTFA}\,\delta^{13}C_{BSTFA})}{N_{compound}} \quad (2)$$

where $\delta^{13}C_{compound}$ is the corrected $\delta^{13}C$ value of the individual compound, $\delta^{13}C_{BSTFA(compound)}$ is the measured value of the individual derivatized compound, $\delta^{13}C_{BSTFA}$ is the isotopic composition of BSTFA determined using the EA-IRMS, N_{BSTFA} is the added carbon number of BSTFA and $N_{BSTFA(compound)}$ is the carbon number of the derivatized compound.

2.3.8. *Method validation.* Extraction recoveries (HLB disks and Strata C18-E cartridges) were evaluated using morphine-spiked wastewater (3 µg/mL, in duplicate) before (sample spike) and after the extraction (eluate spike). The extraction recoveries (in %) were then calculated based on peak area (A) as follows:

$$ER\ (\%) = \frac{A_{sample\ spike}}{A_{eluate\ spike}} \times 100 \quad (3)$$

Three experiments were conducted to investigate possible isotopic fractionation during sample preparation: two with tap water and one with wastewater. Briefly, 1.5 L of tap and wastewater were spiked with morphine (tap water: 0.05 mg/mL and 0.5 mg/mL; wastewater: 0.5 mg/mL), filtered (GF/D and GF/C) and loaded onto a pre-conditioned AttractSPE™ Disk HLB. After loading, disks were washed (50 mL of 5 % methanol in Milli-Q) and dried (vacuum). Morphine was then eluted (50 mL of methanol), acidified

(pH 4 – HCOOH) and purified using a Strata C18-E cartridge. The purified eluate was dried (40 °C, N_2) and stored at -20 °C for further processing by flash chromatography. The analyses were performed and analyzed similarly to the wastewater samples (sections 2.3.3.–2.3.6.).

3. Results and discussion

3.1. **Method performance.** From previous analysis[16] it was determined that obtaining a sufficient amount of morphine for GC-C-IRMS analysis would require extracting approximately 50 L of raw wastewater, whereas, for other compounds, it would require >500 L. Therefore, for this proof of concept, we only extracted morphine. Extraction efficiency was within SANTE/11312/2021 guidelines (HLB disks: EF = 80 %; Strata C18-E: EF = 85 %)[18]. However, a further clean-up was necessary to remove additional interfering compounds (**Figure S2**), performed using flash chromatography.

Four fractions with resolvable amounts of morphine were obtained for stable isotope analysis. Though flash chromatography could potentially lead to isotopic fractionation [19], the $\delta^{13}C$ values obtained for each fraction (average ± standard deviation: -33.7 ± 0.2 ‰) were within measurement uncertainty (1 ‰), indicating that no measurable fractionation had occurred. However, a slight difference (1.8 ‰) observed between $\delta^{13}C$ values of non-processed morphine standard (-36.7 ‰) and the same standard spiked in tap and wastewater (-34.9 ± 0.86 ‰), suggests that slight fractionation occurred during sample preparation, although the measurement error was in the same range (1 ‰).

3.2. **Street drug analysis.** For synthetic drugs, isotopic characterization can link batches with a common source of supply or production, as isotopic composition depends on precursor material used and changes due to kinetic isotopic effects that occur during synthesis[20–22]. In

this study, the $\delta^{13}C$ values of the two amphetamine samples, A1 and A2, were –40.0 ‰ and –42.6 ‰, respectively (**Table 2**). Both values are considerably lower than the amphetamine standard (-30.2 ‰) and $\delta^{13}C$ values reported in the literature (-28.5 to -12.0 ‰; **Table S1**). Considering numerous possible precursors and synthetic routes, e.g., "Leuckart synthesis, the reductive amination of phenyl-2-propanone and nitrostyrene route"[20], as well as synthesis/extraction routes for precursor production, such difference in $\delta^{13}C$ values is expected. For example, Cormic *et al.*[23] reported highly variable $\delta^{13}C$ values for amphetamine precursors (synthetic and of natural origin) used as a nitrogen source ($\delta^{13}C$ ranged from -54.5 to -16.9 ‰). However, further speculation on the observed differences in $\delta^{13}C$ values requires additional study, *i.e.,* data on isotopic nitrogen composition[24].

The $\delta^{13}C$ values for morphine in opium samples and heroin were between -35.8 and -29.7 ‰ (**Table 2**) and agree with the literature data (**Table S2**). However, the values are lower compared to $\delta^{13}C$ values obtained for the poppy itself ($\delta^{13}C$ =-29.4 to -25.7 ‰)[25]. Regarding the origin of poppy, which is used for illicit drug production, the primary producing locations are Southwest Asia, Southeast Asia, Mexico and South America[26]. Thompson *et al.*[11] observed that opium/heroin from Southwest Asia can be distinguished from the other three regions based on the higher morphine $\delta^{13}C$ values (**Table S2**). A similar observation was also made by Ehleringer *et al.*[11]. Based on these findings, the raw material used for the heroin ($\delta^{13}C$ value of morphine = -29.7 ‰) and the opium resin (OR1) with a $\delta^{13}C$ value of morphine = -30.9 ‰ analyzed in this study (**Table 2**), suggest an origin from Southwestern Asia. Nevertheless, additional data, including $\delta^{15}N$ values and an extensive database of authentic samples, are necessary to validate such a hypothesis [11,14,27,28].

Interestingly, the morphine contained in the second analyzed opium resin (OR2) exhibited a $\delta^{13}C$ value of -35.8 ‰, which appears to be the most negative value reported. Low $\delta^{13}C$ values are commonly connected with compounds of synthetic origin. For example, depletion of ^{13}C was found in synthetic aroma compounds, *e.g.,* vanillin and truffle flavorings[29,30]. Similarly, in this study, the morphine standard, confirmed to be of synthetic origin by the supplier (Sigma-Aldrich), had a $\delta^{13}C$ value of -36.7 ‰. It can also be speculated that the opium resin (OR2) with a morphine $\delta^{13}C$ value of -35.8 ‰ was prepared from synthetic morphine and resin, likely intentionally to mimic natural opium. This finding is further supported by the fact that codeine, another known opium alkaloid, was not detected in this particular resin, although it was present in the other analyzed opium street sample. The $\delta^{13}C$ value of codeine (-29.7 ‰) in OR1 was higher in comparison with the $\delta^{13}C$ value of the known synthetic codeine standard (-38.4 ‰). This finding is also proof that synthetic opioids are depleted in ^{13}C.

The $\delta^{13}C$ value of morphine in heroin also offers an opportunity to trace the origin of the reagents (*e.g.,* acetic anhydride or glacial acetic acid) used to convert morphine into heroin through acetylation[31]. In heroin, 17/21 carbon atoms are derived from the plant product (morphine), while the remaining four are derived from the acetalization reagent. Accordingly, mass balance considerations allow reconstruction of the $^{13}C/^{12}C$ composition of the reagent used to produce the heroin[23,24,31]. In this study, its $\delta^{13}C$ value was -34.4 ‰ and could prove valuable in subsequent studies aimed at distinguishing heroin samples based on the geographic origin of the morphine and that of the clandestine production site [31].

3.3. Wastewater analysis. An average $\delta^{13}C$ value of -33.7 ‰ for morphine in raw wastewater falls within the interval of the $\delta^{13}C$ values obtained for morphine in street drugs (-35.8 to -29.7 ‰). Given that Yen *et al.*[32] showed that the isotopic composition of carbon and nitrogen in morphine is maintained during drug metabolism, we speculate that isotopic analysis of drug residues in wastewater may be linked with the origin of illicit drugs. The $\delta^{13}C$ value of morphine in wastewater represents the lower limit of $\delta^{13}C$ typically obtained for morphine in opium, morphine and heroin samples (**Table 2**). Therefore, it could be speculated that morphine-based drugs originate in the Slovenian drug market from locations where more negative $\delta^{13}C$ values are observed, *i.e.,* South America, Southeast Asia and Mexico. However, in the case of wastewater, medical morphine (not analyzed) should be considered since it was the second most commonly prescribed opiate in Slovenia in 2022[33]. To further evaluate its contribution, an isotopic analysis of medical morphine is needed.

Given that wastewater analysis only provides the average $\delta^{13}C$ values of drugs used in the study area as no data on individuals can be obtained[34], it has the potential to detect changes in drug supply. It also could serve as an early warning system, complementing drug profiling data. Such tracking changes will be significant as the drug supply changes. For instance, Afghanistan, formerly a major heroin producer, recently declared a ban on poppy production. This ban will likely have repercussions on the production and distribution of morphine-based drugs[35]. By regularly employing isotopic composition analysis of drug residues in wastewater, particularly at the international level, similar to SCORE, such temporal and spatial changes can be detected and used for forensic intelligence.

Aside from morphine-based drugs, data on the geographic origin of raw material used to produce other semi-synthetic and natural drugs can be obtained, among which cocaine and cannabis (tetrahydrocannabinol, THC) would be of particular interest considering their widespread and growing use[35]. It may also be used to identify changes in the type of synthetic drugs available. As Carter *et al.* [36] showed, the synthetic origin of amphetamines (street drug samples) could be identified based on $\delta^{13}C$ and $\delta^{15}N$ values and drug batches linked to common sources.

In summary, despite providing limited data on drug residues (morphine) in wastewater, this study indicates that isotopic composition analysis of drug residues in wastewater may be used as a complementary source of forensic intelligence. However, such applicability still requires further research, and as already mentioned, aside from determining the isotopic composition of carbon, other light elements, such as nitrogen, oxygen and hydrogen, should be considered. More importantly, alternative extraction and pre-concentration methods to tedious solid-phase extraction of high volumes of wastewater must be explored, *e.g.,* passive or active-passive sampling. Finally, research studies that systematically measure changes in the isotopic composition of illicit drug residues over time and space would be valuable in supporting law enforcement efforts. This study that combines the stable isotope approach, a well-established method, with WBE represents an innovative application with the potential to detect shifts in the trafficking and supply of illicit drugs. As the landscape of drug-related issues evolves, such an approach could be crucial in advancing forensic intelligence related to illicit drugs.

Table 2. $\delta^{13}C$ values obtained in street drugs, laboratory analytical standards and raw wastewater.

Sample	Analyte	Determined $\delta^{13}C$ value (‰)
Amphetamine 1 (A1) – white powder (street drug)	Amphetamine	-39.99
Amphetamine 2 (A2) – white powder (street drug)	Amphetamine	-42.59
Opium 1 (OR 1) – resin (street drug)	Morphine	-30.91
	Codeine	-29.65
Opium 2 (OR 2) – resin (street drug)	Morphine	-35.83
Heroin – brown powder (street drug)	Morphine	-29.67
	Heroin	-30.62
Laboratory analytical standards	Amphetamine	-30.23
	Morphine	-36.67
	Codeine	-38.40
Raw wastewater	Morphine	-33.66

ASSOCIATED CONTENT

Supplementary material

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Notes

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Combining a Stable Isotope Analysis with a Wastewater-Based Epidemiological Approach to Complement Illicit Drug Profiling

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Supplementary information

1. MATERIALS AND METHODS

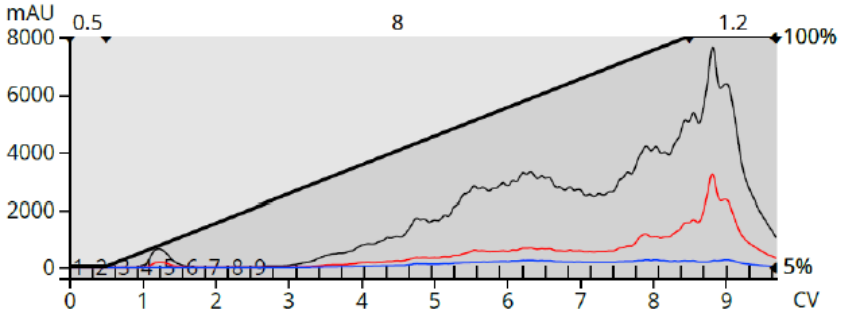

Figure S1. Chromatogram (absorbance in dependency of column volumes) of wastewater extract at λ= 198 – 400 nm (black), λ= 226 nm (red) and λ= 288 nm (blue). The bold line represents the applied elution gradient.

2. RESULTS AND DISCUSSION

a)

b)

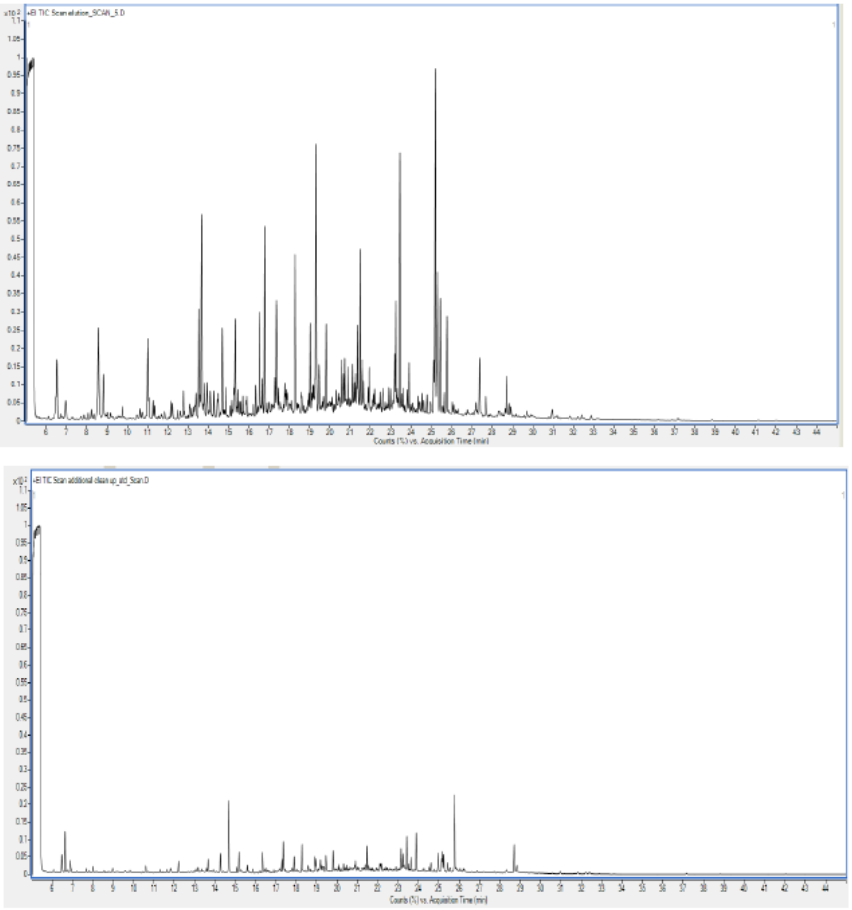

Figure S2. Chromatograms (GC-MS/MS: scan mode) of eluates, that were obtained after extraction of spiked wastewater (10 µg/mL) with HLB disks alone (a) and after additional extraction using Strata C18-E (b)

Table S1. Reported $\delta^{13}C$ values of amphetamine

Sample	$\delta^{13}C$ value of amphetamine (‰)	Synthesis route/precursors	Reference
Amphetamine – industrial source of precursors	−26.7 to −26.0	'nitrostyrene' route ($\delta^{13}C$ value of precursors: -26.5 to -24.5 ‰)	[1]
Amphetamine – natural source of precursors	-28.5 to -28.2	'nitrostyrene' route ($\delta^{13}C$ value of precursors: -28.9 to -24.5 ‰)	[1]
Amphetamine – clandestine production	-28.4	Non-benzaldehyde-derived source ($\delta^{13}C$ value of precursor phenyl-2-propanone: -27.9 ‰)	[1]
18 amphetamine samples	-26.0 to -12.0	Six known sources	[2]

Table S2. Reported $\delta^{13}C$ values of morphine extracted from illicit drugs

Sample	$\delta^{13}C$ value of morphine (‰)	Geographic origin and corresponding average $\delta^{13}C$ values (‰)	Reference
Opium			
Powder, gum, and latex	−33.5 to −29.1	Mexico: −32.1 ‰ South America: −32.5 ‰ Southeast Asia: −32.1 ‰ Southwest Asia: −30.5 ‰	[3]
Morphine			
Not specified	−33.4	Colombia	[4]
Not specified	−32.4 to −31.9	Colombia	[5]
Heroin			
Powder	−32.3 to −30.9	Mexico: −32.0 ‰ South America: −32.2 ‰ Southeast Asia: −32.3 ‰ Southwest Asia (−30.9)	[3]
Powder	−33.9 to −29.9	Mexico (−32.5) South America (−33.3) Southeast Asia (−32.5) Southwest Asia (−30.0)	[6]
Powder	−31.7 to −29.3	Unknown source (−31.5)	[7]

		Mexico (−30.6) South America (−31.0) Southeast Asia (−30.4) Southwest Asia (−29.8)	
Powder	−32.6	Not specified	[8]

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3.4 Drug Residues in the Aqueous Environment: Occurrence and Ecotoxicity

3.4.1 Occurrence, fate and determination of tobacco (nicotine) and alcohol (ethanol) residues in waste- and environmental waters

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Due to increasing production and consumption, partial removal during wastewater treatment, and potential ecotoxicity, residues of licit and illicit drugs are classified as contaminants of emerging concern (Chapter 1.4 Environmental Perspective). Accordingly, many reviews have already been published summarizing the methods used to determine drug residues in aquatic matrices, the occurrence and fate of drug residues in waste and environmental waters, and their adverse effect on various (aquatic) organisms [78], [130], [132], [141]–[144], [157], [168]. However, these reviews focus mainly on residues of illicit drugs and pharmaceuticals and do not summarize data for the otherwise most used licit drugs worldwide, *i.e.,* tobacco (nicotine) and alcohol (ethanol) (Chapter 1.4.1 Analytical methods used to determine drug residues in the aqueous environment), which residues (in case of nicotine) have already been proven ecotoxic (Chapter 1.4.4 Effects on aquatic organisms).

This comprehensive review summarizes 102 papers published in the past two decades investigating the occurrence and fate of most commonly determined nicotine (nicotine, cotinine, HCOT) and alcohol (ethyl sulfate and ethyl glucuronide) residues in waste- and environmental waters. Additionally, methods used for their determination are presented with challenges and future recommendations.

For nicotine and alcohol residue analysis, wastewater was usually sampled as a composite sample, while grab samples are commonly used for environmental waters. Since grab samples cannot provide data on time-dependent fluctuations of concentrations, the investigation of other sampling techniques is encouraged, *e.g.,* passive sampling. SPE followed by RP-LC-MS/MS was typically used to determine nicotine residues, while alcohol residues were determined using an ion-pair reagent and direct injection onto the RP-LC-MS/MS. In order to reduce the amount of matrix entering the MS and lower LOD/LOQ in the case of alcohol residue determination, analytical approaches need to be improved, *e.g.,* by including an extraction step and avoiding using an ion-pair reagent.

Targeted drug residues were determined in aquatic matrices in ng/L to µg/L range. As expected, the highest concentrations were determined in wastewater (raw>treated>reclaimed), followed by surface waters, groundwater and drinking water. Compared to nicotine residues (85 % of revised studies), much less data on the occurrence of alcohol residues is available. Moreover, studies mainly focus on waste- and river waters, indicating more data on environmental waters are still needed.

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Occurrence, fate and determination of tobacco (nicotine) and alcohol (ethanol) residues in waste- and environmental waters

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ABSTRACT

This review includes one hundred and two peer reviewed papers that focus on metabolic residues of the two most used licit drugs globally, nicotine (nicotine, cotinine, trans-3'-hydroxycotinine – HCOT) and alcohol (ethyl sulphate and ethyl glucuronide), in waste- and environmental waters. Sampling strategies and analytical methods are also summarised and discussed. Although grab sampling is the most widely applied method for collecting environmental samples (74% cases), wastewater samples are typically composite samples collected automatically at the wastewater treatment plants (66% cases). Sample preparation and analysis usually include solid-phase extraction (SPE) followed by reverse-phased liquid chromatography with tandem mass spectrometry detection (RP-LC-MS/MS) for nicotine residues. In contrast, alcohol residues are commonly determined via direct injection onto the LC-MS/MS using an ion-pair reagent to improve retention, leaving room for method improvement, e.g., introducing a suitable extraction procedure to achieve lower detection limits and quantification. In comparison to alcohol residues, more studies look into nicotine residues (85% of the studies). Concentration ranges for nicotine, cotinine, HCOT and ethyl sulphate were < 424,000, < 42,300, 50–52,000 and 500–33,000 ng/L in wastewater influents and 15–32,000, < 13,000, 15–1,552 and < 500 ng/L in effluents, while nicotine (12.6–947 ng/L) and cotinine (17–62 ng/L) were detected in reclaimed waters. Among environmental waters, the highest concentrations of nicotine residues were measured in surface waters (nicotine: < 9,340 ng/L, cotinine: < 6,582 ng/L and HCOT: 14–777 ng/L), while their concentrations in groundwater and drinking water were generally in the low ng/L range. This review also reveals the discrepancy between the number of studies in developed countries (90%) compared to developing countries and the need for more studies in the former, where most wastewater flows untreated into the environment.

1. Introduction

Increasing production and consumption, insufficient elimination during wastewater treatment, and potential toxicity at environmental levels classify drugs of abuse and their metabolites among emerging pollutants [1]. Nicotine is the principal tobacco alkaloid (accounting for 98% of total alkaloids in tobacco), commonly introduced into the human body via tobacco smoking [2,3]. Its popularity, addictiveness and non-pharmacological effects of tobacco smoking (e.g., social factors and depression) make it one of the most used licit drugs, with 1.3 billion users worldwide [3,4]. Nicotine can also enter the body through nicotine replacement therapies (patches, gums), e-cigarettes and other nicotine-containing plants (members of the *Solanaceae* family, such as potatoes, tomatoes, eggplants, chilli peppers and members of the *Camellia sinensis* family, such as black and green teas) [2,5,6].

Once in the body, nicotine is metabolised mainly to primary metabolites nicotine N'-oxide (4–7%) and cotinine (70–80%), which are then further metabolised, leaving only 10–15% of cotinine excreted unchanged. The main cotinine metabolite is *trans*-3'-hydroxycotinine, HCOT (33–40% of cotinine) [7]. Once in the environment through excretion after consumption, wash-out from cigarette butts/ashes and tobacco plantations, nicotine and its metabolites undergo several processes, such as transformation (e.g. biological, chemical, photodegradation), leaching, transportation and interaction with soils and sediments (Fig. 1). At the same time, their presence can negatively affect non-targeted organisms [8,9]. Plant-derived alkaloids, such as nicotine, are also known to have a large number of effects on bacterial and fungal pathogens, suggesting that their presence in the environment poses a

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threat to the base of the aquatic food web [10]. As a known insecticide, nicotine can also adversely affect aquatic invertebrates [10,11]. For example, Lilius et al. [12] reported intoxication and immobilisation of *Daphnia magna* (EC_{50} =0.035 mmol/L) and *Daphnia pulex* (EC_{50} =0.00857 mmol/L mmol/L).

Alcohol (ethanol) is also an addictive psychoactive substance used by over 2.3 billion people worldwide [3]. It is usually consumed as an alcoholic beverage and is an integral part of the social life of many cultures [3]. Once ingested, alcohol is rapidly metabolised in a two-stage oxidation process to acetaldehyde and further to acetic acid, with about 5% excreted as ethanol [13,14], while < 1% undergoes Phase-II metabolism (conjugation reactions) to form ethyl glucuronide and ethyl sulphate [13,15]. Despite their low excretion profiles, ethyl glucuronide (0.032%) [16] and ethyl sulphate (0.012–0.022%) are clinically and forensically used as markers of recent alcohol intake [17]. However, both compounds can be present in urine due to consuming non-alcoholic beverages (e.g. non-alcoholic wine, grape juice) or using alcohol-based mouthwash and hand sanitiser. At the same time, ethyl glucuronide is formed from ethanol in urine by *Escherichia coli* [18,19]. Despite the presence of alcohol residues in the environment, little data on their ecotoxicity exists (Fig. 1). Many researchers have addressed nicotine and, to a lesser extent, alcohol residues in the aquatic environment (Fig. 2), including their occurrence, fate and toxicity [1,10,20–25], but to date, no comprehensive review exists regarding their presence and determination in waste- and environmental waters. We believe such a review is warranted.

Reference studies were identified by searching freely available web search engine that provides access to various bibliographic databases of scientifc publications (e.g. Google Scholar) published over the past two decades (from 2002 onwards, when the first US nationwide study on the occurrence of broth range (95) of organic water contaminants was published by Kolpin et al. [24]). Keywords such as licit drug, tobacco, nicotine, alcohol, wastewater, surface water, river, lake, seawater, groundwater, drinking water and consumption were used in the search. The inclusion criteria for the articles were set as follows: aquatic matrices and reporting concentrations, while the exclusion criteria were: non-aquatic matrices, reporting only mass loads or focusing on ecotoxicity. There were no limits regarding the country of origin of the studies. However, only studies written in the English language were taken into account. From selected studies, study location (country), water matrix (e.g., wastewater – influent, effluent, reclaimed water, surface water – river/lake/seawater, groundwater, drinking water), concentration levels of nicotine and alcohol residues, removals during (waste)water treatment (if included) and analytical method used were extracted.

In total, this review summarises 102 papers published in peer reviewed journals investigating the occurrence, distribution and fate of nicotine and alcohol residues in wastewater influents and effluents, reclaimed water, surface waters, groundwater and drinking water, with the majority of the studies (90%) coming from developed rather than developing countries as defined by the United Nation classification [26]. It also reviews the methods used for their determination, current analytical challenges, and future recommendations.

2. Physico-chemical properties

The distribution and fate of nicotine and alcohol residues in the environment depend on many factors such as their concentration, pH, temperature, level of sunlight and physico-chemical properties (Table 1). Knowledge of their physico-chemical properties is important since they can predict their presence in various environmental compartments [27,28].

For example, nicotine and alcohol residues with organic carbon-water partition coefficients ($logK_{oc}$) of < 2.5 (Table 1) suggest their high mobility in soils; octanol-water partition coefficients ($logK_{ow}$) of < 4 indicate their hydrophilicity, and high water solubility (S_w >32,000 mg/L at 25 °C) all imply their presence in the aqueous phase rather than adsorbed to suspended matter or sediment [5,29–31]. However, the actual partition may differ from the predicted due to the influence of environmental conditions, e.g., temperature and pH and other physico-chemical properties such as the dissociation constant (pKa), interactions with other dissolved species and sorption processes [32–34]. For example, dissociation ultimately affects a compound's sorption [6] as well as its aqueous mobility [28]. At environmentally relevant pHs (pH 5–9), nicotine (pKa 4.5–8.8; Table 1) and its metabolites will be in a neutral form, but protonation is possible, while alcohol residues with lower pK_a (2.8–4.7) will be mostly negatively charged, and usually, ionised compounds do not adsorb due to their increased solubility [32]. Despite this, interactions between positively charged functional groups and negatively charged sediments or mineral surfaces may occur [10].

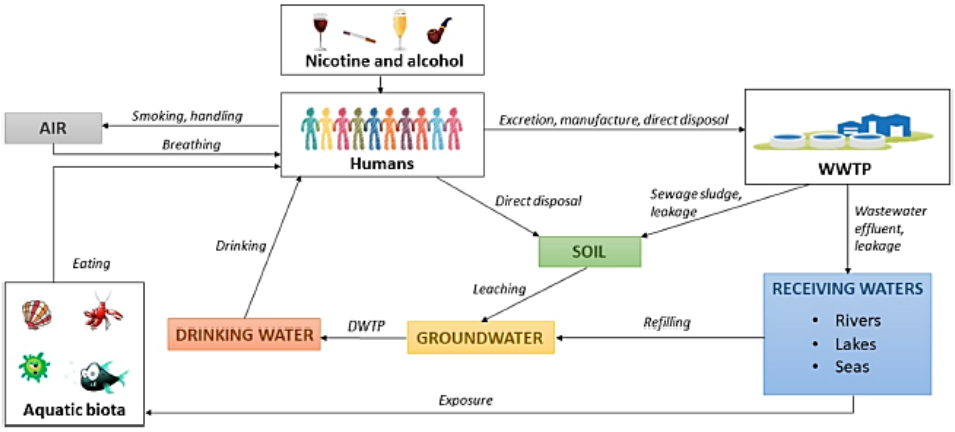


Fig. 1. Entrance and circulation of nicotine and alcohol residues in the environment (WWTP – wastewater treatment plant, DWTP – drinking water treatment plant) [1,40,90,111].

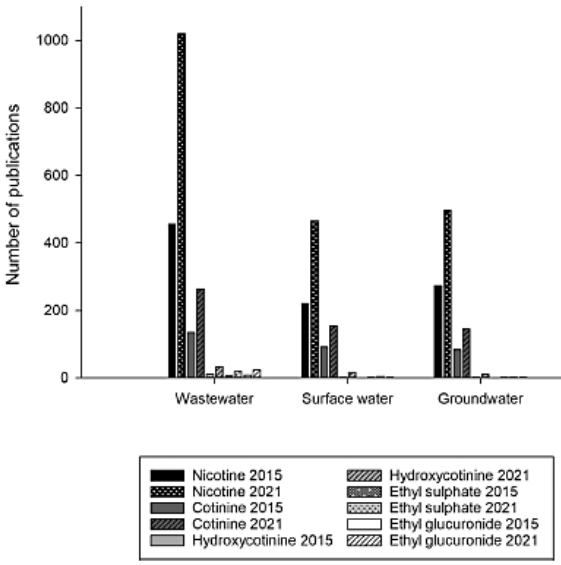


Fig. 2. The number of publications in 2015 and 2021.

Volatilisation from water surfaces is not expected to be a significant fate process based upon their estimated Henry's Law constant (K_H: 8.4×10^{-12}–1.5×10^{-8} atm × m3/mole, at 25 °C 1) [31]. Also, direct photochemical degradation of compounds in water bodies exposed to sunlight is unlikely since only absorbed light may lead to chemical changes [35]. As nicotine and alcohol residues do not absorb wavelengths above 290 nm [31] and light at wavelengths below 295 nm is not transmitted through the atmosphere (transmittance in the atmosphere decreases with decreasing wavelength in the UV–VIS spectra) [35], indirect photochemical reactions with reactive species, such as ·OH, $^1O_2$, H_2O_2, and e^-_{aq} (photodegradation products of humic substances and NO_3^-) most likely limits the persistence of the compounds [36]. Finally, with their apparent hydrophilicity and low bio-concentration factor (BCF: 1.6–5.2; Table 1), their potential to bioaccumulate is low, which is reflected in the literature [30,31,37,38].

In summary, from their physico-chemical properties, nicotine and alcohol residues are expected to be in the aqueous phase, making studies of their presence in water matrices important. Their removal through sorption, volatilisation and direct photolysis is unlikely. However, indirect photochemical reactions are expected, and although nicotine and alcohol residues do not bioaccumulate, their continued presence in the environmental waters is of concern due to possible adverse effects on non-target organisms [1].

3. Biodegradation

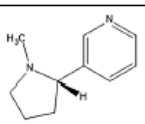
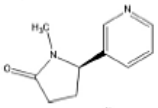
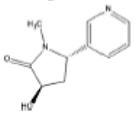
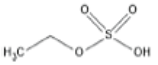
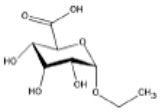
Biodegradation is the breakdown of compounds by microorganisms (enzymatic reactions) [39]. It is influenced by environmental conditions such as the presence of oxygen (anaerobic/aerobic conditions), temperature, pH (affecting the bioavailability of ionisable compounds) and chemical structure of the compounds (presence of functional groups and their number) [32,40]. In wastewaters, the biodegradability of nicotine and alcohol residues has been mainly studied in the laboratory using wastewater (incubation experiments) or bench-scale reactors, which simulate sewer conditions by enabling the formation of biofilms (Table S1). Studies show that suspended microbes degrade mainly ethyl glucuronide [41–43]. Banks et al. [43] also found that aerobic and anaerobic biofilms enhance the degradation of nicotine and alcohol residues, while anaerobic biofilms preferentially increase the degradation of HCOT and ethyl sulphate. It is also clear from the literature that a discrepancy exists between laboratory-scale studies and studies using pilot-scale sewer pipes (section or actual sewer pipes fed with real wastewater) or actual sewers. In contrast to laboratory-scale experiments, nicotine, cotinine, and HCOT are relatively stable in pilot-scale and actual sewers, where their formation was observed (exception: degradation of HCOT), possibly due to de-conjugation [39,44]. Buerge et al. [11] also found that nicotine and its metabolites are highly degraded (90–99%) in wastewater in the presence of activated sludge.

Surprisingly, only the biodegradation of nicotine and cotinine in estuarine and coastal seawater samples has been studied, in this case in an incubation experiment by Benotti and Brownawell [45]. The authors observed that nicotine (half-life: 0.68–9.7 days) is degraded more rapidly than cotinine (half-life: >40 days). Enhanced degradation of compounds under investigation was observed in samples collected in more eutrophic waters (coastal water samples), suggesting that bacterial abundance and species composition are important factors in the biodegradation of the compounds in seawater. The important role of stream sediment microbial communities in the biotransformation of nicotine and cotinine is also supported by Bradley et al. [46].

4. Sampling

Most reviewed studies use composite (66% of studies on wastewater) or grab (74% of studies on environmental waters) sampling (Table S2). The main problem of grab sampling is that it only reflects a "snapshot" of analyte concentrations and not time-dependent fluctuations [47–49]. Equally, average values obtained by composite sampling can miss specific events or spikes, such as changes in water flow (time- and volume-

Table 1
Chemical structures and physico-chemical properties of selected nicotine and alcohol residues.

Compound (CAS No.) Category IUPAC name Elemental formula	Chemical structure	Molecular mass (g/mol)	pKa	Log K_{ow}	S_w (mg/L, at 25 °C)	K_{oc} (L/kg)	K_H (atm × m^3/mole, at 25 °C)	BCF
Nicotine (54–11–5) *tobacco alkaloid* 3-[(2 S)− 1-methylpyrrolidin-2-yl]pyridine $C_{10}H_{14}N_2$		162	8.0–8.5 [31,116]	1.0–1.17[39, 117]	1×10^6 (miscible) [39]	91– 100 [31,118]	3.0×10^{-9} [31]	3.27–3.45 [118]
Cotinine (486–56–6) *nicotine metabolite* (5 S)− 1-methyl-5-pyridin-3-ylpyrrolidin-2-one $C_{10}H_{12}N_2O$		176	4.7 – 8.8 [31,50]	0.07– 0.34[31]	48,910– 1×10^6 (miscible) [31,39]	79.6–130 [31,118]	3.3×10^{-12}[31]	2.05–2.47 [118]
Trans-3′-hydroxycotinine (34834–67–8) *nicotine metabolite* (3 R,5 S)− 3-hydroxy-1-methyl-5-pyridin-3-ylpyrrolidin-2-one $C_{10}H_{12}N_2O_2$		192	4.5	Between − 1.45 and − 1.2[31, 39]	859,700[39]	70.5 [118]	8.35×10^{-12}[31]	1.60–2.02 [118]
Ethyl sulphate (540–82–9) *ethanol metabolite* Ethyl hydrogen sulphate $C_2H_6O_4S$		126	− 2.1[38]	Between − 2.49 and − 0.11[38, 39]	31,500 – 1×10^6 [31,39]	2.38[118]	1.45×10^{-8}[118]	5.18[118]
Ethyl glucuronide (17685–04–0) *ethanol metabolite* (2 S,3 S,4 S,5 R,6 R)− 6-ethoxy-3,4,5-trihydroxyoxane-2-carboxylic acid $C_8H_{14}O_7$		222	2.84–3.45 [31]	Between − 2 and − 1.6 (predicted value) [31]	281,000 (predicted value)[31]	39.4[118]	2.38×10^{-10}[31]	5.18[118]

pK_a – acid-dissociation constant, $logK_{ow}$ – logarithm of octanol-water partition coefficient, S_w – water solubility, K_{oc} – organic carbon-water partition coefficient, BCF – bio-concentration factor, KH – Henry's coefficient

proportional sampling), chemical inputs and the influence of precipitation [47], while sampling position (depth, location) [45] and analyte stability [49] should also be considered. As an alternative, passive sampling, for example, a polar organic chemical integrative sampler (POCIS), has been used to determine alcohol and nicotine residues in WWTP effluent, river water, lake water and seawater [47–49]. However, accurately determining the compound uptake rate, which is affected by various environmental conditions, like temperature and water matrix, is a limitation of the method [47]. Besides POCIS, other passive samplers, including diffusive gradients in thin films (DGT) technology, have been developed but have yet to be applied to monitor nicotine and alcohol residues in different waters.

5. Analysis of water matrices

Most analytical methods for determining nicotine residues in water are multi-residue methods (Table S2). Compared to single-group analysis, more information is gained in one run, which reduces the time and cost of the analysis [50]. However, an extensive range of LOD/LOQ is typical for such methods, as they include many analytes with different physico-chemical properties [51]. In contrast, ethyl sulphate is usually analysed separately or with ethyl glucuronide (Table S2).

5.1. Sample preparation

Solid-phase extraction (SPE) using manly Oasis HLB cartridges is a commonly utilised sample preparation technique to determine nicotine residues in aqueous matrices (Table S2). It provides cleaner extracts of the samples and enables the analytes to be concentrated, which is essential when analytes are in low concentrations, e.g., in surface water, groundwater, and finished drinking water [52]. Other than SPE, continuous liquid-liquid extraction (CLLE) has been used for surface water and groundwater matrices to a lesser extent [24,53–55]. However, obtaining acceptable recoveries can be challenging, especially in multi-residue methods [56,57].

As an alternative, direct injection liquid chromatography was used to analyse wastewater (influent, effluent) and even surface water samples [2,41,52,58–63]. Compared to SPE, direct injection reduces sample preparation time, increases reproducibility, minimises sample contamination, and reduces the number and volume of solvents needed [52,64], resulting in lower sensitivity [2]. In a similar approach and to eliminate the need for SPE, Chiaia et al. [64] explored large-volume injection, LVI (injection volume: 1,800 µL) in wastewater influent. Although the authors observed comparable performance to SPE, the method requires a special injector upgrade kit and is prone to matrix effects, which may explain why it is not widely applied [64]. In a different approach, the ELISA (enzyme-linked immunosorbent assay) was used by Nicolardi et al. [65] to determine nicotine residues in wastewater influent and surface waters. ELISA is based on antigen-antibody reaction using enzyme-linked conjugate and enzyme substrate and is commonly used to identify and quantify compounds in biological samples [66]. However, its application to waters has some limitations regarding the cross-reactivity of structurally related compounds. For example, overestimating cotinine levels due to HCOT cross-reaction was observed by Nicolardi et al. [65].

For alcohol residues, only direct injection of the samples has been reported (Table S2). However, other sample preparation techniques, e.g., supported liquid extraction and liquid-liquid extraction into acetonitrile, are being explored [67].

5.2. Separation techniques

Due to the hydrophilic nature of nicotine and alcohol residues (Table 1), liquid chromatography (LC) is the separation method of choice (Table S2). For nicotine residues, reversed-phase (RP) columns are commonly used, especially since these columns retain a broader range of analytes in terms of polarity (suitable for multi-residue methods) [68]. Hydrophilic interaction chromatography (HILIC), used for polar compounds with low logK_{ow}, was explored and successfully applied for cotinine [69]. The mobile phase used to elute nicotine residues in RP chromatography consists of a binary water and organic solvent (methanol, acetonitrile) system. The addition of either formic acid (0.015–0.5%), acetic acid (0.1–0.5%), ammonium formate (up to 10 mM), ammonium acetate (1–20 mM) or ammonium fluoride (2 mM) is also reported (Table S2) to improve sensitivity and peak shape [70]. Gas chromatography (GC) has also been used to determine nicotine and cotinine [24,53–56,71–73] using (5%-phenyl)-methylpolysiloxane stationary phase columns, although Crouse et al. [56] are the only ones to apply derivatisation (methylation) with trimethylsilyldiazomethane (TMSD) in order to determine cotinine.

Unlike nicotine residues, alcohol residues are poorly retained on conventional RP (C_8 and C_{18}) [74,75] and HILIC [75] columns. Their interaction with hydrophobic column sorbent can be improved by adding an ion-pair reagent (ion-pair chromatography), which adsorbs onto the surface of the stationary phase and interacts electrostatically (positive charge) with alcohol residues (anionic nature) [17,42,75]. The majority of methods applied ion-pair chromatography (Table S2) using one of three different ion-pair reagents: dihexylammonium acetate (DHAA), dibutylammonium acetate (DBAA), and tetrabutylammonium bromide (TBA), either to the mobile phase [42,58,59,74,76,77] or to the sample [17,75]. A comprehensive study on ion-pair reagents (type, amount and addition to mobile phase or sample) was performed by Andres-Costa et al. [17], who observed that the best peak shape and the highest sensitivity were achieved with TBA (0.5 M). However, adding ion-pair reagents to the mobile phase is not recommended when using MS since higher amounts of the non-volatile ion-pair reagent can build up on the ion source and suppress the signal [17,75]. Several studies overcame the need for an ion-pair reagent by using a column with a trifunctional C_{18} alkyl stationary phase with a ligand density that promotes the retention of polar compounds [15,78]. Similar to nicotine, the mobile phase used to elute alcohol residues consist of a binary mixture of water and organic solvent (methanol, acetonitrile), to which formic acid (0.1%) or acetic acid (0.1%) is added to improve peak shapes [17].

5.3. Detection

Nicotine and alcohol residues are typically detected using a mass spectrometer (MS) when coupled to LC [24,53–55,70,73,79–84] or GC [24,53–56,71–73] and UV–VIS in the case of ELISA [65]. In the case of MS, due to its high selectivity and sensitivity [62], a tandem mass spectrometry (MS/MS) is utilised, incorporating a triple quadrupole (QqQ) or a hybrid triple quadrupole/linear ion trap (QTRAP) mass analyser operating in multiple reaction monitoring (MRM) mode (Table S2). High sensitivity enables direct injection analysis and even diluted samples to decrease matrix effects [52,62]. There are limitations related to MRM. For example, the number of common transitions for co-eluting isobaric compounds increases with the number of analytes. There can also be significant differences in intensity between transitions, with only one transition being visible [52,85], which is problematic since three confirmation points, i.e., two transitions as well as retention time, are required for identification by LC-MS/MS according to Commission Decision 2002/657/EC [86]. Structural information can be obtained using information-dependant acquisition (IDA), making identification more reliable [52,87].

High-resolution mass spectrometry (HRMS), such as Time-of-flight (TOF) [62,75,85,87–91] and Orbitrap [68,92,93], have been used to analyse different water matrices (WW influent, effluent, reclaimed, surface water, groundwater and drinking water). In all cases, sufficient sensitivity is obtained (nicotine residues – low ng/L, ethyl sulphate – 200 ng/L). Also, full scan acquisition allows the detection of other, non-targeted compounds such as transformation products when screening based on accurate masses [89].

5.4. Performance of analytical methods

Generally, LOD and LOQ are in the low ng/L–low µg/L range for nicotine and alcohol residues (Table S2). However, caution is needed since different studies use different methods, i.e., the blank, the calibration curve, a standard solution, and a spiked sample, to determine the LOD. Quantification can be problematic due to matrix effects (signal suppression/enhancement by co-eluting compounds) when analysing environmental waters and particularly when analysing wastewater. Electrospray ionisation (ESI), which is a commonly used interface for MS/MS (Table S2), is especially susceptible to this effect [52]. The use of isotopically-labelled internal standards (IS) can be used for compensation for this effect [52] and, indeed, is the most common way of determining nicotine and alcohol residues in waste- and environmental waters (Table S2). Nevertheless, it is clear from the review that not all the published studies included isotopically labelled internal standards for each compound. For example, Bartelt-Hunt et al. [47] quantified cotinine using $^{13}C_3$-caffeine as an IS. Matrix-matched calibration can compensate for matrix effects [2,62,85,87,93–96], but in this case, it cannot fully account for the matrix effects, as the matrix varies even in similar matrices, e.g., wastewaters composition can differ daily [96]. For this reason, Metcalfe et al. [96] and Wu et al. [50] used standard addition. Montesdeoca-Esponda et al. [97] used external calibration to determine nicotine in groundwater, which is not recommended as the matrix effect is not taken into account.

6. Occurrence in waste- and environmental waters

Nicotine and alcohol residues, because of their constant release, physico-chemical properties (high water solubility, low hydrophobicity) and partial elimination during wastewater treatment, are found in ng/L to µg/L in waste- and environmental waters (Table S3). In comparison to alcohol residues, more studies focus on nicotine and its metabolites (85% of reviewed studies). Alcohol residues were only studied in wastewaters, and according to this literature review, no studies looked at alcohol residues in rivers, lakes, seawater, groundwater and drinking water.

The review also finds that nicotine and alcohol residues have been studied mainly in developed countries (90% of reviewed studies) of Europe, North America, different parts of Asia and Australia (Table S3). Only a few studies have been conducted in developing countries, including Nigeria, Turkey, China, South Africa, Vietnam and Egypt, where water quality, e.g., in Nigeria, is unregulated [92]. The unbalanced number of studies (Fig. 3) makes it difficult to compare developed and developing countries. However, the available studies generally show comparable ranges (Table S3).

6.1. Occurrence in wastewater and removal efficiency

Reported influent concentrations of nicotine, cotinine, HCOT and ethyl sulphate were < 424,000 ng/L, < 42,300 ng/L, 300–52,000 ng/L and 500–33,000 ng/L, with the highest concentrations observed in Poland (nicotine), Spain (cotinine), Korea (HCOT) and Belgium (ethyl sulphate). Interestingly, Kumar et al. [59] found no significant seasonal variation in nicotine residues' influent concentrations (variation <5%), which is unexpected, given that the temperature of wastewater in the summer contributes to higher biological activity and degradation. The authors suggest that this is because rainfall lowers the temperature of the wastewater. In contrast, Spongberg and Witter [98] report seasonal

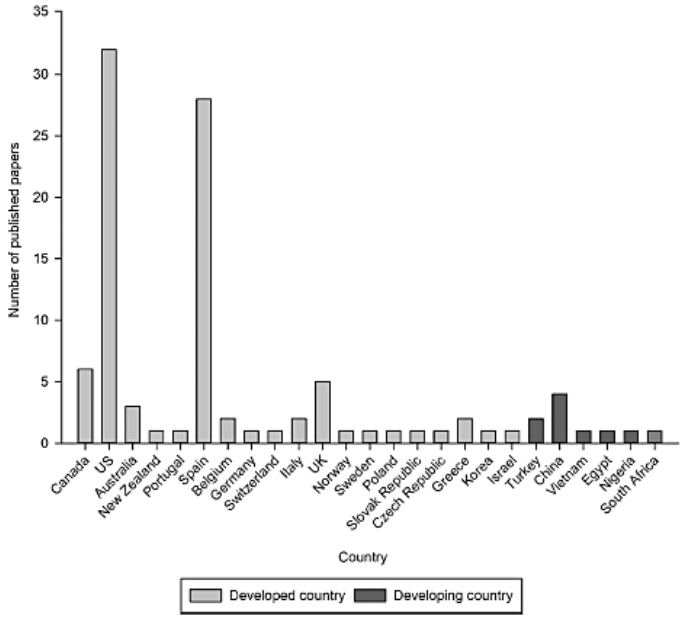


Fig. 3. Number of published papers related to their country of origin (developing country as classified by the United Nations [26]).

variations in cotinine levels (14.2–1 581.1 ng/L), with the highest amounts reported in winter and the lowest in spring.

The removal efficiency of nicotine and alcohol residues depends on the treatment process employed at the WWTP, operational parameters (hydraulic retention time, HRT), their physico-chemical properties (solubility and volatility) and environmental conditions, e.g., rainfall, temperature and pH [20,99–101]. Different removal efficiencies were obtained for nicotine (>3%), cotinine (>46%), HCOT (>66%) and ethyl sulphate (>80%) for different treatment processes (Table 2). The highest removals (>90%) were reported for membrane bioreactors – MBR, Bardenpho process (conventional activated sludge coupled with nutrient removal: 5-stage biological treatment units consisting of consecutive anaerobic/aerobic zones), reverse osmosis membranes (for cotinine) and lagoon constructed wetland treatment system (for cotinine). Different stages of treatment seem to eliminate the compounds to different extents. For example, secondary and tertiary treatments were the most efficient in removing nicotine, cotinine and ethyl sulphate [59, 100,102,103], while not surprisingly, given their physicochemical properties, the removal efficiency of primary treatment (sedimentation) was < 1% [59,100]. No seasonal variation (< 5%) in the removal of nicotine and ethyl sulphate was observed [59].

Overall, incomplete removal means that nicotine and alcohol residues are still present in the effluent in ng/L to μg/L [20]. As can be seen from Table S3, levels of nicotine, cotinine, HCOT and ethyl sulphate in wastewater effluents range from 15 to 32,000 ng/L, < 18,000 ng/L, 15–1,552 ng/L, and < 500 (LOQ) ng/L, respectfully. The highest concentrations of nicotine residues were detected in Spain (Table S3). As expected, generally lower concentrations in effluent than in influent were reported (Table S3).

Table 2
The removal efficiency of nicotine residues and ethyl sulphate in WWTPs, employing different wastewater treatments.

Treatment process	Removal efficiency			
	Nicotine [ref.]	Cotinine [ref.]	HCOT [ref.]	EtS [ref]
Activated sludge	57–> 99%[2, 52,61,99,108, 109,116,119, 120]	46–> 99% [2,11,52,61, 88,99,101, 108,109,119, 120]	66–99% [11,61, 120]	80–> 99% [61]
MBR	> 99%[59]	93–98%[59, 121]	> 99% [59]	> 99% [59]
Bardenpho*	> 99%[59]	96–98%[59]	> 99% [59]	> 99% [59]
Trickling filters	85–98%[51,99]	82–> 85%[51, 99]	–	–
Reverse osmosis membranes	49–63%[117]	94–96%[117]	–	–
Chemical flocculation and activated carbon Processes	3–73%[120]	–	–	–
Lagoon constructed wetland treatment system	–	> 99%[122]	–	–

*Bardenpho – conventional activated sludge, coupled with nutrient removal: 5-stage biological treatment units consisting of consecutive anaerobic/aerobic zones
HCOT – hydroxycotinine, EtS – ethyl sulphate, MBR – membrane bioreactor, UV – ultra-violet

6.2. Reclaimed water

Reclaimed water is the product (effluent) of wastewater treatment that can then be, after additional treatment (e.g., disinfection), used for irrigation in regions where there is water scarcity [79]. Even with this additional treatment, studies show that reclaimed water remains a potential source of pollutants [79,90]. So far, reclaimed water was analysed only in studies conducted in developed countries (Spain – Gran Canaria and the USA; Table S3). In these studies, nicotine (12.6–947 ng/L) and cotinine (17–62 ng/L) were detected with 100% frequency.

6.3. Surface water

Only nicotine residues have been investigated in surface waters, where they were detected in the ng/L to µg/L range (Table S3). Most studies focus on river water, where nicotine (< 9,340 ng/L), cotinine (< 6,582 ng/L) and HCOT (14–777 ng/L) were detected. The highest amount of nicotine was measured in surface water in Nigeria and likely derives from agricultural [92]. In the case of metabolites, the highest concentrations were found in Spain (cotinine) and Korea (HCOT). Generally, nicotine was detected in a comparable range in developed (< 8,187 ng/L) and developing (< 9,340 ng/L) countries, while cotinine was detected with lower concentrations in developing countries (< 6, 582 ng/L in developed countries vs 12.5–47 ng/L in developing countries; Table S3). Aside from consumption trends, concentrations depend on river flow, dilution, degradation and sorption processes, and sampling time (seasonal variation) [53,104,105], although Hua et al. [106] observed no seasonal variation of cotinine in the upper Detroit River (Canada). Studies [47,56,99,107] also found that WWTPs are a significant point source of nicotine residues entering rivers with higher levels of nicotine (upstream: < 4.8–378 ng/L, downstream: 3.6–350 ng/L) and cotinine (upstream: 2.4–29.8 ng/L, downstream: 2.9–210 ng/L) observed downstream of the WWTPs (Table S3). However, other studies [108,109] found significantly higher nicotine concentrations in downstream samples than in the effluent, indicating other possible sources, such as untreated wastewater discharge.

Unfortunately, data for lakes are available primarily for lakes in Canada and the US (Table S3). Similar to rivers, levels of cotinine (< 15.4 ng/L) and HCOT (< 77 ng/L) can be connected to direct WWTP discharge or river water transporting wastewater effluent into the lake [11,108,110]. Buerge et al. [11] did detect cotinine (2.6 ng/L) in a remote Alpine mountain lake (Switzerland), which was explained by aerial transportation and photodegradation of nicotine to cotinine. As a consequence of lifestyle and the influence of other contaminated waters (e.g., surface runoff, irrigation), nicotine (average: 432.6 ng/L) and cotinine (average: 71.7 ng/L) were also detected in 11 wetlands in Eastern Spain [89]. Only a few studies, mainly in the US, have looked at nicotine residues in seawater, showing that aside from WWTPs discharge, agricultural and industrial activities, as well as tourism, may contribute to the presence of nicotine (15.2–1,770 ng/L) and cotinine (4–1,070 ng/L) in investigated seawaters.

6.4. Groundwater

Contaminants can be introduced to groundwater by infiltration of wastewater, contaminated surface water or groundwater and leaching through soil (Fig. 1). Compared to surface waters, groundwater is less tested for contaminants since surface waters contain higher concentrations of analytes due to WWTPs' discharges. Accordingly, results can be an early warning signal of potential groundwater contamination [70, 111,112]. Nicotine and cotinine were detected in groundwater in the low ng/L range (nicotine: < 164 ng/L, cotinine: < 130 ng/L; Table S3). In some cases, their detection was connected to the study design. For example, González Alonso et al. [113] detected cotinine only in samples downgradient from landfills (< 130 ng/L), showing groundwater quality degradation by mixing with wastewater from the landfill. Seasonal variation of nicotine in groundwater samples (water gallery, wells) was studied by Montesdeoca-Esponda et al. [97], but no significant variation was found. Compared to developed countries, nicotine was detected in concentrations up to 3,530 ng/L in Nigeria, indicating higher groundwater pollution.

6.4.1. Drinking water

Although the levels of nicotine and alcohol metabolites in drinking water are unregulated (e.g., in Europe by the Drinking Water Directive 2020/2184 [114]), their presence in drinking water (and water from which drinking water is derived) is of concern due to their potential effect on human health through chronic exposure [70,71,115]. High consumption, ubiquitous presence and incomplete removal during drinking water treatment mean that nicotine and cotinine are, in general, detectable in drinking water at levels < 23 ng/L (Table 3). In only one US study, was cotinine detected in higher amounts (500 ng/L) [80].

7. Conclusion and future recommendations

As this review shows, nicotine and alcohol residues continue to find their way into waste- and environmental waters, where they have been detected in the ng/L to µg/L range. Despite being in low concentrations and having low bioaccumulation potential, monitoring their occurrence remains important given their potential toxic effects. To address the issue, composite and grab sampling are applied to collect waste- and environmental waters, while continued development of passive samplers, such as diffusive gradients in thin films type samplers, is encouraged. Once extracted from the water matrices by SPE, nicotine residues are typically analysed using reverse-phase LC-MS/MS. In contrast, alcohol residues are usually analysed by direct injection onto LC-MS/MS, and their retention is modified using an ion-pairing reagent. However, refining current analytical approaches, especially for alcohol residues, remains an option. Accordingly, the extraction procedure of alcohol residues needs further investigation to minimise the amount of matrix entering the MS in order to be able to detect their presence in surface, ground-, and drinking water. Also, signal suppression in MS resulting from using a non-volatile ion-pair reagent means that using a column that promotes the retention of polar compounds should be prioritised. Such a method may enable multi-compound analysis, i.e., alcohol residues and other compounds of interest. This review found much less information on the occurrence, fate, and distribution of alcohol residues than nicotine residues (85% of the reviewed studies addressed nicotine residues) and that studies are mainly performed in wastewater and river water (connected to WWTPs outflows) and data on alcohol residues in natural waters are missing from the literature. Accordingly, to obtain a more comprehensive overview of the prevalence of nicotine and alcohol residues, more studies on the occurrence of nicotine and alcohol residues in environmental waters are needed,

Table 3
Removal efficiency of nicotine and cotinine in DWTPs, employing different water treatments.

Drinking water treatment employed at DWTP	Removal efficiency	
	Nicotine	Cotinine
Conventional treatment *(pre-chlorination, coagulation, flocculation, sand filtration, ozonisation, GAC filtration, final post-chlorination)*	79%–> 99% [102,123]	57–94% [73,105, 123–125]
Advanced treatment *(ultrafiltration, UV disinfection, reverse osmosis and remineralisation with calcite)*	94 ± 4% [123]	99 ± 0.3% [123]
Not reported	86%–> 99% [126]	52–60% [126]

GAC filtration – granular-active carbon filtration, UV – ultra-violet

especially in developing countries where most wastewater flows untreated into the environment and where such studies are lacking.

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.

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Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.teac.2022.e00164.

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3.4.2 Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment

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Incomplete removal during wastewater treatment means drug residues are continuously introduced into the environmental waters through wastewater effluent. Once in the environment, they can potentially affect aquatic ecosystems adversely. Many studies have already addressed the efficiency of various wastewater treatment technologies for removing drug residues and the occurrence of drug residues in environmental waters (mainly effluent-receiving river waters). However, additional studies are still needed considering the proposed EU Directive concerning urban wastewater treatment (Chapter 1.4.2 Wastewater) and the consideration of individual drug residues for inclusion in the 4th Watch List under the Water Framework Directive (Chapter 1.4.3.1 Surface waters). Furthermore, ecotoxicity studies are warranted to adequately assess environmental risks posed by drug residues in environmental waters (Chapter 1.4.4 Effects on aquatic organisms).

To fill the knowledge gap, this study aimed to determine the efficiencies of Slovenian WWTPs of various sizes and configurations during different seasons (spring, summer and winter) in removing various residues of licit and illicit drugs. For the first time, the removal efficiencies were reported for moving bed biofilm reactor (MBBR). In addition, drug residues were explored in receiving rivers using modified analytical methods developed to determine drug residues in wastewater (Chapter 3.2.2 Investigation of drugs of abuse in educational institutions using wastewater analysis) and a prediction approach based on generating dilution factors. Finally, toxicity towards green algae (*Chlamydomonas reinhardtii*) was studied using a growth inhibition test and environmental risk assessment was made for studied rivers based on measured concentrations of drug residues and effect concentrations predicted using ECOSAR.

Drug residues were detected in wastewater influents and effluents in ng/L to µg/L range. On average, the highest removal efficiencies were observed for nicotine residues (>97 %), while methadone residues were removed to a lower extent (<30 %). The treatment technology employed at the WWTP was recognized as the most critical factor influencing the removal of drug residues, with similar removal observed for activated sludge and MBR and poorer removal of cotinine, cocaine and benzoylecgonine in MBBR. The removal of drug residues at various WWTPs affects their occurrence in river waters, where they were found in the ng/L range. Similar values were obtained with the water analysis and prediction method, indicating that the prediction of environmental concentrations in effluent-receiving rivers can be used as an alternative to labor- and cost-intensive water analysis. Although no pronounced biological effect of drug residues (as an individual or in selected mixtures) was observed on green algae, drug residues may still affect other species, including algae. Indeed, environmental risk assessment predicted that nicotine, methadone, EDDP, morphine and MDMA could affect aquatic organisms at concentrations detected in the studied rivers and warranted further monitoring and regulatory actions.

The study results were presented at four scientific conferences, *i.e.,* Chem2Change, Environmental Chemistry towards Global Change, the 2nd Online ACE Seminar on Chemistry and the Environment Led by Early-Career Scientists; the 26*th* International

Symposium on Separation Sciences; the 15th International Symposium on the Interactions Between Sediments and Water; and the 22*nd* European Meeting on Environmental Chemistry, and were summarized within seven working reports to the final users, *i.e.,* Slovenian WWTPs.

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Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment

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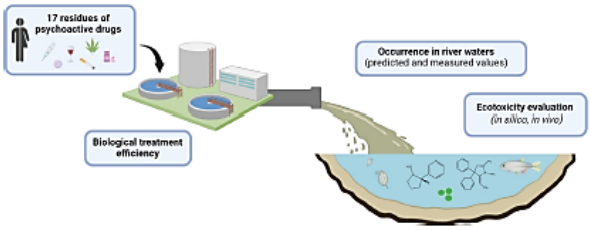
HIGHLIGHTS

- Removal of psychoactive drug residues with activated sludge and MBR were comparable.
- MBBR was less efficient at removing nicotine and cocaine residues.
- Predicted and measured levels in river water were in good agreement.
- *Chlamydomonas reinhardtii* growth was uninhibited by drug residues at 1 mg/L.
- Five drug residues exceeded in silico predicted PNEC.

GRAPHICAL ABSTRACT



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ABSTRACT

Continuous consumption combined with incomplete removal during wastewater treatment means residues of psychoactive substances (licit drugs, medications of abuse and illicit drugs) are constantly introduced into the aquatic environment, where they have the potential to affect non-target organisms. In this study, 17 drug residues of psychoactive substances were determined in wastewater influent, effluent and in receiving rivers of six Slovene municipal wastewater treatment plants employing different treatment technologies. Variations in removal efficiencies (REs) during spring, summer and winter were explored, and ecotoxic effects were evaluated using in silico (Ecological Structure-Activity Relationships software–ECOSAR) and in vivo (algal growth inhibition test) methods. Drug residues were detected in influent and effluent in the ng/L to μg/L range. In receiving rivers, biomarkers were in the ng/L range, and there was good agreement between measured and predicted concentrations. On average, REs were highest for

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nicotine, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), cocaine residues, and amphetamine (>90 %) and lowest for methadone residues (<30 %). REs were comparable between treatments involving activated sludge and membrane bioreactors, while the moving biofilm bed reactor (MBBR) removed cotinine, cocaine, and benzoylecgonine to a lesser extent. Accordingly, higher levels of nicotine and cocaine residues were detected in river water receiving MBBR discharge. Although there were seasonal variations in REs and levels of drug residues in receiving rivers, no general pattern could be observed. No significant inhibition of algal growth (*Chlamydomonas reinhardtii*) was observed for the tested compounds (1 mg/L) during 72 h and 240 h of exposure, although effects on aquatic plants were predicted in silico. In addition, environmental risk assessment revealed that levels of nicotine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), morphine, and 3,4-methylenedioxymethamphetamine (MDMA) pose a risk to aquatic organisms. Since nicotine and EDDP can have acute and chronic effects, the authors support regular monitoring of receiving surface waters, followed up by regulatory actions.

1. Introduction

Once administered, psychoactive drugs cross the blood-brain barrier and act upon the central nervous system affecting mental processes such as perception, consciousness, cognition, mood and emotions (Viana et al., 2012; WHO, 2022). They are consumed licitly for recreational purposes (e.g., nicotine and alcohol), medicinally (e.g., morphine, ketamine, codeine, and methadone), or illicitly (e.g., cocaine, amphetamines, and heroin) (Viana et al., 2012). Like most drugs, they are excreted from the body in urine, faeces, sweat and saliva as either the parent compound or as metabolites (drug residues) and enter the sewer system where, in most cases, they are delivered to a local wastewater treatment plant, WWTP (Jin et al., 2022; Mohan et al., 2021; Zuccato et al., 2005). Once at the WWTP, they are removed to differing degrees (from negative removal efficiencies, RE, to >99 %) depending on influent concentration and their physicochemical properties, treatment technology and environmental parameters (Deng et al., 2020; Di Marcantonio et al., 2020; Evgenidou et al., 2015; Hedgespeth et al., 2012; Jin et al., 2022; Verovšek et al., 2022; Yadav et al., 2017). Consequently, drug residues, e.g. parent compounds and their metabolites, are found in receiving surface waters (e.g., rivers, lakes, and seawater) in the ng/L–μg/L range, making wastewater effluent a major source of drug residues in the environment (Evgenidou et al., 2015; Jin et al., 2022; Verovšek et al., 2022).

The presence of psychoactive drug residues in receiving surface waters raises ecotoxicological concerns due to their psychoactive properties, especially since their presence in the environment through continuous release (pseudo-persistence) poses a risk to non-target organisms (Ebele et al., 2017; Mohan et al., 2021; Rosi-Marshall et al., 2015). Medium to long-term exposure may result in chronic effects (ecological and evolutionary) even at low environmental concentrations (ng/L range). Moreover, their simultaneous presence may lead to additive or synergistic effects (Evgenidou et al., 2015; Jin et al., 2022). Unfortunately, research on their effects on aquatic organisms is limited regarding the number of drug residues tested and the variety of aquatic organisms exposed (Mohan et al., 2021). However, available data indicate that drug residues could harm aquatic organisms. For example, plant-derived substances, such as cocaine, cannabinoids, opioids, nicotine, and amphetamines, have antimicrobial properties (Baran et al., 2020; Radulović et al., 2013; Rosi-Marshall et al., 2015). Nicotine, cocaine and tetrahydrocannabinol (THC) were shown to affect invertebrates, such as water fleas (*Daphnia magna* and *Daphnia pulex*) and mussels (Zebra mussel, *Dreissena polymorpha*), while cocaine was proven to affect vertebrates (European eel, *Anguilla anguilla*) (Binelli et al., 2012; De Felice et al., 2019; Gay et al., 2013; Oropesa et al., 2017; Parolini and Binelli, 2014). So far, no published studies have addressed the ecotoxicological effect of psychoactive drug residues on algae, although it is widely known that the disturbance of algae as primary producers in the aquatic food chain affects higher trophic levels (Geis et al., 2000).

This study aimed to fill knowledge gaps by determining (i) the efficiency of six Slovenian WWTPs differing in size and configuration (including MBBR) for removing psychoactive drug residues; (ii) their presence in receiving waters determined using liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) and predicted based on effluent concentrations and river flows; (iii) their potential aquatic toxicity using an algal growth inhibition test (*Chlamydomonas reinhardtii*) for the first time; and (iv) a risk assessment based on measured concentrations of drug residues in receiving rivers and effect concentrations estimated using Ecological Structure-Activity Relationships software (ECOSAR).

2. Methods

2.1. Compounds of interest

Seventeen residues of licit drugs, medications of abuse and illicit drugs were targeted in wastewater (influents and effluents) and receiving river waters (Table 1). All details regarding reagents are provided in the Supplementary material (see SM: 1.1. Chemicals and Materials).

2.2. Sampling and sample preparation

Six WWTPs varying in catchment size (25,414–270,305 inhabitants) and type of treatment technology (activated sludge – AS, sequential batch reactor – SBR, SBR with UV disinfection, membrane bioreactor – MBR and moving biofilm bed reactor – MBBR) were included in the study (Table S2). Wastewater influent and effluent samples (24-h composites) were collected using time- or volume-proportional sampling taking hydraulic-retention times (HRTs) into account. Receiving waters (Table S2) differing in hydrological conditions, e.g., dilution factor (4.22–887), expressed as the ratio between the receiving river water and wastewater effluent flow, were collected as grab samples from the riverbank, approximately 100 m downstream of the WWTPs outflows. In the case of one WWTP, collection of receiving river water was not possible (Table S2). All water samples were collected in spring and summer (2019) and winter (2020) and stored at −20 °C until analysis. Full details about the WWTPs and receiving waters are given in the SM (1.2. Sampling).

Spring sample analysis is based on previously published methods (Verovšek et al., 2021a, 2021b). Pre-concentration of compounds was achieved using solid-phase extraction (SPE) with Oasis MCX cartridges, followed by analysis using UPLC-MS/MS. In the case of nicotine and alcohol residues, which were present in much higher concentrations, the direct injection method was used. Due to contamination of the MS when using direct injection and ion-pairing reagent (Verovšek et al., 2022), the method was later optimised and used to analyse the summer and winter samples. Both methods are described in the SM (1.3. Sample preparation: waste- and surface waters). In this case, waste- (influent: 125 mL, effluent: 250 mL) and river (0.5 L) water samples were spiked with isotopically-labelled internal standards (ISs) of each drug residue (Table S1). For the determination of alcohol residue (ethyl sulphate), liquid-liquid extraction (LLE) with acetonitrile was used. Nicotine residues were extracted from wastewater influent using supported liquid extraction – SLE (ISOLUTE SLE+, 400 μL, Biotage, Sweden) and from effluent and river water using SPE (Oasis PRIME MCX, 150 mg/6 mL, Waters, Milford, MA, USA). Residues of illicit drugs and medications of abuse were extracted from waste- and river water by SPE (Oasis PRIME MCX). In order to determine THC-

Table 1
Compounds of interest (human metabolic residues).

Psychoactive drug	Compound of interest	Abbreviation
Licit drugs		
Nicotine (tobacco)	Nicotine[a]	NIC
	Cotinine[a]	COT
	Trans-3′-hydroxycotinine[a]	HCOT
Ethanol (alcohol)	Ethyl sulphate	EtS
Medications of abuse		
Morphine	Morphine[a]	MOR
Codeine	Codeine	COD
Methadone	Methadone	MTHD
	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine[a]	EDDP
Ketamine	Ketamine	KET
Illicit drugs		
THC	11-Nor-9-carboxy-Δ9-tetrahydrocannabinol[a]	THC-COOH
Cocaine	Cocaine[a]	COC
	Benzoylecgonine[a]	BE
	Cocaethylene	COE
Amphetamine	Amphetamine[a]	AMP
Methamphetamine	Methamphetamine	MAMP
Ecstasy	3,4-Methylenedioxymethamphetamine[a]	MDMA
Heroin	6-Acetylmorphine	6-AM
Algal growth inhibition test (spike of mixture):		
MIX_1: Nicotine and EDDP		
MIX_2: Nicotine, cotinine, HCOT, EDDP and benzoylecgonine		
MIX_3: Nicotine, cotinine, HCOT, morphine, EDDP, cocaine, benzoylecgonine, amphetamine and MDMA		

[a] Compounds used in algal growth inhibition test (individual spike).

COOH, an additional clean-up was introduced (Strata NH_2, 200 mg/3 mL, Phenomenex, Torrance, California, USA).

2.3. Chemical analysis and method validation

All samples were analysed using reverse-phase UPLC-MS/MS. The method is described in full in the SM (1.5. Sample analysis), and the methods' performance was evaluated by determining the following parameters: linearity, limits of detection (LOD), limits of quantification (LOQ), filtration recovery (FR), extraction recovery (ER), matrix effect (ME), accuracy and repeatability on at least two concentration levels. Artificial wastewater influent and effluent, potable water and blank TRIS acetate phosphate (TAP) medium were used as blank matrices for validation (see SM: 1.6. Method validation). Methods used for analysis were validated in the frame of the Sewage analysis CORe group – Europe (SCORE) interlaboratory comparison study (SCORE, 2022).

2.4. Removal efficiency calculation

Removal efficiency (RE) was calculated according to Eq. (1).

$$RE\,(\%) = 100 - \left(\frac{c_{eff}}{c_{inf}} \times 100\right) \quad (1)$$

where c_{eff} is the concentration of the target compound (ng/L) in wastewater effluent and c_{inf} is the concentration of the target compound in wastewater influent. In the case where 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.

2.5. Predicting environmental concentration of drug residues in receiving rivers

An approach based on generating dilution factors for predicting the environmental concentration of down-the-drain chemicals in surface waters was adapted from Keller et al., 2014. The concentration of drug residues in receiving surface waters was predicted using calculated dilution factors (Table S2) and measured concentrations in wastewater effluents (Tables S11–S13). Estimated concentrations were compared with those measured in the receiving rivers (Tables S11–S13).

2.6. Algal growth inhibition test

Ten drug residues (Table 1) were selected based on their occurrence in wastewater and predicted ecotoxicity (ECOSAR) and spiked (individually and as mixtures) in TAP medium (Table S3). An algal growth inhibition test was then conducted following OECD Test No. 201 guideline (OECD, 2022) with minor modification of prolonged exposure with individual drug residues. The guideline consists of the following three validation criteria: (i) growth rate of at least 0.92 per day, (ii) coefficients of variation between each test day <35 % and (iii) coefficients of variation for individual control cultures during whole test <10 %. Each week of the experiment, green algae (*Chlamydomonas reinhardtii*) culture was prepared in agar (2 g of agar mixed with 250 mL sterile TAP medium). The stock culture was prepared by mixing a portion of the agar culture into a liquid TAP medium and incubated under controlled conditions for three days. The stock culture was inoculated into the test chambers (100 mL glass Erlenmeyer® flasks, sterilised) filled with 10 mL TAP medium to achieve an initial algae concentration of 2×10^4 cells/mL. For cell counting, an aliquot of each liquid culture (50 μL) was transferred to a 96-well plate (Brand GMBH + CO KG, Germany) and injected into a flow cytometer (MACSQuant Analyser 10: Miltenyi Biotec, Germany). Inoculated TAP medium was spiked with ten drug residues (individually or in a mixture) at a nominal value of 1 mg/L. Non-spiked inoculated TAP medium was used for the negative control, and TAP medium with added methanol and acetonitrile (separately and in a mixture) as a control for the effect of solvents of drugs' stock standard solutions (Table S1) on algal growth. All tests were performed in an algae growth chamber (LTH, Slovenia) at room temperature (22 ± 1 °C) under constant light (80–120 μE/m^2s, Sylvania GRO-Lux F 18 W/GRO-T8) and shaking (80 rpm, GFL 3017, Germany). Total exposure time was 240 h for individual drug residues and 72 h for mixtures of drug residues. Algal growth was measured by flow cytometer after 24, 48, 72, and 240 h (each time in triplicate). Specific growth rate (Eq. (2)) and inhibition of growth rate (Eq. (3)) were calculated for each time interval. The levels of drug residues spiked in the TAP medium were determined (at 0, 72, and 240 h) as follows: a portion of the TAP medium was sequentially centrifuged (14,000 RCF, 3 min), spiked with ISs of each targeted analyte and filter-centrifuged using modified nylon centrifugal filters (0.2 μm, 14,000 RCF, 3 min, VWR, Vienna, Italy). The drug residues were then extracted either by SLE or LLE into acetonitrile (see SM: 1.4. Sample preparation: TAP medium).

The specific growth rate (μ) for an individual period was calculated as the logarithmic increase in the cell density (Eq. (2)), where μ_{i-j} is the specific growth rate (cell/mL) at times i to j (hours), X_i is the cell density at the time i and X_j is the cell density at time j. The specific growth rate was calculated for individual measurement within the individual replicate.

$$\mu_{i-j}\left(h^{-1}\right) = \frac{\ln X_j - \ln X_i}{t_j - t_i} \quad (2)$$

The inhibition of growth rate (I_r) for individual measurement within an individual replicate was calculated in percentages as presented in Eq. (3), where μ_C is the average specific growth rate (h^{-1}) in the control group, and μ_T is the growth for individual measurement.

$$I_r(\%) = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (3)$$

2.7. Environmental risk assessment

An environmental risk assessment (ERA) addresses the ecological threat associated with drug residues in receiving waters. In this study, the ERA is based on 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 (European Directive

93/67/EC, 1488/94 and 98/8/EC, Part III) and US Environmental Protection Agency (EPA) guidelines (US EPA, 2022) by calculating risk quotients (RQ) according to Eq. (4):

$$RQ = \frac{MEC}{PNEC} \quad (4)$$

The measured environmental concentration (MEC) is the average and maximal amount of each drug residue in the receiving water body. When the measured concentrations were < LOQ, the LOQ was used in the calculation. The predicted no-effect concentration (PNEC) was calculated by dividing the lowest concentration for a single species short-term (median lethal concentration – LC_{50} or median effect concentration – EC_{50}) or the long-term (no-observed-effect concentration – NOEC) effect concentration with the assessment factor – AF (Table S5). As no experimental data is available for the majority of drug residues except for nicotine (HSDB, 2022), the EC_{50}/LC_{50} were predicted (Table S5) using ECOSAR software (v2.2), which predicts the toxicity of new/untested compounds based on their structural similarities with compounds with known experimental effect levels and physicochemical properties. The lowest experimental EC_{50}/LC_{50} offered in ECOSAR was used for nicotine. For all residues, the NOEC was calculated by dividing the chronic value (ChV) derived using ECOSAR with √2 (European Directive 93/67/EC, 1488/94 and 98/8/EC, Part III). An AF of 1000 was applied to address acute effects and an AF of 50 to assess chronic risks (European Directive 93/67/EC, 1488/94 and 98/8/EC, Part III). The RQ expressing risk to aquatic organisms were compared to levels of concern (LOC) determined by the US EPA (Table S6) (US EPA, 2022), where RQs >1 indicate an acute risk for aquatic plants and RQ >0.05 represents an acute risk to aquatic animals. An RQ >1 represents a chronic effect on aquatic animals. Only an acute risk assessment was made for aquatic plants.

2.8. Statistical analysis

Univariate and multivariate analysis was used to explore differences in removal efficiencies (variation between WWTPs and seasonal variations) and in the occurrence of drug residues in receiving waters (variation between rivers and seasonal variations). A Student's *t*-test (when data normality and equality of variance were assumed), Welch's t-test (when data normality but equality of variance is not assumed) or Mann-Whitney Rank Sum Test (when the normality of the data was not assumed) at the 95 % confidence level ($\alpha = 0.05$) were used to evaluate the differences between two groups. The normality of the data was tested using the Shapiro-Wilk test and the equality of variance using the Brown-Forsythe test (95 % confidence level, $\alpha = 0.05$). The variation within the dataset was explored using principal component analysis – PCA (unsupervised), while orthogonal projection to latent squares-discriminant analysis – OPLS-DA (supervised) was used to determine the importance of the variables (drug residue) in the projection (VIP) score (95 % confidence interval). Overfitting of the OPLS-DA model was excluded using a permutation test ($n = 100$). For PCA and OPLS-DA, the data were either UV or Par scaled, and logarithmic transformation was applied where necessary. In the algal growth inhibition test, the differences in the inhibition of growth rate between the negative control (inhibition set on 0) and the samples (spiked with solvents and drug resides) were evaluated using repeated measures (RM) one-way ANOVA with Dunnett's multiple comparisons post-hoc test (95 % confidence interval, $\alpha = 0.05$). Statistical evaluations were performed using SigmaPlot 14.0, Origin 2020, GraphPad Prism 9 and Simca 15.0.

3. Results and discussion

3.1. Method validation parameters

A linear response ($R^2 > 0.99$) was observed between LOQ–1000 ng/mL for all drug residues except methadone (LOQ–500 ng/L) in artificial wastewater influent (Table S7), between LOQ and at least 300 ng/L in artificial wastewater effluent (Table S8), between LOQ and at least 250 ng/L in potable water (Table S9) and between LOQ and at least 500 ng/L in TAP medium (Table S10). The LOD and LOQ were in the ng/L range (Tables S7–S10), except for LOD/LOQ for licit drug residues in artificial wastewater influent (LOD: 109.5–1166 ng/L and LOQ: 155–3341 ng/L), ethyl sulphate in artificial wastewater effluent (LOD: 739 ng/L and LOQ: 2419 ng/L) and potable water (LOD: 884.9 ng/L and LOQ: 2947 ng/L). Signal suppression or enhancement (Tables S7–S10) was observed in artificial wastewater influent (ME: −7–46.2 %) wastewater effluent (ME: −13.7–15.7 %), potable water (ME: −84.7–32.6 %) and TAP medium (ME: −18–8.5 %). The relative extraction recoveries (Tables S7–S10) were 19–112 % in artificial wastewater influent (the lowest for ethyl sulphate), 29–110 % in artificial wastewater effluent (the lowest for morphine), 23–108 % in potable water (the lowest for ethyl sulphate) and 65–107 % in TAP medium (the lowest for THC-COOH). Despite lower recoveries and higher matrix effects in exceptional cases, good accuracies were obtained for all compounds of interest in tested matrices (86–110 %; Tables S7–S10). Only ethyl sulphate showed an accuracy of 76 % at the lowest spike in potable water. Instrumental and inter-day repeatability was ≤20 %RSD.

3.2. Occurrence in wastewaters

Fourteen out of the 17 targeted drug residues had a high (≥80 %) detection frequency – DF (percentage of the samples containing drug residue >LOD) in the wastewater influents (Fig. S1), while DF of ketamine (8.3 %) and 6-acetylmorhine (20 %) was low (≤20 %). Quantities of drug residues in wastewater influents are, among others (e.g., excretion rate), closely related to the level of drug use (Yadav et al., 2017). The levels of drug residues in aqueous samples sampled in spring, summer and winter are given in Tables S11–S13. As tobacco and alcohol (ethanol) are the two most commonly used drugs in Slovenia, i.e., approximately 24 % and 68 % of the population between 15 and 64 years of age, respectively (NIJZ, 2019a), high detection (DF = 100 %) and high measured concentrations (756–60,900 ng/L) of their residues were expected and in comparable concentrations (μg/L) to that reported in the literature (Verovšek et al., 2022). The medication of abuse with the highest concentration was morphine (9.40–1634 ng/L), agreeing with its higher prescription rate than other studied medications, i.e., in 2019, morphine was prescribed at least 6500 times, codeine 2400 and methadone 30 times (NIJZ, 2019b). In wastewater, morphine may also originate from other sources, such as the metabolism of codeine and heroin (Gracia-Lor et al., 2017). Among illicit drug residues, benzoylecgonine had the highest concentration (180–2900 ng/L), followed by cocaine (54–1096 ng/L) and THC-COOH (up to 736 ng/L).

Nine out of 17 targeted drug residues in wastewater effluent had a high DF (>80 %, Fig. S1), while ethyl sulphate (1.6 %) and amphetamine (2.4 %) had low DFs. Ketamine showed higher DF in wastewater effluents (27 %) than influents (8.3 %), which may be explained by the 3–4 times lower LOD/LOQ obtained for wastewater effluent (Tables S7–S8). Similarly, Bijlsma et al. (2012) found ketamine mainly in wastewater effluents of five Dutch WWTPs ($DF_{influents}$ = 22 %, $DF_{effluents}$ = 88 %). In our study, 6-acetylmorphine was not detected. The levels of drug residues in wastewater effluent depend on their removal efficiency during wastewater treatment (Yadav et al., 2017). As expected, most drug residues had lower concentrations in wastewater effluents than influents (Tables S11–S13), agreeing with the literature data (Yadav et al., 2017; Verovšek et al., 2022). Licit drug residues were detected in wastewater effluents up to 1075 ng/L (nicotine), residues of medications of abuse up to 494 ng/L (morphine) and illicit drug residues up to 218 ng/L (MDMA).

3.3. Removal efficiencies

The highest average REs (>90 %) were observed for nicotine residues, THC-COOH, cocaine residues, and amphetamine, with methadone residues

Table 2
Individual drug residue removal efficiencies (average and range).

Drug residue	Average RE (range) [%]	Drug residue	Average RE (range) [%]
Ethyl sulphate	75 (30–96)	THC-COOH	98 (88–99)
Nicotine	99 (87–99.999)	Cocaine	97 (62–99.9)
Cotinine	97 (83–99.8)	Benzoylecgonine	94 (53–99.9)
HCOT	99 (85–99.99)	Cocaethylene	95 (66–99.7)
Morphine	88 (−6–99)	Amphetamine	97 (78–99.7)
Methadone	27 (−59–74)	Methamphetamine	51 (35–95)
EDDP	20 (−87–86)	MDMA	47 (−155–90)
COD	53 (−64–98)	6-acetylmorphine	42 (29–56)

EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine, RE – removal efficiency, THC-COOH – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol.

having the lowest RE (<30 %; Table 2). The result agrees with already published studies (Baker and Kasprzyk-Hordern, 2013; Bijlsma et al., 2012; Ekpeghere et al., 2018; Nefau et al., 2013; Nguyen et al., 2018; Postigo et al., 2008; Postigo et al., 2010; Terzic et al., 2010). Negative REs for morphine, methadone residues, codeine and MDMA (Table 2) indicate that these drug residues were present in higher amounts in wastewater effluent than the influent. This difference can be explained by the formation/transformation of the compounds during wastewater treatment, e.g., the transformation of parent compound/precursor, deconjugation of glucuronide conjugates or transformation of conjugated metabolites into parent compound and by an inadequate pairing of wastewater influent and effluent samples (sampling not in range of HRT) (Bijlsma et al., 2012; Subedi and Kannan, 2014; Terzic et al., 2010; Yadav et al., 2019).

The differences in the RE of the six WWTPs for the studied compounds were explored using PCA (Fig. 1). Only WWTP_6 formed a distinct group. For the projection, the difference in removal of cotinine, cocaine and benzoylecgonine was important (OPLS-DA), with significantly lower removals obtained for WWTP_6 (cotinine: $U_{statistic} = 14$, $p \leq 0.001$, $\alpha = 0.05$; cocaine; $U_{statistic} = 0$, $p \leq 0.001$, $\alpha = 0.05$; benzoylecgonine: $U_{statistic} = 0$, $p \leq 0.001$, $\alpha = 0.05$). The dependence of RE on commonly applied wastewater treatment technologies are already well known (Yadav et al., 2017) and in terms of efficiency is as follows: MBR > AS > trickling filters (Baker and Kasprzyk-Hordern, 2013; Kasprzyk-Hordern et al., 2009; Petrovic et al., 2009; Verovšek et al., 2022). The present study observed no difference (grouping) in REs between MBR and AS (Fig. 1). To the best of our knowledge, the removal of drug residues by MBBR was studied for the first time and indicated poorer removal of cotinine, cocaine, and benzoylecgonine.

Seasonal variation in RE is expected since environmental factors, namely wastewater temperature and dissolved oxygen, besides treatment processes and operational parameters, play an important role in removing drug residues by (micro)biological processes (Yadav et al., 2017). Indeed, differences in RE regarding season were observed (PCA: small level of

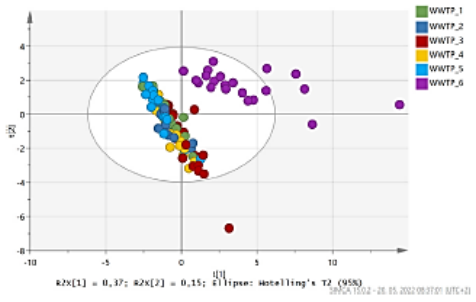

Fig. 1. PCA scatter plot showing variation in REs' dataset.

grouping) for five out of the six studied WWTPs (Fig. S2), with important differences in the RE of nicotine residues (WWTP_4 and WWTP_5), codeine (WWTP_1 and WWTP_6), cocaine (WWTP_3), and benzoylecgonine (WWTP_4). In the case of codeine, cocaine, and benzoylecgonine, significantly higher REs were obtained in summer compared to winter and, in some cases (codeine – WWTP_6, benzoylecgonine – WWTP_4), in comparison to spring (Table S14). Higher REs during summer are expected because higher wastewater temperatures (14.2–25.3 °C compared to 9.90–18.3 °C in spring and 9.4–15.3 °C in winter) generally enhance microbial activity (Castiglioni et al., 2006). In contrast, nicotine residues (nicotine: WWTP_4 and WWTP_5, cotinine and HCOT: WWTP_4) showed significantly higher REs in spring than in summer (Table S14). However, although seasonal variations in REs were observed, they were only significant for specific drug residues at specific WWTPs (Tables S11–S13), suggesting that seasonal RE variation is compound and WWTP specific.

3.4. Occurrence in receiving river waters

The presence of drug residues in receiving waters (Tables S11–S13) is expected due to their occurrence in the effluent (see Occurrence in wastewaters). Nine out of 17 drug residues had high DF (>80 %, Fig. S1), with DF ≤ 20 % obtained for ethyl sulphate (1 %), ketamine (14 %), THC-COOH (8 %) and amphetamine (12 %). 6-acetylmorphine was not detected. Nicotine, HCOT, cocaine residues, amphetamine and methamphetamine, had a higher DF in receiving river waters than wastewater effluents (Fig. S1), which can be explained by either the much lower LOD/LOQ (2–17-times) obtained for river water (Tables S8–S9) or by the input of drug residues from sources other than WWTPs, namely the clandestine release of illicit drugs/precursors into the soil, nicotine wash-out from cigarette butts and ashes and leaking sewer system (Barbosa et al., 2016; Castiglioni et al., 2015). Otherwise, as expected, due to dilution (Table S2), lower levels of drug residues were detected in receiving waters compared to wastewater effluents (Tables S11–S13). In general, licit drug residues were detected in receiving river waters in concentrations up to 312 ng/L (nicotine), residues of medications of abuse in concentrations up to155 ng/L (EDDP) and illicit drug residues in concentrations up to 190 ng/L (BE).

The variations in the occurrence (level) of psychoactive drug residues were explored in terms of location (different rivers with different dilution factors) and season (spring, summer and winter) using multi-variant analysis. The scatter plot (Fig. 2) shows how R_6 (receiving effluent from WWTP_6) is grouped separately. For the projection, the difference in the occurrence of nicotine residues and benzoylecgonine was important (OPLS-DA), with significantly higher concentrations measured in R_6 (nicotine: $U_{statistic} = 229$, $p \leq 0.001$, $\alpha = 0.05$; cotinine: $U_{statistic} = 9$, $p \leq 0.001$, $\alpha = 0.05$; HCOT: $U_{statistic} = 100$, $p \leq 0.001$, $\alpha = 0.05$; benzoylecgonine: $U_{statistic} = 0$, $p \leq 0.001$, $\alpha = 0.05$). The result can be explained by the lower removal of cotinine and cocaine residues and its lower dilution factor (R_6: 4.22–14.4, all studied rivers: 4.22–887), making the influence of wastewater effluent on the quality of receiving river water more significant.

Seasonal variation in levels of drug residues in the receiving waters is shown to be connected to variations in WWTP discharge, REs, dilution (i.e., river flow) and environmental conditions affecting the degradation of compounds (water temperature and exposure to sunlight) (Baker and Kasprzyk-Hordern, 2013; Mendoza et al., 2014). We observed seasonal variations (PCA: grouping of the samples) for R_3, R_5 and R_6 (Fig. S3). For the projections, nicotine residues, morphine, codeine, methadone, EDDP, methamphetamine and cocaethylene were important variables (OPLS-DA), with significantly higher concentrations obtained in winter and spring (Table S15). Exceptions were nicotine residues in R_6, where significantly higher concentrations were observed in the summer and cocaethylene concentrations in R_3, which were significantly higher in spring. Surprisingly, no seasonal variation (e.g., no grouping) was observed for R_1 and R_2 when data were analysed using PCA (Fig. S3). Similar to REs, seasonal variations in drug residues in receiving waters are also compound and receiving water-specific (significant variation observed for a limited number of compounds in different river waters).

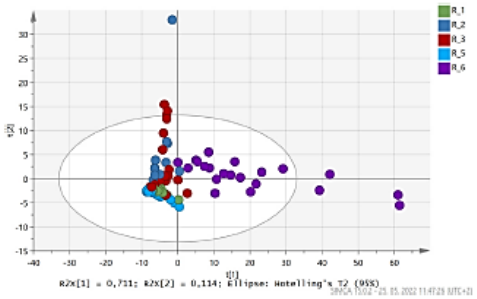

Fig. 2. Scatter plot (PCA) of samples with data on the occurrence of drug residues in the studied rivers (R_1 to R_6).

3.5. Drug residues in river water: measured vs predicted values

A comparison between measured and estimated levels of drug residues in river waters revealed that differences are within an order of magnitude (the average ratio between measured and estimated values is 10.06: 1, without WWTP_5). With only a few exceptions, measured values are higher than predicted (Fig. 3) and are likely sampling related. Sampling water from the river bank means that the complex hydrodynamics of the river section is not captured. Also, it should be noted that the river water samples were taken as grab samples. Grab sampling makes obtaining a representative river water sample more challenging (Verovšek et al., 2022). The assumption that the hydrodynamics of the river is not captured is further supported by the fact that the differences between measured and estimated concentrations are more pronounced in rivers with higher flow rates. The differences may also be related to uncertainties in the river discharge data and the representativeness of the selected stations. Such discrepancies are most noticeable for WWPT_5, where the dilution is most pronounced (WWTP_5: 108–887, other WWTPs: 4.22–121), and the nearest available hydrological station is located approximately 10 km downstream. At this site, differences between measured and estimated values are also the greatest (max ratio measured *vs* estimated values: 5691). All three nicotine residues stand out in particular, which may also be related to untreated wastewater or sources other than WWTP discharge (see Occurrence in receiving river waters). Despite some deviation, the results demonstrate that predicting environmental concentrations when reliable data on river flows are available can be an alternative to analysing river water, especially considering the cost of analysis and at locations where sampling is difficult.

3.6. Algal growth inhibition test results

Cell growth was measured in spiked samples (individually or as a mixture of drug residues) and the negative controls (Table S16). The results show that cell growth in the spiked samples was similar to that in the negative controls (Fig. 4), with negative inhibition (growth enhancement) during 0–24 h (Table S17), while no pronounced enhancing or inhibiting effect was observed with prolonged exposure (72 h and 240 h). Although based on predicted EC_{50} (Table S19), EDDP (EC_{50} = 0.04 mg/L) and THC-COOH (EC_{50} = 0.05 mg/L) were expected to affect algal growth (spiked concentrations were above predicted EC_{50}; Table S5), no significant difference in inhibition of growth rate was observed when spiked individually ($p > 0.05$, $\alpha = 0.05$). The result may be explained by (i) a reduction in the concentration of drug residues (Table S18), which may have occurred due to their transformation, adsorption (test vessel or algal cells) and uptake into algal cells (Elersek et al., 2021; Castiglioni et al., 2013) and (ii) insensitivity of the tested species (*Chlamydomonas reinhardtii*) to a particular drug residue (Rojíčková and Maršálek, 1999). Given that this is the first study looking at the effects of drug residues on green algae, no comparison with the literature is possible.

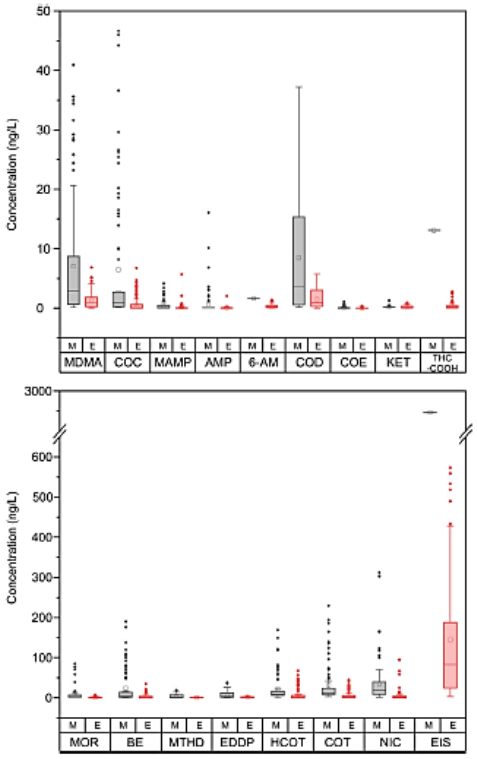

Fig. 3. Comparison and distribution (median, mean, P25–P75, min-max values and outliers) of measured (M) and estimated (E) concentrations of individual compounds in river water.

3.7. Environmental risk assessment

The ECOSAR results are given in Tables S19–S24. Although interactions between different psychoactive compounds may induce synergistic and additive effects on aquatic organisms (la Farré et al., 2008), only the effect of individual drug residues was predicted in this study. The results show that only EDDP at average (47 ng/L, RQaverage = 1.18) and maximal (155 ng/L, RQmax = 3.87) measured concentrations pose an acute risk for aquatic plants in R_2 during winter sampling.

Nicotine was the only measured drug residue in river water (except for R_5) with predicted acute effects on aquatic animals at average (3.87–185 ng/L, RQaverage = 0.06–0.92) and maximal (8.92–312 ng/L, RQmax = 0.09–1.56) levels during all seasons. The results agree with the toxic potential of nicotine reported in studies conducted in Spanish (RQ > 1, maximal concentration: 2500 ng/L (Oropesa et al., 2017)) and Italian receiving waters (RQ > 1, all sampling sites: 670–6430 ng/L [Riva et al., 2019]).

Among the medications of abuse, acute risks to aquatic animals of methadone were predicted in R_2 in winter (average concentration: 20.8 ng/L, RQaverage = 0.06; maximal concentration: 65.8 ng/L, RQmax = 0.19). Also, acute effects were predicted for EDDP at average (6.42–47.0 ng/L,

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3.4.3 Occurrence and sources of residues of drugs of abuse in an urban aquifer: chemical analysis and solute transport modelling

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Since groundwater is an essential source of freshwater worldwide, its quality is highly important and regulated under several legislations. Despite growing concern about groundwater contamination by CEC, existing legislation does not cover these. However, several are already included in the EU voluntarily Groundwater Watch List, namely pharmaceuticals and PFAS. As representatives of CEC, drug residues also have the potential to deteriorate groundwater, a potential source of drinking water quality. So far, only a few studies addressed their occurrence in aquifers (Chapter 1.4.3.2 Groundwater). Accordingly, this study aimed to investigate the occurrence, distribution and potential sources of the residue of licit and illicit drugs in an urban aquifer located beneath the city of Ljubljana, Slovenia. For that purpose, municipal wastewater samples (n=28), samples from aquifer-recharging rivers (n=4) and groundwater samples (n=22) were analysed using SPE followed by RP-LC-MS/MS (Chapter 3.4.2 Removal of residues of psychoactive substances during wastewater treatment, their occurrence in receiving river waters and environmental risk assessment). In addition, the distribution of drug residues was predicted using a solute transport model.

Most commonly, nicotine (<45.7 ng/L), cotinine (<5.86 ng/L), HCOT (<0.528 ng/L) and benzoylecgonine (<0.572 ng/L) were detected. The more frequent occurrence of drug residues was observed in samples downgradient from the main urbanisation area, while they were mainly absent in samples obtained near the river. The result indicates that the leaky sewer system is the primary source of drug residue in the studied aquifer, while the river water that recharges the aquifer and contains drug residues does not significantly contribute to their presence. A good agreement between the measured concentrations and the predicted groundwater distribution was observed, suggesting that modelling can be used to predict the occurrence of drug residues in urban aquifers.

Even though low concentrations of drug residues were found in the study, their occurrence in groundwater and known pharmacological activity emphasize the importance of their inclusion into groundwater monitoring for a better evaluation of possible impacts on the environment and even human health.

The study results were presented at one scientific conference, *i.e.,* the 18th International Conference on Chemistry and the Environment.

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Occurrence and sources of residues of drugs of abuse in an urban aquifer: Chemical analysis and solute transport modelling

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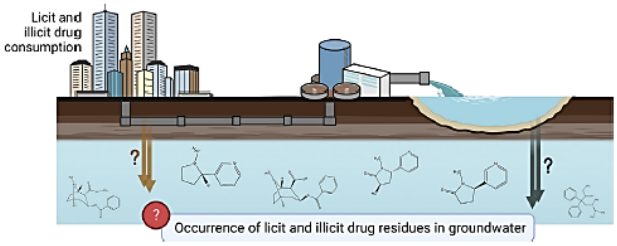
HIGHLIGHTS

- Sources and occurrence of drug residue in an unconfined aquifer were studied.
- Nicotine and cocaine metabolic residues were in groundwater in ng/L.
- Drug residues were more prevalent downgradient from urbanisation.
- Good agreement exists between measured and modelled data.
- A leaky sewer system is the primary source of drug residues in the aquifer.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the occurrence and potential sources of residues of drugs of abuse in an urban aquifer beneath the City of Ljubljana using water analysis and a solute transport model designed to predict nitrogen distribution. Samples were collected from three sources: 28 wastewater samples (24-h composites), 4 aquifer-recharging river samples (grab), and 22 groundwater samples. The samples were analysed for residues of commonly (ab)used licit drugs (nicotine and alcohol), medications of abuse (morphine, methadone, codeine, and ketamine), and illicit drugs (tetrahydrocannabinol – THC, cocaine, amphetamines, and heroin) using liquid-liquid (alcohol residue) and solid-phase extraction, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, we used solute transport modelling to predict the spatial distribution of drug residues in the aquifer and their potential sources. Nicotine (up to 45,7 ng/L), cotinine (up to 5.86 ng/L), trans-3′-hydroxycotinine (up to 0.528 ng/L) and benzoylecgonine (up to 0.572 ng/L) were the most commonly detected drug residues in groundwater, followed by cocaine (<LOQ). In comparison, methadone (0.054 ng/L) was detected only once. A higher prevalence of residues of drugs of abuse was observed in samples obtained at the south-eastern edge of the aquifer, downgradient from the main zone of urbanisation, agreeing with model predictions. Although drug residues were detected in river water, modelling suggests that the city's leaky sewer system is the primary source of drug residues.

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1. Introduction

Groundwater represents the primary source of fresh water for domestic, agricultural and industrial purposes globally (IAH, 2022). Compared to surface water, it is generally considered to be of good quality and usually requires only minimal treatment when used as a source of drinking water (Foster et al., 2013). However, population growth and urbanisation can significantly degrade groundwater quality, for example, through contamination with anthropogenic compounds (Li et al., 2021). In the European Union (EU), the chemical status of groundwater is regulated under the Water Framework Directive 2000/60/EC (Directive 2000/60/EC, 2000) and the Groundwater Directive 2006/118/EC (GWD 2006/118/EC, 2006). These directives provide a minimum list of substances, ions and indicators of human activity, e.g., arsenic, cadmium, lead, mercury chloride, ammonium, sulphate, nitrate, trichloroethylene and tetrachloroethylene, and parameters, such as conductivity, pH and oxygen content that must be regularly monitored in order to maintain groundwater quality. However, numerous other anthropogenic compounds, including emerging contaminants, have the potential to contaminate groundwater (Ebele et al., 2020; Durcik et al., 2023; Verovšek et al., 2022). These so-called emerging contaminants are substances detected in the environment but are not currently included in routine monitoring programs at the EU level and whose behaviour and ecotoxicological effects are not well understood (NORMAN, 2023).

Included among emerging contaminants are drugs of abuse and their metabolites (referred to collectively as drug residues), which are widespread and pseudo-persistent in the surface waters due to their continual consumption and release mainly through polluted wastewater effluent (Ebele et al., 2017; Pitarch et al., 2016; Verovšek et al., 2023; Yadav et al., 2017). Many substances have been shown to affect aquatic organisms at different trophic levels, even at environmental concentrations within the range of ng/L to μg/L (Bunch and Bernot, 2011; Capaldo et al., 2019). In response, several substances, namely cannabinol, cocaine and its metabolite benzoylecgonine, ephedrine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and tetrahydrocannabinol (THC), were considered for inclusion in the 4th Watch list under the Water Framework Directive in 2022 to gather sufficient data to evaluate risks more reliably (Gomez Cortes et al., 2022).

Less well studied are the levels of drug residues in groundwater but given their physicochemical properties that facilitate their transportation through water, they have the potential to enter groundwater through different pathways, such as leaky sewage systems, septic tanks, effluent discharged directly to the ground, and infiltration of effluent-polluted surface water (Estévez et al., 2012; Stuart et al., 2012). Upon entering groundwater, drug residues may subsequently enter finished drinking water, potentially posing a risk to human health (Wadley, 2016). Therefore, it is crucial to have a comprehensive understanding of the occurrence, transport, and fate of drug residues in groundwater for effective water resource management (Lapworth et al., 2019).

Notwithstanding this fact, conducting extensive groundwater analyses across an aquifer to obtain such data is often impractical and resource-intensive. As a result, research on this topic remains limited to only a few EU member states and the USA (Jurado et al., 2012; Lapworth et al., 2019; Verovšek et al., 2022; Yadav et al., 2017). As a possible solution, a modelling approach, such as a solute transport model developed for tracking nitrates in groundwater (Janža et al., 2020; Pietrzak, 2021), could be used as a support to estimate drug residue concentrations (and other pollutants), identify potential sources, and predict their transport within the aquifer.

In this study, we investigated the presence of 17 drug residues in an unconfined intergranular aquifer (Ljubljansko polje, Slovenia), serving 330,000 inhabitants of the city of Ljubljana and its surrounds (VOKA-SNAGA, 2019). We also analysed the aquifer-recharging river (Sava) and influent from the city's main wastewater treatment plant. Furthermore, we used a solute transport model to predict the sources and the distribution of drug residues in the aquifer.

2. Experimental

2.1. Compounds of interest

A total of 17 metabolic drug residues of licit drugs, medications of abuse and illicit drugs were targeted in the study. These included nicotine, cotinine (nicotine metabolite), trans-3′-hydroxycotinine (HCOT; nicotine metabolite) and ethyl sulphate (EtS; alcohol/methanol metabolite), morphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP; methadone metabolite), codeine and ketamine, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH, cannabis/THC metabolite), cocaine, benzoylecgonine (cocaine metabolite), cocaethylene (cocaine metabolite produced when co-consumed with alcohol), amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA – ecstasy) and 6-acetylmorphine (heroin metabolite). For additional data, see Supplementary Information (Tables S1 and S2).

2.2. Study area

The Ljubljansko polje alluvial aquifer is located below the mainly urbanised, low-lying area within the municipality of Ljubljana, Slovenia. Over half of the area is developed, while two-fifths are used for agricultural purposes (Rejec Brancelj et al., 2004). The aquifer comprises highly permeable Quaternary sand beds and gravel which are partially conglomerated. The thickness of these sediments varies from 2 to 100 m and is susceptible to pollution due to the high permeability of the deposits (Rejec Brancelj et al., 2004; Janža et al., 2020).

The aquifer is primarily recharged from the Sava River in the northwestern part and drains back into the river in the eastern part. The average water table depth is 25 m (Rejec Brancelj et al., 2004; Janža et al., 2020). The Ljubljana central wastewater treatment plant (Ljubljana WWTP) serves 270,305 people and is situated at the very far boundary of the aquifer (south-eastern part of the aquifer, Fig. 1). It discharges its effluent into the Ljubljanica River, which then flows into Sava downstream from the aquifer. This fact and given direction of flow in the aquifer, treated wastewater has limited potential to infiltrate groundwater in the studied area. Accordingly, only raw wastewater was examined in this study. The average exfiltration rate of raw wastewater from the entire sewer system was estimated to be 0.21 L/s/km (Pestotnik et al., 2019).

2.3. Sample collection

Raw wastewater samples were collected at the inlet of Ljubljana WWTP as time-proportional 24-h composites during one week in March–April over four consecutive years (2019–2022), while in 2021 samples were obtained in June (Table S3). Sampling was performed in the frame of Sewage analysis CORe group – Europe (SCORE) international monitoring on stimulants consumption (SCORE, 2023) and partially as part of a study on the occurrence of drug residues in waste- and environmental waters (Verovšek et al., 2023).

Four grab samples were also collected from the Sava riverbank at the northern part of the aquifer, which is the primary recharge area for the aquifer (Fig. 1). In order to obtain an idea of the temporal trends of drug residues in the Sava River, samples were collected randomly – one (RW1) in July 2022 and three (RW 2–4) in October 2022.

Groundwater samples (GW 15–22) were collected in June 2019 from eight observation wells in the south-eastern region of the Ljubljansko Polje aquifer adjacent to the Ljubljana WWTP. In addition, 14 groundwater samples (GW 1–14) were collected from observation wells distributed throughout the aquifer during October–November 2022 to determine the spatial pattern of drug residues in groundwater (Fig. 1). To ensure the collection of representative groundwater samples, the wells were purged by pumping out three well volumes before sampling. The obtained samples were then collected and frozen at −20 °C. The depth of the screen sections in the wells where sampling was conducted is presented in Table S4. However, obtaining representative depth-specific groundwater samples in

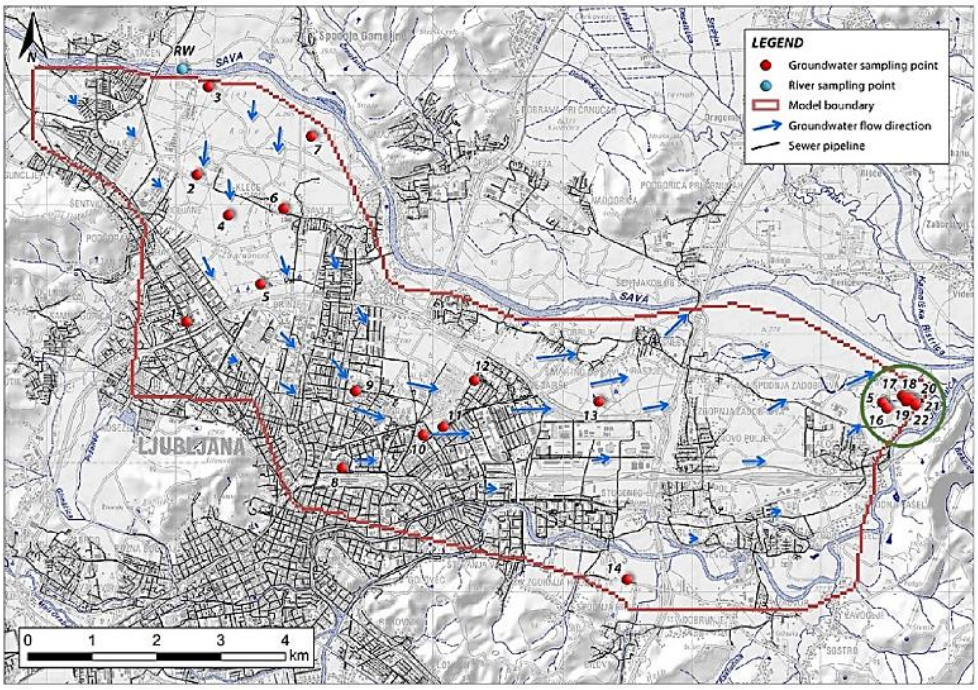

Fig. 1. Location of the Sava River sampling site (RW) and groundwater sampling points (1−22). Green circle: Location of Ljubljana WWTP.

unconsolidated and coarse-grained aquifers, such as the one in the study area, can be challenging due to the high risk of vertical circulations occurring in the annular space around the well screen or casing (Ducommun et al., 2013). Consequently, a multilevel sampling technique for collecting depth-specific groundwater samples was not employed, and the vertical distribution of the analysed drug residues within the aquifer was not addressed.

2.4. Sample preparation and analysis

Samples were analysed following the method of Verovšek et al. (2021, 2023). Briefly, samples were spiked with labelled internal standards of each analyte. Alcohol residues (EtS) were determined by extracting 1 mL of Waste-, surface and groundwater with acetonitrile using liquid-liquid extraction (LLE). All other residues were extracted from 125 mL (wastewater) and 0.5 L (surface and groundwater) of the samples using solid-phase extraction (SPE: Oasis MCX, 150 mg/6 mL; Waters, Milford, MA, US). Analysis was performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Two columns were utilised, namely the Supelco Ascentis® Express C18 (50 × 2.1 mm, 2 μm; Supelco, Pennsylvania, US) for THC-COOH determination and the Acquity UPLC HSS T3 (100 × 2.1 mm, 1.8 μm; Waters Corporation, Massachusetts, US) for the analysis of all other residues. Mobile phases comprised either 0.1 % formic acid/methanol to determine EtS, Milli-Q/methanol for THC-COOH or 0.1 % formic acid/0.1 % formic acid in methanol for all other compounds. Compounds were ionised using electrospray ionisation (ESI) in the positive mode except for EtS and THC-COOH, which were determined in the negative mode. All data were acquired using multiple reaction monitoring (MRM). Concentrations of analytes were obtained by internal quantification.

Performance of the method was evaluated in terms of linearity, limits of detection (LOD) and quantification (LOQ), relative extraction recovery, matrix effect, accuracy and repeatability (See Verovšek et al., 2023). Validation parameters were addressed by spiking artificial raw wastewater and tap water on at least two concentration levels. A linear response ($R^2 \geq 0.99$) was observed for most analytes between LOQ–1000 ng/L and LOQ–250 ng/L in artificial raw wastewater and tap water, respectively, with LOD/LOQ in the ng/L range. Poorer sensitivity was observed only in case of nicotine ($LOD_{raw\ wastewater}$ = 109–1170 ng/L and $LOQ_{raw\ wastewater}$ = 155–3340 ng/L) and alcohol ($LOD_{raw\ wastewater}$ = 739 ng/L, $LOQ_{raw\ wastewater}$ = 2420 ng/L; $LOD_{tap\ water}$ = 885 ng/L, $LOQ_{tap\ water}$ = 2950 ng/L) residues. Signal suppression or enhancement (−85 % to 46 %) was observed in both water matrices. Extraction recoveries were 68–112 %, except for ethyl sulphate, for which recoveries were lower (19–37 %).

2.5. Evaluation of nicotine sources

The ratio ($R_{NIC/COT+HCOT}$) between nicotine and its metabolites was calculated using Eq. (3):

$$R_{NIC/COT} = \frac{c_{NIC}}{c_{COT} + c_{HCOT}} \quad (3)$$

where c_{NIC}, c_{COT} and c_{HCOT} represent measured nicotine, cotinine and trans-3′-hydroxycotinine (HCOT) concentrations, respectively. Ratios were calculated only in samples with measured concentrations above the limit of quantification (LOQ). The calculated value was compared against the theoretical (0.186), which is the sum of the free and conjugated forms of nicotine (13 %), cotinine (30 %) and HCOT (44 %) excreted in the urine

(Castiglioni et al., 2015). When $R_{NIC/COT+HCOT}$ exceeds the theoretical value, the nicotine present in samples originates from other sources aside from consumption, e.g., washouts from cigarette butts (Rodríguez-Álvarez et al., 2014; Shao et al., 2021; Zheng et al., 2017).

2.6. Solute transport modelling

The solute transport model used in this study is based on the mass balance and pressure impact model developed by the Geological Survey of Slovenia (Janža et al., 2020). The model was developed to quantify the impact of a leaky sewer system and agriculture on the nitrate concentration in groundwater and to support the planning and optimisation of groundwater management measures to improve groundwater quality.

In this study, it is assumed that the simulated spatial distribution pattern of nitrate concentration in groundwater could also be used to interpret the behaviour of other substances in wastewater that act as conservative contaminants in the aquifer. As a simulation for drug residue loads, the nitrogen load only from the leaky sewer system (Fig. 2) was used as the input, as raw wastewater in this sewer system has been proven to contain measurable amounts of drug residues (Verovšek et al., 2021, 2023). Agricultural inputs were not considered since, in Slovenia, the application of sewage sludge (but not wastewater), although permitted for farming purposes (Decree 62/08 and 44/22 – ZVO-2, 2008), is strictly regulated and limited (ARSO, 2016). Neither retardation nor degradation processes were considered in the model.

2.7. Statistical analysis

Significant differences in the calculated $R_{NIC/COT+HCOT}$ between waste-, surface and groundwater samples were determined using univariate analysis (Mann-Whitney Rank Sum Test or Student's *t*-test). The normality of the data was tested using the Shapiro-Wilk test and the Equality of variance by the Brown-Forsythe test. The confidence level was 95 % ($\alpha = 0.05$). Patterns in spatial distribution drug residues were studied by applying unsupervised multivariate analysis (principal component analysis – PCA) at the 95 % confidence level ($\alpha = 0.05$), using UV scaling of the data. Statistical analysis was performed using SigmaPlot 14.0 and SIMCA 15.0.

3. Results and discussion

3.1. Occurrence in raw wastewater

All drug residues were detected at least once in raw wastewater (Table S5). High detection frequency of nicotine (tobacco) and alcohol residues was expected in wastewater due to their legality status and high prevalence in Slovenia, i.e., one in five adults (18–74 years old) smokes tobacco (NIJZ, 2021a). Slovenia is also among the leading countries within the European World Health Organization (WHO) in terms of registered alcohol consumption (10.6 L of pure alcohol was consumed per capita aged 15–64 in 2021) (NIJZ, 2021a, 2023). Moreover, they were in the highest concentration among the studied drug residues (average concentration range: 2420–29,600 ng/L), with levels similar to that reported in other studies,

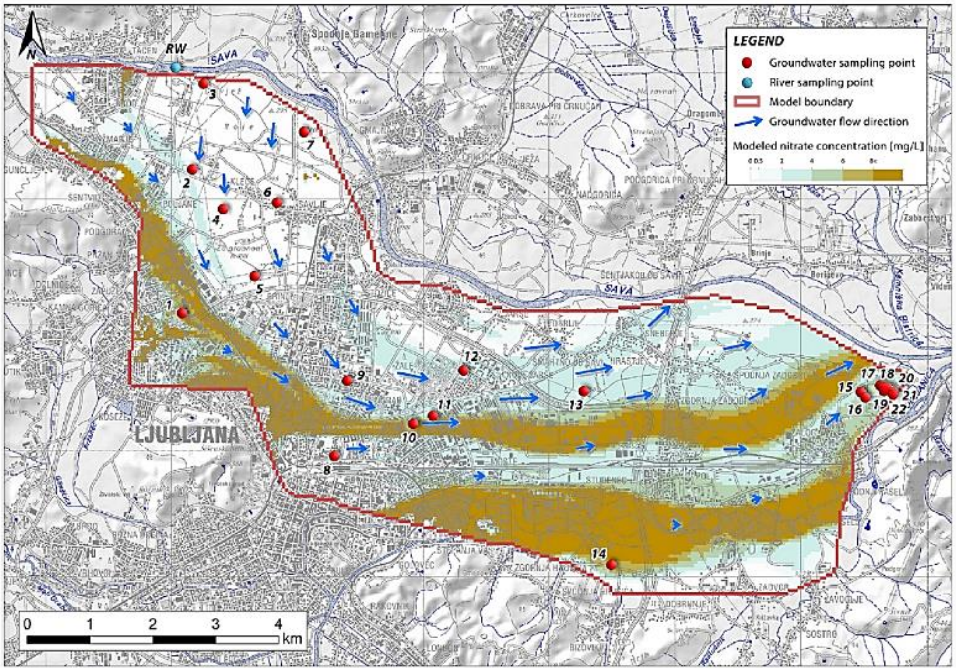

Fig. 2. Map showing the spatial distribution of modelled nitrate concentration in groundwater using nitrogen loads (Janža et al., 2020). The locations of river water (RW: blue dot) and groundwater sampling points (red dots) are also indicated.

i.e., <424,000 ng/L (nicotine), <42,300 ng/L (cotinine), 300–52,000 ng/L (HCOT) and 500–33,000 ng/L (ethyl sulphate) (Verovšek et al., 2022). Also, high concentrations are expected in the case of nicotine as its presence in wastewater may relate to nicotine use and discarded cigarette butts (Castiglioni et al., 2015; Roder Green et al., 2014; Verovšek et al., 2022). Indeed, the calculated $R_{NIC/COT+HCOT}$ (Table S6) exceeded the theoretical value by at least four times, confirming the presence of other sources of nicotine in the wastewater samples.

Medications of abuse were also detectable in wastewater. This finding is likely a result of the availability of parent compounds, i.e., methadone, morphine, codeine and ketamine, as prescription and over-the-counter medications (e.g., codeine) in Slovenia (NIJZ, 2019a). In fact, except for ketamine (detection frequency, DF = 48 %), residues of medications of abuse were detected in all wastewater samples in ng/L to low μg/L (Table S5), which agrees with the detection and concentration levels of these compounds in other studies (see review by Chen et al., 2021), i.e., <383 ng/L (methadone), <342 ng/L (EDDP), 19–1750 ng/L (morphine), 22–4780 ng/L (codeine) and <447 ng/L (ketamine). The levels of morphine, which was in our study determined in the highest concentration (820 ng/L), reflects its high prescription rate in Slovenia; morphine was the most prescribed opiate in 2019 and the third most prescribed in 2021 (NIJZ, 2019a, 2021b). Also, morphine can derive from the metabolism of other drugs, such as codeine and heroin (Gracia-Lor et al., 2017).

During the study period, all examined illicit drugs were known to be available on the Slovenian illicit drug market and reported to have been used in Slovenia (NIJZ, 2019b, NIJZ, 2021a). The highest average concentrations were obtained for benzoylecgonine (2630 ng/L), followed by cocaine (944 ng/L) and THC-COOH (526 ng/L). Compared to these amounts, the levels of the other drug residues were much lower (LOD–160 ng/L). Their residues in wastewater, in the ng/L–μg/L range (Table S5), also agree with those reported in other studies, i.e., <6160 (benzoylecgonine), <2710 (cocaine), <1270 (THC-COOH), and others mainly in ng/L range (see review by Chen et al., 2021).

3.2. Occurrence in Sava River

According to the literature, the primary source of the studied substances in river water is wastewater effluent, which is typically discharged from WWTP into rivers (Rodayan et al., 2016). Although there are no WWTPs in the studied area that discharge directly into the Sava River, treated (in total: 97.9×10^6 m^3) and untreated (in total: 57.8×10^6 m^3) wastewater enters the Sava River upstream from the studied location through wastewater-receiving tributaries (SiStat, 2023). Their concentrations were in the low ng/L range (Table 1), agreeing with those obtained in other river water studies (Chen et al., 2021; Verovšek et al., 2022). Nicotine residues, methadone residues and benzoylecgonine were detected in all river samples, while cocaine, MDMA and codeine were detected only once, with codeine being below the limit of quantification. The impact of sources of nicotine other than consumption (Table S6) was similarly pronounced in wastewater and the river ($U_{tst} = 26.0$, $p = 0.093$, $\alpha = 0.05$). This observation may be explained by sampling the Sava River in an urbanised area where rainwater, which washes nicotine from surfaces, is discharged into the municipal sewer instead of the river. Also, drug residues in the Sava River are based on grab sampling, which can only provide data on the presence of drug residues at the time and place of sampling. Regardless, it still provides data on the general occurrence of drug residues in the river, which was sufficient for this study.

3.3. Occurrence in groundwater

As already stated, several pathways exist by which drug residues can enter an aquifer, such as wastewater infiltration and effluent-receiving surface water. However, their actual occurrence in groundwater depends on soil characteristics, environmental parameters, transport phenomena (e.g., sorption and ion exchange), biodegradation and their physicochemical properties (Estévez et al., 2012; Stuart et al., 2012). Based on soil data (Section 2.2), the presence of drug residues in wastewater (Table S5) and

Table 1
Concentration (ng/L) of drug residues detected in samples from the Sava River and groundwater.

Matrix	Sample	Nicotine	Cotinine	HCOT	Cocaine	Benzoylecgonine	MDMA	Methadone	EDDP	Codeine
River water (Sava)	RW 1	7.24	2.25	3.78	<LOD	0.667	0.262	0.305	0.592	<LOD
	RW 2	3.36	1.06	2.90	<LOD	0.344	<LOD	0.061	0.218	<LOD
	RW 3	8.26	1.36	3.20	0.120	0.646	<LOD	0.054	0.165	<LOD
	RW 4	7.20	1.32	3.04	<LOD	0.458	<LOD	0.098	0.240	<LOQ
Groundwater	GW 1	4.44	<LOD	<LOD	0.110	0.224	<LOD	<LOD	<LOD	<LOD
	GW 2	4.36	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 4	0.834	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 5	5.50	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 6	0.504	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 8	2.40	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 9	0.486	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 10	8.25	<LOQ	<LOD	<LOD	0.139	<LOD	<LOD	<LOD	<LOD
	GW 11	9.04	0.111	<LOD	<LOD	0.320	<LOD	<LOD	<LOD	<LOD
	GW 12	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
	GW 13	<LOD	0.105	<LOD	<LOD	0.142	<LOD	<LOD	<LOD	<LOD
	GW 14	4.22	1.114	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	GW 15	1.09	0.214	0.169	<LOD	0.466	<LOD	<LOD	<LOD	<LOD
	GW 16	32.2	3.12	0.304	<LOQ	0.49	<LOD	<LOD	<LOD	<LOD
	GW 17	22.8	5.86	0.528	<LOQ	0.464	<LOD	<LOD	<LOD	<LOD
	GW 18	6.36	2.08	0.412	<LOD	0.284	<LOD	<LOD	<LOD	<LOD
	GW 19	20.8	3.78	0.22	<LOD	0.292	<LOD	<LOD	<LOD	<LOD
	GW 20	17.0	2.84	0.282	<LOD	0.302	<LOD	<LOD	<LOD	<LOD
	GW 21	45.6	2.86	0.528	<LOQ	0.46	<LOD	<LOD	<LOD	<LOD
	GW 22	11.2	1.29	0.228	<LOQ	0.572	<LOD	0.053	<LOD	<LOD
Detection frequency (DF) in groundwater samples:		82 %	55 %	36 %	27 %	59 %	0 %	4.5 %	0 %	0 %

The concentrations provided are from one sample measurement.
LOD_{COC} = 0.021 ng/L, LOD_{COT} = 0.022 ng/L, LOD_{EDDP} = 0.011 ng/L, LOD_{HCOT} = 0.12 ng/L, LOD_{MDMA} = 0.027 ng/L, LOD_{MTHD} = 0.0075 ng/L, LOD_{NIC} = 0.033 ng/L, LOQ_{COC} = 0.072 ng/L, LOQ_{COT} = 0.072 ng/L (Verovšek et al., 2023).
EDDP – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HCOT – trans-3′-hydroxycotinine, MDMA – 3,4-methylenedioxymethamphetamine.

the Sava River (Table 1) and their physicochemical parameters (Table S7): high hydrophilicity (log K_{ow} < 4) and water solubility (S_w > 8.4 mg/L), and generally low adsorption-desorption distribution coefficient (K_d < 300 L/kg)), it is expected that these substances will be present in the groundwater of the studied aquifer. Nicotine and cotinine residues were the most commonly detected (Table 1), a finding similar to published data (Focazio et al., 2008; Godfrey et al., 2007; Montesdeoca-Esponda et al., 2021; Stuart et al., 2012) where nicotine was reported to be highly prevalent (DF = 82 %, concentration: up to 45.6 ng/L. The $R_{NIC/COT+HCOT}$ value for groundwater (Table S6), unlike that calculated in the Sava River (t = 2.54, p = 0.0296, α = 0.05) and wastewater (U_{stat} = 0.00, p < 0.001, α = 0.05), indicates that sources other than nicotine consumption are more important in groundwater. This finding is supported by the fact that nicotine was detected in 32 % of groundwater samples, but metabolites were below the limit of detection (LOD). It is also interesting to note that cotinine but not nicotine was detected in one groundwater sample. While studies on nicotine degradation in soil and groundwater are unavailable, biodegradation may occur as bacteria (such as *Pseudomonas*) in the environment can break down nicotine into cotinine (Gurusamy and Natarajan, 2013). In the case of alcohol, ethyl sulphate remained undetected despite its presence in the wastewater influent (Table S5). However, the limit of detection (LOD) at 885 ng/L and the limit of quantification (LOQ) at 2947 ng/L of EtS are relatively high, which may contribute to its remaining undetected in the groundwater samples (Verovšek et al., 2023).

Given their low sorption properties (K_d < 300 L/kg, Table S7) and their presence in wastewater (Table S5) and river water (Table 1), it is expected that only morphine and codeine, among all medications of abuse studied, are detectable in the groundwater. According to literature data, codeine has been detected in Spanish (400 ng/L) and Nigerian groundwater (2443 ng/L) (Ebele et al., 2020; Teijon et al., 2010). Interestingly, in our study, only methadone was detected (0.053 ng/L) in one sample near Ljubljana WWTP (Table 1). However, methadone (7.4 ng/L), as well as its metabolite EDDP (0.4 ng/L), were present in the groundwater of Barcelona (Jurado et al., 2012).

Despite its ubiquitous presence in wastewater influent (Table S5), THC-COOH was not detected in groundwater (Table 1), agreeing with the findings of Jurado et al. (2012). The result may be explained by its high hydrophobicity and sorption properties (Table S7). Among stimulants, only cocaine (DF = 27 %, concentrations mainly below LOQ) and benzoylecgonine (DF = 59 %, concentrations up to 0.572 ng/L) were detected. Higher DF and concentrations of benzoylecgonine in comparison to cocaine are expected in environmental waters due to pronounced difference in urine excretion rate, i.e., 30 % vs 1–9 % of the cocaine dose (Gracia-Lor et al., 2016) and degradation of cocaine during percolation through soil (Lesser et al., 2018).

Although MDMA was detected in wastewater (Table S5) and Sava River water (Table 1), it was <LOD in groundwater samples (Table 1). The result is expected since MDMA is known to degrade during percolation through the soil by biotic and abiotic processes (Pal et al., 2011). Interestingly, MDMA was detected (3.9 ng/L) in groundwater in Spain (Jurado et al., 2012).

3.4. Sources of contaminants in groundwater

Evaluation of possible sources of drugs of abuse was performed using a modelling approach. Two primary sources of contamination were evaluated: the Sava River and the city's sewer system. According to relative modelled concentrations (Table S8), high concentrations of the substance system are expected to be found in groundwater samples downgradient from the urbanised area in the south-eastern part of the aquifer. Indeed, the highest number and concentrations of compounds were determined in the eastern part of the aquifer near Ljubljana WWTP (Fig. 3). The difference between those and other samples obtained throughout the aquifer was observed when applying PCA (Fig. S1), i.e., most of the samples obtained near Ljubljana WWTP (blue) are separated from samples obtained from the rest of the aquifer (orange).

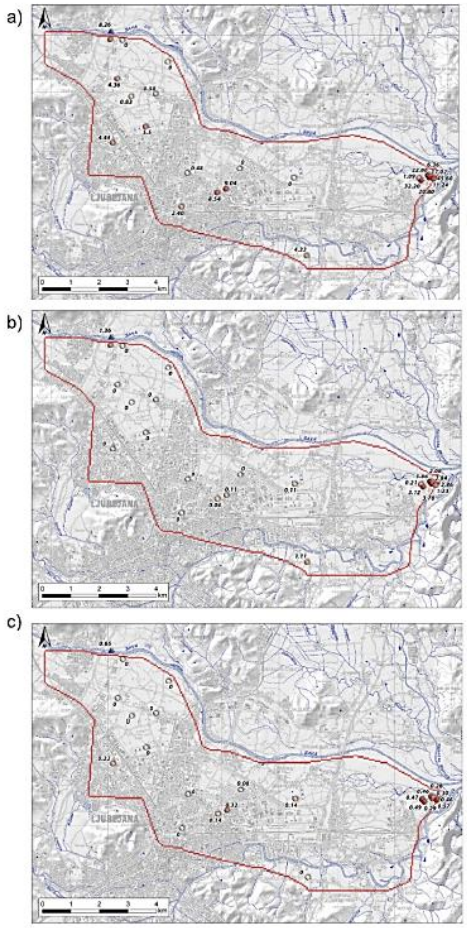

Fig. 3. Measured concentrations of nicotine (a), cotinine (b) and benzoylecgonine (c). Circles present groundwater sampling points and triangle Sava River sampling point (October 2022). The darker circles indicate higher measured concentrations. Value 0 denote <LOD.

Moreover, the model simulated higher concentrations of residues of drugs of abuse to be found in groundwater samples 1,10, and 14 (Table S8), as these sampling locations are in the area of greatest impact from contamination from the leaky sewer system. In alliance with simulation, samples 1, 10, 11 (one cluster) and 14 (second cluster) were grouped separately based on measured concentrations of drug residues (Fig. S2). More importantly, the modelled and experimental data show the absence of drug residues (including nicotine) in groundwater samples obtained near the Sava River (GW 3 and 7). The result indicates that despite being the primary source of recharge for the aquifer, the Sava River is unlikely to be the groundwater's primary source of these compounds.

Overall, a good agreement between modelled and measured concentrations were obtained, with a leaky sewer system being the primary source of

residues of drugs of abuse in the groundwater. The results also demonstrate how the solute transport model, assuming a correlation between the distribution pattern of nitrogen, anthropogenic pollutants and population density, can predict spatial distribution and potential sources of compounds in groundwater and aid in decision-making regarding groundwater management.

4. Conclusions

This study reveals the presence of drug residues in waste and river water at significant concentrations, while in groundwater, drug residues were in low concentrations (ng/L), with nicotine, cotinine, HCOT, and benzoylecgonine the most prevalent. Notably, the absence of detectable drug residues in samples obtained adjacent to the river indicates that the river is not a significant contributor to their levels in the groundwater. Moreover, higher groundwater pollution with drug residues was observed downgradient of groundwater flow, i.e., at the southeast edge of the aquifer near the WWTP, indicating that the city's leaky sewer system is their primary source. The study also found that solute transport modelling can be a reliable support in estimating the occurrence and distribution of drug residues in groundwater, as the experimental data aligned with predicted results. Additionally, valuable data on their sources can be obtained by modelling. Despite being detected in low concentrations, finding drug residues in groundwater along with their known pharmacological activity highlights the importance of their monitoring in water systems to understand better their sources and potential impacts on the environment and public health.

CRediT authorship contribution statement

Taja Verovšek: Conceptualisation, Methodology, Formal analysis, Investigation, Writing – Original Draft. Matija Janža: Conceptualisation, Methodology, Formal analysis, Investigation, Writing – Original Draft. David Heath: Conceptualisation, Methodology, Writing – Original Draft. Ariana Šuštarič, Methodology, Helena Prosen: Writing – Review & Editing. Ester Heath: Conceptualisation, Writing – Review & Editing, Supervision.

Data availability

Data will be made available on request.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164364.

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Chapter 4

Overall Conclusions

WBE applications in the Slovenian general population

Using licit and illicit drugs strongly affects public health. In order to efficiently deal with drug use and prevent its consequences, it is first necessary to obtain information on drug consumption trends in the population. Commonly used approaches like self-reporting surveys and medical and police reports have many weaknesses. To overcome these, other complementary approaches are needed.

Since its first application in 2005 (Milan, Italy), WBE has flourished and gained international recognition through the annual SCORE monitoring. Although monitoring began in 2011, Slovenia participated for the first time in 2017, providing data for Ljubljana, the capital of Slovenia. In the following year, data were provided for three Slovenian municipalities. However, the wastewater samples were analyzed by established SCORE partners, which meant developing an in-house analytical method was crucial for the permanent inclusion of Slovenia in SCORE monitoring. Accordingly, within the thesis, analytical methods were developed for determining licit and illicit drug residues in aquatic matrices using LC-MS/MS.

The development of these methods led to Slovenia's continuous participation in SCORE and the inclusion of the WBE approach in the national drug use monitoring scheme in Slovenia, where slight differences between wastewater analysis data and data obtained by other sources were observed. For example, survey data (2018) indicated a similar prevalence of cocaine, amphetamine and ecstasy among Slovenes aged 15-64, while SCORE 2019 data showed cocaine strongly prevailed. The difference in the obtained data is most likely the result of different methodologies, *i.e.,* surveys are conducted only every few years, have a time lag in reported data, and are often subjective, with respondents tending to underreport drug use. In contrast, wastewater analysis provides rapid and objective data and, as a complementary approach, enables a broader overview of the drug situation in the country, including NPS.

It can be seen from the thesis that using WBE can overcome problems associated with conventional data gathering on NPS, *i.e.,* discovering which NPS are present on the market without obtaining actual data on prevalence or use. With targeted analysis, the use of NPS in a targeted population can be quantified, while by introducing suspect screening into the workflow, rapid data on the identity of newly emerged NPS can be obtained. Accordingly, wastewater analysis tracking changes in NPS use has been developed in the context of an international study, which was carried out for the first time during the New Year period 2019/2020, and whose long-term purpose is to establish monitoring of the international proportion according to the example of SCORE.

This study covers Slovenia's inclusion in the third international NPS monitoring campaign (New Year period 2021/2022) by providing samples from three Slovenian municipalities. Eutylone, 3-MMC and mitragynine were detected in Slovenia, with 3-MMC having one of the highest mass loads detected within the study. Slovenia's participation is important as it provides timely data on NPS use that can support Slovenian public health, similar to SCORE monitoring. Since NPS monitoring is still in its infancy, further participation and evaluation of the methods and results are needed to reach the level of SCORE, *e.g.,* to form best-practice protocol and perform inter-laboratory studies.

Specific populations and vulnerable groups

Aside studying drug use in general populations, the non-invasive nature of WBE makes it highly suitable for studying specific/vulnerable populations, such as young people. In the case of young people, educational institutions have already been recognized as settings to study the motivations behind drug use. Drug trend data among young people are typically gathered through surveys and, in some cases, actual drug testing. Although both approaches pose some ethical issues, drug testing is especially problematic as it is intrusive and may cause long-term psychological and social harm without reducing drug use among routinely tested youth. Moreover, it may even encourage punitive actions rather than early prevention.

In order to augment the existing survey data, WBE has been employed in educational institutions. However, these previous studies primarily focused on a limited number of compounds (conventional illicit drugs, medications of abuse and NPS–targeted analysis) mainly in high education institutions. In this respect, this thesis represents a step forward by looking at the prevalence of residues of licit (alcohol and nicotine), medications of abuse and illicit drugs in the wastewater of 40 wastewater samples from different Slovenian educational institutions covering primary to tertiary levels and different geographic locations. In addition, 23 wastewater samples covering institutions of different types were screened for NPS. This work, therefore, presents the most extensive and comprehensive WBE study of its kind. Moreover, drug prevalence in primary schools was addressed for the first time, offering a unique insight into NPS and illicit drug presence in settings where such data have not been obtained even by surveys (there is only data for cannabis).

Licit drugs, medications of abuse (morphine and codeine), illicit drugs (most commonly cannabis and cocaine), and NPS (*e.g.,* unequivocally identified synthetic stimulants) were present in all types of educational institutions. Sampling was identified as a significant weakness of the study, *i.e.,* good knowledge of the sewer system was required to obtain wastewater only from the targeted educational institution. In contrast, the sampling strategy had to be adopted to avoid inconsistent wastewater flow. Also, obtaining wastewater from the entire institution meant it was impossible to differentiate between drug consumption in specific groups present at the institution, *i.e.,* to obtain data on drug use exclusively for pupils/students. Accordingly, a slight discrepancy was observed when comparing wastewater analysis data with data obtained from surveys, targeting exclusively pupils and students. Regardless, the data clearly showed that drugs are present even in environments otherwise considered "drug-free", such as educational institutions, emphasizing the need for drug monitoring in such settings. Also, they open up the possibility of using WBE as a non-invasive alternative to drug testing, not only for targeting the use of conventional illicit drugs but also for scanning for various NPS. Accordingly, the obtained data can also be used in establishing educational and prevention programs.

Based on the data obtained within this thesis, it can be seen that due to methodological differences in approaches, regardless of the population targeted, there will always be a

discrepancy between WBE data and the data obtained from conventional socio-epidemiological studies. Accordingly, **hypothesis 1 (H1): "Determining drug biomarkers in wastewater influent can confirm reported socio-epidemiological data on trends in drug consumption in Slovenian communities"** was **rejected**. Nevertheless, WBE still provides objective and rapid data on trends in drug use in the population, providing valuable and timely information on the drug situation on the national and international levels. However, specific data, such as data on consumption trends of individuals (*e.g.,* the average dose used, number of drug users in the catchment) and purity of drugs available on the illicit market, cannot be obtained using wastewater analysis as a stand-alone approach. Accordingly, WBE will continue to be used to complement other socio-epidemiological approaches.

WBE: Uncertainties in evaluating drug use

Methodologically, WBE is well established for studying illicit drug use in the general population (municipalities). A best practice protocol has been developed, and comparability of the results has been ensured within the SCORE inter-laboratory studies. Regardless, the approach still has shortcomings, *e.g.,* when parent compounds are selected as biomarkers, drug use may be overestimated due to the contribution of disposed unused drugs, waste from drug production or biomarker loads in wastewater. Without a suitable alternative, parent compounds are commonly used to estimate drug use even within SCORE, *e.g.,* for amphetamines. Therefore, additional analytical methods are needed to reduce such uncertainties, e.g., methods capable of identifying the disposal of unused drugs. The feasibility of two approaches that can predict the origin of biomarkers was studied as part of this dissertation: enantiomeric profiling and isotope-ratio mass spectrometry (IRMS) of light elements, more precisely, carbon.

Enantiomeric profiling using chiral LC-MS/MS has already been used to address the origin (consumption or disposal) of amphetamines in wastewater samples. The analysis has been performed on a limited number of samples with unusually high mass loads, and enantiomeric profiling based on chiral derivatization was investigated. The data showed that chiral derivatization is a suitable alternative to chiral LC for enantiomeric profiling of drug residues in wastewater. Enantiomeric profiling detected dumping events, but only when illicit drugs under investigation are available exclusively in racemic form (*e.g.,* MDMA). However, for drugs with a more diverse synthesis and excretion enantiomeric profile, such as amphetamine and methamphetamine, enantiomeric profiling could supplement WBE consumption data by providing information on drug potency and consequently propose the most probable synthesis route.

The possibility of tracing the origin of organic compounds to detect the disposal of unused drugs using the stable isotope approach combined with WBE was investigated for the first time in this dissertation. The isotopic composition of carbon ($^{13}C/^{12}C$) was analyzed in morphine extracted from analytical standards, street drugs (morphine and heroin) and wastewater. Although it was impossible to differentiate between consumption and disposal, the data shed light on a new area where stable isotope/wastewater analysis could potentially contribute to a better understanding of the drug market, *i.e.,* by complementing data obtained by drug profiling. It can be speculated that changes in the isotopic ratios of the light elements in drug residues present in wastewater could also provide an early warning system regarding changes in the supply of drugs on the illicit drug market. For example, in the case of natural and semi-synthetic drugs (e.g., cannabis, cocaine and heroin), GC-C-IRMS analysis of drug residues in wastewater may also be used to track changes in the geographic origin of plant material used for their production.

However, despite the progress reported in this thesis, more research is needed to support such a claim and take advantage of this potentially powerful new approach.

It can be seen that both enantiomeric profiling using chiral derivatization and stable isotope analysis can supplement WBE data. However, given that only enantiomeric profiling could distinguish between consumption and disposal in specific cases, *i.e.,* drugs with uniform synthesis and excretion profiles. Therefore, **H2: "Enantiomeric profiling and stable isotope-ratio analysis can differentiate illicit drug consumption and direct disposal"** was **partially confirmed**.

The ecological impact of drug residues in the aquatic environment

No doubt, licit and illicit drug use has a major impact on the individual and society, but its impact on the environment is no less important. On the contrary, cultivation, production and drug use have been shown to contribute to the overall carbon footprint and pollute water, soil and air, leading to biodiversity loss. In line with this and the fact that drug use is increasing worldwide, the UNODC also identified drug residues as a growing threat to the environment and encouraged the implementation of studies that address their occurrence in the environment and toxicity.

Considering the physicochemical properties of drug residues, *i.e.,* high hydrophilicity, the aquatic environment is considered the most vulnerable, with treated wastewater being the primary source of drug residues. Accordingly, many studies have assessed the removal efficiency of drug residues in conventional wastewater treatment processes, namely activated sludge, SBR, Bardenpho and MBR. However, the data's inconsistency and the lack of available data for otherwise commonly used drugs (*e.g.,* THC) and treatment processes (*e.g.,* moving bed biofilm reactor, MBBR) shows that removing drug residues during wastewater treatment is only partially understood. More studies are needed in light of that and the fact that the EU Directive on urban wastewater treatment foresees the establishment of limit values for CEC, including psychoactive substances in treated wastewater.

A significant part of this thesis focuses on the removal efficiency of drug residues in Slovenian WWTPs of different sizes and employing different treatment processes, namely activated sludge, SBR, SBR with UV disinfection, MBR and MBBR. Among studied treatment technologies, the efficiency of MBBR in removing drug residues was tested for the first time. Although, on average, most of the studied drug residues were effectively removed (>90 % removal), complete mineralization was not achieved, **confirming H3: "Conventional wastewater treatment technologies do not fully mineralize drug residues"**. Moreover, nicotine and cocaine residues were removed to a lower extent in MBBR than in other treatment processes, with comparable efficiency in removing drug residues. Drug residues were also found in various amounts in effluent-receiving rivers, reflecting their degree of removal during treatment.

The widespread occurrence and pseudo-persistence of drug residues in surface waters such as rivers, lakes and seawater (received by wastewater) is problematic as it has been shown that drug residues can harm aquatic organisms from bacteria to vertebrates. The ubiquity and potential ecotoxicity of drug residues have also led to the consideration of seven drug residues for inclusion in the 4th Watch list under the Water framework directive in 2022, namely cannabinol, cocaine, benzoylecgonine, ephedrine, methamphetamine, MDMA and THC. However, more data are needed on the occurrence and ecotoxicity of these and other drug residues at the environmental level to evaluate the risks they pose to aquatic ecosystems properly.

This thesis investigated the ecotoxicity of residues of commonly abused licit and illicit drugs using *in vivo* (algal growth inhibition test) and *in silico* (ECOSAR) methods. The

impact of drug residues on green algae (*Chlamydomonas reinhardtii*) as a representative of the primary producer in the aquatic food web was tested for the first time. No effect on algae growth was observed at a concentration level (1 mg/L) considered high compared to expected concentration levels in surface waters (literature data) and measured levels in Slovenian effluent receiving rivers (range ng/L). However, only one type of algae was tested, and the effect may still be seen in other types. Especially since a negative effect at concentration 1 mg/L was predicted *in silico* (ECOSAR) for EDDP and THC-COOH.

In environmental risk assessment, ECOSAR was also used to evaluate risks posed by the levels of drug residues detected in Slovenian effluent receiving rivers. At the measured concentrations, the effect on aquatic organisms was predicted for nicotine, methadone, EDDP, morphine, and MDMA, which leads to the **partial rejection** of **H4: "Levels of drug residues in studied receiving river waters do not pose a risk to the aquatic organisms"**, *i.e.,* some drug residues may pose a risk to the aquatic organisms at levels measured. Considering *in silico* data, further *in vivo* studies would be warrented.

The ubiquitous presence in surface waters and their known pharmacological effects indicate that drug residues have a high potential to affect human health, for example, through exposure to polluted drinking water. For many people, groundwater is their vital, high-quality source of drinking water. For this reason, its quality is regulated at the EU level under various directives, which do not currently cover CEC that are otherwise increasingly present in the environmental waters. Such CEC are also drug residues, among which cocaine, despite the limited amount of data (data available only for groundwater of some member states within the EU), is already considered to be frequently present in the EU groundwater and has therefore been proposed for inclusion in the volunteer Groundwater Watch List (GWWL). However, the low amount of data reduced its priority for inclusion, indicating the need for additional studies addressing the occurrence of cocaine (as well as other drug residues) in groundwater.

In this thesis, drug residues were studied for the first time in an unconfined intergranular urban aquifer (Ljubljansko polje, Ljubljana, Slovenia) using both chemical analysis and a solute transport modelling approach. Among targeted drug residues, nicotine and cocaine residues were the most commonly detected. However, the significant result was that chemical analysis confirmed the model, *i.e.,* that residues were distributed downgradient from the main urbanized area, indicating that the leaky sewer system is their primary source in the studied aquifer. The data indicate that although wastewater effluent is commonly considered the primary source of drug residues in the environment (including groundwater), raw wastewater (sewage leakage from the sewer system) should also be considered when addressing urban aquifers.

To summarize, determining drug residues in waste and environmental waters provides data on epidemiological and environmental aspects of drug production and use, which are closely related and require a multidisciplinary approach when being studied. This thesis used different methods, *i.e.,* chemical analysis, ecotoxicity tests modelling, and assessing risks in order to (i) determine drug residues in aquatic matrices, (ii) estimate licit, illicit drug and NPS use in general and specific populations in Slovenia utilizing WBE, (iii) supplement obtained WBE data by providing information on the origin of drug residues, (iv) evaluate removal efficiency of drug residues during various conventional biological wastewater treatments, (v) determine the occurrence of drug residues in environmental waters and (vi) to evaluate associated risks.

Overall, the work represented by this thesis has made significant contributions to both science and society. Importantly, it has provided timely data on changes in spatiotemporal trends in licit and illicit drug use in Slovenia, augmenting our understanding of drug use trends at the international level.

Additionally, it has comprehensively assessed the application of target and non-target wastewater analysis to small, vulnerable populations, such as educational institutions. Notably, it provides insight into the presence of drugs in institutions offering different levels of education, like primary schools, where it is difficult to ethically gather data on illicit drug use using conventional approaches, e.g., surveys. The dissertation has also made strides in assessing the disposal of unused drugs by performing enantiomeric profiling through chiral derivatization. Furthermore, it marks the novel approach of combining the stable isotope approach (using IRMS specifically to obtain $\delta^{13}C$ of drug residues) with WBE to supplement drug profiling data.

It has also provided additional data on removing drug residues during wastewater treatment, specifically for the first time, their removal in an MBBR. Moreover, it has evaluated the prevalence of drug residues in surface waters and assessed associated environmental risks. The thesis has also explored the potential effects of drug residues on green algae—primary producers in the aquatic food web. Lastly, it has investigated (water analysis and modeling) and showed the presence of drug residues in groundwater within an aquifer, an essential source of freshwater.

These contributions are documented in ten peer-reviewed scientific publications, three national reports, and nine working reports. Furthermore, the research findings have been presented at nine scientific conferences (both orally and in writing) and disseminated to the public through more than 15 events, including interviews, articles, and popular science lectures.

However, it is essential to emphasize that this work is far from complete and opens up new avenues of research in wastewater epidemiology. These avenues include researching the application of stable isotopes and further investigation of the environmental impact of drug residues.

Chapter 5

Future Perspectives

Methodological improvements

Although the WBE has been used to study licit and illicit drug use for nearly two decades and is already well-established for studying trends in most common drug use in the general population, several aspects of the approach require further research. The first is the methodology. More studies are needed to identify new biomarkers and evaluate their applicability, *i.e.,* assess their pharmacokinetics and stability to overcome the uncertainties associated with using parent compounds as biomarkers, as in the case of using pholedrine to assess methamphetamine use. This data is also needed for quantifying NPS in wastewater (*e.g.,* in the frame of international NPS monitoring) since data on the metabolism and excretion of NPS is currently limited, which makes estimating their consumption difficult.

Additional research is also needed to reduce the uncertainty related to estimating the size of the targeted population. Although many attempts have already been made to reduce this uncertainty by evaluating fluctuations in population, proposed methods, namely the use of mobile phone trace data and the determination of hydro-chemical parameters, remain unsatisfactory. For example, mobile phone trace data are not readily available in all countries (including Slovenia), while hydro-chemical parameters, such as COD and BOD, reflect human metabolism and industrial and agricultural activities. Accordingly, studies shifted to specific population biomarkers (*e.g.,* metabolites such as cotinine or hormones such as cortisol) research, and this area of research is expected to expand further. Especially since currently proposed population biomarkers show shortages, such as low in-sewer stability and variability in excretion rate.

WBE: applications in general populations

Assuming further support (*e.g.,* by EMCDDA), SCORE monitoring is expected to continue to provide data on trends in drug use internationally since, contrary to surveys, it provides timely and objective data that can track changes yearly, which can be used as an early warning of changes in drug use. In agreement with data obtained by other sources, shifts in targeted drugs can be predicted following the example of ketamine, which was included in the monitoring for the first time in 2022 based on indications of increasing illicit use. Similarly, international monitoring of NPS is expected to continue, within which best practice protocols and interlaboratory studies will need to be developed. Slovenia's continued participation in SCORE and NPS monitoring is beneficial from both national and international perspectives. Accordingly, in the New Year period 2022/2023, Slovenia continued participating in NPS monitoring by providing samples from seven municipalities. However, future participation depends on funding, which is currently funded by ARIS

project N1-0143, "Novel approaches for the estimation of the use of psychoactive pharmaceuticals and illicit drugs by wastewater analysis".

In the future, the use of wastewater analysis is expected to continue to expand into other fields besides estimating population drug use. As demonstrated in this thesis, determining the isotopic composition of light elements in drug residues present in raw wastewater, once expanded upon, may be used to support forensic analysis to complement the field of drug profiling since the analysis shows potential for revealing changes in illicit drug market supply. This thesis, which for the first time determined the $\delta^{13}C$ in morphine present in wastewater, highlighted many areas for improvement. Of particular importance would be simplifying the extraction of drug residues from large volumes of wastewater, *i.e.,* by studying the applicability of passive sampling and expanding the analysis to other isotopes of light elements commonly used to track the origin of organic compounds (nitrogen, oxygen and hydrogen). Any future work will also require a study of potential isotopic fractionation that could bias the results and build a suitable isotopic database of authentic samples.

Another promising application of WBE is using wastewater analysis to assess the spread of viruses (and other diseases) in the population, as has already been exploited during the COVID-19 pandemic. Furthermore, WBE can be used to explore the exposure of a population to compounds that are not intended for human consumption (*e.g.,* pesticides, plasticizers and other household and industrial chemicals) and, in that way, complement HBM (no such study has been done in Slovenia so far). Also, such application is currently tested within an ongoing Partnership for the Assessment of Risks from Chemicals (PARC) project, which is co-funded by the EU and aims to assess safety challenges of well-known, emerging and novel pollutants in line with EU goals towards a pollution-free environment.

WBE: applications in specific populations

Many gaps still need to be addressed when applying WBE to study drug use in specific populations. First, difficulties in wastewater sampling (adjusting to inconsistent wastewater flow) must be addressed to obtain a representative sample and provide good insight into the type and extent of the drug used in a particular isolated population. As an alternative to active sampling, passive and active-passive samplers, *i.e.,* samplers, which use a pump to draw water through the sampler and accumulate targeted compounds onto the sorbent, can be tested for this purpose. However, even in the case of passive samplers, a good knowledge of the sewage system and sampling strategies is required, as passive samplers must be immersed/exposed to wastewater at all times (not to dry out), which is often problematic in case of sampling wastewater from small sub-catchments.

Sampling only a part of a certain sub-catchment or facility should also be explored, as it may offer insight into drug use in a specific part of the targeted population. For example, in educational institutions, sampling wastewater from the entire facility means that the detected drugs can be related to their use in any group of the people present in the institution, *i.e.,* pupils/students, staff or visitors. By exploiting the existing sewerage infrastructure for the targeted collection of wastewater from toilets intended exclusively for pupils/students, the WBE data on drug use in the younger population obtained in this way could be extrapolated to drug use in the younger population. They would better complement the epidemiological data on drug use in this age group. Also, such a setting may allow studying differences in drug use between men and women.

Aside from methodology, an effort should be made to explore possibilities of implementing wastewater analysis in specific populations as a non-invasive alternative to existing methods, *e.g.,* drug testing, should be made. In the case of educational institutions, this would probably mean long-term monitoring of drug use in educational institutions by

analyzing wastewater and assessing positive and negative outcomes, such as controlling and regulating drug use in environments considered "drug-free".

In addition to educational institutions, recreational and professional athletes are one such setting where wastewater analysis can be used to track drug misuse. Currently, doping control is based on drug testing, which is, due to the nature of the method (random testing), only able to spot drug misuse of individuals and cannot fully capture trends in drug misuse in sports. Contrarily, wastewater analysis can be used as a screening test for first doping detection, which can be used as an orientation for further testing. However, since no such applications exist, ethical considerations, *e.g.,* stigmatization of the whole group tested by wastewater, should be carefully considered. In addition to spotting drug misuse, wastewater analysis applying suspect screening or a non-target approach could be used to detect trends in newly abused psychoactive substances in sports.

Drug residues in the environmental waters and associated risks

Despite what this thesis has achieved, additional studies are needed to assess the occurrence of psychoactive substances in environmental waters. Alternatives to grab sampling, which is the most commonly applied technique to sample surface waters, need further exploration, *e.g.,* passive and active-passive sampling. The problem is that grab sampling can only provide data on the occurrence of substances in studied water at the time of sampling, and no time-dependent data on their occurrence can be obtained. Also, studies looking into an expanded list of psychoactive drugs and their metabolites in environmental waters are needed to evaluate further studies addressing environmental risk, *e.g.,* within the Watch List or Groundwater Watch List under the Water Frame Directive. Also, suspect screening and non-target analysis should be considered to broaden the list of compounds that may occur in the environment but remain undetected by targeted analytical approaches. Conducting studies on drug residues in environmental waters is also needed in developing countries and regions with limited wastewater treatment. Here, the absence of treatment, resulting in higher concentrations of drug residues entering the environment, poses a significantly greater risk to ecosystems and human health.

Aside from aquifers, it would be interesting to study the possible effects of drug residues in other subterranean water ecosystems, such as caves, since they represent sensitive and fragile ecosystems, especially since Slovenia is famous for its karst regions. Similar regions also exist around the world. However, to fully understand the transport, distribution and fate of drug residues in the subsurface and *spelio* aquatic environments, the stability of compounds during transport through the soil and within water bodies needs to be addressed.

Furthermore, studies evaluating the impact of drug residues on wildlife are also warranted since their effects on aquatic organisms at environmental levels and no-effect concentrations remain unknown. Considering that and the fact that many drug residues are chiral molecules, for which it is known that enantiomers exhibit different toxicity, a significant avenue of research would involve estimating stereoselective toxicity for adequately assessing the environmental risks posed by drug residues. Moreover, the ecotoxicological test should be performed for subterranean organisms of various trophic levels since no or only a minimal amount of such data exists, although such organisms are especially important for the fragile karst ecosystem. Finally, more studies should also be conducted on determining the occurrence of drug residues in drinking water and assessing the risk they pose to human health.

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T. Verovšek, I. Krizman-Matasic, U. Blaznik, A. Hočevar-Grom, T. Kosjek, E. Heath, "Wastewater-based assessment of drug consumption in Slovenia" in M. Jadl, A. Hočevar-Grom, A. Drev, A. Belščak Čolaković: Report on the drug situation 2020 of the Republic of Slovenia, National Institute of Public Health, Ljubljana, 2020, pp. 64-69. ISSN 1855-8003. https://www.nijz.si/sites/www.nijz.si/files/publikacije-datoteke/np_2020_ang_15_12_20_obl-1.pdf.

Conference Contribution Abstracts

T. Verovšek, M. Janža, D. Heath, A. Šuštarič, H. Prosen, E. Heath, "Residues of drugs of abuse in an urban aquifer: chemical analysis and solute transport modelling" in: *ICCE 2023, the 18th International Conference on Chemistry and the Environment*, Venice, Italy, 2023;

T. Verovšek, I. Krizman-Matasic, A. Celma, L. Bijlsma, F. Hernández, D. Heath, E. Heath, "Addressing the occurrence of drugs of abuse and new psychoactive substances in educational institutions using wastewater analysis" in: *ICCE 2023, the 18th International Conference on Chemistry and the Environment*, Venice, Italy, 2023;

T. Verovšek, A. Šuštarič, M. Laimou-Geraniou, Maria, 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, D. Heath, E. Heath, "Chiral derivatisation with gas chromatographic-tandem mass spectrometric detection: enantiomeric profiling of amphetamines in wastewater" in: *ISSS 2022, the 26th International Symposium on Separation Sciences*, Ljubljana, Slovenia, 2022;

A. Šuštarič, T. Verovšek, H. Prosen, T. Eleršek, E. Heath, "Determination of selected licit and illicit drug residues in surface waters and their impact on green algae *Chlamydomonas reinhardtii*" in: *ISSS 2022, the 26th International Symposium on Separation Sciences*, Ljubljana, 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;

T. Verovšek, I. Krizman-Matasic, V. Kramarič Zidar, M. Stražar, M. Levstek, B. Cimrmančič, S. Lukšič, N. Uranjek, U. Blaznik, A. Hočevar-Grom, A. Celma Tirado, L. Biljsma, F. Hernández, D. Heath, E. Heath, "Assessing the prevalence of licit drugs, medications of abuse and illicit drugs in educational institutions using wastewater analysis" in: *Testing the Waters 5 Conference 2021*, Brisbane, Australia, 2021;

T. Verovšek, I. Krizman-Matasic, D. Heath, E. Heath, "Wastewater Analysis Assessment: Prevalence of Drugs of Abuse in Educational Institutions" in: *EMEC 21, the 21st European Meeting on Environmental Chemistry*, Novi Sad, Serbia, 2021;

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, material science, Ljubljana, Slovenia, 2021;

I. Krizman-Matasic, T. Verovšek, A. Hočevar-Grom, U. Blaznik, A. Drev, E. Heath, "A wastewater based epidemiological study of licit and illicit drugs among schoolchildren and students in Slovenia" in: *Lisbon addictions 2019: the 3rd European Conference on Addictive Behaviours and Dependencies*, Lisbon, Portugal, 2019;

T. Verovšek, I. Krizman-Matasic, T. Kosjek, E. Heath, "Development and application of the method for determination of selected licit and illicit drugs biomarkers in wastewater" in: the *11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day*, Planica, Slovenia, 2019.

Reports

E. Heath, T. Verovšek, "Poročilo analiz vzorcev odpadnih vod na vsebnost prepovedanih in dovoljenih drog na področju centralne čistilne naprave Kranj", IJS Work report, 2023;

E. Heath, T. Verovšek, "Poročilo analiz zbranih vzorcev odpadnih vod na vsebnost prepovedanih in dovoljenih drog na področju centralne čistilne naprave Domžale – Kamnik", IJS Work report, 2022;

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Public Dissemination

- **Interview:** V vodi puščamo prstne odtise (*Eng.*, Fingerprints in water), Delo, 65 (118), 13, ISSN 0350-7521, May 2023;
- **Article:** Ostanki drog motijo življenje: čistilne naprave ne odstranjujejo ostankov zdravil (*Eng., Drug residues affect life: drugs are not efficiently removed by wastewater treatment plants*), Misterij, 30, 358, 31-32, ISSN 1318-1777, May 2023;
- **Interview:** Mamila v odpadnih vodah: narašča uporaba kokaina (*Eng., Illicit drugs in wastewater: increasing use of cocaine*), RTV Slovenia 1, Poročila ob 13h, 2 April 2023;
- **Interview:** Epidemiologija odpadnih vod (*Eng., Wastewater-based epidemiology*), Radio 1, prvi program, Aktualna tema, 31 March 2023;
- **Interview:** Droge v odpadnih vodah (*Eng.*, *Drugs in wastewater*), TV Slovenia 1, Dnevnik 1, 31 March 2023;
- **Interview:** Droge v odpadnih vodah (*Eng.*, *Drugs in wastewater*), TV Koper, Primorska kronika, 31 March 2023;
- **Article:** Uporaba kokaina v Novem mestu narašča (*Eng., The use of cocaine in Novo mesto is increasing*), Dolenjski list, e-publication, ISSN 1581-0550, 29 March 2023;
- **Interview:** Razširjenost drug po Sloveniji (*Eng., The prevalence of drugs in Slovenia*), Kanal A, Svet na Kanalu A, 28 Marec 2023;
- **Interview:** Analiza odpadnih voda (*Eng., Wastewater analysis*), POP TV, 24ur, 28 March 2023;
- **Interview:** 20 najviše uvrščenih mest po porabi kokaina (*Eng., 20 highest ranked cities based on cocaine consumption*), Planet TV, 28 March 2023;
- **Article:** Razširjenost dovoljenih in prepovedanih drog: rezultati analize odpadnih vod slovenskih izobraževalnih institucij (*Eng.*, *Prevalence of licit and illicit drugs: wastewater analysis data from Slovenian educational institutions*), Ventil: revija za fluidno tehniko in avtomatizacijo, no. 27(6), pp. 374-376, ISSN 1318-7279, December 2021;
- **Article:** Odpadne vode ne prikrivajo (*Eng., Wastewater does not hide*). Novice IJS (News JSI), no. 199, pp. 12-15, ISSN 1581-2707, December 2021;
- **Interview:** Odpadne vode razkrivajo razširjenost drog (*Eng., Wastewater reveals drug use*). Delo, no. 239, pp. 14, ISSN 0350-7521, 14 October 2021;
- **Interview:** Trde droge v odpadnih vodah šol (*Eng., Hard drugs in school wastewater*): Kanal A, Svet na kanalu A (*Eng., World on Channel A*), 9 October 2021;
- **Interview:** Droge v izobraževalnih institucijah v Sloveniji (*Eng., Drugs in Slovenian educational institutions*): Znanost na cesti (*Eng.*, *Science on the street*), 30 September 2021;
- **Interview:** Droge med mladostniki (*Eng., Drug use in young population*): Znanost na cesti (*Eng., Science on the street*), 30 September 2021;
- **Interview:** Epidemija odvisnosti (Eng.*, Addiction epidemic*): RTV Slovenija 1, Tednik (*Eng., Weekly*), 24 May 2021;
- **Interview:** Ostanki mamil v odpadnih vodah (*Eng., Drug residues in wastewater*): RTV Slovenija, oddaja 1. Dnevnik, 26 March 2019.

Biography

Education and Research Activities

- **2018–Present:** Jožef Stefan International Postgraduate School, Ljubljana: Doctoral study program Ecotechnologies;
- **2013–2016:** University of Ljubljana, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia: Master's degree in Analytical chemistry. *Thesis title: Določanje hlapnih spojin v propolisu (Eng., Determination of volatile compounds in propolis)*;
- **2010–2013:** University of Ljubljana, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia; Bachelor in Analytical Chemistry. *Thesis title: Določanje kovin v medu (Eng., Determination of metals in honey*).

Foreign Work and Study Experiences

- **2021** (1 month): Institut Universitari de Plaguicides i Aigües (IUPA), Universitat Jaume I, Castellón de la Plana, Spain

Awards, Scholarships and Grants

- **2023:** Ph.D. Students and young Post-docs grant; 18th International Conference on Chemistry and the Environment (ICCE) 2023, Venice, Italy;
- **2022:** The award for the best oral presentation by a young researcher; 26th International Symposium on Separation Sciences (ISSS) 2022, Ljubljana, Slovenia;
- **2021:** Meeting scholarship funded by the Association of Chemistry and the Environment (ACE); European Meeting on Environmental Chemistry (EMEC) 2021, Novi Sad, Serbia;
- **2019:** The award for the best presentation of research achievements in terms of scientific quality and their applicability; 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day.

Research Projects

- Project L1-9191 (Illicit drugs, alcohol and tobacco: wastewater-based epidemiology, treatment efficiency and vulnerability assessment of water catchments);
- Project FrAPI (Funkcionalni dodatki v hrani in krmi: Antioksidacijski Potencial in Izvor)
- Project N1-0143 (Novel approaches for the estimation of the use of psychoactive pharmaceuticals and illicit drugs by wastewater analysis)

Peer Review

- **2021-Present:** Reviewer for Environmental Science and Pollution Research