REMOVAL OF CONTAMINANTS OF EMERGING CONCERN USING ALGAL PHOTOBIOREACTORS

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Doctoral Dissertation
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ODSTRANJEVANJE PRIORITETNIH ORGANSKIH ONESNAŽIL V ALGNIH FOTOBIOREAKTORJIH

Doktorska disertacija

Supervisor: Prof. Dr. Ester Heath

Ljubljana, Slovenia, August 2021

It is change, continuing change, inevitable change, that is the dominant factor in society today. No sensible decision can be made any longer without taking into account not only the world as it is, but the world as it will be.

Isaac Asimov

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Dragi prijatelji, ki stojite mi ob strani.

To je tudi vaše delo.

Abstract

Contaminants of emerging concern (CEC) are organic pollutants occurring in wastewater and consequently enter the environment. An alternative to conventional wastewater treatment are algal photobioreactors, which treat wastewater with low energy input while producing nutrient- and energy-rich algal biomass and treated water, which both have the potential to be reused. Literature review yields knowledge gaps about the removal and fate of CEC in algal photobioreactors. Also, further studies are needed to establish risk assessment, future regulation and the safe reuse of algal biomass and reclaimed water in a circular economy. The goal of this thesis was to study CEC, including pharmaceuticals and personal care products, pesticides and bisphenols in laboratory and outdoor algal photobioreactors. For this reason, existing GC-MS and LC-MS/MS based methods were modified to analyse selected CEC in the aqueous phase of algal photobioreactors, and GC-MS/MS methods for the analysis of the aqueous and biomass phase were developed.

The removal and partitioning of 18 bisphenols in laboratory-scale photobioreactors were studied. The overall removal after 13 days was similar in both experiments: 72 ± 2% and 73 ± 5% removed in pH non-adjusted and pH adjusted series, respectively. Total removal ranged from 25 ± 5% to ≈100% and correlated with Chlorophyll *a* and compound log P. Culture pH also governed the removal of 2,2'-BPF, BPC2, BPAF, BPA and BPC. The significance of this work is in providing insight into the removal and effects of physicochemical parameters and pH on the fate of CEC in algal photobioreactors.

Furthermore, the removal of 28 CEC was compared between a mixed culture of algae and bacteria and a pure algal culture. The removal of bisphenols was higher in the mixed culture (87 ± 5%) than the pure culture (55 ± 7%), while similar removals (85 ± 28% and 79 ± 23%) were obtained for pharmaceuticals. For pesticides, removal (64 ± 21% and 90 ± 11%) was higher in the pure culture. In addition, transformation products of ibuprofen and metoprolol were also detected in both cultures. The importance of this study is in the comparison of 28 CEC removal between a mixed culture and a pure algal culture.

Finally, a pilot-scale high-rate algal pond treating real wastewater containing selected CEC was studied. Removal of BPA was 80%, whereas BPS proved more recalcitrant with an average removal of 32%. Methylparaben, 2,4-dihydroxybenzophenone and oxybenzone reached 92%, 76% and 80% removal, respectively. The most abundant compounds were naproxen and ibuprofen, but their removal efficiency was only 60% and 51%, respectively. This study is novel as it provides information on the removal of CEC estimated under realistic conditions, vital for developing algal photobioreactors for wastewater treatment.

Findings show that algal photobioreactors are a promising way to remove CEC from wastewater. Most CEC removals in algal photobioreactors are comparable to conventional biological wastewater treatment, while algal photobioreactors offer the potential of water and biomass reuse. That said, the work remains ongoing as some CEC remain recalcitrant (e.g. BPS, carbamazepine), and optimisation of operating parameters, along with the development of suitable pre- or post-treatment, is vital. The future of efficient wastewater treatment with algal photobioreactors and regulation depends on the continued study of CEC and their occurrence, removal, fate, and toxicity.

Povzetek

Prioritetna organska onesnažila so snovi, ki se nahajajo v odpadni vodi in z iztoki iz čistilnih naprav vstopajo v okolje. Alternativno biološko čiščenje predstavljajo algni fotobioreaktorji. Ti z minimalnim vložkom energije poleg očiščene vode proizvajajo algno biomaso, oboje pa ima potencial za ponovno uporabo. Pregled literature je pokazal pomanjkljivosti v poznavanju učinkovitosti odstranjevanja in kroženja prioritetnih organskih onesnažil v algnih fotobioreaktorjih. Cilj doktorske naloge je bil proučevati odstranjevanje in kroženje izbranih onesnažil: zdravilnih učinkovin in sestavin iz izdelkov za osebno nego, neonikotinoidnih pesticidov in bisfenolov v laboratorijskih in pilotnih fotobioreaktorjih. Za proučevanje pojavnosti, učinkovitosti odstranjevanja in kroženja izbranih onesnažil smo prilagodili obstoječe analizne metode, ki temeljijo na plinski ali tekočinski kromatografiji sklopljeni z masno spektrometrijo. Prav tako smo razvili nove analizne metode za analizo izbranih prioritetnih onesnažil v vodni fazi in v biomasi alg.

Proučevali smo odstranjevanje in kroženje osemnajstih bisfenolov v laboratorijskih fotobioreaktorjih. Celokupna učinkovitost odstranjevanja bisfenolov je znašala 72 ± 2%, ko pH kulture ni bil nadzorovan, oziroma 73 ± 5%, ko je bil. Učinkovitost odstranjevanja posameznih bisfenolov je znašala od 25 ± 5% do $\approx$100% in je korelirala s klorofilom a. Rezultati so pokazali, da je pH vrednost v kulturi vplivala na učinkovitost odstranjevanja izbranih BP (2,2'-BPF, BPC2, BPAF, BPA in BPC). Delo je pomembeno zaradi poznavanja učinkov fizikalno-kemijskih parametrov in pH na odstranjevanje onesnažil.

Nadalje smo proučevali odstranjevanje onesnažil v fotobioreaktorjih z mešano kulturo alg in bakterij in čisto kulturo alg. Rezultati so pokazali, da so se bisfenoli v mešani kulturi (87 ± 5%) odstranili v večji meri kot v čisti kulturi (55 ± 7%), medtem ko je bila učinkovitost odstranitve zdravilnih učinkovin podobna (85 ± 28% and 79 ± 23%), pri pesticidih pa večja v čisti kulturi (64 ± 21% in 90 ± 11%). Ta študija primerja učinkovitost odstranjevanja osemindvajsetih onesnažil z mešano kulturo alg in bakterij in čisto kulturo alg.

V zadnji fazi smo proučevali onesnažila v pilotnem fotobioreaktorju. Ocenili smo 80% odstranitev BPA, BPS pa se je izkazal za slabše razgradljivega (32%). Metilparaben, 2,4-dihidroksibenzofenon in oksibenzon so dosegli 92%, 76% oziroma 80% učinkovitost odstranjevanja. Naproksen in ibuprofen sta dosegla 60% oziroma 51% učinkovitost odstranjevanja, pri čemer sta bili ti dve učinkovini prisotni v najvišjih koncentracijah. Izsledki dajejo informacije o odstranjevanju onesnažil v realnih pogojih in so izjemnega pomena za optimizacijo in nadaljnji razvoj fotobioreaktorjev.

Izsledki doktorskega dela kažejo, da predstavljajo algni fotobioreaktorji obetaven način odstranjevanja prioritetnih organskih onesnažil iz odpadne vode. Uspešnost odstranjevanje večine spojin je primerljivo s klasičnimi biološkimi čistilnimi napravami, potreben pa bo nadaljen razvoj za boljšo odstranitev slabše odstranljivih onesnaževal kot sta npr. BPS in karbamazepin. Poleg tega pa fotobioreaktorji ponujajo prednost ponovne uporabe virov, kot sta biomasa in voda. Nadaljnje študije pojavnosti, kroženja, odstranjevanja, strupenosti in ocene tveganja prioritetnih organskih onesnažil na tem področju bodo prispevale k učinkovitem čiščenju odpadne vode z algnimi fotobioreaktorji in oblikovanju zakonodaje.

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Abbreviations

AA ... Acetic Acid
ACN ... Acetonitrile
AS ... Activated Sludge
BAF ... Bioaccumulation Factor
BCF ... Bioconcentration Factor
BOD ... Biological Oxygen Demand
BPs ... Bisphenols
BSTFA ... Bis-(trimethylsilyl)-trifluoroacetamide
C_0 ... initial CEC concentration
CAS ... Chemical Abstracts Service
CEC ... Contaminants of Emerging Concern
CI ... Chemical Ionization
CRM ... Certified Reference Materials
DDT ... Dichlorodiphenyltrichloroethane
ECHA ... European Chemicals Agency
EFSA ... European Food Safety Authority
EI ... Electron-Impact
EMA ... European Medicines Agency
EQS ... Environmental Quality Standards
ESI ... Electrospray Ionization
EtAc ... Ethyl Acetate
GC ... Gas Chromatography
Hex ... Hexane
HPLC ... High Performance Liquid Chromatography
HRAP ... High Rate Algal Ponds
HRT ... Hydraulic Retention Time
IT ... Ion Trap analyser
lab-PBR ... Laboratory-scale Photobioreactors
LC ... Liquid Chromatography
LLE ... Liquid-Liquid Extraction
LOD ... Limit of Detection
log K_{aw} ... Logarithm of the air-water partitioning coefficient
log P ... Logarithm of the octanol-water partitioning coefficient
LOQ ... Limit of Quantitation
m_0 ... Initially spiked amount of CEC
MBBR ... Moving Bed Bioreactors
MBR ... Membrane Bioreactor
MeOH ... Methanol
MRM ... Multiple Reaction Monitoring
MS ... Mass Spectrometry
MS/MS ... Tandem Mass Spectrometry

MSTFA	N-methyl-N(trimethylsilyl)-trifluoroacetamide
MTBSTFA	N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide
MW	Molecular Weight
O-PBR	Outdoor Photobioreactors
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
pK_a	Acid dissociation constant
PPCP	Pharmaceuticals and Personal Care Products
QA	Quality Assurance
QC	Quality Control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
R	Removal
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RMOA	Regulatory Management Option Analysis
SBR	Sequencing Batch Reactors
SI	International System of Quantities and Units
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
TOF	Time-Of-Flight analyser
TP	Transformation Product
TPBR	Tubular Photobioreactors
t-TDI	Temporary Tolerable Daily Intake
UPLC	Ultra-Performance Liquid Chromatography
US EPA	United States Environmental Protection Agency
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant

Chapter 1

Introduction

Contaminants of emerging concern (CEC) are a diverse group of organic contaminants discharged to the environment by way of wastewater. As humankind is facing risks from such contaminants and the demand for resources is increasing, efficient wastewater treatment will likely need to be coupled with resource reuse in the future. Algae-based wastewater treatment systems can provide a combination of both. However, the removal efficiency of contaminants in such systems is adequately understood. In the following subchapters, CEC, wastewater treatment, methodological approaches, and quality of measurements will be discussed.

1.1 Contaminants of Emerging Concern

Humankind has, through its technological development, learned to extract or synthesize and use many different compounds. These compounds and their by-products, in some cases, have affected our environment in unforeseen and unwanted ways, especially from the industrial revolution onwards. Although CEC (also referred to as emerging contaminants or emerging pollutants or similar terms) may not be new in the environment, their environmental occurrence has been investigated only recently due to awareness of potential adverse ecological and human health impacts. CEC are typically present in the environment in trace levels, and only recent advances in analytical instrumentation have allowed their detection in increasingly low concentrations (ng/L and even pg/L) [1]. As a group, they include active components of human and veterinary pharmaceuticals, illicit drugs, personal care products, pesticides, hormones, flame retardants, plasticizers and other compounds, as well as their metabolites and transformation products (TPs) [1], [2]. Although many CEC are xenobiotic compounds, there are exceptions, for example, estrone and estradiol. They are also typically not included in routine monitoring programmes, although they may be candidates for future regulation. Their inclusion will, according to the NORMAN network, depend “on research on their (eco)toxicity, potential health effects and public perception and monitoring data regarding their occurrence in the various environmental compartments” [3]. Mixtures of different CEC and their TPs are especially of concern as they may cause additive effects [4]. Unlike persistent organic pollutants (POPs), CEC are not intrinsically persistent, but many are ubiquitous and are considered pseudo-persistent due to their continuous discharge to the environment [5], [6]. Different types of wastewater (e.g. runoff, municipal and industrial) represent the main influx of CEC to the environment due to inadequate removal during wastewater treatment [2], [7], [8], which is concerning as they may cause ecological harm [9]. They may also pose a risk to humans when considering the reuse of wastewater treatment products (e.g. reclaimed water, sludge) for activities like agriculture. Thus, knowledge on the fate of CEC during wastewater treatment and their associated risks in the environment will enable improvements to algal wastewater treatment and reuse of the byproducts.

Physicochemical parameters of CEC are a significant predictor of their behaviour in the environment or during wastewater treatment. For example, the octanol-water partitioning coefficient (log P) is a measure of the polarity of a compounds' hydrophobicity and is defined as the ratio of analyte concentration in n-octanol and water at equilibrium. Values of log P above 4 mean that compounds will be more readily adsorbed to the soil, sediment or accumulate in tissues than be dissolved in water [10]. Furthermore, the acid dissociation constant (pK_a) values indicate the pH value above which acidic compounds will be dissociated (acidic functional groups like -OH and -COOH will donate a proton and become negatively charged) to a higher degree. Consequently, they will be more mobile in the aqueous phase as they are generally more hydrophilic than their neutral counterparts [11]. Solubility in water (in mg/L at 25 °C) is related to polarity and may limit the occurrence of a compound in the aqueous phase. The logarithm of the air-water partitioning coefficient (log K_{aw}) is based on Henry's Law Coefficient and is a measure of the distribution of a compound in either air or water at equilibrium.

When discussing organic pollutants, important phenomena are also bioconcentration, bioaccumulation and biomagnification. According to Arnot and Gobas, bioconcentration describes the uptake of a compound in water through respiratory and dermal surfaces and is expressed as a bioconcentration factor (BCF) [12]. Bioaccumulation, however, also includes the dietary uptake of a compound and is expressed as a bioaccumulation factor (BAF) [12]. Both BCF and BAF are expressed in L/kg units, i.e., the concentration in water is normalized to the tissue concentration. Biomagnification is the successive increase of a compound's concentration at higher levels of the food chain. Because of the inclusion of dietary exposure, biomagnification is included in BAF. Bioconcentration and bioaccumulation are the net results of a compound's uptake and elimination (by respiratory exchange, faeces, metabolism and growth dilution)[12]. According to the EU REACH Regulation, a compound is bioaccumulative when the BCF is higher than 2000 and very bioaccumulative when the factor is higher than 5000 [13]. Data on predicted solubility, log Kaw, BCF and BAF of the studied CEC is presented in Tables S1 – S3. Such data can help to predict the fate of CEC during wastewater treatment and in the environment.

1.1.1 Pharmaceuticals and personal care products

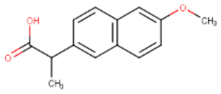
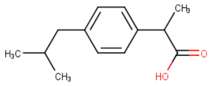
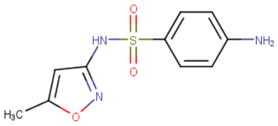
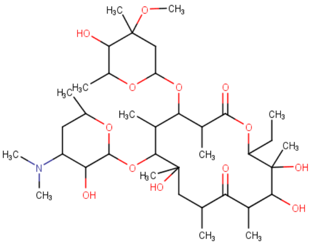
Active pharmaceutical ingredients or pharmaceuticals are "any substance or mixture of substances intended to be used in the manufacture of a medicinal product and that, when used in its production, becomes an active ingredient of that product intended to exert a pharmacological, immunological or metabolic action with a view to restoring, correcting or modifying physiological functions or to make a medical diagnosis" [14]. This dissertation addresses the following groups of pharmaceuticals: analgesics (naproxen, ibuprofen), antibiotics (erythromycin, clarithromycin, sulfamethoxazole), hormones (estradiol, estrone), beta blockers (metoprolol) and anticonvulsants (carbamazepine). Representatives of both macrolide and sulphonamide antibiotics and hormones have been included in the EU Water Framework Directive Watch List [15]–[17] in order to gain firm data on their occurrence in the surface waters and to support future legislation. The occurrence of other pharmaceuticals (analgesics, beta blockers, anticonvulsants) in the environment is also of concern as they are continually released to the environment through wastewater and may have adverse effects on living organisms.

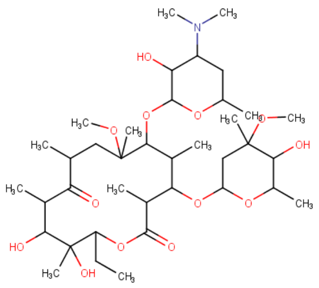
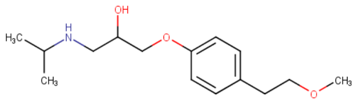
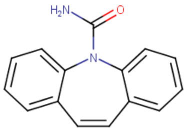
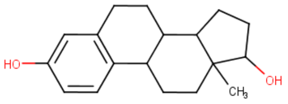
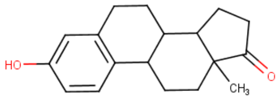
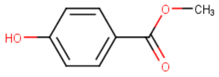
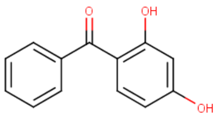
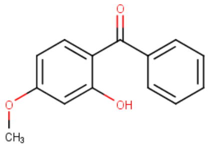
Personal care products are common household products for health, beauty and cleaning purposes, while some of their ingredients are considered CEC. Included compounds belong to two groups; preservatives (methylparaben) and UV-blockers (2,4-

dihydroxybenzophenone and oxybenzone). Parabens [18] and benzophenones [19] make their way into wastewater and further to the environment through the everyday use of these products. These compounds are of concern as they may have potential endocrine disruptive effects on organisms and humans.

Pharmaceuticals and personal care products (PPCP) are often studied and discussed together in the scientific literature. Their predicted log P values range from 0.79 (sulfamethoxazole) to 4.31 (estrone), indicating that estrone has the highest affinity to partition to sediments and biomass (Table 1). Predicted pK_a values indicate that naproxen ($pK_a = 4.2$) and ibuprofen ($pK_a = 4.9$) will have their acidic groups ionized at neutral pH, which will mean that they will be predominantly present in the aqueous phase. Other compounds will be mostly unaffected by pH, e.g. carbamazepine ($pK_a = 16$) which will deprotonate at much higher values and will be in its neutral form at neutral pH. Their predicted solubility ranges from 0.3 to 4777 mg/L, but since they appear in the environment at trace levels, solubility is not a limiting factor for occurrence in aqueous matrices. According to their log K_{aw}, they are also unlikely to volatilize from the aqueous phase to the air. None of the PPCP compounds discussed herein can be considered bioaccumulative due to their low BCF (15 – 436 L/kg) (Table S1).

Table 1: Pharmaceuticals and personal care products, their group, chemical structures and main physicochemical properties: molecular weight (MW), octanol-water partitioning coefficient (log P) and acid dissociation constant (pK_a) as predicted by MarvinSketch [20] and CAS identifiers. Compounds are arranged according to group classification and log P.

Name (group)	**Chemical structure**	**MW**	**log P**	**pK_a***	**CAS**
Naproxen (analgesic)		230.26	2.99	4.2	22204-53-1
Ibuprofen (analgesic)		206.29	3.84	4.9	15687-27-1
Sulfamethoxazole (antibiotic)		253.28	0.79	6.2	723-46-6
Erythromycin (antibiotic)		733.94	2.6	12.4 - 14.6	114-07-8

Name (group)	**Chemical structure**	**MW**	**log P**	**pK_a***	**CAS**
Clarithromycin (antibiotic)		747.96	3.24	12.5 - 14.5	81103-11-9
Metoprolol (beta blocker)		267.37	1.76	14.1	51384-51-1
Carbamazepine (anticonvulsant)		236.27	2.77	16	298-46-4
Estradiol (hormone)		272.39	3.75	10.3	50-28-2
Estrone (hormone)		270.37	4.31	10.3	53-16-7
Methylparaben (preservative)		152.15	1.67	8.5	99-76-3
2,4-dihydroxy benzophenone (UV blocker)		214.22	3.48	7.1 - 8.7	131-56-6
Oxybenzone (UV blocker)		228.25	3.62	7.1	131-57-7

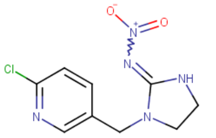
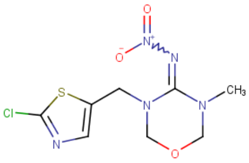
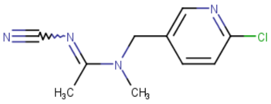
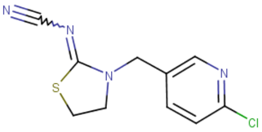
*predicted pK_a values are stated for the neutral species of the molecule, pK_a values for protonated amine groups are omitted

1.1.2 Pesticides

Pesticides are compounds used to control unwanted organisms, notably in agriculture, to protect crops and improve productivity. Records of pesticide usage date back to Roman times, but up until the 20th century, these first-generation pesticides were limited to plant extracts and inorganic compounds, some of which are still in use today, e.g. copper (II) sulfate [21]. However, with the development of organic chemistry, synthetic organochlorine pesticides like DDT became widespread in the 1940s and remained in use for several decades [21]. Rachel Carson was one of the first people to raise public awareness on xenobiotics and helped inspire the global environmental movement with her book *Silent spring* (1962), wherein she pointed to the damage done to the environment and humans by ignorant overuse of pesticides [22]. While pesticide legislation constantly evolves in the developed world [23], pesticides are high production volume chemicals, with global use, and according to the United Nations Food and Agriculture Organization (FAO), more than 4 million tons were produced worldwide in 2018 [24]. Pesticides are mainly applied to agricultural surfaces, from where they make their way into the aquatic environment via agricultural runoff.

Neonicotinoid pesticides bind to nicotinic acetylcholine receptors and block them, causing paralysis and subsequent death, where insect pests are the targets [25]. However, concerns regarding their adverse effects on untargeted organisms like bees and other pollinators and other ecological concerns have increased. Four neonicotinoid pesticides (Table 2) were part of the EU Water Framework Directive Watch List in 2015 [15] and 2018 [16] and were included in this dissertation. They have relatively low predicted log P values (0.53 to 2.06), making them unlikely to adsorb or bioaccumulate. Also, they have a relatively high solubility in water (232 to 7172 mg/L), which does not limit their occurrence in the aqueous environment. These two parameters mean that they will mainly be present in the aqueous phase in the environment. They are also not likely to deprotonate due to the lack of acidic functional groups; however, their amine groups may become protonated at acidic pH values (<6). According to their log K_{aw} (Table S2), they are unlikely to volatilize from the aqueous phase to the air and are not considered bioaccumulative (BCF: 1 – 21 L/kg).

Table 2: Neonicotinoid pesticides, chemical structures and their main physicochemical properties (octanol-water partitioning coefficient - log P and acid dissociation constant - pKa as predicted by Marvin suite (ChemAxon, 2019)) and CAS identifiers. Compounds are arranged according to log P.

Name	**Chemical structure**	**MW**	**log P**	**p K_a**	**CAS**
Imidacloprid		255.66	0.53	15.6	138261-41-3
Thiamethoxam		291.71	1.07	/	153719-23-4
Acetamiprid		222.68	1.11	/	160430-64-8
Thiacloprid		252.72	2.06	/	111988-49-9

1.1.3 Bisphenols

Bisphenols (BPs, Table 3) are a group of compounds characterized by two hydroxyphenyl groups bound by a hydrocarbon bridge and also having diverse chemical groups, such as phenyl, methyl, halogens and others, located on the bridge and phenol rings [26], [27]. Bisphenol A (BPA), the most well-known of the group, was first synthesized in 1891 [28], and although its estrogenic properties were first reported by Dodds and Lawson in 1938 [29], it did not stop its later use in the manufacture of epoxy coatings and polycarbonate plastics in the 1950s. Because doubts about the safety of BPA at the time were put to rest by acute toxicity tests, the production of BPA and its end products continued for decades [28]. BPA remains a high-volume production chemical, with production in Europe estimated to be 1,150,000 tons in 2015 [30] and more than 8,000,000 tons worldwide in 2016 [31]. There is a lack of detailed production data for other BPs. The European Chemicals Agency (ECHA) provides some approximate data (Table 3) on the production and import of BPs in the EU [32]. It is evident that BPA is still the most produced and used bisphenol and has many registered intermediate and derivative compounds [32]. Second is BPS, with 10,000 – 100,000 tons produced per year. In third place is the reaction mixture of the isomers 2,2'-BPF, 2,4'-BPF and 4,4'-BPF, together amounting to 1,000 – 10,000 tons. Others are registered in lower amounts, and their tonnage data is not available, or even more interestingly, data for BPFL and BPAP are registered as confidential.

Table 3: Production/import estimates of BPs as registered with ECHA [32].

Abbreviation	**CAS**	**Registered production/ import data per year [ton]**
BPA	80-05-7	100,000 – 1,000,000
2,2'-BPF	2467-02-9	1,000 – 10,000 [1]
2,4'-BPF	2467-03-0	1,000 – 10,000 [1]
4,4'-BPF	620-92-8	1,000 – 10,000 [1]
BP26DM	5613-46-7	10 – 100
BPAF	1478-61-1	100 – 1,000
BPAP	1571-75-1	Confidential [2]
BPB	77-40-7	N/A [3]
BPBP	1844-01-5	N/A [3]
BPC	79-97-0	10 – 100
BPC II	14868-03-2	N/A [3]
BPE	2081-08-5	N/A [3]
BPFL	3236-71-3	Confidential [2]
BPM	13595-25-0	0 – 10
BPP	2167-51-3	N/A [3]
BPPH	24038-68-4	N/A [3]
BPS	80-09-1	10,000 – 100,000
BPZ	843-55-0	N/A [3]

[1] – data for reaction mixture of the isomers 2,2'-BPF, 2,4'-BPF and 4,4'-BPF.

[2] – data about registered production/importation is classified as confidential.

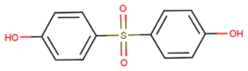
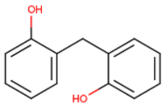
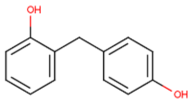
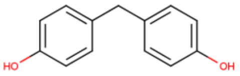
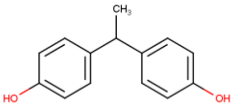
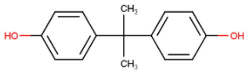
[3] – data not available on the ECHA webpage.

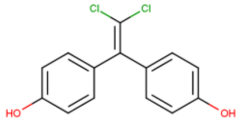
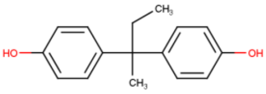
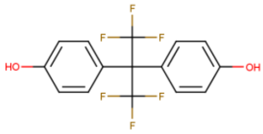
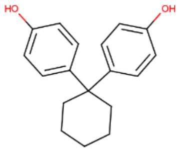
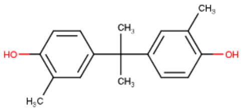
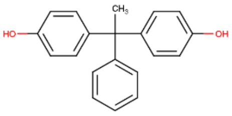
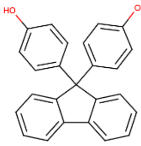
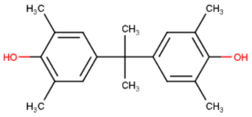
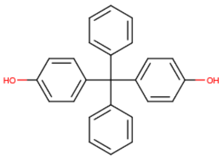
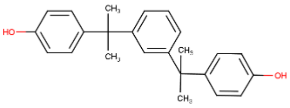
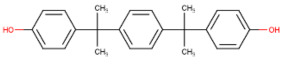
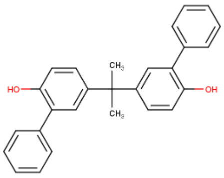
In the 1990s, the hypothesis of endocrine disruption gained credibility, and BPA was shown to exhibit endocrine disruptive properties at low doses, well below the USA safety standard of 50 µg/kg/day at the time [28]. Until 2005, more than 100 studies were performed, highlighting the effects of low concentration exposure to BPA. A meeting of 38 experts on endocrine disruptors and BPA in 2006 resulted in the Chapel Hill consensus statement that "prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behaviour of laboratory animals" [33]. They also reported that human exposure to BPA is found in the biologically active range in 95% of people and that there is great cause for concern as similar effects as in laboratory animals are likely to occur. Myers et al. (2009) explain that the reason for the complexity of EDC toxicity lies in their nonmonotonic response curves (meaning they do not exhibit a linear dose-response relationship) [34]. One of the causes for this kind of response is cell receptor up-regulation by low doses, while high doses cause receptor down-regulation. Other important mechanisms include different gene regulation at low or high ECD doses, hormonal feedback mechanisms and different ECD complex mixture effects.

Today, the applications of BPA include its use as a monomer in the production of polycarbonate, epoxy resins, polysulfone, polyacrylate, polyetherimide and as an additive in thermal paper, polyvinyl chloride and other products [35]. The widespread use of BPA means that, although technically nonpersistent, it is ubiquitous in the environment and present in human and animal tissues [36]. BPA analogues (Table 3) were placed on the market to replace BPA in some of its applications [26], [27]. The replacement of BPA with an alternative could be a response to the public awareness and restrictive regulation of BPA but could also be application-specific according to the required characteristics of the polymer [27]. Due to structural similarities of BPs, there is concern that BPA alternatives have similar endocrine disrupting effects as BPA – a fact corroborated for 4,4'-BPF, BPS, BPE, BPB, BPAP, BPFL, BPAF by studies on their hormonal activity [37]–[42]. These findings indicate that extreme care should be taken in their continued use and discharge.

Main BP physicochemical properties are shown in Table 3. The estimated values of log P of bisphenols range from 2.32 to 7.34. These values mean that, for example, 2,2'-BPF, 2,4'-BPF, 4,4'-BPF, BPA, BPE and BPS (log P $<$ 4) will favour the water phase. In contrast, BPs including BPC II BPB, BPAF, BPAP, BPC, BP26DM, BPBP, BPFL, BPM, BPP, BPPH and BPZ (log P $>$ 4), will likely have a higher tendency to adsorb to tissues, sediment and organic carbon in their neutral form [27]. Predicted pK$_a$ values indicate that a proportion of BPS (pK$_a$ = 7.4 - 8.0) will have acidic groups ionized at neutral pH and will primarily be present in the aqueous phase. Others will be less affected by dissociation at environmental pH due to higher pK$_a$ values (8.2 – 10.5). The water solubility of BPs ranges from 0.003 mg/L to 1774 mg/L. Even though some of these values are relatively low, solubility is not a bottleneck for BPs occurrence in water [27]. According to their low log K$_{aw}$, they are unlikely to volatilize from the aqueous phase to the air. The BCF of the BPs were found to range from 3.5 to 1970, indicating that BPM and BPP could potentially bioaccumulate (Table S3). Although BPs are regarded as a group, they have diverse properties, leading to differences in their fate during wastewater treatment. These differences make them an ideal group to study CEC occurrence and fate during wastewater treatment.

Table 4: Bisphenols, their chemical structures and physicochemical properties (octanol-water partitioning coefficient - log P and acid dissociation constant - pK$_a$ as predicted by Marvin suite [20]) and CAS identifiers. Compounds are arranged according to log P.

Name	Chemical structure	MW	log P	pK$_a$	CAS
BPS		250.27	2.32	7.4 - 8.0	80-09-1
2,2'-BPF		200.24	3.46	8.2 - 10.1	2467-02-9
2,4'-BPF		200.24	3.46	9.8 - 10.4	2467-03-0
4,4'-BPF		200.24	3.46	9.8 - 10.4	620-92-8
BPE		214.26	3.74	9.8 - 10.4	2081-08-5
BPA		228.29	4.04	9.8 - 10.4	80-05-7

Name	Chemical structure	MW	log P	pK$_a$	CAS
BPC II		281.13	4.29	8.9 - 9.5	14868-03-2
BPB		242.32	4.49	9.8 - 10.4	77-40-7
BPAF		336.23	4.77	9.1 - 9.7	1478-61-1
BPZ		268.36	4.91	10.1 - 10.7	843-55-0
BPC		256.35	5.07	9.7 - 10.3	79-97-0
BPAP		290.36	5.18	9.8 - 10.4	1571-75-1
BPFL		350.42	5.99	10.5 - 11.1	3236-71-3
BP26DM		284.40	6.1	9.6 - 10.2	5613-46-7
BPBP		352.43	6.31	9.6 - 10.2	1844-01-5
BPM		346.47	6.72	9.8 - 10.4	13595-25-0
BPP		346.47	6.72	9.8 - 10.4	2167-51-3
BPPH		380.49	7.34	9.5 - 10.1	24038-68-4

1.1.4 Legislation in the European Union

Regulation 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and establishing a European Chemicals Agency (ECHA) is an important act for the regulation of chemicals in the industry [13]. REACH establishes procedures for collecting information on substances and the assessment of hazards and possible restrictions. Of the compounds included in this dissertation, only BPA is on REACH's Candidate List of Substances of Very High Concern (SVHC) because of its reproductive toxicity and endocrine disrupting properties; but it is restricted only in thermal paper [43].

The Water Framework Directive (WFD) was established in 2000 for water management and protection on the level of river basins [44] with Directive 2000/60/EC [45]. According to Article 16, the EU is to adopt measures for reduction and/or phasing out of discharges, emissions and losses of pollutants presenting a risk to or via the aquatic environment. As a result, a list of priority substances in the field of water policy was established in 2001 with Decision 2455/2001/EC, including 33 substances shown to be of major concern for European waters [46]. The list is also included in the WFD by Directive 2008/105/EC, which also introduces Environmental Quality Standards (EQS) that limit permissible concentrations of priority substances in surface waters and biota. These EQS are expressed as an annual average to protect against long-term exposure and as maximum allowable concentrations to protect against short-term exposure. The Directive also included a requirement for EU member states to establish an inventory of emissions, discharges and losses for priority substances [47]. In 2013, Directive 2013/39/EU additionally amended the List of priority substances, including 45 substances, their revised EQS for water and biota, and introduced the concept of a Watch list of substances for EU-wide monitoring [48] in surface waters. The Watch list is critical in supporting future actions, as information on CEC occurrence is often scarce. The first WFD Watch list was established in 2015 with Commission implementing decision 2015/495 including 17 compounds, among them hormones (estradiol, estrone and ethinylestradiol), macrolide antibiotics (erythromycin, clarithromycin and azithromycin) and neonicotinoids (imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid) [15]. It also determined analytical methods and maximum acceptable detection limits for the analysis of the Watch list compounds in water samples. The Watch list was updated in 2018, containing 15 compounds belonging to three previously mentioned groups [16]. Most recently, the Watch list was updated in 2020 and currently includes 19 compounds, among them also the antibiotic sulfamethoxazole) [17].

While EU legislation does not directly regulate CEC in wastewater, the Industrial Emissions Directive [13] instructs that EU member states determine emission limit values for air and water pollutants listed in Annex II (which includes different CEC, POP, biocides, and compounds from Annex X of the WFD – *List of priority substances in the field of water policy*). The Industrial Emissions Directive covers several industrial sectors and waste management, but it excludes urban wastewater treatment. The latter is covered by the Urban Wastewater Treatment Directive [49], which only sets limits for macropollutants, e.g. nitrogen and phosphorus.

To facilitate and regulate the reuse of reclaimed water for so-called fertigation (a portmanteau of irrigation and fertilization), the EU has also laid down Regulation 2020/741, which will apply from 26th June 2023 [1]. This Regulation classifies reclaimed water by quality and permitted agricultural use, determines quality requirements (microbiology, BOD, TSS, and turbidity), as well as monitoring frequency and validation parameters. In Annex II of this Regulation, point 6 allows for additional or stricter quality requirements “when necessary and appropriate to ensure adequate protection of the environment and of human and animal health, in particular when there is clear scientific evidence that the risk originates from reclaimed water and not from other sources" and mentions that these requirements may also concern CEC [1]. Even so, Regulation 2020/741 does not set specific limits for CEC or WFD priority substances regarding fertigation, although this will likely change in the future. Similarly, reuse of sludge from domestic and urban WWTP in agriculture is regulated by Council Directive 86/278/EEC, whose purpose is to promote "the use of sewage sludge in agriculture in such a way as to prevent harmful effects on soil, vegetation, animals and man" [2]. The Directive regulates the limit values for heavy metals (cadmium, copper, nickel, lead, zinc, mercury and chromium) in sludge and soil and lays down rules for application, sampling and analysis. Like Regulation 2020/741, however, it does not regulate permissible levels of CEC or WFD priority substances in sludge, which is likely to change with further evidence and awareness of their potential for harm. While unregulated at present, the valorisation of algal biomass is in line with the principles of the EU's Circular Economy Action Plan [3]. Therefore, regulations for its reuse in agriculture and energy production (e.g. biogas, biodiesel) are expected to be prepared in the following years.

The lack of unified EU legislation regulating the discussed CEC in wastewater effluent seems a glaring oversight in the face of the fact that the main influx of CEC to the environment is through wastewaters [2], [7], [8]. Instead of a unified EU approach to regulating CEC in wastewater, this is the business of every Member state. EU legislation focuses on receiving bodies – surface, ground and drinking water and while this is necessary, not having an EU-wide regulation of CEC in wastewaters gives the impression of skipping a step. The control over CEC pollution will have to be established in the future as only managing CEC efflux of industrial point sources, and municipal WWTP on the EU level will improve environmental emissions and water quality across Europe. However, more information on CEC's occurrence, fate and toxicity is needed for efficient regulation.

1.1.4.1 Pharmaceuticals and personal care products

Because of their very purpose and effects, pharmaceuticals go through a lengthy trial period, where they are pharmacologically evaluated before they go on the market. The regulatory network for medicines in the EU is based on National Competent Authorities of the Member States cooperating with a central European Medicines Agency (EMA) and the European Commission [50], [14]. The pharmaceuticals included in this dissertation are both over-the-counter and prescription medicines that have become essential, yet their constant emission may have potential adverse effects on the environment. Pharmaceuticals are highly regulated until their consumption; however, after excretion and entry into the sewage system, their regulation is non-existent. Although their possible downstream environmental effects have recently raised concerns (e.g. formation of antibiotic-resistant bacterial strains), there are currently no regulations in place that would limit allowable industrial or municipal discharges of pharmaceuticals to the environment. In Article 8c of Directive 2008/105/EC, the intention of a strategic approach to reducing discharges, emissions and losses of pharmaceuticals into the aquatic environment is stated [47]. In accordance with this, the *Strategic approach to pharmaceuticals in the environment* was adopted by the European Commission in 2019, where the main actions are to a) increase awareness and promote prudent use of pharmaceuticals, b) support the development of pharmaceuticals intrinsically less harmful for the environment and promote greener manufacturing, c) improve environmental risk assessment and its review, d) reduce wastage and improve the management of waste, e) expand environmental monitoring, and f) fill other knowledge gaps [51], [52]. The inclusion of pharmaceuticals on the WFD Watch list means that it is essential to continue to collect information in support of future legislation and actions [15].

Ingredients of personal care products are less strictly regulated than pharmaceuticals, but some may pose risks to humans due to direct exposure. Examples include methylparaben (a preservative) and triclosan (an antimicrobial) used in cosmetic products. Both are on the ECHA Endocrine Disruptor Assessment List due to their potential for endocrine disruption. However, the status of their assessment is "Under development". Meanwhile, they are still allowed ingredients in cosmetic products, with a maximum allowable concentration of 0.4% and 0.3%, respectively, under Regulation 1223/2009 on cosmetic products [53]. Although oxybenzone is on the Endocrine disruptor assessment, it is on the "Allowed UV filters" list under Regulation 1223/2009 [53]. Methylparaben, oxybenzone and 2,4-dihydroxybenzophenone are also authorized as additives in the production of plastic materials and articles intended to come into contact with food, with a specific migration limit of 6 mg/kg for the two UV filters and no limit for methylparaben [54].

While adverse effects of PPCP in the environment are suspected, their discharge through wastewater is unregulated. More information on their toxicity to humans and organisms in the environment is needed to achieve stricter regulation or outright ban, where needed.

1.1.4.2 Pesticides

Neonicotinoid pesticides were developed in the 1990s and were approved for use in plant protection products in the EU: acetamiprid and thiacloprid (approved in 2005), clothianidin (2006), thiamethoxam (2007) and imidacloprid (2009) [55]. However, due to large numbers of honeybee deaths, so-called colony collapse disorders and deaths of other pollinators, the use of clothianidin, thiamethoxam and imidacloprid was limited in the EU in 2013 [56]. In addition, further studies on the toxicity towards pollinators [57], [58] and a peer-review of the scientific evidence about clothianidin, thiamethoxam and imidacloprid by the European Food Safety Authority (EFSA) [59] led to them being banned for all outdoor use in 2018 [60]–[62]. Acetamiprid was found to have a low risk to bees, and its approval was renewed in 2018 [63]. The approval for use of thiacloprid was withdrawn in 2020 due to the outcome of a peer review of its risk assessment, resulting in its *de facto* ban [59]. However, neonicotinoids are still used outdoors under certain circumstances. EU legislation allows for Emergency Authorization for use of these banned neonicotinoids "where such a measure appears necessary because of a danger which cannot be contained by any other reasonable means" in Article 53 of Regulation 1107/2009 [64]. Still, this mechanism has potential for abuse [65], and EU records show that neonicotinoids have been authorized for emergency use 210-times from 2016 to 2021 [66].

The EU quality standard for pesticides and their metabolites, degradation, and reaction products is 0.1 µg/L individually or 0.5 µg/L in total for drinking water [67] and groundwater [68]. However, within the legislation, individual pesticides are not specified. Slovenian legislation on drinking water parameters includes the same criteria, and monitoring of drinking water is performed yearly, although neonicotinoids are not included [69]. The discharge of pesticides through wastewater is not regulated.

1.1.4.3 Bisphenols

Increased public awareness about the adverse effects of BPA resulted in restrictive legislation in North America and Europe. This increased awareness led to the retailers and the plastics industry's proactive move to offer "BPA-free" alternatives [4]. Given that the main source of human exposure to BPA is food, several regulations were adopted in the EU. For instance, EFSA performed its' first risk assessment of BPA in 2006 and set a temporary Tolerable Daily Intake (t-TDI) of 50 µg/kg bw/day [5]. In 2015, the t-TDI was lowered from 50 to 4 µg/kg bw/day after an additional risk assessment [6]. As a precaution, the European Commission also restricted the manufacture, sale and import of plastic infant feeding bottles containing BPA in 2011 [7]. The specific migration limits of BPA and BPS from food contact materials are defined as 0.05 mg/kg by Commission Regulation 10/2011 [8]. BPA was also banned in cosmetic products [9] but is allowed in "toys intended for use by children under 36 months or in other toys intended to be placed in the mouth" if it does not exceed a migration limit of 0.04 mg/L [10]. Under the REACH regulation, the European Commission (2016) also restricts the proportion of BPA in thermal paper to be no more than 0.02% by weight after 2nd January 2020 [11]. However, these restrictions are connected to direct human exposure and do not affect the emissions through wastewater.

In the original version of Directive 2008/105/EC on EQS in surface water and biota, BPA was included in Annex III - Substances subject to review for possible identification as priority substances or priority hazardous substances [12]. With later amendments in 2013, Annex III was deleted from Directive 2008/105/EC, and BPA was not included as a priority substance due to insufficient supporting evidence [13]. At present, BPA, BPS and BPB are on the ECHA's Endocrine disruptor assessment list [14], where BPA has been recognized as endocrine disrupting in 2017 for human health and the environment and is pending regulatory management option analysis (RMOA), while the assessment status of BPS and BPB regarding endocrine disruption is still "under development".

Although BPA discharge through wastewater is not regulated by EU legislation, the Slovenian Decree on the emission of substances and heat when discharging WW into surface waters and the public sewage system limits the allowed concentration of BPA in wastewater when directly or indirectly discharged to surface water or the municipal sewer to 0.16 mg/L [15]. It also limits the concentrations of other pollutants, many of them obsolete (e.g., DDT, PCB, organochlorine and pesticides), while other CEC discussed in this dissertation are omitted. The result is that the wastewater discharge of BPs is insufficiently regulated, even though they are suspected or have been proven to have endocrine disrupting effects. More regulative measures will likely be taken following further assessment of their toxicity occurrence and fate in wastewaters and the environment.

1.2 Wastewater treatment

1.2.1 Conventional treatment

Wastewater sources may be generalized into four categories: (1) rainwater (runoff), (2) domestic wastewater, (3) agricultural water and (4) industrial wastewater [70]. All these sources are contaminated with various macro- and micropollutants, and appropriate treatment is necessary for their release to the environment or reuse. Wastewater treatment consists of several stages that are applied as per the purpose, requirements and capabilities of the WWTP, listed in increasing order of sophistication: preliminary treatment, primary treatment, secondary treatment and tertiary treatment, as reviewed by Ullah et al. (2020) [71]. Preliminary treatment usually involves physical treatment designed to remove larger, coarse solids from the influent wastewater and is achieved using screens, grinders and grit chambers. After this, wastewater may still contain settleable and non-settleable solids, along with particulate organic matter. Primary treatment consists of flotation systems, primary involving sedimentation, neutralization tanks and equalization [71]. Flotation systems remove suspended solids, while sedimentation tanks are used for removing settleable solids, where adding dissolved air flotation and coagulant, respectively, may boost treatment efficiency. On the other hand, neutralization and equalization tanks may also be used for neutralization and homogenization, respectively, if prevention of shock loading is necessary [71].

Secondary treatment aims to remove any remaining solids and any remaining dissolved organic matter (e.g. measured as biological oxygen demand – BOD) in the primary influent. Biological treatment employs many anaerobic processes that may use either suspended, attached or combined bacterial biomass [71]. The conventional activated sludge (AS) process is the most common process for biological wastewater treatment. It requires an aeration tank, in which primary-treated influent and recycled sludge are aerated, and microorganisms digest the organic matter and produce flocculates, and a settlement tank, where treated water and sludge are separated [72]. Like AS, sequencing batch reactors (SBR) can operate in parallel and are designed to achieve aeration, sludge settlement, decanting and refilling in one tank [72]. Moving bed bioreactors (MBBR) use carriers that move about freely in the mixed liquor where they can attach and grow biomass. They are characterized by lower energy input and smaller reactor volumes due to the higher surface area available for the growth of microorganisms, negligible hydraulic losses and the higher resistance of microorganisms towards toxic shock while producing less sludge [73].

Membrane bioreactor (MBR) treatment systems use a combination of an aeration tank like in AS, but instead of separating sludge with a settler, filtration of mixed liquor through a membrane is employed. MBR systems allow higher biomass concentrations since the filtration system prevents the biomass from being washed away and prevents pathogenic bacteria and viruses passing through the treatment system, resulting in a high-quality effluent, but at high installation and maintenance costs [73]. The efficiency of biological processes may be influenced by factors such as wastewater pH, temperature, reactor hydraulic retention time (HRT), microorganisms used, dissolved oxygen, food-to-microorganisms ratio, toxic substances, inoculum and feed pre-treatment [74]. Where secondary biological treatment is not effective, chemical processes may be used independently or to supplement it, such as coagulation or flocculation (to remove colloidal particles), chemical precipitation (to treat inorganic pollutants), chemical oxidation (to mineralize putrescible compounds), electrochemical processes and ion-exchange processes [71].

Tertiary treatment processes are used to remove nutrients and micropollutants. Such treatments are based on adsorption (removal from the liquid phase by an adsorbent), solvent extraction (removal based on solubility and partitioning, for recycling), membrane filtration (for removal of suspended, in decreasing pore size: ultrafiltration, nanofiltration and reverse osmosis, which is also suitable for removing dissolved substances), distillation (for recovery of solvent in industrial wastewater), advanced oxidation processes (for degradation of micropollutants by reactive oxygen species) and disinfection (for removal of pathogens) [71]. These processes are influenced by pH, temperature, contact time, surface area, technical suitability and concentration of contaminants. Although tertiary treatment solutions may provide an extremely high-quality effluent, their application is constrained by cost [71].

Sludge is a significant byproduct of biological and chemical wastewater treatment processes, and its safe, cost-effective disposal remains a significant problem. In many cases, before disposal, the sludge is anaerobically digested to produce economically important biogas. In addition, biological and physicochemical processes are also being used for sludge disposal and valorisation, including pyrolysis, ultrasonication and fermentation [75]. Once dewatered, sludge is either transported away to be incinerated in dedicated plants (often to the dissatisfaction and detriment of the local population) or landfilled [70].

1.2.2 Occurrence and removal of contaminants of emerging concern in conventional wastewater treatment

Wastewater treatment plant influent and effluent contain a plethora of substances, among them many CEC. Influent and effluent concentrations of CEC covered in this dissertation (Section 1.1) are summarized in Tables 5 – 7. These Tables show a considerable variation in the abundance of the listed CEC in wastewater influent and effluent and their removal worldwide.

Among PPCP in influent (Table 5), naproxen and ibuprofen are detected in the highest concentrations (up to 210 µg/L and 56.5 µg/L, respectively), while others range from <LOQ to µg/L concentrations. In effluent, concentrations of naproxen, ibuprofen, sulfamethoxazole, metoprolol, estrone, carbamazepine and oxybenzone have been found to reach several µg/L, with the highest detected concentrations reaching 5.8 µg/L (metoprolol). Metoprolol is more recalcitrant, with removals ranging from negative to intermediate (from -332% to 53%), with a similar trend for carbamazepine (removal from -138% to 82%). On the other hand, ibuprofen and naproxen are more biodegradable with removals from 83% to 99% and from 64% to ≈100%, respectively. Negative removals of CEC occurring in WWTP are not an uncommon phenomenon. They are hypothesized to be the consequence of several possible factors: 1) fluctuations in concentration not accounted for by the sampling strategy, 2) not considering the HRT and therefore mismatching influent and effluent loads when calculating removal, 3) desorption of analytes from the solid phase in wastewater, 4) lack of degrading bacteria in the activated sludge, and 5) analyte back-transformation from conjugated compounds [76].

Neonicotinoid pesticides were detected in WWTP influent in lower concentrations than PPCP, ranging from <LOQ to 166 ng/L, which is expected as they are mainly applied to crops. In effluents, imidacloprid reaches concentrations of 54 ng/L. Their removal ranges from 11% – 100%, albeit only a few studies investigate their removal in WWTP (Table 6).

The most abundant and frequently occurring BP in wastewater influent is BPA, followed by BPS and 4,4'-BPF, reaching up to 20.4 µg/L, 1.7 µg/L and 1.2 µg/L in wastewater influent, respectively, (Table 7) [76]–[80]. However, 2,2'-BPF, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPZ, BPM and BPP have also been shown to occur in wastewater influent, although less frequently and in lower concentrations (BPE found up to 744 ng/L) [76], [77], [79]–[82]. The highest detected effluent concentrations were found for BPA, BPS, 4,4'-BPF, 2,2'-BPF and BPBP and can reach 6.2 µg/L (4,4'-BPF). The removal of BPs may also range from negative to almost complete removal (from -36% to 97% for BPA). When writing this thesis, there was no available data on the occurrence and removal of 2,4'-BPF, BPC II, BPFL, BP26DM and BPPH in wastewater treatment.

Table 5: Influent and effluent concentrations of PPCP in WWTP presented as mean concentrations from all included WWTP and their mean removal in a single study. Influent and effluent concentrations are presented as mean concentration and range (min - max).

Compound	**Location**	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**ref.**
Naproxen	3 Chinese WWTP	13 <LOQ - 31	5 <LOQ – 14	65	[83]
	90 EU WWTP	/	27 <LOQ - 958	/	[84]
	1 US WWTP	/	/	96	[85]
	5 US WWTP	35,700 9300 - 210,000	30 <LOQ - 150	≈100	[86]
	1 Greek WWTP	133 <LOQ – 648	<LOQ	≈100	[87]
	1 Singaporean WWTP	6275 3560 - 7762	2274 2063 - 2415	64	[88]
	1 Chinese WWTP	8 2 - 10	1 1 - 2	85	[89]
Ibuprofen	50 USA WWTP	/	460 <LOQ - 4200	/	[90]
	90 EU WWTP	/	81 <LOQ - 2129	/	[84]
	1 Portuguese WWTP	3800 <LOQ - 52,201	/	/	[91]
	3 Chinese WWTP	300 35 - 406	50 <LOQ - 99	83	[83]
	1 US WWTP	/	/	99	[85]
	5 US WWTP	22,300 8,600 - 56,500	56 13 - 92	99	[86]
	1 WWTP in Singapore	33,548 29,075 - 55,975	1001 865 - 1072	97	[88]

Compound	**Location**	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**ref.**
	1 Chinese WWTP	219 8 - 262	20 8 - 36	91	[89]
Sulfamethoxazole	3 Chinese WWTP	25 <LOQ - 95	9 <LOQ - 22	64	[83]
	90 EU WWTP	/	142 >LOQ - 1147	/	[84]
	50 US WWTP	/	910 <LOQ - 2900	/	[90]
	1 US WWTP	/	/	-36	[85]
	1 German WWTP	348 191 - 476	208 59 - 718	42	[92]
	1 Greek WWTP	88 <LOQ - 507	20 <LOQ - 80	77	[87]
	1 Chinese WWTP	48 30 - 62	23 8 - 38	53	[89]
Erythromycin	Italian WWTP	46 /	15 /	67	[93]
	1 Greek WWTP	<LOQ <LOQ - 320	<LOQ	/	[87]
Clarithromycin	Italian WWTP	200 /	280 /	-40	[93]
	1 US WWTP	/	/	-73	[85]
Metoprolol	3 Chinese WWTP	59 <LOQ - 248	120 <LOQ - 280	-103	[83]
	50 US WWTP	/	410 <LOQ - 660	/	[90]
	1 German WWTP	4148 2407 - 5489	4422 3166 - 5762	-9	[92]
	1 Australian WWTP	379 /	178 /	53	[94]
	1 Greek WWTP	96 <LOQ - 474	415 <LOQ - 780	-332	[87]
	1 Chinese WWTP	14 34 - 29	42 <LOQ - 174	-189	[89]

Compound	**Location**	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**ref.**
Carbamazepine	90 EU WWTP	/	832 <LOQ - 4609	/	[84]
	50 US WWTP	/	97 <LOQ - 240	/	[90]
	Italian WWTP	570 /	370 /	35	[93]
	1 US WWTP	/	/	-92	[85]
	5 US WWTP	115 34 - 350	21 <LOQ - 62	82	[86]
	1 German WWTP	1536 815 - 2436	1614 1020 - 2309	-7	[92]
	2 US WWTP	193 61 - 588	289 91 - 731	-63	[95]
	1 Australian WWTP	637 /	694 /	-9	[94]
	1 WWTP in Singapore	324 323 - 340	309 262 - 336	5	[88]
	1 Chinese WWTP	2 2 - 3	5 5 - 6	-138	[89]
Estradiol	5 Korean WWTP	4 <LOQ - 4	<LOQ	≈100	[96]
	5 Brazilian WWTP	143 <LOQ - 776	48 <LOQ - 397	66	[97]
Estrone	1 Portuguese WWTP	55 <LOQ - 177	/	/	[91]
	1 US WWTP	/	/	94	[85]
	5 US WWTP	97 <LOQ - 160	3 <LOQ - 30	97	[86]
	1 Singapore WWTP	<LOQ <LOQ	<LOQ <LOQ	/	[88]
	5 Korean WWTP	47 32 - 70	6 <LOQ - 24	87	[96]
	5 Brazilian WWTP	566 <LOQ - 3050	242 <LOQ - 2080	57	[97]

Compound	**Location**	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**ref.**
Methylparaben	3 Chinese WWTP	240 21 - 446	3 <LOQ - 18	99	[83]
	1 Chinese WWTP	567 211 - 1002	14 3 - 24	98	[98]
	1 Chinese WWTP	166 1 - 246	18 3 - 72	89	[89]
2,4-dihydroxy benzophenone	5 Indian WWTP	62 20 - 113	2 0 - 8	94	[77]
	1 Greek WWTP	<LOQ	<LOQ	/	[87]
Oxybenzone	3 Chinese WWTP	9 <LOQ - 24	3 <LOQ - 9	70	[83]
	3 Norwegian WWTP	/	497 10 - 1915		[82]
	5 Indian WWTP	262 120 - 548	151 43 - 352	46	[77]
	1 WWTP in Singapore	1734 1220–2617	93 60 - 140	95	[88]

Table 6: Influent and effluent concentrations of neonicotinoid pesticides presented as mean concentrations from all included WWTP and their mean removal in a given study. Influent and effluent concentrations are presented as mean concentration and range (min-max).

Compound	**Location**	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**Ref.**
Imidacloprid	8 US WWTP	/	22 12 - 48	/	[99]
	1 Hungarian WWTP	/ 61 - 80	54 /	22	[100]
	16 Spanish WWTP	19 1 - 166	/	≈100	[101]
	1 US WWTP	55 /	49 /	11	[102]
Thiamethoxam	8 US WWTP	/	1 <LOQ - 1.4	/	[99]
	1 Hungarian WWTP	/ 16 - 24	15 /	20	[100]
	1 US WWTP	<LOQ	<LOQ	/	[102]
Acetamiprid	8 US WWTP	/	2 <LOQ - 5	/	[99]
	1 Hungarian WWTP	/ 1 - 3	1 /	23	[100]
	1 US WWTP	4 /	2 /	50	[102]
Thiacloprid	1 US WWTP	<LOQ	<LOQ	/	[102]

Table 7: Influent and effluent concentrations of BPs in WWTP presented as mean concentrations from all included WWTP and their mean removal in a single study. Influent and effluent concentrations are presented as the mean concentration and the data range (min-max) [ng/L].

	Location	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**Ref.**
BPA	7 Chinese WWTP	1921 189 - 20,400	224 16 - 1100	83	[76]
	3 Norwegian WWTP	/	1150 81 - 4611	/	[82]
	5 Indian WWTP	60 15 - 184	5 1 - 14	82	[77]
	5 New York State WWTP	72 <LOQ - 8550	39 <LOQ - 3380	43	[78]
	3 Chinese WWTP	1300 56 - 5850	34 <LOQ - 123	97	[83]
	5 US WWTP	296 60 - 600	13 <LOQ - 44	96	[86]
	1 Singapore WWTP	5734 5325 - 6969	812 619 - 981	86	[88]
	2 Romanian WWTP	/ 644 - 9140	/ 6 - 75	95	[80]
	4 Chinese WWTP	434 211 - 802	228 100 - 330	42	[79]
BPS	7 Chinese WWTP	86 <LOQ - 746	1 <LOD - 4	98	[76]
	3 Norwegian WWTP	/	277 1 - 1138	/	[82]
	5 Indian WWTP	15 7 - 32	3 1 - 4	81	[77]
	5 Slovenian WWTP	21 2 - 41	<LOQ	≈100	[81]
	5 New York State WWTP	29 <LOQ - 707	26 <LOQ - 444	13	[78]
	2 Romanian WWTP	/ 48 - 1688	<LOQ	99	[80]

	Location	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**Ref.**
	4 Chinese WWTP	101 53 - 177	45 <LOQ - 73	55	[79]
4,4’-BPF	7 Chinese WWTP	44 <LOQ – 166	11 <LOD - 35	57	[76]
	3 Norwegian WWTP	/	1336 31 - 6169	/	[82]
	5 Indian WWTP	10 3 - 28	1 1 - 2	96	[77]
	5 Slovenian WWTP	16 5 - 37	<LOQ	≈100	[81]
	5 New York State WWTP	46 <LOQ-1170	66 <LOQ-556	-36%	[78]
	2 Romanian WWTP	<LOQ	<LOQ	/	[80]
	4 Chinese WWTP	298 120 - 605	246 115 - 620	7	[79]
2,2’-BPF	3 Norwegian WWTP	/	925 81 - 2166	/	[82]
BPE	7 Chinese WWTP	7 <LOQ - 98	9 <LOD - 75	-25	[76]
	5 Slovenian WWTP	84 3 - 238	2 0 - 6	98	[81]
	2 Romanian WWTP	/ 29 - 744	/ <LOQ	≈100	[80]
	4 Chinese WWTP	204 88 - 431	151 74 - 400	23	[79]
BPAF	7 Chinese WWTP	2 <LOQ - 13	1 <LOD - 17	/*	[76]
	3 Norwegian WWTP	/	3 1 - 4	/	[82]
	5 Indian WWTP	3 2 - 3	<LOQ	≈100	[77]
	5 Slovenian WWTP	17 5 - 30	8 7 - 10	53	[81]

	Location	**Influent** [ng/L]	**Effluent** [ng/L]	**Removal** [%]	**Ref.**
	4 Chinese WWTP	80 55 - 141	83 55 - 129	-10	[79]
BPB	5 Indian WWTP	3 1 - 6	2 1 - 2	/*	[77]
	5 Slovenian WWTP	9 0 - 28	1 0 - 2	91	[81]
	2 Romanian WWTP	<LOQ	<LOQ	/	[80]
	4 Chinese WWTP	276 120 - 433	179 88 - 351	34	[79]
BPC	5 Slovenian WWTP	6 1 - 12	2 /	/*	[81]
	2 Romanian WWTP	<LOQ	<LOQ	/	[80]
BPZ	5 Indian WWTP	3 3 - 3	<LOQ	/*	[77]
	5 Slovenian WWTP	151 2 - 403	44 3 - 86	71	[81]
	4 Chinese WWTP	323 167 - 488	211 9 - 349	21	[79]
BPAP	5 Indian WWTP	2 2 - 2	<LOQ	/*	[77]
	5 Slovenian WWTP	75 <LOQ - 177	5 <LOQ - 10	93	[81]
	4 Chinese WWTP	155 110 - 225	100 <LOQ - 188	34	[79]
BPBP	3 Norwegian WWTP	/	985 6 - 2926	/	[82]
BPP	5 Indian WWTP	4 2 - 6	0 0 - 0	93	[77]
BPM	4 Chinese WWTP	<LOQ <LOQ	4 <LOQ - 20	/*	[79]

/ - data not available due to sampling strategy or other constraints

* - concentrations too low to accurately calculate removal

1.2.3 Wastewater treatment with algal technologies

Wastewater treatment technologies have seen a rise in the development of nature-based solutions for wastewater treatment, some of them resulting in successful applications, e.g. constructed wetlands [103], [104]. Promising alternatives to biological wastewater treatment are algal-based technologies that rely on algae communities and aerobic heterotrophic microorganisms (primarily bacteria) to treat different wastewaters. Depending on treatment needs, different types of ponds may be used. Facultative and maturation ponds may also be considered waste stabilisation ponds and are used as tertiary treatment for biodegradable oxygen demand (BOD) and pathogen removal [7].

Conversely, high rate algal ponds (HRAP) may be used to remove BOD and nutrients and allow a high rate of algal growth. They are shaped like a raceway, with a paddlewheel for mixing the water. HRAP systems can be used for secondary wastewater treatment and are a potential alternative to conventional biological wastewater treatment technology [105]. When compared to activated sludge reactors, HRAPs have long HRT (HRT: $3 - 8$ h vs. $1 - 14$ days, respectively) and facilitate photodegradation due to their shallow depth ($0.1 - 0.5$ m) [7] and large surface area, i.e., 1 ha or more in full-size HRAP systems [105], [106]. Construction and operational costs of HRAPs for secondary wastewater treatment are one quarter to one-third that of conventional activated sludge treatment [107]. They also do not require aeration as algae provide oxygen and organic acids beneficial for bacteria, producing CO_2 and vitamins. However, competition can occur when nutrients are limited [7]. An alternative design is a tubular photobioreactor (TPBR), which has two open pools on the extremes, between which the medium is flowing through transparent tubes made of polymers; however, flow is still driven by paddlewheels in the pools [108].

Although many resources (e.g., water, energy, food and nutrients) are exploited linearly (extract – use – dispose), the reuse of resources by recycling wastewater is in line with circular economy principles. One of the main advantages of utilising micro-algae-based solutions is nutrient- and energy-rich algal biomass production. The biomass may be exploited, most notably as a feedstock, i.e., for polymer, energy (e.g., biogas or biodiesel) and fertiliser production [109]–[112]. Cultured algae might also be used as a feed supplement for animals, although this would depend on the characteristics of the wastewater [113]. Notably, the valorisation of biomass and reclaimed wastewater is in line with the principles of the EU's Circular Economy Action Plan [114]. The specific characteristics (previous paragraph) of algal wastewater treatment technologies do not make them applicable for every situation, e.g. WWTP of large cities that have to treat a large amount of wastewater are not likely to adopt it. However, where enough area can be spared, and long HRT can be applied according to the amount of wastewater inflow, the use of algal wastewater treatment may prove a valuable asset.

1.2.4 Removal of contaminants of emerging concern with algal technologies

Removal of CEC from wastewater in algal photobioreactors can result from a combination of biodegradation, photodegradation, adsorption and volatilization, depending on the compounds' physicochemical properties [7], [115]. Biodegradation of CEC in algal results from the metabolism of algae and bacteria, either intracellularly or extracellularly [116]. According to Liu et al. (2021), biodegradation of CEC may take place according to three scenarios: (1) microalgae do not directly degrade the compound but provide a favourable environment for bacteria, promoting biodegradation, (2) bacteria and algae both significantly and directly contribute to the biodegradation of CEC, (3) algae and bacteria synergistically degrade CEC, where one can degrade the intermediate products of the other or vice versa [117]. Co-metabolic biodegradation is accomplished by non-specific enzymes produced to assimilate other organic compounds [117]. However, the more intricate workings of expression of removal mechanisms in algal photobioreactors are not understood.

Photodegradation is a likely removal mechanism due to high light exposure of algal ponds and occurs through direct photolysis. Indirectly, algal systems may also improve degradation due to high oxygen production and the possible production of reactive oxygen species [7], [115]. Removal by adsorption and uptake is expected for more hydrophobic compounds (log $P > 4$) [7], [115]. Algae can also efficiently remove nutrients (inorganic and organic nitrogen and phosphorus) from water by incorporating them into their biomass. Due to the removal of CEC and the production of high-quality effluent and algal biomass and its reuse, algal wastewater treatment technologies are receiving considerable attention in recent years [2], [115], [118].

Although not the primary concern of this work, laboratory studies on TPs of included CEC formed in algal photobioreactors will be briefly summarised. Knowledge of TPs is important as they may exhibit recalcitrance and biological activity equal or higher than their precursor [119]. Regarding PPCP, Xiong et al. (2016) postulate that epoxidation and hydroxylation by Phase I enzymes are important in the metabolism of carbamazepine by *Chlamydomonas mexicana* and *Scenedesmus obliquus*, based on determined TPs [120]. Ding et al. (2017) have shown that ibuprofen is likely metabolized by hydroxylation, dehydroxylation, acylation, demethylation, carboxylation and glucuronidation [121]. Furthermore, the same group has performed a similar study on naproxen, yielding hydroxylation, demethylation, decarboxylation and conjugation with tyrosine, glucuronide, and oxalic acid [122].

Studies on algal TPs of neonicotinoid pesticides were not found during the writing of this thesis. However, regarding BPs, an early study by Hirooka et al. (2005) includes identifying monohydroxy BPA as an intermediate, which was then degraded in a culture of *Chlorella fusca*, thereby reducing estrogenic activity [123]. Later, Nakajima et al. (2007) showed that BPA is transformed to different BPA-glycosides by several microalgal species [124]. Tetrabromo-BPA transformation by sulfation, glycosylation, O-methylation and debromination by microalgae has also been observed [125]. Wang et al. (2017) found that BPA is transformed by glycosylation, oxidative hydroxylation, methylation and cleavage into monophenols, with a combination of these processes resulting in various TPs [126]. Ding et al. (2020) also studied the biotransformation of BPS and suggested the conjugation of oxalic acid, dehydroxylation, demethylation and disulfidation as likely pathways [127]. Finally, Ding et al. (2021) identified the loss of phenol rings, aspartate conjunction, reduction and carboxylation as pathways in the metabolism of BPFL [128].

These studies show that CECs are subject to Phase I metabolism (oxidation, reduction or hydrolysis), Phase II metabolism (conjugation), and a combination of both, yielding many possible TPs. Both Phase I and II metabolism detoxify and aid excretion by making toxic compounds more polar. There is also the possibility of transformation products having similar or higher biological activity than their precursors. However, none of the above studies reports such occurrences.

1.2.4.1 Laboratory-scale photobioreactors

Several authors have studied the removal of PPCP (Table 8) and BPs (Table 9) in laboratory-scale photobioreactors (Figure 1). Most often, single strains of algae are grown in simple media, while some studies use a mixture of algae [129]–[131] or different types of wastewater (municipal, anaerobic, secondary wastewater) [129], [130], [132]–[136], to approach real-life conditions in wastewater treatment. HRT ranges from 2 to 40 days, and

C_0 spans the µg/L to mg/L range, with the latter not relevant for wastewater, where such high concentrations are not found (Table 5 – Table 7). The most often studied PPCP in algal bioreactors are carbamazepine (9 studies) and sulfamethoxazole (8 studies), while the least studied are hormones and benzophenones, which have only been the subject of a single study in each case (Table 8). Carbamazepine and metoprolol are recalcitrant in laboratory photobioreactors with removals ranging from 0% to 50%, with two exceptions [132], [137], where they reached 90% and 93% removal, respectively. These findings agree with their reported recalcitrance in conventional wastewater treatment (Table 5). The analgesics ibuprofen and naproxen, and the macrolide antibiotics typically show good biodegradability in photobioreactors (generally 26% - 99%), while sulfamethoxazole shows some recalcitrance (0% - 86%). The limited available data for hormones and benzophenones makes it impossible to draw any conclusions (Table 8).

There is also a distinct lack of laboratory studies on removing neonicotinoid pesticides in photobioreactors at the time of the writing of this thesis. Laboratory-scale studies on BPs are summarised in Table 9. BPA is the most studied BP in laboratory-scale batch cultures of different algae species, often spiked with high concentrations (up to mg L^{-1} range) [138]–[141], which are not relevant for real wastewaters. The removals in these studies range from 18% to 99%, with removals generally lower with ascending concentrations of initially spiked BPA (Table 9). Most studies report removals from the aqueous phase of algal cultures and seldom investigated BPA present in the biomass phase. Eio et al. (2015) and Liu et al. (2010) studied BPA in algal biomass and reported less than 1% of the initially spiked amount being present in the biomass, while Sole and Matamoros (2016) found up to 6% [136], [138], [142].

To date, few studies have investigated the removal of BPs other than BPA in algal photobioreactors. Solé and Matamoros (2016) investigated the removal of BPA, BPAF and 4,4’-BPF in addition to three other endocrine disrupting compounds (EDC) from secondary-treated effluent inoculated with free and immobilised algae. Removals of 46%, 80% and 87% were achieved for BPA, BPAF and 4,4’-BPF, respectively, each spiked at an initial concentration of 10 µg L^{-1} after 10-day incubation [136]. They also found 17% of BPAF in the biomass. Peng et al. (2014) observed the 99% removal of tetrabromo-BPA in 10 days (C_0 = 435 µg L^{-1}) and the formation of its transformation products [125]. Ding et al. (2020) observed removal of BPS at initial concentrations of 0.5 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}, reporting 95%, 34% and 33% removal, respectively [127]. Lastly, Ding et al. (2021) also observed the toxic effects, removal and biotransformation of BPFL [128]. This limited number of studies means there little knowledge on the removal and fate of BPs other than BPA in algal photobioreactors, despite their detection in wastewater and the aquatic environment. While outdoor studies pilot-scale photobioreactors are more directly applicable [143], in order to overcome the knowledge gap on removal pathways and mechanisms, laboratory-scale studies remain essential.

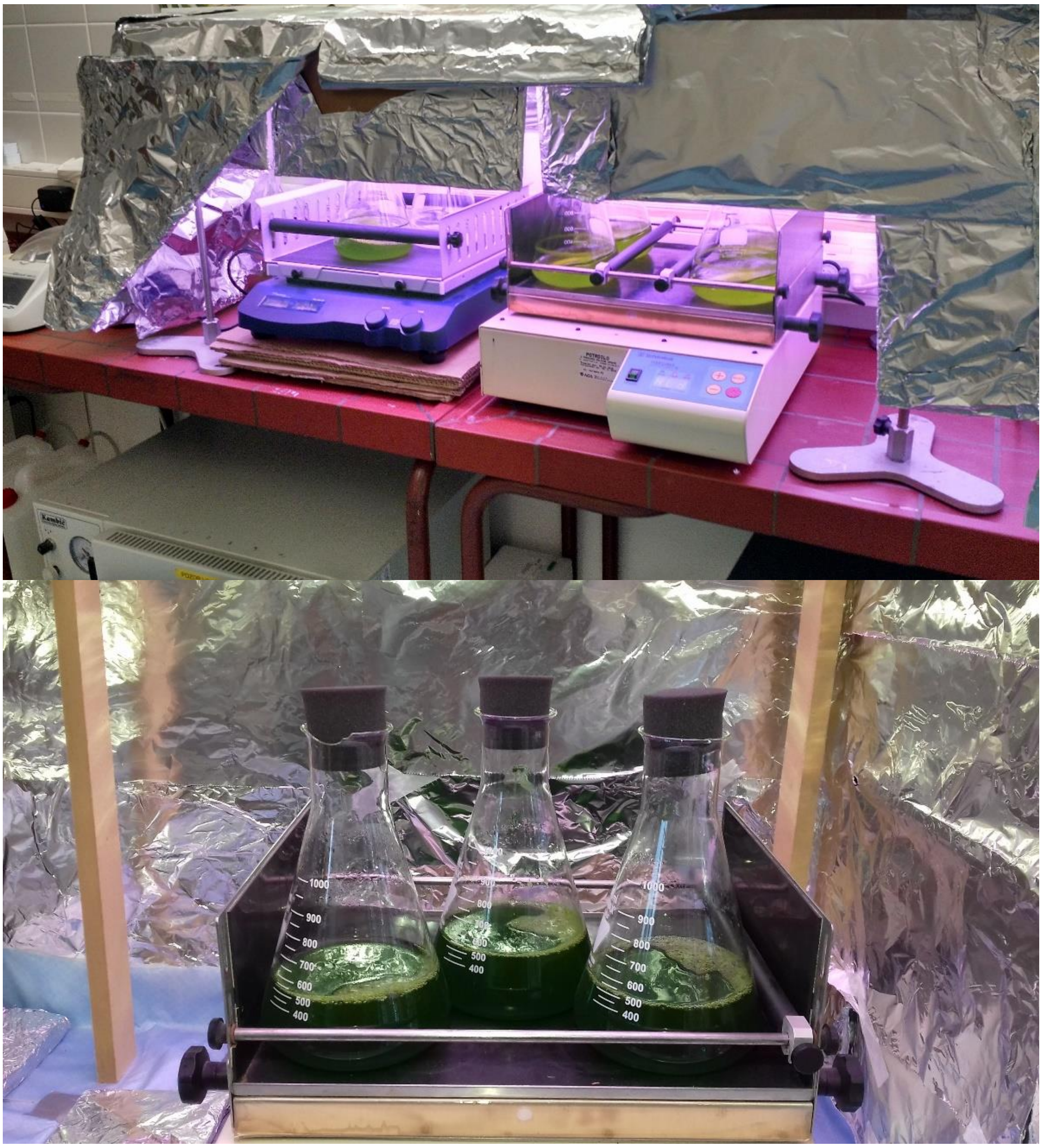


Figure 1: Examples of laboratory-scale batch photobioreactors.
(Photos: Franja Prosenc, David Škufca)

Table 8: Review of laboratory-scale algal studies of PPCP removal. Growth medium, algal species in the photobioreactor, hydraulic retention time (HRT), initial CEC concentration (C_0), and removal (R) are listed for each given compound.

Compound	**Medium**	**Species**	**HRT [day]**	**C_0**	**R [%]**	**Ref.**
Naproxen	urban WW	undefined	2	4177 ± 128 ng/L	28	[129]
	urban WW	undefined	3-4	4177 ± 128 ng/L	24 - 52	[129]
	D1	*Navicula* sp.	21	0.5 mg/L	95	[137]
	BG-11	*Chlorella vulgaris*	3	10 µg/L	0	[144]
	D1	*Cymbella* sp. and	30	1, 10 and 100 mg/L	95, 80 and 60	[122]
	BG11	*Scenedesmus quadricauda*	30	1, 10 and 100 mg/L	60, 65 and 2	[122]
Ibuprofen	ana. WW	*Chlorella sorokiniana*	31	317 ± 33 µg/L	99	[132]
	Urine	*Chlorella sorokiniana*	23	317 ± 33 µg/L	99	[132]
	synt. WW	mix	10	5 µg/L	51 ± 6	[130]
	urban WW	mix	10	5 µg/L	99 ± 1	[130]
	urban WW	undefined	2	8955 ± 959 ng/L	26 - 91	[129]
	urban WW	undefined	3-4	8955 ± 959 ng/L	81 - 94	[129]
	D1	*Navicula* sp.	21	0.5 mg/L	97	[137]
	D1	*Navicula* sp.	15	1, 10, 50 mg/L	60, 27 and 20	[121]
	mun. WW	mix	25	50 µg/L	60%	[133]
Sulfamethoxazole	lake water + F/2	*Nannochloris* sp.	14	10 µg/L	<40	[145]
	second. WW	*Spirogyra* sp.	20	200 µg/l	14±5	[134]
	second. WW	*Zannichellia palustris*	20	200 µg/l	20±2	[134]

Compound	**Medium**	**Species**	**HRT [day]**	**C_0**	**R [%]**	**Ref.**
	Ferty Basic Fertilizer	*Acutodesmus obliquus*	7	250 µg/L	43	[146]
	D1	*Navicula* sp.	21	0.5 mg/L	86	[137]
	BBM	Scenedesmus obliquus	11	0.05, 0.1, and 0.2 mg/L	16, 22, and 29	[147]
	synt. WW	*Haematococcus pluvialis, Selenastrum capricornutum, Scenedesmus quadricauda* and *Chlorella vulgaris*	40	20, 50 and 100 µg/L	74	[148]
	BG-11	*Chlorella vulgaris*	3	10 µg/L	0	[144]
	WC	*Microcystis aeruginosa*	12	10 µg/L	70	[149]
	WC	*Synechococcus* sp.	12	10 µg/L	80	[149]
	WC	*Chlamydomonas reinhardtii*	12	10 µg/L	0	[149]
Erythromycin	synt. WW	*Chlamydomonas acidophila*	14	2300, 14, 2.3 µg/L	20, 99, 99	[150]
Clarithromycin	synt. WW	*Chlamydomonas acidophila*	14	800, 40 and 8 µg/L	42, 99, 99	[150]
	synt. WW	*Haematococcus pluvialis, Selenastrum capricornutum, Scenedesmus quadricauda* and *Chlorella vulgaris*	40	20, 50 and 100 µg/L	76	[148]
Metoprolol	ana. WW	*Chlorella sorokiniana*	31	181 ± 62 µg/L	90	[132]
	urine	*Chlorella sorokiniana*	23	181 ± 62 µg/L	50	[132]
	BG-11	*Chlorella vulgaris*	3	10 µg/L	0	[144]
	WC	*Microcystis aeruginosa*	12	10 µg/L	0	[149]

Compound	**Medium**	**Species**	**HRT [day]**	**C_0**	**R [%]**	**Ref.**
	WC	*Synechococcus* sp.	12	10 µg/L	40	[149]
	WC	*Chlamydomonas reinhardtii*	12	10 µg/L	0	[149]
Carbamazepine	Lake Mead water + F/2	*Nannochloris* sp.	14	10 µg/L	<10	[145]
	ana. WW	*Chlorella sorokiniana*	31	117 ± 17 µg/L	10	[132]
	urine	*Chlorella sorokiniana*	23	117 ± 17 µg/L	5	[132]
	synt. WW	mix	10	5 µg/L	<20	[130]
	urban WW	mix	10	5 µg/L	<20	[130]
	BBM	*Chlorella sorokiniana*	12	1 µg/L	19	[151]
	BBM	*Chlorella vulgaris*	12	1 µg/L	24	[151]
	BBM	*Chlorella saccharophila*	12	1 µg/L	28	[151]
	BBM	*Coelastrella* sp.	12	1 µg/L	36	[151]
	BBM	*Coelastrum astroideum*	12	1 µg/L	20	[151]
	BBM	*Desmodesmus* sp. RUC2	12	1 µg/L	71	[151]
	BBM	*Desmodesmus* sp. 2-6	12	1 µg/L	31	[151]
	BBM	*Scenedesmus* sp.	12	1 µg/L	39	[151]
	BBM	*Scenedesmus obliquus*	12	1 µg/L	38	[151]
	BBM	*Chlamydomonas mexicana*	10	1, 10 and 25 mg/L	<35	[120]
	BBM	*Scenedesmus obliquus*	10	1, 10 and 25 mg/L	<28	[120]
	D1	*Navicula* sp.	21	0.5 mg/L	93	[137]
	synth. WW	*Chlamydomonas acidophila*	14	160 µg/L	≈30	[150]

Compound	**Medium**	**Species**	**HRT [day]**	**C_0**	**R [%]**	**Ref.**
	BG-11	*Chlorella vulgaris*	3	10 µg/L	0	[144]
	mun. WW	*Chlorella vulgaris* and *Scenedesmus obliquus*	25	50 µg/L	60	[133]
Estradiol	synt. WW	mix	15	1 µg/L	≈80	[131]
Estrone	synt. WW	mix	15	1 µg/L	≈ 65	[131]
Oxybenzone	BBM	*Scenedesmus obliquus*	10	0.1, 0.5, 1, 2, 3, mg/L	≈25	[152]

second. WW = secondary treated wastewater,
synt. WW = synthetic wastewater, mun.
WW = municipal wastewater,
ana. WW = anaerobic wastewater,
D1, BG-11, WC, F/2, BBM – standard synthetic media for growing algae

Table 9: Review of laboratory-scale studies of BPs removal. Compound (comp.), growth medium, algal species in the photobioreactor, hydraulic retention time (HRT), initial concentration (C_0), and removal (R) are listed for a given compound.

Compound	**Medium**	**Species**	**HRT [day]**	**C_0**	**R [%]**	**Ref.**
BPA	MSM	*Chlorella sorokiniana*	7	10, 20 and 50 µg/L	39, 31 and 21	[138]
	FW04	*Monoraphidium braunii*	4	2, 4 and 10 mg/L	39, 48 and 35	[139]
	BBM	*Chlamydomonas mexicana*	10	1, 5, 10, 25 and 50 mg/L	39, 38, 17, 16, 8	[140]
	BBM	*Chlorella vulgaris*	10	1, 5, 10, 25 and 50 mg/L	28, 25, 14, 14 and 6	[140]
	artificial seawater with F/2	*Stephanodiscus hantzschii*	16	0.01, 0.10, 1, 3, 5, 7 and 9 mg/L	88, 99, 92, 61 and 48	[141]
	F/2	*Navicula incerta*	4	0.001, 0.01, 0.1, 1, 5 mg/l	38, 35, 31, 9 and 4	[142]
	ultrafiltration effluent	*Nannochloris* sp.	7	192 µg/L	46	[135]
	secondary WW	*Chlorella* sp. and *Nitzschia acicularis*	10	10 µg/L	43 ± 6	[136]
	BG-11	*Desmodesmus* sp. WR1	10	1, 3, 5.5 and 13.5 mg/L	57, 25, 18 and 26	[126]
	BBM	*Picocystis* sp.	5	25, 50 and 75 mg/L	72, 53 and 40	[153]
	Zarrouk	*Graesiella* sp.	5	25, 50 and 75 mg/L	53, 37 and 28	[153]
	BG11	*Chlorella vulgaris*	10	0.5, 5 and 10 mg/L	95, 70 and 55	[127]
BPS	BG11	*Chlorella vulgaris*	10	0.5, 5 and 10 mg/L	95, 34 and 33	[127]
	D1	*Navicula* sp.	5	0.5, 5 and 10 mg/L	95, 55 and 60	[154]
4,4'-BPF	secondary WW	*Chlorella sp.* and *Nitzschia acicularis*	10	10 µg/L	1 ± 1	[136]
BPAF	secondary WW	*Chlorella sp.* and *Nitzschia acicularis*	10	10 µg/L	69 ± 5	[136]
BPFL	BG-11	*Chlorella vulgaris*	10	0.5, 1 and 5 mg/L	>90	[128]

1.2.4.2 Pilot-scale photobioreactors

Efforts have already begun to characterise the removal of various types of CEC using algal treatment technologies in outdoor pilot-scale photobioreactors. While studies on laboratory-scale reactors are common, those involving pilot-scale photobioreactors for wastewater treatment are rare [2], likely due to the scarcity of these systems and the complexity of conducting experiments in them. A pilot-scale HRAP in Ajdovščina, Slovenia, is shown in Figure 2.

Matamoros et al. (2015), who studied the removal of 28 CEC in two HRAPs fed with urban wastewater (V = 0.5 m^3 and HRT of 4 and 8 days), reported on a wide range of CEC removals, with higher removals in the warm season and on the effect of HRT on removal only during the cold season [115]. Similarly, Gentili and Fick (2017), who investigated the removal of 79 pharmaceuticals (HRAP: V = 0.65 m^3 and a 7-day HRT), reported a correlation between light intensity inside the culture and CEC removal [155]. In another study, Villar-Navaro et al. (2018) compared the removal of 81 pharmaceuticals during conventional activated sludge treatment to their removal in an HRAP (V = 9.6 m^3 and an HRT of 6 days) [5]. The authors found that although overall removal was similar, the composition of individual compounds in the effluent varied, indicating different removal mechanisms in the two systems. García-Galán et al. (2020), in their study of the removal of 12 pharmaceuticals and 26 of their metabolites (HRAP: V = 0.47 m^3 and HRT of 4.5 days), found that initial primary treatment did not affect removal, which on average ranged from negative removals (carbamazepine) to >80% (sulfamethoxazole) [156]. The authors also found that algae removed certain compounds to a greater extent than activated sludge and that there was no difference when it came to removing recalcitrant compounds like carbamazepine and metoprolol. Vassalle et al. studied the removal of 11 CEC in a system combining an up-flow anaerobic sludge blanket reactor with an HRAP (V = 0.2 m^3 and HRT of 8 days) [157]. The removal rates ranged from 42% (BPA) to 95% (estrone). Vassalle et al. (2020) have also studied the removal of PPCP in HRAP (V = 0.47 m^3 and HRT of 4.5 days), with removals ranging from -29% (oxybenzone) to 45% (naproxen) [158]. Removal of PPCP and pesticides has recently been studied also in a TPBR. Garcia-Galan et al. and Vassalle et al. observed removals of ibuprofen (33% - 70%), carbamazepine (11% - 15%), oxybenzone (40%) and imidacloprid (-230%) in TPBR (V = 11.7 m^3 and HRT of 5 days) [108], [159]–[161]. Similarly, Hom-Diaz et al. (2017) observed the removal of naproxen (10% - 70%) and ibuprofen (99% - ≈100%) in a smaller TPBR (V = 1.2 m^3 and HRT of 8 or 12 days) [162].

An overview of PPCP removal in outdoor algal photobioreactors is given in Table 10. Compounds range from well removed (naproxen, ibuprofen, erythromycin and clarithromycin) to recalcitrant (carbamazepine and 2,4-dihydroxybenzophenone), while others display a wide range of removals (sulfamethoxazole, oxybenzone). Among neonicotinoid pesticides, only imidacloprid removal has been studied, and its removal from agricultural runoff in a TPBR was ≈-230%, possibly due to back-transformation or deconjugation [161]. Similarly, the fate of BPA removal was investigated in only two HRAP studied. BPA is also the only BP studied in outdoor photobioreactors. The studies found 42% removal [157] and 72% - 85% in the warm season [115]. However, as there is limited data, it is hard to make any firm conclusions. Also, while full-scale HRAP installations exist [105], [106], these systems have not yet been characterized regarding CEC removal.

Figure 2: A pilot-scale HRAP in Ajdovščina, Slovenia. (Photo: Ester Heath)

Table 10: Review of pilot-scale studies of CEC removal. Reactor volume and type, hydraulic retention time (HRT), initial concentration (C_0), and removal (R) are listed for a given compound.

compound	**reactor**	**HRT** [day]	C_0 [ng/L]	**R** [%]	**ref.**
Naproxen	470 L HRAP	4	≈5000	83	[115]
	470 L HRAP	8	≈5000	89	[115]
	9600 L HRAP	6	10972 ± 4627	64	[5]
	205 L HRAP	8	≈160	54	[157]
	470 L HRAP	4.5	≈3000	45	[158]
	1200 L TPBR	8	2945±368	10	[162]
	1200 L TPBR	12	25043±1726	70	[162]
Ibuprofen	470 L HRAP	4	≈9000	99	[115]
	470 L HRAP	8	≈9000	99	[115]
Ibuprofen	9600 L HRAP	6	30539 ± 10456	88	[5]
	470 L HRAP	4.5	4731 ± 7029	67	[156]
	470 L HRAP	4.2	4288 ± 6478	74	[156]
	205 L HRAP	8	≈60	46	[157]
	8500 L TPBR	5	≈60	≈33	[108]
	11700 L TPBR	5	≈400	70	[160]
	1200 L TPBR	8	41450±2431	99	[162]
	1200 L TPBR	12	52091 ± 675	≈100	[162]
Sulfamethoxazole	650 L HRAP	7	250 ± 75	6	[155]
	9600 L HRAP	6	391 ± 277	11	[5]
	470 L HRAP	4.5	383 ± 295	49	[156]
	470 L HRAP	4.2	345 ± 264	88	[156]
Erythromycin	9600 L HRAP	6	173 ± 136	82	[5]
Clarithromycin	9600 L HRAP	6	1054 ± 1060	97	[5]
Metoprolol	9600 L HRAP	6	128 ± 207	76	[5]
	470 L HRAP	4.5	93 ±67	≈30	[156]
Carbamazepine	650 L HRAP	7	219±92	-14	[155]
	9600 L HRAP	6	215 ± 51	6	[5]
	470 L HRAP	4.5	21 ± 11	-24	[156]
	470 L HRAP	4.2	22 ±11	-18	[156]
	8500 L TPBR	5	510	≈15	[108]
	11700 L TPBR	5	≈700	11	[160]
Estradiol	205 L HRAP	8	≈20	7	[157]
Estrone	205 L HRAP	8	≈80	55	[157]
Oxybenzone	470 L HRAP	4	≈4000	97	[115]
	470 L HRAP	8	≈4000	99	[115]
	470 L HRAP	4.5	≈250	-29	[158]

compound	**reactor**	**HRT** [day]	**C_0** [**ng/L**]	**R** [%]	**ref.**
	11700 L TPBR	5	≈50	40	[159]
2,4-dihydroxy-benzophenone	470 L HRAP	4.5	≈250	-22	[158]
Methylparaben	470 L HRAP	4	≈1000	59	[115]
	470 L HRAP	8	≈1000	75	[115]
Imidacloprid	11700 L TPBR	5	≈23	-230	[161]
BPA	470 L HRAP	4	≈60	72	[115]
	470 L HRAP	8	≈60	85	[115]
	205 L HRAP	8	≈90	42	[157]

1.2.5 Contaminant of emerging concern removal comparison

A comparison of removal efficiencies of PPCP from WWTP, laboratory-scale photobioreactors (lab-PBR) and outdoor photobioreactors (O-PBR) is presented in Figure 3, from the aggregated data in Tables 5, 8 and 10. Many PPCPs show an overlap in removal when comparing the three data sources; however, it should be stressed that influent complexity, CEC concentrations and treatment conditions vary significantly between studies. Lab-PBR values are difficult to compare to either WWTP or O-PBR. The reason is the broad range of concentrations investigated and that the studies were performed under stable laboratory conditions. Although median removal values of naproxen, ibuprofen, sulfamethoxazole and oxybenzone are lower in O-PBR than in WWTP, their ranges are comparable. Although the data is limited, removal efficiencies are higher in O-PBR than in WWTP for erythromycin, clarithromycin and metoprolol. Carbamazepine appears equally recalcitrant in either. Available estimates of removal of estradiol, estrone, 2,4-dihydrohybenzophenone, and methylparaben are lower in O-PBR than in WWTP.

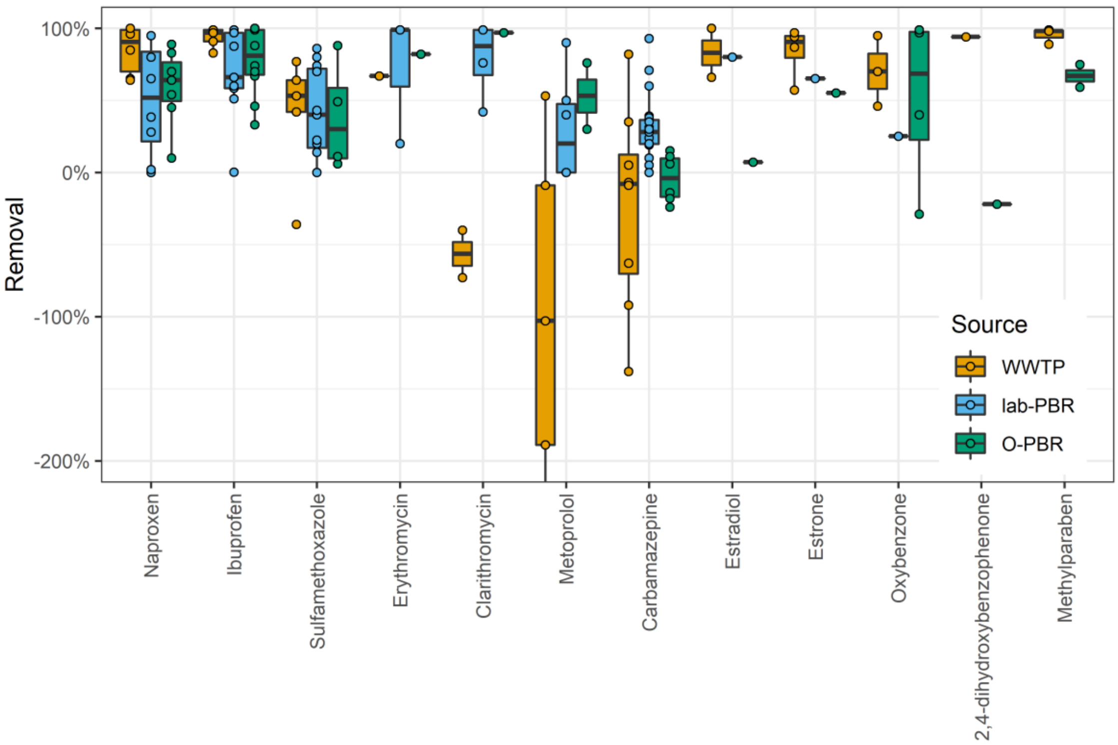


Figure 3: Removal efficiencies of PPCPs in wastewater treatment plants (WWTP, orange), laboratory-scale photobioreactors (lab-PBR, blue) and outdoor photobioreactors (O-PBR, green).

There are few studies concerning removing neonicotinoid pesticides in O-PBR and WWTP and a complete lack of data concerning lab-PBR, so recognizing trends and comparisons is difficult. Nonetheless, removal of imidacloprid in WWTP ranges from 11% to ≈100%, while only one study touches upon its removal in O-PBR, observing only negative removals, on average -230% [161], suggesting that algal photobioreactors may not be suitable for removal of such CEC.

Likewise, only a comparison of BPA is possible between O-PBR and WWTP regarding BPs, as there is a lack of data for other BPs (Figure 4). While the median removal value for O-PBR is lower than that of WWTP, the ranges overlap and have a similar low point if we exclude the negative removal value in WWTP.

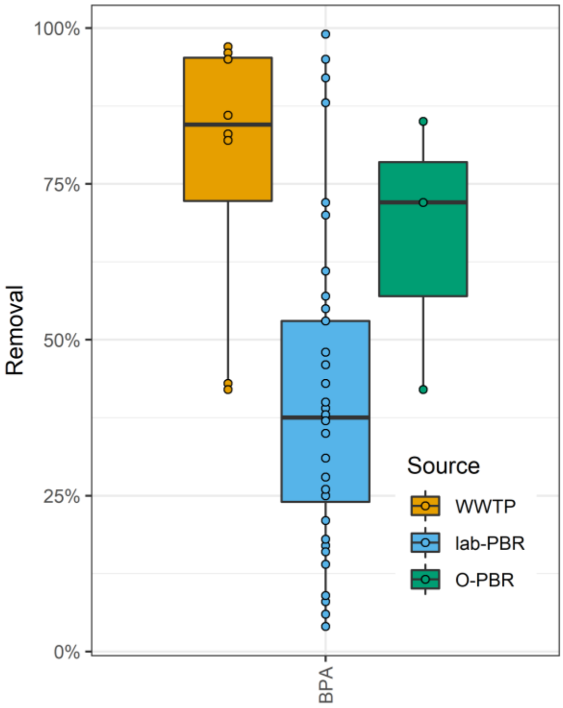


Figure 4: Removal efficiencies of BPA in wastewater treatment plants (WWTP, orange), laboratory-scale photobioreactors (lab-PBR, blue) and outdoor photobioreactors (O-PBR, green).

There are differences in the reported removal of CEC between WWTP and O-PBR. For example, O-PBR has been shown to exhibit lower removals of estradiol, estrone, 2,4-dihydrohybenzophenone and methylparaben; similar removals of naproxen, ibuprofen, sulfamethoxazole, carbamazepine, oxybenzone and BPA, and higher removal of erythromycin, clarithromycin and metoprolol than WWTP. Data is currently scarce, and although pilot-scale studies are performed in algal wastewater treatment systems, treatment still needs to be optimized. Algal photobioreactors show promise for CEC removal from wastewater with simultaneous resource recovery, where their requirements (large area and high HRT) permit their operation.

Unfortunately, studies on CEC removal in algal photobioreactors are too few to draw firm conclusions on CEC removal and their fate, and further studies are necessary for the development of wastewater treatment in algal photobioreactors. Also, the reliability of experimental results depends not only on the experimental set-up but also on the correct use of methodological approaches, which is the topic of the following chapters.

1.3 Methods for the determination of contaminants of emerging concern

Different approaches regarding the sampling, storage, sample preparation and instrumental analysis of environmental and biological samples will be presented in this section. Quality assurance and quality control concepts will also be summarized.

1.3.1 Sampling

Sampling is one of the first steps in any environmental analysis. Therefore, it is crucial that sampling is planned by considering four primary factors, according to Zhang [163]. The first one is to define the project objectives, meaning that we need to establish the goals of our analysis (e.g. establishing a baseline for concentration in a lake, searching for a hotspot, assessing human exposure by human biomonitoring). The second factor considers the spatial and temporal variability of the sampled matrix (e.g. variability of analyte concentration along a river, water levels during different seasons). Third, we must consider sampling and analysis costs, limiting the number of samples we can collect and analyse. Lastly, certain non-technical factors, such as sampling convenience, matrix availability, and regulations (e.g., human biological samples must be considered. Albeit the objectives are the main factor, all must be considered when determining the number, volume or mass of samples and where, how and when they will be collected [163]. Obtaining a representative sample is also essential since, according to the US EPA, representativeness is "a measure of the degree to which data accurately and precisely represent a characteristic of a population, a parameter variation at a sampling point, a process condition, or an environmental condition" and is dependent on the proper design of sampling [164].

In practice, environmental samples are acquired in different ways according to the nature of the matrix. Aqueous samples may be sampled by grab sampling, composite sampling and passive sampling [163]. Grab sampling is cheap, fast and relatively simple, but it only reflects a snapshot of the conditions at that time, and fluctuations can be missed. Composite sampling (time or flow proportional) sampling provides composite samples with programmable automatic sampler devices. Time proportional sampling is achieved by sampling at set intervals over time, while flow-proportional sampling is achieved by varying sampling volume or frequency according to flow [165]. Both provide a good overview of water composition over time, but the sampling apparatus is expensive, requires maintenance and a power source. An alternative is to use a passive sampling device (PSD), most commonly polar organic chemical integrative sampler (POCIS) [166], [167]. Such a device is typically composed of a receiving phase (sorbent), sandwiched between two polyether-sulfone (PES) membranes and held together by two stainless steel rings [167]. It is then immersed for a set time, providing a flow-integrated water sampling by accumulating CEC in the sorbent. A POCIS is simple to use and suitable for the long-term sampling of trace pollutants [166]. However, the sorption rate of CEC to POCIS is affected by several ambient conditions: temperature, pH, dissolved organic matter and salinity. Because of these issues, POCIS is not widely used for monitoring and is still under development [166].

Solid samples in suspension, e.g. sludge and algal biomass, may need settling before sampling and later dewatering using different techniques, e.g. centrifugation, drying, lyophilisation. Solid samples such as soils and sediments are usually sampled with a grab sampler or a core sampler if a vertical profile is desired. There are usually minor short-term variations of contaminants at a given depth in soil and sediments, while vertical variations are common. Stratification, subsampling, mixing and sieving are ways of achieving a representative solids sample [163].

1.3.2 Sample storage

Proper handling and storage of samples are crucial for preserving the analyte of interest and the integrity of the results. The consequences of improper handling and storage might include contamination by container leaching, sorption of the analyte to the container, oxidation and photochemical degradation, and biodegradation.

Preservation strategies depend on the matrix and analytes. Liquid samples are generally stored at -20°C or (wastewater) and 4°C if processed quickly (surface water) [168]–[170]. Surface water and wastewater samples may be acidified, or preservatives may be added to halt any (biological) degradation processes before samples can be stored [169]. Before extraction, aqueous samples could also be filtered or centrifuged to remove any particulate matter, which might cause clogging of the solid-phase extraction (SPE) cartridges or other interference [170]. Solid environmental samples (soil, sediment, sludge or algal biomass) and tissue samples are usually homogenized and dried or preferably freeze-dried [170]. Freeze-drying takes place at low temperature and low pressure, by which we can avoid the breakdown of thermally unstable analytes.

1.3.3 Sample extraction

Extraction is the procedure of separating the analyte from the matrix. Usually, it also involves pre-concentration of the analyte and may serve as a stage to exchange the solvent phase before analysis. For the extraction of liquid environmental samples containing CEC, the most commonly used technique is SPE, followed by liquid-liquid extraction (LLE) [169]. In the case of solid and biological samples, solid-liquid extraction (SLE) is often used, possibly with QuEChERS or SPE, for purification of the extract [26], [170].

SPE has mostly replaced LLE due to having a high preconcentration factor, low consumption of solvents, high recovery as well as being simple, quick and cheap [26], [169]–[172]. In SPE, the liquid sample is percolated through the solid sorbent, causing analytes to be retained on the sorbent [163]. The four steps of SPE are usually: 1) conditioning, 2) loading of sample, 3) washing and 4) elution. Commonly used SPE adsorbents in the analysis of CEC include reversed phase (Oasis HLB and C18), anion-exchange (Oasis MAX) and cationic exchange (Oasis MCX and Strata CX) [26], [169]–[172] sorbents and are selected according to CEC physicochemical properties and matrix characteristics. Molecularly imprinted polymers (MIP) are synthesized through the copolymerization of a functional monomer and a crosslinker in the presence of the analyte molecule that serves as a template [173]. After polymerization, the template molecules are removed, and the MIP is left with cavities that have suitable shape, size and functional groups for retention of the target molecule [173]. MIPs are showing promise as adsorbents in SPE cartridges, as they have high selectivity and sensitivity [174], [175] but have not achieved widespread use due to the lengthy process of MIP development and production and that they are not commercially available for many compounds [173].

1.3.4 Separation

Chromatography denotes various techniques that allow the separation of components in a complex mixture [176]. Generally, the sample is dissolved in a mobile phase (which can be gas, liquid or supercritical fluid), which is forced through a stationary phase either fixed on a sorbent or coated on the inner surface of the chromatographic column. Due to the different physicochemical properties of analytes, components in the sample are distributed to different degrees between the mobile and stationary phase and components that are weakly retained by the stationary phase exit the column faster and vice versa. Due to these differences in retention, components ideally separate into discrete bands that can be further processed by analysers [176]. Techniques commonly used for the separation of CEC are gas-liquid chromatography or shorter, gas chromatography (GC) and liquid chromatography (LC) [169], [170].

1.3.4.1 Gas chromatography

The separation of organic compounds by GC is dependent on the retention of analytes on the stationary phase, governed by the interplay of the degree of their volatility and polarity [176]. Volatility is a requirement for GC separation as the mobile phase is a gas. Because many CEC are relatively non-volatile, they need to be derivatised before gas-chromatographic separation. Derivatization before the analysis may be used to 1) increase volatility and decrease polarity, 2) increase thermal stability, 3) increase detector response (e.g. for electron-capture detectors), 4) improve separation and reduce tailing (Zhang, 2006). CEC are most commonly derivatized by silylation (with bis-(trimethylsilyl)-trifluoroacetamide – BSTFA, N-methyl-N(trimethylsilyl)-trifluoroacetamide – MSTFA or N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide - MTBSTFA) and acetylation (with acetic acid anhydride – AA) [26], [169], [170].

The mobile phase or carrier gas must be chemically inert. Most often, helium is used, but argon, nitrogen and hydrogen can be used as well. The sample is injected into the heated inlet, where it vaporizes. The analytes are then introduced into the column, which is why analytes for GC separation need to be volatile or derivatized to a volatile form prior to analysis. Capillary columns for GC separation are made of fused silica, the inner surface coated with a liquid stationary phase that has to be non-volatile and thermally and chemically stable. Capillary columns are coiled inside the instrument and range in length from 10 to 100 m, with an inner diameter from 0.18 mm to 0.32 mm and a film thickness from 0.1 µm to 1 µm. The choice of stationary phase is dependent on the polarity of analytes – the higher the affinities, the stronger the retention. The polarities of the analytes and column should closely match to optimise elution. The GC column is housed in an oven that controls the temperature of the column precisely. A compound will elute reasonably quickly when the oven temperature is equal to or slightly above its boiling point. When analytes have a broad range of boiling points, the program can be adjusted to increase the temperature with time (continuously or in steps) to ensure an optimal separation and total analysis time. The flow rate may be modified to adjust the analyte bandwidth and separation efficiency [176].

1.3.4.2 Liquid chromatography

Liquid chromatography (LC) is a frequently used separation technique used in the analysis of CEC in environmental and biological samples [169], [170]. At first, liquid chromatography was performed with hand-packed glass columns, through which the flow rate of elution solvents was slow. Nowadays, LC refers to high-performance liquid chromatography (HPLC), which allows higher mobile phase pressures and smaller sized particles in the column to achieve better separation [176].

In LC, a sample is injected and carried by a liquid mobile phase through the column to the detector. LC is different from GC because the analytes interact with the mobile phase. Usually, two miscible solvents are used (e.g. methanol and water) as the mobile phase to achieve the desired polarity. Depending on the mixture properties, analyte elution can be isocratic (solvent mixture has a constant composition, e.g. 50/50) or gradient (two or more solvents are mixed in varying ratios during elution). LC relies on separation in straight stainless steel or polymer columns, packed with small porous microparticles, coated with adsorbents. The column packing is selected (similarly to GC) according to analyte properties. Different normal-phase, reversed-phase, ion-exchange and adsorption chromatography stationary phases may be chosen. In normal-phase chromatography, the stationary phase is polar, and the mobile phase is non-polar. The opposite is true for reverse-phase chromatography, where the latter is more versatile due to easy manipulation of the polarity of aqueous mobile phases. Ultra-performance liquid chromatography (UPLC) differs from HPLC by the smaller particle size in the column, the higher pressure of mobile phase and consequently improvements in time of analysis and resolution [176].

1.3.5 Detection

After separation by GC or LC, CEC in the sample are most commonly detected by mass spectrometry (MS)-based analysers. Different types of MS analysers can provide information about structural elucidation (fragments) of molecules as well as the qualitative and quantitative composition of samples [176]. Before broader applications of MS, popular detection techniques in environmental analysis coupled with GC included the flame ionization detector (FID) or electron capture detectors (ECD), while for LC, ultraviolet-visible spectrophotometry, diode array detector and fluorescence detector were commonly used [170]. While the listed detectors are still in use for specific applications, MS is preferentially used in CEC trace analysis due to its sensitivity, selectivity and reliability of the compound's identification compared to other detectors. Both GC and LC techniques coupled with MS are used to separate CEC, with LC techniques gaining popularity in the last years due to LC’s ability to analyse a broader range of compounds directly in aqueous samples, without derivatization [26].

After chromatographic separation, MS analysis begins by introducing the sample into the vacuum environment of the MS instrument through the inlet system. Analyte molecules are converted into ions in the ion source and then transported into the mass analyser, where ions are selected according to their mass-to-charge ratio (m/z) and their abundance determined in the detector. The resulting mass spectra can then be analysed by computer software [176]. Different ion sources and mass analysers will be summarized as the most defining components of analytical instrumentation. Figure 5 is a simplified diagram of the inner workings of an MS.

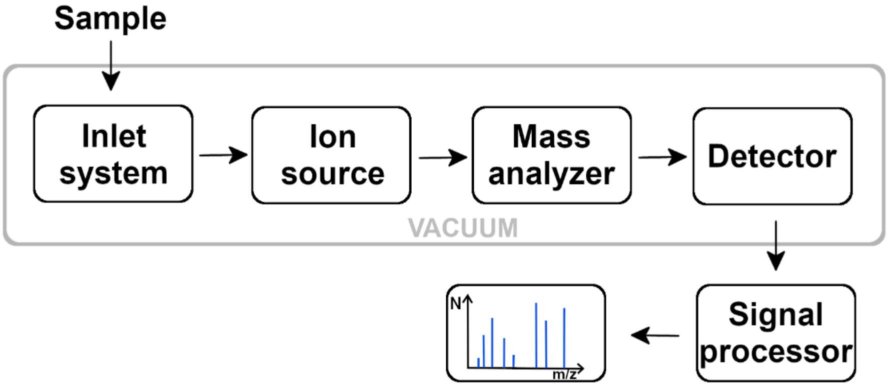


Figure 5: Diagram of an MS.

1.3.5.1 Ion sources

The formation of gaseous analyte ions is the start of every mass spectrometric analysis. Ion sources can be divided into gas-phase and desorption sources. With gas-phase sources, samples are first vaporized and then ionized. Desorption sources directly convert solid- or liquid-state samples into gaseous ions, which is useful when analysing non-volatile or thermally unstable analytes. Alternatively, ion sources can be divided into “hard” and “soft” sources. So-called “hard” sources will transfer sufficient energy to analyte molecules to cause extensive molecular ion fragmentation, giving information about functional groups and structural information. Soft ion sources produce little fragmentation but provide information about the molecular mass of the analyte [176].

Gas-phase ion sources are used for GC, and the sample must be vaporized. The most common ionization technique in connection with GC is electron-impact (EI) ionization (Figure 6), a so-called “hard” ionization source. In this technique, the sample is ionised by bombarding it with a beam of energetic electrons emitted by a filament. The electron beam causes the molecules to lose electrons due to electrostatic repulsion, resulting in positive ions that are highly energetic. These ions then fragment into smaller daughter ions due to subsequent relaxation. Because a constant electron impact energy of 70 eV is used in such sources, it yields very reproducible spectra of molecular fragments. This reproducibility is why there are many mass spectra libraries based on this ionization method, which aids the identification of compounds (e.g. National Institute of Standards and Technology – NIST library). However, the molecular ion can be absent in EI, making it hard to determine the molecular mass. Volatilization of the sample can also mean that analyte molecules might be thermally decomposed. EI is limited to the ionization of molecules smaller than 1000 Da. However, EI produces high ion currents, which improves sensitivity. Chemical ionization (CI) is a “soft” method. It uses reagent gasses, e.g. methane, propane, isobutane, ammonia, which are ionised when bombarded with electrons become ionised. These then react with other reagent molecules. These highly reactive ions are formed in excess and ionise the analyte molecules by either proton or hydride transfer, producing $(M+H)^+$ or $(MH-H)^+$ ions, respectively.

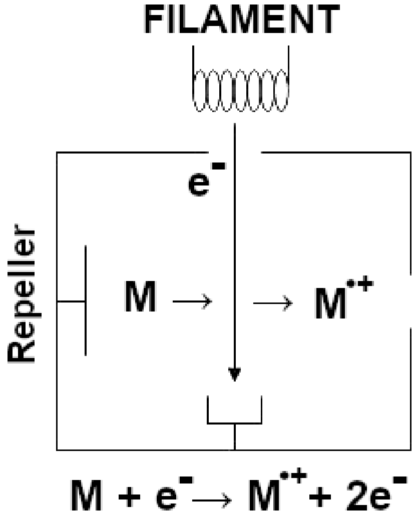


Figure 6: A diagram of an electron-impact ion source and the general ionization reaction, adapted from [177].

Desorption ion sources use different methods to introduce energy into the sample [176]. Electrospray ionization (ESI), a widely used ionization technique used in LC, has become one of the most important techniques for ionising non-volatile CEC in environmental and biological matrices. It takes place at atmospheric pressures and temperatures. A solution of the sample is pumped through a steel capillary surrounded by a cylindrical electrode. Here the solvent is evaporated, and the charge is attached to the droplets of the sample. When the charge is sufficiently concentrated, the sample droplets are torn apart into smaller droplets until the solvent is removed and only charged analyte molecules remain. A helpful feature is that spectra of large biomolecules (e.g. proteins) may have several adjacent molecular peaks that differ by one charge. Large biomolecules' average charge state increases linearly with molecular mass, making it possible to calculate molecular mass from peak distribution. Usually, there is little fragmentation with ESI [176].

The presence of other matrix components in the ion source can cause “ion suppression” in the case of LC-MS techniques. Competition for the ionisation process by other compounds causes the MS signal to be less than that of a pure compound of the same concentration. Salts, buffers, variable matrix components may cause variation in ion suppression between different samples, resulting in unreliable quantitative results. Sample purification techniques, matrix-matched calibration and stable isotope internal standards are employed to reduce interference by this phenomenon [178].

1.3.5.2 Analysers

Mass analysers are devices, which separate ions with different mass to charge ratios (m/z), according to Skoog et al. (2017) [176]. Sensitivity is an essential characteristic of mass analysers and is defined by the number of ions the analyser allows to pass through to yield a signal. Another defining performance characteristic of mass analysers is resolution, which is the ability of the mass analyser to distinguish ions with slight differences in mass. This ability is usually presented as resolving power in by full peak width at one-half maximum (FWHM), where the measured mass is divided by peak width at half maximum of the peak [179]. Low-resolution analysers typically have a resolving power around 10^3 FWHM, while high resolution mass analysers can reach resolving powers up to 10^6 FWHM [179]. A complementary characteristic of high-resolution mass analysers is mass accuracy (given in ppm), which is calculated by the following equation, where m_i is a measurement of a single accurate mass and m_a represents the exact mass of an analyte in Da [180]:

$$\Delta m_i = \frac{(m_i - m_a)}{m_a} \times 10^6 \, [ppm] \tag{1}$$

The basic MS analyser for CEC quantitation is the quadrupole analyser constructed of four parallel metal rods, positioned in a square if viewed in cross-section [176]. Constant or varying voltages on the rods allow the transmission of ions with specific *m/z* to the detector. A so-called scan across m/z values is performed by varying the voltage, resulting in a mass spectrum. Alternatively, specific *m/z* values can be monitored in rapid succession (selected ion monitoring, SIM), which generally improves detection limits [176]. The issue of SIM is that it is not very selective, as other ions with similar *m/z* values may be transmitted and detected, resulting in interference. A more sophisticated approach is using triple quadrupole analysers, operating in tandem (MS/MS), where the first and third quadrupole is similar to single quadrupole analysers, while the second is a collision cell. There, ions can be induced to fragment by collisions with an inert gas (nitrogen or argon), called collision-induced dissociation. The advantage of triple quadrupoles is higher selectivity over single quadrupole analysers since it is likely that two precursor ions with the same *m/z* will produce a product ion with different *m/z* after fragmentation. This is taken advantage of in a technique called multiple reaction monitoring (MRM). In MRM, the first and third quadrupoles operate on *m/z* ratios of precursor and product ions of the target analytes, respectively, thereby providing a reliable identification [178]. Triple quadrupoles are the most commonly used MS analysers in combination with GC or LC separation for targeted environmental analysis due to their selectivity, sensitivity and ease of use [26], [169]–[172]. Quadrupole and triple quadrupole analysers generally provide unit-mass resolution, with a FWHM of about 10^3 [179]. Ion trap (IT) analysers use static and radiofrequency voltages on three hyperbolic electrodes to trap ions. Ions are then selected and released according to their *m/z* values to create a mass spectrum or select for specific *m/z* values. These analysers are unique in that they can repeatedly isolate and fragment trapped ions (MS^n) and have the potential to achieve high sensitivity.

High-resolution MS (HRMS) analysers are used for applications like proteomics and metabolomics for the identification of analytes and structural elucidation [176], [181]. Time-of-flight analysers (TOF) use high voltage to accelerate ions through a drift tube. Depending on their *m/z* values, the ions have different velocities and take different amounts of time to travel to the detector. While the sensitivity of instruments with TOF analysers is relatively low, they give accurate mass measurements (< 3 ppm) of analytes and have high resolving power of $\geq 10^4$ FWHM. In this way, they can provide reliable identification and structural information of compounds in a sample.

Even though stand-alone mass analysers are used, far better analytical capabilities are achieved with hybrid systems. One example is an analyser, where instead of the third quadrupole, there is a TOF, resulting in a so-called qTOF, providing HRMS capability. Another example is the QTrap analyser, where the third quadrupole is also an ion trap, where additional fragmentation steps can be performed [178]. Orbitrap analysers are the latest development in the field of mass analysers and are composed of two outer cup-shaped electrodes and an inner spindle-shaped electrodeIt traps ions in an orbital motion around the spindle due to the variations in the electric field between the electrodes [181]. Orbitrap analysers are characterized by their high resolution $\geq 10^5$ FWHM and mass accuracy of $\leq$ 1 ppm, enabling very accurate identification of analytes [181], [182].

1.4 Quality of measurements

The quality of the measurements made in an analytical laboratory is supported by two systems: quality assurance (QA) and quality control (QC). QA is defined as “all those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy with given requirements for quality” [183]. In the case of analytical measurements, five actions need to be performed for QA: 1) assuring traceability of measurement results, 2) validation of the analytical methods used, 3) evaluation of measurement uncertainty, 4) the use of certified reference materials (CRM) and 5) participation in inter-laboratory studies. On the other hand, QC is defined as “the operational techniques used to fulfil the requirements of quality” [183]. QC involves mainly: 1) determination of precision by way of replicate samples, 2) detection of impurities and interferences by way of blank samples and 3) determination of the accuracy with the use of reference material analysis. In the following subchapters, concepts regarding traceability, method validation and uncertainty will be summarized.

1.4.1 Traceability

For consistent and valid measurement results, comparisons to reference standards must be established, and the uncertainty of these comparisons must be known. This concept is called traceability and provides the basis for comparability of results, regardless of their origin. It is also linked to the International System of Quantities and Units (SI), which defines universal units used to express measurements in science and elsewhere. For establishing traceability, several steps must be performed [184]:

1) To specify the measurand and required uncertainty: The exact measurand or quantity measured must be defined accurately, especially if different measurements are present in different forms (e.g., isotopes, isomers, enantiomers). Also, measurement conditions must be stated if they are not at standard temperature and pressure (20 °C, 1 atmosphere) and a statement regarding whether the measurement involves the amount of a compound recovered from the matrix or the total amount believed to be present is also required.

2) Choice of a suitable method: With an understanding of the measurand, we must select a method for the measurement. The choice may include regulatory requirements for particular methods, customer requirements, cost, experience and equipment availability.

3) Validation of the method: It is vital to check for the selectivity, bias and consequently the fitness of this method for our measurements.

4) Identification of the relative importance of different influence quantities, which affect the measurement we perform. If a quantity affects our measurements (e.g. temperature), it is essential to take measures to monitor and control it and associate it to the uncertainty of the measurement.

5) Use of appropriate reference standards: Calibration with the correct reference standards in conjunction with validated methods is vital for the traceability of measurement and establishing accurate results.

6) Estimation of the uncertainty: Assessment of the overall uncertainty of the results is needed to confirm that it meets end-user requirements.

1.4.2 Validation

Method validation defines the analytical requirements and then determines whether the method fulfils those requirements. Included in this are the performance and suitability of the method. In the course of method validation, several characteristics must be evaluated, according to Eurachem [185]:

1) Selectivity is "the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour" [185]. It is usually investigated by studying the ability of the method to measure selected analytes in samples that contain interferences that are predicted to be present in the regular use of the method.

2) Limit of detection (LOD) is the lowest analyte concentration detectable, while limit of quantitation (LOQ) is the lowest concentration at which the analyte may be quantitated with a certain level of confidence. LOD and LOQ of our method are estimated by measuring blank samples (or samples with a low concentration of analyte) that were treated with the selected method. Calculation methods include estimation by the standard deviation or noise-to-signal ratio.

3) The working range is the interval of concentrations over which our method will provide results with an acceptable uncertainty. The lower border (the LOQ) and the upper border are concentrations at which significant anomalies in sensitivity are observed. Between these two points, the response of the instrument most usually follows a linear relationship.

4) Analytical sensitivity denotes the instrument response concerning the measured quantity.

5) Trueness is an expression of the systematic error and the consequent bias of a method. In other words, it shows how close the mean of an infinite (in theory) number of results is to a reference value.

6) Precision is the representation of the random error of the method and the consequential spread of measurements. It can be presented as a standard deviation for several replicate measurements of the same sample.

7) Ruggedness is the capacity of a method to remain unaffected by minor but deliberate variations in method parameters and indicates the method's reliability.

8) Measurement uncertainty considers all recognised effects on the result. It is expressed as an interval of values attributed to the measured value.

1.4.3 Uncertainty

Every step in the procedure of measurement is associated with a certain amount of uncertainty. As described by the Eurachem guide *Fitness for Purpose of Analytical methods* [185], measurement uncertainty characterizes the dispersion of values of a quantity attributed to a measurand and should consider all recognised effects operating on the result. Uncertainty of measurement does not imply doubt about its validity but enables increased confidence in its validity due to accounting for errors. The uncertainty reflects the lack of knowledge of the true value of a measurement, which can never be known. The measurement always estimates the true value because of uncertainty from random errors and imperfect correction for systematic errors. Sources of uncertainty may include an incomplete definition of the measurand, non-representative sampling, inadequate knowledge about influence quantities, finite instrument resolution, inexact standards or reference materials, assumptions and approximations and personal bias. Uncertainty of a step in the method can be represented as standard uncertainty. Combined standard uncertainty is calculated from different sources of uncertainty, where the individual error components are expressed as standards uncertainties. Expanded uncertainty is calculated by multiplying the combined standard uncertainty by a coverage factor.

Chapter 2

Aims and hypotheses

A thorough review of the literature highlights a significant gap in the knowledge regarding the removal of CECs in algal photobioreactors. For example, literature review shows that experiments are commonly performed at very high concentrations of CEC (mg/L), which is not relevant for wastewaters, where they typically are found in ng/L to µg/L levels. In addition, their presence in algal biomass is often overlooked but may be important for potential biomass reuse as hydrophobic CEC may partition to biomass by adsorption and uptake. Also, the studies on the removal of CEC in photobioreactors are scarce, and accordingly, not all groups of CEC are equally well studied. A good example are BPs, a model group of CEC, where mainly BPA and to a smaller extent also 4,4'-BPF and BPAF are covered. However, most BPs are yet to be included in such studies. In addition, physicochemical parameters and culture conditions that influence the removal of compounds are not well covered, although they have a crucial role in determining treatment efficiency. Finally, most studies are performed on the laboratory scale, and only a handful of pilot-scale algal photobioreactors treating wastewater under realistic conditions have been published, providing limited knowledge about the removal of CEC under realistic conditions.

Accordingly, this thesis focuses on the removal, fate and occurrence of CEC in laboratory and pilot-scale algal photobioreactors. The aim is to understand better the removal of CEC and their cycling in algal photobioreactors for wastewater treatment and finally, to improve algal wastewater treatment and further the safe reuse of reclaimed water and biomass. In order to fill these knowledge gaps, the goals were to:

- Develop, optimise and validate sensitive and reliable analytical methods for determining CEC in the aqueous and biomass phases in algal photobioreactors. Here, BPs are studied as a model group since they exhibit varying physicochemical properties that govern their fate during treatment and in the environment.

- Apply validated analytical methods to evaluate the removal of CEC in laboratory-scale algal photobioreactors at relevant concentrations.

- Compare CEC removal under different culture conditions (e.g. monocultures vs. mixed cultures, pH) and the influence of CEC physicochemical parameters on their removal in laboratory-scale algal photobioreactors.

- Study CEC removal in a pilot-scale HRAP to gain knowledge about its treatment efficiency under realistic conditions.

These methods were then used to test the following hypotheses:

H1: Laboratory-scale algal photobioreactors can remove CEC to below quantifiable levels.

H2: CEC partitioning depends on their properties (log P, pK_a) and culture conditions (pH).

H3: Mixed consortia of algae and bacteria are more efficient at removing CEC from wastewater than a pure algal culture.

H4: Outdoor HRAP can remove CEC from municipal wastewater to below quantifiable levels.

Chapter 3

Publications

3.1 Determination of 18 bisphenols in aqueous and biomass phase of high rate algal ponds: Development, validation and application

The paper "Determination of 18 bisphenols in aqueous and biomass phase of high rate algal ponds: Development, validation and application" by D. Škufca, A. Kovačič, T. Griessler Bulc and E. Heath was published in the journal *Chemosphere* (IF 2021: 7.086) in May 2021 (doi: 10.1016/j.chemosphere.2021.129786). I was solely responsible for method development and optimization, validation, experimental work, data analysis, and manuscript drafting.

This study intends to address two aims of this thesis: 1) to develop, optimise and validate analytical methods to analyse BPs as a model group of CEC in the aqueous and biomass phase of algal wastewater treatment, and with this to 2) study CEC removal efficiency in a pilot-scale HRAP. This work is crucial for furthering analytical methodology and understanding the removal of BPs in HRAP.

Extraction of 18 BPs from the aqueous phase was tested with Oasis MCX and HLB solid phase extraction cartridges, and the former was chosen due to yielding a cleaner extract. The addition of 30% MeOH to the aqueous phase was also employed to improve the recovery of hydrophobic BPs. In addition, the complete workflow for extraction and purification of 18 BPs from biomass was developed. Biomass samples were lyophilised before extraction. For extraction solvent, a mixture of MeOH/CAN (20:80, v/v) resulted in the lowest coextracted material. The extract was solvent exchanged to EtAc/Hex (25:75, v/v) and loaded onto Bond Elut Carbon/PSA SPE cartridges for further clean-up. Due to fatty acids interfering with chromatography, two fractions were eluted: first, 17 BPs were eluted with 3.6 mL of MeOH/EtAc (20:80), and secondly, BPS was eluted with 3.6 mL of MeOH/EtAc/AA (20:78:2). Methods were validated according to the EURACHEM/CITAC guide. *The Fitness for Purpose of Analytical Methods* and measurement uncertainty was estimated. The developed methods were used to analyse aqueous and biomass samples from a pilot-scale HRAP treating wastewater in Ajdovščina, Slovenia. In the aqueous phase of the influent, we detected BPA at a mean concentration of 329 ng/L (min-max: 190 – 1070 ng/L), BPS at 144 ng/L (min-max: 92 – 179 ng/L) and 4,4'-BPF just above the LOQ at 21 ng/L. The removal efficiency of BPA was 80%, whereas only 32% of BPS was removed, indicating recalcitrance of BPS in HRAP.

This study improved analytical knowledge for quantifying a wide range of BPs in the biomass and aqueous phases of algal wastewater treatment and was used to analyse samples from an HRAP designed for treating actual wastewater.

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Determination of 18 bisphenols in aqueous and biomass phase of high rate algal ponds: Development, validation and application

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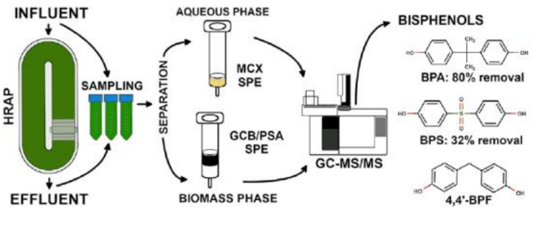
HIGHLIGHTS

- Methods for determining bisphenols in aqueous and biomass phase developed.
- Recoveries range from 78%–106% in both phases.
- Relative expanded uncertainties were 16%–66% at the lowest tested concentration.
- Methods have been applied to wastewater from a pilot-scale HRAP.
- Removal of BPS in HRAP reported for the first time.

GRAPHICAL ABSTRACT



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ABSTRACT

High rate algal ponds (HRAP) are an alternative to conventional wastewater treatment with the potential for wastewater and biomass reuse. In this study, we report the development and validation of methods for analysing 18 bisphenols (BPs) in the aqueous and biomass phase of HRAP. For aqueous phase samples, obtained LLOQ ranged from 10 to 30 ng/L, and recoveries from 78% to 106%. The relative expanded uncertainty was highest at the lowest spiking level (100 ng/L) and ranged from 27% to 66% (BPA), while for the biomass, the LLOQ ranged from 25 to 75 ng/g dw, recoveries from 84% to 103%. The uncertainty ranged from 16% to 37% (BPA). On average, the influent contained 329, 144, and 21 ng/L of BPA, BPS and 4,4′-BPF, and the effluent 69 ng/L, 94 ng/L and <LLOQ, respectively. Only BPA was quantified in the algal biomass. The average removal of BPA was 80%, whereas the removal efficiency of BPS was 32%. To our knowledge, this is the first study analysing a wide range of BPs in both aqueous and biomass phase of HRAP treating real wastewater.

1. Introduction

Endocrine disrupting compounds (EDC) are defined as chemicals (natural and synthetic) that can "*interfere with the production, processing, and transmission of hormones in the body and disrupt the normal functioning of the endocrine system*" (Vogel, 2009). Among known EDC, bisphenol A (BPA) has received much attention and is regulated for several applications, including baby bottles (Kovačič et al., 2020). However, it is only one of a group of compounds collectively referred to as bisphenols (BPs, Table 1). As the name implies, these compounds are characterised by having two bridged hydroxyphenyl functionalities. Many BPs also have the potential to

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Table 1
Abbreviations of BPs, physicochemical properties (octanol-water partitioning coefficient (log Kow) and acid dissociation constant (pKa) as predicted by Marvin suite (ChemAxon, 2019)) and CAS identifiers of BPs.

Abbreviation	IUPAC name	log K_{ow}	p K_a	CAS
BPA	4-[2-(4-hydroxyphenyl)propan-2-yl]phenol	4.04	9.8–10.4	80-05-7
2,2′-BPF	2-[(2-hydroxyphenyl)methyl]phenol	3.46	8.2–10.1	2467-02-9
2,4′-BPF	2-[(4-hydroxyphenyl)methyl]phenol	3.46	9.8–10.4	2467-03-0
4,4′-BPF	4-[(4-hydroxyphenyl)methyl]phenol	3.46	9.8–10.4	620-92-8
BP26DM	4-[2-(4-hydroxy-3,5-dimethylphenyl)propan-2-yl]-2,6-dimethylphenol	6.1	9.6–10.2	5613-46-7
BPAF	4-[1,1,1,3,3,3-Hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol	4.77	9.1–9.7	1478-61-1
BPAP	4-[1-(4-hydroxyphenyl)-1-phenylethyl]phenol	5.18	9.8–10.4	1571-75-1
BPB	4-[2-(4-hydroxyphenyl)butan-2-yl]phenol	4.49	9.8–10.4	77-40-7
BPBP	4-[(4-hydroxyphenyl)-diphenylmethyl]phenol	6.31	9.6–10.2	1844-01-5
BPC	4-[2-(4-hydroxy-3-methylphenyl)propan-2-yl]-2-methylphenol	5.07	9.7–10.3	79-97-0
BPC II	4-[2,2-dichloro-1-(4-hydroxyphenyl)ethenyl]phenol	4.29	8.9–9.5	14868-03-2
BPE	4-[1-(4-hydroxyphenyl)ethyl]phenol	3.74	9.8–10.4	2081-08-5
BPFL	4-[9-(4-hydroxyphenyl)fluoren-9-yl]phenol	5.99	10.5–11.1	3236-71-3
BPM	4-[2-[3-[2-(4-hydroxyphenyl)propan-2-yl]phenyl]propan-2-yl]phenol	6.72	9.8–10.4	13595-25-0
BPP	4-[2-[4-[2-(4-hydroxyphenyl)propan-2-yl]phenyl]propan-2-yl]phenol	6.72	9.8–10.4	2167-51-3
BPPH	4-[2-(4-hydroxy-3-phenylphenyl)propan-2-yl]-2-phenylphenol	7.34	9.5–10.1	24038-68-4
BPS	4-(4-hydroxyphenyl)sulfonylphenol	2.32	7.4–8.0	80-09-1
BPZ	4-[1-(4-hydroxyphenyl)cyclohexyl]phenol	4.91	10.1–10.7	843-55-0

be endocrine disrupting, which has been corroborated for 4,4′-BPF, BPS, BPE, BPB, BPAP, BPFL, and BPAF (Desdoits-Lethimonier et al., 2017; Feng et al., 2016; Le Fol et al., 2017; Russo et al., 2018; Xiao et al., 2018; Zhang et al., 2017) and they have also been detected in the environment (Chen et al., 2016; Wang et al., 2017). Although BPs are often treated as a group, they differ in the physicochemical properties due to the presence of different functional groups, which affects their behaviour (e.g., adsorption and uptake) in the environment and during wastewater treatment (Chen et al., 2016).

Wastewater is the primary source of BPs in the environment, which is why their removal from wastewater is key to lowering emissions (Noszczyńska and Piotrowska-Seget, 2018). A high rate algal pond (HRAP) system is a promising technology for biological wastewater treatment. This is because a HRAP relies on low energy inputs (as opposed to activated sludge reactors, no aeration is required for HRAP) and long hydraulic retention times (HRT, 1–14 days for HRAP *vs* to 3–8 h for activated sludge reactors) to achieve high removal efficiency of nutrients and emerging contaminants (Norvill et al., 2016). There is also the potential to harvest the algal biomass and valorise it for fertiliser or biofuel production (Craggs et al., 2014; Norvill et al., 2016). The removal of nutrients (e.g. nitrogen and phosphorus) from wastewater by algae may produce a higher quality of effluent than conventional wastewater treatment plants (WWTP) without tertiary treatment (Craggs et al., 2014). There is much ongoing research in producing biofuels (Li et al., 2018; Rahman et al., 2017), biopolymers (Rahman et al., 2015), fertilisers (Li et al., 2018; Zhang et al., 2014) and other products from algal biomass as well as reuse of wastewater for irrigation (Wu et al., 2014). The valorisation of wastewater and reuse of resources also means that wastewater treatment with HRAP systems is in line with the European Commission's Circular Economy Action Plan, adopted in March 2020 (European Commission, 2020).

Currently, only two studies cover the removal of BPA in pilot-scale algal wastewater treatment systems. These include (1) Matamoros et al. (2015), who looked at the removal of BPA in addition to 25 other CEC from wastewater in a pilot-scale HRAP, and (2) Vassalle et al. (2020), who investigated the removal of BPA and ten other CEC in the aqueous phase of a pilot-scale HRAP. Several groups have investigated the removal of BPA at the laboratory scale in artificial media, spiked with concentrations ranging from 1 to 50 mg/L (Eio et al., 2015; Gattullo et al., 2012; Guo et al., 2017; Ji et al., 2014; Li et al., 2009) and with different wastewaters as medium (Abargues et al., 2013; Bai and Acharya, 2019; Liu et al., 2010). However, only three laboratory-scale studies have looked at using microalgae to investigate the removal of BPA alternatives, i.e., BPAF, 4,4′-BPF and tetrabromobisphenol A (Peng et al., 2009, 2014; Solé and Matamoros, 2016), indicating a lack of knowledge about BPA alternatives.

Despite the progress, the following knowledge gaps remain: i) the analysis of BPs other than BPA in HRAP; ii) analysis of both the aqueous and biomass phases; iii) understanding the fate and removal at environmentally relevant concentrations, and iv) analytical method validation. Method validation is an integral part of analytical methods, as it shows fitness for its intended purpose and ensures the reliability of results, which many of the published studies lack. It is accomplished by several tests, by which we determine important method performance characteristics that correspond to its intended use: selectivity, limits of detection and quantitation, working range, sensitivity, trueness, precision and measurement uncertainty (Magnusson and Ã–rnemark, 2014).

Our work aimed to develop GC-MS/MS methods to analyse 18 BPs in wastewater treated by algal technologies. An extensive validation has been performed in aqueous and algal biomass phase, and measurement uncertainty estimated. The methods cover environmentally relevant concentrations in the aqueous and biomass phase (ng/L and ng/g, respectively). The validated methods were applied to samples collected from a pilot-scale HRAP treating wastewater, and the removal of studied BPs estimated. We intend to use this analytical method as a foundation for further studies on the mass balance and fate of BPs.

2. Materials and methods

2.1. Standards and reagents

Bisphenol A (>97%) was purchased from Merck (Germany), 2,2′-BPF, 4,4′-BPF, BPAF, BPB, BPE, BPC, BPM, BPBP, BP26DM, BPC2, BPZ, BPAP, BPS, BPFL (>98%) were obtained from Sigma-Aldrich (USA), and 2,4′-BPF (>98%) was purchased from Tokyo Chemical Industry Co. Ltd. (Japan). Information about each compound is available in Table 1, and their structures in the supplementary material (Table S1). Isotopically labelled [$^{13}C_{12}$] 4,4′-BPF ($^{13}C_{12}$-BPF), BPS ($^{13}C_{12}$-BPS), BPB ($^{13}C_{12}$-BPB) were purchased from CanSyn Chem. Corp. (Canada), and deuterated BPA (BPA-d_{16}) from Isotec (USA). The derivatising agent N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, $\geq$ 99.0%), the catalyst pyridine (99.8%), and hydrochloric acid (37%, HCl) were purchased from Sigma-Aldrich (USA). Ethyl acetate (EtAc), methanol (MeOH), acetonitrile (ACN), hexane (Hex), acetic acid (AA) and formic acid (FA) were purchased from J. T. Baker (Netherlands). Ultrapure water was prepared with a MilliQ-water purification system (>18.0 MΩ cm^{-1} at 25 °C Millipore, USA).

Stock solutions were prepared in MeOH (≈ 1 mg/L for standards and ≈0.2 mg/L for internal standards). Working solutions were prepared by serial dilution of the stock solution and stored at 4 °C (Kovačič et al., 2019).

2.2. Method development

Analytical methods were modified, developed and optimised, based on existing methods. The method for determining BPs in the aqueous phase and chromatographic separation was adopted from Kovačič et al. (2019), and the method for extracting BPs from algal biomass was based on Regueiro and Wenzl (2015a).

2.3. HRAP operation

Samples were collected from a pilot-scale HRAP, located at Ajdovščina WWTP, Slovenia (WGS coordinates: 45.87584 N, 13.90627 E). The HRAP has a volume of 3000 L and a surface area of 12.7 m^2. Every 12 h, 10% of the treated water volume was pumped into a biomass settler and an equal amount of fresh primary influent added to the HRAP, resulting in an HRT of 5 days.

2.4. Sample preparation

Samples (50 mL) of influent wastewater (influent) and HRAP effluent (effluent) were taken in triplicate each day at approximately 7.00 a.m. and 7.00 p.m. for five days. Once in the laboratory, each sample was centrifuged for 20 min at 6000 RCF. The supernatant (aqueous phase) was then decanted into a separate tube, and both phases were stored at −20 °C. Because of the lack of certified reference material (CRM) for BPs in wastewater related matrices, samples for method development, validation, and matrix-matched calibration were prepared in-house as described in the SI (Blank sample preparation). All laboratory glassware was precleaned and pyrolysed at 400 °C for 4 h to reduce contamination.

2.4.1. Aqueous samples

The aqueous phase was transferred into a glass flask, and MeOH (21 mL) was added to obtain a 30% MeOH modifier solution. Each sample was then spiked with the internal standard mixture (final concentration in the sample: 500 ng/L of BPA-d$_{16}$, 13C12-BPF, $^{13}C_{12}$-BPS and $^{13}C_{12}$-BPB each, 25 μL of 1 μg/mL solution) and filtered (glass fibre, GF/C filter, 1.2 μm pore size; Whatman, UK). Between samples, glassware was rinsed with water and pure MeOH to avoid carryover. The samples were then acidified (0.5 mL of 7% HCl) and loaded onto MCX Prime (60 mg, 3 cc; Waters, USA) SPE cartridges on a negative pressure manifold. Afterwards, 5 mL of 20% MeOH (v/v) was used to rinse the sample flask. The cartridges were then washed with 3 mL of ultrapure water and dried for 45 min under negative pressure. The analytes of interest were eluted using 1.8 mL 5% FA in EtAc (v/v). The samples were then dried under N$_2$ and derivatised by adding MSTFA (50 μL) and pyridine (50 μL) and incubating them at 80 °C for 1 h prior to analysis.

2.4.2. Biomass samples

The biomass phase was lyophilised using a Martin Christ Gamma freeze dryer (Germany). The internal standard (25 μL of 1 μg/mL solution) and 8 mL of ACN/MeOH (80:20) were added to each sample, which was then homogenised/dispersed with a homogeniser (Miccra D-1, ART Prozess- & Labortechnik GmbH & Co. KG, Germany) for 0.5 min at 25.000 rpm and shaken for 30 min on an orbital shaker at 220 rpm. The samples were then sonicated in an ultrasonic bath for 30 min. Samples (10 mL) were filtered using 0.45 μm PTFE syringe filters (Macherey Nagel, Germany) into glass tubes and dried under N$_2$ at 80 °C. Each sample was then dissolved in 4.5 mL EtAc/Hex (25:75) and purified using Bond Elut Carbon/PSA (250 mg, 3 cc, Agilent, USA) SPE cartridges conditioned with 2.5 mL MeOH/EtAc and 2.5 mL EtAc/Hex. The elution step was performed using 2 × 1.8 mL of MeOH/EtAc (20:80), and subsequently with 2 × 1.8 mL of MeOH/EtAc/AA (20:78:2) for the elution of BPS. Both fractions were dried and derivatised as described for the aqueous samples.

2.5. GC-MS/MS analysis

All samples were analysed using gas chromatography (GC, model 7890B, Agilent, USA) with tandem mass spectrometry (MS/MS, model 7000, Agilent, USA), based on the method of Kovačič et al. (2019). The separation was achieved using a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, USA) with helium as the carrier gas (constant flow mode: 1 mL/min). Samples (1 μL) were injected in splitless mode at 270 °C. The GC oven temperature program was as follows: initial temperature of 120 °C was ramped at 20 °C min^{-1} to 200 °C and held for 6 min, then ramped to 300 °C at 10 °C min^{-1} and held for 3 min. The transfer line was set to 280 °C, and the compounds were ionised in electron impact (EI) mode at 70 eV. Qualification and quantitation were performed using multiple reaction monitoring (MRM). The total runtime was 24 min.

2.6. Method validation

Method validation (aqueous and biomass phases) was performed according to the EURACHEM/CITAC guide (Magnusson and Â–rnemark, 2014). Theoretical limits of quantification were calculated by multiplying the standard deviation s_0 by a factor of ten, divided by the square root of the number of parallels. The standard deviation s_0 was obtained by analysing ten parallels of samples spiked with a concentration close to predicted LLOQ (20 ng/L and 50 ng/g (dw) for aqueous and biomass phases, respectively). The LLOQ for each compound chosen in the study was the lowest acceptable calibration point. A calibration curve covered the working range based on three parallels of each calibration point. The method was validated at three spiking levels for both aqueous (L: 100 ng/L; M: 1200 ng/L, and H: 2500 ng/L) and biomass (L: 250 ng/g; M: 3000 ng/g, and H: 6250 ng/g). The calibration points and spiking levels are given in Table S2. Trueness was estimated as method recovery by comparing six parallels per spike level with post-extraction spiked blank samples as the reference. Instrumental precision was assessed with three consecutive injections of the same sample. Repeatability was estimated by performing the method in six parallels *per* concentration level and intermediate precision (within-lab reproducibility) using two additional repetitions, one of them by a different analyst. Ruggedness was tested for the biomass phase by testing the influence of the amount of biomass (2 mg, 20 mg and 40 mg) on compound quantitation. Finally, the matrix effect was estimated by comparing the signal of post-extraction spiked blank samples with pure standards.

2.6.1. Uncertainty

The uncertainty budget comprises standard uncertainties of recovery, intermediate precision, calibration and instrumental precision for the aqueous samples. The pooled SD of triplicate total suspended solids (TSS) measurements were also included for biomass. The combined uncertainty was calculated from the standard uncertainties according to the Rule of propagation of uncertainty described by GUM (Jcgm, 2008). Expanded uncertainty (U_e) was expressed as the relative uncertainty at each spiking level and calculated by multiplying the combined uncertainty with a coverage factor of 1.96 to achieve a level of confidence of 95%.

2.7. Data analysis

The physicochemical properties of the BPs (log Kow, pKa) were predicted based on compound structure, using the Marvin Suite (ChemAxon, 2019). Peak areas were obtained using the Mass Hunter Quantitative Analysis software (Agilent, USA), and a Kruskal-Wallis test was used to determine significant differences between samples ($\alpha = 0.05$). Tests were performed using the R programming language (R Core Team, 2020) in the R Studio environment (RStudio Team, 2020), and the software packages "tidyverse" (Wickham et al., 2019), "xslx" (Dragulescu and Arendt, 2020) and "rstatix" (Kassambara, 2020) were used to analyse the data. GIMP was used for the creation of the graphical abstract (The GIMP Development Team, 2020).

3. Results and discussion

3.1. GC-MS/MS optimisation

After determining the retention times (R_t) of the individual compounds, potential precursor ions were selected, followed by optimising the CE to obtain maximal product ion abundances. Two multiple transitions (quantification and qualification) were selected for each compound (Table 2). Thus, four to five identification points were obtained for each compound according to the European Commission Decision 2002/657/EC (European Commission, 2002). The analytical parameters are available in Table 2.

Table 2
Parameters for GC-MS/MS analysis of BPs – retention time (R_t), MRM Transition 1, used for quantification and MRM Transition 2, used for qualification.

		Transition 1 (quantification)			Transition 2 (qualification)		
compound	R_t	precursor	product	CE	precursor	product	CE
2,2′-BPF	9.56	344	329	3	329	147	3
BPAF	10.00	480	411	3	411	395	10
2,4′-BPF	10.54	344	329	3	241	223	18
4,4′-BPF	11.49	344	329	3	329	179	3
$^{13}C_{12}$-BPF	11.49	356	341	3	341	185	10
BPE	11.74	358	343	5	343	193	3
d_{16}BPA	11.98	386	368	8	368	197	23
BPA	12.05	372	357	5	357	191	3
BPC	12.66	400	385	35	385	205	3
$^{13}C_{12}$-BPB	12.77	398	369	10	369	197	23
BPB	12.77	386	357	5	357	191	3
BP26DM	13.99	428	413	13	413	219	3
BPC2	14.17	424	409	10	374	359	33
BPZ	15.40	412	369	5	369	203	3
BPS	16.27	394	379	35	229	165	30
$^{13}C_{12}$-BPS	16.26	391	235	25	235	171	30
BPAP	16.63	434	419	13	419	340	5
BPM	18.90	490	475	18	475	309	3
BPP	20.90	490	475	18	475	309	3
BPBP	21.58	496	419	10	331	252	25
BPPH	21.75	524	509	23	509	267	3
BPFL	23.30	494	479	3	494	329	5

3.2. Sample preparation method development

3.2.1. Aqueous phase

Extraction of the aqueous samples was tested with both HLB Prime and MCX Prime SPE cartridges (Waters, USA). It was observed that a higher proportion of impurities were retained using the MCX Prime (Fig. S1), a reversed-phase/ion-exchange sorbent, while HLB Prime is a reversed-phase sorbent. A greater number of interfering compounds are likely retained on the MCX Prime phase due to the additional electrostatic forces. In contrast, BPs are retained and eluted by nonspecific hydrophobic binding interactions, resulting in a cleaner extract (Regueiro and Wenzl, 2015b). Furthermore, less polar BPs had lower recoveries because of adsorption to the filter (Figs. S2 and S3). Accordingly, we tested increasing amounts of MeOH addition (0%–30%) as a modifier in the sample. We found that the addition of 30% MeOH (v/v) to the sample, resulted in significantly higher recovery of the less polar BPs ($p < 0.05$ for BPC, BP26DM, BPBP, BPFL, BPP, BPM and BPPH), while not reducing the recovery of the more polar BPs (Fig. 1). Further development of the method for the aqueous phase was not considered necessary at this point.

3.2.2. Biomass phase

To ascertain the benefits of sample lyophilisation, we analysed three parallels of fresh and lyophilised spiked samples, at three sampling times over nine days. The recovery of BPs was significantly higher ($p < 0.05$) when the biomass was lyophilised for 2,2′-BPF and BPZ at all three sampling times, for BPS, 2,4′-BPF, BPE, BP26DM and BPM at two sampling times and for BPC2, BPB, BPC, BPAP and BPPH at one sampling time (Fig. S4). A higher amount of dry organic extract was obtained when extracted fresh for two out of three samples ($p < 0.05$, Fig. S5). Due to improved recovery and reduced total extracted material, lyophilisation was used for sample preparation. Drying the sample also made solvent exchange easier before loading the SPE cartridges.

The effect of adding different amounts of MeOH, ACN and FA to the extraction solvent on recovery was also tested. The addition of larger amounts of MeOH (60%) and FA (2% and 4%) did not significantly increase the recovery of BPs ($p > 0.05$, Fig. 2) but did significantly increase the amount of dry extract (up to 71% more, $p < 0.05$, Fig. S6). An extraction solvent consisting of MeOH/ACN (20/80, v/v) resulted in the lowest amount of coextracted material. The addition of a second extraction cycle contributed on average < 5% to overall recovery and was therefore not included in further sample preparation (not shown).

Initially, when loading the sorbent phase with EtAc/Hex (50:50, v/v) approximately 90% of BP26DM and >50% of BPS were not retained by the sorbent but were in the breakthrough fraction, which led us to test loading solvents containing amounts of 25%–75% Hex. The optimal loading solvent was 25:75 (v/v) EtAc/Hex.

The elution solvent was tested using different combinations of MeOH (0%–100%), EtAc (0%–100%) and AA (0%–5%) (not shown). Fractions of loading solvent and eluate were collected to determine the recovery of individual BPs in those fractions and determine optimal elution solvent composition and volume. From the results, it was apparent that BPS, as well as other interferences, most notably fatty acids, are only eluted when AA is added. Also, the presence of long-chain fatty acids, namely octadecadienoic acid and α-linolenic acid as (identified using the NIST library with a probability of 98.3% and 85.9%, respectively) interfere with the peak shape of BPA (Fig. S7) and that it was not possible to elute BPS and the interfering fatty acids from the SPE phase separately. The latter is presumably due to ionic interactions with the PSA (Regueiro and Wenzl, 2015a). When AA was added, these ionic interactions were interrupted, and the compounds eluted. Fatty acids are known to

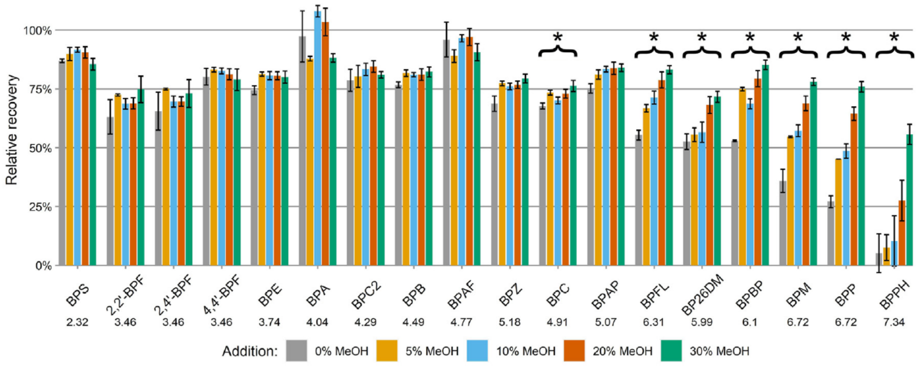

Fig. 1. The effect of MeOH addition as a modifier on the recovery of 18 BPs in the aqueous phase. Results are normalised to post-extraction spiked blank samples for reference. Compounds are arranged from lowest to highest log Kow, noted under the compound name. * - Statistically significant difference ($p < 0.05$).

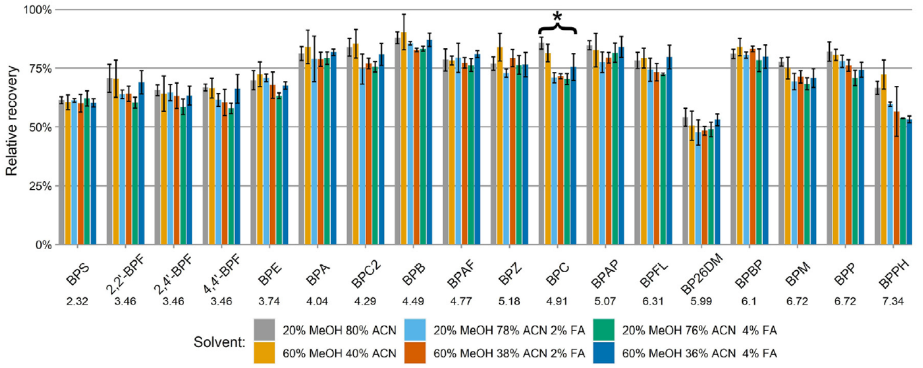

Fig. 2. Recovery of BPs by the composition of extraction solvents. Results are normalised to post-extraction spiked blank samples for reference. Compounds are arranged from lowest to highest log Kow, noted under the compound name. * - Statistically significant difference ($p < 0.05$).

negatively affect gas chromatographic separation (Guan et al., 2009; Hajšlová and Zrostlıkova, 2003; He and Liu, 2007; Saito et al., 2004; Schenck et al., 2002; Shimelis et al., 2007). To overcome this, we eluted 17 BPs with 3.6 mL MeOH/EtAc (20/80), and BPS separately with 3.6 mL MeOH/EtAc/AA (20/78/2).

3.3. Method validation

3.3.1. Aqueous phase

The working range from the LLOQ to 5000 ng/L was linear for all 18 BPs ($R^2 > 0.992$) for all compounds. The LLOQ ranged from 10 to 30 ng/L, with BPA and BPC2 having the highest LLOQ, namely 30 ng/L (Table S3). The trueness is the average recovery of BPs in the aqueous phase, ranged from 78% to 106% (Table S4). The two compounds with the lowest mean recovery at all three spiking levels were BPS (L – 84%, M – 85%, H – 78%) and BPPH (L – 89%, M – 86%, H – 78%), which have log K_{OW} values on the low and high end of the spectrum in this study (2.32 and 7.34, respectively). Except for BPA at spiking level L with −60% (Table S4), the matrix effect was negative in the aqueous phase and ranged from −35% (BPFL at spiking level L) to +15% (BPAF at spiking level L). However, the matrix effect does not play a significant role due to the use of matrix-matched calibration.

Instrumental precision for the three spiking levels was as follows: L – from 0.5% (BPAP) to 11% (BPPH), M – from 0.3% (BPAP) to 6% (BPB) and H – from 0.3% (4,4′-BPF) to 7% (BP26DM). The repeatability ranged from 1.4% to 8% except for BPA (19% at spiking level L). Intermediate precision ranged from 10% to 21%, again except for BPA (32%) at spiking level L (Table S4). All BPs had a markedly worse intermediate precision at spiking level L.

Uncertainty, expressed as the relative expanded uncertainty, ranged from 26% (BPE) to 66% (BPA) at spiking level L, from 11% (4,4′-BPF) to 24% (BP26DM) at spiking level M and from 8% (BPA) to 26% (BP26DM) at spiking level H (Table S5). Due to intermediate precision being a prominent component of uncertainty, BPs had a higher uncertainty at spiking level L.

Although Solé and Matamoros (2016) determined removal in batch experiments with algae and estimated LLOQs in the aqueous phase for BPA, BPAF and 4,4′-BPF as 190, 180 and 190 ng/L, respectively, using 100 mL of sample. While the authors did not report measurement uncertainty, they did report repeatability (<15% for all three BPs. Kovačič et al. (2019)) determined LLOQs from 0.3 to 17.4 ng/L for BPs in wastewater while using a larger sample volume of 250 mL. Thus, our determined LLOQ are consistent with published data, considering the smaller sample volume.

3.3.2. Biomass

The working range (LLOQ to 12,500 ng/g) showed good linearity ($R^2 > 0.990$) and ranged from 25 to 75 ng/g with BPFL being an exception (125 ng/g, normalised to a nominal sample mass of 20 mg dw, Table S6). Analyte recovery ranged from 84% to 103% overall, with an RSD ranging from 2% to 11% (BPA at spiking level L, Table S7). The matrix effect was mostly positive for BPs in the biomass phase and ranged from −13% (BPC2 at spiking level L) to +17% (BPP at spiking level L, Table S7). The instrumental precision ranged from 1% to 11% overall, with the highest three values in the L spiking level, and repeatability ranging from 2% to 9%, except for BPA (11%) at spiking level L. Intermediate precision ranged from 10% to 21%, except for BPA at spiking level L with 32%. All BPs had a markedly lower intermediate precision at spiking level L (Table S7). Ruggedness testing showed that neither 2 mg, 20 mg nor 40 mg of biomass affected the quantification of BPs ($p > 0.05$), except in the case of 4,4′-BPF, where there was a significant difference between 2 mg and 40 mg of biomass at spiking level M ($p < 0.05$). Uncertainty expressed as relative expanded uncertainty ranged from 16% (BPE) to 37% (BPA) at spiking level L, from 21% (BPC and BPM) to 29% (2,2′-BPF and BPAF) at spiking level M and from 19% (BPZ, BPC, BPAP, BP26DM, BPM, BPP and BPPH) to 22% (BPA) at spiking level H (Table S8). It is known, that BPA determination is problematic at low levels due to background contamination owing to its ubiquity, and this can explain why repeatability, intermediate precision and uncertainty, as well as the LLOQ, were higher for BPA at the lowest spiking level in both the aqueous and biomass phase.

Solé and Matamoros (2016) estimated LLOQs for BPA, BPF and BPAF in algal biomass as 1 μg/g, 1.9 μg/g and 0.8 μg/g respectively, albeit in fresh biomass, meaning that their LLOQs are similar to the LLOQs reported in this study, which were determined on a dry weight basis. The authors used a sample mass similar to this study (≈20 mg dw), judging by their reported TSS. Also, comparable LLOQs were determined for BPs in sewage sludge from a conventional WWTP in New York, namely 19.9, 99.5, 5, 12.4, 19.9, 12.4, 19.9 and 12.4 ng/g dw for BPA, BPF, BPS, BPAF, BPAP, BPP, BPB and BPZ, respectively, using solid-liquid extraction and purification with an MCX (Waters, USA) SPE cartridge (Xue and Kannan, 2019). However, the authors used larger amounts of sample (0.1–0.2 g dw).

While the aqueous phase extraction method has similar performance characteristics in terms of LLOQ, the biomass phase method is less sensitive, having LLOQ in the ng/g range. This reduced sensitivity is due to sampling, which necessitated the need for centrifugation and results in a low sample mass of the biomass phase.

3.4. HRAP sample analysis

The developed methods were applied to samples collected from a pilot-scale HRAP, treating wastewater (mixed municipal and industrial wastewater) from the primary settler of the Ajdovščina WWTP (42,000 PE, Slovenia). Of the 18 BPs, we only detected BPS, 4,4′-BPF and BPA above the LLOQ. Bisphenol A occurred in the highest concentrations, ranging from 191 ng/L to 1070 ng/L (mean: 329 ng/L) in the influent and from 43 ng/L to 114 ng/L (mean: 69 ng/L) in the effluent. Bisphenol S ranged from 92 ng/L to 179 ng/L (mean: 144 ng/L) in the influent and 51 ng/L to 111 ng/L, (mean: 94 ng/L) in the HRAP. 4,4′-BPF was above the LLOQ only in three samples in the influent, ranging from <LLOQ to 22 ng/L (Table 3). In the biomass, we could only quantitate BPA, specifically in one sample from HRAP and one of influent. Similarly, in the literature BPA is the most common and abundant bisphenol found in wastewater, followed by BPS and 4,4′-BPF (Karthikraj and Kannan, 2017; Sun et al., 2017; Xue and Kannan, 2019).

Table 3

Concentrations of BPs detected in the aqueous and biomass phase of the HRAP in influent (INF) and effluent (EFF), presented is the mean concentration in ng/L or ng/g, respectively, the range, RSD of the mean and detection frequency (Df).

		Aqueous [ng/L]		Biomass [ng/g]	
compound		INF	EFF	INF	EFF
BPA	Mean	329	69	665	60
	range	190–1070	43–114	665	60
	RSD	76%	33%	/	/
	Df	100%	90%	100%	100%
BPS	mean	144	94	< LLOQ	< LLOQ
	range	92–179	51–111		
	RSD	15%	18%		
	Df	100%	100%	0%	0%
4,4′-BPF	mean	21	<LLOQ	< LLOQ	< LLOQ
	range	20–22	<LLOQ		
	RSD	4%	/		
	Df	100%	70%	0%	0%

The only BP detected in the biomass (Table 3) was BPA, which was below the LLOQ except in one influent biomass sample (665.2 ng/g; TSS = 50 mg/L) and one sample from HRAP (59.7 ng/g; TSS = 507 mg/L). In these two samples, 10% and 29% of BPA, respectively, of total BPA (aqueous + biomass) in the sample was present in the biomass phase. As BPA was < LLOQ in the majority of the HRAP biomass samples, we could only approximate that in most cases, the majority of BPA (>70%) was present in the aqueous phase. Sole and Matamoros, reported up to 6% of BPA retained on algae biomass when spiking BPA at a concentration of 10 μg/L into the algal culture (Solé and Matamoros, 2016). While BPS was quantified in the aqueous phase, it was < LLOQ in the biomass, likely owing to its log K_{ow} (2.32) being lower than that of BPA (4.04) (Wang et al., 2019). While according to the log K_{ow} of 4,4′-BPF (3.46), one would expect it to be present in biomass as well; however, the limiting factor was its low influent concentration.

The removal efficiency is the difference between total influx and efflux of each of the BPs in the aqueous phase. During the sampling period, 80% of BPA introduced into the HRAP was removed, whereas only 32% of BPS was removed. To our knowledge, this is the first study reporting the removal of BPS in HRAP. BPA and BPS were < LLOQ in most effluent biomass samples, suggesting that removal was due to degradation and not adsorption. When compared to the literature data, we see similar removals for BPA, i.e., 72% (HRT = 4 days) and 85% (HRT = 8 days), reported in Spanish HRAPs (Matamoros et al., 2015). In the study by Vassalle et al. (2020), BPA removal in Brazilian HRAPs was only 41.5% (HRT = 8 days). BPA removal is also comparable to the average removal reported in several conventional WWTPs in India (44%–100%), while for BPS a wide range of removals were reported, i.e. 0%–97% (Karthikraj and Kannan, 2017). The removal of BPA, reported in Chinese conventional WWTPs, ranged between ≈55% and ≈90%, while BPS was almost completely (≈100%) removed (Sun et al., 2017). Similar results for BPS are also reported from WWTPs in Slovenia, with a reported 100% removal (Česen et al., 2018). However, Xue and Kannan (2019) recently reported a low removal of BPA (mean removal 52% and 34%) and no removal of BPS (- 11%–1%) in two conventional WWTPs in New York. Higher removal of BPA would match the respective log K_{ow} values of BPA and BPS (4.04 and 2.32, respectively) to a greater degree, assuming that hydrophobicity plays a role in removal.

4. Conclusions

This study developed two complementary methods for quantifying BPs in the aqueous and biomass phase samples from a pilot HRAP treating municipal and industrial wastewater. The methods involved a SPE extraction technique and required only small sample volumes. Extraction and clean-up were based on using MCX Prime SPE cartridges for the aqueous samples and with GCB/PSA cartridges for the biomass phase. The samples were analysed using GC-MS/MS. The methods were thoroughly validated, measurement uncertainty assessed, and LLOQ determined in the low ng/L range for aqueous phase samples and in the ng/g range for biomass phase samples. Both methods performed well regarding linearity, trueness and precision. The estimated measurement uncertainties are generally highest for the lowest spiked concentrations in the aqueous phase and were more equally distributed in the biomass phase. Measurement uncertainty is the highest for BPA at the lowest spiking levels, likely due to background contamination. By improving the validation and uncertainty aspects of the methods, we aimed to provide a basis for a reliable determination of BPs in algal wastewater treatment technologies. With this, we developed methods for quantification of a wide range of BPs in algal wastewater treatment with well-developed quality assurance aspects.

Samples from a pilot-scale HRAP treating real wastewater were analysed with the developed methods, to demonstrate applicability. We quantified 4,4′-BPF, BPS and BPA (in order of increasing mean concentrations) in the aqueous samples. Bisphenol A was quantified in two of the biomass samples. During the study period, the average removal of BPA and BPS from the aqueous phase was 80% and 32%, respectively. To our knowledge, this is the first time that the removal of BPS has been estimated in an algal wastewater treatment system. Finally, while BPs represent only a small fraction of the contaminants that are likely present in wastewater and algal biomass, this study is a step toward determining potentially dangerous waste-borne CEC in a circular economy.

CRediT authors statement

David Škufca: Conceptualization; Methodology; Investigation; Formal analysis, Visualization, Writing - Original Draft; **Ana Kovačič:** Validation, Methodology, Writing - Review & Editing; **Tjaša Griessler Bulc:** Funding acquisition, Resources, Writing - Review & Editing; **Ester Heath:** Conceptualization, Supervision, Project administration, Funding Acquisition, Writing - Review & Editing. 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.chemosphere.2021.129786.

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3.2 Removal and fate of 18 bisphenols in lab-scale algal bioreactors

The paper “Removal and fate of 18 bisphenols in lab-scale algal bioreactors” by D. Škufca, F. Prosenc, T. Griessler Bulc and E. Heath was published in the journal Science of the Total Environment (IF 2021: 7.963) in August 2021 (doi: 10.1016/j.scitotenv.2021.149878). The first authorship was shared between D. Škufca and F. Prosenc, who were jointly responsible for experimental design, experimental work, visualisation and manuscript writing. I was solely responsible for sample preparation, instrumental analysis, data analysis and visualisation of bisphenols’ data.

While several studies examine the removal of BPA at high concentrations in algal photobioreactors, removal at wastewater relevant concentrations of other BPs is not well known. Algal technologies present an opportunity to reuse reclaimed water, and in wastewater, knowledge about the fate of model CEC is vital to prevent hazards. This study aimed twofold: 1) to examine the partitioning of BPs to the aqueous and biomass phase and 2) investigate the influence of pH on their fate and removal.

To achieve this, we designed an experiment with a pure culture of *C. vulgaris*, grown in BBM with a pH adjusted (pH ≈7) and pH non-adjusted series, in which we observed a characteristic rise of pH values (pH ≈10.5) when pH was not adjusted. Results showed that BPs with higher log P (BPC, BPFL, BPBP, BPP, BPM, BPPH; log P $\geq$ 5) tend to partition to biomass two hours after spiking in both pH adjusted and pH non-adjusted series. Overall, a smaller amount of initially spiked BPs remained in the biomass (2.3 ± 1.6% in the pH non-adjusted, and 5.6 ± 0.4% in the pH adjusted series) than remained in the aqueous phase (26 ± 6% in the pH non-adjusted series and 22 ± 2% in the pH adjusted series). Overall removal was similar in both cases, with an average removal of 72 ± 2% in the pH non-adjusted and 73 ± 5% in the pH adjusted series. However, total removals of 2,2'-BPF, BPC2 and BPAF were statistically significantly higher in the pH adjusted experiment, while the removal of BPA and BPC was significantly higher in the pH non-adjusted experiment. The above differences could partly be explained by the high pH (reaching pH 10.5) in the pH non-adjusted series, causing a proportion of the BPs to become ionised, which lowers hydrophobicity and decreases adsorption onto and uptake into the biomass. A positive correlation was found between compound log P and total removal in the pH adjusted and pH non-adjusted series, meaning BPs with higher log P were generally more efficiently removed due to higher uptake. BPS was the most recalcitrant compound between tested BPs, with only 26 ± 4% and 25 ± 5% being removed from the pH adjusted and pH non-adjusted series, respectively. The removal also correlated with chlorophyll *a* concentration for most BPs, meaning that algae played a crucial role in their removal, while culture pH also governed the removal of specific compounds.

This work is significant as it studies the fate of a wide range of BPs in algal photobioreactors. Compound physicochemical parameters and culture pH are then used to explain their partitioning to the aqueous and biomass phase and removal of BPs.

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Removal and fate of 18 bisphenols in lab-scale algal bioreactors

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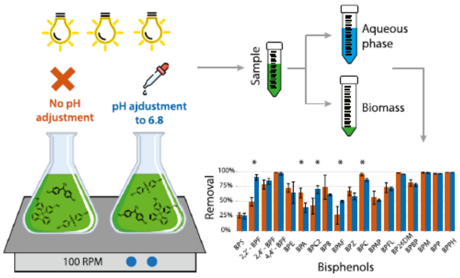
HIGHLIGHTS

- Removal of bisphenols in photobioreactors ranged from 25% to 99%.
- Final removal correlated with compound log P.
- pH adjustment influenced the removal of 2,2′-BPF, BPA, BPC2, BPAF, BPC.
- After 13 days, residual bisphenols were mainly present in the aqueous phase.

GRAPHICAL ABSTRACT



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ABSTRACT

The removal of 18 bisphenols at wastewater relevant concentrations (μg L^{-1} range) was investigated and compared between *Chlorella vulgaris* cultures with pH adjusted to 6.8 and pH non-adjusted cultures where pH raised to above 10. Bisphenols with a high partition coefficient (log P > 6) partitioned to biomass soon after spiking, whereas bisphenols with a low partition coefficient (log P < 4) remained largely in the aqueous phase. Hydrophobic bisphenols and BPF isomers were removed to a large degree in pH adjusted conditions, while BPS and BPAF were the most recalcitrant. The overall average removal after 13 days was similar in both experiments, with 72 ± 2% and 73 ± 5% removed in pH non-adjusted and pH adjusted series, respectively. The removal correlated with chlorophyll *a* concentration for most bisphenols meaning that algae played a crucial role in their removal, while culture pH also governed the removal of some compounds.

1. Introduction

Bisphenols (BPs, details in Table S1) are a group of compounds used in the production of polymers, many of which been recognised to exhibit toxic effects such as cytotoxicity, genotoxicity, reproductive toxicity and endocrine disruption (Chen et al., 2016). Wastewater is one of the main sources of BPs entering the environment, and efficient wastewater treatment is key to lowering their emissions to the environment (Noszczynska and Piotrowska-Seget, 2018) as the presence of contaminants of emerging concern (CEC) may have ecological impacts (Muñoz et al., 2009). The most abundant and frequently occurring BP in wastewater is BPA, followed by BPS and 4,4′-BPF, reaching up to 20,400 ng L^{-1}, 1688 ng L^{-1} and 1170 ng L^{-1} in wastewater influent,

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respectively (Chiriac et al., 2021; Karthikraj and Kannan, 2017; Qian et al., 2021; Sun et al., 2017; Xue and Kannan, 2019). However, 2,2′-BPF, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPZ and BPP have also been shown to occur in wastewater, albeit less frequently and in lower concentrations up to 744 ng L^{-1} (BPE) (Cesen et al., 2018; Chiriac et al., 2021; Karthikraj and Kannan, 2017; Qian et al., 2021; Sun et al., 2017; Thomas et al., 2014). These studies show that while BPs other than BPA are present in wastewater, their occurrence and fate remain largely unknown. On the level of the European Union, there is no umbrella legislation limiting emissions of BPs with wastewater effluents to the environment, which is concerning. However, individual member states may or may not have regulations limiting the concentration of BPA in wastewater (e.g., 160 μg L^{-1} in Slovenia for wastewater effluent emission to the environment Republic of Slovenia (2012)).

At the same time, research of wastewater treatment technologies faces new challenges, as the need for nature-based solutions (Castellar et al., 2021) and resource recovery from wastewater and its treatment by-products becomes apparent (European Commission, 2020). Algal technologies offer the advantage of simultaneous wastewater treatment and resource recovery and lower operational cost compared to conventional activated sludge (CAS) treatment (Craggs et al., 2014). As algae take up nutrients from wastewater and produce lipids, resulting in nutrient- and energy-rich biomass, they also form symbiotic relationships with bacteria and exploit different metabolic routes that may aid in degradation of pollutants (Norvill et al., 2016).

Several studies have investigated the removal of BPA in laboratory-scale batch cultures of different algae species in artificial media, often spiked with high concentrations (up to mg L^{-1} range) of BPA (Eio et al., 2015; Gattullo et al., 2012; Guo et al., 2017; Ji et al., 2014; Li et al., 2009), which are not relevant for real wastewaters. The removals in these studies vary with the spiked concentration of BPA. Generally, lower initial concentrations (e.g., 10–100 μg L^{-1}) of BPA resulted in higher removals (up to 93% in 10 days) (Guo et al., 2017; Li et al., 2009), while higher initial BPA concentrations (1–50 mg L^{-1}), yielded removals between 8 and 48% in up to 10 days (Eio et al., 2015; Gattullo et al., 2012; Ji et al., 2014). A few other studies investigated algae-based removal of BPA in combination with other contaminants of emerging concern (CEC) and achieved 100% removal from municipal wastewater in algae cultures with added oxygen at an initial concentration of BPA of 10 μg L^{-1} (Abargues et al., 2013) and 46% removal from autoclaved municipal wastewater at an initial concentration of BPA of 192 μg L^{-1} (Bai and Acharya, 2019).

Most studies report removals from the aqueous phase of algal cultures and seldom investigated BPs present in the biomass phase. Eio et al. (2015) and Liu et al. (2010) studied the amount of BPA in algal biomass and reported less than 1% being present in the biomass, while Sole and Matamoros et al. (2016) found up to 6% of BPA in the biomass.

To date, few studies have investigated the removal of BPs other than BPA in algal photobioreactors. Solé and Matamoros (2016) investigated the removal of BPA, BPAF and 4,4′-BPF in addition to three other endocrine disrupting compounds (EDC) from secondary-treated effluent inoculated with free and immobilized algae. The authors achieved 46%, 80% and 87% removal of BPA, BPAF and 4,4′-BPF, respectively, spiked at an initial concentration of 10 μg L^{-1} after 10-day incubation. They also found 17% of BPAF in the biomass. Peng et al. (2014) observed the biodegradation (99% removal in 10 days; init. conc. 435 μg L^{-1}) and transformation products of tetrabromobisphenol A. Lastly, Ding et al. (2020) observed biotransformation of BPS at initial concentrations of 0.5 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}, reporting 95%, 34% and 33% removal, respectively. Despite a handful of relevant studies, there is a lack of knowledge regarding the removal and fate of BPs other than BPA in algal photobioreactors, even though many have been detected in wastewater and the aquatic environment. While studies performed in real-life treatment wastewater systems are more applicable (Škufca et al., 2021b), in order to overcome the knowledge gap on the mechanisms of removal, laboratory scale studies conducted under controlled conditions and with sufficient replicates are imperative.

The aim of this study was to investigate the removal and fate of a wide range of BPs with *Chlorella vulgaris*, specifically to: (1) study the removal of 18 BPs at wastewater relevant concentrations in laboratory-scale algal photobioreactors, (2) determine the partitioning and fate of BPs in aqueous and biomass phases during the experiment, and (3) determine differences in the removal of BPs in pH adjusted and pH non-adjusted cultures.

2. Materials and methods

2.1. Standards and reagents

NANOCOLOR tube tests for photometric analysis of nutrients (test numbers 0–88, 0–55, 0–78) were purchased from Macherey-Nagel (Germany). Bisphenol A (4-[2-(4-hydroxyphenyl)propan-2-yl]phenol, BPA) (>97%) was purchased from Merck (Germany), 2-[(2-hydroxy phenyl)methyl]phenol (2,2′-BPF), 4-[(4-hydroxyphenyl)methyl]phenol (4,4′-BPF), 4-[1,1,1,3,3,3-Hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol (BPAF), 4-[2-(4-hydroxyphenyl)butan-2-yl]phenol (BPB), 4-[1-(4-hydroxyphenyl)ethyl]phenol (BPE), 4-[2-(4-hydroxy-3-methylphenyl)propan-2-yl]-2-methylphenol (BPC), 4-[2-[3-[2-(4-hydroxyphenyl)propan-2-yl]phenyl]propan-2-yl]phenol (BPM), 4-[2-[4-[2-(4-hydroxyphenyl)propan-2-yl]phenyl]propan-2-yl]phenol (BPP), 4-[(4-hydroxyphenyl)-diphenylmethyl]phenol (BPBP), 4-[2-(4-hydroxy-3,5-dimethylphenyl)propan-2-yl]-2,6-dimethylphenol (BP26DM), 4-[2,2-dichloro-1-(4-hydroxyphenyl)ethenyl]phenol (BPC2), 4-[1-(4-hydroxyphenyl)cyclohexyl]phenol (BPZ), 4-[1-(4-hydroxyphenyl)-1-phenylethyl]phenol (BPAP), 4-(4-hydroxyphenyl)sulfonylphenol (BPS), 4-[2-(4-hydroxy-3-phenylphenyl)propan-2-yl]-2-phenylphenol (BPPH) and 4-[9-(4-hydroxyphenyl)fluoren-9-yl]phenol (BPFL) were obtained from Sigma-Aldrich (>98%, USA), and 2-[(4-hydroxyphenyl)methyl]phenol (2,4′-BPF) was purchased from Tokyo Chemical Industry Co. Ltd. (>98%, Japan). Information about CAS identifiers and predicted physicochemical properties of each compound is available in Table S1. Isotopically labelled [$^{13}C_{12}$] 4,4′-BPF ($^{13}C_{12}$-BPF), BPS ($^{13}C_{12}$-BPS), BPB ($^{13}C_{12}$-BPB) were purchased from CanSyn Chem. Corp. (Canada), and deuterated BPA (BPA-d_{16}) from Isotec (USA). The derivatising agent N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, ≥99.0%), the catalyst pyridine (99.8%), and hydrochloric acid (37%, HCl) were all purchased from Sigma-Aldrich (USA). Ethyl acetate (EtAc), methanol (MeOH), acetonitrile (ACN), hexane (Hex), acetic acid (AA) and formic acid (FA) were purchased from J. T. Baker (Netherlands). Ultrapure water was prepared with a MilliQ-water purification system (>18.0 MΩ cm^{-1} at 25 °C Millipore, USA).

2.2. Experimental setup

A monoculture of *Chlorella vulgaris* was used in the experiment and Bold's Basal Medium (BBM, PhytoTechnology Laboratories, USA) as culture medium. Standard BBM contains: 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2 \cdot 2\,H_2O$, 75 mg L^{-1} $MgSO_4 \cdot 7\,H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg L^{-1} NaCl, 50 mg L^{-1} EDTA, 31 mg L^{-1} KOH, 4.98 mg L^{-1} $FeSO_4 \cdot 7\,H_2O$, 1 mL L^{-1} concentrated H_2SO_4, 11.42 mg L^{-1} H_3BO_3, 8.82 mg L^{-1} $ZnSO_4 \cdot 7\,H_2O$, 1.44 mg L^{-1} $MnCl \cdot 4\,H_2O$, 0.71 mg L^{-1} MoO_3, 1.57 mg L^{-1} $CuSO_4 \cdot 5\,H_2O$ and 0.49 mg L^{-1} $Co(NO_3)_2 \cdot 6\,H_2O$ (Andersen, 2005). Glucose was added (0.5 g L^{-1}) to support mixotrophic conditions, and the pH was adjusted by adding 34% v/v HCl dropwise until the pH reached 6.8, i.e., the initial pH of BBM medium. Algae were grown in 1 L wide-neck glass Erlenmeyer flasks. To each flask was added 487.5 mL of BBM and 12.5 mL of the *C. vulgaris* seed culture, resulting in a total volume of 500 mL with a cell density of 2.5×10^6 cells mL^{-1} at the start of the experiment. Flasks were incubated on an orbital shaker (100 RPM), and irradiance was provided using fluorescent Osram Fluora lamps, yielding approximately

41 ± 6 μmol m^2 s^{-1} of photosynthetically active radiation. At each sampling time, parameters such as culture temperature, pH, oxidation-reduction potential (ORP) and dissolved oxygen (DO) were measured in the cultures using a WTW multi-parameter portable meter MultiLine® Multi 3620 IDS with corresponding probes (Xylem Analytics, Germany).

In order to study the removal of BPs, four series of experimental reactors were employed in triplicates: abiotic control (BBM + BPs, without algae, illuminated, without pH adjustment) to account for abiotic removal of BPs, biotic blank control (BBM + algae + pH adjustment, without BPs) as a control for BPs contamination and to see if algae growth is affected at this concentration of BPs, pH adjusted series (BBM + BPs + algae + pH adjustment to ~6.8) and pH non-adjusted series (BBM + BPs + algae, without pH adjustment). For practical reasons, all possible controls (e.g., abiotic control with pH 7 and 10, with and without illumination) could not be included in the experimental design. BPs were spiked to the individual photobioreactors by adding 100 μL of working standard solution containing 5 μg mL^{-1} of each BP in pure MeOH, meaning that the initial mass (m_0) of 500 ng of each BP was added to the culture, resulted in an initial concentration of 1000 ng L^{-1} of each of the 18 BPs in the culture medium. Samples (50 mL) were taken at 2, 24, 72, 144, 216, 312 h at approximately 10 a.m. Time 0 represents the nominal starting mass of BPs (m_0) in the Figures, relative to which the removal was calculated as described in Section 2.6. Each sample was centrifuged for 20 min at 6000 RCF (CENTRIC MF 48, Domel, Slovenia) to separate the supernatant (aqueous phase) from the pellet (biomass phase). Both phases were stored at −20 °C prior to analysis.

To monitor the removal of total nitrogen (TN), total phosphate (TP), and total organic carbon (TOC), samples (5 mL) were taken at time = 0 and 312 h, centrifuged for 25 min at 6080 RCF (Universal 320, Hettich, Germany) and the supernatant stored at −20 °C until analysis.

2.3. Algal growth

Algal growth was monitored by measuring absorbance at 684 nm and calculating cell density from the linear equation of the calibration curve (Chia et al., 2013). Additionally, chlorophyll *a* concentrations (Chl *a*) were measured in the cultures, according to existing methodology (Segovia Bifarini et al., 2020). Briefly, 5 mL samples from each parallel were centrifuged at 5240 RCF for 25 min, the supernatant was removed, and 8 mL of ethanol (95%) was added to each sample. This was followed by ultrasound-assisted (1000 W, Elmasonic S 180H, Elma, Germany) extraction for 3 min. Samples were then incubated in the dark at 60 °C for 60 min, ultrasonicated for 2 min, and centrifuged at 6080 RCF for 10 min. The absorbance was then measured at 649 nm, 664 nm and 750 nm. Chl *a* concentration was calculated using the equation for 95% ethanol from Lichtenthaler and Buschmann (2001).

2.4. Sample preparation for bisphenol analysis

Sample preparation was conducted as described in Škufca et al. (2021a). Briefly, the aqueous phase was transferred into a glass flask and 21 mL of MeOH was added. An internal standard mixture (final concentration in the sample: 500 ng L^{-1} of BPA-d_{16}, 13C12-BPF, $^{13}C_{12}$-BPS and $^{13}C_{12}$-BPB each, 25 μL of 1 μg mL^{-1} solution) was also added and the sample filtered (GF/C filter, 1.2 μm pore size; Whatman, UK). The samples were then acidified (0.5 mL 7% HCl) and loaded onto MCX Prime solid phase extraction (SPE) cartridges (60 mg, 3 cm^3; Waters, USA). After washing with 5 mL of 20% MeOH and 3 mL of ultrapure water, the SPE cartridges were dried for 45 min. Analytes were eluted using 1.8 mL 5% FA in EtAc (v/v) and the eluate dried under N_2.

The biomass phase was lyophilised using a Martin Christ Gamma freeze dryer (Germany). Internal standard (25 μL of 1 μg mL^{-1} solution) and 8 mL of ACN/MeOH (80:20) were added to each sample. Samples were homogenised (Miccra D-1, ART Prozess- & Labortechnik GmbH & Co. KG, Germany) for 0.5 min at 25.000 RPM and shaken for 30 min on an orbital shaker at 220 RPM. Afterwards, the samples were sonicated in an ultrasonic bath at the highest setting (550HT, VWR, USA) for 30 min. Extracts were filtered using 0.45 μm PTFE syringe filters (Macherey Nagel, Germany) and dried under N_2 at 80 °C. Extracts were dissolved in 4.5 mL EtAc/Hex (25:75) and purified with Bond Elut Carbon/PSA (250 mg, 3 cm^3, Agilent, USA) SPE cartridges. The elution step was performed using 2 × 1.8 mL of MeOH/EtAc (20:80), and subsequently with 2 × 1.8 mL of MeOH/EtAc/AA (20:78:2) for elution of BPS. Both the aqueous and biomass phase were derivatised with 50 μL MSTFA and 50 μL pyridine and incubated at 80 °C for 1 h.

Bisphenols were analysed using gas chromatography (GC, model 7890B, Agilent, USA) with tandem mass spectrometry (MS/MS, model 7000, Agilent, USA). Separation was achieved using a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, USA) with helium as the carrier gas. Samples were injected in splitless mode at 270 °C. The compounds were ionised in electron impact (EI) mode at 70 eV and detected using multiple reaction monitoring mode. The total runtime was 24 min. More details on analytical methods are available in Tables S2–S6.

2.5. Analysis of nutrients and BOD_5

Nutrients (dissolved nitrogen – DN, dissolved phosphorous – DP, dissolved organic carbon – DOC) were measured at time = 0 and 13 days in the supernatant using NANOCOLOR tube tests 0–88, 0–55, 0–78, respectively, according to manufacturer's instructions. Total nitrogen (TN) and total phosphorous (TP) were also measured in whole cultures to obtain information about the assimilation of nutrients into the culture (TN – DN, and TP – DP). Assimilated nutrients as a percentage of dry weight (dw) of biomass was calculated as mass of assimilated nutrient divided by mass of lyophilised biomass per given volume. Soluble BOD5 (sBOD5) was measured in 97 mL of sample at time = 0 and 13 days using the WTW OxiTop measuring system (Xylem Analytics, Germany) for five days at 20 °C as per manufacturer's instructions.

2.6. Data analysis

Physico-chemical properties of BPs (log P, pKa) were predicted based on compound structure, using the Marvin Suite (ChemAxon, 2019). The chromatograms were quantified using the Mass Hunter Quantitative Analysis software (Agilent, USA). Data analysis was performed using R programming language (R Core Team, 2020) in the R Studio environment (RStudio Team, 2020). The software packages "tidyverse" (Wickham et al., 2019), "xslx" (Dragulescu and Arendt, 2020) and "rstatix" (Kassambara, 2020) were used to analyse the data. The Kruskal-Wallis one-way analysis of variance was used to determine significant differences between groups of samples, while Spearman's rank correlation test was used to test for correlation. In Figures and text, residual BPs are given as relative mass proportion and calculated as measured mass in each phase (aqueous or biomass) at a given time divided by initial nominal spiked mass (m/m_0, with m_0 being 500 ng, equal to a spiked concentration of 1000 ng L^{-1} *per* compound). Removal was calculated relatively to m_0 as total removal, using the following equation:

$$Removal\ (\%) = \frac{(m_0 - (m_a + m_b))}{m_0} \times 100$$

The mass of a given BP in the aqueous phase is m_a, while m_b is the mass in the algal biomass. The sum of the two for a given BP represents the total residue in the photobioreactor. On the other hand, overall removal refers to sum removal of all 18 BPs in both the aqueous and biomass phase. Measured concentrations below the lower limit of quantitation (LLOQ) were substituted with 1/2 of the LLOQ (Table S2).

3. Results and discussion

3.1. Culture growth

Algal growth was comparable in the biotic control and experimental series with and without pH adjustment as shown by the Chl *a* concentration and biomass density (Figs. S1 and 1A, respectively). No statistically significant differences in Chl *a* concentrations were found between the pH adjusted, pH non-adjusted, and biotic control series in any of the time points ($p > 0.05$, Kruskal-Wallis test), indicating that the addition of BPs and pH adjustment did not influence this parameter. The dry weight of algal biomass reached 0.7 g L^{-1} on day 13, which corresponded to 6.4×10^7 cells mL^{-1} and 15.2 μg mL^{-1} of Chl *a*. It should be noted, however, that Chl *a* does not strictly correlate with cell density and can vary with different growth conditions, but it is still widely used as a surrogate to monitor algal growth due to the simplicity of measurement (Eio et al., 2015; Ji et al., 2014; Zhang et al., 2021). Growth slowed down after day 3, but the cultures did not reach the stationary phase during the experiment. ORP in pH adjusted series was above 200 mV throughout the experiment, while in the pH non-adjusted series, it was slightly lower (>140 mV; data not shown), showing that oxidising conditions were favourable in both cases for abiotic degradation of compounds. The DO concentrations ranged from 6.6 mg L^{-1} to 8.4 mg L^{-1} in the pH adjusted, pH non-adjusted and blank control series throughout the experiment with no significant difference in DO concentrations between them (data not shown). The DO levels in these experiments did not inhibit algal growth, which can occur in *C. vulgaris* at DO concentrations ≥31 mg L^{-1} (Kazbar et al., 2019). The pH in the pH non-adjusted experiment (Fig. 1B) started increasing straight away, rose to above pH 10 at 48 h, and remained high throughout the experiment. Similarly, in the pH adjusted experiment, it spiked to approximately pH 9.7 at 48 h but then settled at around 7 after a pH adjustment was made on day 2.

The removal of nutrients and $sBOD_5$ from BBM medium in different series is presented in Table S7 and in the associated discussion. By the end of the experiment (13 days), TN represented 5.1 ± 0.6% and 5.5 ± 1.8% of biomass dw in pH adjusted and pH non-adjusted series, respectively, while TP represented 2.1 ± 0.4% and 0.9 ± 0.2% of biomass dw in pH adjusted and pH non-adjusted series, respectively. Assimilation of TP by the biomass was higher in pH adjusted series, which was due to inorganic phosphate (Pi) being in a more bioavailable form ($H_2PO_4^-$ and HPO_4^{2-}) at pH 7 (pH adjusted series) than at pH >10 (PO_4^{3-}, pH non-adjusted series) (Su, 2021).

3.2. Mass balance and removal of BPs

The mass balance and removal of BPs was investigated in *C. vulgaris* cultures with pH adjustment (pH 6.8) and without pH adjustment (pH reaching >10). In addition, the removal was also investigated in abiotic controls to calculate the contribution of abiotic processes (e.g., adsorption to glassware, photodegradation, oxidation) to the removal. Abiotic controls showed that most BPs were not removed in the absence of algae during the experiment, apart from BP26DM, BPM, BPP and BPPH (Fig. S6). Preliminary experiments showed that up to 49% of the three most hydrophobic BPs (BPM, BPP and BPPH, log P ≥ 6.72) of the initial spike could be recovered by rinsing the glassware of abiotic controls with MeOH after the end of the experiment (not shown). This indicates that a large proportion of these BPs can remain adsorbed to glassware throughout the experiment in the absence of algal biomass. The absence of BP26DM on the glassware walls suggests that this compound is removed to a large degree during the experiment due to photodegradation, while the same may not be said for the other BPs (Prosenc et al., 2021). Kovacic et al. (2019) found BP26DM to be the least stable of the tested BPs in stored samples.

Distribution of BPs between the aqueous and biomass phase during the experiment is shown in Fig. 2. A low proportion of BPs (<50% m_0) in the aqueous phase at the initial sampling ($t = 2$ h) was observed for some BPs with high log P (BPC, BPFL, BPBP, BPP, BPM, BPPH; log P ≥ 5). This observation can be explained, in part, by higher proportion in the biomass (>15% m_0) of BPFL, BPM, BPBP, BPP and BPPH, which indicate that BPs with higher log P tend to partition to biomass soon after spiking in both, pH adjusted and pH non-adjusted series, while they may also partially be adsorbed to the glassware walls as discussed in the previous paragraph. Differences in the distribution of BPs between the two phases can be compared between pH non-adjusted and pH adjusted series in Fig. 2A and B, respectively (also Fig. S7). Generally, for most BPs, pH adjustment did not influence the distribution between the two phases at the end of the experiment (13 days or 312 h). However, in the aqueous phase, some BPs remained in higher proportion in pH non-adjusted series (e.g., 2,2′-BPF, BPC2, and BPAF), while others remained higher in pH adjusted (e.g., BPC and BPA). In biomass, lower proportions (<10% m_0) of all BPs were present in biomass in pH non-adjusted series, while in the pH adjusted series, a higher proportion (≥10% m_0) was observed for some BPs (BPAP, BPZ, BPBP, BPAF, BPC2 and BPFL) (Fig. 2, also Fig. S7).

The concentrations of remaining BPs in each phase (aqueous and biomass) are compared between pH adjusted and pH non-adjusted series at the end of the experiment in Fig. 3. For most BPs, there was no

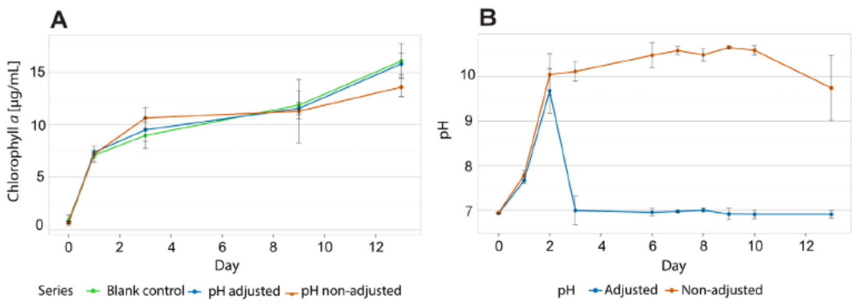

Fig. 1. Chlorophyll *a* concentration over time in different cultures (A) and measured pH in pH-adjusted and pH-non-adjusted experiments (B). The pH in the pH-adjusted series (blue line) is the pH measured in the cultures each day before adjusting the pH to 6.8. $n = 3 \pm$ SD. No statistically significant differences ($p > 0.05$, Kruskal-Wallis test) in Chl *a* concentration between pH adjusted, pH non-adjusted, and blank control series were observed at any given time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

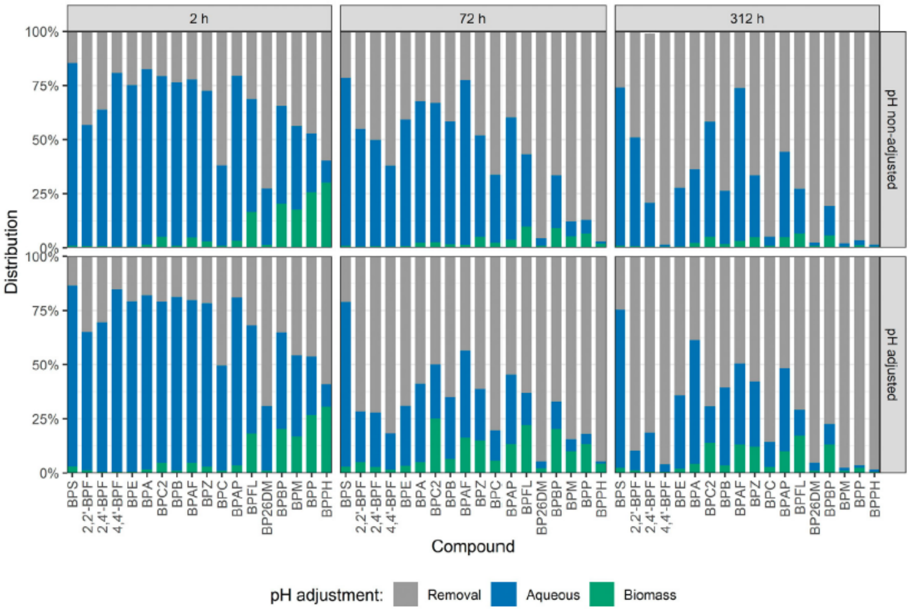

Fig. 2. Distribution of BPs between aqueous and biomass phase at 2 h, 72 h and 312 h in pH non-adjusted (top) and adjusted (bottom) experiments. The compounds on the horizontal axis appear by increasing log P value.

significant difference in remaining concentrations in the aqueous phase between pH adjusted and pH non-adjusted series, apart from a few exceptions (Fig. 3A). 2,2′-BPF, BPC2, and BPAF remained in higher concentrations in the aqueous phase in pH non-adjusted series, while BPC and BPA remained higher in pH adjusted series. In biomass, higher concentrations of BPs were observed in pH adjusted series as compared to pH non-adjusted series (Fig. 3B). BPC2, BPAF, BPZ, BPAP, BPFL, and BPBP occurred in concentrations higher than 150 ng g^{-1} in pH adjusted series, while in pH non-adjusted series all BPs occurred in concentrations below that.

The above differences in aqueous and biomass concentrations can be explained by the high pH (reaching pH 10.5) in the pH non-adjusted series, causing a proportion of the BPs (weak acids with predicted pK_a ranging from 7.4 to 10.5) to become ionised, which lowers hydrophobicity and decreases adsorption onto and uptake into the biomass (Matamoros et al., 2016). Overall, a smaller amount of all the initially spiked BPs remained in the biomass (2.3 ± 1.6% of m_0 in the pH non-adjusted, and 5.6 ± 0.4% of m_0 in the pH adjusted series) than in the aqueous phase (26 ± 6% of m_0 in the pH non-adjusted series and 22 ± 2% of m_0 in the pH adjusted series) at the end of the experiment.

Total removal (aqueous + biomass) of BPs varied in both, pH adjusted and non-adjusted series (Fig. 4). More BPs were removed to a high degree (>90%) in the pH non-adjusted series as compared to the pH adjusted series (six vs. five). At the same time, there was also more BPs that were poorly removed (<50%) in pH non-adjusted series as compared to pH adjusted series (four vs. three).

A positive correlation was found between compound log P and total removal in the pH adjusted and pH non-adjusted series at t = 13 days (312 h) (Spearman's rank correlation, $R = 0.54$, $p = 0.021$ and $R = 0.5$, $p = 0.035$, respectively; Fig. S2), meaning that BPs with higher log P were generally more efficiently removed. Overall removal of all 18 BPs was similar in both series, with an average removal of 72 ± 2% in the pH non-adjusted and 73 ± 5% in the pH adjusted series. However, total removals of 2,2′-BPF, BPC2 and BPAF were statistically significantly higher ($p < 0.05$, Kruskal-Wallis test) in the pH adjusted experiment, while the removal of BPA and BPC was significantly higher in the pH non-adjusted experiment ($p < 0.05$, Kruskal-Wallis test, Fig. 4). It can also be observed that total removal of some BPs (e.g., 2,2′-BPF, BPC2, BPAF) was faster in the pH adjusted series (Fig. 4). The pH values above an acidic compounds' pK_a will cause a proportion of the compound to become ionised, which decreases its uptake (Matamoros et al., 2016). While this may explain the removal differences in part, we believe many other factors influence removal, such as compound stability, steric effects, uptake, presence of surfactants and degrading extracellular enzymes and culture conditions (Liu et al., 2016; Lu et al., 2015; Varjani and Upasani, 2017).

Biodegradation of micropollutants may occur intracellularly, extracellularly or as a combination of both (Xiong et al., 2018) and with lower uptake, intracellular biodegradation is likely limited. Tadkaew et al. (2010) have shown that removal efficiency of diclofenac, ketoprofen and ibuprofen dropped with increasing pH in a membrane bioreactor since a larger proportion of the compounds was ionised and were not removed. This may explain the lower removal of 2,2′-BPF, BPC2 and BPAF in the pH non-adjusted experiment, but not why the converse was true for BPA and BPC. This finding, however, does not explain why the removal of other BPs was not similarly impacted by pH. One other reason might be that different degradation pathways exist for different BPs or that certain BPs may be degraded both extracellularly and intracellularly, while others may be degraded predominantly intracellularly and are dependent on uptake.

BPS was the most recalcitrant compound, with only 26 ± 4% and 25 ± 5% removed in the pH adjusted and pH non-adjusted series, respectively (Fig. 4). Similar removals were observed by Prosenc et al. (2021) in a batch culture of *C. vulgaris* and mixed algal-bacterial consortium. BPS has been found to be non-biodegradable under aerobic conditions by the native river- and seawater microorganism consortia (Danzl et al., 2009; Ike et al., 2006). Ding et al. (2020) have observed removal and biotransformation of BPS at high concentrations in a culture of *Chlorella vulgaris* (0.5 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}). They reported

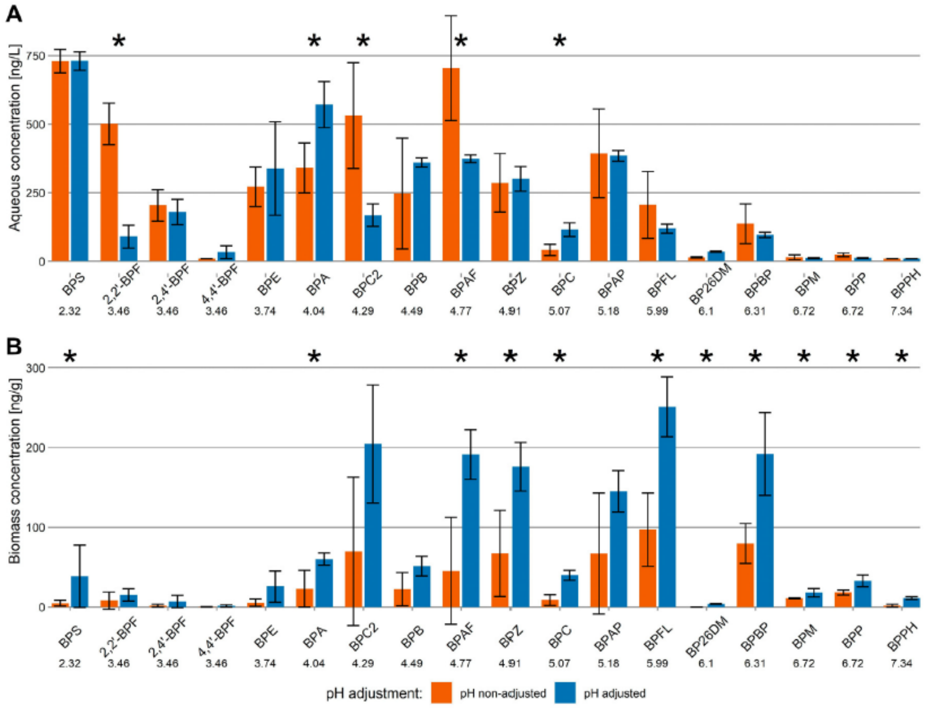

Fig. 3. BPs concentrations in the aqueous phase (A) and dry weight of biomass (B) phase at the end of the experiment ($t = 312$ h). The compounds on the horizontal axis appear by increasing log P value, noted below compound abbreviation. * - denotes statistically significant differences ($p < 0.05$, Kruskal-Wallis test) between the pH non-adjusted and pH adjusted experiment.

95% removal at the lowest concentration after 1 h and 89% removal during the same time in the abiotic control. This, together with no further increase in the removal over 144 h, leads us to believe that a mechanism other than biodegradation was responsible for such high removals. In mixed algal culture, 4,4′-BPF, BPA and BPAF were removed by biodegradation, while adsorption and uptake were found for BPA and especially BPAF (up to 17% on day 3), which is in accordance with this study. Similarly, the majority of residual BPs was present in the aqueous phase (Solé and Matamoros, 2016). However, the removal of BPs is likely higher in real wastewater as it was shown that microalgae grown in simple medium are less successful in removing ibuprofen and caffeine (51% and 17%, respectively) than algae grown in real wastewater (99% for both), which also contains a rich consortium of bacteria that may enhance biodegradation (Matamoros et al., 2016).

Fast uptake of the most hydrophobic compounds and their consequent biodegradation explain why they (log $P > 5$; BPC, BP26DM, BPBP, BPM, BPP, BPPH, except BPAP and BPFL) reached removal >75% in both series. 2,2′-BPF had significantly lower removal in the pH non-adjusted series as compared to pH adjusted series (49 ± 7% vs. 90 ± 5%, respectively), which could be explained by its low pKa (8.2), meaning that a large proportion was likely ionised at $pH > 10$ and not taken up by algae.

To investigate the relationship between the residual BPs and Chl *a* concentration, a linear regression model was fitted to the plot of the two parameters (Fig. 5). 2,2′-BPF, BPC2 and BPAF in pH non-adjusted and BPA in the pH adjusted experiment had slope coefficients greater or equal than -0.01 and low R^2 values (0.044, 0.300, 0.014 and 0.058, respectively), which means that these BPs were removed independently of Chl *a* concentration (and therefore, biomass concentration) and were not biodegraded to a significant degree. The same is true for the slope coefficient of BPS in both experiments (0.002 for pH non-adjusted and 0.008 for pH adjusted). The removal of other BPs showed some dependence on the Chl *a* content, indicating biodegradation by algae. The high R^2 values obtained in the linear regression model of other BPs indicate a good fit between Chl *a* concentration and compound removal in the case of some hydrophobic BPs in the pH non-adjusted experiment (e.g., BPZ, BPBP, BPM, BPP, BPPH with $R^2 \geq 0.75$). Solé and Matamoros (2016) have similarly determined concentrations of 4,4′-BPF, BPA and BPAF to be negatively correlated with TSS in a mixed algae culture.

4. Conclusions

The removal and fate of 18 BPs were investigated in *C. vulgaris* cultures at wastewater relevant concentrations with and without pH adjustment, by separately analysing the aqueous and biomass phase. Removal of individual BPs ranged from 25 to 99%, with overall removal reaching 73% after 13 days. The adjustment of pH influenced the removal of five BPs (2,2′-BPF, BPA, BPC2, BPAF, BPC), whereas others were not influenced by pH adjustment. Of all the initially spiked BPs (18 $\mu g\ L^{-1}$ in total), up to 26% remained in the aqueous phase, while only up to 6% remained in the biomass phase. In most cases, the removal of individual BPs correlated with the growth of algal biomass, while final removals depended on compounds' hydrophobicity. This work, however, was performed in a controlled environment (constant temperature and light, optimal medium). For this reason, studies approaching real-life wastewater conditions are needed for reliable quantification of the mechanisms in wastewater. Further research on transformation products of CEC in algal bioreactors and their toxicity will also show the feasibility of this approach to wastewater treatment. Additionally,

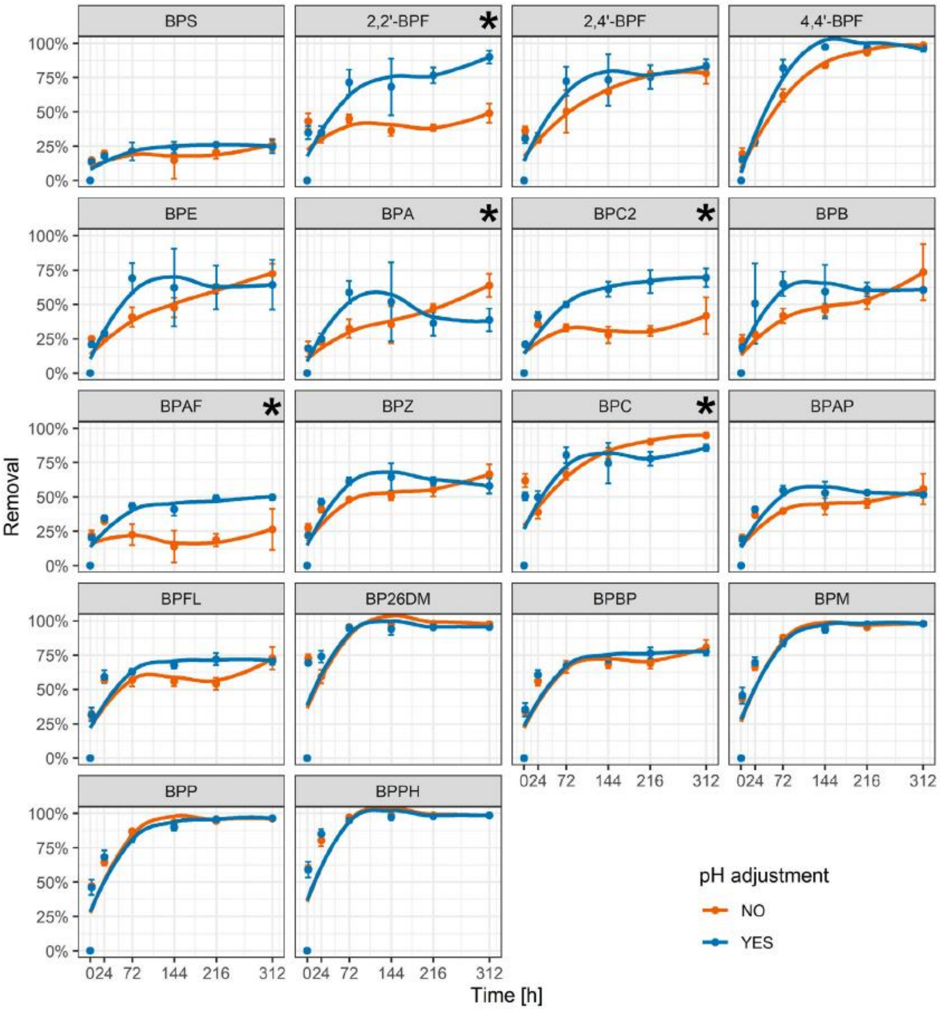

Fig. 4. Total removal (aqueous + biomass) of bisphenols in pH adjusted and pH non-adjusted experiment. The compounds appear in order of increasing log P value, shown are mean values ± SD (n = 3). * - denotes statistically significant differences ($p < 0.05$, Kruskal-Wallis test) in removal between the pH adjusted and non-adjusted series at the end of the experiment (312 h).

the influence of downstream processing of reclaimed water and algal biomass on the residual contaminants remains to be investigated and would also contribute to the safe reuse of algae-based products from wastewater treatment.

CRediT authorship contribution statement

David Škufca: Conceptualization, Methodology, Validation, Investigation, Data Curation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing; **Franja Prosenc:** Conceptualization, Methodology, Validation, Investigation, Data Curation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing; **Tjaša Griessler Bulc:** Project administration, Supervision, Funding acquisition, Resources, Writing - Review & Editing; **Ester Heath:** Supervision, Project administration, Resources, Funding Acquisition, Writing - Review & Editing.

First two authors contributed equally to this study.

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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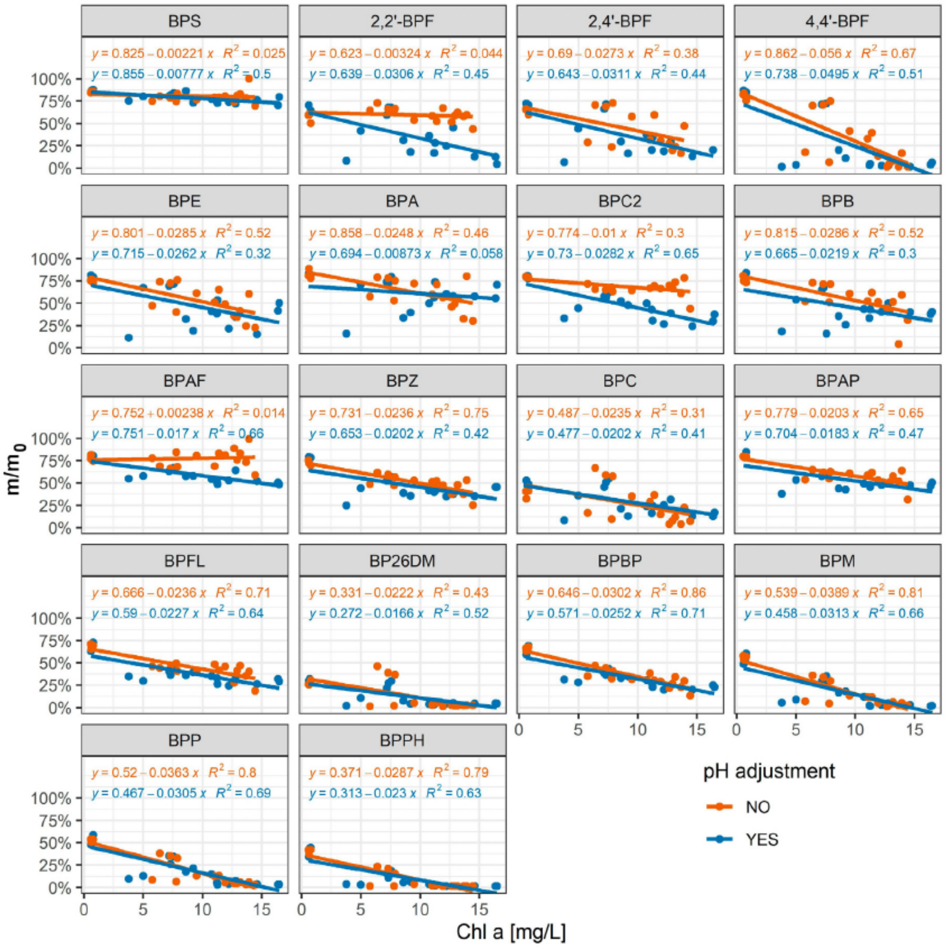

Fig. 5. Relative proportion of total residual BPs correlated with Chl *a* concentration, with linear model equations and coefficients of determination. The compounds are listed by increasing log P value.

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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.2021.149878.

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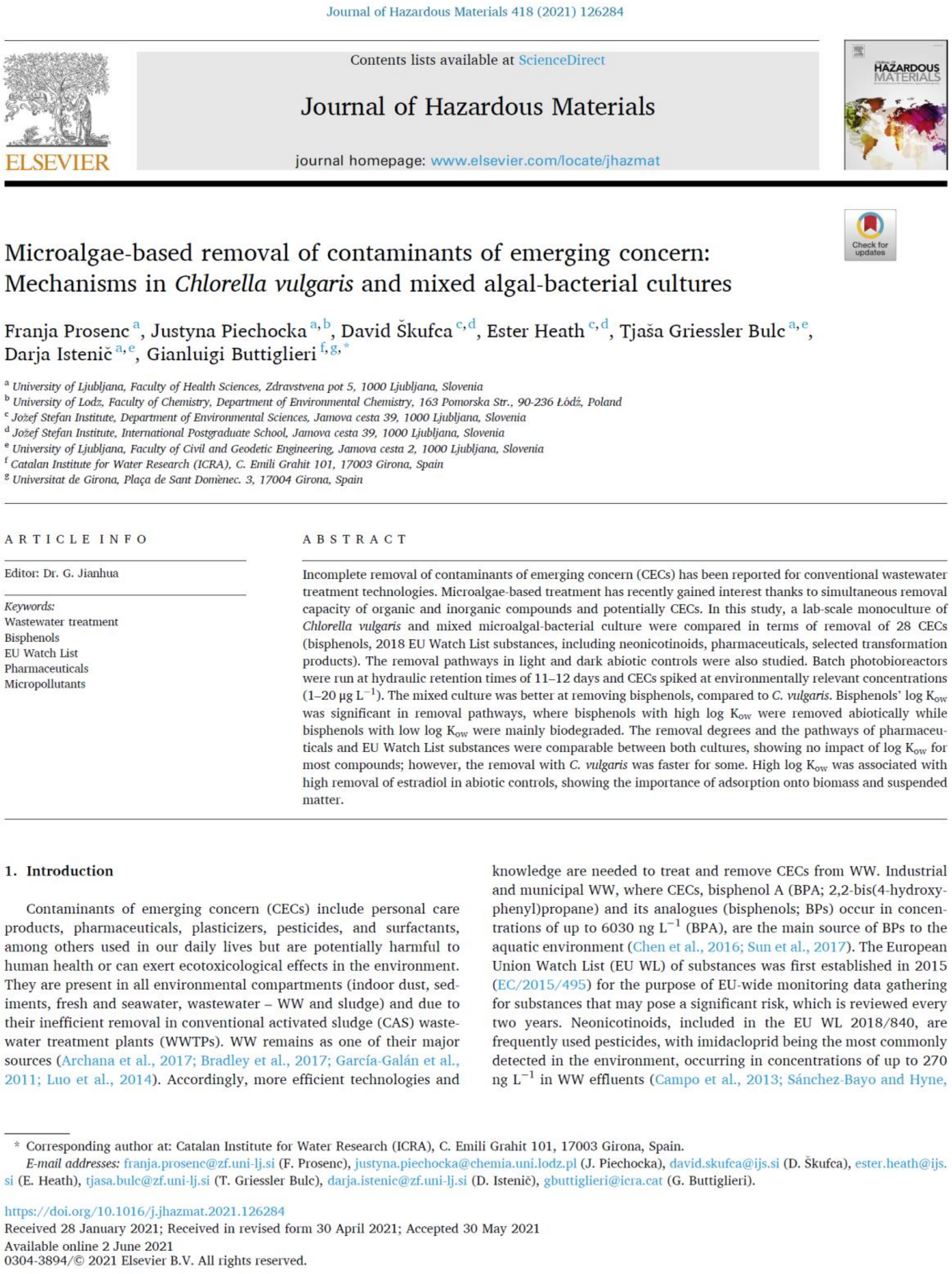
3.3 Microalgae-based removal of contaminants of emerging concern: comparison between Chlorella vulgaris culture and mixed algal-bacterial culture

The paper "Microalgae-based removal of contaminants of emerging concern: comparison between *Chlorella vulgaris* culture and mixed algal-bacterial culture" by F. Prosenc, J. Piechocka, D. Škufca, E. Heath, T. Griessler Bulc, D. Istenič and G. Buttiglieri was published in the *Journal of Hazardous Materials* (IF 2021: 10.588) in May 2021 (doi: 10.1016/j.jhazmat.2021.126284). I independently carried out all the analytical work for bisphenols and contributed to writing the manuscript. I have also contributed to sampling and sample preparation.

This study compared CEC removal in monocultures in simple medium vs. mixed cultures of microalgae and bacteria in wastewater medium under laboratory conditions. Microalgae-based wastewater treatment has recently gained interest thanks to the potential for simultaneous removal of organic and inorganic compounds and CECs, with concurrent resource recovery. While there are studies on simple laboratory-scale and more complex pilot-scale experiments, the connection between the two is not well known. The novelty of this work is in the comparison of the removal of 28 CEC in laboratory photobioreactors using a mixture of microalgae and bacteria from an HRAP grown in municipal wastewater or a pure culture of *C. vulgaris* grown in BBM.

Bisphenols were generally removed to a higher degree in the mixed culture than with pure *C. vulgaris*. However, BPS proved recalcitrant in both. WFD compounds thiamethoxam, erythromycin, clarithromycin, estradiol, and estrone were highly removable in mixed culture, while imidacloprid, acetamiprid and thiacloprid showed higher average removal in pure *C. vulgaris* culture. Of the included pharmaceutical compounds, sulfamethoxazole, metoprolol and ibuprofen have proved highly degradable in both cultures. Carbamazepine was recalcitrant in both cultures. Likewise, it has been observed to be recalcitrant in conventional WWTP. Both *C. vulgaris* and the mixed cultures could remove most of the considered 28 CEC at environmentally relevant concentrations, even outcompeting CAS treatment in the case of sulfamethoxazole, erythromycin, clarithromycin and metoprolol. Biotic removal was further confirmed via detection of transformation products for selected compounds (metoprolol, ibuprofen, sulfamethoxazole and carbamazepine). For example, metoprolol acid was found in higher concentrations in the mixed (20 µg/L) than in the pure culture (5 µg/L). Conversely, ibuprofen carboxylic acid was found in higher concentrations in the pure culture (18 µg/L vs. 2 µg/L, respectively), indicating different mechanisms in the two cultures.

The study showed that microalgae-based treatment is an effective alternative method for removing certain CEC from wastewater. However, results showed that additional treatment solutions should be sought to achieve complete removal (mineralisation of CEC), emphasising known recalcitrant compounds of emerging concern (e.g., BPS, arbamazepine).



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Microalgae-based removal of contaminants of emerging concern: Mechanisms in *Chlorella vulgaris* and mixed algal-bacterial cultures

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ABSTRACT

Incomplete removal of contaminants of emerging concern (CECs) has been reported for conventional wastewater treatment technologies. Microalgae-based treatment has recently gained interest thanks to simultaneous removal capacity of organic and inorganic compounds and potentially CECs. In this study, a lab-scale monoculture of *Chlorella vulgaris* and mixed microalgal-bacterial culture were compared in terms of removal of 28 CECs (bisphenols, 2018 EU Watch List substances, including neonicotinoids, pharmaceuticals, selected transformation products). The removal pathways in light and dark abiotic controls were also studied. Batch photobioreactors were run at hydraulic retention times of 11–12 days and CECs spiked at environmentally relevant concentrations (1–20 μg L^{-1}). The mixed culture was better at removing bisphenols, compared to *C. vulgaris*. Bisphenols' log K_{ow} was significant in removal pathways, where bisphenols with high log K_{ow} were removed abiotically while bisphenols with low log K_{ow} were mainly biodegraded. The removal degrees and the pathways of pharmaceuticals and EU Watch List substances were comparable between both cultures, showing no impact of log K_{ow} for most compounds; however, the removal with *C. vulgaris* was faster for some. High log K_{ow} was associated with high removal of estradiol in abiotic controls, showing the importance of adsorption onto biomass and suspended matter.

1. Introduction

Contaminants of emerging concern (CECs) include personal care products, pharmaceuticals, plasticizers, pesticides, and surfactants, among others used in our daily lives but are potentially harmful to human health or can exert ecotoxicological effects in the environment. They are present in all environmental compartments (indoor dust, sediments, fresh and seawater, wastewater – WW and sludge) and due to their inefficient removal in conventional activated sludge (CAS) wastewater treatment plants (WWTPs). WW remains as one of their major sources (Archana et al., 2017; Bradley et al., 2017; García-Galán et al., 2011; Luo et al., 2014). Accordingly, more efficient technologies and knowledge are needed to treat and remove CECs from WW. Industrial and municipal WW, where CECs, bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) and its analogues (bisphenols; BPs) occur in concentrations of up to 6030 ng L^{-1} (BPA), are the main source of BPs to the aquatic environment (Chen et al., 2016; Sun et al., 2017). The European Union Watch List (EU WL) of substances was first established in 2015 (EC/2015/495) for the purpose of EU-wide monitoring data gathering for substances that may pose a significant risk, which is reviewed every two years. Neonicotinoids, included in the EU WL 2018/840, are frequently used pesticides, with imidacloprid being the most commonly detected in the environment, occurring in concentrations of up to 270 ng L^{-1} in WW effluents (Campo et al., 2013; Sánchez-Bayo and Hyne,

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2014). Hormones, estrone and 17 ß-estradiol are excreted by all humans and animals, but also found in products, such as detergents, shampoos, lotions, and cosmetics (Guedes-Alonso et al., 2014; Roudbari and Rezakazemi, 2018). They can exert endocrine-disrupting effects in aquatic organisms and humans at low concentrations (<0.1 ng L^{-1}) (Roudbari and Rezakazemi, 2018; Ting and Praveena, 2017). In municipal WW, estrone and estradiol occur in concentrations of up to 35 and 25 ng L^{-1}, respectively (Cui et al., 2006; Laganà et al., 2004; Lishman et al., 2006). Pharmaceuticals are a wide group of chemicals, diverse in chemical structure and mechanisms of action. Some of them are recalcitrant to biodegradation and incompletely removed from WW during treatment (Archana et al., 2017; García-Galán et al., 2020a; Gros et al., 2007; Kostich et al., 2014). Levels of pharmaceuticals found in WWTP effluents typically range from high ng L^{-1} to low μg L^{-1} (*e.g.*, ibuprofen was found in concentrations of up to 4200 ng L^{-1} in municipal WW and the macrolide antibiotic erythromycin up to 7.2 μg L^{-1} in hospital WW), and in rivers occurred at levels at least one order of magnitude lower (Al-Maadheed et al., 2019; Gros et al., 2007; Kostich et al., 2014).

Microalgae-based WW treatments are mixed systems incorporating microalgae, bacteria, and other microorganisms. They have been gaining interest due to the capacity of microalgae to grow in low-quality water, utilise nutrients from WW, and remove organic matter, heavy metals and indicators of faecal contamination (Arbib et al., 2013; García et al., 2006; Markou et al., 2014). Microalgae perform photosynthesis and generate oxygen, required by heterotrophic bacteria in WW. In return, heterotrophic bacteria produce carbon dioxide *via* respiration, which is utilised by microalgae. In this way, microalgae and bacteria form a mutualistic relationship, which may help remove contaminants (Gonçalves et al., 2017; Segovia Bifarini et al., 2020). Despite significant research on nutrient removal from WW using algae-based technologies, only a few studies have focused on the potential of microalgae to remove CECs. Although most algae-based systems, such as high-rate algae ponds (HRAPs) have not been specifically designed to remove these contaminants, their long hydraulic retention time (HRT), typically measured in days compared to a few hours for a CAS system may prove beneficial for the removal of more recalcitrant compounds (García-Galán et al., 2020a). Several studies addressing the removal of CECs with microalgae achieved good results for certain compounds. For example, Vassalle et al. (2020) achieved 91% and 95% removal of estradiol and estrone in an up-flow anaerobic sludge blanket (UASB) reactor followed by HRAP, respectively, while García-Galán et al. (2020a), managed to achieve 85% removal of sulfamethoxazole in a pilot-scale HRAP. Except for BPA and a few other BPs, no research has been performed on algae-based removal of wide range of BPs, as well as neonicotinoids, other than imidacloprid. The enhanced effect of microalgae compared to bacteria-only has so far been reported by Matamoros et al. (2016) for seven CECs including two pharmaceutical compounds. The authors were able to demonstrate, for example, a 40% removal of caffeine by naturally occurring bacteria in WW *versus* 90% removal in a HRAP, containing microalgae *Chlorella* sp., *Scenedesmus* sp., and naturally occurring bacteria in WW. Nevertheless, the exact mechanisms of their removal and the respective roles of bacteria, microalgae, and potentially other microorganisms are still not fully understood. The removal processes may include biodegradation, biotransformation, by bacteria and/or algae, chemical oxidation, sorption and photodegradation. This processes largely depend on the physical-chemical properties of the studied contaminants (Blum et al., 2018; Matamoros et al., 2016). For example, contaminants with higher water solubility have higher mobility and hence higher bioavailability and higher exposure to chemical and photochemical degradation under long HRTs (several days) (Blum et al., 2018; García-Galán et al., 2020b). To our knowledge, algal and mixed algal-bacterial cultures have not been systematically compared for removal of a wide range of CECs at environmental concentrations. Moreover, the contribution of abiotic processes was rarely addressed in previous studies. To overcome this knowledge gap, laboratory scale studies conducted under controlled conditions are imperative.

This research aimed to study and compare the removal potential of microalgae in either a monoculture (*Chlorella vulgaris*) or mixed culture (mixed microalgal-bacterial biomass) batch conditions to remove 28 CECs of three groups of substances (namely BPs, substances from the EU WL 2018/840, and pharmaceuticals) in laboratory-scale photobioreactors. The CECs were selected based on their presence in municipal WW, priority of study (*i.e.*, EU WL substances), and their physicochemical properties (Table S1). Additional aims of the experiments were to delineate the processes involved in the removal of these compounds, whether the removal was due to biotic or abiotic processes, and to study the emergence and the removal of transformation products (TPs) of selected substances.

2. Materials and methods

2.1. Chemicals and reagents

Three classes of chemicals were used in the removal experiments, namely BPs: BPA, BPE, BPAP, BPC2, BPZ, BP26DM, BPM, BPAF, BPC, BPB, BPFL, BPAP, 2,2′ BPF, 4,4′ BPF, 2,4′ BPF, and BPS; substances from the EU WL 2018/840: macrolide antibiotics (clarithromycin, erythromycin), neonicotinoids (acetamiprid, imidacloprid, thiacloprid, thiamethoxam), hormones (estrone, estradiol); and pharmaceuticals: ibuprofen, metoprolol, sulfamethoxazole, carbamazepine. In total, 28 compounds were spiked in the removal experiments. A list of substances, their IUPAC names, CAS numbers, and partition coefficients (log K_{OW}) can be found in the Supplementary Information (SI), Table S1. The compounds investigated in this study are included in the second EU WL (EC/2018/840), however, it should be noted that a new EU WL was published during the preparation of this paper (EC/2020/1161). The origin and purity of compounds used is specified in the SI, Section 1.2.

2.2. Stock solutions and storage

Stock solutions of each BP were prepared in methanol (MeOH) (≈1 mg L^{-1} for standards of BPs, ≈2 mg L^{-1} for BPA and ≈0.2 mg L^{-1} for internal standards). Stock standard solutions for EU WL compounds and pharmaceuticals were prepared in MeOH (Honeywell Fluka) at concentration 100 mg L^{-1} and 1000 mg L^{-1}, respectively. All stock solutions were stored in darkness at − 20 °C until use, but no longer than 2 months. Each compound (200 μL) was added to a vial and the solvent mixture evaporated under nitrogen. The solution was then reconstituted in 10 mL of water (2 mg L^{-1} and 20 mg L^{-1} for EU WL compounds and pharmaceuticals, respectively, MIX solution) and vortexed to ensure complete dissolution. An aliquot (1 mL) of MIX solution was then spiked to each bioreactor (adapted from Gusmaroli et al., 2020) to obtain the experimental design initial concentration (indicated in Section 2.4).

2.3. Cultures and culture media

The experiments were performed using two types of culture: monoculture of *C. vulgaris* purchased from AlgEn, Algal Technology Centre (Slovenia) and mixed microalgal-bacterial biomass (indicated as mixed culture from now on) obtained from an operating pilot-scale HRAP (WWTP Ajdovščina, Slovenia, 45°52′32″N 13°54′20″E) treating municipal WW. The HRAP was inoculated with *C. vulgaris* culture and had been in operation for eight months before the mixed culture was obtained and used in the experiments. The bacterial community originated from the WW inflow. Total suspended solids (TSS) and nutrient load in the influent and effluent can be found in the SI, Section 1.7, Table S3.

Experiments with *C. vulgaris* were performed in Bold's Basal Medium (BBM) (PhytoTechnology Laboratories), whereas the experiments with mixed culture were performed in the medium of the HRAP influent. Both media were spiked with stock solutions of listed compounds (MIX solution in water for EU WL substances and pharmaceuticals, and individual stock solutions for BPs in MeOH) in experimental bioreactors and abiotic controls, or corresponding volume of MeOH in biotic controls.

2.4. Experimental setup and sample collection

Two sets of experiments were performed in 1 L bioreactors, which were glass reagent bottles with plastic screw caps, and with initial biomass (either *C. vulgaris* or mixed culture) concentration of TSS 70 ± 14 mg L^{-1}. The contents of each flask were stirred using a magnetic stirrer (200 rpm) and exposed to a 16 h: 8 h light:dark photoperiod at a light intensity of 59 ± 3 μmol m^{-2} s^{-1}. Illumination was ensured from all sides (top, left, right, front and back) with the help of reflective aluminium foil. All experiments were performed in triplicate. Abiotic controls were performed in both, 16 h: 8 h light:dark photoperiod and under dark conditions (24 h). Abiotic controls for *C. vulgaris* and mixed culture experiments were performed in autoclaved BBM and WW, respectively, and are referred to as *C. vulgaris* matrix or mixed culture matrix. Pharmaceuticals were spiked into reaction mixtures at 20 μg L^{-1}, EU WL substances at 2 μg L^{-1}, and BPs (apart from BPA) at 1 μg L^{-1}, and BPA at 2 μg L^{-1}, to match levels found in WW (Česen et al., 2019; Chen et al., 2016; Sun et al., 2017). Biotic controls were set up in a similar way as experimental bioreactors, but without the addition of CECs. This was to indicate whether the presence of CECs at spiked concentrations affect the growth of the cultures. Experimental conditions are shown in Table 1.

Table 1
List of experimental conditions.

Type	Biomass	CECs	Light/dark regime
Experimental bioreactors	*C. vulgaris* or mixed culture	Yes	16 h: 8 h light: dark
Biotic control	*C. vulgaris* or mixed culture	No	16 h: 8 h light: dark
Abiotic control (dark)	No	Yes	24 h dark
Abiotic control (light)	No	Yes	16 h: 8 h light: dark

Representative samples (60 mL) were collected aseptically at times 0 h, 1 h, 16 h, 24 h, 40 h, 48 h, 4 days, 7 days, 9 days, and 11 or 12 days. The duration of the experiment was based on HRTs typical for HRAPs (4–12 days) (García et al., 2000; Hom-Diaz et al., 2017; Plouviez et al., 2019). As the volume in the cultures got smaller, the stirring was adjusted accordingly. Sample preparation for each group of CECs is described in the SI, Section 1.3. The samples were analysed according to the procedure and for the parameters described in Section 2.5.

To check the background concentration of CECs and to evaluate possible matrix effects, additional samples were aseptically collected before starting the experiments. Typically, 20 mL were taken from the bioreactor using a sterile syringe and immediately filtered through Millex-HV Hydrophilic PVDF filters (47 mm, 0.45 μm) (Merck Millipore) to separate the liquid phase from the biomass. The samples were stored in 20 mL amber bottles and kept at − 20° until analysis.

2.5. Analytical methods

2.5.1. Analysis of contaminants of emerging concern

CEC were analysed in the aqueous phase at times 0, 1 h, 16 h, 24 h, 40 h, 4 or 5 days, 7 days, and 11 or 12 days. BPs were analysed by means of SPE-GC/MS as described in Kovačič et al. (2019), Škufca et al. (2021). Further details on the analytical method can be found in the SI, Section 1.4, and Table S2. Substances from the EU WL were analysed by means of online SPE-UHPLC-MS/MS as described in Gusmaroli et al. (2018). More information can be found in the SI, Section 1.5. The analysed pharmaceuticals and selected TPs (TPs of ibuprofen: 1-hydroxy ibuprofen, 2-hydroxy ibuprofen, ibuprofen carboxylic acid; TPs of sulfamethoxazole: 4-nitro sulfamethoxazole, desamino sulfamethoxazole, N-acetyl sulfamethoxazole; TPs of carbamazepine: 10,11-epoxycarbamazepine, 2-hydroxy carbamazepine; TPs of metoprolol: O-desmethyl metoprolol, metoprolol acid, α-hydroxymetoprolol) were analysed by UHPLC-QqLIT-MS/MS using direct injection (Gros et al., 2012; Ferrando-Climent et al., 2012; Rubirola et al., 2014, and Kassotaki et al., 2016), as described in detail in the SI, Section 1.6.

2.5.2. Absorbance and total suspended solids

Bioreactor liquors were analysed for the following parameters: absorbance at 684 nm to monitor daily algal biomass growth (Macherey-Nagel NANOCOLOR® VIS); TSS (mg L^{-1}) to monitor the total biomass growth (bacteria & algae) were measured as per Standard Methods (APHA, 2005) at times 0, 2, 7, 11 or 12 days. TSS of the background medium (BBM or HRAP influent) was subtracted from the total TSS in order to compare the growth between both cultures. Absorbances at 600 nm and 684 nm in abiotic controls were measured to monitor any potential contamination with bacteria or algae, respectively. Air temperature surrounding the bioreactors was monitored at every sampling. More details are provided in the SI, Section 2.4 (Fig. S8, S9, S10).

2.6. Data analysis

The results are presented as percentage removal (Eq. (1)):

$$Removal \quad (\%) = \frac{c_{spiked} - c_t}{c_{spiked}} 100 \tag{1}$$

Where c_{spiked} is the concentration of compound spiked to reaction mixtures, measured in the spiking solution (in case of pharmaceuticals and EU WL substances) or nominal spiked value, plus the concentration measured in the matrix (in case of BPs), and c_t is the concentration measured in the reaction mix at time *t*. Compounds occurring on the horizontal axis in all plots are arranged by an increasing log K_{ow}. The removal for BPA, thiacloprid and estrone in *C. vulgaris* culture, and thiacloprid and metoprolol in mixed culture was normalised to the concentration measured at time 0 (c_{spiked}). This was done to avoid presenting negative removals due to the presence of these compounds were in the matrix at time 0 before spiking, at a concentration higher than the average concentration measured in the spiking solution. These compounds are indicated with a star symbol on the plots.

For each compound, kinetic profiles, degradation rate constants, and half-lives were determined. Each compound was fitted with first order equation (Eq. (2)). For compounds yielding $R^2 < 0.75$, second order equation (Eq. (3)) was trialled. Additionally, half-lives of compounds were calculated (Eq. (4))

$$lnC_t = lnC_0 - k \times t \tag{2}$$

$$\frac{1}{C_t} = \frac{1}{C_0} + k \times t \tag{3}$$

$$t_{1/2} = \frac{ln2}{k} \tag{4}$$

Where C_t is the concentration of a particular compound at time *t*, C_0 is the concentration at the start of the experiment (time 0), *k* is the biodegradation rate constant, and *t* is time.

Compounds with removals of < 25% were classified as poorly removable, 25–65% as intermediately removable and > 65% as highly removable. The classification of compounds was done for intelligibility purposes and for identifying candidate compounds suitable for treatment with algae-based technologies. For calculation purposes, concentrations <limit of detection (LOD) = 0, and measured concentrations <limit of quantification (LOQ) = LOQ/2. Regression analysis was performed to define the relationship between log K_{ow} and removal efficiency. For this purpose, Pearson's correlation coefficient, r(df) was

obtained, where df is degrees of freedom. Data and statistical analyses were performed using MS Office Excel 16.37 (Microsoft).

3. Results and discussion

3.1. Removal of bisphenols

In mixed culture, all BPs, except for BPS, were highly removable (>67% of BPs) within 11 days, 12 BPs even achieved excellent removal, *i.e.*, > 90% (Fig. 1A), while BPS was poorly removed in these conditions (18% in 11 days). Removal was partly due to abiotic factors. Removals after 11 days in the abiotic controls ranged from 0 to ≈ 100% (Fig. S1B and C) and correlated with log K_{ow}, *i.e.*, r (14) = 0.86 for light and 0.76 for dark conditions, $p < 0.001$. The removal of BPFL, BPBP, BPM and BPAF can be largely attributed to abiotic processes under light and dark conditions. BPFL, BPBP, and BPM were, therefore, excluded from Fig. 1 and are presented in Figs. S1 and S2. The amount of removed BPAF from mixed culture matrix ranged from 73% to 75%, while BPFL, BPBP, and BPM were removed by > 89% (Fig. S1B and C). BP26DM was removed 46% in mixed culture matrix in dark conditions, whereas an additional 53% were removed in light conditions (99% in total). Therefore, BP26DM was most probably, at least partially, photodegraded in mixed culture as well. Alternatively, it can be assumed that BPS, BPA and BPB were solely, and 2′4 BPF, 4′4 BPF and BPE largely removed by biotic processes (<20% removal in abiotic controls *vs* >95% removal in mixed culture). Partial biotic removal can be assigned to all the remaining BPs.

In the *C. vulgaris* culture, 4,4′ BPF, BPC, BPFL, BP26DM, BPBP, and BPM (Fig. 1B and S2) were highly removable (>74% in 12 days). It is noticeable that there was a significant variability in the removal efficiencies (RSD from 1% to 141%). BPC2 and BPAF were poorly removable (<25%) and BPS was the most recalcitrant (<3% in 12 days). The remaining seven BPs exhibited intermediate removal (35–60%). Interestingly, the removal in *C. vulgaris* culture correlated significantly with the log K_{ow} (r (14) = 0.70, $p < 0.05$) as did the abiotic removal in autoclaved BBM (r (14) = 0.85 and 0.84, $p < 0.001$, for light and dark conditions at 11 days, respectively). Abiotic removal ranged from 0 to ~85% (Fig. S2B and C). The removal of BPFL, BPBP, and BPM (>77%) as well as BPAF and BPAP (16–32%) in both abiotic controls was proven to be fully attributed to abiotic processes. BP26DM was removed by 48% in abiotic dark conditions, and by 67% in abiotic light conditions, therefore, photodegradation played a less significant role in *C. vulgaris* matrix (Fig. S2). The difference in photodegradation of BP26DM in the two matrices (BBM and WW) can be explained by a potential presence of photosensitisers in WW, such as dissolved organic matter (DOM), which can make compounds more photosensitive (Wang and Chen, 2018; Wenk et al., 2019). The remaining BPs were removed ≤ 32% in abiotic controls. Biotic removal can be partially assigned to all compounds and from these results it appears that abiotic removal is the main degradation route.

Overall, the removal of BPs was on average by 32% more efficient in mixed culture than in *C. vulgaris* culture (Fig. 1, Figs. S1 and S2), therefore, the contribution of bacteria and/or other microalgae and microorganisms present in mixed culture was significant. The temporal variation of removal also differed, namely in the mixed culture the removal of BPs with lower K_{ow} increased after four days while in the *C. vulgaris* culture higher removal efficiencies were obtained after seven days. This is in line with biomass development, which was faster in mixed culture reaching approximately 300 mg L^{-1} TSS after two days, while the *C. vulgaris* culture needed seven days to achieve the same concentration (Fig. S8). Removal of BPAF, BPFL, BP26DM, BPBP, BPM, *i.e.*, those BPs removed mainly by abiotic processes, was similar in both cultures and could be attributed to adsorption and/or biosorption. These BPs have high log K_{ow} (≥ 4.77), which favours adsorption to suspended solids and lipophilic surfaces, *e.g.*, algae cells (Kiki et al., 2020; Matamoros et al., 2015). For some BPs, the removal at the beginning of the experiment was negative (down to −19%), meaning that the concentration measured at time 0 was higher than the nominal spiked concentration. This could be due to sampling error or analytical uncertainty.

To investigate the kinetics of BPs' removal, concentrations *versus* time were fitted to first order kinetic model. The degradation rate constant (k) and half-life of each BP are summarised in Table 2. The degradation rate constant ranged from 0.12 to 0.44 day^{-1} and from 0.05 to 0.40, corresponding to the half-life ($t_{1/2}$) values between 5.78 and 1.59 days, and between 13.13 and 1.75 days in mixed culture and *C. vulgaris* culture, respectively. The degradation kinetic data showed that the degradation process corresponds to the first order kinetics with R^2 values ranging from 0.86 to 0.99, indicating a good fit to first order kinetics for most BPs. Kinetic fitting was not possible for BPs that were poorly degraded (BPS), or their concentration remained stable (BPAF). Kinetic parameters of most BPs in microalgal cultures are largely unavailable in the literature, therefore, comparison with our data was not possible.

Studies addressing the removal of BPs in algal cultures or HRAPs are infrequent and most often only address BPA, while other BPs are omitted, despite the fact that they occur in both WW and surface waters (Česen et al., 2019, 2018). For example, Matamoros et al. (2015), achieved 85% removal of BPA from urban WW during the warm season and 78% during the cold season in a pilot scale HRAP with mixed culture. Their results are comparable to this study (82%), whereas Vassalle et al. (2020) achieved a lower removal (41.5%) in a pilot scale HRAP. A sufficiently high influent concentration of a pollutant may play a role in stimulating biodegradation (Matamoros et al., 2015), which would explain the lower removals observed by Vassalle et al. (2020) who began with a much lower influent concentration (82 ng L^{-1}) than the one used in this study (2 μg L^{-1}). Solé and Matamoros (2016) reported removal efficiencies for BPA, BPF and BPAF, of 43%, 69%, and 0% after 10 days,

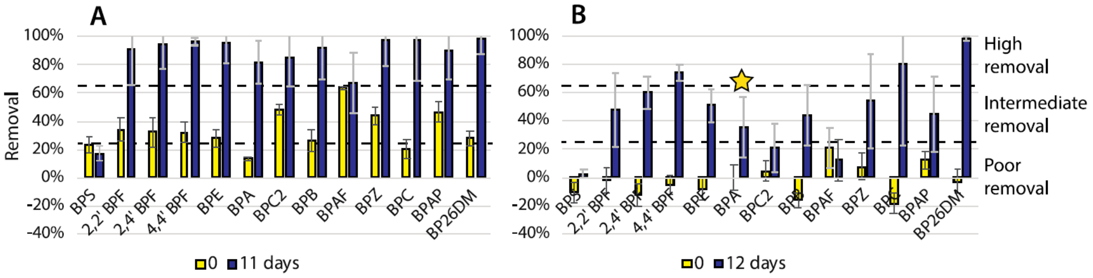

Fig. 1. Removal (%) of BPs in a culture of (A) mixed culture and (B) *C. vulgaris*. Star symbol above BPA indicates that the removal was normalised to the measured concentration at time 0. N = 3, error bars = RSD. The compounds on the horizontal axis appear by increasing log K_{ow} value, BPs removed solely by abiotic means (BPFL, BPBP, BPM) are excluded from this figure and are presented in Fig. S1 and S2. The classification of compounds to "high", "intermediate", and "poor removal" was done for intelligibility purposes only.

Table 2
Rate constants (k [day^{-1}]), half-lives ($t_{1/2}$ [days]), and R-squared values (R^2) for first order kinetic profiles of BPs, EU WL substances, and pharmaceuticals. For compounds that were not removed, were removed in shorter time than 16 h, or did not follow first order kinetics, these parameters are indicated as non-applicable (NA). Compounds with $R^2 < 0.75$ were trialled with second order kinetic model but did not reveal a fit.

Compounds	Mixed culture			*C. vulgaris* culture		
	k (day^{-1})	$t_{1/2}$ (days)	R^2	k (day^{-1})	$t_{1/2}$ (days)	R^2
BPS	NA	NA	NA	NA	NA	NA
2,2′ BPF	0.23	3.04	0.87	0.05	13.13	0.90
2,4′ BPF	0.20	3.48	0.98	0.08	8.49	0.94
4,4′ BPF	0.22	3.10	0.98	0.11	6.14	0.97
BPE	0.22	3.17	0.97	0.06	11.11	0.93
BPA	0.15	4.51	0.91	NA	NA	NA
BPC II	0.12	5.78	0.78	NA	NA	NA
BPB	0.21	3.32	0.97	0.07	9.63	0.94
BPAF	NA	NA	NA	NA	NA	NA
BPZ	0.29	2.41	0.96	0.08	8.75	0.93
BPC	0.32	2.14	0.95	0.18	3.90	0.99
BPAP	0.18	3.75	0.95	0.06	12.03	0.85
BPFL	0.18	3.80	0.78	NA	NA	NA
BP26DM	0.44	1.58	0.86	0.40	1.75	0.93
BPBP	0.27	2.58	0.83	NA	NA	NA
BPM	0.43	1.60	0.91	0.22	3.21	0.88
Imidacloprid	0.08	9.02	0.92	0.20	3.44	0.81
Thiamethoxam	0.15	4.73	0.93	NA	NA	NA
Acetamiprid	0.06	11.55	0.78	NA	NA	NA
Thiacloprid	0.06	11.55	0.91	0.14	5.07	0.83
Erythromycin	0.47	1.49	0.98	0.14	4.98	0.78
Clarithromycin	0.37	1.89	0.95	0.09	7.60	0.92
Estradiol	1.21	0.57	0.77	NA	NA	NA
Estrone	1.19	0.58	0.75	0.12	5.55	0.89
Sulfamethoxazole	0.13	5.35	0.91	NA	NA	NA
Metoprolol	0.23	3.04	0.89	0.50	1.38	0.91
Carbamazepine	NA	NA	NA	NA	NA	NA
Ibuprofen	1.69	0.41	0.96	0.42	1.66	0.99

respectively, in lab-scale photobioreactors inoculated with mixed culture from HRAP. These removals were much lower than the ones obtained in this study, the biggest discrepancy was for BPAF with 67% removed in this study compared with no removal in the cited study. There were several differences in the experimental design of these studies (medium, inoculum, spiking concentration), therefore, a sole reason for these differences could not be depicted.

In the case of *C. vulgaris* culture, Ji et al. (2014), achieved 38% and 25% BPA removals in BBM at much higher initial concentrations (1 and 5 mg L^{-1}, respectively). This is comparable to this study (35%), although with a much lower initial concentration (2 µg L^{-1}). Zhou et al. (2014) investigated the removal of BPA, amongst other CECs, with *C. vulgaris* and other pure algal cultures inoculated into sterilised municipal WW, and obtained 99% removal in 7 days at approximately 20 µg L^{-1}, which is comparable to the removal in mixed culture (82%) but not *C. vulgaris* culture in this study (35%). Ji et al. (2014), Li et al. (2009), reported abiotic removal of BPA of 15–20% in BBM and artificial seawater enriched with f/2-medium, respectively, which is slightly higher than in this study ($\leq$10%). They attributed this result to photodegradation, however, in this study, photodegradation did not play a part in the removal of BPA, as proven by no increased removal observed in light abiotic control (Fig. S1B and C, Fig. S2B and C). Zhang et al. (2009), who investigated *C. vulgaris*-enhanced photodegradation of BPF achieved ~20% removal in 4 h at an initial concentration of 2 mg L^{-1}. In this study, the overall removal of BPF isomers was 48–74% in 12 days; however, no removal was observed within 16 h. Again, their experimental design (spiking concentration, light intensity, presence of ferric ions and humic acid) differed significantly from the present study.

The removal of BPA and other BPs by conventional WW treatment technologies, such as CAS, typically ranges from negative to total removal. Microalgae-based treatment, especially with mixed culture, is therefore, at least as efficient if not better than CAS, although HRT is considerably longer, *e.g.*, days *vs.* hours (Karthikraj and Kannan, 2017; Sun et al., 2017).

3.2. Removal of EU 2018/840 Watch List substances

Substances from the EU WL were variably removed in both, mixed and *C. vulgaris* cultures. In mixed culture, thiamethoxam, erythromycin, clarithromycin, estradiol and estrone were highly removable (100% in 11 days, Fig. 2A). Imidacloprid, acetamiprid, and thiacloprid were intermediately removable (40 – 65%). Imidacloprid, and to a large extent thiamethoxam and estradiol, were abiotically removed (Fig. S3B and C) in mixed culture matrix, whereas erythromycin was removed $\geq$ 50% in abiotic controls in light and dark conditions in 11 days. The remainder was removed by biotic processes. None of the compounds were light sensitive in mixed culture matrix. The removal of the remaining compounds in abiotic control was < 20% (Fig. S3). Biotic removal can, therefore, be fully assigned to clarithromycin, acetamiprid, thiacloprid, and estrone. In some instances, compounds exhibited negative removals (down to –24% for clarithromycin and down to –5% for the rest). This could be due to conjugation/deconjugation phenomena of metabolites. Conjugation is used by organisms to detoxify xenobiotics and it involves the covalent addition of a molecule to a compound (Mir-Tutusaus et al., 2021). In mixed cultures, real WW was used as a medium, meaning that some of these compounds could have been present in a conjugated form, and therefore not targeted by the analytical method used in this study, which later got deconjugated and appeared in concentrations higher than spiked concentrations.

In the *C. vulgaris* culture (Fig. 2B), seven out of eight compounds were highly removable (>65% in 12 days). The only intermediately removable compound was clarithromycin (62%). Abiotic removal was high for erythromycin (>72% in 11 days) in light and dark abiotic controls (Fig. S4B and C) and close to 0% for the rest of the compounds. None of the EU WL substances were found to be light sensitive in the *C. vulgaris* culture (Fig. S4). Biotic removal in *C. vulgaris* culture can, therefore, be fully assigned to all compounds apart from erythromycin, which had an estimated biotic removal between 26% and 28%. Estrone and clarithromycin exhibited negative removals, namely –38% and –6%, respectively. While conjugation/deconjugation phenomena might be the case in real WW, this is highly unlikely in a synthetic medium. Negative removals could have occurred due to previously mentioned reasons. However, estrone is a major transformation product of estradiol, and it is possible that as estradiol got rapidly removed, it transformed to estrone, which, in turn, increased in concentration on day 1 (Kassotaki et al., 2019).

The results show that the cultures were comparable in biotic removals of the EU WL substances, with 82% and 86% average removals observed in mixed and *C. vulgaris* cultures, respectively. No enhancing contribution to removal was observed for bacteria and/or microalgae, apart from neonicotinoids imidacloprid and acetamiprid, which were better removed in *C. vulgaris* culture, while clarithromycin was removed to a higher extent in the mixed culture. The kinetics of the removal of EU WL substances, revealed that most correspond to the first order kinetics with R^2 values ranging from 0.75 to 0.98 (Table 2). The degradation rate constants ranged from 0.06 to 1.21 day^{-1} and from 0.09 to 0.20, corresponding to the half-life ($t_{1/2}$) values between 0.57 and 11.55 days, and between 3.44 and 7.60 days in mixed culture and *C. vulgaris* culture, respectively. Kinetic fitting was not possible for estradiol in *C. vulgaris* culture, which was removed within 16 h and thus providing only two time points with concentration above 0. The concentration of thiamethoxam and acetamiprid in *C. vulgaris* culture remained more or less stable until 120 h (5 days), and then decreased rapidly by the next sampling at 168 h (7 days). This removal dynamics did not fit either first or second order kinetics.

Only a handful of studies address algal removal of substances from the EU 2018/840 WL. García-Galán et al. (2020b), for example,

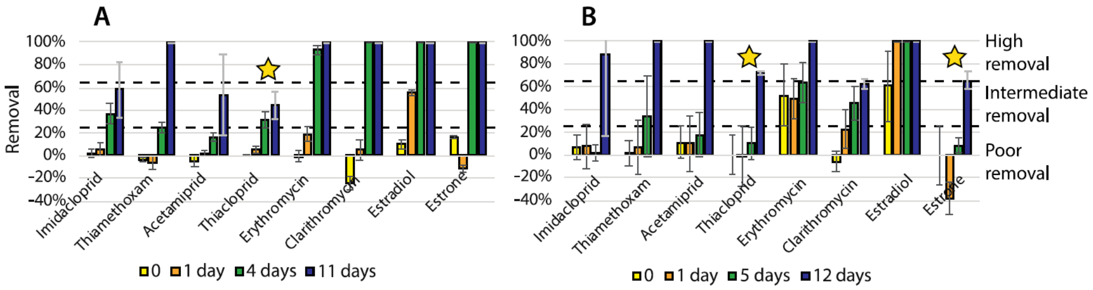

Fig. 2. Removal (%) of EU WL substances in a culture of (A) mixed culture and (B) *C. vulgaris*. Star symbols above thiacloprid indicate that the removal was normalised to the measured concentration at time 0. N = 3, error bars = RSD. The compounds on the horizontal axis appear by increasing log K_{ow} value.

investigated the removal of imidacloprid, amongst others, in an agricultural runoff with mixed culture in a pilot-scale photobioreactor (PBR), and observed negative removals (−240% in average). In this study, imidacloprid was removed 59%, 88% and 90–100% in mixed culture, *C. vulgaris* culture, and abiotic controls in mixed culture matrix, respectively. In abiotic controls in *C. vulgaris* matrix, the removals were negative (down to −15%), however much smaller than in the García-Galán et al. (2020b) study. This difference could be due to imidacloprid spiked in the reaction mixes in its pure form, whereas in the agricultural runoff, it could have occurred in a conjugated form and became unconjugated in the PBR (García-Galán et al., 2020b). Thiamethoxam, acetamiprid, and thiacloprid were not detected in the agricultural runoff, analysed by García-Galán et al. (2020b), or reported in any other study addressing algae-based removal. A TP of thiamethoxam – clothianidin (Hladik et al., 2014), was followed but not detected (LOD = 0.07 ng L^{-1}) in this study.

Hom-Diaz et al. (2017) were able to remove 85% of erythromycin from toilet WW in a pilot-scale PBR with mixed culture, while Jaén-Gil et al. (2018) only achieved 33% removal from toilet WW using pure cultures of *Chlorella sorokiniana* and *Chlamydomonas reinhardtii* in laboratory-scale studies. The results of this study agree with Hom-Diaz et al. (2017), *i.e.*, a high removal (100%) in both, mixed and *C. vulgaris* cultures. However, abiotic removal of erythromycin was relatively high (50% and 74%). confirming macrolide antibiotics instability in aqueous solution (Hassanzadeh et al., 2007; Jaén-Gil et al., 2018).

Clarithromycin was poorly removed from toilet WW with *C. sorokiniana* and *C. reinhardtii* cultures (16%) as reported by Jaén-Gil et al. (2018). However, Kiki et al. (2020), Zhou et al. (2014), observed 76% and 100% removal of clarithromycin from synthetic WW and real sterilised WW with *C. vulgaris* cultures, respectively, in accordance with this study (100% and 62% removals in mixed and *C. vulgaris* cultures, respectively). Kiki et al. (2020) also reported significant photodegradation (24–27%) of clarithromycin in 40 days in synthetic WW. In abiotic controls in mixed culture matrix, the removals were initially negative (down to −74%), however, in abiotic light control, clarithromycin seemed to be removed by day 11, whereas in dark abiotic control it remained negative, indicating that photodegradation might have taken place in this matrix (Figs. S3 and S4).

Zhou et al. (2014) reported 87% removal of estrone from real sterilised WW with *C. vulgaris*, in accordance to the observations reported herein (100% and 66% in mixed and *C. vulgaris* cultures, respectively). Vassalle et al. (2020), achieved only 55% removal of estrone from raw WW in their pilot-scale HRAP with mixed culture. This was increased to 95% by initially treating the influent in an UASB reactor. Estradiol was even more poorly removed in HRAP (7%) but the removal efficiency increased to 91% when initially treated in an UASB (Vassalle et al., 2020). In this study, estradiol was quickly removed, *i.e.*, in mixed culture 81% was removed in 16 h, and 100% in 3.9 days, whereas in *C. vulgaris* culture 99% was removed in less than an hour. However, abiotic removal was high in the mixed culture matrix (*i.e.*, 97–98% removed in 11 days), in contrast to *C. vulgaris* matrix, with no abiotic removal. High removal in abiotic controls in mixed culture matrix, suggests that estradiol is removed abiotically and adsorbed onto biomass and suspended matter, as confirmed by its log K_{ow} (3.75). This could be why it was almost completely removed in autoclaved WW containing suspended matter, but not efficiently removed in the *C. vulgaris* matrix (BBM with no suspended matter). The effect of log K_{ow} was observed by García-Galán et al. (2020b) and Kiki et al. (2020) but not in Vassalle et al. (2020). However, log K_{ow} is only one possible factor that affects compound removal.

Removals of EU 2018/840 WL substances in CAS WWTP vary significantly. For example, neonicotinoids' removal ranges from 3.3% for imidacloprid to 23.2% for acetamiprid (Iancu and Radu, 2018; Sadaria et al., 2016), while estrone and estradiol removals range from 55% to 87%, with higher removals observed in older sludge (Ifelebuegu, 2011). The removal of erythromycin and clarithromycin varies significantly from − 189.9% for clarithromycin to 97.4% for erythromycin (Zhang et al., 2017). These data suggest that microalgae-based treatment is more effective than CAS for most EU WL substances.

3.3. *Removal of pharmaceuticals*

In this study, pharmaceutical compounds removal efficiencies were comparable in mixed and *C. vulgaris* cultures. Sulfamethoxazole, metoprolol, and ibuprofen were highly removable (>78%) in mixed culture (Fig. 3A), however, carbamazepine was only poorly removed (12%, Fig. 3A). These compounds were partly removed due to abiotic processes but with no apparent correlation to log K_{ow} (Fig. S5). In the abiotic controls, removal ranged from 13% to 40% (Fig. S5B and C). Metoprolol was removed the least under both, light (13%) and dark conditions (14%). Its complete removal (100%) in the mixed culture can, therefore, be attributed to biotic processes (similarly for sulfamethoxazole and ibuprofen), whereas for carbamazepine almost entirely to abiotic removal (some TPs occurred in low concentrations, Fig. S7). None of the compounds were found to be light-sensitive in the mixed culture matrix (Fig. S5).

In the *C. vulgaris* culture, sulfamethoxazole, metoprolol, and ibuprofen were highly removable, > 66% (Fig. 3B). Even though similar final removal rates were obtained in this study, faster degradation was observed for these compounds in *C. vulgaris* culture than in the mixed culture. Carbamazepine was intermediately removed under these conditions (34% in 12 days) and, to a greater extent, than in mixed culture (12% in 11 days). In the abiotic controls, removal ranged from 10% to 45% in 11 days (Fig. S6B and C), again without any apparent correlation to log K_{ow}. Removal of sulfamethoxazole, metoprolol, and ibuprofen is partly attributable to biotic removal, more particularly biotransformation, indicated by high removal by *C. vulgaris* (66%, 100%, and 100%, respectively), low removals in abiotic conditions (38%, 10%, and 34%,

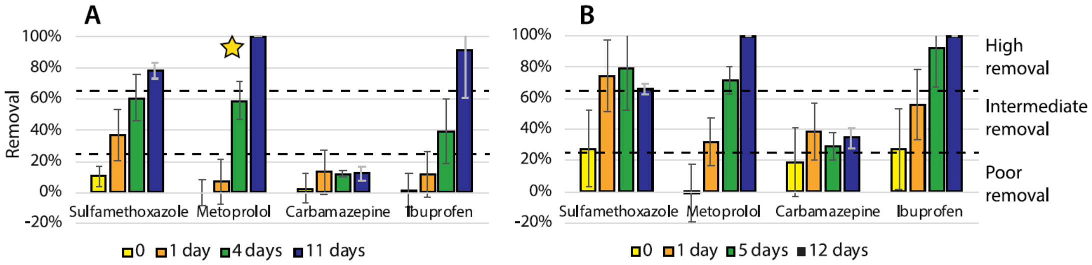

Fig. 3. Removal (%) of pharmaceuticals in a culture of (A) mixed culture and (B) *C. vulgaris*. Star symbol above metoprolol indicates that the removal was normalised to the measured concentration at time 0. N = 3, error bars = RSD. The compounds on the horizontal axis appear by increasing log K_{ow} value.

respectively), and the presence of their TPs (Fig. S7). The removal of carbamazepine was largely abiotic (Fig. S6); however, some TPs (10,11-epoxycarbamazepine and 2-hydroxycarbamazepine) were detected in *C. vulgaris* culture in low concentrations (up to 0.17 μg L^{-1}).

The removal efficiencies and mode of removal (abiotic or biotic) were comparable in both cultures, with average removals of 71% and 75% in mixed and *C. vulgaris* cultures, respectively, indicating no enhancing contribution of bacteria and/or microalgae in terms of achieved removal. However, sulfamethoxazole, metoprolol, and ibuprofen were removed more quickly by *C. vulgaris*, indicating a potential different biodegradation route in *C. vulgaris*. The kinetics of the removal of pharmaceuticals, revealed that most correspond to the first order kinetics with R^2 values ranging from 0.89 to 0.99 (Table 2). The degradation rate constants ranged from 0.13 to 1.69 day^{-1} and from 0.42 to 0.50, corresponding to the half-life ($t_{1/2}$) values between 0.41 and 5.53 days, and between 1.38 and 1.66 days in mixed culture and *C. vulgaris* culture, respectively. Kinetic fitting was not possible for carbamazepine, which exhibited poor removal in both cultures, and for sulfamethoxazole in *C. vulgaris* culture, where its concentrations remained relatively stable beyond 16 h.

Selected TPs of pharmaceutical compounds were studied in both cultures. The TPs detected at highest concentration were metoprolol acid and ibuprofen carboxylic acid (Fig. 4). Other TPs are shown in Fig. S7. In both cultures, metoprolol was removed entirely within 168 h (7 days). However, a higher removal rate was observed in *C. vulgaris* culture with only 6.3 μg L^{-1} remaining after 40 h (1.7 days). whereas

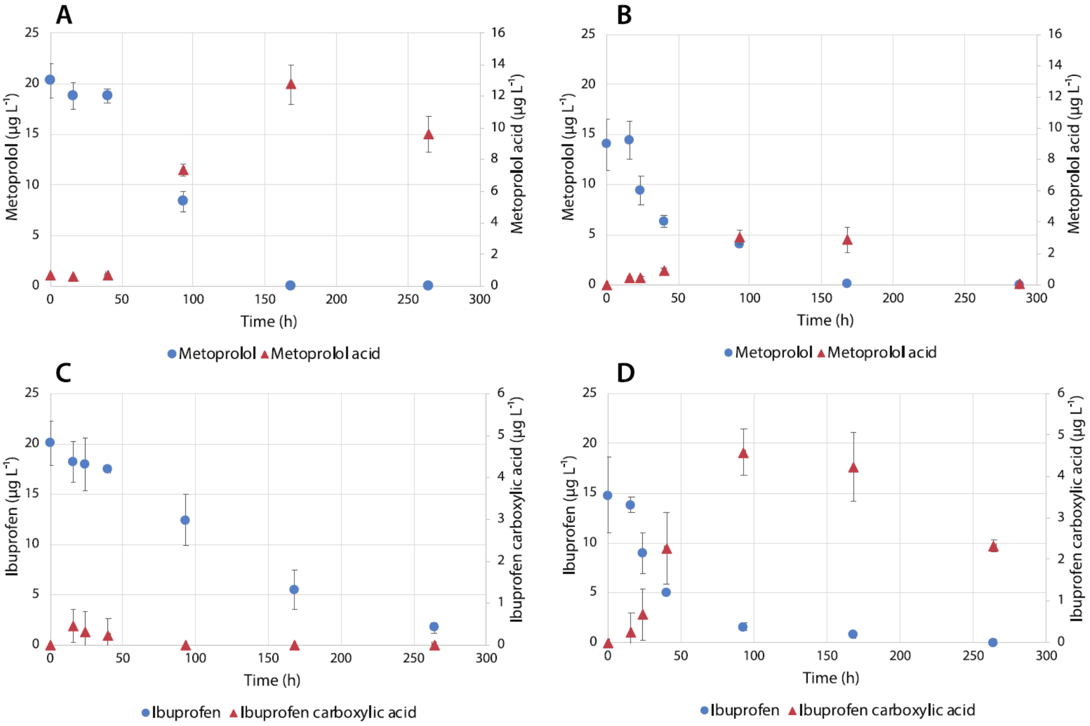

Fig. 4. Change in concentrations of parent compounds (PC) and transformation products (TP) of selected pharmaceuticals over time in a culture of mixed culture and *C. vulgaris*. Metoprolol (PC) and metoprolol acid (TP) in (A) mixed and (B) C. vulgaris culture. Ibuprofen (PC) and ibuprofen carboxylic acid (TP) in (C) mixed and (D) *C. vulgaris* culture. N = 3, error bars = SD.

18.7 μg L^{-1} remained in the mixed culture. Interestingly, the occurrence of metoprolol acid was higher in mixed culture (<12.7 μg L^{-1}) compared to *C. vulgaris* culture (2.9 μg L^{-1}) (Fig. 4A and B). This clearly indicates that metoprolol was mainly biotransformed to metoprolol acid in the mixed culture and underwent further biodegradation (and/or different biotransformation route) in *C. vulgaris* culture. None of the other analysed TPs of metoprolol (O-desmethyl metoprolol and α-hydroxymetoprolol) were detected (Fig. S7), however, other non-analysed TPs are also possible (Jaén-Gil et al., 2021; Rubirola et al., 2014). In both cultures, the level of metoprolol acid decreased after 168 h (7 days), possibly indicating further slower biodegradation. Nevertheless, in *C. vulgaris* culture metoprolol acid, reported for the first time in this study, was removed entirely but remained high in mixed culture at the end of the experiment (at almost 10 μg L^{-1}), which is in line with reported persistence of this TP in CAS (Rubirola et al., 2014).

Ibuprofen was more rapidly removed by *C. vulgaris* than mixed culture (Fig. 4C and D). In this case, the TP ibuprofen carboxylic acid occurred at a higher concentration in *C. vulgaris* culture compared to mixed culture, indicating an efficient biotransformation of ibuprofen in *C. vulgaris* culture. The peak concentration (4.6 μg L^{-1}) in *C. vulgaris* culture was at 93 h (3.9 days), whereas the peak (0.5 μg L^{-1}) in mixed culture occurred at 16 h (0.7 days). After 168 h (7 days), ibuprofen carboxylic acid started to decrease in *C. vulgaris* culture, possibly indicating further biodegradation and/or biotransformation. 2-hydroxy-ibuprofen was also observed (Fig. S7) in the mixed culture (peak concentration: 2.9 μg L^{-1} after 4 days), whereas it was present in lower concentrations in *C. vulgaris*, clearly indicating different biotransformation routes in the two cultures. 1-Hydroxy-ibuprofen TP was also investigated but was not detected in either culture (data not shown).

Additional TPs of all investigated pharmaceuticals were detected in both cultures, occasionally up to 2.9 μg L^{-1} (Fig. S7). Biotransformation can, therefore, be confirmed for all investigated pharmaceuticals, even for the poorly removed carbamazepine, however, to a smaller extent. In the abiotic controls, the concentrations of the TPs were lower than in the corresponding matrix (data not shown).

Algae-assisted removal of sulfamethoxazole was investigated by García-Galán et al. (2020a) and Kiki et al. (2020). No bioaccumulation, low biosorption (1–4%) and photodegradation (5–8%) of sulfamethoxazole by *C. vulgaris* were observed, with 74% being removed after 40 days from spiked synthetic WW. Kiki et al. (2020) attributed this to biodegradation and is comparable to that obtained in this study (66–78%). In the present study, abiotic removal was more considerable (≤40%). In García-Galán et al. (2020a) study of a pilot-scale HRAP treating municipal WW, 51% and 86% of sulfamethoxazole was removed with and without a primary settler, respectively. In the present study, the TPs desamino sulfamethoxazole and N-acetyl sulfamethoxazole were observed in the *C. vulgaris* culture (Fig. S7B). N-acetyl sulfamethoxazole was determined in the mixed culture (Fig. S7A). similar to that observed by García-Galán et al. (2020a). These results suggest that biotic removal by biodegradation/bioassimilation is an important route by which sulfamethoxazole is eliminated.

The reported removal of metoprolol in municipal WW in a pilot scale HRAP study was poor (<36%) and highly variable (García-Galán et al., 2020a). Metoprolol acid was detected in all cases, although its occurrence could not be attributed to biotransformation since metoprolol was hardly or not present in the influent. Metoprolol acid is also a TP of atenolol (another β-blocking agent), which could be present in WW (Radjenović et al., 2008). In this study, metoprolol was wholly removed in both cultures (Fig. 3, S5, S6) and the presence of metoprolol acid was probably a result of biotransformation by bacteria and/or algae (Fig. 4). In aerobic activated sludge batch experiments, metoprolol was removed within 4 days at an initial concentration of 10 μg L^{-1}, while at higher concentration the removal efficiency decreased (Rubirola et al., 2014). This contrasts with removal efficiencies observed in WWTPs, where it is speculated that removal is lower due to short HRTs, *i.e.*, 6–8 h (Maurer et al., 2007). The authors report that metoprolol was not eliminated during conventional WW treatment, but that around 54% was removed using a membrane bioreactor (Rubirola et al., 2014).

In the case of carbamazepine, García-Galán et al. (2020a) found no removal from municipal WW in neither of their pilot-scale HRAPs, with and without a primary settler. Additionally, carbamazepine proved recalcitrant in lab-scale studies involving mixed culture comprising *Chlorella* sp., *Scenedesmus* sp., and bacteria (Matamoros et al., 2016). Its recalcitrance has also been reported in CAS (Hai et al., 2018). Matamoros et al. (2015) achieved 62% removal in a pilot-scale HRAP (HRT = 8 days) during the warm season, and 34% during the cold season. In the present study, carbamazepine was poorly to intermediately removed mainly *via* abiotic processes, although two TPs (10.11 epoxycarbamazepine and 2-hydroxy-carbamazepine) were detected in both cultures (< 0.2 μg L^{-1}).

In the case of ibuprofen in pilot scale HRAPs, García-Galán et al. (2020a) removed 79% from municipal WW, whereas Matamoros et al. (2015) removed between 34% and 99% during cold and warm seasons from urban WW, respectively. Hom-Diaz et al. (2017) removed 98.5% from a toilet WW treated in a pilot scale photobioreactor. As regards to CAS, Ferrando-Climent et al. (2012) reported the complete removal of ibuprofen. These results are in substantial agreement with this study with removals of 91% and 100% in mixed and *C. vulgaris* cultures, respectively. The exception is a study by Zhou et al. (2014). where the authors report negative removals by freshwater microalgae, including *C. vulgaris,* and CAS lab-scale treatments. Subashchandrabose et al. (2011) concluded that microalgae could increase ibuprofen removal by cellular uptake or release of exudates that aid the biodegradation process. Biodegradation was also the primary removal mechanism in a microalgal bioreactor for treating domestic WW and conventional treatment processes (*e.g.*, CAS and membrane bioreactors; Hom-Diaz et al. (2017); and references therein). In two pilot-scale HRAP studies, the identified TPs included 1-hydroxy ibuprofen, 2-hydroxy ibuprofen, and ibuprofen carboxylic acid (García-Galán et al., 2020a; Matamoros et al., 2016). Matamoros et al. (2016), in particular, found a higher amount of hydroxy ibuprofen over ibuprofen carboxylic acid and predicted that biodegradation occurs under prevailing aerobic conditions. 1-hydroxy ibuprofen was never detected in this study. However, 2-hydroxy ibuprofen and ibuprofen carboxylic acid were identified, with ibuprofen carboxylic acid prevailing in the *C. vulgaris* culture (Fig. 4). Based on the results, presented herein and data from published studies, microalgae-based treatment has been shown to be at least, if not more, effective in the removal of pharmaceuticals and the here evaluated TPs, compared to CAS.

3.4. Results summary

The kinetic profiles of compounds revealed that most removals followed first order kinetics and yielded a linear correlation of natural logarithms of compounds' concentrations *versus* time with $R^2 > 0.75$. R^2 values, first order rate constants and half-lives of compounds are reported in Table 2. For compounds that were not or poorly removed (BPS, BPAF, carbamazepine) the fit to kinetic model was redundant, whereas for compounds that were removed in less than 16 h and thus provided less than three points of temporal variation (*e.g.*, estradiol in *C. vulgaris* culture) the fit was not possible. Some compounds, varied in concentration through time, possibly due to adsorption and desorption phenomena, or exhibited stable concentrations with being removed only towards the end of the experiment, and did not fit first or second order kinetics. In summary, the removal of most compounds followed first order kinetics. Half-lives confirmed that BPs were more quickly removed in mixed culture as compared to *C. vulgaris* culture. Neonicotinoids (imidacloprid, thiacloprid) had shorter half-lives in *C. vulgaris* culture, whereas antibiotics (erythromycin, clarithromycin) had shorter half-lives in mixed culture. For pharmaceuticals, half-lives were variable between two cultures, with shorter half-life for metoprolol in *C. vulgaris* culture, and shorter half-life for ibuprofen in mixed culture.

All compounds, classified by degree of removal (poor, intermediate and high) and mode of removal (biotic, partial biotic, abiotic or no removal) are gathered in Table 3. For most compounds, there is no available literature on the removal routes in algae-based treatment of these CECs or the corresponding roles of microalgae and/or bacteria in the removal. Amongst the investigated CECs, the enhancing effect of microalgae in mixed culture has so far only been reported in the removal of ibuprofen, with 45% more removed in mixed culture *versus* bacteria-only (Matamoros et al., 2016). In summary, mixed culture was removing BPs to a larger extent as compared to *C. vulgaris* culture. Fifteen BPs were better removed in these conditions, whereas only six were highly removed in *C. vulgaris* culture. Additionally, eleven BPs were removed solely or partially due to biotic processes in mixed culture, compared to seven in *C. vulgaris* culture. Both cultures, on the other hand, were noticeably similar for EU WL substances and pharmaceuticals, by both, the degree and the mode of removal. However, *C. vulgaris* was faster in removing some compounds, namely imidacloprid, thiamethoxam, acetamiprid, thiacloprid, estradiol, sulfamethoxazole, carbamazepine, and ibuprofen, indicating possible different routes of biotransformation and/or biodegradation.

Table 3
Degree (poor, intermediate, high) and mode (biotic/abiotic) of removal for all investigated compounds in mixed and *C. vulgaris* cultures. Each row represents a separate group of compounds (from the top of each degree/ mode of removal: BPs, EU WL substances, pharmaceuticals).

	Mixed culture	***C. vulgaris* culture**
Poorly removed (0 – 25%)	BPS	BPS, BPC2, BPAF
	/	/
	carbamazepine	/
Intermediately removed (25 – 65%)	/	2,2′ BPF, 2,4′ BPF, BPE, BPA, BPB, BPZ, BPAP
	imidacloprid, acetamiprid, thiacloprid	clarithromycin
	/	carbamazepine
Highly removed (65 – 100%)	2,2′ BPF, 2,4′ BPF, 4,4′ BPF, BPE, BPA, BPC2, BPB, BPAF, BPZ, BPC, BPAP, BPFL, BP26DM, BPBP, BPM	4,4′ BPF, BPC, BPFL, BP26DM, BPBP, BPM
	thiamethoxam, erythromycin, clarithromycin, estradiol, estrone	imidacloprid, thiamethoxam, acetamiprid, thiacloprid, erythromycin, clarithromycin, estradiol, estrone
	sulfamethoxazole, metoprolol, ibuprofen	sulfamethoxazole, metoprolol, ibuprofen
Biotic removal	BPS, BPA, BPB	/
	clarithromycin, acetamiprid, thiacloprid, estrone	imidacloprid, thiamethoxam, acetamiprid, thiacloprid, clarithromycin, estradiol, estrone
	/	/
Partial biotic removal	2,2′ BPF, 2,4′ BPF, 4,4′ BPF, BPE, BPC2, BPZ, BPC, BPAP	2,2′ BPF, 2,4′ BPF, 4,4′ BPF, BPE, BPA, BPZ, BPC
	erythromycin	erythromycin
	metoprolol, sulfamethoxazole, ibuprofen, carbamazepine	sulfamethoxazole, metoprolol, ibuprofen, carbamazepine
Abiotic removal	BPAF, BPFL, BP26DM, BPBP, BPM	BPFL, BPBP, BPM, BP26DM, BPAF, BPAP, BPC2
	imidacloprid, thiamethoxam, estradiol	/
	/	/
No removal	/	BPS, BPB
	/	/
	/	/

4. Conclusions

Both, *C. vulgaris* and a mixed microalgal-bacterial batch cultures, could remove most of considered 28 compounds of emerging concern at environmentally relevant concentration, even outcompeting CAS treatment in some cases. Biotic and abiotic contribution to total removal was evaluated for all the compounds. Biotic removal was further confirmed *via* detection of biotransformation products for selected compounds (*e.g.*, metoprolol, ibuprofen, sulfamethoxazole, estradiol).

Bisphenols were removed to a higher degree in mixed culture than in *C. vulgaris* culture, with log K_{ow} playing an important role in their abiotic removal (higher log K_{ow}, higher removal). Conversely, the removal of pharmaceuticals and EU Watch List substances was similar in both cultures, in the degree and the mode of removal, even though *C. vulgaris* culture was faster in removing some compounds (*e.g.*, imidacloprid, acetamiprid, sulfamethoxazole, ibuprofen) and showed different transformation pathways, suggesting that increasing algae concentration in full scale algae-based treatment systems (*e.g.*, HRAP) can improve their removal rate and efficiency. The results of this study also suggest that environmentally relevant spiked concentrations (1–20 $\mu g\ L^{-1}$) did not inhibit the growth of mixed and *C. vulgaris* cultures.

Microalgae-based treatment can be an effective alternative method for removing several compounds of emerging concern. However, for certain compounds that are more recalcitrant (*e.g.*, BPS, BPB, carbamazepine), additional treatment solutions should be sought. Further research is needed to evaluate removal pathways, not addressed in this study, such as bioaccumulation, biosorption, and/or mineralisation of individual CECs in microalgae-based systems. Additionally, the corresponding roles of microalgae, bacteria, and other present microorganisms in consortia, formed in open systems, such as HRAP should be further evaluated. Moreover, the environmental safety of algae-treated effluent as well as biomass for potential further use (*e.g.*, biofertilizer, bioenergy or other value-added products) should be explored.

CRediT authorship contribution statement

Franja Prosenc: Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization; **Justyna Piechocka**: Investigation, Writing - review & editing; **David Škufca**: Investigation, Writing - original draft, Writing - review & editing; **Ester Heath**: Resources, Writing - review & editing, Funding Acquisition; **Tjaša Griessler Bulc**: Resources, Writing - review & editing, Funding Acquisition; **Darja Istenič**: Conceptualization, Resources, Writing - review & editing; **Gianluigi Buttiglieri**: Conceptualization, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

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.jhazmat.2021.126284.

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3.4 Phycoremediation of municipal wastewater: Removal of nutrients and contaminants of emerging concern

The paper “Phycoremediation of municipal wastewater: Removal of nutrients and contaminants of emerging concern” by D. Škufca, A. Kovačič, F. Prosenc, T. Griessler Bulc, D. Heath and E. Heath was published in the journal Science of The Total Environment (IF 2021: 7.963) in August 2021 (doi: 10.1016/j.scitotenv.2021.146949). Method development and optimisation, validation and sample preparation and analysis were shared between the co-authors A. Kovačič and F. Prosenc and myself. I was solely responsible for sampling, data analysis and drafting the manuscript.

This work’s goal was to study nutrient and CEC removal efficiency in a pilot-scale outdoor HRAP. HRAP are an alternative to conventional wastewater treatment that could contribute to the circular economy by valorising reclaimed water and algal biomass. Average removal of total organic carbon, total nitrogen, NH_4-N, total phosphorus and PO_4-P was 67%, 76%, 87%, 41% and 46%, respectively and is comparable to literature data. The average removals of the compounds were 60% for naproxen, 51% for ibuprofen, 92% for methylparaben, 76% for 2,4-dihydroxybenzophenone, and 80% for oxybenzone. These removals were comparable to reported ranges for removals in conventional WWTP and other pilot-scale HRAP. Differences in removals between the studied CEC partly result from their different physicochemical properties, which may influence biodegradation and uptake processes, the latter being lower or non-existent when ionisable compounds are present in anion form. The results show how naproxen and ibuprofen have lower distribution coefficients (log D) at the average pH value in the HRAP due to them having the lowest predicted pK_a values compared to the other included CEC.

This work is significant because it contributes to the knowledge of basic operating parameters, nutrients and CEC levels were measured, and their removals were determined in a pilot-scale HRAP treating municipal wastewater under realistic conditions. These results also suggest that more acidic compounds are removed to a lesser extent in the slightly alkaline conditions in the HRAP. While treating wastewater in HRAP with subsequent biomass valorisation agrees with the principles of closing material cycles, incomplete removal of recalcitrant CEC means that further optimisation and possibly additional treatment are needed, e.g., pre- or post-treatment with efficient biomass harvesting.

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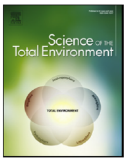

Short Communication

Phycoremediation of municipal wastewater: Removal of nutrients and contaminants of emerging concern

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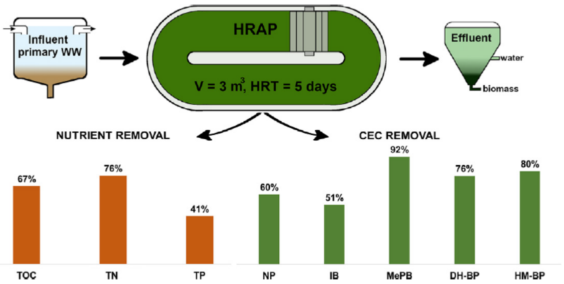
HIGHLIGHTS

- A pilot-scale high-rate algal pond was used to treat municipal wastewater.
- Nutrients removal was 76% for total nitrogen and 41% for total phosphorus.
- CEC removal ranged from 51% to 92%.
- CEC treatment efficiency comparable to activated sludge

GRAPHICAL ABSTRACT



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ABSTRACT

High-rate algal ponds (HRAP) are an alternative to conventional wastewater treatment that could contribute to the circular economy by valorising reclaimed water and algal biomass. This short communication presents the removal of nutrients and contaminants of emerging concern (CEC) in a pilot-scale HRAP treating primary municipal wastewater. Average removal of total organic carbon, total nitrogen, NH_4-N, total phosphorus and PO_4-P, was 67%, 76%, 87%, 41% and 46%, respectively. In addition, 12 CECs were detected in the influent and five in the effluent. Removals were as follows: naproxen 60%, ibuprofen 51%, methylparaben 92%, 2,4-dihydroxybenzophenone 76% and oxybenzone 80%, and are comparable with literature data for conventional wastewater treatment and other HRAPs. In conclusion, studies of nature-based solutions such as HRAPs show encouraging results but need further research to understand the risks and requirements of safe wastewater and biomass reuse.

1. Introduction

Contaminants of emerging concern (CEC) are "*naturally occurring, manufactured or manmade chemicals or materials found in the environment, whose toxicity or persistence are likely to affect the metabolism of a living being significantly*" and include, for example, pharmaceuticals, personal care products, pesticides, plasticisers, flame retardants, and hormones (Tolboom et al., 2019). Different types of wastewater (e.g. runoff, municipal, and industrial) represent the main influx of CEC to the environment due to inadequate removal during wastewater treatment (Norvill et al., 2016; Sutherland and Ralph, 2019; Tolboom et al., 2019). Although most CEC are biodegradable, some are pseudo-persistent due to their continuous output (Villar-Navarro et al., 2018).

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High rate algal ponds (HRAP) are being investigated as a potential alternative to conventional biological wastewater treatment technology (Craggs et al., 2012). When compared to activated sludge reactors, HRAPs have long hydraulic retention times (HRT: 3–8 h vs 1–14 days, respectively) and facilitate photodegradation due to their shallow depth (0.1–0.5 m) (Norvill et al., 2016) and large surface area, i.e., 1 ha or more in full-size HRAP systems (Craggs et al., 2012; Sutherland et al., 2020). They also do not require aeration since algae provide the oxygen and organic acids needed by the bacteria, which provide CO_2 and nutrients for algae (Norvill et al., 2016).

Although most resources (e.g., water, energy, food, and nutrients) are exploited in a linear way (extract – use – dispose), the reuse of resources by recycling wastewater is in line with circular economy principles. One of the main advantages of utilising micro-algae-based solutions is nutrient- and energy-rich algal biomass production. The latter may be exploited, most notably as a feedstock, i.e., for polymer, energy (e.g., biogas or biodiesel) and fertiliser production (Brennan and Owende, 2010; Park et al., 2011; Rahman et al., 2015, 2017). Also, the construction and operational costs of HRAPs for secondary wastewater treatment are one quarter to one-third that of conventional activated sludge treatment (Craggs et al., 2014). Importantly, the valorisation of biomass and reclaimed wastewater is in line with the principles of the EU's Circular Economy Action Plan (European Commission, 2020). However, unexpected contamination with hazardous substances may arise in recycled resources, materials and goods if risks are not identified and remedied.

Efforts have already begun to characterise the removal of various types of CEC using algal treatment technologies. However, while studies on laboratory-scale reactors are common, those involving pilot-scale HRAPs with only five published studies are rare (Tolboom et al., 2019). Matamoros et al. (2015), who studied the removal of 28 CEC in two HRAPs fed with urban wastewater ($V = 0.5$ m^3 and HRT of 4 and 8 days), reported on a wide range of CEC removals, with higher removals in the warm season and on the effect of HRT on removal only during the cold season. Similarly, Gentili and Fick (2017), who investigated the removal of 79 pharmaceuticals (HRAP: $V = 0.65$ m^3 and a 7-day HRT), reported a correlation between light intensity inside the culture and CEC removal, on average varying from negative removals (e.g. alprazolam, biperiden, and cyproheptadine) to >90% (e.g. atenolol, bisoprolol, and carbamazepine). In another study, Villar-Navarro et al. (2018) compared the removal of 81 pharmaceuticals during conventional activated sludge treatment to their removal in a HRAP ($V = 9.6$ m^3 and an HRT of 6 days). The authors found that although overall removal was similar, the composition of individual compounds in the effluent varied, indicating different removal mechanisms in the two systems. García-Galán et al. (2020), in their study of the removal of 12 pharmaceuticals and 26 of their metabolites (HRAP: $V = 0.5$ m^3 and HRT of 4.5 days), found that initial primary treatment did not affect removal, which on average ranged from negative removals (e.g. carbamazepine, salicylic acid) to >90% (e.g. metronidazole, acetaminophen, and norfluoxetine). They found that algae removed certain compounds, e.g., venlafaxine and metronidazole, to a greater extent than activated sludge and that there was no difference when it came to removing recalcitrant compounds like carbamazepine and metoprolol. More recently, Vassalle et al. (2020) studied the removal of 11 CEC in a system combining an up-flow anaerobic sludge blanket reactor with a HRAP ($V = 0.2$ m^3 and HRT of 8 days). The removal rates ranged from 65% (paracetamol) to 95% (estrone), with lower removal for gemfibrozil (39%) and bisphenol A (43%).

A comparison of the data reveals similar removal trends despite the variations in HRAP design and HRT, study duration, and the target CEC. For example, HRT ranged from 4 to 8 days, and HRAP volume ranged from 0.2 m^3 to 9.6 m^3. The average removal of naproxen (NA) and ibuprofen (IB) ranged from 54% and 46% (Vassalle et al., 2020) to 83% and 99% (Matamoros et al., 2015), respectively, while the removal for carbamazepine, as an example of a recalcitrant compound, ranged from −24% (García-Galán et al., 2020) to 62% (Matamoros et al., 2015). It is also apparent that HRAPs treating wastewater are affected by season, light intensity, pre-treatment and other factors, although more data is needed.

Given the few studies on this topic, further investigation is needed to fill the significant knowledge gap regarding CEC removal in HRAP systems (Tolboom et al., 2019). This short communication presents preliminary findings on CECs and nutrient removal from wastewater in a pilot-scale HRAP treating primary municipal wastewater. This study forms part of a much broader project studying nature-based solutions for wastewater treatment and the valorisation of treatment by-products.

2. Materials and methods

Full details about reagents and standards used are in the Supplementary information (SI).

2.1. Sampling and setup

Experiments were performed in an HRAP pilot plant (Fig. 1) situated at a municipal Wastewater Treatment Plant (WWTP) in Ajdovščina, Slovenia. The HRAP has a volume of 3000 L and a surface area of 12.7 m^2. Mixing is accomplished via a paddlewheel, and the pond is fed with wastewater from the primary settler. The microbial community, comprised of inoculated *Chlorella vulgaris* and native microalgae and bacteria, was established over one year of operation. The HRAP was operated in a semi-continuous manner, i.e., every 12 h, 10% of its volume was transferred to a secondary algae settler. An equal amount of fresh influent was then added to the pond. The HRT was five days.

Grab samples (1000 mL) of influent and effluent were collected at 7:00 and 19:00 each day, between 29/7/2019 and 2/8/2019. The pH, oxygen saturation (O_2%), temperature (T), and electrical conductivity (EC) were measured on-site using a multimeter (HQ30D, Hach, US). Total suspended solids (TSS) were determined according to the Standard Method 2540 (Rice et al., 2017). Daily biomass productivity was calculated from the TSS difference of the influent and effluent and normalised to the surface area of the HRAP. After sampling, 300 L of wastewater was transferred from the HRAP to the settler and replaced with 300 L of influent. The cycle was repeated every 12 h for 5 days, resulting in 10 samples of influent and effluent. All samples were frozen at −18 °C until analysis.

2.2. Analysis

Chlorophyll *a* (Chl *a*) levels were measured according to an adapted protocol from Segovia Bifarini et al. (2020). Briefly, three parallels of 10 mL samples were centrifuged, the supernatant was removed, and the pellets stored at −18 °C. After thawing, 8 mL of ethanol (95%) was added to each sample, followed by ultrasound-assisted extraction for 15 min. Samples were then incubated in the dark at 60 °C for 60 min, centrifuged at 8000 RPM for 10 min and absorbance measured at 649 nm, 664 nm and 750 nm. Chl *a* concentration was calculated using the equation for 95% ethanol from Lichtenthaler and Buschmann (2001). Nutrients were measured in the influent and effluent using Nanocolor cuvette tests (Macherey-Nagel, Germany) in filtered samples, namely total organic carbon (TOC), total nitrogen (TN), nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), ammoniacal nitrogen (NH_4-N), total phosphorus (TP) and phosphate phosphorus (PO_4-P).

Target compounds were determined following the methods of Česen et al. (2018) and Kovačič et al. (2020). Briefly, each sample (300 mL) was consecutively filtered through GF-2 (Macherey-Nagel, Germany) and 1.2 μm cellulose nitrate filters (Sartorius, Germany), acidified with concentrated HCl and loaded onto an Oasis HLB Prime cartridge (3 cm^3, 60 mg, Waters, USA). The analysis was performed using gas chromatography with mass selective detection (GC–MS, 7890B/5977A, Agilent, US),

Fig. 1. On-site photograph of the HRAP (photo: Darja Istenič).

and compounds were identified and quantified using selected ion monitoring (SIM) (Tables S2 and S3). Data acquisition and processing were performed using MassHunter Quantitative software (Agilent, US). Removal of nutrients and CEC was calculated according to the Eq. (1), based on Matamoros et al. (2015):

$$Removal = \frac{1}{n}\sum_{i=1}^{n}\frac{\left(\overline{Ci}-Ce\right)}{\overline{Ci}} \times 100\% \qquad (1)$$

where $\overline{Ci}$ is the average influent concentration during the sampling, and Ce is the effluent concentration at each sampling (n = 10). Influent and effluent concentrations were analysed for statistically significant differences with the non-parametric Wilcoxon signed-rank test. Additional details about the analytical methodology are given in the Supplementary information (SI).

3. Results and discussion

3.1. HRAP characteristics

Results show that pH and TSS differed the most between the influent and effluent (Table 1). Algae content in the biomass was variable, shown by the percentage of Chl *a* in TSS, which ranged from 0.8% to 2.6% (Fig. S3), and could affect the suitability of biomass for downstream processes. The average biomass productivity was 15.7 ± 5.4 g TSS/m^2 day, which is lower than reported by Matamoros et al. for a HRT of 4 days (24 ± 8 g TSS/m^2 day), but comparable for a HRT of 8 days (13 ± 2 g TSS/m^2), using a similar methodology (Matamoros et al., 2015).

3.2. Nutrient removal

The levels of analysed nutrients in influent and effluent are summarised in Fig. 2 and Table S5. An average TOC removal of 67% meant that the effluent concentrations were higher overall than the emission limits (10–60 mg/L) set by the EU for direct discharge into surface water (The European Commission, 2004), indicating the need for further optimisation of the HRAP performance or post-treatment of effluent. The average removal of TN was 76%, mostly due to uptake into biomass. While most of the TN in the influent was present as NH_4-N, volatilisation is not a significant removal mechanism since pH values did not exceed pH 9 (Park and Craggs, 2011). Biomass uptake also explains the removal of TP (41%). In similar pilot-scale HRAP, average removals were 72% for TN, and 35% for PO_4-P (García-Galán et al., 2020), 73% for TN and 43% for TP (García et al., 2006), 76% for dissolved inorganic nitrogen and 42% for dissolved reactive phosphorus, which are comparable to removals presented herein (Sutherland et al., 2020).

3.3. Removal of contaminants of emerging concern

Twelve of the 48 analysed CEC were quantified in the influent and five in the influent and effluent (Table S6). Due to matrix interferences, seven of the compounds present in influent could not be quantitated in the effluent, suggesting that the method used may not be suitable for analysing more complex algal pond samples. Since we submitted this article, we have published a study that addresses this issue, by developing and validating a method for analysing bisphenols in this matrix (Škufca et al., 2021). The average removals of the compounds were 60% for naproxen (NP), 51% for ibuprofen (IB), 92% for methylparaben (MePB), 76% for 2,4-dihydroxybenzophenone (DH-BP), and 80% for oxybenzone (HM-BP) (Fig. 3 and Table S6). To our knowledge, this is the first study reporting the removal of DH-BP and HM-BP in an outdoor HRAP treating wastewater. These results are comparable to reported ranges for removals in conventional WWTP, which are 43%–99% for NP, 60%–100% for IB, 82%–91% for MePB, for 88%–99% DH-BP and 64%–98% for HM-BP (Česen et al., 2018; Luo et al., 2014; Wang and Wang, 2016).

Table 1
HRAP influent and effluent characteristics.

		pH	O_2%	T [°C]	EC [μS/cm]	TSS [mg/L]	Chl *a* [mg/L]
Influent	Mean ± SD	6.5 ± 1.3	6 ± 4	24.3 ± 2.7	1055 ± 272	110 ± 49	–
	Min–max	4.5–8.8	4–18	21.6–28.8	675–1398	30–164	–
Effluent	Mean ± SD	7.9 ± 0.5	17 ± 23	25.8 ± 4.2	1003 ± 53	442 ± 104	6.3 ± 1.3
	Min–max	7.2–8.6	4–75	19.6–32.0	915–1075	295–684	4.0 ± 8.5

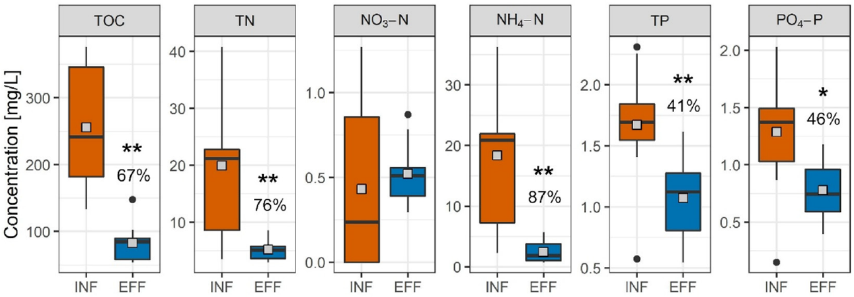

Fig. 2. Box-and-whiskers plots of TOC and nutrient concentrations in influent (INF) and effluent (EFF). Grey square represents mean, average removal efficiency is annotated above the effluent plot, except for NO$_3$-N, where removal was non-existent. Statistically significant differences between the influent and effluent concentrations are denoted with ** - $p < 0.01$, * - $p < 0.05$.

Our results are also comparable to the literature data regarding CEC removal in HRAP. For instance, Matamoros et al. (2015) reported a removal efficiency of NP, IB, MePB and HM-BP of 83%, 99%, 59% and 97%, respectively, in July with a HRT of 4 days. This finding is the inverse of our findings, where MePB had the highest and NP and IB had the lowest removals. Villar-Navarro et al. (2018) report slightly higher removals than in this study, with NP and IB being removed 79% on average in July at an HRT of 6 days and the same goes for García-Galán et al. (2020), who report 79% for IB at a HRT of 4.5 days. Only Vassalle et al. (2020) found a slightly lower removal, as NP was on average removed 54% and IB 46% in a HRAP with an HRT of 8 days. In outdoor tubular photobioreactors with a volume of 1200 L and HRT of 8 days, removal of IB was higher, reaching 99%, while NP removal was lower than IB—only 10% (Hom-Diaz et al., 2017). The cited studies were performed in Spain and Brazil, which are especially suitable for HRAP due to their sunny climate. For comparison, Ajdovščina, Slovenia, receives approximately 1250 kWh/m^2 per year, while Barcelona, Spain and Belo Horizonte, Brazil receive approximately 1750 kWh/m^2 per year in terms of direct normal solar irradiation (S.r.o., 2020).

We can also compare our findings to laboratory-scale studies. However, these usually happen under much more controlled conditions with known microalgae species. De Wilt et al. (2016) have reported removal efficiencies of IB of less than 20% after five days by *Chlorella sorokiniana*, albeit at a much higher starting concentration (317 μg/L). Matamoros et al. (2016) report ≈30% IB removal when treating with a mixed algae culture with *Chlorella* sp. and *Scenedesmus* sp., and more than 80% removal after six days when adding wastewater, both at a starting concentration of IB of 5 μg/L. López-Serna et al. (2019) studied the removal of NP and IB in a laboratory wastewater treatment setup involving anaerobic and anoxic reactors as well as a miniature HRAP. They report NP and IB removal of 44% and 94% at an HRT of 4 days. These results are comparable and higher, respectively to those reported in this study. HM-BP was removed by ≈21% (at a starting concentration of 100 μg/L) and by ≈17% (at a starting concentration of 3000 μg/L) after six days of incubation with *Scenedesmus obliquus* (Lee et al., 2020). NP was removed by ≈40% by *Cymbella* sp. and *Scenedesmus quadricauda* after four days (Ding et al., 2017). While different species and strains of microalgae may play a role in the removal efficiency of CEC, it appears that the presence of bacteria in wastewater promotes and enhances the removal of CEC in algal photobioreactors (Matamoros et al., 2016). According to Liu et al. (2021), this may happen due to one of three scenarios: 1) while microalgae do not participate directly in biodegradation, they offer a favourable environment for bacteria that degrade CEC; 2) both microalgae and bacteria directly degrade the CEC; and 3) synergic cooperation, where bacteria can degrade the intermediate products of microalgae and vice-versa.

Differences in removals between the studied CEC result from their different physicochemical properties, which may influence biodegradation and uptake processes, the latter being lower or non-existent when ionisable compounds are present in anion form (Matamoros et al., 2016). The results (Fig. S4) show how NP and IB have lower distribution coefficients (log D) at the average pH value (Table 1) in the HRAP, due to

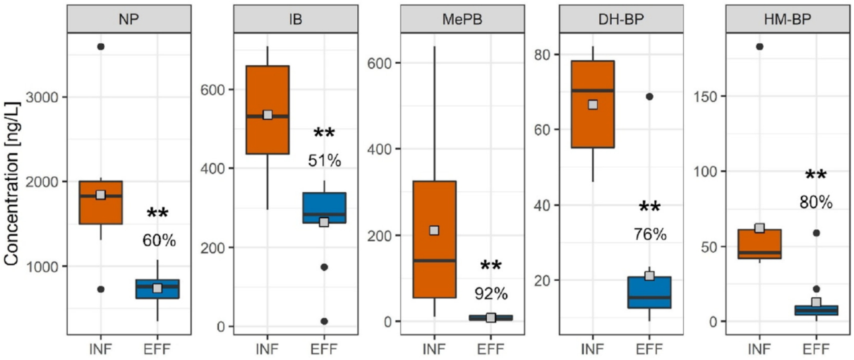

Fig. 3. Box-and-whiskers plots of NP, IB, MePB, DH-BP and HM-BP concentrations in influent (INF) and effluent (EFF). Grey square represents the mean; average removal efficiency is shown above the effluent plot. Statistically significant differences between the influent and effluent concentrations are denoted with ** - $p < 0.01$.

them having the lowest predicted pK_a (4.2 and 4.9, respectively) when compared to MePB, HM-BP and DH-BP (pK_a values of 8.5, 7.1 and 7.1, respectively). These results also suggest that more acidic compounds are removed to a lesser extent in the (usually) slightly alkaline conditions in the HRAP. Conversely, for compounds unaffected by pH, adsorption or biomass uptake may occur, depending on their hydrophobicity. Therefore, to determine the mass balance for CEC during wastewater treatment and identify hazards, algal biomass analysis is essential.

4. Conclusions

Basic operating parameters, nutrients and CEC levels were measured, and their removals determined in a pilot-scale HRAP treating municipal wastewater. Nutrients were removed to a satisfactory degree (41% - 87%), while TOC removal efficiency needs to be optimised. Only 12 of the 48 analysed CEC were quantified in the influent, and of these 12, only five were quantifiable in the effluent. The removal of the studied CEC was comparable to conventional treatment and published removal data for HRAPs. The available literature data showed that the removal of CEC in HRAPs differs depending on physicochemical parameters, HRT and seasonal influences, lighting conditions, and pre-treatment. There is also a need to develop improved methods for analysing CEC in HRAP effluent since this highly complex matrix may interfere with analysis at low concentrations. Analysis of CEC in the algal-bacterial biomass also needs to be performed to close the overall mass balance. While treating wastewater in HRAP with subsequent biomass valorisation agrees with the principles of closing material cycles, poor removal of recalcitrant CEC means that further optimisation and possibly additional treatment are needed, e.g., pre- or post-treatment with efficient biomass harvesting. The potential risks associated with CEC residues in biomass must also be assessed since it could affect valorisation processes such as biopolymer, fertiliser or energy (e.g., biodiesel and biogas) production.

CRediT authorship contribution statement

David Škufca: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Ana Kovačič:** Validation, Methodology, Writing – review & editing. **Franja Prosenc:** Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Tjaša Griessler Bulc:** Funding acquisition, Resources, Writing – review & editing. **David Heath:** Conceptualization, Resources, Writing – review & editing. **Ester Heath:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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.2021.146949.

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Chapter 4

Conclusions

This thesis contributes to knowledge on the occurrence, removal and fate of CEC in algal photobioreactors. In addition to elucidating existing knowledge on CEC removal in laboratory photobioreactors, their removal in an outdoor pilot-scale HRAP was also studied. The contributions to science are presented within four scientific papers published in SCI international journals. Results were also presented at seven national and international conferences as oral and poster presentations (see Bibliography).

To test the hypotheses postulated in this thesis, LC-MS/MS, GC-MS and GC-MS/MS methods were optimised and validated to analyse a wide range of CEC residues in the aqueous phase of laboratory photobioreactors and pilot-scale outdoor HRAP. In addition, methods for analysing BPs in the aqueous and biomass phase of algal photobioreactors were developed and validated. The results confirm the robustness of the methods, LOQ in low ng/L, high recovery and accuracy. Notably, these methods allowed the study of 18 BPs in the aqueous and biomass phases at wastewater relevant concentrations.

✓/✗ The first hypothesis, “H1: Laboratory-scale algal photobioreactors can remove CEC to below quantifiable levels” (<LOQ), is partially accepted since:

- Removal of CEC in laboratory-scale algal reactors to concentrations <LOQ was achieved in the following cases:
    - PPCP: metoprolol, erythromycin and estradiol were removed <LOQ in the mixed and pure culture. Also, clarithromycin and estrone were removed <LOQ in the mixed culture and ibuprofen in the pure culture photobioreactors.
    - Neonicotinoid pesticides: thiamethoxam was removed <LOQ in both cultures and acetamiprid only in the pure cultures.
    - BPs: in total (aqueous and biomass phase), only BPPH was removed to <LOQ in either the pH adjusted or pH non-adjusted series, while 4,4’-BPF was removed <LOQ only in the pH non-adjusted series.
    - When studying the removal of BPs in pure and mixed cultures in photobioreactors, only BPM was removed <LOQ in the mixed culture.
- In most cases, CEC removal in laboratory-scale photobioreactors was >LOQ with removal ranging from 12% (BPAF, carbamazepine) to 99% (4,4’-BPF).

✓ The hypothesis “H2: CEC partitioning depends on their properties (log P, pK_a) and culture conditions (pH)” is accepted, as:

- Low initial (t = 2 h) aqueous amounts (< 50% m_0) were observed for BPs with high log P (BPC, BPFL, BPBP, BPP, BPM, BPPH; log P ≥ 5), indicating that BPs with higher log P tend to partition to biomass soon after spiking in both, pH adjusted and pH non-adjusted series.
- In the aqueous phase, 2,2’-BPF, BPC2, and BPAF (pK_a values 8.2, 8.9 and 9.7, respectively) remained in higher concentrations in pH non-adjusted series at the end of the experiment, as a result of a higher degree of dissociation of these compounds at the elevated pH in the medium (pH > 10).
- Lower amounts (< 10% m_0) of all individual BPs were present in biomass at the end of the experiment in pH non-adjusted series (pH ≈7), while higher amounts were observed (from 10% to 16% m_0) in the case of BPAP, BPZ, BPBP, BPAF, BPC2 and BPFL (pKa values 9.8, 10.1, 9.6, 9.1, 8.9 and 10.5 and log P values 5.18, 4.91, 6.31, 4.77, 4.29 and 5.99, respectively) in the pH adjusted series (pH >10). This result is very likely a result of dissociation and consequently higher solubility of these compounds at higher pH (pH > 10) in the pH non-adjusted series. Log P values of BPs range from 2.32 to 7.34, meaning that the middle of the range (log P: 4 – 6) is influenced most by pH-dependent dissociation, as the more hydrophilic (log P < 4) and more hydrophobic (log P > 6) intrinsically have more affinity for the aqueous or biomass phase, respectively.
- A positive correlation was found between compound log P and total removal in both, the pH adjusted and non-adjusted series, at the end of the experiment. This finding is significant because it indicates that CEC with increasing log P, which are expected to be present in biomass, are more efficiently removed.
- Total removals of 2,2'-BPF, BPC2 and BPAF, were statistically significantly higher in the pH adjusted experiment, while the removal of BPA and BPC was significantly higher in the pH non-adjusted experiment. The higher removal is explained for 2,2'-BPF, BPC2 and BPAF by high pH values, causing a proportion of the compound to become dissociated, decreasing its uptake and removal. However, it does not explain the lower removal of BPA and BPC at the same conditions.

✓/✗ The third hypothesis “H3: Mixed consortia of algae and bacteria are more efficient at removing CEC from wastewater than a pure algal culture” is partially accepted, as:

- The mixed culture was more efficient at removing certain CEC than the pure culture, including:
    - Clarithromycin was removed to a greater extent in the mixed culture (≈100%) than in the pure *C. vulgaris* culture (62 ± 5%).
    - Estrone was removed to a higher degree in the mixed culture (≈100%) than in the pure *C. vulgaris* culture (66 ± 8%).
    - The mixed culture was more efficient at removing most BPs, reflected in overall removal, which was 32% higher in the mixed culture (87 ± 20%) than in *C. vulgaris* culture (55 ± 28%).

- However, for most studied PPCP and pesticides, increased efficiency of mixed cultures was not observed:
    - Overall removal in the mixed and pure cultures was similar for PPCP (85 ± 28% and 79 ± 23%, respectively) and higher in the pure culture for pesticides (64 ± 21% and 90 ± 11%, respectively). The average removal of neonicotinoids imidacloprid and acetamiprid was 30% and 47% higher, respectively, in the pure *C. vulgaris* culture than in the mixed culture.
- Carbamazepine remained recalcitrant in both the mixed (12 ± 5% removal) and pure cultures (34 ± 7% removal). This compound is also known to be recalcitrant in conventional biological wastewater treatment. BPS also proved recalcitrant in both the mixed cultures (18 ± 5% removal) and pure (2 ± 3% removal) cultures; however, BPS was shown to be degradable in conventional WWTP.

✗ Hypothesis "H4: Outdoor HRAP can remove CEC from municipal wastewater to below quantifiable levels" is rejected as none of the CEC observed in the pilot-scale HRAP were removed to <LOQ:

- High removal efficiencies of 92%, 76% and 80% were estimated for methylparaben, 2,4-dihydroxybenzophenone and oxybenzone, respectively. Ibuprofen and naproxen had 60% and 51% removal, respectively, which is at the low end of the reported range for algal photobioreactor removal for these compounds. It is likely that naproxen and ibuprofen, because of their relatively low estimated pKa values of 4.2 and 4.9, were ionized at the average pH of the HRAP (pH 7.9), causing a large proportion to be dissociated, resulting in lower uptake and removal.
- In the HRAP, at the time of sampling, only three BPs (BPA, BPS and 4,4'-BPF) were determined in the influent. BPA and BPS were removed with 80% and 32% efficiency, respectively, leaving average concentrations of 69 ng/L and 94 ng/L in the effluent. 4,4'-BPF was barely above LOQ in two influent samples, so removal could not be accurately calculated.
- The remaining CEC included in the analytical method could not be quantified due to their presence in influent in low concentrations or matrix interferences, indicating the need to optimise analytical methods for CEC determination in complex matrices such as algae-based wastewaters.

Comparison of BPs' removal obtained in both laboratory studies shows that overall removal of BPs in the mixed and pure culture reaching 87 ± 20% and 55 ± 28%, respectively and pH adjusted and non-adjusted reaching 70 ± 21% and 69 ± 23%, respectively, was comparable. The mixed culture reached the highest average overall removal, which shows that mixed cultures may be more promising for CEC removal. However, studies with pure cultures are important since they offer insights into removal mechanisms in a controlled environment. Such an example is the determination of transformation pathways of CEC during algal wastewater treatment.

When comparing CEC removal in the lab-scale studies to the pilot-scale study, differences in the removal of ibuprofen can be observed, as its' average removal was 91% and ≈100% in the mixed and pure laboratory-scale photobioreactors, respectively, and only 51% in the pilot-scale HRAP. For BPs, removals were similar, as average removal of BPS and BPA ranged from 2% to 26% and from 35% to 82% in the laboratory-scale photobioreactors and was 32% and 80% on average in the pilot-scale HRAP, respectively. Even so, optimization of operating parameters and suitable pre- and post-treatment is expected to improve CEC removal, which would be especially desirable for recalcitrant compounds like carbamazepine and BPS.

For some CEC, higher removal was observed in laboratory photobioreactors than is reported for conventional biological WWTP. Removal of sulfamethoxazole (78 ± 5% and 66 ± 3% in mixed and pure cultures, respectively), erythromycin (≈100% in both cultures), clarithromycin (≈100% and 62 ± 5% in mixed and pure cultures, respectively), and metoprolol (≈100% in both cultures) was higher than typically observed in conventional biological WWTP. This finding shows that algal photobioreactors may not only keep up with WWTP regarding CEC removal but may also exceed their removal performance in some cases. Regarding macropollutant removal, study of the pilot-scale HRAP showed that while total nitrogen and total phosphorus were in accordance with emission limits set by the EU for direct discharge into surface water. At the same time, TOC exceeded these values, indicating optimization is needed for the operating parameters of the studied HRAP.

This dissertation increases our knowledge of removing a wide range of CEC, including PPCP, neonicotinoid pesticides and BPs, in wastewater treatment with algal photobioreactors. The removal fate of 18 BPs in algal photobioreactors was studied, where compound physicochemical parameters and culture pH were used to explain partitioning between the aqueous and biomass phases and the removal of BPs. Furthermore, the removal of 28 CEC was compared in laboratory photobioreactors using a mixture of microalgae and bacteria from an HRAP grown in real wastewater and a pure culture of *C. vulgaris* grown in BBM. Finally, this work provides a deeper understanding of nutrient and CEC removal in a pilot-scale HRAP treating municipal wastewater under realistic operating conditions.

In general, algae-based photobioreactors show promise as an alternative to conventional biological wastewater treatment because of comparable removal, low operating costs and the advantage of producing nutrient- and energy-rich biomass. On the downside, algal photobioreactors require longer HRT and larger areas for treatment. However, this drawback may be mitigated with the potential benefits of biomass and reclaimed water reuse. However, many questions remain to be answered before algal photobioreactors and their products are widely applied. Therefore, further studies on CEC removal and optimisation of algal photobioreactors for wastewater treatment are needed. Appropriate legislation will also have to evolve hand in hand with risk assessment of recycling reclaimed water and algal biomass to ensure their safe reuse.

4.1 Future work

Scientists working on wastewater treatment with algal photobioreactors still have many gaps to fill, especially regarding CEC removal, which is crucial for achieving the full benefits of algae-based technologies. As none of the CEC studied in the outdoor HRAP had been removed to <LOQ, further optimization of algal wastewater treatment process parameters (e.g. HRT, total volume, depth, volume per day treated, daily nutrient and TOC loads, microorganism community structure) are needed to increase CEC residue removal efficiency as well as biomass productivity. Perhaps the development of novel photobioreactor designs and principles will enable more efficient wastewater treatment. In addition, the removal of CEC in the laboratory and pilot-scale photobioreactors should be compared to full-scale HRAP, as the effects of up-scaling are not known. At present, CEC occurrence and removal have been studied only in laboratory and pilot-scale systems. Also, to improve CEC removal, ideally reaching their complete mineralisation, research on combining algal photobioreactors with suitable pre- or post-treatments including biological processes and advanced oxidation processes will have to be assessed to remove recalcitrant CEC like carbamazepine.

When complete mineralisation is not reached, it is crucial to identify TPs formed during algal wastewater treatment and their toxicity. Also, complex mixtures of CEC and their metabolites and TPs present in wastewater treatment systems represent an additional layer of complexity. Study of transformation pathways might also expose risks or point to favourable conditions for the degradation of CEC. To assist this work, the development of in silico methods will likely be significant as an aid to future experimental work by modelling algal systems. For instance, given the structure of a given compound and known transformation pathways of other CEC, such software would predict TP and aid in the suspect screening of new CEC residues. Software for estimating toxicity and half-lives of algal TPs of CEC will also be of great value. Programs exist that can estimate parameters of either conventional wastewater treatment or molecular properties of CEC, but more specialised programs providing potential solutions to the problems we face in algal wastewater treatment are needed.

While reuse of byproducts is a potential benefit of algal wastewater treatment, a significant knowledge gap remains concerning the fate and impact of residual CEC in downstream reuse processes, e.g. irrigation with treated water and biomass as a fertilizer or for energy and polymer production. It is currently unknown whether contaminated biomass may be used safely for biofuel, fertilizer or polymers and the associated risks. Therefore, knowing the fate of CEC in downstream processes is crucial and will enable legislation to regulate and guide the reuse of products based on proper risk assessment. Perhaps the use of transgenic algae strains for wastewater treatment and resource production (e.g. enhanced production of enzymes for removal of CEC, enhanced cellular accumulation of nutrients, lipids, pigments, programmed flocculation for ease of harvesting) will increase the efficiency of algal treatment.

Many questions remain to be answered to understand the fate of CEC residues in algae-based wastewater treatment fully. However, the circular reuse of resources will likely fuel further research such as this in the future, for the continued existence of humankind might depend on the development of sustainable technologies.

Appendix A

Supplementary information

A.1 Physicochemical parameters of CEC

Table S1: Physicochemical properties of PPCP, predicted by the EPI Suite based on their structures [186]. CAS – Chemical Abstracts Service identification number, solubility – solubility od compound in water at 25 °C, log Kaw – air-water partitioning coefficient, BCF – bioconcentration factor, BAF – bioaccumulation factor

name	CAS	solubility [mg/L]	log Kaw	BCF	BAF
naproxen	22204-53-1	144.9	-7.9	131.8	131.8
ibuprofen	15687-27-1	41.0	-5.2	436.3	437.0
erythromycin	114-07-8	0.5	-26.7	9.3	9.3
clarithromycin	81103-11-9	0.3	-27.2	15.3	15.3
sulfamethoxazole	723-46-6	3942.0	-10.4	1.5	1.5
estradiol	50-28-2	82.0	-8.8	35.1	35.1
estrone	53-16-7	146.8	-7.8	13.1	13.1
metoprolol	51384-51-1	4777.0	-11.2	8.0	8.0
carbamazepine	298-46-4	17.7	-8.4	19.3	19.3
2,4-dihydroxybenzophenone	131-56-6	413.4	-9.0	9.2	9.2
oxybenzone	131-57-7	68.6	-6.2	66.3	66.3
methylparaben	99-76-3	5981.0	-6.8	3.9	3.9

Table S2: Physicochemical properties of neonicotinoid pesticides, predicted based on their structure using the EPI Suite [186]. CAS – Chemical Abstracts Service identification number, solubility – solubility od compound in water at 25 °C, log Kaw – air-water partitioning coefficient, BCF – bioconcentration factor, BAF – bioaccumulation factor

name	CAS	solubility [mg/L]	log Kaw	BCF	BAF
imidacloprid	138261-41-3	7172.0	-13.2	1.0	1.0
thiamethoxam	153719-23-4	2862.0	-12.6	1.2	1.2
acetamiprid	160430-64-8	222.0	-5.5	20.8	20.8
thiacloprid	111988-49-9	231.9	-8.0	9.4	9.4

Table S3: Physicochemical properties of BPs, predicted based on their structure using the EPI Suite [186]. CAS – Chemical Abstracts Service identification number, solubility – solubility od compound in water at 25 °C, log Kaw – air-water partitioning coefficient, BCF – bioconcentration factor, BAF – bioaccumulation factor

abbreviation	CAS	solubility [mg/L]	log Kaw	BCF	BAF
BPS	80-09-1	1774.0	-13.0	3.5	3.4
2,2 BPF	2467-02-9	617.6	-9.7	33.8	33.8
2,4 BPF	2467-03-0	617.6	-9.7	33.8	33.8
4,4 BPF	620-92-8	617.6	-9.7	28.0	28.0
BPE	2081-08-5	321.6	-9.6	45.6	45.6
BPA	80-05-7	146.2	-9.4	172.7	172.8
BPC II	14868-03-2	140.6	-10.6	227.5	227.6
BPB	77-40-7	45.1	-9.3	170.2	170.3
BPAF	1478-61-1	0.8	-7.6	639.3	643.0
BPZ	843-55-0	16.7	-9.4	271.4	272.1
BPC	79-97-0	14.6	-9.3	112.6	112.6
BPAP	1571-75-1	2.4	-10.6	249.7	250.1
BPFL	3236-71-3	0.01	-12.3	896.7	1288.0
BP26DM	5613-46-7	1.4	-9.2	83.4	83.5
BPBP	1844-01-5	0.04	-11.9	385.1	416.4
BPM	13595-25-0	0.1	-10.2	1970.0	9097.0
BPP	2167-51-3	0.1	-10.2	1970.0	9097.0
BPPH	24038-68-4	0.003	-11.7	192.8	585.9

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Journal Articles Related to the Thesis

D. Škufca, A. Kovačič, T. Griessler Bulc, and E. Heath, “Determination of 18 bisphenols in aqueous and biomass phase of high rate algal ponds: Development, validation and application,” Chemosphere, vol. 271, p. 129786, May 2021. https://doi.org/10.1016/j.chemosphere.2021.129786

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F. Prosenc, J. Piechocka, D. Škufca, E. Heath, T. Griessler Bulc, D. Istenič, and G. Buttiglieri, “Microalgae-based removal of contaminants of emerging concern: Mechanisms in Chlorella vulgaris and mixed algal-bacterial cultures,” J. Hazard. Mater., vol. 418, p. 126284, Sep. 2021. https://doi.org/10.1016/j.jhazmat.2021.126284

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Other articles

A. Kovačič, D. Škufca, M. Zupanc, J. Gostiša, B. Bizjan, N. Krištofelc, M. Sollner Dolenc, and E. Heath, “The removal of bisphenols and other contaminants of emerging concern by hydrodynamic cavitation: From lab-scale to pilot-scale,” *Sci. Total Environ.*, vol. 743, p. 140724, Nov. 2020. https://doi.org/10.1016/j.scitotenv.2020.140724

Articles in preparation

B. Žener, L. Matoh, P. Rodič, D. Škufca, E. Heath, and U. Lavrenčič Štangar, “Removal of 18 bisphenols co-present in aqueous media by effectively immobilized titania photocatalyst”. Submitted to Journal of Environmental Chemical Engineering (August 2021)

Conference contributions

T. Griessler Bulc, D. Istenič, F. Prosenc, D. Škufca, E. Heath, N. Atanasova, A. Krivograd-Klemenčič, M. Pintar, V. Zupanc. Closing material flows by wastewater treatment with green technologies. In: PUCHER, Bernhard (Ed.). Implementing nature based solutions for creating a resourceful circular city. COST Action CA17133 workshop, 13-15 February 2019, Vienna. Vienna: University of Natural Resources and Life Sciences, 2019. Str. 34-35. http://www.circular-city.eu/images/pdf_download/Proceedings_COST_WS_13-15Feb.pdf

D. Škufca, F. Prosenc, A. Kovačič, M. Pflieger, T. Kosjek, T. Griessler Bulc, E. Heath. Removal of bisphenols in laboratory-scale algal bioreactors. In: Book of Abstracts. 2nd IWA Conference on Algal Technologies for Wastewater Treatment and Resource Recovery [and] 2nd IWA Specialist Group Conference in Wastewater Pond Technology, IWAlgae2019, July 1 & 2, 2019, Valladolid, Spain., 2019. pp. 532-533.

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Biography

Education and research activities:

- 2017 – present: Jožef Stefan International Postgraduate School, Ljubljana, Slovenia: PhD student of Ecotechnology
- 2017: Internship at the Chair of Zoology, Department for Biology, Biotechnical Faculty, University of Ljubljana, Slovenia
- 2014 – 2016: University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia: Master's degree in Ecology and Biodiversity entitled "Monitoring of physiological state biomarkers of water lice from cave and surface water environments"
- 2010 – 2014: University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia: Bachelor's degree in Biology

Foreign work and study experience:

- 2014 – 2015 (5 months): Erasmus exchange at the Vrije Universiteit Amsterdam, the Netherlands

Fellowships and scholarships:

- 2017: Jožef Stefan scholarship
- 2014: Erasmus Plus Exchange scholarship
- 2006 – 2011: Zois scholarship

Research projects:

- J2- 8162: Closure of Material Pathways in Urban Wastewater Treatment with Green Technologies,
- L7-1848: Photocatalytic water treatment - development of immobilised catalysts and compact reactor systems