



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

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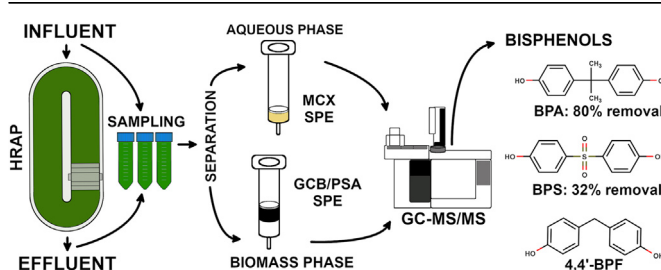
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HIGHLIGHTS

- 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.

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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 K_{ow}) and acid dissociation constant (pK_a) 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 Årénmark, 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 [¹³C₁₂] 4,4'-BPF (¹³C₁₂-BPF), BPS (¹³C₁₂-BPS), BPB (¹³C₁₂-BPB) were purchased from CanSyn Chem. Corp. (Canada), and deuterated BPA (BPA-d₁₆) 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 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 ($>18.0 \text{ M}\Omega \text{ cm}^{-1}$ at 25°C Millipore, USA).

Stock solutions were prepared in MeOH ($\approx 1 \text{ mg/L}$ for standards and $\approx 0.2 \text{ mg/L}$ 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 pre-cleaned 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, $^{13}\text{C}_{12}$ -BPS and $^{13}\text{C}_{12}$ -BPB each, 25 μL of 1 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$ 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 $2 \times 1.8 \text{ mL}$ of MeOH/EtAc (20:80), and subsequently with $2 \times 1.8 \text{ mL}$ 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 \times 0.25 mm \times 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 $20^\circ\text{C min}^{-1}$ to 200°C and held for 6 min, then ramped to 300°C at $10^\circ\text{C min}^{-1}$ 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 Årénmark, 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), “xlsx” (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.

compound	R_t	Transition 1 (quantification)			Transition 2 (qualification)		
		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

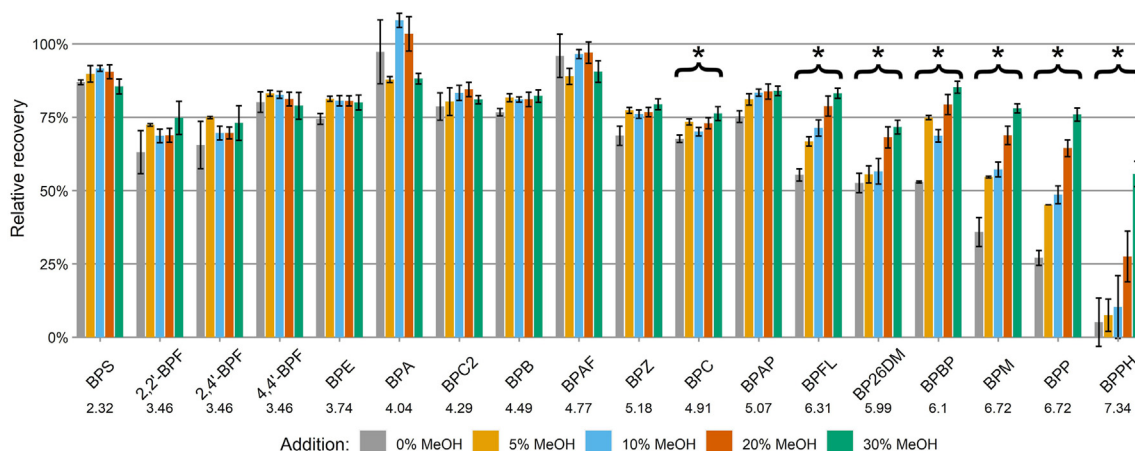


Fig. 1. The effect of MeOH addition as a modifier on the recovery of 18 BPs in the aqueous phase. Results are normalised to post-extraction spiked blank samples for reference. Compounds are arranged from lowest to highest log Kow, noted under the compound name. * - Statistically significant difference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

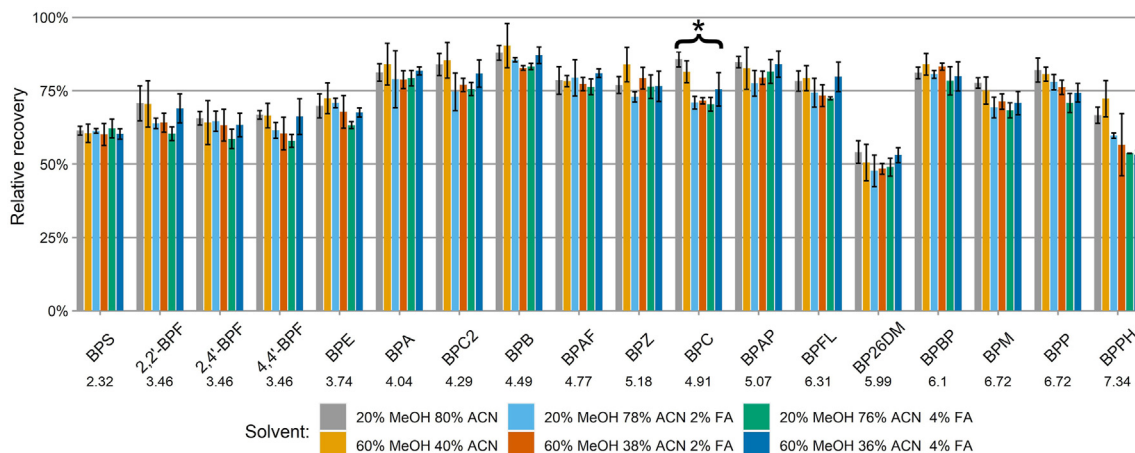


Fig. 2. Recovery of BPs by the composition of extraction solvents. Results are normalised to post-extraction spiked blank samples for reference. Compounds are arranged from lowest to highest log Kow, noted under the compound name. * - Statistically significant difference ($p < 0.05$).

negatively affect gas chromatographic separation (Guan et al., 2009; Hajšlová and Zrostlikova, 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 Kow 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).

The only BP detected in the biomass (Table 3) was BPA, which

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).

compound		Aqueous [ng/L]		Biomass [ng/g]	
		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%

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 $\approx 55\%$ and $\approx 90\%$, while BPS was almost completely ($\approx 100\%$) 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 LOQ 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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