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Kinetics and Biotransformation products of Bisphenol F and S during Aerobic Degradation with Activated Sludge

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ABSTRACT

Bisphenol F (BPF) and bisphenol S (BPS) are becoming widespread in the environment despite the lack of information regarding their fate during wastewater treatment and in the environment. This study assessed the biodegradation kinetics of BPF and BPS during biological wastewater treatment with activated sludge using GC-MS/MS, and the identification of biotransformation products (BTPs) using LC–QTOF-MS. The results showed that BPF and BPS degrade readily and unlikely accumulate in biosolids or wastewater effluent ($c_i = 0.1$ mg L$^{-1}$, half-lives < 4.3 days). The first-order kinetic model revealed that BPF ($k_t = 0.20 – 0.38$) degraded faster than BPS ($k_t = 0.04 – 0.16$) and that degradation rate decreases with increasing initial concentration of BPS (half-lives 17.3 days). The absence of any additional carbon source significantly slowed down degradation, in particular, that of BPS (lag phase on day 18 instead of day 7). The machine-learning algorithm adopted as part of the non-targeted workflow identified three known BTPs and one novel BTP of BPF, and one known and ten new BTPs of BPS. The data from this study support possible new biodegradation pathways, namely sulphation, methylation, cleavage and the coupling of smaller bisphenol moieties.
Bisphenol A (4-[2-(4-hydroxyphenyl)propan-2-yl]phenol, BPA) has been in commercial use since 1957 as a monomer in the production of polycarbonate plastics and epoxy resins. However, concerns over its toxicity, particularly its endocrine disrupting activity, have led many to call for an outright ban on its use, specifically in food contact materials. BPA, however, is only one among several bisphenols such as 4,4'-dihydroxydiphenyl-methane (BPF) and 4,4'-sulphonyldiphenol (BPS), which have become widespread in consumer products and various environmental and human media and exhibit similar endocrine disrupting activity. A significant source of bisphenols in the environment are wastewater treatment plant (WWTP) effluents and the land application of sewage sludge. Concentrations of BPA range from 3–84,110 ng L\(^{-1}\) in influent, n.d.–3100 ng L\(^{-1}\) in the effluent, and 3–3670 ng g\(^{-1}\) dw\(^{-1}\) in sludge, while BPF ranges from n.d.–117 ng L\(^{-1}\), n.d.– 65 ng L\(^{-1}\) and n.d.–384 ng g\(^{-1}\) dw\(^{-1}\), and BPS from n.d.–435 ng L\(^{-1}\), n.d.– 27 ng L\(^{-1}\) and n.d.–600 ng g\(^{-1}\) dw\(^{-1}\), respectively. Among the different technologies used to treat wastewater, biological treatment with activated sludge (AS) has been shown to completely remove BPA. Thus, a considerable effect on the environmental fate of BPF and BPS by bacterial communities is expected and should also be studied, including identification of degradation mechanisms and biotransformation products (BTPs).

Biodegradation of BPA involves oxidative skeletal rearrangement and hydroxylation of the aromatic ring following ring cleavage, and a similar biotransformation of BPF and BPS is expected. However, the different linkage of the BPF and BPS phenolic rings may affect their biodegradability. To date, only a few studies have focused on the biodegradation of BPF and BPS, mainly using single bacterial strains (Table SI-1). However, these conditions are not representative of the natural environment or conditions in WWTPs, where microbial consortia degrade organic pollutants. Currently, only one study of biodegradation of BPF and BPS, by a microbiological community in seawater, and two studies looking at the fate of BPS in soil and AS have been published. Also, specific biodegradation pathways have been proposed only for BPF using Sphingobium yanoikuyae strain FM-2 isolated from river water, in which degradation proceeds via a Baeyer–Villiger reaction, and ring fission followed by ring hydroxylation in the case of Cupriavidus basilensis isolated from a composite soil. Zühlke et al. suggest that the formation of phosphate conjugates by Bacillus amyloliquefaciens (isolated from AS) is another possible detoxification mechanism for BPF. Sphingobium fuliginis OMI (isolated from the rhizosphere of the giant duckweed Spirodela polyrhiza) is also capable of degrading BPF and BPS via hydroxylation and meta-cleavage and is the first report of the aerobic biodegradation of BPS. Other studies show that BPS degrades in forest and farm soils as a result of ortho-cleavage and recent kinetic studies of BPS indicate it is susceptible to degradation in AS or by a Sphingomonas sp. strain NP5.
isolated from AS\textsuperscript{21}. Overall, BPS appears to be more resistant than BPF, especially when only isolated strains were studied (Table SI-1).

Despite progress, the fate of BPF and BPS in WWTPs remains poorly understood\textsuperscript{16}, and no studies have used non-target screening for BTPs of BPF or BPS using bacterial consortia (AS) and accurate mass high-resolution mass spectrometry for their identification and characterisation. This paper seeks to address this knowledge gap by determining the biodegradation kinetics of BPF and BPS during biological treatment with AS using gas chromatography-tandem mass spectrometry (GC-MS/MS), and the identification of BTPs using liquid chromatography-high-resolution quadrupole time-of-flight mass spectrometry (LC-QTOF-MS).

**MATERIALS AND METHODS**

**Reagents and chemicals**

General information about BPF and BPS, reagents and materials, and a description of standards preparation, is presented in the Supplementary information (SI): Chapter SI-1.

**Batch biodegradation experiments**

Aerobic biodegradation was conducted using a modified Zahn-Wellens test\textsuperscript{22,23}. Briefly, batch biodegradation experiments (Table 1) were performed in the dark in 0.5 L glass bottles with a total volume of 400 mL. Each test was aerated using an aquarium pump (4W, 4.0 L min\textsuperscript{-1}). Compounds were added at 0.1, 1, 2, 5 and 10 mg L\textsuperscript{-1} for the kinetics experiment and 10 mg L\textsuperscript{-1} for studying BTP formation. Series A, B, C, D, BL-A and BL-C contained 10 mL of freshly settled AS obtained from an operating mechanical-biological sequencing batch reactor of a nearby WWTP designed to treat 65,000 population equivalents. Two experimental conditions were applied: 1) A, B and BL-A contained nutrient-rich medium (330 mL), and 2) C, D and BL-C contained mineral-rich medium (330 mL) without a carbon source (Table SI-4). Controls E and F contained only deionised water. Biological activity was inhibited by adding 6% formaldehyde into B, D and E to act as controls for A and C, respectively in order to account for potential abiotic losses such as sorption. Except for these controls and blanks, all series were prepared in parallel (A1/2 and C1/2). Experimental parameters (biomass concentration, temperature, pH, evaporation factor) were measured as described in SI-3.3.

During the kinetic experiments, samples (4-mL) were collected at $t = 15$ min (to assure homogeneity) and then at 24-hour intervals over 14-days. Based on kinetic experiments BTP samples were collected at $t = 15$ min and then at 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 days for BPF and at 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18 days for BPS. Each sample was centrifuged at 9000 rpm for 20 min and the supernatant filtered through 0.2 µm regenerated cellulose filters (Phenomenex, USA). Aliquots of
individual extracts were transferred to GC (0.1 or 0.5 mL) or LC (0.5 mL) vials and spiked with isotope-labelled internal standards: $^{13}\text{C}_{12}$-BPF and $^{13}\text{C}_{12}$-BPS ($V = 25 \mu\text{L}; 10 \mu\text{g mL}^{-1}$) or BPA-d$_{16}$ ($V = 25 \mu\text{L}; 20 \mu\text{g mL}^{-1}$). For quantification purposes, the solvent was removed with $\text{N}_2$ (40 °C), derivatised with 50 µL of MSTFA and 50 µL of pyridine for 1 h at 80 °C and diluted with 0.4 mL of ethyl acetate prior to GC-MS/MS analysis. For BTPs screening, the extracts were stored at -20°C and analysed directly by LC-QTOF-MS.

Instrumental analysis

Kinetic experiment samples were analysed using a 7890B series GC coupled to an Agilent 7000 series triple quad MS/MS, (Agilent Technologies, USA)$_{24}$. The analysis is described in detail in SI-3.4.1, Chapter 3. Information on quantification, retention times (RT) and MS/MS optimised conditions are summarised in Table SI-3, while matrix-matched validation using isotopically labelled internal standards was performed as described in SI-3.5. Limits of quantification (LOQ) for BPF and BPS were 0.02 or 0.1 mg L$^{-1}$, respectively, and the average concentrations of analytes in the blanks were < LOQ. Non-target screening for the identification of the formed BTPs was conducted using an Agilent 1290 Infinity UPLC coupled to an Agilent 6530 Accurate-Mass QTOF (Agilent Technologies, USA). An Agilent Jet-Stream electrospray source was operated in negative ionisation mode only since no transformation products of bisphenols were identified in our previous studies. The instrument was run in data-dependent acquisition mode with a mass range of 50 to 1000 m/z and a scan rate of 2 scans s$^{-1}$ for MS and 5 scans s$^{-1}$ for MS/MS (SI-3.4.2, Chapter 3). MassHunter software (Version B.07.00, Agilent Technologies) was used for data analysis.

Data processing and analysis

Data processing and visualisation were performed using Python (version 3.6)$_{25}$ and R-based software (version 3.6.1)$_{26}$. All data were fitted using a first-order kinetic model by nonlinear least-squares analysis$_{27}$. The suspect and non-target screening workflows used in this study were modified from previous studies$_{8,28–30}$. The optimised approach resulted in enhanced confidence in the detection and identification of BTPs. Initially, suspect screening was performed using an expanded csv-database of possible BTPs$_{8}$, and the features were extracted using MassHunter Qualitative Analysis Software (version B.07.00, Agilent Technologies). A non-targeted approach was conducted and optimised using MZmine (version 2.52)$_{31}$ and R$_{32}$. Briefly, MZmine was used to detect and differentiate MS features as follows: mass detection (centroid algorithm), chromatogram building, chromatogram deconvolution (noise amplitude algorithm), deisotoping, filtering according to peak width (0.03 to 2 min), peak alignment (RANSAC algorithm) and gap-filling (the same m/z and RT). For each investigated compound, two lists of mass features, one with broader (lower noise level and peak height) and one with restricted
limits (higher noise level and peak height) were generated and processed in R using the modified Shiny app tindeResting, which uses a Random Forest machine-learning algorithm to predict the most interesting mass features\textsuperscript{32}. This approach also enabled the ability to filter the relevant mass features from a smaller or larger number of false features. Predictions were based on mass feature intensity (> 1000), RT (> 0.5 min), presence/absence in the first sample, controls and blanks, presence in both aliquots, the response ratio between peak height in control/peak height in the sample (< 1), and the prediction value (> 0.1). The resulting m/z values were extracted using the Formula Prediction algorithm in MZmine. The identification of potential BTPs resulting from both workflows was based on accurate mass and isotopic patterns in MS mode. For each match, the MS/MS fragmentation pattern and accurate mass of the respective product ions were analysed. Tentative structures were postulated based on the mass shift of the BTP compared to the parent compound, elemental formulae and by comparing MS/MS fragmentation spectra with the parent compounds and other BTPs. Identification was based on the following criteria: (a) a maximal mass variation of ± 10 ppm between the measured and theoretical parent ions; (b) a maximal mass variation of ± 20 ppm for product ions; (c) an isotope pattern score of at least 70%; (d) the measured double bond equivalent values (DBE) matched the postulated structure; (e) absence/presence in control and blank samples; (f) absence/presence in both aliquots and parallels; and (g) by following the biotransformation time profile. Levels of confidence were assigned to identified BTPs following the scale and requirements as proposed by Schymanski et al.\textsuperscript{33}.

RESULTS AND DISCUSSION

Degradation kinetics

In controls, no significant changes in levels of BPF (87 ± 8%) and BPS (89 ± 10%) were observed during the first ten days. After ten days, a 30% loss of parent compound was observed, indicating abiotic removal. Neither BPF nor BPS was present in the blanks. Experimental parameters are given in SI-4. Figure 1 shows the degradation kinetics for BPF and BPS in nutrient-rich media (A). A nonlinear least-squares analysis produced reasonably good fits, namely $R^2 = 0.72$–0.97 (Table SI-6). The results showed that BPF degrades faster ($k_t = 0.20$–0.38, half-lives = 2.5–3.5 days) than BPS ($k_t = 0.04$–0.16, half-lives 4.3–17.3 days) and that their $c_i$ affects degradation kinetics. For BPF, 53–64% was degraded within 24 h for all tested $c_i$, then after a plateau phase and depending on $c_i$, a complete removal was observed. Levels of BPF were < LOQ (1-20% remaining) by day 5 at $c_i = 2$ mg L\textsuperscript{-1}, by day 8 at $c_i = 0.1$ and 5 mg L\textsuperscript{-1} and by day 10 at $c_i = 1$ and 10 mg L\textsuperscript{-1} (Fig. 1), in accordance with the $k_t = 0.20$–0.38 (Table SI-6). Zühlke et al.\textsuperscript{20} also found that at a certain $c_i$, the formation of BTP could activate bacteria responsible for compound degradation. The degradation rate for BPF in this study is slower compared to those reported in the literature (Table SI-1), where complete degradation was achieved within 24 h at a $c_i \leq$
200 mg L$^{-1}$. One explanation for this discrepancy could be the authors’ use of optimal, isolated bacteria compared to a more realistic microbiological community used in this study.

In line with our study, the opposite effect is shown for the more persistent BPS, for which the removal is higher in bacterially rich communities. At a $c_i = 0.1$ to 5 mg L$^{-1}$, the level of BPS was < LOQ at day 10, as was the case in one parallel batch ($k_t = 0.12$, half-lives = 5.8 days) at $c_i = 10$ mg L$^{-1}$, whereas in a second parallel > 50% remained at day 13 ($k_t = 0.04$, half-lives = 17.3 days). Therefore, $c_i$ had a greater effect on BPS degradation compared to BPF (Fig. 1). The observed discrepancy between the parallels could be due to the toxic effects of BPS at higher $c_i$. These results are in agreement with Zdarta et al.34, who also noted a decrease in the degradation rate of BPF and BPS with increasing $c_i$ by the fungal enzyme *Trametes Versicolor* laccase, immobilised on spongin. In contrast, Huang et al.16, report the complete removal of BPS from AS in a continuous flow bioreactor at a $c_i = 50$ mg L$^{-1}$ after 6 days. However, strong variations in experimental conditions and employed organisms make comparing the data from different studies difficult.

### Biotransformation products identification

Bisphenol F and S BTPs formed in nutrient (A), and mineral (C) rich medium at $c_i=10$ mg L$^{-1}$ by aerobic AS microbes were identified and compared (SI-4). The results are presented in SI-4. No significant losses were observed for BPF (< 10%) over 11 days and BPS (< 20%) over 18 days in the controls (BP-B, -D, -E and -F). The exception is control F, where > 30% BPS was degraded within 14 days. The identified BTPs were not detected in the controls and blanks, showing that the removal of BPF and BPS in conditions A and C was the result of biodegradation. Their postulated formulas and corresponding confidence levels, as proposed by Schymanski et al.33, are presented in Table 2.

All proposed BTPs that met the necessary identification criteria and were detected in samples from at least one sampling time; chromatograms and MS/MS spectra are given in SI-4.2.2. Broader limits in MZmine resulted in a higher number of identified BTPs when the relevant mass features were mainly filtered using the machine-learning algorithm. A semi-quantitative analysis, carried out by calculating the ratio of the peak areas of the BTPs to that of the internal standard, provided information on the time trend of BTPs formation (Fig. 2) and allowed biodegradation pathways to be postulated (Fig. 3 and 4). In this case, the averages of both parallels are not presented; rather, Fig. 2 shows parallels with the highest relative quantities of BTPs. All other relevant information is shown in Fig. SI-5 and SI-6.

**Bisphenol F** eluted at RT 9.20 min as the deprotonated molecule ([M-H]$^-$, 199.0765) and was completely degraded by day 5. Three BTPs (BPF-215, BPF-213 and BPF-279) were identified in nutrient (A) rich media and four (BPF-215, BPF-213, BPF-279 and BPF-229) in mineral (C) rich media (Table 2). BTPs appeared between day 2 and 3, reached a maximum by day 4 and were undetectable by day 6.
The spectrum of BPF-215 ([M-H]−, 215.0719) shows the neutral loss of water and is indicative of aliphatic hydroxylation. Based on the calculated relative quantities, BPF-213 ([M-H]−, 213.0558) most likely forms by the oxidation of BPF-215. Its transformation to BPF-229 ([M-H]−, 229.0525), identified at a confidence level L4 (Fig. 3), possibly forms via a Baeyer-Villiger reaction, as suggested by Inoue et al.35. A small peak at RT 7.90 min had the m/z ([M-H]−, 215.0758) characteristic of a hydrolysed BTP. However, MS/MS fragmentation was not triggered, and the ppm error was high (>10). A novel minor BTP, BPF-279 ([M-H]−, 279.0349) was also identified and the presence of a sulphate (SO3) functional group suggests a sulphate-conjugated BTP.

Bisphenol S was detected as the deprotonated molecule ([M-H]−, 249.0227) at RT 7.10 min. In this case, degradation was complete by day 7 under condition A and by day 18 under condition C (Fig. 2). Although the lag phase for BPS (day 7 or 18) was longer than for BPF (day 5), the concentration of both compounds declined significantly within two days in both nutrient (A) and mineral (C) rich media. Interestingly, despite the sudden and complete reduction in the amounts of BPF and BPS, BTP formation varied depending on degradation kinetics, applied conditions and exposure time. Of the eleven BTPs identified (Table 2), only hydroxylated BPS-265A is consistent with that reported for degradation with the Sphingobium fuliginis OMI14, while BTPs with characteristic ring cleavage were not observed1. Between RT 1.20–1.42 min, four S–C cleavage BTPs were identified: three (BPS-93, BPS-157, and BPS-173) in nutrient- and four (BPS-93, BPS-157, BPS-173 and BPS-189) in mineral-rich media (Table 2). The first three had similar kinetic profiles under both conditions reaching a maximum concentration at day 7 in Series A and day 17 in Series C, followed by their gradual decomposition. The inclusion of oxygen was observed in two compounds: BPS-265A ([M-H]−, 265.0181) at RT 5.90 min and BPS-265B ([M-H]−, 265.0186) at RT 8.60 min. Their RT and MS/MS spectra suggest that they are not positional isomers, but rather, BPS-265A is a hydroxylated form of BPS and is probably the major BTP formed under both conditions. However, it was detected only during slower transformation (C) of BPS and was completely degraded within 15 days. Alternatively, the formation of BPS-265B follows that of cleaved BTPs and is likely formed by coupling of smaller BPS moieties. The presence of BPS-263 ([M-H]−, 263.0383) suggests the linking of a methyl group, as one of the possible biotransformation reaction36 from the methylation of BPS, while BPS-279A ([M-H]−, 279.0342) and BPS-279B ([M-H]−, 279.0350) are isomers formed by a combination of hydroxylation and methylation. Although the positional isomerisation is assumed from their common product ions, neither the exact position of the hydroxyl- nor methyl groups could be determined (confidence level L3). Similar to BPF, BPS-329 ([M-H]−, 328.9891) is probably a result of sulphate conjugation. In contrast, the spectra and RT 10.00 of BPS-341 ([M-H]−, 341.0499) indicate the presence of a phenol group, which was also observed for BPF by Zdarta et al.34 during degradation with immobilized Trametes versicolor laccase.
Biodegradation Pathway. Figure 3 shows the biodegradation pathway of BPF, as proposed in this study. The pathway is the same as that suggested for Sphingobium yanoikuyae, where hydroxylation of the bridging carbon leads to complete mineralisation via the Baeyer-Villiger reaction. Recent studies have reported similar biodegradation pathways common to other bisphenols (Bisphenol A, C, E, B, P, AF, and Z), namely aromatic hydroxylation and meta- or ortho-cleavage of BPF and BPS in soil and sediments and phosphate conjugation of BPF by Bacillus amyloliquefaciens from AS. In this study, hydroxylation also forms part of the biodegradation pathways for BPS. In addition, the results show that degradation occurs by sulphation in the case of BPF and BPS and for BPS via methylation, cleavage of the S-C bond between the phenyl rings and the coupling of smaller moieties (Fig. 3 and 4). The appearance and disappearance of BTPs imply the ability of certain bacteria in AS to slowly transform the parent compound by attaching smaller moieties until other bacteria, capable of removing the parent compounds and BTPs prevail (Fig. 2). We propose that the described biodegradation pathways for both compounds do not differ under the two applied conditions, but the detection of specific BTPs rather depends on the time of sampling. However, in the absence of any carbon source other than BPF or BPS (C), degradation is slower, especially in the case of BPS. Also, despite not having identified lower m/z BTPs of BPF, it is reasonable to assume that they are formed in both conditions but were lost due to rapid degradation and possible mineralisation. This assumption is supported by the relative quantities of the parent compounds and BTPs, i.e., BTP formation << parent compound degraded.

In summary, rapid biodegradation under aerobic conditions suggests that BPF and BPS (c_i = 0.1 mg L^{-1}, half-lives < 2.5 and 4.3 days) will at environmental concentration most likely not accumulate in biosolids or wastewater effluent. The results reveal a new degradation pathway for BPF, based on sulphate-conjugated BTP. The slower degradation of BPS allowed for the identification of 11 BTPs, of which ten are novel and provide new evidence of additional biodegradation pathways. Importantly, new insights into the biodegradation of bisphenols and knowledge of novel biodegradation pathways will play an essential role in understanding the fate of bisphenols during biological water treatment and in the environment.

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Conflicts of interest

None.

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