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Research Paper

Biotransformation pathways of pharmaceuticals and personal care products (PPCPs) during acidogenesis and methanogenesis of anaerobic digestion

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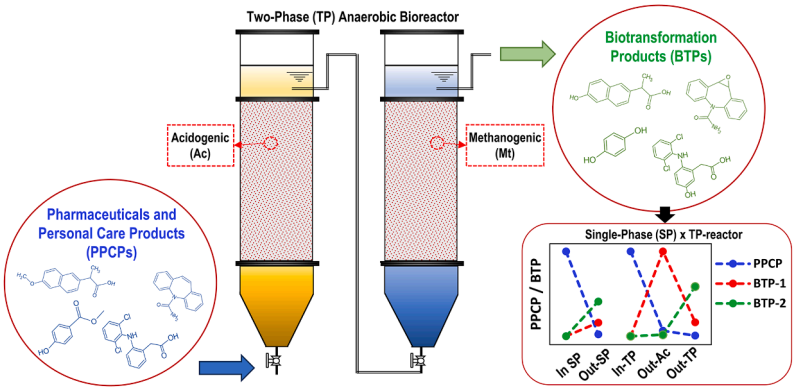
HIGHLIGHTS

- 14 BTPs were confidently identified from ten investigated parent PPCPs.
- Acidogenesis plays a crucial role on the PPCPs biotransformation.
- Hydroxylation reaction was the main biotransformation pathway.
- Two-phase reactor enhances the biodegradability of DCF, IBU and CBZ.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceuticals and personal care products (PPCPs) exhibit varying biodegradability during the acidogenic and methanogenic phases of anaerobic digestion. However, there is limited information regarding the end products generated during these processes. This work investigates the biotransformation products (BTPs) generated in a two-phase (TP) acidogenic-methanogenic (Ac-Mt) bioreactor using advanced suspect and nontarget strategies. Fourteen BTPs were confidently identified from ten parent PPCPs including carbamazepine (CBZ), naproxen (NPX), diclofenac (DCF), ibuprofen (IBU), acetaminophen (ACT), metoprolol (MTP), sulfamethoxazole (SMX), ciprofloxacin (CIP), methylparaben (MPB) and propylparaben (PPB). These BTPs were linked with oxidation reactions such as hydroxylation, demethylation and epoxidation. Their generation was related to organic acid

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production, since all metabolites were detected during acidogenesis, with some being subsequently consumed during methanogenesis, e.g., aminothiophenol and kynurenic acid. Another group of BTPs showed increased concentrations under methanogenic conditions, e.g., hydroxy-diclofenac and epoxy-carbamazepine. The most PPCPs showed high removal efficiencies (> 90 %) – SMX, CIP, NPX, MTP, ACT, MPB, PPB, while DCF, CBZ and IBU demonstrated higher persistence - DCF (42 %); CBZ (40 %), IBU (47 %). The phase separation of anaerobic digestion provided a deeper understanding of the biotransformation pathways of PPCPs, in addition to enhancing the biodegradability of the most persistent compounds, i.e., DCF, CBZ and IBU.

1. Introduction

The presence of pharmaceuticals and personal care products (PPCPs) in municipal wastewater and throughout the biological treatment processes has been frequently reported over the last few decades. Furthermore, the risks associated with their environmental exposure to humans are increasingly being elucidated [1–3]. For instance, the widespread presence of antibiotics in the environment has been extensively reported as a factor promoting the emergence of antimicrobial resistance in various environmental matrices [4]. Additionally, the release of bisphenol-A into waters has been linked to male infertility [5], while prenatal exposure to pesticides and phenols has been consistently associated with birth outcomes and health disorders [6].

PPCPs, when passing through the biological reactors, can be completely mineralized or undergo various processes of biotransformation mediated by microorganisms. Biotransformation products (BTPs) can preserve the original chemical structure of the parent compound, and even present a greater toxic or inhibitory potential on the microbiota [7]. Despite considerable progress in understanding the factors involved in the biological removal of PPCPs in wastewater treatment plants (WWTPs), there are still important knowledge gaps regarding the specific biotransformation pathways of these chemicals. These pathways are crucial for fully understanding how PPCPs are removed from WWTPs and optimizing the biological treatment processes.

Among biological treatment processes, anaerobic digestion (AD) stands out for its lower cost compared to aerobic systems, and greater applicability in warm climate countries, given the absence of the need to input surplus energy [8]. Nevertheless, conventional anaerobic reactors are not optimized to eliminate PPCPs, and there is a need for new treatments and strategies to address this challenge. Anaerobic biofilm reactors, such as anaerobic moving bed biofilm reactors and anaerobic fixed bed biofilm reactors, have shown superior removal of organic micropollutants due to their capacity to form biofilm in the system compared to the traditional configurations [9,10]. Biofilm formation promotes the development of a microbial community that exhibits increased resilience to environment stress condition affecting its metabolic processes [11], including the presence of PPCPs.

AD phase separation within a two-stage anaerobic system, where the initial stage primarily favors acidogenic conditions, followed by a subsequent stage favoring a methanogenic condition, has been documented in various studies to enhance the production and recovery of biomethane [12–14]. Additionally, it leads to the generation of biohydrogen and valuable byproducts such as volatile organic acids and solvents during acidogenesis [15,16]. This process is also promising because it selects fermentative bacteria capable of biotransforming PPCPs in the initial acidogenic stage. This, in turn, reduces the influent load at the methanogenic stage, creating a favorable environment for archaea metabolism that is more sensitive to these chemicals [17].

The literature addressing BTPs in anaerobic systems remains sparse, primarily focusing on systems involving isolated bacteria strains or parental PPCPs concentrations significantly higher (mg L^{-1}) than those typically found in WWTPs (ranging from ng L^{-1} to μg L^{-1}). However, finding BTPs is a challenging task that needs to be addressed for holistically characterize these wastewaters [18], despite there is not well-standardized methodologies yet. Consequently, highly efficient sample preparation techniques, with high concentration capabilities are required, coupled with advanced high resolution mass spectrometry (HRMS)-based techniques. Therefore, the integration of target, suspect and non-target strategies becomes essential to achieve maximum reliability and confidence in the elucidation of the molecular structures of BTPs [19].

In a recent investigation, a through evaluation of the long-term operation of two reactors was conducted, focusing on the removal of PPCPs over time. The findings underscored the feasibility of employing phase separation as a strategy to enhance PPCPs biodegradation [20]. However, the total elimination efficiency of micropollutants in the anaerobic digestion is not sufficient to track the biotransformation pathways that occur in each stage (acidogenesis and methanogenesis) and elucidate the reaction mechanisms involved in the PPCPs removal. The present study aims to provide insights into the BTPs originating from PPCPs during anaerobic digestion, emphasizing the specific contributions of acidogenesis and methanogenesis in the biotransformation pathways for each compound. For that purpose, we compared two distinct systems – a single-phase methanogenic- and a two-phase acidogenic-methanogenic fixed-film bioreactor. We focused on ten PPCPs with a wide range of physicochemical properties and uses, which are ubiquitous and frequently detected in wastewater: carbamazepine, naproxen, diclofenac, ibuprofen, acetaminophen, metoprolol, sulfamethoxazole, ciprofloxacin, methylparaben and propylparaben. This study represents the pioneering effort to investigate biotransformation routes in continuous anaerobic two-phase acidogenic-methanogenic systems, opening up the way for enhancing the removal of BTPs from PPCPs in biological wastewater treatment plants.

2. Material and methods

2.1. Anaerobic bioreactors configuration and operating conditions

The experimental apparatus to evaluate the biotransformation of PPCPs was composed of two different lab-scale anaerobic fixed-film bioreactors (AFBR) configurations – a single-phase (SP-AFBR) methanogenic and a two-phase (TP-AFBR) sequential acidogenic-methanogenic (Ac-Mt) reactor (Fig. S1). The career for biomass adhesion consisted of polyurethane foam – apparent density of 23 g L^{-1}, specific surface area of 43.8 m^2 g^{-1}, and porosity of 92 % [21].

The two methanogenic reactors were inoculated with granular biomass from a full-scale UASB (Up-flow Anaerobic Sludge Blanket) reactor, while the acidogenic biomass was developed through acid treatment (pH 3, HCl 5 mol L^{-1}) and organic load shock (24 g-COD L^{-1} d^{-1}) during two days to favor the selection of fermentative bacteria [22]. Both systems (SP- and TP-AFBR) were operated with a hydraulic retention time of 12 h (6 h in the Ac-AFBR and 6 h in the Mt-AFBR), temperature controlled at 30 °C, and organic loading rate of 2.3 ± 0.2 g-COD·L^{-1}·d^{-1}. The bioreactors were continuously fed for 302 days with a substrate whose composition is detailed in Table S1. The operation of the reactors was divided into two operational stages – a control phase without the addition of the selected PPCPs (phase I - 133 days), and another with the addition of PPCPs (phase II - 169 days).

2.2. Target PPCPs and reference standards

The selected PPCPs are micropollutants of different therapeutic classes and physicochemical properties (log K_{ow} ranging from 0.5 to 4.0, as detailed in Table S2). They are frequently found in municipal wastewater, whose main route of contamination is through human usage. These chemicals serve as representative examples of their respective classes, namely - the antiepileptic drug carbamazepine (CBZ); the non-steroidal anti-inflammatory drugs naproxen (NPX), diclofenac (DCF), ibuprofen (IBU), and acetaminophen (ACT); the beta-blocker metoprolol (MTP); the antibiotics sulfamethoxazole (SMX) and ciprofloxacin (CIP); and the preservatives methylparaben (MPB) and propylparaben (PPB).

All analytical standards used for analysis were of high purity grade (>95 %). Reference isotopically labeled internal standards (IS) were also acquired to correct the matrix effect of the samples in the different bioreactors and samples during the analytical step, and they were listed in Table S3. The feeding substrate for the bioreactors was prepared twice a week, and at the end of each preparation, the PPCPs were spiked to achieve a target concentration of 10 μg L^{-1}. For this, a stock mix solution of PPCPs was prepared by dissolving the pure analytes at a concentration of 100 mg L^{-1} and stored in amber flasks at −20 °C until the time of spiking in the reactor feed medium.

2.3. Sample collection and preparation

Liquid samples from both the influent and effluent of each bioreactor were systematically collected twice a week. This frequent sampling routine allowed for a comprehensive evaluation of the variation in PPCPs removal over time. Additionally, at the conclusion of each operational phase, samples were acquired specifically for the purpose of analyzing BTPs.

First, the liquid samples underwent sequential double filtration (1.2 μm and 0.45 μm), and the pH was adjusted to 6.5 ± 0.2 using formic acid or ammonium acetate for a final volume of 100 mL. Then, a solid phase extraction (SPE) procedure was performed utilizing HLB cartridges (Oasis HLB, 200 mg, 6 mL). These cartridges were pre-conditioned with 6 mL of methanol (HPLC-grade) followed by 6 mL of ultrapure water. Subsequently, the samples were passed through the extraction cartridges with the aid of a vacuum system, ensuring an approximate flow rate of 2 drops per second. Subsequent to sample loading, a washing step was conducted using 12 mL of ultrapure water and then the cartridges were dried under a vacuum (~55 kPa) for 15 min. Elution was carried out by passing 6 mL of methanol, and the resulting extract was dried under a gentle flow of nitrogen gas. The dried extract was reconstituted with 500 μL de methanol, and prior to the day of MS analysis, 500 μL of ultrapure water and a pool IS solution was added to a concentration of 20 μg L^{-1}.

For the analysis of BTPs sorbed on the biomass, 0.25 g of sludge from each reactor was subjected to an ultrasonic solvent extraction, following a procedure described elsewhere [23]. First, 2 mL of methanol was added to the biomass, which was vigorously stirred by vortex, and then 15 min of ultrasonication followed by 5 min of centrifugation at 10,000 rpm were performed. The supernatant extract was stored in glass tubes. The same procedure was repeated three more times – once with 2 mL of methanol and twice with 2 mL of acetonitrile. The final 8 mL- extract was dried completely by evaporating flowing nitrogen gas and resuspended in 50 mL of ultrapure water. Finally, for the analysis of the BTPs, the same protocol described for the liquid samples was followed.

Procedural blank samples, consisting of ultrapure water, were processed following all aforementioned steps detailed. This comprehensive procedure aimed to ensure that there was no background contamination during the sample treatment [24]. Additionally, the influent and effluent samples without the addition of PPCPs (operational phase I) were used as a negative control to confirm that the potentially identified BTPs come from the target PPCPs and not from other metabolic sources.

2.4. Instrumental analysis of PPCPs and their BTPs

Instrumental analysis was carried out using a Waters Acquity UHPLC system coupled to a Q-Exactive QOrbitrap instrument (Thermo Scientific) with an electrospray interface (ESI). Acquisition of 10 μL of samples was performed using both data-independent (DIA, low energy function at 4 eV and high energy function at 35 eV collision energy) and data-dependent modes (DDA, scan at 4 eV with 5 MSMS acquisition per scan at 35 eV collision energy) in both positive (PI) and negative (NI) ionization modes. Chromatographic separation for both PI and NI was using a CORTECS C18 column (2.1 ×100, 2.7 μm). Gradient for PI was using 0.1 % formic acid solution as aqueous phase and MeOH 0.1 % formic acid solution as organic phase (B), while for NI was using both H_2O (aqueous) and MeOH (organic phase, B) with 5 mM ammonium acetate. Gradient in PI started with 5 % B, until 75 % at min 7, 100 % at min 10, kept at 100 % until min 17 and down to 5 % at min 18, with a total run time of 20 min. For NI gradient started at 5 % B, with 50 % at min 3, 90 % at min 6, 100 % at min 13, kept at 100 % until min 17 and down to 5 % in min 18, with a total run time of 20 min

The Orbitrap system was equipped with an electrospray ionization interphase (ESI), operating at 3000 V in positive and 2800 V in negative ionization modes, 350 °C capillary temperature, 40 sheath gas flow, 10 auxiliary gas flow, 100 of maximum spray current, 350 °C probe heater temperature and 60 SLens RF level, with a full scan mass spectra recorded over the range of 67–1000 *m/z* with a resolving power of 60000, while for MSMS resolving power was 30,000.

2.5. Suspect screening performance

Initially, a list of suspected potential BTPs was compiled based on previous literature that explored the biodegradation of specific PPCPs targeted in our study (Table S4). These investigations encompassed a variety of biological systems, including aerobic, anaerobic, or anoxic redox conditions. These studies involved pure strains or mixed cultures, with batch or continuous bioreactors and considered a wide range of initial concentration of PPCPs (from μg L^{-1} to mg L^{-1}). Using this list as a starting point, we manually searched their monoisotopic masses in the LC-HRMS raw data. For those where the mass of the parent ion was found, spectra (retrieved from the DDA) were compared with the one available in online databases (i.e., mzCloud and Massbank).

2.6. Nontarget screening with Compound Discoverer software

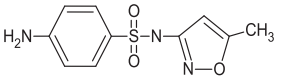
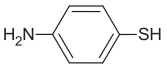
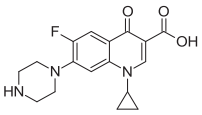
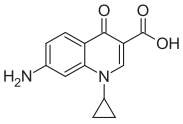
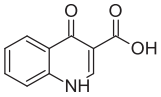
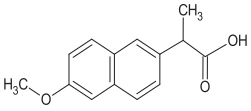
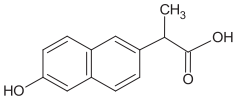
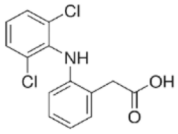
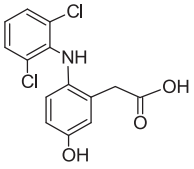
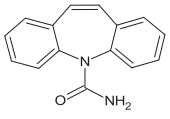
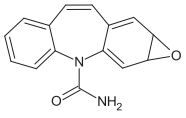
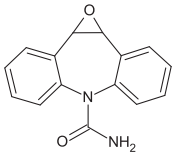
Following the examination of the comprehensive list of possible BTPs, we aimed to perform a nontarget analysis using Compound Discoverer (Thermo Scientific) in order to identify additional BTPs that were not previously reported. Detailed information including the used parameters are available in the **Section S6** of Supplementary Material. All potential candidates were also obtained from DDA since our goal was to obtain clean spectra to avoid as maximum as possible false positive assignations.

3. Results and discussion

3.1. Identification of BTPs: molecular formula and proposed chemical structure

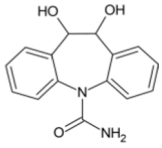
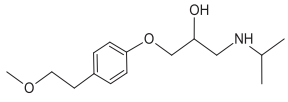
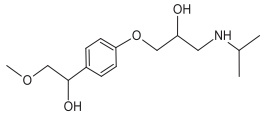
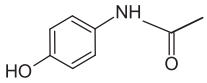
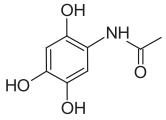
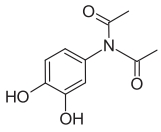
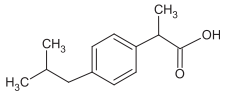
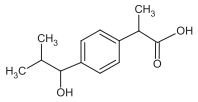
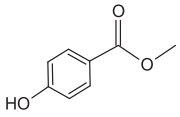
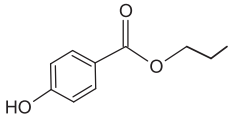
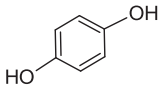
Fourteen BTPs were successfully and confidently identified from the ten investigated parent PPCPs. The suggested molecular structures are detailed in Table 1. Notably, using a suspect screening strategy based on the list of protonated ($[M+H]^+$) and deprotonated ($[M-H]^-$) masses of suspected potential metabolites reported in previous literature (Table S4), twelve BTPs were tentatively identified. In addition to the suspect approach, employing a nontarget strategy revealed an additional pair of BTPs, concretely BTP-183 and BTP-209. The MS/MS spectra of each BTP are listed in Fig. S3.

Table 1
Summary of the BTPs identified during anaerobic digestion in the SP- and TP-AFBR, including their chromatographic retention time (Rt), mass of the protonated or deprotonated adduct, molecular formula, and suggested chemical structure.

PPCP – parent compound	BTP Code	Rt (min)	Monoisotopic Mass	Adduct *m/z*	Formula	Chemical Structure
Sulfamethoxazole (SMX)	-	3.9	253.0521	$[M+H]^+$ 254.0594	$C_{10}H_{11}N_3O_3S$	
SMX	BTP−125	2.0	125.0299	$[M+H]^+$ 126.0372	C_6H_7NS	
SMX	BTP−126	3.4	126.0139	$[M+H]^+$ 127.0212	C_6H_6OS	
Ciprofloxacin (CIP)	-	3.0	331.1332	$[M+H]^+$ 332.1405	$C_{17}H_{18}FN_3O_3$	
CIP	BTP−244	3.7	244.0848	$[M+H]^+$ 245.0920	$C_{13}H_{12}N_2O_3$	
CIP	BTP−189	4.5	189.0426	$[M+H]^+$ 190.0499	$C_{10}H_7NO_3$	
Naproxen (NPX)	-	7.4	230.0943	$[M+H]^+$ 231.1016	$C_{14}H_{14}O_3$	
NPX	BTP−216	3.1	216.0786	$[M-H]^-$ 215.0714	$C_{13}H_{12}O_3$	
Diclofenac (DCF)	-	5.2	295.0167	$[M-H]^-$ 294.0094	$C_{14}H_{11}Cl_2NO_2$	
DCF	BTP−311	4.3	311.0116	$[M-H]^-$ 310.0043	$C_{14}H_{11}Cl_2NO_3$	
Carbamazepine (CBZ)	-	6.3	236.0950	$[M+H]^+$ 237.1022	$C_{15}H_{12}N_2O$	
CBZ	BTP−252	5.6	252.0899	$[M+H]^+$ 253.0972	$C_{15}H_{12}N_2O_2$	
CBZ	BTP−252 −2	5.3	252.0899	$[M+H]^+$ 253.0972	$C_{15}H_{12}N_2O_2$	

(*continued on next page*)

Table 1 (*continued*)

PPCP – parent compound	BTP Code	Rt (min)	Monoisotopic Mass	Adduct *m/z*	Formula	Chemical Structure
CBZ	BTP−270	5.9	270.1004	$[M+H]^+$ 271.1077	$C_{15}H_{14}N_2O_3$	
Metoprolol (MTP)	-	3.3	267.1834	$[M+H]^+$ 268.1907	$C_{15}H_{25}NO_3$	
MTP	BTP−283	3.0	283.1784	$[M+H]^+$ 284.1856	$C_{15}H_{25}NO_4$	
Acetaminophen (ACT)	-	2.4	151.0633	$[M+H]^+$ 152.0706	$C_8H_9NO_2$	
ACT	BTP−183	1.7	183.0532	$[M+H]^+$ 184.0608	$C_8H_9NO_4$	
ACT	BTP−209	4.4	209.0688	$[M+H]^+$ 210.0766	$C_{10}H_{11}NO_4$	
Ibuprofen (IBU)	-	5.4	206.1307	$[M-H]^-$ 205.1234	$C_{13}H_{18}O_2$	
IBU	BTP−222	5.1	222.1256	$[M-H]^-$ 221.1183	$C_{13}H_{18}O_3$	
Methylparaben (MPB)	-	4.2	152.0473	$[M-H]^-$ 151.0401	$C_8H_8O_3$	
Propylparaben (PPB)	-	5.4	180.0786	$[M-H]^-$ 179.0714	$C_{10}H_{12}O_3$	
MPB, PPB, ACT, IBU	BTP−110	2.8	110.0368	$[M+H]^+$ 111.0441	$C_6H_6O_2$	

The confirmation of the structure of each formed BTP was based on the similarities with fragmentation patterns found in databases such as Massbank and MzCloud [25,26]. For instance, Fig. S4 shows the fragmentation pattern of the BTP-189, also known as kynurenic acid or hydroxyquinaldic acid, in this study and in Massbank. This BTP presents two main fragments with *m/z* 162.0547 and 144.0440 ($[M+H]^+$) (https://massbank.eu/MassBank/RecordDisplay?id=MSBNK-Fiocruz-FIO00645&dsn=Fiocruz), which coincide with the neutral losses CO and H_2O, respectively, leading to confirmation of this compound as a BTP.

Analogously, hydroxy-diclofenac (BTP-311), presents the *m/z* fragments 266.0146 and 230.0379 (https://massbank.eu/MassBank/RecordDisplay?id=MSBNK-Eawag-EQ339752&dsn=Eawag), referring to the neutral losses of CO_2 (carboxylic acid) and Cl (Fig. S3j). Other compounds that showed a high degree of similarity with previously reported MS2 spectra were: BTP-252–2 (epoxy-carbamazepine - https://massbank.eu/MassBank/RecordDisplay?id=MSBNK-Eawag-EA091614&dsn=Eawag) (Fig. S3m), BTP-270 (dihydroxy-carbamazepine - https://massbank.eu/MassBank/RecordDisplay?id=MSBNK-Athens_Univ-AU266801&dsn=Athens_Univ) (Fig. S3n), and BTP-222 (hydroxy-ibuprofen - https://www.mzcloud.org/dataviewer.aspx#Creference7300#T11554#c#2683164) (Fig. S3u).

The confirmation of the identified BTPs was also supported by a

comprehensive interpretation of the MS/MS spectra, as depicted in Fig. S3. Examples of characteristic fragmentation patterns for specific BTPs are further described. SMX shows a typical fragmentation pattern with the neutral loss of $C_4H_6N_2O$ (Fig. S3a), which represents a cleavage of the isoxazole ring and a loss of amine in the parent molecule. This results in the most intense first fragment *m/z* 156.01158, which loses an SO and SO_2 bonds to generate the *m/z* fragments 108.0448 and 92.04959, respectively. BTP-126 (4-hydroxythiophenol) exhibits CO and SH_2 neutral losses to generate the most intense fragments of *m/z* 99.02638 and 65.03884, respectively (Fig. S3c). Another interesting example is a typical fragmentation pattern of the NPX molecule (Fig. S3g), which undergoes decarboxylation and demethylation, to generate *m/z* fragments 185.09645 and 170.07288, respectively. BTP-216 (demethyl-naproxen) loses a molecule of CO_2 (carboxylic acid) to generate the fragment with the highest intensity *m/z* 171.08145 (Fig. S3h).

3.2. Elucidation of the BTPs biotransformation pathways and reactions mechanisms

From the tentatively identified BTPs, it was feasible to propose potential biotransformation pathways for each compound among the identified BTPs. These pathways emphasize the primary chemical reactions involved, and they are visually represented in Fig. 1.

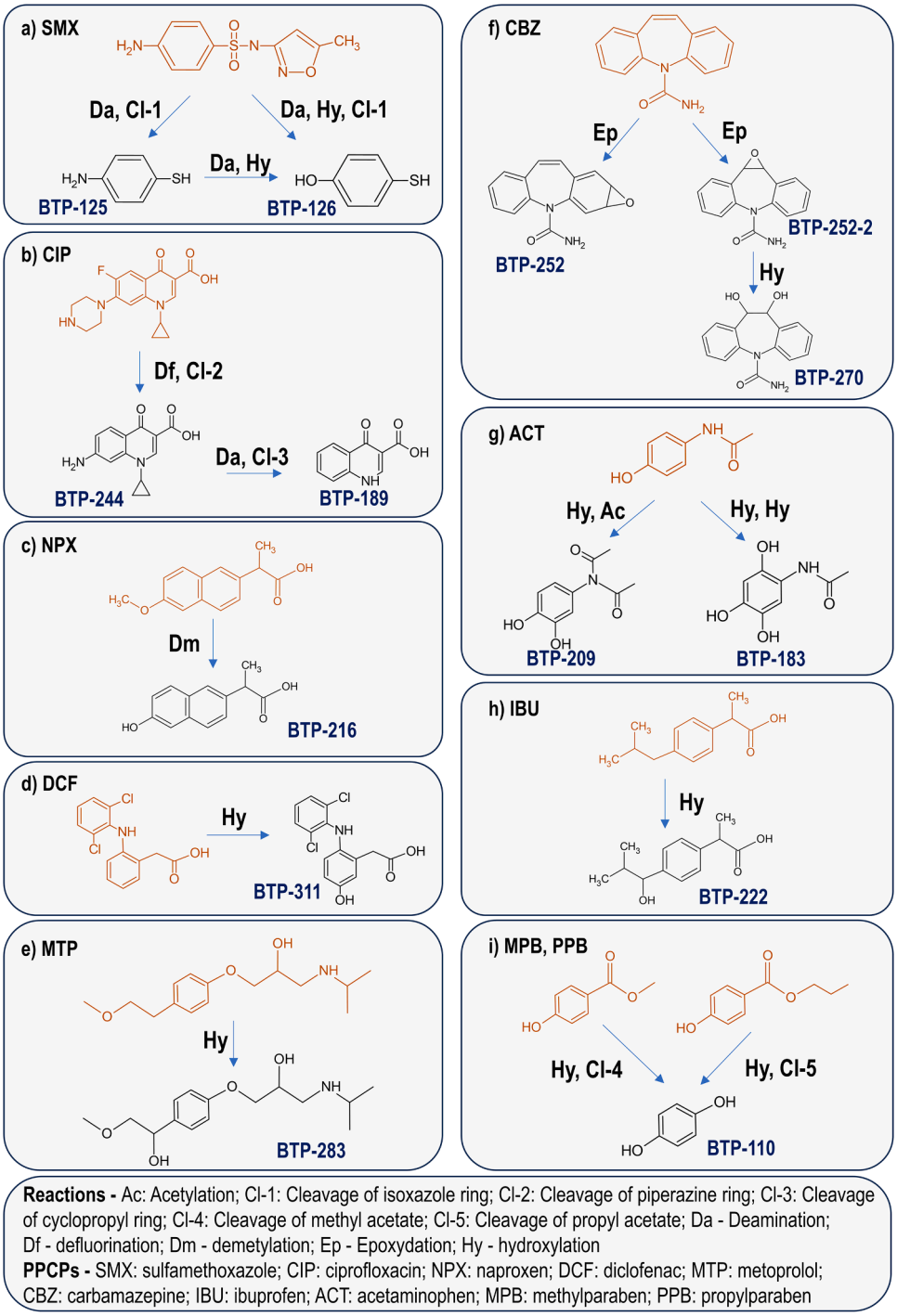


Fig. 1. Proposed biotransformation pathways for the studied PPCPs. The arrows highlight the key reactions, with detailed information provided in the caption.

For SMX (Fig. 1a), both BTPs, namely BTP125 and BTP126, originate from the cleavage of the isoxazole ring and deamination in the parent molecule. Also, BTP-126, could be derived from the hydroxylation of BTP-125 or SMX, with the hydroxylation process potentially linked to the intracellular enzyme cytochrome action P450 (CYP450) [27]. These BTPs were previously reported in anaerobic systems. BTP125, also known as 4-aminothiophenol, was identified by Wei et al. [28] as a product arising from the deamination of sulfanilamide within an anaerobic membrane bioreactor. BTP-126 (sulphanylphenol) was reported by Tang et al. [29] as a consequence of oxidative deamination and N-S bond cleavage in the SMX molecule. The cleavage and anaerobic degradation of the isoxazole ring under anaerobic conditions have been documented in several works [30–34], providing evidence for the biotransformation capability of SMX.

For the biotransformation of CIP (Fig. 1b), initial observations reveal a cleavage of the piperazine ring and a defluorination in the formation of BTP-244 (7-amino-1-cyclopropyl-quinoline). These findings are consistent with those reported by Zhang et al. [35] across various environmental conditions, including aerobic, anaerobic, and sulfate-reducing conditions. BTP-190 is generated through the oxidative removal of the cyclopropyl group and a deamination of BTP-244. This metabolite has been previously reported by Pan et al. [36] during the biodegradation of CIP driven by *Thermus sp.* isolated from pharmaceutical sludge. Jia et al. [33] assessed the CIP biodegradation in an anaerobic sulfate-reducing bacteria sludge system and identified the main mechanisms of biotransformation, including the cleavage of the piperazine ring, oxidative defluorination and hydroxylation. Interestingly, there was no subsequent cleavage of the quinolone structure, which was found to be recalcitrant in the anaerobic process.

In the context of non-steroidal anti-inflammatory drugs (NSAIDs), specifically NPX (Fig. 1c), a demethylation process was identified in the parent molecule. This demethylation is catalyzed by demethylases from various enzyme families, including the likely involvement of tetrahydrofolate-dependentO-demethylase [37]. Lu et al. [38] also observed the generation of this metabolite during NPX biodegradation by *Pseudoxanthomonas sp.* DIN-3, isolated from a biological activated carbon process. For other NSAIDs such as DCF, IBU, and ACT, hydroxylation reactions were observed, leading to the formation of BTPs 311 (Fig. 1d), 222 (Fig. 1h), 209, and 183 (Fig. 1g). Luis Malvar et al. [39] detected hydroxy-diclofenac (BTP-311) and hydroxy-ibuprofen (BTP-222) in anaerobically-digested sludge. Additionally, Poirier-Larabie et al. [40] and Jia et al. [41] comparatively evaluated the degradation of DCF and IBU, in anaerobic and aerobic conditions, respectively. In both studies, BTPs 311 and 222 were only detected in the aerobic condition due to heterotrophic oxidation. Nevertheless, in the anaerobic environment, BTPs were not detected by the negligible biodegradation of the studied pharmaceuticals (removal efficiency <10 %). BTPs 183 (dihydroxy-acetaminophen) and 209 (acetyl-hydroxy-acetaminophen) derived from ACT have not been previously reported, to the best of our knowledge. Specifically, for BTP-209, in addition to hydroxylation, an acetylation reaction was observed in the molecule. This acetylation is commonly detected in the anaerobic generation of metabolites such as acetyl-sulfamethoxazole [42], or acetyl-sulfanilamide [28,29]. Other BTPs from ACT biotransformation have been previously reported (Table S4), but were not identified in our study. ACT generally has a high removal efficiency in wastewater treatment plants [43–45], showing that PPCPs can present different biotransformation pathways, depending on the type of biological treatment applied and the microorganisms involved in the process.

The BTPs of CBZ (Fig. 1f), identified as 10,11-dihydro-10,11-epoxy-CBZ (EP-CBZ – BTP-252–2) and 10,11-dihydro-10,11-dihydroxy-CBZ (DiOH-CBZ – BTP −270) are commonly reported metabolites resulting from the epoxidation and subsequent hydroxylation of CBZ. This occurs through various biological processes involving bacterial strains [46], fungi [47], the human body [48], and organisms such as zebrafish [49] or trout [50]. Miyata-Nozaka et al. [48] pointed out that the first epoxidation reaction is likely catalyzed by cytochrome P450 (CYP) enzymes, with the predominant isoforms being CYP3A4 and CYP3A5. Furthermore, EP-CBZ undergoes further metabolization by epoxide hydrolase to produce DiOH-CBZ. While the isomeric form of epoxy-CBZ (BTP-252) was evidenced in this study, with a fragmentation pattern quite similar to that of BTP-252–2 but at different chromatographic retention times, it should be noted that this particular BTP has not yet been previously reported in the literature.

In the case of MPB and PPB, a consistent biotransformation pattern was observed (Fig. 1**i**). In this process, the parent molecule is cleaved at the same C-C position, resulting in the loss of a methyl-acetate and propyl-acetate, respectively. Subsequently, hydroxylation occurs, leading to the formation of hydroquinone (BTP-110). While this BTP has been previously reported as a metabolite of parabens degradation [51], it has also been identified in the degradation of acetaminophen [52,53] and ibuprofen [37]. Therefore, its presence in anaerobic bioreactors may be attributed to any of these PPCPs, requiring further studies to elucidate the main route of its generation. The biodegradation of MPB and PPB in biological systems by aerobic mixed microbes has been associated with the formation of p-hydroxybenzoic acid, which is further biotransformed to benzoic acid and phenol [54]. While benzoic acid was identified as a metabolite in the analyzed samples, it was discarded as BTP because it was also detected in the negative control samples of the reactors (without the addition of PPCPs). This suggests that this chemical may originate from different metabolic pathways.

In the case of beta-blocker MTP, hydroxylation was also observed in the molecule resulting in the formation of BTP-283, or hydroxy-MTP (Fig. 1e). The presence of this compound was documented by Souchier et al. [55] and Rubirola et al. [56] in urban WWTPs with an aerobic biological system, being fully biodegraded. In summary, considering all the compounds studied, the anaerobic biotransformation of PPCPs predominantly led to oxidation reactions. These reactions included hydroxylation, deamination, demethylation and epoxidation. Notably, hydroxylation was the most common occurring reaction, since it can be driven by multiple microbial enzymes [57].

3.3. BTPs generated during acidogenesis and methanogenesis

Fig. 2 illustrates the variation in chromatographic area observed in the influent (Inf.) and effluent (Eff.) samples from both single-phase (SP) and two-phase (TP) bioreactors – Inf. SP, Eff. SP, Inf. TP, Eff. Ac-TP, Eff. Mt-TP. The values used to generate the graphs in Fig. 2 are detailed in Table S5. Most of the studied PPCPs exhibited high removal rates in both systems (>90 %) – SMX, CIP, NPX, MTP, ACT, MPB, PPB, while DCF, CBZ and IBU showed higher persistence, with removal efficiencies of less than 50 %. Indeed, these three pharmaceuticals have shown high recalcitrance (biodegradation efficiency ranging from null to 20 %) in different anaerobic treatment processes, including anaerobic sludge digesters [43,58], UASB [59,60], anaerobic membrane bioreactors [61,62], and anaerobic fluidized membrane bioreactors [63]. Comparison between SP and TP anaerobic fixed-film bioreactors revealed noticeable differences in removal percentages for DCF (SP: 11 %, TP: 42 %), CBZ (SP: 28 %, TP: 40 %), IBU (SP: 14 %, TP: 47 %), indicating that the acidogenic phase of TP bioreactors significantly contributes to the removal of these compounds. It is noteworthy that the addition of PPCPs did not affect the performance of the bioreactors in terms of organic matter removal, as can be seen in Fig. S5.

For the previously mentioned highly biodegradable compounds (SMX, CIP, NPX, MTP, ACT, MPB, PPB), it is also verified that acidogenesis is a crucial step in their biotransformation. This is evidenced when comparing the removal of compounds in the acidogenic bioreactor (Ac-TP-AFBR) with the removal in the SP-AFBR (Fig. 2). The results show very similar trends, except for MPB and PPB, where acidogenic removal represented approximately 48 % of that observed in the SP reactor.

Certain BTPs are generated during the acidogenic phase and

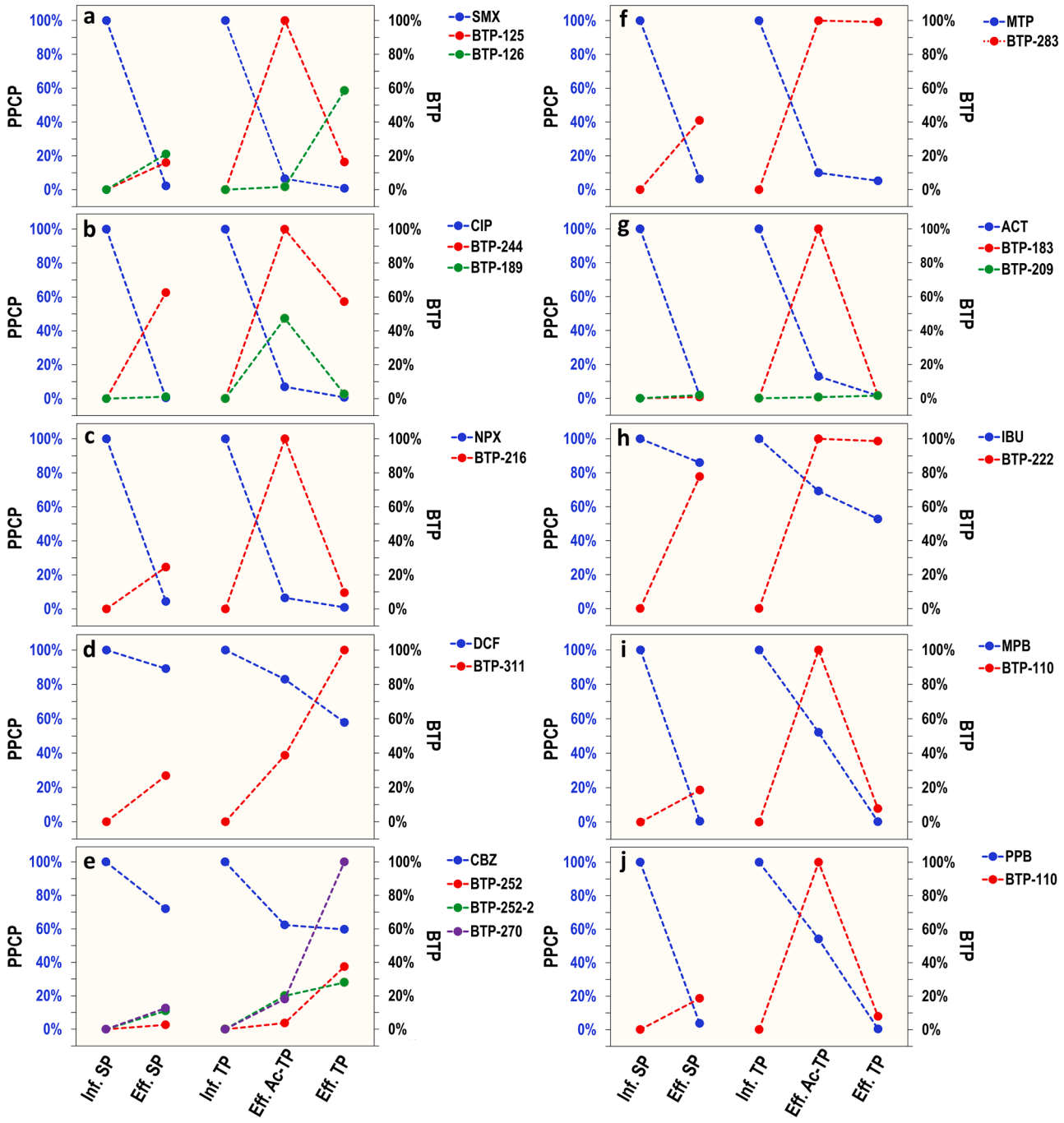


Fig. 2. Relative variation of PPCPs and their respective BTPs in the influent (Inf.) and effluent (Eff.) samples of each system – single-phase (SP) and two-phase (TP) (Acidogenic – Ac + Methanogenic – Mt) anaerobic bioreactors.

subsequently utilized during methanogenesis. Specifically, the removal efficiencies in the TP-methanogenic reactor and their respective parent compound were as follows: BTP-125 (84 %, SMX), BTP-244 (43 %, CIP), BTP-189 (94 %, CIP), BTP-216 (90 %, NPX), BTP-183 (98 %, ACT), and BTP-110 (92 %, MPB, PPB). This utilization process effectively diminishes the concentrations of these BTPs, reducing them to levels equivalent to or slightly lower than those observed in the effluent of the SP-reactor. In the SP-reactor, the acidogenic stage is not as evident due to the syntrophic associations that occur between fermentative bacteria and methanogenic archaea [64] driving the biological reactions for the biomethane production, and therefore not allowing the accumulation of volatile fatty acids in the system. In any case, as acidogenesis occurs in both systems (SP and TP-reactor), the presence of these BTPs was observed in both reactors.

Another subset of BTPs was generated in the acidogenic condition but exhibited a concentration increase due to phase separation within the TP-reactor effluent (Eff. Mt-TP). This group comprises BTP-126 (SMX), BTP-311 (DCF), BTP-252 (CBZ), BTP-252–2 (CBZ), BTP-270 (CBZ), and BTP-209 (ACT). Oxidation through hydroxylation during methanogenesis may be directly related to enzymes linked to the oxidation of short chain volatile fatty acids (valeric, butyric and propionic, as can be seen in Fig. S6) to acetate during acetogenesis [65]. The formation of BTP-209 in the TP-reactor could potentially be linked to the enzymatic activity of acetate kinase (EC 2.7.2.1) which is active in phosphate acetylation during acetoclastic methanogenesis [66]. Finally, a third group of BTPs was generated during acidogenesis but remained

unaffected by the methanogenesis: BTP-283 (MTP) and BTP-222 (IBU).

Analyzing the identified BTPs in the sludge of each bioreactor (Table S5), most of the compounds either were not detected in the sludge biomass or were found in only a negligible fraction of the parent compound. Therefore, the sorption mechanism of the PPCPs on the sludge was not preponderant for their biotransformation pathways.

The results proved that acidogenesis plays a pivotal role in the biotransformation of PPCPs during anaerobic digestion, as evidenced by the generation of all BTPs under acidogenic conditions (Fig. 2). The separation into acidogenesis and methanogenesis phases enabled targeted enhancement of fermentative and methanogenic microbial enzymes, respectively. These enzymes facilitate the oxidation of organic matter and PPCPs through reactions such as hydroxylation, demethylation, acetylation, epoxidation, and cleavage.

4. Conclusions

The adopted analytical strategy by applying suspect and non-target analysis proved to be feasible to accurately detecting fourteen BTPs during acidogenesis and methanogenesis. The biotransformation of PPCPs was closely linked to the fermentative production of organic acids, as all BTPs were identified in the acidogenic condition. Some of these were further consumed during methanogenesis, such as aminothiophenol, demethyl-naproxen and hydroquinone. However, others were enhanced by methanogenic enzymatic action, for example sulphanylphenol, hydroxy-diclofenac and epoxy-carbamazepine. The biotransformation pathways identified basically referred to oxidation reactions, such as hydroxylation, deamination, demethylation and epoxidation. The phase separation of anaerobic digestion into acidogenesis and methanogenesis showed promise for improving the PPCPs biodegradation in wastewater, especially the more recalcitrant compounds like carbamazepine, ibuprofen and diclofenac. However, it is essential to closely monitor potential risks associated with the biotransformation products generated in this system.

Environmental Implication

This study addresses the biotransformation pathways of ten widespread contaminants of emerging concern, including some highly used pharmaceuticals and personal care products (PPCPs), during the acidogenic and methanogenic phases of anaerobic digestion. Their fate in biological treatment systems is an issue of relevant importance, as these compounds have notable impacts on the environment. In our work, the phase separation into acidogenesis and methanogenesis have provided a deeper understanding of the biotransformation pathways of PPCPs, clarifying the contribution of each phase to the generation of their respective biotransformation products, as well as enhancing their elimination in specific cases.

CRediT authorship contribution statement

Rodrigo B. Carneiro: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Rubén Gil-Solsona:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Jessica Subirats:** Writing – review & editing. **Esteban Restrepo-Montes:** Methodology, Investigation. **Marcelo Zaiat:** Resources, Writing – review & editing, Funding acquisition. **Álvaro J. Santos-Neto:** Conceptualization, Resources, Supervision, Funding acquisition. **Pablo Gago-Ferrero:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Data availability

Data will be made available on request.

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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.2024.135444.

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