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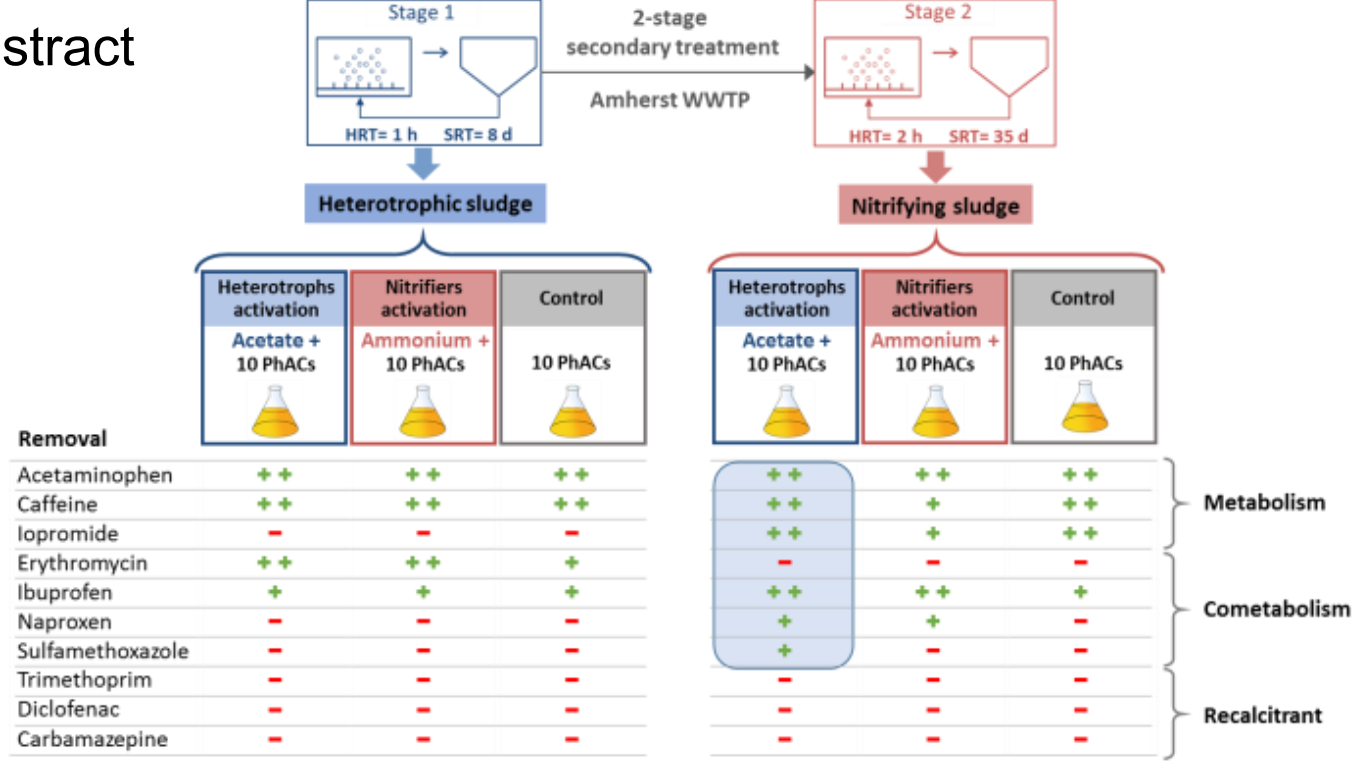
# Graphical Abstract



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Lorena Gonzalez-Gil[1,2‡], Eduardo Fernandez-Fontaina[1,3‡], Randolph R. Singh[3], Juan M. Lema[1], Marta Carballa[1*] and Diana S. Aga[3]

[1]CRETUS Institute, Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2]Defence University Centre, Spanish Naval Academy, Plaza de España, 36920, Marín, Spain

[3]Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY, 14260, United States

[‡] Both authors have equally contributed to this work

*Corresponding author: Marta Carballa (marta.carballa@usc.es)

## HIGHLIGHTS

- The role of heterotrophs and nitrifiers on the (co-)metabolism of PhACs was explored.
- Longer SRT-sludge has a higher capacity to biotransform most of the selected PhACs.
- Feeding composition affects biological activities and thus the co-metabolism of PhACs.
- Slow-growing heterotrophs are crucial for the biotransformation of some PhACs.

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[1]CRETUS Institute, Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

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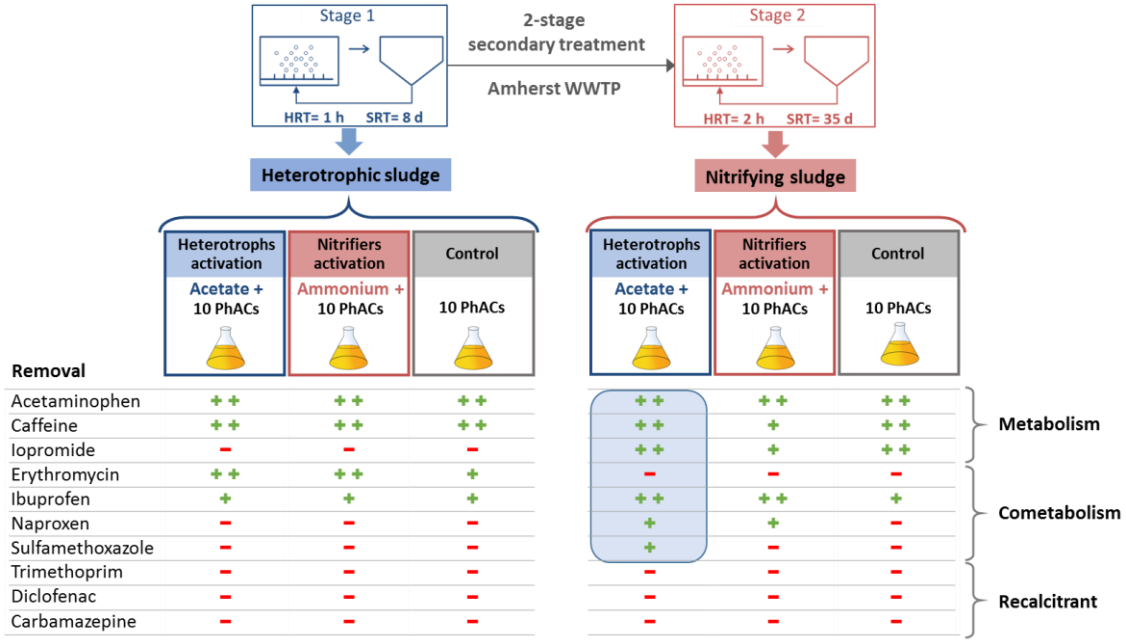
## GRAPHICAL ABSTRACT

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- Acetaminophen, caffeine, and iopromide could be growth substrates of heterotrophs.

**ABSTRACT**

The role of heterotrophic and nitrifying microorganisms in the (co-)metabolic biotransformation of 10 pharmaceutically active compounds (PhACs) was investigated. To this aim, biotransformation assays were performed with heterotrophic and nitrifying sludge developed separately in a two-stage full-scale activated sludge system. Each stage was operated at different inflow wastewater characteristics and sludge retention times (on average 8 d and 35 d). The biotransformation capacity of each sludge was evaluated in the absence of primary substrate and in the presence of acetate and ammonium, to independently elucidate the co-metabolic role of heterotrophs and nitrifiers present in both sludges. Trimethoprim, diclofenac and carbamazepine were recalcitrant (removal < 5% after 1 d; biotransformation rate < 50 µg/g VSS·d) under all the tested conditions. High concentrations of caffeine, acetaminophen and iopromide were quickly biotransformed (> 80% after 1 d; > 800 µg/g VSS·d) in the absence of primary substrates. The heterotrophic sludge only showed a co-metabolic effect towards erythromycin, which increased its biotransformation rate between 43-53% when acetate and ammonium were supplied. In contrast, when stimulated, nitrifiers and slow-growing heterotrophs present in the nitrifying sludge co-metabolically biotransformed acetaminophen, ibuprofen and naproxen to a significant extent. Sulfamethoxazole was recalcitrant, except when the nitrifying sludge was fed with acetate (> 800 µg/g VSS·d), suggesting that slow-growing heterotrophs co-metabolically biotransformed it. This study provides evidence that biotransformation of PhACs depends on several metabolic activities, as the heterotrophic activity of the nitrifying sludge, which are not only determined by the SRT but also by the feeding composition.

**KEYWORDS:** biodegradation; heterotrophs; cometabolism; nitrifiers; organic micropollutants; wastewater treatment plant.

## 1. INTRODUCTION

Organic micropollutants, such as pharmaceutically active compounds (PhACs), are continuously being released into the environment from effluents of wastewater treatment plants (WWTPs) (Tran et al., 2018). Despite their relatively low concentrations, these compounds pose deleterious ecological effects. For instance, the persistence of antibiotics in the environment has been linked to the emergence of antibiotic-resistant bacteria and the spread of resistance genes in the environment (Pruden et al., 2013). The presence of these pollutants in drinking water sources is also of concern and concentration limits will likely be established in the near future (EC, 2018; EPA, 2016). Consequently, their release through WWTP effluents will be also restrained.

Significant research efforts have been conducted to tackle the removal of organic micropollutants in WWTPs. The identification and isolation of different bacterial strains capable of degrading several organic micropollutants co-metabolically in the presence of primary substrates (Larcher and Yargeau, 2011; Almeida et al., 2013; Liu et al., 2013; Zhou et al., 2013; Men et al., 2017) or metabolically, as growth substrate (De Gusseme et al., 2011; Summers et al., 2012; Zhang et al., 2013), suggest that the composition of microbial communities in WWTPs can influence the pathway and extent of micropollutant biotransformation. However, the underlying biological mechanisms (metabolism or co-metabolism) and the driving factors behind the biotransformation of most organic micropollutants in activated sludge reactors are still unclear (Fischer and Majewsky, 2014; Nsenga and Meng, 2019), hampering the development of models to predict their removal efficiencies and concentrations in WWTP effluents (Tran et al., 2013).

Several studies have investigated the influence of operational parameters and the type of biomass on the biotransformation of micropollutants in activated sludge systems (Suárez et al., 2010; Khunjar et al., 2011; Fernandez-Fontaina et al., 2012; Achermann et al., 2018;

Lakshminarasimman et al., 2018; Tran et al., 2018; Kennes-Veiga et al., 2021). Yet, most of the differences found in their removal efficiency remain unexplained (Tran et al., 2018). Part of these variabilities has been attributed to the change of the sludge retention time (SRT). Operating activated sludge reactors at longer SRT (Vieno et al., 2007; Suarez et al., 2010) results in more diverse bacterial populations and a higher proportion of autotrophic ammonia oxidizing microorganisms (AOMs) due to their low specific growth rates. Along with the SRT, the feeding composition (e.g., C:N ratio and degradability of the carbon source) and the hydraulic retention time (HRT) are key for the growth of the different microbial populations, as they determine organic and nitrogen loading rates, and consequently the relative composition of heterotrophs and nitrifiers in activated sludge (Gallé et al., 2019; Nguyen et al., 2021). In addition, the type of growth substrate may alter the catabolic enzymes expressed by a community, thus influencing the metabolic and co-metabolic capacity to biotransform PhACs (Nguyen et al., 2021). Nevertheless, the effect of the feeding composition is by far the less unexplored parameter in activated sludge systems.

Nitrifying conditions (long SRT and low C:N ratio) seem to be more effective than heterotrophic conditions (short SRT and high C:N ratio) for the biotransformation of some micropollutants (Suarez et al., 2010; Fernandez-Fontaina et al., 2012), particularly those that undergo oxidative transformations (Helbling et al., 2012). Although most studies associate biotransformation with the main metabolic activity of the sludge, according to some authors, the higher removal rates observed at high SRT cannot be strictly linked to the action of nitrifiers (Batt et al., 2006; Fernandez-Fontaina et al., 2012; Men et al., 2017; Achermann et al., 2018). Actually, some studies suggest that improved removal of PhACs in nitrifying reactors is not due to the presence of nitrifiers but to the expansion in the composition of the heterotrophic bacterial community developed (Falås et al., 2012; Fernandez-Fontaina et al., 2016). This is consistent with the ecological theory, which postulates that greater diversity

independent of metabolic function, associated with longer SRTs, correlates positively with overall rates of transformation (Johnson et al., 2015).

As highlighted by Tran et al. (2018), there are many PhACs with a removal range between 0-100%. This lack of reproducibility among WWTPs and activated sludge systems reveals that the biological mechanisms and factors behind the biotransformation of most PhACs remain unclear. In this regard, the present study aims to clarify the role of heterotrophic and nitrifying microorganisms developed at different operational conditions (i.e., SRT, HRT and feeding composition) on the metabolic or co-metabolic biotransformation of PhACs. Moreover, it is pursued to elucidate if heterotrophs or nitrifiers are also relevant in the biotransformation of PhACs when they do not represent the main microbial activity of the sludge. This is an essential knowledge to promote the biotransformation capacity of activated sludge systems and to predict the behaviour of PhACs in WWTPs.

## 2. MATERIALS AND METHODS

### 2.1 Selected organic micropollutants

All biotransformation assays were spiked with acetaminophen (ACM), caffeine (CAF), carbamazepine (CBZ), diclofenac (DCF), anhydro-erythromycin (ERY), ibuprofen (IBP), iopromide (IOP), naproxen (NPX), sulfamethoxazole (SMX) and trimethoprim (TMP). The methanol present in the spike of PhACs was evaporated completely before introducing the biomass into the corresponding experimental flask. Higher concentrations than those typically found in raw sewage were used (1 mg/L) to be able to assess if biotransformation could occur through metabolism or co-metabolism and to facilitate the identification of transformation products. According to Su et al. (2015), biotransformation kinetic rates would not be significantly impacted at these concentrations.

## 2.2 Activated sludge

The activated sludge used in this study was collected from a full-scale WWTP in the city of Amherst, NY, USA, treating more than 110 000 $m^3$/d. The water line has two aerobic biological units supplied with oxygen in series (scheme provided in Fig. S1 of in Supplementary Information): stage 1 removes organic matter (75% of the influent biological oxygen demand- $BOD_{inf}$ = 68 mg/L) and operates at a HRT of 1 h and SRT of 8 ± 4 d, while stage 2 has the purpose of achieving nitrification and removal of slowly biodegradable organic matter (BOD decreases approximately from 17 mg/L to 6 mg/L), and operates at a HRT of 2 h and SRT of 35 ± 14 d. Each stage has its own settler (to avoid mixing of both sludges) leading to the development of two different types of biomass. The activated sludge in stage 1 (AS1) will be predominantly heterotrophic, while the sludge in stage 2 (AS2) should develop highly nitrifying bacterial community.

Four samples of AS1 and AS2 were collected from the Amherst WWTP at different days to measure their heterotrophic and nitrifying activities. Within 6 h after collection, respirometric assays were performed in a 300 mL continuously stirred vessel, at room temperature (22 °C) and with activated sludge at similar concentrations to those found in stage 1 and 2 reactors (approximately 1.0 g VSS/L of AS1 and 0.5 g VSS/L of AS2). The activated sludge was previously aerated for 48 h to allow the complete removal of residual substrates. After saturation with air, the endogenous respiration rate was first determined before spiking the substrate for each assay. Then, 200 mg COD/L of sodium acetate or 20 mg N/L of ammonium chloride and 34 mg C/L of sodium bicarbonate were injected as substrate. The decrease in dissolved oxygen (DO) with time was monitored with a YSI Model 54A oxygen meter. Specific oxygen uptake rates due to acetate oxidation and to ammonia oxidation were calculated based on linear regression analysis to determine the heterotrophic and nitrifying activities, respectively.

### 2.3 Biotransformation assays

Three experiments with PhACs were performed with both activated sludges (AS1 and AS2) under different feeding conditions (Table S1). In total, six biotransformation assays were conducted in 2 L flasks (Fig. S1):

- **Control**. No primary substrate was added in this experiment to assess the biotransformation of PhACs in the absence of readily biodegradable substrates.
- **Activation of heterotrophs**. Sodium acetate was supplied as readily biodegradable organic substrate, applying organic loading rates of 921 and 150 mg COD/g VSS·d to the AS1 and AS2, respectively.
- **Activation of nitrifiers**. Ammonium chloride was added at nitrogen loading rates of 15 and 120 mg N/g VSS·d to AS1 and AS2, respectively. Sodium bicarbonate was added at a rate of 32 and 255 mg C/g VSS·d, respectively, in order to maintain a C:N ratio of 1:7.

In addition to these biotransformation assay, two control assays without PhACs were performed to evaluate their possible inhibitory effect over the activity of AS1 and AS2. The biomass concentration set in all the experiments (Table S2) were based on the real concentration measured in the oxygen transfer basins of stage 1 (1.0 g VSS/L) and stage 2 (0.5 g VSS/L). Moreover, AS1 and AS2 were aerated for 48 h to allow for the complete removal of residual substrates. Then, feeding was carried out in fed-batch conditions at feeding rates according to the maximum heterotrophic and nitrifying activities determined by respirometry (Table 1), thus maintaining the selected bacterial population active and preventing substrate limiting conditions (Table S1).

The experiments were run at room temperature (22 ºC) for 5 days and sufficient aeration was provided to keep DO concentration close to saturation (8.5 mg/L). Since these parameters may exert an influence on the biotransformation of organic micropollutants (Stadler et al.,

2015), they were maintained constant and equal in all experiments in order to study the sole effect of the feeding conditions. On average, the pH value throughout the assays was 8.0 ± 0.5. The performance of the experiments was monitored by measuring several conventional parameters: pH, DO, ammonia, nitrite, volatile and total suspended solids (VSS and TSS), dissolved organic carbon and chemical oxygen demand (COD).

The specific heterotrophic activity of these experiments was assessed in terms of Organic Removal Rate (ORR), which was calculated as the difference between the organic loading rate applied and the concentration of dissolved organic carbon measured at the end of the assay in COD units and divided by the concentration VSS and the assay duration. The specific nitrifying activity was assessed in terms of Nitrification Rate (NR), which was calculated as the maximum slope in the $N\text{-}NO_3^-$ production divided by the concentration of VSS of the assay.

Between 9-11 samples of the liquid phase were taken over time during the 5-day experiments to quantify the concentration of PhACs. Even if PhACs were not determined in the solid phase, we can generally assume minor sorption onto the biomass (< 5-10% of the total amount of PhAC) because all target compounds used in this study are known for their low solid-liquid partition coefficients (Table S3 in Supplementary Information) and low biomass concentrations were used in the experiments with AS1 (0.99-1.15 g VSS/L) and AS2 (0.51-0.55 g VSS/L). Moreover, biotransformation rates are usually estimated with the dissolved concentration of micropollutants (Pomiès et al., 2013), which is in constant equilibrium with the sorbed fraction; thus, the analysis of the results will not be impaired.

## 2.4 Analytical methods

### *2.4.1 PhACs and transformation products*

The concentrations of PhACs and several transformation products (TPs) were determined using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Quantification

was based on isotope dilution method using pharmaceutical analogues that are labelled with either deuterium or C-13 stable isotopes (Table S4) purchased from Cambridge Isotope Lab and Sigma Aldrich. Samples of 1.5 mL were collected from the biotransformation assays, filtered through 0.45 µm polypropylene filters and stored at 4 ºC. The separation was carried out using an Agilent 1100 Series HPLC (with a degasser and quaternary pump system), equipped with a Thermo Beta Basic-18 column (100 mm x 2.1 mm and 3µm particle size). The column temperature was maintained at 25 °C. A 20 µL sample was injected; the mobile phase consists of 0.3% formic acid and acetonitrile at a flowrate of 0.2 mL/min. The optimized elution method started at 10% acetonitrile and 90% formic acid maintained for 2 min. Then, the solvent was linearly ramped to 95% acetonitrile and 5% formic acid for 12 min; this composition was maintained for 9 min, then was brought down to 10% acetonitrile and 90% formic acid, and kept for 8 min to equilibrate the column back to its initial condition. Detection of analytes was carried out by an Agilent 6410 Triple Quad LC-MS/MS using an electrospray source in positive ionization mode. The mass spectrometer had a 12 L/min sheath gas flow ($N_2$) at 300°C that desolvated the sample in the source, as it was sprayed from the capillary at 45 psi and 4000 V. Data were processed using the Mass hunter$^{TM}$ software package that came with the LC-MS/MS instrument. Peak identification and analyte quantification were accomplished by monitoring retention times, the ratio of the quantifier and qualifier ions obtained under multiple reaction monitoring (MRM), and the relative signal of the analyte's base peak with its corresponding isotopically-labelled surrogate. Information about the optimized MRM transitions, collision energies used, instrument detection limits, and the expected retention times for each compound are listed in the Supplementary Information (Table S4).

### *2.4.2 Conventional parameters*

Conventional parameters of wastewater treatment were determined throughout the biotransformation experiments. The solution pH was determined using an Accumet AR20 pH/conductivity meter. Dissolved oxygen (DO) was determined with a YSI Model 54A oxygen meter. Ammonia nitrogen was determined spectrophotometrically following Bower/Holm-Hansen method (Bower and Holm-Hansen, 1980). Nitrite, nitrate, volatile and total suspended solids (VSS and TSS) concentrations were determined according to Standard Methods (APHA, 2005). Dissolved organic carbon was determined using a Shimadzu analyzer (TOC-5000). Chemical oxygen demand (COD) was estimated from dissolved organic carbon results according to the ratio of 2.66 mg COD/mg C between the theoretical COD and the organic carbon of sodium acetate.

## 2.5 Biotransformation rate and statistical analysis

To increase statistical sensitivity, pairwise comparisons are based on the biotransformation rate (estimated with 9-11 experimental samples) rather than removal efficiencies of PhACs. Assuming that PhACs follow pseudo-first-order kinetics (Tran et al., 2018), biotransformation rates ($r_b$, µg PhACs/L·d) can be calculated according to Eq. 1.

$$r_b = \frac{dC_W}{dt} = k_{biol} \cdot X_{VSS} \cdot C_W \qquad (1)$$

where,

$k_{biol}$ is the biodegradation kinetic constant (L/ g VSS ·d)

$C_W$ PhACs concentration in the water phase (µg/L)

$X_{VSS}$ Volatile suspended solids concentration (g VSS/L)

Biotransformation rates of PhACs were determined by regression analysis of concentration data against time using the LINEST function of Excel. The uncertainty of the biotransformation rate is calculated in terms of the standard error of the regression. Since pseudo-first-order kinetics depend on the biomass concentration, to have more comparable

results, specific biotransformation rates ($r_{sb}$, µg PhAC/gVSS·d) were preferred in this study (Eq.2).

$$r_{sb} = \frac{r_b}{X_{VSS}} \qquad (2)$$

Significant differences ($p \leq 0.05$) between two specific biotransformation rates were calculated by the Student's t-test (Andrade and Estevez-Perez, 2014).

## 3. RESULTS AND DISCUSSION

### 3.1 Heterotrophic and nitrifying activities

#### *3.1.1 Biological activities of the sludge*

The respirometry tests performed to determine the biological activities of the two types of activated sludge from the Amherst WWTP (Table 1) confirmed that the biomass collected from stage 1 (AS1, SRT = 8 d) was predominantly heterotrophic (727 ± 62 mg $O_2$/g VSS·d), displaying very low nitrifying activity (9 ± 1 mg N-$NH_4^+$/g VSS·d). On the contrary, nitrifying activity was much higher (117 ± 12 mg N-$NH_4^+$/g VSS·d) in the activated sludge from stage 2 (AS2, SRT = 35 d) with lower heterotrophic activity (152 ± 71 mg $O_2$/g VSS·d). Heterotrophs in AS2 are responsible for degrading part of the recalcitrant organic matter not consumed by AS1 in stage 1. Therefore, as expected, the difference in the sludge age and feeding characteristics lead to different microbial compositions and activities in the activated sludge from bioreactors of stage 1 and 2.

**Table 1.** Operational conditions in Stage 1 (organic matter degradation) and Stage 2 (nitrification) of the biological treatment of Amherst WWTP and metabolic activities of the activated sludge from each stage (AS1 and AS2, respectively) determined by the respirometry tests detailed in section 2.2.

| | | Heterotrophic sludge (AS1) | Nitrifying sludge (AS2) |
|---|---|---|---|
| Operational conditions | HRT (h) | 1 | 2 |
| | SRT (d) | 8 | 35 |
| Microbial activities | Heterotrophic (mg $O_2$/g VSS·d) | 727 ± 62 | 152 ± 71 |
| | Nitrifying (mg N-$NH_4^+$/g VSS·d) | 10 ± 1 | 117 ± 12 |
| | Endogenous (mg $O_2$/g VSS·d) | 232 ± 27 | 72 ± 30 |

### *3.1.2 Heterotrophic and nitrifying activities in the biotransformation assays*

Heterotrophic and nitrifying populations in the two types of sludge (AS1 and AS2) were preferentially activated by feeding acetate and ammonium during the biotransformation experiments with PhACs. The specific activities along the six biotransformation assays were assessed in terms of Organic Removal Rate (ORR) and Nitrification Rate (NR) (Fig. 1). More details about the concentrations of ammonium, nitrite, nitrate and dissolved organic carbon are displayed in Fig. S2 and Fig. S3 of the Supplementary Information. As expected, the heterotrophic sludge developed at low SRT (AS1) reached the highest ORR (665 mg COD/g VSS·d) in the experiment fed with acetate (heterotrophic conditions), which corresponds to 72% removal of the total organic loading rate applied (921 mg COD/g VSS·d) (Fig. 1). As shown in Fig. S2, this effectiveness is higher at the beginning of the experiment when remaining nitrogen from the sludge is present. Yet, despite supplying extra nitrogen could increase the heterotrophic activity, it could also enhance nitrifiers, and thus hinder our objective. Consequently, a minor NR was achieved in the experiments performed with AS1; only a slight increase is observed when ammonium was supplied. The nitrifying sludge developed at high SRT (AS2) showed a clear maximum of ORR (125 mg COD/g VSS·d) under heterotrophic conditions (acetate supply), corresponding to an organic matter removal

efficiency of 83%, and a maximum NR of 37 mg N/g VSS·d under nitrifying conditions, one order of magnitude higher than that achieved with AS1.

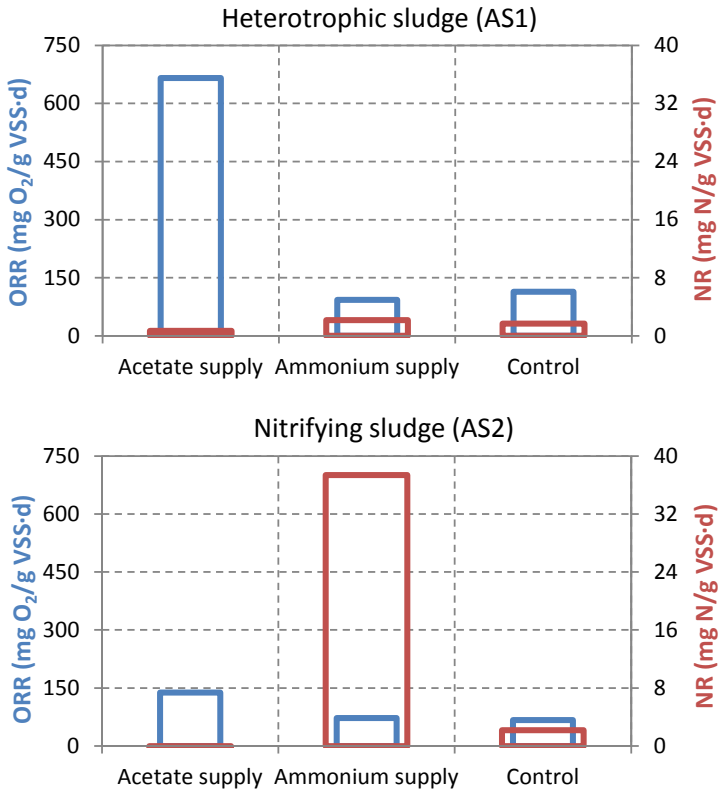


**Fig. 1.** Average organic removal rate (ORR) and nitrification rate (NR) in the biotransformation experiments performed with heterotrophic (AS1) and nitrifying sludge (AS2) under different feeding conditions: acetate supply (activation of heterotrophs), ammonium supply (activation of nitrifiers) and no substrate addition (control).

Prior to the biotransformation experiments, AS1 and AS2 were aerated for 48 h to allow complete removal of residual substrates. The remaining concentrations of organic matter and ammonium nitrogen after this aeration period were very low: 7.2 mg N-$NH_4^+$/L and 22.1 mg DOC/L in AS1 and <0.01 mg N-$NH_4^+$/L and 9.7 mg DOC/L in AS2. Therefore, although almost no readily biodegradable substrate was present in the control experiments, microbial lysis released organic matter and ammonium nitrogen (Fig. S2 and Fig. S3), which could explain the low but still noticeable ORR and NR of the control assays (Fig. 1). Similarly, the heterotrophic activity observed under nitrifying conditions (no organic carbon was supplied)

and the nitrifying activity detected under heterotrophic conditions (no nitrogen was supplied) with AS1 and AS2 (Fig. 1) might be mostly due to microbial lysis. Finally, it should be highlighted that the main biological activities of AS1 and AS2 were very similar in the control assays performed with and without PhACs. According to bibliographic data, it was not expected a substantial inhibitory effect of the selected PhAC concentrations in the activated sludge activities (Dokianakis et al., 2004; Wang and Gunsch et al., 2011; Katipoglu-Yazan et al., 2013; Katsou et al., 2016).

According to the previous results (Table 1 and Fig. 1), the control assays with PhACs will be useful for elucidating the metabolic or co-metabolic biotransformation capacity of a heterotrophic sludge (AS1) versus a nitrifying sludge (AS2) at low biological activities. Moreover, Fig. 1 reveals that the main metabolic activity of AS1 (heterotrophic) and AS2 (nitrifying) was clearly promoted by feeding acetate and ammonium, respectively. Therefore, the comparison between the biotransformation results obtained in these assays with the control will provide insights on the heterotrophic and nitrifying co-metabolism of the selected PhACs. Moreover, it was demonstrated that heterotrophs are also present in AS2 and nitrifying bacteria in AS1 (Table 1 and Fig. 1); thus, the co-metabolic role of these secondary sludge activities on the biotransformation of PhACs could be investigated in the experiments with acetate and ammonium supply to AS2 and AS1, respectively.

### 3.2 Biotransformation of PhACs by the heterotrophic and nitrifying sludge

Fig. 2 shows the specific biotransformation rates of the 10 selected PhACs in the control assays (no feeding) performed with the heterotrophic sludge (AS1) and the nitrifying sludge (AS2). According to their behaviour, the PhACs can be classified into three groups: (*i*) compounds quickly biotransformed (> 800 µg/g VSS·d and removal > 80% after 1 d) at least in one of the sludges, i.e., ACM, CAF, and IOP; (*ii*) compounds slowly biotransformed (100-400 µg/g VSS·d and removal 10-40% after 1 d) at least in one of the sludges, i.e., IBP, ERY

and NPX; and (*iii*) persistent PhACs (< 50 µg/g VSS·d and removal < 5% after 1 d) in both activated sludges, i.e., SMX, CBZ, DCF and TMP.

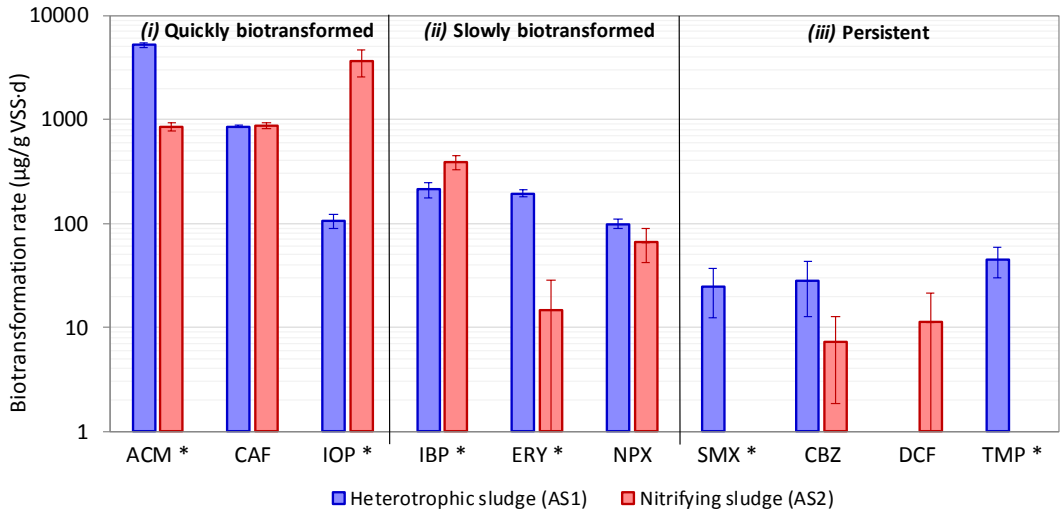


**Fig. 2.** Biotransformation rate of PhACs attained by the heterotrophic (AS1) and nitrifying (AS2) activated sludge in the control experiments (no addition of primary substrate). Error bars represent standard error values of the biotransformation rate calculated considering 10-11 experimental points for AS1 and 9 experimental points for AS2. The asterisks highlight those PhACs whose biotransformation rates (with AS1 and AS2) are significantly different ($p \leq 0.05$).

### *3.2.1 Biotransformed PhACs*

Overall, the biotransformation results obtained in this study for PhACs of group *(i)* and group *(ii)* agree with those reported in real WWTPs (Tran et al., 2018). In fact, among the 60 emerging micropollutants reviewed by Tran et al. (2018), ACM followed by CAF, presented the highest biodegradation kinetic constant. In the case of IBP, it varied among the moderate-high range, and the biotransformation kinetics of IOP, ERY and NPX was considered moderate, although IOP often achieved high removal efficiencies.

As shown in Fig. 2, except for CAF and NPX, the biotransformation rate of group *(i)* or group *(ii)* of PhACs depend on the sludge type (differences were statistically significant according to Student's t-test; $p \leq 0.05$); that is to say, they were affected by the microbial

composition of the biomass. The biotransformation rates achieved by the heterotrophic sludge (AS1) were significantly higher than those obtained by the nitrifying sludge (AS2) in the case of ACM and ERY; in fact, ERY was not removed at all by AS2. On the contrary, quicker biotransformation in the control experiment performed with AS2 was observed for IBP and IOP, which was hardly removed by AS1. Therefore, depending on the compound, biotransformation could be favoured either under heterotrophic or nitrifying conditions. However, as previously mentioned, the differences among AS1 and AS2 are not only due to the SRT but also to the differences in the feeding characteristics (AS2 is fed with the resulting effluent treated with AS1), which might also affect the microbial composition and the enzymatic activities. Consequently, the effects observed on the biotransformation of PhACs cannot be directly linked to the SRT. In fact, the relationship between SRT and biotransformation is still a controversial topic in the literature. For instance, a positive correlation between longer SRT and better biotransformation of PhACs has been reported for IBP (Clara et al., 2005), NPX (Suarez et al., 2010; Falås et al., 2012), and IOP (Batt et al., 2006). However, the opposite trend, where shorter SRT resulted in higher biotransformation, was also reported for IBP (Vuono et al., 2016; Gallé et al., 2019), NPX and CAF (Vuono et al., 2016). These discrepancies in the literature suggest that the SRT is not the only governing factor that should be considered in delineating the microorganisms responsible for the biotransformation of PhACs (Gallé et al., 2019). Other factors, such as substrate composition, might change the metabolic activities of the microbial community, and thus their biotransformation capacity (Fischer and Majewsky, 2014).

### *3.2.2 Persistent PhACs*

Although some significant differences were found in the biotransformation rates (Fig. 2) of PhACs from group (*iii*), the values were very low and almost no biotransformation (< 5% after 1 d) was observed. Therefore, it can be stated that all PhACs from group (*iii*) presented a

similar behaviour with both types of activated sludge. Moreover, as shown in Table 2, except for SMX which will be further discussed in the next section, CBZ, DCF and TMP were also recalcitrant under all tested conditions. The recalcitrance of these three compounds is widely supported in the literature. For instance, the anti-epileptic drug CBZ proved to be very persistent in WWTPs and the environment (Onesios et al., 2009; Tran et al., 2018). Even at long SRTs, obtained in biological treatments designed for nutrient removal or in membrane bioreactors, no biotransformation of CBZ has been observed (Radjenovic et al., 2009; Suarez et al., 2010; Fernandez-Fontaina et al., 2012).

Regarding the anti-inflammatory drug DCF, the reported removal efficiencies support its low biotransformation potential in conventional activated sludge systems (Joss et al., 2006; Nguyen et al., 2019) and throughout WWTPs (Gallé et al., 2019). However, in some particular circumstances, the biotransformation of DCF seems to be improved; for instance, at long SRTs in membrane bioreactors (Vieno and Sillanpää, 2014), and under nitrifying conditions (Suarez et al., 2010; Fernandez-Fontaina et al., 2012). These results cannot be generalized, since other authors found opposite trends. According to Gallé et al. (2019), the removal of DCF seems to be quite independent of operational parameters.

Likewise, although the antibiotic TMP has been usually regarded as difficult to remove in activated sludge systems (Verlicchi et al., 2012) and throughout WWTPs (Onesios et al., 2009), some studies have obtained relevant biotransformation efficiencies at diverse conditions: high nitrifying activities (Fernandez-Fontaina et al., 2012; Men et al., 2017); heterotrophic conditions (Khunjar et al., 2011); a membrane bioreactor with high SRT (Göbel et al. 2007) and an activated sludge system with low SRT and phosphorus accumulating organisms (Lakshminarasimman et al., 2018);

Our results, and the comparison with other studies, confirm that neither heterotrophic nor nitrifying activities may improve the biotransformation of some PhACs. This behaviour is

observed when the PhACs have a highly stable chemical structure (e.g., CBZ), or when their biotransformation depends on physiological capabilities and/or microorganisms not limited by the SRT or the feeding composition (e.g., DCF and TMP).

### 3.3 Stimulation of the co-metabolic biotransformation of PhACs by heterotrophs and nitrifiers

The results obtained with the control assays (Fig. 2) are not enough to attribute the biotransformation of PhACs neither to heterotrophs (the main metabolic activity of AS1) nor to nitrifiers (the main metabolic activity of AS2), since both populations are present in AS1 and AS2 (Fig. 1). Moreover, the biotransformation mechanisms (i.e., metabolism or co-metabolism) behind the removal of the selected PhACs remain unknown. Therefore, to clarify the role of heterotrophs and nitrifiers, their co-metabolic activities were promoted by separately feeding both activated sludges with acetate and ammonium (Table S1). It should be noted that a co-metabolic effect implies an increase in the biotransformation rate of PhACs when the metabolic (enzymatic) activity of the biomass is promoted by adding a specific growth substrate. However, if this effect is not observed, co-metabolic biotransformation cannot be ruled out, since the reaction rate increases linearly with the enzymatic activity, but just until a threshold concentration of enzyme (Frey and Hegeman, 2007; Purich, 2010). Additionally, the biotransformation rate of a compound is not only limited by the metabolic activity of the biomass, but also by other factors, such as reversibility of biological reactions (Gonzalez-Gil et al., 2018; Gonzalez-Gil et al., 2019).

Following the classification of PhACs made in Fig. 2, the fate of compounds belonging to group (*i*), (*ii*) and (*iii*) in all the biotransformation assays is depicted in Fig. 3, Fig. 4 and Fig. 5/Fig. S6, respectively. The corresponding biotransformation rates are summarized in Table 2. By comparing the biotransformation results obtained in the control assays (no primary substrate) with the experiments fed with acetate or ammonium, a significant ($p \leq 0.05$) co-

metabolic effect (concomitant increase in the biotransformation rate and biological activity) was observed for ACM, IBP, NPX and SMX with AS2, and for ERY with AS1.

**Table 2.** Biotransformation rates (µg/g VSS·d) of PhACs by the heterotrophic (AS1) and nitrifying (AS2) sludge in the control assays and under heterotrophic and nitrifying conditions.

| | Biotransformation rate (µg/g VSS·d) | | | | | |
|---|---|---|---|---|---|---|
| | Heterotrophic sludge (AS1) | | | Nitrifying sludge (AS2) | | |
| | Control | Heterotrophic conditions | Nitrifying conditions | Control | Heterotrophic conditions | Nitrifying conditions |
| ACM | 5202 ± 200 | 4656 ± 400 | 5102 ± 400 | 852 ± 80 | 1950 ± 130 | 1837 ± 70 |
| CAF | 860 ± 30 | 478 ± 40 | 840 ± 50 | 866 ± 60 | 1004 ± 70 | 458 ± 50 |
| IOP | 105 ± 20 | 80 ± 6 | 87 ± 10 | 3647 ± 1000 | 1627 ± 500 | 1210 ± 300 |
| IBP | 212 ± 30 | 150 ± 30 | 192 ± 30 | 389 ± 60 | 2006 ± 300 | 1921 ± 100 |
| ERY | 195 ± 10 | 280 ± 30 | 298 ± 20 | 15 ± 10 | 62 ± 20 | 93 ± 30 |
| NPX | 99 ± 10 | 44 ± 9 | 81 ± 7 | 66 ± 20 | 616 ± 30 | 363 ± 80 |
| SMX | 25 ± 10 | 38 ± 10 | 29 ± 9 | – | 858* ± 300 | 16 ± 10 |
| CBZ | 28 ± 10 | 36 ± 10 | 37 ± 10 | 7 ± 5 | 7 ± 7 | 9 ± 9 |
| DCF | – | 3 ± 9 | 1 ± 6 | 11 ± 10 | 11 ± 10 | 31 ± 10 |
| TMP | 44 ± 10 | 55 ± 10 | 48 ± 10 | – | – | – |

*Biotransformation rate was calculated after the 2-days lag phase observed in Fig. 8.

### *3.3.1 PhACs quickly biotransformed*

As depicted in Fig. 3, among the compounds in group *(i)*, the supply of a growth substrate (acetate or ammonium) only exhorted a significantly positive effect in the biotransformation rate of ACM with AS2, which raised from 852 µg/g VSS·d in the control up to 1950 µg/g VSS·d in the presence of acetate. Oppositely, a co-metabolic effect was not observed for ACM in the assays with AS1, and neither for CAF nor for IOP in the assays with AS1 and AS2. The lack of a co-metabolic effect in combination with the fast biotransformation rates attained in the control assays, make plausible the usage of these compounds as carbon and energy sources by bacteria present in AS1 and AS2. Although more detailed studies are needed to support this hypothesis, bacterial strains capable of using ACM as sole carbon, nitrogen and energy source have been isolated from activated sludge in several

studies (De Gusseme et al., 2011; Zhang et al., 2013). Regarding CAF, its quick disappearance was followed by the formation of the N-demethylated metabolites paraxanthine and theobromine, which in turn biotransformed later (Fig. S4). Although the TPs of paraxanthine and theobromine were not identified, further N-demethylation of these metabolites is possible leading to the formation of xanthine and formaldehyde that can enter metabolic pathways in bacteria (Summers et al., 2012). A similar pattern in the formation of both metabolites was observed under all tested conditions; hence, the same biochemical mechanism is responsible for the biotransformation of CAF in activated sludge, regardless of the sludge characteristics and biological activities. Based on all these experimental observations, it is reasonable to assume that ACM, CAF and IOP were biotransformed via direct metabolism. However, the role of co-metabolic biotransformation cannot be ignored because organic carbon and nitrogen are also available in the control assays due to microbial cell lysis (Fig. S2). Yet, the low co-metabolic activities under these conditions might not explain their fast biotransformation.

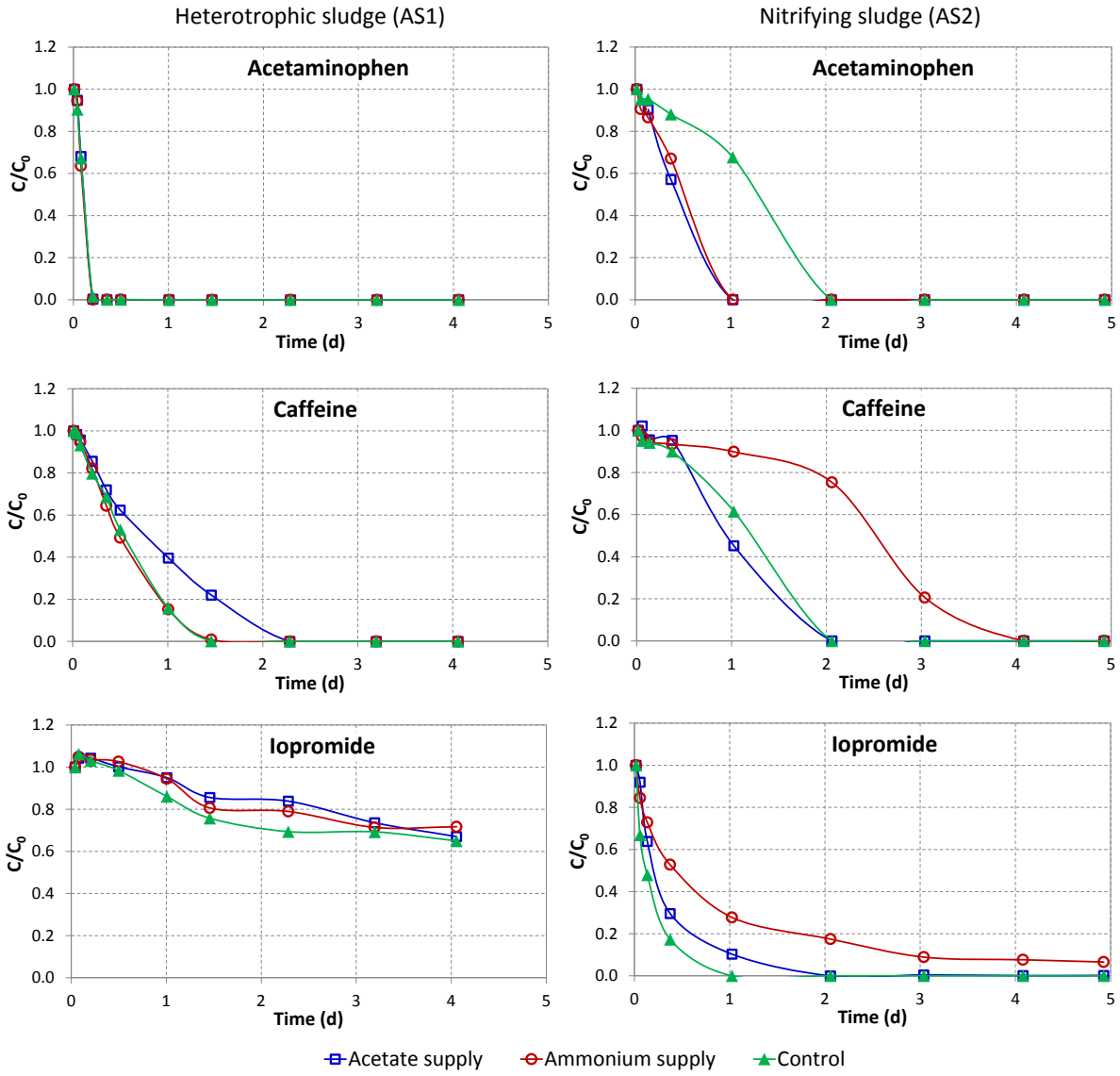


**Fig. 3.** Fate of PhACs that are quickly biotransformed (> 800 µg/g VSS·d and removal > 80% after 1 d) in at least one of the control assays (no additional substrate) performed with AS1 and AS2. The fate under heterotrophic conditions (acetate supply) and nitrifying conditions (ammonium supply) are also shown.

Whether these PhACs are biotransformed via metabolism, co-metabolism, or both, it remains unclear if the responsible microorganisms are heterotrophs or nitrifiers. Co-metabolism can be conducted by both heterotrophs and nitrifiers, while metabolic transformations of organic micropollutants are usually linked to the growth of heterotrophic bacteria; to date, there are no available studies on metabolism by autotrophic organisms

(Nsenga and Meng, 2019). Therefore, both heterotrophs from AS1 and AS2 might be involved in the metabolic biotransformation of ACM, CAF and IOP. Accordingly, Tran et al. (2013) found that heterotrophs are more favourable in the biotransformation of ACM and CAF. However, in the case of ACM, the increase in its biotransformation when ammonium was supplied to AS2 (Fig. 3) suggest that nitrifying activity also contributes to the co-metabolic biotransformation of ACM. Rattier et al. (2014) found that nitrifiers play a role in the biotransformation of ACM and CAF, but other populations might be also involved since under the presence of allylthiourea only a partial decrease in their removal was observed.

In the case of the iodinated X-ray contrast media, IOP (Fig. 3), it was biotransformed at significantly faster rates with AS2 (3647 µg/g VSS·d in the control) when compared to AS1 (105 µg/g VSS·d in the control), as previously shown in Fig. 2. The addition of primary substrates, namely acetate and ammonium, did not have a positive effect in its biotransformation (Fig. 3 and Table 2). The higher biotransformation observed with AS2 agrees with the experiments performed by Batt et al. (2006) in the Amherst WWTP, which showed that nitrifying sludge might be responsible for the biotransformation of IOP. However, they observed that IOP biotransformation in AS2 just decreased from 97% to 86% when allylthiourea was added to inhibit AOMs; thus, it can be hypothesised that other microbial populations of AS2, such as slow-growing heterotrophic bacteria might be key in its biotransformation. Actually, Liu et al. (2013) have already isolated heterotrophic bacteria (*Pseudomonas* sp. *I-24*) from activated sludge capable of degrading IOP. The findings by Kormos et al. (2011) also reinforce the hypothesis of slow-growing heterotrophic bacteria developed at high SRT as main players in the biological removal of IOP, since they have determined high biotransformation efficiencies of IOP (> 80%) in a WWTP with nitrification/denitrification operating at long SRT (16 d). Moreover, Achermann et al. (2018)

found that biotransformation rates of oxidative reactions increased with SRT, as it is the case of IOP.

The proposed biotransformation pathway of IOP (Fig. S5), which is based on previous studies (Perez et al., 2006), indicates that the primary alcohols in the side chains of IOP are oxidized to carboxylic acid leading to the formation of the detected metabolites TP805A and TP805B, which were further oxidized in the remaining primary alcohol group to give TP819. A secondary alcohol is then oxidized to form a ketone group (TP817A). All these metabolites eventually disappeared after 4 days, but the rest of the metabolites measured (Table S4) were not detected. Achermann et al. (2018) suggest that oxidation reactions might be promoted at high SRT and low concentrations of easily degradable carbon (e.g. in AS2) due to an increase in the abundance or diversity of monooxygenase enzymes, such as cytochrome P450, thereby excluding the nitrifying activity as a primary driver of this higher biotransformation.

Finally, it should be noted that the addition of primary substrates to either activate heterotrophs or nitrifiers of AS2 decreased the biotransformation rate of IOP (Fig. 3), which fell from 3647 µg/g VSS·d to 1627 and 1210 µg/g VSS·d, respectively (Table 2). Similarly, when AS1 was fed with acetate and AS2 with ammonium, CAF biotransformation slowed down with respect to the corresponding control assays (Fig. 3 and Table 2). Competitive inhibition between the main growth substrate and PhACs could explain this behaviour. That is to say, active sites of enzymes catalysing the transformation of IOP and CAF might be more occupied when the main growth substrate is available, which of course has a higher affinity by the active site. This behaviour was already reported for CAF in a soil column (Foolad et al., 2015) and for several PhACs in a moving bed biofilm reactor fed with acetate (Liang et al., 2019). Likewise, the presence of readily biodegradable organic matter has been shown to suppress PhAC biotransformation rates under nitrifying and denitrifying conditions (Su et al., 2015).

### *3.3.2 PhACs slowly biotransformed*

The compounds that are slowly biotransformed (group *ii)* appear to undergo co-metabolism. Results in Fig. 4 and Table 2 show a significant ($p \leq 0.05$) steep increase in the biotransformation rate of the anti-inflammatory IBP (389 µg/g VSS·d in the AS2 control assay) that occurred when heterotrophs and nitrifiers of AS2 were fed with acetate (2006 µg/g VSS·d) and ammonium (1921 µg/g VSS·d), respectively. Therefore, it can be assumed that both slow-growing heterotrophic and nitrifying microorganisms of AS2 biotransform IBP by co-metabolism. Different studies support these finding: Zhou et al. (2013) isolated several heterotrophic bacterial strains of Spinghomonas capable of degrading IBP in the presence of high concentrations of primary carbon substrates; Almeida et al. (2013) noticed that the biotransformation rate of IBP by the bacteria *Patulibacter americanus* increased with the primary carbon source concentration (higher biomass growth rate); Fernandez-Fontaina et al. (2012) detected a correlation between nitrification and biotransformation rates of IBP. Additionally, Tran et al. (2009) demonstrated the involvement of both AOMs (by inhibition with allylthiourea) and heterotrophic bacteria (by inhibition with sodium azide) in the biotransformation of IBP, which was recently confirmed by Peng et al. (2019). Therefore, our hypothesis is consistent with previous reports: both slow-growing heterotrophs and nitrifiers co-metabolically biotransform IBP. Although a co-metabolic effect was not observed in the assays with AS1, a notable biotransformation occurred in all of them. This may suggest that low activities of heterotrophs with a rapid growth can also biotransform IBP, as demonstrated by Kennes-Veiga et al. (2021).

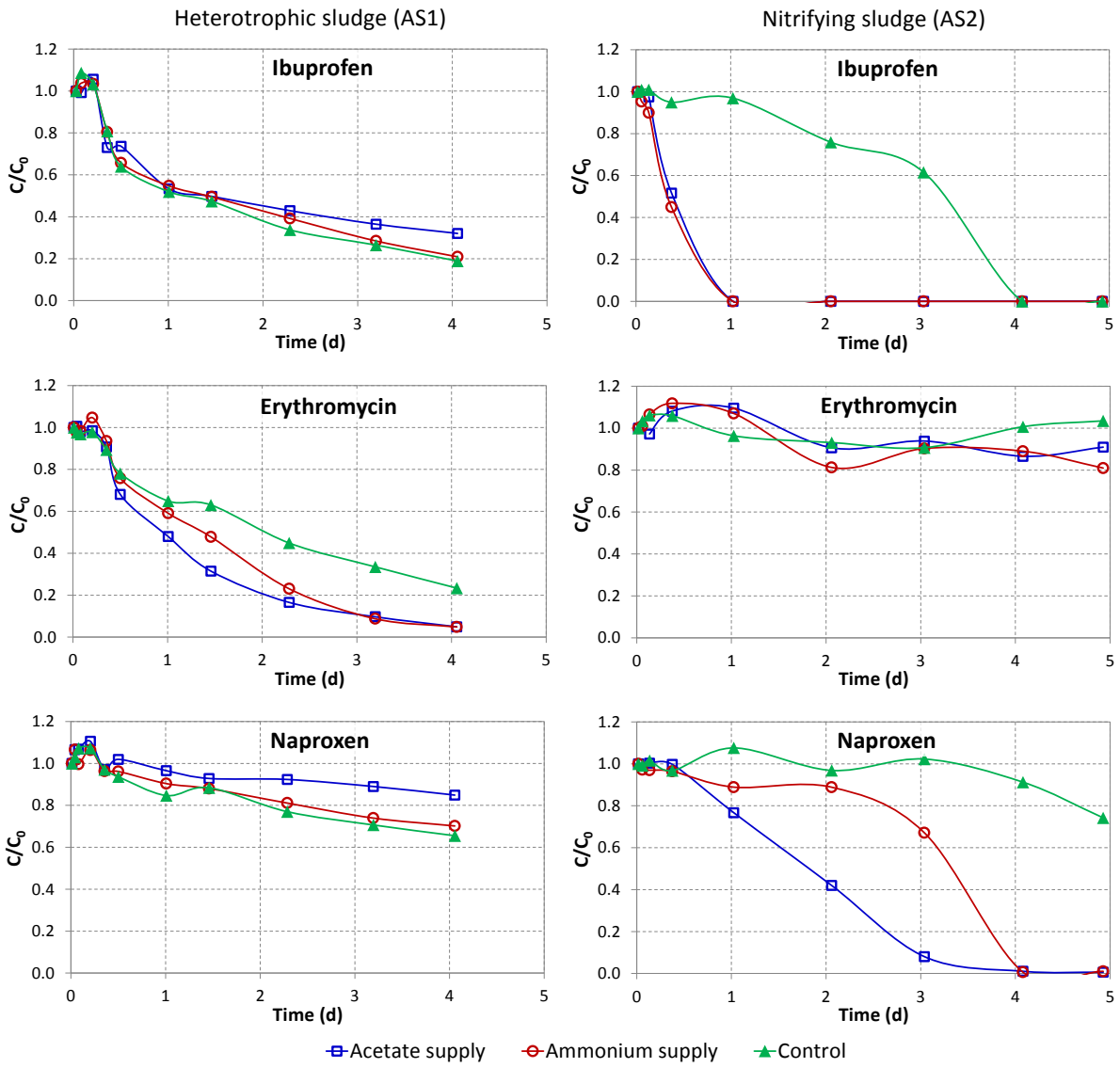


**Fig. 4.** Fate of PhACs that are slowly biotransformed (100-400 µg/g VSS·d and removal 40-10% after 1 d) in at least one of the control assays (no additional substrate) performed with AS1 and AS2. The fate under heterotrophic conditions (acetate supply) and nitrifying conditions (ammonium supply) are also shown.

The biotransformation rate of the macrolide ERY significantly increased (by more than 40% and $p \leq 0.05$) with respect to the control assay with AS1 (195 µg/g VSS·d) when the heterotrophic activity was promoted by feeding acetate (280 µg/g VSS·d) (Fig. 4 and Table 2). A similar behaviour was observed by Kennes-Veiga et al. (2021), who reported a co-metabolic correlation between heterotrophic activity and biotransformation kinetics of ERY.

This suggests that heterotrophs of AS1 are able to co-metabolically remove ERY, although the role of nitrifying bacteria cannot be completely ruled out, as the biotransformation rate of AS1 also increased significantly (above 50% and $p \leq 0.05$) when ammonium was fed (298 µg/g VSS·d) (Fig. 4 and Table 2). Likewise, the involvement of nitrifiers in the biotransformation of ERY was already reported (Suárez et al., 2010; Fernandez-Fontaina et al., 2012). However, the nitrifying activity in AS1 is very low (9 mg $N\text{-}NH_4^+$/g VSS·d, Table 1), even when ammonium was supplied (Fig. 1). Similarly, ERY showed persistent behaviour in all the biotransformation assays with AS2 (nitrifying sludge). Therefore, the increment in the biotransformation rate of ERY by AS1 in the presence of ammonium cannot be directly linked to a higher nitrifying activity. It could be hypothesised that other microbial communities, directly or indirectly dependant on ammonium, might be responsible for this behaviour.

In the case of the anti-inflammatory NPX, almost a 10-fold increase was achieved in the biotransformation rate when heterotrophs were supplied with acetate with respect to the control assay with AS2 (66 µg/g VSS·d) (Fig. 4 and Table 2). This statistically significant ($p \leq 0.05$) difference highlights a main co-metabolic role of slow-growing heterotrophic bacteria, in agreement with the results obtained by Quintana et al. (2005) in a membrane bioreactor. Likewise, after an initial lag-phase (Fig. 4), the biotransformation rate was 5-fold faster under nitrifying conditions than in the AS2 control (Table 2). Thus, nitrifiers might be also involved in NPX biotransformation. Some studies found that AOMs could biotransform NPX since its removal was inhibited by allylthiourea (Margot et al., 2016), while others did not observe any effect of AOM inhibitors (Men et al., 2017). These findings suggest that NPX biotransformation depends on different populations or common enzymes involved in the central metabolism of several microorganisms (Men et al., 2017). Therefore, it cannot be ruled out that NPX might undergo different co-metabolic biotransformation pathways.

Despite O-demethylation being a common biotransformation pathway in activated sludge systems (Quintana et al., 2005), the presence of the corresponding TP (O-desmethylnaproxen) was not observed in our experiments.

### *3.3.3 Persistent PhACs*

The persistence of CBZ, DCF and TMP was not modified by either promoting the heterotrophic or nitrifying activities (Fig. S6). Surprisingly, the antibiotic SMX, which was persistent in both control assays (Fig. 2), was completely biotransformed when heterotrophs from AS2 (SRT = 35 d) were fed with acetate after a lag-phase of 2 days (Fig. 5) (differences with the control were statistically significant according to Student's t-test; $p \leq 0.05$). However, no significant ($p > 0.05$) removal was observed when acetate was supplied to AS1 (Fig. 5), which had a 4-fold higher heterotrophic activity (Fig 2). Therefore, it can be concluded that SMX co-metabolic biotransformation is not dependent on heterotrophic activity in general, but on slow-growing heterotrophic activities, as recently demonstrated by Peng et al. (2019) in an activated sludge reactor with heterotrophic activity and a long SRT (30 d). Likewise, Fernandez-Fontaina et al. (2016) found that stimulating the heterotrophic activity present in nitrifying activated sludge enhances the biotransformation of SMX. Additionally, while low removal efficiencies (< 20%) of SMX were obtained by Joss et al. (2006) with conventional activated sludge, higher biotransformation rates were achieved in membrane bioreactors with characteristic long SRTs (Radjenovic et al., 2009). In contrast, other authors, such as Zhou et al. (2019), also reported biotransformation of SMX by ammonia oxidizers.

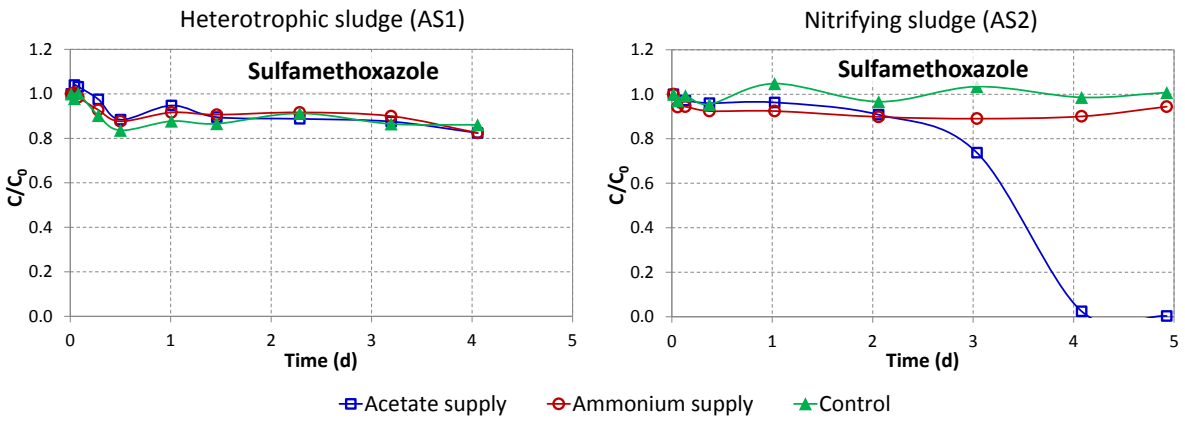


**Fig. 5.** Fate of SMX in the control assays (no additional substrate), under heterotrophic conditions (acetate supply) and under nitrifying conditions (ammonium supply) performed with AS1 and AS2.

## 4. CONCLUSIONS

The strategy followed in this study allowed to stimulate the main and the secondary heterotrophic and nitrifying activities present in both a heterotrophic (developed at low SRT and high C:N ratios) and a nitrifying (developed at high SRT and low C:N ratios) sludge, by changing the feeding characteristics. This novel approach provided new insights about the metabolic and co-metabolic role of heterotrophs and nitrifiers in the biotransformation of some PhACs in activated sludge systems. Moreover, it was an appropriate strategy to elucidate if biotransformation of PhACs was conducted by the main or secondary activities of sludge, which is still a disregarded topic in literature.

In summary, CBZ, DCF and TMP were recalcitrant under all conditions tested, hence their biotransformation seems to not depend on the heterotrophic or nitrifying activity. The heterotrophic sludge (AS1) favoured the biotransformation of ACM and ERY, while the nitrifying sludge (AS2) presented a higher capacity to biotransform IOP, NPX, IBP and SMX. Therefore, it seems that AS2 possess an enhanced degradation capability in comparison to AS1. The latter cannot only be attributed to the role of nitrifiers, but also to the slow-growing heterotrophic microorganisms. Consequently, our results highlight that the main metabolic

activity of the sludge might not be responsible for the co-metabolic biotransformation of PhACs.

Overall, this study demonstrates that not only the SRT, but also the feeding composition exerts a huge influence on the microbial composition and biological activities of the activated sludge and, consequently, on its capability to biotransform PhACs. Future research should focus on the identification of the specific slow-growing heterotrophic strains that biotransform PhACs and the factors that promote their growth, to optimize the performance of WWTPs in terms of organic micropollutant removal.

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# Supplementary Information

# Feeding composition and sludge retention time both affect (co-)metabolic biotransformation of pharmaceutical compounds in activated sludge systems

Lorena Gonzalez-Gil[1,2‡], Eduardo Fernandez-Fontaina[1,3‡], Randolph R. Singh[3], Juan M. Lema[1], Marta Carballa[1*] and Diana S. Aga[3]

[1]CRETUS Institute, Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2]Defence University Centre, Spanish Naval Academy, Plaza de España, 36920, Marín, Spain

[3]Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY, 14260, United States

[‡] Both authors have equally contributed to this work

*Corresponding author: Marta Carballa (marta.carballa@usc.es)

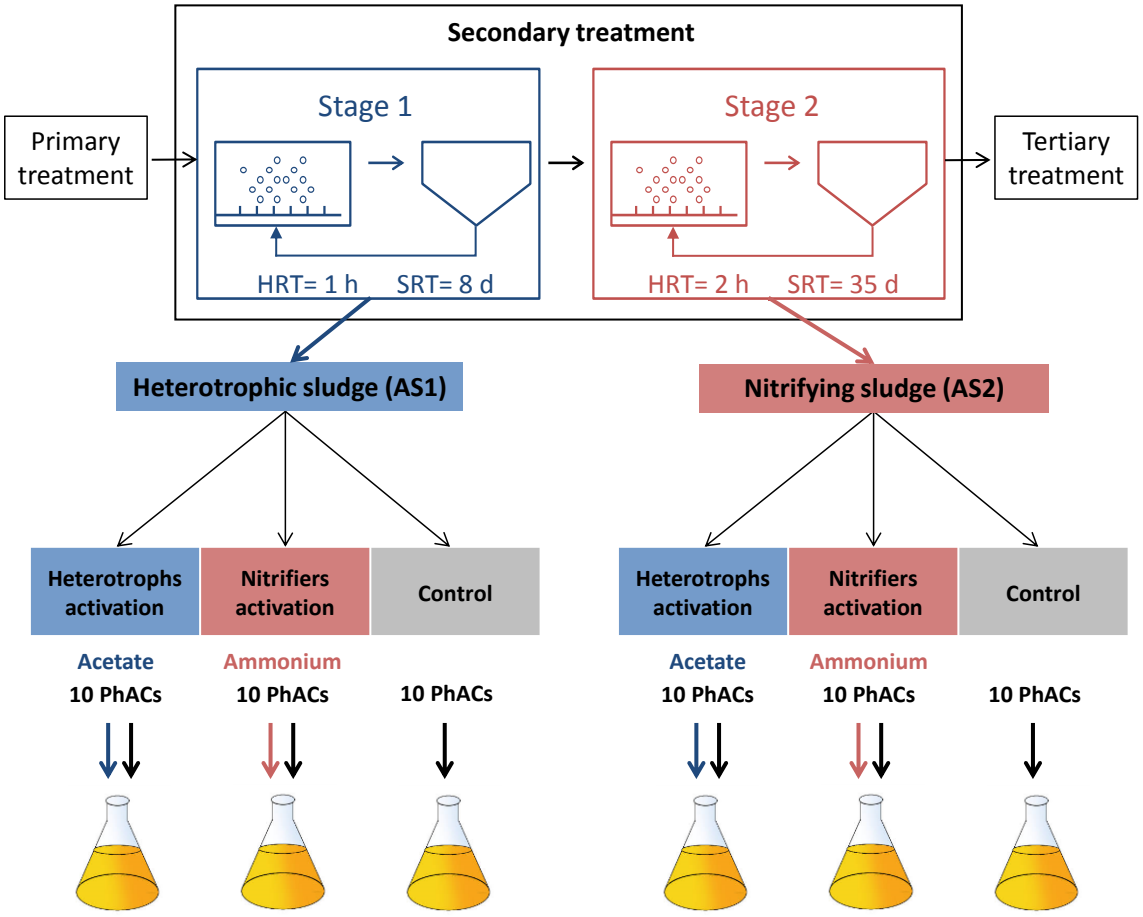


**Fig. S1.** Experimental set-up of the biotransformation assays performed with two types of activated sludge: AS1 (heterotrophic sludge) and AS2 (nitrifying sludge), developed in the two-stage secondary treatment of Amherst (NY, USA) WWTP.

**Table S1.** Feeding characteristics of the biotransformation assays. The assays were initiated with the remaining concentrations of organic matter and ammonium nitrogen after an aeration period of 48 h: 7.2 mg N-$NH_4^+$/L and 22.1 mg DOC/L in AS1 and <0.01 mg N-$NH_4^+$/L and 9.7 mg DOC/L in AS2.

| | | Main substrate | Loading rate | Sodium bicarbonate |
|---|---|---|---|---|
| Heterotrophic sludge (AS1) | Control | – | – | – |
| | Heterotrophic conditions | Sodium acetate | 921 mg COD/g VSS·d | – |
| | Nitrifying conditions | Ammonium chloride | 15 mg N/g VSS·d | 32 mg C/g VSS·d |
| Nitrifying sludge (AS2) | Control | – | – | – |
| | Heterotrophic conditions | Sodium acetate | 150 mg COD/g VSS·d | – |
| | Nitrifying conditions | Ammonium chloride | 120 mg N/g VSS·d | 255 mg C/g VSS·d |

**Table S2.** Biomass concentrations in the biotransformation experiments.

| | | VSS (g/L) | TSS (g/L) |
|---|---|---|---|
| Heterotrophic sludge (AS1) | Control | 0.997 ± 0.170 | 1.340 ± 0.147 |
| | Heterotrophic conditions | 1.148 ± 0.094 | 1.618 ± 0.120 |
| | Nitrifying conditions | 1.047 ± 0.217 | 1.430 ± 0.252 |
| Nitrifying sludge (AS2) | Control | 0.545 ± 0.020 | 0.844 ± 0.034 |
| | Heterotrophic conditions | 0.458 ± 0.018 | 0.758 ± 0.032 |
| | Nitrifying conditions | 0.505 ± 0.064 | 0.848 ± 0.081 |

**Table S3**. Solid-liquid partition coefficients ($K_d$) of the selected PhACs.

| Compound | $K_d$ (L/kg TSS) |
|---|---|
| Acetaminophen | 0.4[a] |
| Caffeine | < 30[g] |
| Carbamazepine | 1.2[b]; 28-66[e]; 135[c] |
| Diclofenac | 16[b]; 16[e]; 118[c] |
| Erythromycin | 10-28[c]; 74[c] |
| Ibuprofen | 6-50[d]; 7[b]; 72[e] |
| Iopromide | 11[b] |
| Naproxen | 13[e] |
| Sulfamethoxazole | 50[d]; 77[c] |
| Trimethoprim | 119-193[g]; 253[c]; 208[f] |

a) Jones et al. (2002); b) Ternes et al. (2004); c) Radjenovic et al. (2009); d) Abegglen et al. (2009); e) Urase and Kikuta (2005); f) Göbel et al. (2005); g) Stevens-Garmon et al. (2011).

**Table S4.** Details about the LC-MS/MS method employed for the detection and quantification of the selected PhACs and their metabolites.

| Analyte | Type | Retention time (min) | MRM transitions | | Collision energy (eV) | | Instrument Quantitation limit (µg/L) |
|---|---|---|---|---|---|---|---|
| | | | Quantifying | Qualifying | Quantifying | Qualifying | |
| Acetaminophen (ACM) | Parent compound | 3.5 | 152 → 110 | 152 → 65 | 14 | 29 | 0.02 |
| Caffeine (CAF) | Parent compound | 5.5 | 195 →138 | 195 → 42 | 18 | 33 | 0.07 |
| *D9-TMP* | *Internal standard* | 5.5 | 198 → 53 | 198 → 138 | | | ND |
| Paraxanthine | CAF metabolite | 3.4 | 181 → 124 | 181 → 96 | 20 | 20 | ND |
| Theobromine | CAF metabolite | 2.8 | 181 → 108 | - | 22 | - | ND |
| Carbamazepine (CBZ) | Parent compound | 13.5 | 237 → 194 | 237 → 179 | 18 | 37 | 0.01 |
| *$d_{10}$-Carbamazepine* | *Internal standard* | 13.5 | 247 → 204 | 247 → 202 | 21 | 37 | ND |
| Diclofenac (DCF) | Parent compound | 16.1 | 296 → 250 | 296 → 214 | 10 | 34 | 0.18 |
| 4-hydroxy-diclofenac | DCF metabolite | - | 312 → 231 | 312 → 266 | 39 | 35 | ND |
| $NO_2$-diclofenac | DCF metabolite | - | 323 → 279 | 323 → 249 | 5 | 25 | ND |
| $NO_3$-diclofenac | DCF metabolite | - | 339 → 295 | 339 → 259 | | | ND |
| Erythromycin-$H_2O$ (ERY) | Parent compound | 12.3 | 716 → 158 | 716 → 116 | | | 0.10 |
| *$^{13}C$-Erythromycin* | *Internal standard* | 12.3 | 735 → 159 | 735 → 83 | 29 | 60 | 0.04 |
| Ibuprofen (IBP) | Parent compound | 16.3 | 207 → 161 | 207 → 117 | 6 | 37 | 0.49 |
| Hydroxy-IBP | IBP metabolite | - | 221 → 177 | - | 8 | | ND |
| Methyl- IBP | IBP metabolite | - | 220 → 161 | - | | | ND |
| Hydroxy-methyl- IBP | IBP metabolite | - | 236 → 178 | - | | | ND |
| Carboxy-methyl- IBP | IBP metabolite | - | 228 → 128 | - | | | ND |
| Carboxy-2-methyl- IBP | IBP metabolite | - | 264 → 205 | - | | | ND |

MRM – multiple reaction monitoring; ND – not determined because there are no commercially available standards for metabolites

**Table S4 (cont.).** Details about the LC-MS/MS method employed for the detection and quantification of the selected PhACs and their metabolites.

| Analyte | Type | Retention time (min) | MRM transitions | | Collision energy (eV) | | Instrument Quantitation limit (µg/L) |
|---|---|---|---|---|---|---|---|
| | | | Quantifying | Qualifying | Quantifying | Qualifying | |
| Iopromide (IOP) | Parent compound | 2.8 | 792 → 573 | 792 → 559 | 33 | 39 | 4.00 |
| TP819 | IOP metabolite | 2.4 | 820 → 587 | 820 → 714 | 35 | 29 | ND |
| TP817A | IOP metabolite | 2.4 | 818 → 701 | 818 → 713 | 27 | 27 | ND |
| TP805A | IOP metabolite | 2.2 | 806 → 559 | 806 → 686 | 39 | 31 | ND |
| TP805B | IOP metabolite | 2.2 | 806 → 573 | 806 → 701 | 33 | 27 | ND |
| TP787A | IOP metabolite | - | 788 → 671 | 788 → 712 | 27 | 25 | ND |
| TP759 | IOP metabolite | - | 760 → 671 | 760 → 684 | 23 | 23 | ND |
| TP731A | IOP metabolite | - | 732 → 613 | 732 → 453 | 27 | 39 | ND |
| TP731B | IOP metabolite | - | 732 → 626 | 732 → 467 | 23 | 41 | ND |
| TP729A | IOP metabolite | - | 729 → 613 | 730 → 457 | 27 | 41 | ND |
| TP701A | IOP metabolite | - | 702 → 613 | 702 → 454 | 25 | 43 | ND |
| TP701B | IOP metabolite | - | 702 → 627 | 702 → 468 | 17 | 37 | ND |
| TP643 | IOP metabolite | - | 644 → 517 | 644 → 613 | 19 | 21 | ND |
| Naproxen (NPX) | Parent compound | 14.8 | 231 → 185 | 231 – 115 | 10 | 60 | 0.11 |
| O-desmethylnaproxen | NPX metabolite | - | 215 → 171 | - | 10 | | ND |
| Sulfamethoxazole (SMX) | Parent compound | 11.8 | 254 → 108 | 254 → 92 | 22 | 25 | 0.03 |
| Trimethoprim (TMP) | Parent compound | 8.3 | 291 → 230 | 291 → 123 | 22 | 22 | 0.01 |
| *$d_9$-Trimethoprim* | *Internal standard* | 8.3 | 300 → 234 | 300 → 123 | 25 | 25 | ND |

MRM – multiple reaction monitoring; ND – not determined because there are no commercially available standards for metabolites

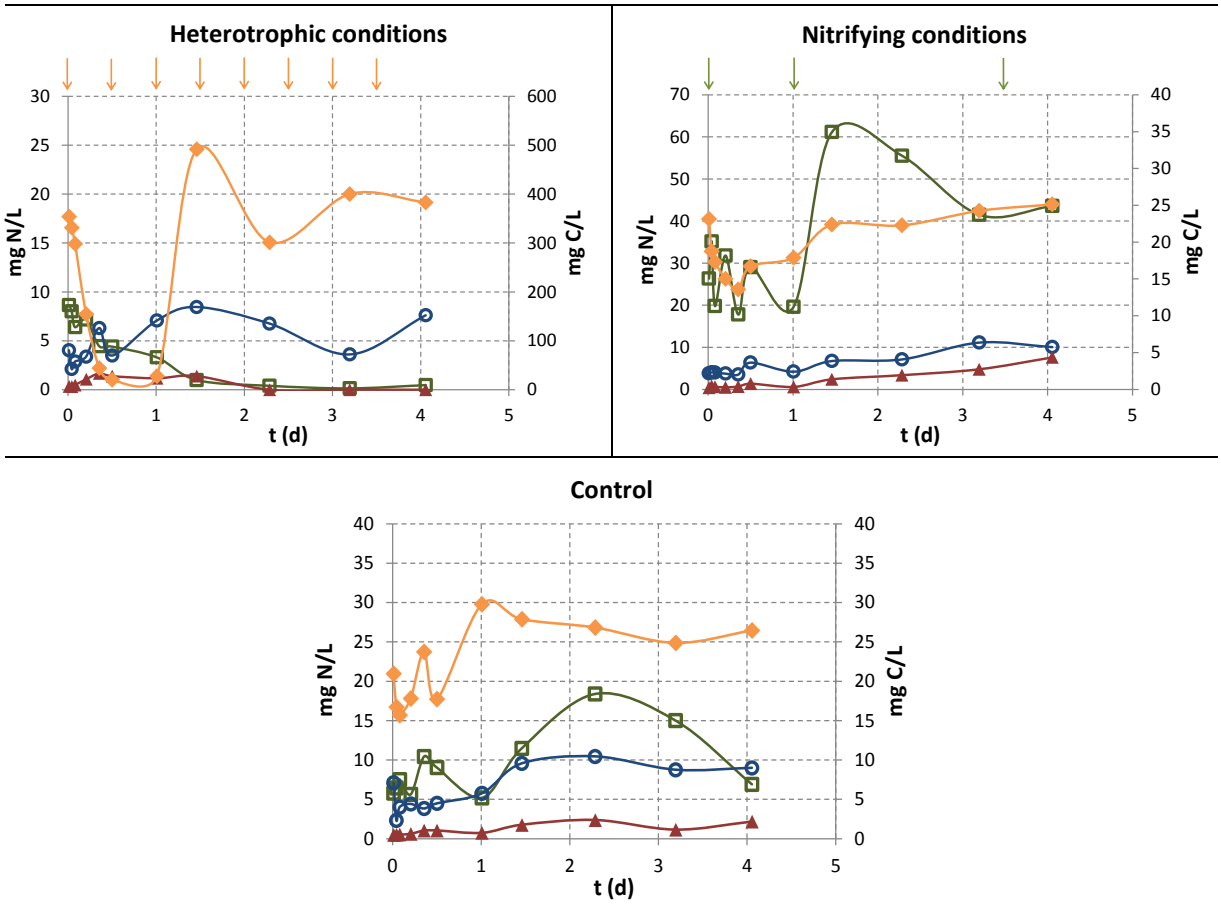


**Fig. S2.** Concentrations of ammonium (□), nitrite (▲), nitrate (○) and dissolved organic carbon (◆) during the batch experiments performed with the heterotrophic sludge (AS1) developed in stage 1 of the Amherst WWTP. Heterotrophs were stimulated by feeding acetate (↓), nitrifiers by supplying ammonium and bicarbonate (↓) and control conditions were maintained without the addition of any primary substrate. It should be noted that ammonium and dissolved organic carbon were usually measured just before adding the corresponding substrate, hence a peak in the concentration is not observed below the arrows.

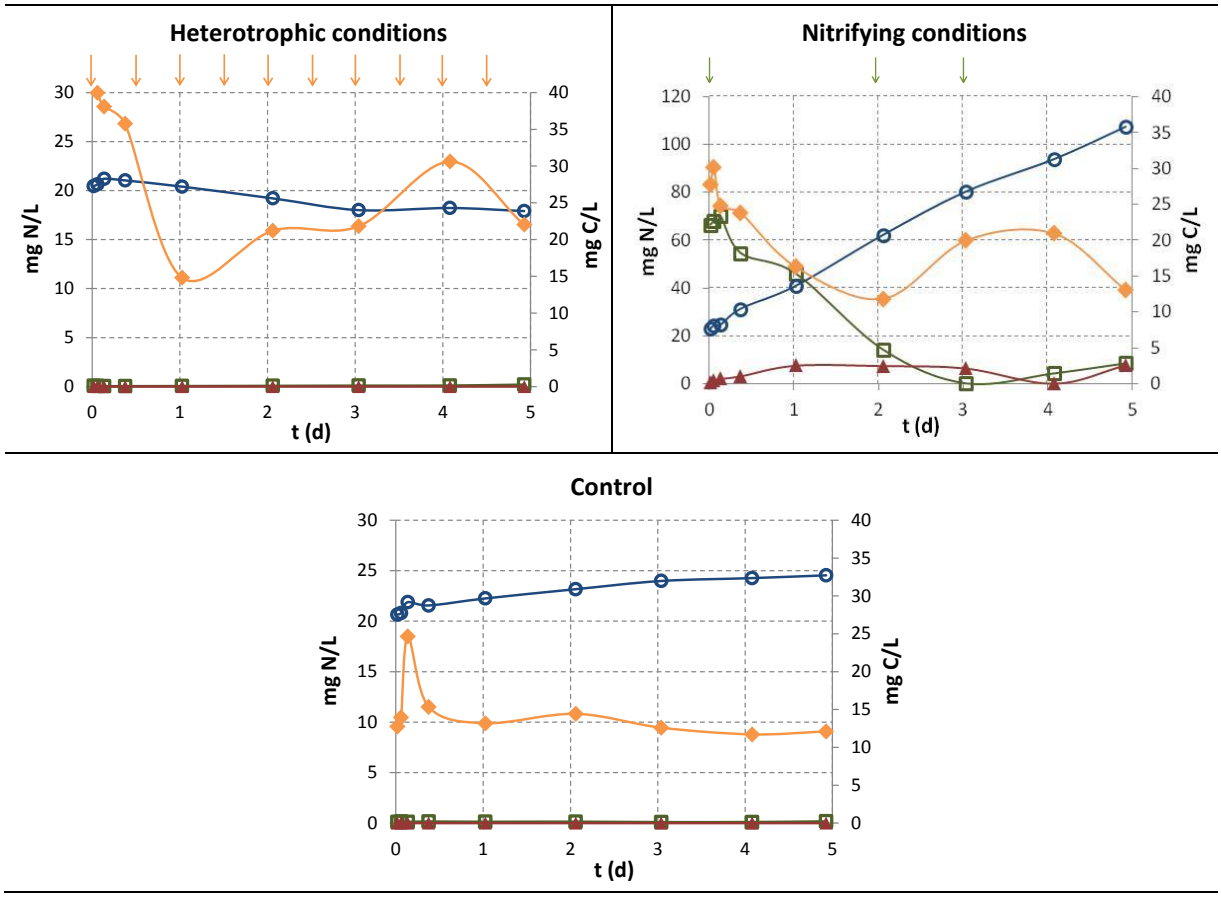


**Fig. S3.** Concentrations of ammonium (□), nitrite (▲), nitrate (○) and dissolved organic carbon (◆) during the batch experiments performed with the nitrifying sludge (AS2) developed in stage 2 of the Amherst WWTP. Heterotrophs were stimulated by feeding acetate (↓), nitrifiers by supplying ammonium and bicarbonate (↓) and control conditions were maintained without the addition of any primary substrate. It should be noted that ammonium and dissolved organic carbon were usually measured just before adding the corresponding substrate, hence a peak in the concentration is not observed below the arrows.

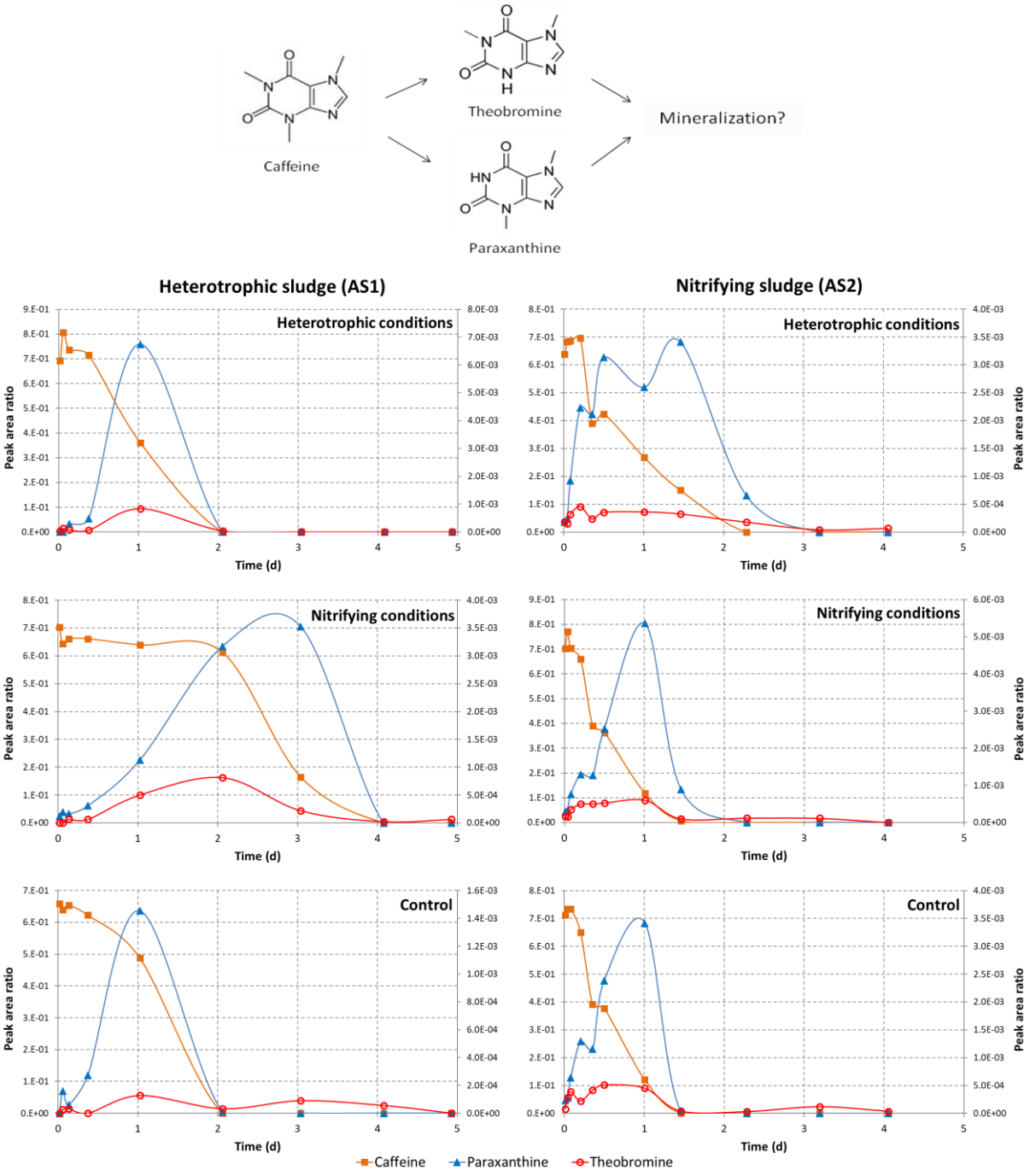


**Fig. S4.** Biotransformation pathway of caffeine and peak area ratios referred to the internal standard ($d^{10}$-CBZ) of caffeine (in the left axis), and its formed metabolites, paraxanthine and theobromine (in the right axis), determined by LC-MS/MS under multiple reaction monitoring (MRM) for the batch experiments performed with AS1 and AS2 under heterotrophic (acetate supply), nitrifying (ammonium supply) and control conditions (no addition of any primary substrate).

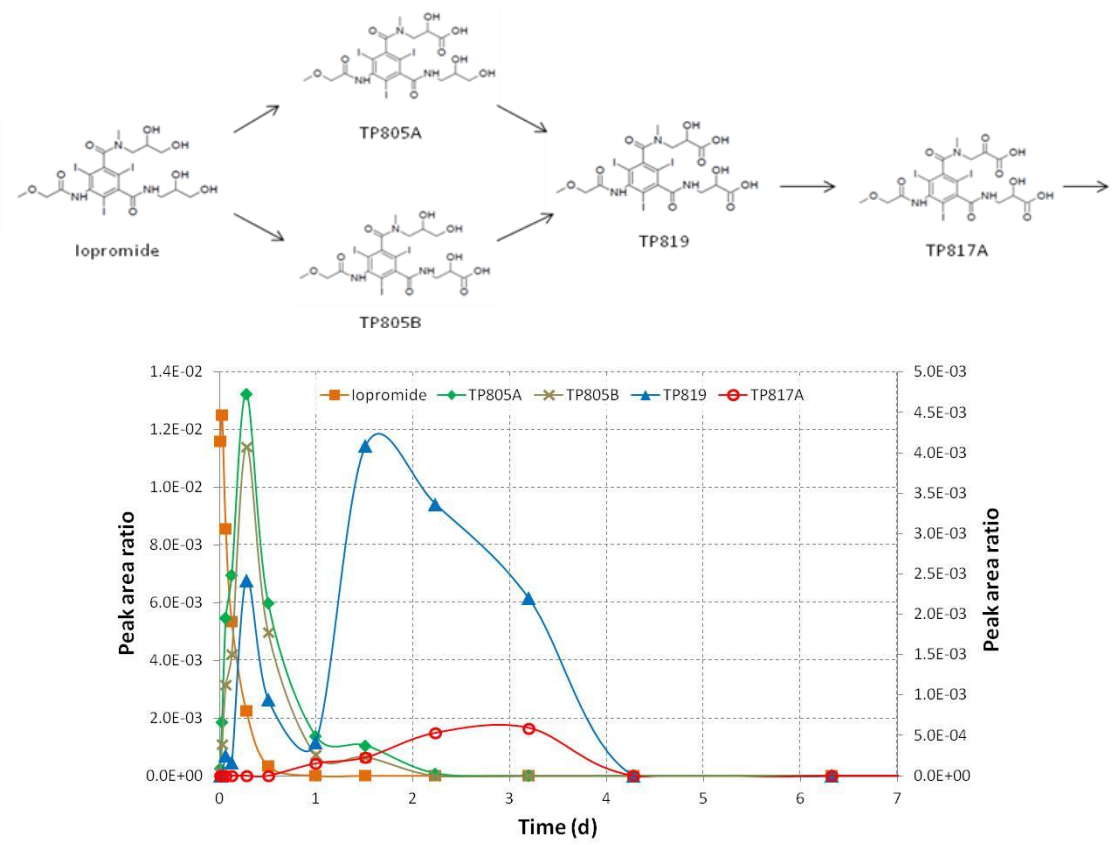


**Fig. S5.** Biotransformation pathway of iopromide and peak area ratios referred to the internal standard ($d^{10}$-CBZ) of iopromide (in the left axis), and its metabolites TP805A and TP805B, TP819, TP817A (in the right axis), determined by LC/MS/MS for the control assay (no primary substrate) with nitrifying sludge (AS2).

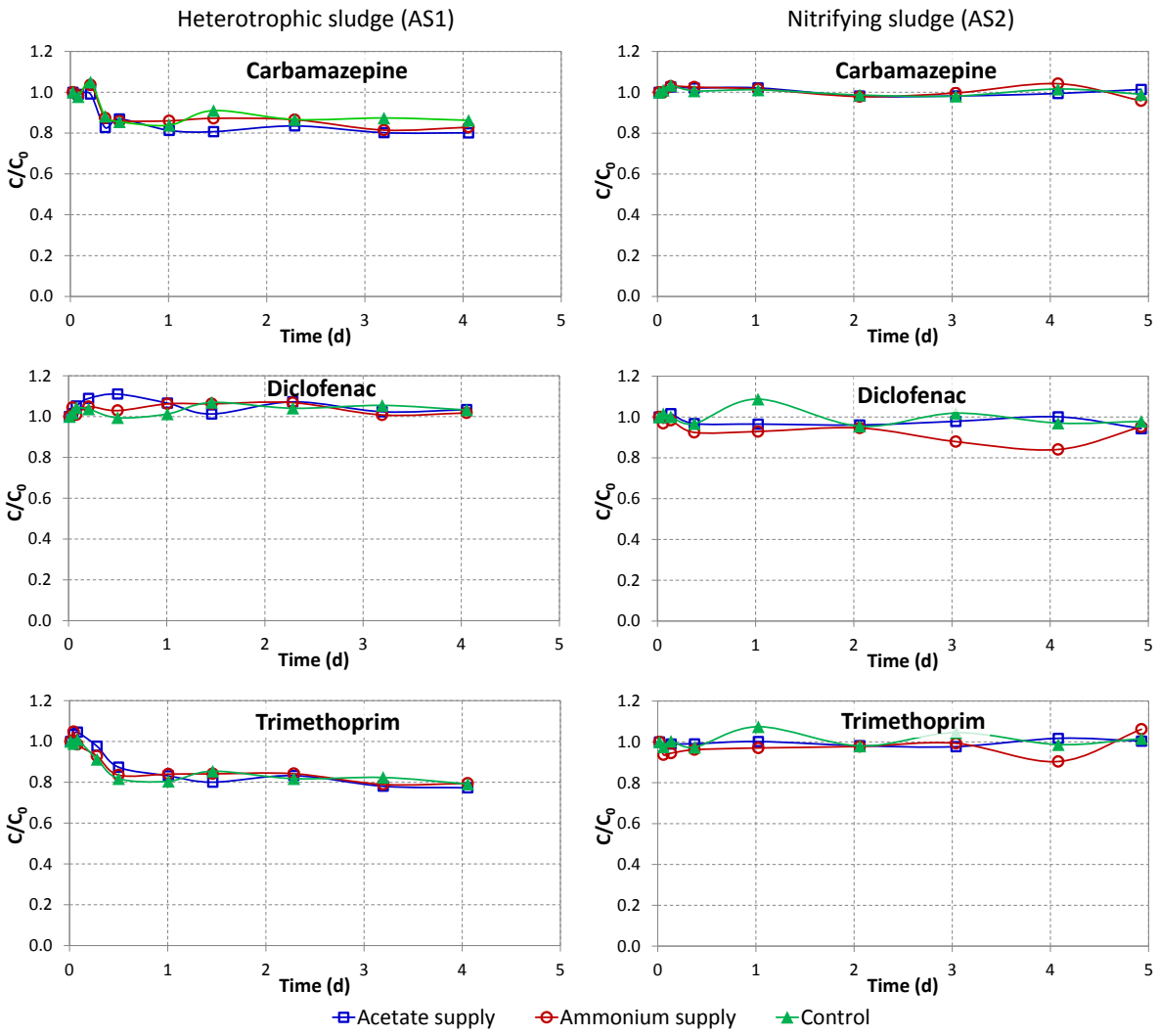


**Fig. S6.** Fate of persistent PhACs (< 50 µg/g VSS·d and removal < 5% after 1 d) in both control assays (no additional substrate) performed with AS1 and AS2. The fate under heterotrophic conditions (acetate supply) and nitrifying conditions (ammonium supply) are also shown.

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