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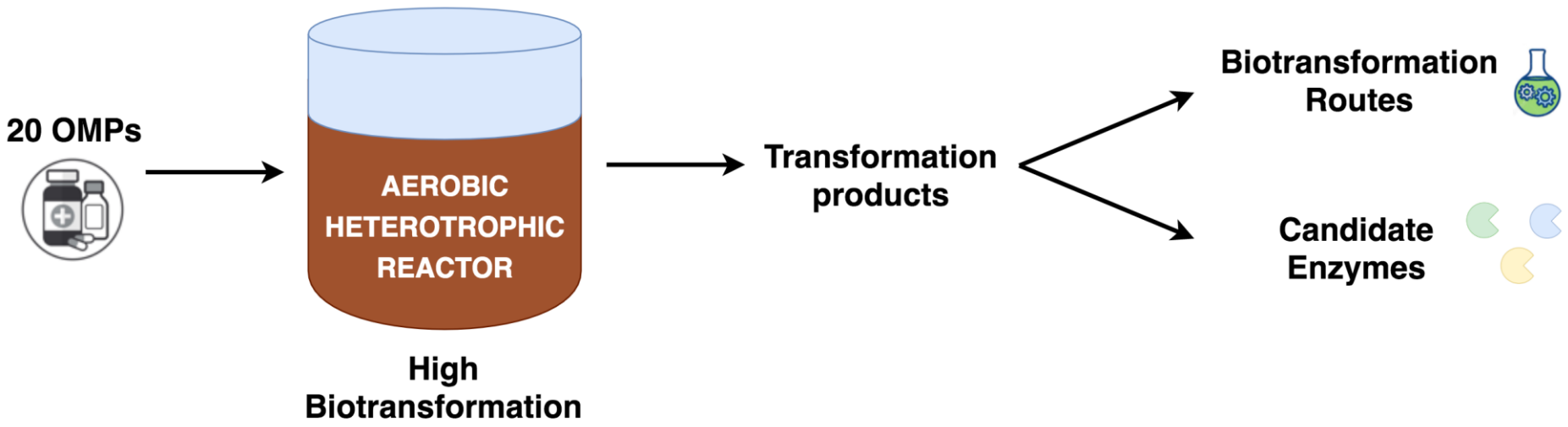
Graphical Abstract

GRAPHICAL ABSTRACT



HIGHLIGHTS

1. Aerobic heterotrophs proved their capacity to extensively biotransform OMPs.
2. Biotransformation was similar to that of activated sludge with nitrogen removal.
3. Oxidation, hydrolysis and conjugation were identified as relevant mechanisms.
4. Oxygenases, dehydrogenases, hydrolases and transferases appear to play a key role.

Heterotrophic enzymatic biotransformations of organic micropollutants in activated sludge

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ABSTRACT

While heterotrophic microorganisms constitute the major fraction of activated sludge biomass, the role of heterotrophs in the biotransformation of organic micropollutants (OMPs) has not been fully elucidated. Yet, such knowledge is essential, particularly when conceiving novel wastewater treatment plants based on a two-stage process including an A-stage under heterotrophic conditions and a B-stage based on anammox activity. Biotransformation of OMPs in activated sludge is thought to mostly occur cometabolically thanks to the action of low specificity enzymes involved in the metabolism of the primary substrates. For a better understanding of the process, it is important to determine such enzymatic activities and the underlying mechanisms involved in OMPs biotransformation. This task has proven to be difficult due to the lack of information about the enzymatic processes and the complexity of the biological systems present in activated sludge. In this paper, a continuous aerobic heterotrophic reactor following 20 OMPs at environmental concentrations was operated to (*i)* assess the potential of heterotrophs during the cometabolic biotransformation of OMPs, *(ii)* identify biotransformation reactions catalysed by aerobic heterotrophs and *(iii)* predict possible heterotrophic enzymatic activities responsible for such biotransformations. Contradicting previous reports on the dominant role of nitrifiers in OMPs removal during activated sludge treatment, the heterotrophic population proved its capacity to biotransform the OMPs to extents equivalent to reported values in nitrifying activated

sludge plants. Besides, 12 transformation products potentially formed through the activity of several enzymes present in heterotrophs, including monooxygenases, dioxygenases, hydrolases and transferases, were identified.

Keywords: Cometabolism; enzymes; heterotrophs; pharmaceuticals; transformation products; wastewater.

1. INTRODUCTION

Activated sludge systems are designed to reduce the biological oxygen demand and the concentration of nutrients, such as nitrogen and phosphorous, in wastewaters. However, they are also capable to remove organic micropollutants (OMPs) such as pharmaceuticals, personal care products and pesticides that reach wastewater treatment plants (WWTPs) through surface runoff and sewers (Besha et al., 2017; Fischer and Majewsky, 2014). The removal efficiency of OMPs in activated sludge systems depends on the nature of the compounds, the operational parameters, the properties of the wastewater and the characteristics of the microbial community (Achermann et al., 2018b).

Most studies on activated sludge systems include heterotrophic and nitrifying activities and provide extensive information on OMPs biotransformation (Lambropoulou and Nollet, 2014; Lema and Suarez, 2017). Moreover, it has repeatedly been shown that nitrifying activity enhances or is even required for substantial biotransformation of many OMPs (Fernandez-Fontaina et al., 2012; Helbling et al., 2012; Tran et al., 2009). As a consequence, it is widely assumed, and for some compounds also experimentally proven, that nitrifiers play a key role in biotransformation. Yet, there are still only a few studies that investigate the specific role the different microbial populations play (Alvarino et al., 2018; Khunjar et al., 2011; Margot et al., 2016; Men et al., 2017), and, in particular, that of the heterotrophic population. At the same time, the heterotrophic activity is gaining interest due to novel WWTPs conceptions, where the A-stage, operating with high loadings and low solid retention times (SRT), exclusively converts readily degradable organic matter, whereas the B-stage is proposed to be operated by an anammox consortium (Liu et al., 2020).

The main removal mechanism for many water-borne OMPs is microbial biotransformation, which is assumed to be of cometabolic nature mostly, due to the very low concentrations at which OMPs are present in wastewater, i.e., in the μg to ng L^{-1} range (Luo et al., 2014; Petrie et al., 2014). Under these conditions, OMPs can hardly provide enough energy to support cellular growth and maintenance and are hence most likely biotransformed by enzymes involved in the biodegradation of primary substrates, which are present at considerably higher concentrations (Alvarino et al., 2018). In activated sludge systems, such enzymes are present in the metabolic network of aerobic nitrifiers and heterotrophs. In the case of nitrifiers, they use simple and well-known substrates, presenting a limited number of candidate enzymes potentially involved in OMPs biotransformation, which facilitates the development of studies trying to elucidate their role. For example, ammonia monooxygenases, present in ammonia-oxidizing bacteria (AOB) and archaea (AOA), have been recognised as key in OMPs biotransformation due to their low substrate specificity (Fernandez-Fontaina et al., 2016; Margot et al., 2016; Rasche et al., 1990; Yi and Harper, 2007), while enzymes of other nitrifying populations, such as nitrite-oxidizing bacteria, have shown a less relevant contribution to the biotransformation of OMPs (Fernandez-Fontaina et al., 2016; Yu et al., 2018). In contrast, aerobic heterotrophs can use a wide variety of organic substrates, sometimes very complex and with different physicochemical properties, which leads to a huge number of candidate enzymes that could contribute to OMPs biotransformation (Achermann et al., 2020; Fischer and Majewsky, 2014; Gulde et al., 2016; Krah et al., 2016). This complexity has resulted in very few studies trying to determine the key enzymes involved in the heterotrophic breakdown of OMPs, despite acknowledging that the heterotrophic biomass governs or enhances the cometabolic removal of many compounds, such as aminopolycarboxylic acids,

sulfamethoxazole, diclofenac, paracetamol and 17-α-ethinylestradiol (Kennes-Veiga et al., 2020; Larcher and Yargeau, 2013; Majewsky et al., 2011, 2010; Tran et al., 2009). Furthermore, it has been reported that heterotrophs and autotrophs can cooperate to remove OMPs and attenuate the presence of intermediate transformation products (TPs). For instance, it has been hypothesized that without an active fraction of heterotrophic biomass, intermediates of estrogens would accumulate in AOB cultures (Shi et al., 2004; Yi and Harper, 2007). Therefore, understanding the capabilities of the heterotrophic population is important even when both nitrifiers and heterotrophs are capable of biotransforming the same compounds because they may perform the biotransformation at different rates and/or follow pathways that lead to distinctly different TPs (Fischer and Majewsky, 2014).

Since OMPs are frequently not fully mineralized during cometabolic biotransformation, the assessment of the TPs is also important because they pose the potential to be as or even more toxic than their parent compounds to the ecosystem (Berkner and Thierbach, 2014; Celiz et al., 2009; Gulde et al., 2016). Besides, TPs identification can be a very helpful tool to determine the reactions occurring in a specific environment and provide hints about the enzymatic activities catalysing OMPs biotransformation. For instance, following this approach, Gulde et al. (2016) demonstrated that N-acyltransferases could be involved in the biotransformation of several amine-containing OMPs in activated sludge.

In this study, we aim to address the contribution of heterotrophs to the biotransformation of OMPs in activated sludge processes and, particularly, their capacity to biotransform a range of OMPs. Besides, we attempt to detect several TPs as a tool to identify key OMP biotransformation reactions catalysed by aerobic heterotrophs and decipher possible enzymatic activities carrying out such

biotransformations. To this end, we evaluated the biotransformation of a set of OMPs with different physicochemical properties in an aerobic heterotrophic reactor and used liquid chromatography coupled to high-resolution mass spectrometry to identify TPs.

2. MATERIALS AND METHODS

2.1. Continuous aerobic heterotrophic reactor

A 5 L continuously stirred lab-scale reactor, connected to a 2 L settler, was operated at 25°C. The reactor was inoculated with sludge from an activated sludge reactor of a WWTP near Santiago de Compostela (Spain) and aerated to ensure oxygen concentrations around 6-7 mg O_2 L^{-1}. The feeding consisted of a synthetic mixture of sodium acetate/acetic acid (in concentrations that ensured operation at neutral pH), ammonium chloride, potassium dihydrogen phosphate, calcium chloride and magnesium sulfate (Table S1). Also, other trace nutrients were added to promote the growth of the aerobic heterotrophic microorganisms (Table S2). The hydraulic retention time (HRT) was set to 1 d and the organic loading rate (OLR) to 0.6 g chemical oxygen demand (COD) L^{-1} d^{-1}. To avoid nitrification, the SRT was kept below 8 days and allylthiourea (ATU) was added to the feeding at a concentration of 5 mg L^{-1}.

The operation of the aerobic heterotrophic reactor was monitored measuring the total suspended solids (TSS), volatile suspended solids (VSS), total and soluble COD, ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and oxygen concentrations, pH and temperature according to Standard Methods (2012). The measurements were performed in triplicate twice per week. Moreover, after four weeks of operation, the changes in OMPs levels and the emergence of potential TPs was determined by taking samples in triplicate from the inlet and outlet of the reactor (considering both the liquid and solid phase) for one week.

2.2. Organic micropollutants

This study focused on 20 environmentally relevant compounds commonly present in wastewater that cover a wide variety of functional groups, physicochemical properties and applications. The compounds were: the antibiotics erythromycin (ERY), roxithromycin (ROX), trimethoprim (TMP), sulfamethoxazole (SMX); the anti-inflammatories ibuprofen (IBP), naproxen (NPX), diclofenac (DCF); the neurodrugs carbamazepine (CBZ), diazepam (DZP), fluoxetine (FLX); the biocide triclosan (TCS); the musk fragrances celestolide (ADBI), galaxolide (HHCB), tonalide (AHTN); the endocrine disruptors estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and the xenoestrogens bisphenol A (BPA), 4-octylphenol (OP) and 4-nonylphenol (NP). The OMPs were purchased from Sigma-Aldrich (Germany), except for the musk fragrances, which were acquired from Ventos (Spain). Depending on the substance, stock solutions were prepared in HPLC-grade acetone or methanol and stored at -20°C. They were added to the feeding at a concentration of 10 $\mu g\ L^{-1}$, except for the musk fragrances and hormones, whose concentrations were 40 and 1 $\mu g\ L^{-1}$, respectively.

2.3. Chemical analysis of the OMPs

Influent and effluent samples were centrifuged at 3500 rpm for 10 min. The supernatant was prefiltered (AP4004705, Millipore) and then filtered again at 0.45 μm (HAWP04700, Millipore). Then, solid phase extraction (SPE) was performed with 200 mL samples and 60 mg Oasis HLB cartridges (Waters, Milford, MA, USA), as described by Fernandez-Fontaina et al. (2013). The quantification of antibiotics (ERY, ROX, SMX, TMP), neurodrugs (FLX, CBZ, DZP) and hormones (E1, E2, EE2) was performed using an Agilent G1312A liquid chromatography instrument with a binary pump and automatic injector HTC-PAL (CTC Analytics) connected to a mass spectrometer API 4000 triple quadrupole (Applied Biosystems). Musk fragrances

(HHCB, AHTN, ADBI), anti-inflammatories (IBP, NPX, DCF), xenoestrogens (BPA, OP, NP) and the biocide (TCS) were quantified using gas chromatography (Varian CP-3900) coupled to an ion trap spectrometer (Varian CG-2100). The quantification procedure was done according to previous studies (Alvarino et al., 2014). All OMPs analyses were performed in triplicate.

The solid phase was frozen and lyophilized. To quantify the OMPs sorbed onto the aerobic sludge, ultrasonic solvent extraction (USE) was performed (Ternes et al., 2005). The USE technique consisted of three sequential extractions with methanol and two with acetone applied to freeze-dried samples of approximately 0.5 g. During each extraction, the samples were sonicated for 15 min and centrifugated at 1500 rpm for 5 min. Then, the supernatants were combined, filtered through glass wool, evaporated (R-205, Büchi) under vacuum conditions (150 mbar) at 35°C and diluted with distilled water. Finally, SPE and micropollutant quantification was performed as described for the liquid phase.

2.4. Chemical analysis of the transformation products

To detect TPs, the samples were again analysed using reversed-phase liquid chromatography coupled to a high-resolution quadrupole Orbitrap mass spectrometer (Q Exactive or Q Exactive Plus, Thermo Scientific). Full-scan MS spectra were acquired both in positive and negative ionization modes and the acquisition of MS2 fragmentation spectra was triggered at m/z values corresponding to masses of suspected TPs.

The sample was loaded onto an Atlantis T3 column (particle size 3µm, 3.0 x 150 mm, Waters) equipped with a guard column (Atlantis T3 VanGuard Cart 3 µm, 3.9x5mm). Nanopure water (Barnstead Nanopure, Thermo Scientific) and methanol (HPLC grade, Fischer Scientific), both with 0.1% formic acid (98-100%, Merck), were used as mobile

phase at a flow rate of 300 μL min^{-1}. Initial conditions (95:5 water/methanol) were maintained for 1 min and then the methanol fraction increased over 16 min to 5:95 water/methanol. These conditions were held for 8 min and then initial conditions were reestablished within 0.1 min and kept for 4.9 min before the next analysis started. The temperature of the column oven was set to 30ºC. Mass spectra were acquired at a capillary temperature of 320ºC and spray voltages of 4kV and -3kV in positive and negative ionization modes, respectively. This method was adapted from previous studies (Achermann et al., 2018b).

The samples were first measured in full scan mode at a resolution of 140,000 at m/z 200 and a scan range of 50-750 m/z in positive and negative switching mode. In a second injection, data-dependent MS2 spectra were recorded at a resolution of 17500 at 200 m/z (isolation window of 1 m/z) using an inclusion list of masses corresponding to suspected TPs. This list was compiled using previously found TPs in the literature, the EAWAG pathway prediction system (EAWAG-BBD, 2020) and by manually applying a range of plausible atomic modifications (hydroxylation, dihydroxylation, demethylation, dehydrogenation, hydrogenation, decarboxylation, etc.).

2.5. Transformation product identification and structure elucidation

A suspect TP screening was performed using the software Compound Discoverer 3.1. (Thermo Scientific). The first part of the workflow comprised retention time (RT) alignment (maximal RT shift: 2 min; mass tolerance 5 ppm) and peak picking (mass tolerance 5 ppm, minimal intensity 10000, maximal peak width: 0.5 min). Then, a subset of features was selected based on comparison with the predefined suspect list. Transformation product candidate peaks had to fulfil the following criteria to be selected for further analysis:

1. Intensity above a certain threshold: maximum area > 25000.

2. The peaks had to have a reasonable peak shape.
3. If a chemical formula was proposed, the observed isotopic pattern had to match the theoretical one.
4. If a MS2 spectrum was available, the fragmentation prediction and the matches to the fragments of the respective parent compound were taken into account. Besides, the FISh scoring node of Compound Discoverer 3.1. was used, rejecting compounds with a score below 50.
5. The peak area of the TPs had to be at least 10 times higher in the effluent than in the influent of the reactor.

Structure elucidation was conducted with Compound Discoverer 3.1. software or manually based on the interpretation of the exact masses, the isotopic pattern of the MS spectra and the MS2 fragments. The outcome and confidence of the structural interpretation differed depending on the availability of structural evidence. To communicate the confidence in the structural interpretation, confidence levels were assigned as proposed by Schymanski et al. (2014): Level 5 (exact mass), Level 4 (unequivocal molecular formula), Level 3 (tentative candidates), Level 2 (probable structure) and Level 1 (confirmed structure). TP evidence, i.e., observed changes in molecular formula and structure, was used to assign corresponding reaction types to the OMPs.

2.6. Calculations of reactor performance

Several indicators were calculated to characterize reactor performance, including the net activated sludge produced, the endogenous biomass decay rate, the nitrogen oxidized, the recirculation ratio and the oxygen requirements.

The net waste activated sludge produced was estimated assuming typical heterotrophic parameters of biomass yield ($Y = 0.4\ g_{biomass}\ g_{substrate}^{-1}$), endogenous decay coefficient

238 (k_d = 0.1 d^{-1}) and cell debris fraction (f_d = 0.15) (Metcalf & Eddy, 2014), as shown in

239 Eq. 1:

240 $$P_{X,VSS} = \frac{Y\ Q\ (S_0 - S)}{1 + (k_d)\ SRT} + \frac{(f_d)\ (k_d)\ Y\ Q\ (S_0 - S)\ SRT}{1 + (k_d)\ SRT} \quad (1)$$

241 where:

242 S_0 influent COD concentration (g L^{-1})

243 S effluent COD concentration (g L^{-1})

244 The endogenous decay rate of biomass in the reactor was also estimated, as indicated in

245 Eq. 2:

246 $$rd = -\mathrm{kd} \cdot \mathrm{X}_{VSS} \quad (2)$$

247 with:

248 X_{VSS} biomass concentration in the bioreactor (g L^{-1})

249 Moreover, as shown below (Eq. 3), the oxidized nitrogen was calculated:

250 $$N_{ox} = TKN_0 - N - \frac{0.12 P_{X,VSS}}{Q} \quad (3)$$

251 with:

252 N_{ox} nitrogen oxidized (mg L^{-1})

253 TKN_0 influent total Kjeldahl nitrogen (mg L^{-1})

254 N effluent N-NH_4^+ concentration (mg L^{-1})

255 $P_{X,VSS}$ biomass as VSS wasted (mg d^{-1})

256 Q influent flow (L d^{-1})

257 To maintain a constant biomass concentration in the reactor, purge was performed daily.

258 Besides, biomass recirculation was performed in a ratio close to 1, typical for activated

259 sludge systems (Metcalf & Eddy, 2014), as indicated in Eq. 4:

260 $$R = \frac{1 - \left(\frac{HRT}{SRT}\right)}{\left(\frac{X_R}{X_{VSS}}\right) - 1} \quad (4)$$

261 with:

X_R biomass concentration in the settler (g L^{-1})

To ensure aerobic conditions, the dissolved oxygen (DO) concentration was controlled. Experimental values over 5 mg O_2 L^{-1} were always maintained in the reactor, following the guidelines of Metcalf and Eddy (2014) for aerobic biological systems during wastewater treatment. The oxygen provided was sufficient to fully oxidize the influent COD (Eq. 5).

$$R_0 = Q(S - S_0) - 1.42P_{X,VSS} = 1.8 \text{ g } O_2 \ d^{-1} \quad (5)$$

3. RESULTS AND DISCUSSION

3.1 Reactor performance

The reactor was operated for 28 days and steady-state conditions were achieved after one week (Table S3). During experimentation, neutral pH was maintained to maximize the activity of the microorganisms as well as to avoid any effects of varying pH levels on OMPs removal. Although frequently receiving little attention, pH is a key parameter affecting OMPs biotransformation. Its increase by just one unit pH can considerably promote the removal of OMPs with cationic-neutral specification and hinder that of compounds with neutral-anionic speciation, as shown by Gulde et al. (2014).

Biomass concentration remained quite stable at 1.4 g L^{-1} by controlling the purge (Fig. 1), performed almost daily from the settler, and the control of the recirculation, set at a ratio of 100% of the influent flow (Eq. 4). The source of organic matter was acetate, which was fully consumed (95-100%), leading to effluent COD concentrations consistently below 0.03 g L^{-1} (Fig. 1). Besides, operation occurred in the absence of nitrification (Fig. 1), indicating that all the nitrogen consumed was used only for

286 microbial growth. In the non-consumed nitrogen, some soluble non-degradable organic
287 nitrogen from endogenous respiration may have been present.
288 The net waste activated sludge produced was estimated at 0.15 g VSS L^{-1} (Eq. 1),
289 including the active heterotrophic biomass formed daily and the cell debris, which
290 accounts for 10-20% of the decayed biomass and cannot be degraded due to their
291 extremely low hydrolysis kinetics (Liu and Wang, 2015). Moreover, there was also an
292 endogenous decay rate of biomass in the reactor, proportional to the active biomass
293 concentration (X_{VSS}), that accounted for 0.14 g VSS L^{-1} d^{-1} (Eq. 2). The endogenous
294 decay represents the cell biomass loss and includes an internal decay (cell level),
295 involving the oxidation of stored substrates to produce energy for cell maintenance, and
296 an external decay (community level), such as cell death or predation by higher
297 organisms (Liu and Wang, 2015). Our reactor presented mostly aerobic heterotrophic
298 bacterial strains, as well as higher life-forms, such as rotifers and protozoans, based on
299 microscopic observation. The high life forms have also been shown to participate in the
300 removal of OMPs, as reported by Gulde et al. (2018) for protozoa, which seem to be
301 involved in the ion trapping of amine-containing compounds and the hydrolysis of
302 select esters and phenylurea compounds.
303 **Figure 1 (A, B and C)**

304 **3.2 Heterotrophic OMPs biotransformation in activated sludge systems**

305 Fig. 2 shows the fate of the selected OMPs in the continuous heterotrophic reactor.
306 Sorption appears to be minimal for most compounds (<10%), except for FLX, for which
307 it accounts for 15-20% of the total mass balance. Results show that 17 out of the 20
308 OMPs were highly removed (above 80%) due to heterotrophic activity and only 3
309 compounds were slightly (TMP) or not removed at all (CBZ, DZP). These results agree

with the biotransformation observed in activated sludge units (Alvarino et al., 2014; Luo et al., 2014; Petrie et al., 2014), except for DCF, for which we obtain higher values than under typical activated sludge conditions (Alvarino et al., 2014; Fernandez-Fontaina et al., 2016). Nonetheless, in other biological treatments, high DCF efficiencies have also been achieved, as in a hybrid biofilm-activated sludge process (Jewell et al., 2016b) and in a nitrifying moving bed biofilm reactor (Torresi et al., 2016). In our experiments, DCF was biotransformed rather extensively, i.e., at 80% (Fig. 2), which could indicate the presence of certain heterotrophic genera capable of biotransforming it more efficiently. In this regard, Nguyen et al. (2019) showed that under exposure to a primary carbon substrate and DCF, certain genera of activated sludge bacteria can significantly increase their abundance, suggesting that they might gain a competitive advantage from its cometabolic removal.

Figure 2

To understand the variability in OMPs biotransformation reported in the literature, Fig. 3 compares the biotransformation performance of our aerobic heterotrophic reactor with that reported under nitrifying conditions exclusively (systems not fed with organic carbon or that have inhibited the heterotrophic activity) and in activated sludge systems presenting both heterotrophic and nitrifying activities. Firstly, it can be observed that heterotrophs reach biotransformations similar to the values of the activated sludge plants, indicating that they can greatly contribute to the removal of the OMPs, and that, for certain compounds, there may not be the need to set up WWTPs with nitrifying activities. There is only a very limited number of studies evaluating the biotransformation of the selected OMPs with exclusively nitrifying populations. In such works, they have also reported high removals for these compounds, indicating that both nitrifiers and heterotrophs have in their metabolic networks enzymes capable of

335 biotransforming the OMPs. However, there are a few compounds that show differences
336 in their behavior between the different conditions. While biotransformation of SMX and
337 TCS is clearly higher under heterotrophic or activated sludge conditions, CBZ and DZP
338 show better efficiencies with nitrifying populations. Moreover, although the average
339 removal values of TMP seem similar under all conditions, it is a compound typically
340 difficult to remove by aerobic heterotrophs (Fernandez-Fontaina et al., 2016), while
341 some nitrifying populations have shown the capacity to biotransform it up to 50% or
342 more (Batt et al., 2006; Fernandez-Fontaina et al., 2012). Furthermore, as discussed
343 previously, heterotrophs display better biotransformation for DCF. Additionally, it is
344 surprising to observe higher average biotransformations for some compounds under
345 exclusively heterotrophic or nitrifying conditions than in activated sludge systems
346 presenting both activities. A possible explanation could be due to differences in the
347 process conditions under which the experiments were performed, such as applying
348 different organic loading rates. However, previous studies have shown that in certain
349 cases mixed consortia of bacteria can provide worse OMPs removals than individual
350 populations because the composition of a mixed bacterial culture can affect their
351 performance (Larcher and Yargeau, 2011).
352 Thus, based on Fig. 3, it can be concluded that nitrifying systems or nitrifying activated
353 sludge systems would not be required to achieve the typical biotransformation extents
354 of the selected OMPs since, in general, the biotransformation is similar for all microbial
355 populations. Nonetheless, it is important to remark that the data collected from the
356 literature was obtained from experiments performed at considerably different
357 operational conditions, which could have affected the biotransformation extents
358 obtained in each case and explain the high standard deviations observed for some
359 compounds.

Figure 3

3.3 Identification of enzymatic biotransformations of OMPs in aerobic heterotrophic conditions

In total, 12 TPs were confidently identified (Table 1 and Table S4) for the 20 investigated OMPs and assigned to three major types of transformation reactions (oxidation, hydrolysis and conjugation). For ADBI and FLX, 2 different TPs were observed, pointing towards sequences of transformation steps or transformations taking place at different functional groups. The structural confidence was considered *probable* (level 2) for 1 TP and *tentative* (level 3) for 9 TPs, whereas 2 TPs could only be assigned an *unequivocal molecular formula* (level 4). The confidence varied mostly depending on the availability of MS and MS2 data, literature information and the molecular structure of the TPs and respective OMPs (structural assignments for TPs containing only C, H and O atoms were generally treated with more caution).

A) Oxidation: Oxidation reactions frequently represent the initial biotransformation of xenobiotics in activated sludge systems. In our reactor, we determined several TPs that were formed through oxidative biotransformation, in the form of hydroxylation, deamination, demethylation and dehydrogenation.

A.1) Hydroxylation: Hydroxylation is commonly reported during OMPs biotransformation since it can be carried out by multiple microorganisms. For instance, AOB and AOA can hydroxylate mianserin (Men et al., 2016), the novel complete AOB (commamox), carbendazim (Han et al., 2019) and heterotrophic microorganisms, EE2 (Khunjar et al., 2011). Our results show heterotrophic hydroxylation of ADBI, HHCB, E1, E2, DCF and NP (Table 1), likely catalyzed by low specificity monooxygenases, such as cytochrome P450 or flavin-containing monooxygenases, or dioxygenases, such

as Rieske-type non-heme-iron dioxygenases (Bjørseth and Angeletti, 1986). The most common biotransformation reaction of steroid hormones, including E1 and E2, is hydroxylation, performed by oxygenases in multiple organisms, such as bacteria, fungi or algae (Pratush et al., 2020). Fragrances can also undergo hydroxylation, as reported for HHCB, in bacteria, algae and fungi, where the initial biotransformation reaction is hydroxylation at different carbon positions (Ding et al., 2020; Martin et al., 2007). In the case of DCF, there is limited information about its biotransformation reactions in WWTPs. However, hydroxylation has been observed as the first reaction in its biotransformation pathway in activated sludge plants, being considered a bottleneck due to its low rate, which could explain the frequently limited removal yield (Bouju et al., 2016). In our reactor, DCF was highly biotransformed (Fig. 2) and the corresponding hydroxylated TP was found, possibly indicating that heterotrophs were able to perform the hydroxylation step efficiently. Differently to DCF, NP hydroxylation in WWTPs has not been reported before, to the best of our knowledge. Nonetheless, in mammals and fish, hydroxylation both in the ring and the alky chain has been observed, possibly due to the action of cytochrome P450 enzymes (Thibaut et al., 2002).

A.2) Deamination: A TP detected for FLX (Table 1) was either *(i)* the product of deamination at the secondary amine group followed by oxidation of the resulting aldehyde or *(ii)* the outcome of initial demethylation, followed by deamination of the resulting primary amine TP and final oxidation of the formed aldehyde. The second pathway seems more plausible since secondary amines typically undergo dealkylation before oxidative deamination. Nonetheless, in certain cases, direct deamination of secondary amines may occur, as in the metabolism of propranolol, where the biotransformation can occur through the desisopropyl primary amine metabolite or by a direct oxidative deamination reaction yielding an aldehyde metabolite and

409 isopropylamine (Beale and Block, 2011). Gulde et al. (2016) observed deaminations for
410 some primary, secondary and tertiary OMPs, such as primaquine, N-
411 demethylvenlafaxine and pyrilamine, but they did not observe FLX deamination as in
412 this study.
413 Assuming the previous demethylation, the deamination step in FLX could be performed
414 by an amine oxidase, catalyzing the oxidative cleavage of the primary amine to an
415 aldehyde while releasing ammonia and hydrogen peroxide. These enzymes are found in
416 multiple organisms controlling the level of amines, participating in multiple pathways,
417 and allowing various amine substrates to be used as sources of carbon and nitrogen in
418 prokaryotes (Messerschmidt, 2010). The following oxidation of the aldehyde to the
419 carboxylic acid is likely catalyzed by an aldehyde dehydrogenase, using oxygen from a
420 water molecule and NAD^+ or $NADP^+$ as cofactors, present in a broad range of anabolic
421 and catabolic pathways and key in the elimination of endogenous and exogenous toxic
422 aldehydes (Riveros-Rosas et al., 2019).
423 The hypothetical N-demethylation of the secondary amine of FLX would form
424 seproxetine as a TP, the most important FLX active metabolite observed in humans and
425 WWTPs. In human metabolism, the biotransformation occurs thanks to cytochrome
426 P450 (Von Moltke et al., 1997), so likely a monooxygenase could be the responsible
427 enzyme in the heterotrophic reactor. The conversion of FLX to seproxetine in activated
428 sludge is very relevant since there is an enantioselectivity preference for (S)-FLX
429 biotransformation. This event leads to (S)-seproxetine formation and (R)-FLX
430 accumulation (unless enantiomerization processes take place), which for some
431 microorganisms can be 10 and 30 times more toxic than (S)-FLX, respectively, and
432 cause an overall increase in toxicity (Andrés-Costa et al., 2017).

A.3) Demethylation: Demethylations are catalyzed by demethylases from a variety of enzyme families, including monooxygenases (Robb et al., 2018) and dioxygenases (Fedeles et al., 2015). N-demethylation is the most common reaction, performed by oxidative demethylases exploiting the weak C-H bonds adjacent to amines and acting on the N-methyl groups. During the process, an oxygen atom is inserted to the C-H bond and then a spontaneous decomposition of the hydroxylated intermediate occurs, forming formaldehyde and a demethylated product. O-demethylation reactions can also occur, particularly during ether cleavage. N-demethylation of OMPs has been reported for several amines, such as pargyline, and O-demethylation has been observed in anaerobic conditions for venlafaxine (Falås et al., 2016) and in activated sludge for TMP, likely due to the action of monooxygenases (Jewell et al., 2016a; Krah et al., 2016).

Besides the possible demethylation of FLX to seproxetine previously mentioned, N-demethylation of the tertiary amine of DZP was detected (Table 1), leading to the formation of nordazepam, a very active metabolite with a long half-life that has been found in humans (Kosjek et al., 2012). In human metabolism, the enzyme carrying out the biotransformation is a cytochrome P450 (Luk et al., 2014), thus, it is likely that in our bioreactor a monooxygenase was also the responsible enzyme.

A.4) Dehydrogenation: Besides hydroxylation, further oxidations were seen for ADBI and NP (Table 1). They could have been catalyzed by alcohol dehydrogenases or oxidases (depending on the electron acceptor), capable to form the carboxylic acid moiety (Phale et al., 2019). These enzymes are present in many organisms, including bacteria, and play a crucial role in many metabolic pathways, as in the reversible reaction where acetaldehyde is converted to ethanol by alcohol dehydrogenase, a key step to regenerate the cofactors required for glycolysis.

B) Hydrolysis: Hydrolysis reactions are carried out by hydrolases, previously observed in activated sludge, as during the biotransformation of atenolol by bacterial amidohydrolases and azoxystrobin by protozoan hydrolases (Achermann et al., 2018b). The reported hydrolases in literature belong to several catalytic classes, including amidases, esterases, phosphatases and peptidases, among others (Krah et al., 2016). Here, we report for TMP a hydrolytic displacement of a primary amine moiety (Table 1), a novelty compared to reported TMP biotransformations, involving mostly demethylation and oxidation reactions (Jewell et al., 2016a). It is hypothesized that the TP formed through hydrolysis of the aminopyrimidine group of TMP (Table 1) could be obtained thanks to aminopyrimidine aminohydrolase (EC 3.5.99.-), involved in the metabolism of thiamine, a vitamin required by organisms from many life kingdoms of life, including bacteria (Lemmer and Nitschke, 1994). Hydrolysis of amine moieties in OMPs is rather rare since they more often undergo N-oxidation, α-C-hydroxylation and conjugation reactions (Gulde et al., 2016). However, this type of biotransformation has been observed before, as in reactions involving chorismic acid (Ganem, 1995), a metabolite present in the shikimic acid pathway, followed by bacteria and other organisms for the biosynthesis of folates and aromatic amino acids.

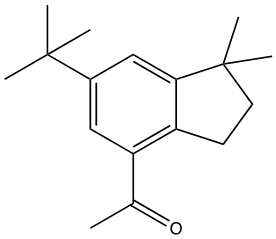
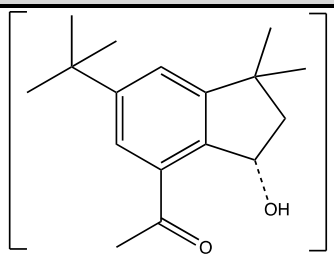
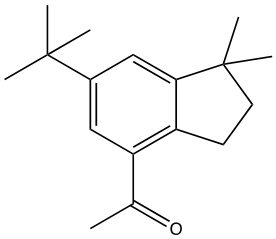
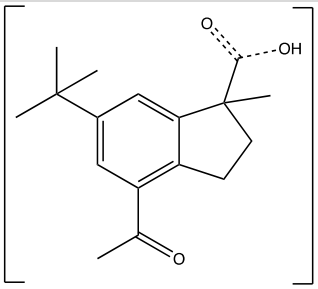
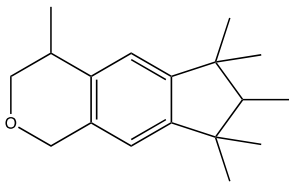
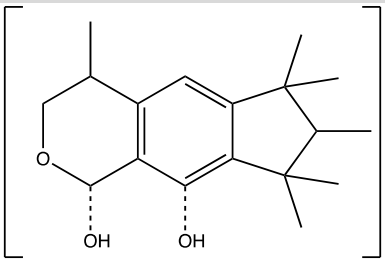
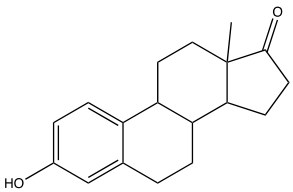
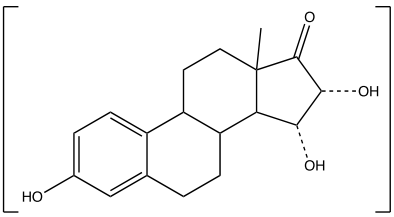
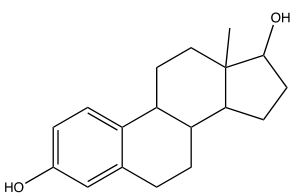
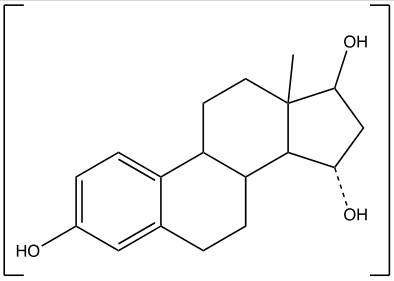
C) Conjugation: Conjugation reactions are anabolic and catalyzed by transferases thanks to the presence of a functional group in the substrate that serves as the anchoring site for a molecule or moiety. Since these types of reactions can occur both with exogenous and endogenous substrates, they play a key role in the metabolism of xenobiotics (Testa and Krämer, 2008).

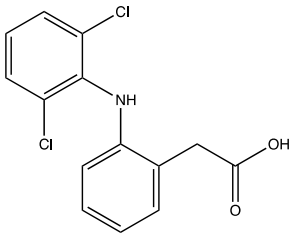
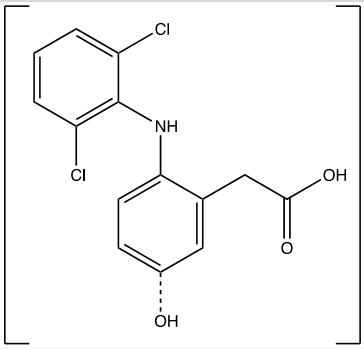
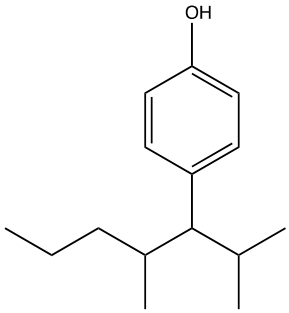
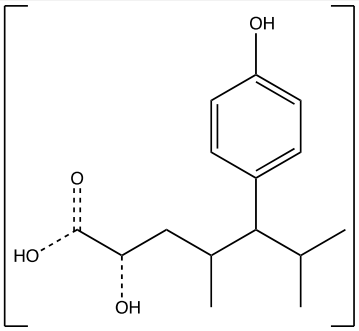
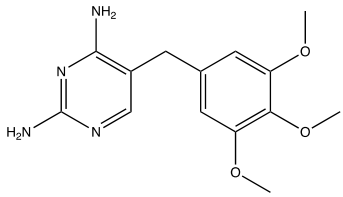
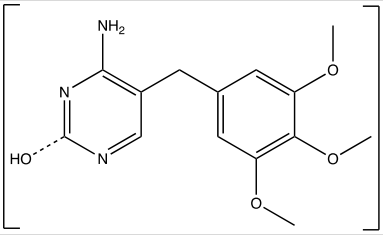
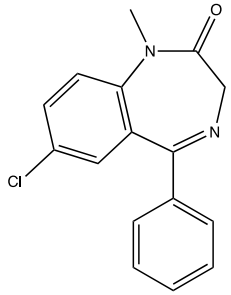
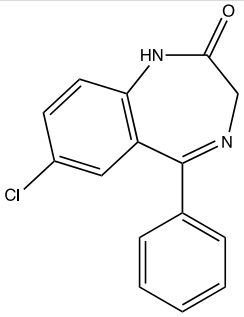
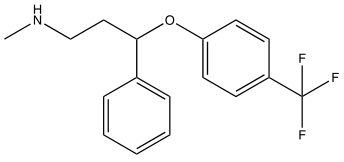
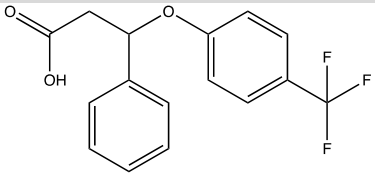
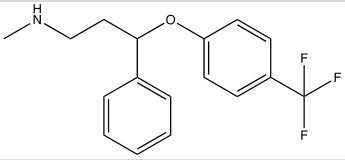
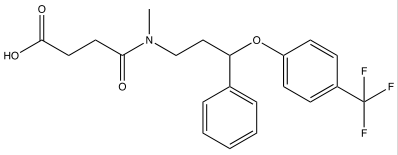
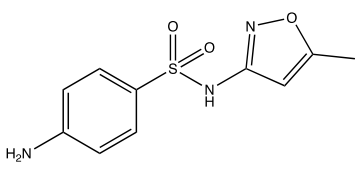
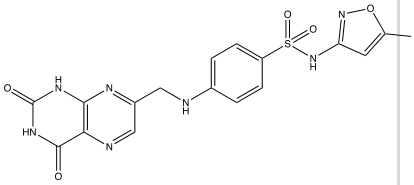
We observed a conjugation reaction at the amine group of SMX (Table 1), leading to a TP found by Achermann et al. (2018a) in the effluent of several WWTPs. The biotransformation pathway consists of a pterin conjugation, an oxidation and a final

hydrolysis reaction. The enzymes involved could belong to the folic acid pathway, such as pterin deaminase and dihydropteroate synthase. If the folic acid synthesis is the responsible pathway, the biotransformation would be linked to bacterial growth and maintenance, which could explain the generally good biotransformation of SMX observed in various environments, including anaerobic, nitrifying and heterotrophic (Achermann et al., 2018a).

Besides, a FLX TP was also formed by conjugation at the amine moiety (Table 1), following an N-acylation reaction (N-succinylation), suggesting N-acyltransferases as the responsible enzymes (Gulde et al., 2016). N-acylation reactions are important in microbial xenobiotic metabolism, with N-succinylation being particularly involved in the biotransformation of primary and secondary amines to secondary and tertiary amines, respectively.

508 **Table 1.** Summary of the suggested TPs structures found in the aerobic heterotrophic
509 reactor, including the confidence level, atom changes relative to the parent OMPs and
510 biotransformation reactions and enzymes likely involved in the process. The TP
511 structures placed in brackets are only suggestions for possible structures. The functional
512 groups bonded with dotted lines could be placed somewhere else in the molecule.

OMPs		Transformation product	Level	Atom change	Reaction	Candidate enzymes
ADBI			3	+ O	Hydroxylation	EC 1.14.- EC 1.13.-
ADBI			3	+ 2O - 2H	Hydroxylation Additional oxidation	EC 1.14.- EC 1.13.- EC 1.1.-
HHCB			4	+ 2O	Hydroxylation	EC 1.14.- EC 1.13.-
E1			4	+ 2O	Hydroxylation	EC 1.14.- EC 1.13.-
E2			3	+ O	Hydroxylation	EC 1.14.- EC 1.13.-

DCF			3	+ O	Hydroxylation	EC 1.14.- EC 1.13.-
NP			3	+ 3O - 2H	Hydroxylation Additional oxidation	EC 1.14.- EC 1.13.- EC 1.1.-
TMP			3	+ O - N, H	Hydrolysis	EC 3.5.99.-
DZP			3	- C, 2H	Demethylation	EC 1.14.-
FLX			3	+ 2O - C, N, 5H	Demethylation Deamination Oxidation	EC 1.14.- EC 1.4.- EC 1.2.1.-
FLX			2	+ 4C, 3O, 4H	Conjugation	EC 2.3.-
SMX			3	+ 7C, 4N, 2O, 4H	Conjugation	EC 2.5.- EC 3.5.4.11

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4. CONCLUSIONS

This study highlights the relevant role of aerobic heterotrophs in the cometabolic biotransformation of OMPs, speaking to their capacity to contribute to the overall removal of OMPs in activated sludge plants and questioning the requirement of maintaining a nitrifying activity. In fact, this study shows that heterotrophs are able to achieve similar extents of biotransformation for the selected OMPs as those reported in several literature studies with activated sludge (with nitrifying, denitrifying and heterotrophic activities) and purely nitrifying systems. Based on the TPs produced, it was possible to identify the main reactions involved in OMPs biotransformation under aerobic heterotrophic conditions as follows: oxidation (hydroxylation, dehydrogenation, deamination and demethylation), hydrolysis and conjugation routes. An overall analysis of all results allows selecting mono- and dioxygenases, dehydrogenases, hydrolases and transferases as some of the main enzymatic activities likely responsible for OMPs biotransformation under heterotrophic conditions. Overall, this work highlights the relevant contribution of heterotrophs to OMPs removal, which are gaining importance in the conception of new WWTPs, and deepens the knowledge on their biotransformation mechanisms.

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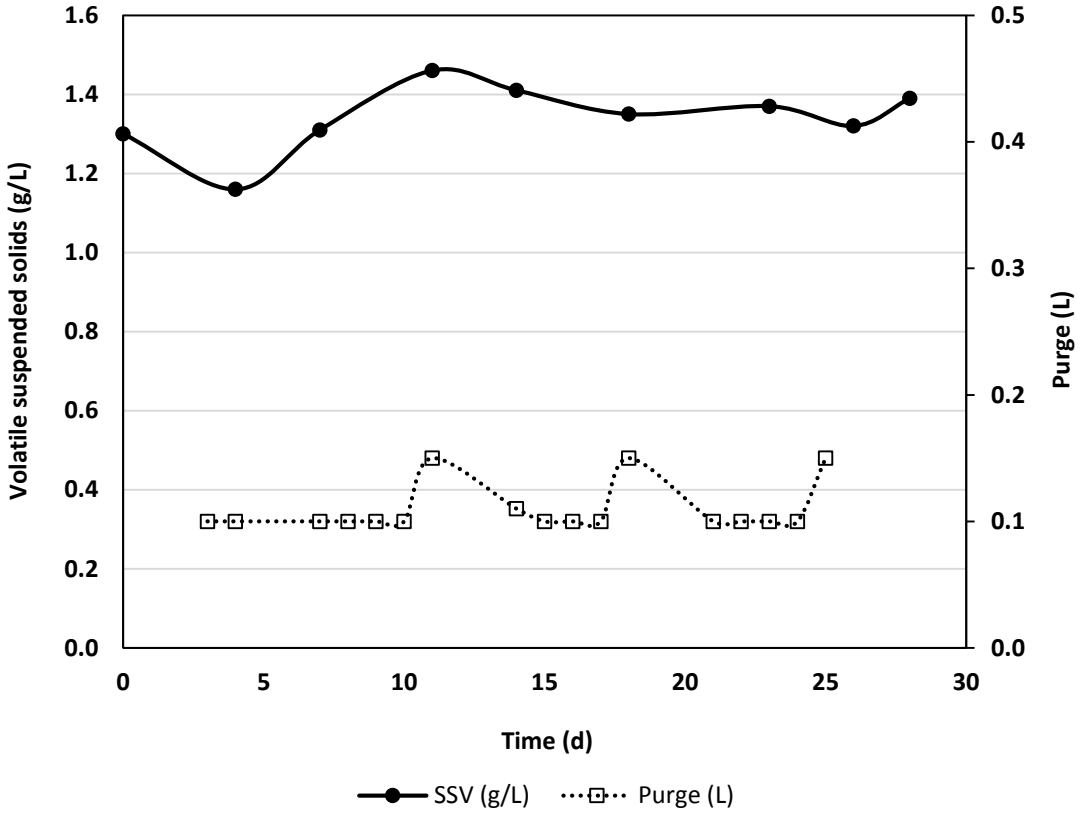
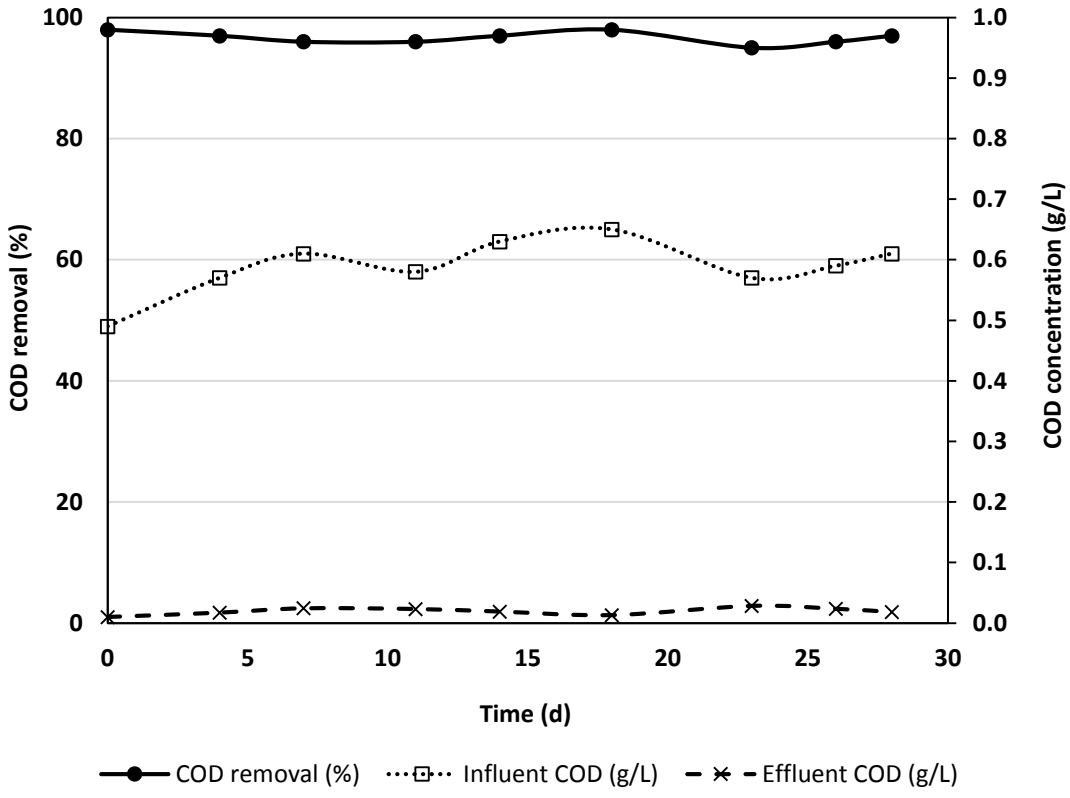
783

Figure

A)


Volatile suspended solids (g/L)
Purge (L)
Time (d)
SSV (g/L)
Purge (L)

B)


COD removal (%)
COD concentration (g/L)
Time (d)
COD removal (%)
Influent COD (g/L)
Effluent COD (g/L)

C)

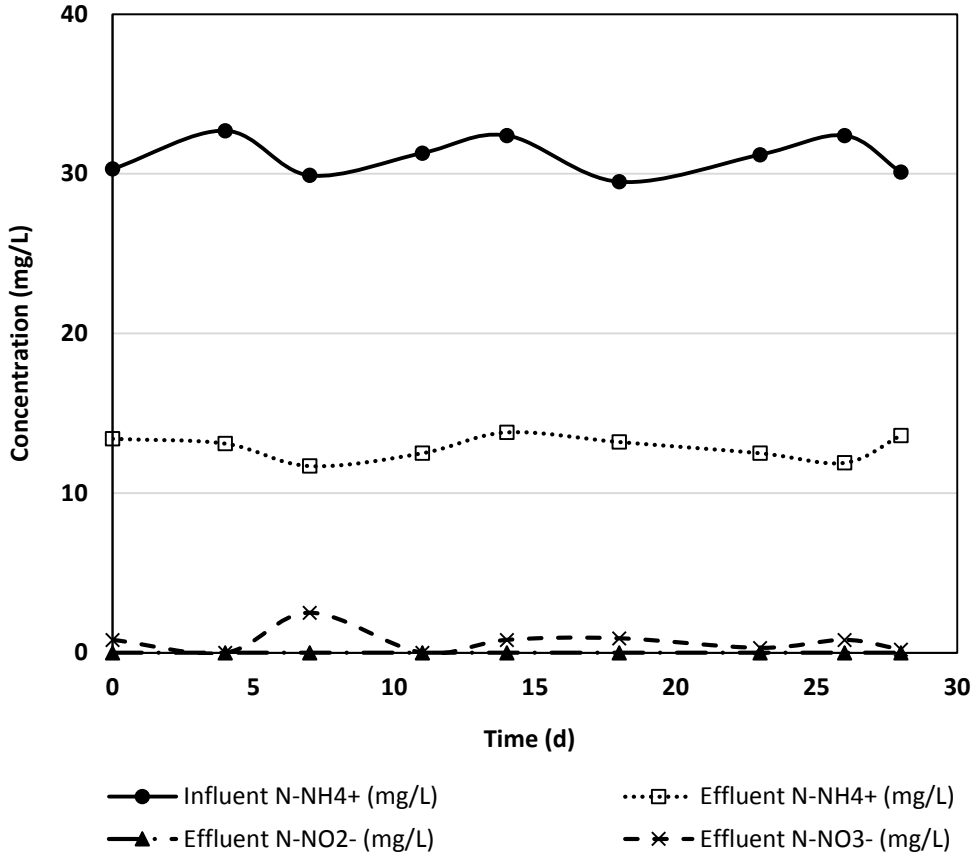


Fig. 1. Operational parameters obtained in the continuous aerobic heterotrophic reactor. A) VSS concentration and purge performed. B) COD removal along with COD influent and effluent concentrations. C) Influent N-NH_4^+ and effluent N-NH_4^+, N-NO_3^- and N-NO_2^- concentrations.

Figure

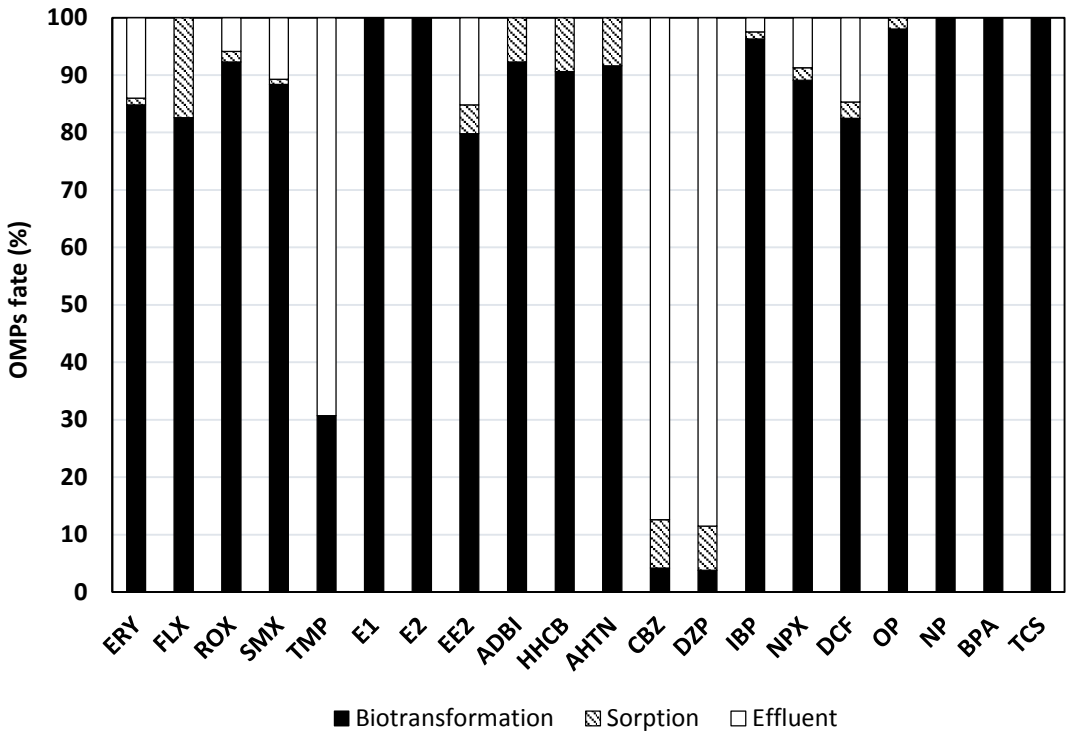


Fig. 2. Fate of the OMPs in the continuous aerobic heterotrophic reactor operated with fixed HRT (1 d) and OLR (0.6 g COD L^{-1} d^{-1}).

Figure

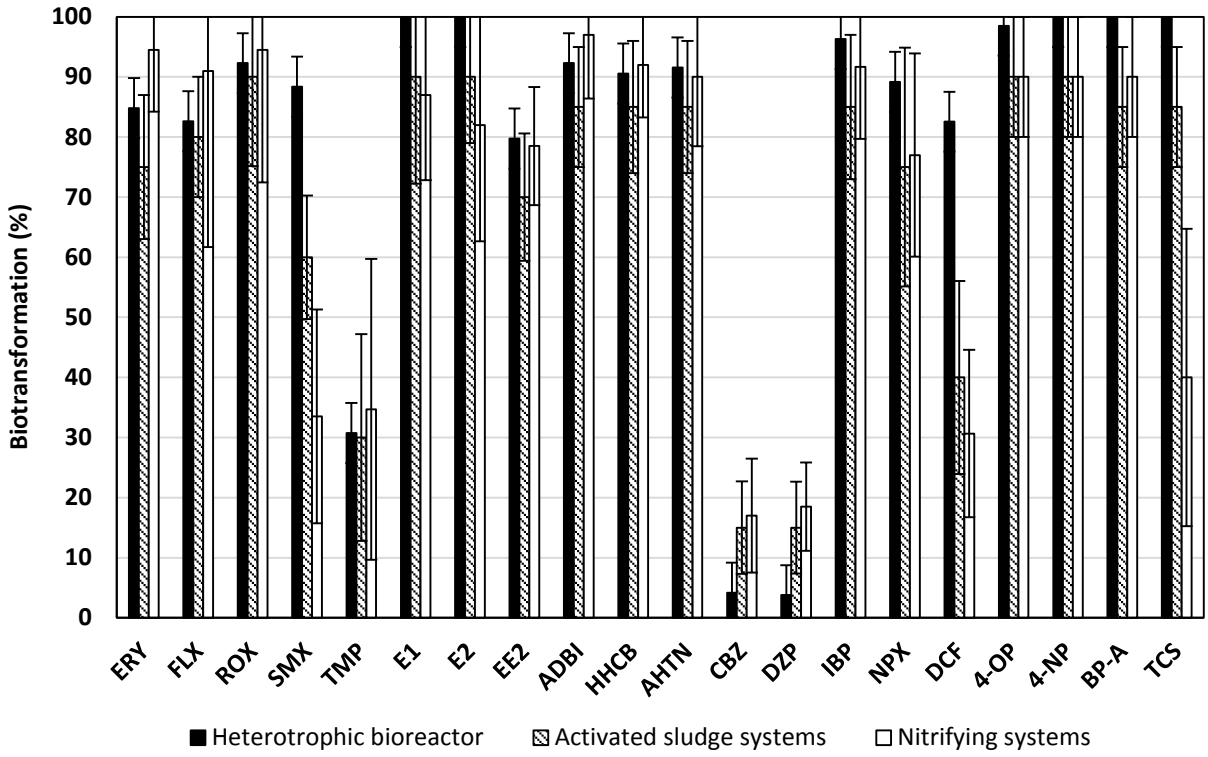


Fig. 3. OMPs biotransformation comparison between our heterotrophic reactor and the average literature-reported values in nitrifying systems and activated sludge systems with heterotrophic and nitrifying activities. The data was obtained from multiple studies (Alvarino et al., 2018a, 2018b; Batt et al., 2006; Fernandez-Fontaina et al., 2016, 2013, 2012; Gardner et al., 2013; Kim et al., 2007; Lee et al., 2015; Luo et al., 2014; Margot et al., 2015; Petrie et al., 2014; Suarez et al., 2010; Tran et al., 2018, 2009; Wang et al., 2020; Yi and Harper, 2007).