# The organic loading rate affects organic micropollutants' cometabolic biotransformation kinetics under heterotrophic conditions in activated sludge

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**Accepted Manuscript**

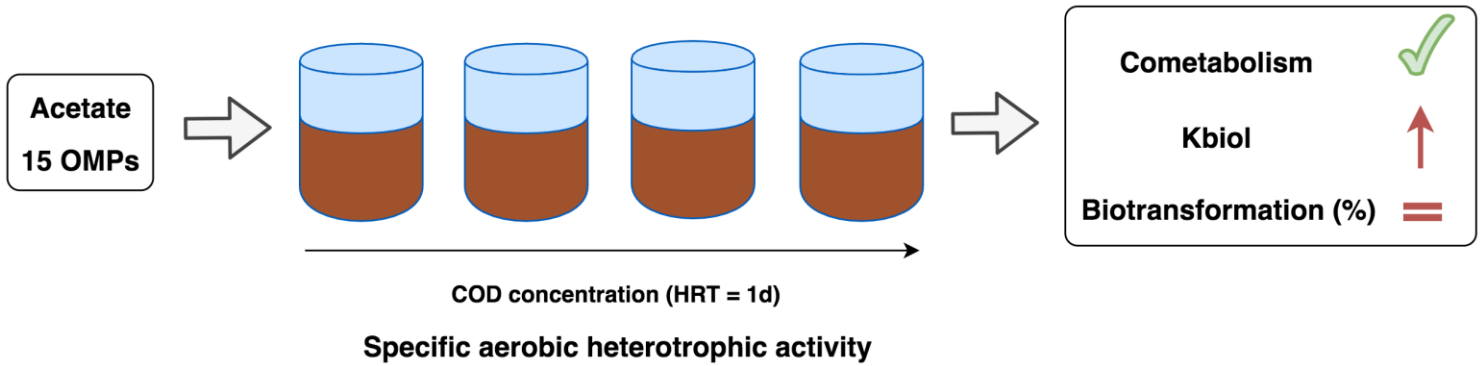
**How to cite:**
Water Research, 189 (2021), 116587
https://doi.org/10.1016/j.watres.2020.116587

## HIGHLIGHTS

1. OMPs cometabolic biotransformation was proved with aerobic heterotrophs.
2. OMPs biotransformation yield did not correlate with the heterotrophic activity.
3. $k_{biol}$ increased with the heterotrophic activity for most compounds.
4. The primary carbon source and the OMPs followed a simultaneous removal.

Graphical Abstract

# GRAPHICAL ABSTRACT



# The organic loading rate affects organic micropollutants' cometabolic biotransformation kinetics under heterotrophic conditions in activated sludge

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## ABSTRACT

Several studies have shown that organic micropollutants (OMPs) are biotransformed cometabolically in activated sludge systems. However, the individual role of heterotrophs in the microbial consortium is still not clear, i.e., there is still a gap regarding the influence of the heterotrophic activity on the cometabolic biotransformation kinetics and yield of the OMPs. Aiming to answer these questions, experiments with increasing primary substrate concentrations were performed under aerobic heterotrophic conditions in a continuous stirred tank reactor operated at several organic loading rates (OLR) with fixed hydraulic retention time. Moreover, the individual kinetic parameters were determined in batch assays with different initial substrate concentrations using the sludges from the continuous reactor. A set of 15 OMPs displaying a variety of physicochemical properties were spiked to the feeding in the ng $L^{-1}$ - µg $L^{-1}$ range. Results reveal that the biodegradation of the primary carbon source and the biotransformation of the OMPs occur simultaneously, in clear evidence of cometabolic behaviour. Moreover, we conclude that the OMPs biotransformation kinetic constant ($k_{biol}$) shows a linear dependence with the OLR of the primary substrate for most of the compounds studied, suggesting that the heterotrophic activity seriously affects the OMPs biotransformation kinetics. However, under typical activated sludge systems operating conditions (hydraulic retention times above 8 h), their biotransformation yield would not to be significantly affected.

**Keywords:** biotransformation kinetic constant; heterotrophs; pharmaceuticals; wastewater treatment plant; yield

## 1. INTRODUCTION

Organic micropollutants (OMPs) including industrial chemicals, pharmaceuticals, personal care products, pesticides and hormones are released daily and carried in residual waters from households, hospitals, industries and agriculture before they reach wastewater treatment plants (WWTPs) (Luo et al., 2014). WWTPs are only capable of removing them to a certain extent, depending on the compound and the process applied, and have become the main source of OMPs release to the environment (Kasprzyk-Hordern et al., 2009).

Since OMPs are present in WWTPs at very low concentrations, in the ng $L^{-1}$ and μg $L^{-1}$ range, they are not capable of supporting microbial growth and, therefore, a primary substrate is required to induce the enzymatic action that allows OMPs biotransformation through cometabolism (Fischer and Majewsky, 2014). As a result, OMPs are biotransformed within the metabolic pathways of the microbial populations present in WWTPs (Alvarino et al., 2018a; Müller et al., 2013). The most common biological treatment in WWTPs is based on an activated sludge system, which typically involves heterotrophic and autotrophic nitrifying activities (Gernaey and Sin, 2013). The combination of nitrifiers and heterotrophs provides a broader range of enzymatic activities, helping to remove a wider variety of OMPs and to a higher extent. For instance, Fernandez-Fontaina et al. (2016) reported that the presence of aerobic heterotrophs enhanced the removal of sulfamethoxazole in activated sludge, and Men et al. (2016) determined that when the nitrifying population was inhibited the biotransformation of bromoxynil was considerably reduced.

The action of heterotrophic bacteria is fundamental in most WWTPs since they are responsible for the biological removal of organic matter (Majewsky et al., 2011). In fact, some facilities are designed only with this purpose and the main microbial activity

they present is heterotrophic (Metcalf & Eddy, 2014). Moreover, the importance of heterotrophs is gaining interest in the novel conceptions of WWTPs, where the A-stages, working at high organic loading rates (OLR) and short solid retention times (SRT), operate basically under heterotrophic conditions (Jimenez et al., 2015). Heterotrophs are fast growers, have short doubling times (K. Kim et al., 2020) and, compared with autotrophs, possess a more efficient metabolism and a higher diversity of organisms (Holtmann and Sell, 2002). Besides, Maeng et al. (2013) and Majewsky et al. (2010) have indicated that heterotrophic bacteria may be involved in the removal yield and degradation rates of OMPs when primary substrates are present. The positive effect of the heterotrophic metabolism on the biotransformation of multiple OMPs has been previously reported, as for diclofenac (Nguyen et al., 2019), 17α-ethinylestradiol (Larcher and Yargeau, 2013) and ibuprofen (Alvarino et al., 2018a), among others. Furthermore, Fernandez-Fontaina et al. (2016) even showed that heterotrophs can be more favourable than autotrophs for biotransformation, as in the case of sulfamethoxazole. Nonetheless, due to the complexity of the heterotrophic metabolism, the contribution they make to the effectiveness and the biotransformation kinetics of the OMPs in WWTPs is still not clear.

To characterize OMPs biotransformation, a pseudo-first order kinetic model is commonly assumed and the resulting biotransformation kinetic constant ($k_{biol}$) largely depends on the physicochemical properties of each particular pollutant (Lema and Suarez, 2017). In fact, Falås et al. (2016) concluded that $k_{biol}$ varies more depending on the nature of the OMPs than on the experimental conditions. However, $k_{biol}$, as a kinetic constant, is also affected by the reactor operating conditions (such as temperature, pH and oxidation reduction potential), the presence and availability of co-substrates and the biochemical versatility of the sludge, as several studies demonstrate (Barceló, 2012;

Petrovic et al., 2013). For instance, Alvarino et al. (2016) reported $k_{biol}$ values of 0.09 and 0.05 L g $VSS^{-1}$ $d^{-1}$ for sulfamethoxazole under aerobic heterotrophic and autotrophic denitrifying conditions, respectively; Gulde et al. (2014) reported $k_{biol}$ increments of one order of magnitude for atenolol at pH 8 compared to pH 6 under activated sludge conditions and Li et al. (2005) reported that 17β-estradiol increased its $k_{biol}$ value from 1.8 to 3.3 L g $VSS^{-1}$ $h^{-1}$ when increasing the temperature from 20 to 35ºC in batch experiments with activated sludge. Nonetheless, conversely, there are so far no conclusive studies regarding the influence of the OLR and the heterotrophic activity on $k_{biol}$.

The role of the OLR in the removal of the OMPs is, in fact, a topic of current and general interest in biological systems, but results still do not show a clear trend. For instance, Kora et al. (2020) showed that increasing the OLR improved the removal of 3 out of 5 OMPs spiked in a methanogenic-aerobic moving bed biofilm reactor and Moya-Llamas et al. (2018) found better biodegradations for 6 OMPs at higher OLRs in a UASB reactor coupled to a MBR. On the contrary, in a moving biofilm bed reactor Abtahi et al. (2018) reported for some OMPs a maximum removal at the highest OLR tested and for others at the lowest OLR and Carneiro et al. (2020) reported a negative impact of increasing OLRs in the biodegradation of citalopram and sulfamethoxazole in anaerobic fix bed biofilm reactors. Finally, differently to these studies, Gonzalez-Gil et al. (2018b) found no correlation between variations in the OLR and the biotransformation of most OMPs in methanogenic digesters.

The objective of the present study is to extend the knowledge behind OMPs biotransformation processes under exclusively aerobic heterotrophic conditions. More specifically, research is focussed on assessing the OMPs cometabolic biotransformation, aiming to determine the relationship between the intensity of the heterotrophic activity

and the OMPs biotransformation rate, with a particular focus on evaluating the effect on $k_{biol}$. The implications of understanding such linkage could be of great importance to design biological systems able to maximize OMPs removal. To reach these goals, a series of experiments in a continuous stirred tank reactor (CSTR) operated at different OLRs were performed, as well as batch experiments set with different initial chemical oxygen demand (COD) concentrations.

## 2. MATERIALS AND METHODS

### 2.1 Organic micropollutants

This study focused on 15 OMPs commonly present in WWTPs that display a wide variety of chemical structures, physicochemical properties and applications (Table S3). The compounds were: the antibiotics erythromycin (ERY), roxithromycin (ROX), trimethoprim (TMP), sulfamethoxazole (SMX); the anti-inflammatories ibuprofen (IBP), naproxen (NPX); the neuro drugs carbamazepine (CBZ), diazepam (DZP); the musk fragrances celestolide (ADBI), galaxolide (HHCB), tonalide (AHTN); the biocide triclosan (TCS) and the endocrine disruptors estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2). 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.

### 2.2 Aerobic Heterotrophic Reactor

A 5 L continuously stirred lab-scale reactor (Figure S1), connected to a 2 L settler, was set at 25°C and operated at four different OLRs (0.2, 0.4, 0.6 and 0.8 g COD $L^{-1}$ $d^{-1}$, that

resulted in 0.4, 0.6, 1.1 and 2.7 g COD g $VSS^{-1}$ $d^{-1}$), selected trying to cover typical conditions of activated sludge WWTPs (Lema and Suarez, 2017; Metcalf & Eddy, 2014). Each experimental stage was maintained for one month and the OLR was changed by varying the organic matter concentration in the feeding while keeping constant the hydraulic retention time (HRT). The reactor was inoculated with sludge from a conventional activated sludge reactor of a WWTP near Santiago de Compostela (Spain). The WWTP was designed for 184000 population equivalents, receives an influent COD ranging between 0.2 - 0.7 g $L^{-1}$ and operates with approximate SRT and HRT values of 10 d and 8 h, respectively. The feeding consisted of a synthetic mixture of sodium acetate and acetic acid as primary carbon source (in concentrations that ensured operation at neutral pH), ammonium chloride, potassium dihydrogen phosphate, calcium chloride and magnesium sulfate (Table S1). Acetate was selected as the carbon source for being an easily biodegradable substrate, optimal for microbial growth, as well as for being directly metabolized through the Krebs cycle (Nelson and Cox, 2017). Even though varying carbon sources lead to the expression of different enzymatic activities and to varying enzymatic regulation levels, the mineralization of organic matter typically requires the involvement of the Krebs cycle. Thus, the use of acetate ensures that the enzymatic activities present in the bioreactor are also present in WWTPs that deal with more complex carbon substrates. Moreover, other trace nutrients were also added to promote the growth of aerobic heterotrophic microorganisms (Table S2). The HRT was set to 1 d to provide sufficient time to the microorganisms to achieve the maximum biotransformation they are capable of, at the same time as minimizing possible changes in the microbial population caused by an HRT too long. Besides, to avoid nitrification, allylthiourea solution was added in the feeding with a concentration of 5 mg $L^{-1}$ and the SRT was maintained below 8 d to minimize the presence of slow-

156 growing microorganisms and favour the heterotrophic activity (Achermann et al., 2018).
157 Aeration was provided to the reactor ensuring oxygen concentrations between 3.5 and
158 7.5 mg $O_2$ $L^{-1}$. After a start-up period of a few days, selected OMPs were spiked in the
159 feeding at a concentration of 10 μg $L^{-1}$, except for the musk fragrances and hormones,
160 whose concentration was 40 and 1 μg $L^{-1}$, respectively. These concentrations were
161 selected according to typical WWTPs influent concentrations (Table S3) (Besha et al.,
162 2017; Clara et al., 2011; Luo et al., 2014; Petrie et al., 2014; Tran et al., 2018a;
163 Verlicchi et al., 2012; Verlicchi and Zambello, 2015).

164 To monitor the operation of the reactor, the conventional parameters shown in section
165 2.4.1. were determined 2-3 times per week. Also, once the steady state was reached at
166 each OLR, inlet and outlet OMPs concentrations were measured (solid and liquid phase)
167 by taking three samples from the feeding and the reactor vessel in three consecutive
168 days. Each of the three inlet samples were taken exactly 24 h (the HRT set in the
169 reactor) before the outlet sample in order to minimize possible slight changes in the
170 influent OMPs concentration.

171**2.3 Batch assays**

172 Batch assays were set to observe if variations in the heterotrophic microbial activity,
173 determined as the maximum COD specific activity, affected the OMPs
174 biotransformation kinetics and, particularly, the $k_{biol}$. The experiments were performed
175 with COD initial concentrations of 0.2, 0.4, 0.6 and 0.8 g $L^{-1}$ by varying the sodium
176 acetate and acetic acid concentration and maintaining the micro and macronutrients
177 used in the aerobic heterotrophic reactor. The biomass was taken from the aerobic
178 heterotrophic reactor working at the respective OLRs to ensure that the microbial
179 population was adapted to the experimental conditions of the assays. For each batch, 18
180 flasks were prepared, three for each time point in order to have triplicates (0, 1, 3, 8, 24

and 48 h). At each of the mentioned times, 3 flasks were taken to determine the concentration of the OMPs. The volatile suspended solids (VSS) concentration was set in all cases to approximately 0.80 g VSS $L^{-1}$ and the temperature and stirring were fixed at 25°C and 150 rpm (Innova 4300 Incubator Shaker – New Brunswick Scientific). Neutral pH was ensured using punctual additions of NaOH or HCl when necessary and an oxygen concentration above 4.5 mg $O_2$ $L^{-1}$ was maintained during experimentation. The conventional parameters shown in section 2.4.1. were determined for each time point, except for the solids and nitrogen concentrations which were measured at times 0, 24 and 48 h. OMPs analysis was performed (solid and liquid phase) by taking samples from the flasks at each time point.

## 2.4 Analytical methods

### 2.4.1 Conventional parameters

Total suspended solids (TSS), VSS, total and soluble COD, ammonium ($NH_4^+$), nitrate ($NO_3^-$) and nitrite ($NO_2^-$) concentration, pH and temperature were measured according to Standard Methods (2012). Dissolved oxygen ($O_2$) concentration was analysed using a multiparameter Hach HQ40d, with a luminescent optical probe (Ritter). In all cases, the analysis was performed in triplicate. Further information can be seen in Table S4.

### 2.4.2 Organic micropollutant analysis

Samples were centrifuged at 3500 rpm for 10 min (except those from the reactor feeding, lacking a solid fraction). Then, the supernatants and the feeding samples were prefiltered (AP4004705, Millipore) and filtered at 0.45 μm (HAWP04700, Millipore). Lastly, solid-phase extraction (SPE) was performed with 200 mL samples and 60 mg Oasis HLB cartridges (Waters, Milford, MA, USA), as described in detail by

Fernandez-Fontaina et al. (2013). The quantification of antibiotics (ERY, ROX, SMX, TMP), neuro drugs (CBZ, DZP) and hormones (E1, E2, EE2) was performed using an Agilent G1312A liquid chromatograph with a binary pump and automatic injector HTC-PAL (CTC Analytics) connected to a mass spectrometer API 4000 triple quadrupole (Applied Biosystems). Musk fragrances (ADBI, HHCB, AHTN), anti-inflammatories (IBP, NPX) and the biocide (TCS) were quantified using a gas chromatograph (Varian CP-3900) coupled with an ion trap spectrometer (Varian CG-2100). All OMPs analyses were performed in triplicate. Further information regarding the recoveries and limits of quantification and detection of the OMPs can be found in Table S5. Overall, the recoveries in the liquid matrix ranged between 70-95% and in the solid matrix between 50-60%, while the limits of quantification and detection ranged between 0.006-0.505 μg $L^{-1}$ and 0.002-0.168 μg $L^{-1}$, respectively.

The solid phase of the samples was frozen and lyophilized. Then, ultrasonic solvent extraction (USE) was performed based on the procedure described by Ternes et al. (2005). The USE technique consisted of three sequential extractions with methanol and two with acetone performed 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 (TurboVap LV, Biotage) flowing nitrogen (200 kPa, 30 °C) and resuspended in water. Finally, SPE and OMPs quantification were performed as described for the liquid phase. The sorption for the OMPs was minimal for all compounds (less than 10% of the total removal), except for the fragrances which showed a 15% of sorption, approximately. These results are consistent with literature information (Alvarino et al., 2018b; Tran et al., 2018b) and, for this reason, throughout the document only the biotransformation of the OMPs will be discussed.

## 2.5 Mass balance and determination of OMPs biotransformation yield and rate

Removal of OMPs was associated with three mechanisms: volatilization, sorption to the sludge and biotransformation. Volatilization and sorption are based on OMPs transfer due to equilibrium processes between the liquid-gas and liquid-solid phases, respectively, while biotransformation consists of the removal of the compounds, which is typically calculated for the dissolved OMPs (Pomiès et al., 2013). The mass balances considered for the liquid and solid phase are expressed as shown in the following equations (Eq. 1 and Eq. 2):

$$\frac{dC_W}{dt} = \frac{F_{in}(t)}{V} - \frac{F_{out}(t)}{V} - \left(\frac{dC_W}{dt}\right)_{volatilization} - \left(\frac{dC_W}{dt}\right)_{sorption} + \left(\frac{dC_W}{dt}\right)_{desorption} - \left(\frac{dC_W}{dt}\right)_{biotransformation} \quad (1)$$

$$\frac{dC_S}{dt} = \frac{F_{in}(t)}{V} - \frac{F_{out}(t)}{V} + \left(\frac{dC_W}{dt}\right)_{sorption} - \left(\frac{dC_W}{dt}\right)_{desorption} \quad (2)$$

with:

$C_W$ dissolved OMPs concentration (µg $L^{-1}$)

$C_s$ OMPs concentration in the solid phase (µg $L^{-1}$)

$F_{in}$ inlet OMPs flow rate (µg $d^{-1}$)

$F_{out}$ outlet OMPs flow rate (µg $d^{-1}$)

$V$ bioreactor volume (L)

Volatilization depends on the physicochemical properties of the OMPs and the operating conditions of the process. In an activated sludge process, volatilization can occur due to stripping, which is controlled by the air flow, the OMPs concentration and the Henry's constant, and due to surface volatilization, which usually is not taken into account as a result of its lower relevance (Ltd I C Consultants, 2001). Volatilization can

be neglected when a compound has a Henry constant below 10 Pa $m^3$ $mol^{-1}$ and its $Hc/K_{ow}$ (Henry's constant divided by the octanol-water partition coefficient) is lower than $10^{-4}$ (Ltd I C Consultants, 2001). This is the case of the 15 OMPs studied in this work (Table S3) and therefore volatilization was not considered in the calculations.

The sorption term consists of an equilibrium between the liquid and solid phase, where OMPs simultaneously undergo sorption and desorption phenomena. Since the OMPs are present at low concentrations, normally a linear model is assumed (Pomiès et al., 2013), as shown below (Eq. 3 and Eq. 4):

$$\left(\frac{dC_W}{dt}\right)_{sorption} = k_{sor} \cdot C_W \cdot X_{TSS} \qquad (3)$$

$$\left(\frac{dC_W}{dt}\right)_{desorption} = k_{desor} \cdot C_S \qquad (4)$$

with:

$k_{sor}$ sorption kinetic constant (L $g^{-1}$ $d^{-1}$)

$k_{desor}$ desorption kinetic constant ($d^{-1}$)

$X_{TSS}$ total suspended solids concentration (g $L^{-1}$)

The partition coefficient ($K_d$), shown in (Eq. 5), is the ratio between the kinetic constants:

$$K_d = \frac{k_{sor}}{k_{desor}} = \frac{C_S}{C_W \cdot X_{TSS}} \qquad (5)$$

$K_d$ partition coefficient (L $g^{-1}$)

Pseudo-first order kinetics are typically used to model the OMPs biotransformation rate ($r_{biol}$), represented in (Eq. 6) (Schwarzenbach et al., 2003):

$$\left(\frac{dC_W}{dt}\right)_{biotransformation} = r_{biol} = k_{biol} \cdot C_W \cdot X_{VSS} \qquad (6)$$

The biodegradation kinetic constants were calculated performing mass balances to the batch experiments, as shown in (Eq. 7):

$$k_{biol}\ (L\ g^{-1}\ d^{-1}) = \frac{C_{W0} - C_{Wt} \cdot (1 + k_d \cdot X_{TSS})}{C_{W0} \cdot X_{VSS} \cdot t} \qquad (7)$$

with:

$C_{w0}$ dissolved OMPs concentration at time 0 (µg $L^{-1}$)

$C_{wt}$ dissolved OMPs concentration at time *t*, where the slope is maximum (µg $L^{-1}$)

The biotransformation yield (Eq. 8) and the specific biotransformation rate (Eq. 9) were calculated as shown in the equations below. Throughout the document, both parameters were taken into account to describe the behaviour of the OMPs. The yield was used to determine the biotransformation efficiency of the compounds and the biotransformation rate provided information regarding the cometabolic behaviour of the OMPs, as well as a better insight of the reactor performance.

$$\text{Biotransformation yield } (\%) = \frac{F_{in} - F_{wout} - F_{sout}}{F_{in}} \cdot 100 \qquad (8)$$

$$\text{Specific biotransformation rate } (\mu g\ g_{VSS}^{-1}\ d^{-1}) = \frac{F_{in} - F_{wout} - F_{sout}}{X_{VSS} \cdot V} \cdot 100 \qquad (9)$$

with:

$F_{wout}$ outlet dissolved OMPs flow rate (µg $d^{-1}$)

$F_{sout}$ outlet OMPs flow rate in the solid phase (µg $d^{-1}$)

The outlet OMPs flow rate in the solid phase ($F_{sout}$) is calculated as shown in (Eq. 10):

$$F_{sout} = C_S' \cdot X_{TSS-effluent} \cdot F_{effluent} + C_S' \cdot X_{TSS-purge} \cdot F_{purge} \qquad (10)$$

where:

$C_S$' OMPs concentration in the solid phase (µg $g^{-1}$)

292 $F$ outlet flow rate (L $d^{-1}$)

293 **2.6 Statistical analysis**

294 To determine if the yields and specific biotransformation rates of the OMPs were
295 statistically different at the tested primary substrate specific biodegradation rates, R
296 software 3.6.2. was used. The statistical tests were performed at a 5% significance level
297 ($p < 0.05$).

298

299

300 **3. RESULTS AND DISCUSSION**

301 **3.1 Biotransformation yield**

302 Fig. 1 shows the biotransformation yield, i.e. the biotransformation percentage, of the
303 selected OMPs at the 4 organic loading rates in the aerobic heterotrophic reactor. The
304 biotransformation extent varied considerably among OMPs, being their behaviour
305 characterized as *(i)* low biotransformation (below 20%) for CBZ and DZP, *(ii)* medium-
306 low biotransformation (20-50%) for TMP, *(iii)* medium-high biotransformation (50-
307 80%) for ERY and *(iv)* high biotransformation (over 80%) for ROX, SMX, IBP, NPX,
308 TCS, ADBI, HHCB, AHTN, E1, E2 and EE2. These results are consistent with
309 literature information (Alvarino et al., 2018b; Fernandez-Fontaina et al., 2016; Khunjar
310 et al., 2011).

311 **Figure 1**

312 It can be observed that the biotransformation yield of most OMPs remained constant
313 throughout the different OLRs (biotransformation differences below 10 percentage

314 points and not statistically significant; $p > 0.05$ ). Only one value for ERY (experiment
315 with 1.1 g COD g $VSS^{-1}$ $d^{-1}$) and another for IBP (experiment with 2.7 g COD g $VSS^{-1}$
316 $d^{-1}$), significantly ($p < 0.05$) deviated (between 15-20 percentage points) to the values of
317 the other three conditions. These events for ERY and IBP cannot be attributed to a
318 specific trend or behaviour. Therefore, overall, the results of Fig. 1 indicate that
319 increasing the aerobic heterotrophic cometabolism did not affect the OMPs
320 biotransformation yield under the conditions tested. Several hypotheses could explain
321 this behaviour. The first one is that the enzymes involved in the OMPs
322 biotransformation might not be increasingly stimulated at higher OLRs. According to
323 Stadman (1970), in catabolic pathways, enzymes can be classified as "constitutive"
324 enzymes, whose concentration is independent of the presence of their substrates, and
325 "inducible" enzymes, that are produced when their immediate substrates or suitable
326 derivatives are present. If the enzymes involved in the biotransformation of the OMPs
327 belong to the first class and were not increasingly stimulated, the yield could have
328 remained stable even at higher OLRs. A second hypothesis is that the maximum
329 cometabolic rate towards OMPs is already reached at the lowest OLR and, therefore,
330 increasing the degradation rate of the primary substrate (acetate) does not have an effect
331 on the OMPs. In this regard, it is commonly assumed that the oxidation rate of a non-
332 growth substrate (OMPs) should always be linked to the oxidation rate of a growth
333 substrate (acetate) proportionally; however, some studies have shown that this is not
334 necessarily always the case (Criddle, 1993; M. H. Kim et al., 2020). Thirdly, the OMPs
335 may have already achieved their biotransformation limits due to thermodynamic
336 constraints. This event could be caused by the reversibility of the biological reactions,
337 leading to a chemical equilibrium between the parent compound and the transformation
338 product, as it has been previously suggested (Gonzalez-Gil et al., 2019). Lastly, the

primary metabolism may increase the specific biotransformation rate, but the HRT may have been high enough to hide this effect, showing the same biotransformation yield in all cases. In common biological treatments, as in activated sludge, the optimal HRT for OMPs removal is 24 h or longer (Boonnorat et al., 2019). Nevertheless, it has been proven that lower times may be sufficient to achieve the maximum biotransformation extent for several compounds (Boonnorat et al., 2019; Ejhed et al., 2018). Overall, the results shown in Fig. 1 are insufficient to determine which of these four hypotheses is more likely, being necessary to evaluate the behavior of the biotransformation rate.

### 3.2 Specific biotransformation rate

In Fig. 2, it can be observed the direct relationship between the OMPs biotransformation rate and the specific sludge activity: the higher the specific biodegradation rate of the growth substrate, the higher the OMPs specific biotransformation rate (Eq. 9). The dependence of the biotransformation rate of the secondary substrates (OMPs) with the biodegradation of the primary substrate (acetate) is a clear prove of cometabolism (Lema and Suarez, 2017). When the concentration of the primary substrate is increased (i.e., a higher OLR is applied), a higher microbial activity of the biomass is achieved. Hence, if an increment of the OMPs biotransformation rate also occurs, it indicates that they are been cometabolized by the same enzymes involved in the metabolism of the growth substrate. In Fig. 2, the differences in the specific biotransformation rates were statistically significant ($p < 0.05$) for all experimental conditions and all OMPs tested.

**Figure 2 (A, B and C)**

Despite all OMPs following a cometabolic biotransformation trend, there are some differences between the compounds, which do not show a homogeneous impact of the OLRs in their biotransformation rates. For instance, while the biotransformation rate of

363 TCS or SMX only slightly increases between the conditions of 0.4 g COD g $VSS^{-1}$ and
364 0.6 g COD g $VSS^{-1}$, other compounds such as ADBI, HHCB and AHTN are much more
365 affected. The explanation for this variability could be related to the enzymatic
366 biotransformations. It is very likely that the OMPs are biotransformed by different
367 enzymes present in the heterotrophic metabolism and that their affinity for such
368 enzymes varies, leading to changes in their cometabolic biotransformation rates. In fact,
369 according to Kim et al. (2020), the cometabolic biotransformation is influenced by the
370 ratio of the initial growth to non-growth substrate concentration and the specificity
371 constant (the kinetic efficiency, that measures how efficiently an enzyme converts
372 substrates into products) ratio of the growth and non-growth substrates, which could be
373 different for each compound.

374 Overall, the specific OMPs biotransformation rates at 2.7 g COD g $VSS^{-1}$ $d^{-1}$ were
375 around 2.5- and 4-times fold the values obtained in the experiment performed at 0.4 g
376 COD g $VSS^{-1}$ $d^{-1}$. Nevertheless, it is noticeable that in comparison to the other
377 compounds, ERY, TMP, E1, E2 and EE2 (Fig. 2) showed reduced rate increments
378 between the experiments performed at 1.1 and 2.7 g COD g $VSS^{-1}$ $d^{-1}$ (although still
379 significantly different ($p < 0.05$)). These lower changes at the higher OLRs could
380 indicate that those compounds are close to reaching a maximum cometabolic
381 biotransformation rate (Gonzalez-Gil et al., 2018), likely determined by some
382 thermodynamic constraints, as suggested in the third hypothesis of section 3.1. In any
383 case, Fig. 2 shows that neither the first hypothesis (enzymatic stimulation was not
384 occurring at higher OLRs) nor the second (the maximum cometabolic rate had been
385 achieved at the lowest OLR) were taking place. Therefore, the best explanation for the
386 results of Fig. 1 is that, although the biotransformation rates varied with the OLR, the
387 HRT was high enough to hinder an effect on the biotransformation yield.

Previous studies have also proven the cometabolism of OMPs in different environments. For example, Fernandez-Fontaina et al. (2012) reported a cometabolic effect in the biotransformation of IBP, ERY, ROX and AHTN, among others, in nitrifying reactors, likely due to the action of ammonia monooxygenase. Similarly, Majewsky et al. (2011) determined that the heterotrophic active fraction was correlated with the removal extent of pharmaceuticals such as DCF and SMX. Interestingly, under anaerobic conditions, González-Gil et al. (2018b) reached a methanogenic activity high enough not to show a relationship with the biotransformation rate of most OMPs, contrary to previous anaerobic studies with lower activities of the primary substrate (Alvarino et al., 2014).

The cometabolic effect proved in this study might imply that reactors operating at higher OLRs could have an improved performance. Higher OLRs are often related to an increased microbial activity and to a larger expression or activity of the enzymes involved in the metabolism of the primary substrates, leading to a higher catalytic activity in the reactor which could enhance the cometabolic biotransformation of the OMPs. Therefore, high load WWTPs are likely to have a better performance in OMPs biotransformation by increasing their yield or, if a limitation has been reached (as in Fig.1), by being able to achieve in a shorter amount of time the same biodegradation yield than low load WWTPs, allowing operations at shorter HRTs.

### 3.3 Influence of heterotrophic activity on the OMPs biotransformation constant

In previous sections, it was shown that a higher heterotrophic activity leads to a higher specific OMPs biotransformation rate, indicating that heterotrophs are key on $r_{biol}$. Based on Eq. 6, it seems that the influence of the heterotrophic activity on $r_{biol}$ occurs through changes on $k_{biol}$. Thus, batch tests were conducted to determine the correlation

between the microbial activity and $k_{biol}$, as well as between the removal of the primary substrate and the pollutants.

Studying the interaction between the OMPs and the growth substrates can help to understand their dynamics and control the fate of the pollutants by properly managing the primary substrates. As shown in Fig. 3, the biotransformation of the OMPs (exemplified for ROX, SMX, NPX and HHCB) occurs simultaneously to the degradation of the primary carbon source (acetate). This finding agrees with the common cometabolic biotransformation theory, where the OMPs, induced by the presence of the primary substrate, enter its metabolic pathway and undergo simultaneous biodegradation (King et al., 1997). OMPs do not yield enough energy to support microbial growth and their biotransformation occurs fortuitously when their chemical structure is modified thanks to the enzymatic action that degrades the primary substrate. Similarly to the findings shown in Fig. 3, Aeming et al. (2019) determined that the removal of 4 endocrine disruptors (fluoranthene, benzo(b)fluoranthene, benzo(a)pyrene and NP) had a synchronal fate to the organic matter during anaerobic digestion and composting. According to the results shown in Fig. 2 and Fig. 3, it is proved that the main driver during the heterotrophic biotransformation of OMPs is the presence of the growth substrate due to cometabolism.

**Figure 3**

As previously indicated, the $k_{biol}$ of a particular compound depends on its physicochemical properties and in the environmental conditions (pH, temperature and oxidation reduction potential, among others). Interestingly, as shown in Fig. 4, we found out that the specific activity of the sludge, caused by the primary substrate, also affects the value of $k_{biol}$. A positive correlation (with an R-square value between 0.80 and 0.96) is observed between $k_{biol}$ and the maximum COD specific activity for all non-

recalcitrant OMPs, with the exceptions of HHCB and ADBI. This proves that $r_{biol}$ is influenced by the microbial activity through the variation of $k_{biol}$. Since it was not seen a limitation in the increasing tendency of $k_{biol}$, which maintained the linearity throughout the assays, it can be speculated that higher $k_{biol}$ values could be obtained at even higher specific COD activities. Nonetheless, we do not have enough data to prove this theory and experiments at higher heterotrophic activities would be required for confirmation.

It is worth comparing the results from Fig. 2 and Fig. 4, which showed some differences. For instance, ERY showed almost 4-fold increases in its specific biotransformation rate (Fig. 2) while its $k_{biol}$ values only showed 2-fold increments (Fig. 4). These discrepancies were also observed in other compounds such as E1 and ROX. One explanation for such behaviour could be that media conditions and especially process conditions, such as operating in batch mode rather than in a continuous manner, could lead to a different intensity in the metabolic responses, in agreement with the results from Park et al. (2018). Besides, microorganisms may have followed different enzymatic routes in the batch and reactor experiments to degrade the substrates due to variations in the initial feeding concentrations, in consonance with previous studies showing that the concentration of the metabolites plays a key role in the pathway selection and metabolic flux (Wegner et al., 2015). Despite these differences, overall both continuous and batch experiments provide consistent and complementary conclusions.

**Figure 4 (A and B)**

Throughout the experiments, the influence of the heterotrophic activity on $k_{biol}$ varied among the OMPs. For instance, in the case of the hormones, while E1 increased its $k_{biol}$ almost 20-fold, E2 only increased it 2-fold and in the case of the fragrances, while

AHTN duplicated its $k_{biol}$, ADBI and HHCB did not show any variation. These findings show that the influence of the heterotrophic activity is substance-specific.

The reported results show that the biotransformation of a particular OMP would depend on the characteristics of the microbial population and their specific performance. From our results, and considering the findings of Majewsky et al. (2010) and Gonzalez-Gil et al. (2018b), it can be concluded that at low microbial activities, the $k_{biol}$ of a compound does not vary, at intermediate activities the $k_{biol}$ increases in a linear manner, and finally, when the microbial activity is sufficiently high, it reaches a plateau and $k_{biol}$ remains constant. Our findings suggest that, for the range of heterotrophic activity studied, the majority of the selected OMPs were in the region where $k_{biol}$ increases linearly. The exceptions of HHCB and ADBI could indicate that they would have required lower or higher microbial activities, proving that $k_{biol}$ is influenced both by the microbial activity and the nature of the compound.

To better understand the influence of the microbial activity on the biotransformation of the OMPs, Table 1 shows an estimation of the biotransformation rates that could be achieved for ROX, NPX, SMX and HHCB (the remaining compounds can be found in Table S6) in a real activated sludge system designed to only remove organic matter, as well as the HRT that these OMPs would require for complete biotransformation according to the experimental $k_{biol}$ values (Fig. 4). For instance, in the case of NPX, assuming an inlet concentration of 10 $\mu g\ L^{-1}$, a $k_{biol}$ equivalent to 0.7 L g $VSS^{-1}$ $d^{-1}$ would provide a biotransformation rate of 14 $\mu g$ NPX $L^{-1}$ $d^{-1}$ and an HRT of 17 h would be required. On the other hand, a $k_{biol}$ of 3.8 L g $VSS^{-1}$ $d^{-1}$ would lead to a biotransformation rate of 76 $\mu g$ NPX $L^{-1}$ $d^{-1}$ and an HRT of 3 h could be sufficient for total NPX removal. The results indicate that higher heterotrophic activities could considerably reduce HRT requirements thanks to improved OMPs specific

biotransformation rates. Moreover, except for DZP and CBZ, which are recalcitrant under heterotrophic conditions, all the OMPs would be almost completely removed after 8 h (Tables 1 and S6). Accordingly, promoting the heterotrophic activity with higher OLRs would not have significant effects on the biotransformation yield at typical HRTs of activated sludge systems, supporting the findings of Fig. 1.

**Table 1.** Biotransformation rate and HRT required for full biotransformation of ROX, NPX, SMX and HHCB in a real WWTP based on the $k_{biol}$ values obtained in the batch assays. The $X_{VSS}$ value used is a typical solids concentration in WWTPs (Metcalf & Eddy, 2014), and the $C_w$ value is in the range of typical influent OMPs concentrations in WWTPs (Luo et al., 2014; Petrie et al., 2014).

| OMPs | Specific biotr. rate (g COD g $VSS^{-1}$ $d^{-1}$) | $k_{biol}$ (L g $VSS^{-1}$ $d^{-1}$) | $X_{VSS}$ (g $L^{-1}$) | $C_W$ ($\mu$OMP $L^{-1}$) | Biotransformation rate ($\mu$g OMP $L^{-1}$ $d^{-1}$) | HRT required for 100% biotransformation (h) |
|---|---|---|---|---|---|---|
| **ROX** | 0.4 | 1.6 | 2 | 10 | 32 | 7.6 |
| | 0.6 | 2.4 | | | 49 | 4.9 |
| | 1.1 | 3.4 | | | 68 | 3.5 |
| | 2.7 | 4.0 | | | 81 | 3.0 |
| **NPX** | 0.4 | 0.7 | 2 | 10 | 14 | 17.1 |
| | 0.6 | 2.8 | | | 56 | 4.3 |
| | 1.1 | 3.4 | | | 68 | 3.5 |
| | 2.7 | 3.8 | | | 76 | 3.2 |
| **SMX** | 0.4 | 1.0 | 2 | 10 | 20 | 12.0 |
| | 0.6 | 2.0 | | | 40 | 6.0 |
| | 1.1 | 2.6 | | | 52 | 4.6 |
| | 2.7 | 2.9 | | | 58 | 4.1 |
| **HHCB** | 0.4 | 5.0 | 2 | 10 | 100 | 2.4 |
| | 0.6 | 3.9 | | | 78 | 3.1 |
| | 1.1 | 6.7 | | | 134 | 1.8 |
| | 2.7 | 5.2 | | | 104 | 2.3 |

## 4. CONCLUSIONS

In this study, experimental data evidenced that higher OLRs lead to higher OMPs biotransformation rates and that the removal of the organic matter and the OMPs occurs simultaneously, proving cometabolism as the main mechanism behind the biotransformation of OMPs under aerobic heterotrophic conditions. The enhancement of the OMPs biotransformation rate occurred due to increases in their respective $k_{biol}$ values, showing that the biotransformation kinetic constant is not only dependant on the compound and the environmental conditions, but also on the intensity of the metabolic activity. Besides, the influence of the heterotrophs in $k_{biol}$ is proved to be compound dependent. On the other hand, the cometabolic trend does not necessarily involve an improvement in the biotransformation yield of the compounds providing that a sufficient HRT is applied. In fact, in this work, it is shown that the effectiveness did not improve at higher specific degradation rates of the primary substrate. Hence, the variation of the WWTP organic load, the management of the organic substrates and the control of the microbial activity appear as key parameters governing OMPs biotransformation.

## 5. ACKNOWLEDGEMENTS

This research was funded by the Spanish Government (AEI) through the COMETT project (CTQ2916-80847-R) and a PhD Xunta de Galicia Grant (ED481A-2018/113, David Kennes). Authors from Universidade de Santiago de Compostela belong to CRETUS Strategic Partnership (AGRUP2015/02) and to Galician Competitive

Research Group (GRC ED431C 2017/29). All these programs are co-funded by FEDER (EU).

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732

Fig_1

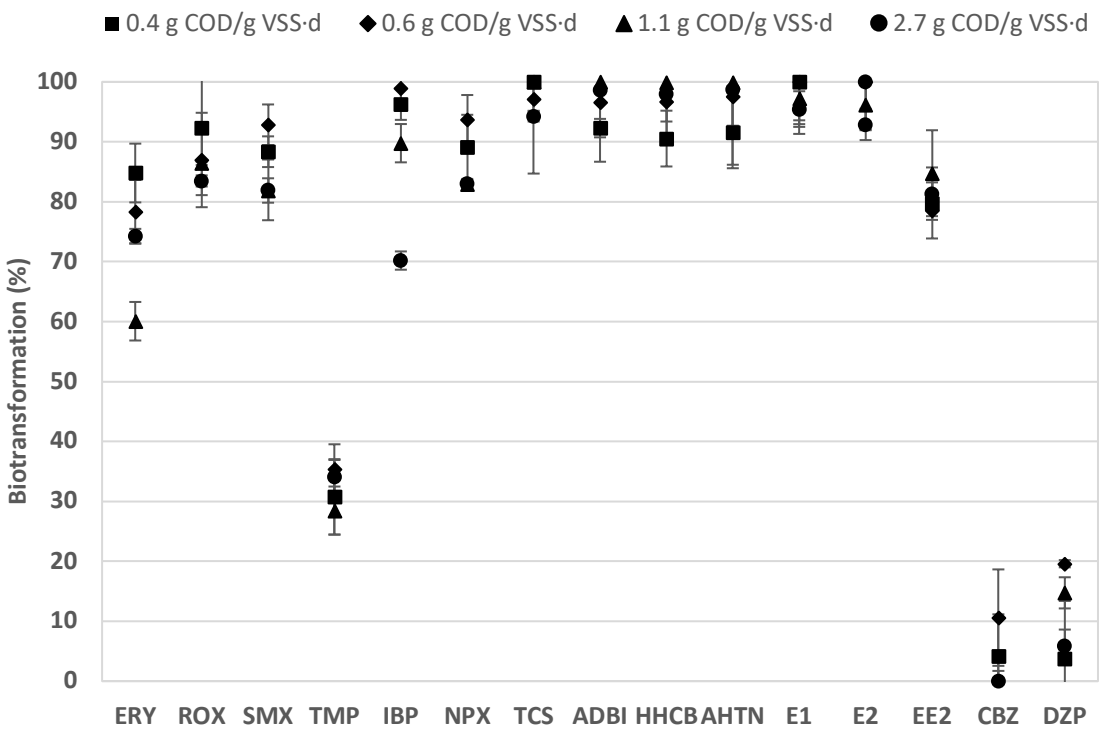
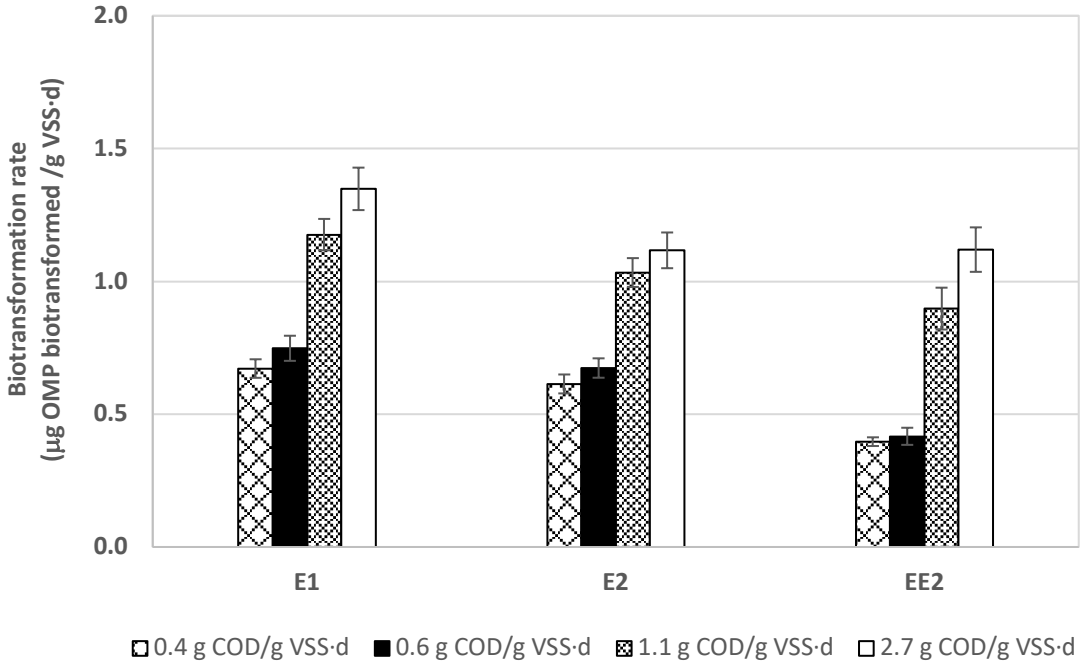


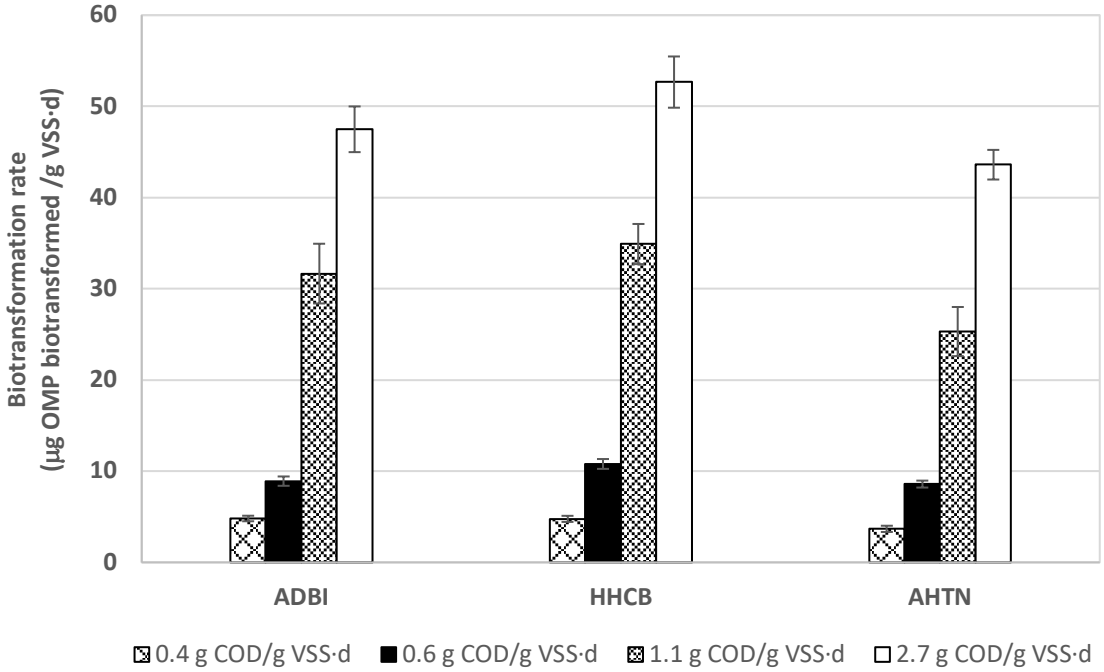

**Fig. 1.** OMPs biotransformation yield achieved in the aerobic heterotrophic reactor operated with fixed HRT (1 day) at 4 different specific organic loading rates of the primary substrate.

Fig_2

A)


Biotransformation rate
(µg OMP biotransformed /g VSS·d)
2.0
1.5
1.0
0.5
0.0
E1
E2
EE2
0.4 g COD/g VSS·d
0.6 g COD/g VSS·d
1.1 g COD/g VSS·d
2.7 g COD/g VSS·d

B)


Biotransformation rate
(µg OMP biotransformed /g VSS·d)
60
50
40
30
20
10
0
ADBI
HHCB
AHTN
0.4 g COD/g VSS·d
0.6 g COD/g VSS·d
1.1 g COD/g VSS·d
2.7 g COD/g VSS·d

**C)**

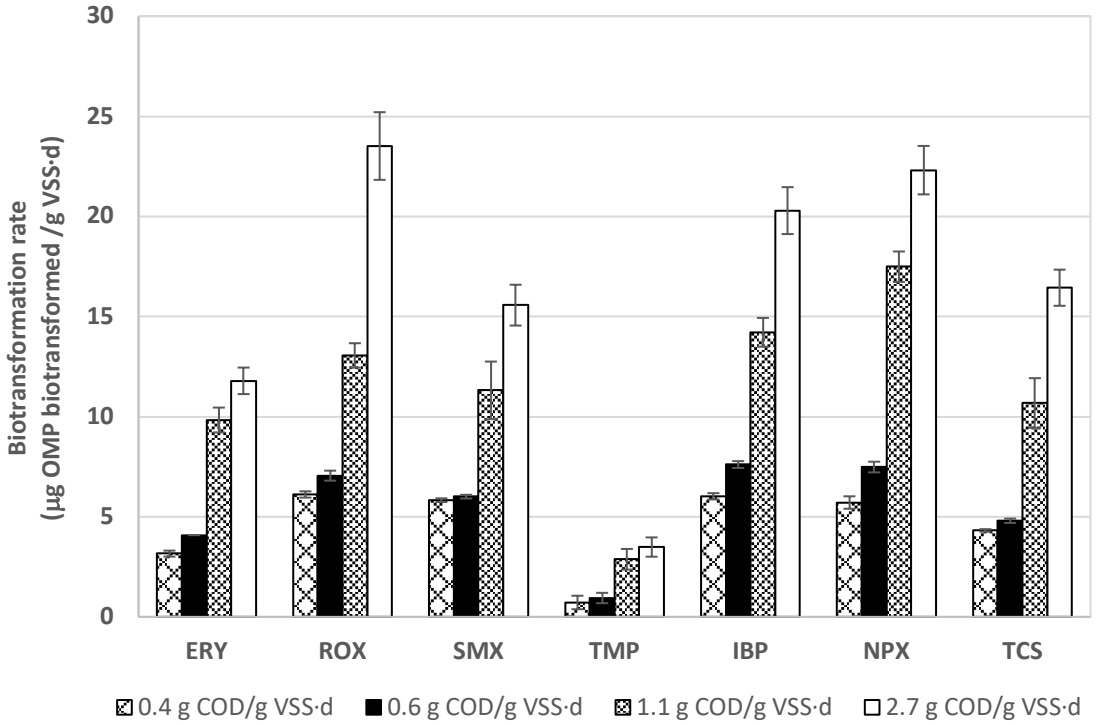



**Fig. 2.** Specific OMPs biotransformation rate (µg OMP g $VSS^{-1}$ $d^{-1}$) at 4 specific biodegradation rates of the primary substrate (g COD g $VSS^{-1}$ $d^{-1}$). A) OMPs fed at 1 µg $L^{-1}$ $d^{-1}$. B) OMPs fed at 40 µg $L^{-1}$ $d^{-1}$. C) OMPs fed at 10 µg $L^{-1}$ $d^{-1}$.

Fig_3

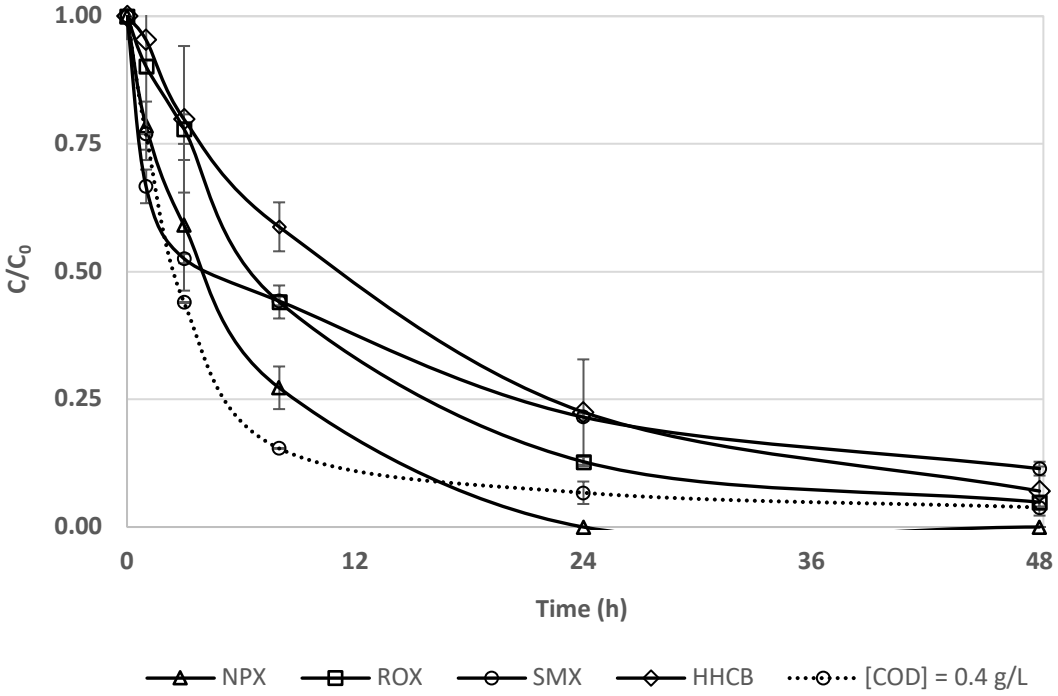
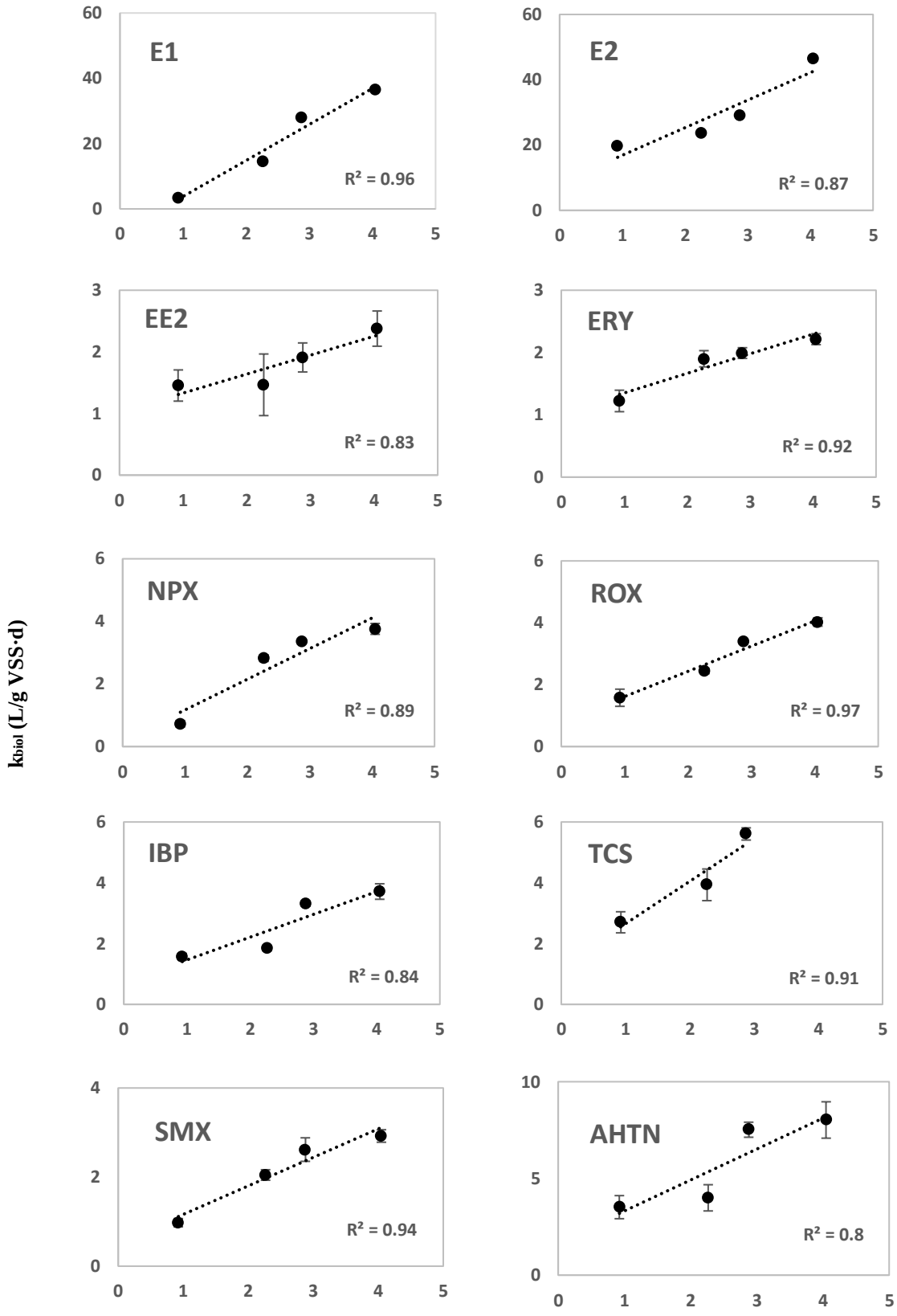


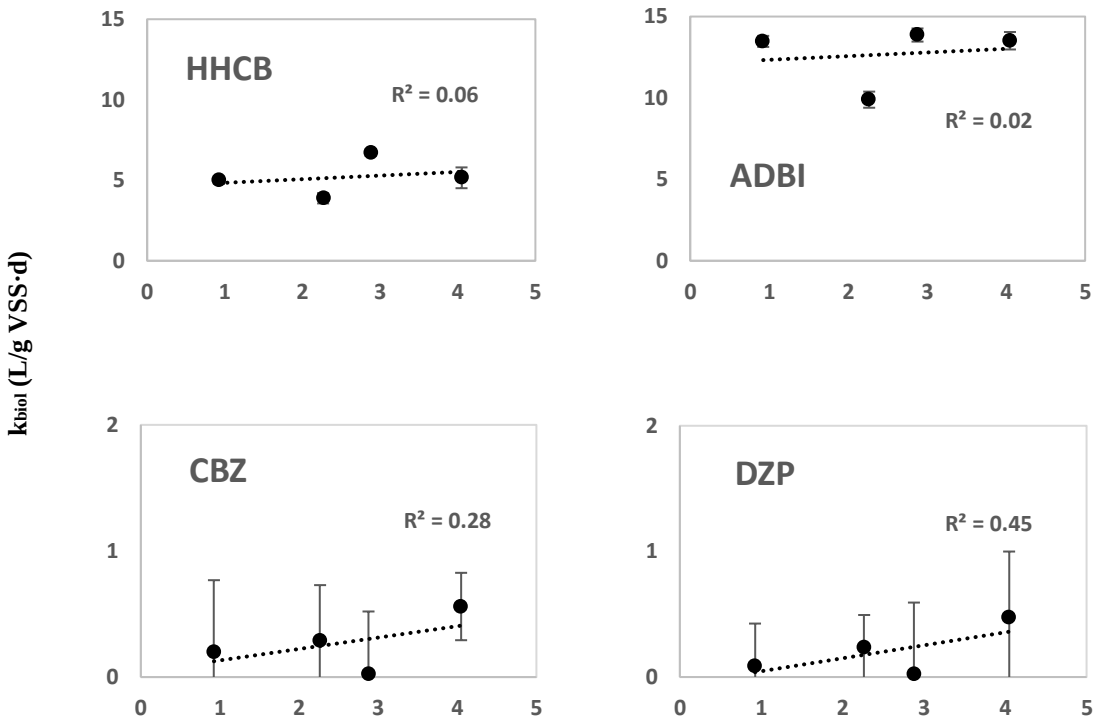

**Fig. 3.** Representation of the biotransformation trend followed by ROX, SMX, NPX and HHCB for the experimental batch with $[COD]_0$ = 0.4 g $L^{-1}$. Similar results were obtained for the rest of OMPs and COD evaluated (data not shown).

Fig_4

A)


E1
R² = 0.96
E2
R² = 0.87
EE2
R² = 0.83
ERY
R² = 0.92
NPX
R² = 0.89
ROX
R² = 0.97
IBP
R² = 0.84
TCS
R² = 0.91
SMX
R² = 0.94
AHTN
R² = 0.8
kbiol (L/g VSS·d)
Maximum specific activity (g COD/g VSS·d)

**B)**




*The $k_{biol}$ of TMP could not be determined.

**Fig. 4.** OMPs biotransformation kinetic constant ($k_{biol}$) *vs* the maximum COD specific activity achieved in each batch assay. A) OMPs that showed a positive correlation. B) OMPs that showed a negative correlation. In each graph is stated the R squared value ($R_2$).