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Influence of metabolism and microbiology on organic micropollutants biotransformation in anoxic heterotrophic reactors

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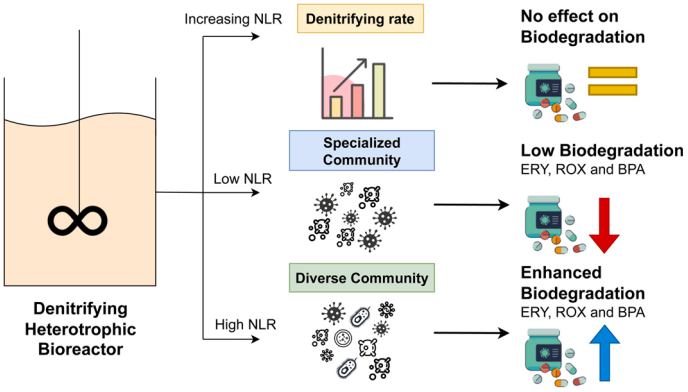
HIGHLIGHTS

- High NLRs increased the microbial biodiversity of the bioreactor.
- Denitrifying heterotrophic activity did not affect OMPs removal efficiency.
- Microbial biodiversity affected the fate of moderately biodegradable OMPs.
- Minor genera seemed to be involved in OMPs biotransformation.

GRAPHICAL ABSTRACT



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ABSTRACT

There is scarce information about the biotransformation of organic micropollutants (OMPs) under anoxic conditions. In this study, a heterotrophic denitrifying bioreactor was set up to study the fate of several OMPs from metabolic and microbiological points of view. Primary metabolic activity was increased by adding progressively higher nitrogen loading rates during the operation (from 0.075 to 0.4 g $N\text{-}NO_3^-$ L^{-1} d^{-1}), which resulted in an important shift in the microbial population from a specialized biomass to a more diverse community. Such a change provoked a significant increase in the removal efficiency of erythromycin (ERY), roxithromycin (ROX) and bisphenol-A (BPA), and some bacterial taxa, such as *Rhodoplanes*, were identified as possible indicators related to the biodegradation of these compounds. The increasing primary metabolic activity in the reactor did not enhance the OMP-specific removal rates, suggesting that the bacterial composition is more influential than cometabolism.

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1. Introduction

1.1. Biotransformation of OMPs in WWTPs

The presence of organic micropollutants (OMPs) in wastewater effluents and wastewater treatment plans (WWTPs) has been extensively reported (Barbosa et al., 2016; Tran et al., 2018). Treated effluents commonly contain concentrations of these pollutants capable of damaging aquatic ecosystems, since conventional WWTPs are not effective enough to remove them completely (Carballa et al., 2004; Tran et al., 2018). Biological treatment is a key step in WWTPs, and more insight is needed to determine the operational and environmental factors involved in the removal of OMPs, such as hydraulic retention time (HRT), sludge retention time (SRT), metabolic activity, redox conditions, etc. (Achermann et al., 2018; Alvarino et al., 2018b; Falås et al., 2016).

Apart from the chemical structure of the pollutants, which determine the interactions with the enzymes, redox conditions significantly influence OMP removal in bioreactors (Alvarino et al., 2018a; Falås et al., 2016), which can lead to different removal efficiencies for a given OMP under aerobic, anoxic or anaerobic conditions. For example, the anti-inflammatory ibuprofen, which is almost completely biodegraded in aerobic systems (Kennes-Veiga et al., 2021; Suarez et al., 2010), behaves as a recalcitrant pollutant in anoxic denitrifying conditions (Polesel et al., 2017) and anaerobic processes (Alvarino et al., 2018b). Comparing these three conditions usually existing in WWTPs, most of the OMPs studied showed higher removals in aerated units, although there are specific cases in which biodegradation takes place to a greater extent in anaerobic reactors. There is less information for anoxic processes, although it is generally assumed that OMP removal is intermediate (25–90%), close to aerobic conditions but to a lesser degree (Alvarino et al., 2018b; Torresi et al., 2018).

1.2. Metabolism-cometabolism

OMPs are usually present at low concentrations (μg L^{-1} or ng L^{-1}) in urban wastewaters. Such trace concentrations are not suitable for the maintenance of microbial growth (Fischer and Majewsky, 2014), with cometabolism being the removal mechanism proposed and demonstrated in different works: i) conventional activated sludge units (Kennes-Veiga et al., 2021); ii) nitrification (Fernandez-Fontaina et al., 2016) or iii) heterotrophic denitrification in moving bed bioreactors (Torresi et al., 2018). In those works, to prove that cometabolism is the main biotransformation mechanism, the dependence of the OMP removal rate on the growth substrate consumption rate was analysed (Kennes-Veiga et al., 2021). It is probable that, in anoxic environments, the same correlation will be found.

Some recent studies went a step beyond, proposing that some enzymes, such as ammonium monooxygenase (AMO) or acetate kinase, which perform key steps during nitrification or the last steps of anaerobic digestion, explain the biotransformation of some OMPs (Fernandez-Fontaina et al., 2016; Gonzalez-Gil et al., 2017). These results indicate that micropollutants are mainly biotransformed in bioreactors through the action of low-specificity enzymes induced by the growth substrates. However, there is still a gap in knowledge about the enzymes responsible for OMP biotransformation in anoxic environments.

Cometabolism may also help to explain the differences in OMP removal between anoxic and aerobic environments. One hypothesis relies on the lower energy generation from nitrate reduction compared with oxygen (Alvarino et al., 2018a). Thus, in anoxic systems, nitrate would not be able to supply enough energy to reach the activation limit to trigger cometabolism. For instance, Sheng et al. (2021) proved that, in nitrifying systems, the presence of ammonia in the typical WWTP concentration range may not be able to provoke the cometabolism of 17ß-ethinylestradiol, which can proceed at higher ammonia concentrations.

1.3. Influence of microbial community composition on OMPs removal efficiencies

Another reason for the different removal efficiencies between the two redox conditions may be related to the microbial composition. The main electron acceptor present in a bioreactor determines its redox condition and has a key influence on microbial composition by selecting different microbial populations (Li et al., 2014), together with the electron donor (usually organic matter). In anoxic environments, it has been reported that the composition of the microbial community may vary enormously depending on the carbon source (Lu et al., 2014; Xu et al., 2018) since some bacterial groups are very specialized and can only grow on specific substrates. For instance, Thomsen et al. (2007) reported that, in the presence of nitrate, *Aquaspirilum*-related bacteria were only able to consume amino acids among all the substrates tested, whereas *Thauera* has proven to be more versatile and able to consume more substrates, such as acetate, propionate and ethanol, apart from amino acids.

The variability found in microbial community composition in WWTPs can explain the differences in OMP removal in similar biological treatment configurations (Helbling et al., 2015; Johnson et al., 2015a; Wolff et al., 2018). Torresi et al. (2017) reported a twofold increase in the biodegradation constants (k_{bio}) for citalopram, trimethoprim, or ibuprofen in a methanol-fed denitrifying bioreactor against an ethanol-fed denitrifying bioreactor. The higher microbial richness found in the methanol reactor coupled with the differences in the microbial composition (especially in the predominant microorganisms) may be the reason for the variation in the biotransformation efficiencies. However, it may be not correct to assume that the predominant group of bacteria is responsible for the main metabolic capabilities of the biological reactor. Indeed, some authors (Saunders et al., 2016; Wolff et al., 2018) stated that, in biological treatment, only a specific group of bacteria (called the core community) perform both metabolic functions and OMP biodegradation. In general, an increase in microbial diversity is considered positive in terms of OMP removal, since the number of bacteria with different capabilities of interacting with and biodegrading such compounds is higher (Johnson et al., 2015a; Stadler et al., 2018).

The aim of this study was to analyse how OMPs are removed under denitrifying anoxic conditions by studying the biotransformation processes in more detail, including metabolic and microbiological approaches. The relationship between the denitrifying activity of the reactor and the removal efficiencies of each micropollutant was assessed to determine if OMPs are removed by a cometabolic process. To elucidate which microbiological factors influence OMP behaviour, the bacterial community composition and specific activity were closely followed.

2. Materials and methods

2.1. Reactor configuration and operational strategy

A lab-scale reactor with a volume of 5 L coupled to a 2 L settler was operated for almost 1.5 years. Biomass was inoculated from a wastewater treatment plant close to Santiago de Compostela, Spain, at a concentration of 1.5 g L^{-1} of volatile suspended solids (VSS). The reactor was gently mechanically stirred to limit the oxygen transference to the bacterial culture. The reactor was operated under anoxic conditions (oxygen concentration was always below 0.05 mg L^{-1}) and at room temperature (between 19 °C and 23 °C).

The operational strategy followed consisted of progressively increasing the F/M (food to microorganism) ratio to check if the OMP biotransformation efficiency was related to the removal rate of the primary substrate fed to the reactor. OMP sampling campaigns were performed once the reactor operation was stable at each of the selected F/M ratios. An increasing concentration of $NaNO_3$ (0.15–2.65 g $NaNO_3$ L^{-1}) was fed to the reactor, keeping the COD/N ratio constant at

approximately 5.5–6, through the addition of sodium acetate (0.26–3.1 g $NaCH_3CO_2$ L^{-1}). Therefore, NO_3^- was always the limiting substrate. The synthetic wastewater fed to the reactor was complemented with 0.025 g L^{-1} KH_2PO_4, 0.05 g L^{-1} Na_2HPO_4 and a trace solution of $FeCl_2$, $ZnSO_4$, $CoCl_2$, $MnCl_2$, $CuSO_4$, KI and H_3BO_3 in a concentration range of 3–150 μg L^{-1} (Suarez et al., 2010). After the first period, part of the sodium acetate was substituted by acetic acid to maintain the pH in the optimal range for denitrification (7.5–8.5).

Fifteen organic micropollutants were chosen for this study: NPX (naproxen), IBP (ibuprofen), DCF (diclofenac), ERY (erythromycin), SMX (sulfamethoxazole), CBZ (carmabazepine), DZP (diazepam), BPA (bisphenol-A), TCS (triclosan), TMP (trimethoprim), CTL (citalopram), ROX (roxithromycin), E1 (estrone), E2 (estradiol) and EE2 (ethinylestradiol). A mixture containing these OMPs dissolved in methanol was added to a 2.5 L glass bottle to obtain a concentration of 100 μg L^{-1} (10 μg L^{-1} in the case of the hormones). This solution was fed to the reactor at a flow rate of 0.5 L d^{-1} and diluted 10-fold with the synthetic wastewater described above to maintain a hydraulic retention time of 1 d. OMP sampling campaigns were performed in four different operational periods (at different F/M ratios).

2.2. Analytical methods

Conventional parameters such TSS, VSS, COD and nitrate were analysed weekly according to standard methods (APHA/AWWA/WEF, 2017), in addition to recording physical parameters such as pH, temperature and dissolved oxygen.

At the end of each operational period, once the reactor was stable at the desired F/M, three different samples were withdrawn from the reactor to determine the fate of the OMPs. For liquid samples, 250 mL was taken from the effluent and from the glass bottle (OMP feed). In addition to the liquid samples, a grab sample of the sludge from the reactor was withdrawn to analyse the adsorbed OMP fraction. Samples were preconcentrated utilizing a solid phase extraction (SPE) method in 3 mL Oasis cartridges. Each sample was finally eluted in 3 mL of a mixture of MTBE (methyl-tertbutyl ether) and methanol.

For OMP analysis in the solid phase, an ultrasonic solvent extraction method was followed (Ternes et al., 2005). This method consisted of successive additions of organic solvents (methanol or acetone) to 0.4–0.5 g lyophilized sludge samples, followed by 15 min sonication and 5 min centrifugation at 1500 rpm. After 5 extraction cycles, the supernatants were combined, evaporated (TurboVap LV, Biotage) and resuspended in Milli-Q water. Finally, SPE was performed following the same procedure as in the case of the liquid phase.

The extracts obtained were analysed in a liquid chromatograph (Agilent G1312A) coupled to a mass spectrometer (API 4000 triple quadrupole, Applied Biosystems). Different ionization methods were followed to analyse the studied compounds: positive electrospray (ESI +) was used for ERY, ROX, TMP, FLX and SMX; negative electrospray (ESI -) was used for IBP, NPX, DCF, CBZ, CTL, BPA and TCS; and atmospheric pressure chemical ionization (APCI) was used for the hormones (E1, E2 and EE2). A binary gradient consisting of 0.1% formic acid (v/v) in water (A) and 100% methanol (B) at a flow rate of 0.7 mL min^{-1} was used for ESI+ and APCI, whereas, in the case of ESI-, the binary gradient composition for elution was 10% methanol (v/v) in water with 5 mM ammonium acetate (A) and 90% methanol (v/v) in water with 5 mM ammonium acetate (B) (Table S1). The recovery efficiencies and the limits of quantification are described in Alvarino et al. (2015).

2.3. Micropollutant mass balance and specific biotransformation rate

Once the reactor reached steady-state conditions in each period, OMP mass balances were applied to the system. In general, OMPs can be removed through three different mechanisms: volatilization, sorption and biotransformation (Eq. (1)).

$$F_{in} = F_{out} + F_{bio} + F_{sorp} + F_{vol} \quad (1)$$

where F_{in}, F_{out}, F_{bio}, F_{sorp} and F_{vol} are the mass flows (μg OMP d^{-1}) corresponding to the influent, effluent (liquid phase only), biodegradation, sorption and volatilization, respectively. In this study, volatilization was not considered since the proposed compounds are not volatile ($H<10^{-5}$ for all compounds according to Suarez et al., 2010) and the reactor is not aerated). For the sorption mass flow, an equilibrium between the sorbed and the dissolved fraction is considered, since the OMPs are spiked continuously into the reactor. Thus, the removal due to sorption would be:

$$F_{sorp} = C_s \cdot (Q \cdot TSS_{eff} + V \cdot \frac{\Delta TSS}{\Delta d}) \quad (2)$$

where C_s is the OMP concentration in the sludge phase (μg g^{-1}), Q is the flow rate (L d^{-1}), TSS_{eff} is the total suspended solids in the effluent (g L^{-1}), V is the reactor volume (L) and $\frac{\Delta TSS}{\Delta d}$ is the growth rate of the biomass in the biological reactor (g L^{-1} d^{-1}).

Combining both Eqs. 1 and 2, F_{bio} can be calculated and applied to calculate both the biotransformation efficiency (Eq. (3)) and the specific biotransformation rate (Eq. (4)) for each pollutant.

$$Biotransformation\ removal\ efficiency\ (\%) = \frac{F_{bio}}{F_{in}} \cdot 100 \quad (3)$$

$$Specific\ biotransformation\ rate\ \left(\mu g\ g_{VSS}^{-1}\ d^{-1}\right) = \frac{F_{bio}}{X_{VSS} \cdot V} \quad (4)$$

where X_{VSS} is the volatile suspended solids in the biological reactor (g L^{-1}).

2.4. 2.4. Statistical analysis

To determine if the OMP removal efficiency was different among the periods, statistical tests were performed using R software 4.1.0 and RStudio (https://www.rstudio.com/) at the 5% significance level, which were also applied to all the correlations and the significance tests reported in Section 2.4.

2.5. Denitrifying activity test

The specific denitrifying activity (SDA) was determined using the manometric test proposed by Buys et al. (2000) and adapted by Santorio et al. (2019). This method is based on the quantification of N_2 generation in the gas phase during the denitrification reaction. Biomass was withdrawn from the reactor in each sampling campaign and inoculated in 120 mL vials (85 mL of liquid phase + 35 mL of gas phase). All tests were performed in triplicate and at 20 °C. Vials were sealed and flushed with helium to remove the air in the headspace and, after an acclimation period in a shaker to reach a constant temperature, were fed with 60–70 mg N-NO_3^- L^{-1} and approximately 300 mg COD L^{-1} to avoid carbon limitation. Headspace overpressure due to N_2 production was followed with a pressure transducer. Once the test was finished, the gas composition along with concentrations of VSS, nitrate and nitrite in the liquid phase were analysed. The equations used for the SDA calculation were described by Santorio et al. (2019).

Additionally, to compare with the SDA calculated from this method, the apparent denitrifying activity (ADA) is calculated using the bioreactor data with the equation below:

$$ADA = \frac{[NO_3^-]_{inf} - [NO_3^-]_{eff}}{HRT \cdot X_{VSS}} \left(\frac{mg\ \ N - NO_3^-}{g_{VSS} \cdot d}\right) \quad (5)$$

where $[NO_3^-]_{inf}$ and $[NO_3^-]_{eff}$ are the inlet and outlet nitrate concentrations, respectively (mg N-NO_3^- L^{-1}, HRT is the hydraulic retention time (d) and X_{VSS} is the biomass concentration (g_{VSS} L^{-1}).

2.6. DNA extraction and 16 S rRNA gene amplicon sequencing

Genomic DNA was extracted from 2 to 3 mL of the mixed liquor using the Nucleospin Microbial DNA extraction kit (Machery-Nagel) according to the instructions of the manufacturer. Duplicates from each operational condition were pooled after quantification and quality control with a Nanodrop system and a Qubit fluorometer (Thermo Fisher). The V3-V4 hypervariable region of the 16 S rRNA gene from bacteria was amplified using Bakt_3 41 F (5′ CCT ACG GGN GGC WGC AG 3′) and Bakt_805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) (Herlemann et al., 2011) and sequenced in AllGenetics & Biology SL (www.allgenetics.eu). Bioinformatic analysis of NGS (Next Generation Sequencing) was performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3).

First, raw sequences were trimmed and filtered to remove low-quality reads. Polished sequences were clustered into operational taxonomic units (OTUs) at a 97% cut-off for sequence similarity and classified against the nonredundant version of the MiDAS database (Dueholm et al., 2021) (https://www.midasfieldguide.org/guide).

The microbial diversity of the community within and between operational periods was conducted using the CLC Microbial Genomics Module and R 4.1.0 software environment with the Vegan package. Three diversity indices were calculated using CLC software: the Chao1 index, which focuses more on species richness, and the Simpson and Shannon indices, which combine both species richness and evenness (Kim et al., 2017).

3. Results and discussion

3.1. Reactor performance

The biological reactor was operated for 540 days through five different periods. First, an acclimation period (AP) to anoxic denitrifying conditions at a nitrate loading rate (NLR) of 0.025 g $N\text{-}NO_3^-$ L^{-1} d^{-1}, which is an average value for domestic wastewater, was maintained in the reactor feed without OMP addition. This served as a first selection step for denitrifying microorganisms in the inoculum. Afterwards, three different periods (P1-P3) were studied in which higher NLRs (0.075, 0.25 and 0.4 g $N\text{-}NO_3^-$ L^{-1} d^{-1}) were successively applied to the reactor. The C/N ratio was set at 5 at the start of the operation and then slightly increased to 5.5–6 to avoid carbon acting as a limiting primary substrate and hampering denitrification. After this acclimation period, the biological reactor was able to remove nitrate almost completely (>99%) during the whole operation, with only trace concentrations of nitrite (<0.1 mg $N\text{-}NO_2^-$ L^{-1}) detected in the effluent. COD removal ranged between 70% and 90% since it was added in excess to ensure complete denitrification (Fig. 1).

Additionally, the biomass was characterized in terms of SDA for each period to compare with the ADA calculated from Eq. (5) (Table 1). An increase in SDA was observed between the acclimation period and period 1, consistent with the nitrogen loading rate increase. The maximum SDA was achieved in P1 and maintained until the end of P2, followed by a decreasing trend, which appears to be correlated with the changes in biomass composition.

3.2. Removal of micropollutants

3.2.1. Removal efficiencies

The removal efficiencies calculated according to Eq. (3) for the micropollutants studied are depicted in Fig. 2 for the different operational periods. Recalcitrant behaviour (< 25%) was detected for IBP, NPX, DCF, EE2, CBZ and DZP (those last two are not shown in the Figure). High removal efficiency (>90%) was found for SMX, CTL, FLX and TCS, with the others (ERY, ROX, E1 + E2 and BPA) showing partial biodegradation (between 25% and 90%).

In general, the removal efficiencies obtained are lower than in aerobic or anaerobic systems. Ibuprofen, as mentioned before, was very well removed (>90%) in activated sludge systems and especially in nitrifying conditions (Fernandez-Fontaina et al., 2016; Kennes-Veiga et al., 2021), in contrast to the present results (~25%). Additionally, higher removal efficiencies in aerobic compared to anoxic conditions were reported for EE2 and NPX (Suarez et al., 2010), while, in the case of DCF, the reported removal efficiency was below 25%. There is no consensus in the literature regarding the removal of DCF, and contradictory data have been reported. For example, sampling campaigns performed in conventional WWTPs reported a huge variation in the

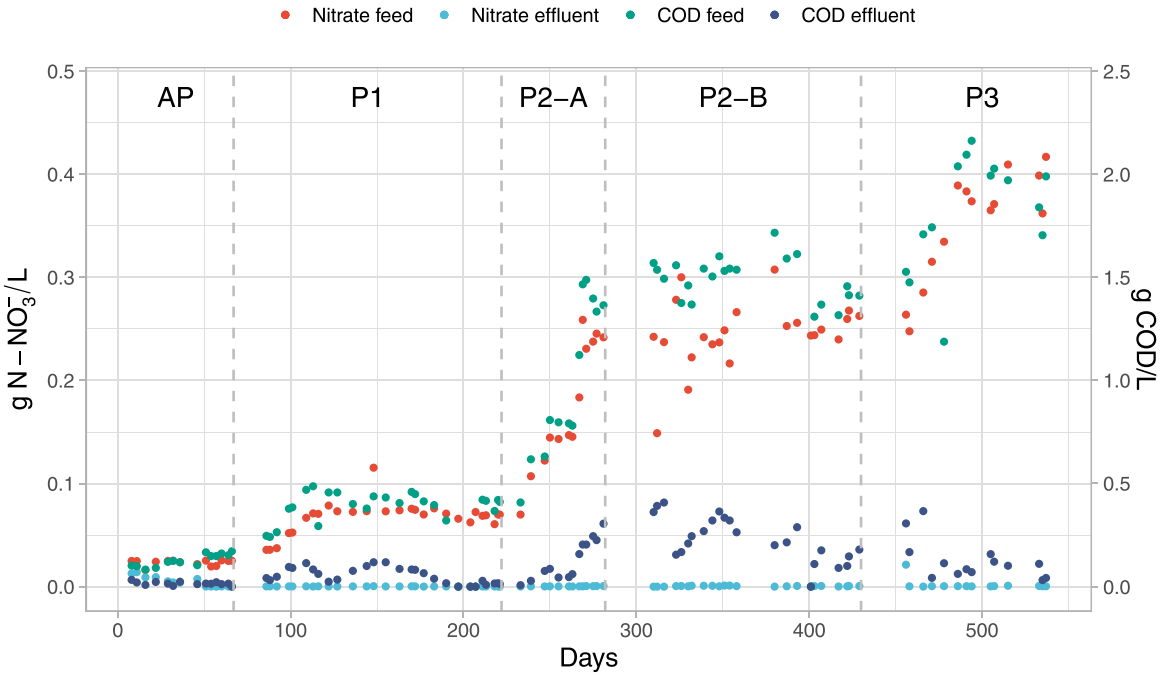

Fig. 1. Removal of nitrate and COD among the whole operation.

Table 1
Reactor feed and pH and denitrifying activities during OMPs sampling periods.

	Acclimation Period	Period 1	Period 2-A	Period 2-B	Period 3
pH Reactor	8.19 ± 0.24	7.99 ± 0.06	8.18 ± 0.05	8.35 ± 0.15	8.16 ± 0.09
F/M	0.11 ± 0.02	0.38 ± 0.03	0.66 ± 0.06	0.52 ± 0.03	0.76 ± 0.08
ADA (mg N-NO_3^- ·$gVSS^{-1}$·d^{-1})	16.50 ± 1.96	64.59 ± 5.75	116.48 ± 4.90	94.13 ± 10.52	163.34 ± 19.13
SDA (mg N-NO_3^- ·$gVSS^{-1}$·d^{-1})	41.27 ± 1.41	343.59 ± 12.04	357.16 ± 8.97	186.41 ± 12.44	207.80 ± 19.03

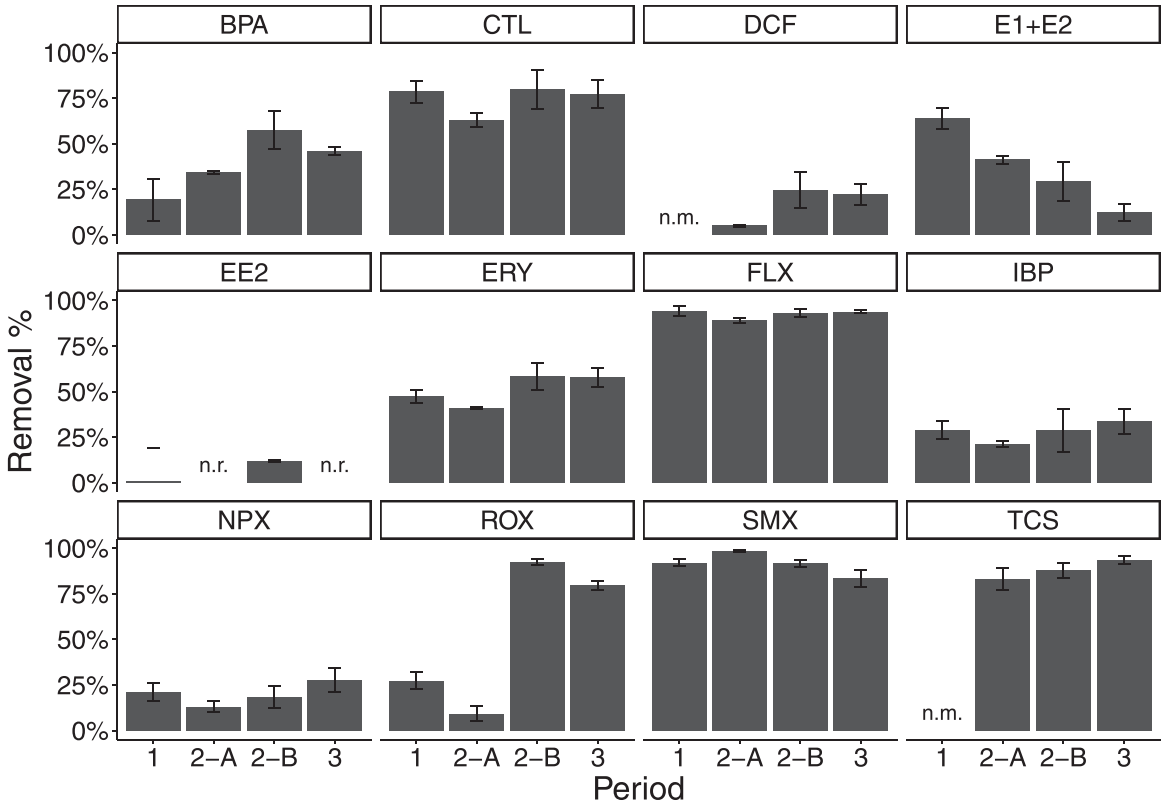

Fig. 2. Removal efficiencies for the studied compounds in each operational period. (n.r.= negative removal, n.m. = not measured). Error bars represent the standard deviation (n = 3).

removal efficiency of DCF (between 0% and 81%), which can be related to the WWTP characteristics (Luo et al., 2014; Tran et al., 2018). However, it is generally considered a poorly biodegradable compound whose biodegradation may be associated with cometabolic mechanisms conducted by certain bacterial taxa (Nguyen et al., 2019). Finally, CBZ and DZP, whose removal efficiencies were approximately 15%, are commonly considered recalcitrant compounds in biological treatments (Alvarino et al., 2018b).

In the present work, SMX was among the highly removed compounds, with removal efficiencies of 92.5% on average, which were significantly higher than previously reported values. For example, in Kassotaki et al. (2018) and in Torresi et al. (2017a), it was classified as moderately biodegradable (20–50% removal) under denitrifying anoxic conditions. FLX was previously reported as a well-removed compound in anoxic conditions. Pomiès et al. (2015) also found high removal efficiencies for FLX and a high tendency to be sorbed on sludge (K_D= 968 $L{\cdot}g_{SS}^{-1}$). In the present study, the removal of FLX due to sorption varied between 5% and 10%, with the remaining 90% biodegraded by the microbial community. The same behaviour was observed for TCS, reaching a high overall removal efficiency (~90%) in the reactor, with approximately 5% removed via sorption and the rest associated with biodegradation.

A moderate removal of ERY was obtained (~50%) during the whole operation, according to other studies performed under similar denitrifying conditions (Polesel et al., 2017; Suarez et al., 2010; Torresi et al., 2017b). Its removal efficiency significantly increased ($p < 0.05$) between P2-A and P2-B. The same behaviour occurred in the case of ROX and BPA. The removal of ROX (> 80% in P2-B and P3) was associated with biotransformation, as previously reported for anoxic conditions (Burke et al., 2014). Regarding the natural hormones (E1 + E2), their removal efficiency in P1 was higher than 50%, similar to other denitrifying systems (Martínez-Quintela et al., 2021; Suarez et al., 2010), but decreased during operation.

In general, the removal efficiencies of recalcitrant compounds did not vary significantly during reactor operation. The same trend was observed for those with high biotransformation efficiencies (SMX, TCS, CTL and FLX). In contrast, some differences were observed for the compounds that were partially removed, such as BPA and ROX, whose removal efficiencies varied in the range of 27–55% and 20–85%, respectively, between P1/P2-A and P2-B/P3. The same behaviour occurs in the case of ERY. However, in the case of natural hormones, a decrease was observed from ~63% at P1 to ~12% at P3. The explanation for the behaviour of BPA, ROX, ERY and the natural hormones will be further discussed in the following sections.

3.2.2. Specific biotransformation rate

The study of the correlation between the main substrate specific removal rate and specific OMP biotransformation rate has been used as a

tool to evaluate whether cometabolism is the main removal mechanism of the selected OMPs (Kennes-Veiga et al., 2021). In this sense, studies performed in aerobic or anoxic conditions have demonstrated the correlation between the increase in the primary substrate metabolic rate and of the specific removal rate of micropollutants for compounds such as ERY, ROX, IBP or TMP (Alvarino et al., 2015; Fernandez-Fontaina et al., 2016; Kennes-Veiga et al., 2021; Martínez-Quintela et al., 2021; Polesel et al., 2017). Following the same procedure, in Fig. 3 we depicted the OMP-specific removal rate (Eq. 4) of the nonrecalcitrant compounds considered in this work in each of the operational periods, which corresponded to different denitrifying activities (P1-P3). However, in the present work, such analysis led to a different observation since no correlation between these parameters was found. The increasing NLR (from 0.075 to 0.4 g N-NO_3^- L^{-1} d^{-1}) applied to the reactor from P1 to P3 did not imply an increase in the OMP-specific removal rate, except for ROX and BPA. In fact, except for these two compounds, the tendency was the opposite: the specific removal rate decreased between P1 and P2-A, but it increased again from P2-B to P3.

The evolution from P2 onwards suggests that the enzymatic activity responsible for OMP biotransformation was maintained during the reactor operation, which may call into question the initial hypothesis regarding the cometabolic biotransformation or the method used to prove it. Several hypotheses may explain these results. First, if the enzymes related to OMP biotransformation are nonspecific or not directly related to the metabolic route of the primary substrate used in this study, the correlation tested in this study may not exist. Thus, the biotransformation of the tested OMPs was not cometabolic. Second, it has been reported that a high growth to nongrowth substrate ratio can inhibit cometabolism due to competitive substrate inhibition (Kim et al., 2020). The affinity constant of the primary substrate is probably much higher than those of the OMPs, leading to a saturation of the active site of the enzymes involved in the cometabolic biotransformation (Kennes-Veiga et al., 2022). In the bioreactor, the initial NLR applied to the system in P1 (0.075 g N-NO_3^- L^{-1} d^{-1}) was probably high enough to saturate those enzymes. Therefore, the maximum OMP-specific removal rate was already achieved in the first period and afterwards maintained during reactor operation. If that was the case, the decrease in the OMP-specific removal rate in P2 was due to the difference in reactor biomass concentration, which increased from 1.2 g_{VSS} L^{-1} at P1 to 2.0–2.5 g_{VSS} L^{-1} in P2. An alternative method to measure cometabolism (radiolabelled micropollutants) or lower NLR are recommended in future studies to better elucidate the main biotransformation mechanism in denitrifying bioreactors.

3.3. Microbial community composition

One sludge sample was taken at the end of each period (Days 66, 221, 283, 430 and 537) to determine the microbial community composition. The total number of reads was 770,387 after trimming and filtering, ranging from 226,863 in the acclimation period to 117,948 reads in P3 (Table S2). Due to the differences in the number of reads, rarefaction curves for the total number of OTUs with respect to the number of reads were calculated. In all cases, the curves reached a plateau, suggesting that the sequencing depth was good enough to be further analysed to explain the diversity in each period (Fig. S1). The reads were clustered at 97% against the MiDAS database, and the number of predicted OTUs was 1524.

The *Proteobacteria* phylum was predominant from AP to P3, with a relative abundance higher than 85%, mainly distributed in the classes *Alphaproteobacteria* and *Gammaproteobacteria* (Fig. 4**a**), in concordance with previous reports in anoxic heterotrophic bioreactors (Lu et al., 2014). These two classes were alternating, with *Alphaproteobacteria* being more abundant in periods AP, P2-B and P3, whereas *Gammaproteobacteria* was more abundant in P1 and P2-A. However, in the last period, their presence decreased significantly to 55%. A kind of specialization occurred when comparing the first periods (the microbial community was distributed in less than 10 classes between AP and P2-A) and the final period, in which the community became more diverse, with a number of other classes (26 different classes were detected between P2-B and P3) appearing, such as *Chloroflexia* (~15%). However, *Alpha* and *Gammaproteobacteria* were still the predominant classes.

Such an increase in biodiversity is more remarkable at lower taxonomic levels, such as the family level (Fig. 4**b**). *Rhodocyclaceae* and *Rhizobiaceae* were the most important families between AP and P2-B (higher than 80% of relative abundance in P1 and P2-A), confirming an evolution oriented to a more specialized biomass. The change in the relative abundance of these two families between AP and P1 may be related to the denitrifying activity of the bioreactor. While most of the genera belonging to the *Rhizobiaceae* are characterized by aerobic metabolism, the members of *Rhodocyclaceae* are more related to denitrifying conditions using acetate as a carbon source (Ginige et al., 2005; Osaka et al., 2006). In the last two periods, the number of different families identified notably increased, with *Rhodocyclaceae* being the most abundant in P2-B but with significantly lower values (43.9%). This trend was more pronounced in P3 (13.4%), with the appearance of *Beijerinckiaceae* (21.8%) and *Roseiflexaceae* (16.4%) as the most abundant. Changes in the biomass concentration (from 2.0 to 2.5 g_{VSS} L^{-1}) possibly caused the variation in the microbial community distribution.

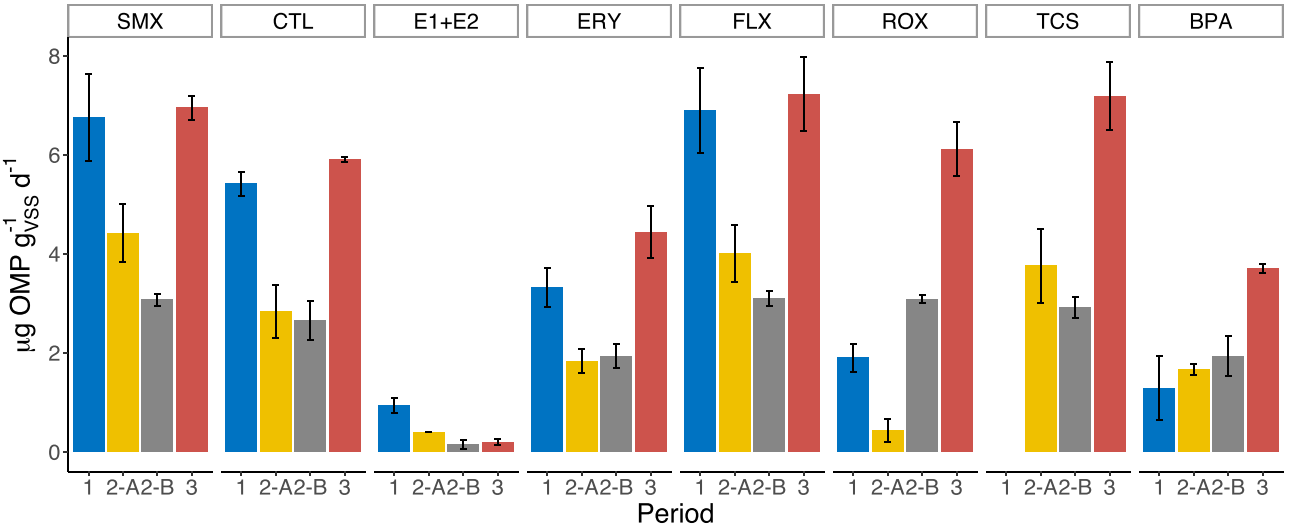

Fig. 3. Specific removal rate of the non-recalcitrant compounds in the three periods of the operation. Error bars represent the standard deviation (n = 3).

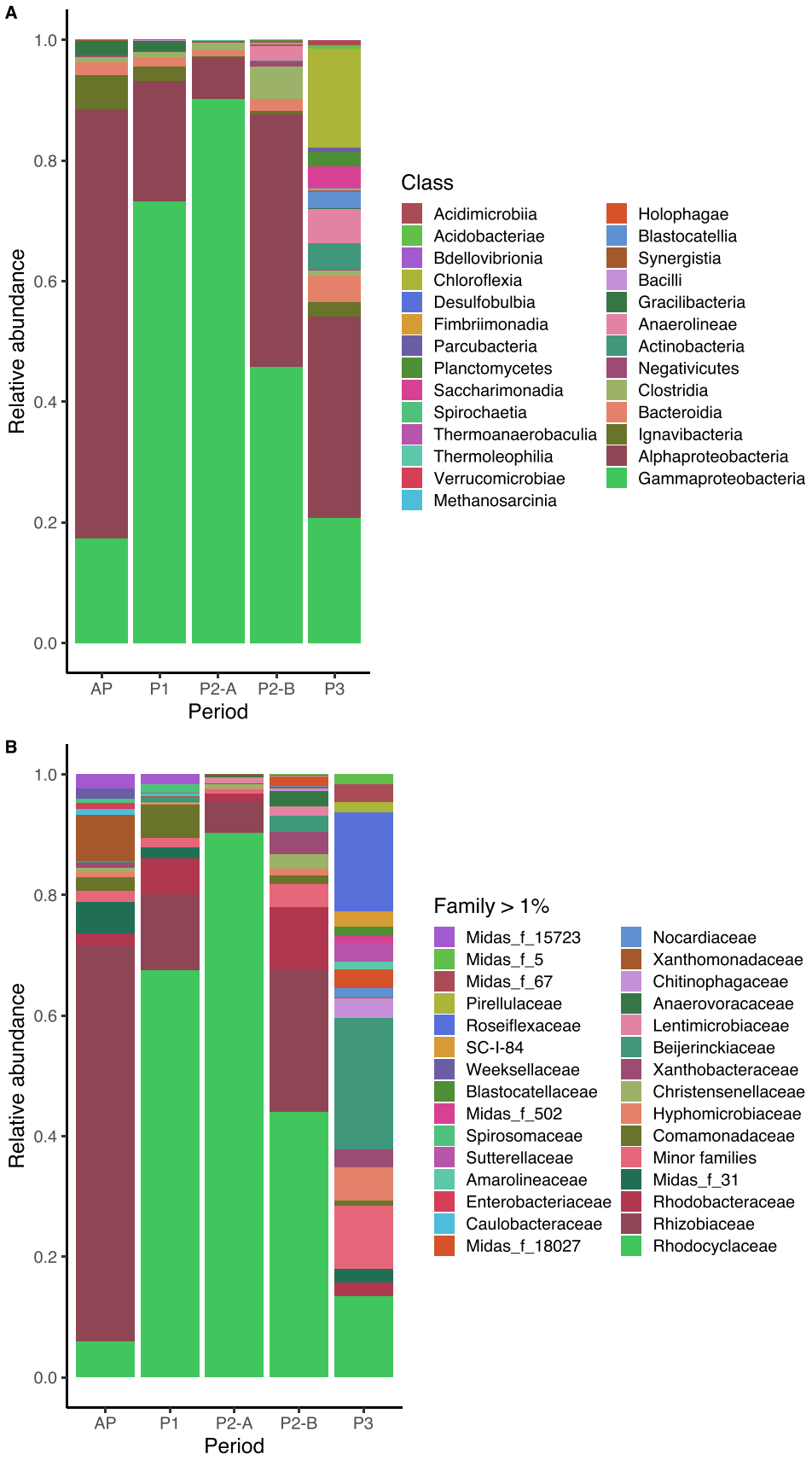

Fig. 4. Bacterial community composition of the reactor within the operational periods. a) Distribution of *Bacteria* at class level. b) Bacteria families with more than 1% of relative abundance.

This shift in microbial composition observed between P2-A and P2-B is not related to the NLR applied, which was maintained at approximately 0.25 g $N\text{-}NO_3^-$ L^{-1} d^{-1} through the second period. However, it seems that *Rhodocyclaceae* is unable to maintain the biological activity of the reactor, showing a decay in their relative abundance from 90.23% to 43.95%, which is an opportunity for other microorganisms, such as *Xanthobacteraceae* (3.55%) or *Beijerinckiaceae* (2.85%), to successfully outcompete while preserving the denitrifying activity in the reactor (ADA). However, such a shift provoked a considerable decrease in the SDA between those periods (Table 1), although they were still capable of performing almost complete denitrification.

This diversity among periods was confirmed by alpha diversity metrics (Table 2). A large variation in the Shannon index when comparing the first three periods with the last two periods can be observed, which does not occur with the Simpson index. This implies that, although the number of species highly increases in P2-B and P3, the emerging OTUs constitute a minority with respect to those present in the first three periods. The Chao index also indicates a significant variation between those periods, which confirms this evolution from an initial biomass with only a few different microorganisms (P1, P2-A) into a more diverse biomass after P2-B. These changes in biodiversity altered the biomass performance in the reactor. Although the nitrogen removal efficiency was not affected during reactor operation, it seems that microbial community specialization led to higher SDA (P1 and P2-A), whereas the increase in biodiversity (P2-B and P3) was responsible for its decrease (Table 1).

In periods 1–2-B, although *Rhodocyclaceae* was the most predominant family, there was no predominant genus. This varied depending on the operational period considered (Fig. S2). *Dechloromonas* and *Dechlorobacter* were the predominant genera during P1 and P2, whereas *Azospira* was the predominant genus in P3. According to the MiDAS database (Dueholm et al., 2021), all of them are able to use nitrite as an electron acceptor as well as oxygen to oxidize acetate. Thus, this variation between genera was probably related to their capacity to adapt to the reactor conditions and, more specifically, to their ability to be the fastest in consuming organic carbon. For instance, some species belonging to the *Azospira* genus can also fix N_2 (Bae et al., 2007), which is highly produced due to the biological activity of the reactor during periods 2 and 3.

3.4. Effect of the microbial community composition on the micropollutants removal

The removal of ERY, ROX and BPA was significantly different ($p < 0.05$) between P1/P2-A and P2-B/P3. As mentioned before, no effect of cometabolism was observed, so the explanation may be related to the change in the microbial composition (Fig. 4). At the initial assessment, the increase in biodiversity between those periods assessed by the observed OTUs (Table S1) and the diversity index (Table 2) was beneficial for the removal of these OMPs. An improvement of the taxonomic richness in a microbial community does not necessarily imply a better efficiency in terms of OMP removal, especially if the new microbial composition has the same metabolic capabilities as the previous one (Johnson et al., 2015a; Torresi et al., 2018). However, it is more likely that the more diverse communities will have wider functional capabilities than the less diverse ones (Johnson et al., 2015b; Stadler et al., 2018). Therefore, it would be expected that the bacteria able to biotransform these micropollutants became more abundant in the reactor in P2-B than in P2-A (and possibly more active in the biological sludge) due to the longer exposure time to those compounds. To check this, a Pearson correlation analysis was performed comparing the relative abundance of the most abundant genera with these OMPs (Fig. 5). Since the removal of the natural hormones (E1 + E2) was also significantly different among the periods, they were included in this analysis. Although it is not possible to ensure that one OMP is biodegraded by a certain genus with the 16 S amplicon sequencing data, these outputs may help to select possible indicators for further studies.

Table 2
Ecological diversity indices measurements.

Sample day	Shannon entropy	Simpson	Chao 1
AP	2.31	0.59	73.54
P1	2.25	0.63	88.88
P2-A	2.92	0.76	111.52
P2-B	4.59	0.92	213.71
P3	6.06	0.97	206.21

The results of Fig. 5 indicate that, except for *Dechlorobacter*, none of the major genera (*Azospira, Dechloromonas, Aquamicrobium,* etc.) described in Section 2.3 (Fig. S2) correlated (neither in positive nor negative mode) with the removal of the selected contaminants. However, some of the minority genera showed a strong positive correlation ($r > 0.9$) with the removal of some of the compounds. *Azorhizobium* positively correlated with the removal of ERY and ROX, while *Rhodoplanes* correlated with ROX and BPA. In both cases, the relative abundance increased significantly ($p < 0.05$) between P2-A and P2-B. For example, the relative abundance of *Rhodoplanes* in P2-A was 0.04% and in P2-B was 2.8%. These outputs support the idea that OMP biotransformation is performed by a group of specialized bacteria that are not necessarily among the most abundant (Saunders et al., 2016; Wolff et al., 2018). In the case of the hormones E1 + E2, no strong correlations were found between them and any of the OTUs identified.

In general, the tendency observed is that genera that positively correlated with the removal of ERY, ROX and BPA correlated negatively with the removal of the natural hormones and vice versa. Consistently, these compounds show an opposite evolution over time with respect to their removal efficiency (Fig. 2). For example, *Brevundimonas* or *Dechlorobacter* were positively correlated with the hormones ($r > 0.8$) and negatively correlated with BPA ($r < -0.8$). This means that, from P1 to P3, their abundance decreased at the same time as the removal efficiency of natural hormones. However, such microbial evolution was positive for the removal of BPA, whose removal efficiency showed an overall positive tendency. This may indicate that some of these genera are involved in the removal of hormones, but they actively compete with other bacterial taxa involved in the removal of the BPA that became more abundant during reactor operation.

When comparing the composition of the microbial community found in this anoxic denitrifying reactor with conventional heterotrophic aerobic bioreactors, there were no large differences. The distribution of the relative abundances can vary depending on the operational factors and the carbon source present in the media, but the same bacterial genera were found (*Acidovorax*, *Dechloromonas*, *Rhodoplanes*, *Thauera*, etc.) (Johnson et al., 2015a; Wolff et al., 2018; Xu et al., 2018). This result is expected, since most microorganisms found in activated sludge are facultative and can use nitrate or oxygen as electron acceptors. Therefore, the differences in the removal efficiency between aerobic and anoxic environments found in compounds such as IBP or NPX may be due to thermodynamic reasons, such as lower oxidation potential (Alvarino et al., 2018a; Kim et al., 2020). Further studies involving a more in-depth analysis of issues such as the microbial community composition in both environments and the study of the enzymes involved in primary metabolism are needed. These analyses, complemented with enzymatic assays to determine the energy required for OMP transformation, will be necessary, among others, to elucidate such differences.

4. Conclusions

A strategy consisting of progressively increasing the nitrogen loading rate fed to the wastewater reactor was applied to evaluate the correlation with the OMP biotransformation rate and check if cometabolic removal could be confirmed. Additionally, the microbial composition in

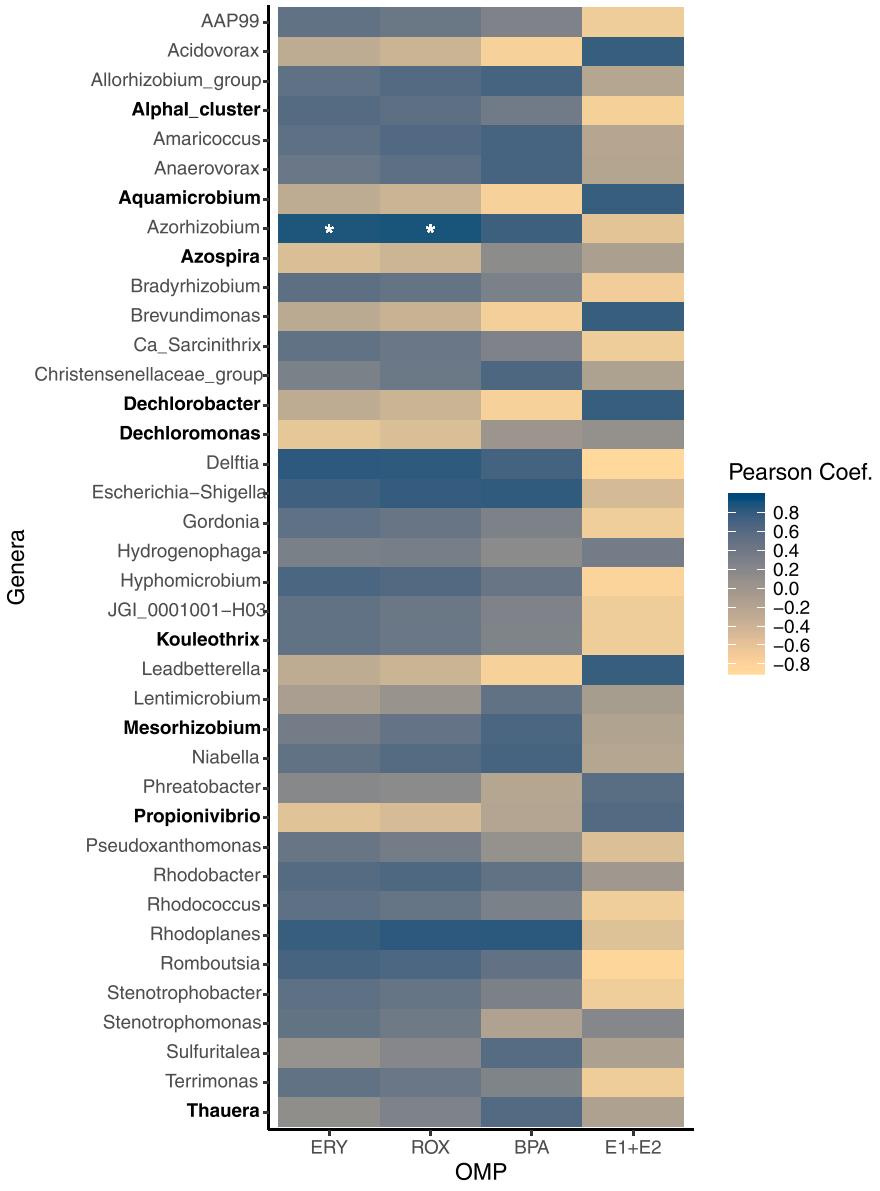

Fig. 5. Pearson correlation heatmap of the most abundant genera identified in the bioreactor (generic MiDAS genera excluded) with OMPs whose removal efficiency was significantly different among periods. Asterisk indicates that the correlation is significant ($p < 0.05$). In bold, genera whose relative abundance was > 5% in any of the periods (P1-P3).

each sampling campaign was characterized to study biomass evolution and enrichment. The main outcomes of the study were as follows:

- A C/N ratio higher than 6 was needed to remove more than 99% of the nitrogen fed to the system. By maintaining this proportion and an adequate supply of P and trace elements, the bioreactor was able to achieve high removal rates for conventional contaminants (~2 g COD L^{-1} d^{-1} and 0.4 g $N\text{-}NO_3^-$ L^{-1} d^{-1}).
- In general, the *Proteobacteria* phylum dominated the bacterial community. The predominant family and genus varied among the periods as the reactor conditions changed (mainly the F/M ratio). Additionally, the microbial community biodiversity greatly increased from period 2-B onwards. This negatively affected the maximum denitrifying activity of the bioreactor but not its efficiency in the removal of COD and N.
- SMX, CTL, FLX and TCS were effectively removed (> 75%) in the bioreactor, whereas ERY, ROX, E1 + E2 and BPA showed moderate removal (25–90%). Biotransformation was the main removal mechanism for the whole set of OMPs, with sorption being only slightly relevant (~ 5%) for FLX and TCS. The rest of the micropollutants fed to the system showed recalcitrant behaviour (< 25%).
- Only for the OMPs removed moderately could a trend be observed during reactor operation. However, it did not show cometabolic behaviour in the sense of increasing nitrogen loading rates leading to higher specific OMP removal rates. This was attributed to the important switch in the microbial composition and to the observation that some genera whose abundance changed during operation showed a strong correlation with the removal of OMPs.
- The increase in taxonomic richness in P2-B and P3 led to a better removal efficiency of ERY, ROX and BPA. *Azorhizobium* and *Rhodoplanes,* two minor genera, positively correlated with the removal of these compounds, suggesting that minority groups may be involved in OMP biotransformation.

This research therefore suggests that the efficiency in OMP removal in denitrifying bioreactors is closer related to the achievement of a more diverse microbial community, than to a further increase in the nitrogen loads. Future research could shed more light into this aspect, going a

step beyond by analyzing key gene expressions to elucidate which enzymes are involved in the OMPs biotransformation.

Environmental Implication

This research paper provides an insight about the removal of several hazardous contaminants (organic micropollutants like pharmaceuticals and endocrine disruptors) in a heterotrophic denitrifying bioreactor. The study not only evaluates the removal efficiencies of these compounds, but it also goes deeper into the impact of the microbial metabolism and composition on their biotransformation. The understanding of micropollutants biotransformation will help to improve their removal in innovative wastewater treatments configurations, which commonly include anoxic environments.

CRediT authorship contribution statement

Miguel Martínez-Quintela: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Sabela Balboa:** Conceptualization, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **José R. Coves:** Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Francisco Omil:** Conceptualization, Methodology, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sonia Suárez:** Conceptualization, Methodology, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The authors do not have permission to share data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129983.

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