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Title: Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A2O) system.

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Keywords: pharmaceutically active compounds; removal efficiency; A2O system; microbial diversity; wastewater treatment.

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Title: Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A²O) system.

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Highlights:

- Removal of 19 pharmaceutically active compounds was assessed in a pilot-scale A^2O
- Removal efficiencies (REs) were calculated under different operating conditions
- Population dynamics of Bacteria, Archaea and Fungi were analyzed by Illumina MiSeq
- REs, operating parameters and microbial populations shifts were linked using MDS

Dear Editor,

We would like to thank you for your feedback of our manuscript Ref. Number **CHEM61562-R1** (Minor revision). Please find below the answer to reviewers' and editor comments, which have been taken into consideration in the new version of the manuscript.

We sincerely hope that the manuscript would be suitable for publication in *Chemosphere* its actual form. In the following answers to reviewer comments, lines indicated are referred to the clean version of the new text file.

Yours sincerely,

Paula Maza,
Corresponding author

AUTHORS' ANSWERS TO COMMENTS FROM EDITORS AND REVIEWERS

Dear authors,

please include your answer to reviewer's #1 comment #3 into the manuscript for the sake of clarity.

Authors's answer - We believe you are actually referring to reviewer #2 comment [3]: "In page 9, lines 137, why the number of samples are different in phase I and phase II?"
We have included the response in the new version of the text (lines 147-154).

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Abstract:

The removal efficiencies (REs) of nineteen pharmaceutically active compounds (PhACs) (six antibiotics-clarithromycin, ofloxacin, sulfadiazine, sulfametazine, sulfamethoxazole and trimethoprim -, four β -blockers -atenolol, metoprolol, propranolol and sotalol- two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators -bezafibrate, fenofibrate and gemfibrozil-, and four psychiatric medications -carbamazepine, diazepam, lorazepam and paroxetine) were ascertained in a pilot-scale anaerobic/anoxic/aerobic (A^2O) system treating urban wastewater, long term operated during two experimental phases using different sets of environmental conditions and operating parameters. Illumina MiSeq sequencing was used to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi communities in the activated sludge. The results showed that mixed liquor suspended solids (MLSS) and food-to-microorganisms ratio (F/M) were operational parameters significantly influencing the REs of five of the analyzed PhACs in the A^2O system. Biota-environment (BIO-ENV) analysis revealed strong correlations between population shifts of the activated sludge community and the REs of PhACs of the different pharmaceutical families. Increased REs of clarithromycin, furosemide, bezafibrate and gemfibrozil were concomitant to higher relative abundances of bacterial phylotypes classified within the *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*), while those of *Betaproteobacteria*, *Chloroflexi* and *Methanomethylovorans* (*Euryarchaea*) correlated positively with the REs of up to seven PhACs belonging to different therapeutic groups.

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1. Introduction

The occurrence and fate of contaminants of emerging concern in aquatic environments and their potential ecotoxicological effects are current issues which have gained relevance in the last decade. Among such contaminants, pharmaceutically active compounds (PhACs) bring forward a special interest among the population (Al Aukidy et al., 2012; Verlicchi et al., 2012). PhACs of different therapeutic classes such as antibiotics, anti-inflammatory and/or analgesic substances, β -blockers, diuretics, lipid regulators or psychiatric medications, occur in raw wastewaters at varying concentrations ranging from ng L^{-1} to $\mu\text{g L}^{-1}$, depending on the prescription habits and usage patterns of each particular country (Kasprzyk-Hordern et al., 2009; Petrie et al., 2015). Among these, anti-inflammatory and/or analgesic pharmaceuticals (AIAPs) such as acetaminophen, ibuprofen or naproxen and the lipid regulators bezafibrate, fenofibrate and gemfibrozil are most commonly detected and at the highest concentrations, reaching values up to several hundred $\mu\text{g L}^{-1}$ (Miège et al., 2009; Tran et al., 2018). Global consumption of PhACs and consequently their incidence in wastewaters are predicted to increase rather than decay in the near future, due to the lengthening of life expectancy of the population (Van Der Aa et al., 2011).

Biodegradation and sorption onto solids are assumed to be the primary mechanisms for the removal of the majority of PhACs in wastewater treatment plants (WWTPs), but the removal rates may considerably differ for each particular compound depending on its chemical characteristics and are also influenced by the operating conditions of the treatment systems (Gao et al., 2012; Jelic et al., 2011; Petrovic et al., 2009; Radjenović et al., 2009). In most cases, the biodegradation of these substances is determined by the presence of primary carbon or nitrogen substrates, as well as the

existence of microorganisms implicated in cometabolic degradation (Tran et al., 2013). Nevertheless, WWTPs have been shown unable to completely remove most of the PhACs present in the influent wastewaters and consequently many of these substances are detected in the treated effluents and stabilized sewage sludge. For example, β -blockers such as atenolol, metoprolol, propranolol, the antiepileptic drug carbamazepine, the AIAP diclofenac, and antibiotics belonging to several chemical classes such as fluoroquinolones, macrolides or sulfonamides, are detected both in influents and effluents of WWTPs worldwide (Gurke et al., 2015; Kasprzyk-Hordern et al., 2008, 2009; Tran et al., 2018; Verlicchi et al., 2012). In addition, the microbial community of activated sludge comprises a high density and diversity of bacterial populations subjected to organic pollutants stresses, making WWTPs optimal scenarios for the spreading of antibiotic resistances and posing important risks for human health due to the transfer of the antibiotic resistance genes (ARGs) from environmental reservoirs to human pathogenic bacterial strains (Hultman et al., 2018). Last, but not least, the presence of antibiotics in wastewaters may affect the diversity and functionality of the complex microbial communities in charge of the biological wastewater treatment, consequently hampering their performance (Deng et al., 2012; Rodriguez-Sanchez et al., 2017).

The concentration of PhACs in treated water often exceeds *the predicted no effect concentrations* (PNECs) for aquatic and soil organisms, so the development of more efficient wastewater treatment configurations with the aim to reduce their concentrations in the treated effluents is a requisite to prevent the dissemination of these substances to water and soil bodies (Kümmerer, 2009). In this sense, several biological treatment technologies (as alternative to the conventional activated sludge system, CAS)

have been evaluated with regard to their efficiency for PhACs removal: membrane bioreactors (MBR), fixed bed bioreactors (FBR), moving bed biofilm reactors (MBBR), aerobic or anaerobic granular sludge (AGS, AnGS), and anaerobic/anoxic/aerobic (A^2O) systems (Lv et al., 2017; Park et al., 2017). However, few studies have attempted to relate the removal efficiencies (REs) of PhACs to the shifts in the structure and diversity of the microbial communities of activated sludge, which in turn are linked to changes of the operational parameters and other uncontrolled variables influencing the WWTPs under real operation conditions.

In this study, the removal efficiencies of nineteen PhACs (six antibiotics- clarithromycin, ofloxacin, sulfadiazine, sulfametazine, sulfamethoxazole and trimethoprim-, four β -blockers -atenolol, metoprolol, propranolol and sotalol- two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators -bezafibrate, fenofibrate and gemfibrozil-, and four psychiatric medications - carbamazepine, diazepam, lorazepam and paroxetine) were evaluated in a pilot-scale A^2O system. The A^2O technology was selected for this study, since this advanced wastewater treatment process is based on a single sludge configuration using a combination of anaerobic, anoxic and aerobic bioreactors in series, which provides better removal efficiency of PhACs compared to CAS (Grandclément et al., 2017; Verlicchi et al., 2012). The A^2O was long-term operated during two experimental phases, using different operation parameters and under different environmental conditions. The Illumina MiSeq sequencing platform was used to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi, in order to find significant links among the observed variability of the relative abundances of

microbial populations, the changes of the environmental/operational variables, and the REs of the targeted PhACs, with the aid of multivariate statistical analyses.

2. Materials and methods

2.1. Description of the wastewater treatment pilot-scale plant and operational conditions

The pilot-scale A²O plant (Fig. S1) was located at the facilities of the WWTP *Murcia Este* (EMUASA, Murcia, Spain) and its characteristics have been previously described in full detail (Gallardo-Altamirano et al., 2018). In brief, the working period covered from May 20th 2016 to March 31th 2017, divided into two differentiated experimental phases: phase I (July 27th - November 13th 2016) and phase II (December 14th 2016- March 31th 2017), which lasted 104 and 105 days, respectively, separated by a transition phase needed for the system to reach stable operation under the parameters chosen for phase II. Average values $\pm$ standard deviations for operational parameters of the A²O system and physical-chemical parameters measured in the influent water samples during phases I and II are summarized in Table S1.

2.2. Wastewater sampling collection for the analysis of physicochemical parameters and PhACs.

The A²O system was sampled at points 1, 2 and 3, as shown in Fig. S1. Daily-composite samples were retrieved three times a week. Chemical oxygen demand (COD), total nitrogen (TN), N-NO₃⁻, N-NH₄⁺ and total phosphorous (TP) were measured with the aid of Merck Spectroquant® kits (Darmstadt, Germany), while biological oxygen demand (BOD₅), total suspended solids in the influent water (TSS), Mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) were measured according to standard methods (Baird and Bridgewater, 2017).

For the analysis of PhACs, eighteen influent and effluent wastewater 24-h composite samples (eight samples in phase I and ten samples in phase II) were collected from sampling points 1 and 3 of the pilot scale plant (Fig. S1). Samples were retrieved both midweek and at the end of the weekend using 500-mL amber PET bottles, as described previously (Gallardo-Altamirano et al., 2018) and were frozen at -20 °C until their analysis. The effluent samples were collected according to the constant HRT (12 h). Initially, the same number of samplings was scheduled for the two experimental phases; however, right after the first sampling of phase II (14/12/16) a heavy rainfall episode took place (15/12/16 -30/12/16) and sampling was suspended, since the exceptional weather conditions could lead to unrepresentative measures of PhACs removal. Paired midweek/end of weekend samplings were resumed in mid-January 2017 under average weather conditions, but the 14/12/16 sample was also analyzed for better reference of the trends of PhACs removal during the whole of experimental phase II.

2.3. Analytical methods for pharmaceutically active compounds

2.3.1. Chemicals and sample treatment

Analytical reference standards (mostly 90% purity, Table S2A) and isotopically labelled compounds for internal standard calibration (Table S2B) were purchased from Sigma-Aldrich (St Louis, MO, USA), Cerilliant (Round Rock, TX, USA), CDN Isotopes (Quebec, Canada), LGC Promochem (London, UK), and Santa Cruz Biotechnology (Dallas, TX, USA). Individual stock solutions of the standards were prepared in MeOH at a concentration of 100 µg mL⁻¹. For calibration and spiking purposes, working solutions (10 µg mL⁻¹ in MeOH) including all tested compounds or labelled compounds were freshly prepared every three months. All solutions were stored in the dark at -

20°C. HPLC-grade solvents (MeOH, ACN, and water) were purchased from Merck (Darmstadt, Germany).

2.3.2. On-line extraction and LC-MS/MS analysis

Pharmaceutical compounds were extracted from wastewater samples using methods previously validated and reported elsewhere (Gros et al., 2009; López-Serna et al., 2010). Briefly, 2.5 mL of sample were pre-concentrated onto a previously conditioned HySphere Resin GP 10 online-SPE cartridge (Spark Holland, Emmen, The Netherlands) and analysed using an automated on-line SPE sample processor SymbiosisTM Pico (Spark Holland) connected in series with a 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer (Sciex, Redwood City, CA, U.S.). An exhaustive extraction procedure was detailed in Gallardo-Altamirano et al. (2018).

The retained analytes were eluted from the cartridge with the LC mobile phase (linear organic gradient of acetonitrile in water with formic acid for positive ionization and ACN:MeOH in water for negative ionization) and transferred to the LC column (Purospher STAR RP-18 endcapped column (125 x 2 mm i.d., 5 µm particle size, Merck, Darmstadt, Germany). The 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer, equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, California, USA), was controlled by the Analyst 1.4.2 Software (Applied Biosystems-Sciex, Foster City, California, USA).

Ionization of the analytes was done using a Turbo Ion Spray source operated in both positive and negative electrospray ionization mode. The negative ionization mode allowed detection of four pharmaceuticals (bezafibrate, furosemide, gemfibrozil and hydrochlorothiazide) including isotopically-labeled compounds. In positive ionization mode, fifteen compounds (atenolol, carbamazepine, clarithromycin, diazepam,

fenofibrate, lorazepam, metoprolol, ofloxacin, paroxetine, propranolol, sotalol, sulfamethoxazole, and trimethoprim) were analyzed including isotopically-labeled compounds. For quantitative analysis, the ESI-MS/MS methods were performed, as described by Gros et al. (2009). MS acquisition was performed in the selective reaction monitoring (SRM) mode, by acquiring two SRM transitions per each target compound and only one for the corresponding surrogate compound. Target compounds and surrogates are listed in Table S3. Selected SRM transitions for each analyte and isotopically-labelled compound and the optimal instrumental conditions set for their analysis are summarized in Table S4.

2.4. DNA extraction, Illumina Miseq sequencing and data processing

Genomic DNA from 4-ml activated sludge samples was extracted as previously described (Gallardo-Altamirano et al., 2018). The genomic DNA pools were sequenced at the facilities of RTL Genomics (Lubbock, Texas, USA, <http://www.researchandtesting.com>), using an Illumina MiSeq platform (Illumina, Hayward, CA, USA). The primer pairs 28F-519R (5'-GAGTTTGATCNTGGCTCAG-3' and 5'-GTNTTACNGCGGCKGCTG-3') (Fan et al., 2012), 517F/909R (5'-GCTAAGSRNCCGTAGC-3' and 5'-CCCCGYCAATTCMTTTRAGT-3) (Maspolim et al., 2015) and FungiQuantF/FungiQuantR (5'-GGRAAACTCACCAGGTCCAG-3' and 5'-GSWCTATCCCCAKCACGA-3') (Liu et al., 2012) were chosen for the partial amplification of the 16S rRNA gene of bacteria and archaea and the 18S rRNA gene of fungi, respectively.

Raw sequencing data were processed using the QIIME software, v. 1.9.1, following the pipeline protocol previously described by Caporaso et al. (2012). The sequences underwent a quality filtering as previously described by Bang-Andreasen et

al. (2017), which removed low-quality reads (Q score ≤ 25), reads with homopolymers longer than 6 nt, ambiguous nucleotides ($n \geq 6$), or consecutive low-quality base calls ($n \geq 5$). Chimeric sequences were removed with USEARCH (Edgar, 2010). Finally, single sequence reads occurring in only one sample were removed of the PHY_table_BIOM for further analyses. Taxonomic *close-reference* assignments based on 97% sequence identity were done with QIIME v. 1.9.1 (Caporaso et al., 2010) using the Greengenes database (version 13_08) for Bacteria and Archaea sequences, while the 18S rRNA eukaryotic SILVA database (version 111) was used for Fungi (Yarza et al., 2017). The descriptive alpha-diversity indices Chao-1, Shannon-Wiener H' , and Simpson were calculated using QIIME. Good's coverage indices were also calculated as described previously (Good, 1953). Sample-size-based and coverage-based rarefaction and extrapolation (R/E) sampling curves with 95% confidence intervals were calculated and plotted using the 'iNEXT' free online tool (Chao et al., 2016; Hsieh et al., 2016), in order to make fair comparisons among samples of unequal size. Heatmaps displaying the relative abundances of bacteria, archaea and fungi in each sample were generated using the vegan package in R v.3.2.0 (<http://www.r-project.org>). The SIMPROF and SIMPER commands of the Primer software (PRIMER-E v. 6.1.18, Plymouth, UK) were used to construct an UPGMA dendrogram with 95% confidence intervals and to estimate the contribution of individual PHYs to the (dis)similarity between groups of samples, respectively, following the methods already described by Maza-Márquez et al. (2016).

2.5 Statistical analyses

IBM SPSS Statistics v. 19 (SPSS Inc., IBM, USA) was used to perform the statistical analyses on the different data sets. The Kruskal-Wallis non-parametric test was selected

to find significant differences among the different groups of samples, using a 95% significance level ($p < 0.05$). When required, Spearman's rank correlation coefficients (ρ) among the different variables were calculated.

All the multivariate statistical analyses were performed using the Primer software (PRIMER-E v. 6.1.18, Plymouth, UK). Non-metric multidimensional scaling (MDS) was used for the ordination of activated sludge samples based on the biotic data sets (relative abundances of PHYs of the targeted groups, derived from the Illumina-sequencing platform). An analysis of similarity (ANOSIM) was also conducted. The ordination of samples was linked to the trends followed by the abiotic variables (REs of the targeted PhACs and environmental/operational variables influencing the A²O system) by means of BIO-ENV (biota-environment) analysis. Full details are described elsewhere (Maza-Márquez et al., 2016).

3. Results and discussion

3.1. Occurrence of PhACs in influent and effluent wastewater samples.

Tables 1 and 2 display the frequency of detection and the concentrations (range, mean, median and relative standard deviation, RSD) of the 19 targeted PhACs in the influent wastewater samples and the effluents of the A²O system during the experimental phases I and II. As shown in Table 1, 13 compounds had $\geq 75\%$ frequency of detection in the influent wastewater in at least one of the experimental phases, of which clarithromycin, ofloxacin, trimethoprim, atenolol, sotalol, furosemide, hydrochlorotiazide, bezafibrate, gemfibrozil, carbamazepine and lorazepam were detected in 100% of the analyzed samples. In contrast, sulfamethazine, sulfamethoxazole, metoprolol, fenofibrate, diazepam and paroxetine were detected in $\leq 50\%$ of samples of both phases, and additionally were measured in very low mean concentrations ($< 20 \text{ ng L}^{-1}$), with the

exception of the antibiotic sulfamethoxazole. According to the Kruskal-Wallis test, there were no statistically significant differences of the concentrations of the assayed PhACs in the influent wastewater between the two experimental phases, except for carbamazepine, which showed a higher mean concentration in phase I (154 ng L^{-1}) compared to phase II (98 ng L^{-1}). The highest mean influent concentrations were observed for the antibiotic ofloxacin, followed in descending order by furosemide, hydrochlorothiazide, gemfibrozil, clarithromycin, and atenolol (Table 1).

Nine PhACs were detected in 100% of the effluent samples, while the concentrations of 5 compounds (sulfamethazine, metoprolol, fenofibrate, diazepam and paroxetine) were below the limits of detection (Table 2). According to the Kruskal-Wallis test, there were statistically significant differences among the two phases for the concentrations of hydrochlorothiazide, gemfibrozil, and bezafibrate. The highest mean values in the effluent were found for ofloxacin, furosemide, hydrochlorothiazide, and gemfibrozil, while the mean concentrations of the remaining PhACs in the effluent were $< 300 \text{ ng L}^{-1}$ in both experimental phases.

The concentrations of all the targeted PhACs in both influent and effluent wastewater fell within the ranges previously reported by several authors (Luo et al., 2014; Tran et al., 2018; Verlicchi et al., 2012), except for the antibiotic ofloxacin, which was measured at concentrations exceeding $100,000 \text{ ng L}^{-1}$ in both influent and effluent water in two samples of phase II (Wednesday 29/03/2017 and Sunday 02/04/2017). Such variations of the concentration of antibiotics in wastewaters are likely related to outbreaks of infection diseases; in this sense, clear seasonality in the concentrations and influent mass loads of ofloxacin was previously reported by Coutu et al. (2013).

The lowest Predicted No Effect Concentration (PNEC) values described in the recent literature (Orias and Perrodin, 2013; Tran et al., 2018; Verlicchi et al., 2012) for all the tested PhACs are shown in Table 2. The average concentrations of eight of the targeted PhACs in the A²O effluent (clarithromycin, ofloxacin, sulfamethoxazole, trimethoprim, propranolol, furosemide, gemfibrozil and lorazepam) fell within the ranges previously reported (Gurke et al., 2015; Kasprzyk-Hordern et al., 2008, 2009; Verlicchi et al., 2012) but were higher than their PNECs, thus posing a risk for aquatic ecosystems (Tran et al., 2018; Verlicchi et al., 2012). In this scenario, implementation of tertiary treatments is desirable to prevent the continuous discharge of these substances into the receiving water bodies.

Table 3 shows the daily mass loads (ML, mg day⁻¹ 1000 inh⁻¹) of the twelve PhACs which were detected in at least 75% of samples of both experimental phases, calculated for the influent and effluent water samples, together with their predicted mass loads (PL) and the corresponding PL/ML influent ratios, calculated as shown in Table S5. Similar influent ML values were found in both experimental phases for each target compound, except for ofloxacin (746 and 13,855 mg day⁻¹ 1000 inh⁻¹ in phase I and II, respectively). The PL/ML ratios were calculated to estimate the accuracy of the PL values, which are considered valid if the PL/ML ratio lies within the 0.5-2.0 range (Ort et al., 2009; Verlicchi et al., 2014). Accordingly, the PL values were acceptable for 6 of the 11 PhACs which were detected in 100% samples of both experimental phases (clarithromycin, trimethoprim, bezafibrate, gemfibrozil, carbamazepine and lorazepam), as well as for propranolol in phase II.

In the A²O effluent samples, the ML values for clarithromycin, atenolol, propranolol, bezafibrate and gemfibrozil were 3-2-fold lower in phase II compared to

phase I (Table 3). Ofloxacin, trimethoprim and hydrochlorothiazide displayed higher ML in phase II compared to phase I, while similar ML values were found for the rest of the targeted PhACs in both phases (Table 3). The variability of the ML values in the effluent was mainly due to the different REs observed in phases I and II, as it will be further discussed in Section 3.2. It has been previously reported that the ML of PhACs in WWTPs influent and effluents are subjected to strong geographic and seasonal variabilities (Jelic et al., 2011; Papageorgiou et al., 2016; Park et al., 2017; Pereira et al., 2015); nevertheless, the data calculated in the present study are comparable with those reported by the aforementioned authors, with the exception of the antibiotic ofloxacin.

3.2. Removal efficiency of PhACs and links with the environmental/operational variables influencing the A²O system

Table 4 shows the REs (range, mean, median and RSD) of the target PhACs throughout the experimental phases I and II in the A²O system. Those compounds with a frequency of detection in samples < 50% and average mean influent concentrations < 20 ng L⁻¹ (sulfamethoxazole, sulfamethazine, sulfadiazine, metoprolol, fenofibrate, diazepam and paroxetine) were excluded from the calculations, since conclusions regarding its REs could not be properly drawn.

According to the Kruskal-Wallis test, statistically significant differences of the REs were observed among phases I and II for 5 PhACs (Table 4). Mean REs were higher in phase II for clarithromycin, bezafibrate, and gemfibrozil (93, 80 and 74%, respectively) compared to phase I (30, 33 and 31%, respectively), while the REs of sotalol and hydrochlorothiazide were higher in phase I (28 and 38%, respectively) compared to phase II (-13 and 0%, respectively). The REs of atenolol, furosemide and trimethoprim were no significantly different among the two experimental phases, while

very low or negative mean REs were observed for ofloxacin, propranolol, carbamazepine, and lorazepam, in agreement with earlier reports in the literature (Ashfaq et al., 2017; Blair et al., 2015; Gurke et al., 2015; Pereira et al., 2015). Negative REs of PhACs are commonly observed in WWTPs and are attributable to the release of molecules adsorbed/enclosed in suspended particles, or the transformation of conjugate forms into the parent compounds via microbial activity (Ashfaq et al., 2017; Blair et al., 2015; Göbel et al., 2007; Verlicchi et al., 2012).

Hydrochlorothiazide and sotalol displayed better removal rates during phase I, mostly favored by the lower concentrations of MLSS ($\rho < -0.70$, Table S6). On the contrary, the REs of bezafibrate, clarithromycin and gemfibrozil, which were significantly higher in phase II (Table 4), were correlated with higher MLSS concentration in the activated sludge, lower F/M ratios, and higher concentrations of NH_4^+ in the influent water (Table S6). In a previous study in the same A^2O system, it was observed that the REs of acetaminophen, ibuprofen, naproxen, and ketoprofen were also improved under the operating conditions of phase II (Gallardo-Altamirano et al., 2018). Operation at high MLSS concentrations and low F/M ratios has been reported particularly favorable for the improvement of the REs of persistent PhACs (Verlicchi et al., 2012). Increased NH_4^+ loading rates were also previously found correlated to better removal rates of micropollutants in WWTPs, an effect which is attributed to the enhancement of the biological activity of nitrifiers, since it is well established that ammonia oxidizers contribute to the biodegradation of a wide range of trace organic compounds in engineered systems, possibly through cometabolic reactions mediated by the ammonia monooxygenase enzymes (reviewed by Kumwimba and Meng, 2019).

The improvement of the REs of bezafibrate, clarithromycin and gemfibrozil in phase II was concurrent with better removal rates of both organic matter and nutrients, compared to phase I (Table S1). In particular, high positive correlations ($\rho > 0.65$) were displayed among REs of the three PhACs and NRR (Table S6). Interestingly, an association among higher NRR and improved removal of PhACs was also established previously in activated sludges of 10 full-scale WTPPs (Helbling et al., 2012).

3.3. Microbial community structure and diversity in the A²O system

The biological treatment of wastewater mainly relies on the ability of heterotrophic bacteria and fungi for the biotransformation of organic substances and the removal of nutrients from effluents. At the same time, Archaea fulfil important roles as part of the syntrophic metabolic networks needed to complete the mineralization of organic compounds under anaerobic conditions (Nobu et al., 2015). The diversity of Bacteria, Archaea and Fungi in the A²O system was surveyed by massive parallel sequencing of the small subunit rRNA genes using the Illumina platform. Sample-size-based and coverage-based R/E sampling curves are shown in Fig. S2, and the summary of the average numbers of reads, richness of PHYs (S), Good's coverage and diversity indices calculated for each microbial group is displayed in Table S7.

After the quality filtering of the raw sequencing data, > 120,000 and > 45,000 16S rRNA reads were obtained for Bacteria and Archaea, respectively, for a total of 8 activated sludge samples analyzed. The number of reads per sample ranged between 7864-28740 for Bacteria and 580-14,642 for Archaea, showing differences of the sequencing depth. A total of 1072 Bacteria PHYs and 89 Archaea PHYs were detected at 97% sequences identity, ranging between 481-661 and 23-61 PHYs per sample, respectively. The R/E curves (Fig. S2) and the values of Good's coverage (>98%, Table

S7) indicated that the achieved sequencing depth sufficed to properly describe the diversity of the bacterial and archaeal communities in the A²O system. The average values of S, Chao-1, H' and Simpson's indices were very similar in both experimental phases for either Bacteria and Archaea. The indices described bacterial communities displaying high species diversity, while in the case of Archaea the diversity was low-to-medium. Both Bacteria and Archaea communities had a low functional organization, according to the average values of the Simpson's index (Table S7).

The diversity of Fungi in the A₂O system was only investigated by Illumina sequencing in samples of phase I, since this group of microorganisms was not detectable in samples of phase II. A total of 4,922 quality filtered fungal 18S rRNA sequences were obtained from 4 samples. One hundred Fungi PHYs were detected at 97% sequences identity, ranging between 28-74 PHYs per sample. The R/E curves (Fig. S2) and the value of Good's coverage ($\geq 96\%$ Table S7) indicated enough sequencing depth for the description of fungal diversity in the A²O samples. The community structure of Fungi was characterized for a low-to-medium species diversity and a low functional organization, according to the average values of S, Chao-1, H' and Simpson's indices (Table S7).

3.3.1. Structure and diversity of the Bacteria communities in the A²O system

The dendrogram in Fig. S3 shows the clustering of the A₂O samples (according to the Bray-Curtis coefficient), based on the relative abundance of bacterial PHYs detected by Illumina sequencing. The global similarity among samples in phases I and II was > 40%. Significant differences were also detected between the experimental phases by ANOSIM analysis (R= 0.84).

A total of 1,072 PHYs with a relative abundance > 0.1% in at least one sample were classified in 27 different Phyla. The bacterial community was dominated by *Proteobacteria*, which displayed the highest average relative abundance in the sludge samples of both phases (60.4 and 64.7%; phase I and II, respectively), followed by *Bacteroidetes* (23.1 and 15.04 %) and *Chloroflexi* (7.7 and 11.4 %) (Fig. 1A-1). Interestingly, unique Phyla were detected in both experimental phases, although at very low relative abundances. Phylotypes affiliated to Phyla TM6 (0.0065%) and OD1 (0.0020 %) were only detected in phase I, while members of the Phyla GOUTA4 (0.0031%), *Gemmatimonadetes* (0.0036 %) and NKB19 (0.0036 %) were exclusive of phase II. Significant differences of the community composition among the two experimental phases were also detected at the Order and Family levels (Fig. 1A-2, A-3).

In order to evaluate which PHYs contributed most to the (dis)similarity between samples of phases I and II, SIMPER analyses were carried out (Table S8). The overall similarity among samples was 61.48% in phase I and 71.09% in phase II. Three PHYs affiliated to the *Comamonadaceae*, *Cytophagaceae*, and *Xanthomonadaceae* (PHYs 962, 406, and 630, respectively) and two PHYs phylogenetic related to *Caldilineaceae* (PHYs 118 and 713) cumulatively contributed > 35% to explain the similarity between phase I samples (Table S8A), while the similarity among phase II samples was explained (> 35%) by PHYs 118, 406 and 962 (Table S8B). SIMPER analysis also showed that PHYs 406 and 962 (Table S8C) were the major contributors to the 47.93% dissimilarity observed between the two experimental phases, cumulative explaining > 18% of the differences.

Proteobacteria, *Bacteroidetes* and *Chloroflexi* have been described as the predominant bacterial groups in aerobic activated sludge of WWTPs, either based on

conventional or advanced technologies (reviewed by Ferrera and Sánchez, 2016; Xia et al., 2018). Of these, *Proteobacteria* are most often found as the prevalent group, particularly in municipal WWTPs (Cyzdik-Kwiatkowska and Zielińska, 2016). Among these, members of the *Comamonadaceae* are regarded as ubiquitous in activated sludge, where they fulfil a variety of functions such as nitrate reduction (Sadaie et al., 2007), biological phosphorus removal (Ge et al., 2015) and biodegradation of complex substrates (Khan et al., 2002).

3.3.2. Structure and diversity of Archaea communities in the A²O system

Fig. S3 displays the Bray-Curtis based dendrogram showing the (dis)similarities between the A²O samples of phases I and II, according to the relative abundance of Archaea PHYs detected by Illumina sequencing. Samples of each experimental phase clustered together in separate groups with a similarity < 20%, while the similarity of the samples within each experimental phase was > 40%. ANOSIM analysis also corroborated the significant differences between experimental phases (R= 0.188).

Fig. 1B (B-1, B-2 and B-3) displays the heatmaps showing the relative abundances of archaeal PHYs in the two experimental phases, at Phylum, Order and Genus level, respectively. *Euryarchaeota* was the dominant Phylum (98.2 and 92.3% average relative abundances, phases I and II, respectively), followed by *Thaumarchaeota* (1.2 and 6.7%) and *Crenarchaeota* (0.1 and 0.4%). Significant differences of the community diversity were also detected among phases I and II at Order and Genus levels (Fig. 1B-2 and B-3). *Methanomicrobiales* were prevalent in phase I (average relative abundance 45.4%), while *Methanobacteriales* dominated in phase II (average relative abundance 49.8%). At the Genus level, phase I was dominated by PHYs closely related to *Methanolinea* (*Methanomicrobiales*) (28.9% average

relative abundance), while *Methanobrevibacter* and *Methanobacterium* (*Methanobacteriales*) were prevalent in samples of phase II (21.8 and 21.2 % average relative abundance, respectively) (Fig. 1B-3).

According to SIMPER analysis (Table S9), the global similarity of the Archaea communities was 61.95% in phase I samples and 48.7% in phase II samples. Three PHYs (PHYs 87, 130 and 29, which were closely related to genera *Methanolinea*, *Methanobrevibacter* and *Methanobacterium*, respectively) contributed 60.8% to explain the similarity between samples of phase I, while PHYs 29, 130, 41 and 87 (closely related to *Methanobacterium*, *Methanobrevibacter*, *Methanomethylovorans* and *Methanolinea*, respectively) contributed 65.06% to the similarity of phase II samples. SIMPER also corroborated the low similarity of the archaeal communities observed between the two experimental phases (47.31% average dissimilarity), with a single PHY (PHY 87, affiliated to *Methanolinea*) contributing a 16.34% explanation to such differences.

Methanobacteriales, *Methanomicrobiales* and *Methanosarcinales* are commonly detected in urban wastewater treatments based on different technologies, including aerated activated sludge systems (Ferrera and Sánchez, 2016). The A²O technology is characterized by aerobic, anoxic and anaerobic zones in series, subjected to internal recirculation. Under such conditions, usually no significant differences of the composition of the bacterial communities are found among the bioreactors with different redox regimes (Gómez-Silván et al., 2014; Kim et al., 2013; Phan et al., 2016). Methanogenic archaea present in the anaerobic zones under optimal conditions to develop their metabolism enter the aerated zones by recirculation, where their contribution to carbon and nitrogen removal is controversial, although some studies

supported their role as enhancers of some biological activities through their symbiotic relationships with Bacteria (Ferrera and Sánchez, 2016).

3.3.3. Structure and diversity of Fungi communities in the A²O system

The dendrogram in Fig. S3 illustrates the clustering of samples (Bray Curtis) according to the relative abundance of the fungal PHYs in the A²O samples from phase I, showing a low similarity (< 20%) among them. PHYs classified in 7 different Phyla/Subphyla were identified. *Ascomycota* was the dominant Phylum (73.4% average relative abundance), followed by *Basidiomycota* (16.2%), *Mortierellomycotina* (*Mucoromycota*, Tedersoo et al., 2018) (4.7%), *Blastocladiomycota* (3.3%), *Zoopagomycota* (1.5%), *Mucoromycotina* (*Mucoromycota*, Tedersoo et al., 2018) (0.63%) and *Chytridiomycota* (0.09%) (Fig. 1C-1). At the Order level, 34 different taxa were detected, dominated by *Ascomycota* of the *Saccharomycetales*, *Eurotiales* and *Hypocreales*, and *Basidiomycota* of the *Trichosporonales* and *Filobasidiales* (26.7, 17.2, 15.7, 7.8 and 5.7% average relative abundance, respectively) (Fig. 1C-2). SIMPER analysis (Table S10) showed that the global similarity between samples was 39.3%, where only 5 PHYs affiliated to the *Hypocreales*, *Trichosporonales*, *Saccharomycetales* and *Eurotiales* were enough to explain > 55% of such similarity.

The results presented here revealed a higher diversity and relative abundance of *Ascomycota* compared to *Basidiomycota* in the A²O system, and in most samples, phylotypes closely related to the *Saccharomycetes* displayed the highest relative abundances within the fungal community (Fig. 1C), in agreement with the knowledge of the diversity of fungi in WWTPs (Maza-Márquez et al., 2016). *Ascomycota* are an extremely diverse group of fungi, accounting for > 65% of these eukaryotic microorganisms in both terrestrial and aquatic environments (Harms et al., 2011).

Several species have been described able to biotransform PhACs, including AIAPs, antibiotics, or β -blockers (Olicón-Hernández et al., 2017).

3.4. Linking the relative abundance of Bacteria and Archaea to the environmental/operational variables of the A²O system

In Fig. 2 (A, B), the activated sludge samples retrieved from the A²O system were ordinated by MDS on the basis of the relative abundance of the populations of Bacteria and Archaea identified by Illumina-sequencing. Fungi were not included in these analyses, since they were only detected in samples of phase I. The vectors on the plots representing the abiotic variables displayed two clear trends throughout the ordinations, in agreement with the different operating conditions characterizing each of the two experimental phases (higher average OT and F/M in phase I, and higher concentrations of COD and BOD₅ in the influent water, higher biomass accumulation (MLSS) and sludge volumetric index (SVI) in phase II) and the increased removal efficiencies of organic matter and nutrients (ORR, NRR, PRR) registered during phase II. These changes in the operational and environmental parameters were strongly related to the shifts of the structure of the microbial communities in the A²O system (Fig. 2), as reported previously for WWTPs by several studies (Ju and Zhang, 2015; Kim et al., 2013; Reboleiro-Rivas et al., 2016).

According to BIO-ENV analysis, among all the tested variables, MLSS, SRT, COD, BOD₅ and NH₄⁺ were those contributing the highest level of explanation (89.1%) to the ordination of samples based on the bacterial community structure (Fig. 2A). The Pearson product-moment correlation coefficients among the vectors in Fig. 2A (Table S11A) showed that MLSS, COD, BOD₅ and NH₄⁺ were positively correlated ($r \geq 0.70$) to the relative abundance of families of the *Alphaproteobacteria* (*Hyphomicrobiaceae*,

Rhodobacteraceae, *Sphingomonadaceae*, unclassified *Rhizobiales*, and unclassified *Sphingomonadales*), *Betaproteobacteria* (*Comamonadaceae*, *Rhodocyclaceae*, and unclassified *Thiobacterales*) and *Chloroflexi* (*Caldilineaceae*). In contrast, the relative abundances of PHYs related to the *Bacteroidetes/Chlorobi* (*Cytophagaceae*, *Saprospiraceae*, Unclassified *Chlorobi*, and Unclassified *Sphingobacteriales*) and *Gammaproteobacteria* (*Chromaticeae* and *Xanthomonadaceae*) showed opposed trends throughout the samples' ordination.

Some of the responses of the bacterial groups to the changes of the operational parameters in the A²O were in agreement with those observed in previous studies. The increase of MLSS in phase II contributed to higher relative abundances of *Caldilineaceae* and *Chloroflexi* populations (Fig. 2A and Table S11A), accordingly to the previous observations in a moving-bed membrane bioreactor (MBMBR) treating municipal sewage (Reboleiro-Rivas et al., 2016). Muszyński et al. (2015) analyzed the bacterial diversity in an A²O system by FISH probing, reporting the positive correlation of the relative abundances of both *Alpha*- and *Betaproteobacteria* with COD and total N of the influent wastewater and their negative correlation with OT, while *Bacteroidetes* of the *Saprospiraceae* followed the opposite trend.

Regarding the relative abundances of Archaea populations at the Genus level, BIO-ENV analysis detected that NH₄⁺ concentration in the activated sludge was the parameter contributing the best explanation to the ordination of samples (46.5%). These results indicate an effect of NH₄⁺ concentration on the succession of both hydrogenotrophic and methylotrophic/acetoclastic methanogens. Strong positive correlations of this abiotic factor ($r > 0.65$) were observed with the relative abundances of most PHYs of the *Methanobacteriales* identified at the genus level, while negative or

negligible correlations were observed for the genera of the *Methanomicrobiales* (with the exception of *Methanoregula*). Higher NH_4^+ concentrations also favored the dominance of *Methanomethylovorans* and *Methanosarcina* versus *Methanosaeta* (Fig. 2B and Table S11B).

Ammonia concentration is regarded as an important parameter for the shaping of the methanogenic communities. NH_4^+ is required as the major N source for most methanogens (Garcia et al., 2000), but at the same time ammonia ions and free ammonia can inhibit methanogenesis (Yenigün and Demirel, 2013). The effect of ammonia on population dynamics and the activity of methanogens has been thoroughly described in anaerobic digesters at much higher average concentrations (usually $>1 \text{ g L}^{-1}$) than those measured in the A^2O system investigated here; however, very similar patterns of community succession in response to ammonia were observed. Previous studies concluded that hydrogenotrophic methanogens are more tolerant to ammonia levels than acetotrophic methanogens, and the obligate acetoclastic *Methanosaeta* are the most sensitive to ammonia (Karakashev et al., 2005; Yenigün and Demirel, 2013). In this regard, *Methanosaetaceae* dominated in sewage sludge digesters operating at low ammonia levels ($0.03\text{-}0.3 \text{ g N-NH}_4^+ \text{ L}^{-1}$), while *Methanosarcinaceae* were prevalent in cattle manure digesters with concentrations of $\text{N-NH}_4^+ >1 \text{ g L}^{-1}$ (Karakashev et al., 2005). Among the hydrogenotrophic methanogens, *Methanobacteriales* increased their abundance versus *Methanomicrobiales* at higher NH_4^+ loading rates in a laboratory-scale two-stage anaerobic digester operated in the range of $0.09\text{-}1.16 \text{ g NH}_4^+ \text{ L}^{-1} \text{ day}$ (Kim et al., 2014).

Nitrososphaera is an ammonia oxidizing archaea (AOA) described in either water or soil environments (Ma et al., 2018; Mosier et al., 2012; Park et al., 2012), and is the

AOA most often identified in WWTPs (Limpiyakorn et al., 2013). In that respect, the average relative abundance of *Nitrososphaera* increased from 1.05 % in phase I to 6.74 % in phase II, and strong positive correlations ($r > 0.65$) were found between the abundance of these AOA and NH_4^+ , TN and NRR (Table S11B). Ammonia-oxidizing bacteria (AOB) have been classically considered the key organisms responsible of nitrification in WWTPs (Yin et al., 2018); however, in the last decades it has been established that AOA of the *Thaumarchaeota* often coexist with AOB in these engineered systems (Limpiyakorn et al., 2013), within an ample range of ammonia concentrations (Yin et al., 2018), and their effective contribution to ammonia oxidation has been recently demonstrated in nitrifying reactors (Roy et al., 2017; Srithep et al., 2018). The results of the present study show that the relative abundance of *Nitrososphaera*-related phylotypes among Archaea was favored by increasing concentrations of ammonia in the A^2O influent, and correlated concomitantly with improved NRR, supporting the relevance of their role in ammonia oxidation. Similar correlation trends were previously found for *Nitrososphaera*-related AOA by Gao et al. (2014).

3.5. Linking population dynamics of Bacteria and Archaea to the REs of PhACs in the A^2O system

MDS and BIO-ENV analyses were used in search of links among the observed changes of the bacterial and archaeal communities and the REs of PhAC in the A^2O system. Fungi were not included in these analyses, since they were only detected in samples of phase I. Besides the data of 7 of the PhACs described in this study (Table 4), the REs of 4 AIAPs (acetaminophen, ibuprofen, naproxen, and ketoprofen) which were previously

reported in the same set of samples (Gallardo-Altamirano et al., 2018) were also included. The samples' ordination plots are shown in Fig. 3.

BIO-ENV analysis revealed strong correlations between the removal of PhACs of different pharmaceutical families (AIAPs, antibiotic, β -blockers, diuretics and lipid-regulator) and the relative abundances of particular bacterial and archaeal populations (Fig. 3 and Table S12). Overall, the trends of the vectors on the plot reflects that the REs of most of the analyzed PhACs were higher in experimental phase II, with the exception of hydrochlorothiazide. BIO-ENV analyses showed that the REs of the PhACs were positively correlated with the increased relative abundance of a core of different bacterial families during phase II (Fig. 3A). Higher relative abundances of *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*) were strongly correlated ($r > 0.75$) with increased REs of clarithromycin, furosemide, bezafibrate and gemfibrozil, while *Betaproteobacteria* (*Comamonadaceae*, *Rhodocyclaceae*, unclassified *Betaproteobacteria* and unclassified *Thiobacterales*) and *Chloroflexi* (*Caldilineaceae*) correlated positively ($r = 0.75-1.0$) with the REs of up to seven PhACs belonging to different therapeutic groups: AIAPs (ibuprofen and naproxen), antibiotics (clarithromycin), diuretics (furosemide), β -blockers (atenolol) and lipid-regulators (bezafibrate, gemfibrozil) (Table S12A). The relative abundances of phylotypes of the *Chitinophagaceae* and *Flavobacteriaceae* (*Bacteroidetes/Chlorobi*) were also correlated to higher REs of ibuprofen and naproxen, while *Gammaproteobacteria* and *Cytophagaceae* abundances displayed a very high positive correlation with the REs of hydrochlorothiazide ($r > 0.9$), the only PhAC which was removed with a significantly higher efficiency under the operating conditions of phase I (Table S12A). Finally, the REs of acetaminophen were strongly and positively correlated ($r > 0.9$) to the relative

abundance of unclassified *Myxococcales* (*Deltaproteobacteria*) and some groups of the *Bacteroidetes/Chlorobi* (Fig 3, Table S12A).

Regarding the archaeal population, MDS and BIO-ENV detected strong correlations ($r \geq 0.60$) between the REs of different AIAPs (acetaminophen, ibuprofen, ketoprofen and naproxen), antibiotics (clarithromycin), β -blockers (atenolol) and lipid-regulators (bezafibrate and gemfibrozil) and the relative abundances of *Methanobrevibacter*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, and unclassified *Methanomicrobiales*. The relative abundance of *Methanomethylovorans* was positively correlated ($r \geq 0.60$) with the REs of ibuprofen, naproxen, clarithromycin, atenolol, furosemide, bezafibrate and gemfibrozil (Table S12B).

The performance of biological wastewater treatment systems for PhACs removal has been thoroughly investigated in systems based on different technologies, but only a few studies provided significant links among their REs and the occurrence/abundance of particular microbial groups, most of which were conducted in laboratory-scale systems using synthetic wastewater spiked with fixed amounts of PhACs, and often using concentrations well over those occurring in real wastewaters. In spite of this differences, the results presented here are consistent with those of previous work.

Alphaproteobacteria and *Betaproteobacteria* have been often linked to the biological removal of PhACs in WWTPs based on different technologies. The relative abundances of populations classified within the *Sphingomonadaceae* were earlier found positively correlated with the REs of the antibiotic trimethoprim in an autotrophic nitrogen removal CANON bioreactor (Rodriguez-Sanchez et al., 2017), and two genera of the *Sphingomonadaceae* (*Sphingorhabdus* and *Novosphingobium*) were pointed out as indicators of conditions favorable for the removal of bezafibrate in both a full-scale and

a pilot-scale WWTP (Wolff et al., 2018). The enrichment in the activated sludge communities of phylotypes related to several genera of the *Comamonadaceae* and *Rhodocyclaceae* was previously observed to occur concomitantly with the removal of bezafibrate and clarithromycin (Wolff et al., 2018), ibuprofen (Li et al., 2016), and trimethoprim (Rodriguez-Sanchez et al., 2017). Isolates taxonomically classified within the *Comamonadaceae* and *Sphingomonadaceae* are also commonly reported able to metabolize/cometabolize different kinds of PhACs under laboratory conditions, either in pure cultures or as part of consortia (Fortunato et al., 2016; Wang and Wang, 2016).

Regarding the links found among the REs of PhACs and other bacterial groups, an increase in the relative abundance of *Flavobacteriaceae* (*Bacteroidetes*) was observed in a pilot-scale MBR fed with synthetic hospital wastewater following the addition of 10 µg L⁻¹ ibuprofen (Tiwari et al., 2019), and populations of this family have been as well associated with the metabolic or cometabolic degradation of ibuprofen in constructed wetlands (Li et al., 2016). Enrichment of *Bacteroidetes* and *Chloroflexi* in an aerated SBR fed with synthetic wastewater amended with a mixture of diclofenac, ibuprofen and naproxen (5 µg L⁻¹ each) was reported by Jiang et al. (2017); however, these authors found that the relative abundance of *Proteobacteria* was at the same time lowered down under the conditions of their study, contrary to the results presented here. Phan et al. (2016) assayed an MBR operated under different sets of conditions for the removal of 30 trace organic contaminants (including gemfibrozil, ibuprofen, ketoprofen, and naproxen) at a concentration of 5 µg L⁻¹, reporting that *Proteobacteria* increased their relative abundance in the presence of the trace organic contaminants, while *Bacteroidetes* (*Cytophagaceae*) mostly thrived in their absence, and concluding that *Betaproteobacteria*, *Caldilineaceae* and *Myxococcales* were more abundant among the

activated sludge community under the operating conditions allowing the effective removal of the micropollutants.

The role of Archaea in PhACs removal in WWTPs has been even more scarcely investigated. Methanogenic archaea are regarded a key component in the biodegradation of pollutants such as hydrocarbons in soil and water, where they thermodynamically favor the degradative processes by acting as a sink of the terminal products H₂ and acetate (Krzmarzick et al., 2018). The acetoclastic genus *Methanosaeta* significantly increased its abundance in diesel-contaminated versus non-contaminated soils (Krzmarzick et al., 2018) and also dominated the community in a WWTP treating industrial wastewater containing azo-dyes (Plumb et al., 2001). *Methanomethylovorans*, a obligately methylotrophic methanogen able to use methanol, methanethiol, methylamines and dimethyl sulfide as substrates (Lomans et al., 1999), has been described as the dominant methanogenic archaea in WWTPs treating high concentrations of antibiotics, pointing at their implication together with sulfate reducing prokaryotes in the latter steps of biodegradation of these compounds under anaerobic conditions (Deng et al., 2012). A similar role of *Methanomethylovorans* in the anaerobic degradation of dyes with sulfonate groups in industrial wastewater was earlier proposed (Plumb et al., 2001).

The relative abundance of *Nitrososphaera*-related phylotypes within the archaeal community of the A²O displayed positive correlations with the REs of furosemide, bezafibrate and gemfibrozil (Fig. 3, Table S12). It was previously shown that the removal of these three PhACs, among others, was significantly hampered following chemical inhibition of ammonia monooxygenase activity in bench-scale MBRs and anthracite-packed biofilters (Maeng et al., 2013; Rattier et al., 2014). In the literature,

the involvement of nitrifiers in the removal of trace organic contaminants from wastewater has been mostly attributed to AOB (Kumwimba and Meng, 2019); however, in a survey of activated sludge samples from 10 full-scale WWTPs, Helbling et al. (2012) concluded that both ammonia oxidation and the removal of PhACs were correlated to the abundance of archaeal, rather than bacterial, *amoA* transcripts.

4. Conclusion

The findings presented here provide new insights on the correlations among plant operation parameters and the assemblage of the activated sludge microbial communities, regarding how these interlinkages influence the removal of PhACs in WWTPs. Robust links were revealed between improved REs of several of the analyzed compounds (acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromycin, trimethoprim, atenolol, furosemide, hydrochlorothiazide, bezafibrate, and gemfibrozil) and the shifts of the relative abundances of different families of *Bacteria* (*Rhodobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae*, *Rhodocyclaceae*, *Caldilineaceae*, *Chitinophagaceae* and *Flavobacteriaceae*) and genera of *Archaea* (*Methanobrevibacter*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, *Methanomethylovorans* and *Nitrososphaera*). Remarkably, the REs of clarithromycin, bezafibrate and gemfibrozil were improved over twofold by the combination of an average MLSS concentration of *ca.* 5 g L⁻¹ with a F/M ratio of 0.21 kg BOD₅ kg MLVSS⁻¹ d⁻¹. These operational parameters are easy-to-control and their adjustment feasible in seek for an improvement of the removal of PhACs in WWTPs.

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Figure Legends

Fig. 1. Heatmaps showing the relative abundances of Bacteria (A), Archaea (B) and Fungi (C) detected by Illumina sequencing in the A²O system operated under different conditions in experimental phases I and II. A1, B1, C1. Classification at Phylum level. A2. Classification at Family level. B2, C2. Classification at Order level. B3. Classification at Genus level. Taxa were excluded from the heatmaps according to the following cut-offs of average relative abundance considering both experimental phases: Bacteria: < 0.5, < 0.6, and < 0.9 % for Phylum, Order and Family, respectively; Archaea: < 0.04 for both Family and Genus; Fungi: < 0.4 for Order.

Fig. 2. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved from the A²O operated under different experimental conditions I and II, according to the relative similarity of Bacteria (A) and Archaea (B) analyzed by Illumina sequencing. In the right column, the MDS plots represent the direction throughout the ordination of the relative abundances of Bacteria, Archaea and Fungi phylotypes, while in the left column, MDS plots represent the abiotic variables measured in the A²O system. BOD₅: biological oxygen demand at 5 days; COD: chemical oxygen demand; F/M: food-to-microorganism ratio; MLSS: mixed suspended solids; OT: operational temperature; TN: Total N; SRT: sludge retention time; SVI: sludge volumetric index. The variables which best explained the distributions of the biotic data according to BIO-ENV analysis are marked with an asterisk (*). Only vectors with length >0.2 are shown.

Fig. 3. A Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the activated sludge samples from the A²O system in the experimental phases I and II, according to the relative similarity of Bacteria (A) and Archaea(B) communities

1098 analyzed by Illumina sequencing. In the right column, the MDS plots represent the
1099 direction throughout the ordination of the relative abundances of the Bacteria and
1100 Archaea phylotypes, while in the left column, MDS plots represent removal rates
1101 (REs%) of acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromycin,
1102 trimethoprim, atenolol, furosemide, hydrochlorothiazide, bezafibrate and gemfibrozil.
1103 Only vectors with length >0.2 are shown.

Title: Linking microbial diversity and population dynamics to the removal efficiency of pharmaceutically active compounds (PhACs) in an anaerobic/anoxic/aerobic (A²O) system.

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Abstract:

The removal efficiencies (REs) of nineteen pharmaceutically active compounds (PhACs) (six antibiotics-clarithromycin, ofloxacin, sulfadiazine, sulfamethazine, sulfamethoxazole and trimethoprim -, four β -blockers -atenolol, metoprolol, propranolol and sotalol- two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators -bezafibrate, fenofibrate and gemfibrozil-, and four psychiatric medications -carbamazepine, diazepam, lorazepam and paroxetine) were ascertained in a pilot-scale anaerobic/anoxic/aerobic (A^2O) system treating urban wastewater, long term operated during two experimental phases using different sets of environmental conditions and operating parameters. Illumina MiSeq sequencing was used to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi communities in the activated sludge. The results showed that mixed liquor suspended solids (MLSS) and food-to-microorganisms ratio (F/M) were operational parameters significantly influencing the REs of five of the analyzed PhACs in the A^2O system. Biota-environment (BIO-ENV) analysis revealed strong correlations between population shifts of the activated sludge community and the REs of PhACs of the different pharmaceutical families. Increased REs of clarithromycin, furosemide, bezafibrate and gemfibrozil were concomitant to higher relative abundances of bacterial phylotypes classified within the *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*), while those of *Betaproteobacteria*, *Chloroflexi* and *Methanomethylovorans* (*Euryarchaea*) correlated positively with the REs of up to seven PhACs belonging to different therapeutic groups.

Keywords: pharmaceutically active compounds; removal efficiency; A^2O system; microbial diversity; wastewater treatment.

1. Introduction

The occurrence and fate of contaminants of emerging concern in aquatic environments and their potential ecotoxicological effects are current issues which have gained relevance in the last decade. Among such contaminants, pharmaceutically active compounds (PhACs) bring forward a special interest among the population (Al Aukidy et al., 2012; Verlicchi et al., 2012). PhACs of different therapeutic classes such as antibiotics, anti-inflammatory and/or analgesic substances, β -blockers, diuretics, lipid regulators or psychiatric medications, occur in raw wastewaters at varying concentrations ranging from ng L^{-1} to $\mu\text{g L}^{-1}$, depending on the prescription habits and usage patterns of each particular country (Kasprzyk-Hordern et al., 2009; Petrie et al., 2015). Among these, anti-inflammatory and/or analgesic pharmaceuticals (AIAPs) such as acetaminophen, ibuprofen or naproxen and the lipid regulators bezafibrate, fenofibrate and gemfibrozil are most commonly detected and at the highest concentrations, reaching values up to several hundred $\mu\text{g L}^{-1}$ (Miège et al., 2009; Tran et al., 2018). Global consumption of PhACs and consequently their incidence in wastewaters are predicted to increase rather than decay in the near future, due to the lengthening of life expectancy of the population (Van Der Aa et al., 2011).

Biodegradation and sorption onto solids are assumed to be the primary mechanisms for the removal of the majority of PhACs in wastewater treatment plants (WWTPs), but the removal rates may considerably differ for each particular compound depending on its chemical characteristics and are also influenced by the operating conditions of the treatment systems (Gao et al., 2012; Jelic et al., 2011; Petrovic et al., 2009; Radjenović et al., 2009). In most cases, the biodegradation of these substances is determined by the presence of primary carbon or nitrogen substrates, as well as the

existence of microorganisms implicated in cometabolic degradation (Tran et al., 2013). Nevertheless, WWTPs have been shown unable to completely remove most of the PhACs present in the influent wastewaters and consequently many of these substances are detected in the treated effluents and stabilized sewage sludge. For example, β -blockers such as atenolol, metoprolol, propranolol, the antiepileptic drug carbamazepine, the AIAP diclofenac, and antibiotics belonging to several chemical classes such as fluoroquinolones, macrolides or sulfonamides, are detected both in influents and effluents of WWTPs worldwide (Gurke et al., 2015; Kasprzyk-Hordern et al., 2008, 2009; Tran et al., 2018; Verlicchi et al., 2012). In addition, the microbial community of activated sludge comprises a high density and diversity of bacterial populations subjected to organic pollutants stresses, making WWTPs optimal scenarios for the spreading of antibiotic resistances and posing important risks for human health due to the transfer of the antibiotic resistance genes (ARGs) from environmental reservoirs to human pathogenic bacterial strains (Hultman et al., 2018). Last, but not least, the presence of antibiotics in wastewaters may affect the diversity and functionality of the complex microbial communities in charge of the biological wastewater treatment, consequently hampering their performance (Deng et al., 2012; Rodriguez-Sanchez et al., 2017).

The concentration of PhACs in treated water often exceeds *the predicted no effect concentrations* (PNECs) for aquatic and soil organisms, so the development of more efficient wastewater treatment configurations with the aim to reduce their concentrations in the treated effluents is a requisite to prevent the dissemination of these substances to water and soil bodies (Kümmerer, 2009). In this sense, several biological treatment technologies (as alternative to the conventional activated sludge system, CAS)

have been evaluated with regard to their efficiency for PhACs removal: membrane bioreactors (MBR), fixed bed bioreactors (FBR), moving bed biofilm reactors (MBBR), aerobic or anaerobic granular sludge (AGS, AnGS), and anaerobic/anoxic/aerobic (A^2O) systems (Lv et al., 2017; Park et al., 2017). However, few studies have attempted to relate the removal efficiencies (REs) of PhACs to the shifts in the structure and diversity of the microbial communities of activated sludge, which in turn are linked to changes of the operational parameters and other uncontrolled variables influencing the WWTPs under real operation conditions.

In this study, the removal efficiencies of nineteen PhACs (six antibiotics- clarithromycin, ofloxacin, sulfadiazine, sulfametazine, sulfamethoxazole and trimethoprim-, four β -blockers -atenolol, metoprolol, propranolol and sotalol- two antihypertensives/diuretics -furosemide and hydrochlorothiazide-, three lipid regulators -bezafibrate, fenofibrate and gemfibrozil-, and four psychiatric medications - carbamazepine, diazepam, lorazepam and paroxetine) were evaluated in a pilot-scale A^2O system. The A^2O technology was selected for this study, since this advanced wastewater treatment process is based on a single sludge configuration using a combination of anaerobic, anoxic and aerobic bioreactors in series, which provides better removal efficiency of PhACs compared to CAS (Grandclément et al., 2017; Verlicchi et al., 2012). The A^2O was long-term operated during two experimental phases, using different operation parameters and under different environmental conditions. The Illumina MiSeq sequencing platform was used to investigate the structure, diversity and population dynamics of bacteria, archaea and fungi, in order to find significant links among the observed variability of the relative abundances of

microbial populations, the changes of the environmental/operational variables, and the REs of the targeted PhACs, with the aid of multivariate statistical analyses.

2. Materials and methods

2.1. Description of the wastewater treatment pilot-scale plant and operational conditions

The pilot-scale A²O plant (Fig. S1) was located at the facilities of the WWTP *Murcia Este* (EMUASA, Murcia, Spain) and its characteristics have been previously described in full detail (Gallardo-Altamirano et al., 2018). In brief, the working period covered from May 20th 2016 to March 31th 2017, divided into two differentiated experimental phases: phase I (July 27th - November 13th 2016) and phase II (December 14th 2016- March 31th 2017), which lasted 104 and 105 days, respectively, separated by a transition phase needed for the system to reach stable operation under the parameters chosen for phase II. Average values $\pm$ standard deviations for operational parameters of the A²O system and physical-chemical parameters measured in the influent water samples during phases I and II are summarized in Table S1.

2.2. Wastewater sampling collection for the analysis of physicochemical parameters and PhACs.

The A²O system was sampled at points 1, 2 and 3, as shown in Fig. S1. Daily-composite samples were retrieved three times a week. Chemical oxygen demand (COD), total nitrogen (TN), N-NO₃⁻, N-NH₄⁺ and total phosphorous (TP) were measured with the aid of Merck Spectroquant® kits (Darmstadt, Germany), while biological oxygen demand (BOD₅), total suspended solids in the influent water (TSS), Mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) were measured according to standard methods (Baird and Bridgewater, 2017).

For the analysis of PhACs, eighteen influent and effluent wastewater 24-h composite samples (eight samples in phase I and ten samples in phase II) were collected from sampling points 1 and 3 of the pilot scale plant (Fig. S1), ~~using 500-mL amber PET bottles as described previously (Gallardo-Altamirano et al., 2018).~~ Samples were retrieved both midweek and at the end of the weekend using 500-mL amber PET bottles, as described previously (Gallardo-Altamirano et al., 2018) and were frozen at -20 °C until their analysis. The effluent samples were collected according to the constant HRT (12 h). Initially, the same number of samplings was scheduled for the two experimental phases; however, right after the first sampling of phase II (14/12/16) a heavy rainfall episode took place (15/12/16 -30/12/16) and sampling was suspended, since the exceptional weather conditions could lead to unrepresentative measures of PhACs removal. Paired midweek/end of weekend samplings were resumed in mid-January 2017 under average weather conditions, but the 14/12/16 sample was also analyzed for better reference of the trends of PhACs removal during the whole of experimental phase II.

2.3. Analytical methods for pharmaceutically active compounds

2.3.1. Chemicals and sample treatment

Analytical reference standards (mostly 90% purity, Table S2A) and isotopically labelled compounds for internal standard calibration (Table S2B) were purchased from Sigma-Aldrich (St Louis, MO, USA), Cerilliant (Round Rock, TX, USA), CDN Isotopes (Quebec, Canada), LGC Promochem (London, UK), and Santa Cruz Biotechnology (Dallas, TX, USA). Individual stock solutions of the standards were prepared in MeOH at a concentration of 100 µg mL⁻¹. For calibration and spiking purposes, working solutions (10 µg mL⁻¹ in MeOH) including all tested compounds or labelled compounds

were freshly prepared every three months. All solutions were stored in the dark at -20°C. HPLC-grade solvents (MeOH, ACN, and water) were purchased from Merck (Darmstadt, Germany).

2.3.2. On-line extraction and LC-MS/MS analysis

Pharmaceutical compounds were extracted from wastewater samples using methods previously validated and reported elsewhere (Gros et al., 2009; López-Serna et al., 2010). Briefly, 2.5 mL of sample were pre-concentrated onto a previously conditioned HySphere Resin GP 10 online-SPE cartridge (Spark Holland, Emmen, The Netherlands) and analysed using an automated on-line SPE sample processor SymbiosisTM Pico (Spark Holland) connected in series with a 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer (Sciex, Redwood City, CA, U.S.). An exhaustive extraction procedure was detailed in Gallardo-Altamirano et al. (2018).

The retained analytes were eluted from the cartridge with the LC mobile phase (linear organic gradient of acetonitrile in water with formic acid for positive ionization and ACN:MeOH in water for negative ionization) and transferred to the LC column (Purospher STAR RP-18 endcapped column (125 x 2 mm i.d., 5 µm particle size, Merck, Darmstadt, Germany). The 4000 QTRAP hybrid triple quadrupole-linear ion trap (QqLIT) mass spectrometer, equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, California, USA), was controlled by the Analyst 1.4.2 Software (Applied Biosystems-Sciex, Foster City, California, USA).

Ionization of the analytes was done using a Turbo Ion Spray source operated in both positive and negative electrospray ionization mode. The negative ionization mode allowed detection of four pharmaceuticals (bezafibrate, furosemide, gemfibrozil and hydrochlorothiazide) including isotopically-labeled compounds. In positive ionization

mode, fifteen compounds (atenolol, carbamazepine, clarithromycin, diazepam, fenofibrate, lorazepam, metoprolol, ofloxacin, paroxetine, propranolol, sotalol, sulfamethoxazole, and trimethoprim) were analyzed including isotopically-labeled compounds. For quantitative analysis, the ESI-MS/MS methods were performed, as described by Gros et al. (2009). MS acquisition was performed in the selective reaction monitoring (SRM) mode, by acquiring two SRM transitions per each target compound and only one for the corresponding surrogate compound. Target compounds and surrogates are listed in Table S3. Selected SRM transitions for each analyte and isotopically-labelled compound and the optimal instrumental conditions set for their analysis are summarized in Table S4.

2.4. DNA extraction, Illumina Miseq sequencing and data processing

Genomic DNA from 4-ml activated sludge samples was extracted as previously described (Gallardo-Altamirano et al., 2018). The genomic DNA pools were sequenced at the facilities of RTL Genomics (Lubbock, Texas, USA, <http://www.researchandtesting.com>), using an Illumina MiSeq platform (Illumina, Hayward, CA, USA). The primer pairs 28F-519R (5'-GAGTTTGATCNTGGCTCAG-3' and 5'-GTNTTACNGCGGCKGCTG-3') (Fan et al., 2012), 517F/909R (5'-GCTYTAAGSRNCCGTAGC-3' and 5'-CCCCGYCAATTCMTTTRAGT-3) (Maspolim et al., 2015) and FungiQuantF/FungiQuantR (5'-GGRAAACTCACCAGGTCCAG-3' and 5'-GSWCTATCCCCAKCACGA-3') (Liu et al., 2012) were chosen for the partial amplification of the 16S rRNA gene of bacteria and archaea and the 18S rRNA gene of fungi, respectively.

Raw sequencing data were processed using the QIIME software, v. 1.9.1, following the pipeline protocol previously described by Caporaso et al. (2012). The

sequences underwent a quality filtering as previously described by Bang-Andreasen et al. (2017), which removed low-quality reads (Q score ≤ 25), reads with homopolymers longer than 6 nt, ambiguous nucleotides ($n \geq 6$), or consecutive low-quality base calls ($n \geq 5$). Chimeric sequences were removed with USEARCH (Edgar, 2010). Finally, single sequence reads occurring in only one sample were removed of the PHY_table_BIOM for further analyses. Taxonomic *close-reference* assignments based on 97% sequence identity were done with QIIME v. 1.9.1 (Caporaso et al., 2010) using the Greengenes database (version 13_08) for Bacteria and Archaea sequences, while the 18S rRNA eukaryotic SILVA database (version 111) was used for Fungi (Yarza et al., 2017). The descriptive alpha-diversity indices Chao-1, Shannon-Wiener H' , and Simpson were calculated using QIIME. Good's coverage indices were also calculated as described previously (Good, 1953). Sample-size-based and coverage-based rarefaction and extrapolation (R/E) sampling curves with 95% confidence intervals were calculated and plotted using the 'iNEXT' free online tool (Chao et al., 2016; Hsieh et al., 2016), in order to make fair comparisons among samples of unequal size. Heatmaps displaying the relative abundances of bacteria, archaea and fungi in each sample were generated using the vegan package in R v.3.2.0 (<http://www.r-project.org>). The SIMPROF and SIMPER commands of the Primer software (PRIMER-E v. 6.1.18, Plymouth, UK) were used to construct an UPGMA dendrogram with 95% confidence intervals and to estimate the contribution of individual PHYs to the (dis)similarity between groups of samples, respectively, following the methods already described by Maza-Márquez et al. (2016).

2.5 Statistical analyses

IBM SPSS Statistics v. 19 (SPSS Inc., IBM, USA) was used to perform the statistical analyses on the different data sets. The Kruskal-Wallis non-parametric test was selected to find significant differences among the different groups of samples, using a 95% significance level ($p < 0.05$). When required, Spearman's rank correlation coefficients (ρ) among the different variables were calculated.

All the multivariate statistical analyses were performed using the Primer software (PRIMER-E v. 6.1.18, Plymouth, UK). Non-metric multidimensional scaling (MDS) was used for the ordination of activated sludge samples based on the biotic data sets (relative abundances of PHYs of the targeted groups, derived from the Illumina-sequencing platform). An analysis of similarity (ANOSIM) was also conducted. The ordination of samples was linked to the trends followed by the abiotic variables (REs of the targeted PhACs and environmental/operational variables influencing the A²O system) by means of BIO-ENV (biota-environment) analysis. Full details are described elsewhere (Maza-Márquez et al., 2016).

3. Results and discussion

3.1. Occurrence of PhACs in influent and effluent wastewater samples.

Tables 1 and 2 display the frequency of detection and the concentrations (range, mean, median and relative standard deviation, RSD) of the 19 targeted PhACs in the influent wastewater samples and the effluents of the A²O system during the experimental phases I and II. As shown in Table 1, 13 compounds had $\geq 75\%$ frequency of detection in the influent wastewater in at least one of the experimental phases, of which clarithromycin, ofloxacin, trimethoprim, atenolol, sotalol, furosemide, hydrochlorotiazide, bezafibrate, gemfibrozil, carbamazepine and lorazepam were detected in 100% of the analyzed samples. In contrast, sulfamethazine, sulfamethoxazole, metoprolol, fenofibrate,

diazepam and paroxetine were detected in $\leq 50\%$ of samples of both phases, and additionally were measured in very low mean concentrations ($< 20 \text{ ng L}^{-1}$), with the exception of the antibiotic sulfamethoxazole. According to the Kruskal-Wallis test, there were no statistically significant differences of the concentrations of the assayed PhACs in the influent wastewater between the two experimental phases, except for carbamazepine, which showed a higher mean concentration in phase I (154 ng L^{-1}) compared to phase II (98 ng L^{-1}). The highest mean influent concentrations were observed for the antibiotic ofloxacin, followed in descending order by furosemide, hydrochlorothiazide, gemfibrozil, clarithromycin, and atenolol (Table 1).

Nine PhACs were detected in 100% of the effluent samples, while the concentrations of 5 compounds (sulfamethazine, metoprolol, fenofibrate, diazepam and paroxetine) were below the limits of detection (Table 2). According to the Kruskal-Wallis test, there were statistically significant differences among the two phases for the concentrations of hydrochlorothiazide, gemfibrozil, and bezafibrate. The highest mean values in the effluent were found for ofloxacin, furosemide, hydrochlorothiazide, and gemfibrozil, while the mean concentrations of the remaining PhACs in the effluent were $< 300 \text{ ng L}^{-1}$ in both experimental phases.

The concentrations of all the targeted PhACs in both influent and effluent wastewater fell within the ranges previously reported by several authors (Luo et al., 2014; Tran et al., 2018; Verlicchi et al., 2012), except for the antibiotic ofloxacin, which was measured at concentrations exceeding $100,000 \text{ ng L}^{-1}$ in both influent and effluent water in two samples of phase II (Wednesday 29/03/2017 and Sunday 02/04/2017). Such variations of the concentration of antibiotics in wastewaters are likely related to

outbreaks of infection diseases; in this sense, clear seasonality in the concentrations and influent mass loads of ofloxacin was previously reported by Coutu et al. (2013).

The lowest Predicted No Effect Concentration (PNEC) values described in the recent literature (Orias and Perrodin, 2013; Tran et al., 2018; Verlicchi et al., 2012) for all the tested PhACs are shown in Table 2. The average concentrations of eight of the targeted PhACs in the A²O effluent (clarithromycin, ofloxacin, sulfamethoxazole, trimethoprim, propranolol, furosemide, gemfibrozil and lorazepam) fell within the ranges previously reported (Gurke et al., 2015; Kasprzyk-Hordern et al., 2008, 2009; Verlicchi et al., 2012) but were higher than their PNECs, thus posing a risk for aquatic ecosystems (Tran et al., 2018; Verlicchi et al., 2012). In this scenario, implementation of tertiary treatments is desirable to prevent the continuous discharge of these substances into the receiving water bodies.

Table 3 shows the daily mass loads (ML, mg day⁻¹ 1000 inh⁻¹) of the twelve PhACs which were detected in at least 75% of samples of both experimental phases, calculated for the influent and effluent water samples, together with their predicted mass loads (PL) and the corresponding PL/ML influent ratios, calculated as shown in Table S5. Similar influent ML values were found in both experimental phases for each target compound, except for ofloxacin (746 and 13,855 mg day⁻¹ 1000 inh⁻¹ in phase I and II, respectively). The PL/ML ratios were calculated to estimate the accuracy of the PL values, which are considered valid if the PL/ML ratio lies within the 0.5-2.0 range (Ort et al., 2009; Verlicchi et al., 2014). Accordingly, the PL values were acceptable for 6 of the 11 PhACs which were detected in 100% samples of both experimental phases (clarithromycin, trimethoprim, bezafibrate, gemfibrozil, carbamazepine and lorazepam), as well as for propranolol in phase II.

In the A²O effluent samples, the ML values for clarithromycin, atenolol, propranolol, bezafibrate and gemfibrozil were 3-2-fold lower in phase II compared to phase I (Table 3). Ofloxacin, trimethoprim and hydrochlorothiazide displayed higher ML in phase II compared to phase I, while similar ML values were found for the rest of the targeted PhACs in both phases (Table 3). The variability of the ML values in the effluent was mainly due to the different REs observed in phases I and II, as it will be further discussed in Section 3.2. It has been previously reported that the ML of PhACs in WWTPs influent and effluents are subjected to strong geographic and seasonal variabilities (Jelic et al., 2011; Papageorgiou et al., 2016; Park et al., 2017; Pereira et al., 2015); nevertheless, the data calculated in the present study are comparable with those reported by the aforementioned authors, with the exception of the antibiotic ofloxacin.

3.2. Removal efficiency of PhACs and links with the environmental/operational variables influencing the A²O system

Table 4 shows the REs (range, mean, median and RSD) of the target PhACs throughout the experimental phases I and II in the A²O system. Those compounds with a frequency of detection in samples < 50% and average mean influent concentrations < 20 ng L⁻¹ (sulfamethoxazole, sulfamethazine, sulfadiazine, metoprolol, fenofibrate, diazepam and paroxetine) were excluded from the calculations, since conclusions regarding its REs could not be properly drawn.

According to the Kruskal-Wallis test, statistically significant differences of the REs were observed among phases I and II for 5 PhACs (Table 4). Mean REs were higher in phase II for clarithromycin, bezafibrate, and gemfibrozil (93, 80 and 74%, respectively) compared to phase I (30, 33 and 31%, respectively), while the REs of sotalol and hydrochlorothiazide were higher in phase I (28 and 38%, respectively)

compared to phase II (-13 and 0%, respectively). The REs of atenolol, furosemide and trimethoprim were no significantly different among the two experimental phases, while very low or negative mean REs were observed for ofloxacin, propranolol, carbamazepine, and lorazepam, in agreement with earlier reports in the literature (Ashfaq et al., 2017; Blair et al., 2015; Gurke et al., 2015; Pereira et al., 2015). Negative REs of PhACs are commonly observed in WWTPs and are attributable to the release of molecules adsorbed/enclosed in suspended particles, or the transformation of conjugate forms into the parent compounds via microbial activity (Ashfaq et al., 2017; Blair et al., 2015; Göbel et al., 2007; Verlicchi et al., 2012).

Hydrochlorothiazide and sotalol displayed better removal rates during phase I, mostly favored by the lower concentrations of MLSS ($\rho < -0.70$, Table S6). On the contrary, the REs of bezafibrate, clarithromycin and gemfibrozil, which were significantly higher in phase II (Table 4), were correlated with higher MLSS concentration in the activated sludge, lower F/M ratios, and higher concentrations of NH_4^+ in the influent water (Table S6). In a previous study in the same A²O system, it was observed that the REs of acetaminophen, ibuprofen, naproxen, and ketoprofen were also improved under the operating conditions of phase II (Gallardo-Altamirano et al., 2018). Operation at high MLSS concentrations and low F/M ratios has been reported particularly favorable for the improvement of the REs of persistent PhACs (Verlicchi et al., 2012). Increased NH_4^+ loading rates were also previously found correlated to better removal rates of micropollutants in WWTPs, an effect which is attributed to the enhancement of the biological activity of nitrifiers, since it is well established that ammonia oxidizers contribute to the biodegradation of a wide range of trace organic

compounds in engineered systems, possibly through cometabolic reactions mediated by the ammonia monooxygenase enzymes (reviewed by Kumwimba and Meng, 2019).

The improvement of the REs of bezafibrate, clarithromycin and gemfibrozil in phase II was concurrent with better removal rates of both organic matter and nutrients, compared to phase I (Table S1). In particular, high positive correlations ($\rho > 0.65$) were displayed among REs of the three PhACs and NRR (Table S6). Interestingly, an association among higher NRR and improved removal of PhACs was also established previously in activated sludges of 10 full-scale WTPPs (Helbling et al., 2012).

3.3. Microbial community structure and diversity in the A²O system

The biological treatment of wastewater mainly relies on the ability of heterotrophic bacteria and fungi for the biotransformation of organic substances and the removal of nutrients from effluents. At the same time, Archaea fulfil important roles as part of the syntrophic metabolic networks needed to complete the mineralization of organic compounds under anaerobic conditions (Nobu et al., 2015). The diversity of Bacteria, Archaea and Fungi in the A²O system was surveyed by massive parallel sequencing of the small subunit rRNA genes using the Illumina platform. Sample-size-based and coverage-based R/E sampling curves are shown in Fig. S2, and the summary of the average numbers of reads, richness of PHYs (S), Good's coverage and diversity indices calculated for each microbial group is displayed in Table S7.

After the quality filtering of the raw sequencing data, > 120,000 and > 45,000 16S rRNA reads were obtained for Bacteria and Archaea, respectively, for a total of 8 activated sludge samples analyzed. The number of reads per sample ranged between 7864-28740 for Bacteria and 580-14,642 for Archaea, showing differences of the sequencing depth. A total of 1072 Bacteria PHYs and 89 Archaea PHYs were detected

at 97% sequences identity, ranging between 481-661 and 23-61 PHYs per sample, respectively. The R/E curves (Fig. S2) and the values of Good's coverage (>98%, Table S7) indicated that the achieved sequencing depth sufficed to properly describe the diversity of the bacterial and archaeal communities in the A²O system. The average values of S, Chao-1, H' and Simpson's indices were very similar in both experimental phases for either Bacteria and Archaea. The indices described bacterial communities displaying high species diversity, while in the case of Archaea the diversity was low-to-medium. Both Bacteria and Archaea communities had a low functional organization, according to the average values of the Simpson's index (Table S7).

The diversity of Fungi in the A₂O system was only investigated by Illumina sequencing in samples of phase I, since this group of microorganisms was not detectable in samples of phase II. A total of 4,922 quality filtered fungal 18S rRNA sequences were obtained from 4 samples. One hundred Fungi PHYs were detected at 97% sequences identity, ranging between 28-74 PHYs per sample. The R/E curves (Fig. S2) and the value of Good's coverage ($\geq 96\%$ Table S7) indicated enough sequencing depth for the description of fungal diversity in the A²O samples. The community structure of Fungi was characterized for a low-to-medium species diversity and a low functional organization, according to the average values of S, Chao-1, H' and Simpson's indices (Table S7).

3.3.1. Structure and diversity of the Bacteria communities in the A²O system

The dendrogram in Fig. S3 shows the clustering of the A₂O samples (according to the Bray-Curtis coefficient), based on the relative abundance of bacterial PHYs detected by Illumina sequencing. The global similarity among samples in phases I and II was >

40% . Significant differences were also detected between the experimental phases by ANOSIM analysis ($R = 0.84$).

A total of 1,072 PHYs with a relative abundance $> 0.1\%$ in at least one sample were classified in 27 different Phyla. The bacterial community was dominated by *Proteobacteria*, which displayed the highest average relative abundance in the sludge samples of both phases (60.4 and 64.7%; phase I and II, respectively), followed by *Bacteroidetes* (23.1 and 15.04 %) and *Chloroflexi* (7.7 and 11.4 %) (Fig. 1A-1). Interestingly, unique Phyla were detected in both experimental phases, although at very low relative abundances. Phylotypes affiliated to Phyla TM6 (0.0065%) and OD1 (0.0020 %) were only detected in phase I, while members of the Phyla GOUTA4 (0.0031%), *Gemmatimonadetes* (0.0036 %) and NKB19 (0.0036 %) were exclusive of phase II. Significant differences of the community composition among the two experimental phases were also detected at the Order and Family levels (Fig. 1A-2, A-3).

In order to evaluate which PHYs contributed most to the (dis)similarity between samples of phases I and II, SIMPER analyses were carried out (Table S8). The overall similarity among samples was 61.48% in phase I and 71.09% in phase II. Three PHYs affiliated to the *Comamonadaceae*, *Cytophagaceae*, and *Xanthomonadaceae* (PHYs 962, 406, and 630, respectively) and two PHYs phylogenetic related to *Caldilineaceae* (PHYs 118 and 713) cumulatively contributed $> 35\%$ to explain the similarity between phase I samples (Table S8A), while the similarity among phase II samples was explained ($> 35\%$) by PHYs 118, 406 and 962 (Table S8B). SIMPER analysis also showed that PHYs 406 and 962 (Table S8C) were the major contributors to the 47.93% dissimilarity observed between the two experimental phases, cumulative explaining $> 18\%$ of the differences.

Proteobacteria, *Bacteroidetes* and *Chloroflexi* have been described as the predominant bacterial groups in aerobic activated sludge of WWTPs, either based on conventional or advanced technologies (reviewed by Ferrera and Sánchez, 2016; Xia et al., 2018). Of these, *Proteobacteria* are most often found as the prevalent group, particularly in municipal WWTPs (Cyzdik-Kwiatkowska and Zielińska, 2016). Among these, members of the *Comamonadaceae* are regarded as ubiquitous in activated sludge, where they fulfil a variety of functions such as nitrate reduction (Sadaie et al., 2007), biological phosphorus removal (Ge et al., 2015) and biodegradation of complex substrates (Khan et al., 2002).

3.3.2. Structure and diversity of Archaea communities in the A²O system

Fig. S3 displays the Bray-Curtis based dendrogram showing the (dis)similarities between the A²O samples of phases I and II, according to the relative abundance of Archaea PHYs detected by Illumina sequencing. Samples of each experimental phase clustered together in separate groups with a similarity < 20%, while the similarity of the samples within each experimental phase was > 40%. ANOSIM analysis also corroborated the significant differences between experimental phases (R= 0.188).

Fig. 1B (B-1, B-2 and B-3) displays the heatmaps showing the relative abundances of archaeal PHYs in the two experimental phases, at Phylum, Order and Genus level, respectively. *Euryarchaeota* was the dominant Phylum (98.2 and 92.3% average relative abundances, phases I and II, respectively), followed by *Thaumarchaeota* (1.2 and 6.7%) and *Crenarchaeota* (0.1 and 0.4%). Significant differences of the community diversity were also detected among phases I and II at Order and Genus levels (Fig. 1B-2 and B-3). *Methanomicrobiales* were prevalent in phase I (average relative abundance 45.4%), while *Methanobacteriales* dominated in

phase II (average relative abundance 49.8%). At the Genus level, phase I was dominated by PHYs closely related to *Methanolinea* (*Methanomicrobiales*) (28.9% average relative abundance), while *Methanobrevibacter* and *Methanobacterium* (*Methanobacteriales*) were prevalent in samples of phase II (21.8 and 21.2 % average relative abundance, respectively) (Fig. 1B-3).

According to SIMPER analysis (Table S9), the global similarity of the Archaea communities was 61.95% in phase I samples and 48.7% in phase II samples. Three PHYs (PHYs 87, 130 and 29, which were closely related to genera *Methanolinea*, *Methanobrevibacter* and *Methanobacterium*, respectively) contributed 60.8% to explain the similarity between samples of phase I, while PHYs 29, 130, 41 and 87 (closely related to *Methanobacterium*, *Methanobrevibacter*, *Methanomethylovorans* and *Methanolinea*, respectively) contributed 65.06% to the similarity of phase II samples. SIMPER also corroborated the low similarity of the archaeal communities observed between the two experimental phases (47.31% average dissimilarity), with a single PHY (PHY 87, affiliated to *Methanolinea*) contributing a 16.34% explanation to such differences.

Methanobacteriales, *Methanomicrobiales* and *Methanosarcinales* are commonly detected in urban wastewater treatments based on different technologies, including aerated activated sludge systems (Ferrera and Sánchez, 2016). The A²O technology is characterized by aerobic, anoxic and anaerobic zones in series, subjected to internal recirculation. Under such conditions, usually no significant differences of the composition of the bacterial communities are found among the bioreactors with different redox regimes (Gómez-Silván et al., 2014; Kim et al., 2013; Phan et al., 2016). Methanogenic archaea present in the anaerobic zones under optimal conditions to

develop their metabolism enter the aerated zones by recirculation, where their contribution to carbon and nitrogen removal is controversial, although some studies supported their role as enhancers of some biological activities through their symbiotic relationships with Bacteria (Ferrera and Sánchez, 2016).

3.3.3. Structure and diversity of Fungi communities in the A²O system

The dendrogram in Fig. S3 illustrates the clustering of samples (Bray Curtis) according to the relative abundance of the fungal PHYs in the A²O samples from phase I, showing a low similarity (< 20%) among them. PHYs classified in 7 different Phyla/Subphyla were identified. *Ascomycota* was the dominant Phylum (73.4% average relative abundance), followed by *Basidiomycota* (16.2%), *Mortierellomycotina* (*Mucoromycota*, Tedersoo et al., 2018) (4.7%), *Blastocladiomycota* (3.3%), *Zoopagomycota* (1.5%), *Mucoromycotina* (*Mucoromycota*, Tedersoo et al., 2018) (0.63%) and *Chytridiomycota* (0.09%) (Fig. 1C-1). At the Order level, 34 different taxa were detected, dominated by *Ascomycota* of the *Saccharomycetales*, *Eurotiales* and *Hypocreales*, and *Basidiomycota* of the *Trichosporonales* and *Filobasidiales* (26.7, 17.2, 15.7, 7.8 and 5.7% average relative abundance, respectively) (Fig. 1C-2). SIMPER analysis (Table S10) showed that the global similarity between samples was 39.3%, where only 5 PHYs affiliated to the *Hypocreales*, *Trichosporonales*, *Saccharomycetales* and *Eurotiales* were enough to explain > 55% of such similarity.

The results presented here revealed a higher diversity and relative abundance of *Ascomycota* compared to *Basidiomycota* in the A²O system, and in most samples, phylotypes closely related to the *Saccharomycetes* displayed the highest relative abundances within the fungal community (Fig. 1C), in agreement with the knowledge of the diversity of fungi in WWTPs (Maza-Márquez et al., 2016). *Ascomycota* are an

extremely diverse group of fungi, accounting for > 65% of these eukaryotic microorganisms in both terrestrial and aquatic environments (Harms et al., 2011). Several species have been described able to biotransform PhACs, including AIAPs, antibiotics, or β -blockers (Olicón-Hernández et al., 2017).

3.4. Linking the relative abundance of Bacteria and Archaea to the environmental/operational variables of the A²O system

In Fig. 2 (A, B), the activated sludge samples retrieved from the A²O system were ordinated by MDS on the basis of the relative abundance of the populations of Bacteria and Archaea identified by Illumina-sequencing. Fungi were not included in these analyses, since they were only detected in samples of phase I. The vectors on the plots representing the abiotic variables displayed two clear trends throughout the ordinations, in agreement with the different operating conditions characterizing each of the two experimental phases (higher average OT and F/M in phase I, and higher concentrations of COD and BOD₅ in the influent water, higher biomass accumulation (MLSS) and sludge volumetric index (SVI) in phase II) and the increased removal efficiencies of organic matter and nutrients (ORR, NRR, PRR) registered during phase II. These changes in the operational and environmental parameters were strongly related to the shifts of the structure of the microbial communities in the A²O system (Fig. 2), as reported previously for WWTPs by several studies (Ju and Zhang, 2015; Kim et al., 2013; Reboleiro-Rivas et al., 2016).

According to BIO-ENV analysis, among all the tested variables, MLSS, SRT, COD, BOD₅ and NH₄⁺ were those contributing the highest level of explanation (89.1%) to the ordination of samples based on the bacterial community structure (Fig. 2A). The Pearson product-moment correlation coefficients among the vectors in Fig. 2A (Table

S11A) showed that MLSS, COD, BOD₅ and NH₄⁺ were positively correlated ($r \geq 0.70$) to the relative abundance of families of the *Alphaproteobacteria* (*Hyphomicrobiaceae*, *Rhodobacteraceae*, *Sphingomonadaceae*, unclassified *Rhizobiales*, and unclassified *Sphingomonadales*), *Betaproteobacteria* (*Comamonadaceae*, *Rhodocyclaceae*, and unclassified *Thiobacterales*) and *Chloroflexi* (*Caldilineaceae*). In contrast, the relative abundances of PHYs related to the *Bacteroidetes/Chlorobi* (*Cytophagaceae*, *Saprospiraceae*, Unclassified *Chlorobi*, and Unclassified *Sphingobacteriales*) and *Gammaproteobacteria* (*Chromaticeae* and *Xanthomonadaceae*) showed opposed trends throughout the samples' ordination.

Some of the responses of the bacterial groups to the changes of the operational parameters in the A²O were in agreement with those observed in previous studies. The increase of MLSS in phase II contributed to higher relative abundances of *Caldilineaceae* and *Chloroflexi* populations (Fig. 2A and Table S11A), accordingly to the previous observations in a moving-bed membrane bioreactor (MBMBR) treating municipal sewage (Reboleiro-Rivas et al., 2016). Muszyński et al. (2015) analyzed the bacterial diversity in an A²O system by FISH probing, reporting the positive correlation of the relative abundances of both *Alpha*- and *Betaproteobacteria* with COD and total N of the influent wastewater and their negative correlation with OT, while *Bacteroidetes* of the *Saprospiraceae* followed the opposite trend.

Regarding the relative abundances of Archaea populations at the Genus level, BIO-ENV analysis detected that NH₄⁺ concentration in the activated sludge was the parameter contributing the best explanation to the ordination of samples (46.5%). These results indicate an effect of NH₄⁺ concentration on the succession of both hydrogenotrophic and methylotrophic/acetoclastic methanogens. Strong positive

correlations of this abiotic factor ($r > 0.65$) were observed with the relative abundances of most PHYs of the *Methanobacteriales* identified at the genus level, while negative or negligible correlations were observed for the genera of the *Methanomicrobiales* (with the exception of *Methanoregula*). Higher NH_4^+ concentrations also favored the dominance of *Methanomethylovorans* and *Methanosarcina* versus *Methanosaeta* (Fig. 2B and Table S11B).

Ammonia concentration is regarded as an important parameter for the shaping of the methanogenic communities. NH_4^+ is required as the major N source for most methanogens (Garcia et al., 2000), but at the same time ammonia ions and free ammonia can inhibit methanogenesis (Yenigün and Demirel, 2013). The effect of ammonia on population dynamics and the activity of methanogens has been thoroughly described in anaerobic digesters at much higher average concentrations (usually $>1 \text{ g L}^{-1}$) than those measured in the A^2O system investigated here; however, very similar patterns of community succession in response to ammonia were observed. Previous studies concluded that hydrogenotrophic methanogens are more tolerant to ammonia levels than acetotrophic methanogens, and the obligate acetoclastic *Methanosaeta* are the most sensitive to ammonia (Karakashev et al., 2005; Yenigün and Demirel, 2013). In this regard, *Methanosaetaceae* dominated in sewage sludge digesters operating at low ammonia levels ($0.03\text{-}0.3 \text{ g N-NH}_4^+ \text{ L}^{-1}$), while *Methanosarcinaceae* were prevalent in cattle manure digesters with concentrations of $\text{N-NH}_4^+ >1 \text{ g L}^{-1}$ (Karakashev et al., 2005). Among the hydrogenotrophic methanogens, *Methanobacteriales* increased their abundance versus *Methanomicrobiales* at higher NH_4^+ loading rates in a laboratory-scale two-stage anaerobic digester operated in the range of $0.09\text{-}1.16 \text{ g NH}_4^+ \text{ L}^{-1} \text{ day}$ (Kim et al., 2014).

Nitrososphaera is an ammonia oxidizing archaea (AOA) described in either water or soil environments (Ma et al., 2018; Mosier et al., 2012; Park et al., 2012), and is the AOA most often identified in WWTPs (Limpiyakorn et al., 2013). In that respect, the average relative abundance of *Nitrososphaera* increased from 1.05 % in phase I to 6.74 % in phase II, and strong positive correlations ($r > 0.65$) were found between the abundance of these AOA and NH_4^+ , TN and NRR (Table S11B). Ammonia-oxidizing bacteria (AOB) have been classically considered the key organisms responsible of nitrification in WWTPs (Yin et al., 2018); however, in the last decades it has been established that AOA of the *Thaumarchaeota* often coexist with AOB in these engineered systems (Limpiyakorn et al., 2013), within an ample range of ammonia concentrations (Yin et al., 2018), and their effective contribution to ammonia oxidation has been recently demonstrated in nitrifying reactors (Roy et al., 2017; Srithep et al., 2018). The results of the present study show that the relative abundance of *Nitrososphaera*-related phylotypes among Archaea was favored by increasing concentrations of ammonia in the A^2O influent, and correlated concomitantly with improved NRR, supporting the relevance of their role in ammonia oxidation. Similar correlation trends were previously found for *Nitrososphaera*-related AOA by Gao et al. (2014).

3.5. Linking population dynamics of Bacteria and Archaea to the REs of PhACs in the A^2O system

MDS and BIO-ENV analyses were used in search of links among the observed changes of the bacterial and archaeal communities and the REs of PhAC in the A^2O system. Fungi were not included in these analyses, since they were only detected in samples of phase I. Besides the data of 7 of the PhACs described in this study (Table 4), the REs of

4 AIAPs (acetaminophen, ibuprofen, naproxen, and ketoprofen) which were previously reported in the same set of samples (Gallardo-Altamirano et al., 2018) were also included. The samples' ordination plots are shown in Fig. 3.

BIO-ENV analysis revealed strong correlations between the removal of PhACs of different pharmaceutical families (AIAPs, antibiotic, β -blockers, diuretics and lipid-regulator) and the relative abundances of particular bacterial and archaeal populations (Fig. 3 and Table S12). Overall, the trends of the vectors on the plot reflects that the REs of most of the analyzed PhACs were higher in experimental phase II, with the exception of hydrochlorothiazide. BIO-ENV analyses showed that the REs of the PhACs were positively correlated with the increased relative abundance of a core of different bacterial families during phase II (Fig. 3A). Higher relative abundances of *Rhodobacteraceae* and *Sphingomonadaceae* (*Alphaproteobacteria*) were strongly correlated ($r > 0.75$) with increased REs of clarithromycin, furosemide, bezafibrate and gemfibrozil, while *Betaproteobacteria* (*Comamonadaceae*, *Rhodocyclaceae*, unclassified *Betaproteobacteria* and unclassified *Thiobacterales*) and *Chloroflexi* (*Caldilineaceae*) correlated positively ($r = 0.75-1.0$) with the REs of up to seven PhACs belonging to different therapeutic groups: AIAPs (ibuprofen and naproxen), antibiotics (clarithromycin), diuretics (furosemide), β -blockers (atenolol) and lipid-regulators (bezafibrate, gemfibrozil) (Table S12A). The relative abundances of phylotypes of the *Chitinophagaceae* and *Flavobacteriaceae* (*Bacteroidetes/Chlorobi*) were also correlated to higher REs of ibuprofen and naproxen, while *Gammaprotobacteria* and *Cytophagaceae* abundances displayed a very high positive correlation with the REs of hydrochlorothiazide ($r > 0.9$), the only PhAC which was removed with a significantly higher efficiency under the operating conditions of phase I (Table S12A). Finally, the

REs of acetaminophen were strongly and positively correlated ($r > 0.9$) to the relative abundance of unclassified *Myxococcales* (*Deltaproteobacteria*) and some groups of the *Bacteroidetes/Chlorobi* (Fig 3, Table S12A).

Regarding the archaeal population, MDS and BIO-ENV detected strong correlations ($r \geq 0.60$) between the REs of different AIAPs (acetaminophen, ibuprofen, ketoprofen and naproxen), antibiotics (clarithromycin), β -blockers (atenolol) and lipid-regulators (bezafibrate and gemfibrozil) and the relative abundances of *Methanobrevibacter*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, and unclassified *Methanomicrobiales*. The relative abundance of *Methanomethylovorans* was positively correlated ($r \geq 0.60$) with the REs of ibuprofen, naproxen, clarithromycin, atenolol, furosemide, bezafibrate and gemfibrozil (Table S12B).

The performance of biological wastewater treatment systems for PhACs removal has been thoroughly investigated in systems based on different technologies, but only a few studies provided significant links among their REs and the occurrence/abundance of particular microbial groups, most of which were conducted in laboratory-scale systems using synthetic wastewater spiked with fixed amounts of PhACs, and often using concentrations well over those occurring in real wastewaters. In spite of this differences, the results presented here are consistent with those of previous work.

Alphaproteobacteria and *Betaproteobacteria* have been often linked to the biological removal of PhACs in WWTPs based on different technologies. The relative abundances of populations classified within the *Sphingomonadaceae* were earlier found positively correlated with the REs of the antibiotic trimethoprim in an autotrophic nitrogen removal CANON bioreactor (Rodriguez-Sanchez et al., 2017), and two genera of the *Sphingomonadaceae* (*Sphingorhabdus* and *Novosphingobium*) were pointed out as

indicators of conditions favorable for the removal of bezafibrate in both a full-scale and a pilot-scale WWTP (Wolff et al., 2018). The enrichment in the activated sludge communities of phylotypes related to several genera of the *Comamonadaceae* and *Rhodocyclaceae* was previously observed to occur concomitantly with the removal of bezafibrate and clarithromycin (Wolff et al., 2018), ibuprofen (Li et al., 2016), and trimethoprim (Rodriguez-Sanchez et al., 2017). Isolates taxonomically classified within the *Comamonadaceae* and *Sphingomonadaceae* are also commonly reported able to metabolize/cometabolize different kinds of PhACs under laboratory conditions, either in pure cultures or as part of consortia (Fortunato et al., 2016; Wang and Wang, 2016).

Regarding the links found among the REs of PhACs and other bacterial groups, an increase in the relative abundance of *Flavobacteriaceae* (*Bacteroidetes*) was observed in a pilot-scale MBR fed with synthetic hospital wastewater following the addition of 10 $\mu\text{g L}^{-1}$ ibuprofen (Tiwari et al., 2019), and populations of this family have been as well associated with the metabolic or cometabolic degradation of ibuprofen in constructed wetlands (Li et al., 2016). Enrichment of *Bacteroidetes* and *Chloroflexi* in an aerated SBR fed with synthetic wastewater amended with a mixture of diclofenac, ibuprofen and naproxen (5 $\mu\text{g L}^{-1}$ each) was reported by Jiang et al. (2017); however, these authors found that the relative abundance of *Proteobacteria* was at the same time lowered down under the conditions of their study, contrary to the results presented here. Phan et al. (2016) assayed an MBR operated under different sets of conditions for the removal of 30 trace organic contaminants (including gemfibrozil, ibuprofen, ketoprofen, and naproxen) at a concentration of 5 $\mu\text{g L}^{-1}$, reporting that *Proteobacteria* increased their relative abundance in the presence of the trace organic contaminants, while *Bacteroidetes* (*Cytophagaceae*) mostly thrived in their absence, and concluding that

Betaproteobacteria, *Caldilineaceae* and *Myxococcales* were more abundant among the activated sludge community under the operating conditions allowing the effective removal of the micropollutants.

The role of Archaea in PhACs removal in WWTPs has been even more scarcely investigated. Methanogenic archaea are regarded a key component in the biodegradation of pollutants such as hydrocarbons in soil and water, where they thermodynamically favor the degradative processes by acting as a sink of the terminal products H₂ and acetate (Krzmarzick et al., 2018). The acetoclastic genus *Methanosaeta* significantly increased its abundance in diesel-contaminated versus non-contaminated soils (Krzmarzick et al., 2018) and also dominated the community in a WWTP treating industrial wastewater containing azo-dyes (Plumb et al., 2001). *Methanomethylovorans*, an obligately methylotrophic methanogen able to use methanol, methanethiol, methylamines and dimethyl sulfide as substrates (Lomans et al., 1999), has been described as the dominant methanogenic archaea in WWTPs treating high concentrations of antibiotics, pointing at their implication together with sulfate reducing prokaryotes in the latter steps of biodegradation of these compounds under anaerobic conditions (Deng et al., 2012). A similar role of *Methanomethylovorans* in the anaerobic degradation of dyes with sulfonate groups in industrial wastewater was earlier proposed (Plumb et al., 2001).

The relative abundance of *Nitrososphaera*-related phylotypes within the archaeal community of the A²O displayed positive correlations with the REs of furosemide, bezafibrate and gemfibrozil (Fig. 3, Table S12). It was previously shown that the removal of these three PhACs, among others, was significantly hampered following chemical inhibition of ammonia monooxygenase activity in bench-scale MBRs and

anthracite-packed biofilters (Maeng et al., 2013; Rattier et al., 2014). In the literature, the involvement of nitrifiers in the removal of trace organic contaminants from wastewater has been mostly attributed to AOB (Kumwimba and Meng, 2019); however, in a survey of activated sludge samples from 10 full-scale WWTPs, Helbling et al. (2012) concluded that both ammonia oxidation and the removal of PhACs were correlated to the abundance of archaeal, rather than bacterial, *amoA* transcripts.

4. Conclusion

The findings presented here provide new insights on the correlations among plant operation parameters and the assemblage of the activated sludge microbial communities, regarding how these interlinkages influence the removal of PhACs in WWTPs. Robust links were revealed between improved REs of several of the analyzed compounds (acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromycin, trimethoprim, atenolol, furosemide, hydrochlorothiazide, bezafibrate, and gemfibrozil) and the shifts of the relative abundances of different families of *Bacteria* (*Rhodobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae*, *Rhodocyclaceae*, *Caldilineaceae*, *Chitinophagaceae* and *Flavobacteriaceae*) and genera of *Archaea* (*Methanobrevibacter*, *Methanoregula*, *Methanosaeta*, *Methanosphaera*, *Methanomethylovorans* and *Nitrososphaera*). Remarkably, the REs of clarithromycin, bezafibrate and gemfibrozil were improved over twofold by the combination of an average MLSS concentration of *ca.* 5 g L⁻¹ with a F/M ratio of 0.21 kg BOD₅ kg MLVSS⁻¹ d⁻¹. These operational parameters are easy-to-control and their adjustment feasible in seek for an improvement of the removal of PhACs in WWTPs.

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Figure Legends

Fig. 1. Heatmaps showing the relative abundances of Bacteria (A), Archaea (B) and Fungi (C) detected by Illumina sequencing in the A²O system operated under different conditions in experimental phases I and II. A1, B1, C1. Classification at Phylum level. A2. Classification at Family level. B2, C2. Classification at Order level. B3. Classification at Genus level. Taxa were excluded from the heatmaps according to the following cut-offs of average relative abundance considering both experimental phases: Bacteria: < 0.5, < 0.6, and < 0.9 % for Phylum, Order and Family, respectively; Archaea: < 0.04 for both Family and Genus; Fungi: < 0.4 for Order.

Fig. 2. Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the samples retrieved from the A²O operated under different experimental conditions I and II, according to the relative similarity of Bacteria (A) and Archaea (B) analyzed by Illumina sequencing. In the right column, the MDS plots represent the direction throughout the ordination of the relative abundances of Bacteria, Archaea and Fungi phylotypes, while in the left column, MDS plots represent the abiotic variables measured in the A²O system. BOD₅: biological oxygen demand at 5 days; COD: chemical oxygen demand; F/M: food-to-microorganism ratio; MLSS: mixed suspended solids; OT: operational temperature; TN: Total N; SRT: sludge retention time; SVI: sludge volumetric index. The variables which best explained the distributions of the biotic data according to BIO-ENV analysis are marked with an asterisk (*). Only vectors with length >0.2 are shown.

Fig. 3. A Non-metric multidimensional scaling (MDS) plots, illustrating the ordinations of the activated sludge samples from the A²O system in the experimental phases I and II, according to the relative similarity of Bacteria (A) and Archaea(B) communities

1099 analyzed by Illumina sequencing. In the right column, the MDS plots represent the
1100 direction throughout the ordination of the relative abundances of the Bacteria and
1101 Archaea phylotypes, while in the left column, MDS plots represent removal rates
1102 (REs%) of acetaminophen, ibuprofen, ketoprofen, naproxen, clarithromycin,
1103 trimethoprim, atenolol, furosemide, hydrochlorothiazide, bezafibrate and gemfibrozil.
1104 Only vectors with length >0.2 are shown.

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Figure 3
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Table 1. Concentration range, mean concentration, median concentration, relative standard deviation (RSD) and detection frequencies of pharmaceutically active compounds (PhACs) in the influent wastewater samples of the A²O system in the experimental phases I (n=4) and II (n=5). The Kruskal Wallis test (*p* < 0.05) was used for statistical analyses. The concentrations of those PhACs marked with an asterisk (*) were significantly different between the two phases. BLD: below the limit of detection.

Therapeutic groups	Compounds	Influent Water (ng L ⁻¹)									
		Phase I (n=4)					Phase II (n=5)				
		Range	Mean	Median	RSD (%)	Freq. (%)	Range	Mean	Median	RSD (%)	Freq. (%)
Antibiotic	Clarithromycin	88 - 859	447	421	82	100	443- 1100	784	881	40	100
Antibiotic	Ofloxacin	1290 - 5710	2610	1720	80	100	1980- 119000	48494	2280	131	100
Antibiotic	Sulfadiazine	BLD - 41	16	11	111	75	BLD - 7	1	0	197	40
Antibiotic	Sulfamethazine	BLD	0	0	0	0	BLD - 6	1	0	209	40
Antibiotic	Sulfamethoxazole	BLD - 873	218	0	200	25	BLD - 592	216	0	138	40
Antibiotic	Trimethoprim	48 - 132	92	93	39	100	75- 164	119	118	30	100
Beta-blocker	Atenolol	279 - 1100	635	581	57	100	378- 905	611	611	35	100
Beta-blocker	Metoprolol	BLD	0	0	0	0	BLD- 17	7	0	137	40
Beta-blocker	Propranolol	BLD - 426	136	59	145	75	1 - 87	35	5	124	100
Beta-blocker	Sotalol	25 - 68	41	36	50	100	27- 46	38	39	21	100
Diuretic	Furosemide	2290 - 4720	3215	2925	33	100	1330- 4090	2792	3210	42	100
Diuretic	Hydrochlorothiazide	2400 - 3630	2790	2565	21	100	1870- 3320	2640	2610	20	100
Lipid-regulator	Bezafibrate	79 - 181	127	125	41	100	96- 223	139	121	35	100
Lipid-regulator	Fenofibrate	BLD	0	0	0	0	BLD	0	0	0	0
Lipid-regulator	Gemfibrozil	1180 - 2330	1625	1495	33	100	1080- 2160	1426	1300	31	100
Psychiatric	Carbamazepine *	117 - 248	154	126	41	100	78- 123	98	88	20	100
Psychiatric	Diazepam	BLD - 2	1	0	200	25	BLD - 1	0	0	162	40
Psychiatric	Lorazepam	183 - 364	241	209	34	100	136- 245	182	174	26	100
Psychiatric	Paroxetine	BLD - 11	5	4	120	50	BLD - 11	2	0	224	20

Table 2. Concentration range, mean concentration, median concentration, relative standard deviation (RSD) and detection frequencies of pharmaceutically active compounds (PhACs) in the effluent wastewater samples of the A²O system in the experimental phases I (n=4) and II (n=5). The Kruskal Wallis test ($p < 0.05$) was used for statistical analyses. The removal of those PhACs marked with an asterisk (*) was significantly different between the two phases. BLD: below the limit of detection. PNEC: predicted no-effect concentrations of the analyzed PhACs, according to the available literature. When the average concentration of a PhAC in the effluent water was higher than its PNEC value, the latter has been boldfaced.

Effluent Water (ng L ⁻¹)												
Therapeutic groups	Compounds	Phase I (n=4)					Phase II (n=5)					PNEC
		Range	Mean	Median	RSD (%)	Freq. (%)	Range	Mean	Median	RSD (%)	Freq. (%)	ng L ⁻¹
Antibiotic	Clarithromycin	117 - 240	168	158	36	100	BLD - 227	55	22	178	60	20 ¹
Antibiotic	Ofloxacin	509 - 5410	2075	1190	108	100	1260 - 128000	51636	1600	133	100	16 ¹
Antibiotic	Sulfadiazine	BLD - 247	64	5	189	50	BLD - 4	1	0	224	20	135 ¹
Antibiotic	Sulfamethazine	BLD	0	0	0	0	BLD	0	0	0	0	5000 ³
Antibiotic	Sulfamethoxazole	BLD - 372	93	0	200	25	0 - 363	115	0	144	40	27 ³
Antibiotic	Trimethoprim	25 - 131	67	56	72	100	83 - 130	105	102	16	100	5.8 ²
Beta-blocker	Atenolol	101 - 558	262	194	78	100	58 - 188	110	107	43	100	20000 ²
Beta-blocker	Metoprolol	BLD	0	0	0	0	BLD - 8	3	0	137	40	100 ²
Beta-blocker	Propranolol	0 - 458	119	9	190	75	BLD - 572	140	9	176	60	50 ²
Beta-blocker	Sotalol	15 - 50	28	24	54	100	37 - 56	43	40	17	100	13000 ²
Diuretic	Furosemide	2170 - 2560	2323	2280	8	100	1340 - 2780	2184	2250	27	100	1000 ²
Diuretic	Hydrochlorothiazide*	755 - 2560	1714	1770	43	100	2110 - 3200	2646	2650	19	100	317000 ²
Lipid-regulator	Bezafibrate*	41 - 104	81	90	34	100	BLD - 54	26	23	75	80	230 ³
Lipid-regulator	Fenofibrate	BLD	0	0	0	0	BLD	0	0	0	0	100 ¹
Lipid-regulator	Gemfibrozil*	781 - 1270	1085	1145	20	100	87 - 680	377	444	65	100	78 ³
Psychiatric	Carbamazepine	85 - 152	127	136	23	100	99 - 148	114	108	18	100	2000 ²
Psychiatric	Diazepam	BLD	0	0	0	0	BLD - 5	1	0	156	40	10 ³
Psychiatric	Lorazepam	185 - 284	216	198	22	100	156 - 245	194	199	19	100	200 ²
Psychiatric	Paroxetine	BLD	0	0	0	0	BLD	0	0	0	0	8800 ²

¹ Verlicchi et al. (2012); ² Orias and Perrodin (2013); ³ Tran et al. (2018)

Table 3. Daily mass load (ML, mg day⁻¹ 1000 inh⁻¹), predicted daily mass load (PL, mg day⁻¹ 1000 inh⁻¹), and PL/ML influent ratios of the twelve pharmaceutically active compounds (PhACs) detected in $\geq 75\%$ of the influent and effluent wastewater samples of the A²O system in experimental phases I and II.

PhACs (by therapeutic group)	ML Phase I		ML Phase II		PL of A ² O System	PL/ML influent ratio	
	Influent	Effluent	Influent	Effluent	Influent	Phase I	Phase II
Antibiotics							
Clarithromycin	128	48	224	16	147	1.15	0.65
Ofloxacin	746	593	13855	14753	1	0.001	0.000
Trimethoprim	26	19	34	30	19	0.72	0.56
Beta-blockers							
Atenolol	182	75	175	32	516	2.84	2.95
Propranolol	39	34	11	12	10	0.26	0.95
Sotalol	12	8	10	40	26	2.18	2.55
Diuretics							
Furosemide	919	664	798	624	192	0.21	0.24
Hydrochlorothiazide	797	490	754	756	185	0.23	0.25
Lipid-regulators							
Bezafibrate	36	23	40	7	36	0.99	0.91
Gemfibrozil	464	310	407	108	410	0.88	1.01
Psychiatric medications							
Carbamazepine	44	36	28	33	29	0.67	1.05
Lorazepam	69	62	52	55	40	0.58	0.77

Table 4. Removal efficiencies (REs, %) of pharmaceutically active compounds (PhACs) and relative standard deviations (RSD) calculated for each compound measured in the A²O system in the experimental phases I (n=4) and II (n=5). The Kruskal Wallis test ($p < 0.05$) was used for statistical analyses. The REs of those PhACs marked by an asterisk (*) was significantly different between the two phases. BLD: below the limit of detection. The compounds with average values $< 20 \text{ ng L}^{-1}$ and a frequency detection $< 50\%$ are not shown.

Therapeutic groups	Compounds	% RE Water									
		Phase I					Phase II				
		Range	Mean	Median	RSD (%)	n	Range	Mean	Median	RSD (%)	n
Antibiotic	Clarithromycin *	-38 - 82	30	37	194	4	74 - 100	93	95	12	5
Antibiotic	Ofloxacin	-319 - 91	-42	31	-448	4	-9 - 45	18	28	133	5
Antibiotic	Trimethoprim	-24 - 56	30	45	121	4	-11 - 29	8	8	216	5
Beta-blocker	Atenolol	-30 - 80	48	71	110	4	69 - 88	81	87	12	5
Beta-blocker	Propranolol	-600 - 100	-90	71	-380	3	-559 - 100	-100	-53	-271	5
Beta-blocker	Sotalol *	-6 - 57	28	31	94	4	-49 - 21	-19	-25	-134	5
Diuretic	Furosemide	-3 - 54	22	19	109	4	-5 - 45	15	13	132	5
Diuretic	Hydrochlorothiazide *	5 - 69	38	39	76	4	-13 - 17	-1	-3	-1202	5
Lipid-regulator	Bezafibrate *	0 - 47	33	43	67	4	61 - 100	80	84	21	5
Lipid-regulator	Gemfibrozil *	13 - 45	31	33	43	4	48 - 92	74	76	23	5
Psychiatric	Carbamazepine	-30 - 66	5	-9	887	4	-28 - -2	-18	-20	-57	5
Psychiatric	Lorazepam	-55 - 49	1	6	3446	4	-19 - 2	-8	-10	-110	5

Supplementary Material

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