Title: Neighbour urban wastewater treatment plants display distinct profiles of bacterial community and antibiotic resistance genes

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ABSTRACT

Urban wastewater treatment plants (UWTPs) are among the major recipients of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs) and antibiotic residues in urban environments. Although during treatment bacteria of human and animal origin are removed, some are able to survive, persisting in the final effluent. The occurrence of these bacteria, especially those harbouring ARGs, may have a direct impact on the quality of the treated wastewater that is returned to the environment. In this study, we aimed to assess if the final effluent bacterial communities of three UWTPs (PT1, PT2, and PT3) located next to each other, were distinct and if such differences were related with the antibiotic resistance profiles.

It was observed that the bacterial community (16S rRNA gene Illumina sequencing) and load of selected ARGs of final effluent differed among the three UWTP, irrespective of sampling time. Members of the families *Aeromonadaceae*, *Campylobacteraceae*, *Veillonellaceae, Weeksellaceae*, and *Porphyromonadaceae* were observed to be positively correlated with some ARGs (*bla*$_{CTX-M}$, *bla*$_{OXA-A}$, *bla*$_{SHV}$) and *intI1* ($p<0.05$), while *Intrasporangiaceae* were observed to be negatively correlated. While *Aeromonadaceae* are recognized relevant ARGs harbours, the other bacterial families may represent bacteria that co-exist with the ARGs hosts, which may belong to minor bacterial groups, omitted in the analyses. These findings suggest the importance of bacterial dynamics during treatment to the ARB&ARGs removal, a rationale that may contribute to design new strategies to apply in the UWTPs to prevent the spread of antibiotic resistance.

KEYWORDS: final effluent; selection; bacterial diversity; antibiotic resistance genes; correlation analyses

1. INTRODUCTION

Urban wastewater treatment plants (UWTPs) have received much attention as major recipients of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs) (Rizzo et al. 2013) and antibiotic residues (Michael et al. 2013) in urban environments. Their capacity to remove ARB and ARGs has also been largely discussed and demonstrated (Manaia et al. 2016; Bengtsson-Palme et al. 2016; Wu et al. 2018). Nevertheless, most authors agree that ARB and ARGs loads released by well-functioning UWTPs may still have a negative impact on the environment, with serious implications to the human health (Berendonk et al. 2015). While it is consensual that it is important to improve wastewater treatment processes in order to maximize the removal of ARB and ARGs, not much is known about the best strategies to achieve such a goal (Berendonk et al. 2015; Manaia et al. 2016; Vikesland et al. 2017). Although wastewater disinfection may promote the reduction of the total microbial loads and of the bacterial diversity, it can create the perfect conditions for the bacterial regrowth of certain groups of bacteria (Manaia et al. 2016; Becerra-Castro et al. 2017; Moreira et al. 2018).

The maintenance or propagation of antibiotic resistance during wastewater treatment may be due to two major driving forces, ARG horizontal gene transfer and/or ARB survival or selection (Berendonk et al. 2015; Vikesland et al. 2017). Despite the importance that horizontal gene transfer may have on ARGs spread, it may be not the most relevant factor to dictate the fate of antibiotic resistance during wastewater treatment (Figueira et al. 2011a; Figueira et al. 2011b; Manaia et al. 2016). The survival or not of specific bacterial lineages during treatment may have consequences on the abundance of ARGs in the final effluent. In this study, we aimed to investigate if ARGs released in the final effluent of a UWTP are related with the diversity of bacteria present in that effluent, at least with some of the groups surviving the wastewater treatment, comprising bacteria frequently associated with humans and animals. In this rationale, it is assumed that the understanding of which bacterial groups are the major vehicles or the ecologic surrogates of ARGs could be a good strategy to identify suitable bacterial indicators of ARGs that survive treatment. This identification could be the basis to manage wastewater treatment processes in order to maximize the reduction or eliminate such bacterial groups. To test the aforementioned hypothesis, we compared the final effluent of three UWTPs, located in the same region (Northern Portugal), and explored whether it was possible to find significant

correlations between the bacterial community composition and the profile of a selected group of ARGs.

2. MATERIALS AND METHODS

2.1. Sampling

Treated wastewater samples were collected in three UWTPs (PT1, PT2, and PT3) located, within a region of approximately 7 km, in Northern Portugal. These UWTPs, serve populations of 150 000 - 170 000 inhabitants equivalent, receive a daily flow of approximately 22 000 m^3 (PT1), 33 000 m^3 (PT2), and 24 000 m^3 (PT3), and all have primary and secondary treatments operating with conventional activated sludge (CAS). PT2 is the only one reporting the reception of hospital effluents and PT3 is the only one with UV disinfection of the final effluent. No information was provided regarding the reception of hospital effluents by PT1 or PT3. Soon after this study, PT1 was temporarily closed for improvement restoration.

Composite samples (24 h) were collected from PT1 and PT2 in four campaigns, in early Spring (average temperature of 9 °C) and early Autumn (average temperature of 17 °C), specifically in three consecutive days (Tuesday - Thursday) in March 2015 (M_2015), October 2015 (O_2015), March 2016 (M_2016), and September 2016 (S_2016). Due to technical problems at PT1, it was not possible to sample this UWTP in March 2016. Grab samples of PT3 were collected in June 2015 (J_2015), July 2015 (JL_2015), September 2015 (S_2015), and March 2016 (M_2016). Samples were stored in ice and transported to the laboratory to be processed within 12 h.

2.2. DNA extraction

Volumes of 100 to 250 mL of final effluent were filtered, in triplicate, through sterile polycarbonate membranes (0.22 µm porosity, Whatman, UK) that were stored at -80 ºC. DNA was extracted from each of the three replicates with the PowerWater® DNA Isolation Kit (MOBIO Laboratories Inc., CA, USA) according to the manufacturer´s instructions. DNA extract´s concentration was measured using the Qubit 3.0 Fluorometer (ThermoFisher

Scientific, USA). A total of 33 DNA extracts, corresponding to 7 independent sampling dates, were stored at -20 °C until further analysis.

2.3. Bacterial community composition

The bacterial community composition was analysed in the 33 DNA extracts, based on MiSeq® Illumina DNA sequencing (Genoinseq, Cantanhede, Portugal) of the V3 - V4 hypervariable region of the 16S rRNA gene using the Bakt_341F 5′ - CCTACGGGNGGCWGCAG - 3′ and Bakt_805R 5 ′- GACTACHVGGGTATCTAATCC - 3’ primers set, according to manufacturer’s instructions (Illumina, San Diego, CA, USA). A filtering based on the reads size and quality trimming was performed using the software PRINSEQ (Schmieder and Edwards 2011). Reads shorter than 200 bp and with average quality scores lower than 25 were excluded. Sequence reads were demultiplexed automatically by the Illumina® Miseq® sequencer using the CASAVA package (Illumina, San Diego, CA, USA) and paired-end reads were merged using a QIIME script. Chimeric sequences were removed using USEARCH v6.1 (Edgar 2010). After the quality control, one of the three replicates of PT3 (September 2015) was removed from the analyses due to the low number of initial reads (n=37 932). Sequences with nucleotide identity higher than 97% were assigned to Operational Taxonomic Units (OTUs) (Edgar 2010) and to taxonomic groups using the Greengenes Database version 13_8 (updated: August 2013) (DeSantis et al. 2006). Singletons, chimeras as well as OTUs assigned to non-bacterial groups, such as chloroplasts or mitochondria, were removed from the data set. After this procedure 3 109 382 good-quality sequences were obtained, grouped into 32 084 OTUs. The downstream analyses were performed after normalization with the cumulative sum scaling (CSS) (Paulson et al. 2014) of the data (except the computation of the alpha diversity metrics). The alpha diversity was evaluated based on bacterial richness (number of OTUs), diversity indices Shannon (Shannon and Weaver 1963), phylogenetic diversity (PD) whole tree (Faith 1992), and Simpson (Simpson 1949), and the richness estimator Chao1 (Chao 1984), using the QIIME 1 pipeline (Caporaso et al. 2010). Alpha diversity was represented as the mean value for each index and the respective standard deviation (SD) values and differences among UWTP effluents calculated by analysis of variance (ANOVA) with post - hoc Turkey’s test ($p < 0.01$). Alpha

rarefaction plot of observed OTUs was performed to verify the sequence coverage. All the plots reached a plateau phase meaning that the final data is a good representation of the bacterial diversity of the wastewater effluent. Beta diversity indices were assessed using the weighted UniFrac metric distances (Lozupone and Knight 2005) from QIIME pipeline (Caporaso et al. 2010) and the results presented as principal coordinates analysis (PCoA). This analysis was also performed by taking into consideration the UWTPs individually in order to assess possible seasonal variations. The relative abundance of the bacterial community composition at different taxonomic levels was compared based on the ANOVA and Tukey-Kramer post-hoc tests ($p < 0.01$) and the p - values were corrected for multiple testing using the Benjamini - Hochberg false discovery rate (FDR) (Benjamini and Hochberg 1995), using STAMP v2.1.3 software (Parks et al. 2014). Pairs of samples with an FDR value < 0.01 were considered significantly different.

The sequence data files that support the findings of this study have been deposited in GenBank within the BioProject PRJNA478220, Sequence Read Archive (SRA) SRS3472441, with the accession numbers SRX4313251-SRX4313282.

2.4. Quantification of antibiotic resistance genes and class 1 integron-integrase

Quantitative polymerase chain reaction (qPCR, StepOne™ Real-Time PCR System, Life Technologies, Carlsbad, CA, USA) was used to measure the abundance (per mL of sample) of seven ARGs (*bla*$_{CTX-M}$, *bla*$_{OXA-A}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *sul*1, *sul*2, and *qnr*S) and class 1 integron - integrase (*intI*1). The 16S rRNA gene was also quantified, as a measure of the total bacterial load, and used to determine ARGs and *intI*1 prevalence values (per 16S rRNA gene copy number). Calibration curves, built based on adequate dilutions of standards of each analysed gene, were produced in parallel with the test samples. Each DNA extract was tested in duplicate for each run. The list of primers and protocols used, based on SYBR Green detection, have been described before (Narciso-da-Rocha et al. 2018). One-way analysis of variance (ANOVA) and Tukey's post-hoc tests were used to assess statistically significant differences ($p < 0.01$) of prevalence and/or abundance of 16S rRNA gene, ARGs or *intI*1 using GraphPad Prism 6.00 software (GraphPad Software, San Diego, CA). Principal component

analysis (PCA) based on the prevalence of ARGs was performed using Canoco 5.01 software (Leps and Smilauer 2014).

2.5. Comparative analyses of bacterial community composition and ARGs and *intI1*

A Redundancy Analysis (RDA) (Wollenberg 1977) was performed to explore possible relationships between the bacterial community structure (families with relative abundance > 1%) and the ARGs and *intI*1 prevalence (target gene copy number / 16S rRNA gene copy number) for all the UWTPs, using Canoco 5.01 software (Leps and Smilauer 2014). The significance of the environmental variables (ARGs and *intI*1) was tested using Monte Carlo permutation tests (999 unrestricted permutations, $p < 0.05$ and $p < 0.01$) after FDR adjustment. The same data set was used to assess Pearson's correlations, testing each UWTP individually, to infer about possible relationships between the bacterial community composition and genes. Pearson's correlation was performed using GraphPad Prism 6.00 software (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Bacterial community

The analysis of the V3 - V4 region of the 16S rRNA gene led to the identification of 32 084 OTUs in the final effluent of the three UWTPs. PT1 showed the highest number of OTUs (7 238 ± 861), followed by PT2 (6 732 ± 1 114) and PT3 (5 704 ± 996). These values were in agreement with the observation that PT3 displayed the lowest Chao1 richness estimator value ($p < 0.01$). However, this difference was not expressed in the Shannon´s and Simpson´s diversity indices, significantly higher in PT3 and PT2 than in PT1 ($p < 0.01$) (Table 1). The whole tree phylogenetic diversity index (PD), which measure the shared phylogenetic distance among taxa in each sample, did not reveal significant differences of the bacterial communities ($p > 0.01$) in the final effluents of the three UWTPs.

The phyla *Proteobacteria* and *Bacteroidetes* predominated in the final effluents of all UWTPs (PT1: 55.2 - 58.8% vs. 21.1 - 25.9%, PT2: 39.1 - 55.0% vs. 13.1 – 14.2%, and PT3: 42.9 - 44.8% vs. 9.4 - 11.3%) (Fig 1). However, the candidate phyla, *TM7* (renamed as

194 Candidatus *Saccharibacteria*) and *OD1* (renamed as Candidatus *Parcubacteria*), made the
195 difference in PT2 and PT3, where they were also among the predominant, with relative
196 abundance values ranging 9.2 - 18.3% and 2.7 - 6.6%, respectively, for PT2, and 5.9 - 12.7%
197 and 8.4 - 14.0%, respectively, for PT3. These phyla were below 3.6% and 3.2%, respectively in
198 PT1. Predominant *Proteobacteria* classes were represented by OTUs affiliated to *Gamma-*
199 (PT1: 9.3 – 11.8%, PT2: 9.3 – 12.5%, and PT3: 7.1 – 10.9%) and *Betaproteobacteria*
200 (PT1: 31.2 – 38.4%, PT2: 16.1 – 26.6% and PT3: 13.6 – 19.7%), in particular of the order
201 *Burkholderiales* (PT1: 23.2 – 30.0%, PT2: 6.2 – 12.6 %, and PT3: 7.4 – 11.0%). *Bacteroidales*
202 was the predominant order of *Bacteroidetes* (PT1: 10.0 – 17.0%, PT2: 3.9 – 6.9% and
203 PT3: 2.5 – 6.7%). It was also noticeable that, at all taxonomic levels, unclassified OTUs were
204 more abundant in PT2 and PT3 than in PT1.
205 Based on the PCoA analysis, the bacterial community observed in the final effluent of the
206 three UWTPs formed different groups, which, according to this analysis, were not influenced
207 by sampling date (Fig. 2a). PT1 displayed the most variable bacterial community composition
208 (Fig. 2a), although PT2 and PT3 were also in separated groups (Fig. 2a). Given the fact that
209 OTUs were defined at a sequence identity value of 97%, it was surprisingly low the number
210 of OTUs shared by all UWTPs, of only 22.0% (Fig. 2b). This number was nevertheless higher
211 than that of unique OTUs, of 18.9%, 16.1%, and 10.8%, in PT1, PT2, and PT3, respectively.
212 Confirming the PCoA (Fig. 2a), PT3 and PT2 shared the highest number of OTUs (37.1%),
213 while PT1 shared 34.3% with PT2 and 26.9% with PT3 (Fig. 2b). In PT1, unique OTUs were
214 mainly *Proteobacteria* (58.4%) and *Bacteroidetes* (30.2%), of the families *Comamonadaceae*
215 and *Flavobacteriaceae*, together with bacteria of the lineage *GZKB119*. The remaining bacterial
216 phyla contributed less than 2.5% for the unique OTUs. In PT2 and PT3 unique OTUs were also
217 mainly *Proteobacteria* (PT2: 43.8%, PT3: 37.8%) and *Bacteroidetes* (PT2: 9.1%, PT3: 7.2%),
218 but other groups were observed, specifically *TM7* (Candidatus *Saccharibacteria*, PT2: 19.6%,
219 PT3: 7.4%) and *OD1* (PT2: 7.7%, PT3: 16.5%). PT3 had also a relevant percentage of unique
220 OTUs identified as *Planctomycetes* (> 5%). In PT2, unique OTUs identified at the family level
221 belonged to *Procabacteriaceae* and *Rhodocyclaceae,* while for PT3 no unique bacterial families
222 stood out.

A more in depth comparative analyses of the bacterial community composition in the final effluent of the three UWTPs considered all families with relative abundance higher than 1% (n=24 families). This comparison showed that PT1 differed from the other two UWTPs by a higher relative abundance ($p < 0.01$) of [*Weeksellaceae*], *Flavobacteriaceae, GZKB119, Porphyromonadaceae, Comamonadaceae*, and *Pseudomonadaceae*, and a lower relative abundance ($p < 0.01$) of *Saprospiraceae* and *Bdellovibrionaceae* (Fig. 3). In PT2, the families *Intrasporangiaceae*, *Chitinophagaceae* and *Procabacteriaceae* were more abundant than in the other two UWTP ($p < 0.01$). PT3 differed from the others UWTPs on a higher relative abundance of *Bdellovibrionaceae* and lower relative abundance of *Sphingomonadaceae* and *Verrucomicrobiaceae* (Fig. 3). Curiously, PT2 and PT3 showed a higher abundance of unclassified families (PT2: 52.6%, PT3: 68.7%) than PT1 (14.7%).

3.2. Antibiotic resistance genes

A set of ARGs (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{OXA-A}$, *bla*$_{SHV}$, *sul*1, *sul*2, and *qnr*S) and class 1 integron-integrase (*intI*1) gene were analysed in terms of abundance and prevalence. The quantification of the 16S rRNA gene is a measure of the bacteria content in all samples and supported the estimation of prevalence values. Similar trends were observed for abundance and prevalence of ARGs and *intI1*, ranked as *bla*$_{CTX-M}$<*bla*$_{SHV}$<*bla*$_{TEM}$<*qnr*S<*sul*2<*bla*$_{OXA-A}$<*intI*1<*sul*1 (Fig. 4). Comparing the different UWTPs, it was observed that PT1 presented significantly higher ($p < 0.01$) abundance and prevalence of *bla*$_{OXA-A}$ and *intI*1 and higher abundance of 16S rRNA gene, *bla*$_{TEM}$, and *qnr*S than PT2 and PT3. In PT3, the gene *bla*$_{SHV}$ was more abundant and *sul*1 more prevalent than in the other plants. PT2 showed a higher prevalence of *bla*$_{SHV}$ and *sul*2 ($p < 0.01$) than the other two UWTPs (Fig. 4a). Despite these significant differences, the average abundance values of ARGs discharged by the different UWTPs differed less than 1.2 log-unit for *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *sul1*, *sul2*, *qnrS,* and *intI1*, being less than 2 log-units for the genes and *bla*$_{OXA-A}$. These results show that the average abundance values of ARGs released by the three UWTPs analysed is very similar, independently of the treatment used, suggesting that this may be associated with the region.

The bacterial community distribution seemed more stable than that of ARGs and *intI1* prevalence values. Even though, the general pattern of distribution was similar, with PT1 clustering apart from PT2 and PT3 (Fig. 4b and Fig. 2a). This observation motivated the search

for possible relationships between the bacterial community composition and structure and the quantified genes in all UWTPs, using a Redundancy Analysis (RDA). According to this analysis, the ARGs and *intI*1 prevalence could explain approximately 84% of the variation observed (Fig. 5). Among the genes showing significant correlation with the bacterial community distribution were bla_{CTX-M}, bla_{OXA-A}, bla_{SHV}, and *intI*1 (Monte Carlo permutations, $p < 0.05$ for bla_{SHV} and *intI*1; $p < 0.01$ for bla_{CTX-M} and bla_{OXA-A}). The genes bla_{OXA-A} and *intI*1 were mainly associated with PT1 samples and members of the families *[Weeksellaceae]*, *Comamonadaceae*, *Flavobacteriaceae*, *GZKB119*, and *Porphyromonadaceae* predominant in that UWTP. In opposition, the genes bla_{CTX-M} and bla_{SHV} were mostly associated with PT2 and PT3 samples and with members of the families *Bdellovibrionaceae*, *Gordoniaceae*, and *Saprospiraceae* (Fig. 5 and Fig. 3).

For a further insight of the multivariate analysis provided by RDA, the comparative analyses presented in Fig. 3 were revisited for inferring about possible statistically significant correlations between specific bacterial families and ARGs and *intI1* prevalence values. This analysis could hint bacterial groups putatively associated with ARGs, either because they harbour some of those genes or because they co-occur with the bacteria that harbour such genes (Ju et al. 2016). The identification of these bacteria is of interest, since being able to survive wastewater treatment, they may contribute to the antibiotic resistance spread. The same analysis could also suggest other groups whose presence might be associated with lower resistance prevalence, in the case of negative correlations. The analysis revealed that members of the families *Aeromonadaceae* and *Campylobacteraceae* (*Proteobacteria*), *Veillonellaceae* (*Firmicutes*), and [*Weeksellaceae]* and *Porphyromonadaceae* (*Bacteroidetes*) were significantly positively correlated with ARGs and *intI1* in different UWTPs. None of these bacterial families was correlated with the *sul1* gene. On the other hand, the *Intrasporangiaceae* were observed to be negatively correlated with most of the ARGs (Fig. 3).

4. DISCUSSION

Some antibiotic resistance features in urban wastewater, such as the most common genes and predominant bacterial phyla seem to follow a general pattern (Gatica et al. 2016; Manaia et al. 2016; Narciso-da-Rocha et al. 2018). However, it is recognized that each UWTP has

283 specificities due not only to biogeographic factors but also to the composition of the influents
284 received, the age of the plant, among others. These factors, may surpass the effect of climate
285 conditions and lead to final effluents with distinct resistance features and community
286 composition. The bacterial community composition may be an important driver in determining
287 the prevalence and patterns of resistance in the final effluent. Indeed, bacteria selection is
288 suggested as an important biological process ruling the fate of ARGs during wastewater
289 treatment, eventually with higher impact on the loads of resistance than horizontal gene transfer
290 (Vaz-Moreira et al. 2014; Bengtsson-Palme et al. 2016; Manaia et al. 2016). In this study, we
291 were interested in comparing the bacterial community and resistance profile in final effluents of
292 three UWTPs. Based on such a comparison we aimed at inferring about possible associations
293 between antibiotic resistance and bacterial community members. Assuming that geographical
294 and socio-economic factors may have a strong effect on the bacterial communities and
295 antibiotic resistance loads in the wastewater effluents, three UWTPs located within a distance of
296 7 km were selected for this comparative study. Interesting differences in these plants are the
297 year of construction (more than 25 years ago for PT1, 15 years for PT2, and 18 years for PT3),
298 the reception of hospital effluents (known only for PT2), and the existence of UV disinfection
299 available at the time of sampling only in PT3. In all UWTPs effluents the predominant bacterial
300 phyla were the same, confirming previous reports that highlight that wastewater samples hold
301 similar bacterial community compositions, at high taxonomic ranks (Munck et al. 2015). As in
302 previous reports, *Proteobacteria* and *Bacteroidetes* were among the major phyla, although in
303 Portugal wastewater samples, *Actinobacteria* are consistently poorly represented (Ye and
304 Zhang 2013; Munck et al. 2015; Binh et al. 2018; Narciso-da-Rocha et al. 2018) (Fig. 1).
305 However, eventually as a result of the history and functioning differences of the three UWTPs,
306 the bacterial community composition clustered into three distinct groups, each represented by a
307 UWTP. Therefore, the current study evidenced that UWTPs located in close regions discharge
308 treated effluents with a distinct profile of taxonomic groups (Fig. 1 and Fig. 2). Indeed, it was
309 observed that in a total of 9 to 12 samples collected in each UWTP, in distinct seasons, with
310 average temperatures 9 °C (early Spring) and 17 °C (early Autumn), the final effluent of each
311 plant fell into the same group, with the effluents of the three UWTPs forming three distinct
312 groups. The UWTPs effect was more notorious than the season effect, for which no significant

differences were observed. This suggests that the microbiota present in the final effluent is somehow characteristic of a given UWTP. This may be related to the previously noted functional stability of wastewater treatment bioreactors, which permit the maintenance of the system, buffering the occurrence of possible perturbations (LaPara et al. 2002).

UWTP PT1 was, among the three analysed, the one with the most distinct bacterial community, with a significantly higher relative abundance of members of the families [*Weeksellaceae*], *Flavobacteriaceae, GZKB119, Porphyromonadaceae, Comamonadaceae*, and *Pseudomonadaceae* (Fig. 2A, Fig. 3). Also, of note was the fact that the phyla *TM7* (Candidatus *Saccharibacteria*) and *OD1* (Candidatus *Parcubacteria*) were more abundant in PT2 and PT3 than in PT1, eventually as a result of the higher bacterial diversity. Members of these phyla have small genomes and reduced metabolic capabilities (Albertsen et al. 2013; Nelson and Stegen 2015), although yielding several genes involved in complex carbon degradation or sulfate reduction (Kantor et al. 2013), which may explain their higher prevalence in the most recent UWTPs (Zhang et al. 2012; Ye and Zhang 2013).

Except for $bla_{\text{OXA-A}}$, with the highest abundance and prevalence values in PT1, the average values of abundance or of prevalence of ARGs and *intI1* did not differ by more than 1.2 log-unit in the different final effluents. This observation suggests that the average loads of ARGs may not differ much in different UWTPs located in the same region. However, the load of ARGs and *intI1* suffered variations over the distinct sampling campaigns, and these were observed to be wider than those registered for the bacterial community composition (Fig. 2A vs. Fig. 4B). Interestingly, the distribution of the relative abundance of bacterial community members and of the ARGs and *intI1* gene prevalence coincided, with the different UWTPs originating distinct groups (Fig. 2A vs. Fig. 4B). This observation seems to confirm the hypothesis that the selection promoted by wastewater treatment, rather than only horizontal gene transfer, may contribute to explain the occurrence of ARGs in the final effluent.

Bacterial families whose prevalence might be correlated with that of ARGs and *intI1* included *Aeromonadaceae* and *Campylobacteraceae* (*Proteobacteria*), and *Veillonellaceae* (*Firmicutes*), whose relative abundance was significantly positively correlated with ARGs prevalence (Fig. 3). Surprisingly, none of these bacterial families was correlated with the *sul1* gene, one of the most widespread genes in the environment. Except for *Aeromonadaceae*, these bacterial groups are

not among the most probable harbours of the analysed ARGs. However, these bacterial lineages may represent bacterial groups that co-exist with the ARGs hosts, in particular because they share similar physiological properties or ecology traits. However, it must be argued that probably groups with relative abundance <1%, whose tracking is difficult with the technique used to characterize the bacterial community, may, eventually, represent the most important carriers of ARGs in wastewater habitats. Further studies based on epicPCR or long-read sequence analyses may bring new insights into this issue (Spencer et al. 2016; Manaia et al. 2018).

5. CONCLUSIONS

The microbiota present in the final effluent is somehow characteristic of a given UWTP and it is not strongly influenced by sampling date or season. In a UWTP, ARGs prevalence presents higher variation than the relative abundance of bacterial families. The average ARGs loads may not differ sharply in different UWTPs located in the same region. And, groups such as *Aeromonadaceae*, *Campylobacteraceae*, *Veillonellaceae, Weeksellaceae*, and *Porphyromonadaceae* were observed to be significantly positively correlated with some ARGs.

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476

Table 1 Range of richness and alpha diversity indices and estimated values (mean value ± SD) for the final effluent of different urban wastewater treatment plants (UWTPs), PT1, PT2 and PT3 analysed over the different sampling campaigns.

UWTPs	Observed OTUs	Richness Estimator	Diversity indices		
		Chao1	Shannon	Simpson	PD whole tree
PT1	5796-7996	9024-12053	7.84-8.85	0.969-0.987	201-259
	7238±861[a]	**10994±1171[a]**	**8.54±0.30[a]**	**0.979±0.008[a]**	**236±22[a]**
PT2	4382-8229	7809-11856	8.57-9.42	0.985-0.991	189-294
	6732±1114[a,b]	**10799±1139[a]**	**8.99±0.27[a,b]**	**0.989±0.002[b]**	**260±30[a]**
PT3	4454-7350	7018-11006	8.74-9.95	0.986-0.996	225-310
	5704±996[b]	**9158±1336[b]**	**9.24±0.48[b]**	**0.991±0.004[b]**	**262±27[a]**

Note: Sample PT3.2 S_2015 was excluded from the analyses due to the low number of reads (n= 37 932). a, b, and c indicate significant differences ($p < 0.01$) between UWTPs.

Figures:

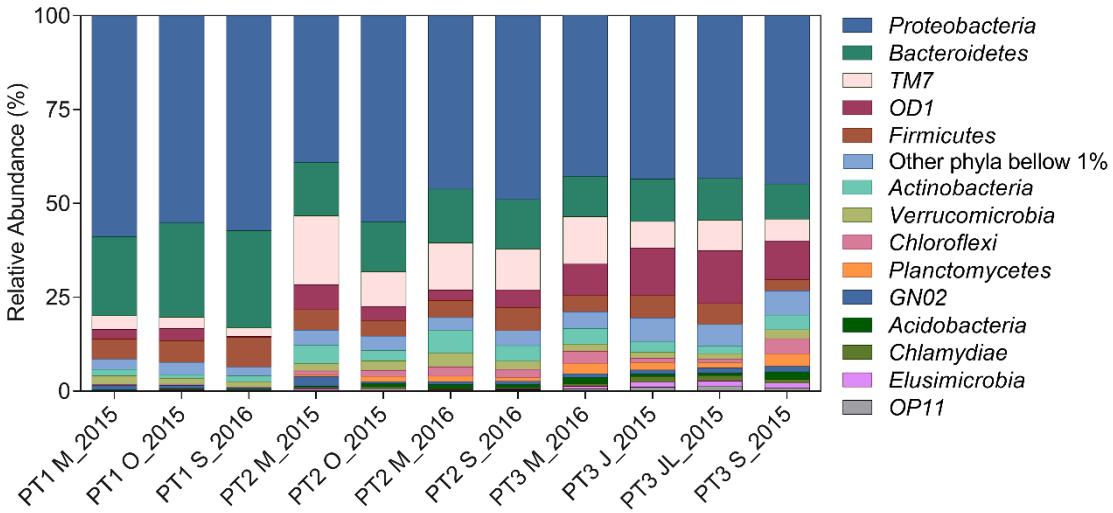
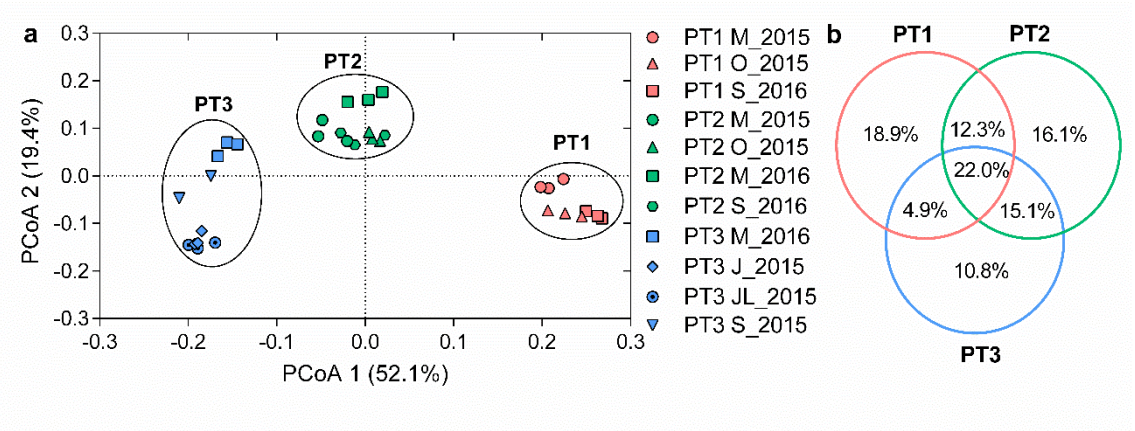


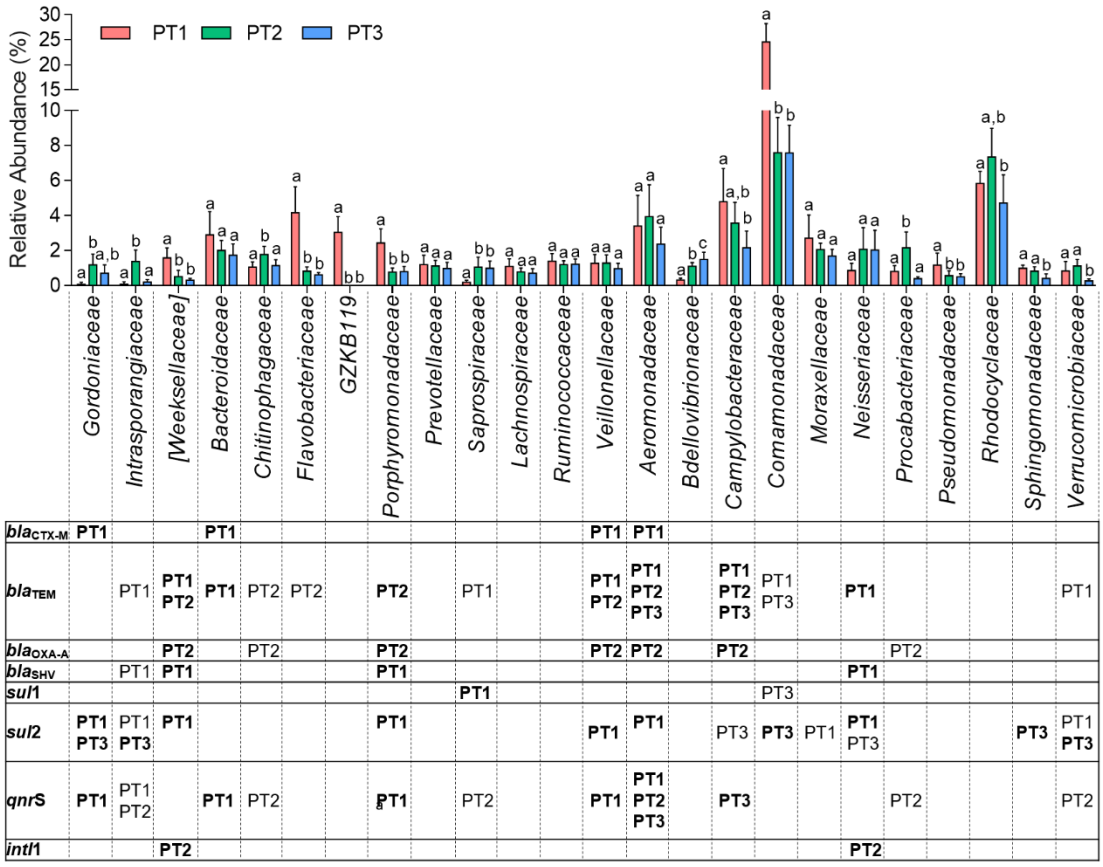
Fig. 1 Relative abundance at bacterial phyla observed in the final effluent of the urban wastewater treatment plants (PT1, PT2 and PT3), in distinct sampling campaigns, each including three consecutive days, in March 2015 (M_2015), October 2015 (O_2015), March 2016 (M_2016), September 2016 (S_2016), June 2015 (J_2015), July 2015 (JL_2015), and September 2015 (S_2015). Taxa with abundance below 1% in all samples were designated as other phyla.



493

494 **Fig. 2** Analysis of the wastewater bacterial diversity. **a)** Principal coordinates analysis (PCoA)
495 based on weighted UniFrac distances between different final effluents of the UWTPs. PT1
496 (pink), PT2 (green), and PT3 (blue), and the respective sampling campaigns: March 2015
497 (M_2015), October 2015 (O_2015), March 2016 (M_2016), September 2016 (S_2016),
498 June 2015 (J_2015), July 2015 (JL_2015), and September 2015 (S_2015). **b)** Venn diagram
499 representation of the percentage of shared and exclusive bacterial OTUs between the different
500 UWTPs.

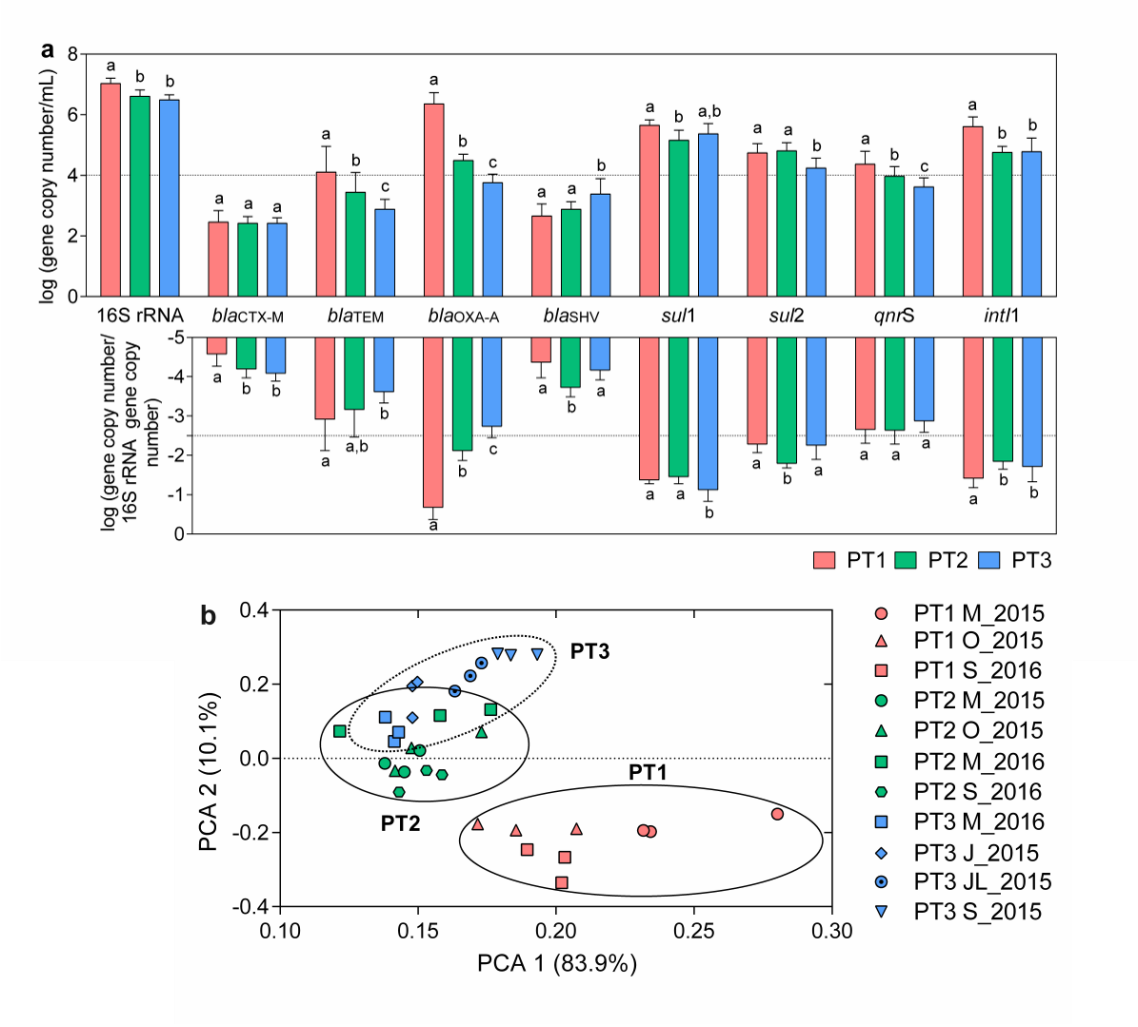
501



502

503 **Fig. 3** Comparison of the relative abundance of families, accounting for more than 1%, identified
504 in the final effluent of the different urban wastewater treatment plants (UWTPs), PT1, PT2, and
505 PT3. The table below the bars represents the Pearson correlation between the relative
506 abundance of a given family in a UWTP and each of the ARGs or *intI*1 gene. a, b, and c indicate
507 significantly ($p < 0.01$) different Tukey-Kramer groups after FDR correction. Positive significant
508 correlations ($\rho > 0.7$; $p < 0.01$) are indicated in bold and negative significant correlations ($\rho < -$
509 0.7; $p<0.01$) are plain text.

510



511

512 **Fig. 4** Quantification of ARGs and *intI*1 in the final effluent of the urban wastewater treatment
513 plants (PT1, PT2, and PT3). **a)** Gene abundance (gene copy number / mL of sample) in the
514 upper part of the figure or prevalence (target gene copy number / 16S rRNA gene copy number)
515 in the bottom. a, b and c indicate significantly ($p < 0.01$) different Tukey´s groups; **b)** Principal
516 component analysis of the distribution of ARGs and *intI1* gene prevalence.

517

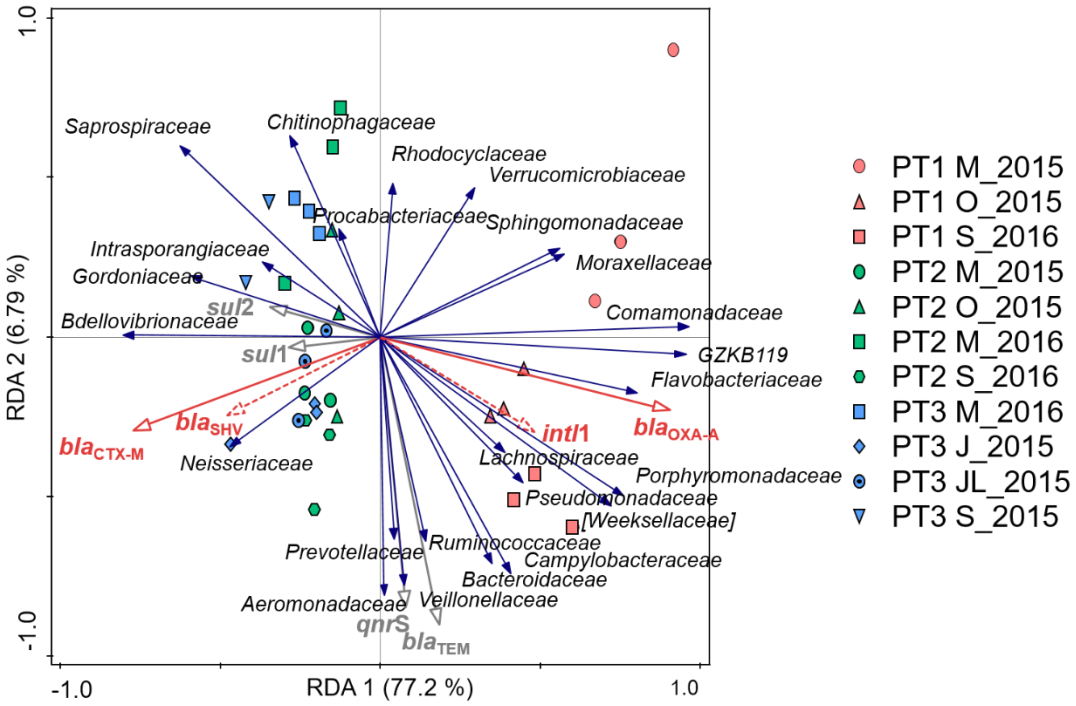


Fig. 5 Redundancy Analysis (RDA) triplot of the bacterial community composition at the family level (relative abundance > 1 %) and environmental variables (ARGs and *intI1* prevalence) in the 32 final effluent samples. Blue arrows indicate the members of the bacterial community. The grey arrows represent environmental variables (ARGs and *intI*1) with no significant correlation, based on the Monte Carlo permutation test after FDR correction. The pointed red and red arrows show the variables with significance lower than 0.05 and 0.01, respectively. PT1 (pink), PT2 (green), and PT3 (blue) regards the UWTPs in distinct sampling campaigns: March 2015 (M_2015), October 2015 (O_2015), March 2016 (M_2016), September 2016 (S_2016), June 2015 (J_2015), July 2015 (JL_2015), and September 2015 (S_2015).