Title: Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant

Authors: Carlos Narciso-da-Rocha[a*](crocha@porto.ucp.pt), Jaqueline Rocha[a*](jrocha@porto.ucp.pt), Ivone Vaz-Moreira[a](ivmoreira@porto.ucp.pt), Felipe Lira[b](felipelira3@gmail.com), Javier Tamames[b](jtamames@cnb.csic.es), Isabel Henriques[c](ihenriques@ua.pt), José Luis Martinez[b](jlmtnez@cnb.csic.es), Célia M. Manaia[a](cmanaia@porto.ucp.pt)

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal

[b] Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Cantoblanco, Madrid, Spain

[c] Biology Department, CESAM, University of Aveiro, Aveiro, Portugal

*, these authors contributed equally for this study.

Corresponding author:

C.M. Manaia, PhD
Escola Superior de Biotecnologia, Universidade Católica Portuguesa
Rua Arquiteto Lobão Vital, 172
4200-374 Porto; Portugal
Tel: +351 22 5580059, Fax: +351 22 5090351
e-mail: cmanaia@porto.ucp.pt

Abstract

Urban wastewater treatment plants (UWTPs) are reservoirs of antibiotic resistance. Wastewater treatment changes the bacterial community and inevitably impacts the fate of antibiotic resistant bacteria and antibiotic resistance genes (ARGs). Some bacterial groups are major carriers of ARGs and hence, their elimination during wastewater treatment may contribute to increasing resistance removal efficiency. This study, conducted at a full-scale UWTP, evaluated variations in the bacterial community and ARGs loads and explored possible associations among them. With that aim, the bacterial community composition (16S rRNA gene Illumina sequencing) and ARGs abundance (real-time PCR) were characterized in samples of raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW), and after a period of 3 days storage to monitoring possible bacterial regrowth (tTWW-RE). Culturable enterobacteria were also enumerated.

Secondary treatment was associated with the most dramatic bacterial community variations and coincided with reductions of ~2 log-units in the ARGs abundance. In contrast, no significant changes in the bacterial community composition and ARGs abundance were observed after UV disinfection of sTWW. Nevertheless, after UV treatment, viability losses were indicated ~2 log-units reductions of culturable enterobacteria. The analysed ARGs (*qnrS*, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$, bla_{TEM}, bla_{SHV}, *sul1*, *sul2*, and *intI1*) were strongly correlated with taxa more abundant in RWW than in the other types of water, and which associated with humans and animals, such as members of the families *Campylobacteraceae*, *Comamonadaceae*, *Aeromonadaceae*, *Moraxellaceae* and *Bacteroidaceae*.

Further knowledge of the dynamics of the bacterial community during wastewater treatment and its relationship with ARGs variations may contribute with information useful for wastewater treatment optimization, aiming at a more effective resistance control.

Keywords: Wastewater treatment, bacterial community dynamics, antibiotic resistance genes, network analysis

1. Introduction

Domestic wastewater has been considered a potential source for the spread of antibiotic resistant bacteria (ARB) (Varela and Manaia, 2013; Berendonk *et al.*, 2015; Manaia *et al.*, 2016; Sharma *et al.*, 2016; Huijbers *et al.*, 2015). During wastewater treatment, bacteria with origin in humans and animals get in close contact with bacteria of environmental origin, participating together in metabolic transformations pivotal for wastewater cleaning, in particular, removal of organic matter, nitrogen and phosphorus compounds (Asano and Levine, 1996; EEA, 2013). Bacteria that previously were in contact with humans or animals might have acquired antibiotic resistance genes (ARGs) and, hence, may act as carriers of those genes to other bacterial community members (Manaia, 2017). The persistence of such carriers, as well as the capacity to transfer ARGs via horizontal gene transfer, may be particularly favoured in some environments, such as wastewater (Rizzo *et al.*, 2013; Manaia *et al.*, 2016). Considering this, the unveiling of potential associations between ARGs and bacterial lineages will be a valuable contribution for better understanding the ecology of antibiotic resistance. The enrichment of this kind of knowledge, based on multiple studies conducted in distinct types of wastewater treatment and worldwide, may contribute to defining general patterns of ARGs-bacterial phylogeny associations, with positive implications on antibiotic resistance control. This was the major motivation for this study, conducted at a full-scale urban wastewater treatment plant (UWTP).

The bacterial communities found in domestic wastewater are rather complex, although with a considerable resemblance, at high taxonomic ranks, among different studies. In general, raw wastewater is dominated by members of the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* and of classes such as *Bacilli*, *Clostridia*, *Bacteroidia*, *Alpha-*, *Beta- or*

Gammaproteobacteria (Vaz-Moreira *et al.*, 2014; McLellan *et al.*, 2010; Ye and Zhang, 2013). These groups comprise bacteria frequently reported as potential antibiotic resistance carriers, such as enterobacteria, enterococci, staphylococci, pseudomonads, among others (McKinney and Pruden, 2012; Alexander *et al.*, 2016; Sousa *et al.*, 2017; Narciso-da-Rocha and Manaia, 2017; Varela *et al.*, 2015; Manaia, 2017). Domestic wastewater treatment combines different processes, often a preliminary settling, a biological treatment, most of the times based on conventional activated sludge processes, and, in some cases, an additional tertiary treatment, which, among other aims, contributes for wastewater disinfection. These processes lead to the sequential removal of suspended solids, organic matter, nutrients and pathogenic microorganisms, among others (Asano and Levine, 1996; EEA, 2013). Inevitably, wastewater treatment leads to important rearrangements in the bacterial community composition and structure (Novo *et al.*, 2013; Varela *et al.*, 2014; Ye and Zhang, 2013; Alexander *et al.*, 2016). As a consequence of the removal of microorganisms, wastewater treatment contributes also to the reduction of the abundance of ARB and ARGs (*per* volume of water) (Chen and Zhang, 2013; Guo *et al.*, 2017; Karkman *et al.*, 2016; Munir *et al.*, 2011; Manaia *et al.*, 2016; Mao *et al.*, 2015; Gao *et al.*, 2012). However, according to different reports, antibiotic resistance prevalence (ARB or ARG *per* total number of bacteria) does not seem to decrease, neither after secondary treatment, nor after disinfection (Hu *et al.*, 2016; Chen and Zhang, 2013; Narciso-da-Rocha *et al.*, 2014; Mao *et al.*, 2015). For instance, Rodriguez-Mozaz *et al*. (2015) observed a decrease of approximately 2 log-units on the abundance of the genes bla_{TEM}, *qnrS* and *sul1* after secondary treatment, while the prevalence (corresponding to the ratio of gene copy number of ARG / 16S rRNA gene) of these genes increased significantly ($p < 0.05$). Also Mao *et al*. (2015) observed that although the abundance of ARGs (*tet*, *sul*, *qnrB*, *ermC*) decreased from the raw inflow to the effluent, in percentage values ranging from 89.0% to 99.8%, the

percentage of bacteria harbouring ARGs that survived disinfection by chlorination was higher than that of total bacteria (assessed based on 16S rRNA gene abundance). These and other results suggest that the dynamics of the bacterial communities may contribute to explain variations on antibiotic resistance prevalence during wastewater treatment (Novo *et al.*, 2013; Varela *et al.*, 2014; Ye and Zhang, 2013; Alexander *et al.*, 2016). Despite the complexity of the whole set of operational parameters and external factors that may influence the wastewater microbiome, studies conducted at full-scale UWTP may contribute to assessing the trends of variation of both the bacterial groups and antibiotic resistance (Manaia *et al.*, 2018).

This study, conducted at a full-scale UWTP, with activated sludge secondary treatment and a tertiary treatment by UV disinfection, aimed to: a) assess the dynamics of the bacterial communities from the raw inflow to the final effluent, and after regrowth and, simultaneously, b) measure the variations of the abundance (*per* volume) and prevalence (*per* total bacteria measured based on the 16S rRNA gene) of a set of ARGs; c) combine both data sets to infer about the bacterial groups, which variation may be associated with the selection or removal of ARB&ARGs.

2. Material and Methods

2.1. UWTP and sampling

This study was conducted at a full-scale UWTP located in Northern Portugal, equipped with activated sludge secondary treatment and UV disinfection as described before (Sousa *et al.*, 2017). The plant serves a population of 170 000 inhabitant equivalent, has an average daily flow of 35 900 m^3, and average daily values of chemical oxygen demand (COD): 222 470 kg; biological oxygen demand (BOD5): 211 100 kg; total suspended solids (TSS): 14 460 kg; total

125 nitrogen (Kjeldahl): 2 550 kg; and total phosphorous: 500 kg (Table S1). The treatment
126 includes a homogenization chamber and a bar screen to remove gross solids; a grit and a grease
127 removal chambers, to remove small solids and fats; a primary settling tank to remove the
128 settleable solids; an activated sludge biological treatment, with recirculation between the
129 aerobic and anoxic tanks for removing the organic load and nutrients (N and P); and an open
130 channel UV system (Trojan, UV3000HO), with 38x8 150 W lamps per channel and a contact
131 time of 11.44 sec, corresponding to a dose of 29.7 mJ/cm^2.
132 Over three sampling campaigns conducted at Tuesdays - 16th June (F1), 14th July (F2), and 15th
133 September 2015 (F3) - grab wastewater samples were collected from raw wastewater after the
134 first settling tank (RWW; 1 L), secondary treated wastewater (sTWW; 6 L), and tertiary treated
135 wastewater collected after UV disinfection (tTWW; 10 L). The samples were collected in
136 sterile flasks, transported to the lab in refrigerated containers and processed within 12 h. For
137 regrowth assays (tTWW-RE), 1 L of tTWW was transferred to a sterile flask and incubated 3
138 days at 20 ºC in the dark. All samples were processed (filtration and DNA extraction) and
139 analysed (bacterial communities and target genes) in triplicate.

140

141 *2.2. Enumeration of culturable enterobacteria*

142 Culturable enterobacteria, the most commonly used indicator to assess microbiological water
143 quality, were enumerated in parallel with selected antibiotic resistant bacteria. A volume of 1
144 mL of wastewater or of the adequate serial dilution was filtered through cellulose nitrate
145 membranes (0.22 μm porosity; Sartorius Stedim Biotech, Göttingen, Germany), placed onto the
146 adequate culture medium and incubated at 30 ºC for 24 h. The culture medium used was the
147 membrane-Fecal Coliform medium (mFC, Difco, Chicago, USA) without antibiotic or

supplemented with ciprofloxacin (1 mg/L; AppliChem, Darmstadt, Germany), cefotaxime (8 mg/L; Sigma-Aldrich, St Luis, USA), or meropenem (4 mg/L; Sigma-Aldrich, St Luis, USA). The antibiotics concentrations used followed the CLSI guidelines (2015), although taking into consideration previous experience of studies with environmental samples and aiming at the future characterization of the isolates (Tacão *et al.*, 2014; Silva *et al.*, 2018). Of note, is the use of 1 mg/L of ciprofloxacin, instead of 4 mg/L, the CLSI minimum inhibitory concentration (MIC). This was justified based on the previous observation that a concentration of 4 mg/L, used for bacterial isolation, which is not the aim of CLSI guidelines, was capable of inducing *gyrA* mutations in *Escherichia coli*. All experiments were performed in triplicate.

2.3. DNA extraction

For total DNA extraction, at least three aliquots of each wastewater sample were filtered through polycarbonate membranes (0.22 μm porosity, Whatman, UK). The volumes of wastewater filtered corresponded to a compromise between expected DNA extraction yield, DNA needs, and filtration capacity of the membrane before collapsing, meaning 25 mL of RWW, 250 mL of sTWW, and 300 mL of tTWW and tTWW-RE. Filtering membranes were stored at -80 ºC until DNA extraction using the PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA) according to manufacturer indications. Samples were also treated with propidium monoazide (PMA) to discriminate between cell membrane damaged and intact cells according to Villarreal *et al.* (2013). DNA extracts were cryopreserved at -20 °C until their use for bacterial community analyses or ARGs quantification.

2.4. Bacterial community analyses

171 The bacterial community composition was analysed based on the hypervariable region V3/V4
172 of the 16S rRNA gene, using Illumina Sequencing (Genoinseq, Cantanhede, Portugal and
173 Parque Científico, Madrid, Spain). For logistics reasons, for RWW and tTWW three replicates
174 of the DNA extract were pooled before community analyses, and for sTWW and tTWW-RE
175 samples the three replicates of DNA extract were analysed independently, and results pooled
176 afterwards. DNA sequences were processed and analysed using QIIME pipeline (Caporaso *et*
177 *al.*, 2010b), after sequences shorter than 200 bp and with average quality scores lower than 25
178 were eliminated. Sequences with average quality lower than 25 in a window of 5 bases were
179 trimmed using the software PRINSEQ (Schmieder and Edwards, 2011). Sequences were
180 demultiplexed automatically by the Illumina® Miseq® sequencer using the CASAVA package
181 (Illumina, San Diego, CA, USA) and after the paired-end reads were merged using a QIIME
182 script. After identification and removal of chimeric reads, sequences were grouped into
183 operational taxonomic units (OTUs) using USEARCH v6.1 (Edgar, 2010) with a phylotype
184 threshold of ≥ 99% sequence similarity. The sequences comprising each OTU were aligned
185 using PyNAST (Caporaso *et al.*, 2010a) and were taxonomically classified using Greengenes
186 Database version 13_8 (updated: August 2013) (DeSantis *et al.*, 2006). As a variable number of
187 sequences was obtained between samples, the cumulative sum scaling (CSS) normalization
188 procedure was used to normalize the results (Paulson *et al.*, 2013). Richness and alpha diversity
189 indices Shannon, phylogenetic diversity (PD) whole tree, and Simpson were calculated after
190 rarefying to 71 576 sequences *per* sample (value of the smallest sample) (Simpson, 1949;
191 Shannon and Weaver, 1963; Faith, 1992).
192

193 *2.5. Quantitative PCR*

Quantitative PCR (qPCR) was used to quantify class 1 integrase genes (*intI1*), selected ARGs (*bla*$_{TEM}$, *bla*$_{OXA-A}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *sul1*, *sul2*, and *qnrS*), and the 16S rRNA gene as a measurement of total bacteria, using the conditions listed in Table S2. The ARGs, conferring resistance to antibiotics of different classes, as beta-lactams, sulfonamides and fluoroquinolones were selected based on their common occurrence in domestic wastewater and widespread distribution in other environmental compartments (Narciso-da-Rocha *et al.*, 2014; Szczepanowski *et al.*, 2009; Zhang *et al.*, 2009; Du *et al.*, 2014; Varela *et al.*, 2016, 2015b). These genes were thus considered as interesting potential surrogates of antibiotic resistance fate during treatment. The *intI1* gene, encoding the class 1 integrons integrase, is abundant in wastewater and is commonly used as a marker for anthropogenic pollution because it is commonly linked to genes conferring resistance to antibiotics and it is found in pathogenic and commensal bacteria of humans and their domestic animals (Gillings *et al.*, 2015). For each wastewater sample, were analysed three independent DNA extracts, and quantification was made based on the Standard Curve method as described in Brankatschk *et al*. (2012) in a StepOne^{TM} Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Possible qPCR inhibition was assessed as suggested by Bustin *et al*. (2009), consisting of the quantification of the target genes in serially diluted samples.

2.6. Statistical analyses

The data referring to quantitative PCR were expressed either as the ratio of the gene copy number *per* volume of wastewater (abundance), a good indication of the treatment stages that may contribute for resistance removal, or *per* 16S rRNA gene copy number (prevalence), which allows inferences about the potential of wastewater treatment to enrich the wastewater

resistome. The prevalence of resistant enterobacteria was calculated as the ratio between the number of culturable bacteria growing on culture medium supplemented with antibiotic, and on antibiotic-free culture medium for the same sample.

One-way analysis of variance (ANOVA) and Tukey's and Bonferroni post-hoc tests (SPSS Statistics for Windows v.24.0; IBM Corp., Armonk, NY, USA) were used to assess statistically significant differences ($p < 0.01$) of prevalence and/or abundance of culturable resistant bacteria or ARGs. The log removal was calculated for ARGs abundance or prevalence as, log removal = $\log X_{untreated} - \log X_{treated}$, where $X_{untreated}$ and $X_{treated}$ referred to the gene abundance or prevalence before and after each treatment, respectively.

The data referring to bacterial community analyses were expressed as the CSS normalized number of reads of each bacterial group or its percentage, corresponding to the ratio between that number and the total number of reads. Statistical differences between alpha metrics were tested using analysis of variance (ANOVA) followed by the post-hoc Tukey HSD method (or the Welch robust test and Games-Howell post-hoc test when homoscedasticity was not reached) using SPSS Statistics for Windows v.24.0 (IBM Corp., Armonk, NY, USA).

Correlations between the relative abundance of bacterial groups at different taxonomic levels were analysed using the statistics software STAMP v2.1.3 (Parks *et al.*, 2014). Statistically significant bacterial community variations were determined using Tukey-Kramer groups test ($p < 0.01$ and effect size below 0.8) (Coe, 2002; Bluman, 2009) using the STAMP v2.1.3 software (Parks *et al.*, 2014). Profile scatter plots were calculated with an interval of confidence of 0.99 using the DP Newcombe-Wilson method (Newcombe, 1998).

Network analysis was performed using statistically significant Pearson's correlation coefficient (Bonferroni corrected p-value < 0.01 and $\rho > 0.98$) of the logarithm-transformed ARG abundance (*per* mL of sample) and logarithm-transformed OTUs abundance. This analysis

involved the OTUs that presented more than 100 reads in each wastewater sample (n = 1011). Network analyses were performed in R environment v3.4.0 using VEGAN package v2.4-4 (Oksanen *et al.*, 2017). Network visualization was conducted on the interactive platform of Cytoscape v3.5.1 (Shannon *et al.*, 2003).

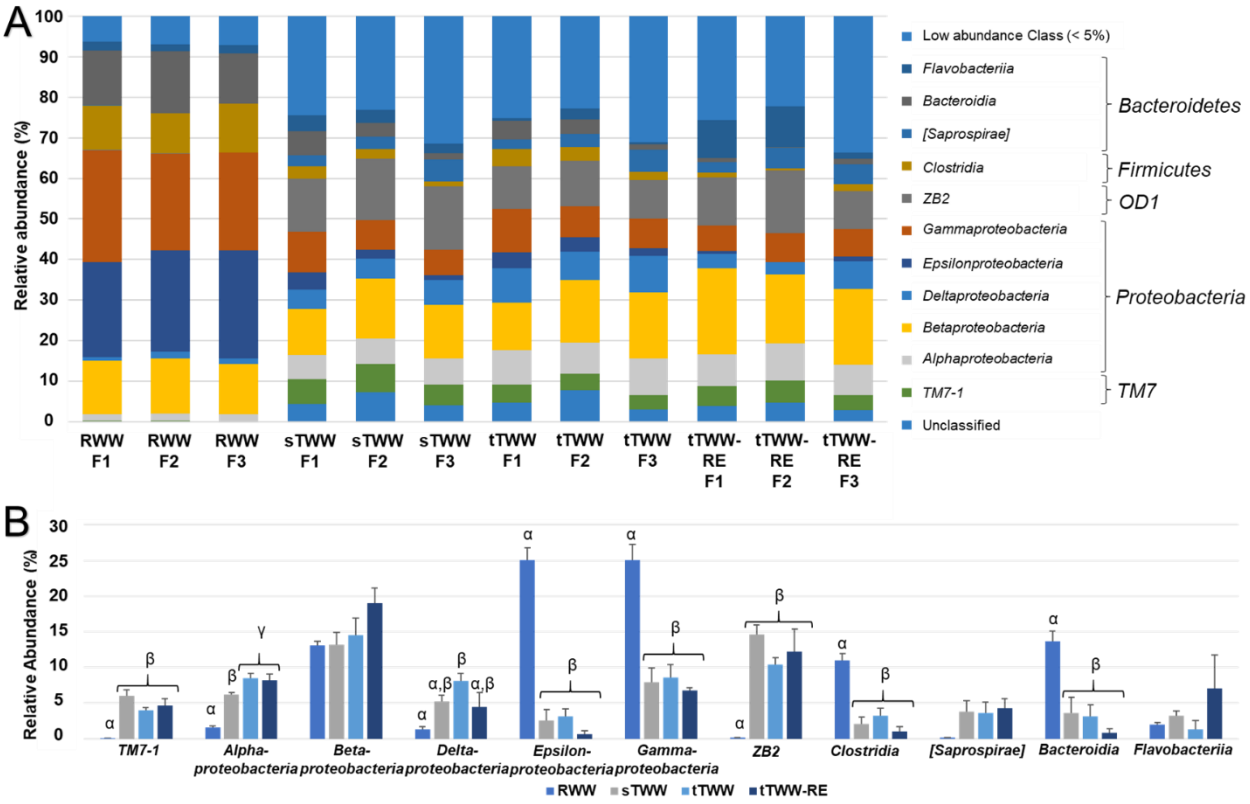
3. Results

3.1. Bacterial communities' dynamics

The comparison of the bacterial community composition in RWW, sTWW, tTWW and after regrowth – tTWW-RE showed that members of the phylum *Proteobacteria* predominated in all types of water (RWW – 66.2%; sTWW – 35.5%; tTWW – 43.3%; tTWW-RE – 39.3%), followed by the *Bacteroidetes* (RWW – 15.8%; sTWW – 13.3%; tTWW – 10.1%, tTWW-RE – 15.1%). Other abundant phyla were *Firmicutes* in RWW (12.3%) and OD1 in TWW, either secondary or tertiary (sTWW – 22.2%; tTWW – 17.0%; tTWW-RE – 18.1%) (Fig. 1A).

Secondary treatment was associated with important variations in the relative abundance of different bacterial classes (Fig. 1A). In particular, with the increase on the relative abundance of members of the classes *Alphaproteobacteria*, *TM7-1* and *ZB2* (increased 4.6%, 5.9% and 14.5%, respectively, $p < 0.01$) (Fig. 1B) and the simultaneous decrease of the classes *Epsilon-* and *Gammaproteobacteria* (decreased 22.5% and 17.2% respectively, $p < 0.01$), and of *Bacteroidia* and *Clostridia* (decreased 10.1% and 8.8%, respectively, $p < 0.01$) (Fig. 1B). Considering the classes that represented more than 5% of the total bacterial community in each sample, significant composition variations after UV disinfection were only observed for *Alphaproteobacteria* that increased 2.3%. In summary, it was the secondary treatment that imposed the most important bacterial community rearrangements and, in general, these

264 rearrangements followed the same pattern in the different sampling campaigns. The low impact
265 of the UV treatment on the bacterial community composition was demonstrated by a high
266 correlation between the profile of bacterial families observed in sTWW and tTWW (R^2 =
267 0.932), while this value was much lower between RWW and sTWW (R^2 = 0.341). The
268 rearrangement of the bacterial community composition after secondary treatment could also be
269 inferred from the significant increase ($p < 0.01$) of the Simpson's alpha-diversity index
270 calculated based on the OTUs abundance, which suggested higher diversity and equitability
271 after the biological activated sludge process (Table S3). However, the bacterial community
272 might also have been affected by the UV disinfection, since it was observed a statistically
273 significant increase ($p < 0.01$) of the PD whole tree index. This index takes into account not
274 only the number of different OTUs but their phylogenetic distance, suggesting that UV may
275 have affected the community at a taxonomic rank below family (Table S3). The storage of
276 disinfected wastewater also led to subtle bacterial community variations, with the advantage of
277 members of the families *Comamonadaceae* and *Flavobacteriaceae* (data not shown).



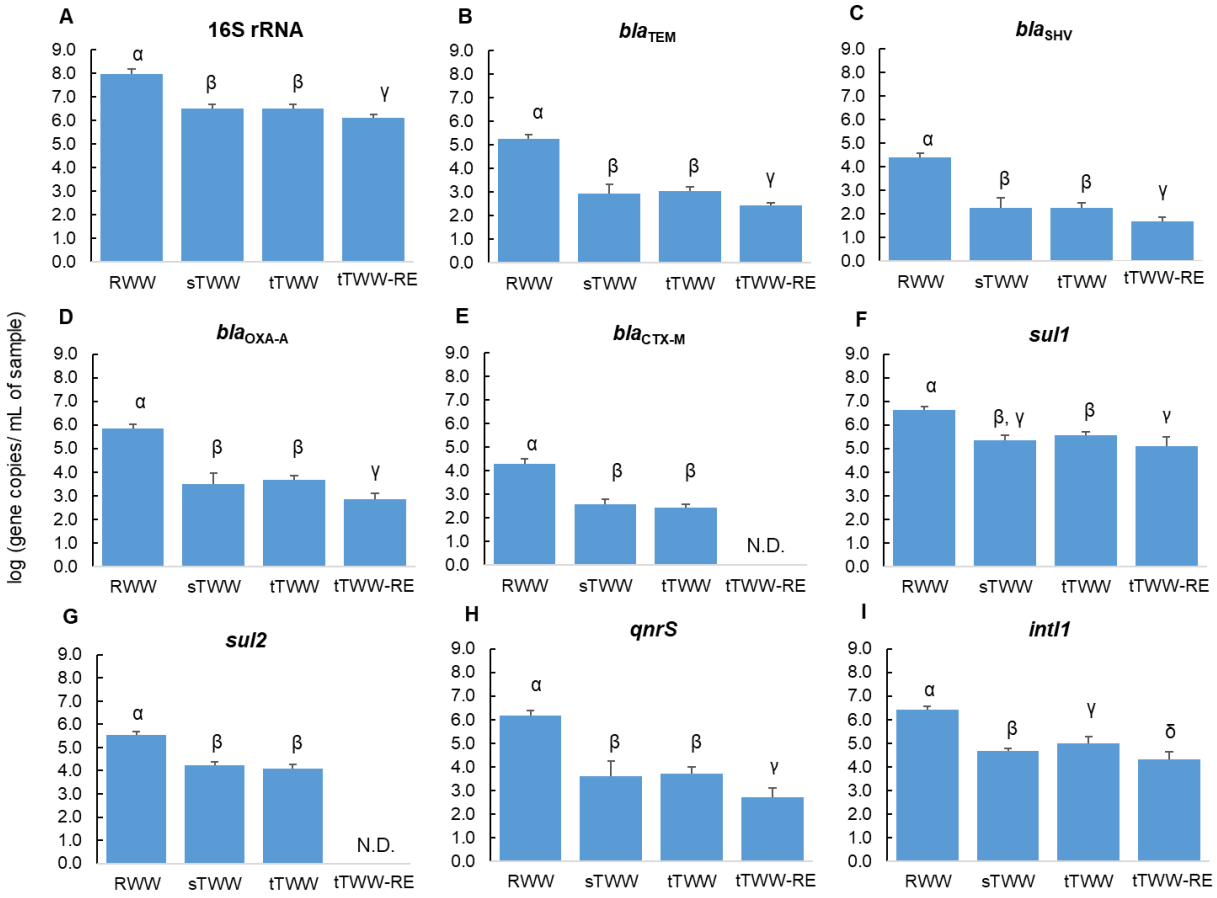
278

279 **Fig. 1** – Wastewater bacterial community composition in different points of the UWTP - raw
280 wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW
281 after 3 days storage in the dark (tTWW-RE) for samples collected at three dates: F1 – June, F2
282 - July, F3 – September. (**A**) Taxonomic classes relative abundance expressed as the ratio
283 between the number of reads of a given class, classified using Greengenes database at 99%
284 identity level, and the total number of reads. Taxa with abundance below 5% in all samples
285 were designated as low abundance classes. (**B**) Bacterial classes whose abundance varied
286 significantly ($p < 0.01$) over treatment. Taxa with relative abundance below 5% in all samples
287 were not included. Data correspond to average values of three sampling campaigns. α, β and γ
288 indicate significantly ($p < 0.01$) different Tukey's groups.

289

3.2. Variation in the abundance and prevalence of antibiotic resistance genes

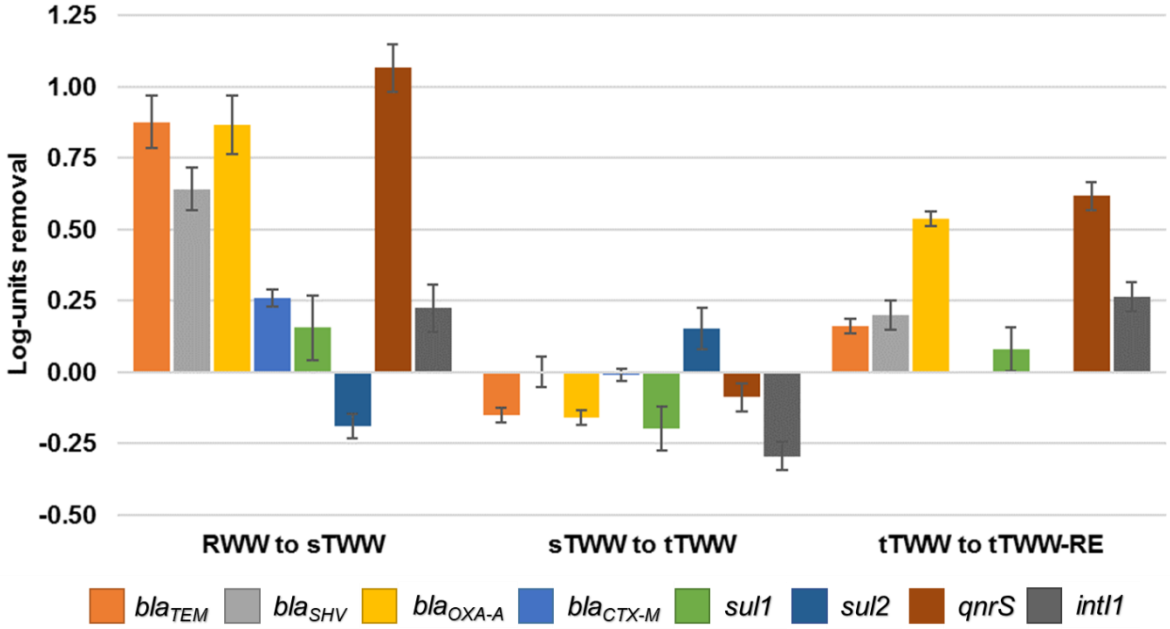
The abundance and prevalence of the class 1 integrase gene (*intI1*) and of seven ARGs, conferring resistance to beta-lactams (bla_{TEM}, bla_{OXA-A}, bla_{SHV}, bla_{CTX-M}), sulfonamides (*sul1*, *sul2*), and fluoroquinolones (*qnrS*) were determined. The abundance (*per* volume of wastewater) of the analysed genes in RWW did not differ significantly in the three sampling dates ($p > 0.01$) and their abundance could be ranked as 16S rRNA > *sul1* > *intI1* = *qnrS* > bla_{OXA-A} > *sul2* > bla_{TEM} > bla_{SHV} = bla_{CTX-M} (Fig. 2). The same hierarchy was observed in terms of prevalence (*per* 16S rRNA gene) (Fig. S1). As observed for the bacterial community composition, secondary treatment was responsible for the sharpest abundance decrease of all the analysed genes (Fig. 2). After secondary treatment, the decrease in the genes abundance varied between 2.57 and 1.31 log-units and, although non-significantly different, the log removal average values could be ranked as *qnrS* > bla_{OXA-A} > bla_{TEM} > bla_{SHV} > bla_{CTX-M} > *intI1* > *sul1* > 16S rRNA > *sul2* (Fig S2). Log removal values based on genes prevalence varied between 1.07 and -0.19 and could be ranked as *qnrS* > bla_{TEM} > bla_{OXA-A} > bla_{SHV} > bla_{CTX-M} > *intI1* > *sul1* > *sul2* (Fig. 3). Accordingly, the prevalence of the *sul* genes did not vary (*sul1*) or significantly increased after secondary treatment (*sul2*) (Fig. S1F).



307

308 **Fig. 2** – Abundance of the genes analysed (gene copy number / mL of sample) in raw wastewater
309 (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days
310 storage in the dark (tTWW-RE). (A) 16S rRNA gene; (B) *bla*$_{TEM}$; (C) *bla*$_{SHV}$; (D) *bla*$_{OXA-A}$; (E)
311 *bla*$_{CTX-M}$; (F) *sul1*; (G) *sul2*; (H) *qnrS*; and (I) *intI1*. Data correspond to average values of three
312 sampling campaigns. α, β, γ, and δ indicate significantly ($p < 0.01$) different Tukey's groups
313 comparing the different types of water. N.D., not determined.

314



315

316 **Fig. 3** – Log-removal values calculated based on gene prevalence (gene copy number/ 16S
317 rRNA gene copy number) in raw wastewater (RWW), secondary effluent (sTWW), after UV
318 disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE). Data correspond
319 to average values of three sampling campaigns.

320

321 After UV disinfection (tTWW), except for *intI1*, non-significantly different values were
322 observed in the abundance or prevalence values in comparison to sTWW (Fig. 2 and Fig. S1).
323 The *intI1* gene was both significantly more abundant (*per* volume of water) and prevalent (*per*
324 16S rRNA gene) in tTWW than in sTWW. In addition, for genes bla_{TEM}, $bla_{\text{OXA-A}}$, *qnrS*, *intI1*
325 and *sul1*, the log removal values after UV disinfection were negative, meaning slight increases
326 on the average values of abundance and prevalence of these genes (Fig. 3 and Fig. S2). To test
327 the hypothesis that some bacterial groups may be damaged after UV disinfection and unable to
328 recover, samples were stored for three days in the dark, to avoid the activation of light-
329 dependent DNA repair systems. Indeed, after 3 days in the dark at 20 ºC, UV-treated

wastewater presented significant decreases on the abundance of the genes 16S rRNA, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-A}$, *sul1*, *qnrS*, and *intI1* (Fig. 2A, B, C, D, F, H, I) and on the prevalence of the genes *bla*$_{OXA-A}$, *qnrS*, and *intI1* (Fig. S1C, G, H).

The analyses of culturable enterobacteria counts contributed also to evaluate the treatment effects. The abundance of enterobacteria resistant to ciprofloxacin or cefotaxime decreased significantly after secondary treatment (Fig. S3). However, enterobacteria resistant to ciprofloxacin, meropenem or cefotaxime were observed to be relatively enriched during secondary treatment, with significant ($p < 0.01$) increases (21% to 28%, 0% to 11%, and 4% to 19%, respectively) on the resistant bacteria prevalence. Curiously, the apparently smooth effect of UV on bacterial inactivation deduced from qPCR data and that could suggest a poor effectiveness of disinfection, was not confirmed by culture-dependent analyses. Indeed, except for ciprofloxacin, the prevalence of resistant bacteria in the secondary effluent was significantly reduced after UV disinfection (28% to 27%, 11% to 1%, and 19% to 9%, respectively).

3.3. Inference about bacterial groups associated with ARGs dynamics

One of the aims of the study was to identify bacterial groups that might be associated with the analysed ARGs. With this aim, a network analysis was performed using Pearson's correlation coefficient of the abundance of ARGs (*per* mL of sample) and the OTUs with more than 100 reads in all wastewater samples (n = 1011). In the network (Fig. 4), only the statistically significant correlations (Bonferroni corrected *p*-value < 0.01 and $\rho > 0.98$) are represented, with most of the OTUs in the network being separated in two major groups, those detected in all wastewater samples (n = 672) and those detected only in TWW samples (n = 320). The first group suggests that the most abundant OTUs persist after the wastewater treatment process,

even if at lower relative abundance (Fig. S4). In contrast, the OTUs detected only in TWW may enter the system via activated sludge and/or be selected during the process, with the potential to increase their relative abundance. Significant correlations were observed between the *intI1* and ARGs quantified in this study and the most abundant OTUs (Fig. 4). In that network cluster, 77 OTUs were observed to be significantly correlated with at least one of the ARGs (Table S4). All these OTUs were detected in all wastewater samples, with exception of two OTUs belonging to the families *Bacteroidaceae* and *Porphyromonadaceae* that were not detected in the tTWW-RE (Table S4).

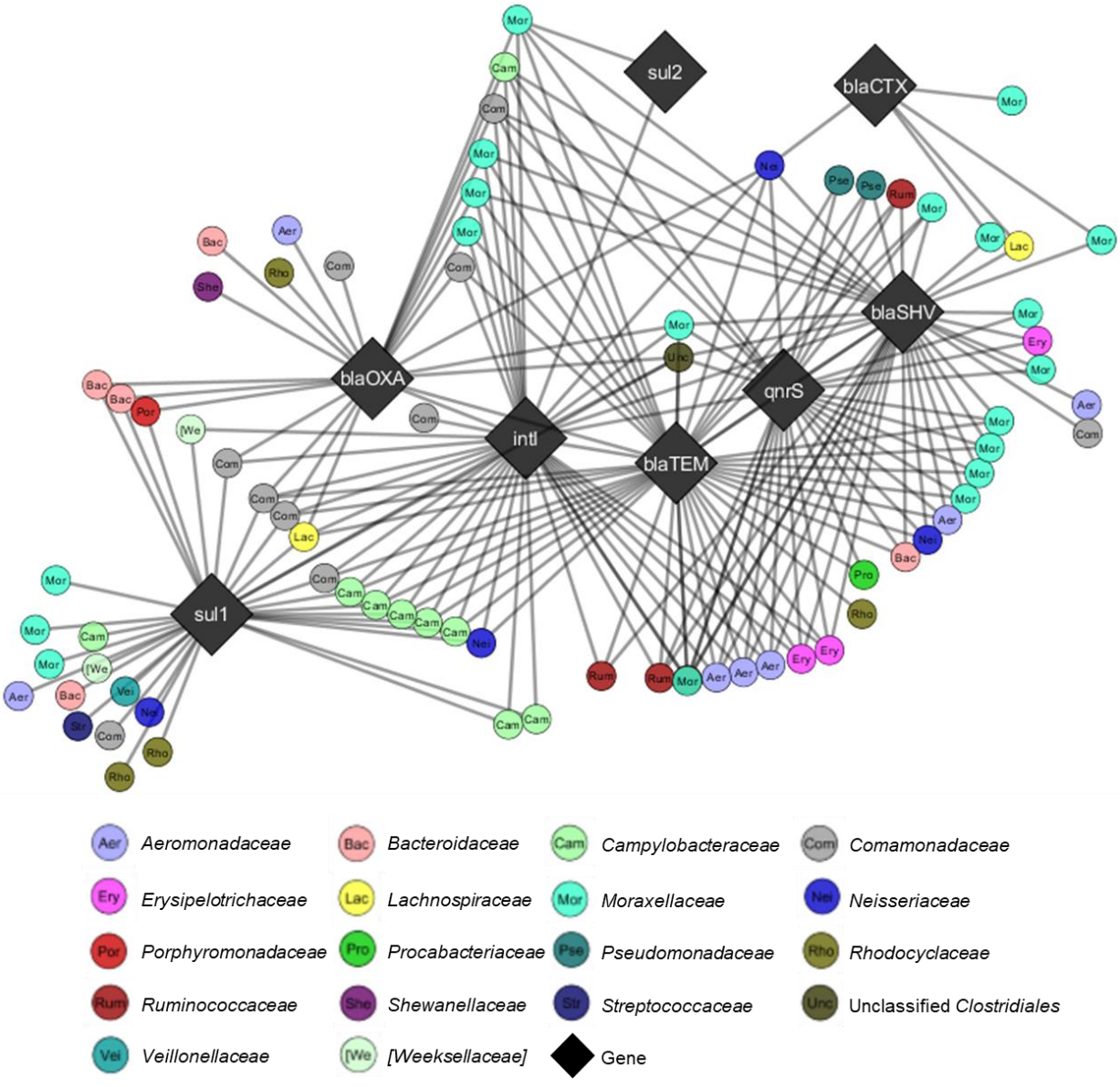


Fig. 4 – Cluster of the network analysis created using statistically significant Pearson's correlation coefficient (Bonferroni corrected *p*-value < 0.01 and ρ > 0.98) of the abundance of ARGs (*per* mL of sample) and the OTUs with counts bigger than 100 reads. The cluster presented in this figure was the largest one and the only one which contained ARGs. The shape of the node refers to genes (diamond) and OTUs (circle). The colour and text inside the circles refer to the Family to which the OTU was attributed.

The relative abundance of the OTUs observed to be correlated with the ARGs was of 5.7% in RWW, 1.3% in sTWW, 1.5% in tTWW and 0.6% in tTWW-RE (Table S4). If considered only the multi-correlated OTUs (correlated with at least 2 ARGs), the total relative abundance values were 3.8% in RWW, 0.8% in sTWW, 0.9% in tTWW and 0.3% in tTWW-RE. The set of families including most of the OTUs correlated with ARGs (*Moraxellaceae*, *Campylobacteraceae*, *Comamonadaceae* and *Aeromonadaceae*) were also those with the highest relative abundance values in the whole community in each type of wastewater (Fig. S4). OTUs classified as members of the phyla *Proteobacteria* (n = 57), *Firmicutes* (n = 12), and *Bacteroidetes* (n = 8) were observed to have important associations with the ARGs (Fig. 4). The family *Moraxellaceae* (19 OTUs) was the only one that presented significant correlations with all the ARGs, being bla_{SHV}, bla_{TEM}, and *qnrS* the genes correlated with the highest number of OTUs (14, 11 and 10, respectively). OTUs affiliated to the families *Campylobacteraceae* (n=9) and *Comamonadaceae* (n=10) were mainly correlated with the genes *intI1*, *sul1* and bla_{TEM}. In addition, most of the *Comamonadaceae* OTUs were also correlated with the $bla_{OXA\text{-}A}$ gene. The families *Aeromonadaceae*, *Bacteroidaceae*, *Neisseriaceae* and *Ruminococcaceae* although with a lower number of OTUs presenting significant correlations with ARGs (7, 5, 4 and 4, respectively) presented a high number of correlations with distinct ARGs, mainly *sul1*

and *bla*$_{\text{OXA-A}}$ for *Bacteroidaceae*, and *intI1*, *qnrS*, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$ for the other families (Table S4; Fig. S4). The genes *bla*$_{\text{CTX-M}}$ and *sul2* were those with the lowest number of correlations with OTUs. The gene *bla*$_{\text{CTX-M}}$ was correlated with 5 OTUs of the families *Moraxellaceae* (n = 3), *Lachnospiraceae* (n = 1), and *Neisseriaceae* (n=1) and *sul2* with one OTU of the family *Moraxellaceae* (Fig. 4; Table S4).

4. Discussion

At the phylum level, the wastewater bacterial community composition is fairly similar in domestic wastewater worldwide, with *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* among the major groups. Noticeably, the low relative abundance of the members of phylum *Actinobacteria* in the present study contrasts with previous publications (McLellan *et al.*, 2010; Ye and Zhang, 2013; Munck *et al.*, 2015). This difference may result from the use of distinct DNA extraction procedures and/or primers selection (Liu *et al.*, 2007; Albertsen *et al.*, 2015). Alternatively, it could be a consequence of stormwater reception, which, in rainy seasons, may avoid the formation of a proto-natural bacterial community in the treatment plant and consequently avoid the establishment of the *Actinobacteria*. However, in other studies using the same DNA extraction procedure and 16S rRNA gene primers, conducted in the same region, with different urban wastewater sources, *Actinobacteria* were not among the major phyla, in contrast to what was observed in river water (Narciso-da-Rocha and Manaia, 2016; Becerra-Castro *et al.*, 2016; Narciso-da-Rocha and Manaia, 2017). This suggests that the low abundance of *Actinobacteria* in wastewater may be a biogeographic specificity.

The bacterial community suffered important rearrangements during wastewater treatment, almost exclusively imposed by secondary treatment (Fig. 1). In the UWTP under study, the

biological treatment comprises an aerobic tank, where the organic matter is degraded and the nutrients, nitrogen, and phosphorus are transformed by aerobic microorganisms; and an anoxic tank, where the activated sludge recirculate and denitrification takes place. These processes involve chemo-, organo-, and lithotrophic microbial groups that lead the metabolic transformations and therefore may justify at least part of the observed bacterial community dynamics. Significant increases of the relative abundance of *Alphaproteobacteria* were observed from RWW to sTWW. Members of this class comprise bacteria with wide metabolic diversity and intense biodegradative activity (Garrity *et al.*, 2005; Krieg *et al.*, 2010), facts that may explain this variation. In addition, also members of the candidate classes *TM7-1* and *ZB2* were observed to increase during treatment. In spite of their supposedly reduced metabolic capabilities and their small genome (Albertsen *et al.*, 2013; Nelson and Stegen, 2015) it is suggested that they present a good fitness during treatment (Ye and Zhang, 2013; Zhang *et al.*, 2012). The relative abundance of other bacteria decreased 2-5 times, being the classes *Epsilon-* and *Gammaproteobacteria* those with the sharpest reductions (Fig. 1). In these classes, members of the *Campylobacteraceae*, *Aeromonadaceae*, *Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae* were those with the highest decreases (data not shown). Given the fact that these groups comprise important human pathogens (Luangtongkum *et al.*, 2010; Toleman *et al.*, 2006; Miró *et al.*, 2010; Jiang *et al.*, 2014), this variation meets one of the key requirements of wastewater treatment – the removal of pathogens. The balanced increase of some bacterial groups and a decrease of others resulted in the higher diversity and equitability indices after the secondary treatment (Table S3), an effect that was not reverted after disinfection as has been reported by other authors (Hu *et al.*, 2012; Ye and Zhang, 2013).

UV-disinfection contributed to reducing the abundance of culturable enterobacteria in ~2 log-units (Fig. S3), a value that was in accordance with the reported values for total or fecal

coliforms removal of 2 – 3 log-units (Guo *et al.*, 2009; Gehr *et al.*, 2003; George *et al.*, 2002). In these studies, UV doses ranging from 5 to 30 mJ / cm^2 were applied to treat wastewater and culturable bacteria counts were used to assess the faecal coliforms removal. The use of culture-independent methods to assess bacterial inactivation by UV or UV combined with other disinfection methods is reported by different authors (Becerra-Castro *et al.*, 2016; Sousa *et al.*, 2017; Lee *et al.*, 2017; Zhang *et al.*, 2015; Munir *et al.*, 2011; Di Cesare *et al.*, 2016). In a study conducted in three UWTPs with different UV-disinfection processes (oxidative ditch / UV, activated sludge / UV, membrane biological reactor / UV), as in the present study, Munir *et al*. (2011) observed reductions of 0 - 0.5 log-units, in the bacterial abundance after disinfection. Di Cesare *et al.* (2016) and Lee *et al*. (2017), using a similar UV dose (25.8 mJ/cm^2 and 27 mJ/cm^2, respectively) to the one we used (29.7 mJ/cm^2) also observed limited effects on the relative abundance of ARGs. The apparent disagreement in the current study between culture-dependent assays, where ~2 log-unit reductions were observed, and culture-independent measurements, suggest that although viable coliforms might be reduced, in general, the microbiota was not strongly affected by disinfection. Even though the dose of radiation used for disinfection (29.7 mJ/cm^2) was similar to that reported by other authors (Di Cesare *et al.*, 2016; Lee *et al.*, 2017), the configuration of the system, which does include a filtration before UV irradiation to remove suspended particles may explain the low removal values observed. The UV radiation quenching due to suspended particles may indeed neutralize the potentially damaging effect to be observed on cells (Brahmi *et al.*, 2010). This possibility may explain why UV radiation did not cause noticeable reductions in the quantity of the 16S rRNA gene or of other genes, with log removal values ranging zero (Fig. S2). However, UV might have generated injured cells, with limited capacity to reproduce. The mechanism of action of the germicide UV radiation involves DNA damages and mutations that, albeit do not

457 kill the cells, limit their viability and future cell division (Hijnen *et al.*, 2006; Dodd, 2012). The
458 use of propidium monoazide (PMA) to distinguish live from dead cells in qPCR measurement
459 has been suggested as a way to assess the proportion of inactivated cells due to cell membrane
460 damages (Nocker *et al.*, 2007; van Frankenhuyzen *et al.*, 2011; Villarreal *et al.*, 2013). Given
461 the mode of action of PMA is it expected that it has a limited value when the target of the
462 disinfection is the DNA since, in the absence of cell membrane damages, PMA will not have
463 increased access to inactivated cells (Nocker *et al.*, 2007). Indeed, the quantification of the 16S
464 rRNA gene in tTWW samples from which DNA was extracted with and without PMA/light
465 activation was identical (data not shown). As an alternative approach to assess the impact of
466 UV disinfection, was analysed the community after storage in the dark (tTWW-RE). This
467 procedure showed that UV disinfection followed by regrowth might lead to alterations in the
468 bacterial community, being the regrowth of members of the families *Comamonadaceae*
469 (*Betaproteobacteria*) and *Flavobacteriaceae* (*Bacteroidetes*) notorious (data not shown). The
470 capacity of *Proteobacteria* to overgrow in disinfected wastewater during storage of has been
471 noted before (Becerra-Castro *et al.*, 2016). However, regrowth depends on disinfection and
472 storage conditions, as well as, on the capacity of bacteria to recover the damages and on their
473 growth rate. Becerra-Castro *et al*. (2016), who stored the UV-treated water under ambient light,
474 reported the regrowth of *Gammaproteobacteria* and, in a lower extent, of *Betaproteobacteria,*
475 suggesting that photolyase systems, totally dependent of light, may be important for the
476 recovery of *Gammaproteobacteria*. Stress recovery after disinfection is also strongly
477 influenced by the amount and diversity of nutrients available in the effluent that will shape the
478 observable changes in the bacterial community. Taking this recovery mechanisms into account,
479 the use of targeted or non-targeted transcriptomic analyses, which can give an overview of the
480 bacterial groups and genes that are active in a bacterial community, are also valuable

approaches to assess the effects of disinfectants (Blazewicz *et al.*, 2013; Liang and Keeley, 2012; Alexander *et al.*, 2016).

The fate of ARGs during wastewater treatment followed, in general, the pattern observed for total bacteria, with the major removal occurring after secondary treatment (Fig. 2). While the literature available is consensual about the reduction of ARGs abundance during wastewater secondary treatment, results on ARGs prevalence vary, with decreases, no variation or even increases, being reported in distinct studies with different plants configurations (Gao *et al.*, 2012; Munir *et al.*, 2011; Narciso-da-Rocha *et al.*, 2014; Rodriguez-Mozaz *et al.*, 2015). For instance, while some studies did not observe significant variations on *tet* genes relative abundance after secondary treatment (Gao *et al.*, 2012), others observed a decrease (Munir *et al.*, 2011; Rodriguez-Mozaz *et al.*, 2015). The same was observed for *sul1* gene with some studies observing no significant variations of gene relative abundance after secondary treatment (Gao *et al.*, 2012; Munir *et al.*, 2011), and others observing an increase (Rodriguez-Mozaz *et al.*, 2015). In this study, it was observed that different ARGs behaved differently over secondary treatment, specifically those encoding beta-lactamases (bla_{TEM}, $bla_{\text{OXA-A}}$, bla_{SHV}, $bla_{\text{CTX-M}}$) and quinolone resistance genes (*qnrS*) *versus* those encoding sulphonamide resistance (*sul1*, *sul2*) and class 1 integrase (*intI1*). While the first group was observed to decrease, the second group did not vary or increased (Fig. 2 and S1). It was hypothesised that a distinct pattern of variation of different ARGs could be explained, at least in part, based on the dynamic of the bacterial community, with notorious decreases during treatment of the bacterial groups most associated with decreasing ARGs. Indeed, these associations could be confirmed for the decreasing ARGs (Fig. 4, Table S4). However, for ARGs with increased or identical prevalence after treatment (*intI1*, *sul1*, and *sul2*), it could be expected to find an association with OTUs whose relative abundance increased after treatment. This supposition was not confirmed based

505 on the network analyses, suggesting the association with minor OTUs (not included in the
506 correlation analyses) or the involvement of horizontal gene transfer (Table S4, Fig. S4). The
507 families *Bacteroidaceae*, *Comamonadaceae*, *Campylobacteraceae*, *Aeromonadaceae* and
508 *Moraxellaceae* were those, among the OTUs with more than 100 reads, presenting important
509 associations with the ARGs analysed (Fig. 4 and S4, Table S4). Noticeably, of these six
510 families, the last three were among those that presented higher reductions after secondary
511 treatment, suggesting the poor survival or high partition with the activated sludge fraction,
512 during secondary treatment. While the exploratory association analysis made do not aim at
513 stating that the analysed ARGs are harboured by the identified bacterial groups, the observed
514 associations may suggest that sources containing these bacteria are also those hosting bacteria
515 with the analysed ARGs, and/or that the conditions favouring the proliferation of those bacteria
516 may be the same that favour these ARGs dissemination. Nevertheless, the results suggest that
517 the major bacterial carriers of ARGs entering the UWTP were removed during secondary
518 treatment, suggesting that the treatment is effective in the removal of some of the major ARGs
519 carriers. Removal during secondary treatment may occur due to out-competition and/or to
520 adsorption onto the sludge particles, which by recirculation may reintroduce ARGs carriers in
521 the system. This is an aspect that deserves future investigation. The adjustment of operational
522 conditions, such as the scheme of sludge recirculation or aeration/anoxic stages in UWTPs,
523 although challenging, may contribute to halt the proliferation or even eliminate preferential
524 ARGs carriers.

525 In summary, it is suggested that the rearrangements of bacterial communities may play a major
526 role in ARGs removal. In addition, an in-depth analysis, searching at the bacterial species or
527 strain levels, for ARGs carriers recognized for being active in horizontal gene transfer and
528 prone for overgrowth (e.g. bacteria belonging to *Gamma*- and *Betaproteobacteria* classes) (Li

529 *et al.*, 2015; Kloesges *et al.*, 2011), need also to be considered for a full overview of resistance
530 dynamics during wastewater treatment. Indeed, bacterial groups with low relative abundance
531 (e.g. < 1%), such as *Enterobacteriaceae* and others, which are not covered by high throughput
532 sequencing analyses as those performed in this study, may be among the important carriers of
533 ARGs, capable of persisting in the final effluents.

534

Supplementary material

Fig. S1 – Prevalence of the genes analysed (gene copie number / 16S rRNA gene copies) in raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE). Data correspond to average values of three sampling campaigns. Gene prevalence: (A) bla_{TEM}; (B) bla_{SHV}; (C) bla_{OXA-A}; (D) bla_{CTX-M}; (E) *sul1*; (F) *sul2*; (G) *qnrS*; and (H) *intI1*.α, β and γ indicate significantly ($p < 0.01$) different Tukey's groups comparing the different types of water. N.D. indicate that data was not determined for the sample.

Fig. S2 – Log-removal values calculated for gene abundance (gene copy number / mL) in raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE). Data correspond to average values of three sampling campaigns.

Fig. S3 – Counts of CFUs per volume of sample (mL) in raw wastewater (RWW), in wastewater subjected to secondary and tertiary treatment (sTWW and tTWW) and in wastewater subjected to tertiary treatment incubated during 3 days (tTWW-RE) of an urban wastewater treatment plant. CFUs were analysed at 24hrs in (**A**) mFC, (**B**) mFC with ciprofloxacin (CIP, 1 mg/L), (**C**) mFC with meropenem (MEM, 4 mg/L), and (**D**) mFC with cefotaxime (CTX, 8 mg/L). α, β and γ indicate significantly ($p < 0.01$) different Tukey's groups comparing the different types of water.

Fig. S4. – Relative abundance for the most abundant OTUs correlated with the ARGs, for the different types of wastewater – raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE).

Table S1 – Chemical and biological parameters of raw wastewater (RWW) and final effluent after UV disinfection (tTWW) for the different sampling campaigns (F1 – June, F2 -July, F3 – September 2015)

Table S2 - Conditions used in qPCR assays.

Table S3 – Alpha diversity indices of the wastewater samples (RWW – raw wastewater, sTWW – effluent of wastewater secondary treatment, tTWW – effluent of wastewater tertiary treatment, tTWW-RE – effluent of wastewater tertiary treatment after 3 day incubation) at different times (F1 – June, F2 - July, F3 – September), calculated based on the average of 10 rarefaction OTU tables.

Table S4 – List of OTUs significantly correlated with the genes of interest and its mean relative abundance in wastewater samples (RWW – raw wastewater, sTWW – effluent of wastewater secondary treatment, tTWW – effluent of wastewater tertiary treatment, tTWW-RE – effluent of wastewater tertiary treatment after 3 day incubation).

Conflicts of interest: none to declare

Authors' contributions: CNR contributed to data analysis and manuscript writing. JR contributed to the execution of the project and data analysis. FL and JT contributed to data analysis. IVM, IH and JLM contributed to project planning and manuscript writing. CMM contributed to project planning, execution, data analysis, and manuscript writing. All authors read and approved the final manuscript.

Data Accessibility: 24 sequence data files that support the findings of this study have been deposited in GenBank within the BioProject with the accession code PRJNA380226.

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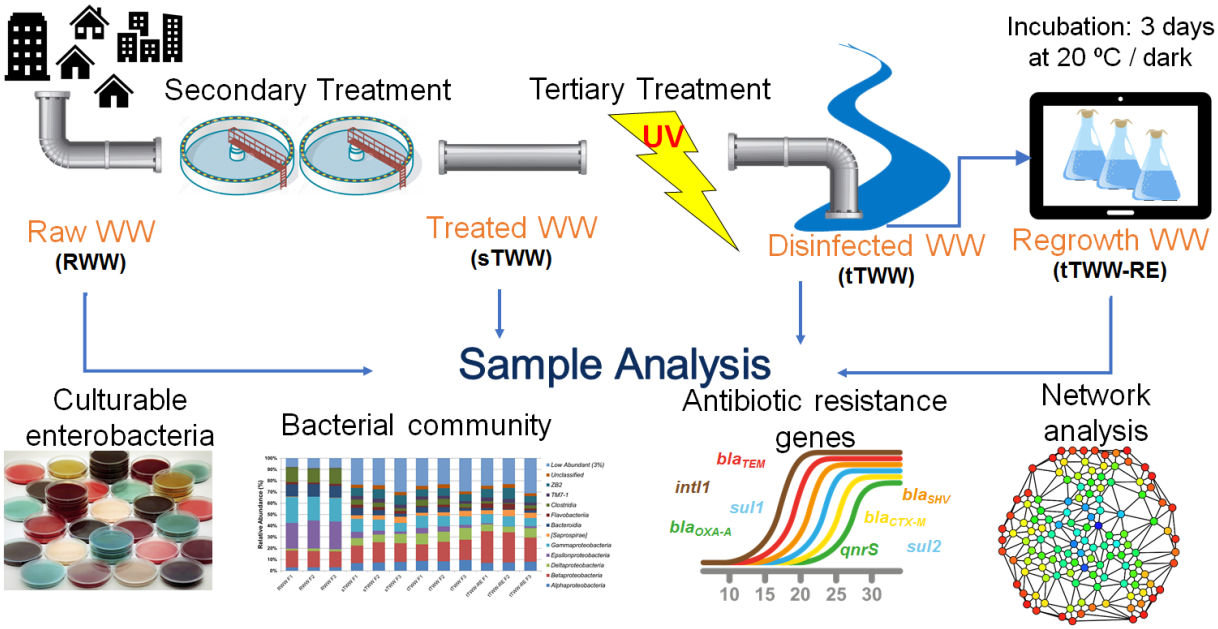
Graphical Abstract



Supplementary Information

Title: Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant

Authors: Carlos Narciso-da-Rocha[a*](crocha@porto.ucp.pt), Jaqueline Rocha[a*](jrocha@porto.ucp.pt), Ivone Vaz-Moreira[a](ivmoreira@porto.ucp.pt), Felipe Lira[b](felipelira3@gmail.com), Javier Tamames[b](jtamames@cnb.csic.es), Isabel Henriques[c](ihenriques@ua.pt), José Luis Martinez[b](jlmtnez@cnb.csic.es), Célia M. Manaia[a](cmanaia@porto.ucp.pt)

Table S1 – Chemical and biological parameters of raw wastewater (RWW) and final effluent after UV disinfection (tTWW) for the different sampling campaigns (F1 – June, F2 -July, F3 – September 2015)

Sample	Campaign	pH	Conductivity 25°C (ms/cm)	COD (mg O_2/L)	BOD5 (mg O_2/L)	TSS (mg/L)
RWW	F1	7.30	904	813	480	268
	F2	7.65	925	932	510	478
	F3	7.73	1128	887	550	568
tTWW	F1	6.52	508	< 50	3	< 10
	F2	6.82	662	< 50	10	< 10
	F3	6.42	611	< 50	4	< 10

The average daily values for total nitrogen (Kjeldahl) and total phosphorous are 2 550 kg and 500 kg, respectively.

COD, chemical oxygen demand; BOD, biological oxygen demand; TSS, total suspended solids.

Table S2 - Conditions used in qPCR assays

Target gene	qPCR Standard	Primers	Primers sequence	Conditions	Efficiency	Limit of quantification (no. of copies)	Primers reference
16S rRNA Gene	*E. coli* ATCC 25922	1114F	CGGCAACGAGCGCAACCC	95 °C for 10 min (1 cycle); 95 °C for 15 s, 55 °C for 20 s and 72 °C for 10 s (35 cycles) Other: 1a	97%	385	[1]
		1275R	CCATTGTAGCACGTGTGTAGCC				
bla_{TEM}	clone bla_{TEM} (pNORM)	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	95ºC 10 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 2a	95%	75	[2]
		blaTEM-R	CTCAAGGATCTTACCGCTGTTG				
bla_{OXA-A}	*E. coli* A2FC14	OXA1B14_fw	CACTTACAGGAAACTTGGGGTCG	95ºC 10 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 2d	99%	64	[3]
		blaOXA1_rv	AGTGTGTTTAGAATGGTGATC				
bla_{SHV}	*E. coli* A4FC7	SHV-FW	CGCTTTCCCATGATGAGCACCTTT	95ºC 10 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 2c	92%	12	[4]
		SHV-RV	TCCTGCTGGCGATAGTGGATCTTT				
bla_{CTX-M}	*E. coli* A2FC14	CTXM-FW	CTATGGCACCACCAACGATA	95ºC 10 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 2a	94%	78	[4]
		CTXM-RV	ACGGCTTTCTGCCTTAGGTT				
sul1	clone *sul1* (pNORM)	sul1-FW	CGCACCGGAAACATCGCTGCAC	95ºC 5 min (1 cycle), 95ºC 15 s - 60ºC 30 s (35 cycles) Other: 3b	93%	240	[5]
		sul1-RV	TGAAGTTCCGCCGCAAGGCTCG				
sul2	clone *sul2*	sul2-FW	TCCGGTGGAGGCCGGTATCTGG	95ºC 5 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 1a	90%	47	[5]
		sul2-RV	CGGGAATGCCATCTGCCTTGAG				
qnrS	clone *qnrS* (pNORM)	qnrSrtF11	GACGTGCTAACTTGCGTGAT	95ºC 5 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 2d	94%	75	[6]
		qnrSrtR11	TGGCATTGTTGGAAACTTG				

intI1	clone *intI1* (pNORM)	intI1-LC1	GCCTTGATGTTACCCGAGAG	95ºC 10 min (1 cycle), 95ºC 15 s - 60ºC 1 min (40 cycles) Other: 2a	91%	75	[7]
		intI1-LC5	GATCGGTCGAATGCGTGT				

1) KAPA SYBR® FAST ABI Prism® qPCR Master Mix; 2) SYBR® Select Master Mix; 3) Fast SYBR™ Green Master Mix; a) 200 nM of primer; b) 300nM; c) 400 nM of primer; d) 600 nM of primer.

Table S3 – Alpha diversity indices of the wastewater samples (RWW – raw wastewater, sTWW – effluent of wastewater secondary treatment, tTWW – effluent of wastewater tertiary treatment, tTWW-RE – effluent of wastewater tertiary treatment after 3 day incubation) at different times (F1 – June, F2 - July, F3 – September), calculated based on the average of 10 rarefaction OTU tables

Sample	Chao1 Index	Dominance	Equitability	Observed OTUs	PD whole tree	Shannon's diversity Index	Simpson's diversity Index
RWW.F1	26340$^{\alpha}$	0.096$^{\alpha}$	0.597$^{\alpha}$	18270$^{\alpha}$	249.4$^{\alpha}$	8.45$^{\alpha}$	0.904$^{\alpha}$
RWW.F2	26880$^{\alpha}$	0.095$^{\alpha}$	0.595$^{\alpha}$	19131$^{\alpha}$	248.8$^{\alpha}$	8.46$^{\alpha}$	0.905$^{\alpha}$
RWW.F3	27088$^{\alpha}$	0.111$^{\alpha}$	0.572$^{\alpha}$	19245$^{\alpha}$	262.4$^{\alpha}$	8.14$^{\alpha}$	0.889$^{\alpha}$
sTWW.F1	16987$^{\beta}$	0.009$^{\beta}$	0.734$^{\alpha,\beta}$	7951$^{\beta}$	221.2$^{\alpha}$	9.51$^{\alpha,\beta}$	0.991$^{\beta}$
sTWW.F2	18763$^{\beta}$	0.010$^{\beta}$	0.706$^{\alpha,\beta}$	9405$^{\beta}$	235.7$^{\alpha}$	9.32$^{\alpha,\beta}$	0.990$^{\beta}$
sTWW.F3	15054$^{\beta}$	0.005$^{\beta}$	0.760$^{\alpha,\beta}$	7909$^{\beta}$	229.8$^{\alpha}$	9.84$^{\alpha,\beta}$	0.995$^{\beta}$
tTWW.F1	37710$^{\gamma}$	0.007$^{\beta}$	0.761$^{\beta}$	23249$^{\alpha}$	416.4$^{\beta}$	11.04$^{\beta}$	0.993$^{\beta}$
tTWW.F2	34722$^{\gamma}$	0.006$^{\beta}$	0.760$^{\beta}$	19050$^{\alpha}$	364.9$^{\beta}$	10.81$^{\beta}$	0.994$^{\beta}$
tTWW.F3	30228$^{\gamma}$	0.003$^{\beta}$	0.808$^{\beta}$	19133$^{\alpha}$	380.1$^{\beta}$	11.49$^{\beta}$	0.997$^{\beta}$
tTWW-RE F1	16707$^{\beta}$	0.034$^{\beta}$	0.625$^{\alpha,\beta}$	8608$^{\beta}$	204.1$^{\alpha}$	8.17$^{\alpha}$	0.966$^{\beta}$
tTWW-RE F2	19929$^{\beta}$	0.022$^{\beta}$	0.618$^{\alpha,\beta}$	11000$^{\beta}$	196.2$^{\alpha}$	8.30$^{\alpha}$	0.978$^{\beta}$
tTWW-RE F3	17735$^{\beta}$	0.004$^{\beta}$	0.777$^{\alpha,\beta}$	8975$^{\beta}$	246.4$^{\alpha}$	10.21$^{\alpha}$	0.996$^{\beta}$

α, β and γ - Statistically significantly different ($p < 0.01$) Tukey's groups.

Table S4 – Relative abundance of OTUs that based on the network analyses were significantly correlated to the genes of interest in wastewater samples (RWW – raw wastewater, sTWW – effluent of wastewater secondary treatment, tTWW – effluent of wastewater tertiary treatment, tTWW-RE – effluent of wastewater tertiary treatment after 3-day incubation)

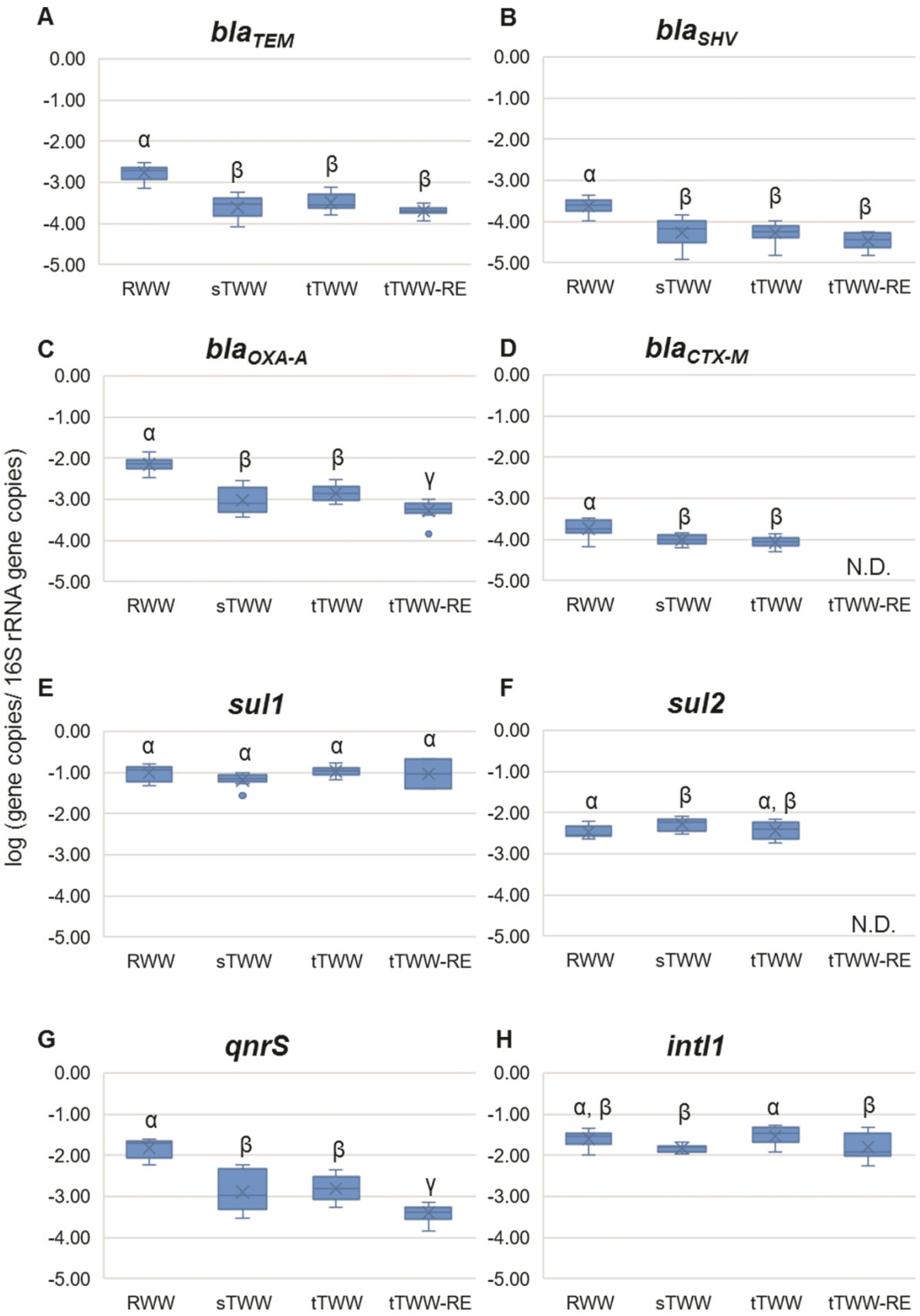
Phylum	**Family**	**OTU ID**	***qnrS***	***bla*** *TEM*	***bla*** *CTX-M*	***bla*** *SHV*	***bla*** *OXA-A*	***intI1***	***sul1***	***sul2***	**RWW (%)**	**sTWW (%)**	**tTWW (%)**	**tTWW-RE (%)**
Bacteroidetes	*Bacteroidaceae*	100					X		X		0.1501	0.0538	0.0542	0.0170
		1118	X	X		X					0.0555	0.0092	0.0108	0.0019
		1246					X				0.0844	0.0195	0.0187	0.0037
		1442					X		X		0.0581	0.0115	0.0095	0.0024
		3252							X		0.0376	0.0042	0.0012	n.a.
		Sub-total									**0.3857**	**0.0982**	**0.0944**	**0.0250**
	Porphyromonadaceae	1374					X		X		0.0397	0.0003	0.0024	n.a.
	[Weeksellaceae]	115							X		0.1374	0.0640	0.0613	0.0308
		700						X	X		0.0669	0.0219	0.0207	0.0088
		Sub-total									**0.2440**	**0.0862**	**0.0844**	**0.0396**
Firmicutes	*Streptococcaceae*	666							X		0.0779	0.0072	0.0108	0.0031
	Lachnospiraceae	1647		X			X	X	X		0.0341	0.0043	0.0034	0.0003
		1840			X	X					0.0406	0.0030	0.0016	0.0016
		Sub-total									**0.1526**	**0.0145**	**0.0158**	**0.0050**
	Ruminococcaceae	1260	X	X		X		X			0.0713	0.0140	0.0148	0.0061
		2127		X		X		X			0.0342	0.0035	0.0071	0.0007
		2755	X	X		X		X			0.0357	0.0089	0.0093	0.0036
		597	X	X		X					0.0737	0.0039	0.0174	0.0016

		Sub-total									**0.2149**	**0.0303**	**0.0486**	**0.0120**
	Unclassified *Clostridiales*	2696	X	X		X	X	X	X		0.0327	0.0047	0.0055	0.0031
	Veillonellaceae	612							X		0.0770	0.0122	0.0160	0.0041
	Erysipelotrichaceae	1187	X			X					0.0561	0.0029	0.0107	0.0013
		1813	X	X		X		X			0.0373	0.0026	0.0079	0.0003
		1967	X	X		X		X			0.0364	0.0038	0.0065	0.0010
		Sub-total									**0.1298**	**0.0093**	**0.0251**	**0.0026**
Proteobacteria	*Comamonadaceae*	127		X			X	X			0.1415	0.0372	0.0509	0.0206
		1286		X				X	X		0.0406	0.0015	0.0049	0.0010
		1414				X					0.0335	0.0032	0.0083	0.0007
		1885					X	X	X		0.0445	0.0061	0.0114	0.0060
		2925						X			0.0364	0.0075	0.0093	0.0065
		3048							X		0.0329	0.0025	0.0060	0.0010
		438	X	X		X	X	X			0.1115	0.0385	0.0334	0.0239
		877					X				0.0635	0.0071	0.0114	0.0051
		93		X			X	X	X		0.1433	0.0498	0.0566	0.0419
		957		X			X	X	X		0.0760	0.0118	0.0140	0.0050
		Sub-total									**0.7237**	**0.1652**	**0.2062**	**0.1117**
	Neisseriaceae	1443	X	X	X	X	X	X			0.0342	0.0028	0.0025	0.0009
		1690	X	X		X					0.0382	0.0035	0.0097	0.0013
		790		X				X	X		0.0637	0.0118	0.0142	0.0031

		819							X		0.0588	0.0028	0.0071	0.0016
		Sub-total									**0.1949**	**0.0209**	**0.0335**	**0.0069**
	Procabacteriaceae	260	X	X							0.1049	0.0261	0.0297	0.0128
	Rhodocyclaceae	1582		X							0.0428	0.0066	0.0025	0.0013
		1701							X		0.0419	0.0083	0.0087	0.0050
		794					X				0.0548	0.0076	0.0098	0.0013
		954							X		0.0585	0.0031	0.0030	0.0013
		Sub-total									**0.1980**	**0.0256**	**0.0240**	**0.0089**
	Campylobacteraceae	1		X				X	X		0.2575	0.1110	0.1346	0.0657
		103							X		0.1410	0.0385	0.0523	0.0130
		1148		X				X	X		0.0969	0.0147	0.0296	0.0039
		21						X	X		0.2036	0.1002	0.0951	0.0550
		3242	X	X		X	X	X			0.0584	0.0094	0.0134	0.0019
		4236		X				X	X		0.0893	0.0168	0.0240	0.0036
		572						X	X		0.1519	0.0214	0.0606	0.0049
		750		X				X	X		0.1089	0.0287	0.0364	0.0067
		868		X				X	X		1.2405	0.3862	0.4962	0.1729
		Sub-total									**1.2405**	**0.3862**	**0.4962**	**0.1729**
Proteobacteria	*Aeromonadaceae*	1389					X				0.0431	0.0075	0.0110	0.0028
		139	X	X		X					0.1300	0.0464	0.0505	0.0195
		1732							X		0.0454	0.0046	0.0131	0.0025
		238	X	X		X		X			0.1271	0.0289	0.0460	0.0069

		512	X	X		X		X			0.1111	0.0458	0.0358	0.0156
		862	X	X		X		X			0.0820	0.0246	0.0229	0.0056
		976				X					0.0794	0.0219	0.0196	0.0043
		Sub-total									**0.6181**	**0.1797**	**0.1989**	**0.0572**
	Shewanellaceae	847					X				0.0663	0.0142	0.0112	0.0056
	Moraxellaceae	1069		X		X	X	X			0.0762	0.0164	0.0097	0.0082
		1084			X	X					0.0671	0.0052	0.0074	0.0040
		1098							X		0.0459	0.0099	0.0031	0.0016
		1154		X			X	X			0.0554	0.0035	0.0044	0.0016
		1203	X			X					0.0568	0.0117	0.0159	0.0036
		1244	X	X		X	X	X		X	0.0560	0.0041	0.0031	0.0013
		1465	X			X					0.0406	0.0059	0.0078	0.0013
		1643							X		0.0359	0.0060	0.0013	0.0003
		2084	X	X		X		X			0.0628	0.0127	0.0099	0.0037
		228							X		0.1185	0.0244	0.0302	0.0080
		2538	X	X		X					0.0327	0.0041	0.0046	0.0007
		2726	X	X		X					0.0357	0.0112	0.0064	0.0043
		326	X	X		X					0.1075	0.0068	0.0076	0.0020
		3573		X		X	X	X			0.0361	0.0073	0.0013	0.0027
		3744			X	X					0.0443	0.0028	0.0019	0.0016
		713	X	X		X					0.0603	0.0049	0.0072	0.0016
		741	X	X		X	X	X			0.0906	0.0261	0.0154	0.0158
		857	X	X		X					0.0981	0.0202	0.0233	0.0053

		98			X						0.1500	0.0393	0.0465	0.0187
		Sub-total									**1.3368**	**0.2367**	**0.2182**	**0.0919**
	Pseudomonadaceae	1192	X	X		X					0.0385	0.0025	0.0058	0.0035
		2029	X	X							0.0331	0.0016	0.0050	0.0015
		Sub-total									**0.0716**	**0.0041**	**0.0108**	**0.0050**
TOTAL											**5.7250**	**1.3001**	**1.5072**	**0.5588**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H
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log (gene copies/ 16S rRNA gene copies)
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-1.00
-2.00
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RWW
sTWW
tTWW
tTWW-RE
α
β
γ
α, β
N.D.

Fig. S1 – Prevalence of the genes analysed (gene copies / 16S rRNA gene copy number) in raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE). Data corresponds to average values of three sampling campaigns. Gene prevalences: (A) bla_{TEM}; (B) bla_{SHV}; (C) $bla_{OXA\text{-}A}$; (D) $bla_{CTX\text{-}M}$; (E) *sul1*; (F) *sul2*; (G) *qnrS*; and (H) *intI1*.α, β and γ indicate significantly ($p < 0.01$) different Tukey's groups comparing the different types of water. N.D. indicate that data was not determined for the sample

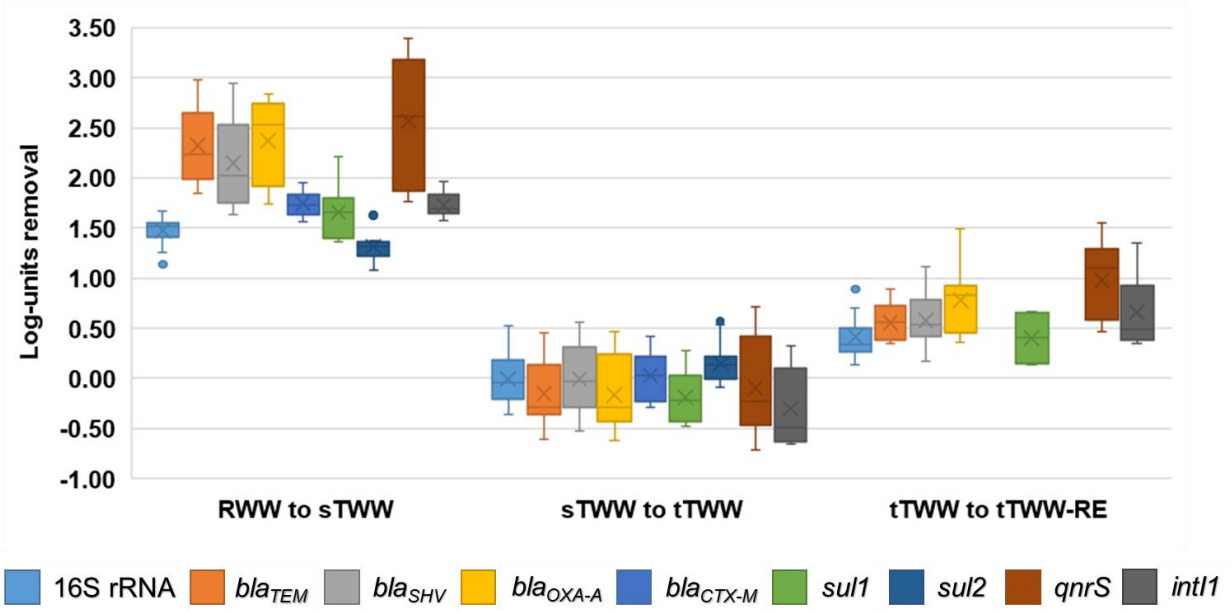

Fig. S2 – Removal values calculated for gene abundance (gene copy number / mL) in raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE). Data correspond to average values of three sampling campaigns.

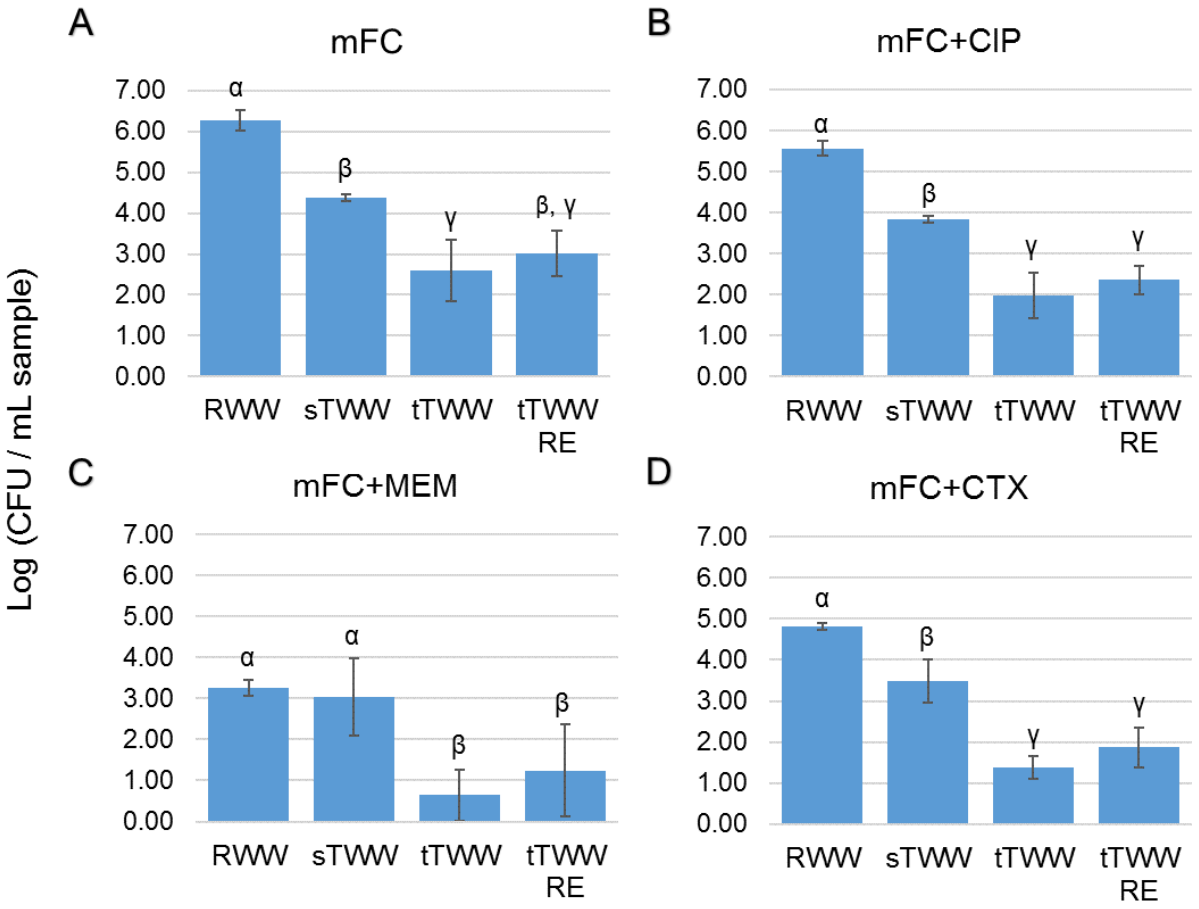

Figure S3 – Counts of CFUs per volume of sample (mL) in raw wastewater (RWW), in wastewater subjected to secondary and tertiary treatment (sTWW and tTWW) and in wastewater subjected to tertiary treatment incubated during 3 days (tTWW-RE) of an urban wastewater treatment plant. CFUs were analysed at 24hrs in (**A**) mFC, (**B**) mFC with ciprofloxacin (CIP, 1 mg/L), (**C**) mFC with meropenem (MEM, 4 mg/L), and (**D**) mFC with cefotaxime (CTX, 8 mg/L). α, β, and γ indicate significantly ($p < 0.01$) different Tukey's groups comparing the different types of water.

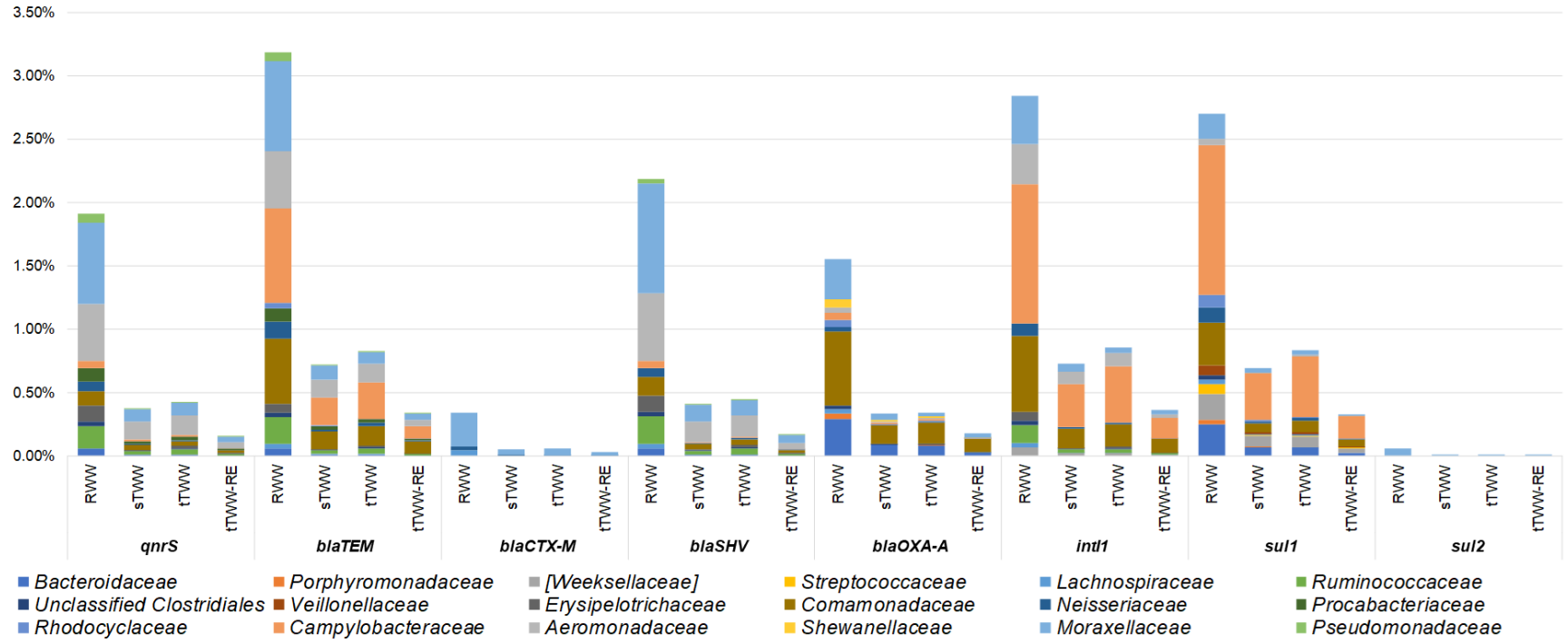

Fig. S4. – Relative abundances for the most abundant OTUs correlated with the ARGs, for the different types of wastewater – raw wastewater (RWW), secondary effluent (sTWW), after UV disinfection (tTWW) and tTWW after 3 days storage in the dark (tTWW-RE).

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