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

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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_{CTX-M}, bla_{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.

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

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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., 2015a, 2015b; 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): 222470 kg; biological oxygen demand (BOD5): 211100 kg; total suspended solids (TSS): 14460 kg; total nitrogen (Kjeldahl): 2550 kg; and total phosphorous: 500 kg (Table S1). The treatment includes a homogenization chamber and a bar screen to remove gross solids; a grit and a grease removal chambers, to remove small solids and fats; a primary settling tank to remove the settleable solids; an activated sludge biological treatment, with recirculation between the aerobic and anoxic tanks for removing the organic load and nutrients (N and P); and an open channel UV system (Trojan, UV3000HO), with 38 × 8 150 W lamps per channel and a contact time of 11.44 s, corresponding to a dose of 29.7 mJ/cm^2.

Over three sampling campaigns conducted at Tuesdays - 16th June (F1), 14th July (F2), and 15th September 2015 (F3) - grab wastewater samples were collected from raw wastewater after the first settling tank (RWW; 1 L), secondary treated wastewater (sTWW; 6 L), and tertiary treated wastewater collected after UV disinfection (tTWW; 10 L). The samples were collected in sterile flasks, transported to the lab in refrigerated containers and processed within 12 h. For regrowth assays (tTWW-RE), 1 L of tTWW was transferred to a sterile flask and incubated 3 days at 20 °C in the dark. All samples were processed (filtration and DNA extraction) and analysed (bacterial communities and target genes) in triplicate.

2.2. Enumeration of culturable enterobacteria

Culturable enterobacteria, the most commonly used indicator to assess microbiological water quality, were enumerated in parallel with selected antibiotic resistant bacteria. A volume of 1 mL of wastewater or of the adequate serial dilution was filtered through cellulose nitrate membranes (0.22 µm porosity; Sartorius Stedim Biotech, Göttingen, Germany), placed onto the adequate culture medium and incubated at 30 °C for 24 h. The culture medium used was the 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.