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Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater

Ana Novo[a], *Sandra André*[b], *Paula Viana*[b], *Olga C. Nunes*[c], *Célia M. Manaia*[a,*]

[a] *CBQF, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal*
[b] *Agência Portuguesa do Ambiente, I.P., 2610-124 Amadora, Portugal*
[c] *LEPAE, Dpto Engenharia Química, Faculdade de Engenharia, Universidade do Porto, 4200-465 Porto, Portugal*

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ABSTRACT

This study was based on the hypothesis that the occurrence of antimicrobial residues and antibiotic resistant bacteria in the sewage could be correlated with the structure and composition of the bacterial community and the antibiotic resistance loads of the final effluent. Raw and treated wastewater composite samples were collected from an urban treatment plant over 14 sampling dates. Samples were characterized for the i) occurrence of tetracyclines, penicillins, sulfonamides, quinolones, triclosan, arsenic, cadmium, lead, chromium and mercury; ii) antibiotic resistance percentages for tetracycline, sulfamethoxazole, ciprofloxacin and amoxicillin and iii) 16S rRNA gene-DGGE patterns. The data of corresponding samples, taking into account the hydraulic residence time, was analyzed using multivariate analysis.

Variations on the bacterial community structure of the final effluent were significantly correlated with the occurrence of tetracyclines, penicillins, sulfonamides, quinolones and triclosan in the raw inflow. Members of the class *Epsilonproteobacteria* presented positive correlations with those antimicrobials, whereas negative correlations were observed with *Beta* and *Gammaproteobacteria* and *Firmicutes*. Antibiotic resistance percentages presented different trends of variation in heterotrophs/enterobacteria and in enterococci, varied over time and after wastewater treatment. Antibiotic resistance was positively correlated with the occurrence of tetracyclines residues and high temperature. A relationship between antibiotic residues, bacterial community structure and composition and antibiotic resistance is demonstrated. Further studies, involving more wastewater treatment plants may help to elucidate this complex relationship.

1. Introduction

Wastewater treatment plants are considered important hotspots for antibiotic resistance spreading (Baquero et al., 2008; Martinez, 2009; Manaia et al., 2012). Three major arguments are often used to sustain this idea. The first is that antibiotic residues and other substances with potential selective pressure, antibiotic resistant bacteria and resistance genes are heavily discharged into the municipal sewage system (Kim and Aga, 2007; Segura et al., 2009; Novo and Manaia, 2010;

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* *Corresponding author*. Tel.: +351 22 5580059; fax: +351 22 5090351.
E-mail address: cmanaia@porto.ucp.pt (C.M. Manaia).

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Luczkiewicz et al., 2010; Kümmerer, 2009; Szczepanowski et al., 2009). The second is that conditions offered to microorganisms during the wastewater treatment process may favor either the selection or the horizontal gene transfer of antibiotic resistance determinants (Szczepanowski et al., 2009; Zhang et al., 2011). The third, is the observation that, worldwide, wastewater treatment, regardless of its efficiency or operational conditions, leads to the production of final effluents containing antibiotic resistant bacteria, sometimes at higher percentages that in the raw inflow (Ferreira da Silva et al., 2006, 2007; Watkinson et al., 2007; Novo and Manaia, 2010; Luczkiewicz et al., 2010; Galvin et al., 2010).

The factors that explain these evidences are at the epicenter of the concerns of microbiologists and wastewater engineers interested in controlling antibiotic resistance increase and spreading into the natural environment. Nevertheless, unfortunately, the knowledge in this area is still limited. Further insights into this topic would contribute to establish adequate control measures and to achieve significant reductions on the prevalence of antibiotic resistant bacteria in treated wastewaters. In general, it is possible to assume that three major categories of factors may influence the fate of antibiotic resistant bacteria during wastewater treatment, the abiotic conditions, the composition and structure of the bacterial community and the presence of possible selective pressure factors. Among the abiotic conditions, factors such as the organic matter load, the temperature or the water flow would be hypothesized as those more relevant. Wastewater bacterial communities comprise mainly members of the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*, most of which are not easily cultivated (Zhang et al., 2012). The dynamics of the different bacterial lineages thriving in wastewater systems may be influenced by a myriad of biotic and abiotic variables (Zhang et al., 2012) and to vary during wastewater treatment. In this respect, selective pressures, exerted by antibiotic residues and heavy metals, even at low concentrations, may be very important (Martinez, 2009; Andersson and Hughes, 2011). Nevertheless, the knowledge in this area is still scant. Indeed, the relationship between bacterial community structure and composition variations and measurable indicators of antibiotic resistance in wastewaters are, to the best of our knowledge, unknown. Further insights into this relationship may contribute to elucidate the role of the so-called uncultivable bacteria on the maintenance and spreading of antibiotic resistance in wastewaters.

A factor widely referred to influence the bacterial communities and promote the proliferation of antibiotic resistant bacteria is the presence of micropollutants, in particular antibiotic residues and heavy metals (Baquero et al., 2008; Martinez, 2009; Skurnik et al., 2010; Graham et al., 2011). Indeed, the occurrence of antibiotic residues in wastewaters is documented (e.g. Segura et al., 2009). Although in literature the potential selective pressure of sub-inhibitory concentrations of antibiotic residues found in wastewaters is often assumed, experimental evidences are rare and difficult to obtain (Graham et al., 2011). Nevertheless, based on *in silico* modeling evidences using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) database, Tello et al. (2012) demonstrated that even at low concentrations antibiotics may exert a selective pressure effect.

In summary, it is possible to conclude that although many publications describe the occurrence of antibiotic residues in wastewaters, and many others explore the occurrence of antibiotic resistance in wastewaters, studies on the influence of one on the extent of the other are, to the best of our knowledge, inexistent. In spite of this apparent gap, over the last decade it became evident that assessments on antibiotic resistance maintenance and spreading in the environment will benefit from multi-parametric analyses, as those often used in ecology studies (e.g. Lopes et al. 2011; Wang et al., 2012). The current study was designed to assess the influence of abiotic factors (e.g. temperature, water flow, antibiotic residues) on the levels of antibiotic resistance and bacterial community structure of the final effluent. Possible correlations between cultivable populations of antibiotic resistant bacteria and the 16S rRNA-DGGE based bacterial community composition were also explored.

2. Materials and methods

2.1. *Sampling*

This study was conducted in a municipal wastewater treatment plant characterized in previous studies (Ferreira da Silva et al., 2006, 2007; Novo and Manaia, 2010). Briefly, this plant serves about 100 000 inhabitants equivalent and beside the municipal sewage, receives about 30% of pre-treated industrial wastewaters (mainly from food-industry and animal farming). Over a period of about 12 h of hydraulic residence time, treatment includes a primary settling tank to remove the settleable solids, an activated sludge biological process, and a secondary settling tank to remove the biomass and other suspended particles. The resultant final treated effluent (annual average of 18 000 m^3 day^{-1}) is discharged into a natural water stream.

A total of 14 24 h composite samples of raw (after the primary settling tank) and treated wastewater was collected in glass sterile bottles and in polypropylene flasks, refrigerated transported to the lab and analyzed within 12 h. Sampling dates comprised three periods, in 2008 and 2009, of two or four consecutive days (from Tuesday to Friday). In order to assess possible seasonal variations, samples were collected in the periods 25–28th November, and 4–5th December (Autumn, P1), in 31st March–3rd April (early Spring, P2) and 21st–24th April (Spring, P3) (Table 1).

2.2. *Enumeration of cultivable bacteria*

The enumeration of total and antibiotic resistant bacteria was made based on the membrane filtration method as described by Novo and Manaia (2010). Briefly, membranes through which were filtered 1–10 mL of adequate decimal dilutions of wastewater samples were incubated on different culture media for enumeration of bacteria: plate count agar (PCA, Pronadisa), 24 h at 30 °C for heterotrophs; m-Faecal Coliforms agar (m-FC, Difco), 24 h at 37 °C for enterobacteria and m-Enterococcus agar (m-Ent, Difco) 48 h at 37 °C for enterococci. The corresponding antibiotic resistant subpopulations were enumerated on the same media supplemented with the