



Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem

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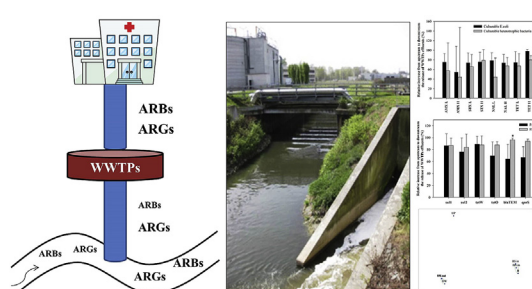
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HIGHLIGHTS

- Brussels WWTPs efficiently remove antibiotic resistant bacteria (ARB).
- Significant increase of ARB was found downstream from the WWTPs outfalls.
- Absolute ARGs abundances are reduced from influents to effluents of both WWTPs.
- Significant increase of ARGs was found downstream from the WWTPs outfalls.
- Some ARGs relative abundance significantly increased in the effluent of WWTPs.

GRAPHICAL ABSTRACT



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ABSTRACT

The main objective of this study was to investigate the antibiotic resistance (AR) levels in wastewater (WW) and the impact on the receiving river. Samples were collected once per season over one year in the WW of a hospital, in the raw and treated WW of two wastewater treatment plants (WWTPs), as well as upstream and downstream from the release of WWTPs effluents into the Zenne River (Belgium). Culture-dependent methods were used to quantify *Escherichia coli* and heterotrophic bacteria resistant to amoxicillin, sulfamethoxazole, nalidixic acid and tetracycline. Six antibiotic resistance genes (ARGs) were quantified in both particle-attached (PAB) and free-living (FLB) bacteria. Our results showed that WWTPs efficiently removed antibiotic resistant bacteria (ARB) regardless of its AR profile. The ARGs levels were the highest in the hospital WW and were significantly reduced in both WWTPs. However, ARB and ARGs abundances significantly increased into the Zenne River downstream from the WWTPs outfalls. The variation in the relative abundance of ARGs through WW treatment differed depending on the WWTP, fraction, and gene considered. The *sul1* and *sul2* genes in PAB fraction showed significantly higher relative abundances in the effluent compared to the influent of both WWTPs. This study demonstrated that WWTPs could be hotspots for AR spread with significant impacts on receiving freshwater ecosystems. This was the first comprehensive study investigating at the same time antibiotics occurrence, fecal

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bacteria indicators, heterotrophic bacterial communities, and ARGs (distinguishing PAB and FLB) to assess AR levels in WW and impacts on the receiving river.

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1. Introduction

Antibiotics have saved millions of human lives since their discovery and application to treating bacterial infectious diseases. However, extensive use of antibiotics has led to an increased prevalence of antibiotic-resistant bacteria (ARB) (Levy and Marshall, 2004). Antibiotic resistance (AR) has been classified by the World Health Organization as one of the three greatest threats to public health in the 21st century and the latest report from the UK Review on Antimicrobial Resistance, published recently (O'Neill, 2016), estimates that the 700,000 annual deaths currently attributable to infections by drug-resistant pathogens will increase, if unchecked, to 10 million by 2050 (Robinson et al., 2016).

Wastewater treatment plants (WWTPs) receive sewage from various sources, including hospitals and households, which are both important sources of antibiotics and ARB (Laht et al., 2014). Hospital effluents, in particular, constitute a special category of waste that is highly hazardous because they contain a myriad of drug residues and infectious agents and are thus an important source of multidrug-resistant bacteria and antibiotics (Rodriguez-Mozaz et al., 2015). Between 20 and 97% of any dose of most antibiotics administered to humans and animals is excreted as an active substance, consequently reaching wastewaters (Jelic et al., 2015). On the other hand, during therapeutic treatment the human gut microbiota is exposed to high concentrations of antibiotics that may stimulate the generation of resistance phenotypes before being released into sewage via human excreta (Servais and Passerat, 2009). In fact, the presence of antibiotics, ARB, and antibiotic resistance genes (ARGs) has been confirmed in many WWTPs worldwide (Michael et al., 2013; Rizzo et al., 2013; Zanotto et al., 2016).

WWTPs are considered important hotspots for the acquisition and spread of antibiotic resistance in the environment and three major reasons are often put forward to sustain this idea: i) the heavy discharge of antibiotic residues, ARB, and ARGs collected in the municipal sewage system; ii) the favorable conditions for both selection and/or horizontal transfer of resistance genes among bacterial cells during the wastewater treatment process; and iii) the widespread observation that WWTP effluents contain high AR levels (sometimes higher than in the raw inflow) (Novo et al., 2013). As a consequence, WWTP effluents are among the most important conduits for the spread of AR to aquatic environments.

Many studies have investigated the fate of antibiotics through wastewater (WW) treatment (Jelic et al., 2015; Michael et al., 2013), whereas many others have focused on the ARG responses to WW treatment, sometimes considering the receiving environment (Ben et al., 2017; Neudorf et al., 2017; Rafraf et al., 2016; Rizzo et al., 2013). Moreover, several studies have analyzed the occurrence and fate of AR bacteria in WWTPs (Bouki et al., 2013; Łuczkiwicz et al., 2010). Despite a considerable amount of research carried out combining the investigation of antibiotics and ARGs (Caucci et al., 2016; Rodriguez-Mozaz et al., 2015; Subirats et al., 2017) as well as ARB and ARGs (Yuan et al., 2015; Zanotto et al., 2016; Zhang et al., 2015) along the WW treatment process, comprehensive studies assessing the fate of antibiotics, ARB, and ARGs in WWTPs and its eventual influence on receiving water bodies are still lacking.

The main objective of this study was to fill this gap investigating

the level of WW contamination by antibiotics, the prevalence of AR in WW, and their effects on the receiving river, focusing at the same time on the fecal bacteria *Escherichia coli*, heterotrophic bacterial communities, and their ARGs distinguishing between particle-attached (PAB) and free-living (FLB) bacterial fractions. *E. coli* was selected as a fecal indicator bacterium that can be exposed to high antibiotic concentrations in the human or animal gastrointestinal tract and acquire resistance before being released into sewer systems and finally reaching WWTPs. The fecal bacteria can thus act as a source of resistance because they can disseminate ARGs to autochthonous bacteria (Baquero et al., 2008). Moreover, continuous release of antibiotics in WWTPs can act as chronic selective pressure able to promote AR (Gullberg et al., 2011).

The specific questions investigated in this study focused on:

- The concentration of antibiotic residues, ARB, and ARGs in raw and treated wastewaters (urban and hospital);
- The impact of WW treatment (secondary and tertiary treatments) on the abundance of antibiotics, ARB, and ARGs
- The eventual impacts of the WWTP effluent release into the receiving river
- The fate of ARGs in WW and receiving river depending on the bacterial fraction considered (PAB and FLB)

To reach these goals, samples were collected over 1 year (one sampling per season) in the WW of a hospital, in the raw and treated WW of the two Brussels (Belgium) WWTPs, as well as at two sites located upstream and downstream from the release of the two WWTP effluents into the receiving river. Culture-dependent and -independent methods were used to estimate the resistance of culturable *E. coli* and heterotrophic bacteria by plate counts containing antibiotics as well as by quantifying the abundance of six genes conferring resistance to the main antibiotic families. The selection of the antibiotics (and the respective genes) was done according to the specific objectives of the study, therefore including relevant clinical antibiotics for which resistance has been reported elsewhere. Since different behaviors are expected in response to the settling stage applied as the first treatment stage in WWTPs, ARGs were analyzed in both PAB and FLB. Most particularly, higher removal rates were hypothesized for ARGs in PAB with respect to the FLB fraction. Finally, considering that the close contact between cells attached to the same particle would increase the probability of an exchange of genetic material encoding resistance, it is important to distinguish the fate of ARGs in these two fractions during WW treatment and in the receiving water bodies.

2. Material and methods

2.1. Study sites and sampling strategy

In this study, the two WWTPs located in the Brussels Capital Region (Belgium) were investigated: the Brussels South (BS) WWTP (360,000 equivalent-inhabitants) in operation since the year 2000 and the Brussels North (BN) WWTP (1.1 million equivalent-inhabitants) in operation since 2007. The two WWTPs function on different technologies. The BS WWTP treatment line includes a primary settling stage (to remove suspended solids) and a

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