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Prevalence of antibiotic-resistant fecal bacteria in a river impacted by both an antibiotic production plant and urban treated discharges

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HIGHLIGHTS

- Domestic effluents increased fecal bacteria concentration in the river.
- Antibiotic production effluents increased the prevalence of antibiotic resistance.
- Multirresistant *E. coli* increased in the river after both industrial and domestic effluents.

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ABSTRACT

In this study, the abundance and spatial dynamics of antibiotic-resistant fecal bacteria (*Escherichia coli*, total coliforms and *Enterococcus* spp.) were determined in water and sediment samples from a river impacted by both antibiotic production plant (APP) and urban wastewater treatment plant (WWTP) discharges. Agar dilution and disk diffusion methods were also used for antimicrobial susceptibility testing. Two antimicrobial agents, cephalexin (25 μg/ml) and amoxicillin (50 μg/ml), were evaluated using the agar dilution method for *E. coli*, total coliforms (TC) and *Enterococcus* spp., whereas the degree of sensitivity or resistance of *E. coli* isolates to penicillin (10 U), ampicillin (10 μg), doxycycline (30 μg), tetracycline (30 μg), erythromycin (15 μg), azithromycin (15 μg) and streptomycin (10 μg) was performed using the disk diffusion method. Real-time PCR assays were used to determine the prevalence of three antibiotic-resistance genes (ARGs). The agar dilution method showed that most *E. coli* isolates and TC were resistant to amoxicillin, especially after receiving the APP discharges. Antibiotic resistances to amoxicillin and cephalexin were higher after the APP discharge point than after the WWTP effluent. The disk diffusion method revealed that 100% of bacterial isolates were resistant to penicillin and erythromycin. Multidrug-resistant bacteria were detected and showed a higher proportion at the WWTP discharge point than those in the APP. Highly multidrug-resistant bacteria (resistance to more than 4 antibiotics) were also detected, reaching mean values of 41.6% in water samples and 50.1% in sediments. The relative abundance of the bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$ and bla_{SHV} genes was higher in samples from the treatment plants than in those collected upstream from the discharges, especially for water samples collected at the APP discharge point. These results clearly demonstrate that both the APP and the WWTP contribute to the emergence and spread of antibiotic resistance in the environment.

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1. Introduction

Following the introduction of antibiotics into medical practice in the second half of the twentieth century, the over- and misuse of these compounds in human and veterinary medicine, animal husbandry, agriculture, aquaculture and food technology have resulted in an increase in antibiotic resistance and multidrug-resistant bacteria (Barbosa and Levy, 2000; Baquero et al., 2008).

Several studies suggest that antibiotic resistance detected in clinical settings is closely associated with mechanisms found in environmental bacteria. In fact, aquatic ecosystems may provide an ideal setting for the acquisition and spread of antibiotic resistance, because they are constantly exposed to anthropogenic environmental changes such as pollution from urban, agricultural, and industrial sources (Koczura et al., 2012, 2013; Korzeniewska et al., 2013; Marti et al., 2013).

Fecal bacteria may reach aquatic ecosystems not only by release of wastewater, but also through surface runoff and soil leaching (Servais

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and Passerat, 2009). The presence of fecal bacteria, including pathogens, in these ecosystems may be harmful to public health with a greater risk when bacteria are resistant to antibiotics. Antibiotic-resistant fecal bacteria can also transfer the resistance to autochthonous bacteria via horizontal gene transfer, which is responsible for the development of antibiotic-resistant bacteria (Davison, 1999). This together with the direct discharge of antibiotics or their metabolites (Hijosa-Valsero et al., 2011) increases selective pressure on bacteria, favoring the emergence and spread of antibiotic resistance.

Previous studies have demonstrated that antibiotic-resistant fecal bacteria may be found in different aquatic ecosystems including rivers (Boon and Cattanach, 1999; Ash et al., 2002; Watkinson et al., 2007a; Tao et al., 2010), estuaries (Parveen et al., 1997), lakes (Edge and Hill, 2005) and coastal areas (Kimiran-Erdem et al., 2007; de Oliveira and Pinhata, 2008). Several studies have also reported high rates of antibiotic-resistant bacteria in wastewater environments (Reinthaler et al., 2003; da Costa et al., 2006; Korzeniewska et al., 2013; Harris et al., 2014). However, the effect of industrial discharges, especially those from antibiotic manufacturing facilities, has only been partly explored. These discharges may be an important reservoir of antibiotic-resistant bacteria and antibiotic resistance genes (ARGs) due to the constant interaction between bacterial populations and antibiotic residues. Given this, the presence of both urban and industrial discharges, separated from each other by less than 1 km makes the study area particularly interesting for studying the effect of anthropogenic activities on the emergence and prevalence of antibiotic resistance.

The aims of this study were therefore to describe the abundance of antibiotic-resistant fecal bacteria and ARGs in water and sediment samples from a river receiving both urban and antibiotic production plant discharges and to study the variation rates of antibiotic resistance and number of bacteria along the river.

2. Materials and methods

2.1. Study site

Water and sediment samples were collected in the Bernesga River downstream from the city of León (Northwest Spain). The urban wastewater treatment plant (WWTP), a conventional activated sludge system treating waste from a population of 250,000 inhabitants including a hospital, is located 1 km downstream of a cephalexin and amoxicillin production plant (APP) with its own activated sludge treatment facility. Six samples were taken from different sites along the river, as well as two samples from the effluents of both treatment plants (Fig. 1) on three different days from August 20th to September 10th, 2010. There are no more discharges in the study area, the nearest one being 10 km upstream from sampling site 1 due to the presence of a small village.

2.2. Sample collection

Water samples were taken with a core along the whole width and depth of the river at each of the sampling sites and pooled to obtain a representative sample at that point. Sediments were also collected with a core, but, because of river morphology, could only be collected in the river areas where they accumulated.

2.3. Flow measurements

To calculate the river flow, current speed and depth were measured every 5 m in transects across the river. A model 2030R flowmeter (General Oceanics Inc.; Miami, FL, USA) was used to measure the current speed. River flow was measured at sampling sites 1 and 6, whose values were used to estimate the flow at the other points.

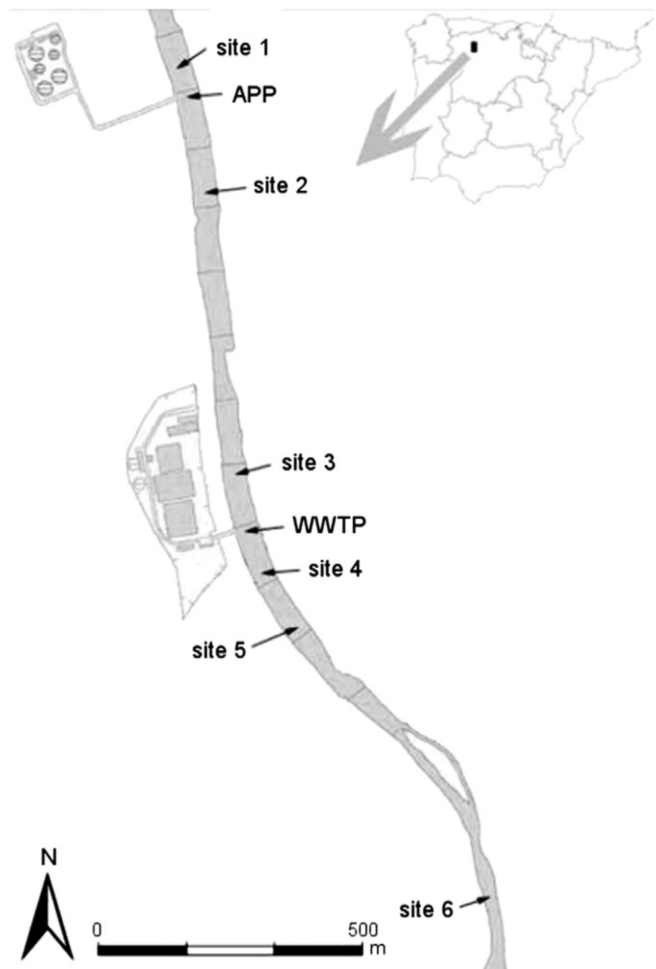

Fig. 1. Location of sampling sites in the Bernesga river. APP: antibiotic production plant; WWTP: urban wastewater treatment plant.

2.4. Enumeration and isolation of bacterial indicators

For the enumeration of *Escherichia coli* and total coliforms (TC), water samples were ten-fold diluted in sterile saline solution (0.4% NaCl) and filtered through a 0.45 μm membrane (Millipore; Darmstadt, Germany). The filters, in duplicate, were then placed on the culture media and incubated at 37 °C for 24 h (*E. coli* and TC) or 48 h (*Enterococcus* spp.) (APHA, 1999). Sediment samples were weighed (100 g each), added to a sterile phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) solution (ratio 50:50 w/v) and shaken to wash out and extract the bacteria; the supernatant was processed in the same way as the water samples (de Oliveira and Pinhata, 2008). For enumeration of sulfite-reducing *Clostridium*, 2 ml of water or sediment supernatant was mixed with the culture medium and incubated in an anaerobic atmosphere for 24 h (APHA, 1999).

Chromocult coliform agar (Merck; Darmstadt, Germany) was used for the isolation of *E. coli* and TC, membrane-filter *Enterococcus* selective (SB) agar (Merck) was used for *Enterococcus* spp., and sulfite polymyxin sulfadiazine (SPS) agar was used for sulfite-reducing *Clostridium* (Merck).

2.5. Antimicrobial susceptibility testing

Two methods were used to test the antimicrobial susceptibility, antibiotic dilution method and disk diffusion method. *E. coli* strain CECT 516 was used as a control following the criteria established by

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