Environmental Pollution 214 (2016) 635–645

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Ciprofloxacin residue and antibiotic-resistant biofilm bacteria in hospital effluent☆

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ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form
6 April 2016
Accepted 9 April 2016

Keywords:
Antibioresistance
Biofilm
Effluent hospital
POCIS
fluoroquinolones

ABSTRACT

Discharge of antimicrobial residues and resistant bacteria in hospital effluents is supposed to have strong impacts on the spread of antibiotic resistant bacteria in the environment. This study aimed to characterize the effluents of the Gabriel Montpied teaching hospital, Clermont-Ferrand, France, by simultaneously measuring the concentration of ciprofloxacin and of biological indicators resistant to this molecule in biofilms formed in the hospital effluent and by comparing these data to ciprofloxacin consumption and resistant bacterial isolates of the hospital. Determination of the measured environmental concentration of ciprofloxacin by spot sampling and polar organic chemical integrative (POCIS) sampling over 2 weeks, and comparison with predicted environmental concentrations produced a hazard quotient >1, indicating a potential ecotoxicological risk. A negative impact was also observed with whole hospital effluent samples using the *Tetrahymena pyriformis* biological model.

During the same period, biofilms were formed within the hospital effluent, and analysis of ciprofloxacin-resistant isolates indicated that *Gamma-Proteobacteria* were numerous, predominantly *Aeromonadaceae* (69.56%) and *Enterobacteriaceae* (22.61%). Among the 115 isolates collected, plasmid-mediated fluoroquinolone-resistant genes were detected, with mostly *aac(6′)-Ib-cr* and *qnrS*. In addition, 60% of the isolates were resistant to up to six antibiotics, including molecules mostly used in the hospital (aminosides and third-generation cephalosporins).

In parallel, 1247 bacteria isolated from hospitalized patients and resistant to at least one of the fluoroquinolones were collected. Only 5 of the 14 species identified in the effluent biofilm were also found in

Abbreviations: BOD_5, Biochemical Oxygen Demand five days; CFU, Colony Forming Unit; CIP, ciprofloxacin; COD, Chemical Oxygen Demand; DAPI, 4′,6′-diamidino-2-phenylindole; DOC, Dissolved Organic Carbon; ESBLs, Extended-Spectrum-Beta-Lactamases; FQs, Fluoroquinolones; HGT, Horizontal Gene Transfer; HQ, Hazard Quotient; IC, Inorganic Carbon; MEC, Measured Environmental Concentration; MIC, Minimum Inhibitory Concentration; NOEC, No Observed Effect Concentration; NT, Total Nitrogen; PEC, Predicted Environmental Concentration; PFGE, Pulsed Field Gel Electrophoresis; PhACs, Pharmaceutically Active Compounds; PI, Propidium Iodide; PMQR, Plasmid Mediated Quinolone Resistance; PNEC, Predicted No Effect Concentration; POCIS, Polar Organic Chemical Integrative Samplers; SEM, Scanning Electron Microscopy; TSS, Total Suspended Solid; TOC, Total Organic Carbon; WWTP, Waste Water Treatment Plant.

☆ This paper has been recommended for acceptance by Klaus Kummerer.

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http://dx.doi.org/10.1016/j.envpol.2016.04.033
0269-7491/

the clinical isolates, but PFGE typing of the Gram-negative isolates found in both compartments showed there was no clonality among the strains.

Altogether, these data confirm the role of hospital loads as sources of pollution for wastewater and question the role of environmental biofilms communities as efficient shelters for hospital-released resistance genes.

1. Introduction

The presence of pharmaceutically active compounds (PhACs) in wastewater has become a major concern for human health and the environment. In the European Union (EU), around 3000 different PhACs are used in human medicine, and many studies have shown their presence in wastewater and surface, ground and drinking water (Kümmerer, 2009; Petrovic et al., 2009; Stackelberg et al., 2004). Hospitals are large consumers of PhACs and use a wide variety of chemical substances such as pharmaceuticals, radionuclides, disinfectants and detergents for health care, diagnostic procedures, disinfection and research (Emmanuel et al., 2005; Kümmerer, 2001; Lindberg et al., 2007). After administration, the active substances, whether metabolized or not by the organism, are excreted, mainly in urine and partially in feces. Consequently, hospital effluents can contain high concentrations of pharmaceutical product, which are generally discharged directly into the public sewer network.

Since the discovery of penicillin by Sir Alexander Fleming 100 years ago, thousands of tons of antibiotics a year are administered to humans and animals. Beta-lactams and fluoroquinolones (FQ) are the most frequently prescribed families of antibiotics (80% of the molecules), and amongst the FQs, ciprofloxacin (CIP) and ofloxacin are the fourth and fifth most prescribed antibiotics in French hospitals (Agence Nationale de Sécurité du Médicament et des produits de Santé, 2014; RAISIN, 2012). Unlike beta-lactam molecules, FQs are able to persist in the environment (Al-Ahmad et al., 1999; Halling-Sørensen et al., 2000; Kümmerer et al., 2000), and several monitoring studies have reported their presence in urban wastewater and surface waters in Switzerland (Alder et al., 2004; Golet et al., 2002), Sweden (Lindberg et al., 2007) and Vietnam (Duong et al.,, 2008). It is likely that hospital effluents contribute largely to the load of antibiotics in waste water treatment plants (WWTP), where evidence shows that they are incompletely removed (Santos et al., 2013; Verlicchi et al., 2015, 2014, 2012).

Hospital effluents contain not only pharmaceutical molecules but also microorganisms from medical care units (patients' excreta and surfaces cleaning). Hospitals are characterized by high bacterial density and diversity, and studies on the bacterial load of hospital wastewaters have reported the presence of several species, mostly from the *Enterobacteriaceae family* (Harris et al., 2014; Tuméo et al., 2008), *Enterococci/Streptococci*, *Staphylococci* and heterotrophic bacteria (Schwartz et al., 2003). The effluents discharged by hospitals and clinics have a lower microbial load than urban wastewater but potentially contain pathogens and/or multidrug-resistant bacteria (Emmanuel et al., 2005; Leprat, 1998; Stalder et al., 2014; Czekalski et al., 2014). In aquatic systems the major fraction of bacterial communities are found in structurally organized surface associated aggregates called biofilms, where bacterial cells are embedded in an extracellular matrix. Owing to the proximity between bacteria, biofilms are considered as ideal reaction chambers for horizontal gene transfer, notably of antibiotic resistance genes (Cattoir et al., 2008; Costerton et al., 1987; Stalder et al., 2014), which results in accelerated evolution and spread of antibiotic resistance (Hausner and Wuertz, 1999; Hennequin et al., 2012). It has also been shown that weak concentrations of antibiotics (sub-MICs) could favor the transfer of resistance in biofilms (Lindberg et al., 2005; Savage et al., 2013). The biofilm matrix itself acts as a barrier against surrounding environmental stress such as antimicrobial agents (Costerton et al., 1987). Paired with the presence of pharmaceutical products, hospital effluent biofilms represent efficient exchange places between bacteria, whatever their origin, clinical or environmental.

In this study, we investigated the presence of ciprofloxacin in hospital effluent over 2 weeks and measured its ecotoxicological potential. In parallel, biofilms were formed *in situ* during this period and we looked for ciprofloxacin-resistant bacteria within these communities. All these data were compared with the quantity of ciprofloxacin consumed by the hospital's patients and to the ciprofloxacin-resistant bacteria isolated from hospitalized patients during the same period.

2. Materials and methods

2.1. Hospital effluent sampling and characterization

The samples were realized within the effluent of the Gabriel Montpied hospital, Clermont-Ferrand (139,500 inhabitants) France (latitude 45.7589632; longitude 3.0941279). It is a medium-size hospital with 1,100 beds that operates 24 h a day with a peak of activity from 7a.m to 1p.m. Water consumption is 13,500–16,000 m^3 monthly, between 409 and 478 L/bed/day. The hospital effluent is directly discharged into the urban sewage network without any external connection and then into the municipal WWTP, “Les Trois Rivières”, which receives and treats about 1,700,000 m^3 a month.

The experimental sampling tank (110 cm–50 cm–50 cm) was located on a line receiving exclusively wastewater from the hospital, upstream of the connection with the pipe recovering rainwater and public network wastewater. The water samples were pointwise collected three times a day, at 9am, 2pm and 4pm, from Monday to Friday for two weeks (04/9-23/2014), gathered in pre-rinsed polypropylene bottles (total volume, 2 L), and immediately transported to the laboratory under cooled conditions (4 °C). Over the same period, the pH, conductivity, temperature and oxygen level of the effluents were measured three times a day *in situ*. Temperature, pH and conductivity were detected with an Orion Star™ apparatus previously calibrated with standard solutions for pH (4.01 and 7.00 at 25 °C) and KCl (0.01 mol/L i.e. 1413 uS/cm and 12.9 mS/cm at 25 °C) for conductivity. The dissolved oxygen level was measured with an oximeter (Sonde oxygène Pro Oda, Ysi). Total suspended solid (TSS) was measured by evaporation of 30 mL of sample at 65 °C for 48H. Photometric test kits (Spectroquant COD cell Test©, Merck, Darmstadt, Germany) were used to determine chemical oxygen demand (COD) according to the standard ISO 15705 established by the Deutsches Institut for Normung. The BOD OxiTop method was used to measure the level of organic oxygen for five days (BOD_5 Biochemical Oxygen Demand five days). Samples were filtered (150 μm) and frozen (−20 °C) for further ion and