Water Research 126 (2017) 232–239

Contents lists available at ScienceDirect

Water Research

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

The fate of carbapenem-resistant bacteria in a wastewater treatment plant

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ARTICLE INFO

Article history:
Received 25 January 2017
Received in revised form
11 July 2017
Accepted 2 September 2017
Available online 22 September 2017

Keywords:
Antibiotic resistance
Environment
Public health
Sewage
Sludge treatment

ABSTRACT

Wastewater treatment plants have been considered potential sources of antibiotic resistance gene exchange and release into the environment. The aim of our study was to quantify environmental and human-associated carbapenem-resistant bacterial populations (CRBPs) across wastewater treatment stages and correlate bacterial counts to physicochemical and other bacteriological parameters in order to see their behaviour in wastewater and sludge and their potential dissemination in the environment. Samples were taken from five sites (treatment stages) of the largest Croatian wastewater treatment plant (20 per site) over 10 months of monitoring. CRBPs were found at all wastewater treatment stages save for the lime-treated, stabilised sludge, which underlines the importance of effluent and digested sludge disinfection. Secondary sludge settling removed 99% of CRBP from the effluent, but the relative proportion of CRBP in the total bacterial count significantly increased in the effluent (0.0020%) and digested sludge (0.0019%) compared to the influent (0.0006%), indicating selection for resistant bacteria in these settings. CRBP counts did not correlate with measured carbapenem concentrations in wastewater, which suggests that antibiotic concentrations were not the reason for CRBP selection. Negative correlation between activated sludge retention time and CRBP indicated that their number could be reduced by increasing the retention time during secondary treatment. Despite the indications that WWTPs select for antibiotic-resistant bacteria, wastewater treatment is very efficient in reducing their absolute numbers, and proper effluent and sludge disinfection can significantly reduce dissemination of antibiotic-resistant bacteria into the environment.

1. Introduction

The increasing number of antibiotic-resistant bacteria (ARB) has become a global health concern. In addition to the regular culprits, such as overuse and misuse of antibiotics in humans and animals, several authors have pointed to wastewater treatment plants (WWTP) as the sites where bacteria develop resistance to antibiotics, proliferate, and spread into the environment (Berendonk et al., 2015; Berglund et al., 2015; Bouki et al., 2013; Rizzo et al., 2013). Activated WWTP sludge is indeed an ideal habitat for bacteria: nutrient-rich, heavily aerated, and fostering the formation of biofilm, which is known to enhance the exchange of genetic material between cells (Donlan, 2002).

However, a recent comprehensive metagenome analysis by Munck et al. (2015) has demonstrated a very limited dissemination of WWTP core resistome to microbial communities outside the WWTP environment. Bengtsson-Palme et al. (2016) suggested that selective pressures other than antibiotic selection might influence the composition of resistance genes in WWTPs and that relevant selection pressures associated with the risk of resistance development cannot be inferred from metagenome analysis alone. This is why they recommend a culture-dependent approach, such as viable cell count of specific bacteria across the sewage treatment process to elucidate its influence on the dissemination of antibiotic resistance.

Carbapenems are considered the most reliable last-resort

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https://doi.org/10.1016/j.watres.2017.09.007
0043-1354/

treatment for infections caused by multidrug-resistant bacteria, yet soaring resistance of Gram-negative bacteria seems to narrow this option rapidly, creating a major healthcare problem worldwide (Meletis, 2016). In Croatia, carbapenem resistance of *Acinetobacter baumannii* clinical isolates soared from 10% in 2008 to 87% in 2015 (CAMS, 2016), and in Sweden it soared from two cases reported in 2008 to 46 cases of carbapenem-resistant *Enterobacteriaceae* in 2014 (Hellman et al., 2014). In February 2017, the World Health Organization (WHO) published its first ever list of antibiotic-resistant "priority pathogens", which specifies 12 families of bacteria that pose the greatest threat to human health. On that list, the carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* rank as "Priority 1: Critical" (WHO, 2017).

Carbapenem-resistant bacteria were found in hospital wastewaters (Ferreira et al., 2011; Zhang et al., 2013; Chandran et al., 2014) and were recently isolated from raw and secondary treated municipal wastewater (Hrenovic et al., 2016). Bengtsson-Palme et al. (2016) expressed particular concern about their finding of carbapenem resistance genes in Swedish WWTP because carbapenemases are rarely found in Swedish clinical isolates.

Even though a number of studies confirm the presence of carbapenem-resistant bacteria in hospital and municipal wastewaters (Ferreira et al., 2011; Zhang et al., 2013; Chandran et al., 2014), they are not quantitative and cannot give an idea about the risk of carbapenem-resistant bacterial population (CRBP) spread from wastewaters and WWTPs to the environment. Moreover, the temperature at which the carbapenem-resistant bacteria from wastewater were cultivated was 35–37 °C (Walsh et al., 2011; Galler et al., 2014; Tanner et al., 2015), which allows the growth of environmental, autochthonous species with intrinsic resistance to carbapenems, such as *Stenotrophomonas maltophilia*. Cultivation at 42 °C suppresses the growth of environmental, autochthonous species (Hrenovic et al., 2017) and is therefore a strong indication of human-associated CRBP.

The aim of our study was to bridge this gap in knowledge by determining (quantifying) both environmental and human-associated CRBPs across wastewater treatment stages and by correlating bacterial counts to physicochemical and other bacteriological parameters in order to see their behaviour in wastewater and sludge and their potential dissemination in the environment.

2. Materials and methods

2.1. Wastewater treatment plant and sampling

Wastewater and sludge samples were collected at the largest Croatian secondary (sewage) WWTP in Zagreb. This WWTP has the capacity of 1,200,000 population equivalents. The sewage wastewater combines domestic, industrial, hospital, and storm wastewaters. Wastewater that passes coarse screens and grease/oil separation (influent) goes to primary settlers for gravity separation (Fig. 1) and next to activated sludge basins (secondary treatment). Effluent is separated from activated sludge in secondary settlers. Surplus activated sludge is mixed with primary sludge and goes to mesophilic anaerobic digestion, after which the sludge is stabilised by dewatering and lime treatment (Fig. 1). Stabilised sludge is disposed of in a landfill.

Samples were taken twice a month across the processing stages from the influent, effluent, activated sludge, digested sludge, and stabilised sludge over 10 months (September 2015–June 2016). In other words, we collected 20 samples per site (processing stage), totalling 100 samples. The samples of the influent and effluent wastewater were 24-h composite samples, while the sludge samples were instantaneous samples.

2.2. Bacteriological analysis

Wastewater and sludge samples for bacteriological analysis were collected in sterile, 250 ml glass bottles and analysed within 2 h. All samples were concentrated on sterile membrane filters (0.45 μm pore size) in triplicate before and after dilution in sterile peptone water. Aerobically grown heterotrophic bacteria (He) were determined on Nutrient agar (Biolife) after incubation at 22 °C for 72 h (APHA et al., 2005) and used as indicators of total bacterial count. The intestinal enterococci (Ie) were determined as indicators of faecal pollution according to HRN ISO 7899-2 (2000). The samples were incubated on Slanetz Bartley agar (Biolife) at 37 °C for 72 h and confirmed on Bile esculin azide agar (Sigma-Aldrich) after incubation at 44 °C for 4 h. Carbapenem-resistant bacterial populations (CRBP) were determined on CHROMagar™ Acinetobacter supplemented with CR102 (CHROMagar, Paris, France) after incubation at 37 and 42 °C for 48 h. Temperature differentiation was used to distinguish the presumably environmental (CRBP37) from the presumably human-associated (CRBP42) population. Supplemented CHROMagar™ allows for growth of carbapenem-resistant *Acinetobacter* sp. and other resistant Gram-negative bacteria, belonging mostly to *Enterobacteriaceae*, *Pseudomonas* spp., and *Stenotrophomonas* spp. (Hrenovic et al., 2017). Bacterial species can be differentiated by colony colour and morphology (see CHROMagar™ Acinetobacter Instructions for use). For the purposes of this research all grown colonies were marked as CRBP. All bacterial counts are expressed as colony-forming units (CFU).

2.3. Physicochemical analysis and carbapenem concentrations in wastewater

The physicochemical properties of wastewater and sludge samples (specified in Tables 1 and 2, respectively) were measured according to the Standard Methods for Examination of Water and Wastewater (APHA et al., 2005).

Samples for wastewater carbapenem concentration measurements were taken in sterile polycarbonate bottles and transferred to the laboratory within 1 h. The samples were passed through 0.2 μm PTFE filters and the concentrations of imipenem, meropenem, and the meropenem metabolite 2-(1-Carboxy-2-hydroxypropyl)-4-{[5-(dimethylcarbamoyl)-3-pyrrolidinyl]sulfanyl}-3-methyl-3,4-dihydro-2H-pyrrole-5-carboxylic acid in the influent and effluent wastewater were measured with ultra-high performance liquid chromatography – quadrupole time-of-flight mass spectrometry (6550 i-Funnel UHPLC Q-TOF MS, Agilent Technologies) using the direct injection method. All chemicals were of high-purity grade; imipenem was purchased from AbcamBiochemicals (Cambridge, USA) and meropenem and the meropenem metabolite from Santa Cruz Biotechnology (Dallas, USA). For quantification we used the MS mode and for qualification the MS/MS mode with three collision energies (10, 20, and 40 V) and the mass range of 50–1000 m/z. The operation conditions in the ESI(+) MS/MS mode were as follows: sheath gas temperature 375 °C, gas temperature 125 °C, heat gas 12 L N2/min, drying gas 15 L N2/min, capillary voltages 3500 V, fragmentor 400 V, and nebuliser 35 psig. The obtained data were further processed with the Agilent MassHunter Workstation software (Quantitative Analysis Version B.07.00/Build 7.0.457.0 for QTOF, Agilent Technologies).

2.4. Statistical analysis

For statistical analyses we used the Statistica 12 software (StatSoft, Inc., Tulsa, USA). The variables were compared using the ordinary Student's *t*-test for independent variables. The correlations between variables were estimated with Spearman's rank