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

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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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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

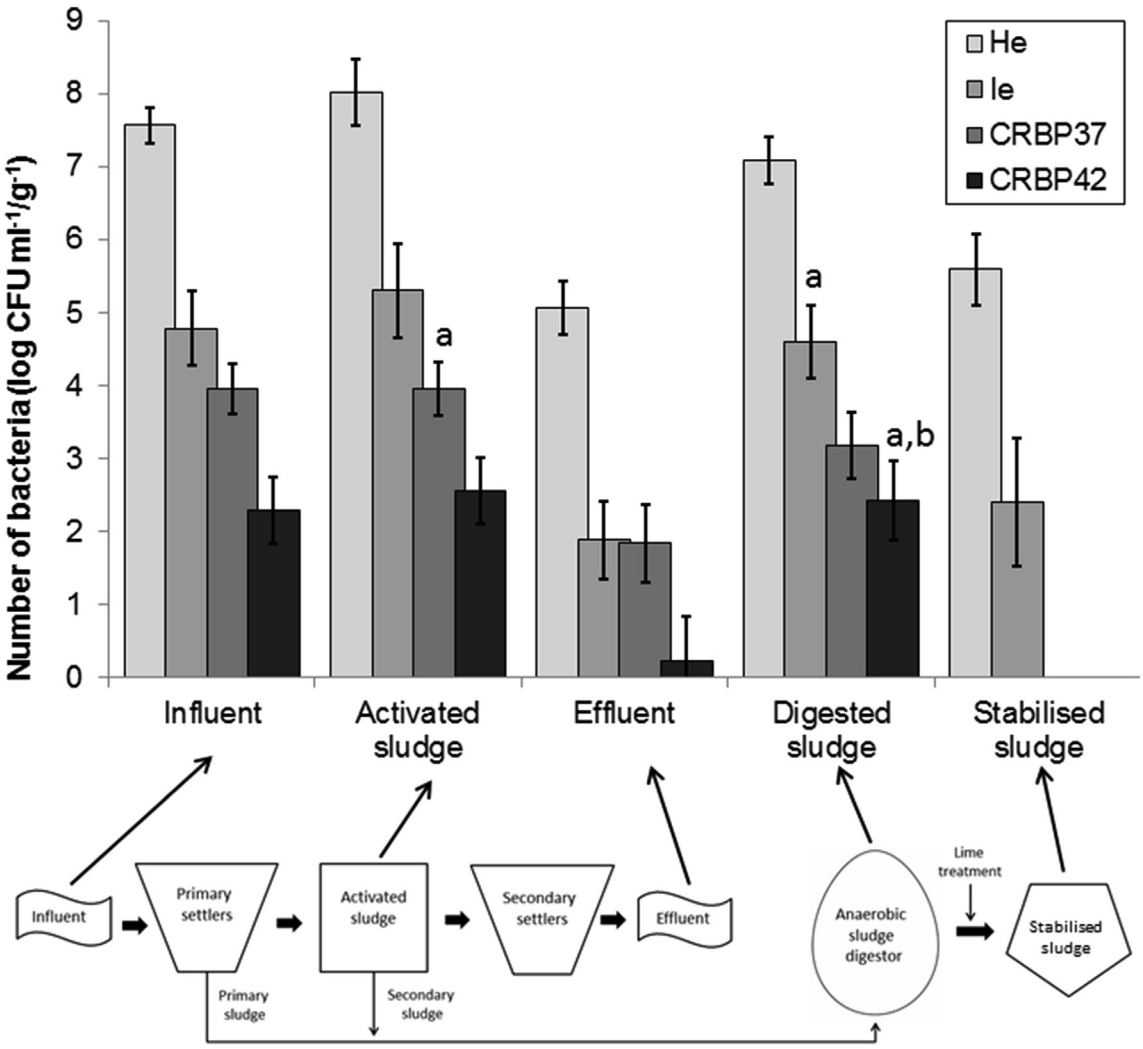

Fig. 1. Bacterial counts (median values ± standard deviation of 20 measurements) at different stages of municipal wastewater processing. The data for stabilised sludge are shown as log CFU g^{-1} since it was a solid sample and were not included in the statistical analysis. a – not significantly different inform the influent; b – not significantly different inform the activated sludge; other values showed significant difference. He – total heterotrophic bacteria, Ie – intestinal enterococci, CRBP37 – carbapenem-resistant bacteria grown at 37 °C, CRBP42 – carbapenem-resistant bacteria grown at 42 °C.

correlation. Statistical decisions were made at a significance level of $p < 0.05$.

Principal coordinate analysis (PCoA) was conducted using an in-house script written in Scilab 5.5.2 (Scilab Enterprises, France) to see the clustering patterns of wastewater and sludge samples. We calculated the pairwise squared Euclidean distances between bacterial counts (expressed as log(CFU+1)) for the five sampling sites (processing stages) to derive a double-centred dissimilarity matrix, which was further factorised using singular value decomposition (SVD). Ordination plots were obtained by projecting the points from ordination space onto the plane defined by the two most significant PCo axes.

3. Results

3.1. Bacteria in wastewater and sludge

Fig. 1 shows bacterial counts at different stages of wastewater processing for 20 samplings. The counts for each stage were relatively constant over the 10 months of monitoring (see Fig. S1–S4). The bacterial counts in the influent wastewater were as follows (in the descending order): He, Ie, CRBP 37, and CRBP 42 (Fig. 1). The same order was found in the effluent wastewater and activated, digested, and stabilised sludge, with the exception that neither CRBP was found in the stabilised sludge (detection limit <1 CFU/g).

The PCoA showed that the influent, activated sludge, and digested sludge samples formed one cluster on the projection plot (Fig. 2), while the effluent and stabilised sludge samples formed two separate clusters. The scores on the main PCo axis and the original variables (log(CFU+1)) showed a very strong negative correlation ($r = -0.930$ to -0.951, $p < 0.05$) for all four bacterial types, indicating that their bacterial counts equally contributed to ordination. Such grouping suggests that the CRBPs were passing through the same WWTP process as the heterotrophic bacteria.

Fig. 3 shows the changes in bacterial counts at three sampling locations within the WWTP relative to the influent. Since the bacterial counts measured at the same time point but at different locations within the WWTP are not mutually related (due to wastewater retention time and the mixing of the influent with excess activated sludge), we decided to estimate the changes in bacterial counts between the processing stages by comparing the 10-month CFU medians for each bacterial type.

Table 1
Physicochemical properties of wastewater and correlations with bacterial counts. Results from 20 measuring are presented.

Sample	Q (m^3 d^{-1})	BOD (mg L^{-1})	COD (mg L^{-1})	N-NH_4 (mg L^{-1})	N-NO_3 (mg L^{-1})	N-NO_2 (mg L^{-1})	N total (mg L^{-1})	P total (mg L^{-1})	P-PO_4 (mg L^{-1})	TS (mg L^{-1})	pH	T (°C)	O_2 (mg L^{-1})	Imipenem (ng L^{-1})	Meropenem (ng L^{-1})	Meropenem Metabolite (ng L^{-1})
Influent																
MIN	2.6×10^5	73.0	109.0	11.2	0.4	0.1	17.6	2.5	1.1	86.0	7.7	10.5	0.0	198.4	20.0	48.6
MAX	5.7×10^5	291.0	421.0	24.3	1.8	1.2	40.3	5.7	2.9	280.0	8.7	19.8	6.1	4958.5	720.8	2348.6
Median	3.2×10^5	170.0	352.5	20.1	0.7	0.2	30.9	4.3	1.9	190.0	8.0	16.5	3.4	2011.8	169.2	156.5
SD	9.0×10^4	57.2	88.5	4.3	0.3	0.2	5.6	1.0	0.5	51.0	0.3	2.5	1.9	1550.9	261.2	730.6
Effluent																
MIN	2.5×10^5	2.0	16.0	0.1	6.1	0.0	9.2	0.7	0.8	0.7	7.2	11.7	7.7	78.4	6.4	6.5
MAX	5.6×10^5	6.2	32.0	3.7	21.5	3.4	28.1	2.6	3.9	10.0	8.0	21.6	10.0	1137.9	823.2	549.4
Median	3.1×10^5	3.2	25.0	0.3	17.3	0.2	19.6	1.9	2.2	5.7	7.5	17.1	8.7	256.1	260.2	37.4
SD	8.9×10^4	1.4	4.1	0.9	4.3	0.8	4.3	0.6	0.7	2.2	0.2	2.6	0.7	347.0	271.0	139.1
Influent																
He	0.032	−0.232	−0.027	−0.320	−0.183	−0.530	0.179	−0.060	−0.166	−0.206	0.075	0.135	−0.136	0.557	0.071	−0.262
Ie	−0.238	0.280	0.117	0.228	0.235	0.040	−0.027	0.277	0.385	−0.116	−0.039	−0.422	0.228	0.396	−0.004	0.455
CR37	0.006	−0.253	−0.017	0.058	0.168	−0.102	−0.082	−0.023	−0.157	0.119	−0.152	0.658	−0.286	0.036	−0.286	0.143
CR42	−0.011	0.069	−0.116	0.176	0.030	−0.153	−0.081	0.090	−0.039	−0.016	0.173	0.115	0.199	−0.229	0.221	0.073
Effluent																
He	−0.174	0.228	0.410	0.101	−0.097	0.377	0.092	0.218	0.215	0.384	0.403	−0.246	−0.006	0.457	0.257	0.264
Ie	0.110	−0.041	0.409	0.579	−0.254	0.653	0.056	0.344	0.119	0.513	0.078	−0.493	0.250	0.093	0.411	0.354
CRBP37	−0.263	0.151	0.050	−0.286	0.278	−0.105	0.169	0.451	0.328	−0.062	0.087	0.236	−0.202	0.079	−0.284	0.393
CRBP42	−0.126	0.161	0.412	0.417	−0.137	0.371	−0.184	0.352	0.142	0.114	0.493	−0.117	0.041	0.239	0.297	−0.075

Shaded fields highlight significant correlations ($p < 0.05$); He – total heterotrophic bacteria, Ie – intestinal enterococci, CRBP37 – carbapenem-resistant bacterial population grown at 37 °C, CRBP42 – carbapenem-resistant bacterial population grown at 42 °C.

Table 2
Physicochemical properties of WWTP sludge types and correlations with bacterial counts. Results from 20 measuring are presented.

Activated sludge	MLSS (g L^{-1})	O_2 (mg L^{-1})	pH	SRT (days)	Digested sludge	pH	T (°C)	RT (days)	Stabilised sludge	pH
MIN	5.2	0.9	6.0	2.7	MIN	7.5	36.2	20.6	MIN	11.6
MAX	10.3	2.4	7.5	11.9	MAX	7.7	36.9	35.6	MAX	12.2
Median	6.6	2.0	7.0	6.1	Median	7.6	36.6	25.7	Median	11.9
SD	1.6	0.3	0.3	2.2	SD	0.1	0.2	4.0	SD	0.2
He	0.489	0.358	0.050	−0.182	He	0.127	−0.179	−0.035	He	−0.155
Ie	0.086	−0.153	0.041	0.232	Ie	0.476	0.221	0.071	Ie	−0.234
CR37	0.712	0.111	−0.142	−0.441	CR37	−0.196	0.094	−0.302	CR37	–
CR42	0.392	0.064	0.017	−0.579	CR42	−0.225	−0.299	−0.381	CR42	–

Shaded fields highlight significant correlations ($p < 0.05$); He – total heterotrophic bacteria, Ie – intestinal enterococci, CRBP37 – carbapenem-resistant bacterial population grown at 37 °C, CRBP42 – carbapenem-resistant bacterial population grown at 42 °C.

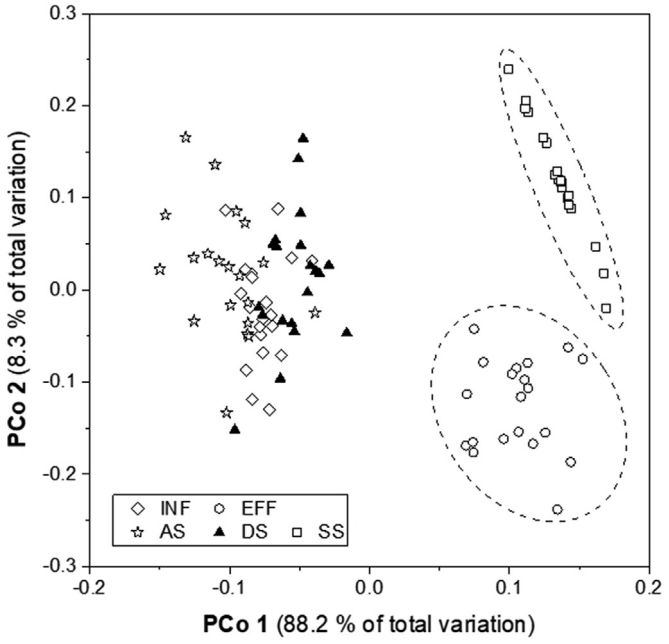

Fig. 2. Ordination of bacterial count data related to different sampling sites within WWTP, according to Principal Coordinate Analysis (PCoA). The first two principal coordinates (PCo 1 and PCo 2) are shown. The percentage of variation explained by individual PCo axis is indicated next to the axis symbol. INF = influent, EFF = effluent, AS = activated sludge, DS = digested sludge, SS = stabilised sludge.

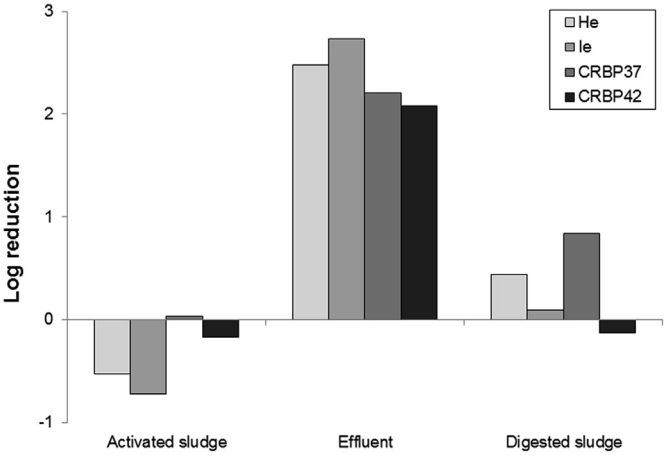

Fig. 3. The reduction of monitored type of bacteria in comparison to influent. The log reduction was calculated as: (log median value of 20 measurements in influent) – (log median value of 20 measurements in respective WWTP stage) for each type of bacteria.

In the activated sludge all but CRBP37 counts significantly increased in respect to the influent (Fig. 1). In the effluent, bacterial counts dropped significantly in respect to the influent: He dropped 99.68%, Ie 99.87%, CRBP37 99.22%, and CRBP42 99.16%. The reduction of CRBP was a result of sludge settling and reduction of the total bacterial count.

In the digested sludge, which passed through anaerobic mesophilic digestion, CRBP42 rose (Fig. 3), but not significantly (Fig. 1). This rise (instead of a drop) suggests that anaerobic mesophilic digestion favours the proliferation of human-associated CRBP. This is corroborated by the significantly higher CRBP42 to total bacterial count ratio in the digested sludge than in the influent, which was not the case with CRBP37 (Fig. 4).

In the stabilised, lime-treated sludge both CRBPs were completely eliminated (100% reduction).

To see whether specific stages of wastewater processing favour the proliferation of certain types of bacteria, we calculated the ratios of Ie and CRBP to He (expressed as log ppm, see Fig. 4). The prevalence pattern in the activated sludge was similar to the influent, but the share of CRBP in the effluent was significantly higher, indicating that the secondary settling after aerobic sludge treatment stimulates CRBP proliferation.

In the digested sludge, the share of CRBP42 was significantly higher when compared to the influent, as was the share of Ie, while the share of CRBP37 did not differ from the influent (Fig. 4). Such result indicates that anaerobic mesophilic sludge digestion favours the proliferation of bacteria of anthropogenic origin, in this case of

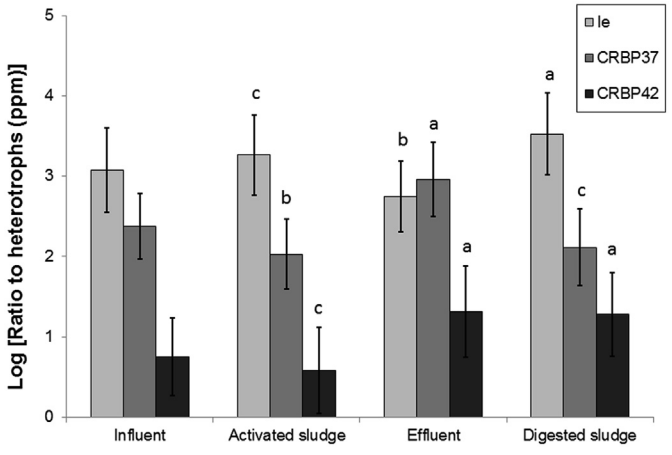

Fig. 4. Prevalence of Ie and CRBPs in total bacterial counts by wastewater treatment stages. Log ppm = $\log10[(CFU_{Ie,\ CR37,\ CR42}/CFU_{He}) \times 10^6]$. a - significantly higher than the influent; b – significantly lower than the influent; c – no significant difference from the influent. He – total heterotrophic bacteria, Ie – intestinal enterococci, CRBP37 – carbapenem-resistant bacteria grown at 37 °C, CRBP42 – carbapenem-resistant bacteria grown at 42 °C.

the human-associated CRBP42, and of Ie as indicators of faecal pollution.

3.2. Physicochemical properties of sludge and wastewater and relation to bacteria

Tables 1 and 2 show the respective physicochemical properties of wastewater and sludge. The influent and effluent water properties varied slightly over the 10 months of sampling, most probably due to occasional storm water influx. Wastewater processing reduced the concentrations of suspended solids and nutrients and increased the concentrations of dissolved oxygen due to activated sludge treatment.

The bacterial counts also varied over the 10-month sampling period (see Fig. 5 for CRBP42 and supplemental Fig. S5–S7 for the rest of the bacteria), and we wanted to see how the variations in CRBP counts correlated with the physicochemical parameters of wastewater and sludge. Influent CRBP37 correlated significantly positively with temperature, and effluent CPRB37 with total phosphorus concentration (Table 1). Effluent CRBP42 correlated significantly positively with pH.

Activated sludge CRBP37 correlated significantly positively with mixed liquor suspended solids, and CRBP42 correlated significantly negatively with sludge retention time (Table 2), suggesting that prolonged aeration could reduce human-associated CRBP in activated sludge.

3.3. Concentrations of carbapenems in wastewater and relation to bacteria

The concentrations of imipenem, meropenem, and the meropenem metabolite in the influent and effluent varied greatly throughout the sampling period (Table 1). Their concentrations significantly dropped in the effluent, indicating that antibiotics are either degraded by wastewater treatment or removed from the effluent by adsorption to activated sludge. We found no significant correlation between CRBP counts and carbapenem concentrations in the influent or effluent.

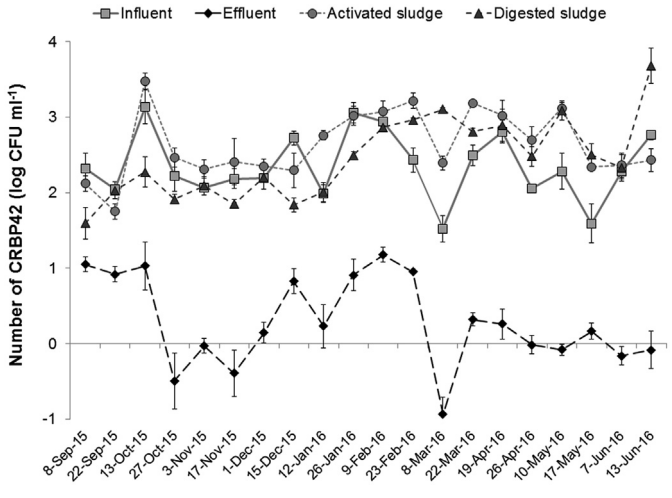

Fig. 5. CRBP42 counts by wastewater processing stages and sampling dates (mean ± SD of triplicate measurement).

4. Discussion

4.1. Abundance of carbapenem-resistant bacteria in WWTP

Considering the large amount of obtained data, the discussion will focus on general conclusions and the CRBPs. The CRBPs were present at all treatment stages save for the stabilised sludge, whose treatment with lime (pH around 12) completely eliminated both. Alkaline disinfection, lime treatment in our case, is completely effective against faecal coliforms, including *E. coli*, and less effective against heterotrophic bacteria (especially the endospore-forming ones) or intestinal enterococci (Ivankovic et al., 2014; Meckes and Rhodes, 2004).

To verify this finding we took one extra sample from the stabilised sludge just before the lime was added. The pH of this sludge was 8.2, and the CRBP37 and 42 counts were 3.9 and 3.5 log CFU g^{-1}, respectively. Counts this high have confirmed that it was lime treatment that removed the CRBPs.

Carbapenems are heavily used in the hospitals all over the world, including Croatia, and the occurrence of carbapenem-resistant isolates is primarily associated with hospital environment and hospital wastewaters (Ferreira et al., 2011; Zhang et al., 2013; Chandran et al., 2014). Our finding of CRBP42, presumably of clinical origin (Goic-Barisic et al., 2016; Hrenovic et al., 2016, 2017), indicates that these bacteria readily passed wastewater treatment stages and reached the final recipient (in our case the Sava River). Similar findings have been reported by Yang et al. (2016) for carbapenemase genes at various processing stages of a Chinese WWTP; their prevalence significantly increased in the activated sludge and dropped with secondary settling. High concentrations of genes were noted in the surplus sludge and in the final product, dewatered sludge, which could increase the risk of propagating carbapenemase genes to endogenous soil bacteria through sludge disposal, fertilisation, or landfill operations. Bouki et al. (2013) have therefore addressed the need to disinfect sludge before the eventual use in agriculture and to prevent the dissemination of antibiotic-resistant bacteria in the environment. Our findings before and after lime treatment confirm this need.

4.2. WWTPs as hotspots for proliferation of drug-resistant bacteria

Antibiotics are released into municipal wastewaters in large quantities through human faeces (incomplete metabolism) (Nagulapally et al., 2009) or extensive use by animal food industry (Kummerer, 2009). WWTPs, especially the ones using activated sludge, have been proposed as hotspots for the development of antibiotic resistance in bacteria inhabiting such systems (Berendonk et al., 2015; Rizzo et al., 2013; Bouki et al., 2013). WWTP influents and effluents contain higher proportions of antibiotic-resistant bacteria (ARB) than surface waters, and conditions at WWTPs favour their proliferation and resistance gene transfer (Kim et al., 2007; Huang et al., 2012; Davies, 2012; Bouki et al., 2013; Rizzo et al., 2013). However, the concentrations of ARB and the associated resistance genes do not correspond to their concentrations in the environment. In fact, they are higher in wastewaters than would be expected from antibiotic wastewater concentrations (Bouki et al., 2013). Al-Ahmad et al. (2009) have suggested that the bacteria that are already drug-resistant do not have a selective advantage in sludge treatment, and that the presence of antibiotics does not favour ARB.

Our findings suggest that CRBPs behave as regular microflora in the WWTP, but their relative proportion in the effluent is higher, which points to positive selection. Our CRBP counts did not correlate with carbapenem concentrations in wastewater, which points to other selection pressures during treatment and/or

changes in total microbial composition, which differs between the treatment stages (Bengtsson-Palme et al., 2016). Beside the effluent, the CRBP count also rose in the digested sludge that passed anaerobic mesophilic treatment. The fact that anaerobic, especially mesophilic, digestion favours the proliferation of ARB has already been proposed by several recent studies (Rahube et al., 2014; Miller et al., 2016).

In the Zagreb WWTP we found a large number of presumably indigenous CRBPs in addition to the human-associated ones. Their relatively stable counts across all processing stages over the 10 months corroborate the latest findings by Munck et al. (2015) where a WWTP community and resistome stability was established over a two-year sampling period. Their results suggest that many resistance genes are present across all processing stages and are unique to WWTPs, yet only a small part of the total resistome is exchanged. As our study did not focus on genes carrying carbapenem resistance, we cannot speculate about gene exchange or selection. In one effluent sample (of 8 September 2015) we did, however, isolate an *Acinetobacter baumannii* that carried the intrinsic, chromosomally located $bla_{\text{OXA-51-like}}$ gene and the acquired plasmid-located $bla_{\text{OXA-23-like}}$ gene (published in a separate article, Goic-Barisic et al., 2017). In an earlier influent and effluent sample of 2014, we isolated *A. baumannii* carrying the same genes (Goic-Barisic et al., 2016). This indicates that the WWTP is constantly receiving and releasing genes encoding carbapenem resistance in the environment. Please note, however, that the Zagreb WWTP is removing more than 99% of human-associated CRBPs that it receives. With disinfection strategies that would be as effective for effluents as they are for digested sludge WWTPs could almost completely prevent the dissemination of resistant bacteria into the environment.

By using the classical cultivation techniques our research complements the findings by Bengtsson-Palme et al. (2016), who reported efficient removal of high influent concentrations of 16 antibiotics from the effluent, presumably by sorption onto sludge particles. In general, the influent resistance genes were halved in the effluent but not substantially in the primary, surplus, or digested sludge (this was also observed by Luo et al., 2014).

Our CRBP counts revealed a similar behaviour (Fig. 3). The carbapenem-resistant bacteria are constantly entering the WWTP, their number increases in the activated sludge and significantly drops in the effluent, most likely because they attach to the activated sludge flocs and are removed by secondary settling. In the activated sludge/secondary settling the relative share of carbapenem-resistant bacteria in total bacterial count increases, indicating they are being selected for (Fig. 4). The removed bacteria pass through anaerobic mesophilic digestion with surplus sludge and are again being selected for (Fig. 4). Finally they are destroyed by lime treatment.

4.3. Selection of resistant bacteria

The common assumption is that the presence of antibiotics in wastewaters favours the proliferation of antibiotic-resistant bacteria (Munck et al., 2015; Andersson and Hughes, 2014; Rizzo et al., 2013; Bengtsson-Palme and Larsson, 2016). This assumption is driven by certain experiments (Liu et al., 2011; Gullberg et al., 2011, 2014) in which resistant bacterial strains had selective advantage over susceptible strains even at antibiotic concentrations way below their minimal inhibitory concentration (sub-MIC). Concentrations that low can be found in wastewaters. This is why Bengtsson-Palme and Larsson (2016) calculated the so-called predicted no-effect concentration (PNEC) for 111 antibiotics from the EUCAST database, above which the selection of resistant strains in the environment is possible.

Reports for WWTP effluents worldwide include many antibiotics, and they are higher than the proposed PNECs, but to the best of our knowledge, our research is the first to report the wastewater and sludge concentrations for imipenem and meropenem. They are much higher than the proposed PNECs of 125 ng l^{-1} for imipenem and 64 ng l^{-1} for meropenem (see Table 1), yet we have found no significant correlation with CRBP counts, even though the effluent CRBP-to-heterotrophs ratio indicates selection for resistant bacteria (Fig. 4). Similar conclusions were drawn by Al-Ahmad et al. (2009), who found no selective advantage for resistant bacteria at sub-MIC concentrations of 11 frequently used antibiotics.

5. Conclusions

We found carbapenem-resistant bacteria at all wastewater treatment stages except in the lime-treated stabilised sludge, which underlines the importance of effluent and digested sludge disinfection in preventing carbapenem-resistant bacteria to reach the environment. Secondary sludge settling removed 99% of carbapenem-resistant bacteria from the effluent. However, the relative proportion of CRBPs in total bacterial count significantly increased in the effluent and digested sludge, which points to their selective advantage over non-resistant bacteria in these settings. On the other hand, CRBP counts did not correlate with carbapenem concentrations in wastewater, which suggests that antibiotic concentrations were not the reason for CRBP selection.

The significant negative correlation between activated sludge retention time and human-associated CRBPs indicates 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 numbers, and proper effluent and sludge disinfection can significantly reduce dissemination of antibiotic-resistant bacteria into the environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2017.09.007.

References

Al-Ahmad, A., Hai A,Unger J,Brunswick-Tietze A,Wiethan, J., Kummerer, K., 2009. Effects of a realistic mixture of antibiotics on resistant and non-resistant sewage sludge bacteria in laboratory-scale treatment plants. Arch. Environ. Contam. Toxicol. 57, 264–273.

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12, 465–478.

APHA, AWWA, WEF, 2005. Standard Methods for Examination of Water and Wastewater, 21th edn. American Public Health Association, New York.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. 86, 140–149.

Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling

antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317.
Berglund, B., Fick, J., Lindgren, P.E., 2015. Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river. Environ. Toxicol. Chem. 34, 192–196.
Bouki, C., Venieri, D., Diamadopoulos, E., 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. Ecotox. Environ. Saf. 91, 1–9.
CAMS, 2016. Antibiotic Resistance in Croatia, 2015. The Croatian Academy of Medical Sciences, Zagreb, pp. 52–54.
Chandran, S.P., Diwan, V., Tamhankar, A.J., Joseph, B.V., Rosales-Klintz, S., Mundayoor, S., Lundborg, C.S., Macaden, R., 2014. Detection of carbapenem resistance genes and cephalosporin, and quinolone resistance genes along with oqxAB gene in *Escherichia coli* in hospital wastewater: a matter of concern. J. Appl. Microbiol. 117, 984–995.
CHROMagar™ Acinetobacter Instructions for use, http://www.chromagar.com/fichiers/144308777914170778 22NT_EXT_055_NOTICE_AC_V9.pdf.
Davies, J., 2012. Sanitation: sewage recycles antibiotic resistance. Nature 487, 302.
Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8, 881–890.
Ferreira, A.E., Marchetti, D.P., De Oliveira, L.M., Gusatti, C.S., Fuentefria, D.B., Corcao, G., 2011. Presence of OXA-23-producing isolates of *Acinetobacter baumannii* in wastewater from hospitals in southern Brazil. Microb. Drug Resist 17, 221–227.
Galler, H., Feierl, G., Petternel, C., Peinthaler, F.F., Haas, D., Grisold, A.J., Luxner, J., Zarfel, G., 2014. KPC-2 and OXA-48 carbapenemaseharbouring Enterobacteriaceae detected in an Austrian wastewater treatment plant. Clin. Microbiol. Infect. 20, 132–134.
Goic-Barisic, I., Hrenovic, J., Kovacic, A., Seruga Music, M., 2016. Emergence of oxacillinases in environmental carbapenem-resistant *Acinetobacter baumannii* associated with clinical isolates. Microb. Drug. Res. 22, 559–563.
Goic-Barisic, I., Seruga Music, M., Kovacic, A., Tonkic, M., Hrenovic, J., 2017. Pan drug-resistant environmental isolate of *Acinetobacter baumannii* from Croatia. Microb. Drug. Res. 23, 494–496.
Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. mBio 5 e01918–14.
Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, e1002158.
Hellman, J., Aspevall, O., Bengtsson, B., Pringle, M., 2014. SWEDRES-SVARM2014. Consumption of Antimicrobials and Occurrence of Antimicrobial Resistance in Sweden (No.14027). Public Health Agency of Sweden and National Veterinary Institute, Solna/Uppsala, Sweden.
Hrenovic, J., Ganjto, M., Goic-Barisic, I., 2017. Carbapenem-resistant bacteria in a secondary wastewater treatment plant. Water sa. 43, 186–191.
Hrenovic, J., Goic-Barisic, I., Kazazic, S., Kovacic, A., Ganjto, M., Tonkic, M., 2016. Carbapenem resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014. Euro Surveill. 21, 21–30.
HRN ISO 7899–2, 2000. Water Quality – Detection and Enumeration of Intestinal Enterococci, Part 2: Membrane Filtration Method.
Huang, J.J., Hu, H.Y., Lu, S.Q., Li, Y., Tang, F., Lu, Y., Wei, B., 2012. Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. Environ. Int. 42, 31–36.
Ivankovic, T., Hrenovic, J., Itskos, G., Koukouzas, N., Kovacevic, D., Milenkovic, J., 2014. Alkaline disinfection of urban wastewater and landfill leachate by wood fly ash. Arh. Hig. Rada. Toksikol. 65, 365–375.
Kim, S., Jensen, J.N., Aga, S.D., Weber, S.A., 2007. Tetracycline as a selector for resistant bacteria in activated sludge. Chemosphere 66, 1643–1651.
Kummerer, K., 2009. Antibiotics in the aquatic environment— a review— Part II. Chemosphere 75, 435–441.
Liu, A., Fong, A., Becket, E., Yuan, J., Tamae, C., Medrano, L., Maiz, M., Wahba, C., Lee, C., Lee, K., Tran, K.P., Yang, H., Hoffman, R.M., Salih, A., Miller, J.H., 2011. Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. Antimicrob. Agents. Chemother. 55, 1204–1210.
Luo, Y., Yang, F., Mathieu, J., Mao, D., Wang, Q., Alvarez, P.J.J., 2014. Proliferation of multi- drug-resistant NewDelhi metallo-β-lactamase genes in municipal wastewater treatment plants in northern China. Environ. Sci. Technol. Lett. 1, 26–30.
Meckes, M.C., Rhodes, E.R., 2004. Evaluation of bacteriological indicators of disinfection for alkaline treated biosolids. J. Environ. Eng. Sci. 3, 231–236.
Meletis, G., 2016. Carbapenem resistance: overview of the problem and future perspectives. Ther. Adv. Infect. Dis. 3, 1521.
Miller, J.H., Novak, J.T., Knocke, W.R., Pruden, A., 2016. Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digestors. Front. Microbiol. 7, 1–11.
Munck, C., Albertsen, M., Telke, A., Ellaban, M., Nielsen, P.H., Sommer, M.O.A., 2015. Limited dissemination of the wastewater treatment plant core resistome. Nat. Comms 6, 8452.
Nagulapally, S.R., Ahmad, A., Henry, A., Marchin, G.L., Zurek, L., Bhandari, A., 2009. Occurrence of ciprofloxacin, trimetho primes ulfamethoxazole, and vancomycin-resistant bacteria in a municipal wastewater treatment plant. Water Environ. Res. 81, 82–90.
Rahube, T.O., Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Duenk, P., Lapen, D.R., Toppa, E., 2014. Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. Appl. Environ. Microbiol. 80, 6898–6907.
Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic re-sistant bacteria and genes spread in to the environment: a review. Sci. Total Environ. 447, 345–360.
Tanner, W.D., Vanderslice, J.A., Toor, D., Benson, L.S., Porucznik, C.A., Goel, R.K., Atkinson, R.M., 2015. Development and field evaluation of a method for detecting carbapenem-resistant bacteria in drinking water. Syst. Appl. Microbiol. 38, 351–357.
Walsh, T.R., Weeks, J., Livermore, D.M., Toleman, M.A., 2011. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect. Dis. 11, 355–362.
WHO – World Health Organization website, 2017, http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1.
Yang, F., Mao, D., Zhou, H., Luo, Y., 2016. Prevalence and fate of carbapenemase genes in a wastewater treatment plant in Northern China. PLoS One 11, e0156383.
Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J.D., Song, H., 2013. Higher isolation of NDM-1 producing *Acinetobacter baumannii* from the sewage of the hospitals in Beijing. PLoS One 8, e64857.