Prevalence of extended-spectrum β-lactamase- and carbapenem-resistance coliforms and genes in municipal wastewater treatment plants in Croatia

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Highlights

- WWTPs reduce but do not eliminate total and resistant *E. coli* and other coliforms
- Good removal of *A. baumanii* and *K. pneumoniae* was reported in most WWTPs
- ESBL genes were only slightly reduced or even enriched after treatment
- CP genes such as bla_{IMP} and bla_{VIM} were frequently enriched during the process
- Concentrations of bla_{IMP} and bla_{VIM} were affected by specific WWTP characteristics

ABSTRACT

Extended-spectrum β-lactamase (ESBL)- and carbapenemase-producing *Enterobacterales* are a critical global health problem and wastewater treatment plants (WWTPs) can promote their spread into the environment; yet their efficacy is not well characterized. Here, we have used conventional culturing to monitor coliform bacteria and quantitative PCR to monitor ESBL (*bla*$_{TEM}$ and *bla*$_{CTX\text{-}M\text{-}32}$) and carbapenemase (CP) genes (*bla*$_{KPC\text{-}3}$, *bla*$_{OXA\text{-}48}$-like, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$) and enteric opportunistic pathogens (EOPs; *E. coli*, *Enterococcus* spp., *A. baumannii*, *K. pneumoniae*) in the influent and effluent of 7 Croatian WWTPs in two seasons. In general, levels of total, cefotaxime- and carbapenem-resistant coliforms were significantly reduced but not eliminated by conventional treatment in most WWTPs. Most WWTPs efficiently removed *K. pneumoniae* and *A. baumanii*, while *E. coli* and *Enterococcus* spp. were reduced but still present in relatively high concentrations in the effluent. ESBL genes were only slightly reduced or enriched after treatment. CP genes, *bla*$_{KPC\text{-}3}$, *bla*$_{NDM}$ and *bla*$_{OXA\text{-}48}$-like, were sporadically detected, while *bla*$_{IMP}$ and *bla*$_{VIM}$ were frequently enriched during treatment and were influenced by specific features of the WWTPs. Our results suggest that improvements in wastewater treatment technologies are needed to minimize the risk of environmental contamination with top priority EOPs and ARGs and the resulting public health.

Keywords: wastewater treatment, ESBL, carbapenemase, antibiotic resistance genes, enteric opportunistic pathogens

1. Introduction

One of the greatest threats to human health in the 21st century is the ineffectiveness of antibiotics in treating bacterial infections. Modern living standards have led to uncontrolled, continuous and ubiquitous use of antibiotics for therapeutic purposes in humans and animals, and for growth promotion and prophylaxis in livestock. This has accelerated the emergence and spread of antibiotic-resistant bacteria (ARB) and their antibiotic-resistance genes (ARGs) in both clinical and non-clinical environments (natural and engineered), and threatens global public health (Ashbolt et al., 2013; Bengtsson-Palme et al., 2018; WHO, 2018; Ben et al., 2019). Of particular concern is the increasing bacterial resistance worldwide to ß-lactam antibiotics such as 3rd generation cephalosporins (extended-spectrum cephalosporins, ESC) and carbapenems. ESC are typically used to treat infections caused by Gram-negative bacteria, but with the increase in these types of infections, their use has increased dramatically and contributed to the emergence of resistant enterobacteria. The most common mechanism of resistance to ESC involves the expression of enzymes called extended-spectrum β-lactamases (ESBLs). ESBLs are commonly found in Gram-negative bacteria, but are very heterogeneous including TEM, SHV, CTX-M and OXA ESBL families (Bradford, 2001). However, the increasing prevalence of infections caused by ESBL-producing enterobacteria has led to the increased use of carbapenems as the crucial antibiotics of last resort used to treat these infections. The most common mechanism of carbapenem resistance involves the production of carbapenemase (CP) enzymes (Suay-García and Pérez-Gracia, 2019). The most clinically important among them are the KPC, NDM, VIM, IMP and OXA-48 types (Walsh, 2010; Nasri et al., 2017; Makowska et al., 2020). Of even greater concern, genes for ESBLs and CPs are commonly found on plasmids, along with genes for resistance to other classes of antibiotics, and spread readily among different bacterial species (Haller et al., 2018; Sib et al., 2020) Therefore, carbapenem-resistant and ESBL-producing *Enterobacterales* have been identified

by WHO as the critical antibiotic-resistant “priority pathogens” that pose the greatest threat to human health due to limited therapeutic options (WHO, 2017.)

Wastewater treatment plants (WWTPs) are considered potential reservoirs for enteric opportunistic pathogens (EOPs) and ARGs, putative hotspots for their horizontal gene transfer (HGT), and sources for their dissemination in the environment (Karkman et al., 2018; Pazda et al., 2019; Wang et al., 2020). WWTPs receive wastewater from a variety of sources, including households and hospitals, so environmental bacteria and pathogenic gut bacteria released with the feces (some of which carry acquired ARGs) can interact and exchange genes horizontally. This HGT of ARGs is the main cause of the spread of resistance in most Gram-negative bacteria and is facilitated in WWTPs by high bacterial densities, high nutrient loads and various types of pollutants, including antibiotics and other selectors of antibiotic resistance (Hembach et al., 2017; Karkman et al., 2018). In general, the abundance of ARB and ARGs is reduced during the wastewater treatment process (Caucci et al., 2016; Wang et al., 2020). However, some ARGs and ARB have been shown to be enriched in treated wastewater compared to raw wastewater, and then released into the aquatic environment (Nasri et al., 2017; Proia et al., 2018; Kumar et al., 2020). Therefore, WWTPs can serve either as a pathway for the spread of antibiotic resistance or as a barrier to limit the release of anthropogenic antibiotic resistance into the environment (Nguyen et al., 2021). Further knowledge on the impact of the wastewater treatment process on the abundance and removal of ARB and ARGs, especially horizontally transmissible and clinically relevant ARGs, is therefore of great importance.

Resistance to ESC and carbapenems among clinical isolates is increasing and reported in many countries (Khan et al., 2018; Lepuschitz et al., 2019; Krilanović et al., 2020). Croatia is one of the countries with high rates of resistance of clinical enterobacteria, especially *K. pneumoniae*, to ESC (53% in 2019; ECDC, 2020). In addition, an increase in carbapenem resistance rates from 2% in 2018 to 12% in 2019 was observed in clinical *K. pneumoniae* isolates in Croatia

(ECDC, 2020). In addition to characterizing clinical ARB, recent studies have also quantified ESBL and CP genes by quantitative PCR in various European WWTPs. For example, the ESBL genes bla_{TEM} and $bla_{CTX\text{-}M}$ were detected in all 16 effluents from WWTPs from 10 countries, and the CP genes $bla_{OXA\text{-}48}$ and and bla_{KPC} were sporadically detected in some effluent samples (Cacace et al., 2019). In contrast, CP genes such as bla_{IMP}, bla_{VIM} and bla_{KPC} were found in some influent samples in different EU countries (Pärnänen et al., 2019). A recent study looking at ESC-resistant coliforms in WWTPs on 5 continents showed that despite good removal of these coliforms in most WWTPs, significant concentrations ($> 10^3$ CFU/mL) were occasionally found in final effluents (Marano et al., 2020). However, little is known about the prevalence of ESC- and carbapenem-resistant coliforms and the corresponding ARGs in Croatian wastewater, which represent a potential dissemination pathway for ESC and carbapenem resistance into natural waters. The aim of this study was therefore to quantify and compare the abundance of ARB/ARGs in the influent and effluent of WWTPs in seven selected Croatian cities over two seasons (winter and summer). The focus was on culturable cefotaxim (ESC)- and carbapenem-resistant coliforms, selected ESBL (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$) and CP genes ($bla_{KPC\text{-}3}$, $bla_{OXA\text{-}48}$-like, bla_{NDM}, bla_{IMP} and bla_{VIM}) and genetic markers specific for EOPs (*E. coli, Klebsiella pneumoniae, Acinetobacter baumannii, Enterococcus* spp.) to provide information as a basis for risk assessment.

2. Materials and methods

2.1. Sample collection

The 24-h composite samples of untreated wastewater (influent) and treated wastewater (effluent) were collected at the municipal wastewater treatment plants (WWTPs) from 7 Croatian cities: Zagreb, Zadar, Karlovac, Vinkovci, Bjelovar, Čakovec and Varaždin (Fig. 1, Table 1). The sampling campaigns were performed during three consecutive days (Tuesday, Wednesday and Thursday) on two separate occasions in 2020 – January/February (winter season) and June/July (summer season). The samples were collected in sterile glass bottles (2.5 L), transported in coolers with ice blocks and processed in a laboratory within 2 h.

Table 1. Characteristics of the cities and wastewater treatment plants (WWTPs) included in the study

	Vinkovci	**Bjelovar**	**Zagreb**	**Čakovec**	**Varaždin**	**Karlovac**	**Zadar**
No. of inhabitants	35,000	40,000	790,017	57,169	58,500	66,823	75,000
Population equivalent of WWTPs	43,000	50,000	1,2000.000	75,000	140,000	98,500	100,000
Mean wastewater flow (m^3/day)*	16,000(W) 10,000 (S)	9840(W) 8867 (S)	307,556(W) 261,620 (S)	9548(W) 9310 (S)	16,500 (W) 15,500 (S)	14,990 (W) 14,605 (S)	9862 (W) 11,012 (S)
Hospitals in catchment	1	1	9	1	1	2	1
No. of hospital beds	262	272	6333	346	968	614	483
No. of biological stages	2	2	<2	≥2	<2	3	≥2

*mean wastewater flow (m^3/day) in two seasons: winter (W) and summer (S)

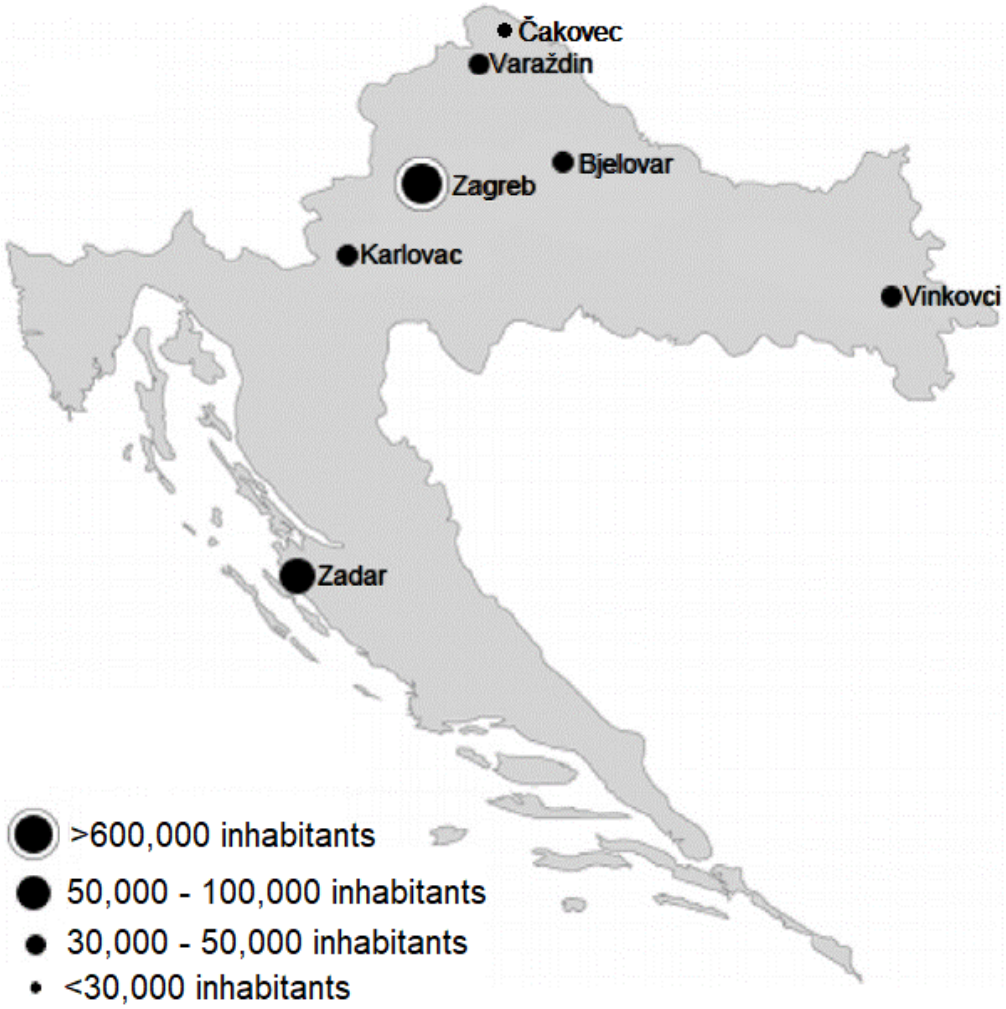

Fig. 1. Map of Croatia with indicated sampling locations

2.2. Physicochemical analyses

Collected wastewater samples were analyzed for their physicochemical characteristics using internationally validated methods (ISO standards; for more details see Table S1). A number of basic parameters was analyzed including temperature, pH, conductivity, total suspended solids, chemical oxygen demand, biochemical oxygen demand, nitrogen forms (total N, ammonium-N, nitrate-N) and total phosphorus.

2.3. Enumeration of culturable coliform bacteria

Samples for microbial cultivation were first serially diluted in 0.85% NaCl (tenfold dilutions up to 1:10,000), and then filtered in triplicate through sterile mixed cellulose ester membrane disc filters (47 mm diameter, 0.22-µm pore size, GE Healthcare, Life Science, SAD) by using vacuum. The filters were then placed on the Rapid′*E. coli* 2 (Bio- Rad, France) agar plates to enumerate total (non-resistant) coliform bacteria and supplemented with 4 mg/L cefotaxime (Sigma-Aldrich, SAD) to enumerate cefotaxime-resistant (CTX-R) coliforms. CHROMagar mSuperCARBA (CHROMagar, France) agar plates were used to enumerate carbapenem-resistant (CR) coliforms. After incubation at 37°C for 24 h, two types of colonies (based on color) were distinquished and enumerated on Rapid′*E. coli* 2 and CHROMagar mSuperCARBA plates – *E. coli* and other non-*E. coli* coliforms (e.g. *Klebsiella, Enterobacter, Citrobacter, Serratia*). Bacteria concentrations for each culture medium and sample were calculated as colony-forming units (CFU) per milliliter of wastewater (CFU/mL).

2.4. DNA extraction and quantitative PCR analyses

For DNA extraction from wastewater, samples (30 - 100 mL of influent and 100 - 700 mL of effluent) were filtered in triplicate through the same membrane filters as mentioned above, and the total community DNA was extracted from filters using the DNeasy Powersoil kit (Qiagen, USA) according to the manufacturer's recommendations. Prior to the extraction procedure, the filters were cut into small pieces with sterile scissors. DNA quality (260/280 ratio) was assessed using a Nanodrop spectrophotometer (BioSpec Nano, Shimadzu, Japan), and DNA quantity using a Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA). All extractions were stored at -20 °C until use.

Quantitative PCR (qPCR) was used to quantify two ESBL genes (bla_{TEM} and $bla_{\text{CTX-M-32}}$), five CP genes ($bla_{\text{KPC-3}}$, bla_{NDM}, $bla_{\text{OXA-48}}$-like, bla_{IMP} and bla_{VIM}) and 16S rRNA gene as marker

for total bacteria. In addition, marker genes for EOPs were also quantified: *ycc*T (*Escherichia coli*), *glt*A (*Klebsiella pneumoniae*), *sec*E (*Acinetobacter baumannii*) and 23S rRNA (*Enterococcus* spp.). The primers targeting these genes and qPCR conditions are listed in Table S2. All qPCR assays were performed on the ABI 7300 Real-time PCR thermocycler (Applied Biosystems, USA) with Power SYBR® Green PCR Master Mix (10 µL, Applied Biosystems, USA), 1 µM of each primer (Table S2) and 2 ng of DNA template in a total volume of 20 µL. The qPCR thermal cycling conditions for ARGs and EOPs were as follows: 95 °C for 15 min, 30 cycles (bla_{VIM}), 35 cycles (*ycc*T, *glt*A, *sec*E and bla_{IMP}) or 40 cycles (bla_{OXA-48}-like, bla_{NDM}, bla_{KPC-3} and 23S rRNA) at 95°C for 15 s, specific annealing temperature for each gene and primer pair (Table S2) for 30 s, and 72°C for 30s, respectively. For quantification of the 16S rRNA gene, thermal cycling conditions were according to López-Gutiérrez et al., (2004).

The plasmids pGEM-T with the corresponding inserts were used as quantification standards for the quantification of the genes bla_{NDM}, bla_{OXA-48}-like, bla_{VIM}, bla_{IMP}, *ycc*T, *sec*E and *glt*A. The pNORM1 plasmid (Rocha et al., 2020) was used to quantify the bla_{TEM} and $bla_{CTX-M-32}$ genes, while the plasmid pUC19 (Heß et al., 2018) was used to quantify the bla_{KPC-3} gene. Plasmid DNA was extracted with ExtractNow™ Plasmid Mini kit (Minerva Biolabs GmbH, Germany) and used after linearization to generate a standard curve (10^2-10^8). For enterococci, the 23S rRNA gene from the reference strain (*Enterococcus avium*) was used as the quantification standard. Negative controls (NTC) were included in each of the assays. Efficiency and accuracy values (Table S2) were determined using six points of serial dilutions of the plasmid carrying ARG. Both samples and standards were analyzed in technical duplicates. Possible qPCR inhibition was assessed by conducting an inhibition test using 10- and 100-fold diluted samples, as previously described (Petric et al., 2011). The detection limit for all target genes was 10^2 gene copies per reaction. Gene abundances were calculated per 1 mL of a sample (absolute abundance) and per number of copies of the 16S rRNA gene (*rrn*) (relative abundance), and

223 results were log transformed. Abundances of *ycc*T gene of *E. coli*, *glt*A gene of *K. pneumoniae*,
224 *sec*E of *A. baumannii* and 23S rRNA gene of enterococci and 16S rRNA gene of total bacteria
225 were reported as cell eqivalents (CE)/mL. In the case of *E. coli*, *K. pneumoniae* and *A.*
226 *baumannii* only one copy of the target gene is present in a cell (Clifford et al., 2012; Gadsby et
227 al., 2015); thus, one copy number corresponds to one cell. However, in case of enterococci and
228 total bacteria, average copy number of 23S rRNA and 16S rRNA genes is five and three,
229 respectively (Stoddard et al., 2015); therefore 23S rRNA and 16S rDNA copies determined by
230 qPCR were dived by 5 and 3, respectively, to convert into CE.
231
232 **2.5. Data analysis**
233 Bacterial and gene concentration data were first log10-transformed. Before deeper
234 analysis, the data were subjected to a Shapiro-Wilk test to assess their normality. This was
235 performed in R Studio v4.0.3. and confirmed that the data followed a normal distribution. Paired
236 t-tests were performed to compare the average concentrations of culturable bacteria, EOPs or
237 ARGs between the influent and effluent of each WWTP and between seasons. In addition,
238 Welch's t-test was assesed to compare the average concentrations of EOPs from influents and
239 effluents in all 7 Croatian WWTPs. These analyses were performed using GraphPad Prism
240 version 8.02 for Windows (GraphPad Software, San Diego, California, USA). Log removal
241 values were calculated by taking the logarithm of the ratio of CFU or relative/absolute gene
242 abundance in influent and effluent water of each WWTP.
243 All further statistical analyses and visualizations were performed in R. Boxplot comparison of
244 relative abundances of resistance/taxonomic genes from effluent in relation to physicochemical
245 WWTP characteristics was performed using the package ‘ggplot2’ (Wickham and Chang,
246 2016). Pearson’s rank correlation tests were performed to evaluate the correlations between the
247 physicochemical parameters and the relative abundance of ARGs or the absolute abundance of
248 gene markers for EOPs. A correlation matrix was constructed using the package ‘corrplot’ (Wei

and Simko, 2017). A non-metric multidimensional scaling (NMDS) analysis was performed to evaluate the distribution of ARGs in WWTPs' influent and effluent in two seasons based on the Bray-Curtis distance calculated using the package 'vegan' (Oksanen et al., 2018). In addition, the Adonis test was applied with the 'vegan' package to analyze the differences in the relative abundance of ARGs between the influent and effluent of each WWTP and between two seasons. All statistical tests were considered significant at $p < 0.05$.

3. Results

3.1. Abundance of culturable coliforms and their reduction by biological treatment in municipal WWTPs of Croatian cities

We quantified non-resistant *E. coli* and non-*E. coli* coliforms as well as CTX-R and CR *E. coli* and non-*E. coli* coliforms in influent and effluent water from 7 different Croatian WWTPs by plating on agar media with and without antibiotics.

The average concentrations of combined coliforms (*E. coli* + non-*E. coli* coliforms) in all influent samples ranged from 1.52 x 10^4 to 1.37 x 10^5 CFU/mL (Fig. 2) and were generally significantly reduced in the effluents (except in Bjelovar) by about 0.97 – 2.22 log units in winter and by about 1.02 – 2.47 log units in summer (except in Čakovec and Varaždin; Fig. 2). As shown in Table S3, *E. coli* concentrations decreased in the effluents to an average of 7.02 x 10^2 CFU/mL in winter, except in Bjelovar, and to 3.06 x 10^3 CFU/ml in summer, except in Čakovec and Varaždin. The concentrations of non-*E. coli* coliforms decreased in the effluents to an average of 1.88 x 10^3 CFU/mL(Table S3), with no significant variations between seasons ($p > 0.05$, paired t-test).

The average CTX-R combined coliform levels in the influents ranged from 1.01 x 10^2 to 4.14 x 10^3 CFU/mL in both seasons (Fig. 2.). These levels were significantly lower in all WWTP effluents (average 1.8 log units in both seasons), except in Bjelovar and Varaždin in winter and in Čakovec and Varaždin in summer. The average concentrations of presumptive CTX-R *E.*

coli ranged from 1.73 x 10^1 to 7.83 x10^2 CFU/mL in the influents of both seasons, while the concentrations of CTX-R non-*E. coli* ranged from 8.37 x 10^1 to 3.36 x 10^3 CFU/ml (Table S3). However, no significant changes were observed between the influent and effluent of CTX-R *E. coli* and CTX-R non-*E.coli* coliforms in Bjelovar and Varaždin WWTPs in winter, and in WWTPs from Varaždin (CTX-R *E. coli* and non-*E. coli*), Vinkovci (CTX-R *E. coli*) and Čakovec (CTX-R non-*E.coli*) in summer.

CR combined coliform counts ranged from 4.05 x 10^1 to 4.81 x 10^3 CFU/mL in the influents, with no significant seasonal variation for each WWTP ($p > 0.05$, paired t-test; Fig. 2, Table S3). CR coliforms were significantly reduced in the effluents of all WWTPs (removal efficiency: 1.39 to 2.72 log units), except for Varaždin WWTP in both seasons.

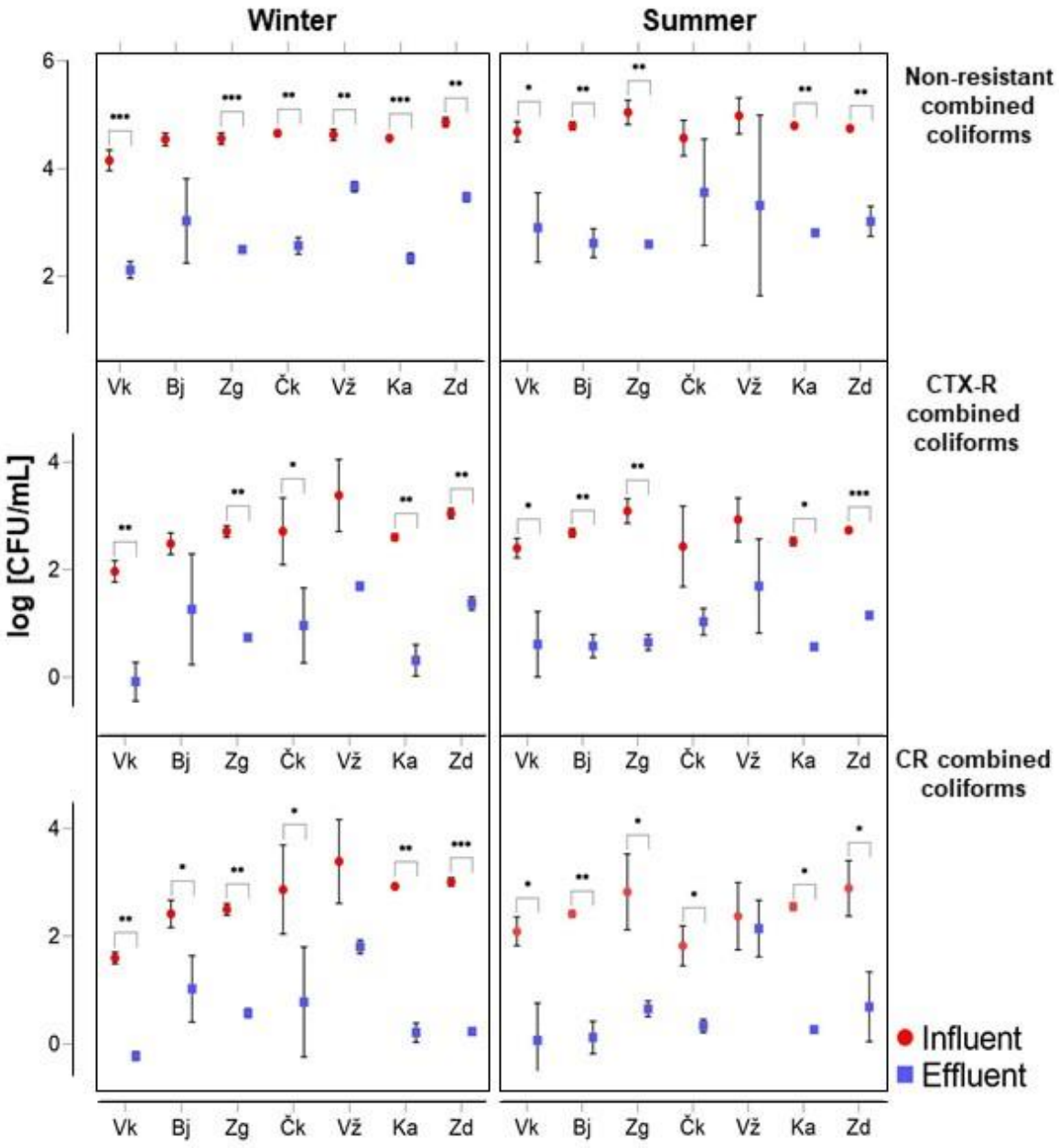

Fig. 2. Quantification of non-resistant combined coliforms (*E. coli* + non-*E. coli* coliforms), cefotaxime-resistant (CTX-R) and carbapenem-resistant (CR) comined coliforms in influent and effluent samples from WWTPs of 7 Croatian cities (Vinkovci, Vk; Bjelovar, Bj; Zagreb, Zg; Čakovec, Čk; Varaždin, Vž; Karlovac, Ka; Zadar, Zd) in winter and summer. A multiple paired t-test was performed on the log transformed data. Asterisks indicate a statistically significant difference between influent and effluent (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3.2. Abundances of EOPs in WWTPs and their removal efficiencies

Apart from the culturable coliforms, the abundance of 16S rRNA gene copies used as a proxy for total bacterial concentration and the abundance of *ycc*T, *glt*A, *sec*E and 23S rRNA genes used as a proxy for total concentration of *E. coli*, *K. pneumoniae*, *A. baumannii* and *Enterococcus* spp., were determined by qPCR in all influent and effluent samples during winter and summer (Fig. 3 and Table S4).

The qPCR-based analyses showed that the mean concentration of total bacteria in the influent samples was approx. 4×10^8 CE/mL in winter and 7×10^7 CE/mL in summer (Fig. 4). Significant decreases in total bacteria counts were achieved at all 7 WWTPs (Fig. 4, Tab. S4), resulting in reductions ranging from 0.13 to 1.92 logs, with no significant seasonal differences ($p > 0.05$, paired t-test). Among the target EOPs, enterococci were most prevalent in the influents (around 10^5 CE/mL in both seasons), followed by *E. coli* (around 10^4 and 10^5 CE/mL in winter and summer, respectively), *A. baumanni* (around 10^4 CE/mL, in both seasons) and *K. pneumoniae* (around 10^3 CE/mL in both seasons) (Fig. 3). Concentrations of *Enterococcus* spp. and *E. coli* in effluents decreased by approx. 1.4 log units and 1.7 log units, respectively (two-seasonal average) (Fig. 3 and Table S4). Concentrations of *K. pneumoniae* were below the detection limit in all effluents in both seasons, as were concentrations of *A. baumanni* in winter and in the majority of summer samples (5/7 WWTPs). In only two WWTPs where *A. baummanii* was detected in the effluent, a reduction of 1.50 (Zagreb WWTP) and 0.59 logs (Varaždin WWTP) was observed (Table S4).

A comparison of *E. coli* concentrations in influent and effluent from all studied WWTPs determined by qPCR of the *ycc*T gene with those determined by plating is shown in Fig. S1. The qPCR-based *E. coli* concentrations were higher (0.5 - 0.99 log units) than the concentrations determined by the culture-based approach in both influent and effluent in both seasons. However, a similar reduction of *E. coli* concentrations due to the conventional

318 treatment was observed for both counting methods (two season average: 1.44 log units - qPCR
319 and 1.83 log units – plating).
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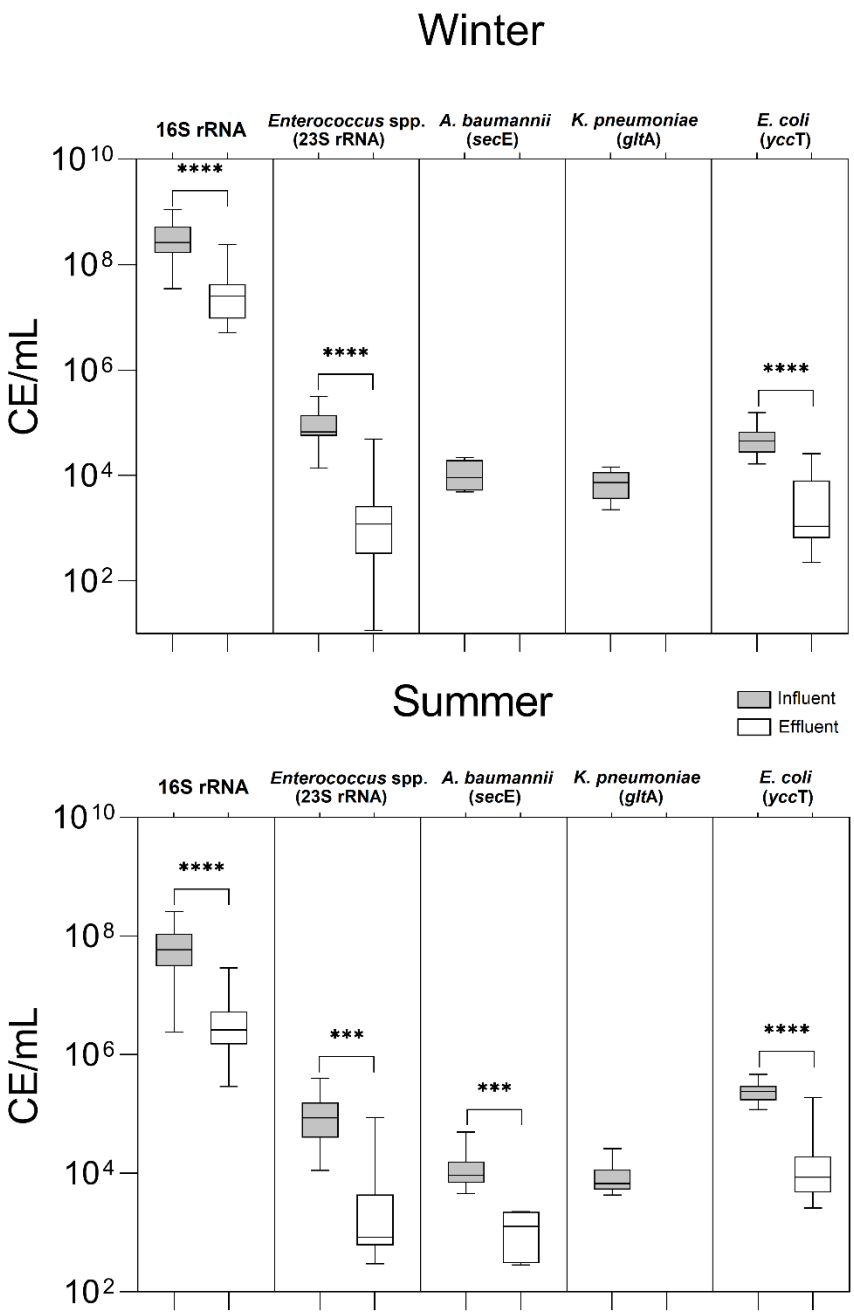

321

322 **Fig. 3.** Absolute abundances of total bacteria (16S rRNA), *Enterococcus* spp. (23S rRNA), *A.*
323 *baumannii* (*sec*E), *Klebsiella pneumoniae* (*glt*A) and *E. coli* (*ycc*T) determined by qPCR of
324 taxon-specific genes (cell equivalents (CE)/mL) in influents and effluents from all 7 studied
325 WWTPs in two seasons. Boxes indicates the median and quartiles, whiskers represent minimal
326 and maximal values. Significance of reduction is assessed by Welch's t-test and is indicated by
327 asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p < 0.0001$). CTX-R, cefotaxime resistant; CR,
328 carbapenem resistant.

3.3. Distribution of ESBL and CP genes in WWTPs

The abundance of two ESBL genes (*bla*$_{TEM}$ and *bla*$_{CTX\text{-}M\text{-}32}$) and five CP genes (*bla*$_{KPC\text{-}3}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{OXA48}$-like and *bla*$_{IMP}$) in total community DNA from 7 investigated WWTP influents and effluents was analyzed by using qPCR (Fig. 4, Table S5). The NMDS plot in Fig. 4A, based on Bray-Curtis similarity, shows that influent and effluent samples were clustered separately based on the relative abundance of the ARGs analyzed (adonis: R2 = 0.70376, $p < 0.001$). In addition, influent and effluent samples from the different sampling periods (winter/summer) were also grouped separately (adonis: R2 = 0.46506, $p < 0.001$).

Fig. 4B shows the distributions (relative abundance) of two ESBL genes in influent and effluent samples from the studied WWTPs. In general, the relative abundance of the two ESBL genes in the influent and effluent samples of all WWTPs was about one order of magnitude higher in summer than in the corresponding winter samples. The *bla*$_{TEM}$ gene was detected in measurable concentrations in almost all samples studied, except in the winter effluent of the WWTPs of two smaller continental cities (Karlovac and Čakovec). The average relative abundance of this gene in the winter influent samples was -3.99 log gene copies/*rrn* copies and -2.91 log gene copies/*rrn* copies in the summer influent samples. In most of the studied WWTPs (4/7), the relative abundance of *bla*$_{TEM}$ in the effluent decreased only slightly compared to the influent in winter, with the lowest removal rate observed for Zagreb (0.17 log units) and the highest for Bjelovar (0.56 log units) (Fig. 4A, Table S5). During the summer, the relative abundance of this gene either slightly decreased after treatment in the WWTPs of four smaller continental cities (Bjelovar, Čakovec, Varaždin and Karlovac; 0.13 – 0.53 log reduction) or even increased in the small city of Vinkovci (0.15 log increase) and in the two larger urban centers (Zagreb and Zadar, 0.11 and 0.49 log increase, respectively). The other ESBLgene, *bla*$_{CTX\text{-}M\text{-}32}$, was detected in all influent samples in winter (-5.54 to -4.47 log gene copies/*rrn* copies), but its concentration in effluent samples from all 7 WWTPs was below the detection

limit (Fig. 4A, Table S5). In contrast, this gene was quantified in all analyzed samples in summer, with no statistically significant difference ($p > 0.05$, paired t-test) between influent and effluent levels (average -3.62 log copies/*rrn* copies). However, when considering the absolute abundance of ESBL genes, larger log reductions were generally observed for both bla_{TEM} (average 1.22) and $bla_{\text{CTX-M-32}}$ genes (average 1.29) than for the relative gene abundances (0.35 and 0.15 average log reduction for bla_{TEM} and $bla_{\text{CTX-M-32}}$, respectively, excluding samples with log increase; Tables S5 and S6).

The distribution of CP genes in the WWTP influent and effluent samples examined is shown in Fig. 4C. $bla_{\text{KPC-3}}$ and bla_{IMP} were detected sporadically in the influent of some WWTPs only in winter (about -4 log gene copies/*rrn* copies), but their concentrations in all summer influents were below the detection limit (Fig. 4C, Table S5). Among the effluents, $bla_{\text{KPC-3}}$ was detected only in the winter effluent of the Bjelovar WWTP, while bla_{IMP} was detected in the effluents of 5 cities (Vinkovci, Bjelovar, Zagreb, Karlovac and Zadar) in winter (average -4.05 log gene copies/*rrn* copies) and 3 cities (Zagreb, Karlovac and Zadar) in summer (average -3.36 log gene copies/*rrn* copies). However, some differences in the removal efficiency of bla_{IMP} were observed when absolute gene abundance was considered. For example, bla_{IMP} removal in Vinkovci WWTP was 0.85 log units in winter (Table S6), while an opposite 0.68 log increase was observed when relative bla_{IMP} abundance was considered (Table S5). The bla_{NDM} gene was detected during winter in influents of 5 continental cities (Vinkovci, Bjelovar, Zagreb, Varaždin and Karlovac; average -5.25 log copies/*rrn* copies), and only in effluents of 2 of these smaller cities (Bjelovar and Karlovac), with relative abundance values slightly higher than in the paired influents (Fig. 4C, Table S5). In contrast, when considering absolute bla_{NDM} gene abundance, a relative decrease (1.27 log units) was observed for the Karlovac WWTP in winter (Table S6). However, in summer, only the influent from the

Bjelovar WWTP had a quantifiable amount of the *bla*$_{NDM}$ gene (about -4 log copies/*rrn* copies), but none of the other 6 influents or all the effluents (Fig. 4C, Table S5).

Gene *bla*$_{OXA-48}$-like was quantified in the influent of all cities except Karlovac in winter (average -5.35 log) and in 5/7 cities in summer (-3.92 to -2.54 log gene copies/*rrn* copies). However, it was below the detection limit in all effluent samples, except in Bjelovar in winter (0.20 log reduction) and Varaždin in summer (0.19 log increase) (Fig. 4B, Table S5). However, when considering the absolute abundance of the *bla*$_{OXA-48}$-like gene, a log reduction of 0.60 was observed for Varaždin WWTP during summer (Table S6). The *bla*$_{VIM}$ gene was detected (average -4.79 log) in the influents of 4 of the 7 studied cities (Bjelovar, Čakovec, Varaždin, Zadar) during the winter season, but only in the effluents of two cities (Bjelovar and Čakovec) it fell below the detection limit, while a slight relative increase was observed in the WWTPs of Varaždin (0.1 log) and Zadar (0.21 log) (Fig. 4B, Table S5). The opposite was observed when considering the absolute *bla*$_{VIM}$ abundance (log reductions of 0.17 and 0.50 in Varaždin and Zadar WWTPs, respectively). The *bla*$_{VIM}$ gene was also quantified in the winter effluents of the Zagreb and Karlovac WWTPs (about -4 log copies/*rrn* copies), but not in the paired influents. During the summer, *bla*$_{VIM}$ was detected in all influents (average -3.84 log copies/*rrn* copies), except in the Karlovac WWTP, and in all effluents, except in the Čakovec WWTP. A decrease of this gene by 0.02 to 1.11 log was observed in two WWTPs (Bjelovar and Zadar), and an increase by 0.12 to 0.53 log was observed in three WWTPs (Vinkovci, Zagreb and Varaždin) (Fig. 4B, Table S5). However, in contrast to the enrichment of this gene in the Vinkovci, Zagreb and Varaždin WWTPs based on relative gene abundance data (Table S5), log reductions of 1.27, 1.08 and 0.46, respectively were found for these WWTPs when absolute gene abundance data were considered (Table S6).

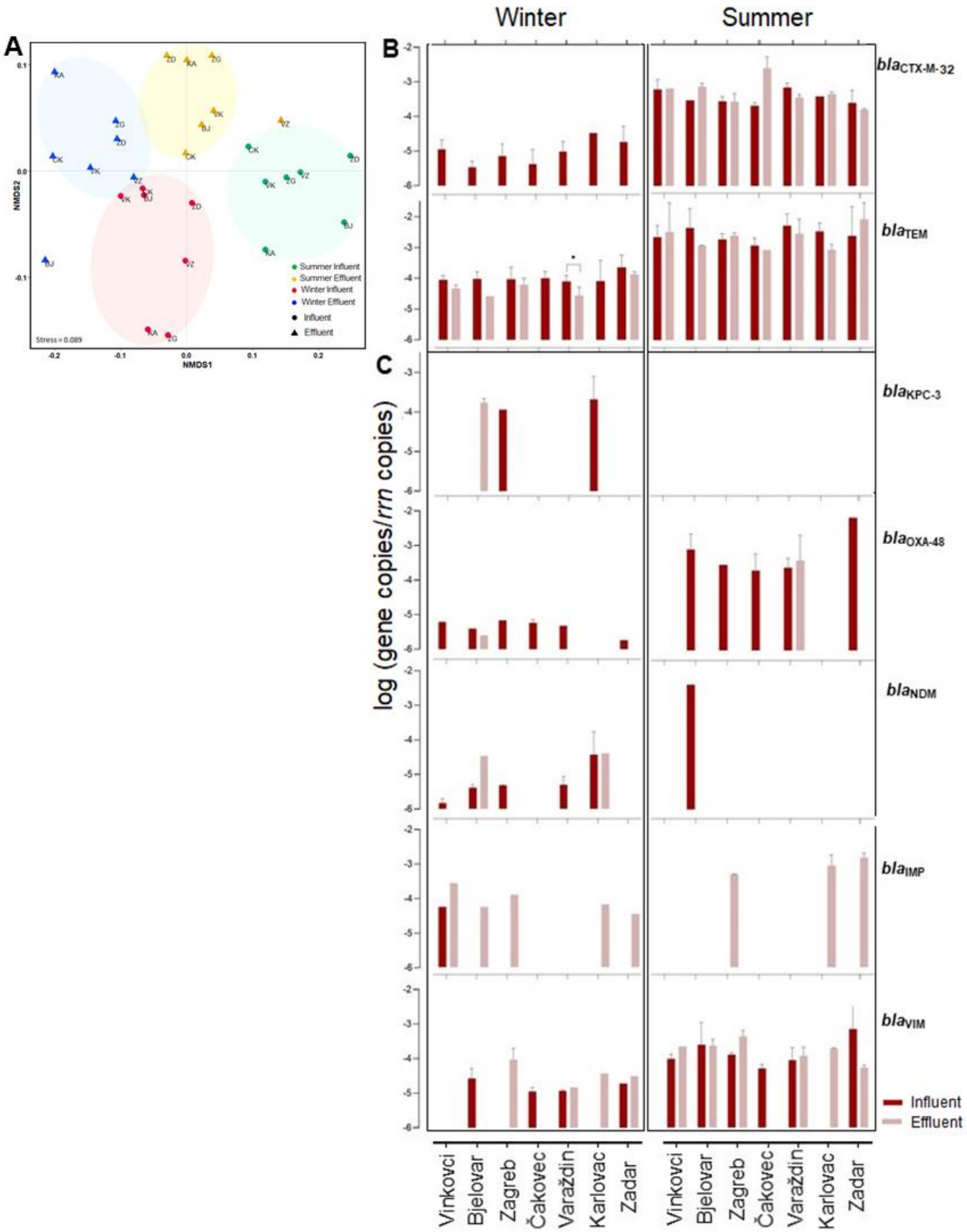
A
B
C
Winter
Summer
NMDS1
NMDS2
Stress = 0.089
Summer Influent
Summer Effluent
Winter Influent
Winter Effluent
Influent
Effluent
log (gene copies/rrn copies)
blaCTX-M-32
blaTEM
blaKPC-3
blaOXA-48
blaNDM
blaIMP
blaVIM
Influent
Effluent
Vinkovci
Bjelovar
Zagreb
Čakovec
Varaždin
Karlovac
Zadar

Fig. 4. Analysis of antibiotic-resistance gene distribution in influent and effluent samples from wastewater treatment plants of 7 Croatian cities (Vinkovci, Bjelovar, Zagreb, Čakovec, Varaždin, Karlovac, Zadar). A) Non-metric multidimensional scaling (NDMS) plot of all samples, labelled by sample type (circles and triangles represent influent and effluent samples, respectively) and by sampling time (green and yellow – summer; red and blue - winter). Relative abundance of B) ESBL (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$) and C) carbapenemase genes ($bla_{OXA\text{-}48}$-like, $bla_{KPC\text{-}3}$, bla_{NDM}, bla_{IMP} and bla_{VIM}) in all samples in winter and summer. Asterisks indicate statistical differences in relative gene abundance between influent and effluent ($p < 0.05$, multiple paired t-test).

3.4. Correlation of ARG/bacterial abundances and physicochemical properties of effluent samples

We performed a Pearson's correlation analysis to investigate the relationship between the target ARGs (relative abundances) and the EOPs (absolute abundances), as well as the culturable coliforms and the physicochemical parameters of the effluent samples from all 7 WWTPs (Fig. 5). The relative abundances of both ESBL genes, bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$, were strongly and significantly correlated with temperature (r = 0.86, $p < 0.001$ and r = 0.90, $p < 0.001$, respectively). In addition, bla_{TEM} correlated significantly with *E. coli* (r = 0.58, $p < 0.05$) and with $bla_{CTX\text{-}M\text{-}32}$ and bla_{VIM} (r = 0.79, $p < 0.001$ and r = 0.54, $p < 0.05$, respectively). A significant negative correlation was observed with bla_{NDM} genes (r = -0.53, $p < 0.05$). Among the CP genes, $bla_{KPC\text{-}3}$ was significantly correlated with BOD_5 (r = 0.74, p < 0.01), suspended solids (r = 0.57, $p < 0.05$) and bla_{NDM} (r = 0.66, $p < 0.01$), and negatively correlated with *E. coli* (r = -0.62, $p <0.05$). The bla_{VIM} showed a significant positive correlation with pH (r = 0.70, p < 0.01) and bla_{TEM} (r = 0.54, $p < 0.05$), but a significant negative correlation with COD (r = -0.69, $p < 0.01$) (Fig. 5). The $bla_{OXA\text{-}48}$-like correlated significantly with enterococci and *A. baumannii* (r = 0.57, $p < 0.05$ and r = 0.71, $p < 0.01$, respectively). In addition to correlations with ARGs, *E. coli* was also significantly correlated with temperature (r = 0.55, $p < 0.05$), while

Enterococcus spp. were significantly correlated with COD (r = 0.55, $p < 0.05$) and suspended solids (r = 0.75, $p < 0.01$) (Fig. 5).

The concentrations of all culturable coliforms tested (non-resistant, CTX-R and CR combined coliforms) were significantly positively correlated with suspended solids (r = 0.55, $p < 0.05$; r = 0.75, $p < 0.01$; r = 0.80, $p < 0.001$, respectively). In addition, both CTX-R and CR coliform bacteria had a significant positive correlation with COD (r = 0.62 and 0.58, respectively; $p < 0.05$), while CR coliforms were additionally significantly correlated with *bla*$_{\text{OXA-48}}$-like and *A. baumannii* (Fig. 5).

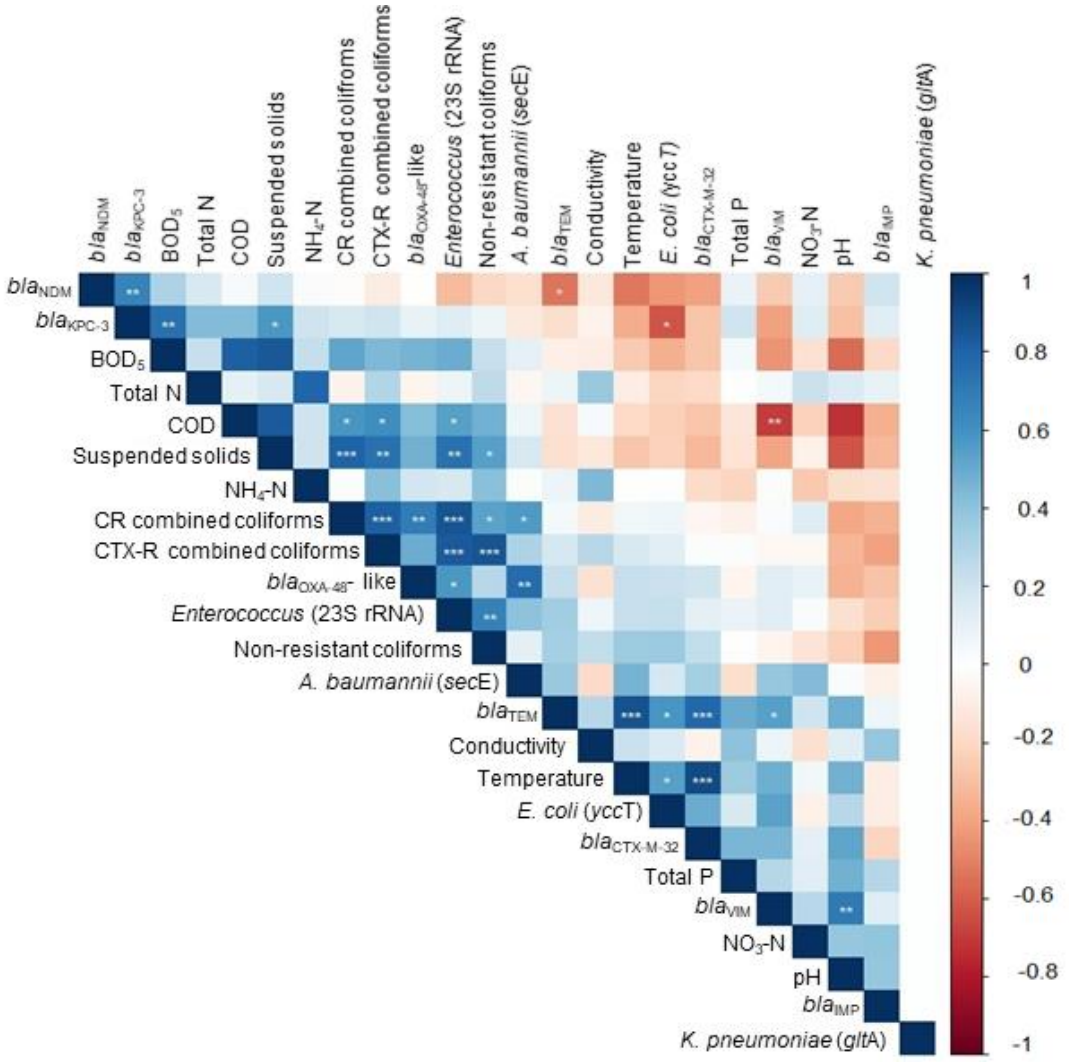

Fig. 5. Pearson's correlation analysis of relative abundance of ARGs (log(gene copies/*rrn* copies)), absolute abundance of EOPs (logCE/mL) and combined coliform bacteria (CFU/mL) and physicochemical parameters of final effluent samples. Cell colors from blue to red indicate positive and negative Pearson correlations, respectively, white indicates no correlation. Asterisks indicate significant correlations (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3.5. Influence of WWTP characteristics on the abundance of ARGs and EOPs

The impact of specific WWTP characteristics on the relative abundance of ARGs and the absolute abundance of EOPs (*E. coli*, *K. pneumoniae*, *A. baumannii* and *Enterococcus* spp.) was investigated by dividing the data obtained for each WWTP parameter into two groups and comparing them using the two-sample t-test. Parameters that showed a significant correlation with the tested gene abundances are the WWTP size, the number of hospitals and hospital beds in the catchment, and COD effluent concentration (Fig. 6).

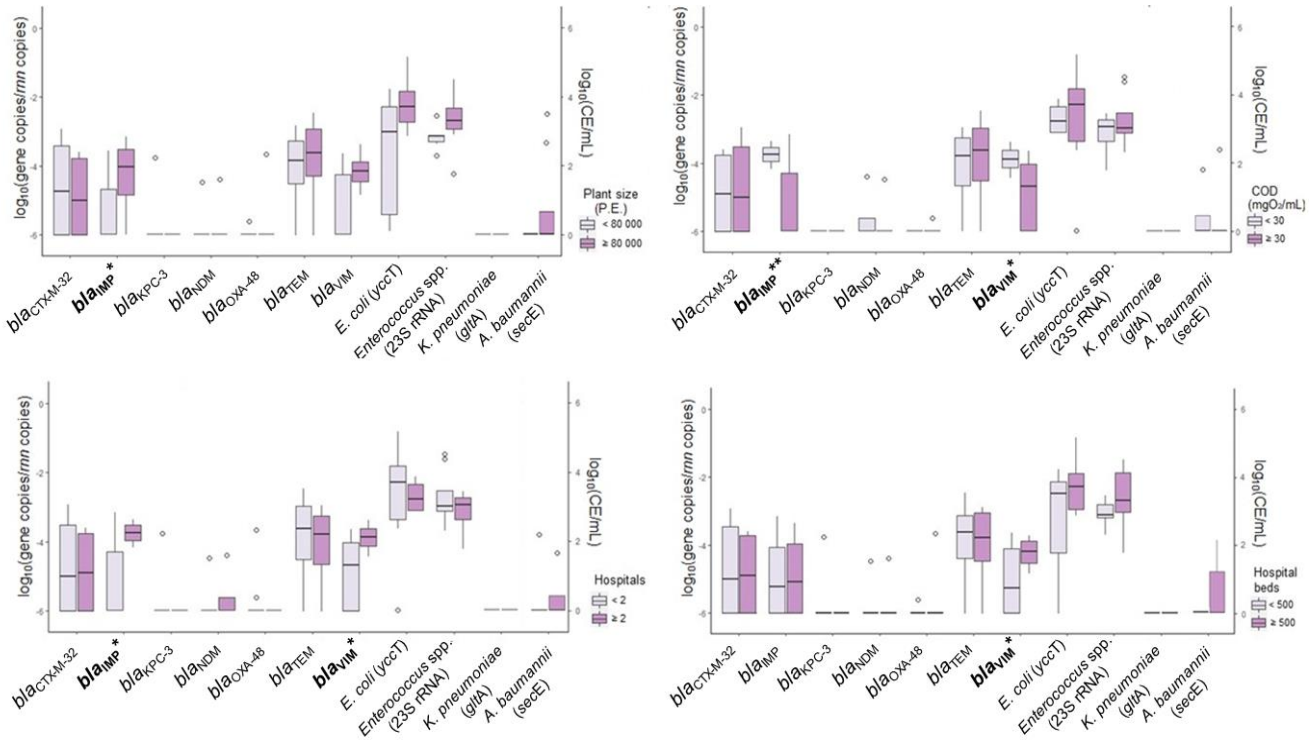

Fig 6. Boxplot comparison of relative abundance of ARGs or absolute abundance of taxonomic genes as proxies for EOPs (*E. coli*, *Enterococcus* spp., *K. pneumoniae* and *A. baumannii*) between selected WWTP parameters from both sampling seasons. Boxes represent quartiles and the median, whiskers are 1.5 x IQR, and circles represent outliers. *indicates significant difference at $p < 0.05$ and ** indicates significant difference at $p < 0.01$ between gene abundances in effluents from two groups of plants.

For example, plant size had a significant effect on the bla_{IMP} gene. The relative abundance of this gene was significantly increased ($p < 0.05$) in WWTPs with more than 80,000 population equivalents. In addition, both bla_{IMP} ($p < 0.001$) and bla_{VIM} ($p < 0.05$) were

significantly increased in plants that had two or more hospitals in the catchment area and COD levels in effluents below 30 mg O_2/L. Also, bla_{VIM} was significantly increased ($p < 0.05$) in WWTP catchments with 500 or more hospital beds (Fig. 6). No significant correlation with resistance or taxonomic gene concentrations was observed for any other parameter examined (Fig. S2).

4. Discussion

The increasing prevalence of resistance to ESC and carbapenems among clinically important pathogens, particularly enterobacteria, is of great concern due to limited antibiotic treatment options worldwide. WWTPs can be an important source for the spread of such ARB and their ARGs as well as EOPs in the environment. Therefore, a better understanding of the fate of ESC- and carbapenem-resistant determinants and various EOPs during wastewater treatment is needed to enable the development of strategies to reduce the risk to public health. Here, we used conventional culturing to monitor coliform bacteria (non-resistant, CTX-R and CR) and qPCR assays to monitor EOPs (*E. coli*, *A. baumannii*, *K. pneumoniae* and *Enterococcus* spp.) and ARGs of special clinical concern (ESBL and CP genes) in 7 Croatian WWTPs during sampling campaigns in winter and summer. All studied WWTPs operated with conventional activated sludge treatment (CAS), but their design capacities (43,000 – 1,2000 000 p.e.) and the number of hospitals/hospital beds in the catchment areas varied in a rather large range.

Analysis of the levels of non-resistant, CTX-R and CR coliforms in the influent and effluent of the WWTPs showed a significant decrease in most of the WWTPs in both seasons. CTX-R and CR coliforms showed a similar average decrease of about 2 log units in both seasons. These results are comparable to those obtained for the removal of CTX-R coliforms in international WWTPs using similar methods (2.1 log reduction for CAS treatment) (Marano et al., 2020). Despite the observed decrease in CTX-R and CR *E. coli* and other coliforms in

effluent samples, the WWTPs of one large (Zagreb) and one small (Varaždin) continental city still discharge up to 10^{11} CTX-R or CR *E. coli* and up to 10^{12} CTX-R or CR other coliforms per day into natural waters. This is consistent with previous studies showing that WWTPs can discharge about 10^{10} – 10^{12} ESC-resistant *E. coli* daily into the aquatic environment, depending on the size of the plant (Bréchet et al., 2014; Kwak et al., 2015). In addition, the concentrations of CTX-R and CR coliforms, especially in winter, were usually among the highest in the influents of the Varaždin WWTP, which can be associated with several factors, including contamination from the local poultry industry. On the other hand, the content of CTX-R and CR coliforms in the influent was not significantly reduced after the treatment, indicating lower effectiveness of the Varaždin WWTP in removing these resistant populations compared to other analyzed plants. In Croatia, there is a regulation on the presence of *E. coli* in municipal effluent discharged to surface waters for bathing and recreational purposes. The *E. coli* concentration should not exceed 10^3 CFU/100 mL in this effluent (Official Gazette of the Republic of Croatia 26/2020). In the present study, culturable (non-resistant) *E. coli* were found in concentrations greater than 10^3 CFU/100 mL (up to 10^5 CFU/100 ml) in all final effluents at both seasons, although the receiving waters of the studied WWTPs are not official bathing areas. Moreover, the concentrations of CTX-R and CR *E. coli* in the effluent of the Varaždin WWTP were above 10^3 CFU/100 mL in both seasons and in Bjelovar in winter. These concentrations were higher than the concentrations of ESC-resistant *E. coli* found in the effluent from Dutch WWTPs (about 10^1 CFU/100 mL; Verburg et al., 2019), but lower than in effluents from Polish WWTPs with different modifications of treatment processes (about 10^4 CFU/100 mL; Osińska et al. (2017). Additionally, positive correlations between parameters such as COD, suspended solids and effluent levels of CTX-R and CR coliform bacteria were observed in this study. Therefore, our data suggest that wastewater treatment processes in Croatian municipal WWTPs need to be

improved and supplemented with disinfection technologies to control the spread of CTX-R and CR *E. coli* and other coliforms via effluents into receiving waters.

Because removal of *E. coli* and other EOPs in WWTPs may be inadequate, discharge of treated wastewater may be a pathway for widespread dissemination of EOPs. We therefore applied qPCR to monitor several EOPs such as *E. coli*, *A. baumannii*, *K. pneumoniae* and *Enterococcus* spp. in 7 WWTPs studied. The results show that most WWTPs generally remove *A. baumanii* and *K. pneumoniae* populations efficiently, as they were rarely or not detected in the final effluents. On the contrary, although *E. coli* and enterococci were generally significantly reduced by biological treatment in the majority of the analyzed WWTPs, they were still present in the effluents in high amounts, in the range of 10^4-10^5 CE/100 mL in both seasons. The exception is the WWTP in the city of Varaždin, where the removal of *E. coli* and enterococci was the lowest and therefore, the concentrations in the effluent were the highest (10^6-10^7 CE/100 mL), in agreement with the data for culturable coliforms. The *E. coli* levels observed in the effluents in this study were comparable to those reported by Heß et al. (2016) and Jäger et al. (2018) based on qPCR of the *uid*A and *ycc*T genes, respectively (10^4-10^5 CE/100 mL). However, the enterococci levels found in the effluents in this study (about 10^5 CE/100 mL) were about 2 orders of magnitude higher than in two German studies based on qPCR of the 23S rRNA gene (Jäger et al., 2018; Hembach et al., 2019). In addition, higher concentrations of *E. coli* determined by qPCR compared to those determined by culturing are to be expected in this study, as qPCR also detects dead cells and cells that are in a ″viable but not culturable″ status. In addition to EOPs, we targeted the 16S rRNA gene as a marker for total bacteria and found that it was present at concentrations of about 10^7-10^{10} CE/100 mL after conventional treatment, which is comparable to previous reports on WWTPs with conventional (Czekalski et al., 2012; Nõlvak et al., 2013; Jäger et al., 2018) and advanced treatment (ozonation) (Heß et al., 2016; Hembach et al., 2019).

Further focus on qPCR quantification of β-lactam resistance genes of major importance in clinical setting (*bla*$_{\text{CTX-M-32}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{KPC-3}}$, *bla*$_{\text{OXA-48}}$-like, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$) showed that the abundance of these genes in influent and effluent varied greatly depending on the type of gene, the sampling location, and the sampling period. β-lactams are one of the most frequently prescribed classes of antibiotics worldwide, including in Croatia, and the most worrisome types of resistance are ESBLs, which confer resistance to ESC, and carbapenemases which confer resistance to carbapenems and all other β-lactams (Paterson and Bonomo, 2005; Sawa et al., 2020). Here, both ESBL genes, *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$, were present in all influent samples, with relative abundance about an order of magnitude lower in winter than in summer. With the exception of *bla*$_{\text{CTX-M-32}}$ in the winter samples, these two genes were predominantly only slightly reduced or even increased after treatment, indicating variation in relative host abundance or possible HGT to new hosts during treatment. Both *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$ genes were detected in effluent samples from different European WWTPs at concentrations one order of magnitude lower than here (Cacace et al., 2019) and in influent and effluent from Polish WWTP at higher concentrations than here (Zieliński et al., 2021). CTX-M-type β-lactamases are the most common types of ESBLs (Mlynarcik et al., 2021) and TEM type β-lactamases are the most common and widespread plasmid-encoded β-lactamase in the environment (Narciso-Da-Rocha et al., 2014; Proia et al., 2018). Plasmids carrying *bla*$_{\text{TEM}}$ often contain *bla*$_{\text{CTX-M}}$ genes (Hembach et al., 2017), which is consistent with the strong positive correlation between *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$ observed in this study. The observation that these two genes correlate strongly with temperature suggests that the proliferation and persistance of bacteria carring these genes is stimulated at warmer temperatures. In addition, a significant positive correlation was found between *bla*$_{\text{TEM}}$ and *E. coli*, suggesting that *E. coli* may be the host of this gene, as previously shown in the literature (Adekanmbi et al., 2020).

569 The most frequent carbapenemases in *Enterobacterales* reported in Europe are KPC,
570 VIM, IMP, NDM and the OXA-48-like enzymes (ECDC, 2013). Of particular concern is the
571 fact that carbapenemases are no longer limited to hospital isolates. In addition to hospitals, they
572 have also been found in long-term care facilities, in the community, in sewage and in receiving
573 waters (Buelow et al., 2018; Proia et al., 2018; Jelić et al., 2019; Sib et al., 2020). Moreover,
574 they continue to spread because their genes, often found in plasmids (Nasri et al., 2017;
575 Freeman et al., 2020), are associated with mobile elements that facilitate their acquisition (e.g.,
576 by clonal and horizontal transfer) and their spread from bacterium to bacterium (Subirats et al.,
577 2017; Zhang et al., 2020). Surveillance among clinical enterobacterial isolates in Croatia
578 showed that the most frequent carbapenemases from 2008-2012 were VIM and NDM, while
579 from 2015-2017, OXA-48 became predominant (Zujić Atalić et al., 2014; Bedenić et al., 2018)
580 In the present study, *bla*$_{\text{KPC-3}}$, *bla*$_{\text{NDM}}$ and *bla*$_{\text{IMP}}$ were sporadically detected in the influent of
581 some WWTPs, mainly in winter (about -3 to -5 log copies/*rrn* copies), which is consistent with
582 reports for some European WWTPs (Subirats et al., 2017; Pärnänen et al., 2019). However,
583 they were rarely detected in effluents, except for *bla*$_{\text{IMP}}$. Interestingly, in seven cases (4 in
584 winter, 3 in summer), *bla*$_{\text{IMP}}$ was found only in the final effluent (about -3 log copies/*rrn* copies)
585 but not in the paired influent, suggesting an enrichment of the bacteria carrying it during
586 wastewater treatment. The opposite was observed for *bla*$_{\text{OXA-48}}$, which was detected mainly in
587 the influent (about -5 log copies/*rrn* copies in winter and -3 log copies/*rrn* copies in summer)
588 but not in the paired effluents of the different WWTPs (except for two WWTPs), indicating its
589 good removal during conventional wastewater treatment. This could be the result of a good
590 removal of *A. baumannii* as a potential host of this gene in the studied WWTPs (qPCR data),
591 since a positive correlation was found between *bla*$_{\text{OXA-48}}$ and *A. baumannii.* This pathogen has
592 already been shown to carry *bla*$_{\text{OXA-48}}$-like genes (Gonçalves et al., 2013;Assem et al., 2017).
593 Finally, the *bla*$_{\text{VIM}}$ gene was detected in the majority of influents (average -4.2 log copies/*rrn*

copies) and effluents (average -4 log copies/*rrn* copies) of the studied WWTPs, being enriched in four WWTPs in winter and in summer. This gene was also found in some influent samples in different European countries (Pärnänen et al., 2019) and in effluents from different German WWTPs (Hembach et al., 2019; Sib et al., 2020), but at lower concentrations (10^1 copies/mL) than in the effluents analyzed in this study (10^2 - 10^3 copies/mL).

Finally, our results also showed that specific WWTP characteristics affect the amount of some ARGs released *via* the final effluent. In contrast to earlier findings (Cacace et al., 2019), we showed that facility size and number of hospitals correlated with concentrations of bla_{IMP} in WWTP effluents, while number of hospitalized patients correlated with concentrations of bla_{VIM} in effluents. However, the concentrations of both bla_{IMP} and bla_{VIM} were found to be inversely correlated with the effluent concentration of COD.

5. Conclusions

This study represents the first comprehensive investigation of the presence and removal of culturable coliforms, specific EOPs and ARGs, which are of utmost importance in the clinical field, in municipal WWTPs of various Croatian cities. We demonstrated that the total bacterial load and the load of culturable coliforms, including CTX-R and CR coliforms, and EOPs such as *E. coli* and *Enterococcus* spp. were reduced but not eliminated during conventional treatment. Our research also showed that target ESBL genes were only slightly reduced or even enriched after treatment. Target CP genes, which can be horizontally transmitted, such as bla_{KPC}, bla_{NDM} and bla_{OXA-48}-like, were sporadically detected in influents and/or effluents, while bla_{IMP} and bla_{VIM} genes were frequently enriched during the process. Therefore, advanced treatment technologies should be employed to reduce the emission of bacteria, including top priority EOPs and ARGs, in effluent and minimize the risk of environmental pollution and resulting public health.

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References

Adekanmbi, A.O., Akinpelu, M.O., Olaposi, A.V., Oyelade, A.A., 2020. Extended spectrum beta-lactamase encoding gene-fingerprints in multidrug resistant *Escherichia coli* isolated from wastewater and sludge of a hospital treatment plant in Nigeria. Int. J. Environ. Stud. 78, 140–150. https://doi.org/10.1080/00207233.2020.1778271

Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G.J., McEwen, S.A., Ryan, J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001. https://doi.org/10.1289/ehp.1206316

Assem, M., Abdala Wifi, M.-N., Elsherif, R., Saad, A., Kadry Ismail, D., Hasain, A., Bassyouni, R., Hussein Gomaa, M.S., 2017. Emergence of Gram-Negative Bacilli with Concomitant *bla*$_{\text{NDM-1}}$- and *bla*$_{\text{OXA-48}}$-Like Genes in Egypt. Am. J. Intern. Med. 5, 1. https://doi.org/10.11648/j.ajim.20170501.11

Bedenić, B., Slade, M., Starčević, L.Ž., Sardelić, S., Vranić-Ladavac, M., Benčić, A., Atalić,

645 V.Z., Bogdan, M., Bubonja-Šonje, M., Tomić-Paradžik, M., Tot, T., Lukić-Grlić, A.,
646 Drenjančević, D., Varda-Brkić, D., Bandić-Pavlović, D., Mihaljević, S., Zarfel, G.,
647 Gužvinec, M., Conzemius, R., Barišić, I., Tambić-Andraševic, A., 2018. Epidemic spread
648 of OXA-48 beta-lactamase in Croatia. J. Med. Microbiol. 67, 1031–1041.
649 https://doi.org/10.1099/jmm.0.000777
650 Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M.H., Zheng, C., 2019. Human health risk assessment
651 of antibiotic resistance associated with antibiotic residues in the environment: A review.
652 Environ. Res. 169, 483–493. https://doi.org/10.1016/j.envres.2018.11.040
653 Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors
654 influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev.
655 42, 68–80. https://doi.org/10.1093/femsre/fux053
656 Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: Characterization,
657 epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14,
658 933–951. https://doi.org/10.1128/CMR.14.4.933-951.2001
659 Bréchet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., Guyeux C., Hocquet,
660 D., Bertrand, X. 2017. Wastewater treatment plant release large amounts of extended -
661 spectrum β – producing *Escherichia coli* into the environment. Clin. Infect. Dis. 12, 1658-
662 1665. https://doi.org/10.1093/cid/ciu190.
663 Buelow, E., Bayjanov, J.R., Majoor, E., Willems, R.J.L., Bonten, M.J.M., Schmitt, H., van
664 Schaik, W., 2018. Limited influence of hospital wastewater on the microbiome and
665 resistome of wastewater in a community sewerage system. FEMS Microbiol. Ecol. 94.
666 https://doi.org/10.1093/femsec/fiy087
667 Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia,
668 P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D.,
669 Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019.

Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039

Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L., Baquero, F., Coque, T.M., 2008. Prevalence and spread of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. 14, 144–153. https://doi.org/10.1111/j.1469-0691.2007.01850.x

Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, 1–10. https://doi.org/10.1093/femsec/fiw060

Clifford, R.J., Milillo, M., Prestwood, J., Quintero, R., Zurawski, D. V., Kwak, Y.I., Waterman, P.E., Lesho, E.P., Mc Gann, P., 2012. Detection of bacterial 16S rRNA and identification of four clinically important bacteria by real-time PCR. PLoS One 7, 1–7. https://doi.org/10.1371/journal.pone.0048558

Czekalski, N., Berthold, T., Caucci, S., Egli, A., Bürgmann, H., 2012. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. Front. Microbiol. 3, 1–18. https://doi.org/10.3389/fmicb.2012.00106

European Centre for Disease Prevention and Control. 2013. Carbapenemase-producing bacteria in Europe: interim results from the European Survey on carbapenemase-producing Enterobacteriaceae (EuSCAPE) project. Stockholm, ECDC. https://doi.org/10.2900/91714.

European Centre for Disease Prevention and Control. 2020. Antimicrobial resistance in the EU/EEA (EARS-Net) – Annual Epidemiological Report 2019. Stockholm, ECDC.

https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2019.pdf (accessed 4 June 2021).

Frahm, E. and Obst, U. 2002. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Of Microbiol. Methods 52, 123-131. https://doi.org/10.1016/S0167-7012(02)00150-1.

Freeman, R., Ironmonger, D., Hopkins, K.L., Puleston, R., Staves, P., Hope, R., Muller-Pebody, B., Brown, C.S., Hopkins, S., Johnson, A.P., Woodford, N., Oliver, I., 2020. Epidemiology of carbapenemase-producing *Enterobacterales* in England, May 2015–March 2019: national enhanced surveillance findings and approach. Infect. Prev. Pract. 2, 100051. https://doi.org/10.1016/j.infpip.2020.100051

Gadsby, N.J., McHugh, M.P., Russell, C.D., Mark, H., Conway Morris, A., Laurenson, I.F., Hill, A.T., Templeton, K.E., 2015. Development of two real-time multiplex PCR assays for the detection and quantification of eight key bacterial pathogens in lower respiratory tract infections. Clin. Microbiol. Infect. 21, 788.e1-788.e13. https://doi.org/10.1016/j.cmi.2015.05.004

Gonçalves, D., Cecilio, P., Ferreira, H., 2013. First detection of OXA-48-like producing *Acinetobacter baumannii* in the faecal flora of nursing home residents in Northern Portugal. ePoster presentation, ECCMID 2013, Berlin, Germany. https://www.escmid.org/escmid_publications/escmid_elibrary/material/?mid=6312 (accessed 29 April 2021)

Haller, L., Chen, H., Ng, C., Le, T.H., Koh, T.H., Barkham, T., Sobsey, M., Gin, K.Y.H., 2018. Occurrence and characteristics of extended-spectrum β-lactamase- and carbapenemase-producing bacteria from hospital effluents in Singapore. Sci. Total Environ. 615, 1119–1125. https://doi.org/10.1016/j.scitotenv.2017.09.217

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017.

720 Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic
721 resistance genes in microbial populations at different municipal wastewater treatment
722 plants in Germany. Front. Microbiol. 8, 1–11. https://doi.org/10.3389/fmicb.2017.01282
723 Hembach, N., Alexander, J., Hiller, C., Wieland, A., Schwartz, T., 2019. Dissemination
724 prevention of antibiotic resistant and facultative pathogenic bacteria by ultrafiltration and
725 ozone treatment at an urban wastewater treatment plant. Sci. Rep. 9, 1–12.
726 https://doi.org/10.1038/s41598-019-49263-1
727 Heß, S., Lüddeke, F., Gallert, C., 2016. Concentration of facultative pathogenic bacteria and
728 antibiotic resistance genes during sewage treatment and in receiving rivers. Water Sci.
729 Technol. 74, 1753–1763. https://doi.org/10.2166/wst.2016.304
730 Heß, S., Berendonk, T.U., Kneis, D., 2018. Antibiotic resistant bacteria and resistance genes in
731 the bottom sediment of a small stream and the potential impact of remobilization. FEMS
732 Microbiol. Ecol. 94. https://doi.org/10.1093/femsec/fiy128
733 Jäger, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G., Schwartz,
734 T., 2018. Reduction of Antibiotic Resistant Bacteria During Conventional and Advanced
735 Wastewater Treatment, and the Disseminated Loads Released to the Environment. Front.
736 Microbiol. 9, 1–16. https://doi.org/10.3389/fmicb.2018.02599
737 Jelić, M., Hrenović, J., Dekić, S., Goić-Barišić, I., Tambić Andrašević, A., 2019. First evidence
738 of KPC-producing ST258 *Klebsiella pneumoniae* in river water. J. Hosp. Infect. 103, 147–
739 150. https://doi.org/10.1016/j.jhin.2019.04.001
740 Karkman, A., Do, T.T., Walsh, F., Virta, M.P.J., 2018. Antibiotic-Resistance Genes in Waste
741 Water. Trends Microbiol. 26, 220–228. https://doi.org/10.1016/j.tim.2017.09.005
742 Khan, F.A., Hellmark, B., Ehricht, R., Söderquist, B., Jass, J., 2018. Related carbapenemase-
743 producing *Klebsiella* isolates detected in both a hospital and associated aquatic
744 environment in Sweden. Eur. J. Clin. Microbiol. Infect. Dis. 37, 2241–2251.

https://doi.org/10.1007/s10096-018-3365-9

Krilanović, M., Tomić-Paradžik, M., Meštrović, T., Beader, N., Herljević, Z., Conzemius, R., Barišić, I., Vraneš, J., Elveđi-Gašparović, V., Bedenić, B., 2020. Extended-spectrum beta-lactamases and plasmid diversity in urinary isolates of *Escherichia coli* in Croatia: a nation-wide, multicentric, retrospective study. Folia Microbiol. (Praha). 65, 649–667. https://doi.org/10.1007/s12223-019-00769-1

Kumar, M., Ram, B., Sewwandi, H., Sulfikar, Honda, R., Chaminda, T., 2020. Treatment enhances the prevalence of antibiotic-resistant bacteria and antibiotic resistance genes in the wastewater of Sri Lanka, and India. Environ. Res. 183. https://doi.org/10.1016/j.envres.2020.109179

Kwak, Y., Colque, P., Byfors, S., Giske, C. G., Möllby, R., Kühn, I. 2015. Surveillance of antimicrobial resistance among *Escherichia coli* in wasteeater in Stockholm during 1 year: does it reflect the resistance trends in the society? Int. J. Antimicrob. Agents 45, 25-32. https://doi.org/10.1016/j.ijantimicag.2014.09.016

Lepuschitz, S., Schill, S., Stoeger, A., Pekard-Amenitsch, S., Huhulescu, S., Inreiter, N., Hartl, R., Kerschner, H., Sorschag, S., Springer, B., Brisse, S., Allerberger, F., Mach, R.L., Ruppitsch, W., 2019. Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals. Sci. Total Environ. 662, 227–235. https://doi.org/10.1016/j.scitotenv.2019.01.179

López-Gutiérrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Methods 57, 399–407. https://doi.org/10.1016/j.mimet.2004.02.009

Makowska, N., Philips, A., Dabert, M., Nowis, K., Trzebny, A., Koczura, R., Mokracka, J.,

2020. Metagenomic analysis of β-lactamase and carbapenemase genes in the wastewater resistome. Water Res. 170, 115277. https://doi.org/10.1016/j.watres.2019.115277

Marano, R.B.M., Fernandes, T., Manaia, C.M., Nunes, O., Morrison, D., Berendonk, T.U., Kreuzinger, N., Telson, T., Corno, G., Fatta-Kassinos, D., Merlin, C., Topp, E., Jurkevitch, E., Henn, L., Scott, A., Heß, S., Slipko, K., Laht, M., Kisand, V., Di Cesare, A., Karaolia, P., Michael, S.G., Petre, A.L., Rosal, R., Pruden, A., Riquelme, V., Agüera, A., Esteban, B., Luczkiewicz, A., Kalinowska, A., Leonard, A., Gaze, W.H., Adegoke, A.A., Stenstrom, T.A., Pollice, A., Salerno, C., Schwermer, C.U., Krzeminski, P., Guilloteau, H., Donner, E., Drigo, B., Libralato, G., Guida, M., Bürgmann, H., Beck, K., Garelick, H., Tacão, M., Henriques, I., Martínez-Alcalá, I., Guillén-Navarro, J.M., Popowska, M., Piotrowska, M., Quintela-Baluja, M., Bunce, J.T., Polo-López, M.I., Nahim–Granados, S., Pons, M.N., Milakovic, M., Udikovic-Kolic, N., Ory, J., Ousmane, T., Caballero, P., Oliver, A., Rodriguez-Mozaz, S., Balcazar, J.L., Jäger, T., Schwartz, T., Yang, Y., Zou, S., Lee, Y., Yoon, Y., Herzog, B., Mayrhofer, H., Prakash, O., Nimonkar, Y., Heath, E., Baraniak, A., Abreu-Silva, J., Choudhury, M., Munoz, L.P., Krizanovic, S., Brunetti, G., Maile-Moskowitz, A., Brown, C., Cytryn, E., 2020. A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. Environ. Int. 144, 106035. https://doi.org/10.1016/j.envint.2020.106035

Mlynarcik, P., Chudobova, H., Zdarska, V., Kolar, M., 2021. In silico analysis of extended-spectrum β-lactamases in bacteria. Antibiotics 10, 1–21. https://doi.org/10.3390/antibiotics10070812

Narciso-Da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014. *bla*$_{\mathrm{TEM}}$ and *vanA* as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system. J. Glob. Antimicrob. Resist. 2, 309–315. https://doi.org/10.1016/j.jgar.2014.10.001

795 Nasri, E., Subirats, J., Sànchez-Melsió, A., Mansour, H. Ben, Borrego, C.M., Balcázar, J.L.,
796 2017. Abundance of carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$) in wastewater
797 effluents from Tunisian hospitals. Environ. Pollut. 229, 371–374.
798 https://doi.org/10.1016/j.envpol.2017.05.095
799 Nguyen, A.Q., Vu, H. P., Nquyen, L. N., Wang, Q., Djordjevic, S. P., Donner, E., Ying, H.,
800 Nghiem, L.D., 2021. Monitoring antibiotic resistance genes in wastewater treatment:
801 Current strategies and future challenges. Sci. Total Environ. 783, 146964.
802 https://doi.org/10.1016/j.scitotenv.2021.146964.
803 Nõlvak, H., Truu, M., Tiirik, K., Oopkaup, K., Sildvee, T., Kaasik, A., Mander, Ü., Truu, J.,
804 2013. Dynamics of antibiotic resistance genes and their relationships with system
805 treatment efficiency in a horizontal subsurface flow constructed wetland. Sci. Total
806 Environ. 461–462, 636–644. https://doi.org/10.1016/j.scitotenv.2013.05.052.
807 Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D.,
808 Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E.,
809 Wagner, H. 2018. Package „vegan“.
810 Osińska, A., Korzeniewska, E., Harnisz, M., Niestępski, S., 2017. Impact of type of wastewater
811 treatment process on the antibiotic resistance of bacterial populations. E3S Web Conf. 17.
812 https://doi.org/10.1051/e3sconf/20171700070
813 Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T.,
814 Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L.,
815 Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T.,
816 Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-
817 Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater
818 treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv.
819 5. https://doi.org/10.1126/sciadv.aau9124

Paterson, D.L., Bonomo, R.A., 2005. Clinical Update Extended-Spectrum Beta-Lactamases : a Clinical Update. Clin. Microbiol. Rev. 18, 657–686. https://doi.org/10.1128/CMR.18.4.657

Pazda, M., Kumirska, J., Stepnowski, P., Mulkiewicz, E., 2019. Antibiotic resistance genes identified in wastewater treatment plant systems – A review. Sci. Total Environ. 697, 134023. https://doi.org/10.1016/j.scitotenv.2019.134023

Petric, I., Philippot, L., Abbate, C., Bispo, A., Chesnot, T., Hallin, S., Laval, K., Lebeau, T., Lemanceau, P., Leyval, C., Lindström, K., Pandard, P., Romero, E., Sarr, A., Schloter, M., Simonet, P., Smalla, K., Wilke, B.-M., Martin-Laurent, F., 2011. Inter-laboratory evaluation of the ISO standard 11063 "Soil quality — Method to directly extract DNA from soil samples." J. Microbiol. Methods 84, 454–460. https://doi.org/10.1016/j.mimet.2011.01.016

Proia, L., Anzil, A., Borrego, C., Farrè, M., Llorca, M., Sanchis, J., Bogaerts, P., Balcázar, J.L., Servais, P., 2018. Occurrence and persistence of carbapenemases genes in hospital and wastewater treatment plants and propagation in the receiving river. J. Hazard. Mater. 358, 33–43. https://doi.org/10.1016/j.jhazmat.2018.06.058

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T.U., Schwartz, T., 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. 8. https://doi.org/10.1016/j.jece.2018.02.022

Sawa, T., Kooguchi, K., Moriyama, K., 2020. Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. J. Intensive Care 8, 1–13. https://doi.org/10.1186/s40560-020-0429-6

Sib, E., Lenz-Plet, F., Barabasch, V., Klanke, U., Savin, M., Hembach, N., Schallenberg, A., Kehl, K., Albert, C., Gajdiss, M., Zacharias, N., Müller, H., Schmithausen, R.M., Exner,

M., Kreyenschmidt, J., Schreiber, C., Schwartz, T., Parčina, M., Bierbaum, G., 2020. Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles. Sci. Total Environ. 746. https://doi.org/10.1016/j.scitotenv.2020.140894

Stoddard, S.F., Smith, B.J., Hein, R., Roller, B.R.K., Schmidt, T.M., 2015. rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic Acids Res. 43, D593–D598. https://doi.org/10.1093/nar/gku1201

Suay-García, Pérez-Gracia, 2019. Present and Future of Carbapenem-resistant *Enterobacteriaceae* (CRE) Infections. Antibiotics 8, 122. https://doi.org/10.3390/antibiotics8030122

Subirats, J., Royo, E., Balcázar, J.L., Borrego, C.M., 2017. Real-time PCR assays for the detection and quantification of carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$) in environmental samples. Environ. Sci. Pollut. Res. 24, 6710–6714. https://doi.org/10.1007/s11356-017-8426-6

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K-H., Gützkow, T., Eichler, W., Pühler, A., Schlüter, A. 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 155, 2306-2319. https://doi.org/10.1099/mic.0.028233-0.

Wei, T., Simko, V., Levy, M., Xie, Y., Jin, Y., Zemla, J., Freidank, M., Cai, J., Protivinsky, T. 2017. R package "corrplot": Visualization of a Correlation Matrix (Version 0.84)

Verburg, I., García-Cobos, S., Leal, L.H., Waar, K., Friedrich, A.W., Schmitt, H., 2019. Abundance and antimicrobial resistance of three bacterial species along a complete wastewater pathway. Microorganisms 7. https://doi.org/10.3390/microorganisms7090312

870 Walsh, T.R., 2010. Emerging carbapenemases: A global perspective. Int. J. Antimicrob. Agents
871 36, S8. https://doi.org/10.1016/S0924-8579(10)70004-2
872 Wang, J., Chu, L., Wojnárovits, L., Takács, E., 2020. Occurrence and fate of antibiotics,
873 antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal
874 wastewater treatment plant: An overview. Sci. Total Environ. 744, 140997.
875 https://doi.org/10.1016/j.scitotenv.2020.140997
876 Wickham, H., Chang. W. 2016. Package "ggplot2". Create Elegant Data Visualisations Using
877 the Grammar of Graphics. Version 2.2.1. 1–189
878 WHO 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery and
879 development of new antibiotics. Geneva: World Health Organization.
880 https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-
881 ET_NM_WHO.pdf (accessed 02 June 2021).
882 WHO 2018. Report on surveillance of antibiotic consumption: 2016-2018 early
883 Implementation. Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO
884 https://www.who.int/publications/i/item/who-report-on-surveillance-of-antibiotic-
885 consumption (accessed 02 April 2021).
886 Zhang, S., Huang, J., Zhao, Z., Cao, Y., Li, B., 2020. Hospital Wastewater as a Reservoir for
887 Antibiotic Resistance Genes: A Meta-Analysis. Front. Public Heal. 8, 1–12.
888 https://doi.org/10.3389/fpubh.2020.574968
889 Zieliński, W., Korzeniewska, E., Harnisz, M., Drzymała, J., Felis, E., Bajkacz, S., 2021.
890 Wastewater treatment plants as a reservoir of integrase and antibiotic resistance genes –
891 An epidemiological threat to workers and environment. Environ. Int. 156.
892 https://doi.org/10.1016/j.envint.2021.106641
893 Zujić Atalić, V., Bedenić, B., Kocsis, E., Mazzariol, A., Sardelić, S., Barišić, M., Plečko, V.,
894 Bošnjak, Z., Mijač, M., Jajić, I., Vranić-Ladavac, M., Cornaglia, G., 2014. Diversity of

carbapenemases in clinical isolates of *Enterobacteriaceae* in Croatia-the results of a multicentre study. Clin. Microbiol. Infect. 20, O894–O903. https://doi.org/10.1111/1469-0691.12635