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Characterization of *Acinetobacter baumannii* from water and sludge line of secondary wastewater treatment plant

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ABSTRACT

The elimination of potentially pathogenic bacteria in wastewater treatment plants (WWTPs) attracts much attention in public health. Reports on the occurrence of the emerging hospital pathogen *Acinetobacter baumannii* in wastewaters do not include a continuous monitoring at all WWTP stages. The objective of this study was to characterize *A. baumannii* recovered from the water and sludge line of the secondary WWTP in Zagreb, Croatia over the period of one year. Recovery of *A. baumannii* was performed using CHROMagar Acinetobacter plates. Antimicrobial susceptibility testing was performed with broth microdilution and results interpreted using EUCAST breakpoints for clinical isolates of *A. baumannii*. Molecular characterization was performed by WGS and cgMLST. The secondary WWTP treating the urban wastewater is constantly receiving viable *A. baumannii* along with genes encoding carbapenem resistance, and emitting them via effluent into the environment. Furthermore, *A. baumannii* from influent are incorporated into activated sludge flocs in aeration basin. *A. baumannii* can survive the technological process of anaerobic mesophilic sludge digestion, and is finally destroyed in alkaline lime-treated stabilized sludge. The majority (102/119) of *A. baumannii* isolates were carbapenem-resistant, while antibiotic-susceptible isolates (17/119) were rarely recovered from all WWTP stages. Carbapenem-resistant isolates belonged to international clonal lineage IC2 carrying OXA-23 and IC1 carrying OXA-72, while the susceptible isolates belonged to IC5 or were unclustered. Increased resistance to antibiotics, together with the appearance of carbapenem- and even pandrug-resistant isolates in effluent as compared to influent wastewater, suggests the need of additional disinfection of effluent prior to its discharge into the natural recipient.

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1. Introduction

Acinetobacter baumannii is a Gram-negative, nonmotile, non-sporing, obligate aerobic coccobacillus. Since the 1990s *Acinetobacter baumannii* appeared as a leading cause of nosocomial infections and hospital outbreaks, but also of sporadic acute community-acquired infections (Dexter et al., 2015). *A. baumannii* causes a variety of infections such as ventilator-associated pneumonia, skin and soft-tissue infections, secondary meningitis, urinary tract infections, surgical wound and bloodstream infections, endocarditis, intra-abdominal abscesses, and is particularly problematic in the intensive care setting (Camp and Tatum, 2010).

A. baumannii has until recently been mostly isolated from hospitalized patients and was rarely from the environment outside of hospital settings. However, over the last decade there have been reports on the existence of *A. baumannii* in environments influenced by human waste. Urban wastewater represents one of the largest proportions of human waste. Much attention is given to the technologies of urban wastewater treatment in order to remove pathogens before its discharge into the natural environment. Urban wastewaters consist of different types of wastewaters that are generated in cities such as domestic, industrial, hospital and storm wastewaters. Of these, hospital wastewaters are recognized as the source of *A. baumannii* of clinical significance. Viable multi-drug resistant (MDR) *A. baumannii* were recovered from untreated hospital wastewater in Brazil, China and Croatia (Ferreira et al., 2011; Zhang et al., 2013; Seruga Music et al., 2017). Recently,

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dissemination of viable *A. baumannii* of clinical significance via the hospital wastewater and urban sewage to river was described in Croatia (Seruga Music et al., 2017).

Viable MDR and carbapenem-resistant *A. baumannii* were recovered from urban wastewater that also contained hospital wastewater, both before and after passage through the secondary wastewater treatment process (Goic-Barisic et al., 2016, 2017; Hrenovic et al., 2016). The above mentioned reports on the occurrence of *A. baumannii* in wastewaters are based on samples collected at one or few sporadic occasions, lacking a continuous monitoring. The secondary type of wastewater treatment process by activated sludge is the most commonly used technology of wastewater treatment worldwide. During the process of wastewater treatment, a surplus sludge is generated. This surplus sludge represents a concentrate of different microorganisms including pathogenic ones. Thus, the surplus sludge should be properly disinfected prior to its disposal in order to prevent the negative impact on the environment. However, in the aforementioned studies, the presence of viable *A. baumannii* was examined only in the line of untreated and treated wastewater, while the line of activated sludge is not examined up to date.

In this study, different stages of the wastewater treatment plant (WWTP) including liquid fractions and sludge were screened over the period of one year for the presence of viable *A. baumannii*. Molecular characterization of isolates was performed in order to elucidate the propagation and fate of *A. baumannii* through the WWTP. These data will be used to initiate strategies for mitigating the propagation of *A. baumannii* via WWTPs effluent into the natural environment, as well as to understand the epidemiology of this emerging human pathogen.

2. Materials and methods

2.1. Wastewater and sludge sampling

Wastewater and sludge were collected at the WWTP in Zagreb, Croatia. The treatment plant is designed for the secondary treatment of urban wastewaters for 1.2 million population equivalents. Urban wastewater from combined sewage systems consists of domestic, industrial, hospital and storm wastewaters. The retention time of wastewater in an underground network of sewers mainly depends on the storm water runoff. The WWTP receives wastewater from all nine clinical hospitals of Zagreb, which are, in accordance with the national legislation, released into the sewage system without pre-treatment (Hrenovic et al., 2016). The total number of beds in Zagreb's clinical hospitals is 4408. The hospital effluent flow rate can be assumed to be equal to the hospital water consumption or a fraction thereof (65–85%) and these values are estimated at 200–1200 L per bed per day (Verlicchi, 2018). It can be assumed that the City of Zagreb generates between 8.8×10^2 and 5.3×10^3 m^3 of hospital wastewater per day. This accounts for an average share of hospital wastewater in Zagreb WWTP influent of 0.3–1.7% (average yearly influent flow rate is 3.2×10^5 m^3/day, Hrenovic et al., 2017b).

After primary treatment at the WWTP, wastewater passes into an aeration basin with activated sludge where the hydraulic retention time varies from 2 to 5 h, with a sludge retention time of 3–11 days. The surplus sludge passes to a mesophilic anaerobic digestion process, where it is kept at 36 °C with a neutral pH and digestion time of 21–36 days. Digested sludge is stabilized by removal of water and treatment with lime, which increases its pH to approximately 12. Non-disinfected effluent water is discharged into the Sava River.

Samples were collected at 19 occasions between July 2015 and June 2016. On each occasion, the sampling was fixed at five stages of the WWTP: influent, effluent, activated sludge, digested sludge, and stabilized sludge. Influent and effluent water samples were 24-h composite samples, while sludge samples were instantaneous samples. Samples were aseptically collected and transported to the laboratory within 1 h.

2.2. Isolation, identification and antibiotic susceptibility profile of A. baumannii

The isolation of *A. baumannii* was performed according to Hrenovic et al. (2016) on CHROMagar Acinetobacter supplemented with 15 mg/L of cefsulodin sodium salt hydrate, both with or without the addition of carbapenem-selective supplement CR102 after the incubation at 42 °C/48 h. Identification of *A. baumannii* was performed by routine bacteriological techniques and the Vitek2 system (bioMérieux), and confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry - MALDI-TOF MS (software version 3.0, Microflex LT, Bruker Daltonics) on cell extracts (Sousa et al., 2014).

The susceptibility to carbapenems (meropenem, imipenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (tobramycin, gentamicin, amikacin), tetracyclines (minocycline), trimethoprim/sulfamethoxazole, and polymyxins (colistin) were determined by minimum inhibitory concentration (MIC) values obtained by the Vitek2 system using the AST-XN05 and AST-N233 testing cards. Colistin resistance was confirmed by gradient dilution E-test (bioMérieux) and broth microdilution method recommended by EUCAST. MICs were interpreted according to the EUCAST (2017) criteria for all antibiotics with defined breakpoints for *Acinetobacter* spp., while for minocycline CLSI (2015) breakpoints were used. Isolates were divided into three groups according to their antibiotic susceptibility profile: susceptible (S) - susceptible to all antibiotics tested; CFQR - resistant to carbapenems and fluoroquinolones; PDR - resistant to carbapenems, fluoroquinolones and colistin. Differences between percentages of resistance of isolates were calculated by using Statistica 12 software (StatSoft, Inc.). A p-value of <0.05 was considered significant.

2.3. Molecular characterization of A. baumannii

From each of the four stages of WWTP, a total of 16 isolates were chosen based on date of sampling and their antibiotic susceptibility profile for further molecular characterization: CR-IN30, CR-IN31, CD-IN39, CR-IN74, CR-S2, CR-S14, CR-S16, CR-D1, CR-D11, CR-D15, CR-D27, CR-EF7, CR-EF8, CR-EF11, CR-EF12, CR-EF31 (Supplementary Table 1). Whole genome sequencing was performed on the isolates and a core genome multilocus sequence typing (cgMLST) of *A. baumannii* isolates was investigated according to Higgins et al. (2017). For the purpose of this study, at least two isolates showing $\leq$12 allelic differences were designated a complex of highly related isolates. Genome sequences were also used to determine traditional 7-loci MLST (http://pubmlst.org/abaumannii/). The genes encoding acquired OXA-type carbapenemases (oxacillinases) were amplified by multiplex polymerase chain reaction (PCR) and their identification confirmed using the whole genome sequencing data. The acquired resistome was determined using genome assemblies and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/). All raw reads generated were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the BioProject accession number PRJEB25650.

2.4. Survival of A. baumannii in anaerobic conditions

The ability of 17 *A. baumannii* isolates recovered from digested sludge (named CR-D10 to CR-D27, Supplementary Table 1) to

survive or grow in anaerobic atmosphere was checked in controlled laboratory conditions. Isolates were pre-grown on nutrient agar (Biolife) plates aerobically (42 °C/24 h), and then exposed to the anaerobic conditions in Anaerocult A system (Merck Millipore) during 30 days at 36 °C. After the period of anaerobic cultivation, the biomass was re-inoculated onto the fresh Nutrient agar, and growth of isolates was checked after aerobic incubation at 42 °C/24 h. The ability of isolates to grow in anaerobic conditions was performed by direct exposure of inoculated plates to the anaerobic conditions in Anaerocult A system at 36 °C/72 h.

3. Results

3.1. Identification and antibiotic susceptibility profile of A. baumannii

During the 19 sampling occasions 95 samples were obtained, and a total of 119 *A. baumannii* isolates were recovered from different stages of the WWTP: 45 (38%) from influent wastewater; 18 (15%) from activated sludge; 20 (17%) from digested sludge; 36 (30%) from effluent wastewater. No *A. baumannii* was recovered from the stabilized lime-treated sludge samples. MALDI-TOF MS analysis gave the reliable score values ranging from 2.005 to 2.418, identifying them as *A. baumannii*.

The majority (102/119, 86%) of isolates demonstrated non-susceptibility to the tested antibiotics, while only 17/119 (14%) were susceptible to all tested antibiotics. Resistant isolates showed a CFQR or PDR antibiotic susceptibility profile (Fig. 1). The CFQR isolates were the most dominant group, comprising 82% of *A. baumannii* isolates recovered from all stages of WWTP, and there were 3% PDR isolates. The PDR isolates were recovered only from activated sludge (one isolate) and effluent wastewater (three isolates). Effluent wastewater contained a significantly lower percentage of susceptible isolates (3%) as compared to other stages of WWTP (13–30%, Fig. 1). When compared to influent wastewater, the percentage of resistant *A. baumannii* generally decreased in activated and digested sludge, but increased for all tested antibiotics in effluent wastewater (Fig. 2). However, a statistically significant difference in resistance was found only for tobramycin ($p = 0.029$) and colistin ($p = 0.000$) in effluent wastewater.

3.2. Molecular characterization of A. baumannii

The molecular characterization of 16 selected *A. baumannii* isolates is given in Tables 1 and 2 and summarised in Fig. 3. The majority of isolates (10/16) belonged to the international clonal lineage 2 (IC2) which were all sequence type (ST)-195 by the Oxford scheme and ST-2 by the Pasteur scheme, 2/16 isolates to IC1 (ST-1), 1/16 to IC5 (ST-79), while 3/16 isolates were unclustered and they did not cluster with any of the ICs (Table 1). The IC2 isolates were recovered at many time points from each stage of WWTP, showing the CFQR or PDR antibiotic susceptibility profile. The CFQR IC1 isolates were recovered only from influent and digested sludge. A single IC5 antibiotic-susceptible isolate was found only in activated sludge, but due to the flow of water and sludge in WWTP, they must have been previously present in influent water but were not detected. Other antibiotic-susceptible isolates from influent, digested sludge and effluent did not cluster with any ICs.

Based on cgMLST profiles of 16 selected *A. baumannii* isolates, a minimum spanning tree was generated based on a core genome of 2390 alleles (Fig. 3). The ten IC2 isolates differed from each other by 1–43 alleles, and included seven isolates in complex 1, which differed by a maximum of 12 alleles suggesting that they are highly related. IC2 also contained complex 2 which comprised two isolates differing in three alleles. A single IC2 isolate showed 15 allelic differences from complex 1. All other isolates were considered singletons including the two IC1 isolates. The distance between the clonal lineages and other isolates was ≥1367 alleles.

The acquired resistome of these isolates is shown in Table 2. All the carbapenem-resistant isolates had an acquired OXA, with OXA-23 present in all IC2 isolates, and OXA-72 in the IC1 isolates. In addition, carbapenem-resistant isolates also possessed resistance genes against macrolides, aminoglycosides, tetracycline, and chloramphenicol (Table 2). Several IC2 isolates also possessed *sul1*. The intrinsic OXAs of the isolates matched what would be expected for IC1 (OXA-69), IC2 (OXA-66), and IC5 (OXA-65). Isolates that cluster together had similar resistomes, with a few exceptions. In complex 1, all the isolates carried *aadA1, aph(3′)-VIa-like, armA, strA, strB, tetB, mphE* and *mseE,* all but one possessed *cat-A1-like* genes. In addition, several other resistance determinants were present in subsets of the isolates, suggesting that they are possibly plasmid encoded and some isolates have lost the plasmid. Carbapenem-

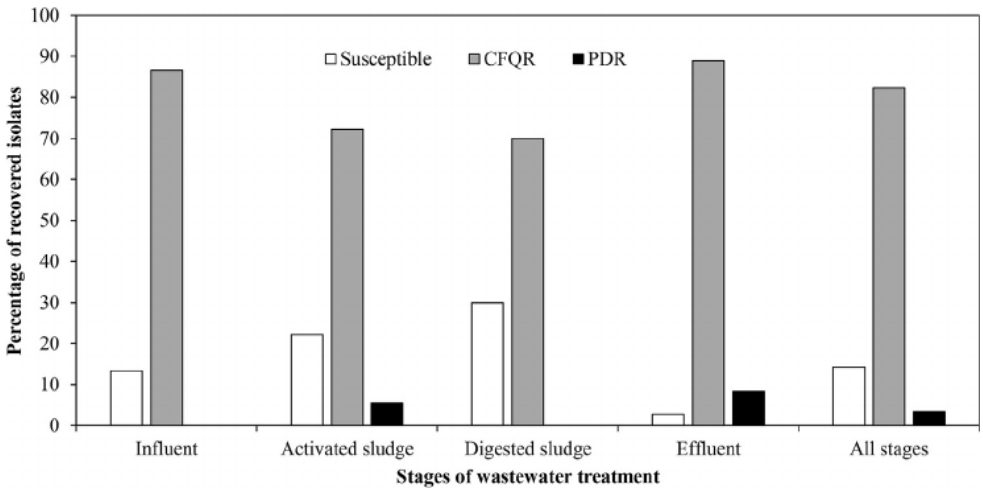

Fig. 1. Antibiotic susceptibility pro le of isolates recovered from different stages of the wastewater treatment process. CFQR - resistant to carbapenems and fluoroquinolones; PDR - resistant to carbapenems, fluoroquinolones and colistin. Number of isolates: influent 45; activated sludge 18; digested sludge 20; effluent 36; all stages (total) 119.

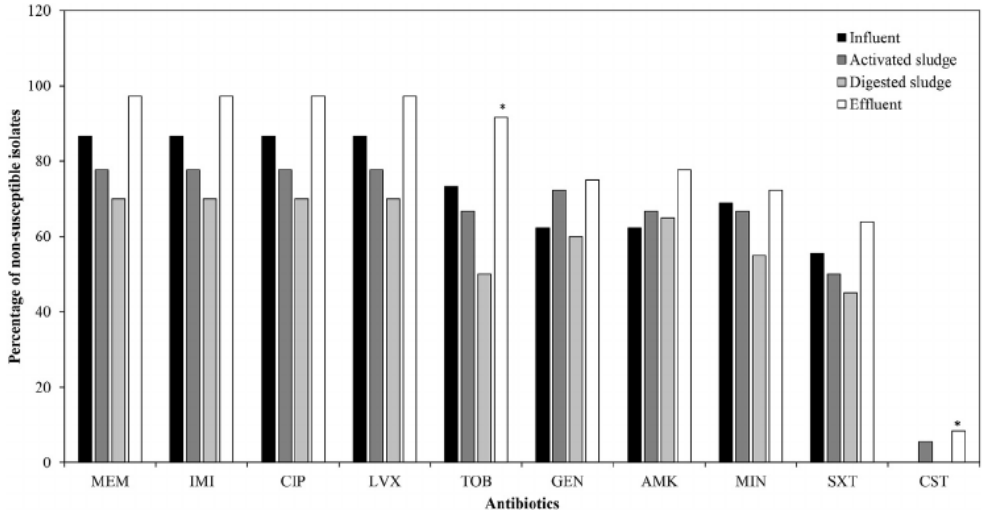

Fig. 2. Percentage of non-susceptible isolates to tested antibiotics [a] per each stage of the wastewater treatment process. [a] carbapenems (MEM-meropenem, IMI-imipenem), fluoroquinolones (CIP-ciprofloxacin, LVX-levofloxacin), aminoglycosides (TOB-tobramycin, GEN-gentamicin, AMK-amikacin), tetracyclines (MIN-minocycline), SXT-trimethoprim/sulfamethoxazole, polymyxins (CST-colistin). * statistically significantly higher than in the influent.

Table 1
Date of sampling, MLST results, and antibiotic susceptibility profile for 16 selected isolates of *A. baumannii* from different stages of the WWTP.
*antibiotics to which isolates remained susceptible are given in brackets; MIN-minocycline, SXT-trimethoprim/sulfamethoxazole, CST-colistin.
Isolates from: influent (IN), effluent (EF), activated sludge (S), digested sludge (D). No *A. baumannii* was recovered from stabilized sludge.

Isolate	Date of sampling	ST-Oxford	ST-Pasteur	Clonal lineage	Antibiotic susceptibility profile
CR-IN30	09.09.2015	ST-195	ST-2	IC2	CFQR (CST)*
CR-IN31	23.09.2015	ST-1523	ST-647	unclustered	S
CR-IN39	28.10.2015	ST-1604	ST-1	IC1	CFQR (MIN, CST, aminoglycosides)
CR-IN74	18.05.2016	ST-195	ST-2	IC2	CFQR (CST)
CR-S2	02.07.2015	ST-195	ST-2	IC2	CFQR (SXT, CST)
CR-S14	24.02.2016	ST-195	ST-2	IC2	PDR (MIN)
CR-S16	23.03.2016	ST-1524	ST-79	IC5	S
CR-D1	02.07.2015	ST-1525	ST-992	unclustered	S
CR-D11	14.10.2015	ST-231	ST-1	IC1	CFQR (MIN, SXT, CST)
CR-D15	13.01.2016	ST-195	ST-2	IC2	CFQR (SXT, CST)
CR-D27	08.06.2016	ST-195	ST-2	IC2	CFQR (SXT, CST)
CR-EF7	09.09.2015	ST-195	ST-2	IC2	PDR
CR-EF8	23.09.2015	ST-195	ST-2	IC2	CFQR (SXT, CST)
CR-EF11	18.11.2015	ST-1526	ST-139	unclustered	S
CR-EF12	02.12.2015	ST-195	ST-2	IC2	CFQR (CST)
CR-EF31	09.03.2016	ST-195	ST-2	IC2	PDR

susceptible isolates possessed only their intrinsic $bla_{\text{OXA-51-like}}$.

3.3. Survival of A. baumannii in anaerobic conditions

The 17 isolates of *A. baumannii* recovered from digested sludge were able to survive on nutrient agar in controlled anaerobic conditions during 30 days at 36 °C, after which they grew normally in aerobic conditions. However, the isolates were not able to grow directly in anaerobic conditions (data not shown).

4. Discussion

The occurrence of viable *A. baumannii* in the secondary type of WWTP was reported previously in influent and effluent water sampled on a few occasions (Goic-Barisic et al., 2016, 2017; Hrenovic et al., 2016). However, until now no systematic monitoring of *A. baumannii* at all stages of WWTPs has been performed, including water and sludge, and no data on the clonality of these isolates exist. In this study, during one-year monitoring (2015–2016) we detected the presence of *A. baumannii* at all stages of the water treatment, with the exception of lime-treated stabilized sludge. We found a predominance of isolates belonging to IC2 carrying the OXA-23 genes, which is mirrored in the prevalence of this clonal lineage and acquired carbapenemase in Europe and beyond (Higgins et al., 2010; Al Atrouni et al., 2016; Ning et al., 2017; Nowak et al., 2017; Pournaras et al., 2017). Indeed this clonal lineage was also found to be predominant in Zagreb hospitals, and is responsible for the majority of nosocomial outbreaks (Vranic-Ladavac et al., 2014). The dominance of *A. baumannii* belonging to ST-195 was also described in 2015 in hospitalized patients, untreated hospital wastewater, urban sewage, and river water in Zagreb (Seruga-Music et al., 2017). One *A. baumannii* isolate belonging to ST-195 and two isolates belonging to ST-231 carrying the OXA-23 and OXA-72 genes, respectively, were recovered in 2016 from soil at an illegal dump site near Rijeka in Croatia (Hrenovic et al., 2017a). During 2014–2015 four *A. baumannii* isolates carrying OXA-23 and one carrying OXA-72 encoding genes related to those described in clinical isolates, were recovered from influent and effluent wastewater at Zagreb WWTP (Goic-Barisic et al., 2016, 2017). This previous evidence together with the

Table 2
Acquired resistance genes and intrinsic bla_{OXA} for 16 selected isolates of *A. baumannii* from different stages of the WWTP.

Isolate	Acquired resistance genes						Intrinsic bla_{OXA}	MLST Ox/Pas
	Macrolide	Aminoglycoside	Tetracycline	Beta-lactam	Chloramphenicol	Sulfonamide	Beta-lactam	
CR-EF12	*mph(E), msr(E)*	*aac(3)-Ia-like, aadA1, aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*	*sul1*	bla_{OXA-66}	ST-195/ST-2
CR-IN74	*mph(E), msr(E)*	*aac(3)-Ia-like, aadA1, aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*	*sul1*	bla_{OXA-66}	ST-195/ST-2
CR-EF7	*mph(E), msr(E)*	*aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}			bla_{OXA-66}	ST-195/ST-2
CR-EF31	*mph(E), msr(E)*	*aac(3)-Ia-like, aadA1, aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*	*sul1*	bla_{OXA-66}	ST-195/ST-2
CR-S14	*mph(E), msr(E)*	*aac(3)-Ia-like, aadA1-like, aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*		bla_{OXA-66}	ST-195/ST-2
CR-S2	*mph(E), msr(E)*	*aac(3)-Ia-like, aadA1, aph(3′)-VIa-like, armA, strA, strB-like*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*		bla_{OXA-66}	ST-195/ST-2
CR-D27	*mph(E), msr(E)*	*aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*		bla_{OXA-66}	ST-195/ST-2
CR-D15	*mph(E), msr(E)*	*aph(3′)-Ic,armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}, bla_{TEM-1D}			bla_{OXA-66}	ST-195/ST-2
CR-EF8	*mph(E), msr(E)*	*aph(3′)-Ic, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}, bla_{TEM-1D}			bla_{OXA-66}	ST-195/ST-2
CR-IN30	*mph(E), msr(E)*	*aac(3)-Ia-like, aadA1, aph(3′)-VIa-like, armA, strA, strB*	*tet(B)-like*	bla_{OXA-23}	*catA1-like*	*sul1*	bla_{OXA-66}	ST-195/ST-2
CR-S16							bla_{OXA-65}	ST-1524/ST-79
CR-IN31							$bla_{OXA-208-like}$	ST-1523/ST-647
CR-D1							bla_{OXA-51}	ST-1525/ST-992
CR-D11	*mph(E), msr(E)*	*aph(3′)-VIa-like*		bla_{OXA-72}			bla_{OXA-69}	ST-231/ST-1
CR-IN39		*aac(3)-Ia-like, aadA1*		bla_{OXA-72}		*sul1*	bla_{OXA-69}	ST-1604/ST-1
CR-EF11							$bla_{OXA-117-like}$	ST-1526/ST-139

results obtained in this study suggest that *A. baumannii* belonging to IC2 and carrying OXA-23 dominate in Zagreb's hospitals, are discharged via untreated hospital wastewater into the urban sewage system, and thus are constantly present in the influent of WWTP. This hypothesis is confirmed by the minimum spanning tree based on cgMLST profiles, which shows that the isolates belonging to IC2 that were temporally (with an interval of 10 months) and spatially distinct were grouped into a closely related cgMLST cluster. The isolates belonging to IC1 carrying OXA-72 were also recovered at different time points and different stages of WWTP. This suggests that there is a continuous inflow of *A. baumannii* isolates belonging to IC2 and IC1 into Zagreb's WWTP receiving the urban wastewater.

The presence of viable *A. baumannii* in activated sludge, digested sludge and effluent suggests the incorporation of *A. baumannii* into activated sludge flocs during the biological treatment of wastewater in the aeration basin. *A. baumannii* inside the activated sludge flocs settles in secondary settlement tanks and undergoes the process of mesophilic anaerobic digestion. *A. baumannii* that are not settled in activated sludge flocs in secondary settlers is then discharged with effluent water into the natural recipient of treated water, the Sava River.

In situ and *ex situ* investigation confirmed the ability of *A. baumannii* to survive the technological process of anaerobic mesophilic sludge digestion. The finding confirms the need of proper management and disposal of sewage sludge generated at WWTP in order to prevent the spread of resistant *A. baumannii* in the environment. Moreover, it indicates the anaerobic environment as a possible ecological niche that enables the survival of this emerging human pathogen. The presence of genes encoding New Delhi metallo-beta-lactamase-1 (NDM-1) closely related to those found in clinical *A. baumannii* isolates were searched at one occasion in a Chinese WWTP receiving domestic and industrial wastewater (Luo et al., 2014). The NDM-1 genes were found in each stage of the WWTP, including the influent, aerobic, anoxic and anaerobic tank, chlorinated effluent, activated sludge, and dewatered sludge. The stabilization of waste sludge by lime treatment had no influence on the concentration of NDM-1 genes. Although the pH of lime-treated sludge was not reported (Luo et al., 2014), it could be assumed that the increase of pH did not impact on the concentration of NDM-1 genes. In our study, no viable *A. baumannii* was found in lime-treated stabilized sludge with a pH of around 12, suggesting the alkaline treatment as an efficient method of disinfection of waste sludge in order to remove the viable *A. baumannii*.

The published literature lacks the concentration of viable *A. baumannii* in environmental samples (Ferreira et al., 2011; Zhang et al., 2013; Hrenovic et al., 2016; Seruga Music et al., 2017), because there is no cultivation media that will allow the selective enumeration of only *A. baumannii*. The recovery of *A. baumannii* is based on the picking of morphologically presumptive colonies and identifying the species by molecular methods. This therefore hinders the accurate calculation of removal efficiency of *A. baumannii* in WWTP. The high proportion of *A. baumannii* recovered from the final effluent (30%) as compared to the raw influent (38% of total isolates) in the same period of monitoring, indicates a low removal efficiency of *A. baumannii* from wastewater in the examined secondary WWTP. Disinfection of the final effluent by chlorination was previously reported being inefficient for the elimination of viable carbapenem-resistant *A. baumannii* and NDM-1 genes (Zhang et al., 2013; Luo et al., 2014). Among the alternative methods of disinfection, alkaline treatment of the waste sludge and final effluent before its disposal into the natural recipient seems a promising

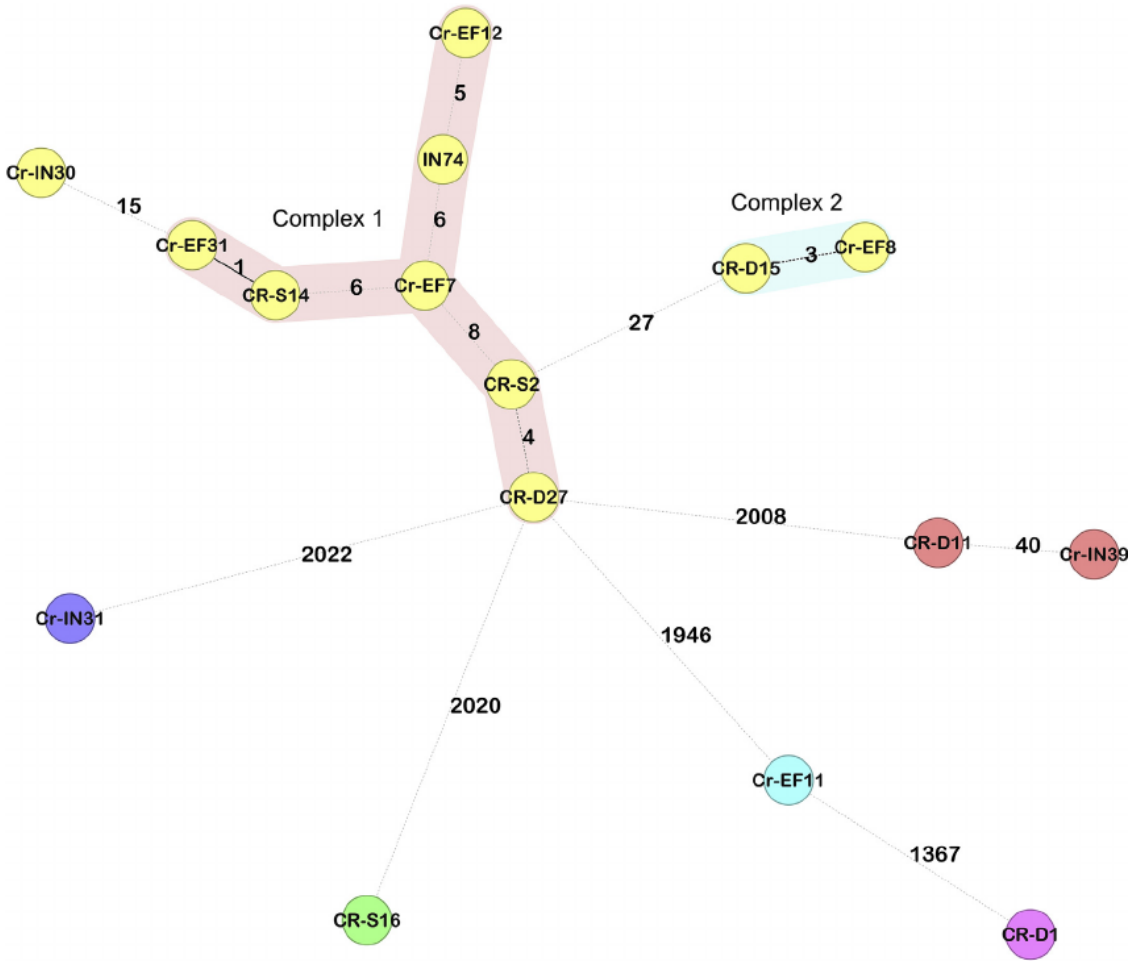

Fig. 3. Minimum spanning tree based on cgMLST allelic profiles of 16 selected *A. baumannii* isolates. Each circle represents an allelic profile based on the sequence analysis of 2390 cgMLST target genes. The numbers on the connecting lines illustrate the numbers of target genes with different alleles. Circles are coloured according to their Pasteur sequence type. A complex was defined as at least 2 isolates showing ≤12 allelic differences.

technology to prevent the propagation of viable *A. baumannii* in the environment.

A. baumannii isolates that showed a CFQR or PDR profile were carbapenem-resistant and represented 86% of all isolates. The same level of carbapenem resistance (87%) was reported for clinical *A. baumannii* isolates from Croatian hospitals in 2015 (CAMS, 2016). WWTPs were proposed as hotspots for the development of antibiotic resistance and proliferation of antibiotic-resistant bacteria (Bouki et al., 2013; Hrenovic et al., 2017b). In this study, an increase of resistance in *A. baumannii* to all tested antibiotics, with a statistically significant increase of resistance to tobramycin and colistin, was found in effluent as compared to influent wastewater. The PDR isolates were recovered only from activated sludge (one isolate) and effluent wastewater (three isolates). Since the effluent is discharged into the Sava River without disinfection, the dissemination of resistant *A. baumannii* in the natural environment through urban wastewater represents a serious concern. However, no significant increase of antibiotic resistance in *A. baumannii* isolates was found in the sludge line as compared to the influent. Moreover, no *A. baumannii* was recovered from the final lime-treated sludge. This suggests that lime-treated stabilized sludge is a safe byproduct of WWTP, but also the need for disinfection of the effluent water prior to its discharge into the environment.

Isolates susceptible to all tested antibiotics were less abundant, but were also found at many time points from each stage of the WWTP. One susceptible isolate belonged to IC5, which was previously considered a pan-American clone (Higgins et al., 2010). Although being a single observation, it points to the epidemiological investigation of the presence and dissemination of IC5 in Europe. Other susceptible isolates did not cluster with any ICs. Although unclustered isolates have been recovered from hospitalized patients (Higgins et al., 2010) and more recently were found in livestock and wild birds (Wilharm et al., 2017), susceptible isolates are very rarely found in Zagreb's hospitals. The nutrient-rich urban wastewater and environmental conditions in the secondary WWTP were shown to also support the persistence of susceptible *A. baumannii*. *A. baumannii* was previously shown to grow and survive in the closed system of aerated effluent from WWTP for over 50 days (Hrenovic et al., 2016). Most probably, the susceptible isolates are also able to survive and multiply in aerated stages of WWTP, which enables their long-term persistence in the urban sewage system, WWTP, and the river as final recipient of treated wastewater. Susceptible isolates were recovered from the water and sludge line harbouring dominant (86%) CFQR isolates carrying OXA-23 and OXA-72. The selective pressure driving horizontal gene transfer in the environmental conditions of WWTP is possibly not strong enough, allowing for the persistence of an antibiotic-susceptible population of *A. baumannii*. These data suggest that

urban sewage system and WWTP could represent a secondary habitat of *A. baumannii*.

5. Conclusions

- influent of urban wastewater at the secondary WWTP contains the viable clinically relevant bacterium *A. baumannii* along with genes encoding for carbapenem resistance
- *A. baumannii* propagate through all stages the WWTP and are released via effluent into the environment
- survival of *A. baumannii* through the technological process of anaerobic mesophilic sludge digestion indicates the anaerobic environment as an ecological niche important in its epidemiology
- the existence of antibiotic-susceptible isolates among the dominant antibiotic-resistant isolates suggests that sewage and WWTP are a secondary habitat of *A. baumannii* outside of the hospital settings
- alkaline lime-treatment of waste sludge is an efficient method to prevent *A. baumannii* disseminating in the environment
- novel methods of effluent disinfection are needed for mitigating the propagation of *A. baumannii* via WWTP effluents into the natural environment.

Transparency declarations

None to declare.

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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.2018.04.057.

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