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Extensively and multi drug-resistant *Acinetobacter baumannii* recovered from technosol at a dump site in Croatia

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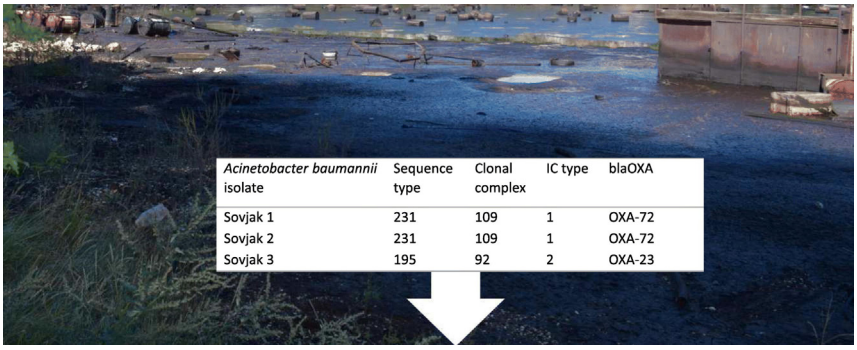
HIGHLIGHTS

- Technosol at the edge of dump site is rich in petroleum hydrocarbons and heavy metals.
- From technosol three isolates of *A. baumannii* were recovered.
- Isolates from technosol are closely related to clinical isolates of *A. baumannii*.
- Illegally disposed hospital waste is the most probable source of *A. baumannii* in technosol.
- Illegal dump sites could represent the public health risk.

GRAPHICAL ABSTRACT



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ABSTRACT

In a karst pit above City of Rijeka in Croatia the hazardous industrial waste was continuously disposed from 1955 to 1990, and later it was periodically used as an illegal dump site. The surface part of a technosol at the edge of dump was analysed mineralogically, geochemically and bacteriologically. From the technosol rich in petroleum hydrocarbons and heavy metals three isolates of *Acinetobacter baumannii* were recovered. Isolates from technosol shared many features that are previously described for clinically isolates: the affiliation to IC1 and 2, multi-drug resistant (MDR) or extensively drug-resistant (XDR) antibiotic resistance profile, carbapenem resistance mediated by bla_{OXA72} and bla_{OXA23} genes, and the expression of virulence factors. In *in vitro* conditions, isolates were able to survive in contact with technosol during 58 days of monitoring. The most probable source of *A. baumannii* in technosol was the illegally disposed hospital waste. Proper management and disposal of human solid waste is mandatory to prevent the spread of clinically important *A. baumannii* in nature.

1. Introduction

Acinetobacter baumannii is an emerging pathogen frequently reported as a cause of hospital outbreaks or sporadic acute community-acquired infections (Dexter et al., 2015). Due to its high frequency of antibiotic resistance and capability to escape the biocidal action of

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antibiotics, *A. baumannii* is highlighted among the so-called "ESKAPE pathogens" (Pendleton et al., 2013). Although *A. baumannii* is not a sporogenic bacterium, it is able to survive in wet or dry inanimate environment during few months (Espinal et al., 2012; Hrenovic et al., 2016).

Human waste is generally known as a source of different pathogens that could be spread in the environment and thus represent a public health risk. Data on the presence of *A. baumannii* in natural environment influenced by human solid waste are very scarce and the role of environmental isolates in the occurrence of human infections is not elucidated. There is only one literature report on the incidental finding of one multi-drug resistant (MDR) *A. baumannii* which is related to a clinical isolate in paleosol influenced by illegally disposed solid waste (Hrenovic et al., 2014). Here we report the finding of three clinically relevant isolates of *A. baumannii* recovered from technosol at a dump site.

2. 2.Materials and methods

2.1. Site location and sampling

The dump site "Sovjak" is located 7 km north west from the centre of Rijeka, the largest port in Croatia. It is situated in a karst pit developed in permeable Lower Cretaceous limestones (Fig. 1). Various hazardous industrial materials were continuously disposed at this site from 1955 to 1990 without tracking system. Until 1966, the site was predominantly used for disposing acid tar from local refinery. After that period and until 1990, the following wastes were disposed: acetylene sludge and waste oils from shipyards, coke coal tar, tank bottom remains from refinery and power plants, different types of spent oils and solvents, crude oil, oily wastewaters and damaged and dangerous goods from the Customs Services (Ribic, 2008). After that period, the dump site was periodically used as an illegal dump site. Ribic (2008) stated that 250,000 m^3 of hazardous waste was presumably deposited at the Sovjak site and that the following four separate layers from top to bottom can be recognized: liquid hydrocarbons, wastewater, soft tar and acid solid tar. Groundwater quality measurements indicated that the site presented a serious threat to human health (Ribic, 2008). The surface part of a technosol at the edge of a dump was collected in October 2016 (Fig. 1a). GPS coordinates and elevation was recorded using Garmin eTrex Vista C device, as follows: X = 5,434,386 m, Y = 4,999,168 m, 301 m asl. The surface part at the edge of a dump represents no longer liquid but solid hydrocarbons mixed with inorganic constituents (Fig. 1b). For bacteriological analyses the sample was aseptically taken in a sterile plastic bottle and processed in the laboratory within 6 h after collection. For mineralogical and geochemical analyses the sample was taken in plastic bags.

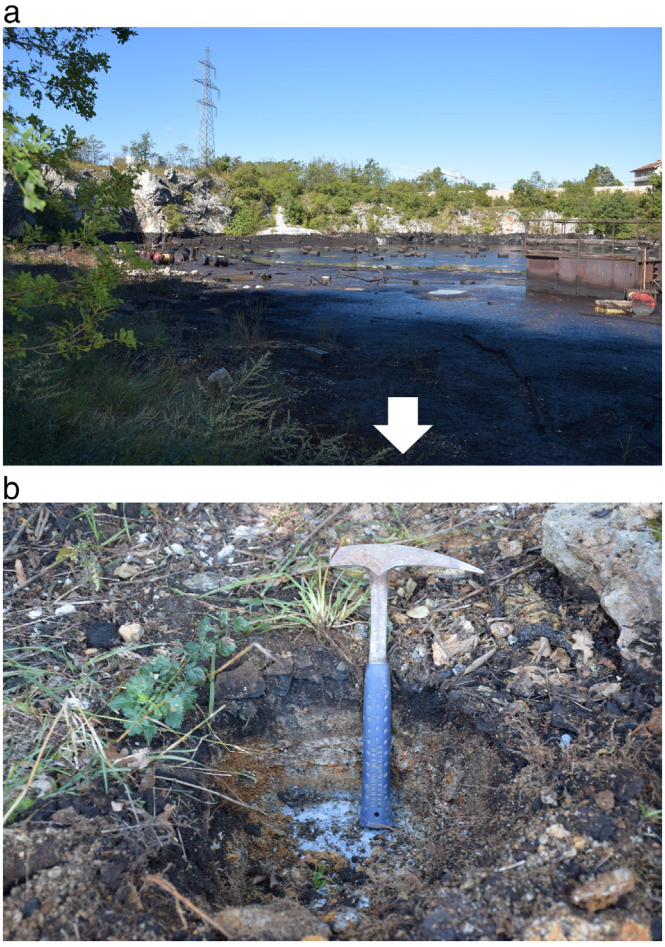

Fig. 1. The dump site "Sovjak" is situated in a karst pit. Technosol sample was taken at the edge of a dump (white arrow shows sampling site, a). The surface part of a technosol was sampled (geological hammer for scale, b).

2.2. Characterization of technosol

A homogenized technosol sample for geochemical and mineralogical analyses was air dried in the laboratory. Major oxides and trace elements were determined by ICP-ES/MS following a lithium borate fusion and dilute acid digestion. Loss on ignition (LOI) was determined by sintering at 1000 °C. The mineral compositions of the technosol sample was determined by X-ray powder diffraction (XRD) using a Philips diffractometer (graphite monochromator, CuK radiation, proportional counter). Trace elements were determined in both original technosol sample and after the extraction of the soluble organic matter. Major oxides and mineral composition were determined in technosol after the extraction of the soluble organic matter. Content of calcium carbonate ($w(CaCO_3)$) was determined volumetrically using the Scheibler apparatus.

The organic matter was analysed in fresh technosol sample as well as in the technosol sample after 58 days of contact with bacteria in laboratory conditions. The extractable/soluble organic matter (EOM) of technosol was determined by 36-hour Soxhlet extraction with dichloromethane-methanol (93:7). The excess of solvent was removed by rotary evaporation at 50 °C and the amount of organic matter was quantitatively determined after drying in a vacuum desiccator. The total sulphur content was determined by Leco SC144DR sulphur analyser. The fractions of EOM were separated by column liquid chromatography. The alkane fraction was subjected to gas chromatography by using Agilent 7890A analyser. Saturated hydrocarbons were analysed by gas chromatography–mass spectrometry by using Agilent 7890A gas chromatograph with quadrupole mass spectrometer Agilent MS 5975C. Biological marker hydrocarbon compounds were used in petroleum fingerprinting.

2.3. Bacteriological analyses

The soil sample was concentrated on the sterile membrane filters with 0.45 μm pore size in triplicate after suspension in sterile peptone water. Aerobically grown total heterotrophic bacteria were determined on Nutrient agar (Biolife) after incubation at 22 °C/72 h (APHA, AWWA,

Table 1
Bacteriological and physical characteristics of technosol sample from which three isolates of *A. baumannii* were recovered.

Parameter	Mean ± SD
Total heterotrophic bacteria (log CFU/g)	6.5 ± 0.1
Intestinal enterococci (log CFU/g)	1.6 ± 0.2
Carbapenem-resistant bacteria cultivated at 37 °C (log CFU/g)	4.1 ± 0.1
Carbapenem-resistant bacteria cultivated at 42 °C (log CFU/g)	1.4 ± 0.1
pH	6.82
Humidity (%)	21.71

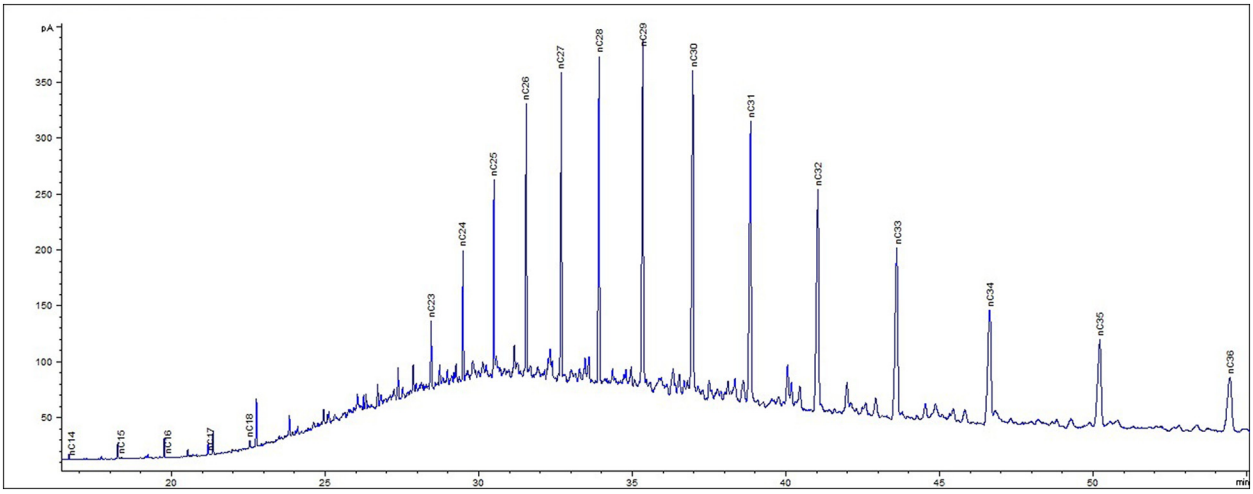

Fig. 2. Gas chromatogram of alkane fraction in fresh technosol. Hydrocarbon distributions was not significantly different after 58 days of contact with *E. ludwigii* and *A. baumannii*.

WEF, 2005). The intestinal enterococci were determined according to HRN ISO 7899-2 (2000). Membrane filters were incubated on Slanetz Bartley agar (Biolife) at 37 °C/72 h and subsequent confirmation of intestinal enterococci was done on Bile esculin azide agar (Sigma-Aldrich) after incubation at 44 °C/4 h. Carbapenem-resistant bacteria were determined on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar), intended for the cultivation of clinically relevant carbapenem-resistant bacteria, after incubation at both 37 °C/72 h and 42 °C/48 h. Cultivation of carbapenem-resistant bacteria was performed at 42 °C to suppress the growth of environmental autochthonous species with intrinsic resistance to carbapenems such as *Stenotrophomonas* spp. which grows only at 37 °C (Hrenovic et al., 2016; Hrenovic et al., 2017). The numbers of total heterotrophic bacteria, intestinal enterococci and carbapenem-resistant bacteria were determined as Colony Forming Units (CFU), logarithmically transformed, and expressed as log CFU per 1 g of wet technosol.

2.4. Isolation and characterization of A. baumannii isolates

Triplicate of 1.0 g of technosol was suspended in and diluted with peptone water. The isolation of *A. baumannii* was performed on CHROMagar Acinetobacter supplemented with 15 mg/L of cefsulodin sodium salt hydrate, both with or without the addition of CR102 after incubation at 42 °C/48 h (Hrenovic et al., 2016). Identification of presumptive colonies was performed by routine bacteriological techniques. Isolates were 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 population structure of *A. baumannii* isolates was determined by multilocus sequence typing (MLST). Fragments of seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD*) were amplified by PCR (ProFlex™ 96-Well PCR System, Applied Biosystems) using specific primers according to the procedures listed in Oxford's MLST database (http://pubmlst.org/abaumannii/). Amplified fragments were sequenced on both strands (commercial service Macrogen Europe, the Netherlands), followed by assembly and manual editing of raw nucleotide sequences by using Sequencher™ 4.7 software (http://www.genecodes.com/). The sequence type (ST) together with allele sequences and profiles were retrieved from the *A. baumannii* MLST website (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_abaumannii_oxford_seqdef).

The antibiotic susceptibility profile was firstly assessed by disk diffusion method. The susceptibility to carbapenems (meropenem, imipenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (tobramycin, gentamicin, amikacin), tetracyclines (minocycline), penicillins/β-lactamase inhibitors (ampicillin/sulbactam, ticarcillin/clavulanic acid, piperacillin/tazobactam), folate pathway inhibitors (trimethoprim/sulfamethoxazole) and polymyxins (colistin) were confirmed according to MICs values obtained by Vitek2 system (Biomerieux) and gradient dilution E-test (AB Biodisk) for colistin. MICs were interpreted according to EUCAST (2017) criteria for all antibiotics with defined breakpoints for *Acinetobacter* spp., while for penicillins/β-lactamase inhibitors and minocycline CLSI (2015) breakpoints were used.

Subsequently, genes encoding OXA-type carbapenemases (oxacillinases) were amplified by multiplex polymerase chain reaction (PCR). Specific primers for $bla_{\text{OXA-40-like}}$ ($bla_{\text{OXA-24-like}}$) and $bla_{\text{OXA-23-like}}$ genes were used (Woodford et al., 2006) under following amplification conditions: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 25 s, 52 °C for 40 s and 72 °C for 50 s with a final chain elongation at 72 °C for 6 min, in a ProFlex™ 3 × 32-well PCR System (Thermo Fisher Scientific). Obtained amplicons of bla_{OXA} genes were sequenced, edited, analysed as described previously (Thompson et al., 1995), and sequences were deposited in GenBank.

2.5. Determination of virulence factors of A. baumannii isolates

Hydrophobicity of bacteria was measured *via* the bacterial adhesion to hydrocarbon assay (Rosenberg et al., 1980). Biofilm formation at the solid/liquid interface was tested according to the crystal violet assay (Kaliterna et al., 2015). After the staining of biofilm with crystal violet, it was solubilized in ethanol and quantified by absorbance at 550 nm. Poor biofilm formation was estimated as $A_{550} < 0.4$. Pellicle formation was confirmed as visible bacterial biofilm formation at air/liquid

Table 2
Chemical composition of technosol after the extraction of soluble organic matter.

Constituent	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	LOI[b]	Sum
Content (wt%)[a]	3.20	0.05	1.33	1.10	0.01	0.54	52.34	0.01	0.02	0.02	41. 3	99.91

[a] wt%, percentage by weight.
[b] Loss on ignition (1000 °C).

Table 3
Content of trace elements in the bulk sample of technosol (1) and after the extraction of soluble organic matter (2).

Element	As	Ba	Cd	Co	Cs	Cu	Mo	Hg	Nb	Ni	Pb	Rb	Se	Sn	U	V	Zn
1[a]	3.6	77	0.8	7.1	0.6	42.2	0.9	0.46	1.5	69.6	89.9	7.0	<0.5	8	3.1	31	2719
2[a]	1.1	22	0.2	3.9	<0.1	40.9	0.4	0.11	0.2	19.9	27.7	1.0	<0.5	1	1.5	16	311

[a] Values are expressed as parts per million.

interface (Nait Chabane et al., 2014). Swarming and twitching surface motility was assessed in polystyrene Petri dishes with Luria-Bertani medium containing 0.5% agarose (Antunes et al., 2011). Swarming motility was observed at the air-agarose interface by direct measuring of the longest diameter of motility. Twitching motility was determined after the removal of the agarose layer, staining the Petri dish with 0.5% crystal violet for 10 min and measuring the longest diameter of motility. Isolates were grouped into categories based on the average values of motility as: <20 mm poor; 20–50 mm intermediate; >50–85 mm highly motile isolates.

2.6. Survival of *A. baumannii* in technosol

The survival of *A. baumannii* in technosol was followed for isolates Sovjak 1 and Sovjak 3. Overnight bacterial cultures were suspended in 30 mL of autoclaved commercial spring water. Into each bacterial suspension in water media, 3.0 g of sterilized or original (non-sterilized) 14-day-old technosol was added. Control tubes were left without the addition of technosol. Media were incubated at 20 °C with 170 rpm during 58 days of monitoring. After the specified period of incubation, tubes were vortexed, subsamples were diluted in sterile saline solution, inoculated onto Nutrient agar plates, and bacterial colonies were counted after incubation at 42 °C/24 h. The number of viable bacteria was determined as CFU, logarithmically transformed, and expressed as log CFU per 1 mL of water suspension. Experiments were performed in technical duplicate with mean values presented. In the original non-sterilized technosol a dominance of one non-*Acinetobacter* colony type was obtained. The colony was characterized by MALDI-TOF MS and the profile of antibiotic resistance was determined by Vitek2 system as described above for *A. baumannii*.

3. Results

3.1. Characterization of technosol

The sample of technosol was pH-neutral with humidity of 22% (Table 1). The content of inorganic matter averaged 24 wt% and soluble organic matter 76 wt% in the fresh technosol sample. The total sulphur content was 2.32 wt%. The bulk composition of EOM was (in wt%): saturated hydrocarbons 33; aromatic hydrocarbons 14; NSO-compounds 8; asphaltenes 46. A pronounced loss of low molecular weight (LMW) hydrocarbons (up to n-C_{23}), as well as unresolved "hump" of complex mixture (UCM) were evidenced in the molecular distribution indicating enhanced processes of evaporatization and biodegradation (Fig. 2). High molecular weight (HMW) hydrocarbon distribution was regular. According to the biomarker scale (Peters et al., 2005), removal of molecular groups or level of biodegradation was heavy to severe. Biomarker analyses revealed the presence of mature petroleum hydrocarbons of marine, algal-bacterial origin.

XRD revealed that calcite is the main constituent of inorganic part of technosol (91 wt% according to Scheibler apparatus) while remaining mineral phases are dolomite, portlandite, vaterite and quartz. The high content of calcite corresponds well with the chemical data (Table 2): the CaO content is 52.34 wt%, clearly indicating that this mineral phase is the dominant mineral phase in the technosol. Mineral composition, as well as the structure of the technosol profile itself (Fig. 1b) may imply that this inorganic material represents a remain of industrial (probably organic) waste that was treated with cement. Namely, portlandite and both polymorphs of $CaCO_3$ (calcite and vaterite) are products of cement hydration (Stepkowska et al., 2003). Very high content of Zn (2719 ppm) was observed in technosol sample (Table 3) implying the potential pollution with heavy metals. Since the content of all analysed trace metals was significantly lower in technosol from which soluble organic matter was extracted (Table 3) we can tentatively conclude that all analysed trace metals were dominantly bound to organic fraction. This is in accordance with Durn et al. (2004) who found that the organic-sulphide fraction (Or) is a dominant carrier of zinc, mercury and cadmium in lime stabilized waste from petroleum industry (Panonian part of Croatia), representing 92%, 78% and 44% of total zinc, mercury and cadmium concentrations.

In the fresh sample of technosol a high number of total heterotrophic bacteria was found (Table 1), indicating its non-toxicity to bacteria. The presence of intestinal enterococci indicated the faecal pollution of technosol. The number of carbapenem-resistant bacterial population was much lower when cultivated at 42 °C as compared to 37 °C. However, carbapenem-resistant bacteria grown at 42 °C suggested the presence of clinical relevant bacteria in technosol (Hrenovic et al., 2016; Hrenovic et al., 2017).

3.2. Characterization of *A. baumannii* isolates

In total, three presumptive colonies of *A. baumannii* were isolated from technosol; one from plate containing the carbapenem-selective supplement CR102 inoculated with 0.01 g of technosol, and two from a plate without the CR102 inoculated with 0.01 g of technosol. Three isolates of Gram-negative coccobacilli gave negative oxidase, positive catalase reaction, with typical orange-red reaction on Kligler Iron Agar. MALDI-TOF MS score values ranged from 2.000–2.086 for *A. baumannii*. According to the MLST analysis by using the Oxford MLST scheme, two isolates (Sovjak 1 and 2) were identified as sequence

Table 4
Multilocus sequence typing according to MLST Oxford scheme, MIC values of tested antibiotics[a], and the presence of genes of bla_{OXA} lineage in three *A. baumannii* isolates originating from technosol.

Isolate	Sequence type	Clonal complex	IC type	MIC values of antibiotics (mg/L)												bla_{OXA}
				MEM	IPM	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	TZP	SXT	CST	
Sovjak 1	231	109	1	≥16^R	≥16^R	≥4^R	4^R	≤1	≤1	32^R	≤1	16^I	≥128^R	≥128^R	≤20	≤0.5	OXA-72
Sovjak 2	231	109	1	≥16^R	≥16^R	≥4^R	4^R	≤1	≤1	16^I	≤1	16^I	≥128^R	≥128^R	≤20	≤0.5	OXA-72
Sovjak 3	195	92	2	≥16^R	≥16^R	≥4^R	4^R	≤1	≤1	>64^R	8^I	16^I	≥128^R	≥128^R	≥320^R	≤0.5	OXA-23

[R] - resistant, [I] - intermediate according to EUCAST and CLSI criteria.

[a] Carbapenems (MEM-meropenem, IMI-imipenem), fluoroquinolones (CIP-ciprofloxacin, LVX-levofloxacin), aminoglycosides (TOB-tobramycin, GEN-gentamicin, AMK-amikacin), tetracyclines (MIN-minocycline), penicillins/β-lactamase inhibitors (SAM-ampicillin/sulbactam, TIM-ticarcillin/clavulanic acid, TZP-piperacillin/tazobactam), folate pathway inhibitors (SXT-trimethoprim/sulfamethoxazole), polymyxins (CST-colistin).

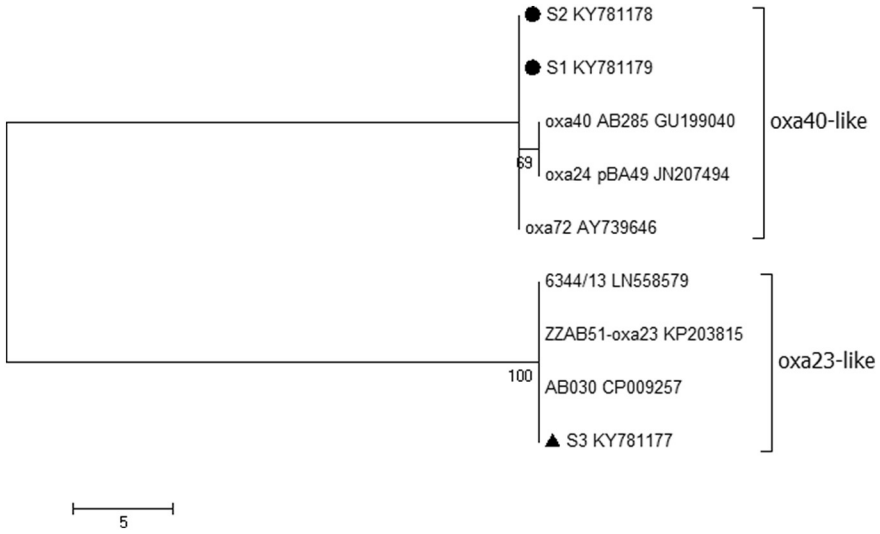

Fig. 3. Unrooted phylogentic tree (NJ method, number of differences) constructed on the basis of bla_{OXA} genes encoding OXA-type carbapenemases. GenBank accession numbers are given next to the name of each *A. baumannii* Sovjak isolate marked as S1, S2, and S3.

type (ST)-231 clustering into the clonal complex 109 (CC109) which corresponds to the international clone 1 (IC1), while one isolate (Sovjak 3) belonged to the ST-195 clustering into the CC92 inside IC2 (Table 4).

Although there was no selective screening for carbapenem-resistance during the isolation of *A. baumannii* from technosol on plate without CR102, all three isolates were carbapenem-resistant (Table 4). The isolates Sovjak 1 and 2 carried bla_{OXA72}, while the isolate Sovjak 3 carried bla_{OXA23} gene (Table 4, Fig. 3). Moreover, isolates shared the resistance to carbapenems, fluoroquinolones, aminoglycosides and penicillins/β-lactamase inhibitors and sensitivity to tobramycin, gentamicin and colistin. Isolates Sovjak 1 and 2 belonging to the IC1 remained susceptible to minocycline, trimethoprim/sulfamethoxazole and colistin. Isolate Sovjak 3 belonging to the IC2 remained susceptible only to colistin. According to the criteria for non-susceptibility to antimicrobial categories (Magiorakos et al., 2012), isolates Sovjak 1 and 2 were classified as multi-drug resistant (MDR) and Sovjak 3 as extensively drug-resistant (XDR).

With regard to virulence features, the isolates Sovjak 1 and 2 belonging to ST-231 were hydrophobic and formed pellicle at air/liquid interface, in contrast to the isolate Sovjak 3 belonging to ST-195 (Table 5). All three isolates formed poor biofilm at the solid/liquid interface. The isolate belonging to the ST-195 showed much lower swarming motility as compared to isolates belonging to the ST-231, while all three isolates showed intermediate twitching motility.

3.3. Survival of A. baumannii in technosol

The survival of *A. baumannii* isolates Sovjak 1 and Sovjak 3 in the natural spring water and in the suspension of 10% of sterilized technosol is shown in Fig. 4. In the spring water, numbers of isolate Sovjak 1 were unchanged up to 20 days of contact, after which slight decrease of bacterial numbers was observed (89% of survival after 58 days of monitoring). Numbers of the isolate Sovjak 3 were constant up to 10 days of contact, and slight further decrease of bacterial numbers resulted in 65% of survival after 58 days of contact. The multiplication of *A. baumannii* isolates was not evidenced in the spring water. In the suspension of 10% of sterilized technosol pure cultures of both *A. baumannii* isolates multiplied, bacterial numbers increased after only one day of contact and continued to increase up to 30 days of contact. After 58 days of contact bacterial numbers were still higher than initial numbers (127% and 125% of survival for isolate Sovjak 1 and Sovjak 3, respectively).

In the original non-sterilized technosol a dominance of one non-acinetobacter colony type was obtained. The identification of this isolate gave MALDI-TOF MS score value of 2.045 for *Enterobacter ludwigii*. *E. ludwigii* was confirmed sensitive to all tested antibiotics except ampicillin and amoxicillin/clavulanic acid. The survival of native *E. ludwigii* and artificially added *A. baumannii* isolates Sovjak 1 and Sovjak 3 in the suspension of 10% of original technosol is shown in Fig. 5. In both systems (with isolates Sovjak 1 and Sovjak 3) numbers of *E. ludwigii* increased after just one day of contact and continued to grow up to 7 days of contact. Slight further decrease of *E. ludwigii* numbers resulted in 121% and 100% of survival after 58 days of contact in the systems with isolate Sovjak 1 and Sovjak 3, respectively. In the original technosol containing the native population of *E. ludwigii*, numbers of both isolates of *A. baumannii* increased up to 3 days of contact. Continuous further decrease of *A. baumannii* numbers resulted in final survival of 29% and 27% for isolate Sovjak 1 and Sovjak 3, respectively. The analysis of organic fraction of non-sterilized technosol after 58 days of contact with *E. ludwigii* and *A. baumannii* did not reveal the significant changes in the content of organic matter or hydrocarbon fractions (Fig. 2).

4. Discussion

Clinical isolates of carbapenem-resistant *A. baumannii* belonging to ST-231 were previously described in South and North America, but also in Europe (Grana-Miraglia et al., 2016). Carbapenem-resistant *A. baumannii* belonging to ST-195 were reported from hospitalized patients in Asian countries (Lean et al., 2016), as well as in Denmark (Hammerum et al., 2015). The emission of XDR *A. baumannii* ST-195

Table 5
Hydrophobicity, biofilm and pellicle formation, swarming and twitching motility of *A. baumannii* isolates.

Isolate	Hydrophobicity (A_{410}%)	Biofilm (A_{550})	Pellicle	Swarming (mm)	Twitching (mm)
Sovjak 1	82.9	0.124	+	64	30
Sovjak 2	80.9	0.071	+	53	22
Sovjak 3	0.0	0.027	−	13	48

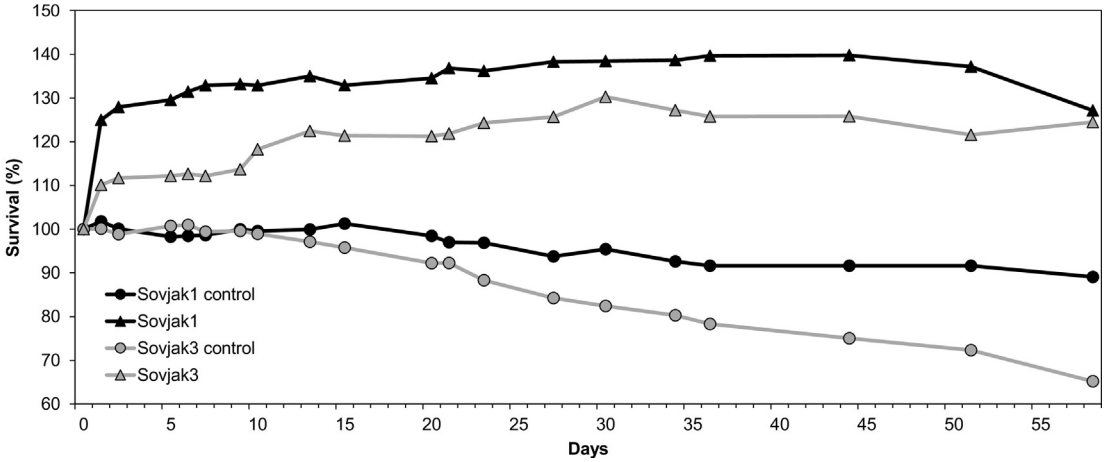

Fig. 4. Survival of *A. baumannii* isolates Sovjak 1 and Sovjak 3 in natural spring water (control) and in natural spring water with added 10% of sterilized technosol. Initial log CFU/mL: 6.8 ± 0.1 Sovjak 1 control; 7.3 ± 0.1 Sovjak 3 control; 6.0 ± 0.0 Sovjak 1; 6.6 ± 0.0 Sovjak 3. Survival of bacteria was calculated as ((log CFU/mL_{time}: log CFU/mL_{start}) * 100), where log CFU/mL_{time} is number of bacteria on day of measurement and log CFU/mL_{start} is initial number of bacteria.

from patients to the hospital wastewater, urban sewage, and the river as the natural recipient of wastewaters was recently described in Croatia (Seruga Music et al., 2017). In Croatia, carbapenem-resistant clinical isolates of *A. baumannii* belonging to IC1 were present from 2002 (Goic-Barisic et al., 2007). From 2009 onwards, *A. baumannii* belonging to IC2 were additionally reported in Croatian hospitals (Franolic-Kukina et al., 2011; Goic-Barisic et al., 2011). Carbapenem-resistance of *A. baumannii* is very frequent in Croatian clinical isolates (87% in 2015, CAMS, 2016) as well as in isolates from wastewater (Goic-Barisic et al., 2016; Hrenovic et al., 2016; Seruga Music et al., 2017). The presence of acquired genes bla_{OXA72} and $bla_{OXA23\text{-like}}$ group was reported in Croatian clinical isolates of *A. baumannii* from 2009 (Franolic-Kukina et al., 2011; Goic-Barisic et al., 2011; Vranic-Ladavac et al., 2014) and in wastewater isolates from 2014 (Goic-Barisic et al., 2016; Hrenovic et al., 2016).

Three isolates of *A. baumannii* recovered from technosol shared many features that are previously described for clinically relevant isolates: the affiliation to IC1 and 2, MDR or XDR antibiotic resistance profile, carbapenem resistance mediated by bla_{OXA72} and bla_{OXA23} genes, and the expression of virulence factors. This suggests the hospital solid waste as a probable source of isolates. Based on the recorded incidence of *A. baumannii* in Croatian hospitals (Goic-Barisic et al., 2007; Franolic-Kukina et al., 2011; Goic-Barisic et al., 2011), the hospital waste could have been illegally disposed at this dump site after 2002 or even after 2009. This hypothesis based on the results obtained in this study implies that *A. baumannii* could survive for more than a decade in technosol, or that the hospital waste was illegally dumped recently.

The examined sample of technosol contained huge amount (76%) of soluble organic matter constituted of petroleum hydrocarbons. In the original sample, signs of natural petroleum bioremediation were evidenced by: high content of asphaltenes, loss of volatile hydrocarbons up to C_{23}, expressed UCM, and proper distribution in a higher molecular range (Gough and Rowland, 1990; Peters et al., 2005). Despite the evident multiplication of *E. ludwigii* and *A. baumannii*, no evident future *in vitro* biodegradation of petroleum was observed. This confirms the resistance of higher molecular weight hydrocarbons to the process of biodegradation. The multiplication of examined heterotrophic bacteria most probable occurred as a result of growth on low molecular weight hydrocarbons which were not evidenced as loss of total weight.

Literature data indicate that bacteria from the genus *Acinetobacter* are able to degrade petroleum hydrocarbons. However, the majority of

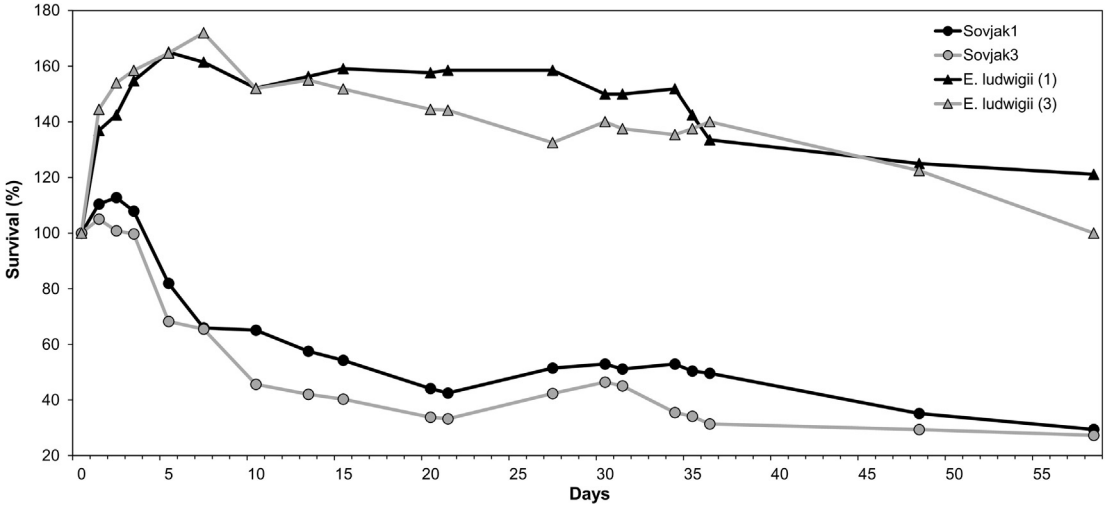

Fig. 5. Survival of *A. baumannii* isolates Sovjak 1 and Sovjak 3 in natural spring water with added 10% of original technosol that contained the native bacterial population. Native population of bacteria was represented by one strain of *Enterobacter ludwigii* and is marked as *E. ludwigii* (1) and *E. ludwigii* (3) in the system with isolate Sovjak 1 and Sovjak 3, respectively. Initial log CFU/mL: 6.8 ± 0.1 Sovjak 1; 7.3 ± 0.1 Sovjak 3; 4.0 ± 0.0 *E. ludwigii* (1) and *E. ludwigii* (3). Survival of bacteria was calculated as ((log CFU/mL_{time}: log CFU/mL_{start}) * 100), where log CFU/mL_{time} is number of bacteria on day of measurement and log CFU/mL_{start} is initial number of bacteria.

determination of the reported species is not precise enough to ensure it is *A. baumannii*. Confirmed isolates of *A. baumannii* have been recovered from soil contaminated with petroleum hydrocarbons and were able to degrade the total petroleum hydrocarbon fractions of crude oil (Sarma et al., 2004). Nevertheless, the hydrophilic human clinical isolate of *A. baumannii* strain RUH 3023 was unable to degrade diesel fuel (Mara et al., 2012). The *A. baumannii* isolates in the pure culture examined in this study multiplied in the contact with technosol (Fig. 4), which suggest that they were able to use the petroleum hydrocarbons as the source of nutrients for multiplication. Hydrophobic isolate Sovjak 1 showed better multiplication as compared to hydrophilic isolate Sovjak 3 (Table 5). Obviously, the *A. baumannii* isolates Sovjak 1 and 3 could degrade the petroleum hydrocarbons, but based on their MDR and XDR profile and acquired oxacillinase genes, described isolates are not potential candidates for bioremediation of contaminated environment. However, *A. baumannii* isolates were overgrown in the competition with *E. ludwigii* present in the technosol (Fig. 5). Based on the evident multiplication of *E. ludwigii* in the contact with technosol, this species was also able to use the petroleum hydrocarbons contained in technosol. Due to the good multiplication in technosol and sensitivity to antibiotics, *E. ludwigii* isolate could find the application in bioremediation of petroleum hydrocarbons. However, the further bioremediation of mature oil present in the examined technosol will be most probable of very low efficiency.

5. Conclusions

This study confirmed the possibility of illegal dump sites of human solid waste being a source of MDR and XDR *A. baumannii*. Isolates of *A. baumannii* that are closely related to clinical isolates are able to survive in anthropogenically influenced environment rich in petroleum hydrocarbons and heavy metals. Awareness should be raised towards the proper mandatory management and disposal of human solid waste to prevent the spread of clinically important *A. baumannii* in nature.

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