Survival of extensively- and pandrug-resistant isolates of *Acinetobacter baumannii* in soils

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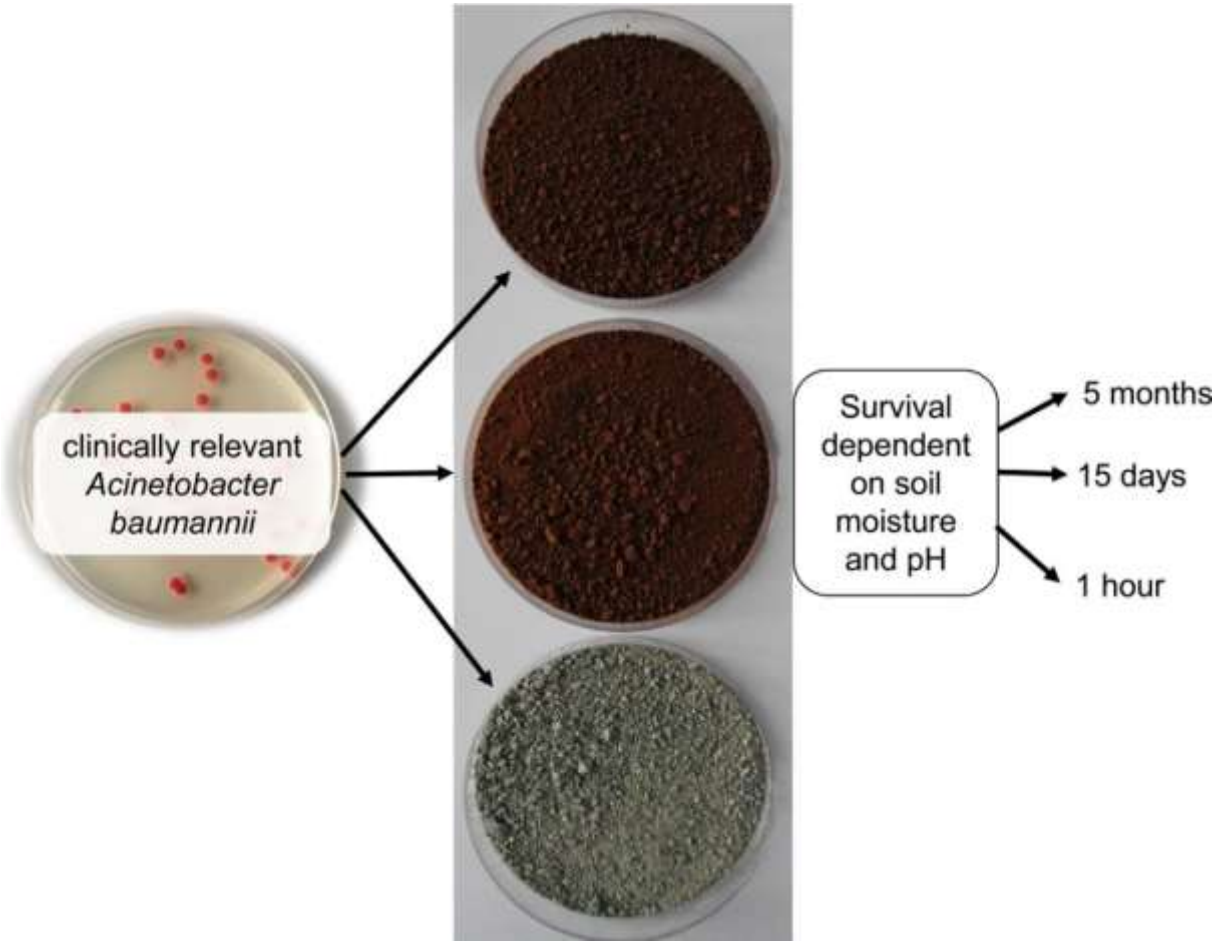
Abstract

The extensively- and pandrug-resistant isolates of *Acinetobacter baumannii* are a leading cause of hospital acquired infections. Aside from reports of *A. baumannii* in wastewaters influenced by hospital effluents, only two studies reported its detection in soils. The lack of data hinders the assessment of soil as the possible environmental reservoir of *A. baumannii*, prediction of the behaviour of this emergent pathogen in soils and their potential consequences. This study examined the survival of clinically relevant isolates of *A. baumannii* in the sterilized and fresh soils of different moisture and pH values. In the alkaline, weakly acid, and strongly acid soil *A. baumannii* survived for five months, up to 15 days, and up to one hour, respectively. Decrease of moisture below 16 wt% can help in the elimination, but the long-term maintenance of viable *A. baumannii* was detected in completely dry alkaline soil. Linear regression model confirmed that the pH value of soil is the most important environmental factor, which determines the survival of *A. baumannii* in soils. Acidification of

soil seems a promising method in the remediation of soils contaminated with emerging human pathogen *A. baumannii*.

Graphical abstract



Keywords: bacteria; environment; moisture; pH; public health; soil.

1. Introduction

Bacterium *Acinetobacter baumannii* is an emerging opportunistic pathogen causing hospital outbreaks in immunocompromised patients worldwide (Towner, 2009). Acute community-acquired human infections occurring outside hospital environment represent a minor proportion of total *A. baumannii* infections (Dexter et al., 2015), but suggest a source of this pathogen outside of the hospital settings. Little is known about the presence of this ESKAPE

pathogen (Boucher et al., 2009) outside the hospital settings, and the role of environmental isolates in the epidemiology of *A. baumannii* is not elucidated.

Viable *A. baumannii* of clinical significance were recovered from hospital wastewaters (Ferreira et al., 2011; Zhang et al., 2013; Kovacic et al., 2017), and consequently from urban wastewaters, wastewater treatment plants (Hrenovic et al., 2016; Higgins et al., 2018), and natural recipients of wastewaters influenced by hospital effluents (Girlich et al., 2010; Seruga Music et al., 2017). Aside from reports of *A. baumannii* in wastewaters influenced by hospitals, only a few studies reported its detection elsewhere in the environment. *A. baumannii* has been unsuccessfully searched for in soils as a source of infection among US service members injured during Operation Iraqi Freedom, probably due to the long storage duration of archived soils (Scott et al., 2007). One *A. baumannii* which was related to a clinical isolate was incidentally found in paleosol influenced by illegally disposed human solid waste (Hrenovic et al., 2014). Three clinically relevant *A. baumannii* were recovered from the technosol developed at the dumpsite (Hrenovic et al., 2017).

The success of *A. baumannii* as an emerging human pathogen is ascribed to its ability to acquire resistance to almost all antimicrobial agents (Towner, 2009). Nowadays, the worrying extensively- and pandrug-resistant clinical isolates are reported worldwide (Roca et al., 2012). In the form of biofilm developed on glass and ceramic surfaces, *A. baumannii* shows reduced susceptibility to common disinfectants (Ivankovic et al., 2017). Moreover, the long-term persistence of *A. baumannii* on dry surfaces of hospital environment represents the source for spread of hospital outbreaks. *A. baumannii* can persist on dry glass or cellulose acetate surfaces for a month (Espinal et al., 2012; Bravo et al. 2016) and endure extreme pH values in defined water media (Dekic et al., 2018). However, these data obtained *in vitro* are not directly applicable to the real conditions in natural environment and substrata from which *A. baumannii* was recovered. One *A. baumannii* isolate was able to survive in low extent 24 h

of contact in 10 wt% water suspension of acid (pH 3.37) palaeosol (Hrenovic et al., 2014). Two *A. baumannii* isolates survived in 10 wt% water suspension of sterilized technosol during 58 days (Hrenovic et al., 2017).

The survival of clinically relevant *A. baumannii* isolates that express the resistance to last-resort antibiotics in autochthonous soils poses a concern and still remains to be determined. The lack of data hinders the assessment of soil as the possible environmental reservoir of *A. baumannii*, prediction of the behaviour of this emergent pathogen in soils and their potential consequences. The aim of this study was to examine the survival of clinically relevant isolates of *A. baumannii* in the sterilized and fresh soils of different moisture and pH values.

2. Materials and methods

2.1. Characteristics of tested A. baumannii isolates

Three isolates of *A. baumannii* deposited at the University of Zagreb, Faculty of Science were chosen for experiments. Two environmental isolates (EF7 and EF8) were recovered from effluent of the Zagreb wastewater treatment plant (Higgins et al., 2018). One clinical isolate (OB4138) was recovered from the patient suffering from hospital-acquired pneumonia at the Special Hospital for Pulmonary Diseases in Zagreb, Croatia (Seruga Music et al., 2017). The antibiotic resistance has been tested by the Vitek2 system (Biomerieux) using the AST-XN05 and AST-N233 testing cards. Colistin resistance was confirmed by broth microdilution method. The minimum inhibitory concentration values (MIC) were interpreted according to the EUCAST (2017) and CLSI (2015) criteria for clinical isolates of *Acinetobacter* spp. All three isolates were non-susceptible to carbapenems, fluoroquinolones, aminoglycosides, minocycline and penicillins/β-lactamase inhibitors (Table 1). Isolates EF8 and OB4138 that

Table 1. MIC values of tested antibiotics[a] and antibiotic resistance profile for three tested isolates of *A. baumannii*.

Isolate	MEM	IMI	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	SXT	CST	Antibiotic resistance profile
EF7	$> 16^{R}$	$> 16^{R}$	$> 4^{R}$	$> 8^{R}$	$> 16^{R}$	$> 16^{R}$	$> 64^{R}$	8^{I}	$\geq 32/16^{R}$	$\geq 128/2^{R}$	$\geq 4/76^{R}$	20^{R}	PDR
EF8	$\geq 16^{R}$	$\geq 16^{R}$	$\geq 4^{R}$	$\geq 8^{R}$	$\geq 16^{R}$	$\geq 16^{R}$	8^{S}	$\geq 16^{R}$	$\geq 32/16^{R}$	$\geq 128/2^{R}$	$\leq 2/38^{S}$	$\leq 0.5^{S}$	XDR
OB4138	$\geq 16^{R}$	$\geq 16^{R}$	$> 4^{R}$	8^{R}	$> 16^{R}$	$> 16^{R}$	$> 64^{R}$	$> 16^{R}$	$16/8^{I}$	$\geq 128/2^{R}$	$\leq 2/38^{S}$	$\leq 0.5^{S}$	XDR

[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/clavulanate); folate pathway inhibitors (SXT- trimethoprim/sulfamethoxazole); polymyxins (CST-colistin). ^{R} - resistant, ^{I} - intermediate according to EUCAST or CLSI criteria.

remained susceptible to trimethoprim/sulfamethoxazole and colistin were classified as extensively drug-resistant (XDR). Isolate EF7 that was also resistant to trimethoprim/sulfamethoxazole and colistin was classified as pandrug-resistant (PDR, Higgins et al., 2018).

2.2. Sampling and characterization of soils

Three soil/palaeosol samples were chosen for experiments. Red palaeosol, Terra Rossa, and gray palaeosol situated on limestone of Cretaceous age were collected at three localities in Istria, Croatia. These soils were chosen for the experiments based on the results of previous investigations (Hrenovic et al., 2014; Durn et al., 2015; Zhang et al., 2018). They are representative for alkaline, weakly acid and strongly acid type of soil/palaeosol. In the text term soil will be used for both soil and palaeosol samples. Soil pits were dug to a depth of 50 cm, and disturbed soil samples were collected from the upper 30 cm of each pit. Samples were aseptically collected in the sterile plastic bags and processed in the laboratory within 24 h after collection.

The pH value was measured with WTWSenTix81 electrode after triplicate suspension (1:2.5) of soil in distilled water. The chemical composition (fraction <2 mm) was determined by the commercial Bureau Veritas Mineral Laboratories, Canada. Major oxides and several trace elements (Ba, Co, Ni, U, V) were determined by ICP-ES/MS following a lithium borate fusion and dilute nitric digestion. Other trace elements were determined by ICP-ES/MS following modified aqua regia digestion (1:1:1 HNO_3:HCl:H_2O). The contents of sulfur and carbon were determined by a Leco analyzer.

The mineral composition of <2 mm and <2 μm fractions were determined by X-ray powder diffraction (XRD) using a Philips diffractometer (graphite monochromator, CuKμ radiation, proportional counter). The <2 μm fraction was separated by sedimentation in

cylinders and quantitatively obtained after the appropriate settling time. The XRD patterns of clay fraction (non-oriented and oriented preparations) were obtained after the following treatments: air drying; Mg-saturation; K-saturation; K-saturation and ethylene glycol solvation; K-saturation and dimethyl sulfoxide solvation; Mg-saturation and ethylene glycol solvation; and heating for two hours at 350 and 550°C after K and Mg saturation. The mineral phases were identified using the Powder Diffraction File (1996) data system and the Panalytical XPert HighScore (v. 1.0d) program package. The identification of clay minerals was generally based on the methods outlined by Moore & Reynolds (1989). The term "illitic material" was used as defined by Środoń (1984) and Środoń and Eberl (1984). The term "MLM" was used for mixed-layer clay minerals in which type of interstratification and constituting clay minerals were not recognized with certainty. Semi-quantitative estimates of minerals were based on the relative intensities of characteristic X-ray peaks and were presented with Xs, but no quantitative value was assigned to each X.

2.3. Experimental set up

For the experiments with pure cultures of *A. baumannii* isolates, a proportion of 300 g of each soil sample in triplicate was sterilized by autoclaving (120℃/20min) in laboratory glasses. A full 10 µL loop of overnight culture of each *A. baumannii* isolate was separately suspended in a flask containing 100 mL of autoclaved commercially available spring water. Each type of soil was inoculated with suspension of each *A. baumannii* isolate. By this procedure, the moisture of soils was adjusted to maximum water holding capacity and soils were simultaneously supplemented with *A. baumannii*. In total 9 systems were set up: 3 *A. baumannii* isolates (EF7, EF8, OB4138) inoculated in 3 soil samples (red palaeosol, Terra Rossa, gray palaeosol). Thoroughly mixed content in glasses was covered with medical gauze

and left to spontaneously dry in the dark at room temperature of 22±2°C. When the moisture of soils dropped to 5 wt%, the soil mixtures continued to dry in a desiccator with silica gel.

During the experiment the soil moisture and number of viable bacteria were monitored. At the beginning of experiment and after the specified time points, the soil samples were well mixed and sub-samples were taken for analyses. The soil moisture was measured gravimetrically by drying the soil at 105°C to constant weight. Number of viable *A. baumannii* was measured after suspending by vortexing one g of soil (3 min/45 Hz) in technical triplicate in sterile physiological solution, following dilution of samples and inoculation onto selective CHROMagar Acinetobacter medium supplemented with CR102 (CHROMagar). After incubation at 42°C/24h the colonies were counted. The abundance of *A. baumannii* was expressed as log colony forming units (CFU) per one g of wet soil.

Experiments with fresh non-sterilized soils were set up in the same manner. In these experiments, additionally the number of total heterotrophic bacteria was measured by the inoculation of samples onto nutrient agar (Biolife) and subsequent incubation at 22°C/72h. Here, it should be mentioned that there were no native bacteria in fresh non-sterilized soils that grew in selective conditions employed for the enumeration of *A. baumannii*. Occasionally during experiment, the randomly chosen colonies developed on CHROMagar Acinetobacter supplemented with CR102 after incubation at 42°C/24h were subjected to identification of species. Identification of bacterial colonies was performed by matrix-assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF MS, software version 3.0, Microflex LT, Bruker Daltonics) on whole cells. All tested colonies were confirmed as *A. baumannii*. Thus, the presence of heterotrophic bacteria in fresh non-sterilized soils did not hinder the enumeration of *A. baumannii*.

2.4. Microscopic analyses

Soil subsamples (fresh and supplemented with *A. baumannii*) were fixed in 2.5% glutaraldehyde in phosphate buffered saline, and prepared for scanning electron microscopy (SEM) using standard techniques. Briefly, after removal of the fixative, the samples were rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide in dH_2O, rinsed again in phosphate buffer and then serially dehydrated with ethanol up to absolute ethanol. Samples were then dried with hexamethyldisilazane (HMDS) and carbon coated before examination at low voltage (0.5 kV) with a Zeiss Ultra PLUS FEG SEM (Carl Zeiss Microscopy, Munich, Germany).

2.5. Statistical analyses

Reduction of *A. baumannii* abundance was calculated as (log CFU/g(start) – log CFU/g(time)). Statistical analyses were carried out using Statistica 13.3 (TIBCO Software, Inc.). For pairwise comparisons factorial ANOVA and Duncan post hoc test were used. Correlations between variables were estimated using Pearson's correlation coefficient. The significance level applied was $p<0.05$. To predict the abundance of *A. baumannii* in soils of different pH and soil water content conditions, linear regression model was calculated.

3. Results

3.1. Characterization of soils

Examined soil samples had pH values ranging from alkaline to acid: 8.43±0.14 for red palaeosol, 5.40±0.21 for Terra Rossa, and 2.51±0.27 for gray palaeosol. Terra Rossa and red palaeosol had similar chemical composition of both major and trace elements (Tables 2 and 3). The only difference was the higher content of MgO and CaO in red palaeosol which corresponds with the mineral composition (Table 4). Compared to Terra Rossa and red

Table 2. Chemical composition of the investigated soils (in wt%).

Sample	SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	TiO_2	P_2O_5	LOI[a]	Sum
Red palaeosol	57.56	15.62	6.16	0.12	1.54	4.62	0.66	1.72	0.88	0.09	10.8	99.77
Terra Rossa	62.52	16.55	6.59	0.20	0.55	0.32	0.51	1.33	1.21	0.14	9.8	99.72
Gray palaeosol	44.42	23.56	3.68	<0.01	2.40	3.00	0.14	3.98	1.27	<0.01	17.3	99.75

[a] loss on ignition (1000°C).

palaeosol, the gray palaeosol was depleted in SiO_2, Fe_2O_3, Na_2O and enriched in sulphur, Al_2O_3 and K_2O (Tables 2 and 3). Trace element contents also differed, as compared to gray palaeosol, red palaeosol and Terra Rossa were enriched in Cd, Cu, Cu and Pb. On the contrary, gray palaeosol had higher contents of Mo, Ni and U, which is in favour of an acidic reductive pedogenic paleoenvironment.

Terra Rossa and red palaeosol samples contained quartz, plagioclase, K feldspar, goethite, haematite, micaceous clay minerals (illitic material and mica), kaolinite, and mixed-layer clay minerals (Table 4). Compared to Terra Rossa, red palaeosol additionally contained chlorite, 14Å clay minerals, dolomite and calcite. The presence of calcite and dolomite (Tables 2 and 4) was the probable cause of alkaline pH of red palaeosol. Illitic material and illite/smectite mixed-layer minerals were the main constituents of gray palaeosol, while quartz, pyrite, gypsum and jarosite were present as minor mineral phases (Table 4). The low pH of gray palaeosol was due to pyrite weathering and the formation of secondary mineral phases, gypsum and jarosite, in Cretaceous palaeosols. The content of carbon was very low in gray palaeosol and much higher in Terra Rossa (Table 3). The content of carbon in Terra Rossa probably corresponds to organic carbon. Since the red palaeosol contains carbonate mineral phases, obtained value for carbon stand for both organic and inorganic carbon in this sample.

3.2. Survival of A. baumannii in sterilized soils

Survival of three isolates of *A. baumannii* (named EF7, EF8, OB4138) in three sterilized soil samples is shown in Fig. 1. The moisture of the soils at the beginning of the experiments was adjusted to maximum water holding capacity and shown as the soil water content in wt%. In the case of alkaline red palaeosol (pH 8.43), the initial abundance of all three *A. baumannii* isolates increased during the first 6 days of contact, but after 14 days of contact sharply

Table 3. Content of sulfur, carbon and selected trace elements in the investigated soils. S and C in wt%; As to Zn in mg/kg.

Sample	S	C	As	Ba	Bi	Cd	Co	Cu	Hg	Mo	Ni	Pb	Sb	U	V	Zn
Red palaeosol	<0.02	1.42	14.9	294	0.5	0.5	19	22.8	0.04	1.3	80	23.3	0.2	2.9	162	55
Terra Rossa	0.02	1.19	16.7	322	0.6	0.4	29.9	53.7	0.05	3.0	84	39.8	0.5	4.9	226	33
Gray palaeosol	2.30	0.04	9.9	250	0.4	0.1	12.1	8.1	0.01	13.2	156	14.4	<0.1	8.3	201	46

Table 4. Semi-quantitative mineral composition of the <2 mm fraction of the investigated soils.

Sample	Quartz	Calcite	Dolomite	Plagioclase	K-feldspar	Goethite	Haematite	Pyrite	Gypsum	Jarosite	Mica/Illitic material	Kaolinite	Chlorite	14 Å mineral[a]	MLM[b]
Red palaeosol	XXX	X	X	X	X	X	X				XX	X	X	X	X
Terra Rossa	XXX			X	X	X	X				XX	XXX			X
Gray palaeosol	X							X	X	X	XXXX[d]				XXX[c]

X - relative abundance of minerals based on X-ray diffraction (no quantitative value is assigned to X).
[a] Smectite and/or vermiculite.
[b] Mixed-layer clay minerals.
[c] Illite/smectite mixed-layer minerals.
[d] Sample contains only illitic material.

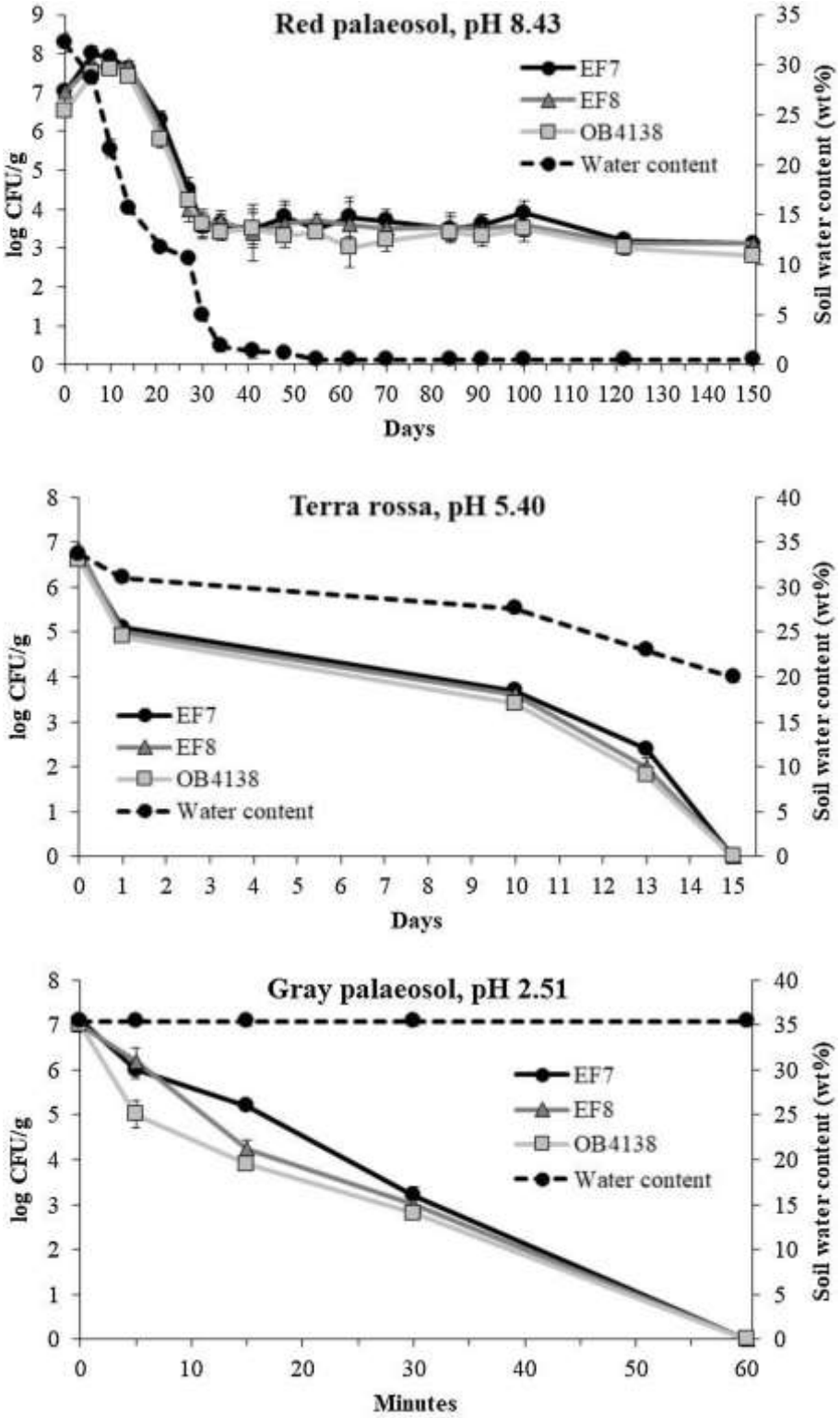


Fig. 1. Survival of three isolates of *A. baumannii* in sterilized soil samples adjusted initially to maximum water holding capacity during 150 days of monitoring.

Mean values of triplicate measurements and standard deviations are presented. Experiments with Terra Rossa and gray palaeosol were interrupted after 15 days and 60 min of contact, respectively due to disappearance of viable *A. baumannii* (< 1 CFU/g of wet soil).

decreased. A decrease of bacterial abundance became stagnant after 30 days and continued up to 150 days of contact. Increase of the initial abundance of *A. baumannii* and persistence of high numbers during the first 14 days of contact occurred at soil water content from 32 to 16 wt%. A sharp decrease of *A. baumannii* abundance (for 4.0 log CFU/g) from 14th to 30th day of contact was statistically significantly connected (r= 0.911) to the decrease of the soil water content from 16 to 5 wt%. Further maintenance of the water content at minimum (<1 wt%) did not result in further decrease of *A. baumannii* abundance, which was maintained at 3.4±0.3 log CFU/g. During this period of maintenance of low *A. baumannii* abundance, occasionally the small colony variants appeared on the selective plates inoculated with all three *A. baumannii* isolates. The cells of *A. baumannii* became quickly immobilized onto particles of sterilized red palaeosol and formed biofilm after the first SEM examination after 6 days of contact (Fig. 2).

In the case of weakly acid Terra Rossa (pH 5.40), decrease of the initial abundance of all three *A. baumannii* isolates after the first day of contact continued up to 15 days of monitoring, when no *A. baumannii* was detected in soil (< 1 CFU/g of wet soil). Decrease of the *A. baumannii* abundance was statistically significantly correlated with the soil water content (r=0.991). However, complete decay of viable *A. baumannii* was observed at high soil water content (20 wt%).

Strongly acid gray palaeosol (pH 2.51) caused the sharp and complete decay of viable *A. baumannii* (< 1 CFU/g of wet soil) within 60 min of contact. Decrease of the *A. baumannii* abundance was not correlated to the soil water content, which stayed at initial 35 wt% during the short duration of the experiment. No statistically significant difference among the survival of three tested *A. baumannii* isolates was observed at the end of experiments with all three soil types.

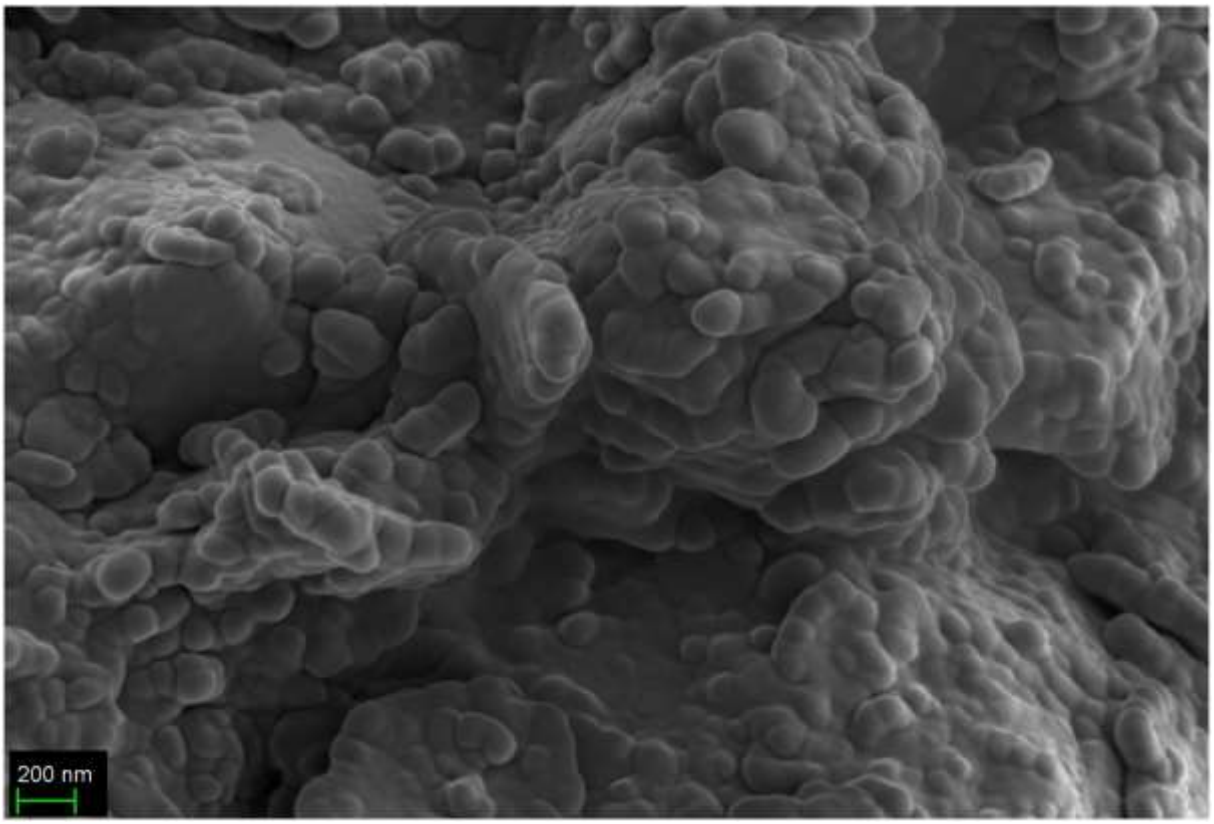


Fig. 2. Scanning electron micrograph of pure culture of *A. baumannii* cells (isolate EF8) immobilized onto particles of sterilized red paleosol after 6 days of contact.

3.3. Survival of A. baumannii in fresh soils

Survival of three isolates of *A. baumannii* in three fresh soil samples containing the native population of heterotrophic bacteria is shown in Fig. 3. As in the previous experiment, there was a statistically significant correlation of *A. baumannii* abundance with soil water content of red palaeosol (r=0.862) and Terra Rossa (r=0.893) during the whole period of monitoring. In the alkaline red palaeosol (pH 8.43), the initial abundance of all three *A. baumannii* isolates slightly increased during the first 5 days of contact, following the sharp decrease after 8 days of contact, and further maintenance of the abundance until 150th day of monitoring. Increase of the initial abundance of *A. baumannii* occurred at soil water content from 30 to 16 wt%, while the sharp decrease of the abundance (for 3.2 log CFU/g) was accompanied (r= 0.925) by the drop of soil moisture from 16 to 5 wt%. The maintenance of the abundance of *A.*

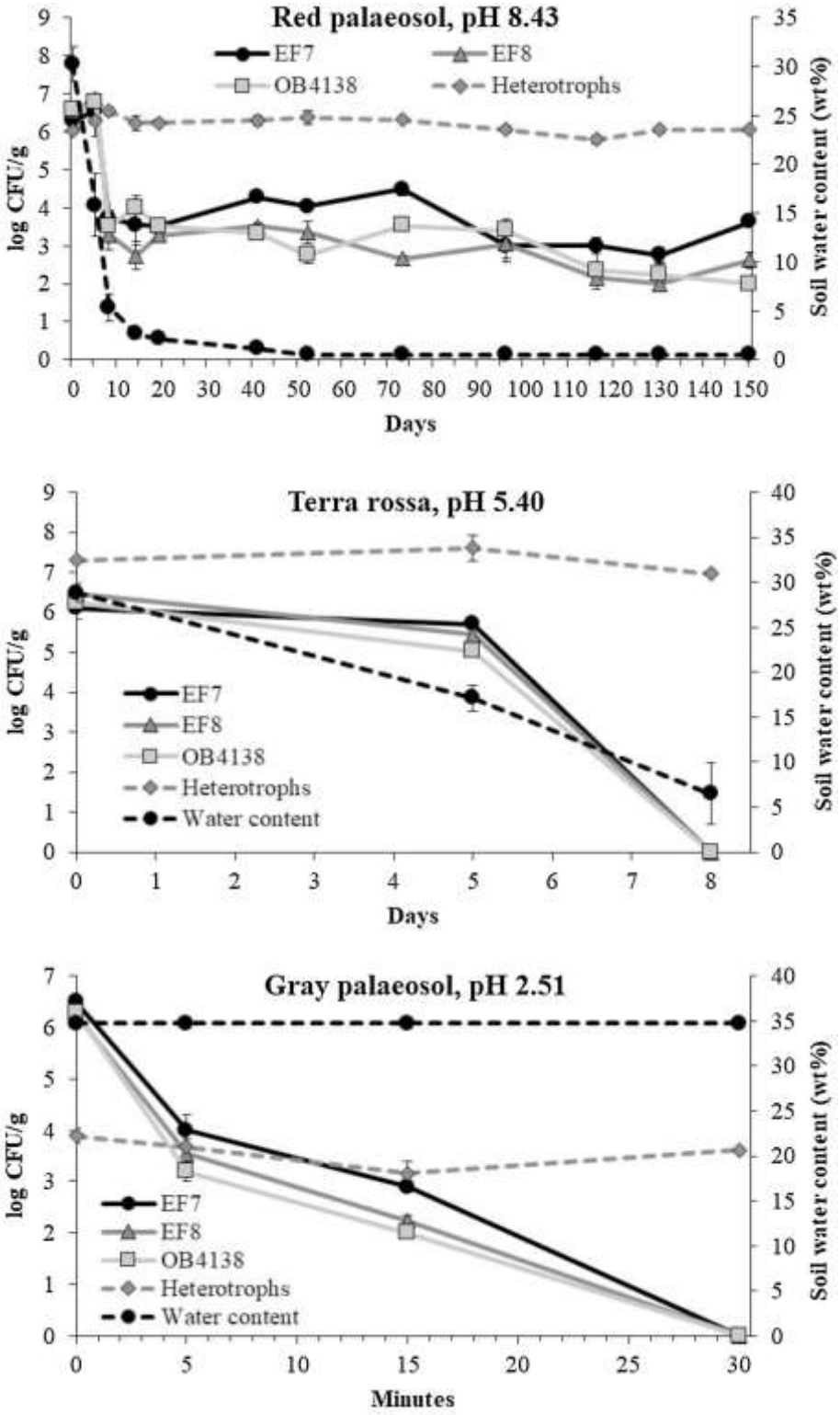


Fig. 3. Survival of three isolates of *A. baumannii* in fresh soil samples containing the native population of heterotrophic bacteria and adjusted initially to maximum water holding capacity during 150 days of monitoring.

Mean values of triplicate measurements and standard deviations are presented. Experiments with Terra Rossa and gray palaeosol were interrupted after 8 days and 30 min of contact, respectively due to disappearance of viable *A. baumannii* (< 1 CFU/g of wet soil).

baumannii at 3.1±0.7 log CFU/g until the end of the experiment was measured at minimum water content of soil (<1 wt%). In contact with weakly acid Terra Rossa (pH 5.40), the slight decrease of the *A. baumannii* abundance during the first 5 days was accompanied by a loss of soil moisture from 27 to 17 wt%. Complete decay (< 1 CFU/g of wet soil) of *A. baumannii* after 8 days of contact was accompanied by the future decrease of the soil water content to 6 wt%. The contact with strongly acid gray palaeosol (pH 2.51) resulted in continuous decrease of *A. baumannii* abundance and complete decay (< 1 CFU/g of wet soil) within 30 min of contact, despite of high soil water content of 35 wt%.

No significant difference in the survival of three tested *A. baumannii* isolates was detected in contact with all three soil types. Regardless of the soil moisture, the abundance of native population of heterotrophic bacteria stayed constant during the period of monitoring in all three soils. There was no statistically significant correlation between *A. baumannii* abundance and heterotrophic bacteria in all three soil types.

The SEM confirmed the presence of native cells immobilized onto particles of fresh soil samples (native soils in Fig. 4). After 5 days of contact with red palaeosol the increase of CFUs of *A. baumannii* (Fig. 3) was accompanied with thick biofilm formed on the soil particles (Fig. 4). Despite decrease or complete loss of CFUs of *A. baumannii* after 5 days and 4h of contact with Terra Rossa and gray palaeosol, respectively, immobilized native bacteria were still present on the particles.

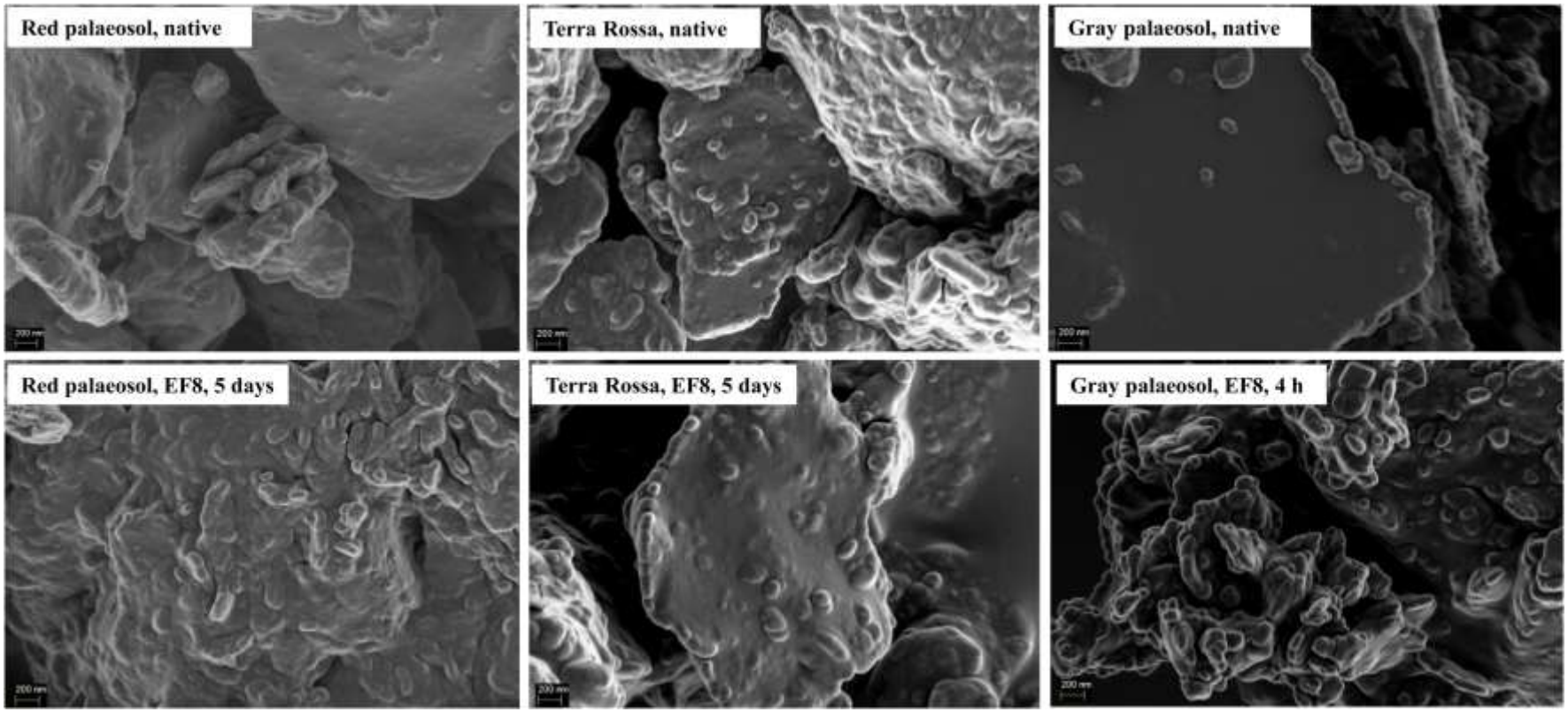


Fig. 4. Scanning electron micrographs of native cells immobilized onto particles of fresh soil samples (native, first row), and immobilized cells onto particles of soils supplemented with *A. baumannii* (isolate EF8) after 5 days of contact with red palaeosol and Terra Rossa or 4 h of contact with gray palaeosol (second row).

3.4. Regression model

Pearson correlation coefficients and scatter plots between the bacterial abundance, pH and soil water content were examined in order to eliminate co-linear variables and reduce redundancy. Values of pH followed by soil water content were the strongest variables that affected bacterial abundance in soils. The impact of heterotrophic bacteria on the abundance of *A. baumannii* in non-sterilized soils was negligible, therefore it was excluded from further analysis. The linear regression equation that predicts the impact of pH on the abundance *A. baumannii* in all tested soils is as follows (adjusted R^2 =71.1%, p=0.000):

$$A.\,baumannii\ (\log CFU/g) = 0.490pH - 1.709$$

The linear regression model for water soil content was calculated for red palaeosol and Terra Rossa separately, while no model could be built for gray palaeosol due to the short duration of the experiment and constant water soil content. The linear regression model that predicts the impact of water soil content (in wt%) in red palaeosol (rp, adjusted R^2 =77.6%, p=0.000) and Terra Rossa (tr, adjusted R^2 =48.4%, p=0.000) on the abundance *A. baumannii* is as follows:

$$A.\,baumannii\ (\log CFU/g) = 0.150rp + 3.222$$

$$A.\,baumannii\ (\log CFU/g) = 0.214tr - 1.398$$

4. Discussion

The *A. baumannii* could be introduced into soils by infiltration of wastewaters or by leaching of human solid waste by storm waters and consequent infiltration in soil (Hrenovic et al.,

2014; Hrenovic et al., 2017). Once in soil, clinically relevant *A. baumannii* represent a source of infection of people that come into contact with soil (Scott et al., 2007). The importance of contaminated soils as an environmental reservoir of *A. baumannii* will be depended on the capability of bacteria to survive in it. Native bacteria in soils are well adapted to the survival in soils influenced by different environmental factors, as shown in this study. The most important environmental factors that will dictate the persistence of non-spore forming *A. baumannii* are the soil moisture and pH value. Otherwise, toxic compounds such as antibiotics, disinfectants and heavy metals could surely negatively affect the survival of *A. baumannii*, but their concentrations, even in anthropogenically influenced soils, are rather below the effective dose (Bernier and Surette, 2013; Hrenovic et al., 2014; Hrenovic et al., 2017). From data presented here, the survival of clinically relevant *A. baumannii* in soils under different moisture regimes and pH values could be predicted.

Red alkaline palaeosol supported the long-term 150-days survival of *A. baumannii* in both sterilized and fresh non-sterilized soil. Multiplication of *A. baumannii* in alkaline soil could be explained by the availability of organic carbon in soil. *A. baumannii* multiplied and showed undisturbed survival in alkaline red palaeosol at soil moisture of 32-16 wt%. Decrease of soil moisture from 16-5 wt% prevented the multiplication and could help in reduction of *A. baumannii* abundance (for 3.6±0.5 log CFU/g), but no complete decay of viable *A. baumannii* could be expected (maintenance at 3.3±0.2 log CFU/g). Small colony variants of *A. baumannii* that were detected during the prolonged exposure to the minimal soil moisture, probably appeared as a consequence of environmental stress. Similar small colony variants of *A. baumannii* were described after the exposure of *A. baumannii* to unfavourable temperatures and pH values (Dekic et al., 2018). Most probable, the biofilm formation onto particles of red palaeosol enabled the survival of viable *A. baumannii* even in completely dried soil (moisture <1 wt%). Disappearance of viable *A. baumannii* in fresh Terra Rossa

within 8 days of contact compared to 15 days of contact with sterilized Terra Rossa is ascribed to the differences in soil water content at the end of monitoring (6 *vs* 20 wt%). As in the case of alkaline red palaeosol, decrease of water content below 16% promoted the decay of viable *A. baumannii*. In linear regression model that predicts the abundance of *A. baumannii* in Terra Rossa, only 48.4% of the variance in *A. baumannii* abundance could be accounted to the variance in water soil content. The result of the model is not surprising, since the pH of Terra Rossa is the factor that contributes greatly to the *A. baumannii* abundance.

In previous studies on the influence of desiccation on the pure culture of *A. baumannii* at designated air humidity on sterilized surfaces, no pH value of the surfaces was reported. If we suppose that the surfaces were about neutral pH, results reported here are comparable to the previous data. *A. baumannii* persisted on dry glass surfaces for 36 days at relative air humidity of 31% (Espinal et al., 2012), and on dry cellulose acetate filter surfaces for 30 days at relative air humidity of 21-27% (Bravo et al., 2016). At this range of humidity, tested isolates of *A. baumannii* also showed undisturbed survival in alkaline soil. However, at the high moisture of weakly acid Terra Rossa (20 wt%) or strongly acid gray palaeosol (35 wt%) complete decay of viable *A. baumannii* was detected. This suggest the pH value of soil as the most important environmental factor which determines the survival of *A. baumannii* in soils.

Extensively- and pandrug-resistant isolates of *A. baumannii* in defined water media survived at neutral to slight alkaline pH (6.9-8.1) for five months, while at pH 2 they only survived up to three hours (Dekic at al., 2018). These observations are comparable to the tolerance of *A. baumannii* to pH in soils. In the alkaline soil (pH 8.43) isolates of *A. baumannii* also survived for five months, while in acid soil (pH 2.51) survived up to one hour. The extensively drug-resistant environmental isolate EF8 and clinical isolate OB4138, as well as pandrug-resistant isolate EF7 showed similar survival in soils. This suggests that antibiotic resistance in soils save of effective antibiotic concentrations and origin of isolate are not

important factors for the survival of *A. baumannii* in soils. The absence of correlation of *A. baumannii* with total heterotrophic bacteria in fresh soils suggests the lack of the possibility of biological control of *A. baumannii* mediated by native soil bacteria. However, acidification of soil seems a promising method in the remediation of soils contaminated with emerging human pathogen *A. baumannii*.

5. Conclusions

- The survival of clinically relevant isolates of *A. baumannii* in soils is not influenced by native heterotrophic bacteria.
- The antibiotic resistance and origin of isolates are irrelevant for the survival of *A. baumannii* in soils.
- In alkaline soil *A. baumannii* can survive for five months. Decrease of the moisture of alkaline soil from 16-5 wt% can help in reduction of *A. baumannii* for 4 log CFU, but long-term maintenance of 3 log CFU/g is expected even at moisture <1 wt%.
- In weakly acid soil decrease of moisture below 16 wt% can help in elimination, but up to 15 days of contact is needed to eliminate 7 log CFU of *A. baumannii*.
- In strongly acid soil *A. baumannii* can survive up to one hour.

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Conflict of interest. None to declare.

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Figure captions

Fig. 1. Survival of three isolates of *A. baumannii* in sterilized soil samples adjusted initially to maximum water holding capacity during 150 days of monitoring.

Mean values of triplicate measurements and standard deviations are presented. Experiments with Terra Rossa and gray palaeosol were interrupted after 15 days and 60 minutes of contact, respectively due to disappearance of viable *A. baumannii* (< 1 CFU/g of wet soil).

Fig. 2. Scanning electron micrograph of pure culture of *A. baumannii* cells (isolate EF8) immobilized onto particles of sterilized red paleosol after 6 days of contact.

Fig. 3. Survival of three isolates of *A. baumannii* in fresh soil samples containing the native population of heterotrophic bacteria and adjusted initially to maximum water holding capacity during 150 days of monitoring.

Mean values of triplicate measurements and standard deviations are presented. Experiments with Terra Rossa and gray palaeosol were interrupted after 8 days and 30 minutes of contact, respectively due to disappearance of viable *A. baumannii* (< 1 CFU/g of wet soil).

Fig. 4. Scanning electron micrographs of native cells immobilized onto particles of fresh soil samples (native, first row), and immobilized cells onto particles of soils supplemented with *A. baumannii* (isolate EF8) after 5 days of contact with red palaeosol and Terra Rossa or 4 h of contact with gray palaeosol (second row).