# Survival of emerging pathogen *Acinetobacter baumannii* in water environment exposed to different oxygen conditions

Svjetlana Dekic[1,*,+], Jasna Hrenovic[1,+], Erna van Wilpe[2], Chantelle Venter[2] and Ivana Goic-Barisic[3]

[1]Faculty of Science, Department of Biology, University of Zagreb, Zagreb, Croatia

[2]Laboratory for Microscopy & Microanalysis, University of Pretoria, Pretoria, South Africa

[3]Department of Clinical Microbiology and University of Split School of Medicine, University Hospital of Split, Split, Croatia and School of Medicine, University of Split, Split, Croatia

+Both authors contributed equally to this manuscript.

* corresponding author: *svjetlana.dekic@biol.pmf.hr*

## Abstract

Bacterium *Acinetobacter baumannii* is a leading cause of hospital infections. Over the last decade, its occurrence in natural environments outside hospital settings has been reported. The aim was to examine the survival of *A. baumannii* in water media exposed to different ranges of oxygen supply in order to predict its behaviour in the environment. The abundance of five *A. baumannii* isolates was monitored in nutrient-depleted and nutrient-rich water media in aerated, intermediate and anaerobic conditions (oxygen saturation 96, 56 and 0%, respectively). *A. baumannii* survived in both media in all tested oxygen concentrations for 50 days. In nutrient-rich water survival of *A. baumannii* was lowest in anaerobic conditions, while in nutrient-depleted water there was no difference in survival regardless of oxygen availability. *A. baumannii* formed translucent small colony variants as the fast response (after 1 day) and dormant cells as the prolonged response (after 14 days) to anaerobic conditions. Transmission electron microscopy (TEM) images showed the outer membrane of coccobacillus dormant cells was up to four times thicker than in regular cells. Once in the environment, *A. baumannii* is able to survive regardless of the availability of dissolved oxygen, which represents a serious public health concern.

**Keywords:** *Acinetobacter baumannii*, anaerobic conditions, dormant cells, oxygen, small colony variants, water

## INTRODUCTION

*Acinetobacter* baumannii is a Gram-negative coccobacillus, non-motile, non-spore forming, non-fermentative, strict aerobe (Towner 2009). Although not an obligate pathogen, *A. baumannii* is nowadays a leading cause of nosocomial infections and hospital outbreaks worldwide. *A. baumannii,* primary sensitive to antibiotics, during the last 30 years has developed resistance to commonly used antimicrobial agents (Roca *et al.* 2012). Today, clinical isolates resistant to last-resort antibiotics such as carbapenems and colistin are being reported worldwide (Towner 2009; Roca *et al.* 2012; Gottig *et al.* 2014). Due to the expression of the virulence factors, *A. baumannii* can survive for prolonged periods on abiotic surfaces in the hospital environment (Espinal *et al.* 2012).

Over the last decade, the occurrence of A. *baumannii* in natural environments outside of hospital settings has been reported. Viable *A. baumannii* of clinical significance were recovered from untreated hospital wastewaters (Ferreira *et al.* 2011; Zhang *et al.* 2013; Kovacic *et al.* 2017) urban

wastewaters and wastewater treatment plants (Hrenovic *et al.* 2016; Higgins *et al.* 2018), as well as from rivers (Girlich *et al.* 2010; Seruga Music *et al.* 2017).

However, very little is known about the environmental factors that influence the survival of *A. baumannii* in waters from which it was recovered. A recent study by Dekic *et al.* (2018) demonstrated the survival of A. *baumannii* under different temperature (—20 to 44 °C) and pH (5-10) regimes as well as nutrient availability. Optimal conditions for the long-term survival of *A. baumannii* were temperatures of 4 and 22 °C regardless of nutrient availability (Dekic *et al.* 2018). Additionally, Hrenovic *et al.* (2016) investigated influent and effluent at the wastewater treatment plant from which *A. baumannii* isolates were recovered. From these data, it could be concluded that *A. baumannii* existed in waters with different nutrient availability at almost no dissolved oxygen (0.09 mg $O_2$/L, 18.6 °C) in influent to the very high oxygen concentration in aerated effluent. The multiplication and survival of *A. baumannii* in the aerated system of autoclaved effluent wastewater was also reported (Hrenovic *et al.* 2016). The composition of effluent wastewater varies from time to time and from place to place, which hinders the deeper insight into the survival of this pathogen in the water environment. Recently, the survival of *A. baumannii* through the technological process of anaerobic mesophilic sludge digestion at the wastewater treatment plant was reported (Higgins *et al.* 2018). These isolates were able to survive on nutrient agar in laboratory-controlled anaerobic conditions during 30 days, after which they grew normally in aerobic conditions. However, no growth of colonies on the inoculated nutrient agar was detected after direct exposure to anaerobic conditions. This finding by Higgins *et al.* (2018) indicated the anaerobic environment as an ecological niche important in the epidemiology of clinically relevant *A. baumannii.* However, the anaerobic conditions in digested sludge and nutrient agar are not much applicable to the conditions in the natural environment. The prediction of the behaviour of this emerging pathogen in waters still could not be established due to the lack of data.

Therefore, the aim of this study was to examine the survival of environmental as well as clinical isolates of *A. baumannii* in different water media exposed to different ranges of oxygen supply.

## MATERIALS AND METHODS

### Characteristics of tested A. baumannii isolates

Five isolates of A. baumannii deposited at the University of Zagreb Faculty of Science were chosen for the experiments (Table 1). Four environmental isolates were recovered from Zagreb wastewater treatment plant (WWTP), one from influent (IN39) and three from effluent (EF7, EF8, EF11) wastewater (Goic-Barisic *et al.* 2017; Dekic *et al.* 2018; Higgins *et al.* 2018). One clinical isolate (OB4138) was recovered from a patient suffering from hospital-acquired pneumonia at the Special Hospital for Pulmonary Diseases in Zagreb, Croatia (Seruga Music *et al.* 2017; Dekic *et al.* 2018). According to the antibiotic susceptibility profile (Magiorakos *et al.* 2012), isolates were grouped into several categories: susceptible to all tested antibiotics (S) - EF11; multidrug-resistant (MDR) - IN39; extensively drug-resistant (XDR) - EF8 and OB4138; pandrug-resistant (PDR) -EF7. MDR isolates are resistant to at least one antibiotic in three or more antibiotic categories, XDR are resistant to at least one antibiotic in all but one or two antibiotic categories, while PDR isolates are resistant to all antibiotics in all tested categories.

**Table 1.** Characteristics of tested *A. baumannii* isolates

| Isolate | Origin | International clonal lineage | Antibiotic susceptibility profile | Reference |
|---|---|---|---|---|
| IN39 | WWTP influent | IC1 | MDR (MIN, CST, aminoglycosides) | Goic-Barisic *et al.* (2017), Dekic *et al.* (2018); Higgins *et al.* (2018) |
| EF7 | WWTP effluent | IC2 | PDR | |
| EF8 | WWTP effluent | IC2 | XDR (SXT, CST) | |
| EF11 | WWTP effluent | unclustered | S | |
| OB4138 | Bronchial aspirate | IC2 | XDR (SXT, CST) | Seruga Music *et al.* (2017), Dekic *et al*. (2018) |

Antibiotics to which isolates remained susceptible are given in brackets; MIN-minocycline, SXT-trimethoprim/sulfamethoxazole, CST-colistin. S-sensitive to all tested antibiotics, MDR-multidrug-resistant, XDR-extensively drug-resistant, PDR-pandrug-resistant.

## Experimental set up

Behaviour of five isolates of A. *baumannii* was tested in two different water media exposed to three types of different oxygen supply. The nutrient-depleted commercially available spring water (SW) was used as well as nutrient broth (Biolife) diluted with distilled water to 1:100 (DNB). In comparison to SW, DNB can be considered nutrient-rich, while it has fewer nutrients than regular nutrient broth. Chemical properties of the tested water media are given in Table 2. A full 10 uL loop of overnight culture of each *A. baumannii* isolate was suspended in a tube containing 10 mL of autoclaved SW or DNB. One mL of suspension was inoculated in duplicate in the 50 mL tubes containing 30 mL of autoclaved SW or DNB. Aerated conditions were achieved by forced aeration of water media with sterile air filtered through the membrane filters and serological pipette. Intermediate oxygen supply was achieved in the stationary loosely capped tubes. Anaerobic conditions were maintained by placing the loosely capped tubes in the commercial Anaerocult A system (Merck Millipore). Prepared inoculated tubes were incubated at 22 ± 2 °C during 50 days of monitoring.

**Table 2.** Chemical properties of the tested media

| Chemical parameters | SW | DNB |
|---|---|---|
| pH | 8.1 | 6.9 |
| Dissolved oxygen (mg/L $O_2$) | 4.6 | 4.6 |
| Chemical oxygen demand (mg/L COD) | 3 | 99 |
| Total organic carbon (mg/L C) | <1 | 44 |
| Total nitrogen (mg/L N) | 0.7 | 13.2 |
| Total phosphorus (mg/L P) | 0.1 | 1.1 |

SW-commercially available spring water, DNB-diluted nutrient broth with distilled water (1:100).

Measurement of oxygen concentrations was done with a WTW Oxi 330i electrode, in duplicate series of tubes to prevent cross contamination. In aerated and intermediate conditions, the dissolved oxygen concentration was constant during 50 days of monitoring: 8.4 mg/L (oxygen saturation of 96%) and 4.6 mg/L (oxygen saturation of 56%), respectively. To generate anaerobic conditions, sachets made of absorptive and highly gas-permeable paper filled with an oxygen-binding mixture were put inside an Anaerocult jar. The addition of water started the reaction, which consisted of oxidation of iron inside the sachets and release of CO2. An oxygen strip indicator in the Anaerocult A system indicated no dissolved oxygen 3 h after the closing of the jar.

In another series of tubes, the number of viable bacteria was monitored at the beginning of the experiment, after 1, 2 and further every 7 days of incubation. After a specified period of time, tubes were shaken, sub-samples were diluted in sterile saline solution, inoculated in technical triplicate onto Mueller-Hinton agar (Biolife) plates, and bacterial colonies were counted after incubation at 42 °C/24 h. The number of viable bacteria was determined as colony forming units (CFU), logarithmically transformed, and expressed as log CFU per one mL of water medium.

### Microscopic analyses

In order to check the purity of bacterial cultures during 50 days of monitoring, Gram staining was routinely preformed each time when the number of CFU was determined in all systems. The original subsamples at the start and at the end of aerobic and anaerobic experiments with DNB were fixed in 2.5% glutaraldehyde in phosphate buffered saline, and prepared for scanning (SEM) and transmission (TEM) electron microscopy using standard techniques. Briefly, after removal of the fixative, the samples were rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide in phosphate buffer, again rinsed in phosphate buffer and then dehydrated in a graded ethanol series up to absolute ethanol. Cells for SEM were dried with hexamethyldisilazane and carbon coated before examination at low voltage (0.5 kV) with a Zeiss Ultra PLUS FEG SEM. The samples for TEM were infiltrated with an epoxy resin and ultrathin sections were examined at 200 kV with a Jeol 2100F TEM.

### Statistical analyses

Statistical analyses were carried out using Statistica 13.3 software (TIBCO Software Inc.). For pairwise comparisons, factorial analysis of variance (ANOVA) and Duncan post hoc test were used. For making correlations, Spearman's rank correlation coefficient was used. Decisions regarding statistical significance were made at $p < 0.05$. For graphical presentation, the percentage of survival was calculated as follows: $((\log CFU/mL_{time}:\log CFU/mL_{start})*100)$, where log $CFU/mL_{time}$ represents the number of bacteria on a day of measurement and log $CFU/mL_{start}$ the initial number of bacteria. In some cases the survival percent exceeds 100%, which signifies bacterial growth.

## RESULTS

### A. baumannii survival

All five tested isolates of A. baumannii successfully survived the aerated, intermediate and anaerobic conditions in both SW and DNB during 50 days of monitoring (Figures 1–3). In nutrient-depleted SW there was no evident multiplication of bacteria. The multiplication of all isolates occurred in nutrient-rich DNB during the first two days of contact in aerated and intermediate conditions, and to a lesser extent during the first day in anaerobic conditions.

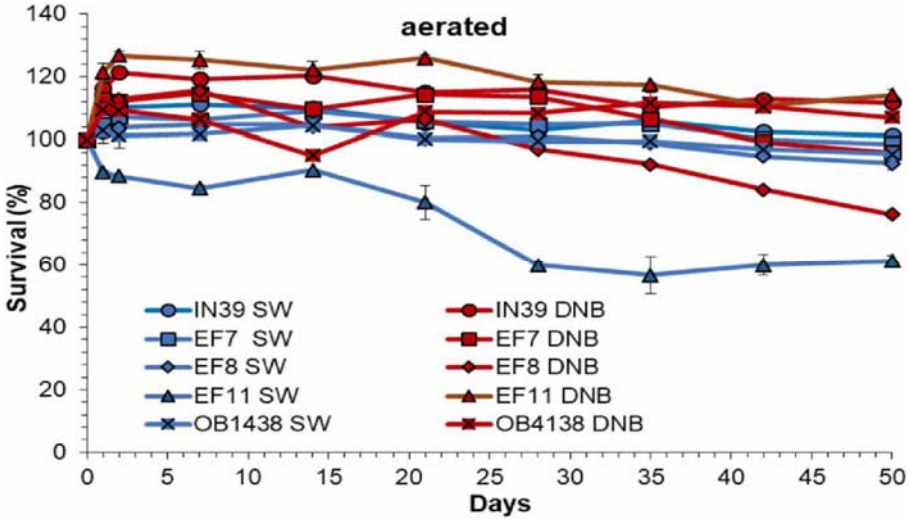


**Figure 1**. Survival of five isolates of A. baumannii in commercial spring water (SW) and diluted nutrient broth (DNB) exposed to aerated conditions (oxygen saturation of 96%) during 50 days. Initial concentration of all isolates was 6.9 ± 0.3 log CFU/mL. Mean values and standard deviations are presented

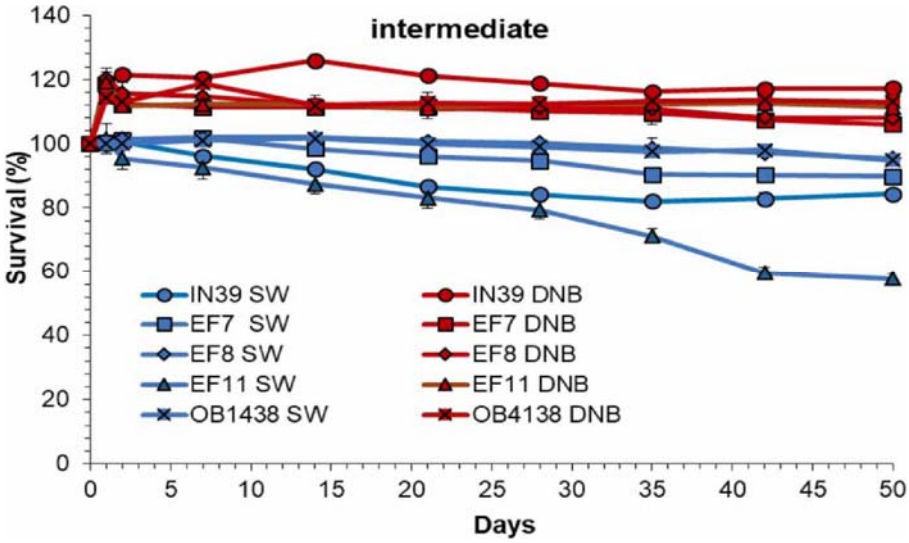


**Figure 2**. Survival of five isolates of A. baumannii in commercial spring water (SW) and diluted nutrient broth (DNB) exposed to intermediate conditions (oxygen saturation of 56%) during 50 days. Initial concentration of all isolates was 6.9 ± 0.3 log CFU/mL. Mean values and standard deviations are presented

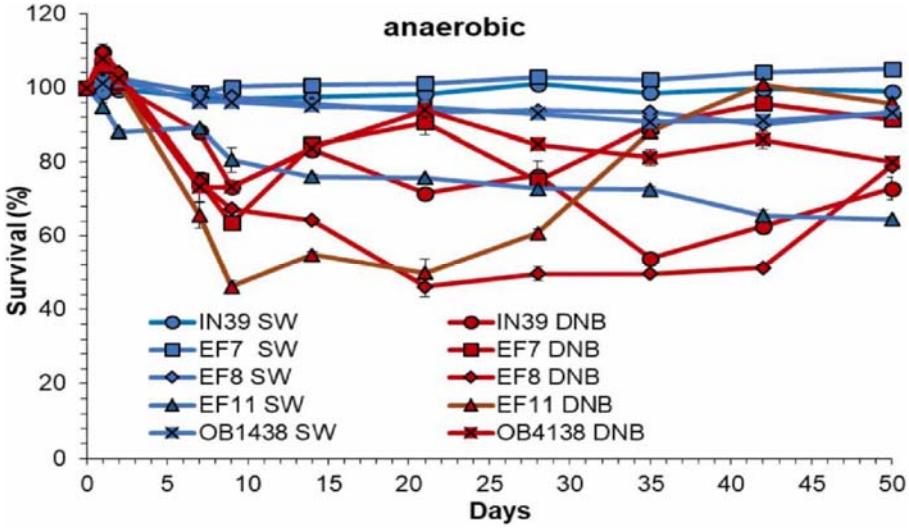


**Figure 3.** Survival of five isolates of A. baumannii in commercial spring water (SW) and diluted nutrient broth (DNB) exposed to anaerobic conditions (oxygen saturation of 0%) during 50 days. Initial concentration of all isolates was 6.9± 0.3 log CFU/mL. Mean values and standard deviations are presented.

In SW, there was no statistically significant difference between the survival of isolates in aerated, intermediate and anaerobic conditions (Figures 1–3). The survival of four isolates (IN39, EF7, EF8, OB4138) in SW after 50 days of contact ranged from 84–105%. The isolate EF11 sensitive to all tested antibiotics had the lowest survival in SW in all tested oxygen conditions as compared to other isolates. After 50 days of contact in aerobic, intermediate and anaerobic conditions, the survival of isolate EF11 in SW averaged 61, 58 and 64%, respectively.

In DNB, there was no difference between the survival of EF11 and other isolates. The survival of all isolates in DNB after 50 days of contact ranged from 73 to 117% in different oxygen conditions. The highest survival was observed in DNB in aerated and intermediate conditions (76–117%) with no statistically significant difference between these conditions (p ¼ 0.075). However, the survival in anaerobic DNB (73–93%) was statistically lower than in both aerated (p ¼ 0.007) and intermediate (p ¼ 0.000) conditions. The survival in DNB was statistically positively correlated with dissolved oxygen concentration (R ¼ 0.448, p ¼ 0.013), whereas the survival in SW showed no statistically significant correlation. In the anaerobic DNB, after the sharp decrease in CFUs during the first nine days, A. baumannii behaved erratically, having irregular survival curve with sudden decreases and increases in concentration of viable bacteria (Figure 3).

Furthermore, A. baumannii from anaerobic conditions formed smaller translucent colonies together with normal opaque ones (Figure 4). Small colony variants irregularly but constantly appeared on the plates of Mueller-Hinton agar inoculated with all isolates either from SW and DNB after only one day of incubation in anaerobic conditions. The identity of translucent colonies as A. baumannii was confirmed by matrix-assisted laser desorption ionisation-time of flight mass spectrometry – MALDI-TOF MS based on the analysis of ribosomal proteins. No translucent colonies were observed in the aerated or intermediate experiments.

**Figure 4.** Translucent smaller colonies beside normal large opaque colonies of A. baumannii, formed on plates inoculated with isolate EF7 in diluted nutrient broth (DNB) exposed to anaerobic conditions (oxygen saturationof0%) for28days.

**Microscopic analyses**

Due to the unusual survival curve, cells from the anaerobic DNB experiment were further analysed and compared to cells from aerobic conditions in DNB. After 14 days and further every 7 days, the subsamples were Gram stained and checked under the optical microscope. Among the normal red-stained A. baumannii cells of coccobacillus shape, the unusual bloated poorly stained cells were observed (data not shown). The SEM analysis of A. baumannii after the exposure to anaerobic DNB conditions showed no surface damage of cells as compared to those at the start of the experiment, but cells were stacked together with the extracellular substances (Figure 5). The TEM showed normal structure of A. baumannii cells after the exposure to aerobic conditions in DNB (Figure 6(a)). After the exposure to anaerobic conditions in DNB, the majority of A. baumannii cells showed damage with signs of lysis, with only spheroplasts remaining in some cases (Figure 6(b)). Aminority of cells retained a dark protoplast with an intact membrane. Cells with thick envelopes, although in the minority, were easily distinguished among the damaged and undamaged cells (Figure 6(c) and 6(d)). These cells of coccobacillus shape remained osmotically stable. We conclude that the cells containing the thick envelopes are dormant cells. Dormant cells were not evidently bigger (1,317 × 1,029 nm) as compared to normal the outer membrane of the control cells from aerobic con-cells (1,181 × 996 nm), but there was an apparent difference conditions averaged 30 ± 4 nm (from 22 to 41 nm, n ¼ 93). The in the thickness of the outer membrane. The thickness of outer membrane of dormant cells from anaerobic conditions was on average 3.3 times thicker, averaging 98 ± 11 nm (from 83 to 111 nm, *n* — 17). TEM images (Figure 6(d)) showed that the thickness of the outer membrane was due to an increase in the electron-dense, most likely, peptidoglycan layer.

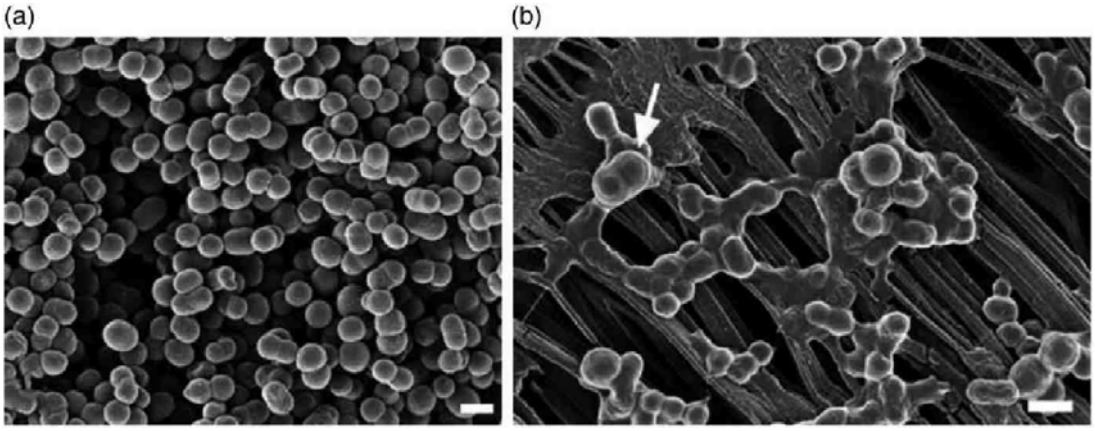


**Figure 5**. Scanning electron microscopy of A. baumannii cells (isolate EF7, DNB). (a) Cells without surface damage at the start of aerobic and anaerobic experiments; (b) cells stacked with extracellular substances (arrow) at the end of the anaerobic experiment (50 days of contact). At the end of the aerobic experiments, the cells were without change (like in A). Microbars: 1 μm.

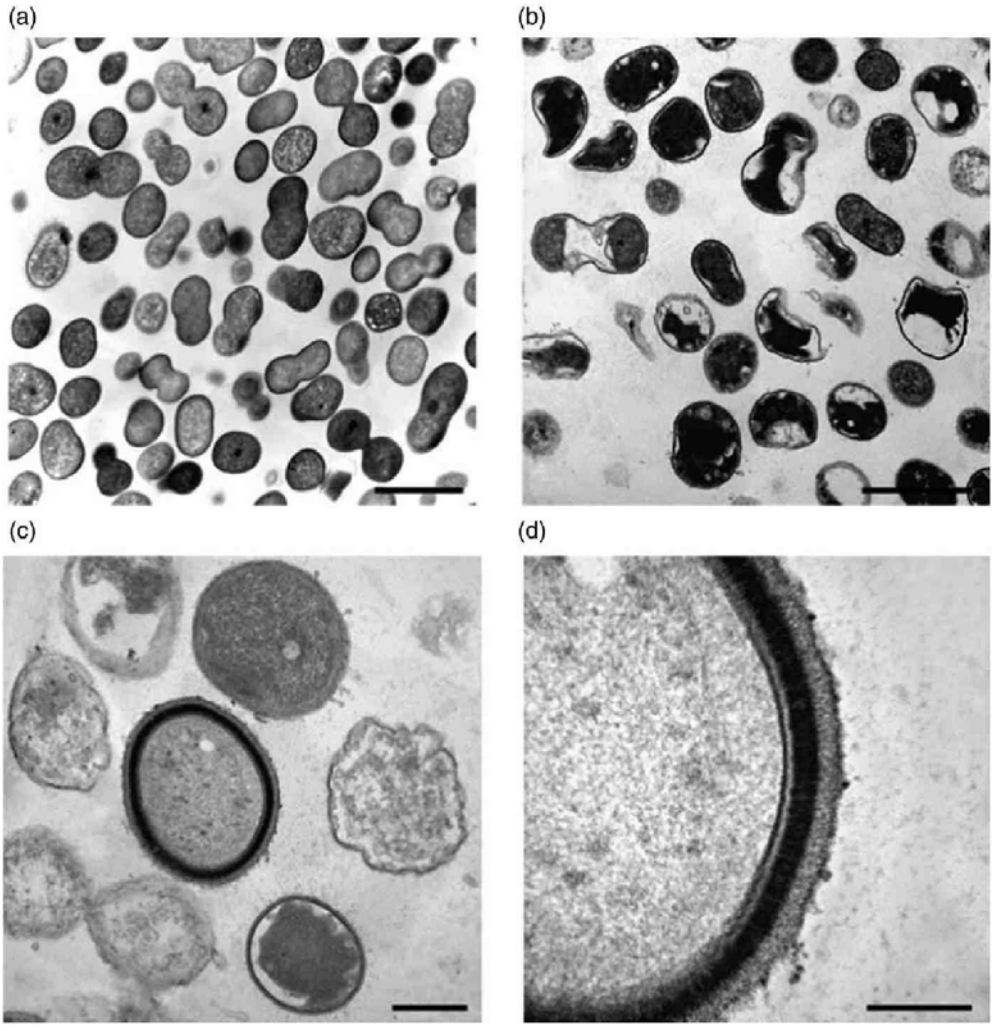


**Figure 6**. Transmission electron microscopy of A. baumannii cells (isolate EF7, DNB). (a) Cells with normal structure at the end of aerobic experiment (oxygen saturation of 96%); (b) damaged cells at the end of anaerobic experiment (50 days of contact, oxygen saturation of 0%); (c) dormant cell from anaerobic experiment; (d) magnified envelope of dormant cell. Microbars: A, B – 2 μm; C – 500 nm; D – 200 nm.

## DISCUSSION

The results presented here show that one clinical and four environmental, but clinically relevant, isolates of *A. baumannii* were able to survive both in nutrient-depleted and nutrient-rich water media in the aerated, intermediate and anaerobic conditions during 50 days. Moreover, this emergent human pathogen multiplied in the nutrient-rich water medium. The monitoring of survival in aerated DNB is consistent with the previous report (Hrenovic *et al.* 2016) of multiplication and good survival of XDR isolates of *A. baumannii* in sterilised effluent wastewater (of chemical composition close to the DNB) within 50 days. This implies that once in the environment, clinically relevant *A. baumannii* are able to persist in a wide range of water environments: from oligotrophic spring water to eutrophic lakes, at full oxygen saturation to complete anaerobic conditions. Although not commonly associated with anaerobic sediments, *A. baumannii* could possibly survive in such environments.

Antibiotic-resistant pathogens like *A. baumannii* are transferred from hospitals into the natural environment via the hospital wastewater (Ferreira *et al.* 2011; Zhang *et al.* 2013; Kovacic *et al.* 2017). In some countries, hospital waste-water is not disinfected, meaning that raw hospital wastewater is discharged directly into a river (Hrenovic *et al.* 2019). In other situations, the hospital wastewater is mixed with domestic wastewater and storm runoff, after which it goes to a wastewater treatment plant. There is evidence that antibiotic-resistant *A. baumannii* survive the wastewater treatment and are discharged into the natural environment (Hrenovic *et al.* 2016; Seruga Music *et al.* 2017; Higgins *et al.* 2018). The time duration of 50 days is sufficient for water masses to spread far from the source point. This fact is disturbing and represents a public health problem. Sporadic acute community-acquired human infections with *A. baumannii* (Dexter *et al.* 2015) are well known, but the source of infection has not been identified thus far. Even anaerobic natural water environments could present a source of *A. baumannii,* where this bacterium could survive for prolonged periods.

The antibiotic sensitive isolate EF11 showed the lowest survival in SW as compared to MDR, XDR or PDR isolates.

In the nutrient-rich urban wastewater, the persistence of antibiotic-sensitive isolates among dominant antibiotic-resistant *A. baumannii* isolates was reported, suggesting the urban sewage is a natural habitat of susceptible *A. baumannii* (Higgins *et al.* 2018). The EF11 isolate recovered from wastewater is therefore probably accustomed to the high nutrient concentration, which explains its lower survival in nutrient-poor water. This may suggest better survival of the problematic antibiotic-resistant isolates in clean oligotrophic waters, but the association between the genetic determinants for the resistance to antibiotics and the resistance to environmental conditions needs to be examined further.

Small colony variants represent a subpopulation with distinctive phenotypic traits as compared to the parent bacterial population (Proctor *et al.* 2006). Besides the atypical colony morphology, small colony variants exhibit unusual biochemical characteristics, which often leads to the mis-identification of the species by using the routine identification techniques. Since the small colony variants are resistant to antibiotics successful in treatment of the parent population, they represent a challenge in clinical microbiology. Small colony variants are described in different bacterial species, and most often in *Staphylococcus aureus* (Seifert *et al.* 2003), as a cause of recurrent human infections. It is hypothesized that small colony variants arise from the parent population as a consequence of the selective pressure of prolonged antimicrobial treatment of infected patients. Translucent colonies formed on LB plates incubated at 37 °C were recently reported in *A. baumannii* (Tipton *et al.* 2015). However, the described translucent colonies were not smaller than the opaque ones, and the translucency could be viewed only under a dissecting microscope with oblique indirect illumination at density of colonies greater than 200 per plate. Contrary to the increased resistance to aminoglycosides of *S. aureus* small colony variants (Seifert *et al.* 2003; Proctor *et al.* 2006), translucent *A. baumannii* colonies showed the increased susceptibility to aminoglycosides (Tipton *et al.* 2015). In our case, small colony variants of *A. baumannii* appeared after the exposure of isolates to anaerobic conditions without contact with antibiotics. Therefore, we presume that the appearance of small colony variants is a consequence of environmental stress caused by depletion of oxygen necessary for the normal metabolism of *A.*

*baumannii.* In addition, Dekic *et al.* (2018) reported the occurrence of small colony variants in *A. baumannii* after the exposure to extreme temperatures and pH. These data imply that *A. baumannii* forms translucent colonies in unfavourable environmental conditions. Translucency of smaller colonies was visible under normal room lighting, differentiating them from the translucent *A. baumannii* colonies reported by Tipton *et al.* (2015). The biochemical characteristics of small translucent colonies were not examined, but the identification of *A. baumannii* was confirmed by MALDI-TOF MS. This implies the necessity of advanced techniques for the identification of A. *baumannii* in addition to routine techniques based on the biochemical characteristics, especially when working with the environmental samples where *A. baumannii* could be exposed to oxygen depleted conditions.

Special concern rises from the fact that *A. baumannii* isolates formed dormant cells in anaerobic conditions, which enhances its survival in an unfavourable environment. Dormancy in non-sporulating bacteria has been recognised for decades (Kaprelyants *et al.* 1993). Exogenous dormancy refers to the reversible state of low metabolic activity, which helps the cells survive for a long time without multiplication. Dormant cells are unable to divide and form colonies on agar plates without a preceding resuscitation phase (Kaprelyants *et al.* 1993). In our case, the resuscitation of dormant cells was provided by incubation of inoculated agar plates in the air, where dormant together with undamaged cells were allowed to form CFUs. Therefore, the initial nine-day drops in CFUs of *A. baumannii* in anaerobic DNB actually did not reflect the formation of dormant cells. But, the sudden increases in CFUs after 14 days of contact could be connected to the formation of dormant cells. The erratic curve of CFUs in DNB exposed to anaerobic conditions could not be explained by the 'bust and boom' survival strategy where weak bacterial cells die in unfavourable conditions and the remaining persister cells live on the expense of dead cells (Bravo *et al.* 2016). Namely, in the oxygen depleted conditions multiplication of *A. baumannii* is unlikely to happen. As judged by relatively high CFUs from the anaerobic experiment, *A. baumannii* isolates did not form the viable but non-culturable cells, which is in agreement with previous records for *A. baumannii* ATCC 19606 T under nutrient-deprived conditions (Bravo *et al.* 2016). Dormancy per se is one of the possible consequences of starvation. However, until now it has not been clear what enables the fraction of the population to respond differently to the starvation stress (Pu *et al.* 2017). In this study, there was no difference in the survival curve of *A. baumannii* in nutrient-poor medium regardless of oxygen concentration. An unusual survival curve was registered only in the experiment with DNB subjected to anaerobic conditions. The nutrient availability in DNB together with exposure to anaerobic conditions governed the erratic behaviour of *A. baumannii* and the formation of dormant cells.

Here, observed dormant cells could be classified as those employing a 'passive defence'; they are different from persister cells employing an 'active defence' to expel antibiotics (Barth *et al.* 2013; Pu *et al.* 2017). The thick envelope of dormant cells probably reduces the permeability of the outer membrane, which enables the minimum metabolism of cells in an unfavourable environment. It should be emphasized that small colony variants should not be equated with the dormant cells of *A. baumannii.* Although not quantified, small colony variants were much more abundant than dormant cells and did not match in either the same time (1 day of contact for small colony variants *vs* 14 days of contact for dormant cells) or trend of appearance. It seems that the small colony variants are a fast response to the adverse environmental conditions, while the formation of dormant cells requires prolonged exposure.

*A. baumannii* is considered a strict aerobe, producing energy by aerobic respiration with oxygen as the terminal electron acceptor, but is unable to grow with nitrate as the terminal electron acceptor (Garrity *et al.* 2005). In routine bacteriology, it is known that *A. baumannii* gives a characteristic weak orange reaction in the depth of Kligler Iron Agar. This suggest the formation of weak or low concentration of acid from glucose, and also could be an indication of fermentation potential. *Pseudomonas aeruginosa* is another strict aerobe confirmed to perform fermentation in the absence of oxygen (Eschbach *et al.* 2004; Schreiber *et al.* 2006). The mechanisms of *P. aeruginosa* fermentation are well investigated; however, the mechanism of *A. baumannii* anaerobic metabolism has not been investigated at all which opens new research opportunities.

To our knowledge, this work provides the first evidence of the survival of A. *baumannii* in anaerobic conditions. The findings call for the future usage of microscopic, biochemical and molecular techniques in order to elucidate the mechanisms of survival of non-spore forming, strictly aerobic, human pathogens in anaerobic environments.

## CONCLUSIONS

*A. baumannii* isolates survived both in nutrient-depleted and nutrient-rich water media in aerated, intermediate and anaerobic conditions (oxygen saturation of 96, 56 and 0%, respectively) for 50 days. Clinical *A. baumannii* isolate behaved the same as environmental isolates of clinical significance. In addition, the antibiotic-sensitive isolate showed the lowest survival in nutrient-depleted water as compared to MDR, XDR or PDR isolates. The availability of dissolved oxygen did not influence the survival of *A. baumannii* in nutrient-depleted water, but enhanced its survival in nutrient-rich water. The formation of small colony variants as the fast response and dormant cells as the prolonged response to anaerobic conditions was observed. The results provide insight into the behaviour of *A. baumannii* and make an important contribution to raise awareness that this emerging pathogen persists in the natural environment, which should lead to appropriate monitoring and control measures to reduce the threat to public health.

## ACKNOWLEDGEMENTS

This study was supported by Croatian Science Foundation (IP-2014-09-5656). Many thanks to Snježana Kazazic´ for enabling the use of the MALDI-TOF MS device.

## REFERENCES

Barth, V. C, Rodrigues, B. A., Bonatto, G. D., Gallo, S. W., Pagnussatti, V. E. & Ferreira, C. A. 2013 Heterogeneous persister cells formation in *Acinetobacter baumannii. PLoS One* 8, e84361.

Bravo, Z., Orruño, M., Parada, C, Kaberdin, V. R., Barcina, I. & Arana, I. 2016 The long-term survival of *Acinetobacter baumannii* ATCC 19606 T under nutrient-deprived conditions does not require the entry into the viable but non-culturable state. *Archives of Microbiology* 198 (5), 399-407.

Dekic, S., Hrenovic, J., Ivankovic, T. & van Wilpe, E. 2018 Survival of ESKAPE pathogen *Acinetobacter baumannii* in water of different temperatures and pH. *Water Science and Technology* 78 (5-6), 1370-1376.

Dexter, C, Murray, G. L., Paulsen, I. T. & Peleg, A. Y. 2015 Community-acquired *Acinetobacter baumannii:* clinical characteristics, epidemiology and pathogenesis. *Expert Review of Anti-Infective Therapy* 13 (5), 567-573.

Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D. & Schobert, M. 2004 Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *Journal of Bacteriology* 186, 4596-4604.

Espinal, P., Martí, S. & Vila, J. 2012 Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. *Journal of Hospital Infection* 80 (1), 56-60.

Ferreira, A. E., Marchetti, D. P., De Oliveira, L. M., Gusatti, C. S., Fuentefria, D. B. & Corção, G 2011 Presence of OXA-23-producing isolates of *Acinetobacter baumannii* in wastewater from hospitals in southern Brazil. *Microbial Drug Resistance* 17 (2), 221-227.

Garrity, G M., Brenner, D. J., Krieg, N. R & Staley, J. T 2005 *Bergey's Manual of Systematic Bacteriology. Volume Two, The Proteobacteria, Part B, The Gammaproteobacteria.* Springer, New York, USA.

Girlich, D., Poirel, L. & Nordmann, P. 2010 First isolation of the blaOXA-23 carbapenemase gene from an environmental *Acinetobacter baumannii* isolate. *Antimicrobial Agents and Chemotherapy* 54 (1), 578-579.

Goic-Barisic, I., Seruga Music, M., Kovacic, A., Tonkic, M. & Hrenovic, J. 2017 Pan drug-resistant environmental isolate of *Acinetobacter baumannii* from Croatia. *Microbial Drug Resistance* 23 (4), 494-496.

Gottig, S., Gruber, T M., Higgins, P. G, Wachsmuth, M., Seifert, H. & Kempf, V. A. 2014 Detection of pan drug-resistant *Acinetobacter baumannii* in Germany. *Journal of Antimicrobial Chemotherapy* 69, 2578-2579.

Higgins, P. G, Hrenovic, J., Seifert, H. & Dekic, S. 2018 Characterization of *Acinetobacter baumannii* from water and sludge line of secondary wastewater treatment plant. *Water Research* 140, 261-267.

Hrenovic, J., Goic-Barisic, I., Kazazic, S., Ganjto, M. & Tonkic, M. 2016 Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014. *Euro Surveillance* 21, 1-10.

Hrenovic, J., Durn, G., Kazazic, S., Dekic, S. & Music, M. S. 2019 Untreated wastewater as a source of carbapenem-resistant bacteria to the riverine ecosystem. *Water SA* 45 (1), 55-62.

Kaprelyants, A. S., Gottschal, J. C. & Kell, D. B. 1993 Dormancy in non-sporulating bacteria. *FEMS Microbiology Reviews* 104, 271-286.

Kovacic, A., Seruga Music, M., Dekic, S., Tonkic, M., Novak, A., Rubic, Z., Hrenovic, J. & Goic-Barisic, I. 2017 Transmission and survival of carbapenem-resistant *Acinetobacter baumannii* outside hospital setting. *International Microbiology* 20 (4), 165-169.

Magiorakos, A. P., Srinivasan, A., Carey, R B., Carmeli, Y, Falagas, M. E. & Giske, C. G 2012 Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* 18, 268-281.

Proctor, R A., von Eiff, C, Kahl, B. C, Becker, K, McNamara, P. & Herrmann, M. 2006 Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology* 4, 295-305.

Pu, Y, Ke, Y & Bai, F. 2017 Active efflux in dormant bacterial cells - new insight into antibiotic persistence. *Drug Resistance Updates* 30, 7-14.

Roca, I., Espinal, P., Vila-Farrés, X. & Vila, J. 2012 The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. *Frontiers in Microbiology* 3, 148.

Schreiber, K, Boes, N., Eschbach, M., Jaensch, L., Wehland, J., Bjarnsholt, T, Givskov, M., Hentzer, M. & Schobert, M. 2006 Anaerobic survival of *Pseudomonas aeruginosa* by pyruvate fermentation requires an Usp-type stress protein. *Journal of Bacteriology* 188, 659-668.

Seifert, H., Wisplinghoff, H., Schnabel, P. & von Eiff, C. 2003 Small colony variants of *Staphylococcus aureus* and pacemaker-related infection. *Emerging Infectious Diseases* 9, 1316-1318.

Seruga Music, M., Hrenovic, J., Goic-Barisic, I., Hunjak, B., Skoric, D. & Ivankovic, T. 2017 Emission of extensively-drug resistant *Acinetobacter baumannii* from hospital settings to the natural environment. *Journal of Hospital Infection* 96 (4), 323-327.

Tipton, K. A., Dimitrova, D. & Rather, P. N. 2015 Phase-variable control of multiple phenotypes in *Acinetobacter baumannii* strain AB5075. *Journal of Bacteriology* 197, 2593-2599.

Towner, K. J. 2009 *Acinetobacter:* an old friend, but a new enemy. *Journal of Hospital Infection* 73, 355-363.

Zhang, C, Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J. D. & Song, H. 2013 Higher isolation of NDM-1 producing *Acinetobacter baumannii* from the sewage of the hospitals in Beijing. *PLoS One* 8, e64857.