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## Emerging human pathogen *Acinetobacter baumannii* in the natural aquatic environment: a public health risk?

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### ABSTRACT

Bacterium *Acinetobacter baumannii* is an emerging human pathogen whose presence in the aquatic environment raises the issue of public health risk. Fish colonization represents the potential route of pathogen transmission to humans. The aim was to examine the colonization of *A. baumannii* to freshwater fish *Poecilia reticulata*. An extensively drug-resistant *A. baumannii* was tested at three concentrations in natural spring water. Additionally, 70 fish from the Sava River (Croatia) were screened for the presence of *A. baumannii*, which was not found in gill swabs or analysed gut. The colonization potential of *A. baumannii* in freshwater fish is dependent upon its concentration in surrounding water. The low concentration of *A. baumannii* in natural waters represents low colonization potential of freshwater fish. The risk for public health exists in closed water bodies where there is constant inflow of water polluted by *A. baumannii* in concentrations above 3 log CFU mL<sup>-1</sup>.

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*Acinetobacter baumannii*;  
bacteria; fish; *Poecilia reticulata*; aquatic environment

## Introduction

Most bacteria of the genus *Acinetobacter* are ubiquitous in the environment, as they can be found in waters, soils and on various inanimate surfaces (Peleg et al. 2008). *A. baumannii* has in the last two decades emerged as a significant human pathogen due to its growing antibiotic resistance, resistance to disinfectants and persistence on biotic and abiotic surfaces in adverse environmental conditions (Espinal et al. 2012). *A. baumannii* is considered an opportunistic pathogen associated with nosocomial infections in immunosuppressed patients (Towner 2009). Carbapenem-resistant *A. baumannii* strains have been declared as number one concern by the World Health Organization (2017). Carbapenems are broad-range  $\beta$ -lactam antibiotics used as a last line of defense against multi-drug-resistant bacteria. However, carbapenem resistance is a growing phenomenon. For example, in Croatia carbapenem resistance of *A. baumannii* increased from 10 % in 2008 to 86 % in 2016 (CAMS 2017). Furthermore, community-acquired infections outside hospital environment have been recorded (Dexter et al. 2015). Clinically significant *A. baumannii* have been recovered from hospital wastewaters (Zhang et al. 2013; Kovacic et al. 2017), wastewater treatment plants (Hrenovic et al. 2016) and the natural

aquatic environment in the Seine River in France (Girlich et al. 2010) and the Sava River in Croatia (Seruga Music et al. 2017). The constant dissemination of clinically significant *A. baumannii* isolates from hospital setting into the environment is evident, therefore the potential pathways of colonization and consequently infection should be explored. In the aquatic environment, bacteria interact with various organisms, some of which are fish used for consumption as well as commercial or recreational purposes (Burger 2002). Some species of the genus *Acinetobacter* such as *A. lwoffii*, *A. johnsonii*, *A. calcoaceticus* as well as *A. baumannii* have been reported as opportunistic fish pathogens (Koziońska et al. 2014; Behera et al. 2017). With regard to the appearance of *A. baumannii* in marine and fresh waters, so far there have been only a few cases of established fish colonization. One *A. baumannii* isolate was recovered from diseased fish in fish farms in China (Xia et al. 2008) and two from India (Rauta et al. 2011; Behera et al. 2017). Additionally, two isolates were recovered in wild fish from the Mediterranean Sea in Algeria (Brahmi et al. 2016). Apart from that, there was also one *A. baumannii* isolate recovered from freshwater fish in a local pet shop in Malaysia (Seong We et al. 2008). These reports indicate that *A. baumannii* is able to infect fish. The reports of emerging fish pathogens *A. lwoffii* and *A. johnsonii* in humans and their association with bacteraemia (Turton et al. 2010; Koziońska et al. 2014), suggests that fish could also represent the route for potential transmission of pathogens to humans. By cleaning fish tanks, handling and consumption of colonized or infected fish, humans could be exposed to *A. baumannii*, which could potentially cause human community-acquired infections. The infection potential of *A. baumannii* in fish (Xia et al. 2008; Rauta et al. 2011; Behera et al. 2017) has been examined in closed system experiments, where *A. baumannii* suspension was directly injected into fish and the LD<sub>50</sub> (dose of bacteria that causes 50 % mortality in the observed population) was estimated at colony forming units (CFU) of 10<sup>8</sup> mL<sup>-1</sup>. However, the injection of viable *A. baumannii* into fish is not applicable to estimate *A. baumannii* potential to colonize fish in the marine or freshwater environment.

The aim of this study was to examine the colonization potential of clinically significant *A. baumannii* in the water medium to fish *Poecilia reticulata* and to screen freshwater fish from the Sava River near the City of Zagreb (Croatia) for the presence of *A. baumannii*. Moreover, the goal was to elucidate whether freshwater fish could be the vector for the spread of clinically significant *A. baumannii* in the aquatic environment thus representing a potential risk to public health.

## Materials and methods

### Ex situ experiment

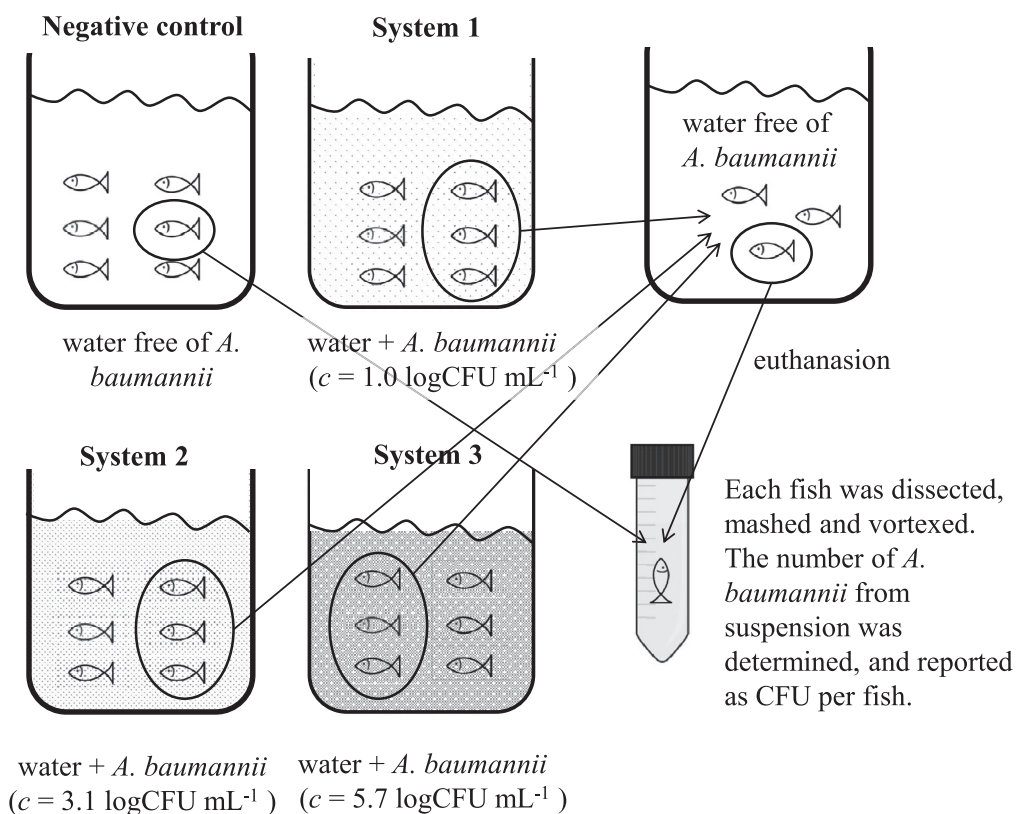
#### *A. baumannii* isolate

*A. baumannii* used in this experiment was recovered in October 2015 from the Sava River downstream of Zagreb, Croatia. This isolate (named Sava 4) was previously described in the study by Seruga Music et al. (2017). It belongs to sequence type 195 inside international clone lineage 2, displays the extensively drug resistance profile due to its susceptibility to only one antibiotic category – polymixins (colistin), and is related to clinical isolates.

#### Experimental set-up

*Ex situ* experiment was conducted using freshwater fish *P. reticulata* (Guppy) which is a small fish species belonging to the family Cyprinidae that is commonly used as a test organism and also in microbiology research (Ryan et al. 2004; Pate et al. 2005).

The set-up of the experiment as a static system is shown in Figure 1. Three different concentrations of *A. baumannii* were prepared in 500 mL of commercial natural spring water (Jana, Croatia) in three glass tanks (system 1–3). The initial concentration of *A. baumannii* in water was set to: 1.0 log CFU mL<sup>-1</sup> in system 1, 3.1 log CFU mL<sup>-1</sup> in system 2, 5.7 log CFU mL<sup>-1</sup> in system 3. The fourth system without added *A. baumannii* into spring water served as a negative control. Six healthy fish of the species *P. reticulata* from laboratory breeding (Table 1) were placed into each system. The



**Figure 1.** Schematic set-up of *ex situ* experiment.

**Table 1.** Measurements of fish caught in the River Sava and fish used in the experiment (\*).

Fish species	Length (cm)	Weight (g)
<i>Alburnoides bipunctatus</i>	8.6 ± 2.8	8.9 ± 6.4
<i>Barbus barbus</i>	10.1 ± 3.7	15.7 ± 14.2
<i>Squalius cephalus</i>	23.4 ± 8.7	231.3 ± 319.7
<i>Poecilia reticulata</i> *	3.7 ± 0.4	0.4 ± 0.2

experiment was conducted with moderate aeration at room temperature (25 °C) for 12 days. Fish were maintained under a 12 h day/12 h night light regime and were fed once every two days with dry feed. During the experiment, signs of infection (loss of mucus, skin lesions or changes in the gill), as well as the survival of *P. reticulata* were regularly monitored.

After the 1st and 12th day of contact, three fish from each system were transferred into fresh commercial natural spring water and left to swim for two hours in order to separate bacteria weakly bound to fish. Subsequently, fish were euthanized by exposure to 0.05 mL L<sup>-1</sup> of MS-222 (tricaine methanesulfonate, Merck, Germany) followed by pithing. Each whole fish was dissected by sterile scissors and transferred into a test tube containing 9 mL of sterilized peptone water. The content was mashed with sterile glass rod, vortexed (5 min/45 Hz, Kartell TK3S, Italy) to obtain homogenous fish suspension (Brahmi et al. 2016) and then left to settle for 1 min.

### **Bacteriological analysis**

The number of *A. baumannii* per one whole fish was determined by filtration of vortexed fish tissue through sterile membrane filters with pore size of 0.45 µm (Sartorius, Germany). Filters were placed on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar, France) and incubated at 42 °C/48 h (Hrenovic et al. 2017). In the water of each tank, number of *A. baumannii* and aerobically grown total heterotrophic bacteria were determined in samples both before and after its dilution in peptone water. Number of total heterotrophic bacteria was determined on Nutrient agar (Biolife, Italy) after the incubation at 22 °C/72 h. The number of *A. baumannii* in both water and fish was determined as CFU, logarithmically transformed and expressed as log CFU mL<sup>-1</sup> of water or log CFU per one whole fish. The number of total heterotrophic bacteria was determined only in water and expressed as log CFU mL<sup>-1</sup> of water. According to the preliminary analysis of used spring water and healthy *P. reticulata* before the experiments were to commence, no *A. baumannii* was detected per 100 mL of water or one whole fish.

### **In situ experiment**

#### **Fish sampling**

Sampled area of the Sava River belongs to the middle rhithron, which is characterized by fish communities consisting mainly of omnivorous chub *Squalius cephalus*, insectivorous spirlin *Alburnoides bipunctatus* and benthivorous barbel *Barbus barbus* (Simonović et al. 2017). The electrofishing generator (Briggs & Stratton, 7.5-kW Vanguard model) was used to provide 600-V direct current. Electrofishing was performed from a rubber boat (3.6 m length), using a 30-cm ring-shaped anode attached to a long fibreglass pole. The cathode was a copper wide positioned under the front half of the boat. All captured fish were immediately transferred to a plastic bucket containing river water, and all fish were processed on shore.

The sampling of fish was conducted in the Sava River at two locations upstream (45°49'11.83"N, 15°49'40.59"E; 45°49'37.22"N, 15°49'24.86"E) and one downstream the City of Zagreb, Croatia (45°44'45.59"N, 16°13'45.51"E) in April 2016 and May 2017.

Gill swabs of all caught fish (70 in total) were taken immediately after the capture of each fish. Eight specimens of *S. cephalus* and eight specimens of *A. bipunctatus* from one location upstream the City of Zagreb were transported in aerated river water to the laboratory within 2 h after collection. Fish were euthanized by exposure to 0.05 mL L<sup>-1</sup> of MS-222 followed by pithing, after which the gut was also analysed.

### **Bacteriological analysis**

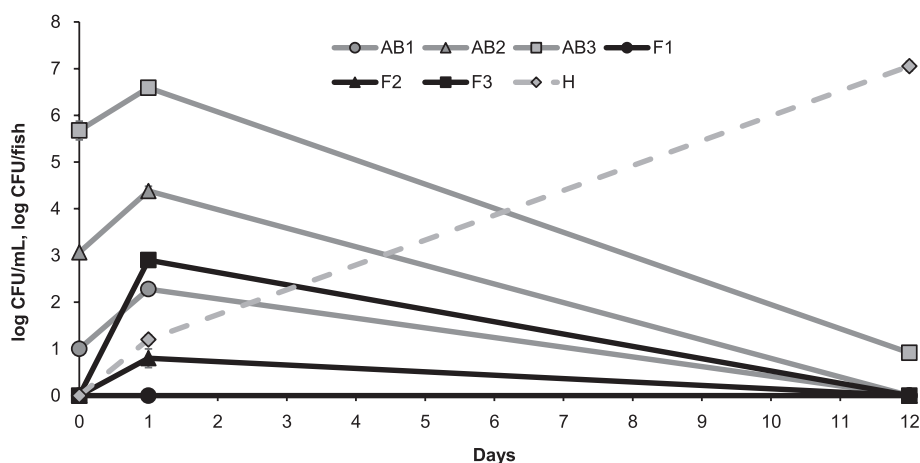
Gill swabs of 70 fish and the gut of 16 fish were analysed for the presence of *A. baumannii*. Gill swabs of caught fish were directly inoculated onto the cultivation plates. Fish gut was dissected, mashed, vortexed in peptone water (Brahmi et al. 2016) and filtered through sterile membrane filters of pore size 0.45 µm. Both gill swabs inoculates and membrane filters were incubated at 42 °C/48 h on CHROMagar Acinetobacter, after which the plates were screened for the presence of *A. baumannii*.

### **Compliance with ethical standards**

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Faculty of Science at which the studies were conducted.

### **Statistical analysis**

Statistical analyses were carried out using Statistica software 13.3 (TIBCO Software Inc.). Absolute numbers of bacteria were logarithmically transformed. The comparisons between variables were done



**Figure 2.** Numbers of *Acinetobacter baumannii* in water and in fish, and numbers of total heterotrophic bacteria in water during 12 days of monitoring. W1–3 – concentration of *A. baumannii* in water (system 1–3); F1–3 – concentration of *A. baumannii* in fish (system 1–3). H – total heterotrophic bacteria in water of all three systems. Mean values of triplicate measurements and standard deviations are presented.

using the ordinary Student's t-test for independent variables. The correlations between variables were estimated using the nonparametric Spearman R test. Statistical decisions were made at a significance level of  $p < 0.05$ .

## Results

### Ex situ experiment

The results of colonization potential of *A. baumannii* to *P. reticulata* are presented in Figure 2. After one day of contact, the initial concentration (1.0, 3.1 and 5.7 log CFU mL<sup>-1</sup>) of *A. baumannii* in water of all three systems increased to 2.3, 4.4 and 6.6 log CFU mL<sup>-1</sup> in system 1, 2 and 3, respectively. No *A. baumannii* was found in fish in the system 1, while 0.8 and 2.9 log CFU of *A. baumannii* per one whole fish were found in system 2 and 3, respectively. There was a statistically significant positive correlation between the concentration of *A. baumannii* in water and in fish in all systems ( $R = 0.89442$ ,  $p = 0.04051$ ).

After 12 days of exposure, the concentration of *A. baumannii* in the water of all three systems was significantly lower in relation to the first day ( $p = 0.00582$ ), with 100 % decrease of *A. baumannii* in systems 1 and 2 and 86 % decrease in system 3 (Figure 2). No *A. baumannii* was recovered from fish after 12 days of exposure in any system.

In the water medium of all three systems, the initial number of total heterotrophic bacteria was -0.3 log CFU mL<sup>-1</sup> (Figure 2). After the first day the number increased to 1.2 log CFU mL<sup>-1</sup>, and it continued to rise significantly ( $p = 0.00008$ ) up to 7.1 log CFU mL<sup>-1</sup> at the end of the experiment in all systems (Figure 2). It appeared that the *A. baumannii* in water was overgrown by other heterotrophic bacteria.

### In situ experiment

A total of 70 fish of three different species were caught in the Sava River. At locations upstream the City of Zagreb 32 specimens of *S. cephalus*, 5 of *B. barbus* and 15 of *A. bipunctatus* were collected in April 2016 and May 2017. Downstream the City of Zagreb 10 specimens of *S. cephalus*, 5 of *B. barbus* and 3 of *A. bipunctatus* were collected in April 2016. Morphometric measurements of fish species are



presented in Table 1. *A. baumannii* was not found in any of the gill swabs (70 fish) or analysed gut (16 fish).

## Discussion

In previous studies, the infection potential of *A. baumannii* to fish was examined by directly injecting the bacterial suspension into fish (Xia et al. 2008; Rauta et al. 2011; Behera et al. 2017). However, these kinds of experiments are not applicable to environmental conditions. In this study, the natural environmental conditions were simulated in closed laboratory system to examine the potential of *A. baumannii* present in water to colonize healthy freshwater fish. The colonization of fish precedes fish infection, and represents a potential route for the spread of this pathogen to humans. The source of sporadic community-acquired *A. baumannii* human infections (Dexter et al. 2015) is not defined up to this date. The contact of humans with colonized or infected fish during cleaning fish tanks, handling or consumption could represent a potential source of human infections.

The pure cultures of *A. baumannii* do not multiply in natural spring water due to the lack of nutrients. However, *A. baumannii* is able to survive for 58 days in natural spring water with only a slight decrease in survival percentage (Hrenovic et al. 2017). In the presented closed system with natural spring water, multiplication of *A. baumannii* occurred during the first day of monitoring. With time, fish metabolize food and excrete metabolic products, which serve as a source of nutrients for *A. baumannii* as well as other heterotrophic bacteria, enabling their numbers to increase. The one-day growth of *A. baumannii* can be explained by the absence of competition with other bacteria that were present in very low numbers in spring water at the beginning of the experiment and the input of nutrients from the fish metabolism. Later in the experiment *A. baumannii* was further overgrown by other heterotrophic bacteria. The competitive overgrowth of *A. baumannii* with *Enterobacter ludwigii* has been previously described in the closed systems containing the suspension of technosol in spring water (Hrenovic et al. 2017).

*A. baumannii* present in water was able to colonize *P. reticulata* after 24 h of contact. However, the heterotrophic bacteria may have affected the colonization potential after 12 days of contact. The colonization potential was dependent on the bacterial concentration in surrounding water. The higher the concentration of *A. baumannii* in water, the greater the potential of colonization. It should be mentioned that neither signs of infection (loss of mucus, skin lesions or changes in the gill) nor mortality of *P. reticulata* was observed during the experiment.

One to four environmental isolates of *A. baumannii* have been recovered from 10 mL of water from both the Seine River downstream Paris (Girlich et al. 2010) and Sava River downstream Zagreb (Seruga Music et al. 2017), which is less than 10 CFU mL<sup>-1</sup> corresponding to *A. baumannii* concentration in system 1 in this study. At this lowest tested concentration, no colonization of *P. reticulata* was observed. Furthermore, *A. baumannii* was not found on gills or in the gut of freshwater fish caught in the Sava River. These data obtained *in situ* and *ex situ* suggest the low colonization potential of *A. baumannii* in healthy freshwater fish. Colonization of fish occurred within one day of contact at *A. baumannii* concentration in water above 3.1 log CFU mL<sup>-1</sup> (10<sup>3</sup> CFU mL<sup>-1</sup>), which is extremely high for natural environment. These data are in agreement with the reported infective dose of 10<sup>8</sup> CFU mL<sup>-1</sup> of *A. baumannii* when injected intraperitoneally into freshwater fish (Behera et al. 2017).

*P. reticulata* were able to repel up to 3.1 log CFU mL<sup>-1</sup> of *A. baumannii* present in surrounding water (Figure 2), which suggests effective antimicrobial defence mechanisms in fish. The excreted mucus and integument are enough in most cases to ensure protection of fish against bacteria present in water. Blood plasma and gastrointestinal tract additionally contain numerous antibacterial factors (Ellis 2001). However, if the skin is damaged bacteria can enter the organism. Since *A. baumannii* is an opportunistic pathogen, the fish have a greater chance of being infected if they are injured or if their immune system is compromised due to the effect of various stress factors such as temperature and salinity changes, parasites, pollution and overcrowding in fish tanks (Bly et al. 1997).

*A. baumannii* is found in the natural environment influenced by human solid or liquid waste (Hrenovic et al. 2016, 2017; Seruga Music et al. 2017). The concentration of *A. baumannii* was not determined in waters where this bacterium was reported as a cause of mortality of farmed fish (Xia et al. 2008; Rauta et al. 2011; Behera et al. 2017). *A. baumannii* could probably be present at increased concentrations in closed water bodies under the influence of human waste, which increases the concentration of bacteria as well as organic matter in the natural environment. In such cases, there is a greater chance for the colonization and consequent infection of freshwater fish. In order to prevent the spread of *A. baumannii* in fish farms, the fish farms should be positioned at a safe distance from the influence of human waste. Furthermore, greater attention should be given to the fish care and cleaning of fishing tools in order to prevent the colonization and consequent infection of fish by *A. baumannii*. Further studies on the colonization potential of *A. baumannii* should be performed on other fish species used for human consumption.

## Conclusion

The colonization of fish in the natural environment by *A. baumannii* is dependent upon bacterial concentration in surrounding water. The low concentration ( $<10$  CFU mL<sup>-1</sup>) of *A. baumannii* in natural waters together with the fish immune system contribute to very low colonization potential of *A. baumannii* in fish, which indicates that freshwater fish from the natural environment are probably poor vectors for the spread of extensively drug-resistant bacteria. However, concentrations of *A. baumannii* above 3 log CFU mL<sup>-1</sup> may result in colonization of freshwater fish, thus representing public health risk.

## Disclosure statement

The authors declare no conflict of interest.

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