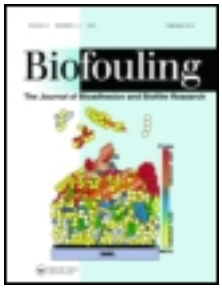

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Resistance of bioparticles formed of phosphate-accumulating bacteria and zeolite to harsh environmental conditions

Tomislav Ivankovic [a], Jasna Hrenovic [a] & Renata Matonickin-Kepcija [b]

[a] Faculty of Science, Division of Biology, Department of Microbiology, University of Zagreb, Zagreb, Croatia

[b] Faculty of Science, Division of Biology, Department of Zoology, University of Zagreb, Zagreb, Croatia

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Resistance of bioparticles formed of phosphate-accumulating bacteria and zeolite to harsh environmental conditions

Tomislav Ivankovic[a]*, Jasna Hrenovic[a] and Renata Matonickin-Kepcija[b]

[a]*Faculty of Science, Division of Biology, Department of Microbiology, University of Zagreb, Zagreb, Croatia;* [b]*Faculty of Science, Division of Biology, Department of Zoology, University of Zagreb, Zagreb, Croatia*

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Extreme environmental conditions, such as pH fluctuations, high concentrations of toxicants or grazing of protozoa, can potentially be found in wastewater treatment systems. This study was carried out to provide specific evidence on how 'bioparticles' can resist these conditions. The term 'bioparticle' is used to describe a particle comprising natural zeolitized tuff with a developed biofilm of the phosphate-accumulating bacterial species, *Acinetobacter junii*, on the surface. The bacteria in the biofilm were protected from the negative influence of extremely low pH, high concentrations of benzalkonium-chloride and grazing by *Paramecium caudatum* and *Euplotes affinis*, even under conditions that caused complete eradication of planktonic bacteria. During an incubation of 24 h, the biofilms were maintained and bacteria detached from the bioparticles, thus bioaugmenting the wastewater. The bioparticles provided a safe environment for the survival of bacteria in harsh environmental conditions and could be used for successful bioaugmentation in wastewater treatment plants.

Keywords: *Acinetobacter junii*; bacteria; biofilm; bioparticle; wastewater treatment; natural zeolite

Introduction

The removal of excessive phosphorus from wastewaters is important to prevent eutrophication of oligotrophic aquatic bodies. The accumulation of phosphate (P) in the cells of P-accumulating bacteria present in activated sludge, a process commonly known as the enhanced biological phosphorus removal (EBPR), has become the preferred means of P removal over the last 40 years (Seviour et al. 2003). Although many full-scale EBPR plants are functional all over the world, there is still no detailed understanding of the microbiology and biochemistry involved. It is feasible that many of these plants are operating sub-optimally, and that improvement in EBPR in full-scale facilities could be achieved through the implementation of knowledge gained from basic research. Bioaugmentation of activated sludge plants with P-accumulating bacteria is one of the possibilities for upgrading existing EBPR wastewater treatment plants (Watanabe et al. 2000; Seviour et al. 2003).

Studies dealing with bioaugmentation often observe that the number of microorganisms introduced decreases shortly after addition to a site. The reasons given for this often include the death of the microorganisms due to abiotic and biotic stresses (Alexander 1999). In a review article on new approaches for bioaugmentation, Gentry et al. (2004) state: 'The abiotic stresses may include fluctuations or extremes in temperature, water content, pH and nutrient availability, along with potentially toxic pollutant levels in contaminated soil. In addition, the added microorganisms almost always face competition from indigenous organisms for limited nutrients, along with antagonistic interactions including antibiotic production by competing organisms, and predation by protozoa and bacteriophages'.

A study dealing with bioaugmentation in a nitrifying sequencing batch reactor showed that protozoan predation was one of the main contributors to the decrease of inoculated denitrifying bacteria and the subsequent failure of bioaugmentation (Bouchez et al. 2000).

Biofilms are widely used in wastewater treatment processes in the form of bacteria immobilized onto suitable carriers. The bed reactors, which are used in every aspect of wastewater purification, exemplify the importance of biofilms (Nicolella et al. 2000). Bacteria immobilized onto carriers offer advantages over suspended planktonic cell systems, such as facilitating cell-liquid separation by sedimentation or filtration, increased number of cells and shorter retention time in the reactors (Nicolella et al. 2000).

P-accumulating bacteria are normally present in small numbers in activated sludge (Seviour et al. 2003). *Acinetobacter junii* has become the model organism for EBPR since it was isolated from an activated sludge with enhanced P removal characteristics (Fuhs & Chen 1975). Since then, several studies have disputed the role of

*Corresponding author. Email: tomislav.ivankovic@biol.pmf.hr

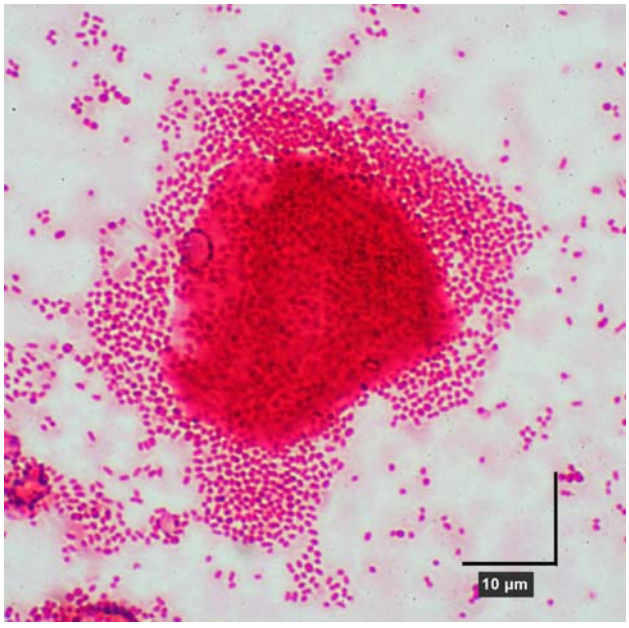

Figure 1. The P-accumulating bacterium *A. junii* immobilized on the particle of natural zeolitized tuff after incubation for 24h in nutrient broth. Scale bar = 10 µm.

Acinetobacter sp. as the main organism responsible for enhanced P removal (Cloete & Steyn 1988; Martin et al. 2006) and other bacteria have been proposed as the main contributors to the EBPR process (Oehmen et al. 2007). *A. junii* was chosen as a model organism in this study for the following reasons: is cultivable under laboratory conditions, Seviour et al. (2003) uphold the importance of *Acinetobacter* sp. in the EBPR process and bioaugmentation of activated sludge with *A. junii* enhances P removal from wastewater (Hrenovic et al. 2010; Ivankovic et al. 2010).

The aim of this study was to test experimental bioparticles with challenges characteristic of wastewater treatment systems, such as fluctuations in pH, high concentrations of toxicants and grazing by protozoa, to determine if bioparticle technology could be an improvement in the bioaugmentation of wastewater treatment systems. In this study, the term bioparticle (Figure 1) describes a particle comprising an aluminosilicate carrier, natural zeolitized tuff rich in clinoptilolite (NZ), with a developed biofilm of the P-accumulating bacterial species, *A. junii*, on the surface.

Materials and methods

Microorganisms

A pure culture of the Gram-negative, heterotrophic, P-accumulating bacterial species, *A. junii*, was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (strain DSM 1532, isolated from activated sludge with EBPR characteristics). Protozoa belonging to the phylum Ciliophora were obtained from activated sludge. Following isolation of a single ciliate with a micropipette, specimens were repeatedly washed with sterile water. *Paramecium caudatum* and *Euplotes affinis* were cultured in filtered effluent water with boiled rice grains. The fresh activated sludge and secondary treatment effluent were obtained from a wastewater treatment plant which purifies the municipal wastewater from the combined sewer system of Zagreb, Croatia. Both species of protozoa were maintained at room temperature (25 °C).

Preparation of the bacterial carrier

NZ bedrock from Bigadic, Turkey was used to prepare the particles to act as the carrier for the bacteria. X-ray powder diffraction estimated that the NZ sample consisted of clinoptilolite (70%), subordinate opal-CT and quartz (10–15% each) and traces of K-feldspar and mica (Hrenovic et al. 2009). The particle size fraction 0.125–0.25 mm was used and prior to the experiments the NZ sample was washed three times with deionized water, dried and sterilized at 105 °C for 16 h.

Simulated wastewater

The base growth medium for the experiments was simulated wastewater of the following composition (in $mg\,l^{-1}$ of distilled water): Na-propionate 300, peptone 100, $MgSO_4$ 10, $CaCl_2$ 6, KCl 30, yeast extract 20 and KH_2PO_4 88. If not otherwise stated, the pH of the medium was set to 7.0 ± 0.2 before autoclaving (121 °C for 20 min).

Preparation of the bioparticles

To seed the particles and establish a biofilm, 1 g of NZ was added to Schott bottles containing 100 ml of nutrient broth (Biolife, Italy). One ml of a suspension of *A. junii* (10^8 CFU ml^{-1}) was subsequently added, and the bottles were sealed and set for aerobic incubation for 6 h at 30 °C in a water bath with agitation (70 rpm). Preliminary experiments (Hrenovic et al. 2009) determined that 6 h was the optimal time for the development of a stable biofilm of *A. junii* on the particles of NZ. After the incubation, bioparticles were washed with sterile 0.3% NaCl solution and introduced to the experiment by simply pouring the wet NZ from one Schott bottle to another under aseptic conditions. The number of *A. junii* immobilized on the bioparticles prepared using this protocol was 2.4×10^9 CFU g^{-1}.

Exposure of bioparticles to realistic environmental conditions

The key measures in the experiments were the number of bacteria and their survival. These measurements were compared between the reactors with planktonic cultures

of *A. junii*, the reactors with bioparticles, and the reactors in which the planktonic culture of *A. junii* and the NZ particles were added separately.

To test the resistance of bioparticles to unfavourable pH, simulated wastewaters with pH values ranging from 2 to 5 were prepared. The pH of wastewater was verified after autoclaving. It differed in the range of ±0.1 pH units from the pH value of wastewater set before autoclaving. One ml of bacterial suspension (10^8 CFU ml^{-1}), 1 g of bioparticles or 1 ml of bacterial suspension and 1 g of NZ particles were added to Schott bottles containing 100 ml of simulated wastewater. The bottles were incubated aerobically (1 l min^{-1} of sterile air) for 24 h at 30 °C in a water bath with agitation (70 rpm).

To test the survival of bioparticles exposed to high levels of toxicant, simulated wastewater with a disinfectant, the cationic surfactant benzalkonium chloride (BAC), was prepared. Concentrations of 0.1, 1.0, 10, 50 and 100 mg l^{-1} BAC were prepared by aseptically adding the disinfectant to Schott bottles with 100 ml of wastewater which had been previously autoclaved. BAC is known to decompose at high temperatures necessitating the later addition. One ml of bacterial suspension, 1 g of bioparticles, or 1 ml of bacterial suspension and 1 g of NZ particles were added to the bottles and incubated aerobically as described above.

To test the behaviour of bioparticles exposed to protozoa, 10 ml of a culture containing ciliates were added to Schott bottles containing 100 ml of simulated wastewater and 1 ml of bacterial suspension, 1 g of bioparticles or 1 ml of bacterial suspension and 1 g of NZ particles. After sealing, the bottles were incubated as described above at a temperature of 25 °C. This temperature was chosen to provide the ideal conditions for the incubation of the protozoa.

To gain an insight into the behaviour of the bioparticles when exposed to extreme environmental conditions, the number of bacteria and bacterial survival were monitored during incubation for 24 h. A set of Schott bottles with 100 ml of simulated wastewater with a pH of 7 or 3 and with 100 mg l^{-1} of BAC were prepared. In each bottle, 1 g of bioparticles was added and the bottles were simultaneously set to aerobic incubation (30 °C, 70 rpm). After the designated incubation time (3, 5, 7, 12 or 24 h) the numbers of planktonic and immobilized cells in the reactors were determined. The adsorption of BAC to NZ and bioparticles with established biofilms was monitored over time in the reactors with 100 mg l^{-1} of BAC. The experiments, described above, were performed in duplicate.

Analytical methods

The planktonic bacteria were quantified as the number of colony forming units (CFU) by serially diluting (10^{-1}–10^{-8}) 1 ml of the supernatant from the bottles and inoculating 0.1 ml on nutrient agar plates (Biolife, Italy). The plates were incubated at 30 °C for 24 h, after which the bacterial colonies were counted and the number of *A. junii* was determined and expressed as CFU ml^{-1}.

The number of bacteria immobilized onto the NZ particles and on the bioparticles was determined by aseptically taking the NZ or bioparticles from the bottle, washing them three times with sterile 0.3% NaCl solution and aseptically placing them in a sterile plastic tube containing 9 ml of sterile NaCl solution. The tube was then shaken vigorously for 3 min on a mechanical shaker (40 Hz). With this procedure (Durham et al. 1994), the immobilized cells were detached from the carrier and remained as planktonic cells in the supernatant. One ml of the supernatant was then serially diluted, inoculated on nutrient agar plates and incubated as described above. The remaining carrier was dried and weighed so the number of immobilized bacteria could be reported as CFU g^{-1}.

In the experiments on protozoan grazing, the number of heterotrophic (planktonic and immobilized) bacteria other than *A. junii* was also determined. *A. junii* and other heterotrophs were differentiated by incubating the inoculated nutrient agar plates under different conditions. At 37 °C for 24 h the *A. junii* grew selectively, with insignificant numbers of other heterotrophs. At 22 °C for 72 h where both types of bacteria grew, the colonies were visually identified and counted as *A. junii* (circular, convex, smooth with entire margins, Garrity et al. 2005) or other heterotrophs. The determinations of CFU were performed in triplicate.

The numbers of *P. caudatum* and *E. affinis* were determined by taking 1 ml of sample from the reactor and counting the number of individuals by direct microscopy (magnification ×50, Jenaval, Carl Zeiss AG, Germany) at the start of the experiment and after incubation for 24 h. Five counts were performed for each experiment, and the number of protozoa was expressed as units ml^{-1}.

The survival of bacteria was determined by comparing the total number of bacteria (immobilized plus planktonic) in the bottles at the start of the experiment and after incubation for 24 h, and was expressed as a percentage. The number of CFUs were logarithmically transformed to normalize distribution and to equalize variances of the measured parameters (Pan et al. 2006). Statistical analyses were performed with transformed data using Statistica Software 9.1 (StatSoft, Tulsa, USA). The samples were compared using one-way analysis of variance with *post hoc* Duncan's new multiple range test. Statistical decisions were made at a significance level of $p < 0.05$.

Concentrations of BAC were measured spectrophotometrically using HACH method 8337 at the start and the measurements were made after 1, 3, 5, 7 and 24h by

aseptically taking the required aliquots. The samples were prefiltered through Sartorius nitrocellulose filters (0.2 µm pore size).

Results and discussion

Bioparticles at unfavourable pH

To determine the optimal pH for the growth of *A. junii*, planktonic cultures were incubated for 24 h in wastewater with pH values ranging from 2 to 10. The optimal pH for the growth of *A. junii* was 7 as indicated by significantly ($p < 0.05$) higher numbers of bacteria when compared to other pH values (Figure 2). At pH values from 6 to 10, the bacteria actively multiplied during the 24 h of incubation. At pH 5, there was a prominent decay of the bacterial population, so a pH value of 6 was set as a borderline for normal growth of *A. junii* in simulated wastewater. At pH 2–4 there were no viable cells in the reactors after incubation for 24 h (Figure 2).

When the bioparticles were added to a simulated wastewater of pH 7, the number of immobilized bacteria stayed constant during the 24 h of incubation (Figure 3). After incubation for 3 h, planktonic bacteria were observed in the wastewater and this was accompanied by a slight decrease in the number of immobilized bacteria. Thus, detachment of bacteria immobilized on the bioparticles resulted in bioaugmentation of the wastewater.

When the bioparticles were added to a simulated wastewater of pH 3, the number of immobilized bacteria decreased during the first 7 h of incubation (Figure 4). The detection of planktonic bacteria after 3 and 5 h of incubation suggested that the bacteria were detaching from the biofilm and subsequently dying in the wastewater at this unfavourable pH. As the number of immobilized bacteria increased significantly ($p < 0.05$) from 7 to 24 h of incubation (Figure 4), it can be hypothesized that the biofilm regained its composition and formed a protective layer, probably from bacterial extracellular matrix and dead cells, which allowed the bacteria in deeper layers of the biofilm to survive and multiply actively.

The bacterial population immobilized in the form of bioparticles displayed resistance to the negative influence of low pH when compared to the planktonic population of *A. junii*. There were no viable planktonic cells in bottles with a wastewater of pH 4, whereas in the bottles with bioparticles the survival of *A. junii* after incubation for 24 h was 87% (Figure 5). In the bottles with bioparticles, the survival of *A. junii* was 50% even at pH 3, and the total decay of the bacterial population was noted only at pH 2 (Figure 5). The addition of NZ particles alone did not favour bacterial survival, the final pH of

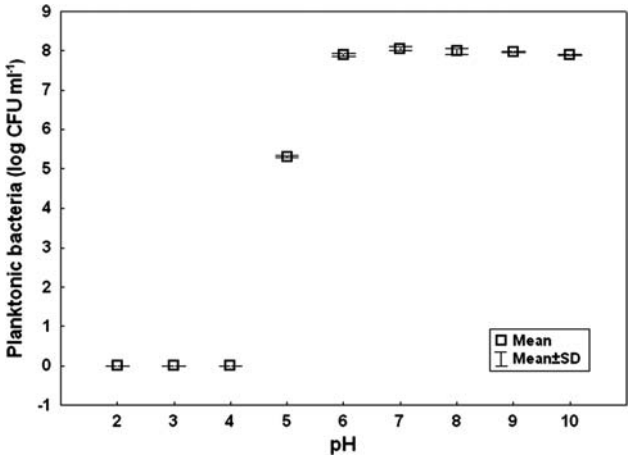

Figure 2. The density of planktonic *A. junii* after incubation for 24 h in the reactors containing wastewater of various initial pH values. The number of bacteria at zero hours (t_{0h}) = 2.91 $\pm 0.35 \times 10^7$ CFU ml^{-1}.

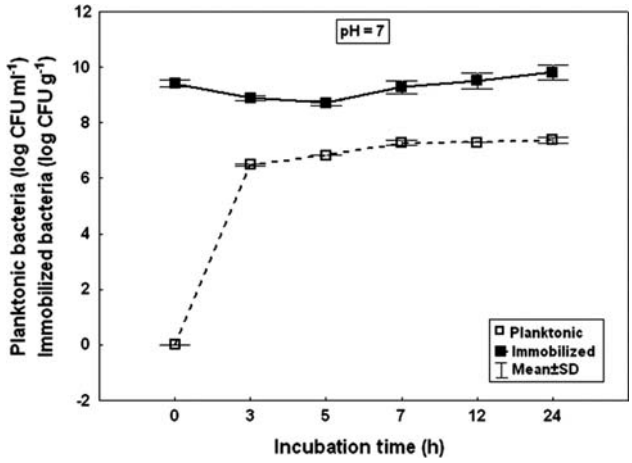

Figure 3. The density of planktonic and immobilized bacteria in the reactors with wastewater of initial pH 7, monitored for 24 h after the addition of bioparticles.

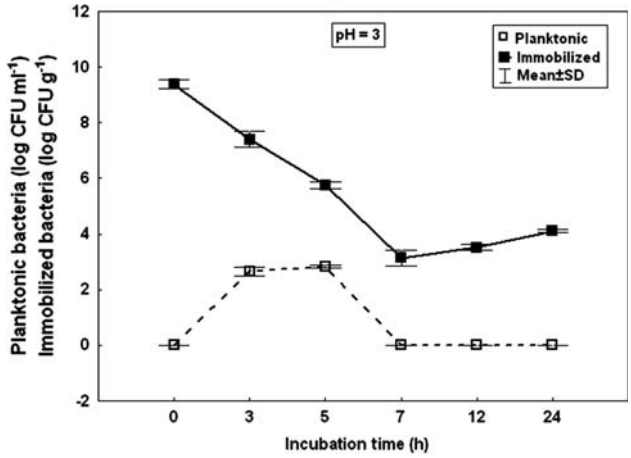

Figure 4. The density of planktonic and immobilized bacteria in the reactors with wastewater of initial pH 3, monitored for 24 h after the addition of bioparticles.

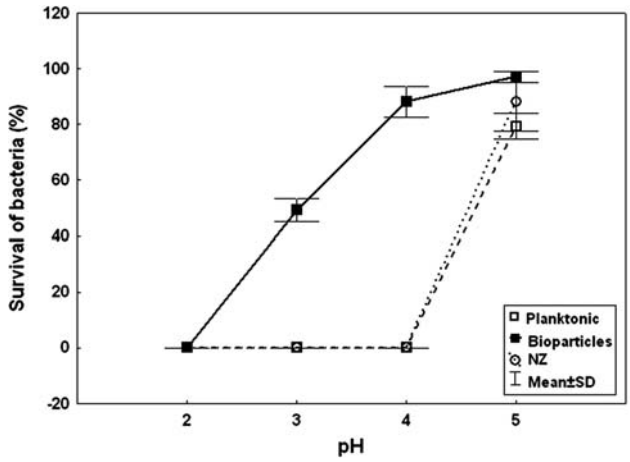

Figure 5. Survival of *A. junii* after incubation for 24 h in wastewater with low pH, incubated as planktonic culture, in the form of bioparticles, and as planktonic culture and NZ particles added separately.

wastewater remained low, with results suggesting that the bacteria were dying before they were able to attach to the NZ particles and form biofilms. Results from this experiment showed that bacteria in the form of bioparticles were protected from low pH due to the preformed biofilm and not due to the presence of the NZ particles themselves.

The planktonic culture of *A. junii* was not resistant to low pH which is consistent with the reported pH range for the growth of *Acinetobacter* sp. from 6 to 8 (Garrity et al. 2005). However, when present as a biofilm on bioparticles, cells of *A. junii* were protected from unfavourable pH. The phenomenon of biofilms being protected from acidic conditions has been described previously in the literature; *Listeria monocytogenes* demonstrated increased resistance to acetic acid when attached to stainless steel (Kuhnert 1999). Experiments on the acid adaption of *Streptococcus mutans* showed that the cell density and the growth of the biofilm modulated acid adaptation, and suggested that the optimal development of acid adaptation involves both low pH induction and cell–cell communication (Li et al. 2001). Analogous results were obtained in a study by Zhu et al. (2001) where cells of *S. mutans* exposed to acid shock survived in far greater numbers when grown in biofilms than in suspension. An additional observation from this study was that the bacteria in biofilms were protected when acid shock was introduced exogenously *via* the growth medium but were not protected in the biofilm when acid shock was induced endogenously *via* sucrose consumption. Results from the above studies indicated that the cells in a biofilm possess a number of novel proteins, of unknown function, that are not produced by planktonic cells (Cotter & Hill 2003). Along with cell–cell interactions, the cells in a biofilm appear to be physically protected from exogenous sources of protons because viable cells are frequently observed in the deepest layers of the biofilm (Cotter & Hill 2003).

Bioparticles exposed to high levels of disinfectant

The cationic surfactant BAC was toxic to *A. junii* when incubated as a planktonic culture (Figure 6). After incubation for 24 h the EC_{50} value obtained was 8.08 mg l^{-1}; toxic chemicals are defined by an EC_{50} value between 1 and 10 mg l^{-1} (Ivankovic et al. 2009). Complete die-off of bacterial population was observed at 50 mg BAC l^{-1} (Figure 6). The toxicities of the other cationic surfactants, dodecylpyridinium-chloride, cetylpyridinium-chloride and hexadecyltrimetyl ammonium-bromide, to *A. junii* were (EC_{50}): 0.38, 0.17 and 0.12 mg l^{-1}, respectively (Hrenovic & Ivankovic 2007; Hrenovic, Ivankovic, et al. 2008). The toxicity of BAC to the Gram-negative bacterium, *Pseudomonas putida*, was 6.0 mg l^{-1} (EC_{50}) or 14.1 mg l^{-1} (EC_{90}) (Sütterlin et al. 2007), which is comparable to the results given in this study. When the bioparticles were added to the reactors, the same BAC concentrations were not toxic to the bacteria; the EC_{50} value was not reached at BAC concentrations of 100 mg l^{-1} (non-toxic chemicals are classified as those with an EC_{50} value > 100 mg l^{-1}). At 50 mg l^{-1} of BAC, the survival of *A. junii* added in the form of bioparticles was 97% (Figure 6). Even at 100 mg BAC l^{-1} the survival of *A. junii* in the form of bioparticles was 88%.

In the reactors containing 100 mg l^{-1} of BAC, the number of bacteria immobilized on the bioparticles decreased slightly during the 24 h of incubation, resulting in a 4% reduction from the starting number (Figure 7). The bioparticles could still be classified as stable,

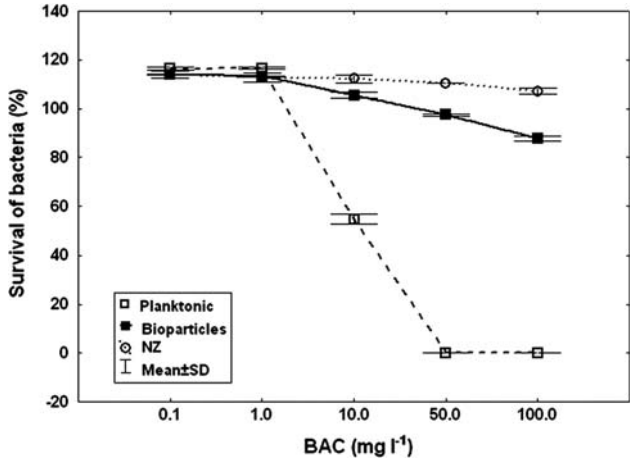

Figure 6. Survival of *A. junii* after incubation for 24 h in wastewater with different concentrations of BAC, incubated as planktonic culture, in the form of bioparticles, and as planktonic culture and NZ particles added separately.

because the biofilm was maintained over 24 h of exposure to high levels of disinfectant (Figure 7). After incubation for 3 h planktonic bacteria, which had detached from the biofilm were observed, and interestingly the planktonic population survived for up to 24 h (Figure 7). Also, when the NZ particles and planktonic *A. junii* were added separately to the wastewater containing BAC, the survival of bacteria was noticeable, with the bacteria multiplying even at 100 mg BAC l^{-1} (Figure 6).

It is clear that the addition of bioparticles or NZ particles to the reactors with BAC changed the conditions to favour the growth of the bacteria. Based on data in the literature, it was concluded that the BAC adsorbed onto the surface of the NZ particles thus diminishing the toxic effect towards *A. junii*. The effect of cationic surfactant sorption on NZ was shown for hexadecyltrimethylammonium bromide (Hrenovic, Rozic, et al. 2008) and BAC (Jevtić et al. 2012). The experiments where sorption of BAC was monitored over 24 h in the reactors where NZ particles and *A. junii* were added separately, as well as in the reactors where bioparticles with preformed biofilm were added, are in agreement with this theory (Figure 8). After incubation for 1 h, 68 and 58% of the starting concentration of BAC was sorbed in reactors with bioparticles and in reactors with NZ particles and planktonic *A. junii* added separately, respectively. The sorption of BAC continued over the entire duration of the experiments, with the final concentrations of BAC after 24 h being 22.2 mg l^{-1} (bioparticles) and 7.0 mg l^{-1} (NZ particles with planktonic *A. junii*). The BAC sorbed more readily to the particles of NZ without biofilm. This resulted in a lower concentration of BAC remaining in the wastewater when reactors with NZ particles and bioparticles were compared, and led to a higher percentage

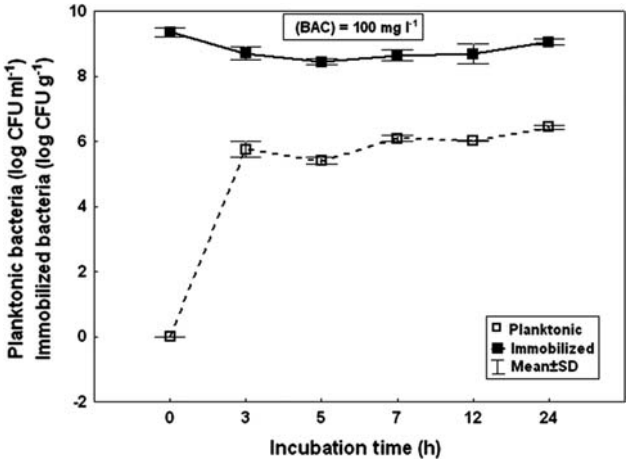

Figure 7. The density of planktonic and immobilized cells in the reactors with wastewater and 100 mg l^{-1} of BAC, monitored for 24 h after the addition of bioparticles.

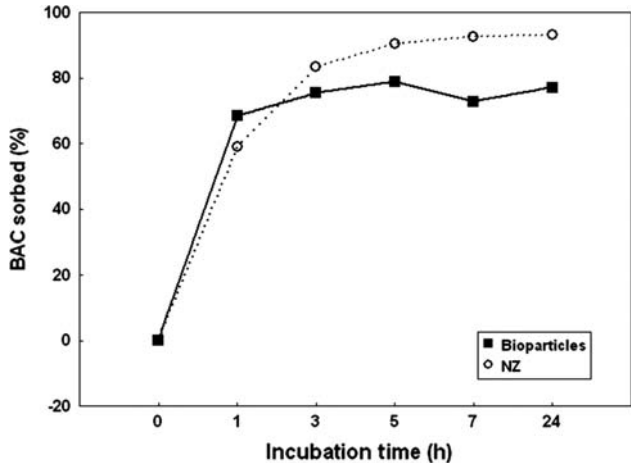

Figure 8. Adsorption of BAC during incubation for 24 h in the reactors with wastewater containing BAC and *A. junii* added in the form of bioparticles, or as planktonic culture and NZ particles added separately. In the control reactor, with planktonic culture of *A. junii* only, the sorption of BAC after 24 h was 0%. The starting concentration (c_{0h}) of BAC = 98.85 mg l^{-1}.

of bacterial surviving. This experiment also demonstrated the protective role of the biofilm for the *A. junii* cells immobilized in the form of bioparticles. After incubation for 24 h, the concentration of BAC in the reactors with bioparticles was considerably above the determined EC_{50}. Despite this, the biofilm remained stable and maintained high numbers of immobilized bacteria. In addition, bacteria were detaching from the biofilm and bioaugmenting the wastewater.

The resistance of bacteria housed in biofilms to antimicrobials is well documented in the literature. A study published by Ceri et al. (1999) demonstrated that for biofilms of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, 100–1000 times the concentration of certain antibiotics were required for efficacy, as compared to the bacteria grown as planktonic populations. Stewart and Costerton (2001) state that, *in vivo*, antibiotics might kill planktonic bacteria shed from the attached population, but fail to eradicate the bacterial cells embedded in the biofilm. Thus the biofilm can act as a nidus, or reservoir, for the recurrence of infection. The antibacterial and antifungal agent, triclosan, reduced the growth of *Salmonella enterica* grown planktonically but cells in a biofilm were tolerant (Tabak et al. 2007). Interestingly, the cells from the biofilm when grown as planktonic cultures showed similar sensitivity to triclosan as the original planktonic culture. The authors concluded that the tolerance of the bacteria in the biofilms to triclosan was attributable to low diffusion of the agent through the extracellular matrix, while changes of gene expression might provide further resistance to triclosan and to other antimicrobials (Tabak et al. 2007). It can be

reasoned that similar mechanisms of resistance were displayed in this study, where cells of *A. junii* were housed within biofilms on the particles of NZ.

Interactions between the bioparticles and the protozoa

The bioparticles were added to reactors with mixed cultures of the ciliates, *P. caudatum* and *E. affinis*, to investigate the survival of *A. junii* under grazing pressure from the protozoa. In wastewater treatment plants with activated sludge processes, ciliate protozoa are considered to play a vital role in reducing the numbers of freely suspended and loosely attached bacterial cells in the bulk liquid *via* grazing. This improves the clarity of the final effluent leaving the treatment plant (Warren et al. 2010). Protozoa have also been reported to control the biofilms of drinking water plants by grazing (Pedersen 1990). Some features of protozoan–bacterial interactions have been well documented, that is, there is considerable evidence to suggest that grazing is not a random process (Simek et al. 1997), and that each ciliate species selects particles as food on the basis on their size, depending on the specific morphological features of the mouthparts of each protozoan species (Fenchel 1986).

As cultures of protozoa were obtained from activated sludge and cultivated in fresh effluent, a certain amount of heterotrophic bacteria were also introduced into the experiment along with the protozoa. This way we could monitor the behaviour of bioparticles in conditions very similar to real wastewater treatment plants where competition between *A. junii* and other heterotrophic bacteria would be present.

The protozoa grazed on *A. junii* cells when cultivated as a planktonic culture. The starting number of *A. junii* cells was reduced tenfold after incubation for 24 h (Table 1). In the reactors with bioparticles, the number of *A. junii* after 24 h was tenfold higher than the starting number, indicating that the bacteria were multiplying actively. It can be assumed that cells of *A. junii* were detaching from the surface of the biofilm on the NZ carrier and bioaugmenting the wastewater, resulting in high numbers of planktonic bacteria (Table 1). When NZ particles and planktonic cultures of *A. junii* were added separately, the grazing pressure from the protozoa was not substantial enough to prevent the bacteria from multiplying actively and forming biofilms on the NZ particles (Table 1). The protection of immobilized *A. junii* from ciliate grazing can be compared to a study by Zubkov and Sleigh (1999), where grazing of bacteria from marine biofilms amounted to 25% of the biofilm on polymeric filters and up to 7% of the biofilm on mineral filters.

The results of this study indicated that protozoa were preferentially feeding on planktonic *A. junii* over planktonic native heterotrophic bacteria as noted from the significant ($p < 0.05$) increase in the native bacterial population after incubation for 24 h (Table 1). Although the incubation temperature of 25 °C was slightly lower than the ideal temperature for growth of *A. junii* (30 °C), this could not have been the only reason for such a significant reduction of *A. junii* when compared to native bacteria, because *A. junii* shows good growth in the temperature range 15–37 °C (Garrity et al. 2005). The reverse trend was observed in the reactors with bioparticles and NZ particles; the total number of *A. junii* after incubation was significantly ($p < 0.05$) higher than the number of heterotrophs (Table 1), indicating that *A. junii* cells in the form of bioparticles were not only protected from the grazing of protozoa but obtained competitive advantages over the native bacteria.

Table 1. Survival and location of bacteria and protozoa in experimental reactors.

Parameter		Planktonic	Bioparticles	NZ
t_0 Total bacteria (CFUs ml^{-1})	*A. junii*	$5.9 \pm 0.3 \times 10^6$	$2.4 \pm 0.9 \times 10^7$	$1.8 \pm 0.2 \times 10^6$
	Heterotrophs		$2.2 \pm 1.4 \times 10^4$	
t_0 Protozoa (units ml^{-1})	*P. caudatum*		24 ± 5	
	E. affinis		10 ± 3	
t_{24} Immobilized bacteria (CFUs g^{-1})	*A. junii*	–	$3.8 \pm 0.3 \times 10^{10}$	$1.2 \pm 0.1 \times 10^{9}$ [B]
	Heterotrophs	–	$5.9 \pm 1.6 \times 10^{10}$	$1.4 \pm 1.6 \times 10^{8}$ [B,D]
t_{24} Planktonic bacteria (CFUs ml^{-1})	*A. junii*	$7.0 \pm 2.0 \times 10^5$	$1.5 \pm 0.2 \times 10^{8}$ [A]	$8.4 \pm 0.7 \times 10^{7}$ [A,B]
	Heterotrophs	$2.4 \pm 0.1 \times 10^{7}$ [D]	$1.3 \pm 0.1 \times 10^{7}$ [A,D]	$2.8 \pm 0.3 \times 10^{7}$ [A,B,D]
t_{24} Total bacteria (CFUs ml^{-1})	*A. junii*	$7.0 \pm 2.0 \times 10^{5}$ [C]	$5.3 \pm 0.3 \times 10^{8}$ [A,C]	$9.5 \pm 0.3 \times 10^{7}$ [A,B,C]
	Heterotrophs	$2.4 \pm 0.1 \times 10^{7}$ [C,D]	$2.3 \pm 1.3 \times 10^{8}$ [A,C,D]	$2.9 \pm 0.1 \times 10^{7}$ [B,C,D]
t_{24} Protozoa (units ml^{-1})	*P. caudatum*	50 ± 19[C]	34 ± 8	42 ± 13
	E. affinis	35 ± 17[C]	51 ± 20[C]	43 ± 20[C]
Final pH		8.23	8.28	7.93

Note: CFUs ml^{-1} of bacteria, units ml^{-1} of protozoan cells at the start (t_0) and after incubation for 24 h (t_{24}). Columns denote whether bacteria were added to the reactors as a suspension (planktonic), in the form of bioparticles (bioparticles) or as a planktonic culture with NZ particles added separately (NZ). [A] = significantly different when compared to reactors with planktonic culture; [B] = significantly different when compared to reactors with bioparticles; [C] = significantly different when compared to start (t_0); [D] = significantly different when compared to *A. junii*.

There were high numbers of heterotrophs immobilized on the bioparticles and NZ particles after incubation for 24 h (Table 1). The number of immobilized native bacteria was significantly ($p < 0.05$) higher in the reactors with bioparticles suggesting that the pre-established biofilm was an attractive environment where the native bacteria attached readily and continued their growth as constitutive members. The presence of native bacteria in a biofilm does not affect the ability of *A. junii* to remove P from wastewater; this was demonstrated in experiments where bioparticles, with a biofilm composed of multiple species, were tested in simulated wastewater and in raw municipal wastewater (Hrenovic et al. 2011).

The protozoa were active during the 24 h of the experiment. The numbers of protozoa after incubation for 24 h were the same or significantly ($p < 0.05$) higher than at the start of the experiment (Table 1). A significant difference ($p < 0.05$) in the rate of multiplication was observed between the two species; the numbers of *P. caudatum* were only significantly higher than at the start in the reactors with planktonic cultures of *A. junii*, while the numbers of *E. affinis* were significantly higher in all of the reactors (Table 1). Results suggest that *E. affinis* was much more successful than *P. caudatum* in grazing on the immobilized bacteria, probably due to the different species-specific feeding modes. Although both species are filter-feeding ciliates, the filtration principles are different. *Euplotes* sp. creates water currents within the membranelle zone which also serves as a filter, resulting in an optimal size of food particle >3 μm (Fenchel 1980a). *Paramecium* sp. also uses membranelles for the generation of water currents, but the filter is an undulating membrane. This filtering mechanism enables *P. caudatum* to retain smaller particles, with an optimal food particle size of ~1 μm (Fenchel 1980a, 1987). Filter feeding ciliates optimized for larger prey sizes have the ability to concentrate food from dilute suspensions (Fenchel 1980b), while species specializing in smaller prey require high food concentrations for population growth (Fenchel 1980a). This implies that *P. caudatum* had the capacity to successfully multiply in reactors with a planktonic population of *A. junii*, whilst *E. affinis* successfully multiplied in reactors with bioparticles and NZ particles as well, where initial concentrations of planktonic bacteria were lower. In addition, *E. affinis* tends to temporarily attach to solid objects during feeding in order to create stronger feeding currents (Fenchel 1987). Lawrence and Snyder (1998) also showed strong feeding responses and activity of *Euplotes* sp. on biofilms. In the current experiments, the 'eager' adherence of *E. affinis* to bioparticles and NZ was observed using light microscopy (Movie S1, Supplementary information). [Supplementary material is available *via* a multimedia link on the online article webpage.] However, these experiments did not provide quantified experimental data which would show a significant impact of the adherence of the protozoa on the bacterial populations.

Conclusions

The bioparticles proved to be resistant to extremes of pH, disinfectant (BAC) and the grazing of protozoa. The biofilm of *A. junii* immobilized on the surface of NZ particles was maintained during incubation for 24 h despite unfavourable growth conditions. During the 24 h of the experiment, bacteria were detaching from the surface of the bioparticles and bioaugmenting the wastewater. The results showed that bioparticles formed from *A. junii* immobilized onto NZ particles provided a safe environment for the survival of the bacteria and that bioparticles show great potential for withstanding the harsh environmental conditions that often cause failure of bioaugmentation.

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