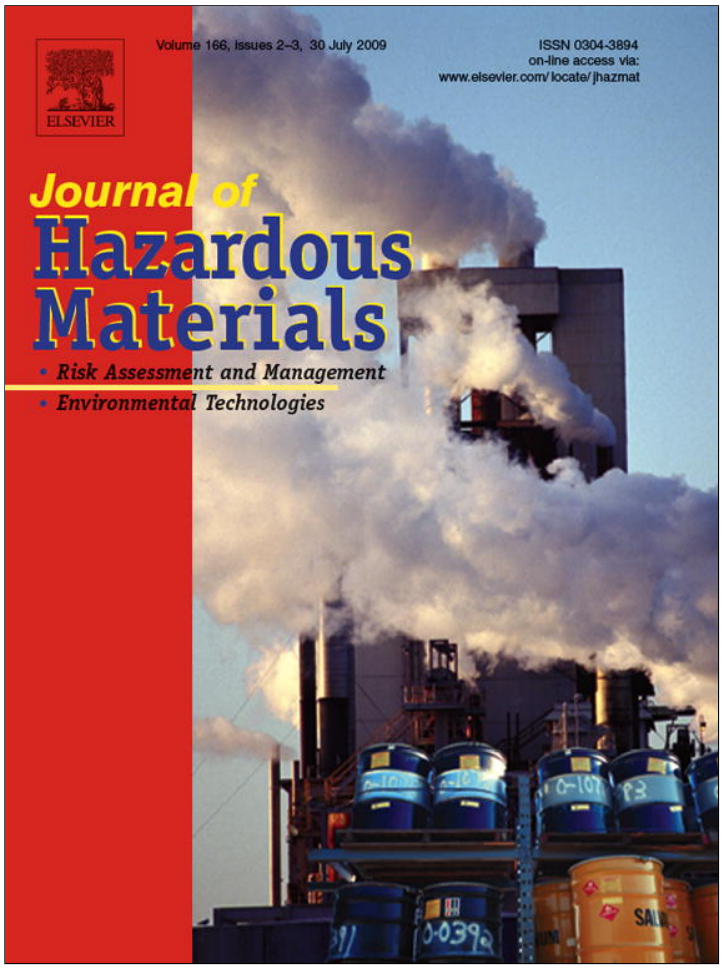

Journal of Hazardous Materials 166 (2009) 1377–1382

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

The effect of mineral carrier composition on phosphate-accumulating bacteria immobilization

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ARTICLE INFO

Article history:
Received 10 September 2008
Received in revised form 11 December 2008
Accepted 11 December 2008
Available online 24 December 2008

Keywords:
Bentonite
Clinoptilolite
Immobilization
Phosphate-accumulating bacterium
Wastewater
Zeta potential

ABSTRACT

The goal of this study was to determine the dynamics and yield of immobilization of the phosphate-accumulating bacterium *Acinetobacter junii* on mineral carriers. As mineral carriers natural clinoptilolite tuff from Turkey (T) and Serbia (S) and natural bentonite (TER), in original and magnesium (Mg)-exchanged form were used. The key feature which determined the extent of immobilization of *A. junii* was the type of carrier; the immobilization yield decreased in order T > TER > S. The number of immobilized cells was significantly higher for the Mg-exchanged carriers when compared to their original counterparts (95 and 75×10^8 CFU g^{-1} for T, 74 and 58×10^8 CFU g^{-1} for TER, 19 and 6×10^8 CFU g^{-1} for S). The Mg-exchanged T and S displayed a prolonged biofilm growth up to 24 h, while the original counterparts reached the mature biofilm after 12 h of incubation. Both forms of TER reached the mature biofilm after 24 h of incubation, due to swelling property of the material. The number of immobilized cells correlated significantly negatively with particle size of the carrier, indicating that particle size is another important feature which determined the extent of immobilization. The Mg-exchange of original carriers resulted in significant increase of the zeta potential. When all of the materials were compared, the increase of the zeta potential of carriers correlated negatively with the number of immobilized cells, suggesting that the zeta potential of material is not a crucial factor which determined the immobilization of cells.

1. Introduction

Eutrofication of aquatic environments caused by excessive release of phosphorus (P) through wastewaters is a worldwide pollution problem. Enhanced biological phosphorus removal (EBPR) has become the preferred process over the chemical P removal from wastewaters. Bacteria from the genus *Acinetobacter* have become the model organism for EBPR [1], although according to recent metagenomic analysis [2] they are present as a minor component of activated sludge and P removal is carried out primarily by uncultivable species, such as *Accumulibacter phosphatis*. However, the capacity of *Acinetobacter* spp. to remove P was the highest among all the P-accumulating isolates from an activated sludge plant [3].

Currently, attention is being drawn to the immobilization of the desired bacteria on various materials as carriers in order to achieve a higher cell density and activity in bioreactors. The P-accumulating bacteria immobilized onto naturally occurring materials showed a good incorporation in the activated sludge biomass [4]. The bacteria immobilized onto carriers can be removed from the EBPR system and disposed together with activated sludge, giving a promising alternative for improving the EBPR process.

In several studies natural zeolite tuff (NZ) [4–7] and clay [8,9] proved to be promising carriers of bacteria. It is not known whether the primary factors controlling the degree of bacterial adsorption are related to the capacity of the solid surfaces to support growth, purely to physicochemical interactions, or to some combination of these factors [10]. One of the factors influencing the bacterial adsorption is the surface charge, described by the zeta potential, of both the carrier and the bacteria [11].

The goal of this study was to determine whether the immobilization of bacteria is dependent on the type, particle size and zeta potential of the carrier. By monitoring the bacterial colonization through time elapsed, we wanted to get an insight into the immobilization process itself.

2. Material and methods

2.1. Bacterium

The culture of P-accumulating bacterium *A. junii* strain DSM 1532 was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH [9].

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doi:10.1016/j.jhazmat.2008.12.064

2.2. Simulative wastewater

A chemically defined water solution was used to simulate the real wastewater. The composition was as follows (in mg L^{-1} of distilled water): Na-propionate 300; peptone 100; $MgSO_4$ 10; $CaCl_2$ 6; KCl 30; yeast extract 20; KH_2PO_4 88. The pH value was adjusted to 7.00 ± 0.04 with 1 M NaOH or 1 M HCl before autoclaving (121 °C/15 min). The pH was measured with WTW 330 pH-meter.

2.3. Carriers

Three types of natural carriers were used in this study. The NZ from Bigadic, Turkey (T) contains approximately 70% of clinoptilolite, subordinate opal-CT and quartz (10–15% of each) and traces of K-feldspar and mica [12], as estimated by X-ray powder diffraction method by comparison with samples in which clinoptilolite content was determined by internal standard method.

The NZ from Igros, Serbia (S) consists of approximately 75% clinoptilolite; several minor constituents (approximately 5% of each) are calcite, analcime (another zeolite group mineral), plagioclase feldspars, mica (biotite and/or celadonite) and quartz.

Terrana® (TER), an active clay mineral, is a commercial material obtained from Süd-Chemie AG and it consists mostly of Ca-bentonite.

The particle sizes of the carriers were: < 0.125 mm (T, S and TER), 0.125–0.25 mm (T2), 0.25–0.5 mm (T3) and 0.5–1.0 mm (T4). Before the start of the experiment carriers were washed three times with approximately 100 mL of demineralized water and dried at 105 °C (T and S) or 60 °C (TER) for 16 h.

The original carriers were subjected to magnesium (Mg)-exchange process, according to a previously published procedure [13]. In short, 10 g of material was treated with 250 mL of 1 M $MgCl_2$ solution. Erlenmeyer flasks were incubated at 30 ± 0.1 °C and mechanically shaken at 200 rpm for 48 h. The materials were then washed with demineralized water until the negative chloride ion test was obtained. The exchanged carriers were also dried at 105 °C or 60 °C for 16 h before the start of the experiment.

2.4. Zeta potential and P-adsorption capacity of the carriers

For measurements of the zeta potential 0.01 g of the material was dispersed in 50 mL of demineralized water. The samples were allowed to stand for 5 min to let the larger particles settle. An aliquot was taken from the supernatant and the potentials were measured using the Zetasizer 3000 – Malvern Instruments, which automatically calculates the electrophoretic mobility of the particles and converts it to the zeta potential using the Smoluchowski equation. The zeta potential of *A. junii* was measured in 10 mM KH_2PO_4 solution as described previously [14], using the same instrument.

The P-adsorption capacity of each carrier was determined by equilibrating a material within a range (0, 5, 50, 100, 500 mg L^{-1}) of P solutions made from KH_2PO_4 [9]. Set of Erlenmeyer flasks containing 1.0 g of the carrier and 100 mL of P solution were incubated at 30 ± 0.1 °C and mechanically shaken at 200 rpm (to assure complete agitation) during 4 days. Two drops of chloroform were added in each flask to prevent microbial growth. Every 24 h a volume of 10 mL of supernatant was filtered through Sartorius nitrocellulose filters (0.2 μm pore diameter) and P concentration was measured spectrophotometrically using the molybdovanadate method (Hach method 8114). After 72 h the P concentration in the supernatant became constant and this was taken as equilibrium time. The deficit of P concentration in the solution was considered to be adsorbed by the carrier. The obtained P-adsorption capacity of carriers was taken as maximum possible to occur in the experiments.

2.5. Experimental procedure

The bacteria were pre-grown on a nutrient agar (Biolife, Italy) for 16 h at 30 ± 0.1 °C. The biomass was then resuspended in 9 mL of sterile 0.3% NaCl solution; the concentration of the bacteria was approximately 10^8 of viable bacterial cells per mililiter. A 1 mL of resuspended biomass was inoculated into Erlenmeyer flasks containing 100 mL of simulative wastewater. In each flask 1.0 g of carrier was added. The flasks were sealed with a sterile gum cap and aerobically incubated in a water bath with shaker at 30 ± 0.1 °C/70 rpm. An aeration rate of 1 L min^{-1} with filtered air was provided. The flasks were incubated for a determined period of time, namely 3, 5, 6, 7, 9, 12 and 24 h. After the elapsed time, the necessary measurements were undertaken.

2.6. Number of bacteria

The number of viable bacterial cells was determined as colony-forming units (CFU). A 1 mL of supernatant was serially diluted (10^{-1} to 10^{-9}) and volumes of 0.1 mL were aseptically inoculated onto nutrient agar (spread-plate method). After incubation (30 ± 0.1 °C/24 h), the bacterial colonies were counted and the number of cells was reported as planktonic CFU mL^{-1}. In order to determine the number of immobilized cells, each carrier was taken from the flask, washed three times with sterile saline solution, and aseptically placed into a tube containing 9 mL of 0.3% NaCl. The sample was crushed with a sterile glass rod and vigorously shaken on a mechanical shaker (40 Hz/3 min). This procedure [12,15] detaches immobilized cells from the carrier, so that they remain as individual cells in the suspension. From such suspension serial dilutions were made, nutrient agar plates were inoculated and incubated as already described. The colonies were counted and the remaining carrier samples were dried and weighed. The number of cells was reported as immobilized CFUs per gram of the dry carrier. All measurements were done in triplicate.

A direct microscopy (Axiovert 200 MAT; Carl Zeiss MicroImaging, Inc.) was performed to confirm the immobilization of cells onto the carriers. Neisser staining was performed to confirm the presence of poly-P granules in the cells.

2.7. Data analysis

The results obtained for the flasks with original and Mg-exchanged carriers were compared. The null hypothesis was that the flasks showed no difference in performance. Ordinary Student's *t*-test was performed and the results were considered significant at the 5% level ($p = 0.05$). The correlation between variables was estimated using the Pearson linear correlation.

3. Results and discussion

3.1. Immobilization dependent on the size of particles

The original and Mg-exchanged T samples of four different particle sizes were tested under same conditions and number of immobilized cells after 24 h of incubation was compared (Fig. 1). The number of immobilized cells correlated significantly negatively with particle size of the T carrier ($r = -0.63$; $p < 0.05$), indicating that particle size of the carrier is an important feature in immobilization of the bacteria. This finding is consistent with the already observed [12,13] decrease of immobilization of bacteria with the increase in particle size of the carrier. Since the smallest particle size of original and Mg-exchanged T samples showed the best results, future experiments were done with this size fraction.

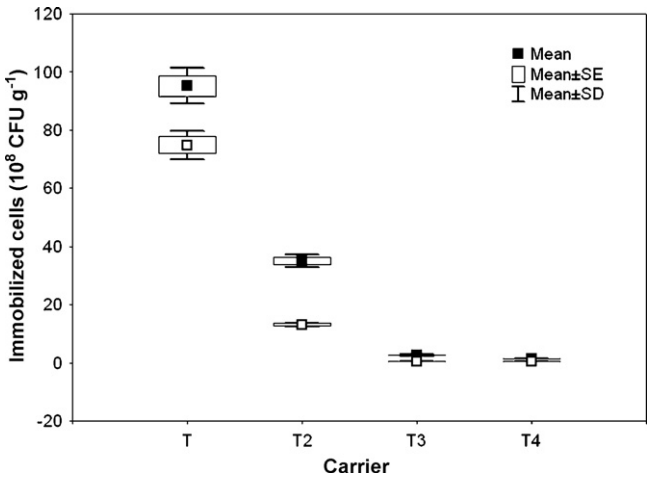

Fig. 1. Number of immobilized cells of *A. junii* on different size fractions of original (empty boxes) and Mg-exchanged (full boxes) zeolite tuff from Turkey after 24 h of incubation. Particle sizes: T < 0.125 mm; T2 0.125–0.25 mm; T3 0.25–0.5 mm; T4 0.5–1.0 mm. [c_0 CFU (10^6 CFU mL^{-1})] = 12.96 ± 3.29.

3.2. Immobilization dependent on the type of carrier

The performance of flasks containing different types of carriers is shown in Table 1. After 24 h of contact time with the carrier, one part of bacteria were present in micro-colonies strongly adsorbed to one another and to the outer surface of the carriers, while the rest of the bacteria remained as planktonic cells in the supernatant. Neisser staining confirmed the presence of intracellular poly-P granules in both immobilized and planktonic bacteria.

The highest number of immobilized cells was achieved with the Mg-exchanged T sample (9.52×10^9 CFU g^{-1}, Table 1). This is somewhat higher than the immobilization rates reported so far for *Acinetobacter* species and other microorganisms immobilized onto mineral carriers: 6.86×10^9 CFU g^{-1} of *A. calcoaceticus* immobilized onto the Mg-exchanged NZ [13]; 5.28×10^9 CFU g^{-1} of *A. junii* immobilized onto the surfactant-modified NZ [12]; 2.9×10^9 CFU g^{-1} of *Acinetobacter* spp. immobilized onto ceramics [16]; 2.5×10^8 CFU g^{-1} of *A. johnsonii* immobilized inside alginate beads [17]; 9×10^8 CFU g^{-1} of *Pseudomonas aeruginosa* immobilized onto the Type-Z biocarrier [15] and 3.6×10^8 CFU mL^{-1} of *Saccharomyces cerevisiae* immobilized onto NZ [5]. Here it can be seen that the clinoptilolite content in the NZ does not have to be the prevailing factor for the immobilization of bacteria. Namely, the T sample having clinoptilolite contents of 70% showed somewhat better immobilization capacity than the NZ sample originating from Croatia [13] having clinoptilolite content of 50%, but significantly higher than the S sample having clinoptilolite content of 75%.

The highest number of cells immobilized onto bentonite was 7.40×10^9 CFU g^{-1} obtained for Mg-exchanged TER. This is similar to 4.79×10^9 CFU g^{-1} and 7.78×10^9 CFU g^{-1} of *A. junii* immobilized onto original and surfactant modified bentonite [18], but significantly lower than the number of *P. putida* adsorbed onto kaolinite (4.1×10^{10} cells g^{-1}) and montmorillonite (3.2×10^{10} cells g^{-1}) [19]. However, the number of *P. putida* was calculated indirectly and perhaps exaggerates the real number of viable bacterial cells immobilized on the carrier.

The extent of the immobilization on different carriers can also be displayed by comparing the ratio of immobilized and planktonic cells (CFU immobilized/CFU planktonic). After 24 h of incubation the ratio was significantly greater in flasks containing the T and TER when compared with S (Table 1), indicating that S is the least suitable carrier. On the contrary, the number of planktonic cells (Table 1) was greater with S than with the T and TER. These results are consistent for both the Mg-exchanged and the original carriers. Probable explanation is in the mechanism of adsorptive growth [20]. At the start of the incubation cells adsorb on the carrier particles and continue their growth. A greater number of cells that were attached initially on the most suitable carriers (T and TER) resulted in the smaller number of cells that were growing planktonically in the supernatant. Increased number of initially attached cells increased the number of immobilized cells and decreased the number of planktonic cells at the end of the incubation, depending on the type of the carrier used.

The total number of cells and number of immobilized cells after 24 h of incubation (Table 1) were significantly higher ($p < 0.05$) in all flasks containing the Mg-exchanged carriers when compared to their original counterparts. This observation is consistent with previous researches [13,19], which showed that the immobilization rate and the increase of the total number of cells increased remarkably when increasing the concentration of Mg^{2+} in the NZ or clay carrier.

The P-adsorption capacities of each carrier are presented in Table 1. The Mg-exchange increased the carrier capacities 11%, 69% and 76% for T, S and TER, respectively. The observation that the Mg-exchange process increases the P-adsorption capacity agrees with the previous observation for other NZ [13,21]. The obtained P-adsorption capacities for NZ (22–84 mg P kg^{-1}) fit in the range of 2–48 mg P kg^{-1} reported for NZ [6,9,21]. The adsorption capacity for TER (40 mg P kg^{-1}) is comparable with the previously obtained 50 mg P kg^{-1} for clay samples [9]. The Mg-exchanged NZ displayed adsorption capacity that was similar to the previously reported 37.5 mg P kg^{-1} for Mg-exchanged NZ [13]. In general, all the tested materials had negligible P-adsorption capacity, and all the P removed from simulative wastewater can be ascribed to the bacterial activity.

The Mg^{2+} ions are particularly important for stable EBPR [22], and increasing the available Mg^{2+} substantially increased the P removal capacity of biomass in a bench scale pilot plant [23]. It

Table 1
Features of the mineral carriers and properties of the flasks containing *A. junii* and mineral carriers measured after 24 h of incubation. Natural zeolite tuff from Turkey (original T, Mg-exchanged TMg); natural zeolite tuff from Serbia (original S, Mg-exchanged SMg); Terrana® (original TER, Mg-exchanged TERMg). Particle size of the carrier: < 0.125 mm; [c_0 CFU (10^6 CFU mL^{-1})] = 12.52 ± 2.03; [c_0 P–PO_4 (mg L^{-1})] = 23.57 ± 0.51.

Parameter	Type of carrier					
	T	TMg	S	SMg	TER	TERMg
Zeta potential (mV)	−23.12 ± 0.57	−21.18 ± 0.40	−17.60 ± 2.52	−14.76 ± 1.70	−15.38 ± 1.32	−11.92 ± 2.56
P-adsorption capacity (mg kg^{-1})	77.5 ± 3.5	84.0 ± 1.4	22.5 ± 4.7	38.0 ± 2.7	40.4 ± 3.6	71.2 ± 4.6
Total cells (10^8 CFU mL^{-1})	1.25 ± 0.02	1.82 ± 0.06	1.55 ± 0.05	2.11 ± 0.15	1.39 ± 0.04	1.56 ± 0.01
Planktonic cells (10^7 CFU mL^{-1})	4.99 ± 0.29	8.65 ± 0.05	14.85 ± 0.01	19.30 ± 0.15	8.01 ± 0.29	8.40 ± 0.40
Immobilized cells (10^8 CFU g^{-1})	74.91 ± 0.01	95.22 ± 0.01	6.14 ± 0.26	18.75 ± 0.51	58.23 ± 6.84	73.97 ± 2.90
CFU immobilized/CFU planktonic	151.08 ± 18.72	106.56 ± 4.17	10.33 ± 0.33	24.39 ± 1.24	139.18 ± 3.89	221.05 ± 19.17
P removed (%)	46.32 ± 0.55	51.99 ± 0.11	40.21 ± 0.21	63.01 ± 2.48	46.91 ± 2.43	52.53 ± 1.86
P-uptake rate (10^{-11} mg P CFU^{-1})	8.45 ± 0.13	6.77 ± 0.15	6.18 ± 0.03	6.86 ± 0.78	8.16 ± 0.26	7.87 ± 0.30
pH	7.89 ± 0.04	7.25 ± 0.04	7.92 ± 0.04	7.71 ± 0.04	7.98 ± 0.04	7.90 ± 0.04

appears that Mg^{2+} along with K^+ are released and taken up simultaneously with P in EBPR systems [23]. In this study the percent of the total P removed was significantly ($p < 0.05$) higher in the flasks with the Mg-exchanged carriers when compared to original carriers (5.67%; 22.8% and 5.62% for T, S and TER, respectively). The Mg-exchange enhanced the P-uptake rates per CFU only for S (Table 1) but not for T and TER, thus no connection between the Mg^{2+} and the difference in P-uptake rates can be established. Probable explanation is that after 24 h of incubation the majority of bacteria were still in the logarithmic, non P-accumulating, phase of growth and the difference in total P removed was the result of P incorporation in the biomass rather than the enhanced P-uptake capacity of *A. junii*. It is obvious that more bacteria can take up more P from wastewater, resulting in the higher percent of P removal.

All of the carriers used in the study shifted the neutral pH-value of simulative wastewater towards the alkaline after the 24 h of incubation (Table 1). Average pH-values were higher in the flasks with original carriers (7.77 ± 0.18) rather than with the Mg-exchanged ones (7.46 ± 0.28) but not significantly ($p > 0.05$). The difference probably comes from the increased metabolic activity in the flasks with the Mg-exchanged carriers due to the higher number of bacterial cells. The pH-values after 24 h of incubation in all the flasks with carriers were not considerably different (<0.5 pH-units) than the pH-value in the control reactor (7.42 ± 0.05) containing solely the pure culture of *A. junii*. This indicates that the clinoptilolite and bentonite did not act as a pH regulator, which agrees with previous observations for other NZ [5,9].

3.3. Immobilization dependent on the zeta potential

In order to determine whether Mg-exchange induced the change in the surface charge of the carriers and if this affected the rate of bacterial immobilization, the zeta potential of all the materials, together with the zeta potential of *A. junii* was measured (Table 1). Results revealed that the net surface charge of the Mg-exchanged carriers was significantly ($p < 0.05$) more positive than the surface charge of the original counterparts. The number of immobilized cells (Table 1) on the Mg-exchanged carriers was greater when compared to the original carriers. Since the pure culture of *A. junii* is negatively charged, electrostatic interactions in adhesion on the carriers are mostly repulsive and have to be overcome by attractive Lifshitz–van der Waals, hydrophobic and specific interaction forces [14]. The Mg^{2+} induced increment in surface charge of the carriers, probably decreased the repulsive forces and enhanced the immobilization of the bacteria by favouring the initial attachment of the cells. Few studies stated that the maximum adsorption of bacterial cells occurred at first 60 min of bacterial exposure to mineral surfaces [19,24], so this initial attachment induced by the difference in zeta potential could be an important step defining the immobilization of bacteria. However, when both the original and Mg-exchanged samples were considered, the decrease in electronegativity of the carriers correlated negatively with the increase in number of immobilized bacterial cells ($r = -0.33$; $p = 0.52$). For example, the zeta potential of S was significantly more positive than T, but the immobilization rates were tenfold lower. This indicates

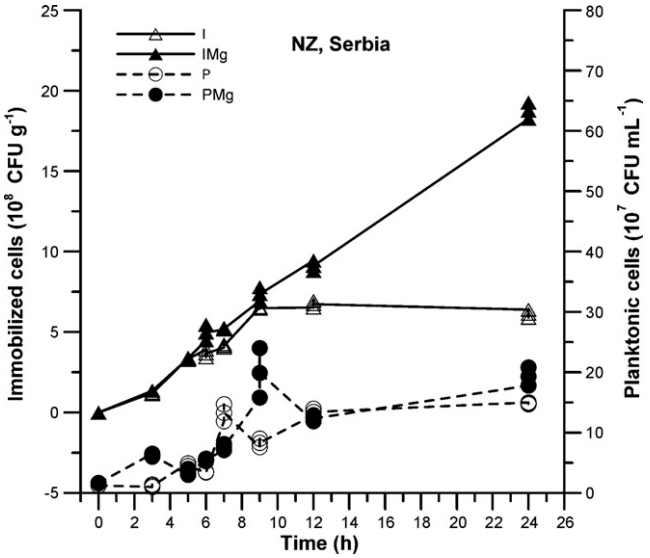

Fig. 3. Number of *A. junii* in the flasks with zeolite tuff from Serbia, measured after the elapsed time of incubation. Full line marks immobilized cells (I original; IMg Mg-exchanged) and dotted line marks planktonic cells (P original; PMg Mg-exchanged). [c_0 CFU (10^6 CFU mL^{-1})] = 14.32 ± 1.84.

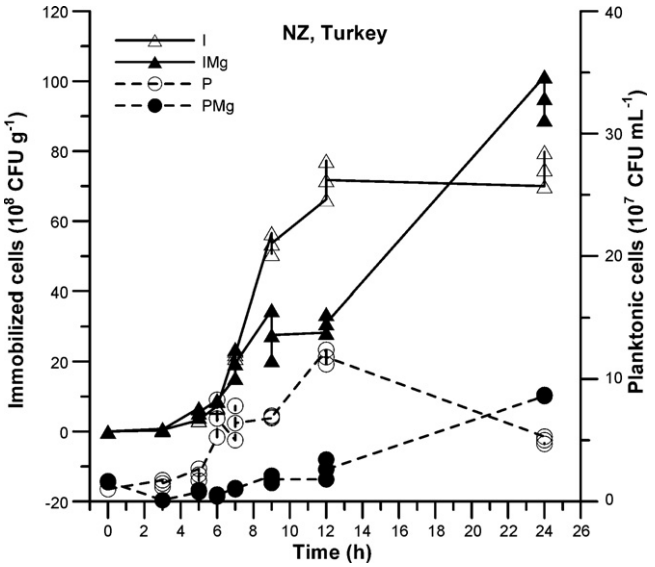

Fig. 2. Number of *A. junii* in the flasks with zeolite tuff from Turkey, measured after the elapsed time of incubation. Full line marks immobilized cells (I original; IMg Mg-exchanged) and dotted line marks planktonic cells (P original; PMg Mg-exchanged). [c_0 CFU (10^6 CFU mL^{-1})] = 11.38 ± 1.39.

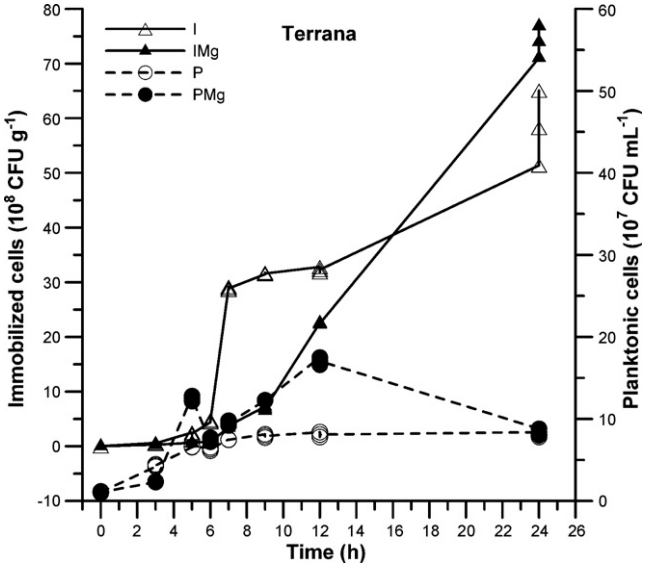

Fig. 4. Number of *A. junii* in the flasks with Terrana®, measured after the elapsed time of incubation. Full line marks immobilized cells (I original; IMg Mg-exchanged) and dotted line marks planktonic cells (P original; PMg Mg-exchanged). [c_0 CFU (10^6 CFU mL^{-1})] = 10.80 ± 0.68.

that the zeta potential might have some influence on the bacterial immobilization, but the primary factor is most probably the type of the material. The Literature citations about zeta potential and immobilization of bacteria are inconclusive. Jiang et al. [19] proposed that the electrostatic properties of mineral surfaces play a vital role in the adsorption of *P. putida* by soil colloidal factions. A study by van Merode et al. [14] showed that the clinical *Enterococcus faecalis* strains with less negative zeta potential adhered significantly better on polystyrene. On the other hand Rao et al. [25] suggested that electrostatic interactions are not the primary factors determining adhesion of *Streptococcus sanguis* and *Actinomyces naeslundii* to apatite minerals. Similar deduction was obtained in a study on immobilization of *A. junii* on surfactant-modified zeolites [12].

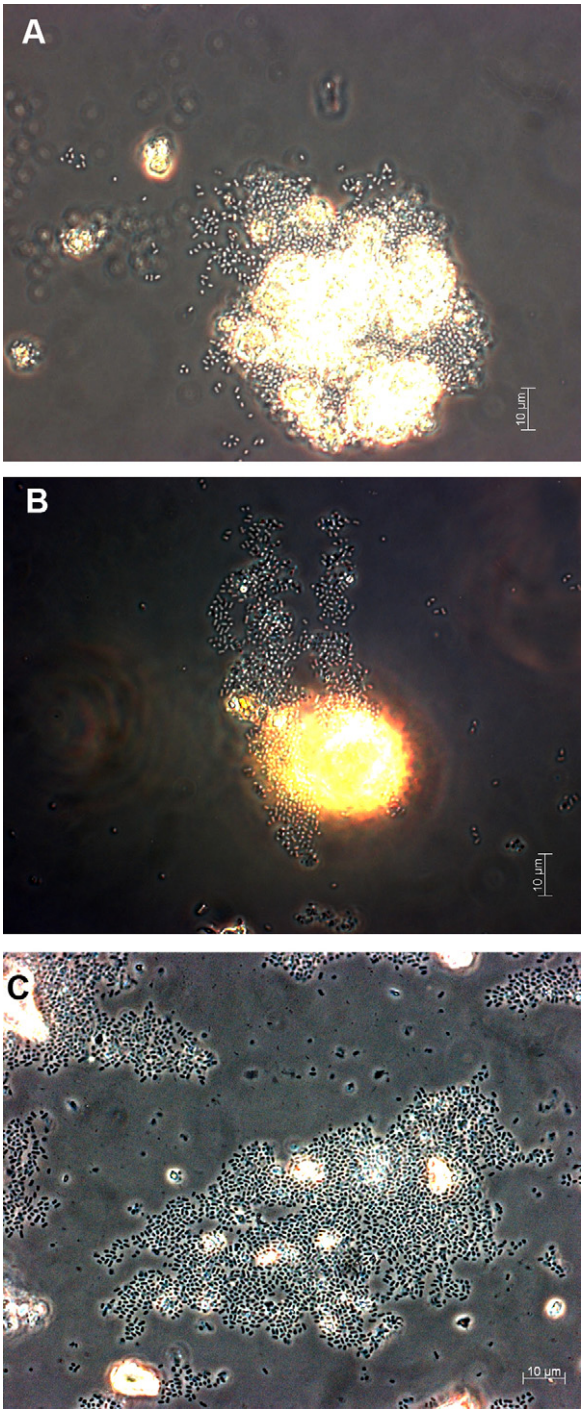

Fig. 5. Immobilized cells of *A. junii* on the zeolite tuff from Turkey (A), zeolite tuff from Serbia (B) and Terrana® (C) after 24 h of incubation.

3.4. Immobilization dependent on the contact time

To get an insight into the mechanism of immobilization itself, the number of cells through time was followed. Increase in the number of planktonic cells did not follow the increase in the number of immobilized cells (Figs. 2–4). This confirms the statement that the biofilm formation is the result of clonal growth of initially attached cells, rather than the recruitment of the planktonic cells.

In comparison with original NZ, the Mg-exchanged NZ carriers displayed a prolonged biofilm growth, as can be seen from the number of immobilized cells versus time plot (Figs. 2 and 3). The number of immobilized cells in flasks containing the original NZ (T and S) did not differ significantly at 12 h and 24 h of incubation, suggesting that 12 h is the time needed to reach the mature stage of the biofilm formation. The Mg-exchanged NZ displayed different trend; the number of immobilized cells was rising up to 24 h of incubation, resulting in higher number of immobilized cells at the end of the incubation. The cells seem to divide more readily on the Mg-exchanged carriers and the credible reason could be the access of the increased amount of Mg^{2+} on the surface of the carriers, exhibiting already mentioned positive influence on the biomass yield.

The TER samples displayed similar trend, but not identical. The number of immobilized cells in flasks with original and Mg-exchanged samples was significantly higher at 24 h than at 12 h (Fig. 4), indicating that a mature state of biofilm formation was not reached after 12 h of incubation as for T and S. The reason for different trends in biofilm formation is in the different nature of material used as carrier of bacteria. TER exhibits specific clay-flocculating properties in water medium and through time the TER particles were swelling, which provided enhanced surface for immobilization after 12 h of incubation, resulting in prolonged biofilm growth.

The difference in the mechanism of immobilization of bacteria arising from the different nature of NZ and clay carriers is shown in Fig. 5. After 24 h of incubation, bacteria were present as a thick biofilm on the surface of NZ, while interlocked as cell aggregates among the small particles of swelled TER.

4. Conclusions

The Mg-exchanged T proved to be the most suitable carrier of *A. junii*. All of the Mg-exchanged carriers achieved significantly higher immobilization rates when compared to their original counterparts. However, the exchange of the original materials with Mg^{2+} probably would not be cost effective if used for full scale wastewater treatment plants. The usage of natural minerals with the Mg^{2+} as the dominant exchangeable cation could be a satisfactory solution.

The number of immobilized cells decreased with the increase in particle size of the carrier and this was consistent with both the Mg-exchanged and the original carriers. Measurements of the zeta potential of both the carriers and the bacterium indicated that the zeta potential exhibited certain, but not crucial influence on the bacterial immobilization. The bacterial immobilization on mineral carriers seems to be defined by the type and particle size of the material used, not by the zeta potential.

Acknowledgements

This research was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (projects nos. 119-1191155-1203 and 119-1191155-1156). Special thanks to Mr.

Gerhard Kummer, Süd-Chemie AG, for providing the Terrana® samples.

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