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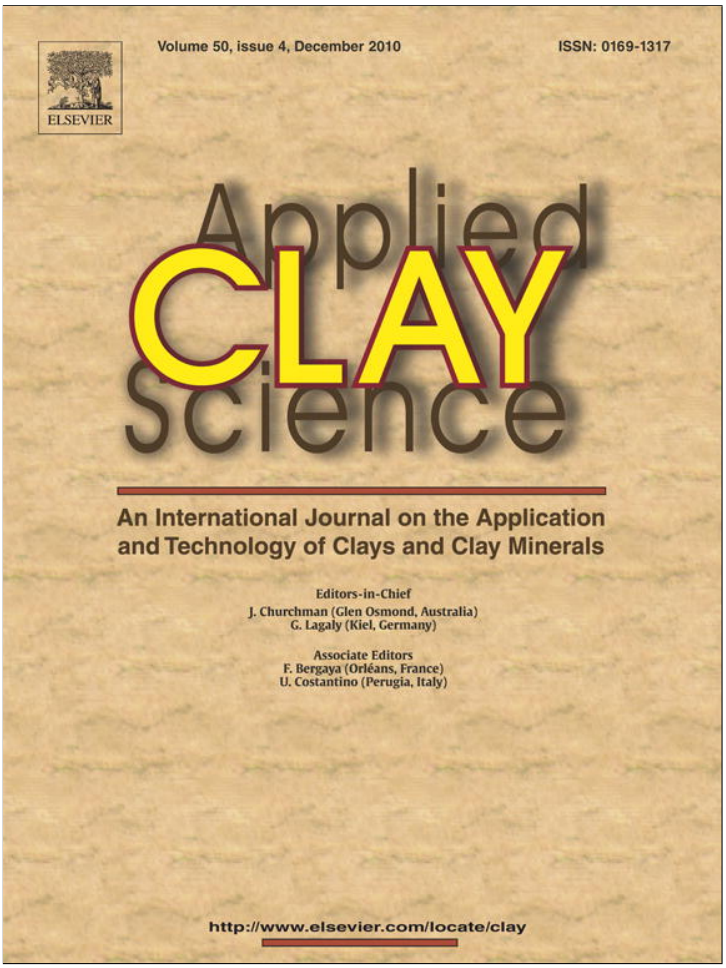

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Sepiolite as carrier of the phosphate-accumulating bacteria *Acinetobacter junii*

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ABSTRACT

Sepiolite in its original (OS) and purified form (PS) was tested as a carrier of the phosphate-accumulating bacteria *Acinetobacter junii*. The numbers of *A. junii* immobilized onto sepiolite were 5.57 and 8.12×10^9 CFU g^{-1} for OS and PS, respectively. The immobilized bacteria were metabolically active and removed phosphate from synthetic wastewater. Better phosphate removal in reactors with immobilized bacteria when compared to reactors with planktonic bacteria was mainly due to the increased bacterial biomass and at a lower extent due to the increased bacterial phosphate-uptake rates. The bioaugmentation of an activated sludge with *A. junii* immobilized on OS significantly improved the phosphate removal from synthetic wastewater when compared to a conventional activated sludge.

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1. Introduction

The immobilization of desired bacteria on suitable materials as carriers is currently gaining much attention in various fields of biotechnology. The immobilization process offers a higher density of bacterial cells, enhanced metabolic activity in the bioreactors and better survival of bacteria during the periods of environmental stresses (Costerton et al., 1999; Hogan and Kolter, 2002). The fluidized bed reactors utilize small fluidized media particles to induce extensive cell immobilization, thereby achieving a high biomass in the reactor, resulting in shortening of the reactor residence time (Shieh and Hsu, 1996). The immobilized *Pseudomonas* sp. were stable for oil degradation and tolerant to 5% NaCl while the planktonic bacteria were not (Emtiazi et al., 2005). Immobilization of bacteria can also be used in construction of biobarriers. In this concept the bacteria are immobilized on a suitable carrier and employed *in-situ* where bacterial activity is used to create a reactive zone for the treatment of contaminated groundwater (Lyew et al., 2007).

The immobilization of desired bacteria on suitable carriers to achieve a higher cell density and activity in bioreactors could be an alternative for improving the biological phosphate removal from wastewater. This process is based on the accumulation of soluble phosphate in wastewater inside the bacterial cells in the form of nonsoluble polyphosphate granules (Seviour et al., 2003). Some naturally occurring minerals were used as carriers of phosphate-accumulating bacteria and showed a good incorporation in the activated sludge biomass (Hrenovic et al., 2003a). Immobilized bacteria that are incorporated this way can easily be removed from the wastewater treatment system and disposed together with the activated sludge. The efficiency of an immobilized system for phosphate removal will depend on a number of factors such as properties of the carrier, bacterial strains being used and number of metabolically active bacteria.

Sepiolite is commercially obtainable, of relatively low cost (150–250 € per tonne) and non-toxic. Main deposits of sepiolite are located in Anatolia in Turkey, Ceelbuur in Somalia, South-central China and Spain with 70% of world reserves and annual output of approximately 1300.000 tons (Alvarez-Ayuso and Garcia-Sanchez, 2003). Sepiolite particles are needle-like (Can et al., 2010). There is an increasing interest in recent years to utilize sepiolite in environmental studies since sepiolite is a good adsorbents of neutral molecules and cations (Alkan et al., 2005a). Sepiolite does not exhibit swelling properties like other clay minerals, which can provide difficulties (clogging of hardware or filters jamming) in treatment systems. Sepiolite is commonly used in the oil refining, wastewater treatment, adsorption of odours, drugs and pesticides (Ersoy and Celik, 2002). The implementation of phosphate-accumulating bacteria immobilized on sepiolite in wastewater treatment plants is promising because of specific physical properties and the high magnesium content. Mg^{2+} along with K^+ ions is particularly important for the process of phosphate accumulation and formation of biomass of phosphate-accumulating bacteria (Rickard and McClintock, 1992; Schonborn et al., 2001). Zeolite tuff and bentonite were used to adjust the lack of Mg^{2+}, Ca^{2+}, K^+ and trace metal content in laboratory scale plants

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with the activated sludge (Hrenovic et al., 2010). Therefore, we supposed that the sepiolite will be a good carrier of immobilized and metabolically active phosphate-accumulating bacteria. The aim of this study was to test the sepiolite as a carrier of phosphate-accumulating bacteria *Acinetobacter junii* to be applied in wastewater treatment system.

2. Materials and methods

2.1. Sepiolite

The sepiolite was obtained from Shijiazhuang World Imp&Exp Trade Co, Ltd, China. Original (OS) and purified (PS) sepiolite were used. The aggregate size of OS and PS was 100–325 mesh (0.044–0.149 mm). PS was obtained by reacting 50 g of OS with 1200 mL of 0.15 M HCl for 4 h at room temperature (22 ± 2 °C). The sample was washed with demineralized water, and the procedure was repeated once more. The pH of dispersion was set to 6.0 followed by reaction with 500 mL of 0.5 M NH_4Cl for 1 h at room temperature. The material was again washed with demineralized water and then treated with 500 mL of 0.5 M $MgCl_2 \times 6H_2O$ at same conditions as above. PS was then washed with demineralized water until the chloride ion test was negative. OS and PS were dried at 50 °C for 24 h.

2.2. Bacteria

The culture of phosphate-accumulating bacteria *A. junii* strain DSM 1532 was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (Hrenovic et al., 2003b).

2.3. Activated sludge

The activated sludge was obtained from the wastewater treatment plant treating municipal wastewater from the combined sewer system of Zagreb, Croatia. Before the start of experiment the sludge was maintained for 3 months in the Armfield Aeration Apparatus to acclimatize to laboratory conditions. The volume of 1 L of the activated sludge was fed with 5 L of raw municipal wastewater every 48 h. The unit was kept at room temperature (22 ± 2 °C) and aeration of 3 L min^{-1} was provided through the entire period.

2.4. Synthetic 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 10; and KH_2PO_4 88. The pH value was adjusted to 7.00 ± 0.04 with 1 M NaOH or 1 M HCl (Kemika, Croatia) before autoclaving (121 °C/15 min).

2.5. Experimental design

For the immobilization of bacteria, *A. junii* was pre-grown on a nutrient agar (Biolife, Italy) for 16 h at 30 ± 0.1 °C. The biomass was then suspended in 9 mL of a sterile 0.05 M NaCl solution. One mL of suspended biomass was inoculated into Erlenmeyer flasks containing 100 mL of synthetic wastewater. The starting number of *A. junii* was 10^{10} CFU L^{-1}. An amount of 1.0 g of carrier was added to each flask. The flasks were sealed with a sterile gum cap and aerobically incubated in a water bath with a shaker (30 ± 0.5 °C/70 rpm, Memmert WNB) for 24 h. Filtered air was provided at an aeration rate of 1 L min^{-1}. After the incubation, the number of planktonic and immobilized bacteria was determined.

The bioparticles were prepared by incubating a suspension of *A. junii* (concentration of 10^{10} CFU L^{-1}) and 2.0 g of OS in Erlenmeyer flasks with 200 mL of sterile synthetic wastewater or 0.05 M NaCl solution. The flasks were incubated as described above. After the incubation the bioparticles consisting of *A. junii* immobilized on OS were gently washed with 0.05 M NaCl and transferred in the reactors with the activated sludge. The experiments with the activated sludge were designed as laboratory scale sequencing batch reactors. A 5 mL of the centrifuged sludge (2000 rpm/3 min) was washed with the sterile 0.05 M NaCl solution and added to bottles containing 200 mL of synthetic wastewater. The control reactor (R1) contained only the activated sludge. The sludge in the second reactor (R2) was bioaugmented with a suspension of *A. junii* at a concentration of 10^{10} CFU L^{-1}. In the third reactor (R3) 2.0 g of OS was added. The other two reactors were supplemented with 2.0 g of bioparticles prepared in wastewater (R4) or 0.05 M NaCl (R5). The bottles were incubated at room temperature for 24 h in a water bath with a shaker (70 rpm). The aeration rate of filtered air was of 1 L per min. The suspended solids and sludge volume index (SVI) were determined for each reactor before and after the incubation.

2.6. Analytical methods

The chemical composition of OS and PS was determined by the commercial ACME Analytical Laboratory, Canada. All elements, except of carbon which was determined by the LECO analyser, were determined by ICP-emission spectrometry following a lithium metaborate/tetraborate fusion and dilute nitric digestion. The mineralogical composition of OS and PS was determined by X-ray powder diffraction (XRD) on a Philips X-pert diffractometer using CuK_α radiation.

The electrophoretic mobility of sepiolite and bacteria was measured in 0.05 M NaCl solution and in synthetic wastewater at different pH values. The pH was adjusted with HCl and NaOH (Kemika, Croatia). The mass concentration of sepiolite was 0.2 g L^{-1} and the concentration of bacteria was 10^{10} CFU L^{-1}. The particles and bacteria were dispersed on a mechanical shaker at 200 rpm for 15 min. The samples were allowed to stand for 5 min to let larger particles to settle. An aliquot taken from the supernatant was used to measure the electrophoretic mobility. The measurements were performed on ZetaPlus Zeta Potential Analyser, Brookhaven Instruments Corporation. The instrument uses electrophoretic light scattering and the Laser Doppler Velocimetry method for determination of particle velocity and, from this, the mobility.

The pH value was measured with the WTW 330 pH-meter. The phosphate concentration in the synthetic wastewater was measured spectrophotometrically in a DR/2500 Hach spectrophotometer using the molybdovanadate method (Hach method 8114). Before the phosphate measurement the samples were filtered using Sartorius nitrocellulose filters with a pore diameter of 0.2 μm. The scanning electron microscopy (Field Emission Scanning Electron Microscope, Jeol JSM 7000 F, Japan) was performed to confirm the immobilization of bacterial cells onto the sepiolite fibres. Neisser staining followed by light microscopy (Olympus CX 21) was performed to confirm polyphosphate granules inside the cells of *A. junii*.

For the determination of planktonic bacteria, 1 mL of supernatant was serially diluted (10^{-1} to 10^{-9}) and volumes of 0.1 mL were aseptically inoculated onto the nutrient agar (spread plate method). After the incubation (30 ± 0.1 °C/24 h), the bacterial colonies were counted and the number of viable cells was reported as CFU L^{-1}. To determine the number of immobilized cells, each carrier was taken from the flask, washed three times with the sterile 0.05 M NaCl solution, and aseptically placed into a tube containing 9 mL of 0.05 M NaCl. The samples were crushed with a sterile glass rod and vigorously shaken on a mechanical shaker (40 Hz/3 min, Kartell TK3S). This procedure (Durham et al., 1994) detaches immobilized cells from the carrier. Serial dilutions were made from these suspensions and nutrient agar plates were inoculated and incubated as already described. After the incubation the colonies were counted

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and the remaining samples were dried and weight. The number of cells was reported as immobilized CFUs per 1 g of the dry carrier. All measurements were done in triplicate. The suspended solids and SVI were determined by standard methods (APHA et al., 2005).

Statistical analyses were carried out using Statistica Software 8.0 (StatSoft, Tulsa, USA). The numbers of bacterial CFU were logarithmically transformed beforehand to normalize distribution and to equalize variances of the measured parameters. The comparisons between samples were done using the one-way analysis of variance (ANOVA) and subsequently the post-hoc Duncan test was performed for the calculations concerning pair-wise comparisons. The correlation between variables was estimated by Spearman correlation analysis. Statistical decisions were made at a significance level of $p<0.05$.

3. Results and discussion

3.1. Characterization of sepiolite

The chemical composition of OS and PS is given in Table 1. The chemical analysis showed that examined OS originating from China had a chemical composition significantly different from sepiolites described in the literature (Newman and Brown, 1987; Alkan et al., 2005b; Can et al., 2010; Garcia-Romero and Suárez, 2010). The most prominent difference i.e. the higher content of CaO in OS was due to the presence of calcite and dolomite. Mineralogical analysis showed that OS contained only 40–50% of sepiolite, with other important constituents (10–15%) being smectite, talc, amphibole, dolomite and calcite while minor constituents were from the serpentine mineral group (Fig. 1). The sepiolite content in OS was considerably<85% and 98% of sepiolite in samples originating from Turkey and Serbia, respectively (Can et al., 2010; Lemic et al., 2005). Therefore, the OS was reacted with diluted HCl to enrich the sepiolite. The purification of OS yielded with reduced content and 50–55% of sepiolite. Sepiolite reflections on the XRD powder pattern of PS had the same shape as for OS indicating that the reaction with diluted HCl removed calcite but did not affect the sepiolite structure (Rodriguez et al., 2003).

The variation of the electrophoretic mobility of OS and PS as a function of pH is shown in Fig. 2. The type of electrolyte had very low influence on the mobility and the isoelectric point. The isoelectric points (pH = 2.0 to 3.2) were comparable to pH = 2.2 for sepiolite from Spain (Knapp et al., 1997), but were much lower than pH = 6.6 for sepiolite from Turkey (Alkan et al., 2005a). These differences could arise from the different origins i.e. purity and chemical composition of the sepiolites. The surface of OS and PS was negatively charged at neutral pH like the surface of *A. junii* (Fig. 2). Therefore, no attractive electrostatic forces could be expected in contact of the bacteria with OS or PS.

Table 1
Chemical composition (in mass %) of original (OS) and purified sepiolite (PS). LOI — loss on ignition, TOT/C — total carbon.

	OS	PS
SiO_2	33.44	47.54
Al_2O_3	0.95	1.25
Fe_2O_3	0.23	0.19
MgO	18.99	22.65
CaO	19.34	9.62
Na_2O	0.08	0.13
K_2O	0.15	0.25
TiO_2	0.03	0.03
P_2O_5	0.04	0.04
LOI	26.4	17.9
Sum	99.69	99.63
TOT/C	5.05	2.10

3.2. Performance of bacteria A. junii in the presence of sepiolite

After 24 h of contact with OS or PS many cells of *A. junii* were immobilized on the surface of the sepiolite fibres by extracellular substances (Fig. 3). The low speed of stirring (70 rpm) applied during 24 h of the immobilization procedure did not break the sepiolite fibres, which usually only occurs after 3 min stirring at 21,000 rpm (Can et al., 2010). The number of *A. junii* immobilized onto sepiolite was 5.57 and 8.12×10^9 CFU g^{-1} for OS and PS (Table 2). These numbers are comparable to 10^9 CFU g^{-1} of *Bradyrhizobium japonicum* and 10^{10} CFU g^{-1} of *Sinorhizobium fredii* immobilized on sepiolite from Spain (Albareda et al., 2008). The rates of immobilization of *A. junii* on sepiolite were also comparable to zeolites: 9.52×10^9 CFU g^{-1} immobilized on Mg-exchanged zeolite (Hrenovic et al., 2009), 5.28×10^9 CFU g^{-1} on surfactant-modified zeolite (Hrenovic et al., 2008) and 2.33×10^9 CFU g^{-1} on natural zeolite (Hrenovic et al., 2005). The number of bacteria immobilized on PS was significantly larger than on OS. This result does not prove that the sepiolite by itself exclusively immobilized bacteria. The bacteria can also be immobilized on other minerals present in the sample (smectite, talc, amphibole, dolomite, and calcite). The specific surface area of sepiolite increased by acid-activation (Myriam et al., 1998), which is the probable reason for the enhanced immobilization on the PS.

The number of planktonic bacteria in the reactors after the incubation (Table 2) was also a parameter describing the extent of immobilization. As described previously as adsorptive growth (Nicola and Lazazzera, 2004), at the start of the incubation the cells were adsorbed on the particles and continue to growth. A larger number of cells that were attached initially on PS and a smaller number of cells grew planktonically in the supernatant and vice versa. Therefore, the ratio of immobilized and planktonic bacteria was significantly higher in the reactor containing PS than in the reactor with OS (Table 2).

The number of total bacteria and the ratio of final and starting number of *A. junii* in the OS and PS-reactors were significantly higher than in the control reactor (Table 2). It was shown that increased concentrations of Mg^{2+} in wastewater increased the multiplication of *A. junii* (Hrenovic et al., 2010). Sepiolite is rich in Mg^{2+} ions and can be a good source of Mg^{2+} ions for *A. junii*, which therefore increase the yield of bacterial biomass.

Sepiolite is a good adsorbent of heavy metal ions (Alvarez-Ayuso and Garcia-Sanchez, 2003), different organic species, neutral molecules and organic cations (Rytwo et al., 1998). However, no references could be found on adsorption of phosphate. We tested sepiolite by incubating a 1.0 g of OS and PS in 100 mL of synthetic wastewater without the addition of *A. junii*. The reduction of the phosphate concentration in the reactors after 24 h of incubation was 1 and 1.5% for OS and PS, and therefore can be considered irrelevant. We conclude that phosphate removed from the wastewater is the result of metabolism of *A. junii* and not due to adsorption by sepiolite itself.

The bacteria in reactors, both immobilized and planktonic, were metabolically active and removed phosphate from the wastewater. After the incubation a high proportion of bacteria contained intracellular granules of polyphosphate. A significantly higher removal of phosphate (Table 2) was achieved with PS (35%) than with OS (28%) or in the control reactor (17%). The phosphate-uptake rates did not differ in the reactors with OS and PS but were significantly higher than in the control reactor (Table 2). Phosphate removed from wastewater correlated statistically significantly with the total CFUs of *A. junii* ($p=0.993$) and with phosphate-uptake rates ($p=0.667$). This indicated that the better phosphate removal in the presence of OS and PS was mainly due to the increased bacterial biomass and to a lower extent due to the increased bacterial phosphate-uptake rate. The increased concentrations of Mg^{2+} in wastewater besides increasing the yield of biomass of *A. junii* also increased the bacterial phosphate-uptake rate (Hrenovic et al., 2010). High Mg^{2+} contents in the OS and PS-reactors were available for

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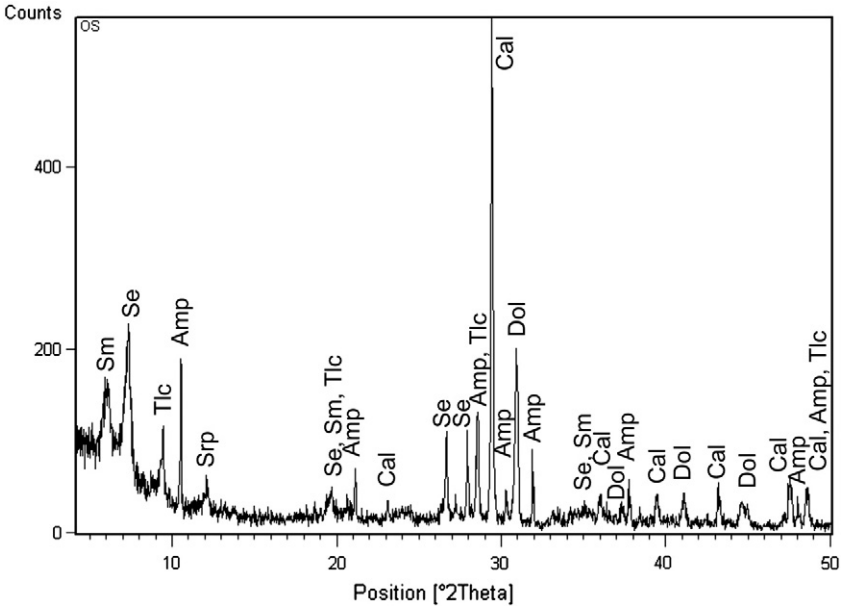

Fig. 1. X-ray powder pattern of original sepiolite (OS). Se — sepolite, Sm — smectite, Tlc — talc, Amp — amphibole, Srp — serpentine, Cal — calcite, Dol — dolomite.

bacterial metabolism by ion exchange and thus enhanced the yield of biomass and phosphate-uptake rate of *A. junii*, resulting in better phosphate removal from wastewater. The extracellular polymers created by the bacterial immobilization onto solid surfaces could also contribute to phosphate removal, then acting as a phosphate reservoir (Cloete and Oosthuizen, 2001).

The final pH values in the OS and PS-reactors were significantly higher than in the control reactor (Table 2). The difference probably comes from the increased metabolic activity due to the higher number of bacterial cells. Although statistically significant, the difference was only 0.3 pH units which indicated that the sepiolite did not act as a pH regulator, which was previously also confirmed for bentonite (Hrenovic et al., 2009). The possible negative influence of pH on the growth and metabolism of *A. junii* can be excluded since these bacteria grow in the pH range 6–8 (Garrity et al., 2005).

3.3. Bioaugmented activated sludge in the presence of sepiolite

When the control reactor (R1) contained only the activated sludge 34% of the starting phosphate concentration was removed after 24 h of incubation (Table 3). The phosphate adsorption of the activated sludge was in an expected range of <50% for conventional biological treatment (Fuhs and Chen, 1975). The phosphate removal in the reactor with the bioaugmented sludge (R2) was improved to 40% (Table 3). This cannot be considered significant when taking in view that the activated sludge is a highly complex biological system and such a difference could be the consequence of many unrecognizable factors, not only the bioaugmentation with *A. junii*. Comparable phosphate removal was obtained when the sludge was bioaugmented with *A. calcoaceticus* removing 19–57% of total phosphorus depending on the type of wastewater used (Hrenovic et al. 2003c). The addition

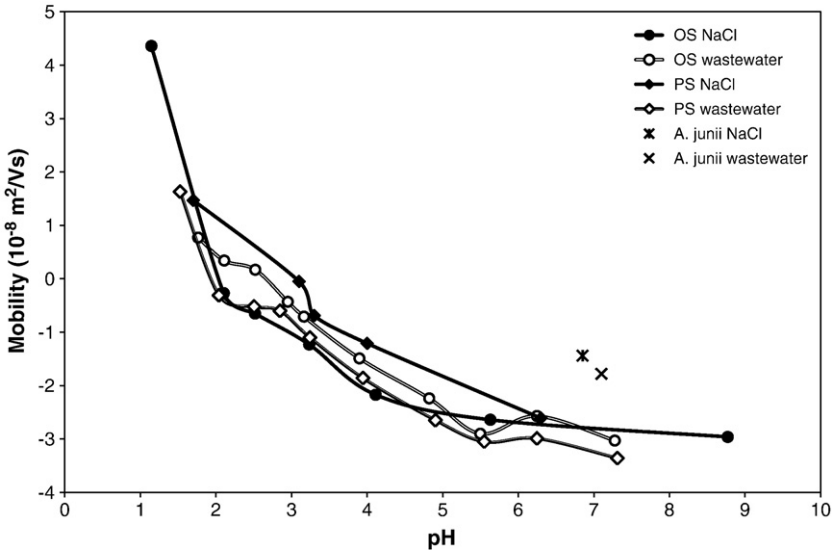

Fig. 2. Electrophoretic mobility of original sepiolite (OS), purified sepiolite (PS) and bacteria *A. junii* as a function of pH in synthetic wastewater and 0.05 M NaCl. Isoelectric points: OS NaCl = 2.0; OS wastewater = 2.1; PS NaCl = 3.2; and PS wastewater = 2.7.

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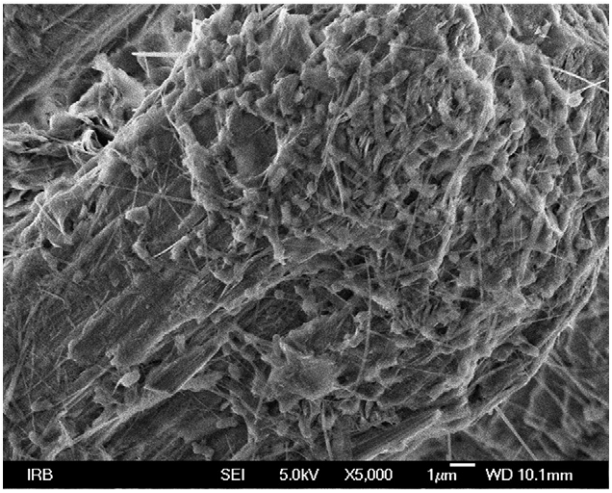

Fig. 3. Immobilized cells of *A. junii* on purified sepiolite (PS).

of only OS to the activated sludge (R3, Table 3) resulted in a final phosphate removal of 35%, which could not be considered as an improvement in comparison with the control reactor.

The activated sludge was bioaugmented with *A. junii* immobilized on OS in synthetic wastewater (R4) and in 0.05 M NaCl (R5). Depending on the media used, the numbers of *A. junii* immobilized on OS differed appreciably. In wastewater where an extensive increase of *A. junii* was expected, the number of immobilized cells was $4.48 \pm 0.67 \times 10^9$ CFU g^{-1}. Because of the high concentration of phosphate in the wastewater, a simultaneous immobilization and accumulation of polyphosphate granules inside the cells of *A. junii* occurred. We assume that the addition of bioparticles prepared in this way will diminish the phosphate removal by the activated sludge, since the bacterial cells were already occupied with polyphosphate. Therefore, we prepared bioparticles in 0.05 M NaCl where no increase and phosphate-uptake of *A. junii* were expected. The number of immobilized cells was significantly lower ($9.42 \pm 0.58 \times 10^7$ CFU g^{-1}), but there were very few polyphosphate granules inside the cells of *A. junii*. The phosphate removal in the reactor R4 was 46% (Table 3), which represented a considerable 12% increase compared with the control reactor (R1) but only a slight improvement of 6% as against the reactor with the activated sludge bioaugmented only with *A. junii* (R2). The highest phosphate removal of 64% was obtained in the reactor R5 (Table 3), which was a significant improvement towards all other reactors. The phosphate removal by bioparticles was not depended on the number of immobilized bacteria since the best removal was obtained with bioparticles prepared in the NaCl solution which contained a twofold lower number of immobilized cells compared to bioparticles prepared in wastewater. The probable reason for such good performance is that *A. junii* immobilized in the NaCl solution were phosphate-depleted and thus exhibited the highest phosphate-uptake when compared to *A. junii* on bioparticles prepared in synthetic wastewater.

Table 2
Performance of reactors containing *A. junii* (control), *A. junii* and original sepiolite (OS) and *A. junii* and purified sepiolite (PS) after 24 h of incubation. [c_0 CFU (10^{10} L^{-1})] = 1.59 ± 0.13; [c_0 P-PO_4 (mg L^{-1})] = 20.43 ± 0.12, significantly different values: ^{A}— compared to control; ^{B} — compared to OS.

Parameter	Control	OS	PS
Immobilized cells (10^9 CFU g^{-1})	–	5.57 ± 0.47	8.12 ± 0.48^B
Planktonic cells (10^{10} CFU L^{-1})	8.41 ± 0.40	4.37 ± 0.25^A	$3.81 \pm 0.20^{A,B}$
Total cells (10^{10} CFU L^{-1})	8.41 ± 0.40	9.93 ± 0.27^A	$11.93 \pm 0.29^{A,B}$
CFU immobilized/planktonic	–	318 ± 10	532 ± 5^B
CFU final/CFU start	5.19 ± 0.29	6.37 ± 0.61^A	$7.91 \pm 0.44^{A,B}$
P-uptake rate (10^{-11} mg P CFU^{-1})	4.05 ± 0.19	5.84 ± 0.16^A	5.87 ± 0.14^A
P removed (%)	16.53 ± 1.02	28.41 ± 1.16^A	$34.59 \pm 0.60^{A,B}$
Final pH	7.56 ± 0.02	7.82 ± 0.02^A	7.86 ± 0.02^A

Table 3
Performance of reactors containing activated sludge (R1), activated sludge bioaugmented with *A. junii* (R2), activated sludge and original sepiolite (R3), activated sludge and bioparticles prepared in synthetic wastewater (R4) and activated sludge and bioparticles prepared in 0.05 M NaCl (R5).

Reactor	Suspended solids (g L^{-1})		SVI (mL g^{-1})		P removed (%)	Final pH
	t_0	t_{24h}	t_0	t_{24h}		
R1	1.80	1.96	85.62	90.62	33.51	7.95
R2	1.66	1.88	90.24	90.42	40.41	7.92
R3	11.58	16.40	10.36	11.58	35.00	8.18
R4	9.50	13.57	8.42	11.79	45.64	8.13
R5	9.82	12.88	12.22	11.64	64.20	8.23

After 24 h of incubation of bioparticles in the activated sludge, a high number of *A. junii* (10^8 CFU g^{-1}) were still immobilized on OS while the bioparticles were solid. This indicates that bioparticles are promising materials for the use in wastewater treatment systems where bacteria from biofilms would continuously augment the sludge. Muyima and Cloete (1995) used alginate beads as carrier of phosphate-accumulating bacteria *A. johnsonii*. The immobilized bacteria were metabolically active and removed phosphate from the system with the activated sludge with some of the cells leaking out of the carrier. The usage of OS as a carrier of *A. junii* in this mode of action needs to be examined in future studies.

The final pH values in reactors with OS and bioparticles (R3, R4 and R5) were up to 0.3 pH units higher than in R1 and R2. The pH value influences the aerobic metabolism of phosphate-accumulating bacteria. The study of Filipe et al. (2001) showed that the phosphate-uptake rates were essentially the same at pH 7.0 and 7.5 but decreased steeply at pH 6.5. Serralta et al. (2006) reported that the biological phosphate uptake was essentially the same at pH = 7.4–8.25 but the rates obtained at pH values>8.25 were significantly lower. These literature data are in agreement with our observation, where the best phosphate removal was obtained in the reactor with the highest pH value of 8.23.

The addition of OS, alone or in the form of bioparticles, showed better metabolic and growth conditions regarded to the amount of suspended particles (Table 3). The increase of this value was distinctly larger in the reactors with OS (4.82, 4.07 and 3.06 g L^{-1} for R3, R4 and R5, respectively) than in the reactors with the activated sludge (0.16 and 0.22 g L^{-1} for R1 and R2, respectively). The OS was very well dispersed in the activated sludge and enhanced the growth of microorganisms increasing the amount of suspended particles. SVI was significantly lower in the reactors with OS, showing better settling properties of the activated sludge when compared to OS free reactors (Table 3). The addition of other clay minerals to activated sludge systems also resulted in a good dispersion of minerals in the activated sludge, which appreciably increased the amount of suspended particles and decreased SVI (Chudoba and Pannier, 1994; Wimmer and Buhl, 1998).

4. Conclusions

Sepiolite from China in its original (OS) and purified (PS) form is a good carrier of phosphate-accumulating bacteria *A. junii*. The number of immobilized bacteria was 5.57 and 8.12×10^9 CFU g^{-1} for OS and PS. The immobilized bacteria were metabolically active and removed phosphate from synthetic wastewater. Enhanced phosphate removal was mainly due to the increased bacterial biomass and to the lower extent increased bacterial phosphate-uptake rates. The bioaugmentation of the activated sludge with *A. junii* immobilized on OS significantly improved phosphate removal from synthetic wastewater

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compared to the conventional activated sludge. The use of phosphate-accumulating bacteria immobilized on OS in wastewater treatment plants is promising.

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