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Requirement of *Acinetobacter junii* for magnesium, calcium and potassium ions

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The goal of this study was to determine the concentrations of Mg, Ca and K ions required for the formation of metabolically active population of phosphate (P)-accumulating bacterium *Acinetobacter junii*. The availability of Mg, Ca and K originating from natural minerals in the conditions of severe shortage of these cations was tested. In the case of shortage of Mg, Ca and K ions in wastewater the P removal was absent due to the decay of *A. junii*. In the cases of Mg or K shortage in wastewater the P removal was negligible due to the decay of *A. junii*, while Ca was not essential for the examined bacterium. The minimal required concentrations of Mg and K in synthetic wastewater were 0.64 mg Mg/mg P and 0.50 mg K/mg P. The natural zeolitized tuffs and bentonite, either in Mg, Ca or K form, successfully replaced the lack of Mg, Ca, K and trace metals in wastewater. The requirement of *A. junii* for examined cations was in the order: Mg>K>Ca.

[**Key words:** Bacteria; Calcium; Magnesium: Phosphate; Potassium; Wastewater]

The activated sludge treatment is the most common way of the purification of wastewaters. During the last 40 years, the enhanced biological phosphate removal (EBPR) became the preferred process of phosphate (P) removal from wastewater. The EBPR process is carried out by the microbial population of P-accumulating bacteria (PAB) present in the activated sludge. These bacteria are able to accumulate the soluble P present in the wastewater as a nonsoluble intracellular polyP in the form of volutin granules. The biochemical models of metabolism of PAB implied that alternating anaerobic and aerobic (or anoxic) conditions are necessary to provoke EBPR. In the absence of oxygen, PAB transport volatile fatty acids into the cell and subsequently convert and store these as poly-hydroxy-alkanoates. The energy for this transport and storage is supplied by the hydrolysis of intracellularly stored polyP to P which is released from cell to the liquid, glycogen degradation and anaerobic tricarboxylic acid cycle. Under following aerobic conditions, stored poly-hydroxy-alkanoates would be catabolised, using oxygen (or nitrate) as electron acceptor to generate energy for the cell growth, maintenance, glycogen formation and polyP synthesis, resulting in the P uptake in a quantity greater than the amount previously released (1, 2). The P accumulated in the PAB in the form of polyP is easily removed from the system with the surplus sludge, resulting in the removal of P from wastewater.

The PAB community are not identical in different EBPR wastewater treatment systems and change from place to place and from time to time. The PAB involve phylogenetically and taxonomicaly diverse groups of bacteria. The cultivable PAB include genera of Gram-negative: *Acinetobacter, Aeromonas, Alcaligenes, Enterobacter, Lampropedia, Moraxella, Pseudomonas* (3-5) and Gram-positive bacteria: *Bacillus, Micrococcus, Microlunatus, Staphylococcus, Streptococcus* (5, 6). Different culture independent methods demonstrated that the P removal in EBPR system is carried out primarily by uncultivable bacterial species (7).

The bacterial strains which are able to accumulate more than 10^{-12} mg P/cell are classified as the PAB (5). The overall P removal from wastewater will depend on the capacity of PAB strain to accumulate the P intracellulary and the abundance of strain in the activated sludge. The PAB from the genus *Acinetobacter* does not have to predominate in the activated sludge community and can comprise less than 10% of the total bacterial population (5, 8). It should be argued that this small percentage in the activated sludge still represents several million cells per gram of biomass. If they have high capacity of polyP accumulation, then they would represent a significant contribution to EBPR. The *Acinetobacter* spp. had the highest capacity to accumulate polyP among all the cultivable PAB isolates (5) and have become the model organism for EBPR since it was isolated from the P-removing activated sludge plant (3). *Acinetobacter* spp. could accumulate up to 100 mg of P per gram of cell protein, which represents more than 10% of dry biomass (8). The PAB species from the genus *Acinetobacter* include: *A. baumanii, A. baylyi, A. bouvetii, A. calcoaceticus, A. gerneri, A. jonsonii, A. junii, A. lwoffi, A. tandoii, A. tjernbergiae, A. towneri* (9). None of the *Acinetobacter* isolates, including the *A. junii* investigated in this study, possess the typical metabolic characteristics of the PAB. Namely they failed to accumulate the poly-hydroxy-alkanoates from extracellular volatile fatty acids in anaerobic conditions of growth. These isolates are however able to accumulate polyP in the absence of extracellular carbon sources in aerobic conditions of growth. In spite of being not dominant or required for EBPR, *Acinetobacter* sp. may have a

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significant impact on P removal at least in the conventional secondary wastewater treatment process.

Cations such as Mg, Ca and K are micronutrients necessary to all microorganisms. The importance of Mg, Ca and K for PAB is even more significant. Many authors pointed out that the availability of Mg, Ca and K ions for activated sludge is essential for EBPR. For example, the poor performance of the full scale plant with EBPR was associated with the seasonal reduction of Mg in influent (10). The severe shortage of these cations in full scale EBPR systems treating domestic wastewater is in general unlikely to occur. But these systems may be exposed to temporal variations of Mg, Ca or K due to the chemical composition of water used or dilution by rainwater. The full scale EBPR systems receiving the industrial wastewater with high amount of P and no Mg, Ca or K also represent a risk for deterioration of EBPR. The Mg, Ca and K are not parameters which are routinely measured in wastewater and relevant information on the concentration of these cations in influent is limited. The Mg, Ca and K were detected in the structure of polyP granules of PAB as counter-ions of negatively charged P anions in the polyP chains (11, 12). The volutin granules contained two types of polyP, playing different roles in the bacterial metabolism: reactive Mg- and K-stabilized polyP (taking part in P release and P uptake processes) and inert Ca-stabilized polyP (stabile during changes in redox conditions). The increased concentration of Mg ions originating from natural zeolitized tuff increased the yield of biomass of PAB *A. calcoaceticus* in pure culture, which resulted in better P removal from wastewater (13).

The aim of this study was to determine the critical concentrations of Mg, Ca and K ions for the formation of biomass and successful P removal in the pure culture of PAB *A. junii*. The availability of Mg, Ca and K originating from natural minerals in the conditions of severe shortage of these cations was tested.

MATERIALS AND METHODS

Bacterium The culture of PAB *A. junii* strain DSM 1532 (former name of the strain *A. calcoaceticus*) was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH and maintained on nutrient agar (Biolife).

Minerals Three types of natural minerals of particle size <0.125 mm were used in this study. The natural zeolite tuff 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 (14), as estimated by X-ray powder diffraction method by comparison with samples in which clinoptilolite content was determined by internal standard method. The zeolite tuff 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 (14). Terrana® (TER) is a commercial bentonite obtained from Süd-Chemie AG (15). The main exchangeable cation in samples of T, S and TER was Ca (14). The mineral samples were subjected to Mg, Ca and K exchange process. A 10 g of mineral was treated with 250 mL of 1 M $MgCl_2 \times 6H_2O$, $CaCl_2 \times 2H_2O$ or KCl solution. Erlenmeyer flasks were incubated at 30 ± 0.5 °C and mechanically shaken at 200 rpm for 48 h. The minerals were then washed with demineralized water until the negative chloride ion test was obtained and air dried. By ion exchange, in total 9 samples of carrier were obtained (Mg, Ca and K rich). The prepared carriers were sterilized at 105 °C for 16 h before the start of the experiment.

Synthetic wastewater Chemically defined water solutions were prepared to simulate the real wastewater. The composition of five types of used wastewaters prepared in demineralized water is shown in Table 1. The pH of wastewater was set to 7.0 ± 0.2 with 1 M NaOH or HCl before autoclaving (121 °C/20 min).

TABLE 1. Composition of synthetic wastewaters.

Component	Type of wastewater				
	A	B	C	D	E
Na-propionate (mg L^{-1})	300	300	300	300	300
$(NH_4)_2SO_4$ (mg L^{-1})	400	400	400	400	400
Na_2HPO_4 (mg L^{-1})	92	92	92	92	92
$MgCl_2 \times 6H_2O$ (mg L^{-1})	0-836	84	84	0	836
$CaCl_2 \times 2H_2O$ (mg L^{-1})	37	0-367	37	0	367
KCl (mg L^{-1})	19	19	0-191	0	191
Trace metal solution (mL L^{-1})	10	10	10	0	10

Trace metal solution contained in mg L^{-1} of demineralized water: $FeSO_4$ 50; $MnSO_4$ 5; $ZnSO_4$ 0.5; $CuSO_4$ 0.5; $CoSO_4$ 0.05; $MoSO_4$ 0.05.

TABLE 2. Number of total cells (CFU), ratio of cell multiplication, phosphate (P) uptake rate per CFU and percentage of P removal in reactors containing wastewater A with different initial concentrations of magnesium.

Initial Mg concentration (mg L^{-1})	Wastewater A			
	Total cells (10^9 CFU L^{-1})	CFU final/ CFU initial	P uptake rate (10^{-11} mg P CFU^{-1})	P removal (%)
0.13	9.73 ± 1.42	0.23 ± 0.02	4.17 ± 0.64	2.09 ± 0.08
11.61	49.33 ± 4.16^{A}	1.21 ± 0.04^{A}	5.50 ± 0.45^{A}	14.76 ± 1.26^{A}
23.21	56.00 ± 3.61^{A}	1.46 ± 0.04^{A,B}	6.63 ± 0.44^{A,B}	19.97 ± 1.22^{A,B}
54.30	79.33 ± 5.86^{A,B,C}	1.90 ± 0.06^{A,B,C}	7.46 ± 0.53^{A,B}	30.74 ± 1.45^{A,B,C}
109.70	85.00 ± 7.55^{A,B,C}	2.20 ± 0.11^{A,B,C,D}	7.68 ± 0.70^{A,B}	33.77 ± 1.78^{A,B,C,D}

[c_0 CFU (10^9 CFU L^{-1})] = 40.20 ± 2.55; [c_0 P-PO_4 (mg L^{-1})] = 18.82 ± 0.44; [c_0 Ca (mg L^{-1})] = 11.70 ± 1.17; [c_0 K (mg L^{-1})] = 9.56 ± 0.66. Significantly different values are: A—compared to concentration 0.13 mg L^{-1}; B—compared to concentration 11.61 mg L^{-1}; C—compared to concentration 23.21 mg L^{-1}; D—compared to concentration 54.30 mg L^{-1}.

Experimental design In the first set of experiment, the performance of *A. junii* depended on the concentrations of Mg, Ca and K ions was tested. Using wastewater A the requirement of *A. junii* for Mg ions in the presence of Ca and K ions was tested. Using wastewater B the requirement of *A. junii* for Ca ions in the presence of Mg and K ions was tested. Using wastewater C the requirement of *A. junii* for K ions in the presence of Mg and Ca ions was tested. Cells of *A. junii* were pregrown on a nutrient agar plates for 24 h at 30 ± 0.1 °C. The biomass was resuspended in 9 mL of sterile 0.3% NaCl solution and 1 mL of suspension was allotted to each flask containing 100 mL of sterile synthetic wastewater. The flasks were sealed and incubated for 72 h at 30 ± 0.5 °C/70 rpm in a water bath with shaker (Memmert WNB22). An aeration rate of 1 L per min with filtered air was provided. The incubation time of 72 h was chosen according to the preliminary experiment with wastewaters A, B and C, where *A. junii* reached the stationary phase of growth after 72 h of incubation.

In the second set of experiment, the natural minerals were tested as a source of Mg, Ca and K ions for *A. junii*. In these experiments the wastewater D with shortage of Mg, Ca, K ions and trace metals was used. Cells of *A. junii* were inoculated in flasks as above described. In each flask a 1.0 g of mineral was added. The control flask contained solely bacteria, with no mineral added. The flasks were incubated in the same conditions as above described. In order to determine the concentration of exchangeable cations (Mg, Ca and K) available from natural minerals, a 1.0 g of mineral was treated in 100 mL of wastewater D. Erlenmeyer flasks were incubated for 72 h at 30 ± 0.5 °C/70 rpm in a water bath with shaker. The measured concentrations of Mg, Ca and K ions released in water were taken as the minimum available for bacteria.

In the third set of experiment, the influence of Mg, Ca, K ions and trace metals on the performance of bioaugmented activated sludge was tested. The fresh activated sludge was obtained from the aeration tank of a municipal wastewater treatment plant and acclimatized for 10 days to wastewater E. The activated sludge was centrifuged (2000 rpm/3 min), washed with sterile 0.3% NaCl and bioaugmented with *A. junii* before the experiments were to commence. The bioaugmented activated sludge was inoculated in 500 mL of wastewater D, wastewater D with addition of 1% of mineral TMg and wastewater E. The flasks were incubated at 22 ± 0.5 °C in the same conditions as above described.

Analytical methods Water samples for the measurements of P and exchangeable ions (Mg, Ca and K) were filtered through Whatman filter units of pore diameter 0.2 μm. The P ($P\text{-}PO_4^{3-}$) concentration in water was measured spectrophotometrically in a DR/2500 Hach spectrophotometer by the molybdovanadate method (Hach method 8114). The concentrations of K^+, Ca^{2+} and Mg^{2+} were determined by atomic absorption spectrometry (AA-6800, Shimadzu). The pH value was measured with WTW 330 pH-meter. For measurements of the zeta potential a 0.01 g of mineral was dispersed in 50 mL of demineralized water. The samples were allowed to stand for 5 min to let the larger particles to 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 number of viable bacterial cells was determined as colony-forming units (CFUs) grown on the nutrient agar after incubation at 30 ± 0.1 °C for 24 h. Planktonic cells were determined by serial dilutions (10^{-1} to 10^{-8}) of 1 mL of supernatant sample and inoculations of nutrient agar plates. In order to determine the number of immobilized cells, each carrier was taken from the flask, washed three times with 300 mL of sterile 0.3% NaCl 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 (16) detaches the immobilized cells from the carrier, so that they remain as individual cells in the suspension. From such suspension serial dilutions were made and nutrient agar plates were inoculated and incubated as already described. After incubation, the bacterial colonies were counted and reported as CFUs per 1 gram of dry carrier. The Neisser stain was

performed to confirm polyP granules in cells of *A. junii*. A direct microscopy (Axiovert 200 MAT; Carl Zeiss MicroImaging, Inc.) was also performed to confirm the immobilization of cells onto the carriers. Mixed liquor suspended solids (MLSS) were determined after drying at 105 °C for 1 h. Sludge volume index (SVI) was calculated after 30 min of sludge settlement. All measurements were done in triplicate.

Statistical analyses Statistical analyses were carried out using Statistica Software 8.0. The numbers of bacterial CFU were logarithmically transformed beforehand to normalize distribution and to equalize variances of the measured parameters. The inter-group comparisons between samples were done using the one-way analysis of variance (ANOVA) and subsequently the post-hoc Duncan test was used 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 $p<0.05$.

RESULTS AND DISCUSSION

The results of the influence of varying concentrations of Mg (in conditions of Ca and K availability) on the formation of biomass and P removal of *A. junii* are presented in Table 2. The final pH values varied from 6.57 in reactor with no Mg addition up to 7.35 in reactor containing the highest concentration of Mg. In the conditions of severe Mg shortage, a decay of bacteria was obtained, while P removal was negligible. By increasing the initial Mg concentration in wastewater to 11.61 mg L^{-1} the formation of new bacterial biomass was obtained, which increased further by increasing the Mg concentration. The P uptake rates per CFU of *A. junii* also increased by increasing the initial Mg concentration in wastewater. The microscopic photographs (Figs. 1A, B) showed the poor bacterial biomass and lack of polyP granules inside the cells of *A. junii* in shortage of Mg and good bacterial biomass formation and the presence of poly P granules in excess of Mg. Good performance of the pure culture of *A. junii* was obtained by influent Mg/P ratio of 0.64 to 5.71 mg Mg/mg P, while negligible P removal was obtained by Mg/P ratio of 0.01. The Mg ions were necessary for the successful P removal from wastewater by providing the formation of biomass of *A. junii* and increasing the P uptake rates per CFU of *A. junii*. This observation is in agreement with the previous one (13), where the

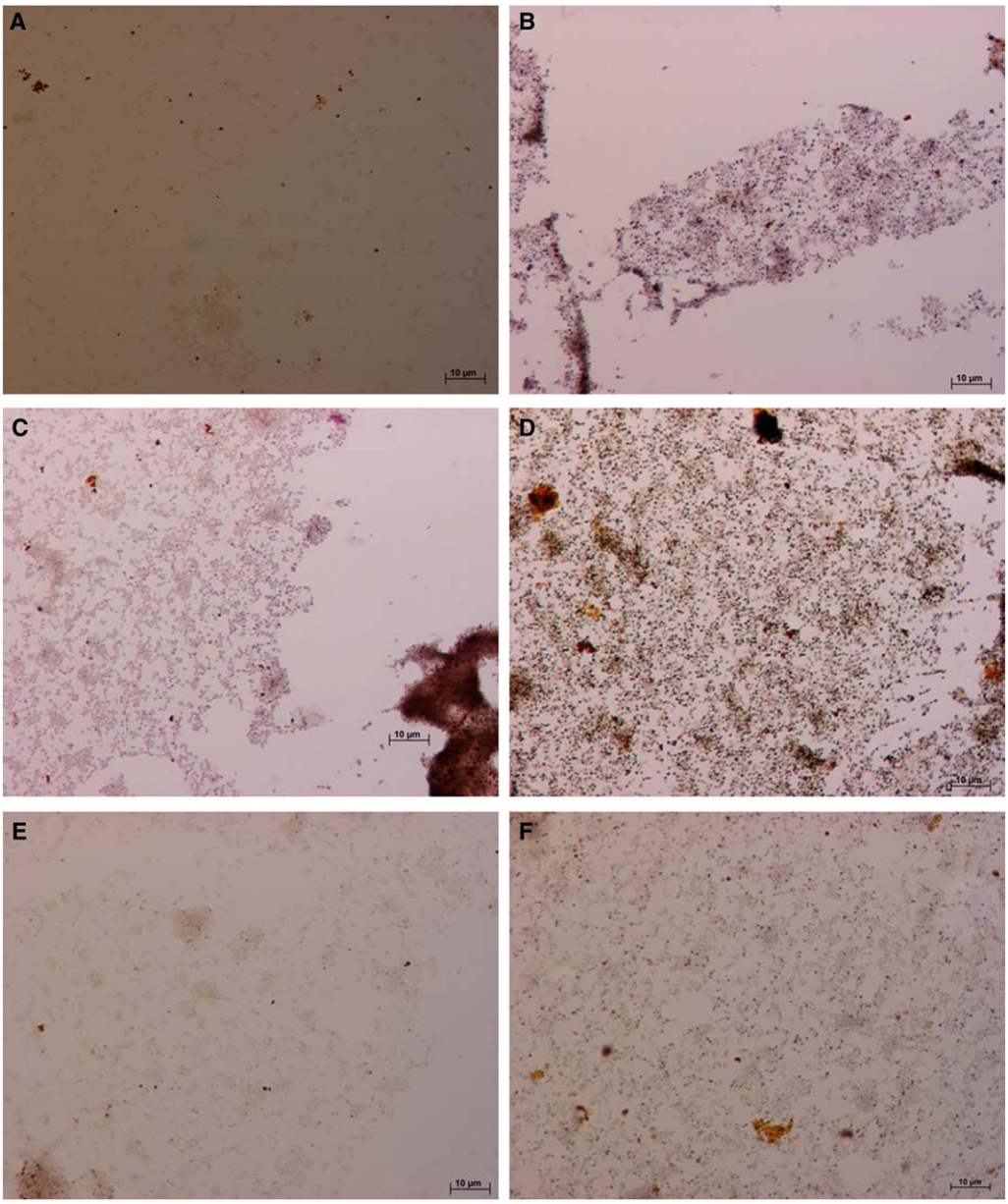

FIG. 1. Dark blue polyphosphate granules after Neisser staining inside the cells of *A. junii* in shortage (left column) and in excess (right column) of magnesium, calcium and potassium.

increased concentration of Mg ions originating from natural zeolitized tuff increased the yield of biomass of PAB *A. calcoaceticus* in pure culture, which resulted in better P removal from wastewater. The required Mg concentration for *A. junii* in our experiments was 11.61 mg L^{-1}, which is close to the 8 mg L^{-1} reported for the EBPR activated sludge (17). By increasing the initial Mg concentration from 11.61 to 23.21 mg L^{-1} the increase of percentage of P removal was (although statistically significant) only about 5% higher. This observation is in accordance with the study of Schonborn et al. (12), where the concentration of Mg in influent wastewater was 15 mg L^{-1} and the increase of Mg in influent up to 31 mg L^{-1} did not enhance the maximum values of P removal than it improved daily fluctuations of P removal.

The results of the influence of varying concentrations of Ca (in conditions of Mg and K availability) on the multiplication and metabolism of *A. junii* are presented in Table 3. The final pH values increased from 7.20 in the reactor with no Ca addition up to 7.57 in reactor with the highest Ca concentration. The number of final CFU and ratio of cell multiplication increased when increasing the Ca concentration in wastewater. The P uptake rates per CFU of *A. junii* were not statistically significantly different among reactors with different initial Ca concentrations. The microscopic photographs (Figs. 1C, D) showed good bacterial biomass formation and presence of polyP granules inside the cells of *A. junii* in shortage of Ca, which was pronounced in the excess amount of Ca. Appropriate performance of the pure culture of *A. junii* was obtained by influent Ca/P ratio of 0.01 to 5.55 mg Ca/mg P. The shortage of Ca in wastewater (in the presence of Mg and K) did not negatively influence the formation of bacterial biomass and as a consequence the P removal from wastewater. However, the presence of increased concentrations of Ca had the positive influence on the performance of pure culture of *A. junii*. Rickard & McClintock (18) reported that EBPR worked neither without Mg nor K, while Ca was not essential for the process. The result of this study agrees with this statement, since a good multiplication of *A. junii* and appropriate P removal was obtained in reactor with the Ca content contained in deionized water.

The results of the influence of varying concentrations of K (in conditions of Mg and Ca availability) on the *A. junii* are presented in Table 4. The final pH values varied from 7.31 in the reactor with no K addition up to 7.71 in reactor with the highest K concentration. In the conditions of severe shortage of K a decay of bacteria was obtained, while P removal was negligible. By increasing the initial K concentration in wastewater to 9.53 mg L^{-1} the formation of bacterial biomass was obtained, which increased by increasing the K concentration. The P uptake rates per CFU of *A. junii* significantly increased by increasing the initial K concentration from 0.27 to 9.53 mg L^{-1} and stayed constant by further increase of initial K concentration. The microscopic photographs (Figs. 1E, F) showed the poor bacterial biomass and lack of polyP granules inside the cells of *A. junii* in shortage of K and good bacterial biomass formation and the presence of poly P granules in excess of K. The K consumption in reactors containing the influent K/P ratio of 0.01; 0.50; 1.01; 2.59 and 4.87 mg K/mg P was 0.27; 0.29; 0.37; 0.44 and 0.63 mg K/mg P, respectively. The obtained consumption of K was close to those obtained for activated sludge with EPBR characteristics, where at influent K/P ratio of 1.25 and 2.5 the consumption of K was 0.21 and 0.47 mg K/mg P, respectively (19). Good performance of the pure culture of *A. junii* was obtained by K/P ratio of 0.50 to 4.87, while negligible P removal was obtained by K/P ratio of 0.01. The K ions were necessary for the formation of biomass of *A. junii* and successful P removal from wastewater. In the EBPR system (19) exposed to severe shortage of K (0.013 mg K L^{-1} and K/P ratio of 0.0009 mg K/mg P), but in the presence of Mg and Ca in the influent the P removal was absent, while the complete P removal was achieved when excess amounts of K were present (18.9 and 37.8 mg K L^{-1} corresponding to K/P ratio of 1.25 and 2.5, respectively). The results of this study are in agreement with this observation that K is an essential micronutrient required for successful P removal from wastewater and that the excess amounts of K have the positive influence on the performance of system.

From Tables 2–4 it can be seen that the increase of Mg, Ca and K ions resulted in a significant increase of P removal from wastewater. According to statistical analysis, the percentage of P removal showed much better correlation with the number of total cells of *A. junii* ($R = 0.942$) than with the P uptake rates ($R = 0.387$). The P removal from wastewater was more the function of increased bacterial biomass than the increased P uptake rates. It can be concluded that the addition of Mg, Ca and K ions enhanced the growth of *A. junii* and as a result increased the P removal from wastewater. The Mg and K were required for the formation of new bacterial biomass while Ca was not.

The zeta potential of minerals and the concentration of released Mg, Ca and K ions after 72 h of contact with synthetic wastewater D are presented in Table 5. All 9 minerals tested had negative zeta potential. The zeta potential of K exchanged samples was significantly more negative than the Mg or Ca exchanged counterparts. Less negative zeta potentials obtained for the Ca and Mg exchanged counterparts are explained by the greater valence of these divalent cations when compared to monovalent K ions (20,21). The main exchangeable cation in all three tested original mineral samples (T, S and TER) was Ca. The ion exchange process was successful to obtain mineral with dominance of Mg, Ca or K. The differences in concentrations of Mg, Ca and K ions released from examined minerals in wastewater D are explained by different cation selectivity of minerals. Namely the affinity of clinoptilolite (minerals T and S) towards investigated cations is in

TABLE 3. Number of total cells (CFU), ratio of cell multiplication, phosphate (P) uptake rate per CFU and percentage of P removal in reactors containing wastewater B with different initial concentrations of calcium.

Initial Ca concentration (mg L^{-1})	Wastewater B			
	Total cells (10^9 CFU L^{-1})	CFU final/ CFU initial	P uptake rate (10^{-11} mg P CFU^{-1})	P removal (%)
0.16	53.33 ± 4.51	1.38 ± 0.06	4.52 ± 0.38	12.53 ± 1.62
12.41	61.17 ± 3.55	1.46 ± 0.02	4.65 ± 0.27	15.37 ± 1.41
24.64	$126.33 \pm 6.66^{A,B}$	$3.41 \pm 0.04^{A,B}$	4.76 ± 0.26	$31.91 \pm 1.44^{A,B}$
57.02	$155.67 \pm 3.21^{A,B,C}$	$4.11 \pm 0.17^{A,B,C}$	4.77 ± 0.10	$39.78 \pm 1.79^{A,B,C}$
104.90	$339.67 \pm 8.62^{A,B,C,D}$	$8.90 \pm 0.11^{A,B,C,D}$	4.83 ± 0.12	$86.76 \pm 1.77^{A,B,C,D}$

[c_0 CFU (10^9 CFU L^{-1})] = 38.73 ± 2.18; [c_0 P-PO_4 (mg L^{-1})] = 18.78 ± 0.08; [c_0 Mg (mg L^{-1})] = 12.30 ± 1.07; [c_0 K (mg L^{-1})] = 9.28 ± 0.48. Significantly different values are: A-compared to concentration 0.16 mg L^{-1}; B-compared to concentration 12.41 mg L^{-1}; C-compared to concentration 24.64 mg L^{-1}; D-compared to concentration 57.02 mg L^{-1}.

TABLE 4. Number of total cells (CFU), ratio of cell multiplication, phosphate (P) uptake rate per CFU and percentage of P removal in reactors containing wastewater C with different initial concentrations of potassium.

Initial K concentration (mg L^{-1})	Wastewater C			
	Total cells (10^9 CFU L^{-1})	CFU final/ CFU initial	P uptake rate (10^{-11} mg P CFU^{-1})	P removal (%)
0.27	25.00 ± 4.36	0.62 ± 0.08	2.86 ± 0.55	3.56 ± 1.07
9.53	51.33 ± 4.39^{A}	1.38 ± 0.03^{A}	5.49 ± 0.50^{A}	14.73 ± 1.28^{A}
18.96	$62.33 \pm 4.16^{A,B}$	1.56 ± 0.01^{A}	5.63 ± 0.37^{A}	$18.62 \pm 1.77^{A,B}$
48.92	$86.67 \pm 7.02^{A,B,C}$	$2.22 \pm 0.06^{A,B,C}$	5.79 ± 0.47^{A}	$26.42 \pm 1.50^{A,B,C}$
96.38	$129.67 \pm 2.52^{A,B,C,D}$	$3.51 \pm 0.21^{A,B,C,D}$	5.86 ± 0.11^{A}	$38.35 \pm 0.66^{A,B,C,D}$

[c_0 CFU (10^9 CFU L^{-1})] = 38.67 ± 2.73; [c_0 P-PO_4 (mg L^{-1})] = 19.00 ± 0.65; [c_0 Ca (mg L^{-1})] = 10.83 ± 1.75; [c_0 Mg (mg L^{-1})] = 11.16 ± 1.42. Significantly different values are: A—compared to concentration 0.27 mg L^{-1}; B—compared to concentration 9.53 mg L^{-1}; C—compared to concentration 18.92 mg L^{-1}; D—compared to concentration 48.98 mg L^{-1}.

TABLE 5. Zeta potential of minerals and concentration of released Mg, Ca and K ions in wastewater D with shortage of Mg, Ca, K and trace metals.

Mineral	Zeta potential (mV)	Wastewater D		
		Mg^{2+} (mg L^{-1})	Ca^{2+} (mg L^{-1})	K^{+} (mg L^{-1})
T Mg	-13.90 ± 0.91	10.01	55.13	0.30
T Ca	-13.86 ± 1.93	3.75	64.17	0.21
T K	$-20.06 \pm 0.90^{A,B}$	4.93	5.71	130.99
S Mg	-12.88 ± 1.43	15.85	30.17	0.25
S Ca	-13.38 ± 0.61	4.41	53.81	0.26
S K	$-16.42 \pm 1.93^{A,B}$	10.62	14.24	116.17
TER Mg	-11.92 ± 2.56	16.24	1.12	10.06
TER Ca	-9.16 ± 1.02^{A}	1.43	21.67	12.08
TER K	$-22.46 \pm 0.54^{A,B}$	1.54	6.49	162.37

Significantly different values are: A—compared to exchange with Mg; B—compared to exchange with Ca.

the order $K^{+}>Ca^{2+}>Mg^{2+}$ (22), while the affinity of bentonite (mineral TER) is in the order $Ca^{2+}>Mg^{2+}>K^{+}$ (23).

The influence of addition of minerals to the wastewater D with shortage of Mg, Ca, K and trace metals is presented in Table 6. In the control reactor without addition of mineral the P removal was absent due to the total decay of bacterial population. This suggests the essential requirement of *A. junii* for trace metals and Mg, Ca and K ions. The final pH values varied from 7.26 to 7.98 in reactors containing minerals and averaged 6.18 in control reactor. Since *A. junii* grows in the pH range from 6.0 to 8.0, the pH can be eliminated as a cause of bacterial decay.

Clinoptilolite tuffs (mainly saturated with K^{+} or NH_4^{+}) are widely used in the field of agronomic and horticultural applications for the purpose of its slow-release fertilization or a combination of ion exchange and mineral dissolution reactions (24). All minerals tested in this study successfully replaced the lack of trace metals, Mg, Ca and K and allowed the formation of metabolically active bacterial population. The performance of bacterial culture was dependent on the type of mineral added and the type of dominant cation in mineral. The performance of bacterial culture in reactors containing minerals was dependent on the number of total CFU, which showed a significantly positive correlation with the percentage of P removal from wastewater ($R=0.888$). The best P removal from wastewater was achieved using mineral T, followed by S and TER and using the Mg exchanged minerals when compared to their counterparts.

The bacterial population in reactors with addition of mineral consisted of planktonic cells in supernatant and immobilized cells on minerals as carriers. In cases of all minerals tested, the bacterial population immobilized onto mineral carriers was numerous to the number of planktonic cells. The extent of bacterial immobilization was primarily dependent on the type of mineral and to a lesser extent on the type of ion exchange. Much better immobilization of bacteria was obtained on T and TER than on S, regardless of the type of ion dominant in mineral. The highest immobilization was obtained with Mg exchanged minerals when compared to the Ca and K exchanged counterparts. The zeta potential of minerals did not show the positive correlation with the number of immobilized cells ($R=-0.014$). The minerals which showed the highest (TMg, 9.61×10^{9} CFU/g) and lowest (SCa, 0.93×10^{9} CFU/g) extent of bacterial immobilization had the similar zeta potential (Table 5). The *A. junii* which had the negative zeta potential of −18.4 mV in the log phase to −21.3 mV in the stationary phase of growth (25) was successfully immobilized onto all negatively charged minerals. This indicates that the key factor for the immobilization of *A. junii* onto mineral is the original structure of mineral. This observation is in agreement with literature data which suggested that the zeta potential is not the primary factor which determined the immobilization of *Streptococcus sanguis* and *Actinomyces naeslundii* to apatite minerals (26) or *A. junii* to natural zeolite tuff and bentonite (15).

The interpretation of the influence of Mg, Ca and K concentrations originating from minerals on *A. junii* was complicated (when compared to the experiments where these micronutrients were added in form of pure chemicals) due to the continuous release of exchangeable ions, which was dependent on the original structure of mineral and the combination of these ions in mineral. The real concentration of these micronutrients is hard to follow in this system, since the ions released form minerals will be simultaneously consumed by bacteria. It is obvious that both planktonic and immobilized cells in shortage of Mg, Ca and K were dependent on the availability of exchangeable ions contained in minerals. The planktonic cells were dependent on the concentration of exchangeable ions released from minerals in wastewater. The number of planktonic cells correlated significantly positive ($R=0.903$) with the concentration of Mg released from minerals in wastewater (Table 5), but not significantly with the concentration of Ca ($R=0.261$) and K ($R=-0.194$). This observation is consistent with the experiments where Mg, Ca and K were added in form of pure chemicals. Namely, the concentrations of Mg released from minerals (1.43–16.24 mg L^{-1}, Table 5) were in the critical range required for normal performance of *A. junii*, while concentrations of Ca released from minerals (1.12–64.17 mg L^{-1}, Table 5) had no negative influence on *A. junii* and concentrations of K released from minerals (0.21–162.37 mg L^{-1}, Table 5) were in the extreme range from those below the required concentrations to the excess concentrations.

The number of immobilized cells did not show significantly positive correlation with the concentrations of Mg ($R=0.270$), Ca ($R=0.338$) or K ($R=0.087$) released in wastewater. This is explained by the fact that immobilized cells were in close contact with minerals and can consume these micronutrients directly from minerals,

TABLE 6. Number of immobilized, planktonic and total cells (CFU), ratio of immobilized and planktonic cells, ratio of cell multiplication, phosphate (P) uptake rate per CFU and percentage of P removal in reactors containing wastewater D and different minerals exchanged with Mg, Ca or K.

Mineral	Wastewater D						
	Immobilized cells (10^{9} CFU g^{-1})	Planktonic cells (10^{9} CFU L^{-1})	Total cells (10^{9} CFU L^{-1})	CFU immobilized/ CFU planktonic	CFU final/ CFU initial	P uptake rate (10^{-11} mg P CFU^{-1})	P removal (%)
Control	–	0	0*	–	0	0	0.72 ± 0.01
T Mg	9.61 ± 0.39	43 ± 6	139 ± 2	562.17 ± 50.87	11.59 ± 0.14	14.52 ± 0.18	98.54 ± 0.68
T Ca	4.83 ± 0.30^{A}	36 ± 4^{A}	84 ± 7^{A}	342.81 ± 55.31^{A}	5.98 ± 0.47^{A}	14.07 ± 1.09	49.06 ± 0.43^{A}
T K	$7.67 \pm 0.72^{A,B}$	36 ± 4^{A}	$113 \pm 11^{A,B}$	580.83 ± 54.57^{B}	$9.39 \pm 0.95^{A,B}$	$9.23 \pm 0.94^{A,B}$	$52.70 \pm 1.71^{A,B}$
S Mg	1.56 ± 0.11	114 ± 6	130 ± 5	34.21 ± 0.76	9.97 ± 0.35	12.28 ± 0.43	74.30 ± 0.49
S Ca	0.93 ± 0.77^{A}	57 ± 3^{A}	66 ± 2^{A}	40.70 ± 1.36^{A}	5.10 ± 0.16^{A}	12.23 ± 0.38	33.61 ± 0.39^{A}
S K	1.51 ± 0.1^{B}	$81 \pm 6^{A,B}$	$96 \pm 5^{A,B}$	$44.52 \pm 3.34^{A,B}$	$8.69 \pm 0.48^{A,B}$	$11.41 \pm 0.62^{A,B}$	$53.70 \pm 0.37^{A,B}$
TER Mg	7.24 ± 0.87	79 ± 4	151 ± 4	228.83 ± 15.21	12.62 ± 0.37	7.33 ± 0.22	54.15 ± 0.75
TER Ca	6.94 ± 0.39	30 ± 8^{A}	99 ± 12^{A}	604.45 ± 126.02^{A}	8.99 ± 1.06^{A}	10.95 ± 1.29^{A}	44.70 ± 0.13^{A}
TER K	6.29 ± 0.31^{A}	$21 \pm 2^{A,B}$	$83 \pm 5^{A,B}$	$827.39 \pm 40.09^{A,B}$	8.34 ± 0.52^{A}	$9.01 \pm 0.56^{A,B}$	$36.41 \pm 0.25^{A,B}$

[c_0 CFU (10^{9} CFU L^{-1})] = 11.90 ± 1.21; [c_0 P-PO_4 (mg L^{-1})] = 20.63 ± 1.71; [c_0 Mg (mg L^{-1})] = 0.14 ± 0.03; [c_0 Ca (mg L^{-1})] = 0.19 ± 0.02; [c_0 K (mg L^{-1})] = 0.26 ± 0.04.
Significantly different values are: A—compared to exchange with Mg; B—compared to exchange with Ca. *$<10^{3}$ CFU L^{-1}.

TABLE 7. Performance of activated sludge bioaugmented with *A. junii* in wastewater D, wastewater D with addition of mineral TMg and wastewater E.

Parameter	Wastewater D	Wastewater D + TMg	Wastewater E
Immobilized cells (10^9 CFU g^{-1})*	–	3.49	–
Planktonic cells (10^9 CFU L^{-1})*	0.25	22.80	28.55
Total cells (10^9 CFU L^{-1})*	0.25	26.29^{A}	$28.55^{A,B}$
CFU immobilized/CFU planktonic	–	382	–
CFU final/CFU initial	0.01	0.89^{A}	$1.06^{A,B}$
P uptake rate (mg P g^{-1} MLSS)	0.50	1.87^{A}	$2.96^{A,B}$
P removal (%)	8.58	33.41^{A}	$57.25^{A,B}$
MLSS (g L^{-1})	3.51	3.77^{A}	$4.01^{A,B}$
SVI (mL g^{-1})	68.38	57.36^{A}	58.95^{A}
Final pH	5.78	6.75^{A}	$7.73^{A,B}$

[c_0 CFU (10^9 CFU L^{-1})] = 25.82 ± 4.59; [c_0 P-PO_4 (mg L^{-1})] = 20.73 ± 0.35; [c_0 MLSS (g L^{-1})] = 3.61 ± 0.02; [c_0 SVI (mL g^{-1})] = 59.19 ± 0.97. Significantly different values are: A—compared to wastewater D; B—compared to wastewater D + TMg. * CFU refers to *A. junii*.

without a need that they be released from minerals in wastewater. The number of immobilized cells on Mg exchanged minerals (Table 6) was close to the number of immobilized *A. junii* on the same minerals (9.52×10^9 CFU g^{-1} for TMg, 1.88×10^9 CFU g^{-1} for SMg and 7.40×10^9 CFU g^{-1} for TERMg) in wastewater containing the salts of Mg, Ca and K, peptone and yeast extract (15). This suggests that the bacterial immobilization is a function of mineral property and not the composition of water medium used for cultivation.

When analysing the performance of single mineral, it is evident that with TCa and SCa (which supplied the highest concentration of Ca with Mg and K below the established optimal concentrations) the poorest multiplication of *A. junii* and percentage of P removal were obtained. Also in case of TERMg addition (where a minimum concentration of Ca was present with Mg and K close to the established optimal concentrations) the best performance of bacterial culture was observed. In reactors containing TMg, TCa, SMg and SCa the K ions were available in traces (Table 5), but this did not disturb the performance of bacterial culture. In these reactors it seems that the *A. junii* was more influenced by the available Mg concentration. Considering all influences of concentrations of exchangeable ions originating from minerals on *A. junii*, the beneficial influence of these ions was in the order Mg>K>Ca.

It can be summarized that in shortage of Mg, Ca and K in wastewater, all 9 tested minerals successfully replaced the lack of these micronutrients and allowed the formation of metabolically active bacterial cells. With the addition of minerals, the P uptake rates were always significantly higher than those observed in experiments with wastewaters B, C and D. A better efficiency of P removal in reactors with mineral addition was the result of bacterial metabolism, since the pure minerals did not adsorb more than 2.5% of P from wastewater. From the practical aspect, it should be emphasised that addition of eight minerals resulted in the percentage of P removal higher than those maximum obtained with addition of Mg salt, one mineral achieved higher P removal than those maximum obtained with addition of Ca salt and seven minerals achieved higher P removal than those maximum obtained with addition of K salt.

The influence of Mg, Ca, K and trace metals on the performance of activated sludge bioaugmented with *A. junii* is shown in Table 7. In wastewater D with shortage of ions a decay of *A. junii* and activated sludge was obtained, resulting in low P removal (8.58%). Addition of mineral TMg to the wastewater D resulted in significantly higher number of *A. junii*, higher concentration of activated sludge measured as MLSS, better settlement of activated sludge indicated as lower SVI and better removal of P from wastewater (33.41%). Better performance of pure culture of *A. junii* obtained in the wastewater D containing the mineral TMg (Table 6) than in bioaugmented activated sludge is explained by the competition of *A. junii* with other microorganisms present in activated sludge at lower temperature of incubation. In wastewater E with excess of ions the highest biomass of *A. junii* and activated sludge was developed, resulting in the best removal of P from wastewaters (57.25%). In the experiments with bioaugmented activated sludge the removal of P from wastewater was dependent on the number of *A. junii* ($R = 0.800$) and P uptake rates of activated sludge ($R = 0.998$). These preliminary experiments showed that the requirement for Mg, Ca and K ions determined in this study is applicable to the real conditions of wastewater treatment, which should be further investigated.

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