

Biosorption of phosphate from synthetic wastewater by biosolids

Research Article

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Abstract: The aim of this study was to determine the potential of phosphate (P) removal from wastewater by biosolids prepared by the immobilization of P-accumulating bacteria onto organic bentonite. Organic bentonite was prepared by treating bentonite clay with quaternary ammonium salt – cetyltrimethylammonium (CTA) bromide. Cation exchange capacity (CEC) of the bentonite was found to be 179.0 meq/100 g of the dry bentonite. The CTA occupied ca. 175% of the CEC. Modification of bentonite with CTA in amounts higher than 55% of the CEC resulted in the change of zeta potential of particles from negative to positive. Only in reactors containing organic bentonite samples occupied with 3.5 and 28% of the CEC was P efficiently removed from wastewater by combined adsorption and bacterial uptake in the biomass. Organic bentonite samples with higher CTA loadings (from 55 to 175% of the CEC) showed bactericidal effects. To enhance P removal from wastewater in the aerated biological system, biosolids consisting of P-accumulating bacteria and organic bentonite can be used, but special attention should be given to the configuration of sorbed CTA molecules and its potential desorption.

Keywords: Bacteria • Bentonite • Biosolids • Phosphate • Wastewater

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1. Introduction

Clay minerals are comprised of small crystalline particles that are formed from silica tetrahedral sheets and from aluminium or magnesium octahedral sheets. Quaternary ammonium cations such as cetyltrimethylammonium (CTA) interact with clays and replace the exchangeable inorganic cations. As a result of the larger size of the CTA cations compared to the size of the replaced cations, the interlamellar distance of the mineral increases and additional space is produced in the particles, facilitating the attraction of other organic compounds [1,2]. Studies with CTA-modified clays showed that they sorbed not only nonpolar organic species but also oxyanions [3].

The activated sludge treatment is the most common way for the purification of wastewater. The process of enhanced biological phosphorus removal from wastewater is based on the metabolic activity of P-accumulating bacteria present in the activated sludge. These bacteria transport extracellular P into the cell and convert it into nonsoluble poly-P [4-6]. Bacteria from the genus *Acinetobacter* have become the model organism for enhanced biological phosphorus removal since it was isolated from the P-removing activated sludge plant [4]. Although *Acinetobacter* spp. was present in extremely low numbers in the activated sludge plant, their capacity to remove P was the highest among all of the P-accumulating isolates [5].

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By the adsorption of P onto CTA-modified clay, larger amounts of P can be removed from wastewater. But, as soon as maximum capacity of the clay is reached, no further retention will occur [7]. Therefore, there is a need to transform/degrade the soluble P present in the wastewater by other mechanisms. Currently, attention is being drawn to the immobilization of P-accumulating bacteria onto a suitable material in order to achieve a higher cell density in bioreactors, and as a result, the better efficiency of the wastewater treatment process [6,8-10].

There appears to be much opportunity for combining the adsorption of P on CTA-modified clays with bacterial accumulation of P in order to improve the P removal from wastewater. To date, there has been no effort to apply the promising CTA-modified clays in the enhanced biological phosphorus removal process. The aim of this study was to determine the interaction of the CTA-modified bentonite (OB) and P-accumulating bacteria from the standpoint of basic science and possible application.

2. Experimental Procedures

2.1 Bentonite

The natural bentonite from Ciba Specialty Chemicals Inc. (NB) was primarily in sodium form, with montmorillonite purity more than 95%. The cation exchange capacity (CEC) of the clay was measured by ammonium acetate procedure [11] and was found to be 179.0 meq/100 g of dry clay. The chemical composition of the NB estimated by the classical scheme of analysis is: 59.5% SiO₂, 11.31% Al₂O₃, 2.23% Fe₂O₃, 3.48% Na₂O, 0.17% K₂O, 2.80% CaO, 2.86% MgO and 18.47% loss of ignition.

2.2 Bacterium

A P-accumulating bacterium *Acinetobacter junii* (DSM 1532) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [6].

2.3 CTA sorption onto NB and preparation of OB

The OB samples were obtained by replacing natural inorganic exchangeable cations of the Na-bentonite with CTA cations through ion exchange reaction. For the synthesis a cationic surfactant, quaternary ammonium salt cetyltrimethylammonium bromide, C₁₆H₃₃N(CH₃)₃Br (Merck) was used. The critical micelle concentration of CTA at 30°C was 0.94 mmol/L as estimated by measuring the conductivity of aqueous CTA solutions of different concentrations. The CTA solutions were prepared with

deionised water. The concentrations of CTA were from 0.91 to 53.5 mmol/L, i.e. above and below the critical micelle concentration. 100.0 ml of the CTA solution and 1.0 g of the NB were shaken on a mechanical shaker (Biosan OS-10) at 150 rpm during 48 h in a thermostat (Mettmert IPP400) at 30.0±0.1°C (above Kraft point, which is 25°C). Thereafter, 20 ml of each mixture was centrifuged (3000 rpm for 5 min) and the supernatant was analysed for residual CTA. The concentration of sorbed CTA was calculated from the mass balance equation as follows:

$$Q_e = (c_0 - c_e) \frac{v}{m}$$

where c_0 and c_e are the initial and equilibrium liquid-phase concentrations of CTA solution (mmol/L), respectively; v the volume of surfactant solution (L), and m the mass of bentonite (g).

The obtained OB were filtered through the Büchner funnel and filter paper (blue band). The samples were washed with distilled water until negative reaction to Br⁻ ions was reached and air dried. Large clumps of the OB were pulverized with a mortar and pestle.

2.4 Zeta potential of NB, OB and bacteria

The zeta potential of the NB and OB was measured using a Zetasizer 3000- Malvern Instruments, equipped with microprocessor unit. This unit automatically calculates the electrophoretic mobility of the particles and converts it to the zeta potential using the Smoluchowski equation. 0.010 g of dry OB sample was dispersed on the mechanical shaker (Biosan OS-10) at 200 rpm/15 min in 50 ml of distilled water. The samples were allowed to stand for 5 min to let larger particles settle. An aliquot taken from the supernatant was used to measure zeta potentials. The zeta potential of bacterium *A. junii* was measured as previously described [12]. The procedure was repeated three times for each measurement.

2.5 Synthetic wastewater

The composition of the synthetic medium used to simulate the sewage was (in mg L⁻¹ of distilled water): Na-propionate 300; peptone 100; MgSO₄ 10; CaCl₂ 6; KCl 30; yeast extract 20; KH₂PO₄ 132. The pH of the synthetic wastewater was adjusted to 7.00±0.02 with 1 M NaOH or 1 M HCl before autoclaving (121°C/15 min).

2.6 Experimental design

Dry particles of the chosen OB were again washed 3 times in the membrane filtration system with 300 ml of deionised water. The wet material was used for experiments. After experiments the particles were

dried (105°C/24 h) and all results were calculated per dry mass of OB.

Experiments were carried out with NB/OB only and with NB/OB and bacteria, in order to define the amount of P removed by sorption onto bentonite particles or by the combined action of bacterial uptake and sorption. Bacteria were pre-grown on the nutrient agar (Biolife, Italy) for 20 h at 30.0±0.1°C. Thereafter the biomass was placed in sterile 0.3% NaCl and dispersed by shaking (2700 rpm/2 min using the test tube shaker Kartell TK3S). One ml of dispersed biomass was inoculated into 100 ml of synthetic wastewater. In each flask 0.5 g of the natural or OB was added. The flasks were sealed with a sterile gum cap and thereafter aerobically agitated (70 rpm) in a water bath (Memmert WNB 7-45) controlled with a thermostat (30.0±0.1°C). The aeration rate of 1 L/min with filtered air was provided during the 24 h of experiment. All experiments were carried out as triplicate tests.

2.7 Analytical methods

Phosphate and CTA measurements were done after filtering the water samples (50 ml) through Whatman filter units with a pore diameter of 0.2 µm. The P (P-PO_4^{3-}) concentration in water was measured spectrophotometrically in a DR/2500 Hach spectrophotometer by the molybdovanadate method (Hach method 8114). The concentration of CTA was determined in a DR/2500 Hach spectrophotometer by the direct binary complex method (Hach method 8337).

The number of viable bacterial cells was determined as colony-forming units (CFU) grown on the nutrient agar after incubation at 30±0.1°C for 72 h. At the end of experiments, the particles of NB and OB were washed three times with 300 ml of sterile 0.3% NaCl, and cell counts were performed in order to determine the number of immobilized cells. Each carrier was aseptically placed in a tube containing 9 ml of sterile 0.3% NaCl, crushed with a sterile glass rod and dispersed by shaking (2700 rpm/10 min). This procedure, similar to [13,14], removed the immobilized cells so that they remained as individual cells on the carrier (compared to the previous multilayer cell coverage of material, more than 10 µm thick), which was confirmed by microscopy. Suspensions of immobilized cells prepared in this way were serially diluted (10^{-1} to 10^{-9}). Volumes of 0.1 ml were plated (spread plate method) onto nutrient agar. After incubation, the bacterial colonies were counted and reported as CFU per one gram of dry NB and OB. At the same time, the CFU count of the supernatant was performed in order to determine the number of free cells per one ml of water. All measurements were done in triplicate with mean values presented. Neisser staining

was performed to confirm poly-P granules in cells of *A. junii* and Gram staining to confirm immobilization of the cells onto NB and OB. Stained samples were examined using an inverted microscope (Axiovert 200 MAT, Carl Zeiss MicroImaging Inc.) at magnification of 1000x.

3. Results and Discussion

3.1 CTA sorption onto NB and zeta potential

Results of the determination of CTA sorption onto NB are presented in Figure 1. The obtained amount of the CTA sorbed on the NB depended on the CTA available in aqueous solution. The maximum sorption of CTA was achieved in the solution of starting concentration 53.50 mmol/L. In this solution, 3.11 mmol CTA/g was sorbed onto NB particles which correspond to the 175% of CEC. The inorganic cations located on the surface of clay minerals can be exchanged with organic cations. For non-swelling silicates, cation exchange takes place only on the external surfaces of the basal planes. For layered silicates like bentonite which swell in water, the above phenomenon occurs also on the internal surfaces. The amount of surfactant bound by a mineral may be several times higher than its CEC. This is regarded as a structure-dependent property [15].

According to the CTA sorption isotherms, the OB chosen for future experiments were loaded with the CTA from starting solution concentrations of: 0.91, 5.19, 10.00, 20.00, 26.70, 40.00 and 53.50 mmol/L, in which CTA occupied ca. 3.5%, 28%, 55%, 110%, 140%, 160% and 175% of the CEC, and were named as OB1-7.

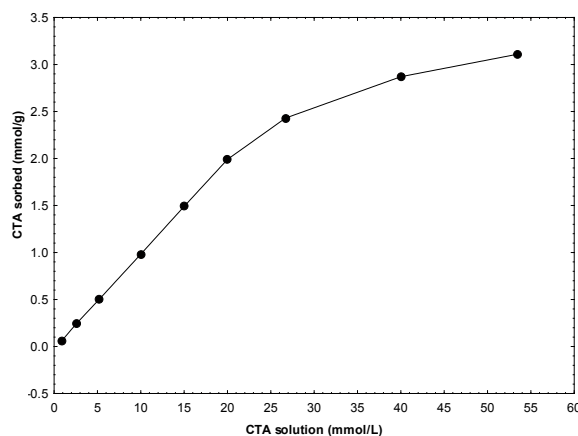


Figure 1. The CTA sorption isotherms after equilibrating the natural bentonite in different CTA solutions.

The results of measurements of zeta potential of

OB are presented in Figure 2. The zeta potential of OB stayed negative up to the sorption of 0.982 mmol CTA/g (55% of CEC) where the zeta potential was -5.5 mV. Zeta potential is a reflection of surface potential. Owing to the intrinsic heterogeneity of clay minerals, the amphiphilic character of the adsorptive and, particularly, the complicated electrostatic interactions, adsorption of ionic surfactants on clay minerals is a complex phenomenon. The concentration where hemimicelles are formed on the kaolinite surface corresponds to the sharp increase in the contact angle and decrease in the zeta potential [16]. At a slightly higher concentration the contact angle decreases again and the zeta potential reverses sign. Harwell *et al.* [17] suggested that bilayered surface aggregates are formed with charged head groups pointing outwards and called these aggregates "admicelles". For these models the surfactant aggregation at the surface plays a major role. This aggregation will be rather similar to micelle formation [18]. Scanning force microscopy results suggest that CTA added to montmorillonite locates itself not only interstitially, but also in external monolayers and, at 70% and 100% CEC additions, as external hemimicelles [19]. At the isoelectric point the surface charge is compensated by the surfactant charge. In our research the isoelectric point was achieved by ca. 70% of CEC. The modification of bentonite with higher CTA loadings resulted in the change of zeta potential of particles from negative to positive. The zeta potential of the pure culture of *A. junii* after 24 h of incubation averaged -18.4 mV. The cell surface charge of *A. junii* is comparable to the subpopulation 1 of *Enterococcus faecalis*, but less negative than subpopulation 2 of *E. faecalis* [12] or *Pseudomonas putida* [20].

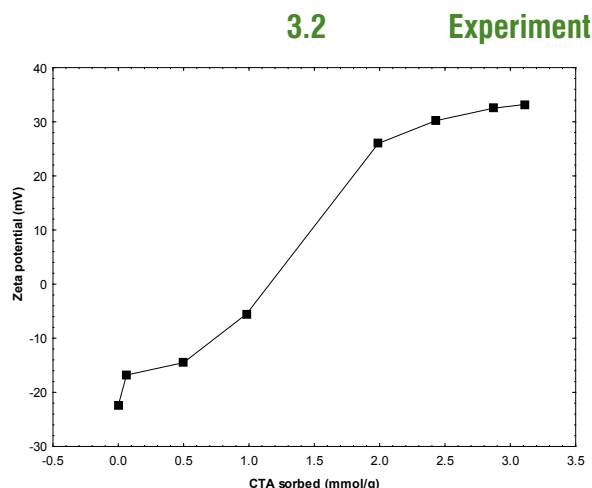


Figure 2. Zeta potential as a function of the CTA sorbed on bentonite particles.

with synthetic wastewater and NB/OB

Little is known about how OB would behave in a complex medium. In mixed solutions of sulphate, nitrate and P competitive anion sorption was reported for P sorption onto the organic zeolite [21]. The results (Table 1) showed that the addition of NB and OB resulted in the P removal from wastewater, but in variable extents. The amount of P removal increased with increasing the CTA loading on OB. The NB sorbed only 2.37% of applied P, while the samples 4-7OB showed significantly higher sorption of P. External hemimicelles and surfactant aggregates would likely be the sites for P uptake by electrostatic attraction. The CTA released into the synthetic wastewater also increased with increasing the CTA loading on OB.

CTA sorbed (mmol/g)	P removal (%)	CTA released (mmol/L)
0.000	2.37	0.00
0.063	3.81	1.37x10 ⁻³
0.500	5.95	1.92x10 ⁻³
0.982	6.43	3.57x10 ⁻³
1.990	28.74	5.21x10 ⁻³
2.430	39.41	7.41x10 ⁻³
2.870	44.43	1.10x10 ⁻²
3.110	72.32	1.48x10 ⁻²

Table 1. Performance of reactors containing organic bentonite in synthetic wastewater ($c_0 \text{ P-PO}_4 \text{ (mg/L)} = 29.87 \pm 1.43$).

3.3 Experiment with synthetic wastewater, NB/OB and bacteria

After 24 h of bacterial cultivation in reactors containing NB or OB, very different interactions were observed (Table 2). With the NB, 1OB and 2OB one part of the total cell population was immobilized onto particles by adsorptive growth (forming the biosolids), while the other part of the bacteria remained as free cells. With the 3-7OB no bacterial growth was detected. The highest number of immobilized cells was obtained with 2OB (7.78×10^9 CFU/g). This is somewhat higher than the reported 6.86×10^9 CFU/g of *A. calcoaceticus* immobilized onto the magnesium-exchanged natural zeolite [10], 5.28×10^9 CFU/g of *A. junii* immobilized onto CTA modified natural zeolite [14], 2.9×10^9 CFU/g of *Acinetobacter* spp. immobilized onto ceramics by the vacuum method [9], 2.5×10^8 CFU/g of *A. johnsonii* cells immobilized inside alginate beads [8] or 9×10^8 CFU/g of *P. aeruginosa* immobilized onto a type-Z carrier consisting of silica alumina matrix containing zeolite [13].

The total number of viable cells was higher in reactors containing 1OB and 2OB than in the reactor containing NB. In contrast, no bacterial growth was observed in reactors containing 3-7OB. The P-uptake

CTA sorbed (mmol/g)	Immobilized cells (10 ⁹ CFU/g)	Free cells (10 ⁷ CFU/ml)	Total cells (10 ⁹ CFU/ml)	P uptake rate (10 ⁻¹⁰ mg P/CFU)	P removal (%)	CTA released (mmol/L)
0.000	4.79	7.3	1.20	0.75	30.77	0.00
0.063	2.95	16.4	1.94	1.54	92.23	5.49x10 ⁻⁴
0.500	7.78	9.6	1.74	1.48	81.98	8.23x10 ⁻⁴
0.982	0.00	0.00	0.00	0.00	7.55	3.02x10 ⁻³
1.990	0.00	0.00	0.00	0.00	30.77	4.12x10 ⁻³
2.430	0.00	0.00	0.00	0.00	40.66	6.86x10 ⁻³
2.870	0.00	0.00	0.00	0.00	44.98	1.02x10 ⁻²
3.110	0.00	0.00	0.00	0.00	74.35	1.37x10 ⁻²

Table 2. Performance of reactors containing organic bentonite and bacteria in synthetic wastewater (c_0 P-PO₄ (mg/L) = 29.88±1.59; c_0 CFU (10⁶ CFU/ml) = 23.25±3.60).

rates per total CFU of *A. junii* were on average the highest in reactors containing 1OB and 2OB than NB. Neisser staining followed by microscopic examination revealed the presence of poly-P granules in the cells of *A. junii*. It is obvious that more active bacteria can take up more P. Therefore, a higher percent of P removal was achieved in the reactor containing 1OB (92%) than in the reactor containing 2OB (82%). In reactors containing 3-7OB no enhancement of P removal was achieved when compared to the reactors containing no bacteria (Table 1). Therefore, it can be summarised that only in reactors containing 1OB and 2OB with CTA sorbed in partial monolayer, was the P removed from wastewater by combined sorption onto the OB and bacterial uptake in the biomass.

3.4 Influence of OB on bacteria

In general, microorganisms are negatively charged and adhere better to minerals with rough and positively charged surfaces [22]. The amount of *P. putida* adsorbed by colloidal clay minerals was in the order of goethite, kaolinite and montmorillonite, suggesting that the electrostatic properties of mineral surfaces play a vital role in the adsorption of bacteria [20]. The maximum immobilization of cells obtained in this study (7.78 x 10⁹ CFU/g) was achieved with the 2OB, which had the negative zeta potential as the NB. The 3OB had negative zeta potential and 4-7OB had positive zeta potential, but did not show immobilization of viable or dead cells. These suggest that in the case of OB, mechanisms other than surface charge of the particles play a role in the process of successful immobilization of cells. The main mechanism which determined the immobilization of bacterial cells was the concentration of active CTA molecules. Namely, the CTA destroys the cell membrane and causes cells to die [23].

In the preliminary 24 h experiments [24], the estimated EC₅₀ values of the aqueous CTA for the inhibition of CFU in the pure culture of *A. junii* was

3.27±1.12 x 10⁻⁷ mol/L and for the inhibition of the P-uptake rates 2.47±0.51 x 10⁻⁶ mol/L. Complete inhibition was observed by the CTA concentration of 10⁻⁵ mol/L and higher. These results proved a high acute toxicity of the free CTA against *A. junii*. In the constructed wetland containing 10 cm radial pacing of the CTA-modified natural zeolite, 100% of *Escherichia coli* was removed from the sewage influent [7].

The CTA sorbed onto the NB is subjected to desorption, depending on the initial surfactant loading, on the type of the mineral substrate and the flow rate [25]. The CTA desorption is greater from the OB modified to surfactant aggregate coverage compared to the monolayer coverage. In the batch experiments a desorption of bilayered CTA from the organic zeolites was 0.6 to 1.8 mmol/L [25]. The potential desorption of CTA molecules from OB, especially those sorbed by weak forces onto the monolayer via hydrophobic alkyl chain interactions, can result in the formation of toxic unbound CTA molecules. The experimental data on the CTA release from OB in the synthetic wastewater (Table 1) demonstrate that the CTA release increased by increasing the CTA loading on OB. The CTA release from OB in the synthetic wastewater containing bacteria was slightly lower than in reactors without bacteria (Tables 1 and 2). These observations can explain the toxicity of the higher CTA loadings of OB against *A. junii* due to the desorption of CTA from OB during 24 h in the well aerated and mixed system. A desorption of the CTA from the OB may be safely ignored for certain applications, such as the permeable barrier for the filtration of groundwater or leachate by low flow-rate conditions [7]. Therefore, a suggested method which will probably protect the CTA release from OB can be the reduction of turbulence in the reactor. The positively charged polar head of the CTA plays a role in the bactericidal action of this compound. It is likely that the OB particles with aggregate CTA coverage act bactericidal themselves.

Although the CTA in aqueous solution is bactericidal, if sorbed onto the OB, *A. junii* can remain viable. The toxicity of the CTA against bacteria is greatly reduced or eliminated if the surfactant is bound to zeolite [7]. In this case, P removal from wastewater can be achieved by the formed biosolids, i.e. combined P sorption onto OB particles and bacterial uptake of P in the biomass.

4. Conclusions

The OB can be used to enhance P removal from wastewater in an aerated biological system, but special attention should be given to the configuration of sorbed CTA molecules and its potential desorption. The OB with CTA sorbed on the bentonite surface by lateral hydrophobic interactions showed bactericidal

effects. The efficient P removal from wastewater can be achieved by using biosolids consisted of OB with partial monolayer CTA coverage and immobilized P-accumulating bacteria, by the combined mechanisms of P adsorption onto OB and bacterial uptake of P in the biomass.

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