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Phosphate uptake by immobilized *Acinetobacter calcoaceticus* cells in a full scale activated sludge plant

NYO Muyima and TE Cloete

Environmental Biotechnology Laboratory, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa

An *in situ* study of the P-uptake ability of *Acinetobacter calcoaceticus* was carried out using the alginate immobilization technique. Immobilized *A. calcoaceticus* cells displayed a high P-uptake ability (>97% P-accumulating cells) when immersed in the aerobic zone of an activated sludge system for 30–240 min. The overall P-accumulation pattern of the anaerobic zone depicted a typical P-release mechanism. However, limited P-accumulation was also observed at this stage. Growth and anaerobiosis were not prerequisites for P-uptake. The immobilized cell retention time in the anaerobic zone did not affect remarkably the inherent P-uptake ability of immobilized *A. calcoaceticus* when exposed to the aerobic stage. P-uptake and release were reversible and depended on the environmental conditions to which immobilized cells were exposed. Immobilization of *A. calcoaceticus* using alginate can be regarded as a reliable method of studying pure cultures in the activated sludge process.

Keywords: *in situ* study; P-uptake; immobilized *A. calcoaceticus*; enhanced biological phosphorus removal; activated sludge

Introduction

Enhanced biological phosphorus removal (EBPR) is a widely used biological nutrient removal process. Alternation of an anaerobic period and aerobic period is a prerequisite for EBPR [1,14,19]. Under anaerobic conditions, activated sludge (AS) releases phosphate and removal of phosphate occurs during aerobic conditions [7,9,12,16]. There are many successful applications of AS systems for EBPR [18]. However, the role of particular P-accumulating bacteria, the mechanisms involved and indeed the functions of the anaerobic stage itself remain unclear [7,10,16]. The uptake and release of phosphate are reversible and this phenomenon seems to be the key to elucidating the mechanism of biological phosphorus removal [14]. The anaerobic stage is thought to play the role of a fermentation zone where acidogenic bacteria transform organic matter into volatile fatty acids [8,15]. These fatty acids may be used by the P-accumulating bacteria and stored in the form of poly-β-hydroxybutyrate (PHB). During the aerobic stage, PHB could be metabolized and the energy liberated thereby partially used to reconstitute the polyphosphate reserves [10]. However, neither the inclusion of an anaerobic zone nor the presence of volatile fatty acids in the feed influences the inherent ability of strains to accumulate polyphosphate [13]. The rate of P-uptake was almost unchanged before and after *A. calcoaceticus* cells were subjected to anaerobic stress for several hours, and P-release occurred under aerobic conditions after the cessation of growth [16].

Acinetobacter has been used extensively as a model organism for optimizing the AS process for EBPR. However, the lack of suitable techniques for *in situ* study of this organism, and others which may play an important role in this regard, has led to a lack of understanding about the mechanism controlling EBPR. In the laboratory, the conditions imposed on the organisms may differ vastly from their natural environment where they are continually exposed to changes in such critical factors as the nature and concentration of substrates [4]. Microorganisms may thus behave differently in a laboratory environment compared to an AS plant. Thus, extrapolation of laboratory scale results to full scale AS plants should be examined cautiously, since often non-recorded factors can affect the observed and/or desired metabolic activity.

In terms of studying particular P-accumulating bacteria *in situ*, immobilization of pure cultures such as *A. calcoaceticus* could be useful, since it would offer the possibility of recovering pure cultures from full scale systems after they have been suspended in the AS system. This would enable the researcher to simulate the effect of different plant conditions on a particular organism without having to interfere with the operation of a full scale plant itself.

The aim of this study was to determine whether immobilized *Acinetobacter calcoaceticus* cells could be used to study the P-uptake ability of these bacteria in the AS process under different conditions.

Materials and methods

The bacterium

An *A. calcoaceticus* ATCC 23055^T stock culture was incubated in 100 ml acetate enrichment medium containing 5 g CH_3COONa, 2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g KH_2PO_4, 0.2 g $CaCl_2 \cdot 2H_2O$, 1 L tap water, and pH 7.0 [4,9] on an Edmund Bühler TH10 rotary shaker, at 160 rpm, and 28° C for 72 h before immobilization.

Correspondence: NYO Muyima, Environmental Biotechnology Laboratory, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa
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Experimental site

The experiments were carried out in a Bardenpho activated sludge system, at the Daspoort sewage works, Pretoria, South Africa. This sewage work is a combination of biological P- and N-removal system with an average flow of 39.7 ML day^{-1} and an average organic concentration of 389 mg L^{-1} COD.

Immobilization technique

The immobilization procedure was carried out under aseptic conditions. A 72-h culture of *A. calcoaceticus* was centrifuged at 8540 rcf for 10 min. The supernatant phase was discarded, and the bacterial pellet was washed with sterile distilled water. It was then resuspended in a sufficient quantity of sterile distilled water to obtain a 10-ml final volume, and dispersed on a vortex mixer. The homogeneous bacterial suspension obtained was mixed aseptically with a sufficient quantity of sterile sodium-alginate solution on a magnetic mixer to obtain 3% (w/v) final concentration. Immobilization was performed using a modification of the technique of Bashan [2]. A sterile 20-ml syringe with a 26-G needle was used to add the mixture dropwise into a 1.1% $CaCl_2$ solution for cross-linking. The sodium-alginate beads entrapping *A. calcoaceticus* cells were washed with sterile tap water.

Experimental procedure

Beads were put into a nylon net bag to facilitate contact between the mixed liquor and the immobilized bacterial cells. The net bag was attached to a metal ring weight to prevent it from floating and to maintain it at a fixed depth (Figure 1).

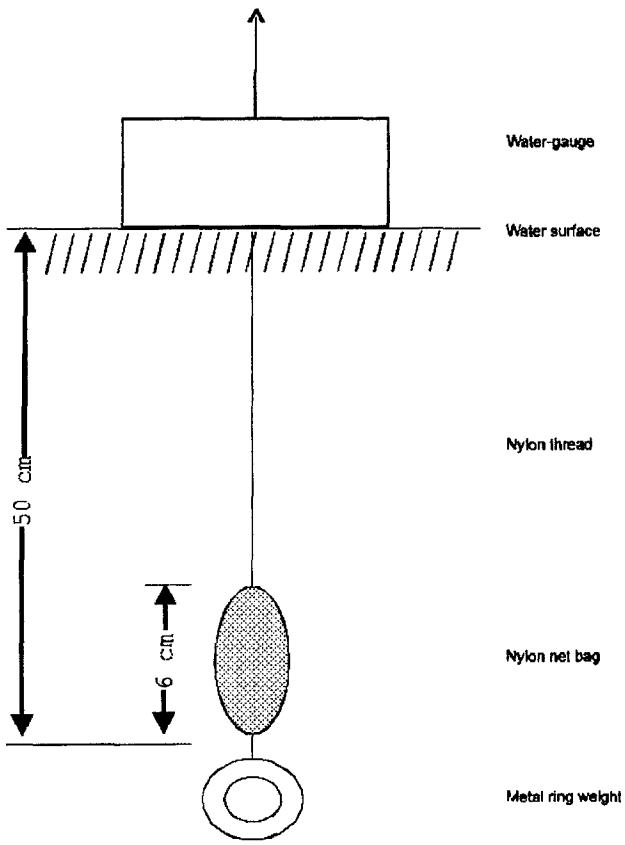


Figure 1 Experimental set-up for the *in situ* studies (side view)

To assess the ability of *A. calcoaceticus* immobilized cells to accumulate polyphosphate in a full scale AS plant, 10 g of the immobilized bacteria were suspended in either the aerobic zone or the anaerobic zone at a depth of 50 cm. After 30, 60, 90, 120 and 240 min respectively, samples were removed from the experimental system. These were washed with sterile tap water and 0.1 g sample beads were broken up by vigorous shaking on a vortex mixer for about 1 min in phosphate buffer to recover bacterial cells for counting bacteria containing polyphosphate granules in the form of volutin. After metachromatic staining [4], the P-accumulating bacteria count was performed using a Nikon (Alphaphot/YS, Japan) 104 light microscope (with phase contrast). The percentages of P-accumulating bacteria per ml were estimated from a count of at least 10 randomly chosen microscope fields [6]. Sodium-alginate beads without bacterial cells and *A. calcoaceticus* immobilized cells which had not been in contact with the AS system were used as controls. These bacteria were inspected immediately after immobilization.

To assess the effect of anaerobic conditions on P-uptake or release, beads containing *A. calcoaceticus* cells were immersed in the aerobic zone for 30 min and then transferred from the aerobic to the anaerobic zone. After 30, 60, 90, 120 and 180 min samples were taken for P-accumulating bacteria counts.

To determine whether there is an optimum cell retention time in the anaerobic zone required to induce optimal P-uptake, five nylon net bags containing 10 g of immobilized bacteria each were immersed into the anaerobic zone respectively. After 30, 60, 90, 120 and 240 min, each bag was transferred from the anaerobic to the aerobic zone for 30 min and then samples were taken for P-accumulating bacteria counts.

Transmission electron microscopy of P-accumulating A. calcoaceticus *cells*

The P-accumulating ability of immobilized *A. calcoaceticus* cells was determined using transmission electron microscopy (TEM). For each observation with TEM, approximately four beads for each treatment were fixed for 24 h in 2% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, rinsed three times (15 min each) in sodium cacodylate buffer, fixed again for 1 h in 1% osmium tetroxide, rinsed three times (15 min each) in sodium cacodylate buffer, and then dehydrated with increasing ethanol solutions as follows: 50% ethanol for 15 min, 70% for 15 min, 90% for 15 min, 100% three times (15 min each). Beads were cut in quarters and soaked in increasing resin concentrations as follows: 33% resin for 1 h; 66% resin for 1 h; 100% resin two times, 18 h and 8 h; then kept in the oven overnight to solidify. Solidified samples were cut using a Reichert-Jung ultratome (Austria) and stained using lead citrate. As observed by Buchan [4,5], phosphate (dense body) was often torn out of the cell during the cutting process.

Results

P-uptake by immobilized A. calcoaceticus *suspended in the aerobic zone*

Immobilized cells of *A. calcoaceticus* accumulated polyphosphate within 30 min after immersion in the aerobic zone of the AS plant. The maximum P-uptake rate (100% cells containing volutin) occurred 180 min after incubation and was maintained up to 240 min of incubation (Figure 2; Plates 1,2). As observed by Streichan and Schön [18], most of the phosphate shown in electron micrographs was accumulated inside the immobilized cells as polyphosphate granules.

The effect of anaerobic conditions on P-uptake and release by immobilized A. calcoaceticus *cells*

A. calcoaceticus immobilized cells also showed P-uptake 30 min after immersion in the anaerobic zone (42% of cells exhibited volutin). However, the uptake rate was lower than that observed in the aerobic zone. Between 60 and 240 min the number of P-accumulating cells decreased indicating P-release (Table 1; Figure 2; Plates 3, 4).

About 93% P-accumulating cells were recorded 30 min after immersion of *A. calcoaceticus* immobilized cells in aerobic conditions, before being transferred to the anaerobic zone (Table 1; Figure 2). When transferred into the anaerobic zone, *A. calcoaceticus* immobilized cells released phosphate gradually between 30 min and 90 min (15% of cells exhibiting volutin). Thereafter, the P-accumulating cell numbers increased again and remained approximately constant up to 180 min (*ca* 50% of cells exhibiting volutin). Although a high rate of P-release (*ca* 85%) was recorded under anaerobiosis, no complete release was observed during the experimental period of 240 min (Table 1; Figure 2; Plates 5, 6).

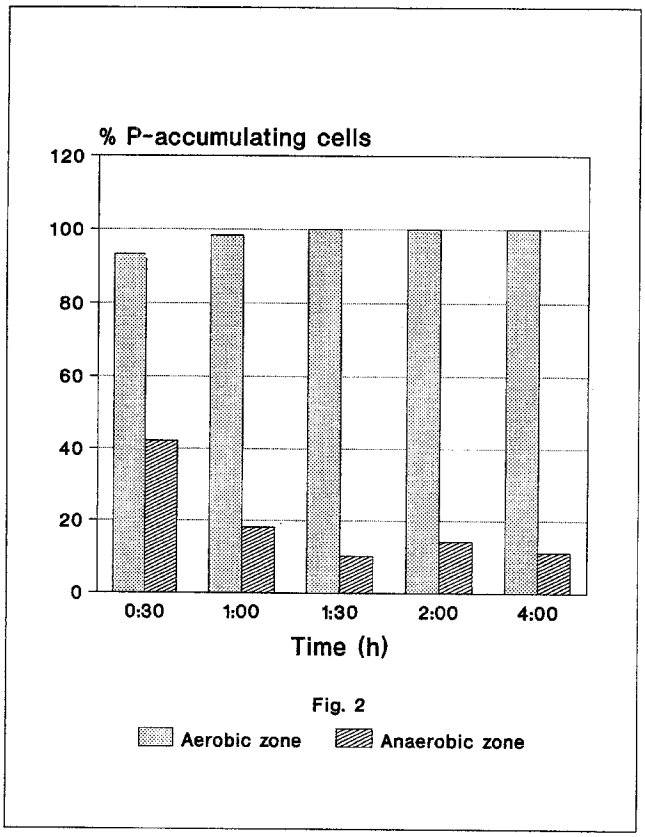


Figure 2 P-accumulating cell percentages after suspension of *A. calcoaceticus* immobilized cells in the aerobic and anaerobic tanks of the Daspoort AS plant

High rates of P-accumulating cells were recorded when P-accumulating bacteria cells were kept in anaerobic conditions before being transferred into the aerobic zone for a minimal uptake time of 30 min. However, no remarkable difference was found between *A. calcoaceticus* immobilized cells kept for 30 min and up to 4 h in anaerobiosis before being transferred into the aerobic zone. This means that there was no P-uptake enhancement in relation to the length of exposure to anaerobic conditions (Table 1). These results support our previous observations on P-uptake by immobilized *A. calcoaceticus* cells immersed in the aerobic zone without being previously exposed to anaerobiosis.

Discussion

P-uptake under aerobic conditions

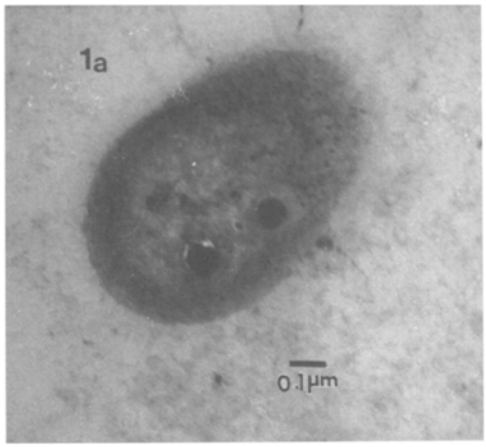
Immobilized cells of *A. calcoaceticus* accumulated phosphate (93.3% of cells exhibited volutin) within 30 min after immersion in the aerobic zone of the AS plant and within 90 min all the cells exhibited volutin (Figure 2; Table 1). This observation agrees with several reports that P-uptake occurs under aerobic conditions [1,9,14,16]. Bosch [3] indicated that growth was not a prerequisite for P-uptake by *A. calcoaceticus*. The results from this study support this, since 30 min after immersion a high number of P-accumulating cells was recorded.

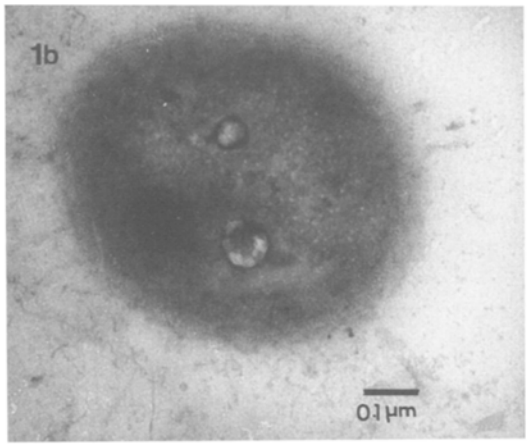
It has been found that *A. calcoaceticus* released phosphate under aerobic conditions after cessation of growth due probably to consumption of substrate [16]. A slow release of phosphate in activated sludge was reported when the period of the aerobic phase exceeded 4 h [17]. Our results are in agreement with these findings since no decrease in P-accumulating cell numbers was recorded indicating that cells did not release phosphate under aerobic conditions within 4 h [16,17].

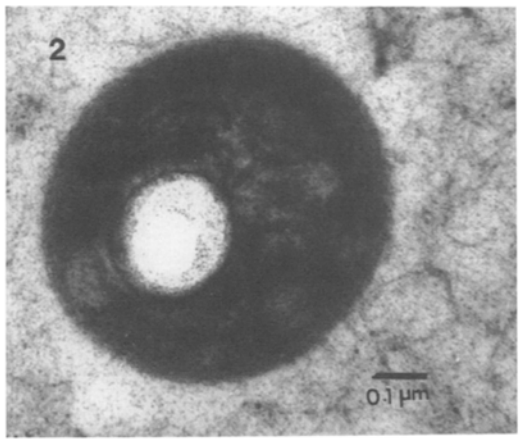
P-uptake and release under anaerobic conditions

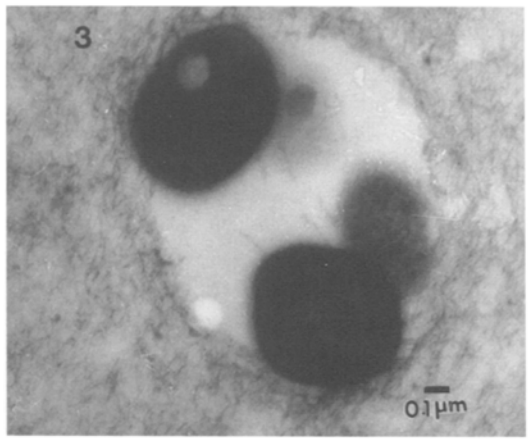
Since *A. calcoaceticus* cells in this study were grown in low phosphate medium, the initial accumulation of polyphosphate under anaerobiosis could have been made possible by energy supplied by oxidation of PHB [20]. Although *A. calcoaceticus* accumulated polyphosphate within 30 min, it displayed a typical P-release pattern between 30 and 90 min (Figure 2). These results are in agreement with empirical observations that under anaerobic conditions P-accumulating bacteria release phosphate [11]. The slight increase of phosphate observed 120 min after incubation may suggest that under certain circumstances P-uptake may occur at a low rate even under anaerobic conditions.

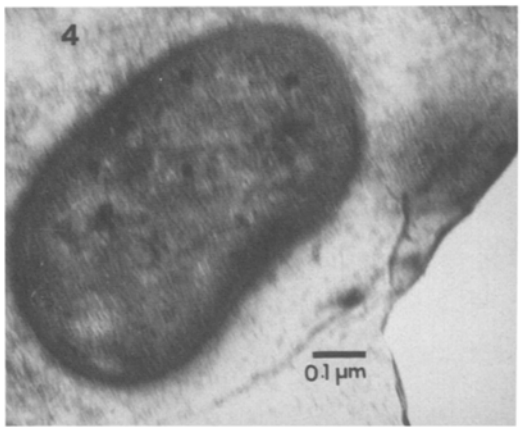
Although *A. calcoaceticus* immobilized cells released phosphate gradually within the initial 90 min in the anaerobic zone of the AS plant, the release of phosphate after a long retention period seemed discontinuous and no com-

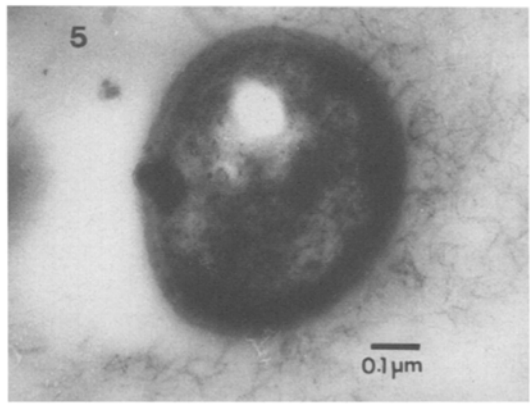


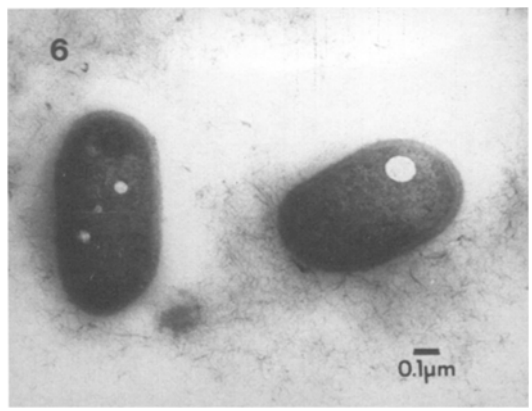












Plates 1–6 1a, b: P-accumulating cells from the aerobic zone, 60 min after suspension. 2: P-accumulating cells from the aerobic zone, 120 min after suspension. Note that the dense body had been torn out of the cell. 3: P-accumulating cells from the anaerobic zone, 30 min after suspension. 4: P-accumulating cells from the anaerobic zone, 60 min after suspension. Note the small dense bodies. 5: P-accumulating cells transferred from the aerobic to the anaerobic zone, 60 min after suspension. 6: P-accumulating cells transferred from the aerobic to the anaerobic zone, 180 min after suspension

Table 1 % P-accumulating cells after suspension of *A. calcoaceticus* immobilized cells into the aerobic and anaerobic tanks of the Daspoort AS plant

Time (min)	Aerobic	Anaerobic	Aerobic to anaerobic[a]	Anaerobic to Aerobic[b]
30	93.3	42.1	78.5	98.4
60	98.3	18.2	31.5	97.3
90	100	10.2	15.5	100
120	100	14.2	52.1	99.6
180	–	–	49.9	–
240	100	11.2	–	97.3

[a] Transfer of *A. calcoaceticus* immobilized cells 30 min after pre-incubation in aerobic zone to anaerobic zone
[b] Transfer of *A. calcoaceticus* immobilized cells from anaerobic zone (ie 30, 60, 90, 120, and 240 min of pre-incubation) to aerobic zone for 30 min incubation
– means that no sampling was made

plete release was attained. It has been reported that *Acinetobacter* cells from the logarithmic stage of growth release phosphate immediately after transfer from aerobic to anaerobic conditions and that the release of phosphates occurs linearly with time during the initial 5 h [16]. It has also been reported that P-release from stationary phase cells was considerably smaller than that from log-phase cells [16]. Since 72-h cultures used in this study were from the stationary phase, our results confirm a low P-release rate. These results also confirmed that P-release under anaerobic stress is not due to lysis of the cells [16]. Since an energy source is required to maintain high intracellular levels of phosphates against a phosphate concentration gradient, *A. calcoaceticus* which cannot form ATP through the glycolytic pathway [16,20], probably released phosphate because of the high phosphate concentration gradient under anaerobic conditions. Under high phosphate concentration gradients, cells passively release phosphate up to a certain point, in order to find an equilibrium with the bulk liquid phosphate concentration. This might explain the fact that a complete release was not observed.

The effects of anaerobic retention time on the P-uptake ability of A. calcoaceticus *cells*

Although *A. calcoaceticus* immobilized cells subjected to anaerobic conditions readily accumulated polyphosphate when transferred to the aerobic zone, the length of the retention time in the anaerobic zone did not influence their P-uptake ability. Our results are in agreement with those of Ohkate [16] who indicated that the rate of P-uptake was not influenced by the anaerobic exposure time. In contrast, Marais [14] reported that the magnitude of phosphate removal appeared to increase with increase in the nominal retention time of the anaerobic reactor.

Since cells which were not previously exposed to anaerobic conditions accumulated phosphate when immersed into the aerobic zone (Figure 2), we conclude that anaerobiosis is not a prerequisite for P-uptake by *Acinetobacter*.

Immobilized *Acinetobacter* cells showed high P-uptake in the aerobic zone and P-release in the anaerobic zone of the AS plant. However, limited P-accumulation was also observed under anaerobic conditions. Growth and anaerobiosis were not prerequisites for P-uptake by immobilized *A. calcoaceticus* cells. The length of the retention time in the anaerobic zone did not influence P-uptake by *A. calcoaceticus*. Although cells in the aerobic stage displayed bigger and/or more polyphosphate granules than those in the anaerobic stage, a great heterogeneity of *A. calcoaceticus* immobilized cells with polyphosphate granules was observed. The alginate immobilization technique can be regarded as a reliable method for the *in situ* study of pure cultures in AS process on the subject of EBPR.

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