Biomass relationship to growth and phosphate uptake of pseudomonas fluorescens, escherichia coil and acinetobacter radioresistens in mixed liquor medium.

Journal of Industrial Microbiology (1996) 16, 364–369

Biomass relationship to growth and phosphate uptake of *Pseudomonas fluorescens, Escherichia coli* and *Acinetobacter radioresistens* in mixed liquor medium

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The ability of *Pseudomonas fluorescens, Escherichia coli* and *Acinetobacter radioresistens* to remove phosphate during growth was related to the initial biomass as well as to growth stages and bacterial species. Phosphate was removed by these bacteria under favourable conditions as well as under unfavourable conditions of growth. Experiments showed a relationship between a high initial cell density and phosphate uptake. More phosphate was released than removed when low initial cell densities (10^2–10^5 cells ml^{-1}) were used. At a high initial biomass concentration (10^8 cells ml^{-1}), phosphate was removed during the lag phase and during logarithmic growth by *P. fluorescens. Escherichia coli*, at high initial biomass concentrations (10^7 cells ml^{-1}), accumulated most of the phosphate during the first hour of the lag phase and/or during logarithmic growth and in some cases removed a small quantity of phosphate during the stationary growth phase. *Acinetobacter radioresistens*, at high initial cell densities (10^6, 10^7 cells ml^{-1}) removed most of phosphate during the first hour of the lag phase and some phosphate during the stationary growth phase. *Pseudomonas fluorescens* removed phosphate more than *A. radioresistens* and *E. coli* with specific average ranges from 3.00–28.50 mg L^{-1} compared to average ranges of 4.92–17.14 mg L^{-1} for *A. radioresistens* and to average ranges of 0.50–8.50 mg L^{-1} for *E. coli.*

Keywords: biomass; growth; phosphate uptake; *Pseudomonas fluorescens*; *Escherichia coli*; *Acinetobacter radioresistens*

Introduction

Phosphorus removal from wastewater can be achieved through chemical precipitation and coagulation, through biological treatment or a combination of both. Methods of phosphorus removal have been reviewed by Yeoman *et al* [22].

Since excess phosphate uptake by bacteria in activated sludge was observed for the first time by Vaker *et al* [20], research on phosphate removal by biological methods has intensified. Optimization and better control of the process will, however, require more information on the ecology and physiology of the polyphosphate-accumulating bacteria.

The role of polyphosphates as an energy reserve under anaerobic conditions in *Acinetobacter* spp has been reported by many workers [3, 11]. Representatives of this genus are the most intensively studied and best known poly-P-bacteria in activated sludge. They are considered the most significant and efficient accumulators of phosphate in activated sludge plants treating municipal wastewater [4, 8]. It is important, however, to recognize the fact that most studies on the population structure of activated sludge have been based on viable counts on agar plates [19]. When using conventional and API 20E test systems, Venter *et al* [21] found that *Acinetobacter* constituted 6% of the population using conventional test identification procedures and 18% using the API 20E test system. Species of *Pseudomonas* constituted 21% of the total when using conventional identification procedures and 15% when using the API 20E test system. *Moraxella* constituted 12% using conventional identification procedures and 4% using the API 20E test system and *Alcaligenes* constituted 18% (conventional identification procedures) and 22% (API 20E test system). Brodisch and Joyner [2] using the Analytical Profile Index (API) system to identify the composition of the microbial communities in the anaerobic, anoxic and aerated stage of three biological phosphate removal plants and two laboratory scale units, reported that organisms of the genera *Aeromonas* and *Pseudomonas* constituted more than 50% of the total aerobic microbial population. In contrast to findings of other workers, bacteria of the genus *Acinetobacter* were present in minor proportions. Cloete and Steyn [5] indicated that *Acinetobacter* spp constituted less than 10% of the total population. Using *Acinetobacter* spp number, volutin volumes, densities and phosphate content, Cloete and Steyn [6] reported that a maximum of 34% phosphate removal in activated sludge could be attributed to removal by *Acinetobacter* spp as polyphosphate. Other organisms or mechanisms therefore have to be involved to account for the observed phosphate removal.

Among the microorganisms responsible for phosphate accumulation identified by Suresh *et al* [18], were *Pseudomonas vesicularis, Acinetobacter lwoffi* and *Acinetobacter calcoaceticus*. Gram-positive organisms, such as *Arthrobacter globiformis*, have also been reported to accumulate phosphate in the order of 20% of their dry cell mass [16]. The ability of *Pseudomonas* and *Escherichia coli* to remove phosphate from activated sludge mixed liquor has also been tested [7]. Currently it is not known whether these organisms could also play a role in enhanced biological phosphorus removal in activated sludge systems.

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Received 11 December 1995; accepted 23 April 1996

Bosch [1], moreover, found that under conditions of increased nutrient availability, phosphate removal increased, possibly due to the resultant increase in biomass and not to an enhanced phosphate-accumulating ability of individual cells. These findings encouraged us to investigate the relationship between biomass and phosphate uptake as well as growth and phosphate uptake of *P. fluorescens, E. coli* and *A. radioresistens.*

Materials and methods

Bacteria

Pseudomonas fluorescens and *Escherichia coli* were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa. *Acinetobacter radioresistens* FO-1 was obtained from Dr Y Nishimura, Institute of Applied Microbiology, University of Tokyo. The organisms were maintained on Nutrient Agar (Biolab, South Africa) and incubated for 48 h at 28°C for *P. fluorescens* and *A. radioresistens* and at 37°C for *E. coli*, before inoculation into 100 ml Nutrient Broth (Biolab).

Mixed liquor preparation

Mixed liquor samples were drawn from the anaerobic zone and aerobic zone of a five-stage Bardenpho activated sludge plant at the Daspoort sewage works in Pretoria. Samples were filtered three times through Whatman No. 1 filter paper. The mixed liquor was then diluted in tap water, to obtain a final concentration of *ca* 30 ml L^{-1} P. The medium was further modified by adding: 5 g L^{-1} sodium acetate (BDH, South Africa), 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Merck, South Africa), 0.18 g L^{-1} KNO_3 (Merck). The final pH was adjusted to 7 with 2 N HCl before autoclaving the medium at 121°C for 15 min. This mixed liquor medium was used in all experiments.

Experimental procedure for estimation of biomass

The agar plate method was used to determine biomass of *Pseudomonas fluorescens, E. coli* and *A. radioresistens* cultured in 100 ml sterile Nutrient Broth and incubated at a shaking speed of 80 rpm for 48 h at the temperature requirements of each species (28°C for *P. fluorescens* and *A. radioresistens*, 37°C for *E. coli*). Low initial biomass inocula (10^2, 10^4, 10^6 cells ml^{-1} for *P. fluorescens* and 10^2, 10^4 cells ml^{-1} for *E. coli*) were obtained by using microbial dilutions within the range of 10^{-2} to 10^{-5}. One millilitre of each bacterial suspension and of 10^{-3} and 10^{-5} dilutions were inoculated into 250-ml sterile mixed liquor. High biomass inocula (10^7 and 10^8 cells ml^{-1} for *P. fluorescens*; 10^5, 10^6, 10^7 cells ml^{-1} for *E. coli* and *A. radioresistens*) were obtained by centrifugation at 8000 rpm for 20 min. Harvested cells were suspended (after washing and suitable dilution) in 250 ml mixed liquor. One millilitre of cultures at each density was removed from the sample and diluted 1:10 with sterile Ringer's solution in the range of 10^{-2} to 10^{-8} to determine the initial biomass. Flasks were then incubated at the appropriate temperature for each organism. Hourly (for the first 8 h and then after 24 h or for a 24-h period) a 1-ml sample was taken for viable count determination and 1 ml for phosphate analysis. All experiments were carried out in duplicate or in triplicate.

Phosphate analysis

The ability of bacteria to remove phosphate from the medium was examined on samples taken hourly for a period of 8 h and again after 24 h and for a 24-h period for the full duration of the experiments. Phosphate uptake was monitored hourly on 1-ml samples removed from the flasks with a syringe and filtered through a 0.22-μm pore-size filter (Millipore, South Africa). A 250-ml sterile mixed liquor sample treated in the same manner was used as control. The filtered sample was diluted 1:10 with sterile Ringers solution and used for phosphate concentration determination, using the P(VM) 14842 test kit (Merck) and an SQ 118 photometer.

Results and discussion

The study performed on the relationship between biomass, growth and phosphate uptake indicated that the ability of these polyphosphate-accumulating bacteria to grow and to remove phosphate from the activated sludge mixed liquor was related to the initial cell densities as well as to growth stages and bacterial species. The patterns of phosphate removal in cultures inoculated with different cell concentrations of these organisms are summarized in Figures 1–5.

During the logarithmic growth phase, bacteria were in a state of balanced growth after which the growth of cells was limited by the exhaustion of available nutrients (Figure 4). In terms of phosphate removal, low initial cell densities (10^2, 10^4 and 10^6 cells ml^{-1} for *P. fluorescens* and 10^2, 10^4 cells ml^{-1} for *E. coli*) released phosphate, once transferred into the mixed liquor (Figure 1 and Figure 3). Release of phosphate was enhanced during active growth. The removal of phosphate took place near the end of the logarithmic growth phase and when the stationary growth phase was reached (Figure 1). Release of phosphate during active growth was associated with the competition between nucleic acid synthesis and polyphosphate for intracellular phosphate [12]. Moreover, it has been confirmed that a higher phosphate concentration in the growth medium results in diffusion of phosphate into the cells, whereas a larger phosphate concentration within the cells results in a tendency for phosphate to diffuse out of the cells [14].

The presence of acetate can also trigger the release of phosphate [8, 13]. Deinema *et al* [10] associated phosphate release with carbon compounds other than acetate. Nevertheless, the release of phosphate may act as a source of energy both for the reestablishment of proton motive force and for substrate storage [8].

Although some phosphate (Figure 1) was removed near the end of logarithmic growth, *P. fluorescens* in low initial biomass cell concentrations (10^2, 10^4, 10^6 cells ml^{-1}) removed most of the phosphate during the stationary growth phase with an average of 5.50 mg L^{-1} P (for 10^2 cells ml^{-1}), 13.50 mg L^{-1} P (for 10^4 cells ml^{-1}) and 22.75 mg L^{-1} P (for 10^6 cells ml^{-1}) after 24 h. Harold [12] also noted enhanced phosphate uptake when growth of *Aerobacter aerogenes* ceased as the result of a nutrient deficiency. The removal of phosphate during the stationary growth phase supports previous findings by Bosch [1] and Streichan [17].

P. fluorescens (1.49×10^7 cells ml^{-1} as initial inoculum) released phosphate at early logarithmic growth phase. Some

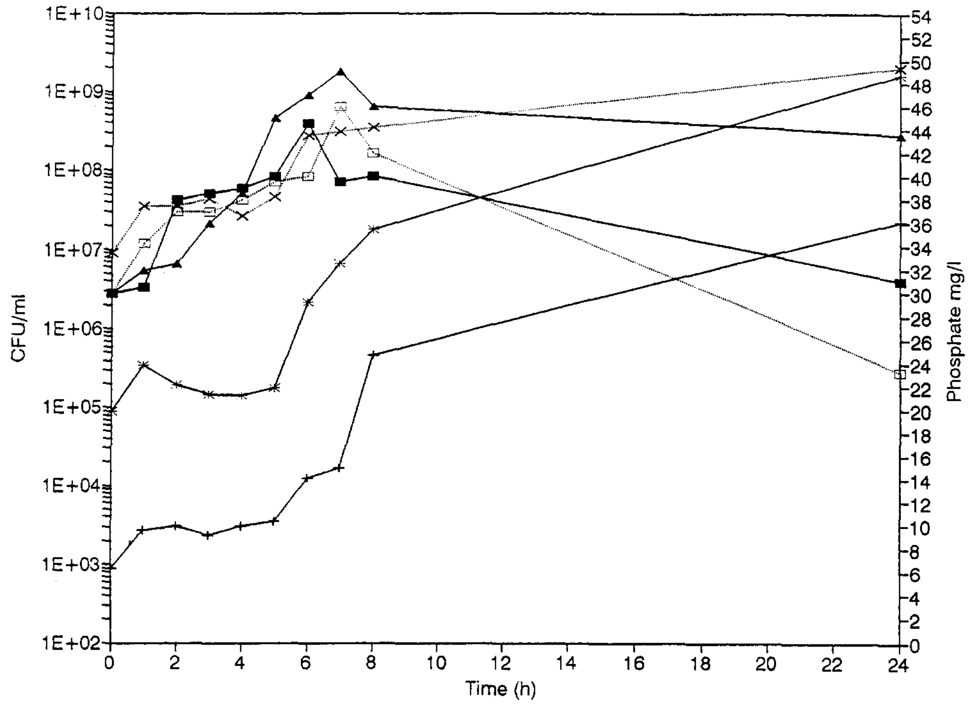

Figure 1 Growth of (A: —+—, b: —✳—, c: —×—) and phosphate uptake (a: —▲—, b: —■—, c: —⊟—) by *P. fluorescens* using different initial cell densities (a: 9×10^2 cells ml^{-1}, b: 9×10^4 cells ml^{-1}, c: 9×10^6 cells ml^{-1}) in activated sludge mixed liquor.

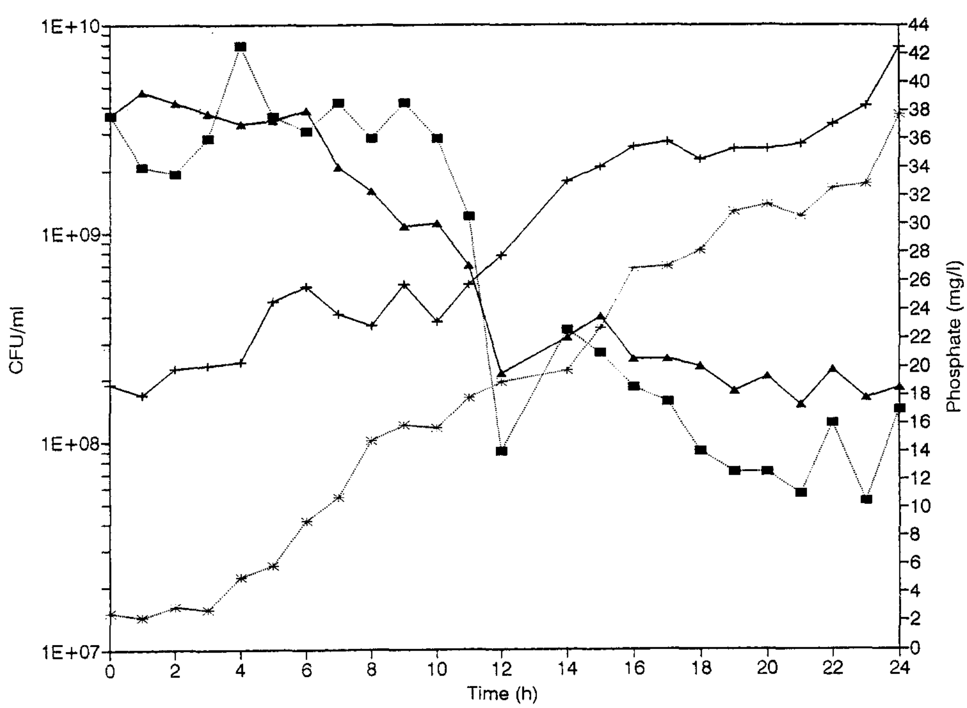

Figure 2 Growth of (d: —+—, e: —✳—) and phosphate uptake by (d: —▲—, e: —■—) *P. fluorescens* using different initial cell densities (d: 1.46×10^7 cells ml^{-1}, e: 1.89×10^8 cells ml^{-1}) in activated sludge mixed liquor.

phosphate was removed during the lag growth phase (Figure 2). Most of the phosphate (28.50 mg L^{-1} after 12 h) was removed during the logarithmic growth phase, in spite of some fluctuations (probably due to the competition between nucleic acid synthesis and polyphosphate for intracellular phosphorus, inducing rapid degradation of accumulated phosphate) [12]. At a high initial biomass concentration of 10^8 cells ml^{-1}, phosphate was removed during the lag growth phase and during the logarithmic growth phase (Figure 2). Removal of phosphate during the lag phase was in accordance with the observation of other workers who stipulated that slow growing cells often contained polyphosphate granules [6, 12] and that there was a relationship between phosphate uptake and the relative number of phos-

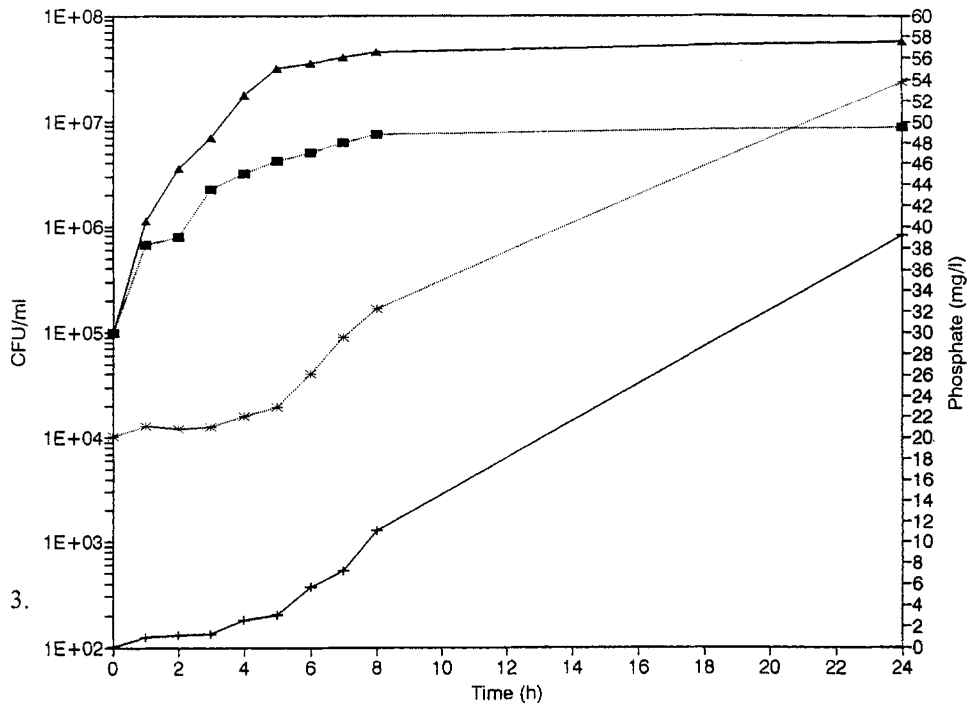

Figure 3 Growth of (a: —+—, b: —✳—) and phosphate uptake by (a: —▲—, b: —■—) *E. coli* using different initial cell densities (a: 1.02×10^2 cells ml^{-1}, b: 1.03×10^4 cells ml^{-1}) in activated sludge mixed liquor.

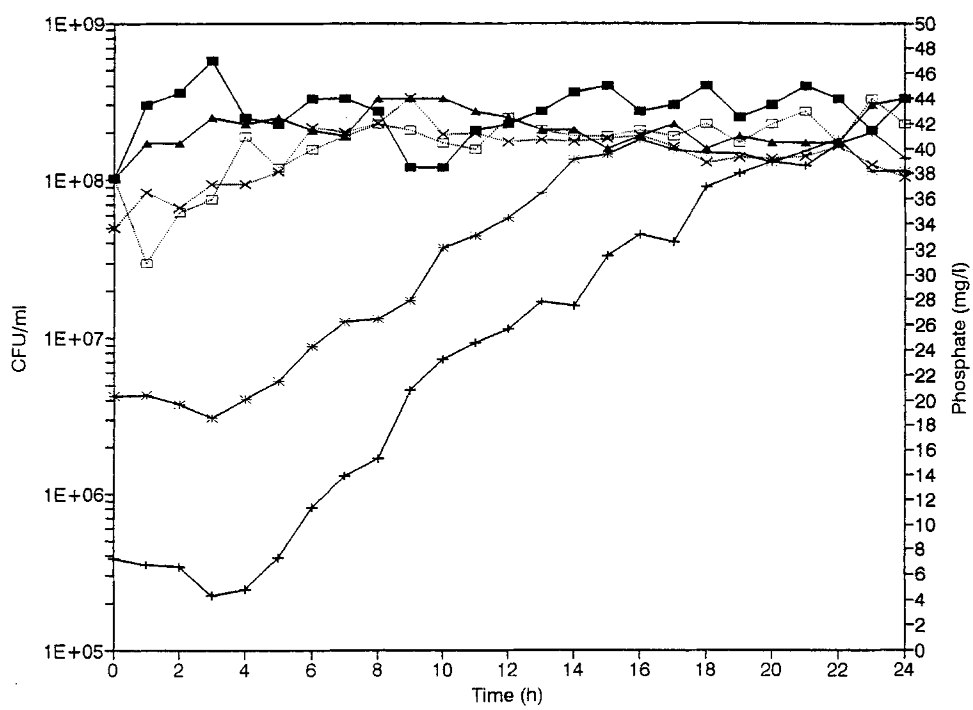

Figure 4 Growth of (c: —+—, d: —✳—, e: —×—) and phosphate uptake by (c: —▲—, d: —■—, e: —⊟—) *E. coli* using different initial cell densities (c: 3.80×10^5 cells ml^{-1}, d: 4.30×10^6 cells ml^{-1}, e: 5.00×10^7 cells ml^{-1}) in mixed liquor medium.

phorus-accumulating bacteria.

Enhanced uptake of phosphate occurred when cells grown under phosphorus limitation were exposed to phosphorus (overplus phenomenon) or under unfavourable conditions after growth ceased. However, enhanced phosphate uptake under favourable conditions during growth, was found in a few bacteria, including *Acinetobacter* strains [9, 15]. This study revealed the removal of phosphate by *P. fluorescens* under favourable conditions of growth. Enhanced uptake of phosphate by growing bacteria may not only be important in the assimilation of phosphate generated by the hydrolysis of phosphate organic compounds later in the treatment process [15], but is also necessary for synthesis of cell components.

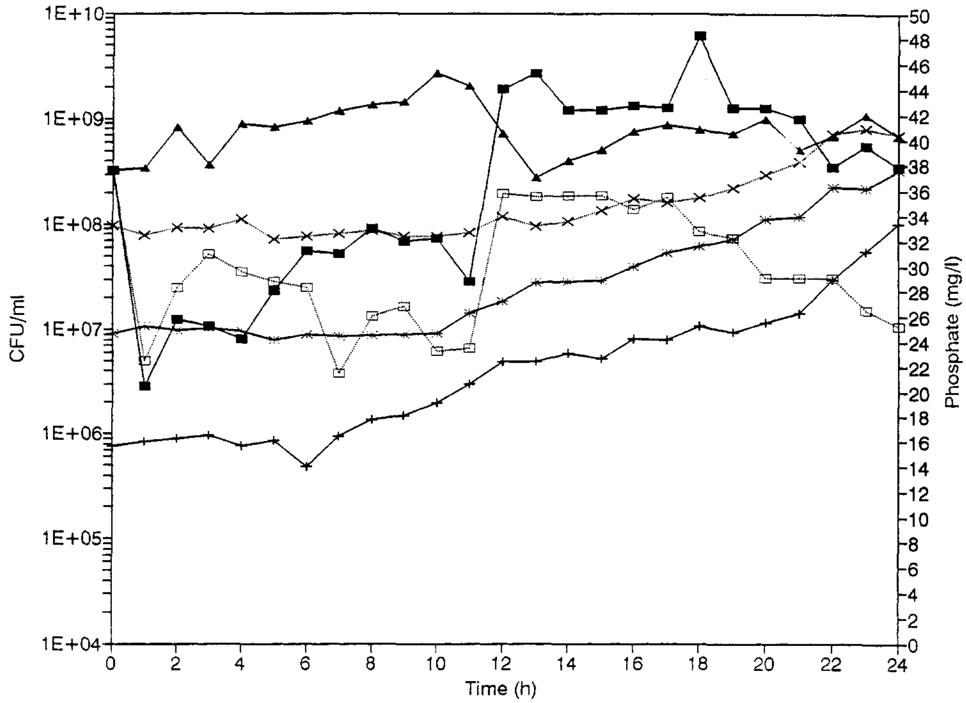

Figure 5 Growth of (a: —+—, b: —✳—, c: —×—) and phosphate uptake by (a: —▲—, b: —■—, c: —⊟—) *A. radioresistens* using different initial cell densities (a: 7.53×10^{-5} cells ml^{-1}, b: 9.20×10^{6} cells ml^{-1}, c: 9.70×10^{7} cells ml^{-1}) in mixed liquor medium.

Escherichia coli removed phosphate during the lag growth phase and this was influenced by the initial cell concentration (Figure 4). The removal of phosphate during the lag growth phase was also noted when using high initial cell concentrations of *Acinetobacter radioresistens* (Figure 5). Using 4.30×10^{6} cells ml^{-1} as initial cell density, *E. coli* removed some phosphate when cells increased in number during the logarithmic phase (Figure 4). Similar observations were made for *A. radioresistens* when using 7.53×10^{5} cells ml^{-1} as initial cell density (Figure 5). Nevertheless in almost all samples, release and uptake of phosphate by *E. coli* and *A. radioresistens* were illustrated by significant fluctuations during growth (Figures 4–5). Additional experiments with high biomass of *E. coli* and *A. radioresistens* are then required in order to confirm the relationship between biomass, growth of these bacterial species and phosphate uptake.

The overall results showed that *P. fluorescens* (Figures 1–2) removed phosphate more than *A. radioresistens* (Figure 5) and *E. coli* (Figures 3–4) and that the biomass and growth stage were important factors affecting phosphate removal.

Acknowledgements

The authors thank the Water Research Commission of South Africa for funding the research.

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