Polyphosphate accumulation by bacteria isolated from activated sludge

M Sidat, F Bux* and HC Kasan
Centre for Water and Wastewater Research, Technikon Natal, PO Box 953, Durban 4000, South Africa

Abstract

Biological phosphorus removal is an acknowledged phenomenon applied to reduce phosphorus concentrations in wastewaters. Under aerobic conditions some heterotrophic organisms store polyphosphate intracellularly which serves as a phosphorus and energy source during periods of phosphorus starvation. The objective of this study was to investigate polyphosphate accumulation by all bacteria isolated from the aerobic zone of a nutrient removal plant. Polyphosphate organisms were isolated on casitone glycerol yeast agar. Procedures for phosphate uptake studies included exposure of each monoculture to anaerobic environments for 2 h followed by aeration for 5 h. Extracellular orthophosphate concentrations were determined after aeration using a Merck SQ118 spectrophotometer. Identification was conducted using various biochemical tests, API 20E and 20NE. *Acinetobacter calcoaceticus* var. *lwoffi*, *Aeromonas hydrophila*, gram-positives and *Pseudomonas* spp. were among the predominant phosphate removers. Neisser staining confirmed that the organisms had stored polyphosphate intracellularly. Findings confirm that *Acinetobacter calcoaceticus* var. *lwoffi* is the predominant micro-organisms involved in enhanced phosphorus uptake.

Introduction

Biological phosphorus removal from wastewater is based on the enrichment of activated sludge with phosphate-accumulating organisms (PAOs). To achieve a phosphorus-removing bacterial population in an activated sludge system, exposure of sludge to alternating anaerobic and aerobic (or anoxic) conditions is necessary (Bdrjanovic et al., 1997). Under anaerobic conditions, P-removing bacteria convert volatile fatty acids (VFAs) synthesised in the zone by fermenters to polyhydroxybutyrate (PHB) which is stored intracellularly. Under aerobic conditions, stored PHB is used to generate cell growth, poly-P synthesis and glycogen formation and maintenance, resulting in the uptake of phosphate (Bdrjanovic et al., 1997).

The dominant bacteria in the activated sludge system are aerobic heterotrophs that degrade and eventually mineralise organic compounds present in wastewater to carbon dioxide and water. It is the small size of bacteria and their resultant large surface area to volume ratio which makes them efficient in terms of nutrient and catabolic exchange (Gray, 1989). Heterotrophic bacterial populations remain relatively stable throughout the plant with various environments in the three zones allowing different bacteria to dominate in terms of metabolic activity (Lötter and Murphy, 1985).

Several early studies have shown that the removal and release of phosphorus within a sludge are the results of the dominance of a single genus of bacteria known as *Acinetobacter* spp. and more specifically a single species, *Acinetobacter calcoaceticus*, was implicated (Buchan 1980, 1983; Horan, 1991; Starkenburg et al., 1993). *Acinetobacter* spp. are able to accumulate more phosphate than is required for cell synthesis; the so-called **luxury phosphate uptake**.

* To whom all correspondence should be addressed:
☎ (031) 204-2346; fax (031) 204-2778; e-mail faizalb@umfolozi.ntech.ac.za
Received 15 April 1998; accepted in revised form 29 September 1998.

Acinetobacter spp. are normally present in activated sludge, but in the minority due to the low growth rate. *Acinetobacter* organisms prefer VFAs, especially acetate, as a growth substrate which are present or can be produced from wastewaters in an activated sludge system. This is achieved by incorporating an anaerobic zone, mostly at the beginning of the aeration tank, where the return sludge meets the incoming wastewater (Starkenburg et al., 1993).

Controversy surrounds the notion that *Acinetobacter* spp. is the predominant micro-organism involved in enhanced phosphorus uptake. Using respiratory quinone profiles some researchers found no correlation between the number of *Acinetobacter* spp. and the extent of phosphorus removal (Cloete and Steyn 1988). Other researchers reported that *Acinetobacter* spp. were predominant when enumerated using the analytical profile index method. For example, Hart and Melmed (1982) estimated *Acinetobacter* spp. at 56% to 66% of the total population, Buchan (1983) reported 48% to 66%, Lötter (1985) 56% to 66%, Lötter and Murphy (1985) ca. 60% to 70% and Kerdachi and Healey (1987) 73%. However, it was shown that *Acinetobacter* spp., as detected by the biomarker diaminopropane, was the dominant organism only in wastewater plants with low organic loading (Auling et al., 1991). Using oligonucleotide probes specific for *Acinetobacter* spp., it was found that the genus formed less than 10% of the total bacterial population (Wagner et al., 1994). Probing techniques, compared to plating techniques, do not overestimate *Acinetobacter* spp. populations (Wagner et al., 1994). Nutrient-rich medium favours growth of gamma-subclass proteobacteria (e.g. enterobacteria) and selects against beta-subclass proteobacteria (Wagner et al., 1993). This is mainly due to the selectivity of media and culture conditions (Wagner et al., 1994). In addition to *Acinetobacter* spp., the Gram-positives are also able to accumulate polyphosphate (Lötter and Murphy, 1985). Bacteria like *Pseudomonas* spp., *Aerobacter* spp., *Moraxella* spp., *Escherichia coli*, *Mycobacterium* spp., *Beggiatoa* spp. and *Klebsiella* spp. also have the ability to accumulate phosphorus at approximately 1 to 3% of the cell dry mass (Bitton,

TABLE 1
PHOSPHATE ACCUMULATING CAPACITY SHOWN BY BACTERIAL MONOCULTURES ISOLATED FROM THE AEROBIC ZONE

Organism	Phosphorus uptake[a] ($mg \cdot \ell^{-1}$)	Viable count $CFU \cdot m\ell^{-1}$	Phosphorus uptake[b] per cell ($mg \cdot cell^{-1}$)
Pseudomonas fluorescens	20.6	$6.0x10^{8}$	$3.43x10^{-11}$
Staphylococcus aureus	5.6	$4.8x10^{8}$	$1.16x10^{-11}$
Enterobacter agglomerans	6.0	$1.12x10^{9}$	$5.35x10^{-12}$
Micrococcus spp.	10.4	$3.7x10^{8}$	$2.81x10^{-11}$
Staphylococcus spp.	4.4	$7.9x10^{8}$	$5.56x10^{-12}$
Pseudomonas testesteroni	13.3	$8.4x10^{8}$	$1.58x10^{-11}$
Staphylococcus epidermidis	10.4	$6.0x10^{8}$	$1.73x10^{-11}$
Bacillus cereus	13.7	$4.3x10^{8}$	$3.18x10^{-11}$
Pseudomonas acidovorans	8.9	$8.9x10^{8}$	$1.00x10^{-11}$
Aeromonas hydrophila	8.6	$5.2x10^{7}$	$1.65x10^{-10}$
Pseudomonas mendocina	19.6	$4.6x10^{8}$	$4.26x10^{-11}$
Pseudomonas putrefaciens	3.3	$5.5x10^{7}$	$6.00x10^{-11}$
Alcaligenes denitrificans	13.8	$3.2x10^{9}$	$4.31x10^{-12}$
Moraxella spp.	7.1	$6.0x10^{8}$	$1.18x10^{-11}$
Moraxella phenylpyruvica	15.8	$7.4x10^{8}$	$2.13x10^{-11}$
Streptococcus spp.	5.2	$3.2x10^{8}$	$1.62x10^{-11}$
Acinetobacter calcoaceticus var. *lwoffi* ATCC	18.4	$1.0x10^{8}$	$1.84x10^{-10}$
CONTROL	17.6	$7.04x10^{7}$	$2.50x10^{-10}$
	-	-	-

Key:
ATCC *Acinetobacter calcoaceticus* var. *lwoffi* obtained from ATCC No.23055
a [P uptake ($mg \cdot \ell^{-1}$)]=[P uninoculated control ($mg \cdot \ell^{-1}$)]-[P sample ($mg \cdot \ell^{-1}$)]
b [P uptake ($mg \cdot \ell^{-1}$)]=[P uptake ($mg \cdot \ell^{-1}$)] / [CFU/mℓ after incubation x 1000]
CONTROL - Uninoculated sterilised ML media

1994). Phosphate uptake has also been reported for other organisms such as *Azotobacter vinelandii* (Tsai et al., 1979) and the fungus *Neurospora crassa* (Cramer et al., 1980).

Bosch (1992) reported that under conditions of increased nutrient availability, phosphate removal increased, possibly due to the resultant increase in biomass and not due to an enhanced phosphate-accumulating ability of individual cells. Experimental evidence has favoured the relationship of biological phosphate uptake to initial biomass concentration, growth stage and bacterial species (Momba and Cloete, 1996). It has been reported that *Acinetobacter* spp. are responsible for the removal of only 34% phosphorus from activated sludge systems (Cloete and Steyn, 1988).

The objective of this research was to investigate polyphosphate accumulation by all bacteria isolated from the aerobic zone of a biological nutrient removal (BNR) plant.

Materials and methods

Sampling

Sludge was obtained from a full-scale Johannesburg type BNR system. Sludge was collected from the aerobic zone of the system in a sterile 1 ℓ Schott bottle containing glass beads which were used to enhance break-up of flocs. The rationale for sampling from the aerobic zone was due to the fact that poly-P organisms occur in greater numbers and are more metabolically active than in the anaerobic zone (Lötter and Murphy, 1985). Sludge was stored at 4°C during transit and processed immediately.

Culture media used for phosphate (PO_4^{3-}) uptake studies

Mixed liquor samples from the anaerobic zone were allowed to settle for 2 h and centrifuged at 3 480 g for 20 min. Supernatant was filtered using Whatman No. 1 filter paper to remove biomass as well as other suspended solids, and 5 $g \cdot \ell^{-1}$ sodium acetate, 0.5 $g \cdot \ell^{-1}$ $MgSO_4.7H_2O$ and 0.18 $g \cdot \ell^{-1}$ KNO_3 were added to the filtrate. Filtrate was diluted with distilled water to render a final phosphate concentration of 38 $mg \cdot \ell^{-1}$. pH was adjusted to 7 with 2 N HCl before autoclaving at 121°C for 15 min (Bosch and Cloete, 1993).

Isolation of mixed liquor (ML) bacteria

Serial dilutions (10^{-1} to 10^{-9}) of the ML sample were prepared. Dilutions (0.1 mℓ) were aseptically plated on casitone glycerol yeast-extract agar (CGYA). Plates were incubated at 22°C for 5 d. Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. Colonies were subcultured on CGYA plates and re-incubated at 22°C for 5 d until monocultures were obtained.

Figure 1

Predominance of gram-negative poly-P genera isolated from the aerobic zone

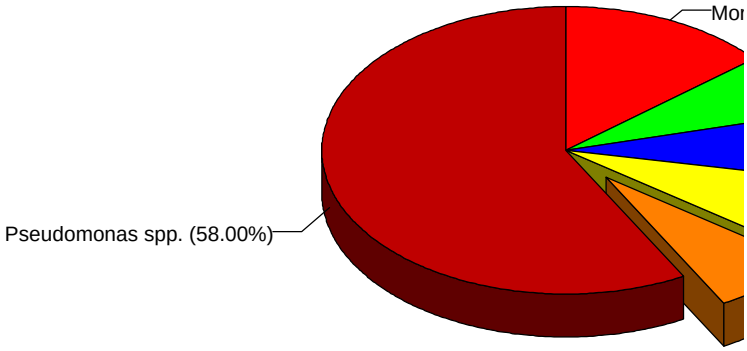
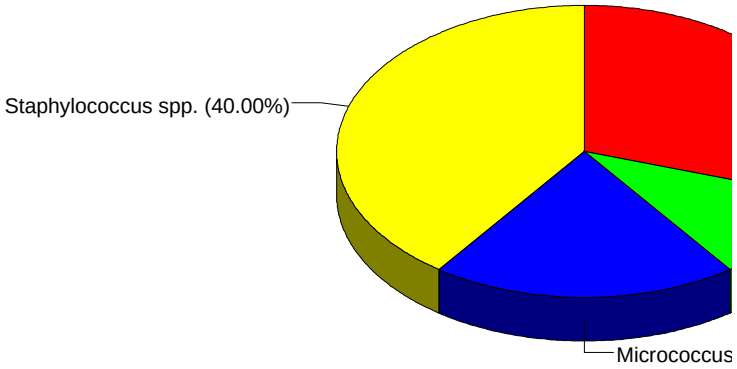


Figure 2

Predominance of gram-positive poly-P genera isolated from the aerobic zone



Phosphate uptake studies using mixed liquor bacteria

Inoculum was prepared by inoculating 10 mℓ of nutrient broth with a colony from each monoculture and incubated at 22°C for 24 h. One mℓ inoculum was serially diluted and plated onto nutrient agar to obtain a viable cell count. Plates were incubated at 22°C for 24 h. Four mℓ inoculum was aseptically transferred to 96 mℓ of ML media-containing flasks (initial orthophosphate conc. was known). Flasks were sealed and incubated anaerobically at 22°C for 2 h (on a shaker at 80 r·min^{-1}). Flasks were then aerated for 5 h by placing on a shaker at 120 r·min^{-1} (T= 22°C), pH noted (7.5) and 10 mℓ medium was extracted and filtered through 0.22 µm filters to determine orthophosphate concentration using Merck SQ118 method number P (VM) 14842 (Bosch and Cloete, 1993). A pH of 7.8 of the supernatant after aerobiosis would have determined that phosphorus had precipitated as either calcium or magnesium salts, which could erroneously account for the decrease in phosphorus concentration in the isolate. Neisser stains were performed to confirm phosphate accumulation. All poly-P organisms were identified using biochemical tests, API 20E and 20NE. Phosphate uptake studies were also conducted on an American Type Culture Collection (ATCC) culture of *Acinetobacter calcoaceticus* var. *lwoffi.* The ATCC culture served as a standard for phosphate uptake studies.

Results and discussion

Isolation and identification of poly-P organisms assisted in understanding the contribution of each isolate to PO_4^{3-} removal from waste waters. Using conventional plating techniques, 39 monocultures were initially isolated from the mixed liquor, subjected to phosphate uptake studies of which only 24 showed ability to accumulate PO_4^{3-}. Fourteen isolates were gram-negative while the other 10 were gram-positive and therefore formed 58% and 42% respectively of total poly-P population isolated from the sludge sample. Lötter (1985) reported that after *Acinetobacter* spp. it was the gram-positives followed by *Pseudomonas* spp. that dominated the aerobic zone of a BNR plant. During this study gram-positives and *Pseudomonas* spp. formed 50% of the total number of monocultures obtained and therefore proved to be the dominant organisms in the sludge sample obtained from the aerobic zone. During the present study, subsequent examination of cultures isolated such as the gram-positives, *Pseudomonas* spp., *Acinetobacter calcoaceticus* var *lwoffi*, *Klebsiella* spp., *Aeromonas* spp., *Moraxella* spp., *Alcaligenes* spp. and *Enterobacter* spp. showed that these bacteria were capable of phosphorus accumulation as polyphosphate. These results confirmed those of previous workers (Lötter and Murphy, 1985; Kavanaugh and Randall, 1994).

Results of PO_4^{3-} uptake studies conducted on the monocultures and ATCC culture of *Acin2etobacter calcoaceticus* var. *lwoffi* are shown in Table 1. The table lists 17 of the 24 isolates that accumulated phosphate. This was due to the fact that only the highest phosphate accumulators were of interest and taken into account. The remaining 7 isolates were regarded as low phosphate accumulators since they accumulated less than 10^{-12} mg PO_4^{3-}·cell^{-1}. Total PO_4^{3-} uptake per cell (mg PO_4^{3-}·cell^{-1}) indicated the highest and the lowest PO_4^{3-} accumulators from the group (Table 1). *Acinetobacter calcoaceticus* and *Aeromonas hydrophila* were found to be the highest PO_4^{3-} accumulators. Although the two organisms were present in the minority i.e. both isolated only once, they accumulated 1.84 x 10^{-10} and 1.65 x 10^{-10} mg PO_4^{3-}·cell^{-1} respectively. *Pseudomonas mendocina, Pseudomonas fluorescens* and *Pseudomonas putrefaciens* accumulated 4.26 x 10^{-11},

3.43×10^{-11} and 6.00×10^{-11} mg $PO_4^{3-} \cdot cell^{-1}$ respectively, and were also classified as high phosphate accumulators.

Results illustrated in Fig. 1 indicate the highest PO_4^{3-} accumulators (gram-negatives) together with their predominance (in %). The results reveal that amongst the gram-negatives, *Pseudomonas* spp. were the predominant organisms and formed 58% of the gram-negative organisms isolated. *Moraxella* spp. formed 14%, *Acinetobacter* sp. 7%, *Aeromonas* spp., *Alcaligenes* spp. and *Enterobacter* spp. each constituted 7% of the total gram-negative population. All these organisms showed the propensity to accumulate PO_4^{3-} but the highest was shown by *Acinetobacter calcoaceticus* and *Aeromonas hydrophila*. These results did not only substantiate findings by Cloete (1997) who reported *Acinetobacter* spp. as having a higher potential to accumulate polyphosphate when compared to the other poly-P organisms, but also support work by Suresh et al. (1985) who reported the genus *Pseudomonodaceae* to be highly active in phosphate uptake and release. These results correlate with the findings of Brodisch and Joyner (1983) who not only reported the predominance of *Pseudomonas* spp. (16.5%) but also the presence of *Aeromonas* spp. (19.5%) and *Acinetobacter/Moraxella* spp. (5.5%) in the aerobic zone of the laboratory-scale activated sludge system. During the present study the population of *Pseudomonas* spp. was reported to be 58% and *Acinetobacter/Moraxella* to be 7 and 14%. These high percentages could be due to subjecting the sludge to a short period of anaerobiosis (i.e. 2 h) which disallowed the complete depletion of nutrients in the cell and thus caused the cells to die.

Lötter (1985) reported gram-negative organisms to be the dominant organisms in the activated sludge system. Results illustrated in Fig. 2 indicate the predominance of gram-positives in the sludge, found to be 42% of the total poly-P population. These results support work by Brodisch and Joyner (1983) who reported the predominance of gram-positive organisms throughout their laboratory-scale activated sludge system. They reported the gram-positive population to be 34.5% in the aerobic zone. The figure also indicates that *Staphylococcus* spp. were the predominant gram-positives, forming 40% of the gram-positive population isolated from the sludge followed by *Streptococcus* spp. (30%), *Micrococcus* spp. (20%) and *Bacillus* sp. (10%). *Bacillus cereus*, *Micrococcus* spp., *Staphylococcus epidermidis* and *Streptococcus* spp. showed reasonably high phosphate-accumulating ability in consecutive descending order respectively (Table 1). This suggests that gram-positive organisms can perform similarly (i.e. accumulate phosphate) to gram-negatives if subjected to appropriate conditions.

Previous researchers such as Buchan (1983), Lötter and Murphy (1985), Cloete et al. (1985), Kerdachi and Healey (1987), Bayly et al. (1989) and Beacham et al. (1990) who conducted studies on full-scale plants have all reported that *Acinetobacter* spp. formed 50 to 70% of the total population isolated from the mixed liquor. Some workers have hypothesised that microorganisms other than *Acinetobacter* spp. play a fundamental role in biological phosphate removal. Some studies with municipal sludge showing enhanced biological phosphate removal (EBPR) indicated that *Acinetobacter* spp. accounted for 1 to 10% of the bacterial communities in laboratory- and pilot-scale activated sludge units (Brodisch and Joyner, 1983; Hiraishi et al., 1989). Wagner et al. (1994) using oligonucleotide probes specific for *Acinetobacter* spp. reported that *Acinetobacter* spp. formed 8 to 10% of the bacterial community in sludge. Results obtained during the present study support the findings of these workers who reported low *Acinetobacter* spp. counts when conducting studies on laboratory-scale activated sludge plants. Based on these findings these researchers also concluded that EBPR did not always depend on the presence of *Acinetobacter* spp.

The low *Acinetobacter* spp. count during this study implied that organisms other than *Acinetobacter* coexisted in order for phosphate removal to occur. Phosphate-accumulating ability of *Pseudomonas* spp., *Aeromonas* spp., *Moraxella* spp., gram-positives , *Enterobacter* spp. and other isolates showed that organisms besides *Acinetobacter* spp. are capable of absorbing orthophosphate and accumulating it as polyphosphate.

Conclusions

Although *Acinetobacter* spp. were present in extremely low numbers, their capacity to accumulate polyphosphate intracellularly was the highest amongst all the isolates. Their estimated low predominance in the sample could be due to the fact that the studies were conducted on a laboratory scale. It can also be concluded that besides *Acinetobacter* spp., other organisms such as gram-positives, *Pseudomonas* spp., *Aeromonas* spp., *Moraxella* spp., *Enterobacter* spp were capable of luxury phosphate uptake. *Acinetobacter calcoaceticus* var. *lwoffi* together with *Aeromonas hydrophila*, gram-positives (namely *Bacillus cereus)* and *Pseudomonas* spp. were regarded as the highest phosphate accumulators during the present study. Future research will focus on the application of rRNA gene probe technology for the characterisation of bacteria responsible for polyphosphate uptake in activated sludge.

Acknowledgments

The authors wish to thank the Water Research Commission of South Africa for financial support. Special acknowledgement is given to the Centre for Water and Wastewater Research for providing facilities for the laboratory work.

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