Anoxic phosphorus removal by denitrifying heterotrophic bacteria

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Abstract The unexplained occurrence of anoxic phosphorus (P) accumulation has largely hampered modeling of nitrification denitrification biological excess P removal (NDBEPR) systems. The aim of this study was, therefore, to isolate and identify denitrifying – P accumulating heterotrophic bacteria (DPBs) from a NDBEPR system in order to evaluate anoxic P accumulation and the specific mechanisms involved. Results of the study showed various heterotrophic bacteria to be capable of anoxic P accumulation utilising nitrate (NO_3) as electron acceptor. While *Pseudomonas* spp. predominated, *Serratia* spp. and *Vibrio* spp. demonstrated the most efficient anoxic P accumulation with 7.10 and 7.29 mgPO_4-P/L removal, respectively, at an initial NO_3 concentration of 13.54 mgNO_3-N/L and P concentration of 16.34 mgPO_4-P/L. Weaker DPBs were also identified which were only capable of accumulating small amounts of P at low initial P and NO_3 concentrations due to weak denitrification capacity. Anoxic P release was also observed due to the presence of acetate.

Keywords Anoxic; denitrification; heterotrophic bacteria; P removal

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Introduction

Phosphorus (P) removal under anoxic conditions has been observed in a number of biological nutrient removal systems and it is becoming apparent that this phenomenon needs to be understood better (Wanner *et al*., 1992; Kuba *et al*., 1993; Barker and Dold, 1996; Kuba *et al*., 1997; Mino *et al*., 1998; Ekama and Wentzel, 1999; Barak and van Rijn, 2000; Dionisi *et al*., 2001). Ekama and Wentzel (1999) found that denitrification kinetics determined for nitrification denitrification (ND) systems vary considerably at times when applied to nitrification denitrification biological excess P removal (NDBEPR) systems. This was largely due to the unexplained occurrence of anoxic P removal and varying active heterotrophic fraction estimates (Ekama and Wentzel, 1999). From a microbiological perspective there is little argument against organisms being able to utilise nitrate (NO_3)/nitrite (NO_2), instead of oxygen, for oxidation of stored polyhydroxyalkonoates (PHA) with subsequent uptake of P (Ahn *et al*., 2000). Although uncertainty exists regarding the organisms involved, it is possible that *Pseudomonas* spp. may play a significant role as these organisms have been reported to accumulate polyphosphate (poly-P) and are possibly predominant denitrifiers (Osborn *et al*., 1989; Kavanaugh and Randall, 1994; Jørgensen and Paulii, 1995; Sidat *et al*., 1999; Drysdale *et al*., 1999). However, for effective modeling of N and P removal in NDBEPR systems, much still needs to be understood about the organisms involved, their biochemistry and the physiological conditions that influence anoxic P removal.

The aim of this study was, therefore, to isolate and identify denitrifying P-accumulating heterotrophic bacteria (DPBs) from a full-scale NDBEPR system in order to evaluate anoxic P accumulation and the mechanisms involved.

Methods

Isolation and identification of heterotrophic bacteria

Two different media were used for the initial enrichment and isolation of potential DPBs.

Acetate mineral media (AMM) was used to isolate potential poly-P accumulating organisms (PAOs) (Jørgensen and Paulii, 1995) while casitone glycerol yeast autolysate (CGY) agar was used for isolation of ordinary denitrifying heterotrophic bacteria (Osborn *et al.*, 1989; Drysdale *et al.*, 1999). Random mixed liquor samples were taken from the anoxic and aerobic zones of the Darvill NDBEPR process situated at Pietermaritzburg, South Africa. The samples were homogenised using glass beads for floc break up and serial dilutions (10^{-1} to 10^{-8}) prepared and plated onto AMM and CGY agar using the spread plate technique. After 5 to 7 d of incubation at 20°C, well defined bacterial colonies were isolated. Subsequent to P uptake screening, positive DPBs or isolates demonstrating anoxic P release, were identified to at least generic level using Gram stains, API 20E, API 20NE, key differential biochemical tests, and cellular and colonial morphological characteristics.

Evaluation of anoxic P accumulation

Potential PAO and denitrifier isolates were initially screened for denitrification using a colorimetric biochemical reduction test (Drysdale *et al.*, 1999). This initial screening step identified denitrifying heterotrophic bacteria, eliminating further unnecessary evaluation of non-denitrifiers which, owing to their inability to utilise NO_3 as a final electron acceptor for cellular respiration, should not be capable of accumulating P under anoxic conditions. Neisser positive (Jenkins *et al.*, 1984) denitrifying isolates were then grown anaerobically in liquid acetate media (24 h at 25°C), to deplete resident poly-P granules (Jørgensen and Paulii, 1995). After incubation the biomass was centrifuged (8,000 rpm, 10 min, 10°C) and washed with distilled water. Cells were re-centrifuged and then suspended in P uptake media of varying P concentrations (3.27, 4.90 and 16.34 mgPO_4-P/L) (Jørgensen and Paulii, 1995) and incubated for a further 6 h. Anoxic conditions were created in the P uptake media via the addition of 2.26, 11.29 and 13.54 mgNO_3-N/L coupled with helium sparging prior to inoculation (Kuba *et al.*, 1993). NO_3 and P concentrations were monitored hourly using a Merck SQ118 photometer while uninoculated broths were used as controls for the experiments.

Results and discussion

Anoxic P accumulation

Anoxic P accumulation was observed in ten heterotrophic bacterial isolates of which *Pseudomonas* spp. predominated. Of these, six isolates proved capable of good P accumulation under anoxic conditions (Table 1; Figure 1) while four were only capable of accumulating P in small amounts at low initial P (4.90 mgPO_4-P/L) and NO_3 (2.26 mgNO_3-N/L) concentrations (Table 1; Graph not shown). *Serratia* spp. and *Vibrio* spp. demonstrated the best anoxic P accumulation with 7.10 and 7.29 mgPO_4-P/L removal, respectively, at an initial NO_3 concentration of 13.54 mgNO_3-N/L and P concentration of 16.34 mgPO_4-P/L.

Table 1 Identification of DPBs demonstrating strong and weak anoxic P accumulation

Isolate reference no. and identification		Initial P (mgPO_4-P/L)	P uptake (mgPO_4-P/L)	Initial NO_3 (mgNO_3-N/L)	NO_3 reduc. (mgNO_3-N/L)
DP1	*Vibrio* spp.	16.34	7.29	13.54	2.92
DP2	*Serratia* spp.	16.34	7.10	13.54	6.22
DP3	*Aeromonas* spp.	16.34	3.86	13.54	3.27
DP4	*Pseudomonas* spp.	16.34	3.29	13.54	3.65
DP5	*Pseudomonas* spp.	16.34	2.05	13.54	3.57
DP6	*Pseudomonas* spp.	16.34	3.43	13.54	4.37
DP7	*Pseudomonas* spp.	4.90	1.51	2.26	1.10
DP8	*Pseudomonas* spp.	4.90	1.51	2.26	0.95
DP9	*Pseudomonas cepacia*	4.90	1.13	2.26	1.25
DP10	*Klebsiella* spp.	4.90	1.05	2.26	1.14

The NO_3 reduction observed confirms that some denitrifying bacteria are capable of P removal using NO_3 instead of oxygen as electron acceptor. It is most probable that these organisms are also capable of aerobic P accumulation but it is uncertain as to how their P removal capacities will vary between anoxic and aerobic conditions. Although anoxic P accumulation tends to be less than that possible under aerobic conditions (Kerrn-Jespersen and Henze, 1993) it uncertain as to how this corresponds with the individual organisms capable of anoxic P removal. Kerrn-Jespersen and Henze (1993) demonstrated that anoxic P accumulation was dependent on the ratio of DPBs to aerobic P accumulators. This may offer some explanation of the difficulties experienced in modeling NDBEPR systems based upon the kinetics of ND and anaerobic/aerobic systems, as the physiological conditions in these systems differ and, therefore, do not necessarily select for the same microbial community structure. Pure culture studies by Sidat *et al*. (1999), demonstrated

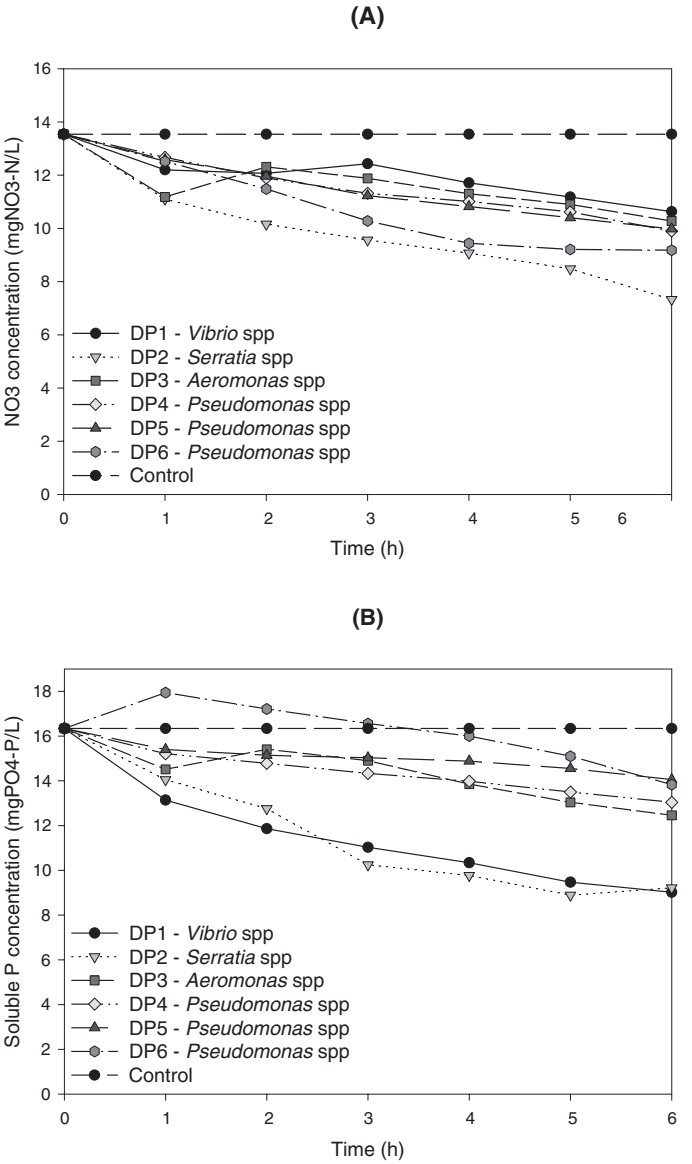

Figure 1 NO_3 reduction (A) and P up take (B) profiles by isolates DP1-DP6 when cultivated in P up take media under anoxic conditions

very similar P removal capacities under aerobic conditions, to those seen in this study under anoxic conditions. Therefore, it is possible that anoxic P uptake may be an effective alternative to aerobic P uptake should the correct conditions be established for selecting DPBs within NDBEPR systems. The weaker DPBs removal capacities varied between 1.83 and 1.05 mgPO_4-P/L removal with 1.25 to 0.95 mgNO_3-N/L reduction. It is possible that the weak anoxic P accumulation observed by these organisms is due to the weak denitrification capacity demonstrated. Weak denitrification would result in lack of available electron acceptors, in turn, restricting oxidation of PHA and subsequent P accumulation. However, these organisms may not necessarily be weak P accumulators under aerobic conditions but are simply limited, due to weak denitrification capacity, under anoxic conditions.

Anoxic P release

Anoxic P release was also observed for seven isolated denitrifying bacteria of which *Pasteurella* spp. released as much as 5.91 mgPO_4-P/L with concurrent reduction of 6.25 mgNO_3-N/L (Table 2; Figure 2). Denitrification was seen to occur simultaneously with P release with NO_3 reduction ranging from 3.31 to 7.46 mgNO_3-N/L and P release ranging between 1.56 and 5.91 mgPO_4-P/L. No single genera was found to be predominant except for two *Moraxella* spp. isolated.

Although four of the isolates demonstrated slight P uptake after initial P release, these organisms cannot be positively linked to excess P removal under anoxic conditions. However, it is interesting to note that P release occurred simultaneously with NO_3 reduction as the presence of NO_3 is known to inhibit P release under anaerobic conditions (Kuba *et al*., 1996; Muyima *et al*., 1997). However, as acetate was present in the P uptake media it is possible that P release was acetate induced (Muyima *et al*., 1997) even though NO_3 was available as an electron acceptor. Researchers have shown that P release is directly dependent on the presence of acetate and not necessarily anaerobic conditions which only stimulate fermentation of substrates to acetate and other volatile fatty acids (Muyima *et al*., 1997). In the absence of an external carbon source these organisms may be capable of anoxic P accumulation as their denitrification capacity was good. Furthermore, slight P uptake observed after initial release may be a result of decline in acetate concentration due to denitrification and intracellular PHA production. As a result acetate would be nonlimiting allowing for P accumulation to proceed. However, the P uptake experiments were not conducted for longer than six hours to verify this possibility. Alternatively, these organisms may be PAOs that are incapable of anoxic P accumulation but able to denitrify under anoxic conditions. This would suggest possible overlap in the functions of PAOs as well as possible alternative biochemical mechanisms involved. This, however, remains uncertain and a more detailed understanding of these organisms is required to fully understand the mechanisms of BEPR occurring in NDBEPR systems.

Table 2 Identification of denitrifying heterotrophic bacteria demonstrating simultaneous denitrification and anoxic P release

Isolate reference no. and identification		Initial P (mgPO_4-P/L)	P release (mgPO_4-P/L)	Initial NO_3 (mgNO_3-N/L)	NO_3 reduc. (mgNO_3-N/L)
DP11	*Pseudomonas fluorescens*	4.90	1.56	11.29	5.26
DP12	*Flavobacterium indologenes*	3.27	2.75	11.29	4.94
DP13	*Pasteurella* spp.	3.27	5.91	11.29	6.25
DP14	*Moraxella* spp.	3.27	2.32	11.29	3.31
DP15	*Achromobacter xylosoxidans*	3.27	1.98	11.29	4.51
DP16	*Moraxella* spp.	3.27	2.64	11.29	6.59
DP17	*Alcaligenes* spp.	3.27	2.06	11.29	7.46

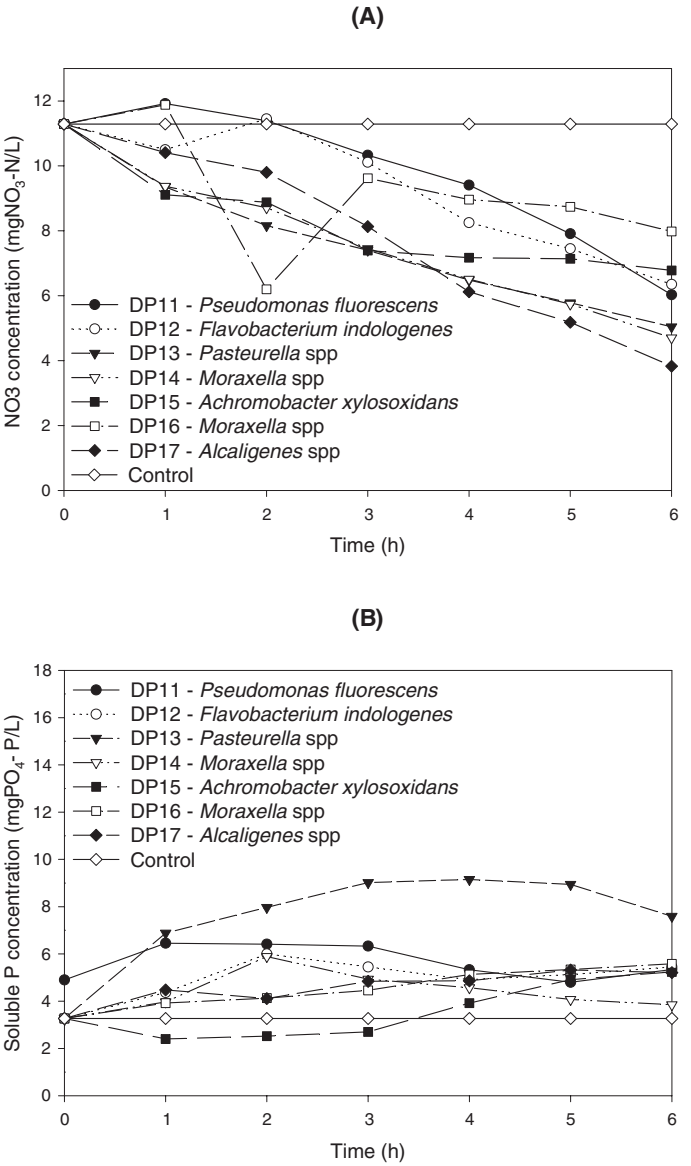

Figure 2 NO_3 reduction (A) P release (B) profiles by isolates DP11-DP17 when cultivated in P uptake media under anoxic conditions

Conclusion

Various heterotrophic bacteria demonstrated a capacity for anoxic P uptake utilising NO_3 instead of oxygen. It is, however, uncertain as to what variation there is between the aerobic and anoxic P removal rates of these organisms. Of the DPBs isolated and identified, *Pseudomonas* spp. predominated while *Serratia* spp. and *Vibrio* spp. demonstrated the most efficient anoxic P accumulation. Weaker DPBs were also identified which were only capable of accumulating small amounts of P at low initial P and NO_3 concentrations due to weak denitrification capacity. These organisms, however, may not necessarily be weak PAOs under aerobic conditions. Anoxic P release was also observed by some denitrifying heterotrophic bacteria, possibly due to the presence of acetate in the P media. These organisms may, therefore, be true DPBs or, alternatively, be denitrifying PAOs that are incapable

of anoxic P accumulation. Results of this study, therefore, indicate the possibility of more than one mechanism involved in BEPR which may be restricted to different bacteria.

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