Microbial community profile of a biological excess phosphorus removal (BEPR) activated sludge system using a cultivation-independent approach

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Abstract

It is generally accepted that biological release of phosphorus in the anaerobic zone of a nutrient removal system and phosphorus accumulation in the subsequent aerobic zone is directly proportional to the quantity of volatile fatty acid or the readily biodegradable COD fraction (f_{bs}) entering the system. This will enrich for polyphosphate accumulating organisms (PAOs) in the system and an increase in biological phosphorus removal will be observed. Enrichment for PAOs during the present study was essentially achieved by increasing both the phosphorus and f_{bs} concentrations (maintaining constant total COD loads) in the influent to the system. Fluorescence *in situ* hybridisation (FISH) using kingdom-, subdivision- and genus-level probes was used to identify and enumerate the bacterial community implicated in biological excess phosphorus removal (BEPR). Hybridisation of up to 78% of the cells (in relation to DAPI staining) with probe EUB338 indicated that a high proportion of the sludge comprised metabolically active bacteria. Bacterial predominance in the BEPR sludge appeared, in descending order, as such: β Proteobacteria (22%); α Proteobacteria (19%); γ Proteobacteria (17%); and, Actinobacteria (11%). Incidence of *Acinetobacter* spp. appeared to be relatively low with counts amounting to < 9% of the total bacterial count. The results indicate that the β and α Proteobacteria are metabolically functional (either directly or synergistically) in BEPR processes and reiterate the functional misconception of *Acinetobacter* spp. in these same systems.

Nomenclature

AE1/2	aerobic reactor/s 1 and/or 2
AN	anaerobic reactor
API	Analytical Profile Index
AX	anoxic reactor
BEPR	biological excess phosphorus removal
BNR	biological nutrient removal
CGYA	Casitone Glycerol Yeast Autolysate Agar
COD	chemical oxygen demand
DAPI	4',6'-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DO	dissolved oxygen
DSVI	dilute sludge volume index
EDTA	ethylenediaminetetra-acetic acid
FISH	fluorescence *in situ* hybridisation
FSA	free and saline ammonia
f_{bs}	biodegradable, soluble COD fraction
HAc	acetic acid
MLSS	mixed liquor suspended solids
NaAc	sodium acetate
OUR	oxygen utilisation rate
PAO	polyphosphate accumulating organism
PBS	phosphate buffered saline
Q_i	influent flow rate
R_s	sludge age
RBCOD	readily biodegradable COD (synonymous with f_{bs})
SRP	soluble reactive phosphorus
S_{bsi}	influent biodegradable, soluble COD
S_{ti}	influent total COD
TKN	total Kjeldahl nitrogen
TP	total phosphorus
VSS	volatile suspended solids
WWTP	wastewater treatment plant
WWW	wastewater works

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Received 29 July 1999; accepted in revised form 19 May 2000.

Introduction

Although BEPR plant operations have been successfully and widely applied, they still experience irregularities with regards to biological phosphorus removal. Mathematical descriptions of the BEPR mechanism have resulted in the construction of a number of simulation models (Dold et al., 1991; IAWQ, 1995). However, these models have usually been constructed via observations under controlled, laboratory conditions. Variability in real operational conditions (influent flow, load and composition) indicates the difficulty in directly applying these models when developing new or upgrading existing WWTP to incorporate BEPR. The biological mechanism, therefore, has to often be supplemented with chemical dosage (with its negative impacts) to ensure the WWTP concerned complies with regulatory P concentrations in their effluents. Advances in process and sanitary engineering during the past three decades have ensured that advanced wastewater treatment processes such as BEPR and nitrogen removal could indeed be constructed. Yet it remains disconcerting to note that a technologically advanced biological process such as BEPR still lacks fundamental understanding from a biochemical and microbiological point of view (Mino et al., 1998a).

Molecular based analysis has emerged as a powerful tool to facilitate a better understanding of the structure and function of the microbial population comprising various ecosystems (Stahl et al., 1988; Gersdorf et al., 1993; Lim et al., 1993; Raskin et al., 1994).

As far as the activated sludge process is concerned, such information could assist process retrofitting, design and modeling as these evolve into more complex mathematical descriptions. Such advances are proving essential as nutrient loads on WWTP increase and as environmental legislation regarding effluent quality becomes more stringent. Mathematical models involving BEPR are beginning to include more specific microbiological and biochemical information from lower levels of organisation contained within the surrogate biomass (Wentzel and Ekama, 1997). However, that portion of the active biomass responsible for phosphorus uptake has not been directly measured within the IAWQ Activated Sludge Model No. 2 (IAWQ, 1995) construct. In this regard, the direct determination of PAOs (quantitative as well as qualitative data) becomes more relevant.

Acinetobacter has traditionally been proposed to play an important role in BEPR by many early workers when applying cultivation-dependent techniques (Fuhs and Chen,1975; Hart and Melmed, 1982; Lötter, 1985). However, culture-independent enumeration techniques such as fluorescent antibody methods (Cloete and Steyn, 1987) and *in situ* hybridisation (Wagner et al.,1993; 1994) reveal that *Acinetobacter* spp. are not predominant in BEPR sludges. It has also been found that cultivation-dependent enumerative methods lead to overestimations of this genus (as well as other members of the γ Proteobacteria). Direct *in situ* techniques are now considered more accurate for quantifying bacteria in a particular ecosystem. Fluorescently labelled oligonucleotide probes have been used with epifluorescence microscopy to identify single cells of specific phylogenetic groups in the environment (Hicks et al., 1992). This approach targets the rRNA within intact bacterial cells using oligonucleotides that are complementary to conserved signature sequences of phylogenetically defined taxa. Such probes have been designed for the following taxonomic levels:

- Domains Archaea, Bacteria and Eukarya (Amann et al., 1990).
- Intermediate family levels, viz. α, β and γ subclasses of the family Proteobacteria (Manz et al., 1992) and the Actinobacteria (formerly referred to as Gram positive bacteria with high G + C genomic content) (Roller et al., 1994).
- Lower genus levels e.g., *Acinetobacter* spp. (Wagner et al., 1994).

Research undertaken to determine the PAO population implicated in BEPR identifies particular bacterial phylogenetic families as being predominant in either sequencing batch reactors, operated in anaerobic/aerobic cyclic conditions, or continuous anaerobic/aerobic conditions to stimulate BEPR. These studies, using FISH technology, indicate the predominance of the β Proteobacteria and Actinobacteria (Blackall et al., 1998; Christensson et al.,1998; Crocetti et al., 2000). Sudiana et al. (1998) found that the β Proteobacteria group predominated in activated sludge communities which had been acclimatised with acetate as the major carbon source under phosphorus limited or rich conditions. Kawaharasaki et al. (1999) found that although the β Proteobacteria were predominant in BEPR sludge fed with acetate, they did not seem to accumulate large amounts of polyphosphate. Instead, up to 85% of the α Proteobacteria, representing only 7% of the total population, were inferred to accumulate large amounts of polyphosphate as revealed by staining with a high concentration of DAPI.

When formulating system response data on phosphate release and uptake, Wentzel et al. (1988) developed an enhanced PAO culture by means of decrements in the sewage fraction to a BNR activated sludge pilot plant, simultaneously increasing the feed acetate fraction to a maximum of 500 mgCOD/l as sodium acetate. They defined an enhanced culture system as one in which the system response could be accredited to the resident PAOs through selection of a suitable substrate (acetate) and set of environmental conditions (sequencing anaerobic/aerobic reactors) which promoted their dominance in the system. This approach would theoretically allow for more qualitative results to be construed than artificially cultivated pure bacterial cultures grown in chemostat systems as growth of other normal competing heterotrophs and natural predation would be curtailed but not totally excluded from the system. PAO behaviour in these systems could therefore be expected to conform closely to their behaviour in normal, 'natural' mixed culture systems. When aerobic cultures were subsequently identified using the phenotypic/biochemical identification API system, it was found that > 90% of the bacterial population consisted of *Acinetobacter* spp. (Wentzel et al., 1988). This finding, among others, led to common acceptance that *Acinetobacter* was indeed the principal organism responsible for poly-P accumulation and P removal from municipal wastewater. Subsequent models (Wentzel et al., 1989a; b) were formulated using *Acinetobacter* as the surrogate PAO.

Considering the limitations of cultivation-dependent enumeration methods, the present study incorporated FISH technology to conduct a detailed microbial community analysis of BEPR activated sludge (P sludge). Similar analysis of the seed inoculum (NP sludge), obtained from the return stream of a full-scale non-BEPR system was conducted. Microbial communities between the two sludge types were subsequently compared to evaluate whether a population shift did occur, thereby revealing those phylogenetic groups implicated in BEPR. Although it has since been shown that *Acinetobacter* is not the dominant organism in BEPR processes (Wagner et al., 1994; Melasniemi et al., 1999), the studies were not based on continuously operated samples of BEPR enhanced cultures fed pure acetate. The current study sought to repeat the work of Wentzel et al. (1988; 1989a;b) with a view to the application of molecular methods for bacterial identification and enumeration.

Materials and methods

Unit set-up

An insulated shipping container was used to house the activated sludge pilot plant (unit manufactured by Dept. of Civil Engineering, University of Cape Town) which was placed, on-site, at Darvill WWW (Pietermaritzburg, KwaZulu-Natal). A submersible pump (50 l/min) was suspended in the sump where settled sewage from the balancing tank enters the head of the full-scale activated sludge system, ensuring that settled sewage was supplied to a refrigerated holding tank (500 l, 2 to 4°C) when required. Sewage was continuously mixed at low energy inputs to minimise aeration.

Unit configuration and layout

The pilot plant was designed and modelled upon the 3-stage Phoredox process and operated at 20°C (±1°C). A schematic design of the pilot plant is given in Fig. 1. Reactor configuration consisted of the following zones: AN (8 l); AX (4 L); AE1 (10 l); and AE2 (10 l). The clarifier (2.5 l), situated downstream of the reactors, was positioned at a 60° angle to the horizontal. Target Q_i was set at 36 l/d; settled sewage was fed directly to the AN zone using a peristaltic pump (Gilson). The s-recycle, pumped (Gilson) from the clarifier to the AN zone, was set at a ratio of 1:1, with respect

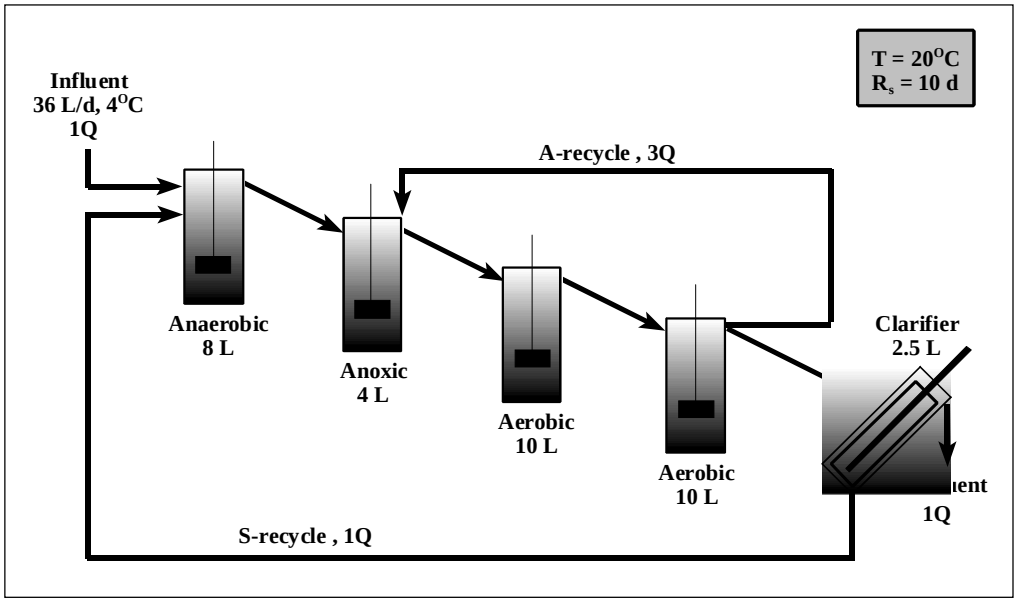

Figure 1
Schematic representation of the pilot plant unit modeled upon the 3-stage Phoredox process

to Q_i. The a-recycle, from AE2 to the AX zone, was pumped (Watson-Marlow) at a ratio of 3:1, with respect to Q_i. System R_s was maintained at 10 d for the duration of experimentation. OUR was measured in AE2 by means of a DO probe and meter (Randall et al., 1991). Air sparged into the aerated zones was controlled such that the aquarium air pumps switched the supply on and off in accordance with the lower and upper set DO points of 2.0 and 5.0 mgO/l, respectively.

Acquisition of seed inoculum

In order to observe any change which may have occurred in the activated sludge microbial community when attempting to enrich for PAOs, mixed liquor (NP sludge) was obtained from the return sludge stream of Amanzimtoti WWW (South Durban, South Africa). This plant is a non-BEPR, single aerobic treatment facility, presumably with little or no contribution (with reference to metabolic function) from the resident PAO population. This assumption was made due to the inability of PAOs to compete effectively with ordinary heterotrophs for COD without selector pressure (anaerobic zone at head of system with S_{bsi} entering the specific reactor). It was therefore assumed that any shifts which occurred in the sludge community when BEPR selection pressure was exerted could be attributed to gradual dominance of the PAOs within the system.

Feed supplementation for enhanced culture development

In order to simulate steady-state conditions, a constant S_{ti} of 500 mg/l was maintained throughout experimentation. However, during the earlier periods of experimentation, this proved difficult since Darvill WWW settled sewage is relatively weak. A lower S_{ti} concentration was accepted during these early stages using NaAc as the sole organic supplement. Orthophosphate, magnesium and sodium bicarbonate (for alkalinity) were also added to new sewage batches throughout experimentation. Anhydrous NaAc (ACE, South Africa) concentrations were incrementally increased to the feed at regular time intervals to a maximum COD equivalent of 500 mg/l. At a concentration of 400 mgNaAc/l as supplemented COD, settled sewage in the feed was diluted with tap water, thereby maintaining an S_{ti} concentration of approximately 500 mg/l. At this stage, macro- and micro-nutrient supplementation to the feed commenced in accordance with the recipe suggested by Wentzel et al. (1988). Ammonium chloride was also added to the feed stock to maintain TKN influent values of approximately 25 mgN/l.

Acid dosing

Wentzel et al. (1988) found that increased P uptake in the aerobic reactors of a BEPR system results in an increase in the pH of the mixed liquor. It was therefore anticipated that pH control would become increasingly significant and critical with increasing acetate dosage. Mixed liquor pH in all four reactors was monitored daily using a portable probe and meter (Beckman). Dilute hydrochloric acid (20 to 300 mM HCl) was dosed to AE1 at a rate of 500 ml/d. Addition of acid successfully maintained the mixed liquor pH of the system below 7.8 thereby negating P removal due to biological chemical precipitation.

Parameters measured

Table 1 describes the parameters routinely measured as well as the methods employed to conduct the various analyses.

Cultivation-dependent plate counts

Aliquots of activated sludge mixed liquor (1 ml) were serially diluted, spread on CGYA solid agar and incubated aerobically for 5 d at 20°C. All plating was performed in duplicate. Plates containing visible colony counts of $30<n<100$ were selected for further studies. The API 20 NE identification system (bioMérieux, France) was used to identify the isolates (data not shown). Isolates corresponding to family- and species-level groups of interest were quantified and expressed as fractions of total plate counts.

TABLE 1
SAMPLING POSITION AND PARAMETER MEASUREMENT

Test	COD[1]	TP[2]	SRP[3]	TKN[4]	FSA[5]	NO_3[6]	V/MLSS[7]	DSVI[8]	OUR[9]	pH[10]
Influent	✺ ✱	✺	✱	✺	✱					
AN		✺ ×								✓
AX		✺ ×								✓
AE1		✺ ×								✓
AE2		✺ ×					✓	✓	✓	✓
Effluent	✺ ✱	✺	✱	✺	✱	✱				

✓ Measurement taken (filtering not applicable);
✺ Unfiltered sample;
✱ Filtered through Millipore 0.45 μm glass fibre syringe filter;
× filtered through Whatman No. 1 filter paper (or equivalent)
[1] Microwave digestion and potentiometric titration
[2] Sulphuric acid/persulphate digestion at 100°C followed by molybdate-vanadate colour development for orthophosphate (*Standard Methods*, 1989)
[3] As 2 above, excluding digestion procedure
[4] According to Skalar AutoAnalyser Industrial Method Cat. No. 155-205
[5] According to Skalar AutoAnalyser Industrial Method Cat. No. 155-205
[6] According to Skalar AutoAnalyser Industrial Method Cat. No. A461-S
[7] Separation of solids by centrifugation, drying in crucible at 105°C and incineration at 550°C
[8] According to Lilley et al. (1997)
[9] YSI DO probe (5739) and automated procedure of Randall et al. (1991)
[10] Beckman portable pH meter
Note: S_{bsi} concentration was determined according to the physical-chemical separation method of Mamais et al. (1993)

TABLE 2
PROBE SEQUENCES AND TARGET SITES FOR *IN SITU* HYBRIDISATION

Probe	Sequence	Target site	% F[1]	Fluor	Reference
EUB338 [2]	5'- GCTGCCTCCCGTAGGAGT -3'	16S	20	Rhodamine	Amann et al., 1990
ACA23a [3]	5'- ATCCTCTCCCATACTCTA -3'	16S	35	Rhodamine	Wagner et al., 1994
AER [4]	5'-CTACTTTCCCGCTGCCGC-3'	16S	35	Fluorescein	Kämpfer et al., 1996
PSE [5]	5'-GTCGGCCTAGCCTTC-3'	23S	20	Fluorescein	Amann et al., 1995
ALF1b [6]	5'- CGTTCG(C/T)TCTGAGCCAG -3'	16S	25	Rhodamine	Manz et al., 1992
BET42a [7]	5'- GCCTTCCCACTTCGTTT -3'	23S	35	Rhodamine	Manz et al., 1992
GAM42a [8]	5'- GCCTTCCCACATCGTTT -3'	23S	35	Fluorescein	Manz et al., 1992
HGC69a [9]	5'- TATAGTTACCTCCGCCGT -3'	23S	25	Rhodamine	Roller et al., 1994

[1] percentage formamide (v/v) in the hybridisation buffer;
[2] EUB338 specific for all hitherto sequenced species affiliated to the kingdom Bacteria (previously referred to as Eubacteria) and phylogenetically distinct from the kingdom Archae (previously referred to as Archaebacteria);
[3] ACA23a, specific for the genus *Acinetobacter*;
[4] AER, specific for the genus *Aeromonas*;
[5] PSE, specific for most true members of the genus *Pseudomonas*;
[6] ALF1b,specific for the α subclass of Proteobacteria and most members of the phylum "spirochetes and relatives", and *Flexistipes sinusarabicus;*
[7,8] BET42a and GAM42a, specific for β and γ subclass of Proteobacteria, respectively;
[9] HGC69a, specific for the Actinobacteria.

TABLE 3
OPERATING DATA FOR BEPR LABORATORY-SCALE UNIT (P SLUDGE) RESULTS EXPRESSED AS MEAN VALUES WITH SAMPLE STANDARD DEVIATIONS IN PARENTHESES

Period	HAc [1] (mg/l)	TP:S_{ti} [2] (mgP/mgS_{ti})	MLSS [3] (g/l)	SRP removal [4] (mgP/l)	P/VSS [5] (mgP/ mgVSS)
A	0	ND	ND	ND	ND
B	100	0.04 (0.01)	1.532 (0.38)	2.3 (1.52)	ND
C	150	0.04 (0.01)	1.587 (0.25)	5.3 (1.86)	ND
D	200	0.06 (0.03)	1.689 (0.28)	16.3 (3.65)	ND
E	300	0.05 (0.05)	2.420 (0.50)	24.5 (2.93)	0.12 (0.03)
F	400	0.05 (0.01)	2.429 (0.23)	23.8 (3.22)	0.15 (0.05)
G	500	0.10 (0.02)	2.421 (0.22)	35.2 (4.16)	0.27 (0.02)

[1] represents feed HAc supplementation only, not S_{ti};
[2] feed TP expressed as ratio of S_{ti};
[3] determined in reactor AE2;
[4] SRP removal = SRP influent - SRP effluent;
[5] $P = P_{uf} - P_f$/VSS (P_{uf} = reactor unfiltered total P concentration; P_f = reactor filtered total P concentration); determined in reactor AE2;
ND = not determined

Sampling and cell fixation

A sample of NP sludge was collected, fixed and analysed upon initiation of the experiment. A grab sample P sludge mixed liquor was collected from the anaerobic, anoxic, and aerobic zones (community analyses were conducted on the contents of individual reactors) during the latter stages of period G (Table 2). Bacterial monoculture controls were grown aerobically at 30°C in nutrient broth and harvested for fixation at mid-logarithmic phase (OD = 0.6 to 0.8 at A_{600}). This ensured a high cellular rRNA content. All samples were fixed for 2 h at 4°C with 3% paraformaldehyde/PBS by the addition of three volumes of fixative to one volume of sample. Cells were then washed in 1 x PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) and then resuspended in PBS/cold absolute ethanol (1:1 v/v). For fixation of Gram positive cells, activated sludge was added to ethanol to a final concentration of 50% (v/v).

Membrane filtration and staining with DAPI

Membrane filtration was carried out as described by Porter and Feig (1980) with the following modification. Cellulose acetate (Millipore, 0.22 µm pore size, 25mm diameter) filters were stained for 24 h in Sudan Black (0.3% w/v in 60% ethanol). Dual staining of cells with DAPI and fluorescent oligonucleotides was modified from the method of Hicks et al. (1992) so that cells were stained after *in situ* hybridisation with DAPI (0.33 µg/ml) for 5 min.

Oligonucleotide probes and hybridisation

Oligonucleotide probes used during the present study are given in Table 2.

Probes were synthesised and labeled with either rhodamine (red) or fluorescein (green) by Roche Molecular Biochemicals. Aliquots of 5 µl fixed sludge were applied to pre-cleaned poly-lysinated slides and allowed to air dry. Spotted cells were then dehydrated by serial immersion through 60%, 80% and 96% (v/v) ethanol (3 min each). Samples of 10 µl hybridisation solution (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01% SDS, 50 ng probe, X % (v/v) formamide) were applied to each spot and incubated for 2 h at 46°C in an isotonically equilibrated humidity chamber. Probe was removed from the slide by rinsing with 2 ml pre-warmed washing solution (20 mM Tris/HCl, 0.01% SDS, 5 mM EDTA, Y M NaCl). The salt concentration, Y, in the washing solution was adjusted to the formamide concentration, X , in the hybridisation buffer according to the formula of Lathe (1985). Slides were rapidly transferred into washing solution and incubated at 48°C for 20 min. Slides were then rinsed briefly with distilled water, air-dried and mounted in VectaShield antifading solution (Vector Laboratories, USA) for viewing by microscopy.

Microscopy and image analysis

Cells were visualised with a Zeiss Axiolab microscope (Carl Zeiss, Germany) fitted for epifluorescence with a 50W mercury high- pressure bulb and Zeiss filter sets 02, 09 and 15. Images were captured using a Sony (Japan) CCD camera. Image analysis was carried out using the Zeiss KS 300 imaging system by measuring the relative area of fluorescence from probe conferred signals and DAPI staining. Since all probes did not confer equal intensities of fluorescence, the threshold for fluorescent emission by each probe was accordingly adjusted for individual samples by interactive use of the image analysis software. Ten microscopic fields under the 40X objective were randomly selected for enumeration of each sample challenged with group- and domain-level probes.

Results and discussion

Reactor operating data for the P sludge, during enhanced culture development, is summarised in Table 2. A more thorough description of the system response to increasing influent HAc and P concentrations can be obtained from Atkinson (1999). As influent HAc concentrations increased an increase in PO_4-P removal was noted, although maximum SRP removal potential may have never been realised due to the lack of prevailing steady-state conditions during the various experimental periods. During the present study, system response to increasing acetate dosages was only recorded for a period of two R_s which appears inadequate to ensure steady-state. The high P/VSS value of 0.38 achieved by Wentzel et al. (1988) was therefore never obtained. The highest P/VSS value obtained during the present study amounted to 0.27 when the culture was fed acetate as the sole carbon source (Table 3). However, the P sludge consistently released P in the AN reactor

TABLE 4
PLATE AND DIRECT CELL COUNTS (BY MEMBRANE FILTRATION) FOR NP AND P SLUDGES

	NP sludge (cells/ml)	P sludge (cells/ml)
Plate counts	3 to 5.2 x 10^7	1.0 to 2.6 x 10^6
DAPI (membrane filtration)	7.2 to 8.0 x 10^{10}	1.1 to 1.6 x 10^{10}
Estimated active bacteria	5.1 to 5.8 x 10^{10}	8.6 x 10^9 to 1.25 x 10^{10}

TABLE 5
PROBE SPECIFIC COUNTS EXPRESSED AS A PERCENTAGE OF TOTAL CELLS STAINED WITH DAPI COMPARED TO CULTURE-DEPENDENT ENUMERATION

	In situ (%)[1]	*In situ* (%)[2]	Plating (%)[3]
ALF1b	11	19	14
BET42a	25	22	13
GAM42a	14	17	66
HGC69a	13	11	0
PSE	< 3	< 4	50
AER	< 2	< 4	2
ACA23a	< 5	< 9	24
EUB [4]	73	78	-

[1] NP sludge FISH counts;
[2] P sludge FISH counts;
[3] P sludge plate counts;
[4] expressed as a percentage of the DAPI stain, i.e. EUB/DAPI ratio

(results not shown) and took up P in the subsequent AE zones (P transformation patterns consistent with those found in conventional BEPR processes). It appears that the system was operating under P limiting conditions during periods D and F due to large amounts of P taken up and inadequate P dosage which may have weakened the BEPR response during these respective periods. It was anticipated that phosphate limitation would not have adversely affected results of the sludge community analyses as samples for fixation and hybridisation were only extracted during period G of experimentation.

Large clusters of polyphosphate-containing cells (Neisser positive) were observed in sludge samples from AE1 and AE2 of the P sludge. As expected, similar staining of the seed NP sludge showed minimal staining of metachromatic inclusions, indicating little or no polyphosphate accumulation. It was interesting to note that Neisser staining of P sludge AX samples indicated large amounts of intracellular polyphosphate inclusions.

The informal name "bacteria" is frequently used loosely to refer to all prokaryotes, but care should be taken to interpret its meaning in a particular context. Because this study employs oligonucleotide probes that are phylogenetically based, one should note that the domain Bacteria (previously referred to as kingdom Eubacteria) are considered phylogenetically distinct from the domain Archae (previously referred to as kingdom Archaebacteria) and in all foregoing discussion reference to bacteria excludes members of the domain Archae (Woese et al., 1990).

Since rRNA content has been shown to be directly proportional to growth rates (Schaechter et al., 1958; Delong et al., 1989; Wallner et al.,1993; Gourse et al., 1996) this study assumes that all cells bearing probe conferred fluorescence are metabolically active. The active bacterial biomass, present in activated sludge samples, was estimated by multiplying the EUB/DAPI ratio and the total cell count as obtained by membrane filtration (Table 3). The EUB/DAPI ratio, listed in Table 4, represents the percentage of total cells that hybridised with probe EUB338 for both P and NP sludges. Results are not given for individual AE, AX and AN zones (in the case of the P sludge) since community composition was found to be stable across the reactors.

Note that the genus level counts (*Pseudomonas* spp., *Aeromonas* spp., and *Acinetobacter* spp.) listed in Table 5 are given as maximal percentages for those few fields observed bearing probe signals and do not represent average counts. The sum total (17%) of the genus level probes applied to the P sludge should therefore not be assumed to imply that the γ Proteobacteria have been accounted for by these three genera.

The microbial community was assessed by measuring the area of cells that displayed probe conferred fluorescence and expressing this as a percentage of the area that bound DAPI. This approach was used as opposed to individual cell counts for *in situ* hybridisations due to the fact that the majority of the activated sludge flocs were very compact. Figure 2 gives an impression of floc compaction as viewed for family representatives belonging to β (red) and γ(green) Proteobacteria. For total cell counts by membrane filtration, mechanical floc disruption was carried out using a mini-beadbeater (Biospec,USA) and 1 mm glass beads. This enabled efficient counting of individual cells without affecting DAPI fluorescence. However, it was found that this treatment led to decreased probe conferred signals during *in situ* hybridisations. This was probably due to cell disruption which resulted in leakage of ribosomes from the cells thereby decreasing the probe target number. Hence, for *in situ* hybridisations, relative area counts were employed instead of individual cell counts.

Based on the assumption that rRNA content is proportional to metabolic activity, the direct approach was found to be more representative of the bacterial community than plate counts. This is revealed by the discrepancies between cultivation-dependent and *in situ* analyses (Table 4). Total plate counts were found to underestimate the metabolically active bacterial population by at least three orders of magnitude. Cultivation-dependent plating methods did not represent the bacterial families and species constituting the sludge proportionally since they overestimated the contribution of the γ Proteobacteria subclass and underestimated

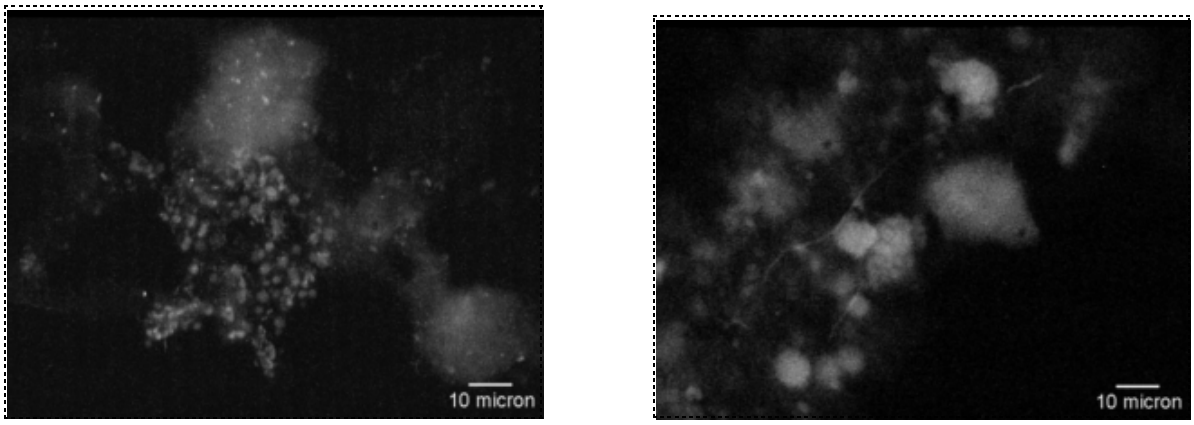

Figure 2
In situ *hybridisation of enhanced culture activated sludge with rhodamine (red) labelled probe BET42a and fluorescein (green) labeled probe GAM42a (X40)*

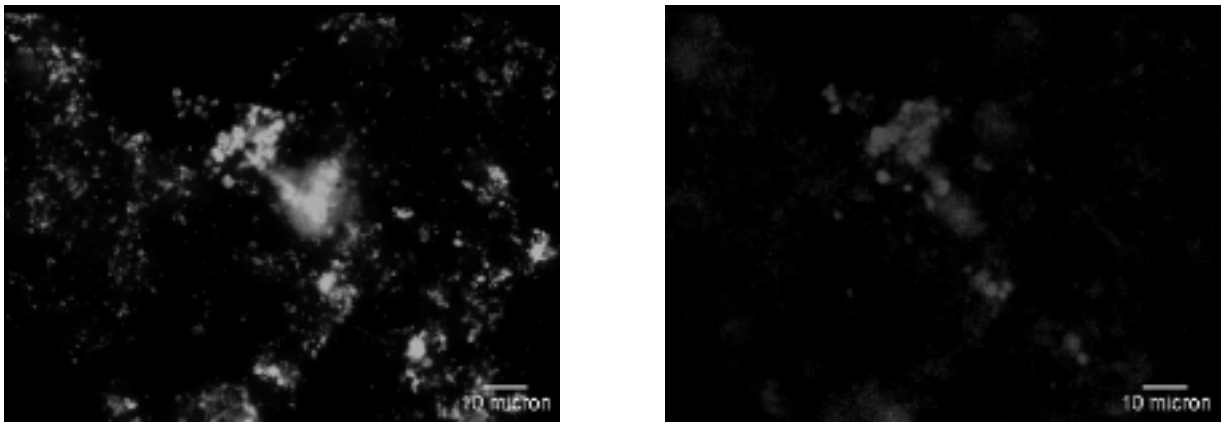

Figure 3
DAPI stain (blue) of enhanced activated sludge (left) and in situ *hybridisation with rhodamine (red) labelled probe ALF1b (right) for the same microscope field (X40)*

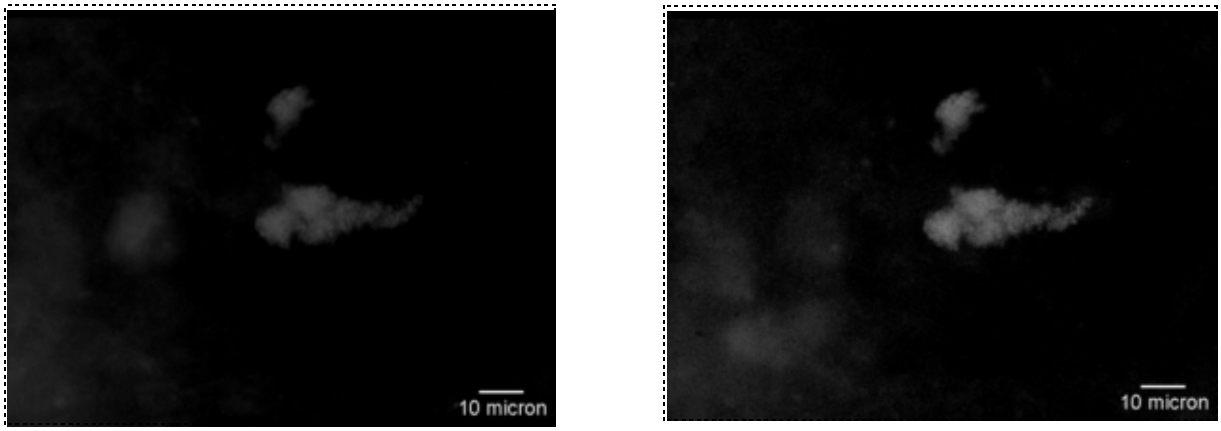

Figure 4
In situ *hybridisation of enhanced culture activated sludge with rhodamine (red) labeled probe ACA23a (left) and fluorescein (green) labeled probe GAM42a (right) for the same microscope field (X40)*

the contribution of the α and β Proteobacteria and the Actinobacteria (Table 5).

A high percentage of P sludge samples that were stained with DAPI also bound the probe EUB338 (78%) indicating that the majority of cells were bacteria that were metabolically active (Table 5). Extracellular polymers, synthesised by the sludge bacterial cells, did not appear to inhibit probe entry into the cells. Cells at the inner and peripheral regions of the flocs showed comparable probe uptake.

While most studies have indicated the predominance of members of the β Proteobacteria and/or Actinobacteria in BEPR sludges (Snaidr et al., 1997; Kawaharasaki et al.,1999; Bond et al., 1999), findings presented here indicate a greater degree of bacterial diversity in the P sludge (Figs. 2 and 3). The β Proteobacteria subclass was found to be present in highest numbers (22%), followed by the α and γ Proteobacteria (19 and 17%, respectively) and Actinobacteria (11%). The sum of the family-level probes for the P sludge (69%) is 9% less than the EUB probe percentage (78%), indicating the percentage of those bacterial families in the P sludge which were not accounted for (Table 4).

Members of the α Proteobacteria have been implicated by Kawaharasaki et al. (1999) as being capable of taking up large amounts of phosphate although they were not found to predominate BEPR sludge. During the present study, the α Proteobacteria were visualised to be clumped in compact spherical aggregates (Fig. 3). A clear shift in this particular population was observed to occur when comparing the NP sludge (11%) to the P sludge (19%). It is possible that the inclusion of an anoxic zone in the reactor configuration may have resulted in this population coming into predominance. This might also explain the discrepancies between the results obtained in the current study and work conducted using SBR's, operated in only aerobic/anaerobic modes, where the contribution of the α Proteobacteria to the total active sludge biomass was found to be minimal.

The application of the probe specific for *Acinetobacter* spp. enabled the visualisation of cocco-bacilli occurring either in chains or clusters that were also identified as members of the γ Proteobacteria (Fig. 4). This study supports findings that *Acinetobacter* spp. are not predominant in BEPR activated sludges in spite of the fact that the γ Proteobacteria were present at an amount of approximately 17% of the total community. The presence of *Acinetobacter* was estimated to be < 9% of the total population. Analysis of the population with a battery of probes for some commonly encountered bacterial species belonging to the γ Proteobacteria revealed that the contribution of *Pseudomonas* spp. and *Aeromonas* spp. in BEPR is also probably limited since they each constituted < 4% of the total sludge community (Table 4). Bacteria belonging to the Actinobacteria were found in the lowest numbers (11%). Filamentous-like clusters which took up probe HGC69a were commonly observed (figure not shown).

A survey of twenty full-scale activated sludge plants operating in Japan (Mino et al., 1998b) showed the β Proteobacteria to be most dominant followed by the α subclass and Actinobacteria, respectively. Studies conducted on a full-scale nutrient removal works operating in South Africa, using the tandem approach of FISH and dot-blots, also implicate the α Proteobacteria in BEPR (Mudaly et al., 2000). DGGE profiles also indicate a high species diversity in BEPR systems (Mino et al., 2000). It is therefore likely that there are several possible population structures capable of mediating BEPR and that BEPR is a result of a cumulative effect where no one genus or species dominates system response.

Conclusions

The enhanced culture community defined during the present study is clearly dispersed over a wide phylogenetic range of at least four bacterial families. Considering that each family comprises many genera and species, and noting that the community belonging to the β and γ Proteobacteria displayed numerous morphotypes, it may be concluded that bacteria capable of excess polyphosphate accumulation probably belong to different phylogenetic groups and cannot be ascribed exclusively to any one bacterial genus or species. This makes the targeting of a discrete PAO community in activated sludge a complicated task. A notable feature of enhanced culture development was the increase in relative numbers of the α Proteobacteria when the P sludge was compared to the seed NP sludge. It is recommended that representative genera within this subclass be investigated further for their ability to take up phosphate *in situ* within the activated sludge community, as well as in batch monoculture studies, to determine their physiology. Findings of this study also corroborate those of others in that *Acinetobacter* spp. were found in very low numbers in the P sludge, suggesting minimal contribution to BEPR. Despite their frequent and relatively high recovery rates on CGYA nutrient agar, *Pseudomonas* and *Aeromonas* spp. were not found to predominate the P sludge community.

Acknowledgements

The authors gratefully acknowledge the financial assistance of the Water Research Commission and the National Research Foundation (previously the Foundation for Research Development). Umgeni Water (Pietermaritzburg, South Africa) is also acknowledged for use of their equipment and for the various analyses conducted.

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