SMART WATER FUND PROJECT

FINAL REPORT

A Novel Process For Removing Phosphorus From Waste Water

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March 2009

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EXECUTIVE SUMMARY

Wastewater treatment plants are generally considered to represent the major point sources for the phosphorus (P) entering rivers and streams, which is responsible for eutrophication and the formation of toxic and environmentally damaging Cyanobacterial (Algal') blooms. Hence over the past 40 years many (EBPR) treatment plants around the world have been designed to remove P microbiologically to levels below those considered likely to support bloom production. These all operate where P and COD are added together and the biomass is recycled continuously through alternative anaerobic (feed): aerobic (famine) phases, considered essential to allow the polyphosphate accumulating organisms (PAO) to dominate. The process we studied was fundamentally different in that it was continuously aerated, and the additions of P and COD (acetate) were temporally separate. We undertook to see if this process was operationally feasible, to determine the influence of changing several operating conditions on its performance and to scale up the process to see if it could serve its intended function, to remove P from clarified effluent from a conventional activated sludge system. Details of the study are given in the Final report. This summary outlines some of the key findings. It is structured as in the final report as a series of project stages, each with its separate aims.

STEP 1 had the following aims:

Can we establish stable EBPR performance in this process carried out at 2litre laboratory scale in a sequencing batch reactor (SBR) fed both a synthetic wastewater and clarified effluent from a conventional treatment plant (both supplemented with acetate as carbon source)? If so, what is the composition of the microbial community which develops, and what is the identity of the PAO dominating under these conditions?

The final report contains detailed data showing that long term stable P removal was achieved, where P levels in the final effluent were generally undetectable at ambient temperature, under conditions where acetate was added at 120mg/l carbon, pH was controlled at pH7.5 and sludge age maintained at 20days. The major PAO populations were identified by a range of molecular methods as Accumulibacter phosphatis, the same PAO shown to dominate anaerobic:aerobic EBPR processes around the world. Furthermore its in situ physiology was as expected of a PAO

However if acetate feed levels were less than 120mg/l carbon, the community composition changed and another population became more dominant. These were eventually identified after some delay as Defluviicoccus, organisms known to pose as possible competitors of the PAO in also being able to assimilate acetate in the feed stage, but not then synthesising plyP in the subsequent famine stage as Accumulibacter does. Instead these accumulate glycogen and are often referred to as glycogen accumulating organisms (GAO). Much of our subsequent work was directed at trying to find operating conditions which favoured the PAO over the GAO, but this work was generally unproductive. In attempts to find a possible alternative carbon source only utilised by the PAO, we showed that these GAO would grow on a much wider range of feed sources. Also present in large numbers were bacteria related to Dechloromonas, which from our data, did not appear to be associated with EBPR decreases. Therefore the work moved on to the next stage.

STEP2 had the following aims:

As Defluviicoccus appeared to pose a potential threat to the reliability of our novel EBPR process, all experiments where operating conditions were altered, required us to carry out detailed microbiological community analyses. We undertook to change sequentially a range of relevant operational parameters. Unlike conventional EBPR process, operating pH did not appear to affect process stability, and almost complete P removal occurred at pH7 and 6.5. In anaerobic:aerobic EBPR systems, as pH falls the GAO are thought to be able to out-compete the PAO, but microbiological data suggested this was not the case with our process. However, it did not run reliably at 30C (as seen with conventional EBPR plants) or 15C. Furthermore dropping the sludge age to 10days affected process stability. Microbiological data supported our earlier hypothesis that

plant failure was generally (but not exclusively) associated with the increased numerical dominance of Defluviicoccus. Details of these experiments and their outcomes are given in the final report.

STEP3 had the following aims;

It was decided at this stage to proceed to the final stage in the process, the construction and operation of a pilot plant (150l), based on the lab scale operational data, and to see whether it would remove P from the clarified effluent of the Carrum ETP activated sludge process. Initial conditions were 120mg/l acetate as carbon, sludge age of 15days, and no temperature or pH control. Neither of the latter two parameters changed much over the SBR cycle. Altogether 5 runs were attempted, and except for run 1, which operated successfully for about 3weeks, in none of the others was satisfactory EBPR performance achieved. Consequently there was no time to study how changes in aeration rate and other critical parameters might influence process stability, but instead efforts were directed at trying to understand why this process so reliable at lab scale was failing here. Again the answer seemed to come from the microbiological analyses, which generally agreed with the chemical data. Under these conditions the microbial community became dominated by a novel bacterium, whose presence in large numbers (up to 50% of all cells) corresponded to EBPR failure. It was not Defluviicoccus the problem organism in the lab scale system, but a prevviouslu undescribed organism which after much work, we managed to identify. Using FISH probes designed by us, we clearly showed that it was entering our pilot plant in the Carrum effluent feed, where it was present in high numbers. With these new FISH probes we also showed it was never present in any of our lab scale reactors, even when EBPR had failed, probably because it was not detectable in the inoculum we used from the Kyneton plant. Nor could we find it in several other non-EBPR and anaerobic;aerobic activated sludge systems. It's in situ phenotype showed it assimilated acetate in the feed stage, but never stained for polyp. However, the process worked very well removing P from the Melton effluent in the laboratory.

INTRODUCTION

Removal of phosphorus from wastewater is essential to prevent formation of toxic algal blooms in receiving waters. These blooms limit the uses to which the recycled contaminated water can be put. Current wastewater treatment plants removing phosphorus microbiologically rely on cycling biomass through aerobic and anaerobic reactors to select microbial communities that accumulate phosphorus. This project evaluated and developed a process which is essentially aerobic, and examined its potential to be installed as an add-on unit to a conventional system to produce an effluent containing < 0.1mg/l phosphorus. If achievable, the treated water will be environmentally safer, and suitable for more diverse uses.

BACKGROUND

Australia is a water limited continent and southern states like Victoria are particularly prone to water shortages, so its rivers, streams, inland oceans and lakes are priceless assets. Unfortunately many of these water bodies suffer intermittently from eutrophication arising from long-term phosphorus (P) pollution, and unsightly and potentially dangerous cyanobacterial or algal blooms are common. Some of these organisms are highly toxigenic, and their health effects range from the relatively trivial to long term carcinogenic. The economic costs associated with cleaning up these blooms, if feasible, are substantial and their impact on general environmental health is enormous. Prevention is much sounder environmentally than attempted cure. As some of the major point sources of P are wastewater treatment plants, most systems now built in inland Australia, and where the treated waters run into environmentally sensitive receiving bodies, are designed to remove P microbiologically. However, effluents from such systems frequently require additional treatment with iron or aluminium compounds to achieve the P levels required by the EPA.

A common feature of enhanced biological phosphate removal (EBPR) plants currently operating around the world is that they all cycle their biomass through alternative aerobic: anaerobic reactor zones. Consequently their construction, or conversion of a conventional wastewater treatment plant to an EBPR system is complex and expensive. If a biomass sample taken from the aerobic reactor is examined microscopically, cells staining for polyphosphate (polyP) are abundant, but no intracellular polyβ-hydroxyalkanoate granules (PHA) can be detected. On the other hand, biomass from the anaerobic reactors stains for PHA but not polyP. These are common features of all EBPR plants regardless of current design and geographical location. It is believed that under anaerobic conditions, the phosphate accumulating organisms (PAO) utilize their intracellular reserves of polyP to generate the energy needed for anaerobic substrate (mainly acetate) uptake into their cells, and the conversion of this acetate into PHA, an energy and nutrient storage compound. Orthophosphate formed from polyP degradation is then released into the medium. Then, under aerobic conditions, where readily utilizable substrates are no longer available to the bacteria, the stored PHA are utilized by the PAO as a carbon and energy source to transport orthophosphate into the cells and convert it to polyp granules. The accepted view is that the PAO have a strong selective advantage under these aerobic: anaerobic operating conditions, because they can grow aerobically in the absence of any exogenous source of nutrients, by using the PHA accumulated under anaerobic conditions.

These EBPR plants have been developed empirically over the past 30 years, but with no clear understanding of how and why they work, or the structure and functions of the communities of bacteria actually removing the phosphorus. Consequently when they fail, as they can do, we do not know the cause or how to rectify the problem. If we understood better the microbiology of these plants then we would be better placed to improve their performance, and protect the rivers, streams and lakes (as well as sensitive marine habitats) from pollution of this kind. The availability of molecular, non-culture dependent methods of community analyses means that acquiring such microbiological information is now feasible. It is possible for example, to resolve the composition of the microbial communities in these systems and to ascribe clear functions to individual populations there. So in our view we can determine which are the microbes actually accumulating P and to use this information to develop methods to monitor the performance of these plants, for possible early warning systems of EBPR failure.

Although the effects of continuous aeration on EBPR have been reported, where the same chemical transformations distinctive of anaerobic: aerobic EBPR appear to occur in the absence of an anaerobic phase (Pijuan et al. 2006), it has yet to be shown that a fully aerobic process can remove P for an extended period of time. The process used in this study is a novel aerated P removing process, based on a process that was first developed by Professor Ron Bayly and Dr John May (May et al. 1997; Bayly and May 1998) as an add on unit for discharge from sewage effluent, storm water, industrial waste and process streams.

The Bayly and May SBR process consisted of cycles as follows:
1. In the FAMINE phase, addition of treated effluent containing P is added to the community which had accumulated C/energy (PHA) reserves. Cells use this reserve to take up the P, lowering the P level in the effluent.
2. In the FEED phase, addition of a C/energy source so that the biomass, under N and P limitation, will preferentially use their intracellular stores of polyP to produce new biomass, release P, and increase their C/energy reserves by PHA synthesis.
3. The effluent, now containing no P, and a portion of the biomass is then wasted from the system before a new cycle is started with the addition of new P containing effluent, and cells with intracellular C reserves ready to take up the P.
A portion of the biomass is reused in step 1, to treat a new batch of P containing effluent. As there is little biologically metabolisable substrate in the effluent feed, only cells that have been conditioned by the accumulation of C/energy reserves are able to take up the P and convert it to intracellular P (Bayly and May 1998) as in a conventional anaerobic: aerobic EBPR process.

The key features are a low concentration of biologically utilisable C compounds in the effluent feed, and low levels of free P after the cells have taken up the P. These conditions will select microbes able to store intracellular polyP and a C/energy source, i.e. PAO. Critically, unlike traditional anaerobic: aerobic processes the addition of C source and effluent P is temporally separated (May et al. 1997; Bayly and May 1998).

During their study (May et al. 1997; Bayly and May 1998) the process was run as an SBR with acetate as a C source, which provided more reliable EBPR performance than when ethanol or methanol was used. Various operating parameters were changed, including feeding the reactor synthetic medium and also effluent from a conventional activated sludge plant. They also investigated the influence of no pH control, stirring rate during start-up, oxygen levels during acetate uptake, oxygen deprivation on acetate uptake, and presence of P and other biologically utilizable substrates at time of C source addition. The number of reactor cycles per day was increased from 3 to 4, improving the throughput of the system, and the N: C ratio optimised. They found the SBR process was appropriate for removal of influent P of 5 mg L^{-1}, and it successfully removed P for periods of up to 133 days (May et al. 1997).

However, this system could only cope with no more than 2 mg L^{-1} additional P in each influent feed before P removal failure, which is not satisfactory if the system is to be receiving effluent from a conventional activated sludge plant, where the effluent P concentration may vary. In the absence then of suitable molecular methods no attempt was made to identify the bacteria removing P in the system, and the biomass samples were only stained with Sudan Black to detect PHB inclusions, and Neisser staining was used to detect polyP to show which chemical transformations were occurring. The authors suggested the identity of the bacteria present was not important. Rather the presence of the appropriate physiological phenotype was the crucial factor, especially with an add-on process, as the inoculum would vary from system to system, and therefore different bacteria may perform these functions.

However, our current understanding of PAO and GAO and the factors that may contribute to competition between the two suggest it is vitally important to understand which bacteria are

functional in this system in removing P, and what operational and other conditions can change the PAO: GAO balance, which can determine process success or failure (May et al. 1997). Additionally the Bayly and May system was originally developed on the basis of observations that *Acinetobacter* in pure culture accumulated PHB under aerobic conditions when supplied acetate but not P. They designed oligonucleotide probes targeting the PHA synthase gene of *Acinetobacter* which they claimed could detect this organism in their community. *Acinetobacter* is no longer considered an important PAO and the potential harmful influence of the GAO on EBPR has only since become apparent. Thus, there is a much greater need now to identify and understand which populations are present.

In this study, a novel aerated process based on that conceived by Bayly and May (May et al. 1997; Bayly and May 1998; unpublished data) was used. The original purpose of this process was as an add-on unit attached to the end of a full scale non-EBPR plant, to remove the P remaining in the treated effluent. Many EBPR plants use chemical dosing to remove any residual effluent P, which as discussed earlier, has several disadvantages. Upgrading conventional plants as EBPR systems is costly, and not always feasible, and such an add-on process which reliably removed P to required levels would be a cheaper and more efficient alternative.

Smart Water funded this work to fully evaluate and develop to a pilot plant scale this fundamentally different EBPR system. It operates aerobically in a single reactor, with all the associated cost savings compared to constructing more conventional multi-stage EBPR systems. One major benefit is that a single reactor system is simpler to operate and control and readily installed as a final tertiary treatment process attached to and fed effluent from an existing conventional activated sludge plant for the purpose of 'polishing' that effluent. From the earlier work at Monash, it seemed suitable for both large and small scale treatment plants to produce a treated water of a quality appropriate for a wider range of applications than would be currently achievable. In our study attention was paid to the choice and level of the carbon source supplementation necessary for its successful operation. In addition to optimising plant configuration and operation, it undertook to examine closely the microbial communities in the EBPR biomass to see if and how they changed with changes in plant operational parameters and performance. It was hoped that such information could then be used as a basis for monitoring the system and provide possible early warning systems for potential plant malfunction.

PROJECT PLAN

This project was divided up into several sequential stages, as outlined below. The experimental work was carried out by two research fellows and a PhD student, and additional funding was obtained from the Australian Research Council (Discovery Grant DP0557646)

Step 1.

a) Can we establish aerobic EBPR in an SBR fed synthetic sewage supplemented with acetate and effluent from a conventional plant also supplemented with acetate, and how do their EBPR capacities and general operational performances compare?

b) Do the microbial communities in these systems vary, especially in terms of the P accumulating bacterial populations there? There is some evidence that the P accumulating populations can vary with feed composition and probably plant operating conditions in conventional EBPR plants. Thus it is important to know whether this is the case with this novel process.

The work described next addresses these issues

1 Materials and Methods

1.1 Operation of the sequencing batch reactors (SBRs)

Laboratory scale SBRs were maintained by Dr Johwan Ahn and Bradley Campbell (La Trobe University). The following information is based on the work they carried out to maintain the SBRs over the course of the project. Details given are of the standard reactor conditions. Where these were changed, appropriate details will be given in the text.

1.1.1 Operation of the laboratory scale SBR

The SBR consisted of a vessel (series 500, LH Fermentation, United Kingdom) and a stirrer drive unit (model 502D, LH Fermentation, United Kingdom). The reactors were stirred at 300 rpm and aerated at 710 mL min^{-1}. The pH was maintained with a pH controller (model 505, LH Fermentation, United Kingdom) and by adding 0.25 M HCl. The partial pressure (pO_2) of the mixed liquor was monitored with a galvanic G2 oxygen electrode (Uniprobe, United Kingdom), and redox potential with an IJ64 redox probe (Ionde, Australia).

Two types of P-containing feeds were used, a synthetic wastewater and clarified effluent from a full scale non-EBPR WWTP. The synthetic wastewater feed was based on the chemically defined medium described by Bayly et al. (1991), made up as two parts and autoclaved separately, which were added to the reactor at 1 X concentration (Part A: 100 X stock solution 5.732 g L^{-1} NH_4Cl, 50 g L^{-1} KCl, 25 g L^{-1} $MgCl_2.6H_2O$, 3.3 g L^{-1} $CaCl_2.2H_2O$, 1.0 g L^{-1} Na_2SO_4, 5.038 g L^{-1} $NaH_2PO_4.2H_2O$. Part B: 1,000 X stock solution 0.5 g L^{-1} H_3BO_3, 0.4 g L^{-1} $MnSO_4.H_2O$, 0.4 g L^{-1} $ZnSO_4.7H_2O$, 0.2 g L^{-1} $FeCl_2.6H_2O$, 0.16 g L^{-1} $H_2MoO_4.H_2O$, 0.1 g L^{-1} KI, 0.04 g L^{-1} $CuSO_4.5H_2O$). Feed modifications were that the P level was reduced to 10 mg P L^{-1}, the ammonium chloride (the N source) was adjusted to a final feed level of 20 – 32 mg N L^{-1}, and the sodium acetate was omitted. The clarified effluent feed was taken from a non-EBPR activated sludge plant in Melton, Victoria, Australia. This effluent generally contained 10 – 12 mg P L^{-1}. It was transported by courier to the laboratory at regular intervals, and kept at 4 °C until use. Its chemical composition varied over the course of these experiments, with total nitrate, ammonia and COD values ranging between 17 – 52 mg L^{-1}, 6 – 23 mg L^{-1} and 6 – 73 mg oxygen L^{-1} respectively. With both feeds, the acetate was added separately at the appropriate time in the SBR cycle (see below) to give a concentration equivalent to 160 mg C L^{-1} in the reactor mixed liquor.

The startup inoculum for the reactors was obtained from MUCT EBPR plants located in Castlemaine and Kyneton, Victoria, Australia. These systems removed P to a concentration of less than 0.5 mg P L^{-1}. The start up of the reactor took 5 days. Using the synthetic medium, the SBR was operated in a

batch mode until the P concentration was less than 0.1 mg P L^{-1} for 24 h, the P feed was then stepwise increased to 10 mg P L^{-1}. N was supplied as NH_4Cl and increased in parallel with the P, from 8 to 32 mg N L^{-1}, and acetate was increased incrementally from 40 to 160 mg C L^{-1}.

An initial C: N ratio of 5:1 was adopted during startup and then gradually increased to 8:1 by decreasing the NH_4Cl levels, where stable performance was obtained, and no N was detectable in the treated effluent. Stable SBR performance was achieved after 15 – 20 days where 10 mg P L^{-1} was almost always completely removed. The same startup protocol was used with the process to treat the clarified effluent from a non-EBPR activated sludge treatment plant, except the level of P in the influent usually ranged from approx. 10 – 15 mg L^{-1}, and the same stepwise increases in the acetate reactor concentration and P levels in the clarified effluent feed were used.

1.1.2 Laboratory scale SBR cycles

The reactors were operated at room temperature (approx. 20 °C) with a cycle time of 8 h. Changes in temperature were controlled by cold fingers fed water via a temperature controlled water bath. Pumping of liquids, airflow and stirring was controlled by electronic timers (type PC787, Arlec, Australia). Each cycle consisted of five stages. In stage 1 (FAMINE stage), P was pumped in during the first five minutes, this stage went for a total of 99 min. In stage 2 mixed liquor was pumped out for 1 min. In stage 3 (FEED stage) acetate was pumped in during the first 4 min, this stage went for a total of 320 min. Stage 4 was the settling stage which went for 45 min, and in Stage 5 the clarified low P liquor was pumped out over 15 min. During stages 4 and 5 the reactor was not stirred or supplied any air. The maximum working volume of the reactor was 1,500 mL, the volume of P-containing feed added to a reactor was 750 mL, and the volumes of mixed liquor and clarified P-depleted liquor removed from the reactor were 25 mL and 725 mL, respectively. The hydraulic retention time was 16 h and the mean cell retention time (sludge age) 20 d.

1.1.3 Variation of operational conditions

Any further variations in operational conditions of the SBRs are detailed in the text. For the remainder of this report the following abbreviations are used (Table 1.1).

Table 1-1 Abbreviations used in text for operating conditions of the laboratory aerated EBPR SBR.

Abbreviation	Condition
Variation in feed source and acetate supplementation	
Feed A	Synthetic wastewater with 160 mg L^{-1} C as acetate
Feed B	Synthetic wastewater with 120 mg L^{-1} C as acetate
Feed C	Clarified effluent with 160 mg L^{-1} C as acetate
Feed D	Clarified effluent with 120 mg L^{-1} C as acetate
Feed E	Clarified effluent with 100 mg L^{-1} C as acetate
Variation in sludge age	
Reactor F	Clarified effluent with 120 mg L^{-1} C as acetate - 20 day sludge age
Reactor G	Clarified effluent with 120 mg L^{-1} C as acetate - 15 day sludge age
Reactor H	Clarified effluent with 120 mg L^{-1} C as acetate - 10 day sludge age
Variation in pH	
pH 6.5	Clarified effluent with 120 mg L^{-1} C as acetate - pH 6.5
pH 7.0	Clarified effluent with 120 mg L^{-1} C as acetate - pH 7.0
pH 7.5[1]	Clarified effluent with 120 mg L^{-1} C as acetate - pH 7.5
Variation in temperature	
Reactor L	Clarified effluent with 120 mg L^{-1} C as acetate - 15 °C
Reactor M	Clarified effluent with 120 mg L^{-1} C as acetate - 15 °C
Reactor N	Synthetic wastewater with 120 mg L^{-1} C as acetate - 15 °C

Reactor O	Clarified effluent with 120 mg L^{-1} C as acetate - 25 °C
Reactor P	Synthetic wastewater with 120 mg L^{-1} C as acetate - 25 °C
Reactor Q	Clarified effluent with 120 mg L^{-1} C as acetate - 30 °C

[1] same condition as Feed G, 15 day sludge age

1.1.4 Chemical analysis of laboratory scale SBR samples

All chemical analyses were performed by Dr J Ahn or B Campbell (La Trobe University). Concentrations of orthophosphate, acetate, and ammonium chloride are expressed in terms of phosphorus (P), carbon (C), and nitrogen (N), respectively.

Analyses of P and ammonium were performed according to standard methods (Clescerl et al. 1996). Acetate was either analysed by ion chromatography (LC-10Ai, Shimadzu, Japan) fitted with an anion column (Shodex KC-811, Showa Denko, Japan) and a diode array detector (SPD-M10AVP, Shimadzu, Japan), or by the Advanced Water Management Centre, University of Queensland, St Lucia, Queensland, Australia.

To determine intracellular levels of PHA, biomass samples were collected at appropriate times during the SBR cycles and either immediately frozen in a mixture of dry ice and methanol, followed by lyophilisation, or was incubated at 100 °C for 24 h with 1:1 chloroform-acidified methanol (10% sulphuric acid) (Ryu et al. 1997). The identification and quantification of PHA methyl esters were conducted in a 1:10 split mode by a gas chromatography system (model 3900, Varian) equipped with a ChromPack capillary column (CP-Sil5CB, Varian) and a flame ionization detector. Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (Aldrich) and sodium 3-hydroxybutyrate (Lancaster, United Kingdom) were used as standards. For quantifying glycogen, sludge samples were autoclaved with 0.6 N HCl at 121 °C for 60 min. After cooling to room temperature and adjusting the pH to 7 with 0.6 M NaOH, glycogen concentration was measured as glucose equivalents by using a hexokinase enzymatic glucose kit (Thermo, Australia).

1.2 DNA extraction methods

1.2.1 The phenol chloroform extraction method of McVeigh et al. (1996)

With the method of McVeigh et al. (1996), 200 µL biomass was centrifuged at 14,000 g for 10 min at 4 °C and supernatant discarded. The pellet was resuspended in 500 µL phenol-chloroform-isoamyl alcohol (PCIA) (25:24:1 v:v:v) (Sigma) and 750 µL 0.12 M sodium phosphate buffer (1 M solution: 0.418 M Na_2HPO_4, 0.163 M NaH_2PO_4, pH 8.0, diluted to 0.12 M with sterile Milli-Q® water) and added to a 2 mL tube containing approx. 0.5 g 0.1 mm diameter glass beads. Samples were homogenised in a mini bead beater™ (Biospec Products, USA) at maximum speed for 60 sec, and centrifuged at 14,000 g for 10 min at 4 °C. The supernatant was carefully removed using a pipette and placed in a clean 2 mL tube kept on ice. Then 750 µL 0.12 M sodium phosphate buffer (pH 8.0) was added to the pellet and the extraction method repeated. Supernatants were adjusted to a volume of 1 mL in each tube with 0.12 M sodium phosphate buffer (pH 8.0), and 1 mL PCIA (25:24:1 v:v:v) (Sigma) added to each. Tubes were mixed by inversion approx. 20 times to form an emulsion, and centrifuged at 14,000 g for 10 min at 4 °C. Supernatant was carefully removed using a pipette and transferred to a clean tube, and 0.6 volumes of 2-propanol (Sigma) were added to the tube. Tubes were inverted several times, and held on ice for 30 min, before being centrifuged at 14,000 g for 20 min at 4 °C. Supernatants were removed and discarded. 500 µL 70% ethanol was added to the pellet and the contents again mixed before being centrifuged at 14,000 g for 10 min at 4 °C, and supernatant carefully removed and discarded. This step was repeated, the supernatant again carefully removed and discarded and the tubes then inverted to allow contents to dry, before precipitated DNA was resuspended in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0), and stored at -20 °C until required.

1.2.2 The NaTCA method of McIlroy et al. (2008b)

With the sodium trichloroacetate (NaTCA) method (McIlroy et al. 2008b), 200 µL biomass was centrifuged at 14,000 g for 10 min at 4 °C and supernatant discarded. The pellet was resuspend in 1,500 uL lysis buffer (3 M NaTCA, 50 mM Tris-HCl pH 8.0, 15 mM EDTA pH 8.0, 1% [w/v] N-lauroylsarcosine, 1% [w/v] polyvinylpyrrolidone (PVP), 10 mM dithiothreitol (DTT), 1.67% [v/v] antifoam (Dow Corning 1520-US)) and approx. 0.5 g 0.1 mm diameter glass beads. Samples were homogenised in a mini bead beater™ (model UF-80A12, Biospec Products, USA) at max speed for 3 min, and then centrifuged at 14,000 g for 5 min at 4 °C. Supernatant was transferred to a fresh tube containing 0.6 volumes 2-propanol (Sigma) and incubated on ice for 15 min. Tubes were then centrifuged at 20,800 g for 15 min. Supernatants were removed and discarded, and 500 µL 70% ethanol added to the resulting pellet. Contents were again mixed before being centrifuged at 20,800 g for 10 min at 4 °C and supernatant carefully removed and discarded. This step was repeated, the supernatant again carefully removed and discarded and the tubes then inverted to allow contents to dry, before the precipitated DNA was re-suspended in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). This was incubated at 35 °C for 20 min and centrifuged at 20,800 g for 4 min. Supernatant containing DNA was carefully removed and transferred to a fresh tube and stored at -20 °C until required.

1.2.3 The UltraClean™ Soil DNA Isolation kit (MoBio Laboratories, Inc) and the sodium xanthogenate method (Tillett and Neilan 2000)

DNA extractions performed by Dr M Beer (La Trobe University) using the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc) were carried out following the manufacturer's instructions using the recommended protocol for maximum yield, and the sodium xanthogenate method detailed by Tillett and Neilan (2000). Modifications to this protocol were that the xanthogenate treatment was carried out in a water bath at 70 ^{o}C for 2 h, and the resulting DNA pellet was washed 3 times with 70% ethanol rather than once.

1.2.4 Assessment of extracted DNA quality by agarose gel electrophoresis

A 1% agarose gel was prepared in 1 X TAE buffer (40 mM Tris-HCl, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0). DNA was mixed with 6 X loading dye (0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 70% glycerol) to a final concentration of 1 X loading dye and loaded into wells, gels were electrophoresed at 70 V for approximately 70 min using a Bio-Rad Universal Powerpack. Gels were stained by immersion in 1 X TAE buffer (40 mM Tris-HCl, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0) containing ethidium bromide (BioRad) at a final concentration of 5 mg mL^{-1} for 10 min, or ethidium bromide was added to the gel before setting at the same concentration. DNA was visualised using a UV Transilluminator (UVP, USA) and images captured using a Nikon Coolpix 995 digital camera. Images of agarose gels were analysed using Adobe® Photoshop®, where DNA concentration was determined by comparing the fluorescence intensity of a band of known DNA concentration from the 2-Log DNA ladder (New England Biolabs) to that of the amplification product.

1.3 16S rRNA clone library construction

1.3.1 PCR amplification of 16S rRNA genes

The 16S rRNA clone library was constructed using primers 27F and 1492R (Table 1.2) at an annealing temperature of 45 °C. Each PCR reaction contained 1 X AmpliTaq Gold® Reaction Buffer, 2.5 U AmpliTaq Gold® DNA Polymerase (both from Applied Biosystems), 2.5 mM $MgCl_2$, 0.2 µM primers, and 200 µM each dNTP (Roche) and made up as a master mix for a final reaction

volume of 25 µL with nuclease free water. The master mix was mixed by pipetting and spun briefly in a microfuge, then 24 µL master mix and 1 µL template DNA were added to 0.2 mL thin walled PCR tubes. Amplifications were carried out in an Applied Biosystems GeneAmp PCR system thermal cycler using the following protocol: initial denaturation for 5 min at 95 °C, followed by 30 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 45 °C and 1 min 30 sec elongation at 72 °C. This was followed by a final elongation of 10 min at 72 °C. Products were stored at -20 °C until required.

Five PCR reactions from each extraction method were carried out and DNA assessed by agarose gel electrophoresis as described in Section 1.2.4. Products from each extraction method were pooled and again visualised by agarose gel electrophoresis. Gel bands were excised using a clean razor and cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Purified DNA was assessed and quantified using agarose gel electrophoresis as described in Section 1.2.4.

1.3.2 16S rRNA cloning

Equal concentrations of purified DNA (Section 2.3.1) were pooled and the resulting mix ligated into pGEM®-T Easy Vector using T4 DNA ligase (both from Promega) and incubated overnight at 4 °C. Ligation mixtures were transformed into JM109 High Efficiency Competent Cells according to the manufacturer's instructions. 60, 80, and 100 µL aliquots were plated onto Luria Bertani (LB) (Tryptone, Yeast Extract, NaCl, all from Oxoid) agar plates containing 100 µg mL^{-1} ampicillin (Sigma) that had been spread with 100 µL of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20 µL of 50 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and 30 min allowed for absorption before use. Plates were incubated overnight at 37 °C and then refrigerated for approximately 4 h before being examined for white insert containing colonies. Single white colonies were re-streaked onto fresh LB agar plates with IPTG and X-Gal, and incubated overnight at 37 °C.

1.3.3 Colony insert PCR

Colonies were screened for inserts using colony PCR with primers M13F and M13R (Geneworks) (Table 1.2) at an annealing temperature of 53 °C. Single colonies were selected and added to 1.5 mL tubes containing 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and 0.1% Triton-X. Tubes were heated at 95 °C for 5 min and 1 µL of the lysate was added to a PCR reaction. PCR reactions contained 1 X Green GoTaq® Green Master Mix (Promega), which contains GoTaq® DNA Polymerase, 1 X Reaction Buffer (pH 8.5), 200 µM each dNTP, and 1.5 mM $MgCl_2$. The master mix was briefly vortexed and spun in a microfuge before use, then added to 0.2 µM of each primer and nuclease free water for a final reaction volume of 25 µL. This was mixed by pipetting and spun briefly in a microfuge. 24 µL Master Mix and 1 µL of lysate mixture were added to 0.2 mL thin walled PCR tubes. Amplifications were carried out in an Applied Biosystems GeneAmp PCR system thermal cycler using the following protocol: initial denaturation for 5 min at 95 °C, followed by 30 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 53 °C and 1 min 30 sec elongation at 72 °C. This was followed by a final elongation of 10 min at 72 °C. Reaction products were stored at -20 °C until required. DNA was assessed and quantified using agarose gel electrophoresis as described in Section 1.2.4.

Single colonies of clones with inserts of the correct size were incubated in 5 mL LB broth overnight at 37 °C with shaking. Plasmids were extracted using the Wizard® *Plus* SV Minipreps DNA Purification Systems (Promega) following the manufacturer's instructions. Plasmid quality and quantity were assessed by agarose gel electrophoresis as described in Section 1.2.4.

Table 1-2 Sequences of primers used in construction of the 16S rRNA clone library

Primer	Sequence	Target[1]	Reference
27F	GAGTTTGATCMTGGCTCAG	*Bacteria* 27 of 16S rRNA gene	Modified from Lane (1991)
519R	GWATTACCGCGGCKGCTG	*Bacteria* 519 of 16S rRNA gene	(Lane 1991)
530F	GTGCCAGCMGCCGCGG	*Bacteria* 530 of 16S rRNA gene	(Lane 1991)
907R	CCGTCAATTCMTTTRAGTTT	*Bacteria* 907 of 16S rRNA gene	(Lane 1991)
926F	AAACTYAAAKGAATTGACGG	*Bacteria* 926 of 16S rRNA gene	(Lane 1991)
1100R	GGGTTGCGCTCGTTG	*Bacteria* 1100 of 16S rRNA gene	(Lane 1991)
1492R	GGYTACCTTGTTACGACTT	*Bacteria* 1492 of 16S rRNA gene	Modified from Lane (1991)
1525R	AAGGAGGTGWTCCARCC	*Bacteria* 1525 of 16S rRNA gene	(Lane 1991)
M13F	CGCCAGGGTTTTCCCAGTCACGAC	pGEM®-T Easy Vector	(Messing 1983)
M13R	TCACACAGGAAACAGCTATGAC	pGEM®-T Easy Vector	(Messing 1983)

[1] *E. coli* numbering system
Note R = A:G, Y = C:T, M = A:C, K = G:T, S = G:C, W = A:T

1.3.4 Sequencing and phylogenetic analysis

Clones containing inserts were screened using one way sequences obtained with sequencing primers M13F or M13R (Table 1.2). Sequencing reactions were performed in 1.5 mL centrifuge tubes containing 300 ng plasmid DNA, 0.8 μM primer, 5% (v/v) DMSO or 1 M betaine and made up to 12 μL with nuclease free water. Samples were electrophoresed on an AB3739*xl* 96-capillary sequencer (Australian Genome Research Facility, University of Queensland, St Lucia, Queensland, Australia).

Sequences were aligned using ClustalW (Larkin et al. 2007) and separated into operational taxonomic units (OTUs) based on 99% relative similarity of their DNA sequence (approx. 600 bp) using the program DNAdist (Felsenstein 1989, Biomanager by ANGIS www.angis.org.au) to create a distance matrix, and DOTUR (Schloss and Handelsman 2005) to separate the clones into OTUs. A representative clone of each OTU was fully sequenced using the sequencing primers M13F, M13R, 519R, 530F, 907R, 926F and 1100R (Table 2.2) with reactions prepared as described above. When necessary sequences were processed through the Long Trace program (www.nucleics.com.au). Contigs of full sequences were assembled using Geneious Pro v3.7.1 (Biomatters Ltd, New Zealand) and checked for chimeras using the programs Bellepheron v3 (DeSantis et al. 2006) and Mallard (Ashelford et al. 2006). Putative chimeras were further checked using Pintail (Ashelford et al. 2005). Sequences were aligned using the ARB software package (Ludwig et al. 2004). Phylogenetic trees were constructed using the maximum likelihood algorithm using the bacteria filter. Bootstrapping analysis was performed with maximum parsimony and 1000 replicates.

The homogenous coverage of the clone library was calculated using the equation

$$C = [1 - (n1 \times N^{-1})] \times 100\%$$

Where C = homogenous coverage
n1 = number of OTUs with only one sequence (based on 99% similarity)
N = total number of 16S rRNA genes analysed.

Species richness was calculated using the equation

$$\text{Species Richness} = \text{Number of OTUs} / C$$

(Singleton et al. 2001; Juretschko et al. 2002; Kong et al. 2007).

1.4 DGGE community profiling

1.4.1 DNA extraction

For DGGE analysis biomass samples were either frozen at -80 °C or fixed in an equal volume of absolute ethanol until extraction. Samples from other full scale plants were taken and mailed via express post to the laboratory, and when received were fixed in an equal volume of absolute ethanol immediately. DNA was extracted from biomass using the phenol chloroform method adapted from McVeigh et al. (1996) as described in Section 1.2.1, and extracted DNA integrity was assessed as described in Section 1.2.4.

1.4.2 PCR amplifications

PCR amplifications for analysis of the 16S rRNA fragments were carried out using the primers 341F + GC clamp and 534R at an annealing temperature of 53 °C (Table 1.3). PCR reactions were performed using 1 X GoTaq® Green Master Mix (Promega), which contains GoTaq® DNA Polymerase, 1 X Reaction Buffer (pH 8.5), 200 μM each dNTP, and 1.5 mM $MgCl_2$. The master mix was briefly vortexed and spun in a microfuge before use, then added to 0.5 μM each primer and nuclease free water for a final reaction volume of 25 μL. This was mixed by pipetting and spun briefly in a microfuge. 24 μL Master Mix and 1 μL of template DNA were added to 0.2 mL thin walled PCR tubes. Amplifications were carried out in an Applied Biosystems GeneAmp PCR system thermal cycler using the following protocol: initial denaturation for 5 min at 95 °C, followed by 30 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 53 °C and 30 sec elongation at 72 °C. This was followed by a final elongation of 10 min at 72 °C. Products were stored at -20 °C until required.

To estimate PCR amplification product concentration and to ensure it was of the required size, PCR product was run on a 3% agarose gel at 100 V for approx. 20 min and DNA visualised and quantified as described in Section 1.2.4.

Table 1-3 Sequences of primers used in PCR amplification for analysis by DGGE.

Primer	Sequence	Target[1]	Reference
341F[2]	CCTACGGGAGGCAGCAG	*Bacteria* 341 16S rRNA gene	(Muyzer et al. 1993)
534R	ATTACCGCGGCTGCTGG	*Bacteria* 534 16S rRNA gene	(Muyzer et al. 1993)
462F[2]	GTTAATACCCTGWGTAG ATGACGG	PAO cluster 462 16S rRNA gene	Adapted from Crocetti et al. (2000)
651R	CCCTCTGCCAAACTCCAG	PAO cluster 651 16S rRNA gene	Adapted from Crocetti et al. (2000)
GC	CGCCCGCCGCGCGCGGCGG	n/a	(Muyzer et al. 1993)

clamp	GCGGGGCGGGGGCACGGGGGG

[1] *E. coli* numbering system, [2]GC clamp was attached at 5´ end
Note R = A:G, Y = C:T, M = A:C, K = G:T, S = G:C, W = A:T

1.4.3 DGGE gel casting

Acrylamide gels were cast using the DCode Universal Mutation Detection System (Bio-Rad). A 1 mm thick 16 cm x 10 cm gel was cast to a final concentration of either 10% or 8% acrylamide for universal and *Accumulibacter* analysis respectively, with the appropriate denaturation gradient (100% denaturation 10 mol L^{-1} urea and 40% [v / v] formamide) (details in text). A 0 % and 100 % denaturation solution was made according to Table 2.4, and mixed in the appropriate ratios to form solutions of the required composition.

Ammonium persulphate (0.09% (v/v) (Bio-Rad) and 0.09% (v/v) *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) (Bio-Rad) were added to each denaturation solution, with 100 µL D-Code dye (0.5% bromophenol blue (w/v), 0.5% xylene cyanol (w/v), 1 X TAE buffer) added to the higher percentage denaturation solution for gradient visualisation. Gels were poured using the DCode Gradient Delivery System, up to the bottom of the comb area, and allowed to polymerise for at least 3 h. The top of the gel, forming the comb area, was then poured with a 0% denaturation solution, to avoid any contamination of DNA in the wells with urea, and allowed to polymerise for 1.5 h.

Table 1-4 Denaturation solutions of denaturing gradient gel electrophoresis (DGGE) gels

Gel	8%		10%	
Denaturation solution	0%	100%	0%	100%
40% Acrylamide: Bis (37.5:1)	20 mL	20 mL	25 mL	25 mL
TAE buffer	2 mL	2 mL	2 mL	2 mL
Formamide	0 mL	40 mL	0 mL	40 mL
Urea	0 g	42 g	0 g	42 g
Milli-Q® water	to 100 mL	to 100 mL	to 100 mL	to 100 mL

1.4.4 Gel electrophoresis and visualisation

Once gels had polymerised, DNA samples were mixed with 2 X loading dye (0.05% bromophenol blue (w/v), 0.05% xylene cyanol (w/v), 70% glycerol) to give a final DNA concentration of approximately 300 ng, determined as detailed in Section 1.2.4, and loaded into wells using tapered 200 µL pipette tips. Gels were electrophoresed for 16 h at 70 V in 1 X TAE buffer (40 mM Tris-HCl, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0).

After electrophoresis gels were carefully removed from the glass plates and placed into a plastic container with 100 mL TAE buffer (40 mM Tris-HCl, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0) containing SYBR® Gold to a final concentration of 0.1% (v/v). Gels were rotated on an orbital mixer for 20 min at room temperature, and were visualised using a UV Transilluminator (UVP, USA), and images captured using a Nikon Coolpix 995 digital camera, using Adobe® Photoshop® software.

DGGE gels were analysed using Gelcompar II, Version 4.5 (Applied-Maths), and dendograms were constructed using the clustering option with the Pearson correlation coefficient, with the optimal position tolerance setting optimised.

1.5 Fluorescence in situ *hybridisation* (FISH)

1.5.1 Cell fixation and storage

Biomass samples were collected and fixed for FISH analysis in ethanol and paraformaldehyde for analysis of Gram positive and Gram negative cells respectively (Daims et al. 2005). Fixation was carried out as described by Daims et al. (2005). Approx. 2 mL biomass was taken from each system at the required sampling time. In the case of full scale plants, samples were transported to the laboratory by express post, and fixed immediately after arrival. For fixation of Gram positive cells, to 1 mL biomass an equal volume of absolute ethanol was added. For fixation of Gram negative cells, 3 volumes of freshly prepared 4% paraformaldehyde were added. Samples were incubated at 4 °C for 3 h, then centrifuged at 5,000 g for 5 min at 4°C to pellet the cells, the supernatant removed and the pellet resuspended in 1 X PBS (137 mM NaCl, 10 mM $Na_2HPO_4.7H_2O$, 2.7 mM KCl, 1.47 mM KH_2PO_4). This process was repeated twice more to remove fixative. Samples were finally resuspended in an equal volume of 100% ethanol and 2 X PBS and stored at -20 °C until required.

1.5.2 Hybridisation

FISH hybridisations were carried out as described by Amann (1995). Probes used and their corresponding formamide concentrations are listed in Table 2.8. Probes were labelled with either Cy5, Cy3, or FLUOS fluorochromes and purchased from Prooligo (Sydney, Australia). Further details can be found at Probebase.net (Loy et al. 2003) or in the initial publications (see Table 2.8). All FISH experiments included the appropriate controls. The EUBmix probes (Amann et al. 1990; Daims et al. 1999) were applied to ensure cell permeability was adequate, and the non-EUB probe (Wallner et al. 1993) used to assess the level of background auto-fluorescence in the sample after hybridisation. Fixed biomass samples were briefly vortexed and approx. 4 µL was applied to Teflon coated microscope slides that had been further coated in Vectabond™ (Vector Laboratories). Slides were dried at 46 °C, and biomass on slides was dehydrated through an ethanol series of 50%, 80%, and 96% for 3 min each and let dry. Hybridisation buffer was freshly prepared (0.9 M NaCl, 20 mM Tris-HCl pH 7.2, 0.01% SDS, x µL formamide, and y µL sterile Milli-Q® water) (Table 1.5) and 10 uL spread over each well of the microscope slide. A 1 µL aliquot of each probe (50 ng μL^{-1}) was added to the hybridisation buffer. Slides were placed in 50 mL falcon tubes containing paper towel moistened with approx. 1 mL hybridisation buffer and incubated in a hybridisation oven (RPN 2510, Amersham Biosciences) at 46 °C for 1.5 h. Following this the hybridisation buffer and any unbound probe was washed off the slides using wash buffer (20 mM Tris-HCl pH 7.2, 0.01% SDS, x µL 5 M NaCl, y µL EDTA, made up to 50 mL with sterile Milli-Q® water) (Table 1.6). Slides were then placed into 50 mL falcon tubes containing remaining wash buffer in a waterbath at 48 °C for 10 – 15 min. After washing slides were dipped in cold distilled water for a few seconds to remove residual wash buffer and air dried with compressed air. Slides were stored at -20 °C if not immediately viewed.

Table 1-5 Formamide and Milli-Q® water volumes in FISH hybridisation buffer to achieve different stringency for FISH analyses (Daims et al. 2005).

µL formamide (x µL)	% formamide	µL Milli-Q® water (y µL)
0	0	1598
100	5	1498
200	10	1398
300	15	1298
400	20	1198
500	25	1098

600	30	998
700	35	898
800	40	798
900	45	698
1000	50	568

Table 1-6 NaCl and EDTA volumes to achieve different stringency in FISH wash buffer (Daims et al. 2005).

% formamide in hybridisation buffer	M NaCl	µL NaCl (x µL)	µL EDTA (y µL)
0	0.900	9000	-
5	0.636	6300	-
10	0.450	4500	-
15	0.318	3180	-
20	0.255	2150	500
25	0.159	1490	500
30	0.112	1020	500
35	0.080	700	500
40	0.056	460	500
45	0.040	300	500
50	0.028	180	500

1.5.3 Microscopic examination of FISH samples

Prior to examination, samples were mounted in approximately 3 µL Vectashield® (Vector laboratories). Slides were examined using a Nikon Eclipse 800 epifluorescence microscope, where Cy3 labelled probes were viewed with the G-2A filter set (Excitation/Emission 510-560/568), FLUOS labelled probes using the FITC filter set (Excitation/Emission 465-495/505), and Cy5 labelled probes using the Cy5/HYQ filter set (Excitation/Emission 620/60). Images were captured with a Nikon DXM 1200 CCD camera using Nikon ACT-1 software. Alternatively, slides were viewed using a Leica TCS SP2 (model DM IRE2) CLSM equipped with Argon, Green HeNe and Red HeNe lasers, and images captured using Leica Confocal Software (Version 2.61).

1.5.4 Semi-quantitative FISH

For semi-quantitative analyses, FISH was carried out as described in Section 1.5.2. Samples were labeled with two probes, each tagged with different fluorochromes. One probe specifically targets the population to be quantified, the other general probe binds to all bacteria (Daims et al. 1999; Daims et al. 2006). The EUBmix probes (Amann et al. 1990; Daims et al. 1999) were used as the general probe set to target all Bacteria. The abundance of the selected population was quantified by measuring the percentage of biomass fluorescing with the population probe expressed as a percentage of the biomass fluorescing with the EUBmix probes. Samples were viewed with the Leica CLSM and images captured using Leica Confocal Software (Version 2.61). Between 20 and 30 (usually at least 30), randomly selected fields of view were captured at 400 x magnification. Image analysis was carried out with Daime image analysis software (Daims et al. 2006) where the percentage biovolume of cells responding to each probe was expressed as a percentage of the total area fluorescing with the EUBmix probes. Mean abundances of populations were compared by the Student *t*-statistic analysis method. Two-tailed comparisons were considered significantly different when $P < 0.05$.

1.6 FISH probe design and validation

FISH probes were designed using the Design Probe and Probe Match functions with ARB software (Ludwig et al. 2004). Sequences closely related to the target sequences were retrieved using BLAST searches (Altschul et al. 1997), chimera checked as described in Section 1.3.4, and added to the ARB database. Possible probes were generated, and checked for matches in the Greengenes (DeSantis et al. 2006), SILVA (Pruesse et al. 2007), and RDP (Cole et al. 2007) databases. BLAST searches (Altschul et al. 1997) were also carried out to determine if there were any sequences containing the proposed FISH probe target site that were not in the databases.

Newly designed probes were validated as described by Daims et al. (2005). Probes were tested against a biomass sample containing the target organism using an increasing formamide gradient of 0 – 70% in 5 – 10% increments. The optimal formamide concentration was determined by assessing the fluorescence intensity of the probe positive signal at each formamide concentration. A formamide concentration of 5% below which the fluorescence of the probe began to decrease was determined as the optimum formamide concentration, determined by measuring the fluorescence intensity of at least 100 cells at each formamide concentration, using Adobe® Photoshop®.

The effect of helper probes on newly designed probes was assessed by hybridising the probe to the biomass sample at a formamide concentration of 30%. Images were taken of hybridisations with and without helper probes. At least 100 cells (20 cells in 5 images) were assessed for fluorescence intensity using Adobe® Photoshop®. The minimum helper probe combination that yielded the highest fluorescence was used.

1.7 Fluorescence in situ *Hybridisation/ Microautoradiography (FISH/MAR)*

1.7.1 Incubations

FISH/MAR was carried out essentially as described by Lee et al. (1999) and Kong et al. (2004), with some modifications. Biomass samples were taken from the reactors and diluted if appropriate with filtered effluent from the same reactor. Samples were placed into 10 mL glass vials with rubber stoppers and shaken vigorously for 20 min (Kong et al. 2004) to ensure aerobic conditions. They were then incubated with sufficient radiolabelled substrate to obtain the desired radioactivity level (for details see appropriate experiments in text) and unlabelled substrate to provide cells with appropriate levels of substrate and minimise the amount of radiolabelled substrate used. All radiolabelled substrates were obtained from GE Healthcare (Australia) (Table 2.7). Samples were incubated with shaking on an orbital shaker at room temperature for appropriate periods. Controls allowing for possible chemography and for background silver grain formation were incorporated into all experiments, using biomass samples pasteurised at 70 °C for 10 min before addition of substrates (Kong et al. 2004). For anaerobic and anoxic incubations, samples were pre-incubated with unlabelled substrate for 2 h. This was to ensure the cells were taking up the substrate to grow, not merely for storage, as their storage capacity would by then be full, and to ensure there was no uptake due to traces of oxygen remaining (Nielsen and Nielsen 2005). Following this, sludge and sludge water were prepared in one vial, and the substrates and electron acceptors in another. Samples underwent three cycles of flushing with oxygen free N for 5 sec then placed under vacuum for 30 sec. Substrates and electron acceptors were then added while N was being flushed into the vials.

Table 1-7 Details of substrates used in MAR incubations.

Radiolabelled Substrate	Specific activity (mCi $mmol^{-1}$)	Unlabelled Substrate
[1-^{14}C] Acetic acid	56	Sodium acetate
1-[2,3-^{3}H] Aspartic acid	36,000	Aspartic acid
1-[G-^{3}H] Glutamic acid	49,000	Glutamic acid

d-[6-^{3}H] Glucose	29,000	Glucose
[1(3)-^{3}H] Glycerol	3,000	Glycerol
[9,10(n)-^{3}H] Oleic acid	10,000	Oleic acid
[9,10(n)-^{3}H] Palmitic acid	51,000	Palmitic acid
^{33}P Phosphate	3,000,000	Sodium dihydrogen orthophosphate
[1-^{14}C] Propionic acid	55	Propionic acid

1.7.2 Biomass fixation

Following all MAR incubations, samples were split in half and to one half was added an equal volume of 100% ethanol, while to the other 3 volumes of freshly prepared 4% paraformaldehyde were added to fix Gram positive and Gram negative cells respectively (Amann et al 1995). Fixed samples were incubated at 4 °C for 3 h and washed 3 times in 1 X PBS (137 mM NaCl, 10 mM $Na_2HPO_4.7H_2O$, 2.7 mM KCl, 1.47 mM KH_2PO_4), except for those incubated with ^{33}P. These were washed 3 times in 0.1 M sodium citrate-HCl buffer (pH 2) (Kong et al. 2004). All samples were resuspended in equal volumes of 100% ethanol and 2 X PBS, and stored at -20 °C until required.

1.7.3 Liquid scintillation counting

Radioactivity in biomass samples were counted with a liquid scintillation counter (Wallac 1450 Microbeta Plus) to confirm the absence of substrate uptake in control samples (Eales et al. 2003) as follows. 100 µL aliquots were centrifuged at 5,000 g for 5 min, the pellets resuspended in sterile Milli-Q® and resuspended pellets and supernatants each mixed with 700 µL Supermix scintillation cocktail (Wallac, Finland) in 24 well sample plates (Wallac, Finland). Plates were shaken on a Wallac Delfia® Plateshake mixer for 30 min, left standing for 30 min in the dark before being counted using the program appropriate for the isotope. Radioactivity levels are expressed as counts per minute (cpm).

1.7.4 FISH procedure

MAR samples were mixed by briefly vortexing, and approx. 30 µL aliquots placed on a 24 x 50 mm gelatine coated cover glass. Biomass samples were homogenized by briefly rubbing them between a coverslip and a glass microscope slide to disrupt flocs and make FISH detection of populations easier. Slides were air dried at room temperature and cells dehydrated through an ethanol series of 50%, 80%, and 96% for 3 min each, as described in Section 1.5.2. Coverslips were allowed to dry, and FISH hybridisation was then carried out as described in Section 1.5.2. After hybridisation, slides were dipped in LM-1 Emulsion (GE Healthcare) pre-warmed to 43 °C for 15 min, for 5 sec under darkroom conditions. Excess emulsion was drained off onto paper toweling for 5 sec, and the back of the coverslips wiped clean. These were allowed to dry at room temperature, and were stored in the dark in desiccators at 4 °C. Optimal times of exposure were determined empirically, where one coverslip was developed each day until cells displayed positive signals in the form of silver grain deposition on their surfaces. Development was performed by immersing coverslips in Kodak D-19 developer (40 g L^{-1} in Milli-Q® water) for 30 sec to 3 min, followed by 1 min immersion in Milli-Q® water (the stop solution), and a 4 min immersion in sodium thiosulphate (30% w/v) as a fixative. Finally coverslips were washed in reverse osmosis (RO) water for 10 min, and allowed to air dry at room temperature. Samples were mounted in Vectashield® (Vector Laboratories) and placed on a glass microscope slide before viewing as described in Section 1.5.3 on a Nikon E800 epifluorescence microscope.

1.8 Cytochemical staining of biomass samples

1.8.1 Staining for intracellular polyP

Biomass samples were stained for intracellular polyP with DAPI. Staining was performed simultaneously with FISH, as described by Kawaharasaki et al. (1999) and Liu et al. (2001). Briefly, after hybridisation with FISH probes, 10 µL 10 ppm DAPI was placed on each well, and slides were left in the dark at room temperature for 10 min. They were then rinsed with distilled water and dried with compressed air before microscopic examination as described in Section 1.5.3 using the UV-2A filter set (Excitation/Emission 330-380/420). Cells containing polyP fluoresced bright yellow/white, and cells containing no polyP fluoresced light blue from the DAPI binding to cell DNA. To confirm DAPI negative cells did not arise from a loss of polyP during FISH hybridisation (Zilles et al. 2002), in some cases DAPI staining was also performed on non-hybridised cells using the staining procedure described above, and prior to FISH hybridisation, when the immersion oil was removed from the slide by absolute ethanol (Liu et al. 2001) for approx. 30 min. FISH was then performed as described in Section 1.5.2.

1.8.2 Staining for intracellular PHA

Staining for PHA was carried out by the method of Ostle and Holt (1982) with some modifications. Approximately 4 µL biomass was spread over each well and slides were air dried. They were then immersed for 10 min in pre-warmed Nile blue A (100 mg L^{-1} in absolute ethanol) at 55 °C, rinsed in RO water and washed in 8% acetic acid (in absolute ethanol) for 1 min, before a final rinse in RO water. Slides were air dried, and samples were mounted and viewed as described in Section 2.6.3 with filter set G2-A. PHA staining was carried out either alone or preceding FISH analyses, when images of cells of interest were captured and their location on the slide recorded. Slides were then immersed in 100% ethanol for 1 h to remove the Nile blue A stain, and FISH hybridisation performed as described in Section 1.5.2 with either Cy3 or FLUOS labeled probes. Cells previously stained for PHA were then re-located and images captured as before.

1.8.3 Staining for membrane integrity

Staining for cell membrane integrity as a reflection of cell viability was carried out by the method of Carr et al. (2003). 1 mL aliquots of biomass were mixed with 40 µL 100 X SYBR® Gold in DMSO (Molecular Probes, USA) and 3 µL propidium iodide (1 mg mL^{-1}) (Molecular Probes, USA), and incubated at room temperature in the dark for 15 min, briefly vortexed and a 10 µL aliquot was placed on a glass microscope slide. A coverslip was placed over each sample and the slide viewed as a wet mount on the Nikon E800 microscope using the UV-2A filter set. Images were captured as previously described in Section 1.5.3. Cells with intact membranes stained with SYBR® Gold appear green, while cells with damaged membranes appear red as the propidium iodide is able to enter the cell and quench the green fluorescence (Ziglio et al. 2002).

1.8.4 Gram staining

Gram staining was carried out as described by Lindrea et al. (1999).

1.8.5 Capsule staining

Capsule staining was carried out as described by Jenkins et al. (1993) using India Ink negative staining.

Table 1-8 List of oligonucleotide FISH probes used in this study

Probe name	Sequence (5´ - 3´)	Target	FA[1] (%)	Reference
EUB338-I[2]	GCTGCCTCCCGTAGGAGT	Most Bacteria	35	(Amann et al. 1990)
EUB338-II[2]	GCAGCCACCCGTAGGTGT	*Planctomycetales*	35	(Daims et al. 1999)
EUB338-III[2]	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	35	(Daims et al. 1999)
Non - EUB	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	n/a	(Wallner et al. 1993)
BET42a	GCCTTCCCACTTCGTTT	*Betaproteobacteria*	35	(Manz et al. 1992)
BET42a-competitor	GCCTTCCCACATCGTTT	Competitor for BET42a	n/a	(Manz et al. 1992)
BONE23a	GAATTCCATCCCCCTCT	Beta1-group of *Betaproteobacteria*	35	(Amann et al. 1996)
BTWO23a	GAATTCCACCCCCCTCT	Competitor probe for BONE23a	35	(Amann et al. 1996)
PAO462b[3]	CCGTCATCTRCWCAGGGTATTAAC	*Candidatus* "Accumulibacter phosphatis"	35	(Zilles et al. 2002)
PAO651[3]	CCCTCTGCCAAACTCCAG	Most members of the *Candidatus* "Accumulibacter" cluster	35	(Crocetti et al. 2000)
PAO846b[3]	GTTAGCTACGGYACTAAAAGG	*Candidatus* "Accumulibacter phosphatis"	35	(Zilles et al. 2002)
RHC439	CNATTTCTTCCCCGCCGA	*Rhodocyclus* spp., most members of the *Candidatus* 'Accumulibacter phosphatis' cluster, *Azospira* lineage	30	(Hesselmann et al. 1999)

RHC175a	TGCTCACAGAATATGCGG	Most members of the *Dechloromonas-Ferribacterium-Quadricoccus-Azonexus*, the *Azospira*, and the *Rhodocyclus-Propionivibrio-Accumulibacter* lineages	nd	(Hesselmann et al. 1999)
RHX991	GCTCTCTTGCGAGCACTC	*Candidatus* 'Accumulibacter phosphatis' (clone R6)	30	(Hesselmann et al. 1999)
Rc988	AGGATTCCTGACATGTCAAGGG	*Rhodocyclus* spp., *Candidatus* 'Accumulibacter phosphatis', and related bacteria	nd	(Crocetti et al. 2000)
ZRA23a	CTGCCGTACTCTAGTTAT	Most members of the *Zoogloea* lineage, not *Z. resiniphila*	35	(Rossello-Mora et al. 1995)
GAM42a[4]	GCCTTCCCACATCGTTT	*Gammaproteobacteria*	35	(Manz et al. 1992)
GAM42a_T1038_G1031[4]	GCCTTTCCACATGGTTT	Representatives of the *Xanthomonas* group	35	(Siyambalapitiya and Blackall 2005)
GAM42a_T1038[4]	GCCTTTCCACATCGTTT	Representatives of the *Xanthomonas* group	35	(Siyambalapitiya and Blackall 2005)
GAM42a_A1041_A1040[4]	GCAATCCCACATCGTTT	Representatives of the *Xanthomonas* group	35	(Siyambalapitiya and Blackall 2005)
GAM42a-competitor[4]	GCCTTCCCACTTCGTTT	Competitor probe for GAM42a	n/a	(Manz et al. 1992)

GAOQ431[5]	TCCCCGCCTAAAGGGCTT	*Candidatus* 'Competibacter phosphatis'	35	(Crocetti et al. 2002)
GB	CGATCCTCTAGCCCACT	*Candidatus* 'Competibacter phosphatis' - group GB	35	(Kong et al. 2002)
GB_G1(GAOQ989) [5,6]	TTCCCCGGATGTCAAGGC	*Candidatus* 'Competibacter phosphatis' - subgroup G1 in Group GB	35	(Crocetti et al. 2002)
GB_G2[6]	TTCCCCAGATGTCAAGGC	*Candidatus* 'Competibacter phosphatis' - Subgroup G2 in Group GB	35	(Kong et al. 2002)
ACA23a	ATCCTCTCCCATACTCTA	*Acinetobacter*	35	(Wagner et al. 1994)
ALF968	GGTAAGGTTCTGCGCGTT	*Alphaproteobacteria*, except *Rickettsiales*	20	(Neef et al. 1999)
TFO_DF862	AGCTAAGCTCCCCGACAT	*Defluviicoccus vanus*	35	(Wong et al. 2004)
DEF438	CGCCTGAGACGATGATGAC	*Defluviicoccus vanus*	20	(Kong et al. 2001)
TFO_DF218[7]	GAAGCCTTTGCCCCTCAG	Cluster 1 *'Defluviicoccus'*-related TFO in *Alphaproteobacteria*	35	(Wong et al. 2004)
TFO_DF618[7]	GCCTCACTTGTCTAACCG	Cluster 1 *'Defluviicoccus'*-related TFO in *Alphaproteobacteria*	35	(Wong et al. 2004)
TFO_DF776	GCTATAGCGTCAGTTACGG	Cluster 1 *'Defluviicoccus'*-related TFO in *Alphaproteobacteria*	30	(Wong and Liu 2007)

TFO_DF629	AGGACTTTCACGCCTCAC	Cluster 2 *'Defluviicoccus'*-related TFO in *Alphaproteobacteria*	nd	(Wong and Liu 2007)
DF988[8]	GATACGACGCCCATGTCAAGGG	Cluster 2 *'Defluviicoccus'* – related TFO in *Alphaproteobacteria*	35	(Meyer et al. 2006)
DF1020[8]	CCGGCCGAACCGACTCCC	Cluster 2 *'Defluviicoccus'* – related TFO in *Alphaproteobacteria*	35	(Meyer et al. 2006)
H966	CTGGTAAGGTTCTGCGCGTTGC	Helper probe for DF988	n/a	(Meyer et al. 2006)
H1038	AGCAGCCATGCAGCACCTGTGTGGCGT	Helper probe for DF988 and DF 1020	n/a	(Meyer et al. 2006)
SPH120	GGGCAGATTCCCACGCGT	Sphingomonas	30	(Eilers et al. 2000)
SBR9-1a	AAGCGCAAGTTCCCAGGTTG	Putative SBR GAO	30	(Beer et al. 2004)
AMAR839	CTGCGACACCGAACGGCAAGCC	*Amaricoccus* spp.	20	(Maszenan et al. 2000)
Nso1225	CGCCATTGTATTACGTGTGA	Betaproteobacterial ammonia-oxidizing bacteria	35	(Mobarry et al. 1996)
Ntspa662	GGAATTCCGCGCTCCTCT	genus *Nitrospira*	35	(Daims et al. 2001)
Ntspa662-comp	GGAATTCCGCTCTCCTCT	Competitor for Ntspa662	n/a	(Daims et al. 2001)
HGC69a	TATAGTTACCACCGCCGT	*Actinobacteria* (high G+C Gram-positive bacteria)	25	(Roller et al. 1994)
Actino_1011	TTGCGGGGCACCCATCTCT	*Tetrasphaera japonica*, enhanced biological phosphorus removal clone Ebpr19, Ebpr20 (*Actinobacteria*)	30	(Liu et al. 2001)

Actino-221	CGCAGGTCCATCCCAGAC	Actinobacterial PAO	30	(Kong et al. 2005)
C1-Actino-221	CGCAGGTCCATCCCATAC	Competitor 1 for Actino-221	n/a	(Kong et al. 2005)
C2-Actino-221	CGCAGGTCCATCCCAGAG	Competitor 2 for Actino-221	n/a	(Kong et al. 2005)
Actino-658	TCCGGTCTCCCCTACCAT	Actinobacterial PAO	40	(Kong et al. 2005)
C1-Actino-658	TCCGGTCTCCCCTACCAC	Competitor probe 1 for Actino-658	n/a	(Kong et al. 2005)
C2-Actino-658	ATTCCAGTCTCCCCTACCAT	Competitor probe 2 for Actino-658	n/a	(Kong et al. 2005)
LGC354A [9]	TGGAAGATTCCCTACTGC	Most *Firmicutes* (Gram-positive bacteria with low G+C content)	35	(Meier et al. 1999)
LGC354B [9]	CGGAAGATTCCCTACTGC	Most *Firmicutes* (Gram-positive bacteria with low G+C content)	35	(Meier et al. 1999)
LGC354C [9]	CCGAAGATTCCCTACTGC	Most *Firmicutes* (Gram-positive bacteria with low G+C content)	35	(Meier et al. 1999)
CFX1223[10]	CCATTGTAGCGTGTGTGTMG	phylum *Chloroflexi* (green nonsulfur bacteria)	35	(Björnsson et al. 2002)
GNSB-941[10]	AAACCACACGCTCCGCT	phylum *Chloroflexi* (green nonsulfur bacteria)	35	(Gich et al. 2001)
PLA46	GACTTGCATGCCTAATCC	*Planctomycetales*	30	(Neef et al. 1998)
PLA886	GCCTTGCGACCATACTCCC	*Planctomycetales*	35	(Neef et al. 1998)
PLA886-comp	GCCTTGCGACCGTACTCCC	Competitor probe for PLA886	n/a	(Neef et al. 1998)
CF319a	TGGTCCGTGTCTCAGTAC	Most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*	35	(Manz et al. 1996)

Aqs997	CTCTGGTAACTTCCGTAC	*Aquaspirillum*	35	(Thomsen et al. 2004)
Comp1Aqs997	CTCTGGCAACTTCCGTAC	Competitor probe for Aqs997	n/a	(Thomsen et al. 2004)
Comp2Aqs997	CTCTGGTCACTTCCGTAC	Competitor probe for Aqs997	n/a	(Thomsen et al. 2004)
Dech454	ACCGTCATCCGCACAGGG	*Dechloromonas* clone (clone 30-ORF28, clone 20-ORF29, and clone 26-ORF26)	35	Dr Michael Beer
Dech472	CCGTCATCCACACCCTGTATTAAA	*Dechloromonas* clone (09-ORF25) and *Zoogloea ramigera*	nd	Dr Michael Beer
DES 841	ACGACAGCGAGAAGTGAC	*Candidatus* 'Defluviisphaera carrumensis' OTU 1 and 2	25	This study
DES 448	AACCCCGCAACCCGTCCT	*Candidatus* 'Defluviisphaera carrumensis' OTU2	40	This study
DES 447	CCGCTTCCCGTCCTCCCT	*Candidatus* 'Defluviisphaera carrumensis' OTU1	nd	This study
DES H821	CCCCACCGTCTAGTTCTC	Helper probe for DES 841	n/a	This study
DES H865	GGCGGAGAATTAACGCG	Helper probe for DES 841	n/a	This study

nd = not determined, n/a = not applicable
[1]FA = formamide concentration in hybridisation buffer, [2 - 10] applied in equimolar amounts as: [2]EUBmix, [3]PAOmix, [4]Gam42a_mix, [5]GAOmix, [6]GBmix, [7]DF1mix, [8] DF2mix, [9]LGCmix, [10]CFXmix
Note R = A:G, Y = C:T, M = A:C, K = G:T, S = G:C, W = A:T

2 The microbiology of the community in an aerated phosphorus removal process

2.1 Introduction

The microbiology of conventional anaerobic: aerobic EBPR systems has been studied extensively. It is now clear the major PAO populations in these processes are the betaproteobacterial *Candidatus* 'Accumulibacter phosphatis' (Hesselmann et al. 1999; Crocetti et al. 2000; Zilles et al. 2002; Kong et al. 2004; Kong et al. 2007). The most commonly seen putative GAO have been identified as *Candidatus* 'Competibacter phosphatis' (Nielsen et al. 1999; Crocetti et al. 2002; Kong et al. 2002; Kong et al. 2006), and *Defluviicoccus* related cells (Wong et al. 2004; Meyer et al. 2006; Burow et al. 2007; Wong and Liu 2007). Furthermore, these have been shown to possess the expected phenotype of a PAO (Kong et al. 2004; Chua 2006; Chua et al. 2006) and GAO (Kong et al. 2005; Kong et al. 2006) respectively, and these phenotypes match the proposed models for EBPR processes (Seviour et al. 2003; Oehmen et al. 2007).

In the aerated P removal process studied here, the selection pressures are different to a conventional anaerobic: aerobic EBPR process. The aerated process is based on the temporal separation of the C and P supplies. This separates the process into two clear phases, the FEED phase where the C source is supplied, and the FAMINE phase where the P containing effluent is supplied. Whether this results in similar transformations to those occurring in a conventional EBPR system is not known. Based on our knowledge of anaerobic: aerobic EBPR systems, the selection in those processes is for organisms able to accumulate the available C source in the anaerobic feed phase and store it as PHA. Then in the aerobic FAMINE phase, in the absence of any exogenous C source, these populations can use their PHA stores to assimilate excess amounts of P and to synthesise polyP, and so P is removed finally in the biomass when sludge is wasted.

2.2 Aims

The aims of this section were to:

- Identify the major PAO and GAO populations in this aerated process using a range of molecular methods.
- Use FISH/MAR to investigate the ecophysiology of the major populations, and determine if similar chemical transformations that take place in a conventional anaerobic: aerobic EBPR process are occurring in this aerated process.

2.3 Operation of the SBR

The SBR was maintained and operated by Dr J Ahn (La Trobe University) as detailed in Section 1.1. All the chemical analyses on biomass samples were also conducted by Dr Ahn as detailed in Section 1.1.4.

2.3.1 FEED phase

At the start of the FEED phase, acetate was added to the reactor as the C source. This stage lasted 320 min. During this time acetate was rapidly assimilated by the organisms, and P release and PHA production and storage occurred, followed by P uptake and PHA utilisation. After the FEED phase, a settling phase of 45 min allowed the clarified low P liquor to be separated and pumped out of the reactor. It was then ready to receive more P containing feed for the next FAMINE stage, and so on.

2.3.2 FAMINE phase

During the famine phase, a synthetic wastewater feed containing P was pumped into the reactor. The FAMINE phase lasted 99 min, during which time P uptake occurred. At the end of the FAMINE phase, biomass containing stored polyP granules was removed from the reactor.

2.4 Chemical analysis of the aerobic EBPR community fed synthetic wastewater containing 160 mg L^{-1} C as acetate

The SBR was operated as described in Section 1.1, initially at an acetate feed concentration of 160 mg L^{-1} C as acetate (Feed A). The main steps of the SBR cycle are shown in Figure 2.1. The P removal efficiency over time is given in Figure 2.2. Data from the start-up phase of this process showed that once established, the P removal efficiency was always close to 100% (Figure 2.2), so that no detectable P could be measured in the final effluent. The data given are from the first 60 days of operation, during which time the initial acetate and P feed levels were gradually increased to allow the community to become established, although a similar stable operation producing no detectable P in the effluent was maintained for more than 2 months.

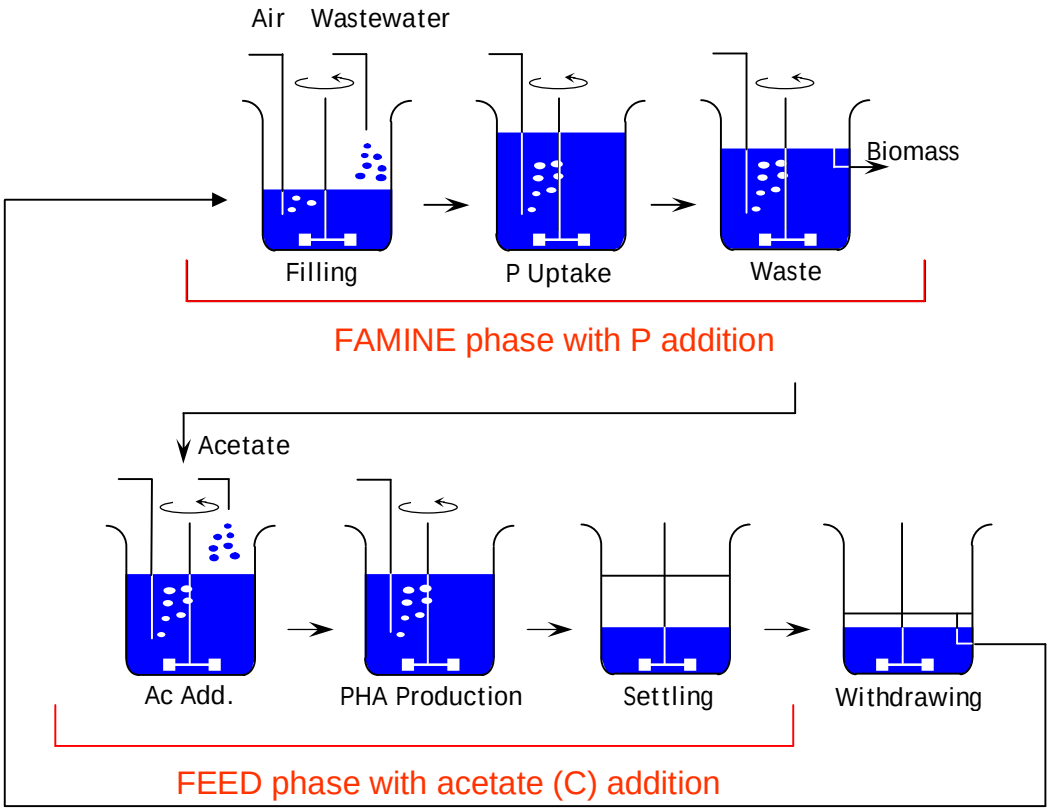


Figure 2.1 Main stages of a cycle of the aerated phosphorus removal process, operated in a sequencing batch reactor (SBR). Diagram courtesy of Dr J Ahn.

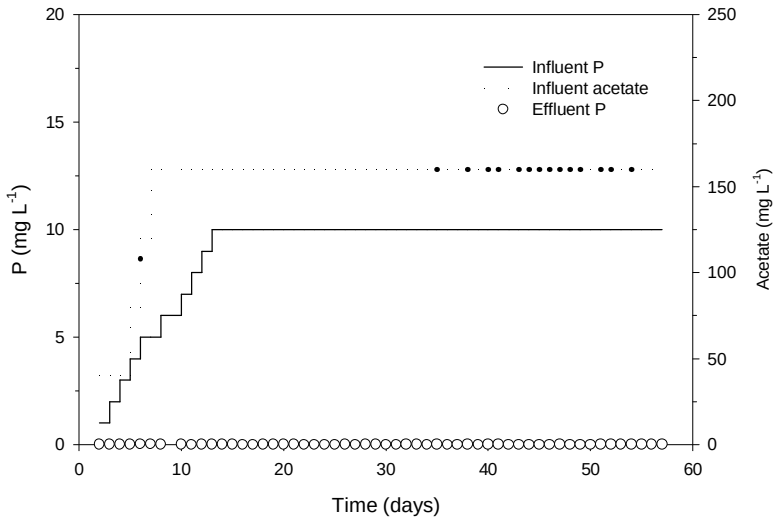


Figure 2.2 Time profile during the start-up period for the aerated EBPR SBR. Profile shows incremental increase of influent P (—), influent acetate (•) and levels of P in the effluent (◦)using synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). (Data provided by Dr J Ahn, La Trobe University).

2.4.1 Chemical transformations in the FEED phase

A typical chemical profile is shown in Figure 2.3. Standard errors are not given, but were always < 2%. During the FEED phase the added acetate was assimilated into the biomass within 44 min of addition, and most was used not for growth but for synthesise of intracellular PHA, under what were P and possibly N limiting conditions, since biomass PHA levels rose in parallel. A carbon mass balance based on existing stoichiometric equations (Smolders et al. 1994) showed that about 98% of the acetate assimilated by this biomass was converted to PHA. GC analysis showed PHB was the only PHA synthesized by this microbial community (data not shown). During acetate assimilation, P was released into the bulk liquid from the biomass at a ratio of 0.12 mmol P release mmol $acetate^{-1}$ utilised, which is similar although slightly lower than that reported for many conventional EBPR systems (Van Loosdrecht et al. 1997; Oehmen et al. 2007). Not all the intracellular biomass polyP was released. Once acetate had been depleted and P was available in the medium, the populations containing PHA were able to respire it aerobically as their C and energy source, a transformation which usually occurs in the aerobic famine stage of conventional EBPR processes (Bond et al. 1995; Seviour et al. 2003; Oehmen et al. 2007), and was reassimilated. PHA levels decreased until all the P had been reutilised and was no longer available for growth. Then the rate of PHB disappearance gradually slowed and eventually ceased before all the intracellular PHB was utilised.

2.4.2 Chemical transformations in the FAMINE phase

When P was added to the reactor in the FAMINE phase it was rapidly assimilated by the P starved cells (Figure 2.3). PHB levels fell in this stage, suggesting it was again being respired to provide energy for P uptake and polyP production. Glycogen levels did not appear to vary detectably over the course of the SBR cycle. Figure 2.4 shows the oxidation/redox profile. After acetate addition and P release the DO level fell to about 5 – 12% saturation, and rose to 80% after acetate depletion. It remained at this level except during settling when it fell to about 6 – 10% saturation, which was not accompanied by any detectable P release (data not

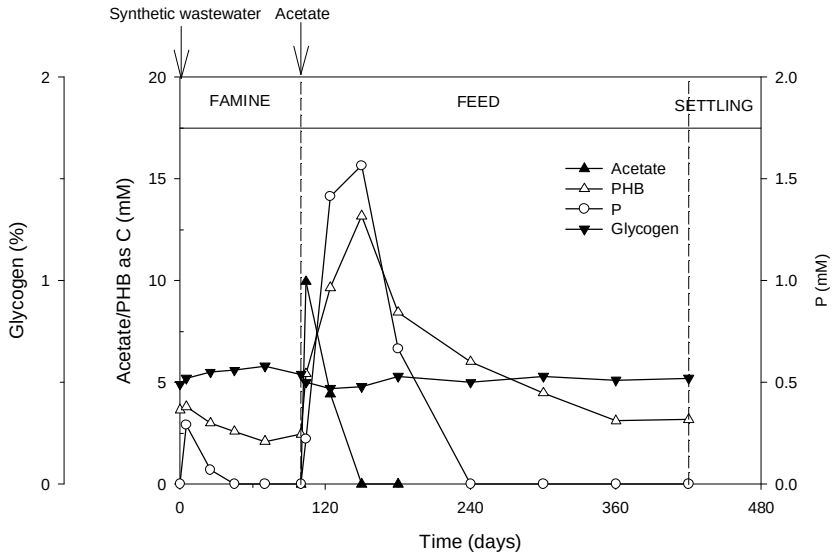


Figure 2.3 Chemical profile of the aerated EBPR SBR cycle. Profile shows cycle when using synthetic wastewater and 160 mg L^{-1} C as acetate (Feed A), showing changes in acetate levels (▲), P content of mixed liquor (○) PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) over a FEED: FAMINE cycle. Arrows indicate where acetate and the synthetic wastewater were added to the reactor. (Data provided by Dr J Ahn, La Trobe University).

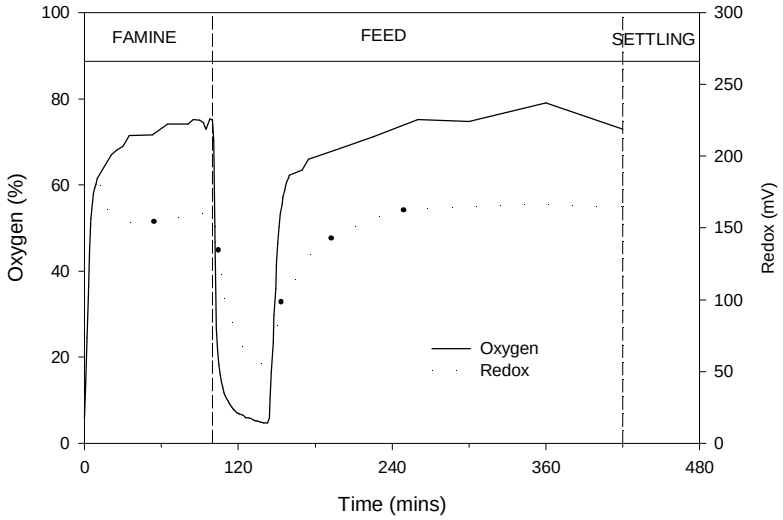


Figure 2.4 Dissolved oxygen (—) and redox potential (•) profiles for the aerated EBPR SBR. Profile is over a FEED: FAMINE period when fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). (Data provided by Dr J. Ahn, La Trobe University). Further details in Figure 2.3.

shown). The redox potential fell to about +50 mV during the acetate feed period, confirming the environment was always an oxidizing one.

2.5 Community composition of the aerated EBPR community fed synthetic wastewater containing 160 mg L^{-1} C as acetate

2.5.1 Community composition by Gram staining

Gram staining of biomass samples taken from this community (detailed in Section 1.8.4) showed mainly Gram negative cells, with a few small Gram positive cells with a coccoid morphology (Figure 2.5a, b). Large clusters of cells with the morphology of *Accumulibacter* clusters stained neither Gram negatively nor Gram positively, but a yellow-brown colour, as was reported earlier for PAO cells (Crocetti et al. 2000) (Figure 2.5b). Viability staining (Section 1.8.3) showed that each of these large clusters contained both viable and non-viable cells (Figure 2.5c, d), and capsule staining (Section 1.8.5) revealed that these clusters and the TFOs were both heavily encapsulated (Figure 2.5e, f).

2.5.2 Community composition by 16S rRNA analysis

A 16S rRNA clone library was constructed in the early part of this study by Dr M Beer (La Trobe University) as described in Section 1.3, using the UltraClean™ Soil DNA Kit (Mo Bio Laboratories, Inc) for DNA extraction as detailed in Section 1.2.4. The phylogenetic tree showing the 33 OTUs generated from this biomass is given in Figure 2.6. It reveals that members of the *Betaproteobacteria* were strongly represented, and a large number of *Dechloromonas* (>50%, data not shown) and *Zoogloea* related clones were present. Smaller numbers of *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Thermus*-related clones also occurred in the clone library, as did clones from the *Cyanobacteria* and *Deinococcus-Thermus* phyla. Only one Gram positive clone was detected in that library, which was a *Firmicutes* related clone. Of particular interest was that no *Accumulibacter* clones, the PAO commonly found in conventional EBPR systems, were detected.

2.5.3 Community composition by FISH analysis

When FISH analysis was conducted on biomass samples taken from the reactor after 28 d of successful P removal, as described in Section 1.5, the following populations were detected.

2.5.3.1 Gram negative Bacteria

2.5.3.1.1 Betaproteobacteria

From FISH analysis it was clear that a large portion of the biomass consisted of *Betaproteobacteria*, appearing largely as clusters of coccobacilli, as well as single cells. These cells fluoresced with the BTWO23a probe targeting Cluster 2 of the *Betaproteobacteria* (Figure 2.7a), while none fluoresced with the BONE23a probe, targeting the Cluster 1 members (Amann et al. 1996). Most of the same cells fluorescing with the BTWO23a probe responded positively to the PAOmix probes (PAO462b, PAO641, PAO846b) targeting the *Accumulibacter* PAO (Figure 2.8a) (Crocetti et al. 2000; Zilles et al. 2002). They also hybridised with the RHC439 (Figure 2.7b), RHC175, and Rc988 probes, targeting the *Rhodocyclus* related organisms described by Hesselmann et al. (1999) and Crocetti et al. (2000). Cells hybridising with the PAOmix probes made up 22.6 ± 4.6% of the biomass volume which fluoresced with the EUBmix probes. These cells were generally coccobacilli, forming large round clusters.

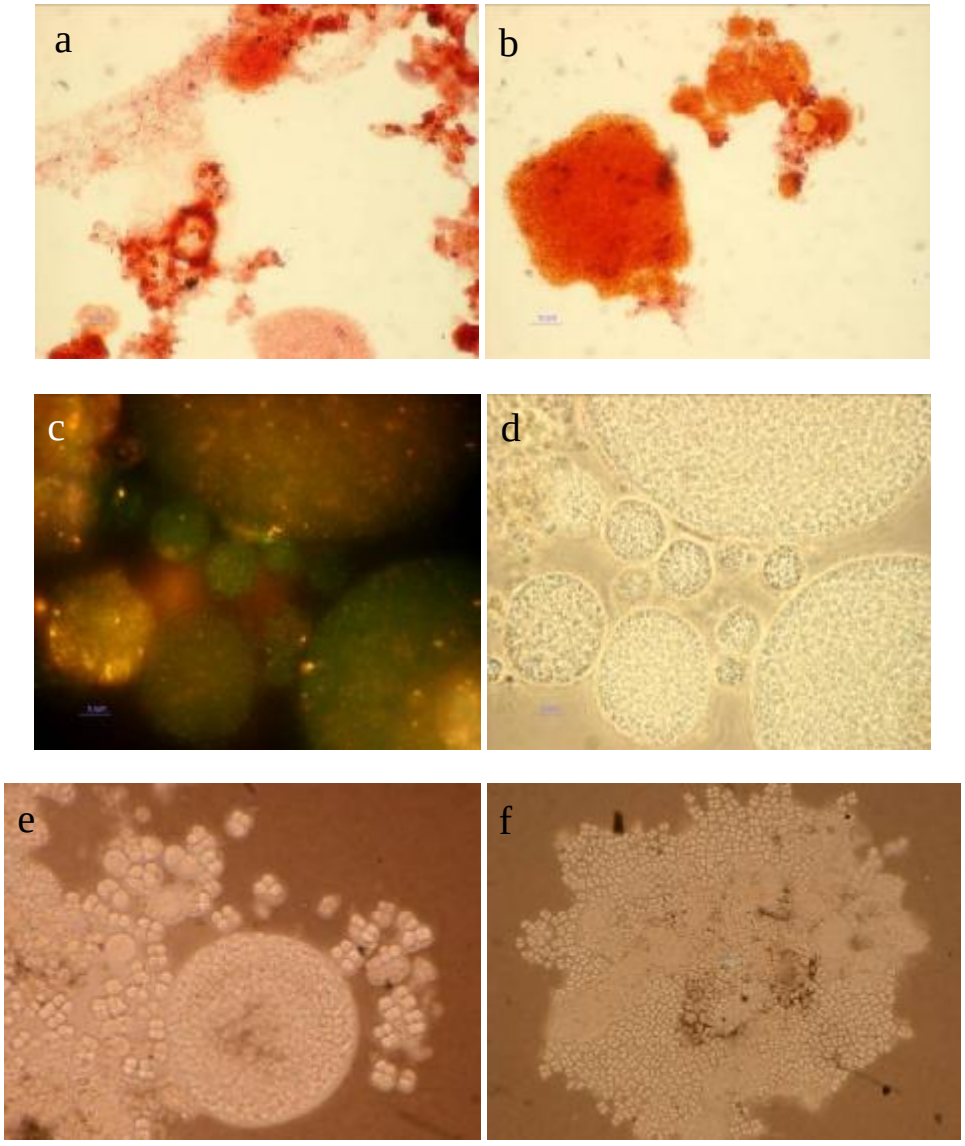


Figure 2.5 Micrographs of biomass from the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a) and b) Gram stains. Note the small Gram positive cocci in a), and the large cell clusters appearing as neither Gram positive nor Gram negative but a yellow/brown colour in b). c) and d) Viability stain of biomass, c) some clusters stain green, indicating intact membranes and viability, and some stain yellow, indicating non viable cells with degraded membranes. d) Same field of view as c) under phase contrast. e) and f) Capsule stains of biomass. Note the clear halos surrounding large cell clusters in e) and TFO in e) and f).

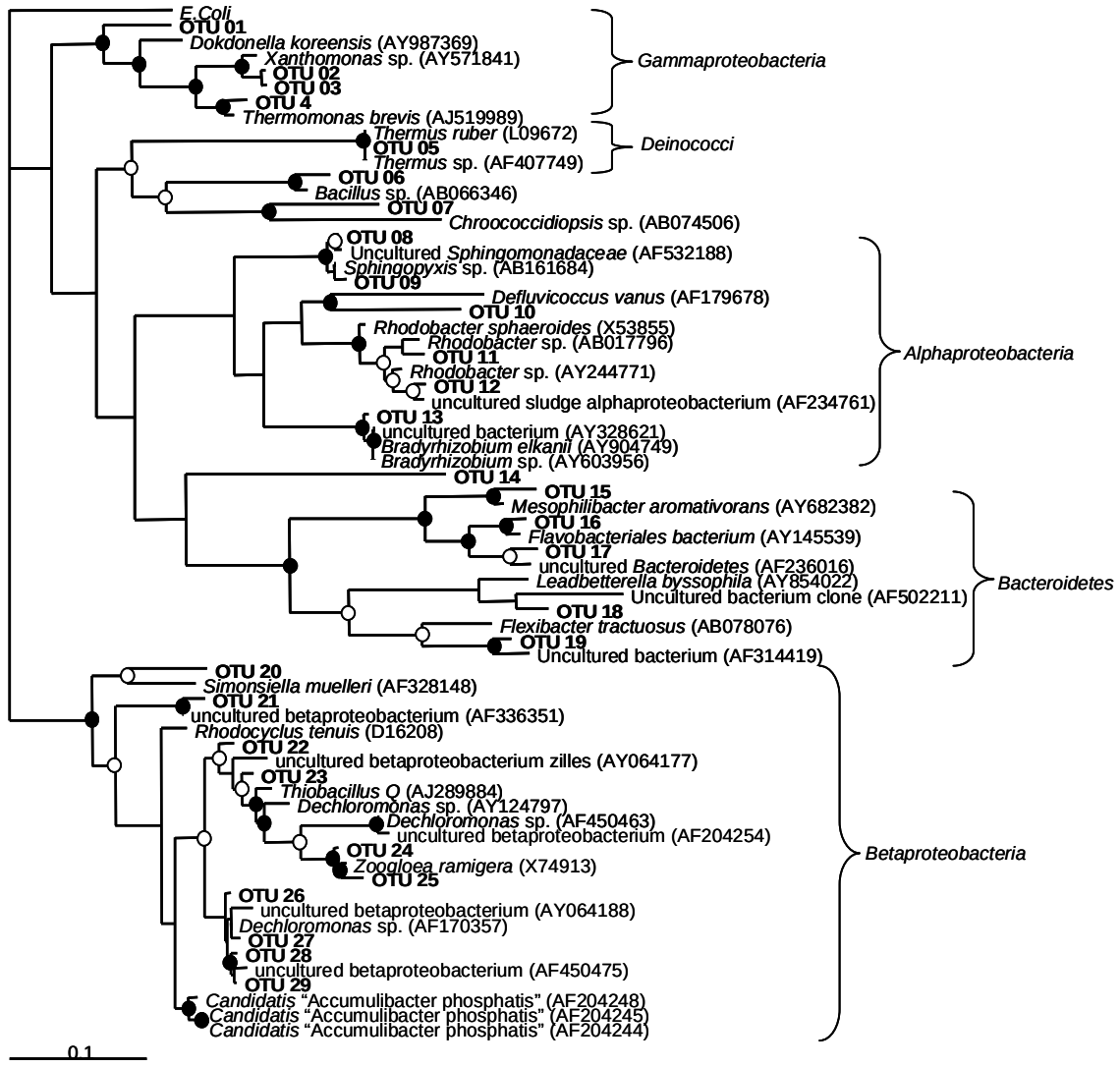


Figure 2.6 Neighbour joining tree of the sequences (OTUs) in a clone library obtained from the aerated EBPR SBR. The SBR community was fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). Library was constructed by Dr M Beer (La Trobe University). Bootstrap values are a percentage of 1,000 analyses (•) indicates values >95%, (◦) indicates values >75%.

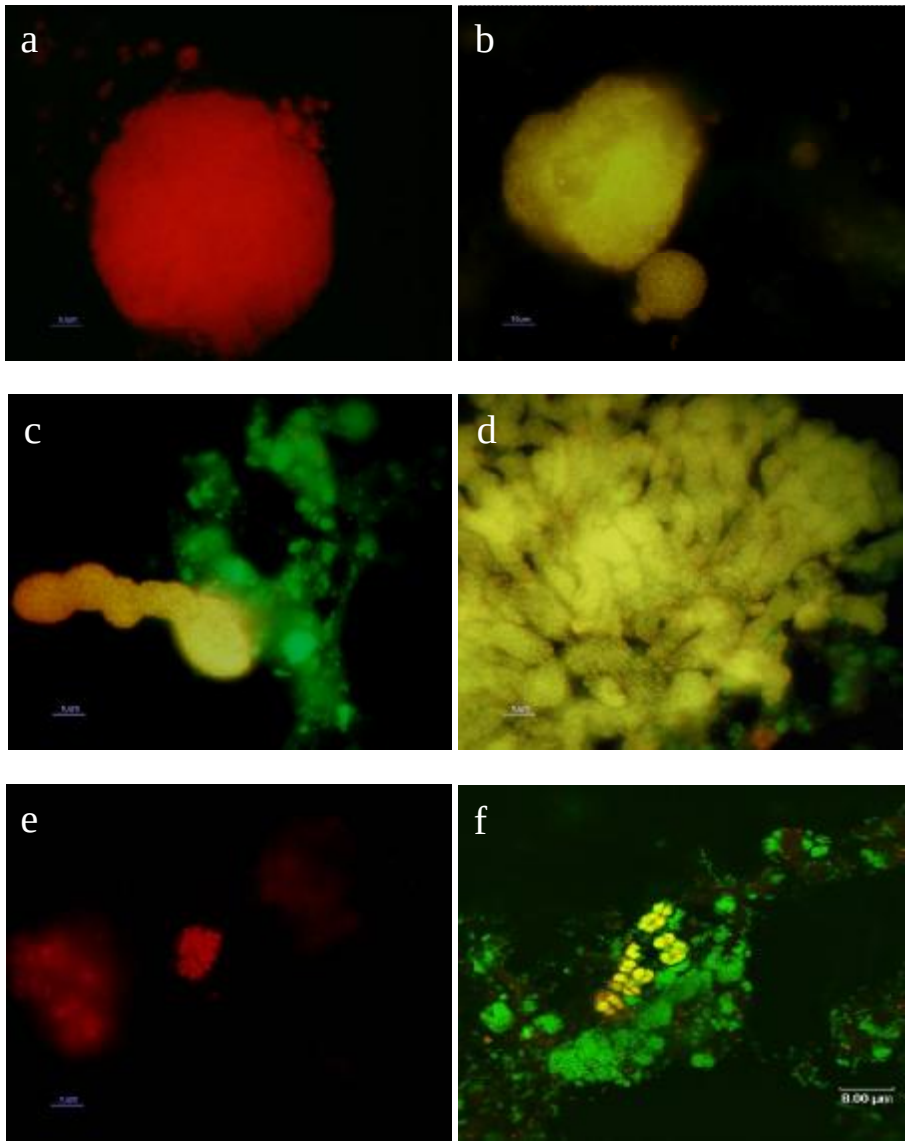


Figure 2.7 FISH and phase contrast micrographs showing populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a) *Betaproteobacteria* cells hybridising with the BTWO23a probe (Cy3). b) *Accumulibacter* related cells hybridising with both the RHC439 probe (Cy3) and PAOmix probes (FLUOS). c) and d) Cells hybridising with EUBmix probes (FLUOS), and c) *Dechloromonas* related cells hybridising with the Dech472 probe (Cy3), d) *Zoogloea* hybridising with the Zra23a probe (Cy3), showing typical *Zoogloea* finger like tendrils. e) Cluster 1 *Defluviicoccus* related cells hybridising with DF1mix probes (Cy3). f) *Sphingomonas* cells hybridising with the SPH120 probe (Cy3) and EUBmix probes (FLUOS). Cells that hybridised with both Cy3 and FLUOS labelled probes appear yellow.

Most of the other *Betaproteobacteria* fluoresced with the Dech454 (Figure 2.8b) and Dech472 (Figure 2.7c) probes designed by Dr M Beer (La Trobe University) during this study, to target the *Dechloromonas* related clones (clone 30-ORF28, clone 20-ORF29 and clone 26-ORF26 for Dech454, and clone 09-ORF25 for Dech472, see Figure 2.10) present in the clone library constructed by him. The Dech454 probe targets *Dechloromonas* spp. and other uncultured bacteria, while the Dech472 targets uncultured *Rhodocyclaceae*, two uncultured environmental bacteria, and *Zoogloea* spp., including *Zoogloea ramigera* (Probecheck, Loy et al. 2008). Clusters fluorescing with the Dech454 probe contributed approximately 16.2 ± 2.5% to the biovolume fluorescing with the EUBmix probes. These FISH probed cells also formed large clusters consisting of small rod shaped cells. The cells in clusters fluorescing with the Dech472 probe were usually much smaller and less spherical in their appearance and far less frequently seen than those targeted by the Dech454 probe. However, this Dech472 probe also targets *Zoogloea* spp., and typical zoogloeal finger-like clusters were sometimes seen in biomass samples (Figure 2.7c). Some cells responding to the Zra23a probe targeting *Zoogloea* (Rossello-Mora et al. 1995) were also detected, and these made up 5.2 ± 0.6% of the biovolume hybridising with the EUBmix probes. These cells were also arranged in clusters, but also as 'finger-like' tendrils characteristic of *Zoogloea* (Rossello-Mora et al. 1995) (Figure 2.7d).

2.5.3.1.2 Alphaproteobacteria

Most of the *Alphaproteobacteria* detected with the ALF968 probe displayed a TFO morphology. These TFO responded to neither the SBR9-1a nor the AMAR839 FISH probes, both of which were designed to detect alphaproteobacterial TFO populations in EBPR communities (Maszenan et al. 2000; Beer et al. 2004). Nor did they respond to the TFO-DF862 or DEF438 probes targeting the Cluster 1 *Defluviicoccus* related TFO in EBPR communities (Wong et al. 2004). A few of the TFO fluoresced with the DF1mix probes targeting Cluster 1 *Defluviicoccus* (Wong et al. 2004) (Figure 2.7e), and with the SPH120 probe targeting the sphingomonads (Eilers et al. 2000) (Figure 2.7f). However, most of the TFO targeted with the broad range ALF968 could not be identified further in the samples examined in the early part of this study.

However, Meyer et al. (2006) described two new probes to target the Cluster 2 *Defluviicoccus* related cells which they found in a poorly performing anaerobic: aerobic EBPR system. These were the DF988 and DF1020 probes. By subjective microscopic estimation, these probes appeared to hybridise with most of the TFO in the biomass samples from the aerated EBPR SBR community. In fact about 7.9 ± 1.0% of the cell volume fluorescing with the EUBmix also fluoresced with the DF988 probe (Figure 2.8c, d). Visually this probe appeared to have a slightly broader target range than the DF1020 probe did, which hybridised with 8.3 ± 1.1% of the biovolume fluorescing with the EUBmix probes. This observation agrees with the earlier data of Meyer et al. (2006), who reported that all cells hybridizing with the DF1020 probe also hybridized with the DF988 probe, but all cells hybridizing with the DF988 probe did not hybridise with the DF1020 probe. A few other alphaproteobacterial TFO not responding to any of the *Defluviicoccus* targeted FISH probes were also present in these biomass samples (data not shown).

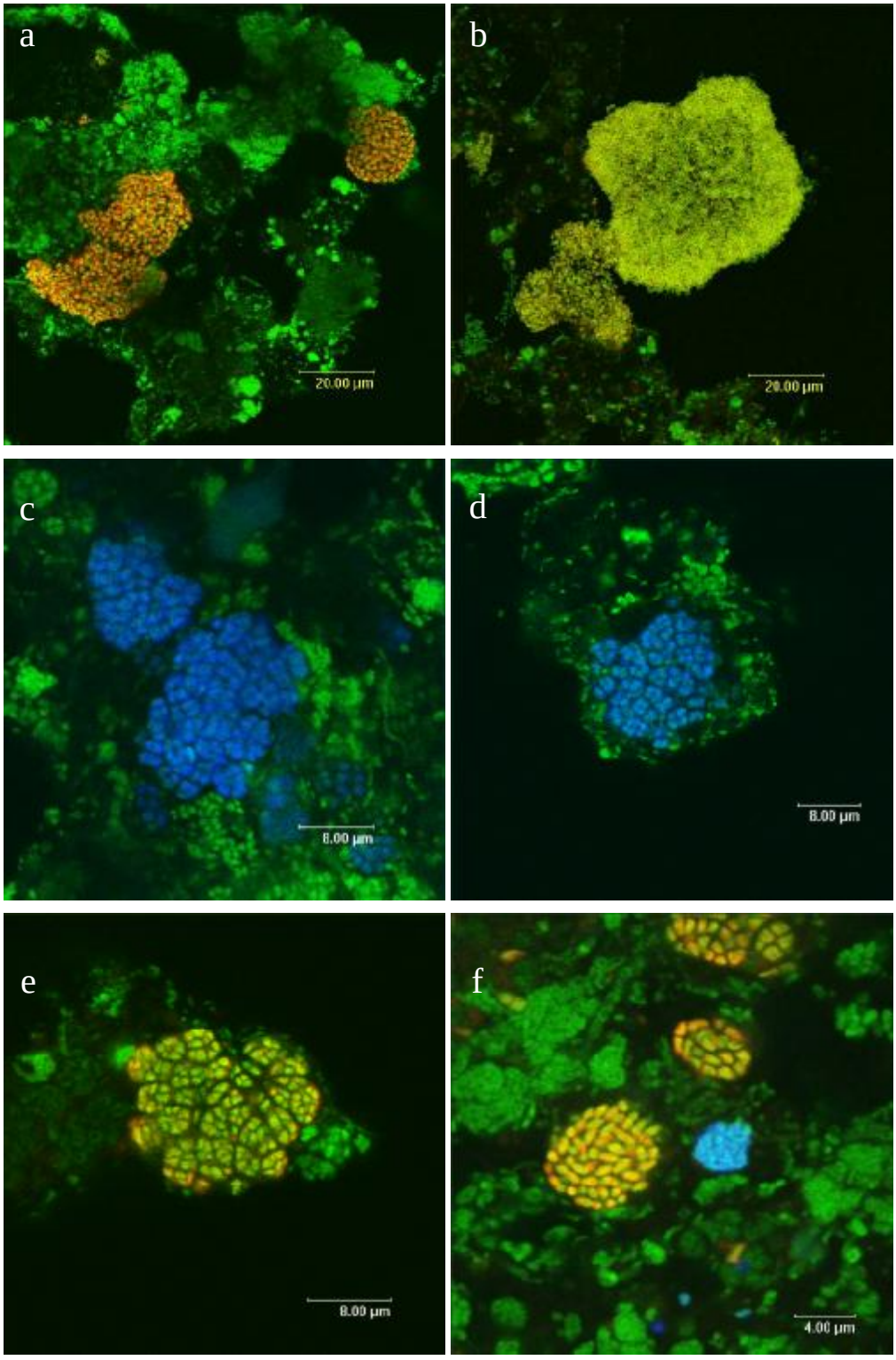


Figure 2.8 FISH micrographs showing the main populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a) *Accumulibacter* clusters hybridising with the PAOmix probes (Cy3). b) *Dechloromonas* cells targeted by the Dech454 probe (Cy3). c) and d) *Defluviicoccus* related cells arranged in tetrads, targeted with the DF988 probe (Cy5). e) *Nitrosomonas* cells hybridising with the Nso1225 probe (Cy3). f) *Nitrosomonas* and *Nitrospira* cells hybridising with the Nso1225 (Cy3) and Ntspa662 (Cy5) probes. All cells also hybridised with the EUBmix probes (FLUOS). Cells that hybridised with both probes appear yellow (EUB and Cy3), and light blue (EUB and Cy5).

2.5.3.1.3 Gammaproteobacteria

Cells responding to the Gam42a probe targeting the *Gammaproteobacteria* (Manz et al. 1992; Siyambalapitiya and Blackall 2005) were also present as clusters of small rods and cocci (data not shown). However, no cells responded to the ACA23a probe targeting *Acinetobacter* spp. (Wagner et al. 1994), confirming they are not important PAO populations in this EBPR process. In addition, only a few cells (<1% of biovolume by visual estimation) responding to the GAOmix or GBmix probes targeting the *Competibacter* GAO (Crocetti et al. 2002; Kong et al. 2002) were seen in any of the biomass samples (Figure 2.9a).

2.5.3.1.4 Nitrifying organisms

Very small numbers (<1%) of ammonia oxidising *Nitrosomonas* (Figure 2.8e) and nitrite oxidising *Nitrospira* were also detected in this community. They appeared in clusters, as expected from previous studies (Wagner et al. 1995; Daims et al. 2001), and were sometimes seen immediately adjacent to each other, as reported frequently elsewhere (Figure 2.8f) (Mobarry et al. 1996; Juretschko et al. 2002).

2.5.3.2 Gram positive Bacteria

2.5.3.2.1 Firmicutes

No cells in the biomass appeared to hybridise with the LGCmix probes, targeting members of the *Firmicutes.*

2.5.3.2.2 Actinobacteria

There appeared to be no *Actinobacteria* present in the biomass that responded to the Actino1011 or Actino221 probes for the actinobacterial PAO, designed to target short rods in full scale EBPR plants (Liu et al. 2001; Juretschko et al. 2002; Kong et al. 2005). However, some small cocci hybridised with the Actino658 probe (data not shown), although they did not display the tetrad morphology of the cells the probe was originally designed against. A few small coccal cells, sometimes in small clusters also hybridised with the HGC69a probe designed to target all members of the *Actinobacteria* (Roller et al. 1994) (see Figure 2.12a).

2.5.3.3 *'Chloroflexi'*

The probes CFX1223 and GNSB-941 target members of the phylum *Chloroflexi* (Björnsson et al. 2002). These probes hybridised with filaments that were present in large amounts based on a visual examination of the biomass but these were not identified further (Figure 2.9b). These did not appear to hybridise with the EUBmix probes.

2.5.3.4 *Planctomycetales*

Members of the *Planctomycetales* were also present, appearing as small coccoid cells which responded to the PLA886 (Figure 2.9c) and PLA46 probes (Neef et al. 1998) (data not shown). Larger irregular shaped cells also fluoresced with these probes (Figure 2.9c), but did not appear to hybridise with the EUBmix probes, which is not surprising since sequences for this group of bacteria are relatively few in the current databases (Cole et al. 2007).

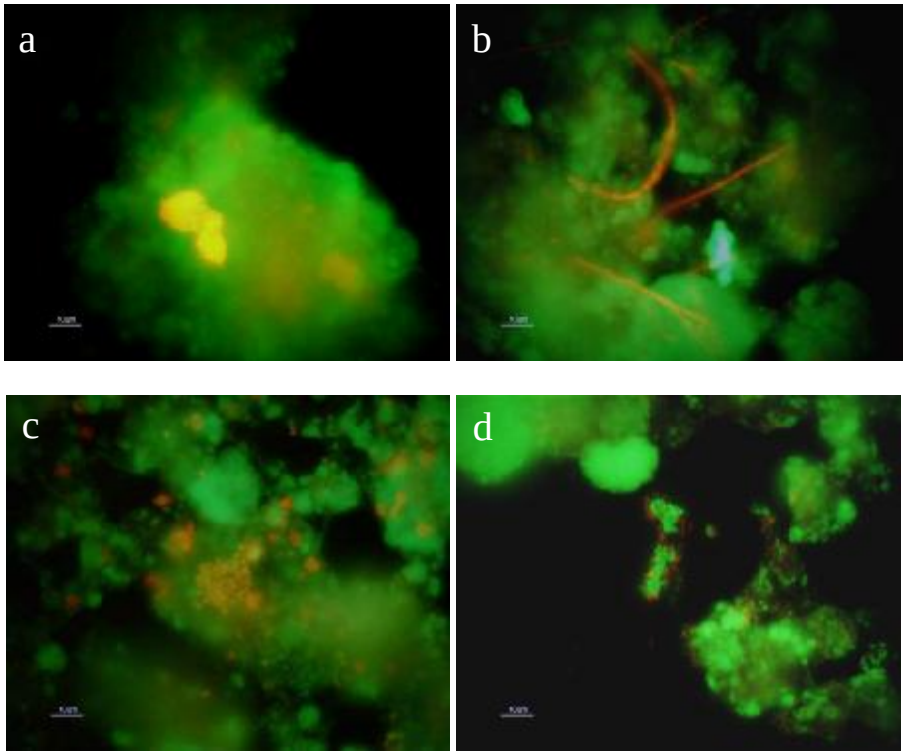


Figure 2.9 FISH micrographs showing populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a – d) Cells hybridising with the EUBmix probes (FLUOS), and a) GBmix probes (Cy3) targeting *Competibacter* cells, b) *Chloroflexi* cells hybridising with the CFX1223 and GNSB-941 probe mix (Cy3). Note these cells do not hybridise with the EUBmix probes. c) *Planctomycetes* cells hybridising with the PLA886 probe (Cy3), note the larger irregular shaped cells again do not hybridise with the EUBmix probes. d) *Flavobacteria* cells hybridising with the CF319a probe (Cy3), note these cells do not hybridise with the EUBmix probes either. Cells that hybridised with both Cy3 and FLUOS labelled probes appear yellow.

2.5.3.5 Other organisms present

Flavobacteria have been reported in Europe as frequent members of the activated sludge community (Kragelund et al. 2008), and were detected here by the probe CF319a as small cells scattered through many of the clustered cells, as well as appearing as short filaments. However, these cells did not appear to hybridise with the EUBmix probes either, as shown in Figure 3.9d. No cells hybridised with the Aqs997 probe targeting the *Aquaspirillum* related bacteria, a group very commonly seen in EBPR activated sludge systems in Europe (Meier et al. 1999; Thomsen et al. 2007).

2.6 Discrepancies between the clone library data and those from FISH analyses

While the FISH results showed the reactor community was dominated by betaproteobacterial *Accumulibacter* cells and alphaproteobacterial *Defluviicoccus* related TFO (Section 2.5.3), neither of these populations were revealed in the clone library constructed by Dr M Beer (La Trobe University). The most likely explanation was the failure of the kit used to extract DNA from the heavily encapsulated cells (see Figure 2.5e, f), or biases associated with the PCR amplification step.

Therefore, a second clone library was constructed using two different extraction methods, the phenol chloroform McVeigh protocol (McVeigh et al. 1996) and the NaTCA protocol (McIlroy et al. 2008b), both of which had been shown to extract DNA from these cells (S. McIlroy, personal communication). DNA was extracted from biomass samples as described in Section 1.2.1 and 1.2.2 using both extraction protocols, and the DNA extracts pooled for clone library generation and analysis as detailed in Section 1.3.

2.6.1 16S rRNA clone library analysis using the McVeigh et al. (1996) and NaTCA (McIlroy et al. 2008b) DNA extraction methods

The clone library obtained with these extraction methods contained 41 OTUs using 99% sequence similarity as the cut off (Stackebrandt and Ebers 2006). A phylogenetic tree showing these OTUs is given in Figure 2.10. One OTU sequence was chimeric and removed from the library. Unlike the UltraClean™ library (constructed by Dr M Beer, Section 1.5.2), this clone library contained two *Candidatus* 'Accumulibacter phosphatis' clones in OTU c79. These clones have identical sequences to those of the uncultured bacterium clone UWMH_9 (Acc. EF565148) of He et al. (2007), which they recovered from a conventional EBPR system. Three Cluster 2 *Defluviicoccus* related clones (OTUs c112, c15, and c101), also appeared. OTUs c112 and c15 were most closely related (99% and 98% similarity respectively) to the uncultured alphaproteobacterial clones pGAO43 and pGAO8 (Acc. DQ146468 and DQ146465) of Meyer et al. (2006), recovered from a propionate fed SBR with failed EBPR capacity. OTU c101 was identical to the uncultured bacterium clone LPB46 (Acc. AF527585) of McMahon et al. (unpublished) from an SBR performing EBPR. However, as with the UltraClean™ clone library, this library also revealed many *Dechloromonas* related clones, in OTUs c9, c59, c61, c71, c85, and c96, and many *Zoogloea* related clones, in OTUs c8, c64, c78, c83, c84, c87, and c92. This relative abundance agrees with the FISH data reported earlier in this section (Section 3.5.3.1.1). Only one Gram positive clone was found (OTU c60), which was 99% similar to *Micropruina glycogenica*, thought to be a putative GAO (Shintani et al. 2000). No *Competibacter* clones were detected, which was not unexpected since the FISH data (see Section 1.5.3.1.3) revealed only a small number (<1% biovolume) present in this community.

At the level of 99% as a threshold of relative sequence similarity of the 16S rRNA gene sequences retrieved, the calculated homogenous coverage

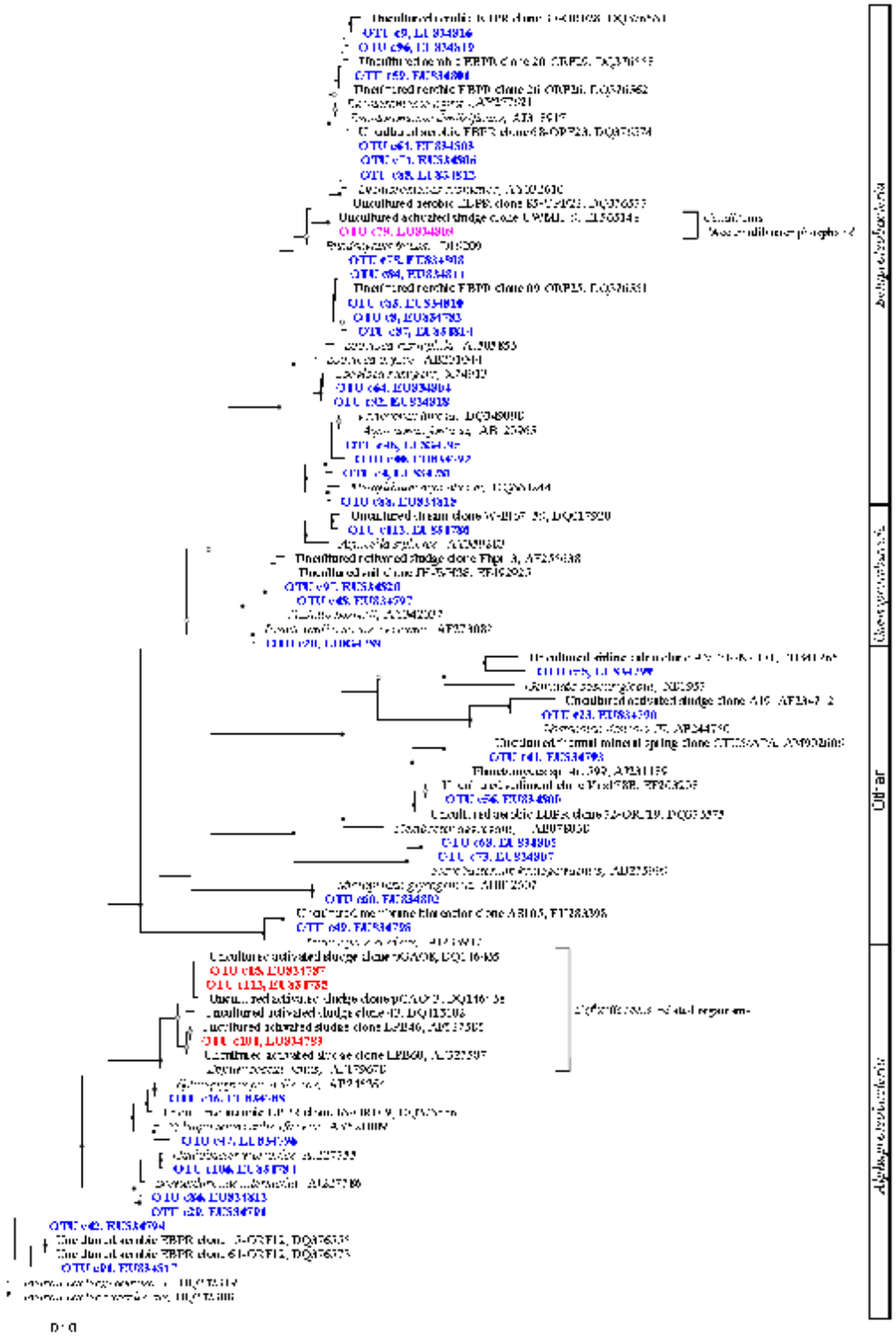


Figure 2.10 Maximum likelihood phylogenetic tree of the sequences (OTUs) in a clone library obtained from the aerated EBPR SBR. The SBR community was fed synthetic wastewater and 160 mg L^{-1} C as acetate (Feed A). Library was generated using the NaTCA and McVeigh DNA extraction methods. Bootstrap values are a percentage of 1,000 analyses (•) indicates values >95%, (◦) indicates values >75%.

(Singleton et al. 2001; Juretschko et al. 2002; Kong et al. 2007) was 76%. This indicates a large fraction of the biodiversity in the reactor was represented in the library. The species richness was also determined as being 53 species.

2.7 Ecophysiology of major populations

The *in situ* phenotypes of the *Accumulibacter* and *Defluviicoccus* related organisms were confirmed as displaying phenotypes similar to those of PAO and GAO respectively, reported in anaerobic: aerobic EBPR systems, using Nile blue A staining for PHA inclusions, and DAPI staining for polyP storage, as detailed in Section 1.9.

2.7.1 PolyP/FISH staining

2.7.1.1 FAMINE phase

DAPI staining showed the *Accumulibacter* positive cells contained polyP at the end of the famine phase (Figure 2.11a, b). Not all cells in each cluster were DAPI positive, which was noted earlier for the *Accumulibacter* clustered cells in anaerobic: aerobic EBPR systems (Zilles et al. 2002; Beer et al. 2006) (Figure 2.11e, f). DAPI staining was also performed without FISH (data not shown) which confirmed that the *Accumulibacter* cells did contain polyP, as it has been reported that the FISH hybridisation process can affect the effectiveness of DAPI staining (Zilles et al. 2002). A few cells hybridising with the HGC69a probes stained both positively (Figure 2.12a, b) and negatively for polyP with DAPI, although the small numbers present suggested they played a minor role in P removal in this aerated system. Other small coccoid cells also stained positively for polyP, although these could not be identified as *Actinobacteria* with the FISH probes used. Importantly, DAPI staining showed the *Dechloromonas* related clusters never stained positively for polyP storage through the SBR cycle (Figure 2.11g, h). Furthermore no TFO, including the *Defluviicoccus* related cells appeared to contain polyP after staining with DAPI (Figure 2.12c, d), although some larger unidentified TFO gave a yellow fluorescence after DAPI staining, consistent with polyP presence (data not shown).

2.7.1.2 FEED phase

DAPI staining showed the *Accumulibacter* positive cells also contained polyP at the end of the FEED phase (Figure 2.11c, d), which agrees with the chemical profile data, showing that polyP released from these cells following acetate addition was reassimilated by the cells. Again not all cells in every cluster were DAPI positive.

2.7.2 PHB/FISH staining

2.7.2.1 FAMINE phase

Nile blue A staining combined with FISH showed that only a few *Accumulibacter* clusters contained PHB at end of the FAMINE phase (Figure 2.13a, b). Again this agrees with the chemical profile data that these populations were using their PHA stores to provide energy to assimilate P and synthesise polyP.

The *Dechloromonas* related cells also appeared to contain PHA at the end of the FAMINE phase (not shown), while the *Defluviicoccus* related TFO and other alphaproteobacterial TFO stained both positively and negatively for PHA in samples taken throughout the FAMINE phase (Figure 2.12e – h).

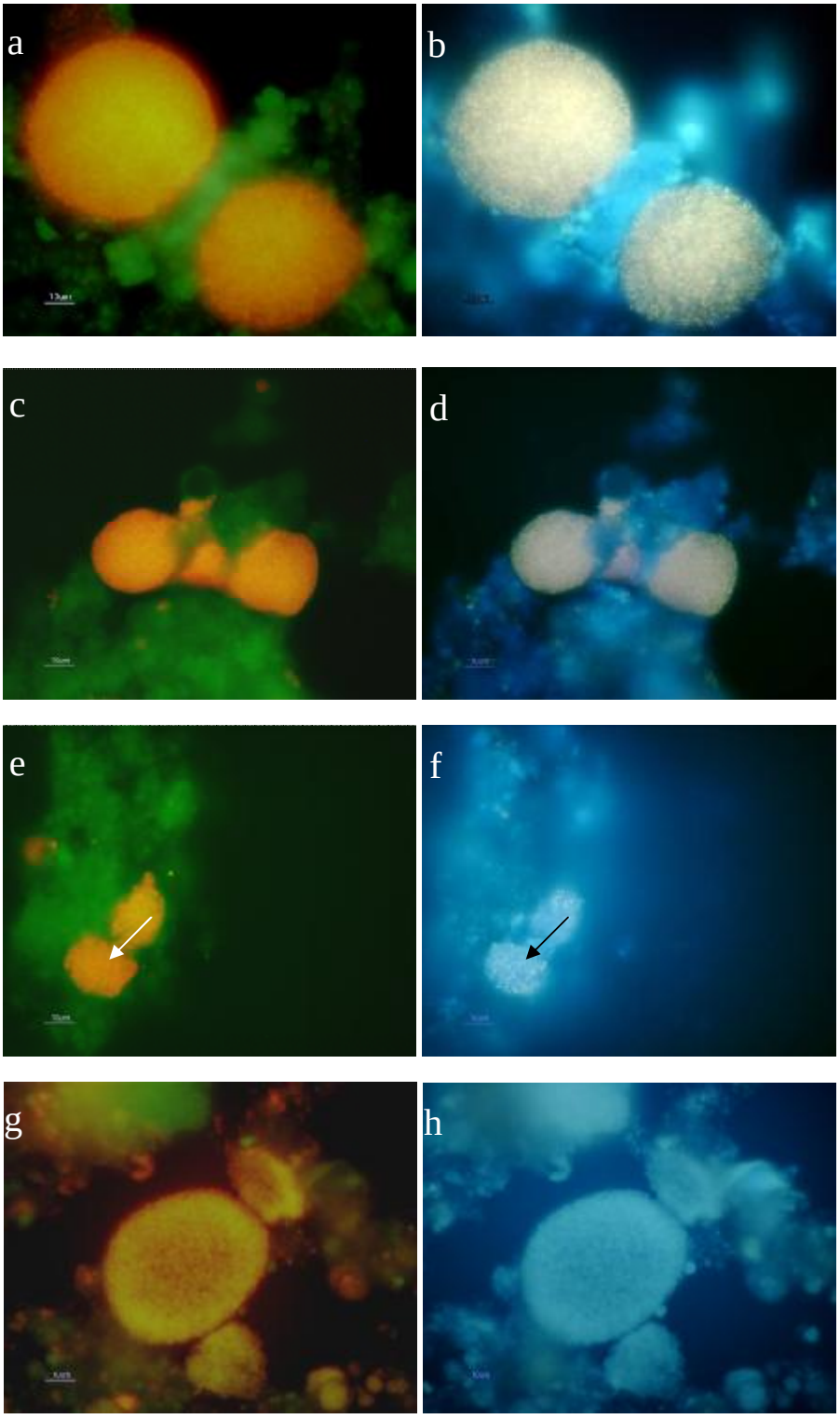


Figure 2.11 Micrographs of FISH and DAPI staining showing populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a, c, e) *Accumulibacter* cells hybridising with the PAOmix probes (Cy3) and EUBmix probes (FLOUS). b, d, f) Same field of view stained with DAPI, showing the *Accumulibacter* positive for polyP storage at the end of the b) FAMINE phase, d) FEED phase, and f) cells in a cluster staining positively and negatively for polyP. g) *Dechloromonas* related cells hybridising with the Dech454 probe (Cy3) and EUBmix probes (FLUOS). h) Same field of view showing the cells negative for polyP storage by DAPI staining. Cells hybridising with both FLUOS and Cy3 probes appear yellow.

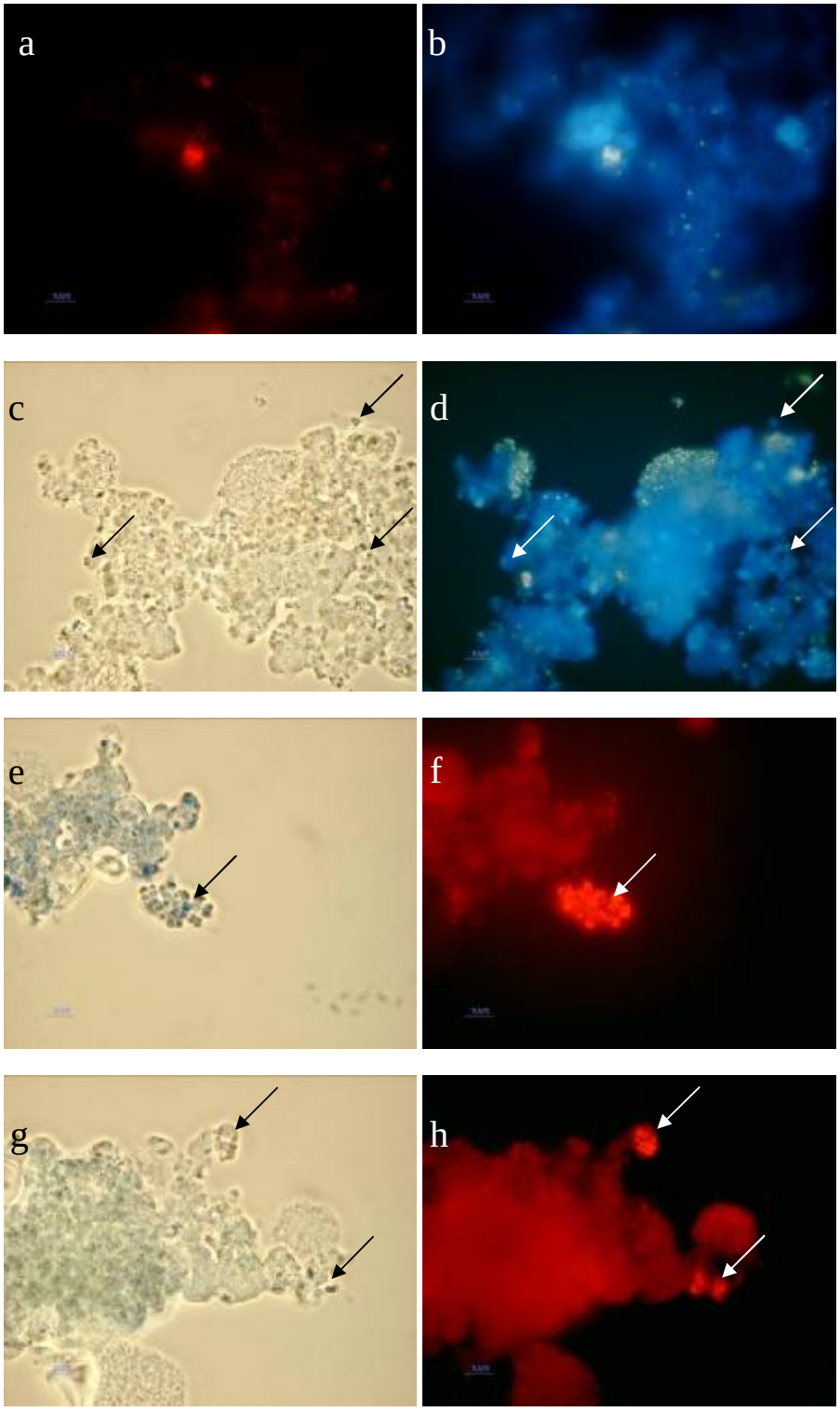


Figure 2.12 FISH and phase contrast micrographs of populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a) *Actinobacterial* cells hybridising with the HGC69a probes (Cy3). b) Same field of view showing the HGC positive cells positive for polyP storage. c, e, g) Phase contrast micrograph showing TFO cells, indicated by arrows. d, f, h) Same field of view showing d) TFO stain negatively for polyP storage with DAPI. f) and h) TFO staining positively for PHA accumulation with Nile Blue A staining.

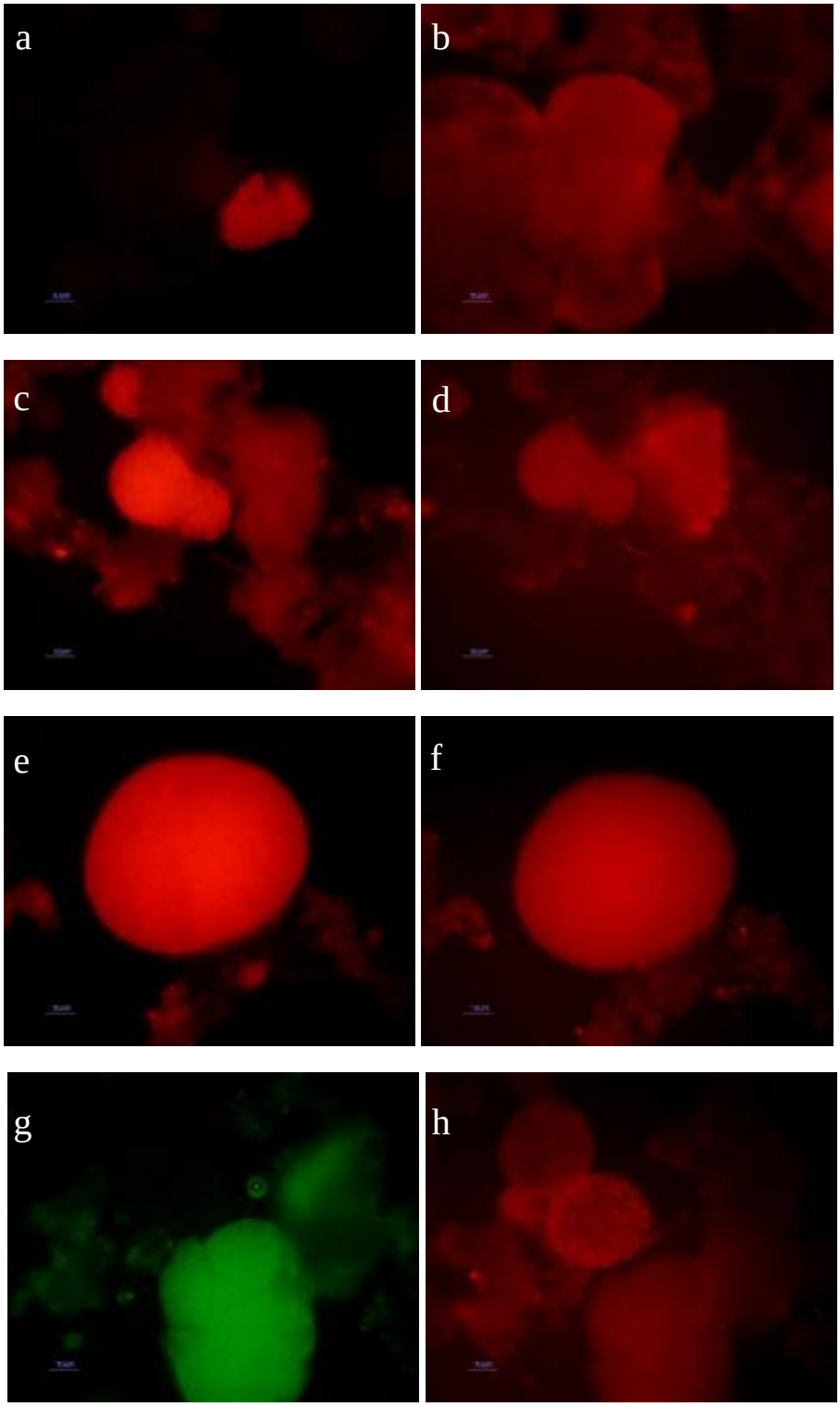


Figure 2.13 FISH micrographs of *Accumulibacter* cells in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a, c, g) *Accumulibacter* cells targeted with the PAOmix probes (Cy3), and g) FLUOS labelled. b, d, f, h) Same field of view stained for PHA accumulation with Nile blue A. Micrographs show *Accumulibacter* cells: b) negative for PHA accumulation at the end of FAMINE phase. d) Positive for PHA accumulation 1 h into FEED phase. f) Positive for PHA accumulation mid FEED phase. h) Negative for PHA accumulation at the end of FEED phase.

2.7.2.2 FEED phase

Nile blue A staining combined with FISH showed the *Accumulibacter* cells also contained PHB at end of the FEED phase. Furthermore, samples taken during this phase showed the *Accumulibacter* positive cells contained PHA after acetate depletion (Figure 2.13c, d) and throughout the FEED phase (Figure 2.13e, f). However, they seemed to stain less strongly for PHB immediately after acetate exhaustion, and towards the end of the FEED phase, where many clusters appeared as PHB negative (Figure 2.13g, h). This observation is also consistent with the chemical profile, and suggests these PAO populations were using their PHB stores during P uptake. Nile blue A staining revealed both the *Dechloromonas* related cells (not shown) and the alphaproteobacterial TFO (Figure 2.12e – h) also appeared to contain PHA at the end of the acetate FEED phase

2.7.3 FISH/MAR analysis of populations

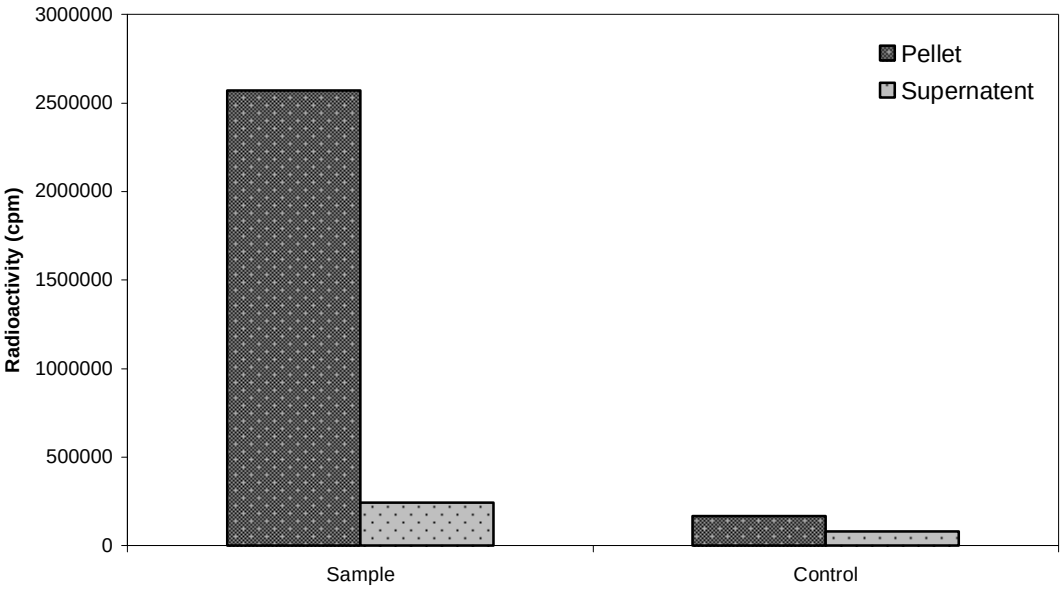
To further confirm that the *Accumulibacter* cells in this system showed a phenotype similar to the PAO phenotype reported in anaerobic: aerobic EBPR systems and were capable of assimilating acetate and P under the FEED and FAMINE conditions, FISH/MAR was used. The same approach was used to show whether the betaproteobacterial *Dechloromonas* related cells and alphaproteobacterial *Defluviicoccus* related TFO displayed a phenotype similar to that of a GAO in anaerobic: aerobic EBPR systems, in being able to assimilate acetate in the FEED phase but not phosphorus in the FAMINE phase. Thus biomass samples were incubated with radioactively labelled orthophosphate, ^{33}P, and ^{14}C acetate, and FISH/MAR analyses carried out.

Incubations were performed under conditions closely mimicking those present in the SBR, as described in Section 1.7. Biomass samples were taken from the reactor just before the addition of acetate or P, and incubated with ^{14}C acetate at a concentration of 6.6 mM, and ^{33}P orthophosphate at a concentration of 0.32 mM, which are the same concentrations found in the reactor. Acetate incubations were with a radioactivity of 0.5 µCi mg suspended solids $(SS)^{-1}$, and ^{33}P at a radioactivity of 10 µCi $mgSS^{-1}$. All the other MAR conditions were established to reflect the reactor conditions as much as possible. Thus, biomass samples were incubated with acetate for 45 min, the time taken for all acetate to be depleted in the reactor, and with ^{33}P orthophosphate for 45 min and 1.5 h, the time taken for all the P to be assimilated during the FAMINE phase and the length of the FAMINE phase, respectively. Slides were incubated for 3, 6, and 9 d. For P incubations, cells giving a positive MAR response with ^{33}P after 3 or 6 d were considered putative PAO, following suggestions of Kong et al. (2004). Cells giving a positive signal with acetate after 3 days were considered positive.

2.7.3.1 Assimilation of acetate during the FEED phase

Liquid scintillation counting confirmed that acetate was assimilated by the total biomass community in samples removed from the reactor during the FEED phase (Figure 2.14a). FISH/Nile blue A staining data also indicated *Accumulibacter* continued to take up acetate for the length of the FEED phase, and were storing it in the form of PHB (see also the chemical data, Section 3.4). The data showed that *Accumulibacter* assimilated acetate under incubation conditions identical to those existing in the SBR (Figure 3.15a, b). Furthermore, it appeared the *Dechloromonas* related cells also assimilated acetate (Figure 2.15e, f), as did the *Defluviicoccus* related TFO (Figure 2.15g, h). *Accumulibacter* and *Dechloromonas* related cells appeared to be more active in assimilating acetate, based on silver grain deposition density per cell (Nielsen and Nielsen 2005). However, some *Accumulibacter* clusters did not appear to assimilate acetate at all under the conditions imposed during this FEED stage (Figure 2.15c, d).

a



b

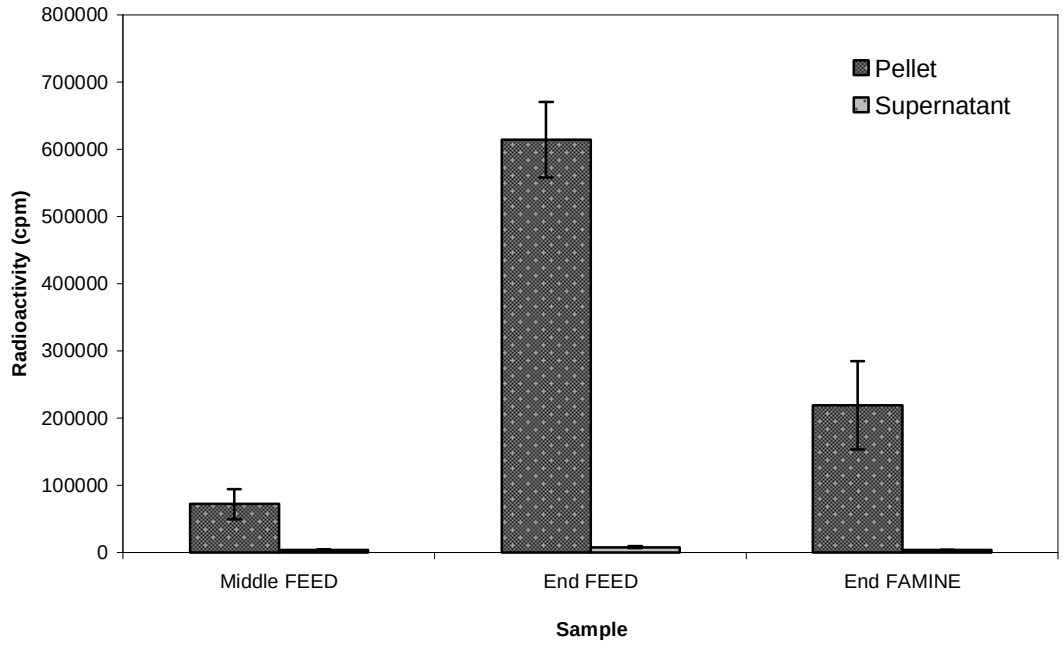


Figure 2.14 Liquid scintillation counts of biomass samples from the aerated EBPR SBR after MAR incubations. Community was fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a) Liquid scintillation counts of ^{14}C labelled acetate in biomass samples after its addition to samples removed from an aerated P removal SBR. b) Liquid scintillation counts of ^{33}P in biomass samples after its addition to samples removed from an aerated P removal SBR during the middle of the FEED phase, at the end of the FEED phase, and the end of the FAMINE phase. Error bars show standard error.

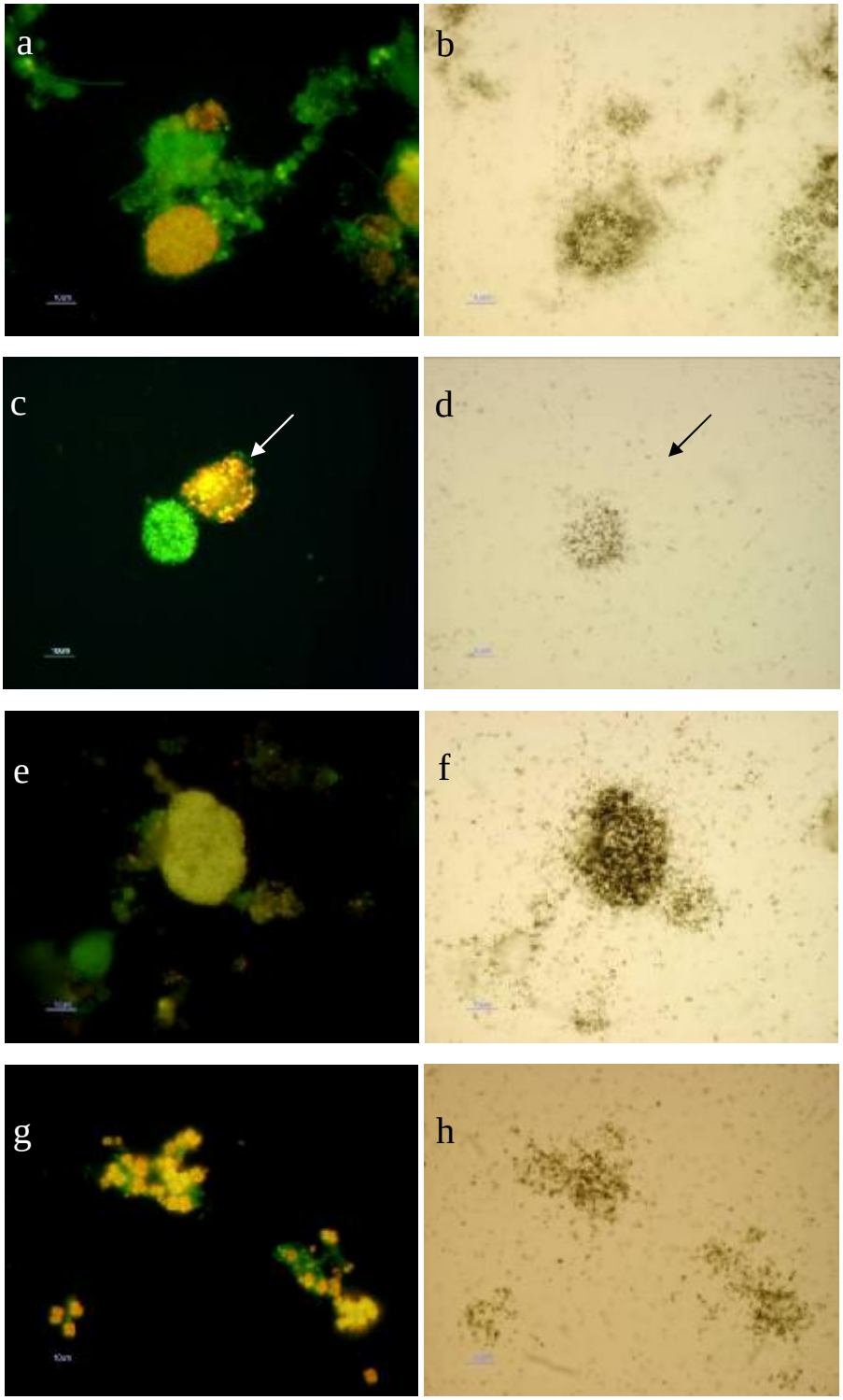


Figure 2.15 FISH micrographs and bright field images showing cells from the aerated EBPR SBR. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A) community after MAR incubations. a, c, e, g) FISH images showing cells hybridising with the EUBmix probes (FLUOS) and: a and c) *Accumulibacter* cells hybridising with the PAOmix probes (Cy3). e) *Dechloromonas* related cells hybridising with the Dech454 probe (Cy3), and g) *Defluviicoccus* related cells hybridising with the DF988 probe (Cy3). b, d, f, h) corresponding fields of view showing: b) *Accumulibacter* cells assimilating acetate, d) *Accumulibacter* cells not assimilating acetate, f) *Dechloromonas* related cells and g) *Defluviicoccus* related cells assimilating acetate. Cells hybridising with both Cy3 (red) and FLUOS (green) labelled probes appear yellow.

2.7.3.2 Assimilation of P during the FAMINE phase

Clusters of *Accumulibacter* cells identified by the PAOmix FISH probes assimilated phosphorus in the FAMINE stage (Figure 2.16a, b), although some cells displayed a heavier silver grain deposition than others during P reutilisation in the FEED phase, and at the end of the FAMINE phase. Not all the *Accumulibacter* cells were able to assimilate phosphorus (Figure 3.16c, d). However, this confirms that the *Accumulibacter* populations in this process possess a phenotype closely resembling that of the *Accumulibacter* populations in conventional anaerobic: aerobic EBPR plants. Liquid scintillation counts (Figure 2.14b) confirmed P uptake by the biomass during both FEED and FAMINE stages as expected.

No other cells including the *Dechloromonas* and *Defluviicoccus* related TFO showed any evidence of P uptake even after a 6 day MAR exposure period, and so these did not behave as PAO, using the criteria of Kong et al. (2004). The MAR data confirmed the DAPI staining results for these populations, and showed the phenotype of the *Dechloromonas* and *Defluviicoccus* related TFO is similar to that of a GAO, in that they assimilated acetate during the FEED phase but not P in the FAMINE phase. This is analogous to their behaviour in conventional anaerobic: aerobic EBPR processes (Seviour et al. 2003; Oehmen et al. 2007; Seviour and McIlroy 2008).

2.8 The effect of decreasing acetate supplementation from 160 mg L^{-1} to 120 mg L^{-1} C as acetate

Previous results showed the aerated process could successfully remove P reliably when fed 160 mg L^{-1} C as acetate with a synthetic feed. To make the process more cost effective, the acetate feed concentration was decreased to 120 mg L^{-1} C as acetate (Feed B), and any changes in community composition were determined. The SBR was otherwise operated as detailed in Section 1.1.

2.8.1 P removal performance of the aerated EBPR community fed synthetic wastewater containing 120 mg L^{-1} C as acetate

Using synthetic wastewater and an acetate supplementation of 120 mg L^{-1} C as acetate (Feed B), the process successfully removed P for 78 d. Effluent P profiles are shown in Figure 2.17.

2.8.2 Community composition of the aerated EBPR community fed synthetic wastewater containing 120 mg L^{-1} C as acetate

2.8.2.1 Community composition as determined by FISH analysis

Samples for FISH analysis were taken after 29 days of successful P removal. FISH analysis showed the major populations were similar to those at 160 mg L^{-1} C as acetate (Feed A). Therefore it was decided the major populations of *Accumulibacter*, *Defluviicoccus* related bacteria and *Dechloromonas* related bacteria would be routinely quantified, in an effort to determine if changing operating conditions affected their relative abundances. Figure 2.18 shows the abundance of these populations in comparison with the populations in the community with Feed A. The *Accumulibacter* again appeared to be the major PAO present (Figure 2.19a), their abundance increasing to 32.6 ± 3.7% of the biovolume when the acetate levels were decreased from 160 mg L^{-1} to 120 mg L^{-1}, while the abundance of *Dechloromonas* related cells had decreased to 10.0 ± 1.1%. This decrease coincides with the replacement of their large clusters seen with Feed A by smaller sized clusters (Figure 2.19b). The *Defluviicoccus* related cells targeted by the DF988 probe (Figure 2.19c) increased to 12.9 ± 2.3% of the biovolume when the acetate concentration was decreased. However, abundance

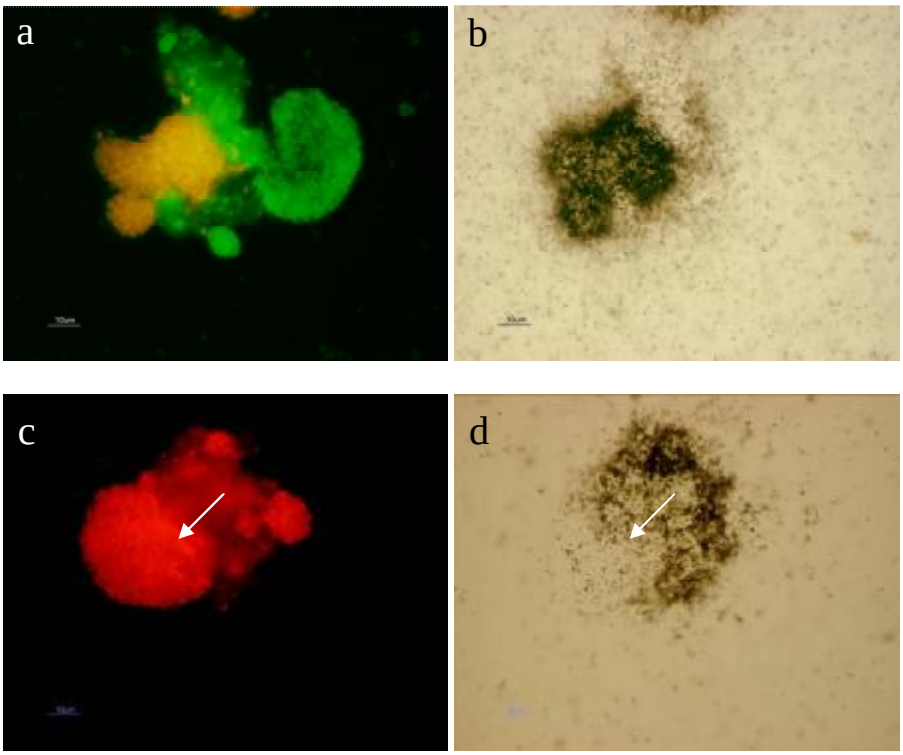


Figure 2.16 FISH micrographs and bright field images showing cells from the aerated EBPR SBR after MAR incubations. Community is fed synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A). a and c) FISH images showing *Accumulibacter* cells hybridising with the PAOmix probes (Cy3), and a) and with the EUBmix probes (FLUOS). b and d) corresponding field of view showing: b) *Accumulibacter* cells assimilating ^{33}P, and d) *Accumulibacter* cells not assimilating ^{33}P. Cells hybridising with both Cy3 (red) and FLUOS (green) labelled probes appear yellow.

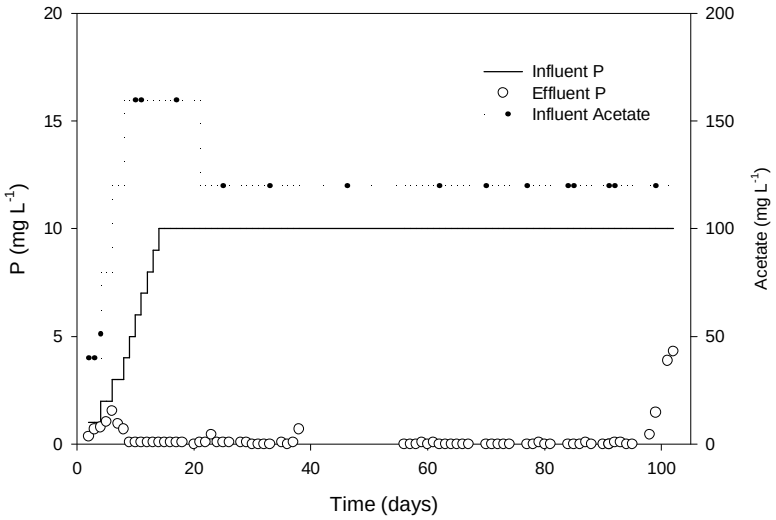


Figure 2.17 Time profile during the start-up period for the SBR. Profile shows incremental increase of influent P (—), influent acetate (•) and levels of P in the effluent (◦) when using synthetic wastewater with 120 mg L^{-1} C as acetate (Feed B). (Data provided by Dr J Ahn, La Trobe University).

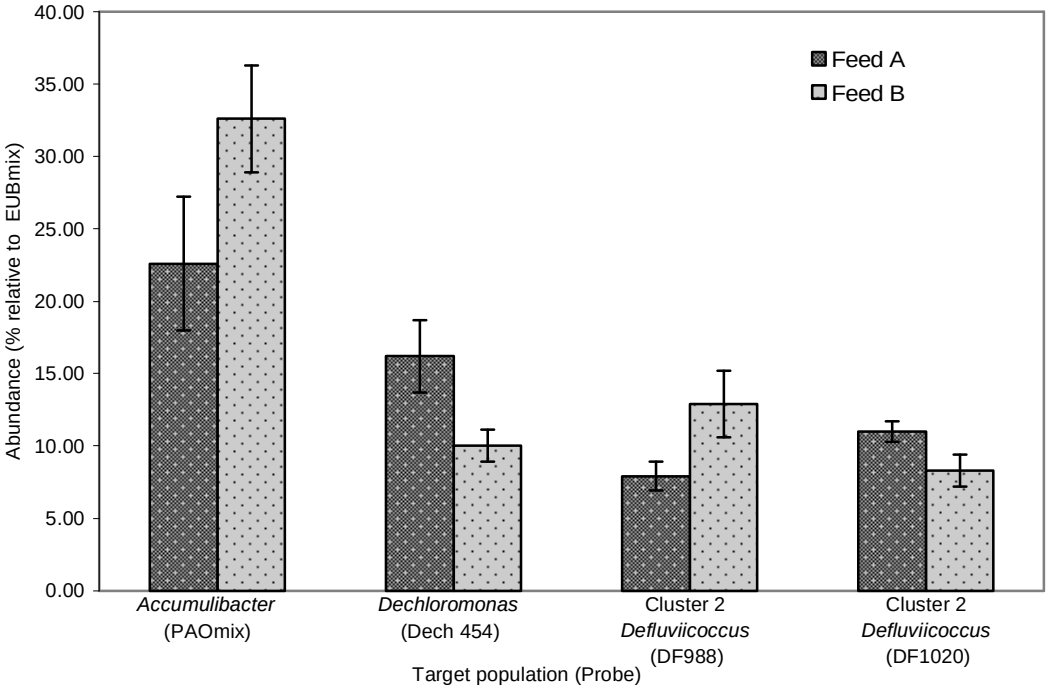


Figure 2.18 FISH analysis of populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 160 and 120 mg L^{-1} C as acetate (Feed A and B). The percentage biovolume of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells is relative to total bacteria fluorescing with EUBmix. Error bars show standard error.

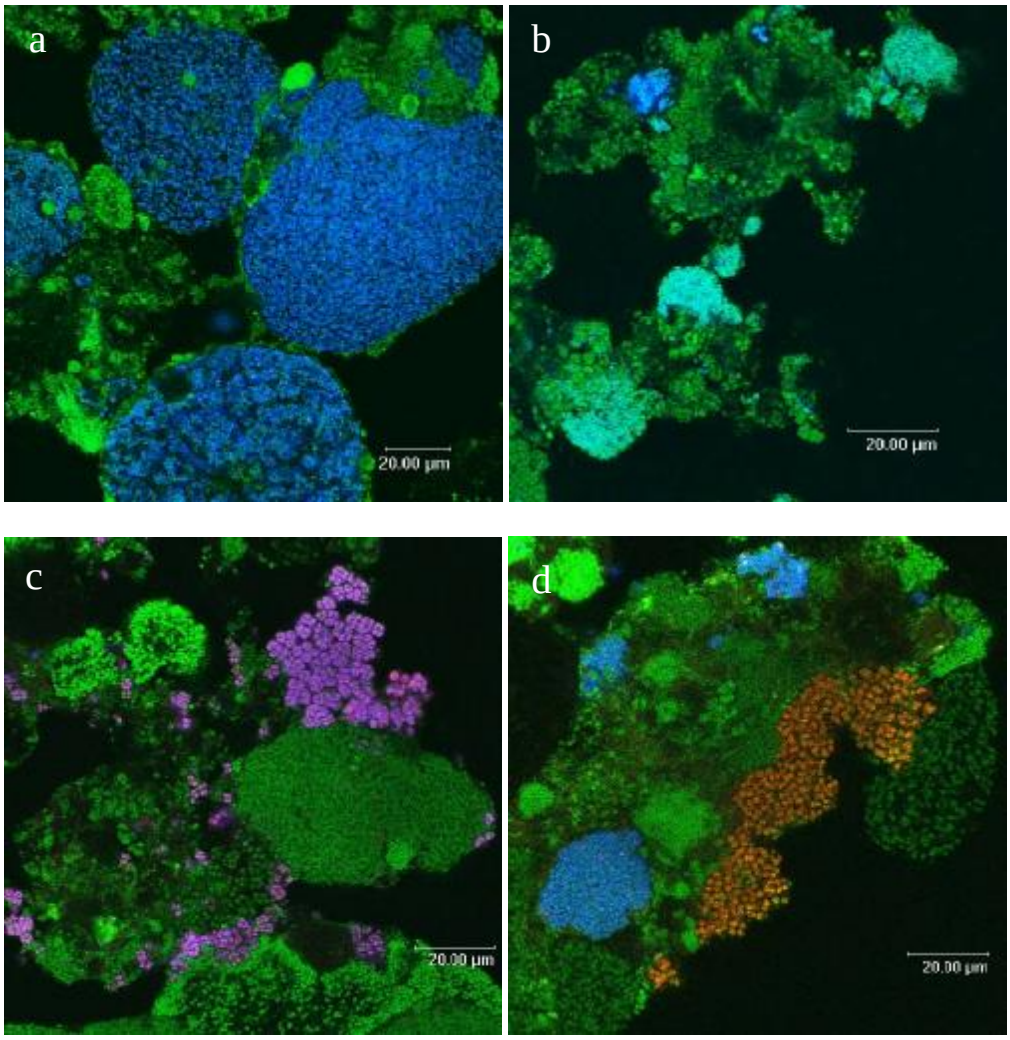


Figure 2.19 FISH micrographs showing main populations in the aerated EBPR SBR. Community is fed synthetic wastewater with 120 mg L^{-1} C as acetate (Feed B). a) *Accumulibacter* clusters hybridising with the PAOmix probes (Cy5). b) *Dechloromonas* cells targeted by the Dech454 probe (Cy5). c) Cluster 2 *Defluviicoccus* related cells targeted with the DF988 (Cy5) and DF1020 probes (Cy3). d) Cluster 1 and 2 *Defluviicoccus* related cells targeted with the DF1 mix probe (Cy3) and DF2mix probes (Cy5) respectively. All cells also hybridised with the EUBmix probes (FLUOS). Cells that hybridised with both Cy3 and FLUOS labelled probes appear yellow, while those hybridising with both Cy5 and FLUOS labelled probes appear light blue.

of cells targeted by the DF1020 probe fell slightly to 8.3 ± 1.1%. A few Cluster 1 *Defluviicoccus* related cells were again present, shown in Figure 2.19d.

Other minor populations present in the community were at similar levels to those found when the process was operated with Feed A, as assessed by visual estimation, shown in Table 2.1.

Table 2-1 FISH analysis of populations present in the aerated EBPR SBR. SBR was fed synthetic wastewater with 160 mg L^{-1} (Feed A) and 120 mg L^{-1} (Feed B) C as acetate.

Target	Probe(s)	Feed A	Feed B
Betaproteobacteria			
Betaproteobacteria	Bet42a	+++	+++
Beta1-group of *Betaproteobacteria*	Btwo23a	+++	+++
Beta2-group of *Betaproteobacteria*	Bone23a	-	-
Rhodocyclus spp.	RHC439	+++	+++
Dechloromonas related bacteria	Dech472	+	Nd
Zoogloea spp.	Zra23a	++	++
Alphaproteobacteria			
Alphaproteobacteria	Alf968	+++	+++
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	+	+
Cluster 1 *Defluviicoccus* related bacteria	DF776	-	-
Cluster 2 *Defluviicoccus* related bacteria	DF629	-	-
Amaricoccus spp.	Amar839	-	-
Sphingomonas	Sph120	+	+
Sphingomonas spp.	Sbr9-1a	+	-
Gammaproteobacteria			
Gammaproteobacteria	Gam42a_mix	+	+
Candidatus 'Competibacter phosphatis'	GAOmix	-	nd
Subgroup 1 and 2 of group GB	GBmix	+	+
Nitrifying organisms			
Nitrosomonas	Nso1225	+	-
Nitrospira	Ntspa662	+	+
Other			
Planctomycetales	Pla46	+++	+++
Planctomycetales	Pla886	+++	+++
Flavobacteria	Cf319a	+++	+++
'Chloroflexi'	CFX1223/GNSB941	++	++
Actinobacteria	HGC69a	+	nd
Tetrasphaera japonica	Actino1011	-	nd
Actinobacterial PAO	Actino221	-	-
Actinobacterial PAO	Actino658	+	+
Firmicutes	LGCmix	-	nd
Aquaspirillum	Aqs997	-	-

nd = no data
– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

2.8.3 Ecophysiology of major populations at lower acetate levels

2.8.3.1 PolyP/FISH staining

DAPI staining again showed the *Accumulibacter* clustered cells stained both positively and negatively for polyP storage with DAPI. Again small cocci were present that were DAPI positive, but as before, they did not hybridise with the Actino658 probe, and these cocci remain unidentified. Some even smaller cocci did hybridise with the Actino658 probe, and some of these were DAPI positive (data not shown).

2.8.3.2 FISH/MAR analysis of populations

MAR incubations were carried out described in Section 1.7 and 2.7.3, except that biomass was incubated with acetate to a final concentration of 5 mM, to reflect the lowered acetate concentration in the reactor.

2.8.3.2.1 Assimilation of acetate during the FEED phase

The *Accumulibacter* PAO cells again showed a positive MAR result for acetate uptake in the FEED stage (Figure 2.20a, b), as did the alphaproteobacterial TFO (identified as *Defluviicoccus* related cells) after 3 and 6 d development (data not shown).

2.8.3.2.2 Assimilation of P during the FAMINE phase

When incubated with ^{33}P, the *Accumulibacter* related PAO again showed both positive (Figure 2.20c, d) and negative results for P uptake after 3 d. The larger *Accumulibacter* clusters gave a distinctive pattern in that the edge of the clusters was positive, while internally cells in these clusters were MAR negative. This artefact was overcome by more thorough disruption of the clusters. Some *Accumulibacter* cells did not take up P, and after 6 d development a few cells that appeared to show a positive MAR signal for P uptake did not hybridise with the PAO mix probes, which could indicate normal physiological cellular uptake for growth by these cells. The *Actinobacteria* targeted by the HGC69a probe gave inconclusive results, although the cells probed appeared to be negative for P uptake (data not shown). The *Defluviicoccus* related alphaproteobacterial TFO again were again negative by MAR for P uptake (data not shown).

2.9 Discussion

The work described in this section was directed at analysing the microbial community of a novel aerated EBPR process, whose operating features were described earlier (Section 2.3). Special importance was placed on identifying the populations involved in P removal and other dominating organisms which might affect the EBPR capacity of this system.

2.9.1 Chemical transformations during the SBR cycle

The data presented here clearly show that the aerated P removal process operating under the conditions detailed removed P reliably from a synthetic wastewater to undetectable levels over an extended period of time. The main feature distinguishing it from anaerobic: aerobic EBPR processes is that the supply of P during the FAMINE stage is temporally separated from the supply of C (acetate) in the FEED stage, whereas addition of these occurs simultaneously in anaerobic: aerobic EBPR processes (Seviour et al. 2003; Oehmen et al. 2007; Seviour and McIlroy 2008). Therefore, when acetate is added, no P and probably no N

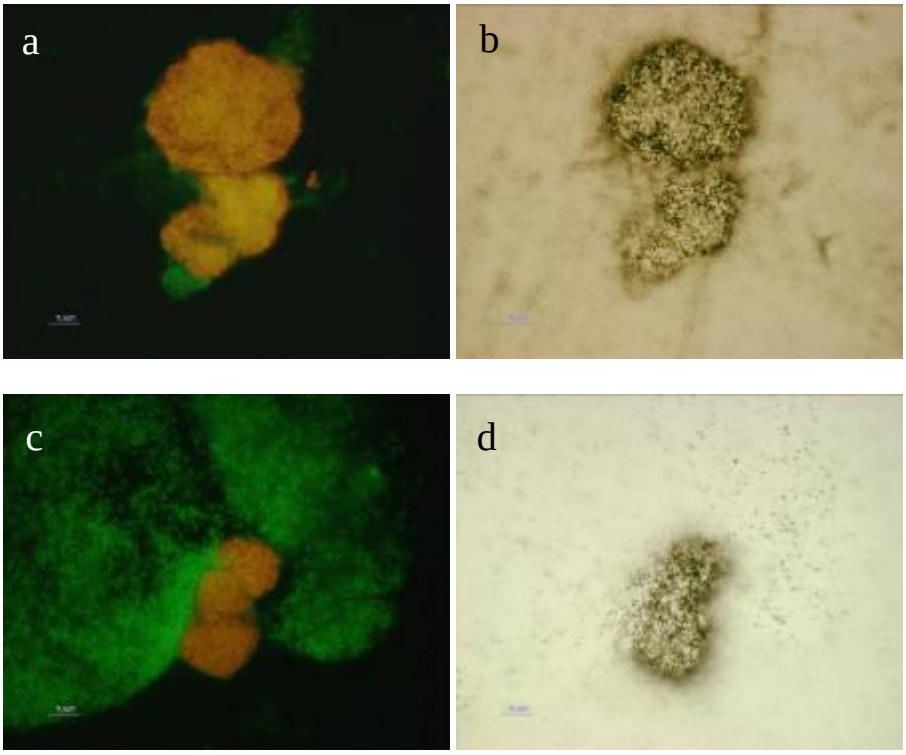


Figure 2.20 FISH micrographs and bright field images showing cells from the aerated EBPR SBR after MAR incubations. Community is fed synthetic wastewater with 120 mg L^{-1} C as acetate (Feed B). a, b) FISH images showing cells hybridising with the EUBmix probes (FLUOS), and a) the PAOmix probes (Cy3), and c) the RHC439 probe (Cy3) targeting *Accumulibacter* related cells. b and d) corresponding field of view showing *Accumulibacter* related cells assimilating b) ^{14}C acetate, and d) ^{33}P. Cells hybridising with both Cy3 and FLUOS labelled probes appear yellow.

is available for cells to grow, and subsequent addition of P occurs when no readily metabolisable COD is available. The conditions imposed on the community in this aerated process provide the PAO populations with a selective advantage in that during the FEED stage when cell growth is not possible, the PAO store the acetate as PHB, which can then be used aerobically in the FAMINE stage for assimilation of P and synthesis of polyP. Similar selective pressures are seen in anaerobic: aerobic EBPR processes, but under different operating conditions.

Therefore this process might be thought to question one of the central dogmas of EBPR, the essential requirement for anaerobic: aerobic cycling of the biomass to allow the PAO to dominate. Yet why such conditions are needed for EBPR is not clear. It has been suggested that under anaerobic conditions, cells are unable to respire acetate and grow, and so PHB synthesis occurs (Seviour et al. 2003; Oehmen et al. 2007; Seviour and McIlroy 2008). The same growth conditions leading to PHB production in this aerated process are established by ensuring P limitation in the medium in the FEED phase. Alternatively, it has been suggested that anaerobic conditions prevent non-PAO aerobic and anaerobic (denitrifying) respiring populations using readily metabolisable COD, acetate, and thus outcompeting the PAO (Oehmen et al. 2007). Yet no convincing experimental evidence to support either hypothesis is available (Seviour and McIlroy 2008).

2.9.2 Does this novel process remain aerobic?

The critical question then is whether this process is strictly an aerobic one. Most importantly, does the community become anaerobic during the addition of acetate in the FEED stage? This would mean that the release of P, a critical step, was really an anaerobic process, as it is in conventional anaerobic: aerobic EBPR processes. Redox and DO measurements from probes immersed in the bulk liquid suggest that the environment in the reactor during acetate addition is always an oxidizing one (Figure 2.4). However, these probes cannot show whether the microenvironments within the flocs are anaerobic, which then might explain the release of P. Microelectrodes are needed to answer this question. However, there is no reason why aerobic P release can not occur from the PAO, and in fact published data (Brdjanovic et al. 1998; Ahn et al. 2002; Guisasola et al. 2004; Pijuan et al. 2005) have shown it does happen in EBPR biomass samples, and the chemical changes described under such conditions are similar in many ways to those seen here (Figure 2.3).

Guisasola et al. (2004) and Pijuan et al. (2005) both showed that an *Accumulibacter* dominated biomass taken from an anaerobic: aerobic EBPR reactor released P aerobically upon continuous exposure to aerobic conditions, and in fact high EBPR capacity was retained for several days under these aerobic conditions (Pijuan et al. 2006). Eventual failure was not because of changes in the microbial composition of the community where the PAO were being out-competed, as earlier hypotheses had suggested, since relative *Accumulibacter* and *Competibacter* numbers, as determined by FISH, did not change upon EBPR failure. Instead, the chemical data (Pijuan et al. 2006) suggested that the process failed because the *Accumulibacter* gradually adjusted their metabolism to aerobic conditions, with a gradual decrease in glycogen turnover during the aerobic FEED and FAMINE stages. This, they suggested, was because the PAO were able to obtain energy and reducing power for growth by aerobically respiring acetate via the TCA cycle instead. Hence there was no requirement to replenish their glycogen reserves. Although the chemical profiles in their studies showed PHA production did not change with a switch to aerobic conditions, the biomass gradually re-assimilated less and less of the P released during the aerobic feed stage, which resulted eventually in EBPR failure. Many of these chemical transformations are similar to the ones described here, except this process did not fail. One critical difference between the two is the feed strategy used. In the experiments of Pijuan et al. (2005; 2006), the C and P were added simultaneously. However, in our aerated process they were temporally separated, ensuring P and C limitation respectively and hence unbalanced growth conditions and storage in the FEED phase. Aerobic PHA respiration then allowed cells to reassimilate the P released during acetate uptake.

2.9.3 Community population analysis

2.9.3.1 16S rRNA clone library analysis of the aerated EBPR SBR community

A clone library from a reactor biomass sample taken after 28 days of complete P removal constructed by Dr M Beer (La Trobe University) using the UltraClean™ Soil kit (Mo Bio Laboratories, Inc) method for DNA extraction gave data inconsistent with the FISH results. Despite more than 100 clones sequenced, neither *Accumulibacter* nor *Defluviicoccus* related sequences were revealed. Similar differences in PAO and GAO community composition between clone library data and FISH analysis have been reported in other EBPR studies (Hesselmann et al. 1999; Meyer et al. 2006). Their absence from clone libraries was thought to reflect the inability of the UltraClean™ Soil DNA kit (Mo Bio Laboratories, Inc) to extract their DNA, because in each case the cells were typically enclosed within heavily encapsulated clusters (Figure 2.5e, f), and while this kit is suited for soil and faecal samples, it is not specific for activated sludge samples (Mo Bio Laboratories 2008). When the phenol chloroform method of McVeigh et al. (1996) and NaTCA (McIlroy et al. 2008b) extraction methods were used in combination a clone library containing 16S rRNA gene sequences from these populations was generated (Figure 2.10). Even then, only two of the 107 clones sequenced were *Accumulibacter* related organisms, and both grouped into a single OTU (>99% shared sequence similarity), while FISH analyses showed they contributed about 23% to total cell biovolume. The scarcity of their sequences must be attributed again to the heavily encapsulated clusters (Figure 2.5e, f), which still resisted these methods for cell lysis. Microscopic examination of biomass after DNA extraction showed that most of them remained intact (S. McIlroy, personal communication), supporting this.

It may be that under the operating conditions used in this aerated process, these clusters are more robust than those formed in anaerobic: aerobic EBPR communities. García-Martín et al. (2006) showed two clusters in *Accumulibacter* encoding genes involved in the synthesis of EPS, essential for their survival in treatment plants, and binding them together in dense flocs which would remain in the system (Wilén et al. 2003). It may be possible that these gene clusters are up regulated under aerobic conditions.

Only three clones of the Cluster 2 *Defluviicoccus* related organisms were obtained, and each comprised a single OTU. Again, bearing in mind the FISH data for these populations (about 10% of the biovolume), they appear to be under-represented, which may be explained in the same way as for *Accumulibacter*. Thus, the extensive EPS capsular material associated with these (Figure 2.5e, f) restricts cell lysis. Alternatively, a bias in the PCR step may allow a preferential amplification of 16S rRNA genes from the other less well represented populations.

Regardless of the DNA extraction methods used, both libraries contained many clones related to the betaproteobacterial *Dechloromonas* and *Zoogloea*, and most sequences were very similar to those of other *Dechloromonas* clones obtained from conventional EBPR systems (Coates et al. 1999; Zilles et al. 2002). This perhaps reflects the relative ease with which these cells are lysed during DNA extraction. Interestingly, the clone libraries suggest that all the DNA extraction methods used in the present study were capable of lysing some Gram positive cells, even though their DNA is traditionally considered more difficult to extract than that of Gram negative cells (More et al. 1994; Head et al. 1998).

The homogenous coverage of species in the community was calculated as 76%, suggesting a large fraction of the reactor biodiversity was represented in the latter clone library, and supporting the suggestion that the DNA extraction method chosen was an appropriate one for lysing representative populations from the biomass. This value is higher than the 19% found by Kong et al. (2007) and slightly higher than the 64% found by Juretschko et al. (2002). Both of these studies however, were carried out on full scale plants, where higher community diversity might be expected (Kong et al. 2007). Kong et al. (2007) calculated they would need to sequence 400 – 500 clones to cover the biodiversity in the full scale WWTP they studied. Based on the 76% coverage obtained in this study, approximately 140 clones (107 were used) would be required to achieve the same. The species richness for this aerated community was calculated as 53, lower

than both Kong et al. (2007) and Juretschko et al. (2002), who found values of 411 and 83 respectively, supporting the claim made above that this community is a more specialised one. However, this number should be considered as a minimum richness value since the PCR primers used may not cover all bacteria (Juretschko et al. 2002; Kong et al. 2007), and some populations were probably not lysed by the DNA extraction process.

2.9.3.2 Population composition and ecophysiology by FISH and MAR/FISH analysis

FISH analysis in combination with DAPI staining showed the major PAO in this aerated community are *Candidatus* 'Accumulibacter phosphatis', contributing to nearly all of the polyP containing cells in the biomass. These have been identified as major PAO in many anaerobic: aerobic EBPR communities (Wagner et al. 1993; Bond et al. 1999a; Hesselmann et al. 1999; Cottrell and Kirchman 2000; Crocetti et al. 2000; Liu et al. 2001; Zilles et al. 2002; Kong et al. 2004; He et al. 2008), regardless of their operational configuration or location. *Accumulibacter* cells in this aerated process appear to share many of the ecophysiological features of their counterparts in anaerobic: aerobic systems. Thus, they both assimilated acetate during the FEED phase and stored this as PHB, and then in the FAMINE phase both took up P in excess amounts using their PHB stores as energy sources. This PAO phenotype was confirmed by FISH/MAR studies and cytochemical staining. Importantly, not all their PHB was degraded by the end of the FEED phase, allowing them to use this as the energy source to assimilate P in excess amounts during the FAMINE phase, a feature confirmed by Nile blue A staining. A few *Actinobacteria* were also identified by FISH, and some of these stained positively with DAPI. However, their small numbers suggest a minor role, if any, for them in this aerated process.

FISH probes targeting other organisms commonly reported in wastewater treatment communities identified many similar bacterial populations in this aerated EBPR process. Not all of these stained positively for polyP or for PHB, and so how they cope with the selective feed: famine conditions imposed on them is not clear. None were found in large numbers. Some populations, including *Planctomycetes,* and *Chloroflexi*, have also been commonly reported in anaerobic: aerobic EBPR plants. Only small numbers of ammonia oxidising *Nitrosomonas* and nitrite oxidising *Nitrospira* were present, suggesting little nitrification was occurring.

It became clear eventually from the FISH data that the *Alphaproteobacterial* TFO seen in such large numbers in this community and not confidently identified in the early FISH analyses, were mainly members of Cluster 2 *Defluviicoccus.* These populations have also been detected in anaerobic: aerobic EBPR systems with poor EBPR capacity (Meyer et al. 2006; Burow et al. 2007). Other betaproteobacterial cells were identified as *Dechloromonas* related cells, using FISH probes designed from sequence data retrieved in the UltraClean™ clone library (Section 2.5.2, Figure 2.6), where their clones dominated. Members of this genus have been reported often in anaerobic: aerobic EBPR systems (Zilles et al. 2002b; Kong et al. 2007), although their functional role there has not always been clear. Neither the *Defluviicoccus* nor *Dechloromonas* related populations stained positively for polyP with DAPI or assimilated P using FISH/MAR, confirming no phosphorus uptake and polyP storage. Yet Kong et al. (2007) did suggest that *Dechloromonas* may behave as a PAO in a full scale EBPR plant.

2.9.3.3 Defining the term 'GAO' in this aerated process

Wong et al. (2004) and Meyer et al. (2006) showed the *Defluviicoccus* related cells they found in anaerobic: aerobic EBPR communities displayed a GAO phenotype, in being able to assimilate acetate and synthesis PHA in the anaerobic feed stage, but not store P as polyP in the aerobic famine stage. The present study shows *Defluviicoccus* and *Dechloromonas* both assimilate acetate in the FEED stage and synthesis PHA, but do not take up P into polyP in the FAMINE stage. However, in this process both FEED and FAMINE stages are carried out under aerated conditions and the chemical data show no evidence that biomass glycogen levels change during the SBR cycle as they do so distinctively in conventional anaerobic: aerobic EBPR processes. Furthermore, in this aerated community, although the *Defluviicoccus* related organisms were major populations, the only PHA detected in the biomass was PHB. This is in contrast to

anaerobic: aerobic EBPR systems where GAO presence leads to the synthesis of PHV (Oehmen et al. 2007). Burow et al. (2008b) found 24% of the total PHA was PHV when Cluster 2 *Defluviicoccus* were present in an anaerobic: aerobic EBPR SBR fed acetate. Thus, the question is raised whether the present definition of a GAO phenotype can be more generally applicable and is a suitable one for the *Defluviicoccus* populations under these aerated conditions, in the absence of any evidence for glycogen accumulation.

Even more questions are raised about this 'GAO' definition when the work of Zhou et al. (2008) and Erdal et al. (2008) is considered. They have both demonstrated that the *Accumulibacter* PAO can switch their phenotype to that of a GAO under conditions where insufficient P is available to support polyP synthesis in the aerobic FAMINE stage, to act as the energy source for substrate assimilation and PHA production in the anaerobic feed stage. Under these conditions they use glycogen instead, which is thus formed from PHA at higher levels than are usually found in the FAMINE stage.

It may be that in light of these findings, the way the GAO term is used for PAO competition currently needs to be changed. This is especially relevant in this aerated P removal process, where the metabolisms of many populations usually found in conventional anaerobic: aerobic EBPR systems may undergo marked adjustments. It may instead be more appropriate to refer to all populations able to assimilate acetate during the FEED phase into PHB, without P assimilation in the FAMINE phase, as PAO competitors.

2.9.4 Effect of decreasing the acetate feed concentration

Decreasing the acetate concentration to 120 mg L^{-1} C as acetate (Feed B) showed this aerated process still removed P reliably. FISH data suggests that *Accumulibacter* in fact preferred the lower acetate concentration, as their relative numbers increased. However, those of the *Dechloromonas* related populations decreased, suggesting they were unable to compete as well for available acetate at the lower level. Relative numbers of *Defluviicoccus* related cells hybridising with the DF988 probe increased, while those targeted by the DF1020 probe decreased. As these probes are designed to bind to the same populations (Meyer et al. 2006), this difference may reflect that cells hybridising only to the DF988 probe were competing more efficiently for acetate. FISH/MAR results showed the ecophysiological features of these community populations did not change at the lower acetate level.

2.10 Conclusion

The SBR EBPR process characterised and described in this section behaves essentially as an aerated EBPR process. Temporally separate aerated FEED and FAMINE phases are in many aspects analogous to the anaerobic: aerobic phases in conventional EBPR systems in terms of how the populations cope with them physiologically and survive there. The chemical transformations involving the cycling of P and C, involving storage of both, are also very similar to those found in anaerobic: aerobic processes, except that C storage in the FEED stage occurs under P limiting conditions and not oxygen limiting ones as in the anaerobic: aerobic process. Glycogen recycling also seems to have a much less important role, if any, as a source of energy and reducing power than in anaerobic: aerobic processes, and there seems less need for cells to replenish their glycogen stores. PHA can be used aerobically to provide the energy for P assimilation in both the FEED and FAMINE stages of this novel process.

The microbial community is also very similar to that reported for most anaerobic: aerobic EBPR systems, in that both are dominated by *Betaproteobacteria*. Several methods were used to confirm that *Accumulibacter* were the major PAO, and *Defluviicoccus* related TFO and *Dechloromonas* related cells both displayed the currently recognised phenotype of a GAO, but in the absence of glycogen cycling. Each assimilated acetate in the FEED stage storing it as PHB, but did not assimilate P in the subsequent FAMINE stage. Other populations detected in the community are also similar to those frequently found in anaerobic: aerobic EBPR communities. Decreasing the acetate concentration did not affect the effectiveness of the system, nor the

community composition, except that the relative abundances of *Accumulibacter*, *Defluviicoccus*, and *Dechloromonas* related cells changed at lower acetate levels. DNA extraction was confirmed as a critical step in these analyses.

Step 2.

Once these conditions were established for EBPR with acetate supplementation, the process was optimized and its reliability examined.

The following questions were addressed:

a) Does EBPR capacity change in response to changes in feed rates (sludge ages), operating temperature and pH? Many studies with conventional EBPR systems (see Document 2) have suggested that EBPR capacity can be affected markedly by changes to these operational parameters.

b) Can the system recover from perturbations to its performance and how quickly?

c) Do the microbial communities and P accumulating populations change with these operational changes? If so how and why?

The following section addresses these questions.

3 The influence of operating conditions on community composition in the aerated EBPR SBR process

3.1 Introduction

Work described in the previous section resolved the composition of the community established in the aerated EBPR SBR process when operated with a synthetic wastewater feed with acetate supplementation concentrations of 160 mg L^{-1} (Feed A) and 120 mg L^{-1} C as acetate (Feed B). The data presented there showed the process removed P reliably at both these acetate concentrations, and although there were changes in the abundance of some of the major microbial populations, the community composition did not change markedly at the lower acetate concentration. Therefore, the next step was to determine the robustness of this process when fed with a clarified effluent from a non-EBPR full scale WWTP, as this was to be the source used if the process was operated at pilot plant and full scale stage, and when operational conditions were changed.

3.2 Aims

The aims of this section were to:

- Investigate how changing operating conditions affected the performance of this aerated P removal process. Source of feed, acetate supplementation concentration, sludge age, pH and temperature were all varied sequentially to determine their influence on plant performance. Community analyses by FISH/MAR, cytochemical staining, and PCR-DGGE were carried out to determine how changes in these operating parameters affected the microbial populations present, and whether these changes could be associated with changes in EBPR performance.

3.3 Reactor operation

The reactors were operated by both Dr J Ahn and B Campbell (La Trobe University), as previously detailed (Section 1.1). Any other changes are detailed in the text. Where clarified effluent was used, it was obtained from Melton WWTP, Victoria, Australia, and generally contained 10 – 12 mg P L^{-1}. Further operational details are given in Sections 2.1 – 2.4. All comparisons are made with the initial process, when the system was operated with Feed A (Section 3.4).

3.4 Effect of changing the feed source on plant performance

Firstly it was decided to see whether this process worked equally well when fed with an effluent from a conventional activated sludge plant as the source of P, and the feed supplemented with 160 mg L^{-1} C as acetate (Feed C). The acetate supplementation concentration was then decreased to 120 mg L^{-1} (Feed D) and 100 mg L^{-1} (Feed E) C as acetate in an attempt to make the process more cost effective.

3.4.1 P removal performance and chemical analysis

The reactors were operated as detailed in Section 1.1, and fed with clarified Melton plant effluent, at pH 7.5, 20 °C, and a sludge age of 20 d. Acetate supplementation was at concentrations of 160 (Feed C), 120 (Feed D), and 100 (Feed E) mg L^{-1} C. Under the conditions of Feed C, the process successfully removed P for 21 d before the conditions were changed to Feed D (120 mg L^{-1} C as acetate). Chemical profile data were taken after 20 d with Feed C. After successfully removing P for 78 d with Feed D acetate levels were again lowered to Feed E (100 mg L^{-1} C as acetate), where the process failed after a further 24 d. Effluent P profiles over this time period are shown in Figure 4.1. A typical chemical profile across the SBR cycle from Feed C is shown in Figure 3.2.

Analysis of this chemical profile shows the same relationships and trends appear to occur with Feed C as with Feed A, which were described and discussed earlier (Section 2.5), although there appeared to be a slight decrease in glycogen levels corresponding to PHA synthesis in the FEED phase. This suggests that the *Accumulibacter* PAO cells were using glycogen as a source of reducing power for PHA production, as was proposed in anaerobic: aerobic EBPR models (Seviour et al. 2003; Oehmen et al. 2007). The subsequent glycogen increases may arise from the PHA and P being utilised for biomass growth and glycogen replenishment, again as suggested in the anaerobic: aerobic EBPR metabolic models (Seviour et al. 2003; Oehmen et al. 2007). Another possibility is that flocs in the biomass become anaerobic during acetate addition, leading to anaerobic PHA production involving glycogen.

3.4.2 Community composition by FISH analysis

Biomass samples for FISH analysis were taken from the reactor operating with Feed C after 21 d of successful P removal, with Feed D after 24 and 48 d of P removal, and with Feed E after 28 d when P removal had failed. Analysis showed the community composition in each case was similar to that of the community established with Feed A (Section 2.5.3). Figure 3.3 shows the abundances of major populations, the *Accumulibacter* (Figure 3.4a), *Dechloromonas* (Figure 3.4b) and *Defluviicoccus* related cells (Figure 3.4e), relative to those fluorescing with EUBmix probes. From the biomass samples taken at day 24 to day 48, with Feed D there was a significant increase and decrease ($p < 0.05$) in the abundances of *Defluviicoccus* (Figure 3.4f) and *Dechloromonas* related cells respectively. A significant ($p < 0.05$) decrease in the abundance of *Accumulibacter* (Figure 3.4a) when acetate concentrations were lowered to 100 mg L^{-1}, and a significant increase ($p < 0.05$) in *Defluviicoccus* related cells abundance (Figure 3.4 e, f) were also recorded with Feed E. FISH analyses also showed many of the same populations were present in the communities regardless of the acetate feed concentration, and whether P was being removed successfully. These include small numbers of *Competibacter* and nitrifying bacteria (Figure 3.4c, d), as shown in Table 3.1.

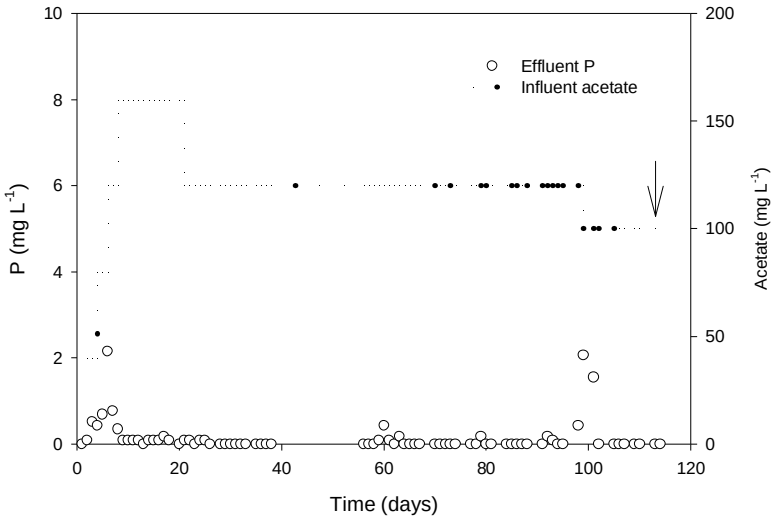


Figure 3.1 Time profile for the aerated EBPR SBR. Profile shows incremental increase of influent acetate (•) during the start-up period and effluent P levels (◦) when using clarified Melton effluent with different acetate supplementation of: 160 mg L^{-1}, 120 mg L^{-1} and 100 mg L^{-1} C as acetate (Feeds C, D, and E). The arrow shows where the P removal started to fail. (Data provided by Dr J Ahn).

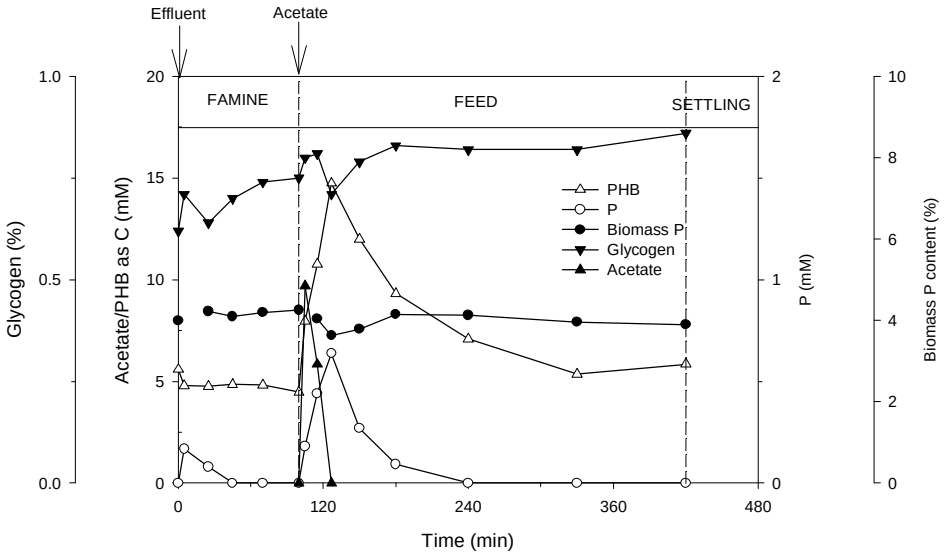


Figure 3.2 Chemical profile of the aerated EBPR SBR. Profile shows a cycle from the stable operational period of the system using clarified Melton effluent and 160 mg L^{-1} C as acetate (Feed C). Changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) are shown. The downward facing arrows indicate where acetate and the clarified effluent were added to the reactor. (Data provided by Dr J Ahn).

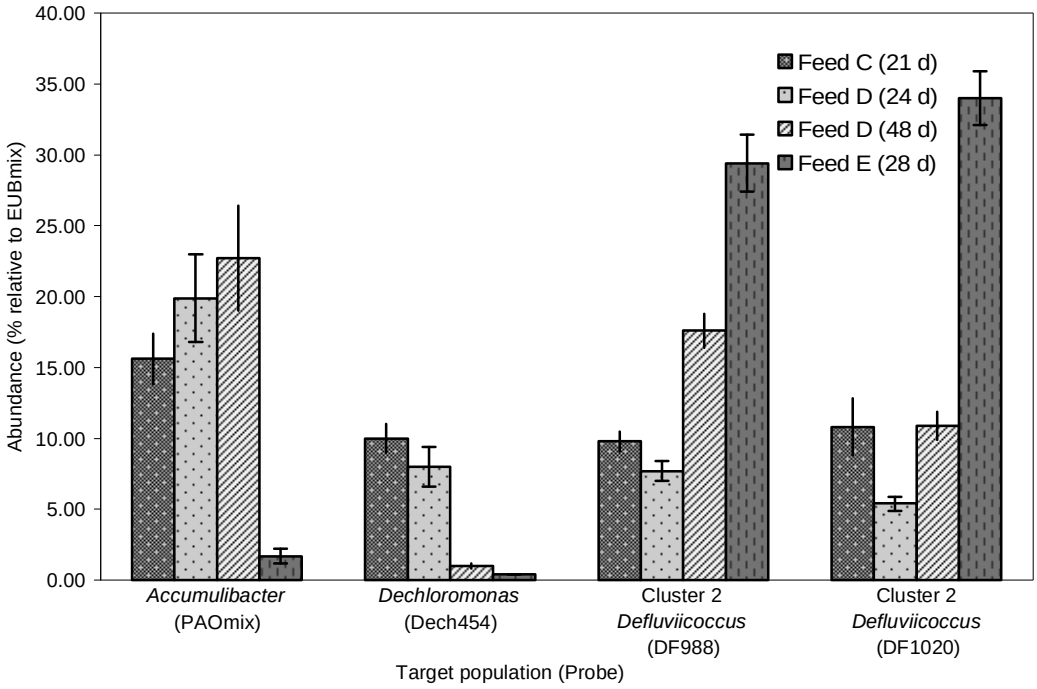


Figure 3.3 FISH analysis of populations in the aerated EBPR SBR at different acetate supplementations. Community is fed clarified Melton effluent at acetate levels of: 160 (Feed C), 120 (Feed D), and 100 (Feed E) mg L^{-1} C as acetate. Abundance is of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells relative to total bacteria fluorescing with EUBmix.

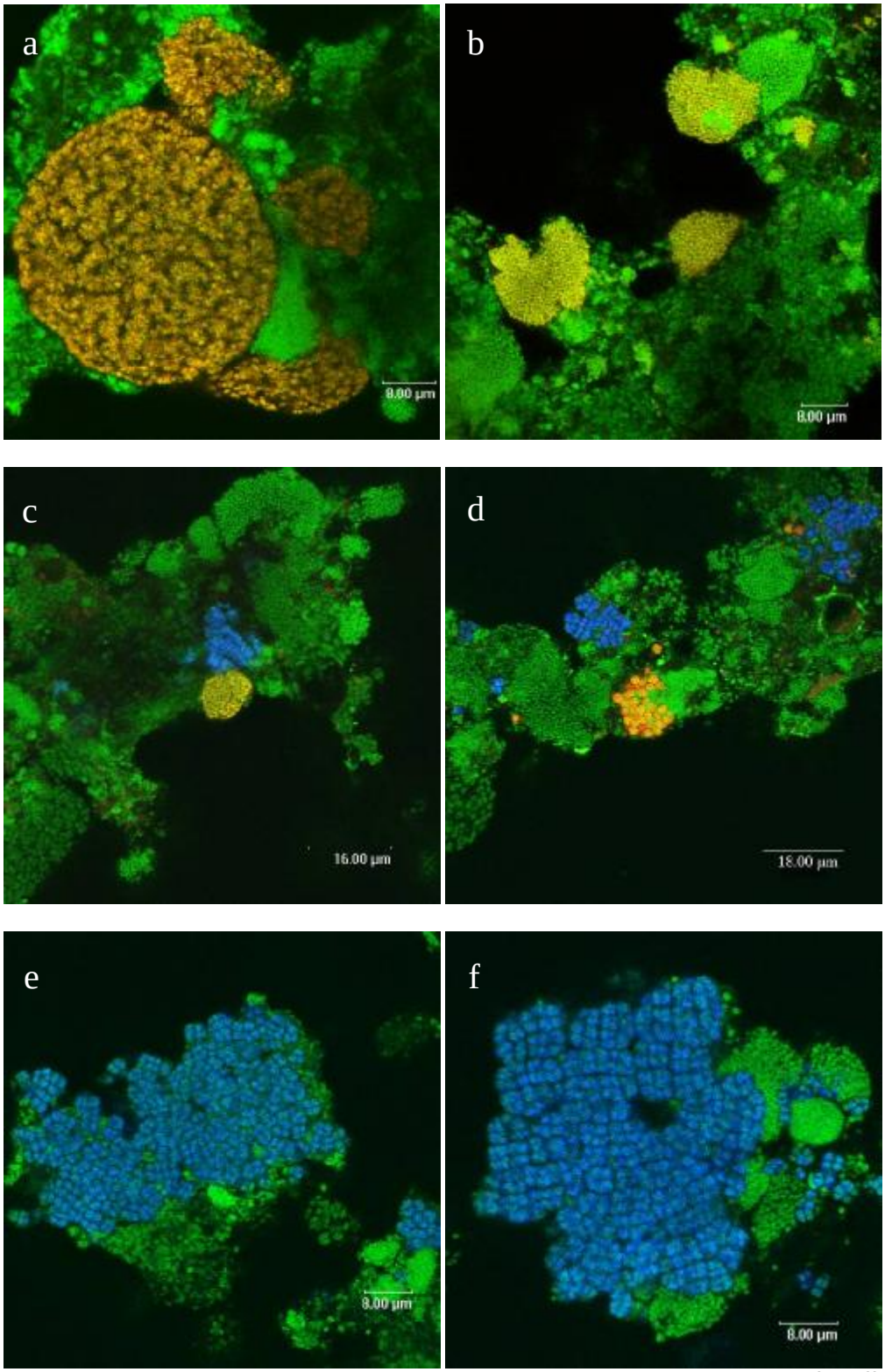


Figure 3.4 FISH micrograph showing main populations in the aerated EBPR SBR community when fed different acetate supplementation levels. Community is fed clarified Melton effluent at: a – e) 160 mg L^{-1} (Feed C), and f) 120 mg L^{-1} C as acetate (Feed D). a) *Accumulibacter* clusters hybridising with the PAOmix probes (Cy3). b) *Dechloromonas* cells targeted by the Dech454 probe (Cy3). c) *Nitrosomonas* and *Nitrospira* cells hybridising with the Nso1225 (Cy3) and Ntspa662 (Cy5) probes respectively. d) *Defluviicoccus* and *Competibacter* related cells hybridising with the DF2mix (Cy5) and GBmix (Cy3) probes respectively. e) and f) *Defluviicoccus* related cells targeted with the DF988 probe (Cy5). All cells also hybridised with the EUBmix probes (FLUOS), cells that hybridised with both probes appear yellow (EUB and Cy3), and light blue (EUB and Cy5).

Table 3-1 FISH analysis of populations present in an aerated EBPR SBR at different acetate supplementation levels. SBR was operated with Melton clarified effluent and acetate supplementation of: 160 (Feed C), 120 (Feed D), and 100 (Feed E) mg L^{-1} C as acetate.

Target	Probe(s)	Sample			
		Feed C (21d)	Feed D (24d)	Feed D (48d)	Feed E (28d)
Betaproteobacteria					
Betaproteobacteria	Bet42a	+++	+++	nd	+++
Rhodocyclus spp.	RHC439	+++	+++	nd	+
Dechloromonas related bacteria	Dech472	+	+	nd	nd
Zoogloea spp.	Zra23a	+	++	nd	-
Alphaproteobacteria					
Alphaproteobacteria	Alf968	+++	+++	nd	+++
Defluviicoccus vanus	Def438	nd	-	nd	nd
Defluviicoccus vanus	DF862	nd	-	nd	nd
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	+	+	+/++	+/++
Cluster 2 *Defluviicoccus* related bacteria	DF629	nd	-	nd	nd
Amaricoccus spp.	Amar839	nd	+	+	nd
Sphingomonas	Sph120	++	+	+/++	+
Sphingomonas spp.	Sbr9-1a	nd	-/+	-/+	nd
Gammaproteobacteria					
Gammaproteobacteria	Gam42a_mix	+	+	nd	+
Candidatus 'Competibacter phosphatis'	GAOmix	-/+	-	nd	+
Subgroup 1 and 2 of group GB	GBmix	++	+	nd	+
Nitrifying organisms					
Nitrospira	Ntspa662	+	+	nd	+
Nitrosomonas	Nso1225	+	-	nd	-
Other					
Planctomycetales	Pla46	+++	+++	nd	+++
Planctomycetales	Pla886	+++	+++	nd	+++
Flavobacteria	Cf319a	+++	+++	nd	+++
'Chloroflexi'	CFX1223/GNSB941	+++	+++	nd	+++
Actinobacteria	HGC69a	+	-	nd	nd
Actinobacterial PAO	Actino221	-	-	-	-
Actinobacterial PAO	Actino658	-	+	nd	-
Firmicutes	LGCmix	nd	-	nd	nd
Aquaspirrilum	Aqs997	nd	-	nd	nd

nd = no data

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

3.4.3 Community composition analysis by DGGE profiling

3.4.3.1 DNA extraction method

16S rRNA DGGE profiles of these communities were generated as described in Section 21.4, using a denaturation gradient of 30 – 70%, with DNA extracted by the UltraClean™ soil DNA kit (MoBio Laboratories, Inc) and the xanthogenate (Tillett and Neilan 2000) methods, detailed in Section 1.2.3.

3.4.3.2 DGGE community profile

DGGE profiles were obtained as described in Section 1.4. The DGGE profile of the 16S rRNA fragments from the communities with Feeds A, C, D, and E are shown in Figure 3.5, and the dendogram showing the profile similarities in communities developing with Feeds C, D, and E is given in Figure 3.6.

Identification of some of the 16S rRNA gene fragments was carried out as detailed in and Appendix B. The *Accumulibacter* clone OTU c79 16S rRNA fragment appeared to be present in communities from Feeds C, D, and E, but not with the earlier Feed A. This fragment was present even after system failure with Feed E, which agrees with the FISH data that *Accumulibacter* populations were still present. *Accumulibacter* clone d62 was not present in the DNA from the community with Feed A, although it was present in DNA from those with Feeds C and D. When the system had failed (Feed E), this band had disappeared. *Dechloromonas* related clone OTU c96 was seen in all the communities, agreeing with the FISH data. *Defluviicoccus* related clone OTU c15 was detected with a very strong intensity in DNA from the community with Feed E when P removal had failed and when FISH data showed *Defluviicoccus* related cells were the dominant population. It was not possible to identify the location of fragments corresponding to *Dechloromonas* related clone OTU c9 or *Defluviicoccus* related clones OTU c101 and c112 from these profiles. The dendogram shows the profiles from Feeds C and E were 95.8% similar, while the profile from Feed D was 94.7% similar to these.

3.4.4 Discussion

The results presented here show that this novel aerated process can remove P equally as well from the clarified Melton effluent in the system supplemented with Feed D (120 mg L^{-1} C as acetate) as it did with Feed C (160 mg L^{-1} C as acetate) and with a synthetic wastewater (Section 3). This suggests the process is quite robust and able to deal with a variable influent feed. However, with Feed E (100 mg L^{-1} C as acetate) the process eventually failed, which the FISH results suggest may be from a much higher relative abundance of Cluster 2 *Defluviicoccus* related organisms.

When acetate concentrations were decreased in Feed C (160 mg L^{-1} C as acetate) to Feed D (120 mg L^{-1} C as acetate) the *Accumulibacter*: DF988 positive *Defluviicoccus* ratio increased from 1.59 to 2.58. *Dechloromonas* related population abundance decreased at the lower acetate concentrations, suggesting these populations did not compete as well with *Accumulibacter* for acetate at the lower levels. On the other hand, the *Defluviicoccus* related populations, initially a minor population when the feed was changed to Feed D, had increased after 48 d. By then the *Accumulibacter:* DF988 positive *Defluviicoccus* ratio had decreased to 1.29, although the system was still working well to remove P. This suggests the *Defluviicoccus* related organisms were becoming more dominant as acetate levels in the feed fell. With Feed E, and failed P removal capacity, *Defluviicoccus* related populations had increased significantly, and the *Accumulibacter:* DF988 positive *Defluviicoccus* ratio was only 0.06. These trends seem to imply that *Defluviicoccus* was out-competing *Accumulibacter* at this lower acetate concentration, which disagrees with the suggestions of Burow et al. (2008b), who presented evidence to suggest that under anaerobic conditions *Accumulibacter* had a higher scavenging ability for acetate than *Defluviicoccus* when acetate concentrations were low, because of a permease-mediated acetate uptake system. From the experiments described here the critical acetate concentration at which *Accumulibacter* could out-compete *Defluviicoccus* under aerated conditions appeared to be around 120 mg L^{-1} C as acetate (Feed D), since the *Accumulibacter* abundance was significantly higher

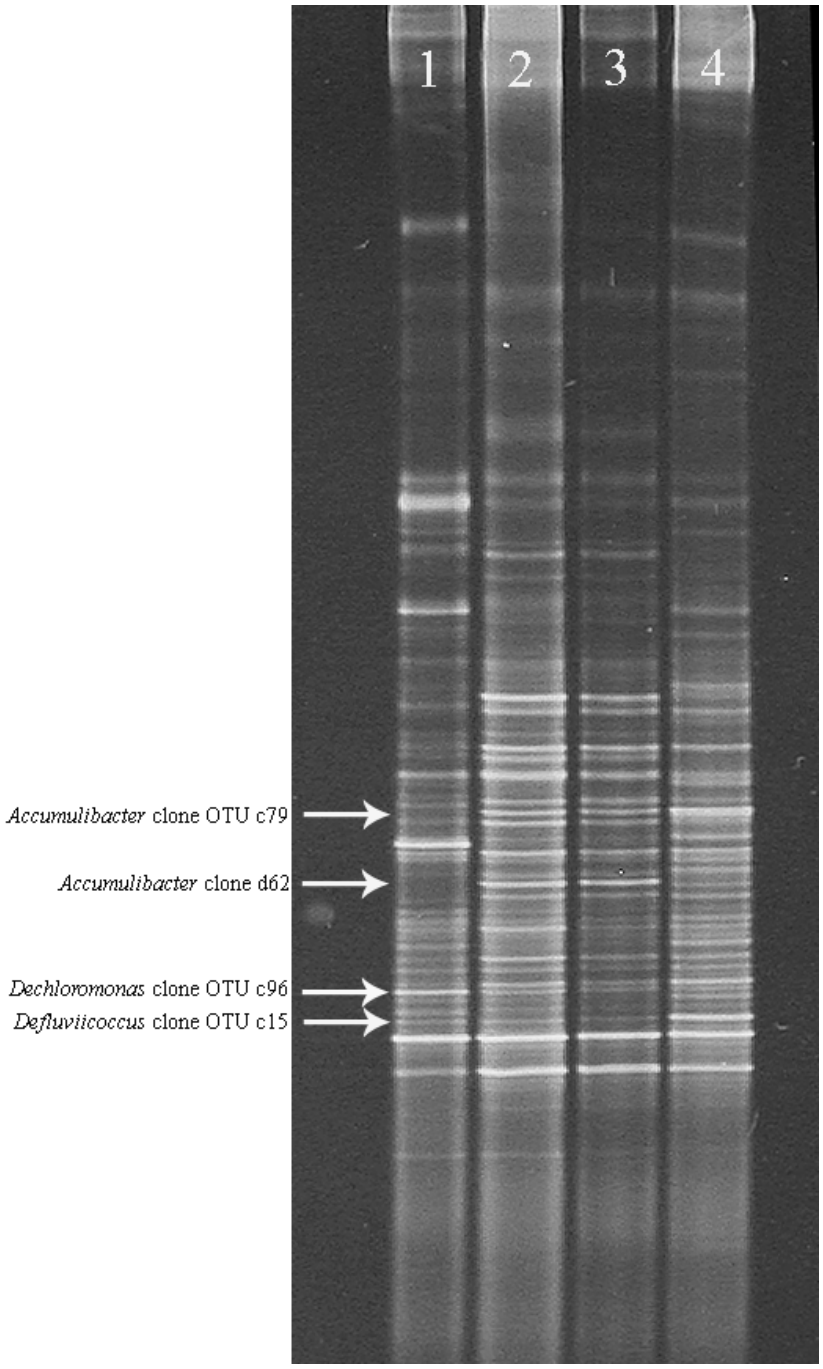


Figure 3.5 PCR-DGGE profiles of partial 16S rRNA gene fragments generated from the aerated EBPR SBR with different levels of acetate supplementation. Profiles used v3 region universal primers. The SBR community was fed: Lane 1) synthetic wastewater and 160 mg L^{-1} C as acetate (Feed A) (standard). Lanes 2 – 5) clarified effluent and: Lane 2) 160 mg L^{-1} C as acetate (Feed C), Lane 3) 120 mg $^{-1}$ C as acetate (Feed D), and Lane 4) 100 mg L^{-1} C as acetate (Feed E).

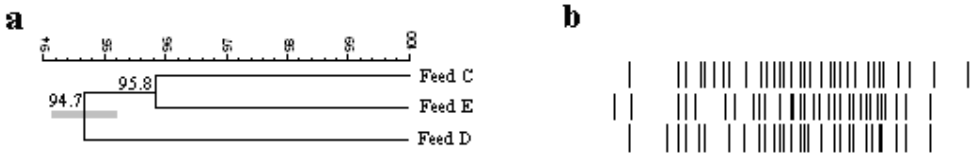


Figure 3.6 Similarity analysis of 16S rRNA DGGE profiles from the aerated EBPR SBR at different acetate supplementations. The SBR was operated with clarified effluent and acetate levels of: 160 mg L^{-1} (Feed C), 120 mg L^{-1} (Feed D), and 100 mg L^{-1} (Feed E) C as acetate. a) Dendogram constructed using the Pearson similarity correlation coefficient displaying similarity of banding patterns between the community 16S rRNA DGGE profiles. Error bars show standard deviation. b) Representation of bands on the DGGE profile from Figure 4.5.

than *Defluviicoccus.* However, as the abundance of *Defluviicoccus* related organisms increased in communities from 28 d to 48 d operation (Figure 4.3), even with supplementation of 120 mg L^{-1} C as acetate they were eventually likely to out-compete the *Accumulibacter*.

DGGE 16S rRNA banding patterns (Figure 3.5), showed the communities with Feed C and Feed E were more similar to each other than that present with Feed D, and suggests that community diversity with Feed E was as high as when the process was removing P with both Feeds C and D.

As this process worked reliably with 120 mg L^{-1} C as acetate and a Melton effluent feed supply, these conditions were selected for all future experiments. The next step was determining the effect of reducing the sludge age of the SBRs.

3.5 Effect of changing the sludge age on plant performance

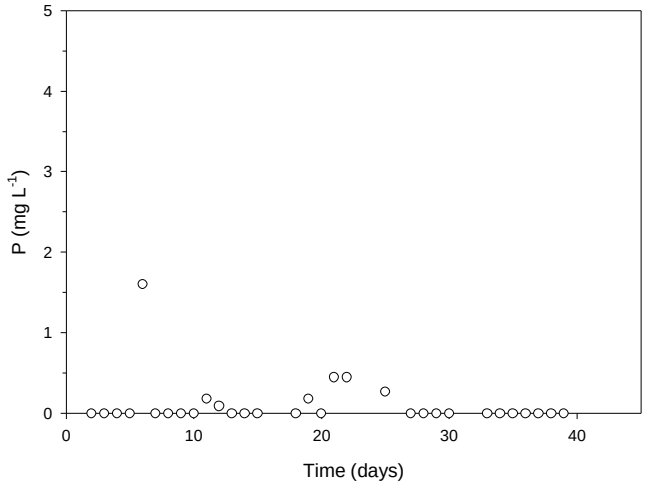
Reducing sludge age increases operating capacity and/or reduces required reactor volume (Seviour et al. 2003). Thus, the effect of reducing sludge age from 20 d to 15 d and 10 d was investigated. Some earlier evidence had suggested that putative PAO proliferate (Zilles et al. 2002; He et al. 2008) and compete better with putative GAO at a lowered sludge age in conventional anaerobic: aerobic EBPR processes (Whang and Park 2006), while Wong and Liu (2006) had suggested that a longer hydraulic retention time and sludge retention time appeared to favour Cluster 1 *Defluviicoccus* related cells in their anaerobic: aerobic SBR system.

3.5.1 P removal performance and chemical analysis

Reactors were operated as described previously (Section 1.1), with Melton clarified effluent and acetate supplementation of 120 mg L^{-1} C as acetate, pH 7.5, and 20 °C. The sludge age of the reactors was varied from 20 d (Sludge age F) to 15 d (Sludge age G), and 10 d (Sludge age H) by varying the amount of biomass wasted from the reactor at the end of the FAMINE phase, and the cycle time was decreased from 8 h to 6 h. Analysis of the clarified Melton effluent feed confirmed the P levels were still around 10 mg L^{-1}, and the COD varied between 40 – 50 mg oxygen L^{-1}.

The effluent P profiles of these reactors operated with different sludge ages are shown in Figure 3.7 and the chemical profiles given in Figure 3.8. Sludge age F (20 d) successfully removed P for 40 d before conditions were changed to Sludge age G (15 d). Effluent P profiles of the SBRs operated with Sludge ages G and H showed that 2 days after sludge ages were reduced P removal failed, but it was quickly re-established in both reactors after approximately 5 days. Sludge age G removed P for another 26 d and Sludge age H successfully removed P for only another 19 d, before conditions were changed.

a



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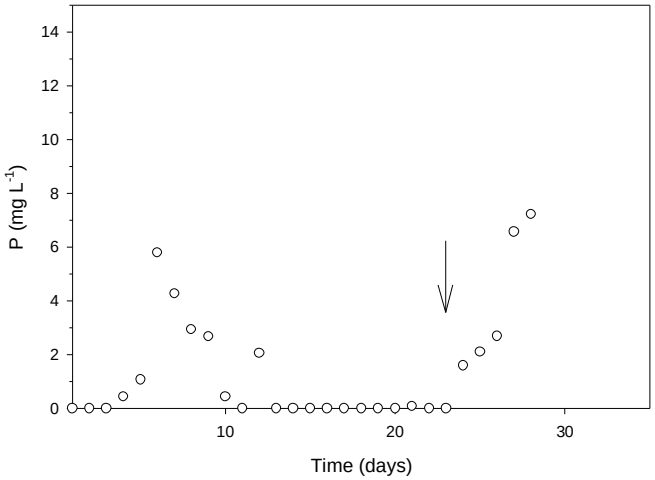

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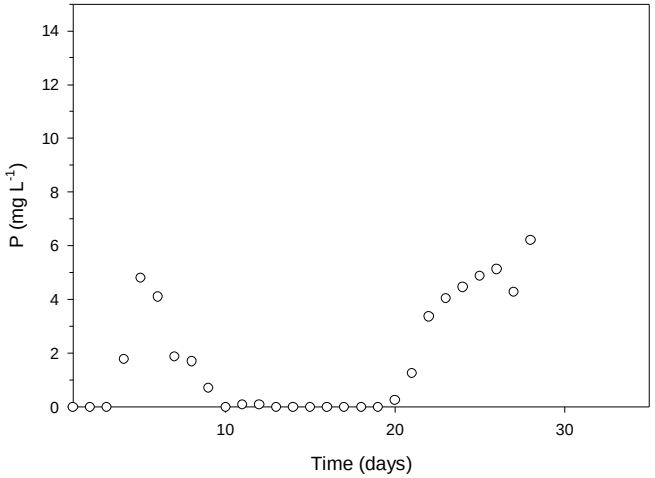
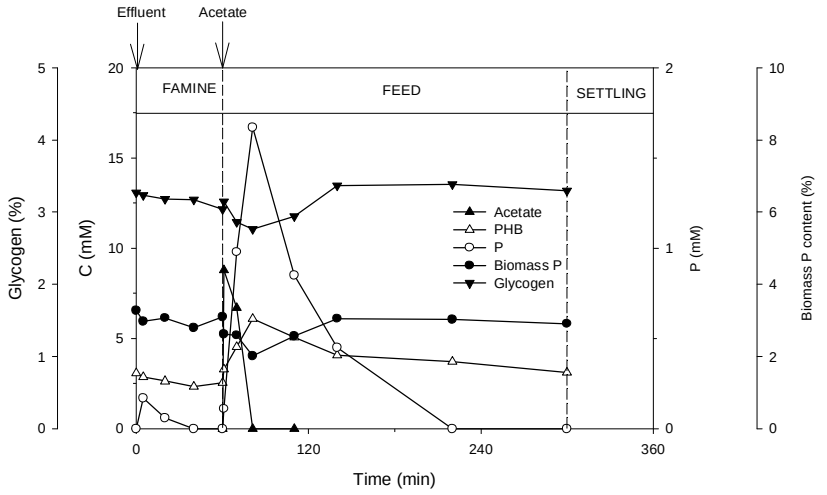


Figure 3.7 Time profile showing levels of P in the effluent of the aerated EBPR SBRs operated with different sludge ages. The SBRs were fed clarified Melton effluent and 120 mg L^{-1} C as acetate with sludge ages of: a) 20 d (Sludge age F), b) 15 d (Sludge age G) (arrow indicates where conditions were changed), and c) 10 d (Sludge age H). (Data provided by Dr J Ahn).

a



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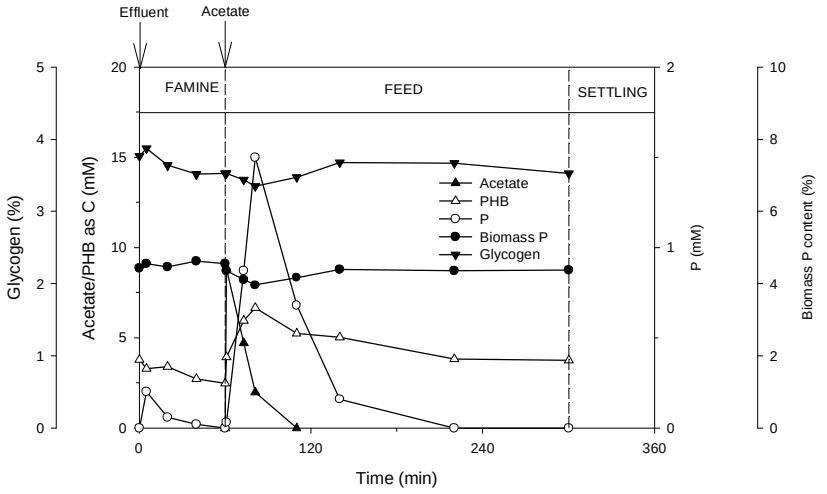

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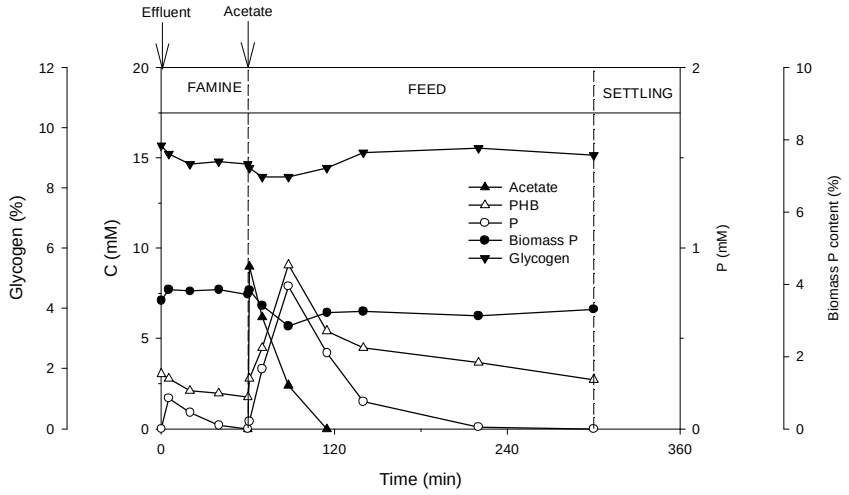


Figure 3.8 Chemical profile of the aerated EBPR SBR cycle at different sludge ages. The SBRs were fed clarified Melton effluent and 120 mg L^{-1} C as acetate at sludge ages of: a) 20 d (Sludge age F), b) 15 d (Sludge age G), and c) 10 d (Sludge age H). Profiles show changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) over a FEED: FAMINE cycle. Downward facing arrows show where acetate and clarified effluent were added to the reactor. (Data provided by Dr J Ahn).

3.5.2 Morphology of *Defluviicoccus* related TFO

Examination of biomass from each of the three reactor communities revealed that the *Defluviicoccus* related cells targeted by the DF2mix probes (Meyer et al. 2006) were present in two different morphologies. In previous experiments (Section 2.5.3, Section 3.4) most cells that responded to the Cluster 2 *Defluviicoccus* targeted probes exhibited the distinctive TFO morphology. All of these TFO cells hybridised with the DF988

probe, and most with the DF1020 probe. However, small cocci were seen in these communities (Figure 3.9), which hybridised only with the DF988 probe. Cells with the typical TFO morphology were still present, hybridising with both the DF988 and DF1020 probes (Figure 3.9), but the cocci responding to only the DF988 probe were now much more abundant. While different morphologies have been reported for Cluster 1 *Defluviicoccus* (Wong et al. 2004), this is the first known report of two morphotypes for the Cluster 2 *Defluviicoccus*.

3.5.3 Community composition by FISH analysis

Biomass samples for FISH analysis were taken from Sludge ages F, G, and H, after 40, 27 and 27 d respectively. Abundance of the major cell populations, relative to those fluorescing with EUBmix probes, is shown in Figure 3.10. The abundance of *Accumulibacter* and *Dechloromonas* related cells were significantly ($p < 0.05$) higher in communities with Sludge age F than in those from Sludge ages G and H. Other populations present in the community were similar to those found under the other previous operating conditions (Section 3.4), as shown in Table 3.2.

3.5.4 Community composition analysis by DGGE profiling

The DGGE profile showing community profiles of 16S rRNA fragments from communities with different sludge ages is shown in Figure 3.11 and the dendogram comparing these is given in Figure 3.12. These profiles were generated with DNA obtained using the combined UltraClean™ soil DNA kit (MoBio Laboratories, Inc) and the Xanthogenate DNA extraction methods (Tillett and Neilan 2000).

The profiles (Figure 3.11) show *Accumulibacter* related clone OTU c79 was again not obviously present in the DNA sample from the Feed A community, which was used as the standard marker for all comparisons. However it appeared to be present in DNA from all other communities, although not obvious in profiles from Sludge age F. *Accumulibacter* related clone d62 could not be confidently identified in any profiles, while *Dechloromonas* related clones OTU c9 and c96 were tentatively identified in all samples. *Defluviicoccus* related clone OTU c15 could not be identified in the profiles from Sludge age F, but was visible in profiles from communities from reactors operated with Sludge ages G and H, although FISH data suggested the presence of *Defluviicoccus* related cells in the communities developed with these sludge ages. Furthermore, *Defluviicoccus* related OTUs c101 and c112 could not be identified, possibly because of the DNA extraction methods used not lysing their cells.

The dendogram shows the profiles from the communities at all three sludge ages were greater than 98% similar to each other.

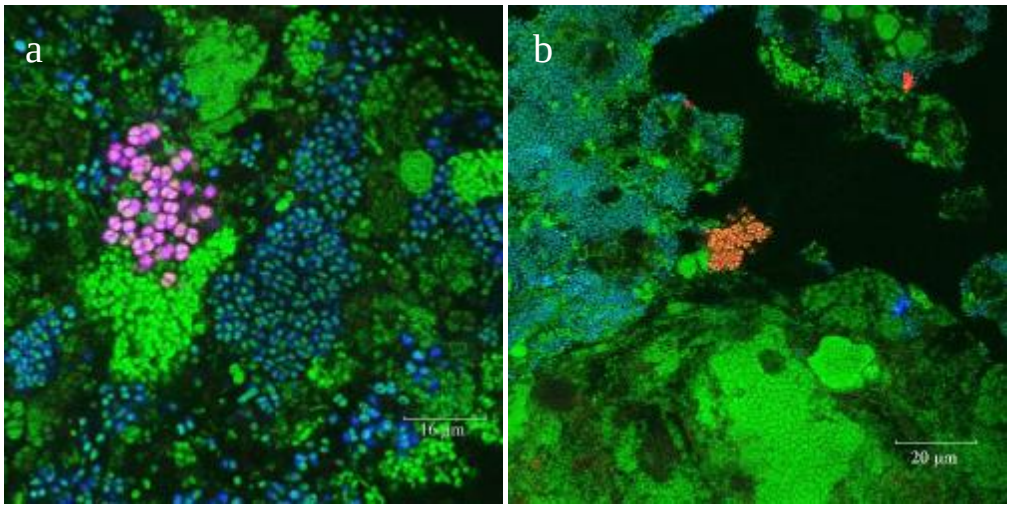


Figure 3.9 FISH micrograph showing the morphology of Cluster 2 *Defluviicoccus* related cells in the aerated EBPR SBR. Community is fed clarified Melton effluent and 120 mg L^{-1} C as acetate at different sludge ages. a and b) Cells hybridising with the DF988 (Cy5) and DF1020 (Cy3) and EUB (FLUOS) probes, in biomass samples taken from a) 10 day sludge age (Reactor H), and b) 20 day sludge age (Reactor F). Cells that hybridised with both probes appear yellow (EUB and Cy3), and light blue (EUB and Cy5), and with all three probes light purple/pink.

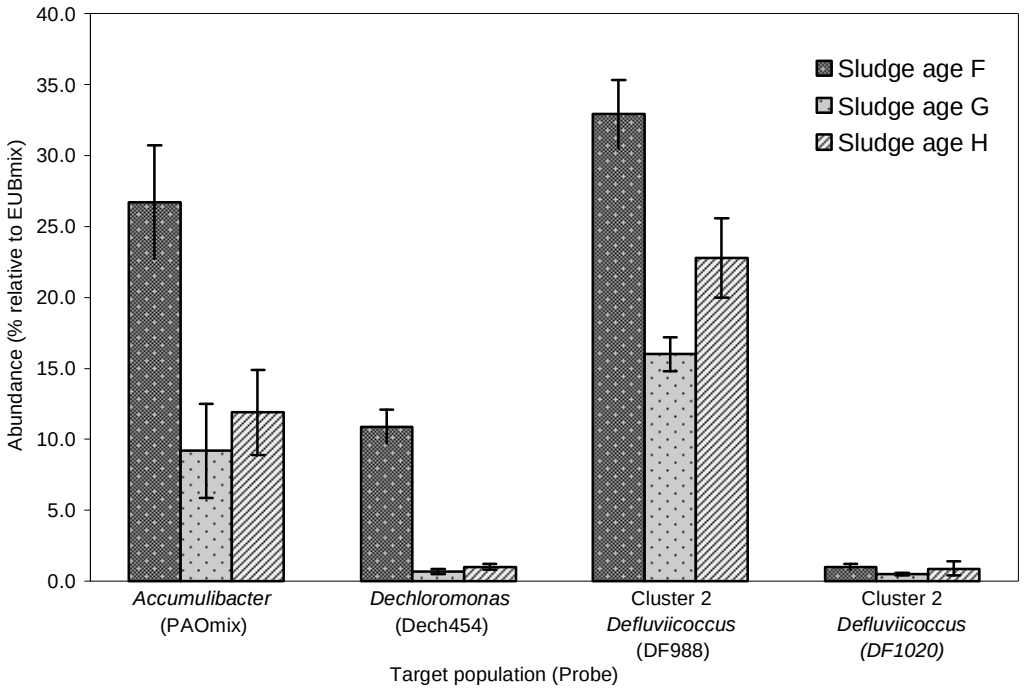


Figure 3.10 FISH analysis of populations in the aerated EBPR SBR at different sludge ages. SBRs were fed clarified Melton effluent and 120 mg L^{-1} C as acetate with sludge ages of: 20 d (Sludge age F), 15 d (Sludge age G), and 10 d (Sludge age H). Percentages are relative to total bacteria fluorescing with EUBmix probes for *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells.

Table 3-2 FISH analysis of populations present in an aerated EBPR SBR at different sludge ages. SBR was operated with clarified Melton effluent and 120 mg L^{-1} C as acetate at sludge ages of: 20 d (Sludge age F), 15 d (Sludge age G), and 10 d (Sludge age H).

Target	Probe	Sample		
		Sludge age F	Sludge age G	Sludge age H
Betaproteobacteria				
Zoogloea spp.	Zra23a	++	+	nd
Alphaproteobacteria				
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	+	+	+
Gammaproteobacteria				
Gammaproteobacteria	Gam42a_mix	++	++	++
Subgroup 1 and 2 of group GB	GBmix	+	+	+
Nitrifying organisms				
Nitrospira	Ntspa662	+	+	+
Nitrosomonas	Nso1225	+	+	+
Other				
Planctomycetales	Pla46	+++	+++	+++
Planctomycetales	Pla886	+++	+++	+++
Flavobacteria	Cf319a	+++	+++	+++
'Chloroflexi'	CFX1223/GNSB941	+++	+++	+++
Actinobacterial PAO	Actino221	-	-	-
Actinobacterial PAO	Actino658	+	-	-

nd = no data

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

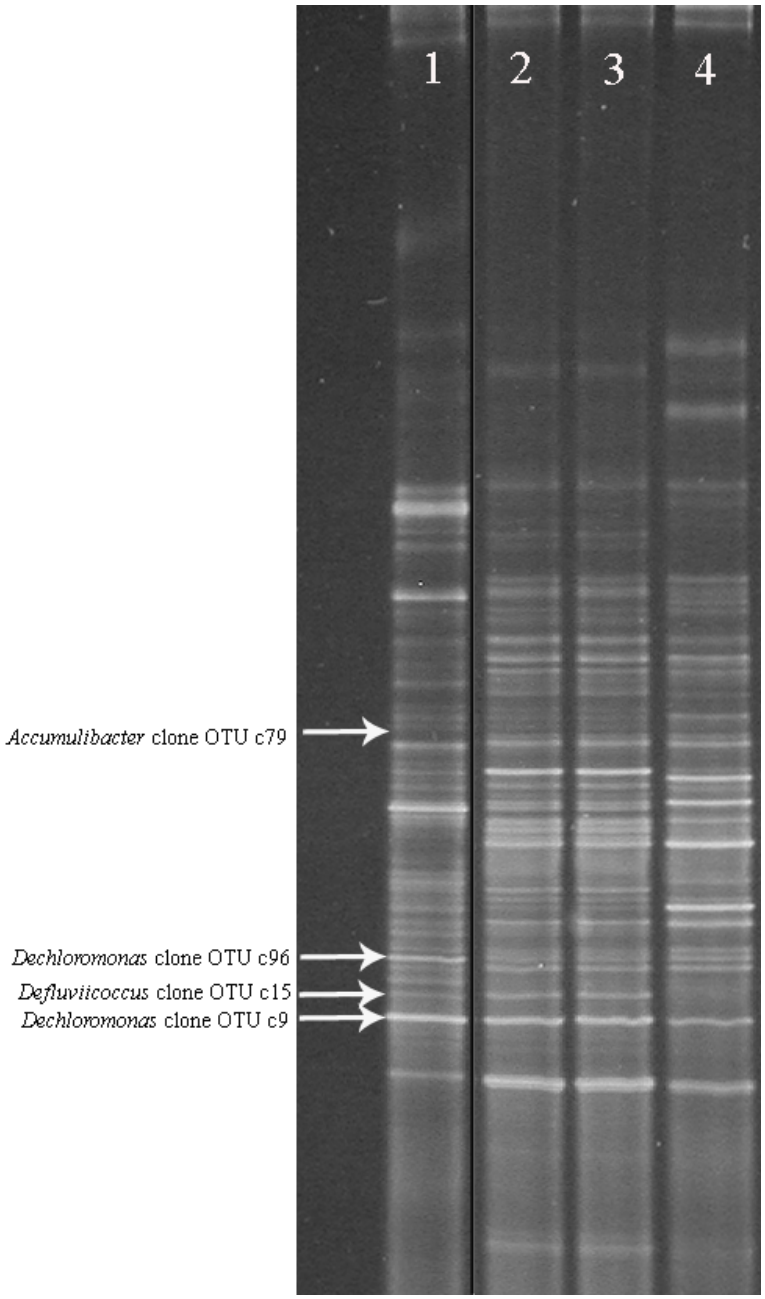


Figure 3.11 PCR-DGGE profiles of partial 16S rRNA gene fragments generated from the aerated EBPR SBR with different sludge ages. Profiles used v3 region universal primers. SBRs were fed Lane 1) synthetic feed with 160 mg L^{-1} C as acetate (Feed A) (standard), Lanes 2 – 4) clarified effluent with 120 mg L^{-1} C as acetate with sludge ages of: Lane 2) 10 d (Sludge age H), Lane 3) 15 d (Sludge age G), and Lane 4) 20 d (Sludge age F).

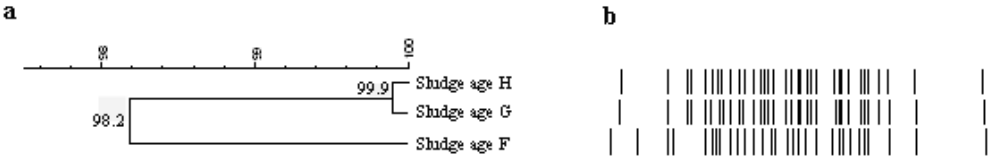


Figure 3.12 Similarity analysis of 16S rRNA DGGE profiles from the aerated EBPR SBR biomass samples operated with different sludge ages. SBRs were fed clarified effluent and 120 mg L^{-1} C as acetate with sludge ages of: 20 d (Sludge age F), 15 d (Sludge age G), and 10 d (Sludge age H). a) Dendogram constructed using the Pearson similarity correlation coefficient displaying similarity of banding patterns between the community 16S rRNA DGGE profiles. Standard deviations were negligible. b) Representation of bands on the DGGE profile from Figure 3.11.

3.5.5 Discussion

3.5.5.1 Chemical transformations

It was suggested (Oehmen et al. 2007) that a low ratio of P release: acetate uptake usually characterises a community with low *Accumulibacter* populations and high GAO populations in anaerobic: aerobic EBPR systems. At a sludge age of 10 d, Sludge age H showed a lower P release: acetate uptake ratio than Sludge ages F and G, and a higher percentage of glycogen in the biomass than in communities from the other two reactors. The abundance of DF988 positive *Defluviicoccus* related organisms in this aerated community was approximately double that of the *Accumulibacter* related cells, agreeing with the proposal of Oehmen et al. (2007), and suggesting these were responsible for the increased glycogen levels. Several other studies have also suggested that the relative activity of PAO and GAO can be estimated after determining this ratio (Saunders et al. 2003; Schuler and Jenkins 2003). Furthermore it is also known to be affected by operating pH and C source (Oehmen et al. 2007). However, the *Accumulibacter:* DF988 positive *Defluviicoccus* abundance ratio in Sludge age H was similar to that of Sludge age G, which had a higher P release: acetate uptake ratio, and showed similar P removal performance to that with Sludge age H. Furthermore, these assumptions (Oehmen et al. 2007) are based on anaerobic: aerobic systems where *Defluviicoccus* is behaving as a GAO, and whether it is appropriate to refer to *Defluviicoccus* as a GAO under these aerated conditions, in the absence of any direct evidence that it is the population that accumulates glycogen, should be considered.

3.5.5.2 Effect of changing sludge age on community composition

Based on banding patterns, DGGE analyses showed the communities with Sludge ages G and H appeared more similar to each other than the community present with Sludge age F. These data agree with the FISH data, which show that even with the changes in sludge age, *Accumulibacter* were always the dominant PAO population, and continued to exhibit the typical PAO phenotype they displayed in previous experiments (Section 2.7). *Defluviicoccus* and *Dechloromonas* related cells were also both present at all sludge ages, except that the *Defluviicoccus* related TFO cell morphology had changed from the typical characteristic TFO to small cocci, and cells with this morphology only responded to the DF988 probe. There was no significant difference in the relative abundances of *Accumulibacter* cells in Sludge ages G and H, although significantly more *Accumulibacter* were present with Sludge age F. Under these operational conditions however, a significantly higher level of *Dechloromonas* and DF988 positive *Defluviicoccus* related cells were seen in Sludge age F than in the others.

The population data here disagree with those of Zilles et al. (2002) and He et al. (2008), who both found a greater abundance of *Accumulibacter* in full scale anaerobic: aerobic EBPR plants operating with a shorter sludge age than those with a longer sludge age. They also disagree with Whang and Park (2006), who proposed that the PAO compete better with GAO at a lower sludge age, although neither population were identified in their system.

The next step was to determine the effect of changing pH on the performance of this aerated EBPR SBR process.

3.6 Effect of changing pH on plant performance

Many studies in anaerobic: aerobic EBPR systems have investigated how changing pH might affect the competition between PAO and GAO (Liu et al. 1996; Bond et al. 1999b; Filipe et al. 2001a; Filipe et al. 2001c; Filipe et al. 2001d; Filipe et al. 2001b; Jeon et al. 2001; Schuler and Jenkins 2002; Serafim et al. 2002; Oehmen et al. 2005a; Zhang et al. 2005; López-Vázquez et al. 2008), but usually with little or no microbiological data to explain the trends they saw. To determine possible effects of pH on this aerated P removal process, the SBRs were operated at several pH levels.

3.6.1 P removal performance and chemical analysis

Reactors were operated as detailed in Section 1.1, again with Melton clarified effluent with acetate supplementation of 120 mg L^{-1}, a sludge age of 15 d, at 20 °C, and the pH was controlled at either 6.5 (pH 6.5) or 7.0 (pH 7.0). The data were compared with those from Sludge age G whose performance at pH 7.5 was described previously in Section 3.5. The effluent P profiles and the chemical profiles for pH 6.5 and 7.0, and 7.5 are shown in Figures 3.13 and 3.14, respectively. Chemical profile data were obtained at day 28 for Reactor 6.5 and day 29 for Reactor 7.0. Effluent P profiles show both reactors successfully removed P for more than 32 d before being shut down. Chemical profile data show the same patterns of transformations occurred in both as seen with the baseline process with synthetic wastewater in Feed A, although at pH 6.5 and 7.0 glycogen levels appeared to decrease slightly in parallel with levels of PHB synthesis, as was seen with data from Feed C (Section 3.4), and Sludge ages F and H (Section 3.5).

3.6.2 Community composition by FISH analysis

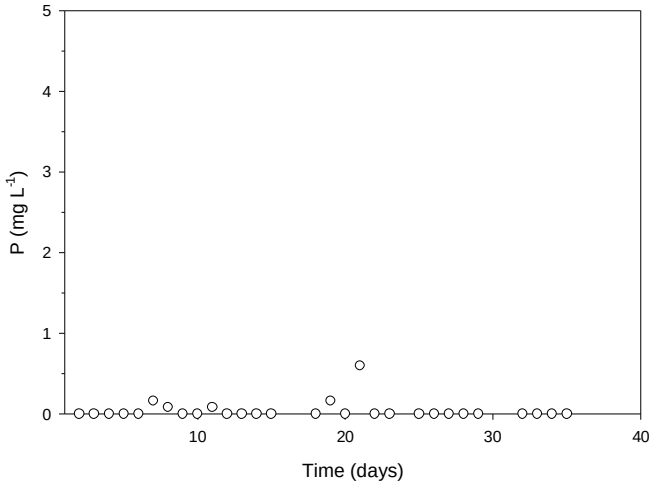
Biomass samples for FISH analysis were taken after 32 d from both reactors. Figure 3.15 shows the relative abundances of key populations. At pH 6.5 and pH 7.0 the relative abundances of *Accumulibacter* were similar, although higher than measured at pH 7.5. The abundance of DF988 positive *Defluviicoccus* related cells increased with pH, and DF988 positive *Defluviicoccus* related cells were significantly ($p < 0.05$) more abundant at pH 7.5 than at the other pHs. At pH 7.5 significantly ($p < 0.05$) fewer *Dechloromonas* related cells were detected by FISH. Other populations in the communities at pH 6.5 and 7.0 are shown in Table 3.3. Those present at pH 7.5 were presented in Table 3.2.

3.6.3 Community composition analysis by DGGE profiling

3.6.3.1 DNA extraction method

DGGE profiles for all future experiments described in this report were generated as described in Section 2.5, using a denaturation gradient of 30 – 70%, with DNA extracted by the phenol chloroform method of McVeigh et al. (1996) (Section 1.2.1).

a



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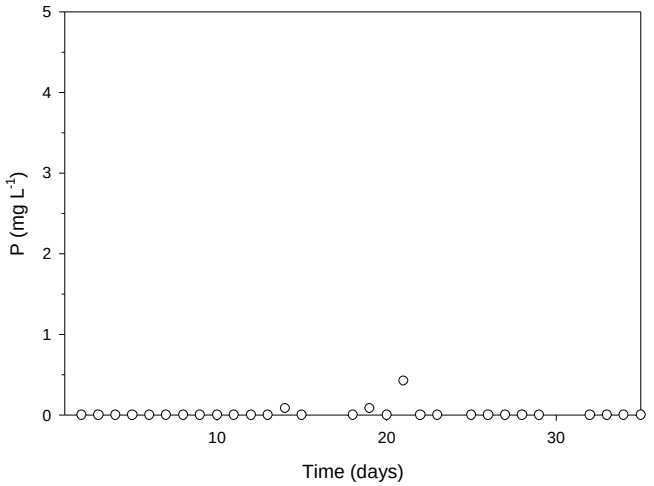

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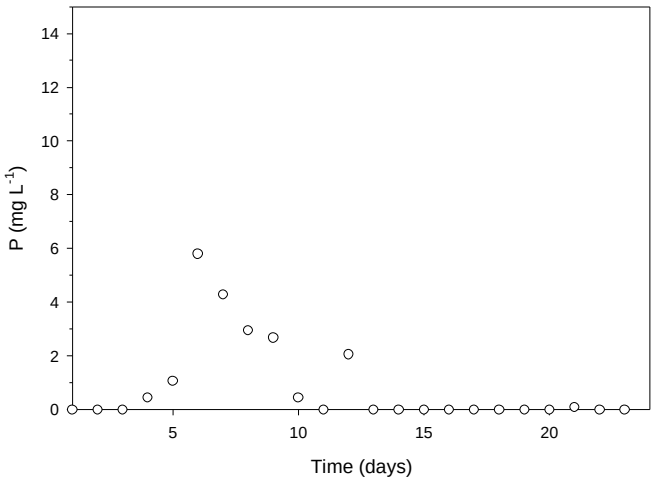
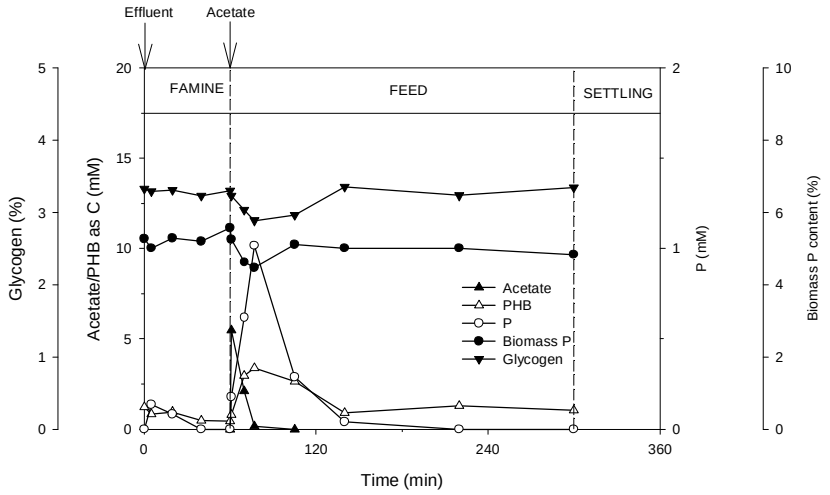
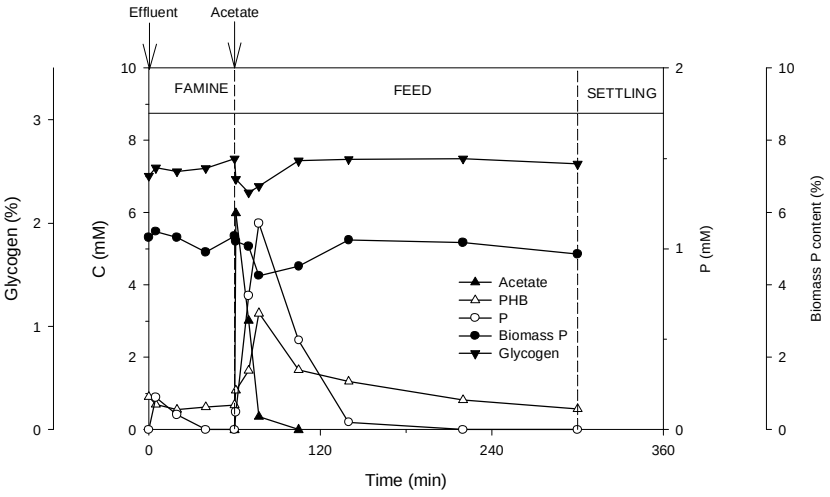


Figure 3.13 Time profile showing levels of P in the effluent of the aerated EBPR SBR at different pH levels. SBRs were operated with clarified Melton effluent and 120 mg L^{-1} C as acetate at: a) pH 6.5, b) pH 7.0, and c) pH 7.5. (Data provided by Dr J Ahn).

a



b



c

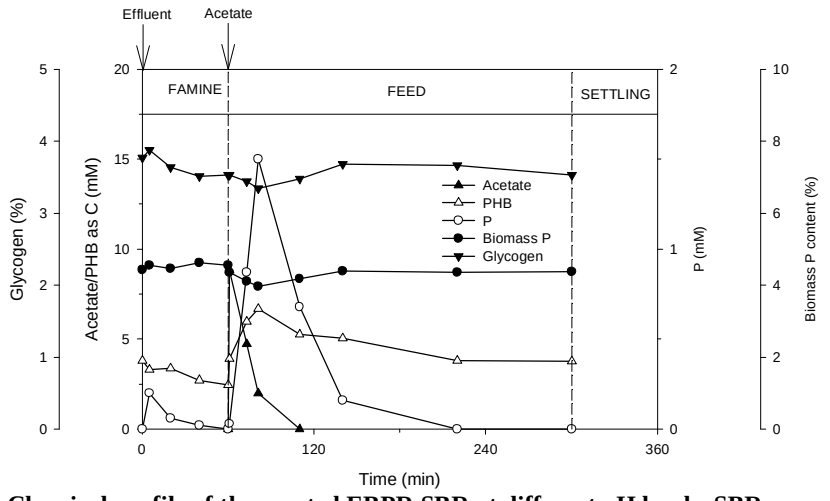


Figure 3.14 Chemical profile of the aerated EBPR SBR at different pH levels. SBRs were fed clarified Melton effluent and 120 mg L^{-1} C as acetate at: a) pH 6.5, b) pH 7.0, and c) pH 7.5. Graph shows the changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) over a FEED: FAMINE cycle. The downward facing arrows indicate where acetate and the clarified effluent were added to the reactor. (Data provided by Dr J Ahn).

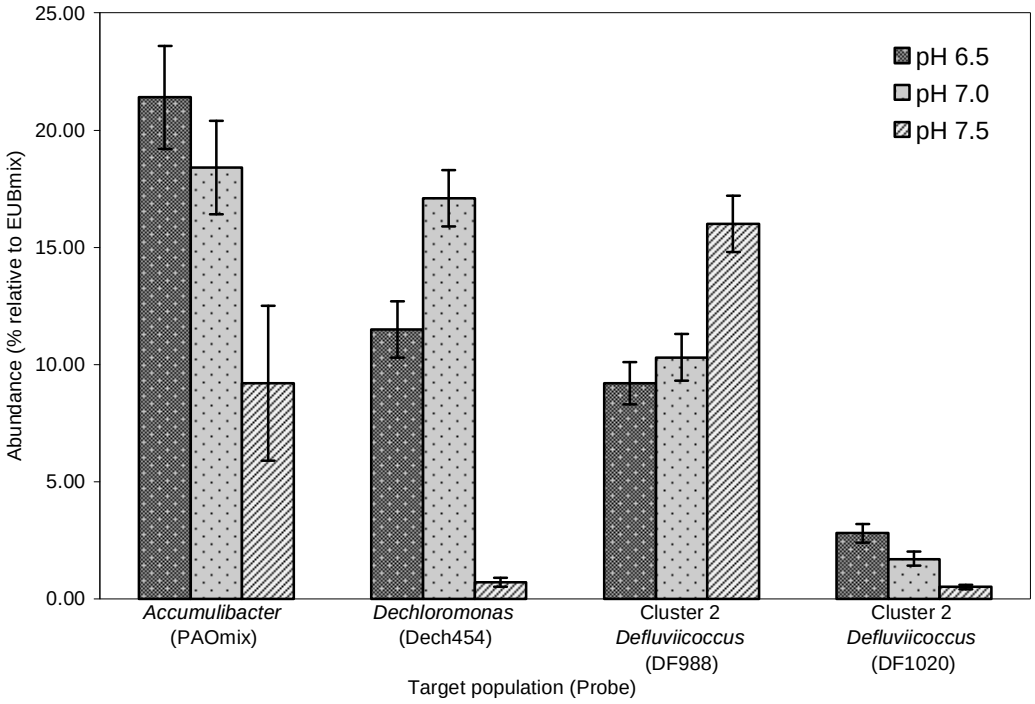


Figure 3.15 FISH analysis of populations in the aerated EBPR SBR at different pH levels. SBRs were fed clarified Melton effluent supplemented with 120 mg L^{-1} C as acetate at: pH 6.5, pH 7.0, and pH 7.5. The biovolume percentage of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells is relative to the total bacteria fluorescing with EUBmix. Error bars show standard error.

Table 3-3 FISH analysis of populations present in the aerated EBPR SBR at different pH levels. SBR was operated with Melton clarified effluent and 120 mg L^{-1} C as acetate at pH levels of 6.5 and 7.0.

Target	Probe	pH 6.5	pH 7.0
Betaproteobacteria			
Betaproteobacteria	Bet42a	+++	+++
Dechloromonas related bacteria	Dech472	-	nd
Zoogloea spp.	Zra23a	-	++
Alphaproteobacteria			
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	+	+
Gammaproteobacteria			
Gammaproteobacteria	Gam42a_mix	++	++
Subgroup 1 and 2 of group GB	GBmix	+	+
Nitrifying organisms			
Nitrospira	Ntspa662	+	+
Nitrosomonas	Nso1225	+	+
Other			
Actinobacterial PAO	Actino221	nd	-
Actinobacterial PAO	Actino658	++	+

nd = not determined

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

3.6.3.2 DGGE community profiles

The 16S rRNA DGGE profiles of the EBPR communities at pH 6.5, 7.0, and 7.5 are shown in Figure 3.17. *Accumulibacter* clones OTU c79 and d62, and *Dechloromonas* related clone OTU c96 could be identified in profiles at all three pH levels. A dendogram showing the similarity in these 16S rRNA patterns is shown in Figure 3.16, showing the profiles from communities from pH 7.0 and 7.5 were 95.3% similar, while the profile from the community at pH 6.5 was 94.2% similar to pH 7.0 and 7.5.

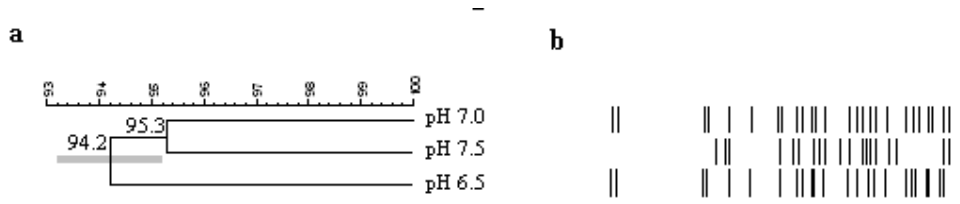


Figure 3.16 Similarity analysis of 16S rRNA DGGE profiles from the aerated EBPR SBR operated with different pH levels. SBRs were fed clarified Melton effluent and 120 mg L^{-1} C as acetate at pH 6.5, pH 7.0, and pH 7.5. a) Dendogram constructed using the Pearson similarity correlation coefficient displaying similarity of banding patterns between the community 16S rRNA DGGE profiles. Error bars show standard deviation. b) Representation of bands on the DGGE profile from Figure 3.16.

3.6.4 Discussion

Several attempts have been made to understand the effects of pH on anaerobic: aerobic EBPR performance, in terms of possible shifts in the PAO and GAO balance at different pH values (Oehmen et al. 2007). The consensus from these studies is that a higher pH favours the *Accumulibacter* PAO over the GAO (Oehmen et al. 2007), although most of the reported work has analysed communities where *Competibacter*, not *Defluviicoccus*, were the GAO. This shift is thought to occur because at the higher pH acetate uptake requires more energy, which the *Accumulibacter* can meet by using their stored polyP as an energy source, while the GAO have to use their glycogen stores (Oehmen et al. 2007). However in this aerated system the opposite is seen, so that the relative abundances of *Accumulibacter* cells decrease as pH increases. Unidentified alphaproteobacterial TFO have been identified in EBPR SBRs as a major population at pH 7.0 but were less abundant at pH 8.0 (Oehmen et al. 2005a). However the data presented here clearly show that the *Defluviicoccus* related TFO abundance in this aerated process increased as pH increased.

This suggests that they are better able to compete with the *Accumulibacter* for acetate as pH increases. Why this might happen is not clear from the chemical profile data, and further work is required to elucidate the metabolic features of both the *Accumulibacter* and *Defluviicoccus* related cells under these present conditions.

The DGGE profile banding patterns showed the communities from reactors run at pH 7.0 and pH 7.5 were more similar to each other qualitatively than from the community at pH 6.5, although all were very similar.

The next series of experiments set out to investigate how varying the operating temperature might affect process performance and associated community structure.

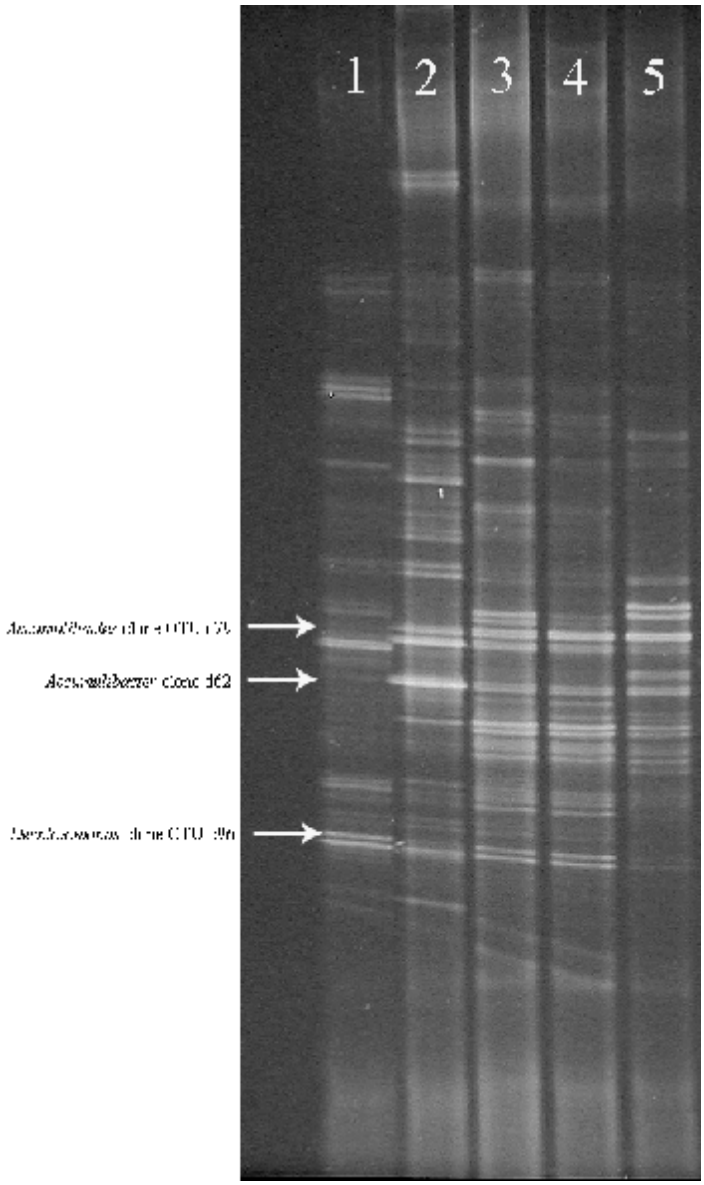


Figure 3.17 PCR-DGGE profiles of partial 16S rRNA gene fragments from the aerated EBPR SBR at different pH levels. Profiles were generated using v3 region universal primers. The SBRs were fed: Lane 1 and 2) synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A) (standards) with DNA extracted using Lane 1) the UltraClean™ Soil DNA kit, and Lane 2) the McVeigh et al. (1996) method. Lanes 3 - 5) clarified Melton effluent with 120 mg L^{-1} C as acetate at: Lane 3) pH 6.5, Lane 4) pH 7.0, and Lane 5) pH 7.5.

3.7 Effect of changing temperature on plant performance

Temperature has often been cited as a possible reason for anaerobic: aerobic EBPR process failure, which generally works much better at temperatures less than 20 °C (Whang and Park 2002; Panswad et al. 2003; Whang and Park 2006), although little microbiological data are available from these studies to indicate why. PAO have dominated communities in plants run at 20 °C (Panswad et al. 2003), while both *Competibacter* and unidentified TFO GAO dominated at 30 °C (Whang and Park 2002; Panswad et al. 2003; Whang and Park 2006). The P uptake rates for PAO were found to be highest at 15 – 20 °C (Brdjanovic et al. 1997; Baetens et al. 1999), while an unidentified GAO dominated sludge was able to assimilate acetate faster then the PAO dominated sludge at temperatures above 20 °C (Whang and Park 2002). Some data suggest *Competibacter* GAO become metabolically inactive below 10 °C (López-Vázquez et al. 2008), but the response to temperature of the *Defluviicoccus* related cells has not been documented.

Therefore, the possible influence of temperature on this aerated P removal system were examined by operating SBRs at 15, 25, and 30 °C.

3.7.1 Operating the reactor at 15 °C

3.7.1.1 P removal performance and chemical analysis

Reactors L and M were both operated at 15 °C as described in Section 2.1, and fed with clarified Melton effluent with acetate supplementation of 120 mg L^{-1} C as acetate, a sludge age of 15 d and pH 7.5. Reactor L removed P for 14 d, and Reactor M removed P for 15 d. Reactor M then recovered P removal capacity after a further 13 d and resumed removing P, although it never reached complete EBPR capacity, and effluent P values ranged from 0 – approx. 2 mg L^{-1}. A chemical profile was taken from Reactor M during the period of P removal after 79 d. Reactor N was operated identically to Reactors L and M, except it was supplied with a synthetic wastewater feed. However, it removed P for only 10 d, and samples used to determine the chemical profile were taken on day 9. Effluent P profiles are shown in Figure 3.18 and chemical profiles for Reactors M and N in Figure 3.19.

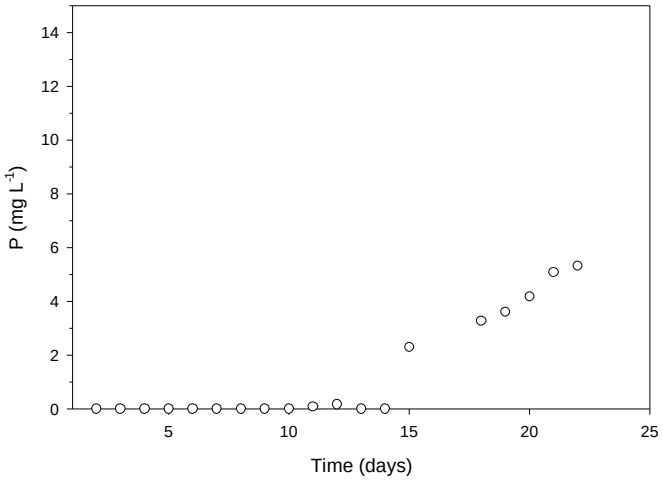
3.7.1.2 Community composition by FISH analysis

Biomass samples for FISH analysis were taken on days 13 and 22 from Reactor L, from Reactor M on days 14, 28, and 43, and from Reactor N on day 8. Figure 3.20 shows the abundance of the populations of interest (selected populations are shown in Figure 4.28). The results show a significant ($p < 0.05$) increase in the abundance of DF988 positive *Defluviicoccus* related cells corresponding to when Reactor L failed. Other populations present in the communities are shown in Table 3.4.

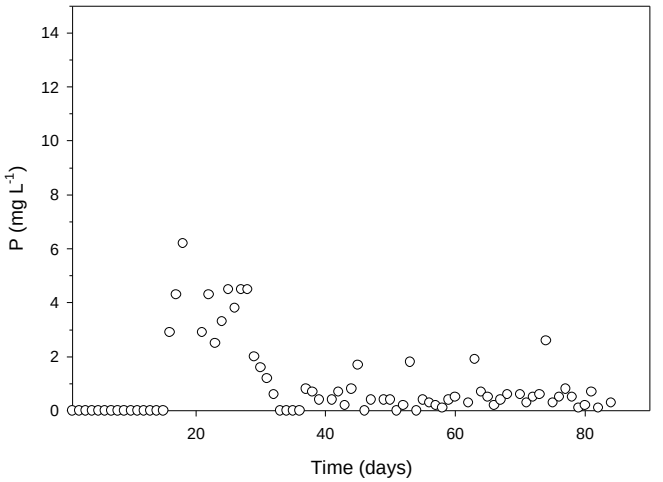
3.7.1.3 Community composition analysis by DGGE profiling

The dendogram comparing DGGE profiles from communities from Reactors L, M, and N are shown in Figure 3.21, and the DGGE profiles from which these were derived are given in Figure 3.22. *Accumulibacter* related clone OTU c79 was detected in profiles from all samples, while *Accumulibacter* related clone d62 was present in the profiles from Reactor M after P removal had recovered, but not when the process was removing P earlier. These data agree with FISH data (Section 3.7.1.2) showing *Accumulibacter* were present in all samples, regardless of whether high P removal was occurring. The dendogram shows the profiles were very similar ($\geq$ 98%) regardless of whether high P removal was occurring.

a



b



c

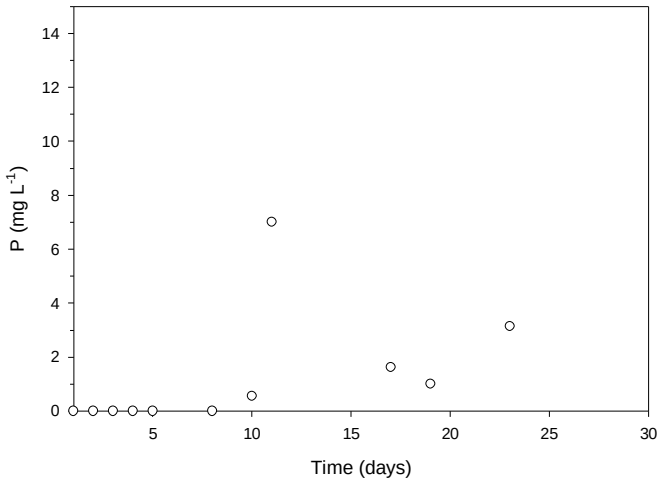
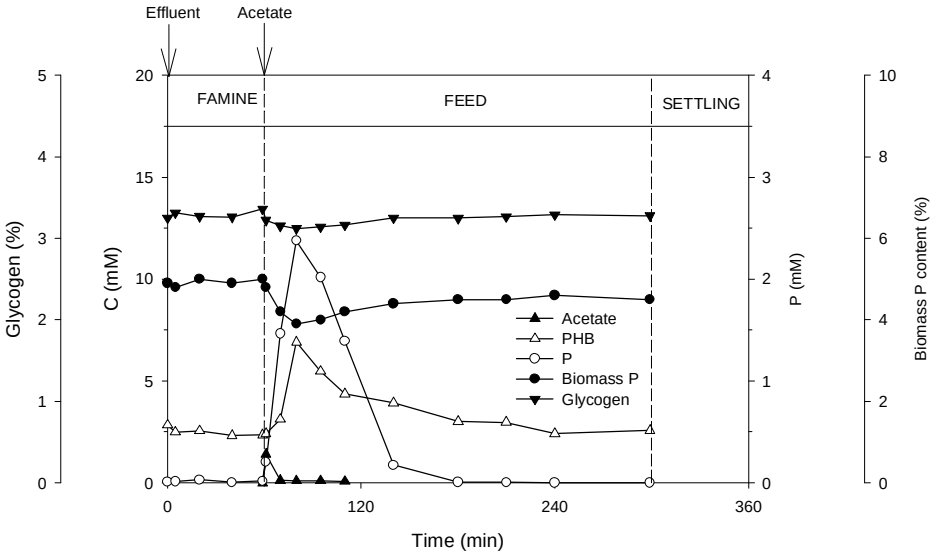


Figure 3.18 Time profile showing levels of effluent P from the aerated EBPR SBR at 15 °C. SBRs were operated with 120 mg L^{-1} C as acetate with: a and b) clarified Melton effluent (Reactors L and M), and c) synthetic wastewater (Reactor N). (Data provided by Dr J Ahn and B Campbell).

a



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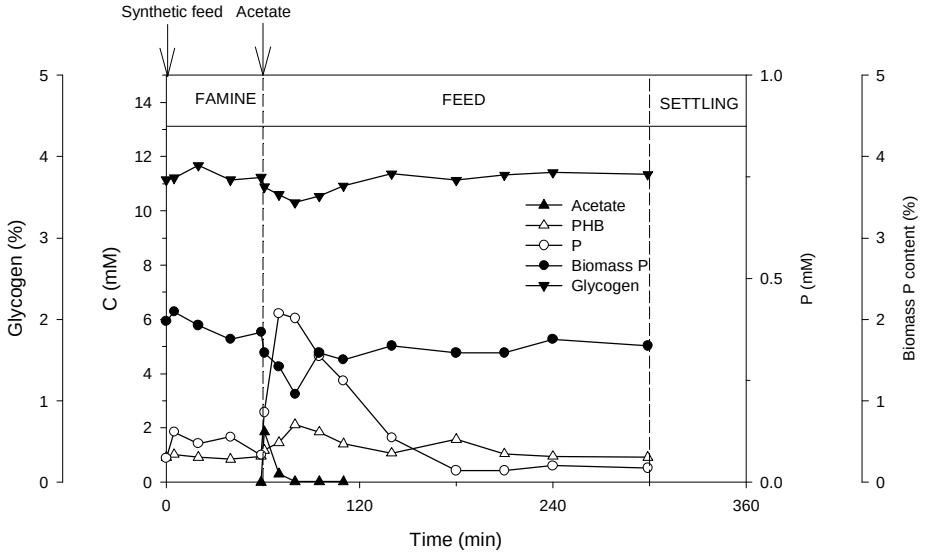


Figure 3.19 Chemical profiles from the aerated EBPR SBR at 15 °C. SBRs were operated using clarified Melton effluent with 120 mg L^{-1} C as acetate using different feed sources of: a) clarified wastewater (Reactor M), and b) synthetic wastewater (Reactor N). Changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) are shown over a FEED: FAMINE cycle. The downward facing arrows indicate where acetate and the clarified effluent or synthetic wastewater were added to the reactor. (Data provided by Dr J Ahn and B Campbell).

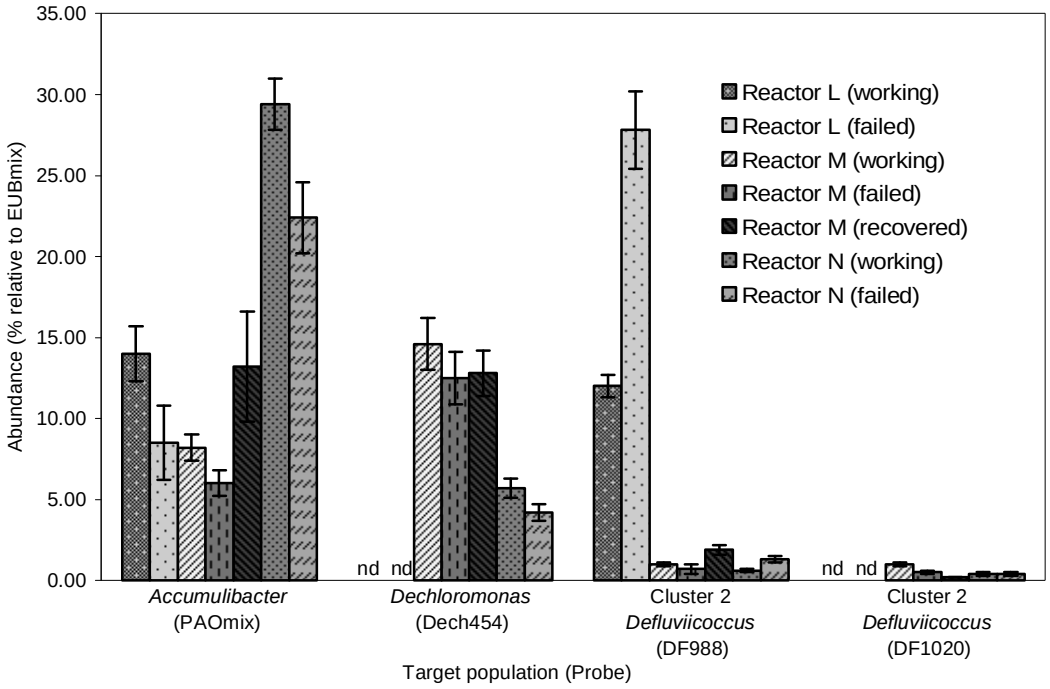


Figure 3.20 FISH analysis of populations in the aerated EBPR SBR operated at 15 °C. SBRs are supplemented with 120 mg L^{-1} C as acetate using feed sources of clarified effluent (Reactors L and M), and synthetic wastewater (Reactor N). The percentage biovolume is relative to total bacteria fluorescing with EUBmix of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells. Error bars show standard error.

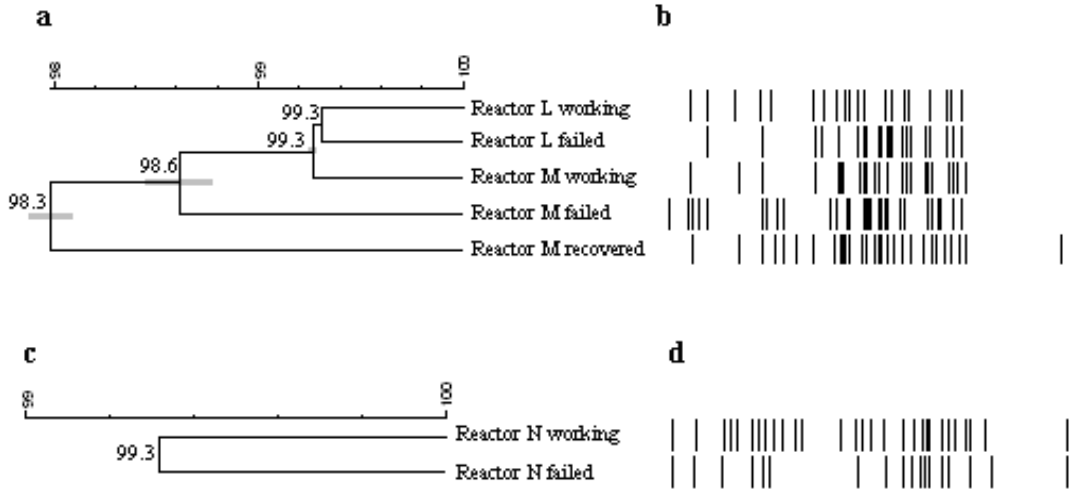


Figure 3.21 Similarity analysis of 16S rRNA DGGE profiles from the aerated EBPR SBR operated at 15 °C. SBRs were supplemented with 120 mg L^{-1} C as acetate. a and c) Dendogram constructed using the Pearson similarity correlation coefficient displaying similarity of banding patterns between the community 16S rRNA DGGE profiles for: a) Melton clarified effluent feed (Reactors L and M) and c) synthetic wastewater (Reactor N). Error bars show standard deviation. b and d) Corresponding representation of bands on the DGGE profile from Figure 3.22.

Table 3-4 FISH analysis of populations present in an aerated EBPR SBR. SBR was supplemented with 120 mg L^{-1} C as acetate at 15 °C using different feed sources of clarified wastewater (Reactors L and M), and synthetic wastewater (Reactor N).

Target	Probe	Reactor L (working)	Reactor L (failed)	Reactor M (working)	Reactor M (failed)	Reactor M (recovered)	Reactor N (working)	Reactor N (failed)
Betaproteobacteria								
Betaproteobacteria	Bet42a	nd	nd	nd	nd	nd	+++	+++
Dechloromonas related bacteria	Dech472	++	++	++	++	+	++	+
Zoogloea spp.	Zra23a	++	++	+++	+++	+++	-	+++
Alphaproteobacteria								
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	nd	nd	-	-	+	+	-
Gammaproteobacteria								
Gammaproteobacteria	Gam42a_mix	+/++	++	++	+	+	+++	+++
Subgroup 1 and 2 of group GB	GBmix	++	++	+	+	+	+	+
Nitrifying organisms								
Nitrospira	Ntspa662	++	++	++	++	++	++	++
Nitrosomonas	Nso1225	+	+	+/++	+	nd	+	+
Other								
Planctomycetales	Pla886	+++	+++	+++	+++	+++	+++	+++
Flavobacteria	Cf319a	++	++	++	++	++	+++	+++
'Chloroflexi'	CFX1223/ GNSB941	+++	+++	+++	+++	+++	+++	+++
Actinobacteria	HGC69a	nd	nd	nd	nd	nd	+	+
Actinobacterial PAO	Actino221	nd	nd	nd	nd	nd	nd	-
Actinobacterial PAO	Actino658	+	+	+	+	+	+	+

nd = no data - not detected, +/– very few, +, few ++, some, +++ common (Wong and Liu 2007).

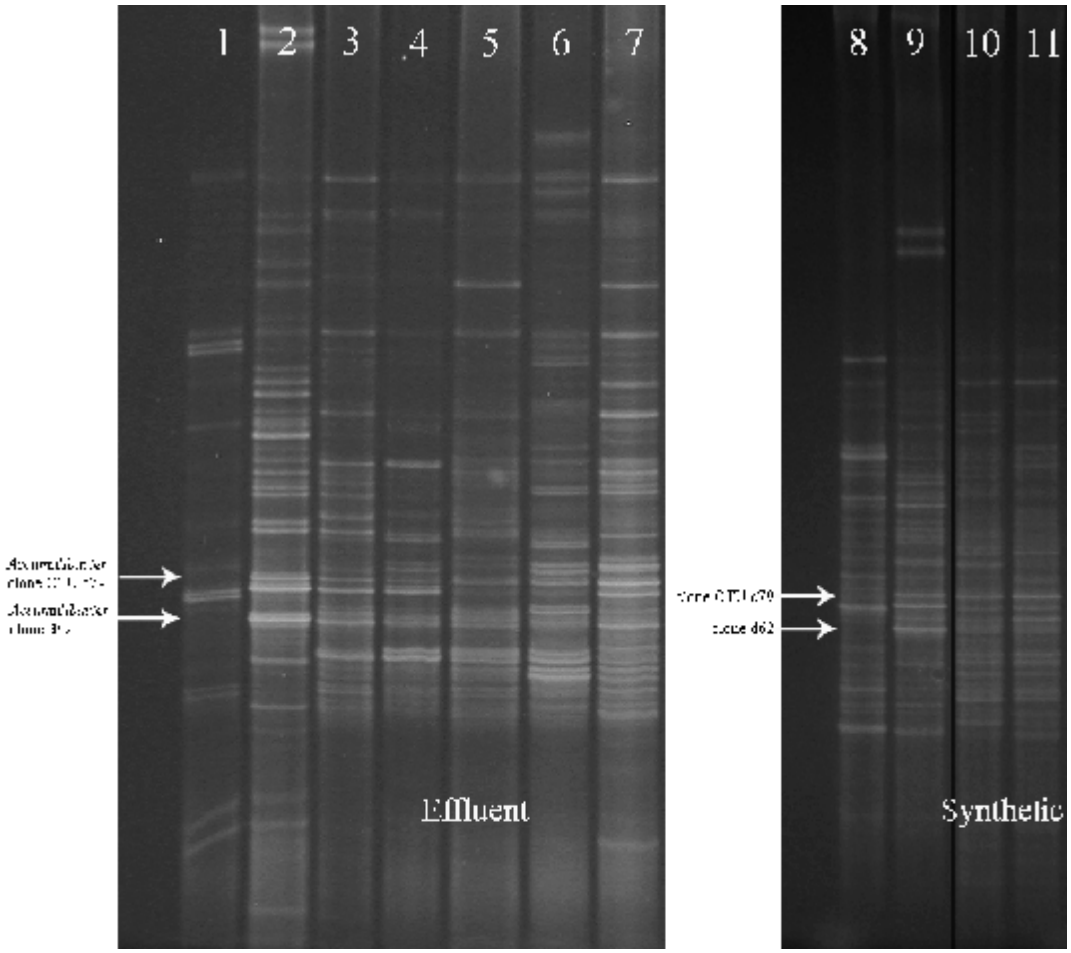


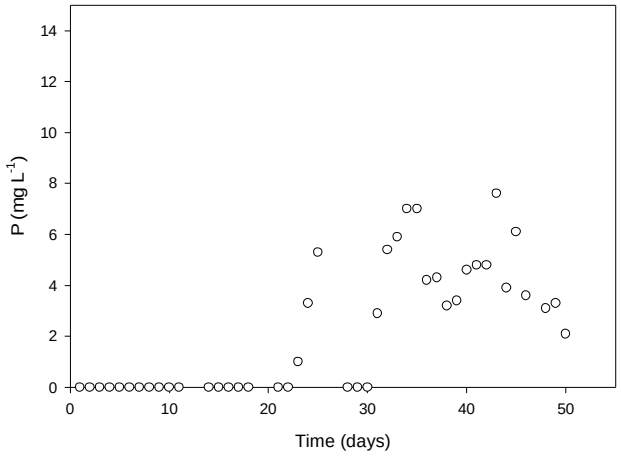
Figure 3.22 PCR-DGGE profiles of partial 16S rRNA gene fragments from the aerated EBPR SBR operated at 15 °C. Profiles were generated using v3 region universal primers. SBRs were fed: Lanes 1 and 2, 8 and 9) 160 mg L^{-1} C as acetate with synthetic wastewater (Feed A) (standards) with DNA extracted using the: Lanes 1 and 8) UltraClean™ Soil DNA kit, and Lanes 2 and 9) the McVeigh et al. (1996) method. Lanes 3 – 7) clarified effluent feed with 120 mg L^{-1} C as acetate at a temperature of 15 °C (Lanes 3 – 5 Reactor L, Lanes 6 and 7 Reactor M) when the process was: Lanes 3, 5, and 7) removing P, and Lanes 4 and 6) not removing P. Lanes 10 and 11) Synthetic feed with 120 mg L^{-1} C as acetate at a temperature of 15 °C (Reactor N) when the process was: Lane 10) removing P, and Lane 11) not removing P.

3.7.2 Operating the reactor at 25 °C

3.7.2.1 P removal performance and chemical analysis

Reactor O was operated as detailed in Section 1.1 with clarified Melton effluent, acetate supplementation of 120 mg L^{-1} C as acetate, and a sludge age of 15 d at pH 7.5. Reactor O removed P for 23 d, while Reactor P was operated using synthetic wastewater and removed P for 33 d. The effluent P profiles are presented in Figure 3.23, and the chemical profile from Reactor P from samples taken on day 31 in Figure 3.24.

a



b

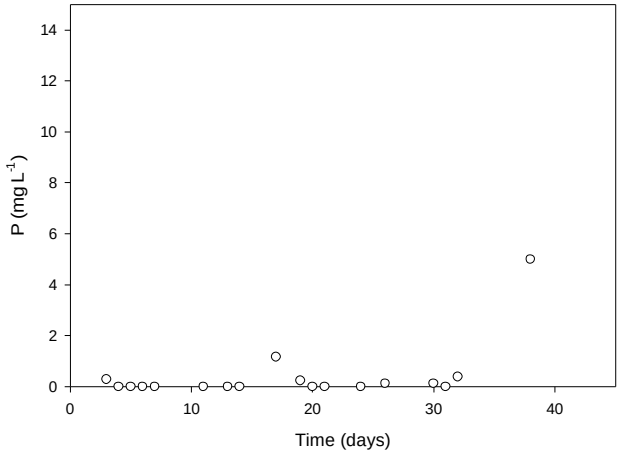


Figure 3.23 Time profile showing effluent P levels from the aerated EBPR SBR operated at 25 °C. SBRs were fed 120 mg L^{-1} C as acetate using different feed sources of: a) clarified Melton effluent (Reactor O), and b) synthetic wastewater (Reactor P). (Data provided by B Campbell).

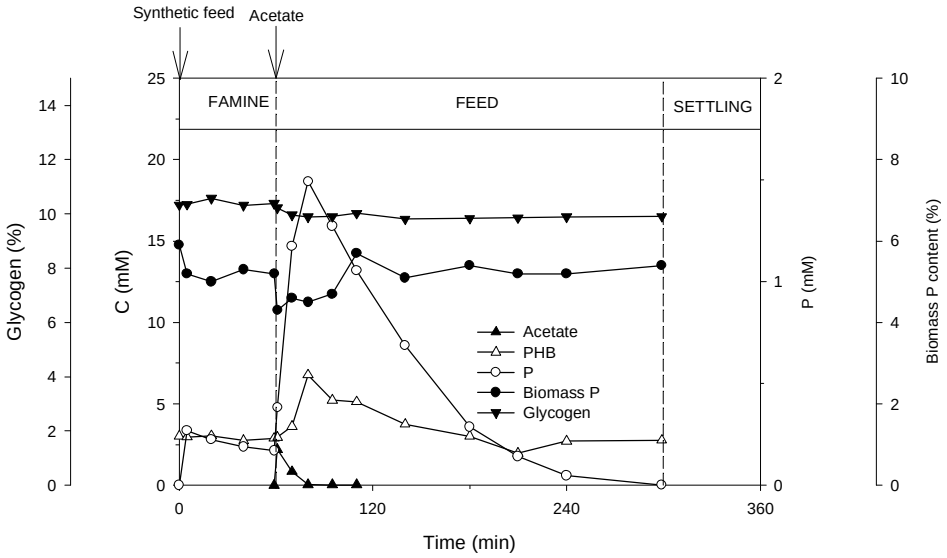


Figure 3.24 Chemical profile of the aerated EBPR SBR cycle operated at 25 °C. SBR was fed synthetic wastewater with 120 mg L^{-1} C as acetate. Changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) are shown over a FEED: FAMINE cycle. The downward facing arrows indicate where acetate and the synthetic wastewater were added to the reactor. (Data provided by B Campbell).

3.7.2.2 Community composition by FISH analysis

Samples for FISH analysis were taken after 21 d and 35 d from Reactor O, and after 31 d from Reactor P. Figure 3.25 shows the abundance of major populations (selected populations are shown in Figure 3.28). There was a significant ($p < 0.05$) decrease in the abundance of *Accumulibacter* when the P removal capacity of Reactor O had failed, and there was significantly ($p < 0.05$) more *Accumulibacter* present in Reactor P. There was also a significantly ($p < 0.05$) higher abundance of *Accumulibacter* related cells then DF988 positive *Defluviicoccus* related cells in this reactor. The other populations detected by FISH are given in Table 3.5.

3.7.2.3 Community composition analysis by DGGE profiling

The dendogram comparing DGGE profiles of 16S rRNA fragments from communities from Reactors O and P is shown in Figure 3.26, and the DGGE profiles are shown in Figure 3.27. Again a fragment representing *Accumulibacter* related clone OTU c79 was present when Reactor O was removing P and when P removal capacity had failed, and in Reactor P. *Accumulibacter* related clone d62 was also present, although sometimes not as readily visible on the profiles. Profiles from both working and failed Reactor O communities appeared very similar to each other (98.7%), although some quantitative differences in the fluorescent intensities of bands from communities when high P removal was and was not occurring could be recognised, where the populations were not identified.

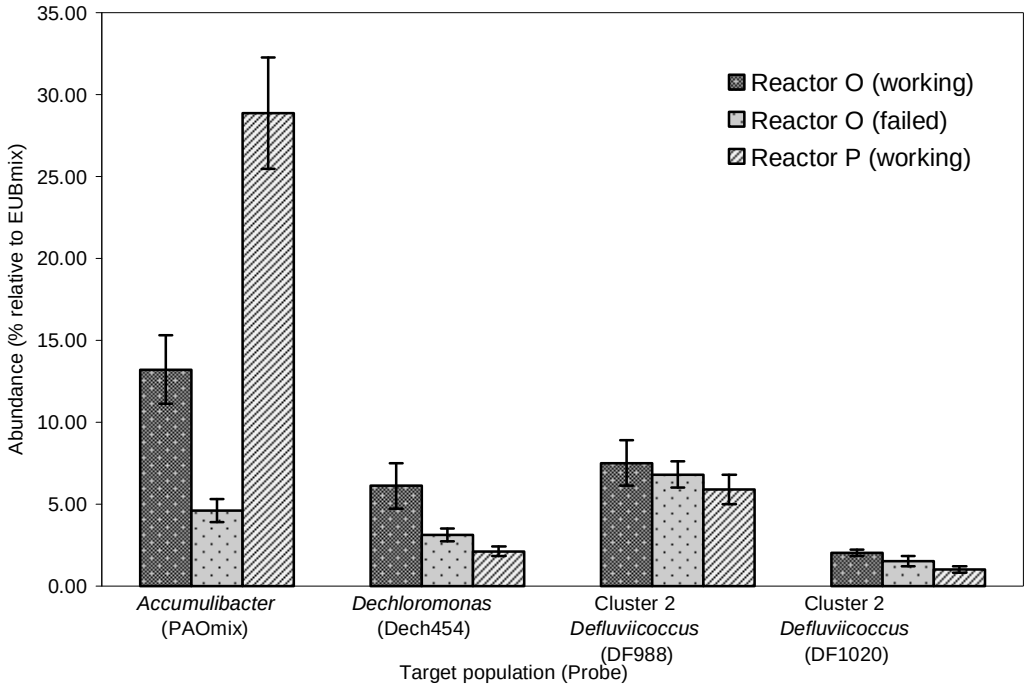


Figure 3.25 FISH analysis of populations in the aerated EBPR SBR operated at 25 °C. SBRs were operated with 120 mg L^{-1} C as acetate and different feed sources of clarified Melton effluent (Reactor O) and synthetic wastewater (Reactor P). The percentage biovolume is relative to total bacteria fluorescing with EUBmix of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells. Error bars show standard error.

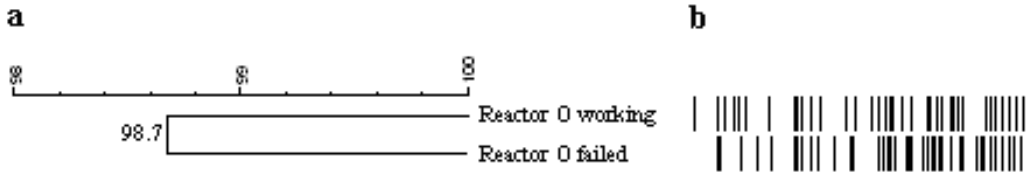


Figure 3.26 Similarity analysis of 16S rRNA DGGE profiles from the aerated EBPR SBR operated at 25 °C. SBRs were operated with 120 mg L^{-1} C as acetate with clarified Melton effluent (Reactor O). a) Dendogram constructed using the Pearson similarity correlation coefficient displaying similarity of banding patterns between the community 16S rRNA DGGE profiles. Standard deviation was negligible. b) Representation of bands on the DGGE profile from Figure 4.27.

Table 3-5 FISH analysis of populations present in an aerated EBPR SBR. SBR was operated with 120 mg L^{-1} C as acetate at 25 °C with different feed sources of clarified Melton effluent (Reactor O) and synthetic wastewater (Reactor P).

Target	Probe	Reactor O (working)	Reactor O (failed)	Reactor P
Betaproteobacteria				
Dechloromonas related bacteria	Dech472	+	+	++
Zoogloea spp.	Zra23a	+/++	+/++	++
Alphaproteobacteria				
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	+	+	+
Gammaproteobacteria				
Gammaproteobacteria	Gam42a_mix	++	+	++
Subgroup 1 and 2 of group GB	GBmix	+	+/++	+
Nitrifying organisms				
Nitrospira	Ntspa662	+/++	+	++
Nitrosomonas	Nso1225	++	+/++	++
Other				
Planctomycetales	Pla886	+++	+++	+++
Flavobacteria	Cf319a	++	++	++
'Chloroflexi'	CFX1223/GNSB941	+++	+++	+++
Actinobacterial PAO	Actino221	nd	nd	-
Actinobacterial PAO	Actino658	++	+	+

nd = no data

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

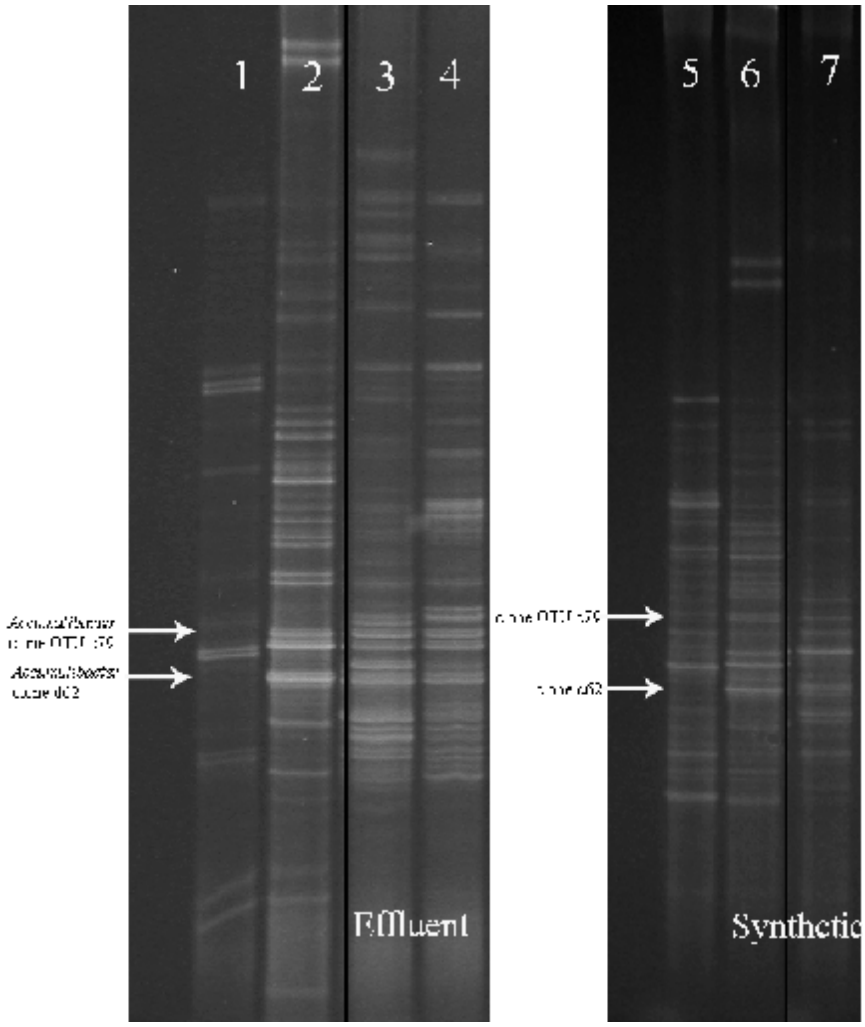


Figure 3.27 PCR-DGGE profiles of partial 16S rRNA gene fragments from the aerated EBPR SBR operated at 25 °C. Profiles were generated using v3 region universal primers. SBRs were operated with: Lanes 1 and 2, 5 and 6) synthetic wastewater with 160 mg L^{-1} C as acetate (Feed A) (standards) with DNA extracted using the UltraClean™ Soil DNA kit (MoBio Laboratories, Inc) (Lanes 1 and 5), and the McVeigh et al. (1996) method (Lanes 2 and 6). Lanes 3, 4 and 7) Profiles of communities fed clarified Melton effluent (Lanes 3 and 4, Reactor O), and synthetic wastewater (Lane 7, Reactor P) with 120 mg L^{-1} C as acetate at a temperature of 25 °C when the process was: Lanes 3 and 7) removing P, and Lane 4) not removing P.

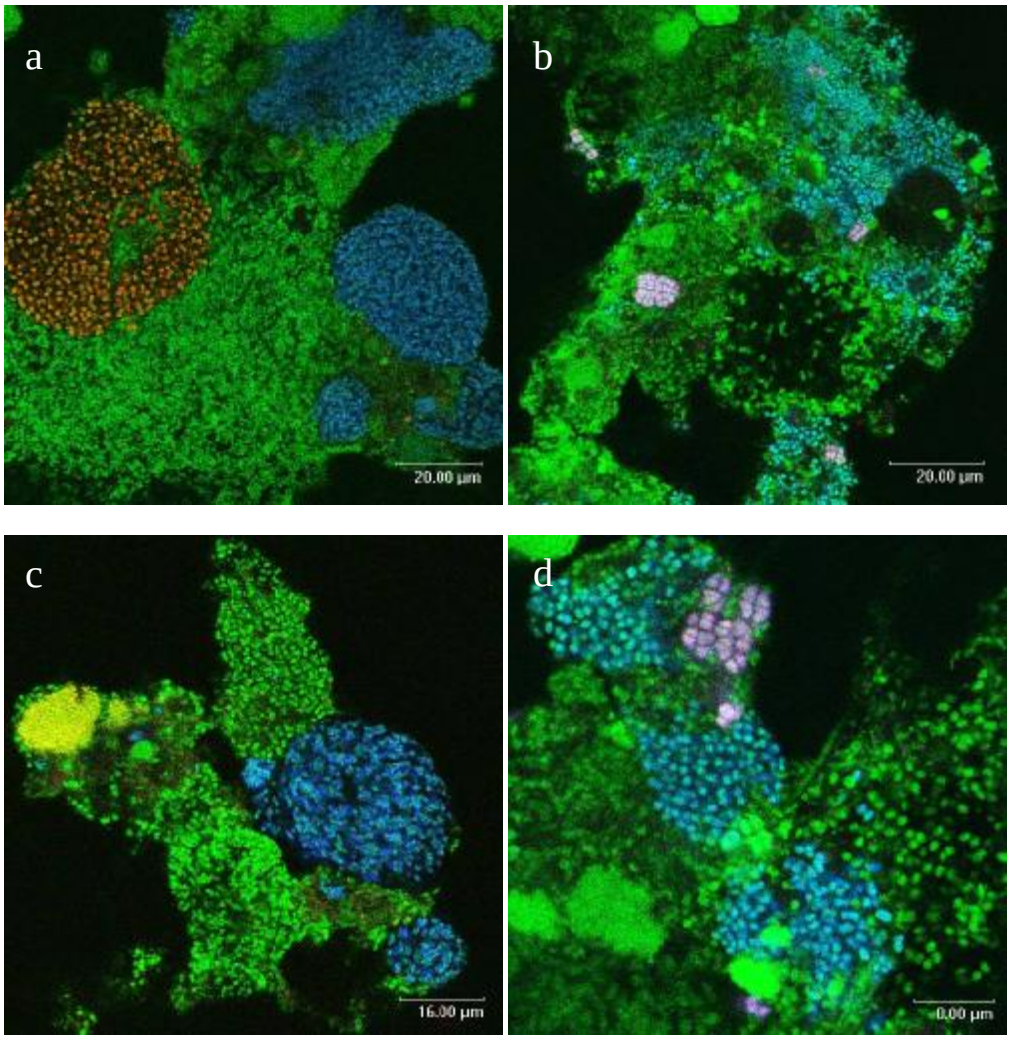


Figure 3.28 FISH micrographs showing populations in the aerated EBPR SBR community at different temperatures. a) *Accumulibacter* clusters hybridising with the PAOmix probes (Cy5) and *Defluviicoccus* related cells hybridising with the DF2 mix probes (Cy3) when the process was removing P at 15 °C fed clarified effluent (Reactor L). b) *Defluviicoccus* related cells hybridising with the DF988 (Cy5) and DF1020 (Cy3) probes when the process was not removing P at 15 °C fed synthetic wastewater (Reactor N). c) *Accumulibacter* clusters hybridising with the PAOmix probes (Cy5) and *Dechloromonas* related cells hybridising with the Dech454 probe (Cy3), and d) *Defluviicoccus* related cells hybridising with the DF988 (Cy5) and DF1020 (Cy3) probes. Micrographs c) and d) are when the process was fed clarified effluent and removing P at 25 °C (Reactor O). Cells that hybridised with both probes appear yellow (FLUOS and Cy3), and light blue (FLUOS and Cy5).

3.7.3 Operating the reactor at 30 °C

3.7.3.1 P removal performance and chemical analysis

The SBR (Reactor Q) was operated as described in Section 1.1, fed clarified Melton effluent with 120 mg L^{-1} C as acetate, a sludge age of 15 d and pH 7.5, at 30 °C. The effluent P profile is presented in Figure 3.29. Reactor Q removed P for only 9 d before failure.

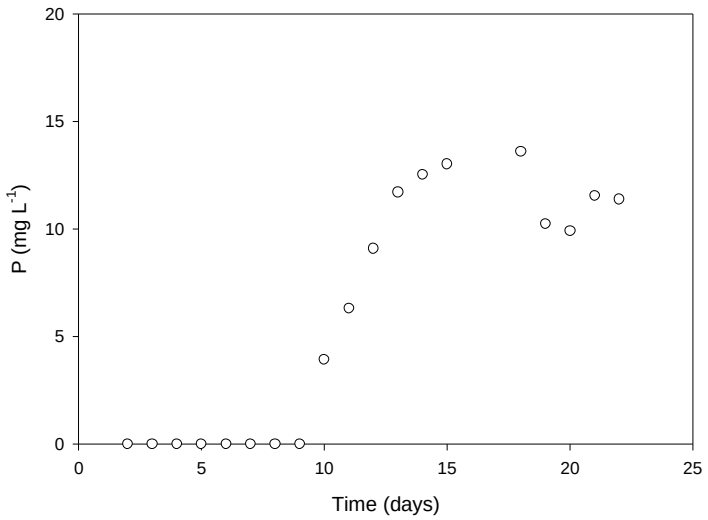


Figure 3.29 Time profile showing levels of P in the effluent for the aerated EBPR SBR operated at 30 °C. SBR was fed clarified Melton effluent with 120 mg L^{-1} C as acetate at 30 °C (Reactor Q). (Data provided by Dr J Ahn).

3.7.3.2 Community composition by FISH analysis

Biomass samples for FISH analysis of Reactor Q were taken on days 13 and 22. Figure 3.30 shows the abundance of major populations, revealing a significant difference ($p < 0.05$) in the abundances of both *Accumulibacter* and *Defluviicoccus* related cells from the start to the end of reactor operation. Table 3.6 shows other populations present in the biomass as assessed by FISH analysis.

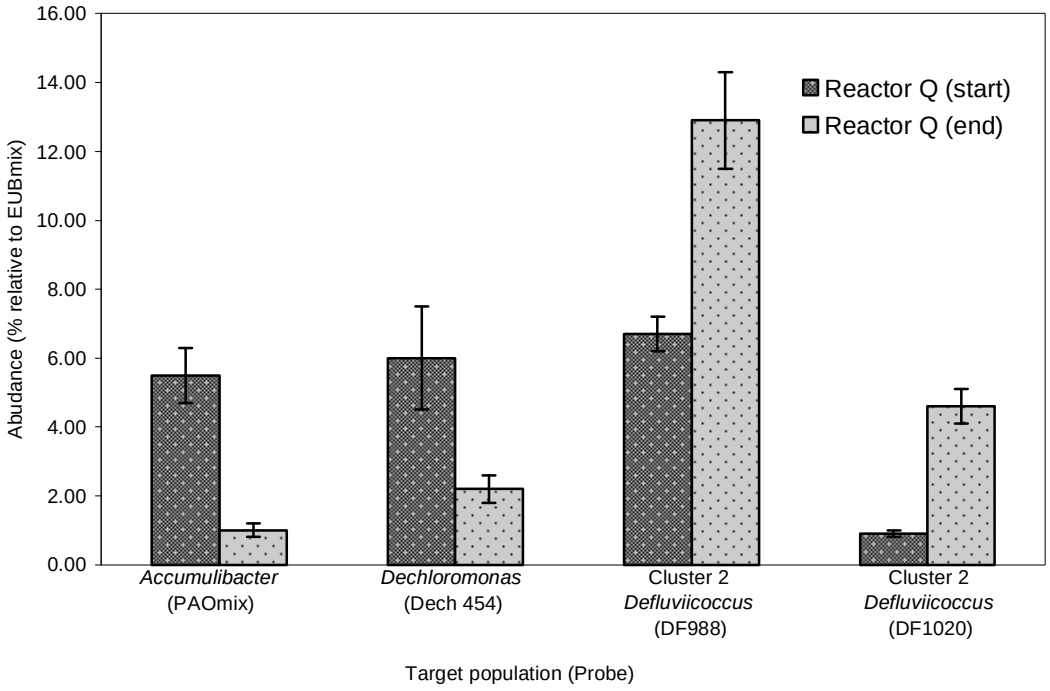


Figure 3.30 FISH analysis of populations in the aerated EBPR SBR operated 30 °C. SBR was fed clarified Melton effluent with 120 mg L^{-1} C as acetate. The percentage biovolume is relative to total bacteria fluorescing with EUBmix of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells. Error bars show standard error.

Table 3-6 FISH analysis of populations present in an aerated EBPR SBR. SBR was operated with clarified Melton effluent and 120 mg L^{-1} C as acetate at 30 °C.

Target	Probe	Reactor Q (13d)	Reactor Q (22d)
Betaproteobacteria			
Dechloromonas related bacteria	Dech472	++	+++
Zoogloea spp.	Zra23a	+++	nd
Alphaproteobacteria			
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	nd	+
Gammaproteobacteria			
Gammaproteobacteria	Gam42a_mix	+++	+/++
Subgroup 1 and 2 of group GB	GBmix	++	+
Nitrifying organisms			
Nitrospira	Ntspa662	nd	+
Nitrosomonas	Nso1225	nd	+
Other			
Planctomycetales	Pla886	+++	+++
Flavobacteria	Cf319a	++	++
'Chloroflexi'	CFX1223/GNSB941	+++	++
Actinobacterial PAO	Actino221	nd	-/+
Actinobacterial PAO	Actino658	+	+

nd = no data

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

3.7.4 Discussion

Results obtained in this section show that at 15 °C (Reactors L, M, and N) the process in three replicated runs could not reliably remove P over an extended period. In Reactor L (operated with clarified effluent) *Accumulibacter* abundance decreased while that of DF988 positive *Defluviicoccus* increased significantly with P removal failure. However, in Reactor M the abundance of *Accumulibacter* and DF988 positive *Defluviicoccus* related cells did not change significantly in the period between P removal failure and recovery. The data from Reactor L seems to disagree with previous literature from anaerobic: aerobic EBPR system that suggest putative GAO dominate only at higher operating temperatures (Oehmen et al. 2007). However, this evidence was based only on communities containing *Competibacter* and not *Defluviicoccus* whose metabolic features, especially in this aerated system, may be markedly different. The appropriateness of referring to *Defluviicoccus* as a GAO under these aerated operating conditions has already been questioned (Section 3.9.3.3). Furthermore, in Reactor M neither *Defluviicoccus* nor *Dechloromonas* related cells, both of which have a feed: famine GAO phenotype in this process, dominated when P removal failed.

At 25 °C (Reactors O and P) the process was equally unsuccessful and removed P for only 23 and 33 days before failure. In Reactor O, *Accumulibacter* relative abundances decreased significantly after P removal failure, although the levels of other populations did not change significantly. In Reactor P a significant difference was seen between abundances of *Accumulibacter* and *Defluviicoccus* related cells, although in the latter their abundance was the same as that in the recovered communities from Reactor O, consistent with the view that process failure did not occur by *Defluviicoccus* out-competing *Accumulibacter* for the acetate.

The process also failed at 30 °C (Reactor Q), when the relative abundance of *Accumulibacter* related cells decreased significantly from day 13 to day 22, with a corresponding significant increase in the abundance of *Defluviicoccus* related cells. This suggests that the *Defluviicoccus* might be out-competing *Accumulibacter* for acetate at this higher temperature. It also agrees with some of the trends in earlier studies with anaerobic: aerobic EBPR systems where *Competibacter* GAO were more able to assimilate substrates (López-Vázquez et al. 2007). Whether this applies to *Defluviicoccus* under the conditions in this study is not known.

Analyses of DGGE 16S rRNA profiles showed communities from reactors run at the same temperature with the same feed source were very similar, regardless of whether high P removal was achieved. However, the community profile from Reactor M DNA when it recovered its P removal capacity clustered separately to the other two DGGE profiles from Reactor M (Figure 3.21).

Results suggest the aerated EBPR process is susceptible to temperature, regardless of the influent feed, and that it probably would not cope well with temperature fluctuations typical of seasonal changes. The usual inability of the process to recover once P removal had failed suggests it is more fragile than earlier suggested (Section 3.4.4). In some cases P removal failure was clearly associated with proliferation of *Defluviicoccus*, yet sometimes the system failed with no relative increase in *Defluviicoccus* abundance. In these cases failures cannot be attributed to *Competibacter* GAO, as their numbers remained at insignificant levels in all the runs. It may be that failure occurs because *Accumulibacter* eventually modify their metabolism in response to aerobic conditions, where the selective pressures needed for EBPR under the aerated conditions no longer operate, as has been suggested previously (Pijuan et al. 2006; Carvalho et al. 2007; Zhou et al. 2008).

3.8 Ecophysiology of Accumulibacter *and* Defluviicoccus *related cells*

DAPI staining showed the *Accumulibacter* cells in all the communities in reactors run under all these different conditions stained positively for polyP storage. However, some *Accumulibacter* cells did not appear to accumulate polyP, as was seen in earlier analyses (Section 2.7.1) (data not

shown). No TFO ever stained positively for polyP storage with DAPI, although again a few small unidentified cocci like those seen earlier (Section 2.7.1) were DAPI positive (data not shown).

Nile blue A staining showed *Accumulibacter* related cells taken from samples from Feed D were positive for PHA storage in samples taken throughout the FEED phase. As with biomass from Feed A, after 30 min of the FEED phase *Accumulibacter, Dechloromonas* and *Defluviicoccus* related TFO cells all stained positively for PHA, although a few *Defluviicoccus* related TFO did not. However, as seen before (Section 2.7.2) toward the end of the FEED phase the *Accumulibacter* cells stained negatively for PHA, while *Dechloromonas* and *Defluviicoccus* related TFO showed a variable response to PHA staining (data not shown).

FISH/MAR results showed that the *Accumulibacter* cells taken from biomass from Feed D were positive for acetate uptake (Figure 3.31a, b), although, as noted before (Section 2.7.3.1), some cells showed a negative response, and some clusters seemed more heavily encrusted with silver grains than others (Nielsen and Nielsen 2005). The *Defluviicoccus* related TFO and *Dechloromonas* related cells also assimilated acetate as shown by FISH/MAR (Figure 3.31 e – h). The *Accumulibacter* cells were the only cells to show evidence of assimilation of P in the FAMINE phase after 3 and 6 days of development (Figure 3.31c, d), suggesting they were the major PAO populations (Kong et al. 2004).

3.9 General discussion and conclusions

Work described in this section was directed at investigating the effects of varying operational conditions of the aerated EBPR SBR on community composition. These included source of P supply, acetate supplementation concentration, sludge age, pH and temperature. In summary, results showed:

- The process was reliably removing P when the feed source was changed from synthetic wastewater to clarified effluent from a conventional activated sludge plant. When the acetate supplementation concentration was lowered to 100 mg L^{-1}, P removal failed, which corresponded to *Defluviicoccus* related cells becoming dominant in the community.

- When the sludge age was lowered from 20 d to 15 d the process continued to remove P reliably, although a second small coccoid morphology of *Defluviicoccus* related cells, not reported anywhere before, were observed.

- Decreasing operating pH from 7.5 to 6.5 led to an increase in the relative abundance of *Accumulibacter* related cells and a corresponding decrease in *Defluviicoccus* related cells, which is the opposite trend to that reported for anaerobic: aerobic EBPR communities in response to pH. Efficient P removal occurred at all three pH values.

- At 15 °C the aerated process always failed, again possibly from the *Defluviicoccus* related cells becoming dominant, although when the experiment was repeated *Defluviicoccus* relative abundances did not increase in the same way, and the process later recovered. When the feed was changed to synthetic wastewater its reliability did not improve. At 25 °C the process was again unstable, and regardless of the feed it removed P reliably for only about 30 d. At 30 °C the process again failed, an event which corresponded to considerable proliferation of *Defluviicoccus* related cells.

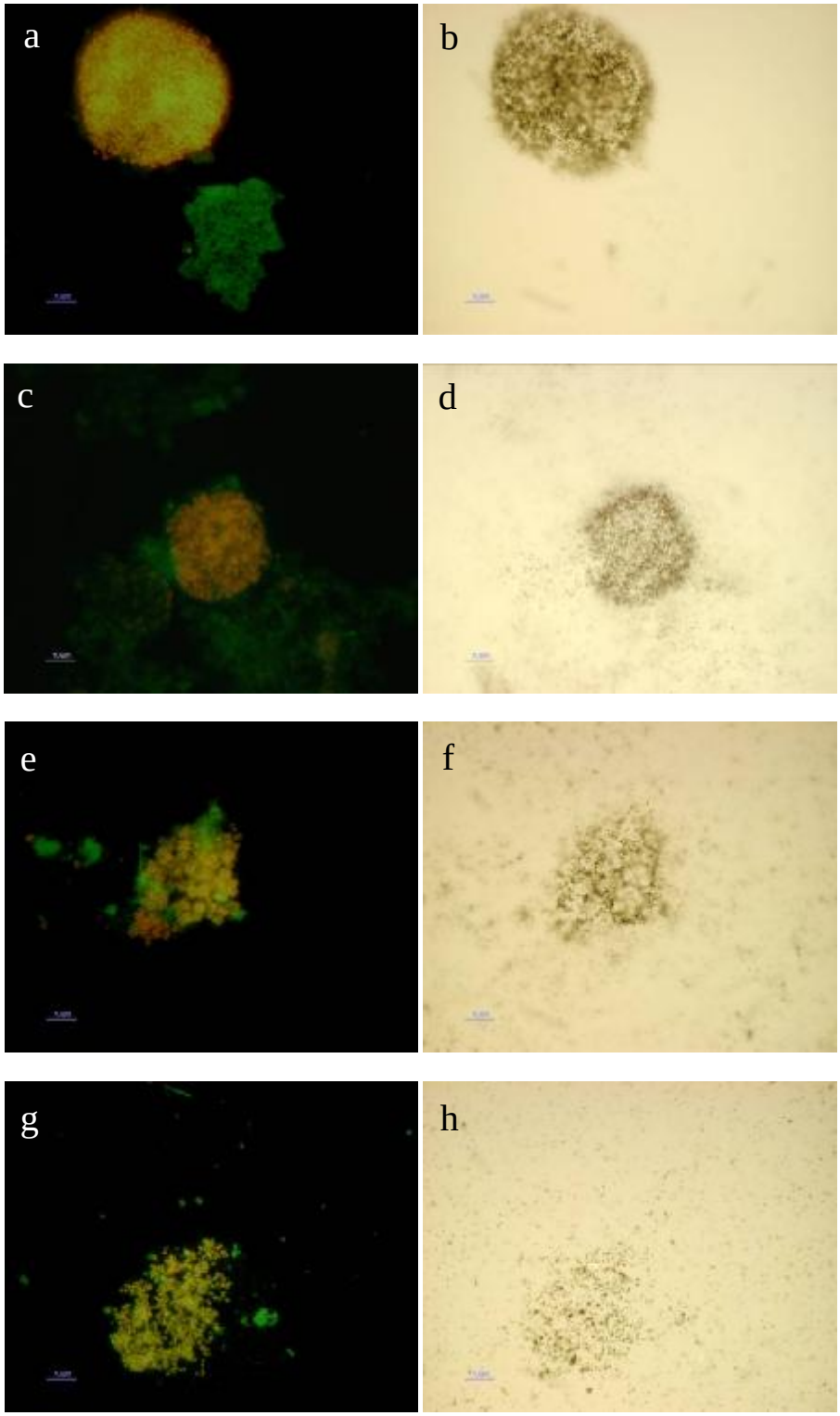


Figure 3.31 FISH micrographs and bright field images showing cells from the aerated EBPR SBR community after MAR incubations. a – d) FISH images showing cells hybridising with the EUBmix probes (FLUOS), and a and c) the PAOmix probes (Cy3) targeting *Accumulibacter* cells. b and d) corresponding field of view showing *Accumulibacter* cells assimilating b) ^{14}C acetate, and d) ^{33}P. e) *Alphaproteobacteria* cells hybridising with the ALF968 probe (Cy3). g) *Dechloromonas* related cells hybridising with the Dech454 probe (Cy3). f and h) corresponding fields of view showing cells assimilating acetate. Cells hybridising with both Cy3 (red) and FLUOS (green) labelled probes appear yellow.

- 16S rRNA DGGE profiles suggested the community compositions did not change markedly in response to any of the operational changes discussed in this section, even after P removal had failed, and FISH/MAR ecophysiological studies showed that *Accumulibacter* always remained the major PAO possessing the expected PAO phenotype. Both *Defluviicoccus* and *Dechloromonas* related cells also aerobically assimilated acetate and synthesised PHB, but never polyP, in the SBR cycle.

Possible reasons to explain these operational data in terms of shifts in the competitive abilities of the *Accumulibacter* and *Defluviicoccus* related cells remain unclear. System failure was not always associated with an increase in *Defluviicoccus* relative abundance. However, as the most likely reason was in the relative efficiencies of acetate assimilation by the two, attempts were made in the next section to see if another C source could be used to replace acetate in the FEED stage where any such advantage imparted to *Defluviicoccus* related cells would no longer operate.

Step 3.

The influence of substrate supplementation with substrates other than acetate was undertaken, to resolve which alternatives might be suitable for the process to achieve stable reliable EBPR performance. The following questions were addressed.

a) Can we achieve the same EBPR performance obtained with acetate supplementation if we use other cheaper carbon sources?

4 Ecophysiology of populations in the aerated EBPR SBR

4.1 Introduction

In the previous section (Section 3) data were presented showing that P removal in this aerated system failed under some operational conditions. Some of these failures in P removal were thought to arise from *Defluviicoccus* related TFO becoming more dominant in the reactor, as P removal failure often corresponded to an increase in their relative abundance and a corresponding decrease in the relative abundance of *Accumulibacter* related PAO. In particular, P removal failed rapidly when the acetate supplementation concentration was decreased to 100 mg L^{-1} (Section 3.4), and in some cases when the process was operated at low (15 °C), and high (30 °C) temperatures (Section 3.7). Therefore FISH/MAR was used to investigate the ecophysiology of these *Defluviicoccus* related TFO, and other populations in the process, in an attempt to better understand their role in this novel P removal process.

4.2 Aims

The aims of this section were to:

- Investigate the aerobic ecophysiology of the *Defluviicoccus* related TFO.
- Determine whether a substrate could be found which was assimilated only by the *Accumulibacter* PAO, in an attempt to control the proliferation of the *Defluviicoccus* related TFO.

4.3 Abundance of major populations

Biomass samples were taken from the aerated EBPR SBR fed clarified effluent and 120 mg L^{-1} C as acetate, operated at 20 °C with a sludge age of 20 d (described in Section 3.5 as Reactor F).

The relative abundances of the major populations in Reactor F are shown in Table 4.1.

Table 4-1 FISH analysis showing the abundance of populations in the aerated EBPR SBR. SBR was fed clarified effluent and 120 mg L^{-1} C as acetate (Reactor F) from the Melton WWTP. Abundance is biovolume percent of *Accumulibacter* PAO and *Dechloromonas* and *Defluviicoccus* related cells relative to total cells fluorescing with EUBmix. Error is standard error.

Target	Probe	Abundance (%)
Candidatus 'Accumulibacter phosphatis'	PAOmix	26.7 ± 4.0
Dechloromonas related bacteria	Dech454	10.9 ± 1.2
Cluster 2 *Defluviicoccus* related bacteria	DF988	32.9 ± 2.4
Cluster 2 *Defluviicoccus* related bacteria	DF1020	1.0 ± 0.2

4.4 FISH/MAR analysis of populations

FISH/MAR was carried out as described in Section 1.8. Samples were taken from the SBR just before the addition of acetate, and substrates (Table 1.7) were added to a final concentration of 2 mM, except for palmitate which was used at 0.5 mM, and a radioactivity of 10 uCi $mgSS^{-1}$, with biomass diluted to 1 gSS L^{-1} using filtered sludge water. Samples were incubated for 4 h with continuous shaking. Slides were incubated for 4 and 7 d, except when palmitate was used and incubation then occurred for up to 28 d to ensure no substrate assimilation was occurring. With propionate, incubations were for only 18 h, since any longer meant that the very heavy silver granule deposition on cells made visualisation of the FISH signal problematic.

4.4.1 Liquid scintillation counts

Liquid scintillation counts were performed as detailed in Section 1.7.3, to check substrate uptake by the biomass samples, ensure the washing step was sufficient to remove unassimilated substrate, and confirm that the pasteurised control cells had not taken up any substrate. Figure 4.1a shows that some substrate uptake by the biomass occurred with all tested substrates, although the assimilation levels for glucose and palmitate appear markedly lower than with the others. Figure 4.1b shows a very high substrate uptake by this biomass for propionate, compared to the control. The residual counts in samples of the supernatants were very low, indicating adequate washing in the wash step. However, the supernatant from the sample incubated with oleate showed a high count (Figure 4.1a), indicating some labelled substrate remained after the wash step. Therefore particular care was taken when examining the control slides for this substrate and comparing these with the test results.

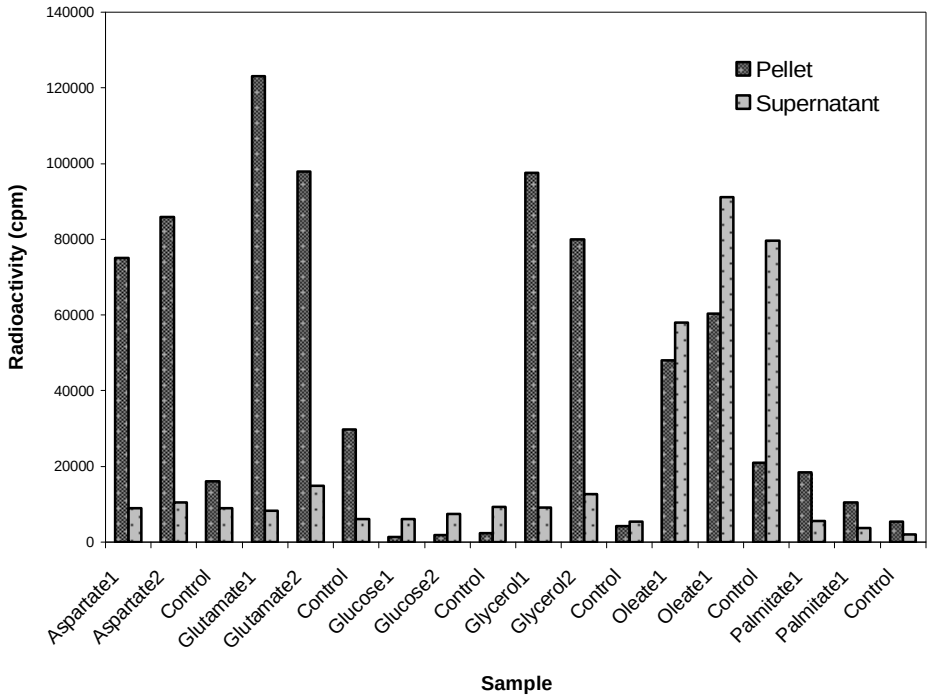
4.4.2 Substrate assimilation by populations

The substrate uptake results for the major populations in this biomass are shown in Table 4.2. At least 100 cells of each population were assessed for each determination. Based on the criteria of Burow et al. (2007) and Kong et al. (2004), cells were designated + if > 90% of clusters exhibited silver grain deposition, -/+ if between 20 - 90% of clusters exhibited silver grain deposition, and - if no silver grain deposition was seen. Cells showing the positive MAR response after 4 d development were considered to assimilate a particular substrate, following suggestions by Kong et al. (2004). No MAR signal after 7 d development was considered a negative result.

4.4.2.1 Aerobic substrate assimilation pattern of *Defluviicoccus* related TFO

Results show the *Defluviicoccus* related TFO aerobically assimilated most substrates examined (Figure 4.2a – g), except for glucose and palmitate, which were in fact not assimilated by any population in the biomass sample. Based on the assumption that under otherwise identical experimental conditions, silver grain deposition densities on individual cells reflect the levels of labelled substrates assimilated by them (Nielsen et al. 2003), assimilation of propionate by the *Defluviicoccus* related cells appeared to be very high (Figures 4.2g, h, 4.3a, b), confirming the liquid scintillation counting data (Figure 4.1). The coccoid cell morphotype targeted only by the DF988 probe was consistently covered with the highest silver grain densities, although their smaller surface area to volume ratio may be partly the reason for this (Figure 4.2a, b). Cells hybridizing with the DF1020 probe assimilated the same range of substrates as the DF988 probed cells, except that they did not assimilate oleate (data not shown).

a



b

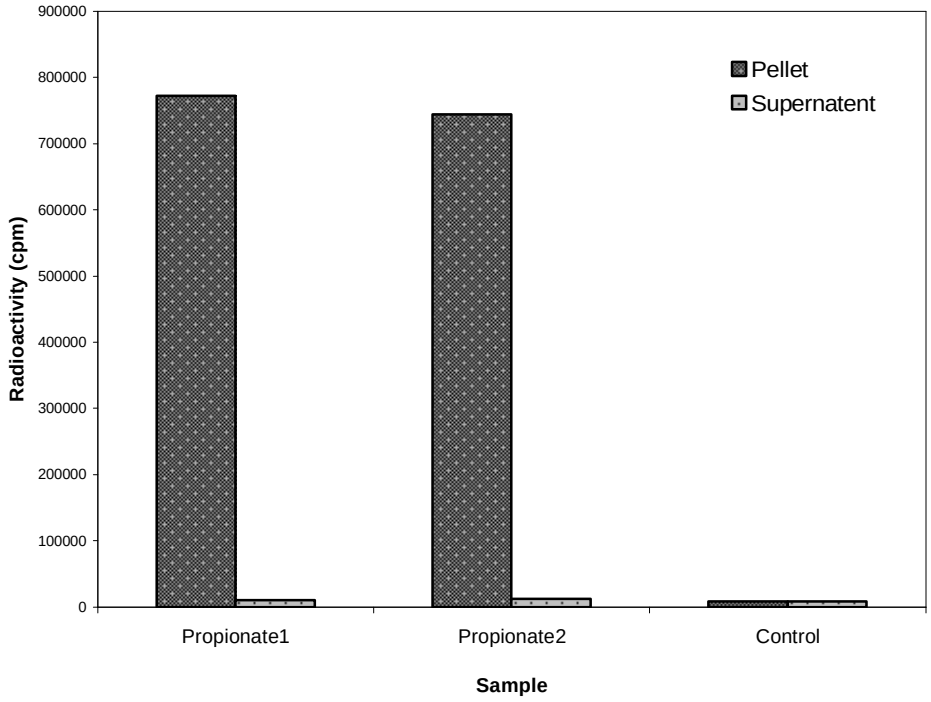


Figure 4.1 Liquid scintillation counts of biomass from the aerated EBPR SBR after MAR incubations. Counts are of: a) ^{3}H labelled substrates and b) ^{14}C labelled propionate in the biomass samples removed from the aerated EBPR SBR after 4 h incubation.

4.4.2.2 Aerobic substrate assimilation pattern of *Accumulibacter* related cells

The *Accumulibacter* related cells targeted by the PAOmix FISH probes assimilated acetate aerobically, as expected (Sections 2.7.3.1, 2.8.3.2.1 and 3.8), as well as aspartate, propionate and glutamate (Figure 4.4a – f). They did not assimilate glycerol (Figure 4.4g, h), oleate, palmitate or glucose (data not shown). As with the *Defluviicoccus* related TFO, assimilation of propionate by this population appeared to be very rapid and considerable (Figure 4.4c, d).

4.4.2.3 Aerobic substrate assimilation pattern of *Dechloromonas* related cells

FISH probed *Dechloromonas* related cells showed a similar aerobic substrate assimilation pattern to that of the *Accumulibacter* PAO, taking up acetate, aspartate, glutamate and propionate, but not oleate or glycerol. However, a positive MAR signal for aspartate and glutamate could only be seen after 7 d development. Again assimilation of propionate appeared to be high (data not shown).

4.4.2.4 Substrate assimilation pattern of Cluster 1 *Defluviicoccus* and *Candidatus* 'Competibacter phosphatis'

Too few cells fluoresced with the DF1mix and GBmix probes to obtain reliable MAR data. However, results suggest propionate (Figure 4.3c – f), acetate and possibly aspartate (data not shown) were assimilated by cells responding to probes targeting both populations.

4.5 Discussion

4.5.1 Ecophysiology of *Defluviicoccus* related cells

Results show the *Defluviicoccus* related cells never stained positively for polyP storage with DAPI, yet were positive for PHB storage when stained with Nile blue A. This agrees with data shown in Section 2.7 that the *Defluviicoccus* related cells display a feed: famine phenotype similar to that of GAO recorded in conventional anaerobic: aerobic systems. More importantly, the MAR data suggest that any attempts to control the *Defluviicoccus* in this novel aerated EBPR process by manipulating the feed composition by replacing acetate with an alternative C source are unlikely to be successful. The only substrate tested that was not utilized aerobically by the *Defluviicoccus* related cells, which was glucose, was also not taken up by the *Accumulibacter* PAO, while *Defluviicoccus* could assimilate glycerol which the *Accumulibacter* did not. Hence, on this evidence replacing acetate with cheaper C sources such as an amino acid rich protein or lipid hydrolysate for example, would seem only more likely to encourage the *Defluviicoccus* related cells at the expense of the *Accumulibacter* PAO.

Dai et al. (2007) have suggested that members of Cluster 1 *Defluviicoccus* might differ in their ecophysiological requirements from other GAO members, being able to assimilate both acetate and propionate at comparatively high rates. However, Burow et al. (2007) showed with FISH/MAR that Cluster 1 and Cluster 2 *Defluviicoccus* in full scale plants showed the same substrate assimilation patterns. Different substrates were tested to those used here, but when the aerobic FISH/MAR results obtained in my study for the Cluster 2 *Defluviicoccus* GAO are compared with these data, they strengthen the view that physiological differences may exist among strains within this group. For example, the Cluster 2 probed *Defluviicoccus* cells in this study rapidly assimilated propionate. Yet Burow et al. (2007) showed under aerobic conditions that cells in samples from full scale anaerobic: aerobic EBPR plants responding to both the DF988 and DF1020 probe mix for Cluster 2 *Defluviicoccus* gave a positive result for propionate only after extended development times. This is consistent with a much slower assimilation rate than for acetate. Such a result is surprising, especially since Oehmen et al. (2005b) and Dai et al. (2007) had shown that their alphaproteobacterial TFO and *Defluviicoccus* related TFO assimilated propionate anaerobically preferentially to acetate. This observation was exploited by Meyer et al. (2006), allowing the TFO in their community to be identified as *Defluviicoccus* by stable isotope

Table 4-2 Substrate utilization patterns of the putative *Accumulibacter* PAO and *Defluviicoccus* and *Dechloromonas* related cells from the aerated EBPR SBR.

Probe/ Substrate	PAOmix	Dech454	DF988 (small cocci)	DF988 (TFO)	DF1020	DF1mix	GBmix
Acetate	+	+	n/a	+	+	+	+
Aspartate	+	(+)	+	+	+	-/+	-/+
Glutamate	+	(+)	+	+	+	-	-
Glycerol	-	-	+	+	(+)	-	-
Oleate	-	-	(-/+)	(-/+)	-	-	-
Propionate	+	+	+	+	+	+	+
Palmitate	-	-	-	-	-	-	-
Glucose	-	-	-	-	-	-	-

() indicate results after 7 d incubation, + indicates more than 90% of clusters exhibited silver grain deposition, -/+ indicates between 20 - 90% of clusters exhibited silver grain deposition, and - indicates no silver grain deposition was seen.

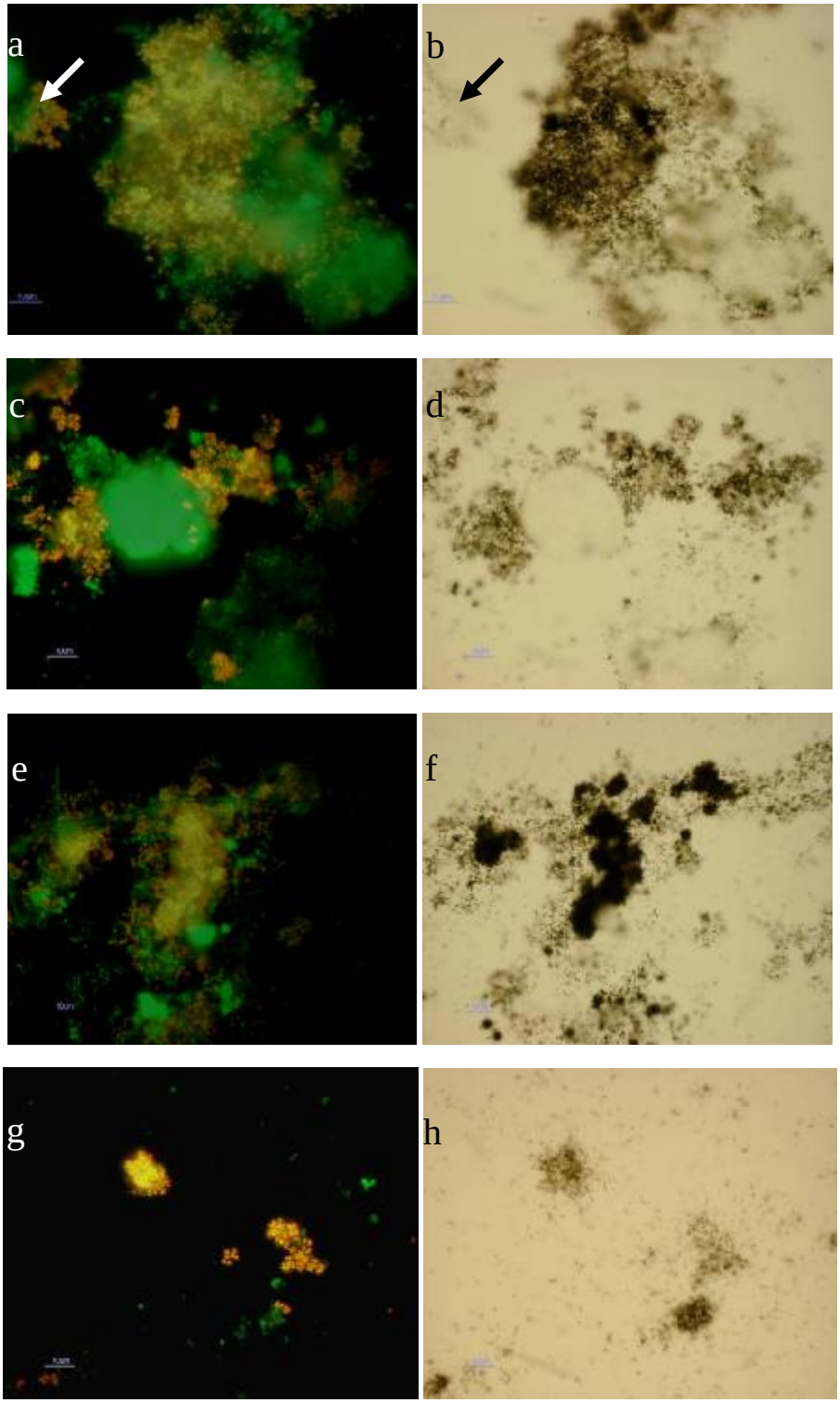


Figure 4.2 FISH and brightfield micrographs showing aerobic substrate assimilation by the *Defluviicoccus* related cells from the aerated EBPR SBR. a, c, e, g) FISH micrographs showing a, c, e) cells hybridising with the DF988 probe (Cy3), g) cells hybridising with the DF1020 probe (Cy3). b, d, f, h) Corresponding fields of view showing cells ability to assimilate aerobically: b) aspartate, note the arrow indicates the weaker uptake by the large *Defluviicoccus* related cocci in tetrads compared to the smaller coccal cells, d) glutamate, f) glycerol, and h) propionate. Cells are also hybridised with the EUBmix probes (FLUOS), and appear yellow when hybridised with FLUOS and Cy3.

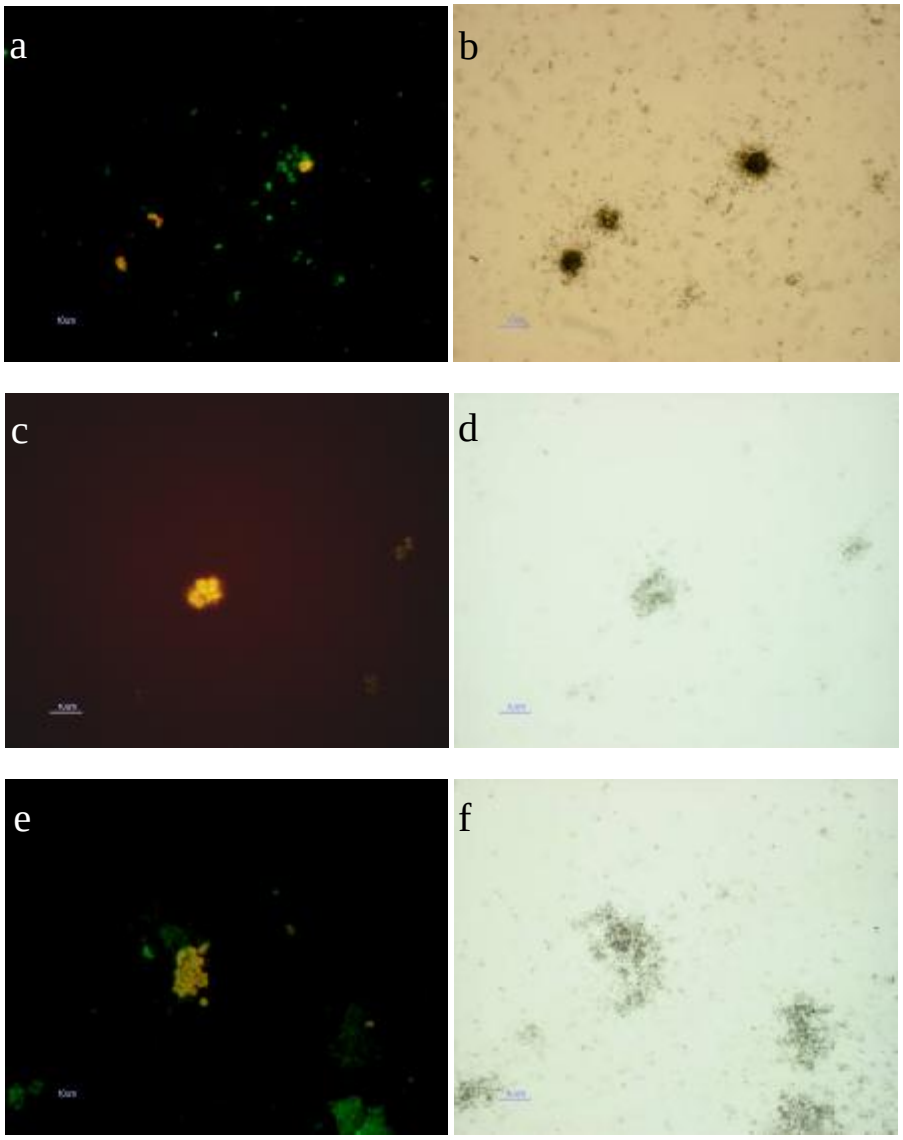


Figure 4.3 FISH and brightfield micrographs showing substrate assimilation by cells from the aerated EBPR SBR. a, c, e) FISH micrographs showing cells hybridising with the a) DF1020 probe targeting Cluster 2 *Defluviicoccus* related cells (Cy3), c) DF1mix probes targeting Cluster 1 *Defluviicoccus* related cells (Cy3), e) GBmix probes targeting *Candidatus* 'Competibacter phosphatis' (Cy3), and EUBmix probes (FLUOS). Cells hybridising with both FLUOS and Cy3 labelled probes appear yellow. b, d, f) Corresponding fields of view showing the cells ability to assimilate propionate.

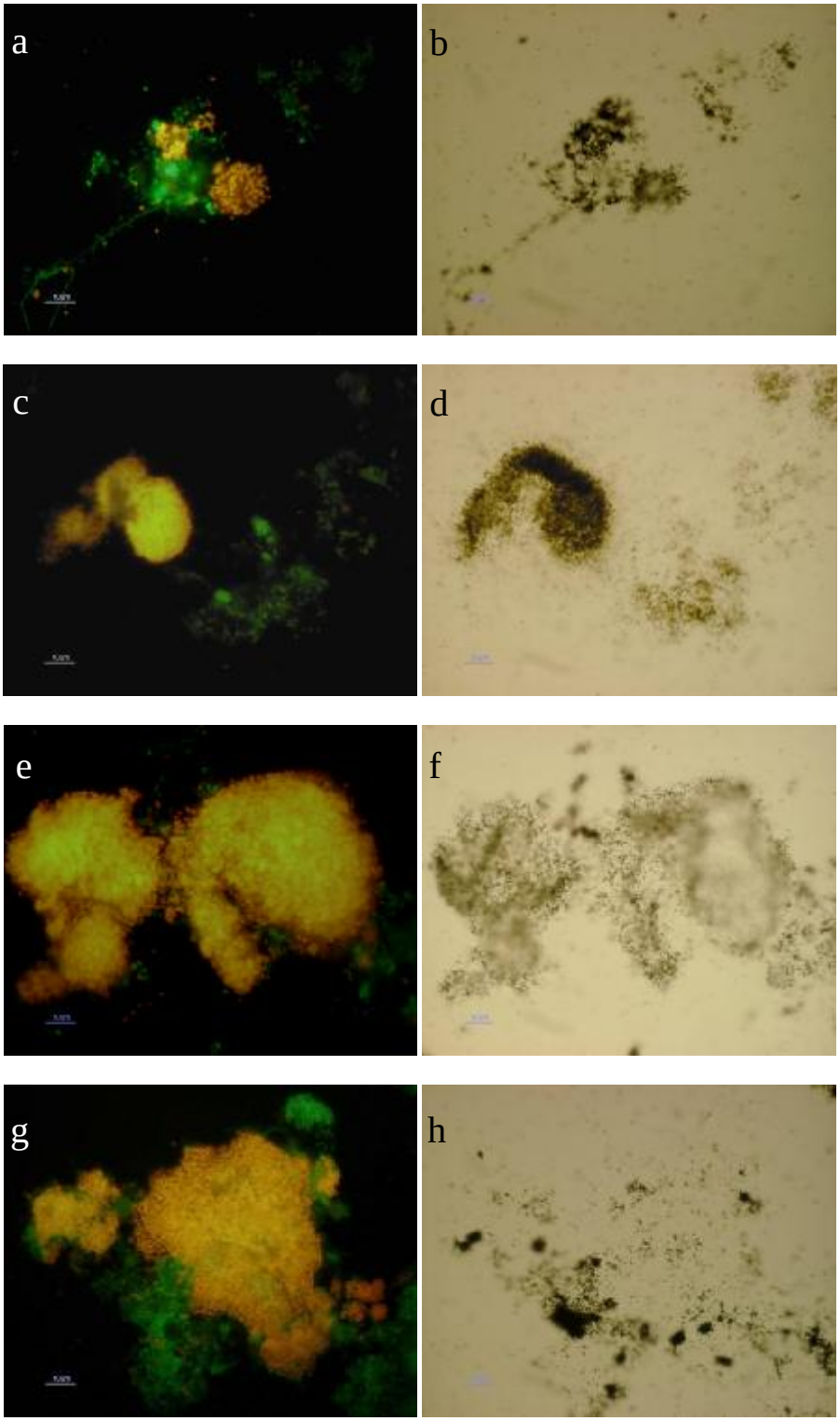


Figure 4.4 FISH and brightfield micrographs showing substrate assimilation by the *Accumulibacter* related cells from the aerated EBPR SBR. a, c, e, g) FISH micrographs showing cells hybridising with the PAOmix probes (Cy3) and EUBmix probes (FLUOS). b, d, f, h) Corresponding fields of view showing the cells ability to assimilate: b) aspartate, d) propionate, and f) glutamate, and h) *Accumulibacter* cells not assimilating glycerol. Cells hybridising with both FLUOS and Cy3 labelled appear yellow.

probing with a ^{13}C-propionate feed. Furthermore, using FISH/MAR, Wong and Liu (2007) also reported both high anaerobic and aerobic propionate uptake in their EBPR community by Cluster 1 *Defluviicoccus*. There were other marked differences between the aerobic ecophysiological data for Cluster 2 *Defluviicoccus* reported here and those obtained by Burow et al. (2007). Unlike the Cluster 2 *Defluviicoccus* characterized here, those in their study also assimilated glucose (albeit relatively weakly, based on silver grain deposition levels), but neither aspartate nor glutamate.

These discrepancies in substrate assimilation patterns among the *Defluviicoccus* related cells may reflect the specificity of the FISH probes used, which target phylotypes, not individual strains. Thus, each may impart fluorescence to several strains sharing their target sites, but differing in their phenotypic properties. On the other hand, the selective pressures brought to bear on the populations in conventional anaerobic: aerobic EBPR processes and this aerated process may select for strains with different ecophysiological traits.

4.5.2 Ecophysiology of other populations

The aerobic substrate assimilation pattern of *Accumulibacter* in this aerated system are similar but not identical to the anaerobic MAR assimilation patterns of *Accumulibacter* seen in full scale systems, where they were able to assimilate acetate, aspartate, glutamate and propionate, but not glucose and palmitate (Kong et al. 2004; Chua et al. 2006; Eales 2006). Even though they shared the same substrate assimilation profile with *Accumulibacter*, the FISH/MAR data presented here, based on silver grain deposition densities, suggest that *Dechloromonas* could not compete as effectively for acetate with it or with the *Defluviicoccus*, which is contrary to that found earlier with Feed A (Section 3.7.3.1). Kong et al. (2007) showed by FISH/MAR that *Dechloromonas* in a full scale EBPR plant showed the same substrate assimilation pattern as those examined in this study, although different FISH probes were used. Data from my study also suggest that the Cluster 1 *Defluviicoccus* and *Competibacter* cells in this aerated process assimilated acetate and propionate, and some cells assimilated aspartate. Other work has shown *Competibacter* cells could assimilate acetate and propionate aerobically, although they did not assimilate aspartate (Kong et al. 2006). The Cluster 1 *Defluviicoccus* related cells also showed similarities in their substrate uptake profiles with those found in full scale systems, and assimilated acetate and propionate, although aspartate was not taken up (Burow et al. 2007; Wong and Liu 2007).

4.6 Conclusion

The explanation as to why increasing *Defluviicoccus* population sizes (Sections 4.4 and 4.7) seems to parallel some falls in P removal capacity in this EBPR process is not clear from these MAR data. Many models evaluating competition between the PAO and GAO for anaerobic: aerobic EBPR systems (Oehmen et al. 2007; Whang et al. 2007) are based on the assumption that *Accumulibacter* does not compete as well for acetate as *Defluviicoccus* does. However, its whole genome sequence (García Martín et al. 2006) tends to suggest that it possesses very effective acetate transport systems, providing it with a strong competitive ability in EBPR processes. Furthermore, the metabolic data of Burow et al. (2008b) suggest a much faster anaerobic acetate assimilation rate by *Accumulibacter*.

Therefore it is still not clear why the *Defluviicoccus* related cells were able to out–compete the *Accumulibacter* related populations when operational conditions in the aerated EBPR SBR were changed, and especially when acetate concentrations were lowered to 100 mg L^{-1}. The *Defluviicoccus* related cells may acquire a competitive advantage under some operating conditions by having a higher aerobic conversion factor for biomass production from their stored PHA than the *Accumulibacter* PAO, as suggested by Whang et al. (2007). Experiments need to be performed with a quantitative method like NanoSIMS, where substrate uptake rates can be estimated at a single cell level (Li et al. 2007). Metagenomic analyses of both Cluster 1 and 2 *Defluviicoccus* strains may also help to clarify these important questions.

The next step in this study was to scale up the laboratory scale SBR process up to 150 L pilot plant stage, receiving effluent from a conventional non-EBPR full scale WWTP.

Step 4.

The final stage in this project was to use the data from steps 1-3 to set up and run a pilot plant (150-200 litres) SBR system under the conditions optimized in the earlier stages to attempt to achieve long term aerobic EBPR using effluent from a conventional treatment plant. The following questions were addressed.

a) Are the results obtained in small scale SBRs achievable at larger scales?

b) Do the microbial community composition and P accumulating populations change with changes in scale/

c) Can we achieve similar stability and long term reliability of this process?

d) Is this process now suitable to test at a larger scale at a full-scale activated sludge plant?

5 Pilot plant studies

5.1 Introduction

Results from previous sections (Section 2 and 3) have shown this aerated EBPR community was capable of removing P to barely detectable levels when supplemented with acetate, regardless of whether it was fed a synthetic wastewater feed or clarified effluent from the Melton non-EBPR activated sludge plant with P levels of approx. 10 – 12 mg L^{-1}. The next step was to scale up this SBR process to pilot plant stage, so it could be attached to the end of a conventional non-EBPR WWTP, and its effectiveness in polishing this effluent to remove P monitored.

5.2 Aims

The aims of this section were to:

- Investigate the community composition of this novel aerated process when scaled up to pilot plant stage, especially to identify the major PAO and GAO populations by FISH, and use DGGE analysis to profile the communities developed under these conditions.

5.3 Pilot plant operation

The pilot plant (Figure 5.1) was situated to treat the clarified effluent leaving the Eastern Treatment Plant (ETP), Carrum, Victoria, Australia. The ETP plant is a Lutzig-Ettinger configured system, with nitrification/denitrification ability, and treats the wastewater from the approx. 1.5 million people in the Eastern suburbs of Melbourne, Victoria, Australia. More information can be found in Table 5.5.

P-containing secondary effluent feed was obtained from the Eastern Treatment Plant (ETP), The secondary effluent had a P level that varied between approx. 8 and 15 mg P L^{-1}. The effluent was supplemented with 15 mg N L^{-1} as NH_4Cl, as it only contained approx. 1 mg N L^{-1}. Based on the synthetic medium of Bayly et al. (1991), $MgCl_2.6H_2O$ (260 mg L^{-1}) and $CaCl_2.2H_2O$ (33 mg L^{-1}) were also added to the ETP effluent to avoid possible Ca^{2+} and Mg^{2+} limitation known to affect EBPR capacity.

The SBR pilot plant consisted of a 200 L plastic reactor vessel with a working volume of 150 L. Contents were mixed with a stirrer (Lightning Mixer EV5P37, I.B. Engineering, Australia) controlled with a variable speed drive (Toshiba VF-S11, I.B. Engineering, Australia), which was operated at 300 revolutions per minute (rpm) during the mixing period. Aeration used a 150 L min^{-1} aquarium air pump (LP-100, Age of Aquariums, Australia), modified with a voltage controller for adjusting aeration rate, connected to an Elastolox (Aquatec-Maxcon Pty Ltd,

Australia) diffuser attached to the reactor base. Mixed liquor pH, dissolved oxygen and redox were monitored with Global Water probes (Kenelec Scientific, Australia), and temperature was monitored with an LM35 precision temperature sensor (Jaycar, Australia). Data from these sensors was logged with an HOBO U12-006 (One Temp, Australia) data logger. SBR cycle sequence control was set with a programmable logic controller (AF-20MR-D, One Temp, Australia). A 2,500 L rainwater tank was filled weekly with the clarified secondary effluent from the ETP plant. The SBR reactor was fed the clarified effluent from a 100 L plastic tank, filled by a submersible pump from the rainwater tank. A simple gravity feed system ensured that 75 mL was added at the appropriate time each SBR cycle.

The start up inoculum for the pilot plant was obtained from the MUCT EBPR plant located in Kyneton, Victoria, Australia. The pilot plant was inoculated with approx. 100 L of RAS with a typical mixed liquor suspended solids (MLSS) of 10,000 mg L^{-1}. Startup involved operating the pilot plant in batch mode with acetate fed in at the operational concentration (120 mg L^{-1} C as acetate) every 6 h for 3 d. An identical start up to that of the laboratory scale SBRs could not be achieved, due to limited accessibility to the pilot plant site. Following this period, full operational cycling was commenced, as detailed below.

Cycle time was 6 h, with each cycle consisting of five stages. Stage 1 (FAMINE): 75 L of P containing effluent was rapidly (1 min) gravity-fed into the reactor, 60 min allowed for P uptake. Stage 2: 2.5 L mixed liquor was removed from the reactor (2 min) to achieve a sludge age of 15 d.

Stage 3 (FEED): 1 min to pump acetate (200 mL) into the reactor and 240 min allowed for acetate utilisation. Stage 4: biomass was allowed to settle for 45 min. Stage 5: 75 L of effluent was removed over a 15 min period. Aeration and mixing were stopped during stages 4 and 5.

The reactor was supplemented in the FEED stage with 120 mg L^{-1} C as acetate, with a sludge age of 15 d, chosen from the laboratory scale experimental data. Temperature and pH were not controlled but were constantly monitored. Figure 5.2 shows a schematic of how the plant was designed to operate.

5.3.1 P removal performance

After the initial run, over a six month period the pilot plant was never able to establish prolonged continued reliable high levels of P removal. The data presented here in detail are those obtained from Run D. However, essentially the same results were obtained from repeated experiments over this operational period, even though process failure times varied with each run. Table 5.1 shows summarised operational data from all five runs of the pilot plant.

During Run D, P removal failure had occurred by day 12. The effluent P profile is given in Figure 5.3. The acetate feed level was increased from 120 mg L^{-1} to 160 mg L^{-1} on day 15, in an attempt to recover P removal capacity. As none was present in the effluent, it was also supplemented with 15 mg L^{-1} N, on day 1 NH_4Cl, Mg^{2+}, and Ca^{2+} were added to the ETP effluent in attempts to overcome possible Mg^{2+} and Ca^{2+} limitation known to affect P removal (Seviour et al. 2003). The MLSS decreased from 5.2 g SS L^{-1} on day 1 to 2.4 g SS L^{-1} by day 23, and volatile SS (VSS) levels decreased from 0.92 g VSS L^{-1} on day 9 to 0.19 g VSS L^{-1} on day 23. A similar marked drop in MLSS/VSS levels was recorded for all the pilot plant runs. The dissolved oxygen level dropped to approx. 5% during acetate addition (Figure 5.4), as seen previously in laboratory scale experiments (Section 2.4), but then increased to 80% saturation. The average temperature during the run was 20 °C, and the pH 6.8.

Figure 5.5 shows a typical chemical profile from run D after it had been operating unsuccessfully for 23 d. The profile shows biomass polyP levels were very low at < 1% (w/w) biomass dry weight, even though acetate was removed rapidly by the community and used for PHB synthesis and possibly for cell growth. No P release or reassimilation was measurable during the acetate feed stage, resulting in no net P removal, and hence EBPR failure. However, PHB levels

Figure 5.1 Photographs of the aerated EBPR SBR pilot plant located at Eastern Treatment Plant, Carrum, Victoria, Australia. Photographs show: a) side view, and b) top view of the 200 L plastic drum and 2,500 L tank containing secondary effluent fed to the SBR

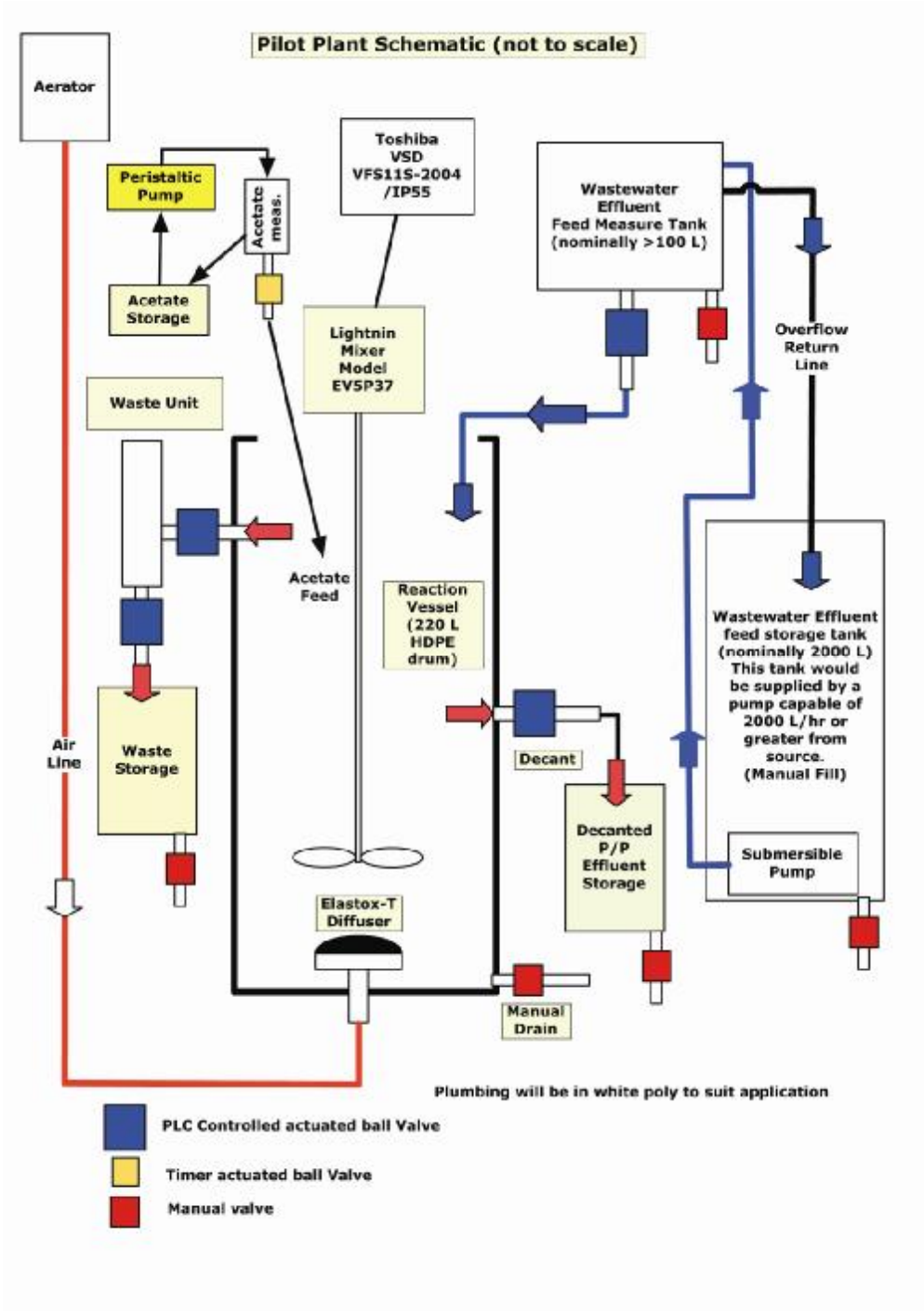


Figure 5.2 Schematic of the aerated EBPR SBR operating in pilot plant mode at Eastern Treatment Plant, Carrum, Victoria, Australia. Diagram courtesy of Ken Lindrea.

Table 5-1 Operational data from Runs A - E of the aerated EBPR SBR pilot plant. Pilot plant was operated at ETP treatment plant from Summer 2007 – Winter 2008 using ETP treatment plant effluent with 120 mg L^{-1} C as acetate. (Data provided by B Campbell).

Run	Days of operation (d)	Successful P removal (d)	MLSS[1] start (g L^{-1}) (day measured)	MLSS final (g L^{-1}) (day measured)	Average pH	Average temp (°C)
A	36	26	1.1 (12)	0.3 (19)	7.5	21.5
B	12	4	4.4 (1)	2.3 (12)	7.0	22.5
C	25	0	5.6 (1)	0.2 (25)	7.2	21.6
D	27	12	5.2 (1)	2.8 (27)	6.8	20.4
E	22	5	9.7 (1)	2.5 (22)	6.8	17.6

[1]MLSS = mixed liquor suspended solids.

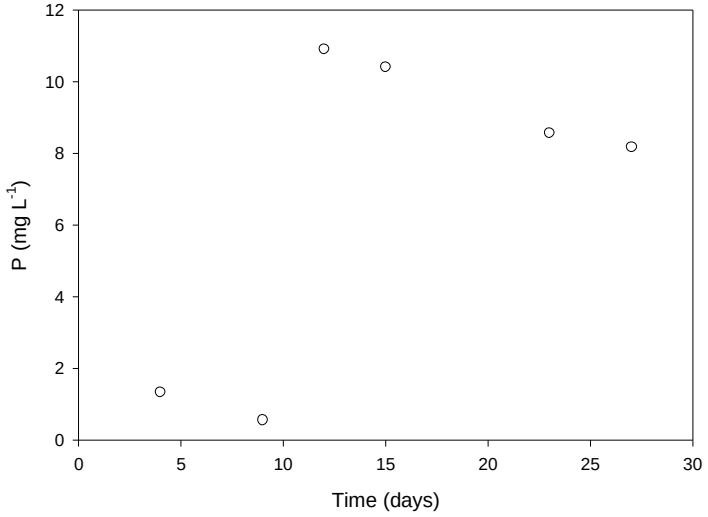


Figure 5.3 Time profile showing levels of P in the final effluent of the aerated EBPR pilot plant. Profile is taken from Run D using ETP treatment plant effluent with 120 mg L^{-1} C as acetate. (Data provided by B Campbell).

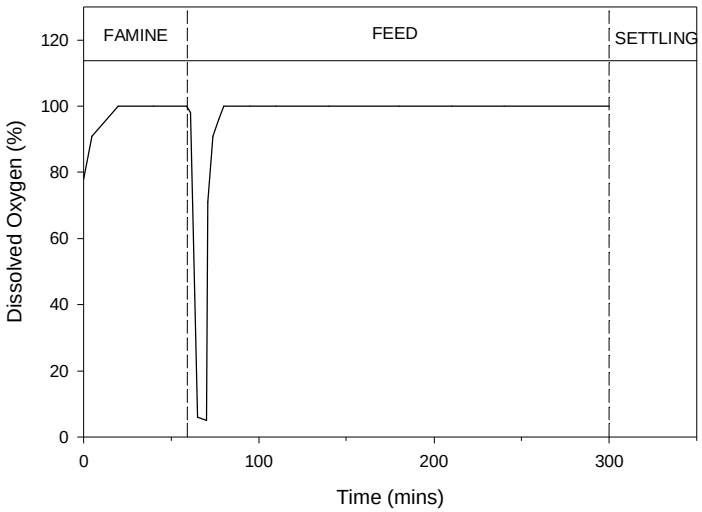


Figure 5.4 Dissolved oxygen profile over a FEED: FAMINE cycle for the aerated EBPR SBR pilot plant. Profile is taken during Run D when fed ETP effluent with 120 mg L^{-1} C as acetate. (Data provided by B Campbell).

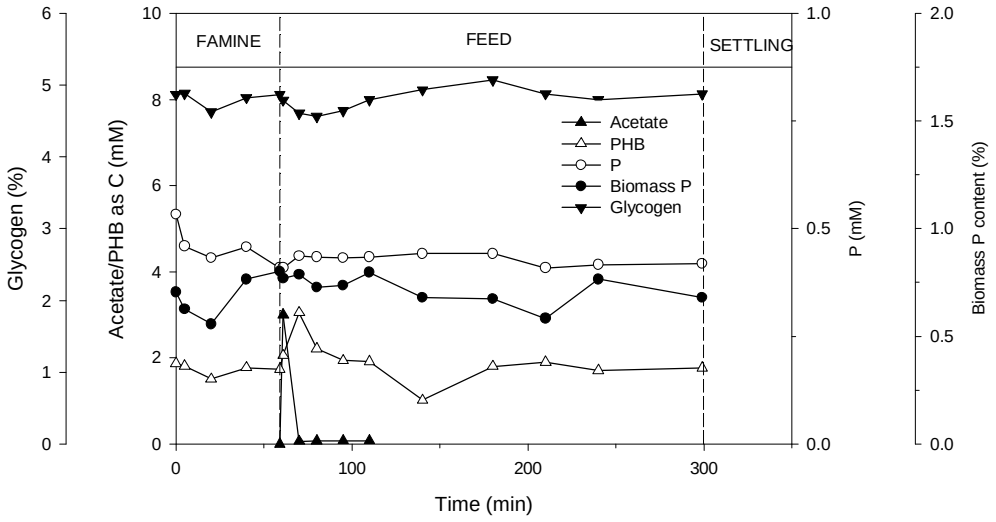


Figure 5.5 Chemical profile of a cycle from the aerated EBPR pilot plant. Profile is taken during Run D using ETP treatment plant effluent with 120 mg L^{-1} C as acetate when it was not removing P after 23 d. Changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) are shown over a FEED: FAMINE cycle. (Data provided by B Campbell).

appeared to increase slightly corresponding to acetate assimilation, and as these fell after acetate exhaustion from the medium, where the PHB was probably being respired by the community as an energy source. Glycogen biomass levels changed little over the SBR cycle, and the profiles were similar to those seen earlier in the aerated lab scale reactors (Sections 2 and 3). This suggests that glycogen transformations so striking in the feed: famine stages of anaerobic: aerobic EBPR processes were not occurring in this reactor to the same extent.

5.4 Community composition of populations in the aerated pilot plant

5.4.1 Community composition by FISH analysis

FISH analyses of the community present on day 1 of Run D revealed that it was similar to that seen in the laboratory scale SBR EBPR community described earlier (Section 2.5, 2.8.2, 3.4 – 3.7), using inoculum from the same source (Kyneton WWTP). Thus, it was dominated by clustered *Accumulibacter* cells, which made up 13.4 ± 0.5% of the total biovolume of cells fluorescing with the EUBmix probes (Figure 5.6a). As before, *Accumulibacter* cells stained positively and negatively with DAPI for polyP storage, suggesting not all were actively functioning as PAO in this pilot plant community (data not shown). A few (<1% total biovolume) actinobacterial cells were also present, and some of these also stained positively for polyP with DAPI (data not shown). Members of the Cluster 2 *Defluviicoccus* related cells were again present (Figure 5.6c), although they made up < 1% of the community biovolume. No Cluster 1 *Defluviicoccus* related cells were detected, and only a few gammaproteobacterial *Competibacter* cells were present, again agreeing with the earlier laboratory scale SBR microbiological data (Section 2.5, 2.8.2, 3.4 – 3.7). Far fewer *Dechloromonas* related cells than were present in the laboratory-scale process (Section 2.5, 2.8.2, 3.4 – 3.7) were detected. Other populations revealed by FISH analysis are given in Table 5.2.

5.4.2 Community composition by FISH analysis of the community not removing P

When P removal had failed, there was a corresponding marked change in the community composition. FISH analyses of a biomass sample taken after 27 d showed that the PAO population had become numerically far less important. Thus, *Accumulibacter* PAO (Figure 5.6b), most of which had stained positively for polyP, had decreased to 4.3 ± 0.6% of the biovolume, and Cluster 2 *Defluviicoccus* related cells were barely detectable. The contributions of other populations of interest to this community are shown in Table 5.2. Most importantly, the community was dominated by large numbers of coccobacilli approx. 2 µM in diameter (Figure 5.6d). These failed to hybridize with the FISH probes designed to target the *Alpha-* and *Betaproteobacteria,* but hybridised with the Gam42a probe for members of the *Gammaproteobacteria.* However, none responded positively to the GB and GAOmix FISH probes designed to target all the *Competibacter* GAO so far described (Crocetti et al. 2002; Kong et al. 2002). FISH analyses of biomass samples taken during the other runs of the pilot plant over a six month period showed that failure of P removal was invariably accompanied by dominance of these large cocci, as shown in Table 5.3.

5.4.3 Community composition by 16S rRNA clone library analysis

Attempts were made to identify these gammaproteobacterial cells using a fluorescence activated cell sorter (FACS) to enrich the population, as this had proved successful in assisting with identifying other EBPR activated sludge populations in our laboratory (McIlroy et al. 2008a). This work was carried out by Simon McIlroy (La Trobe University) and Ken Field (University of Melbourne). The cells of interest were fluorescently labelled with the Gam42a probe. After flow cytometric sorting, the resulting population was made up largely of Gam42a positive cocci (data not shown). A clone library of rRNA sequences from the enriched FACS sorted gammaproteobacterial cells was constructed by Dr S Petrovski (La Trobe University) as detailed in Section 1.3. This clone library revealed that of the ten clones sequenced, nine were members of the *Gammaproteobacteria,* with the other 16S rRNA sequence most closely related to the

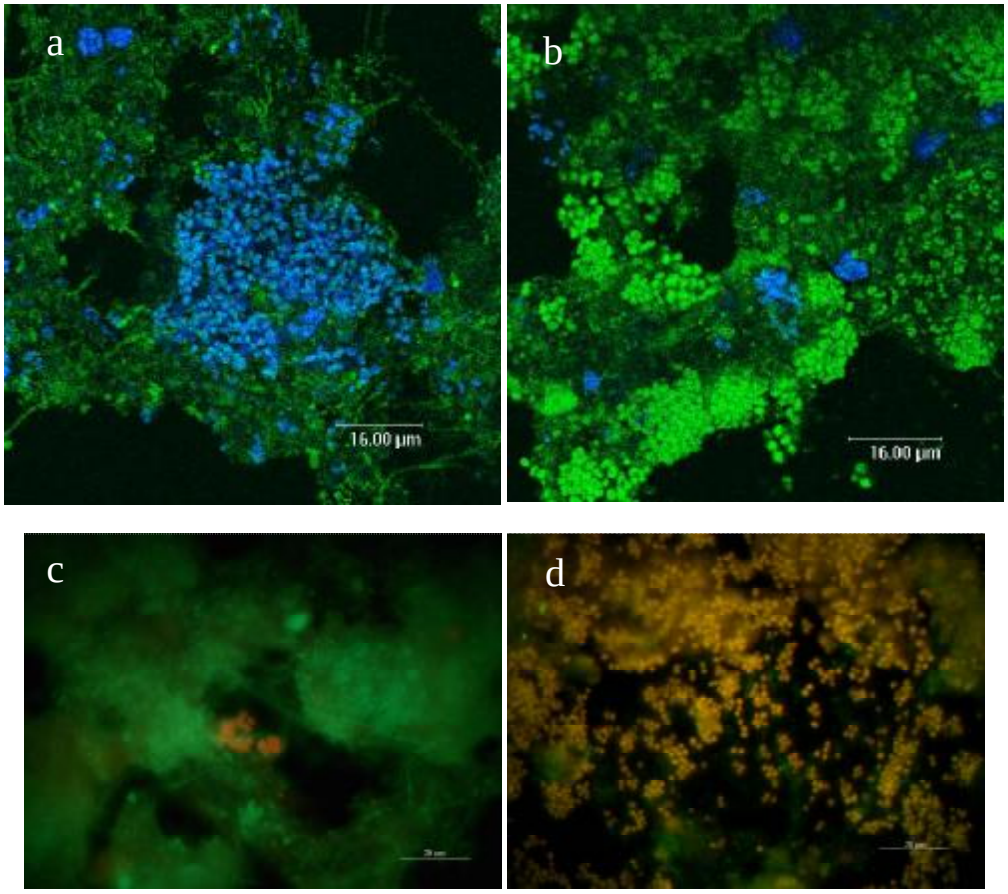


Figure 5.6 Micrographs of cells from the aerated EBPR pilot plant. Community was fed ETP treatment plant effluent with 120 mg L^{-1} C as acetate. a and b) Cells hybridising with the PAOmix probes (Cy5) targeting *Accumulibacter* when the pilot plant was a) removing P and b) not removing P. c and d) Cells hybridising with the c) DF2mix probes (Cy3) and d) Gam42a probe (Cy3) targeting *Defluviicoccus* and *Gammaproteobacteria* respectively when the plant was not removing P. Cells are also hybridised with the EUBmix probes (FLUOS). Cells hybridised with FLUOS (green) and Cy3 (red) appear yellow, with FLUOS and Cy5 (blue) appear light blue.

Table 5-2 FISH analysis of populations present in the aerated EBPR pilot plant when P was being removed and when P removal had failed. Pilot plant was operated using ETP treatment plant effluent with 120 mg L^{-1} C as acetate.

Target	Probe(s)	Removing P (Day 1)	P removal failed (Day 27)
Betaproteobacteria			
Dechloromonas related cells	Dech454	+	+
Alphaproteobacteria			
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	-/+	-
Gammaproteobacteria			
Gammaproteobacteria – cocci cells	Gam42a_mix	-	+++
Gammaproteobacteria – other	Gam42a_mix	+++	+++
Subgroup 1 and 2 of group GB	GBmix	+	+
Other			
Actinobacteria	HGC69a	++	+

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

Table 5-3 FISH analysis of populations present in the aerated EBPR pilot plant over a six month period from Summer 2007 – Winter 2008. Pilot plant was operated using ETP treatment plant effluent with 120 mg L^{-1} C as acetate.

Target	Probe(s)	Run A start	Run A end	Run B start	Run B end	Run C start	Run C end	Run E start	Run E mid	Run E end
Betaproteobacteria										
Candidatus 'Accumulibacter phosphatis'	PAOmix	+++	+++	++/+++	+++	+++	+	+++	++/+++	++
Dechloromonas related cells	Dech454	+++	nd	-	+	+	nd	+	+	-
Alphaproteobacteria										
Cluster 1 *Defluviicoccus* related bacteria	DF1mix	-	-	-	+++	-	nd	+	-	-
Cluster 2 *Defluviicoccus* related bacteria	DF2mix	+	++/+++	++	++	+	-	++	+/++	+/++
Gammaproteobacteria										
Gammaproteobacteria – cocci	Gam42a_mix	+	+++	++	+++	++	+++	+	+	+++
Gammaproteobacteria - other	Gam42a_mix	+	+	+++	+++	++	++	+++	+++	+++
Subgroup 1 and 2 of group GB	GBmix	nd	+	+	+	+	-	+	+	+
Other										
Actinobacteria	HGC69a	+/++	+	+	+	+	+	++	++/+++	++

nd = no data

– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

alphaproteobacterial *Sphingomonas*. All these gammaproteobacterial sequences were closely related to members of the genus *Ectothiorhodospira*, although BLAST (Altschul et al. 1997) searches revealed the sequence similarity was low at only 92%, suggesting they were members of another taxon. Phylogenetic analysis separated these gammaproteobacterial sequences into two OTUs (based on 99% relative sequence similarity cut off), with six sequences in OTU 1 and three in OTU 2, and both clustered closely together, but quite distinct from the cluster containing *Candidatus* 'Competibacter phosphatis' (Figure 5.7).

5.4.4 DGGE analysis of community composition in the aerated EBPR pilot plant

DGGE analysis was carried out as detailed in Section 1.4, using a denaturation gradient of 40 – 80%. Analysis showed that considerable population diversity was present in all the biomass samples taken from the pilot plant communities in the different runs, as judged by the number of fragments in each profile (Figure 5.8). Importantly, these DGGE patterns also showed marked changes under conditions of good and poor EBPR, essentially confirming the FISH data. Thus, profiles showed that bands 4 and 5 (Figure 5.8) corresponding to the 16S rRNA gene fragments recovered from clones of members of OTU 1 and OTU 2 clusters respectively, fluoresced strongly from samples taken when the pilot plant was not removing P, but much less intensely in those taken when P removal was occurring. A fragment corresponding to one of the *Accumulibacter* clone sequences obtained from the laboratory scale aerated reactor operating at 160 mg L^{-1} C as acetate (see Section 3) (Figure 5.8, band 1) was also present in all community samples, with the exception of the final run of the pilot plant, where no P removal could be measured. Bands corresponding to a second *Accumulibacter* clone sequence from the laboratory scale aerated EBPR process operating at 120 mg L^{-1} C as acetate (Figure 5.8, band 2) did not appear in the profiles from any of the community DNA samples.

5.5 FISH Probe design and evaluation

5.5.1 Probe design

FISH probes were then designed as detailed in Section 2.7 to target the 16S rRNA of members of these two OTUs. Two probes, DES 447 and DES 448, were designed initially against members of OTU 1 and OTU 2 respectively, based on their almost complete 16S rRNA sequences. However, when checked with BLAST (Altschul et al. 1997) searches of all deposited sequences, each of the selected probe target sequences was detected in a few partial sequences (< 600 bp) from other populations. These sequences have been incorporated into the phylogenetic tree to show their relative positions (Figure 5.7). They included a partial sequence of a clone (AB205654) obtained from an acetate fed nitrifying community after stable isotope probing, which contained the target site for the DES 448 probe. However, this sequence was overall < 90% similar to the almost full 16S rRNA sequences of both OTU 1 and 2 members, but instead was most closely (93%) related to *Thauera terpenica*, a member of the *Betaproteobacteria*. Therefore, a third probe, DES 841, was designed to target the whole cluster, but to exclude the AB205654 sequence. It was unclear whether two other partial sequences obtained from an EBPR SBR and a DGGE band from an SBR (AF245331 and EF015280 respectively) were also targeted by the DES 841 FISH probe as the sequence data deposited did not include the target site for this probe (indicated by dashed lines in Figure 5.7). As the DES 841 probe target site was located in the low fluorescence region V of the 16S rRNA (Fuchs et al. 1998), two helper probes were designed on either side of its target site to increase probe accessibility (Fuchs et al. 2000), and their sequences are given in Table 5.4.

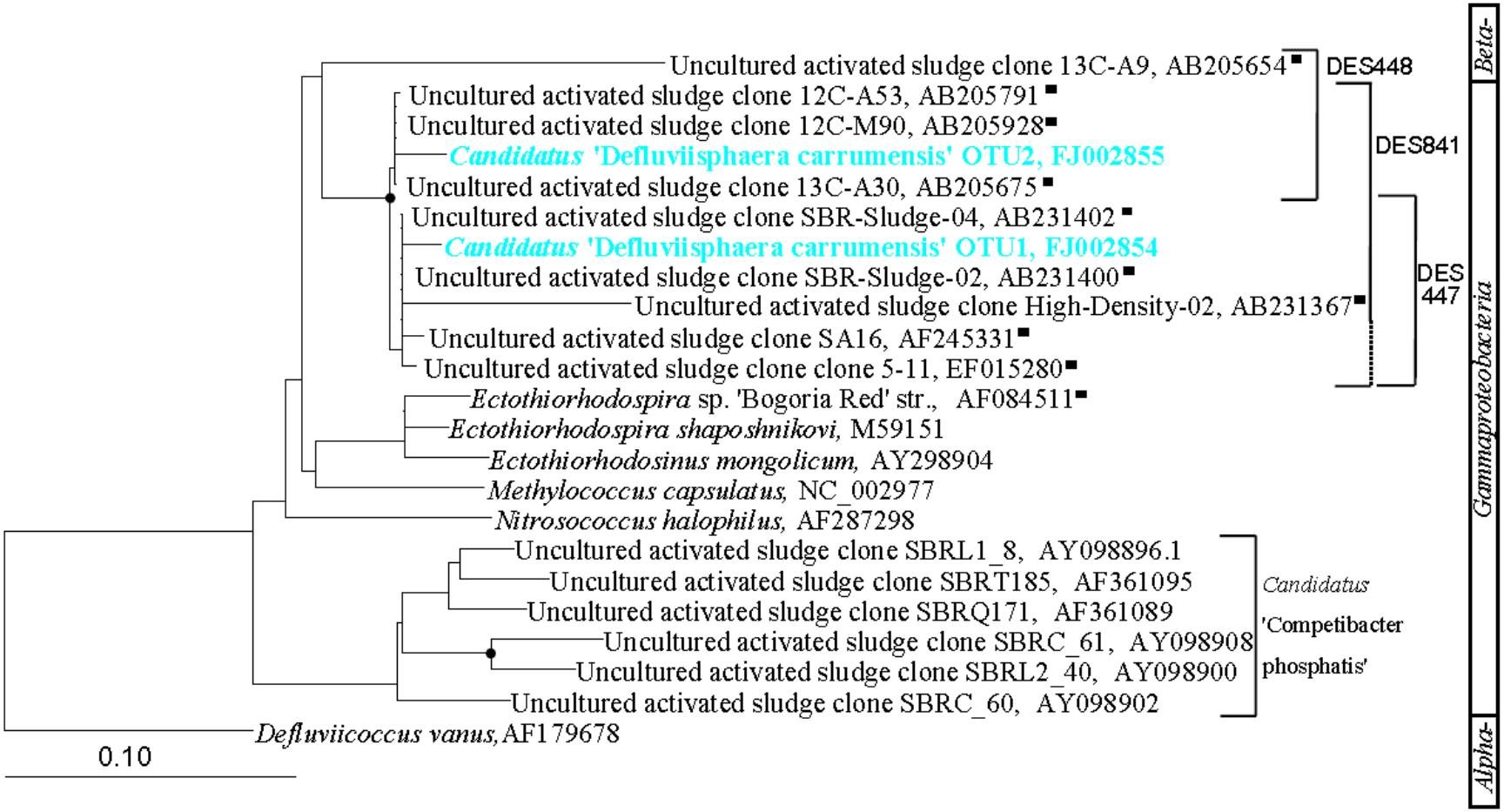


Figure 5.7 Phylogenetic tree (maximum likelihood) of 16S rRNA sequences of gammaproteobacterial clones (represented in bold), and related sequences, obtained from the aerated EBPR pilot plant community. The community was fed ETP treatment plant effluent with 120 mg L^{-1} C as acetate. Coverage of FISH probes designed to target these are indicated. Bootstrap values are from 1000 analyses, (•) indicates values >95%. (▪) indicates partial sequences added after phylogenetic analysis by the parsimony insertion tool of ARB. The scale bar corresponds to 0.1 substitutions per nucleotide position.

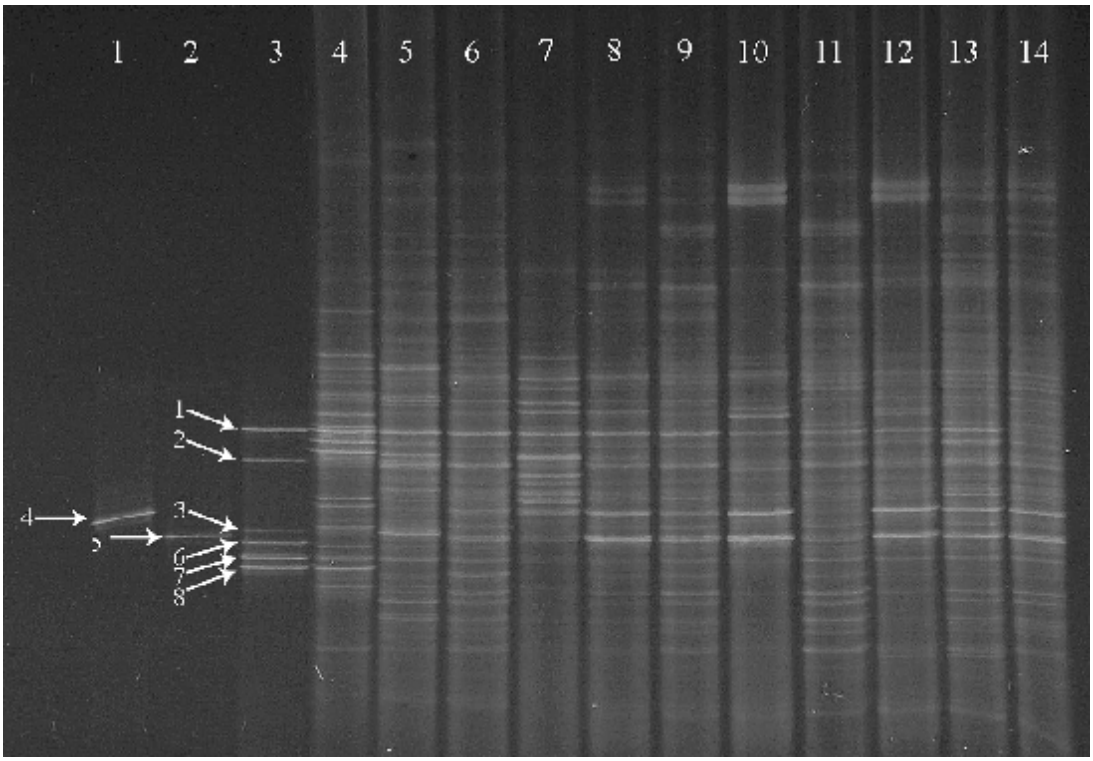


Figure 5.8 16S rRNA PCR-DGGE profiles of communities from the aerated EBPR SBR and pilot plant. The SBRs were operated using ETP treatment plant effluent with 120 mg L^{-1} C as acetate. Lane 1) Gammaproteobacterial clone OTU 2 (Band 4), Lane 2) Gammaproteobacterial clone OTU 1 (Band 5). Lane 3) Internal ladder: Bands 1 and 2 – *Candidatus* 'Accumulibacter phosphatis' clones, bands 3, 6 and 7 – Cluster 2 *Defluviicoccus* related clones, band 8 – *Dechloromonas* related clone, obtained from the aerated EBPR SBR. Lane 4) Community profile from the aerated EBPR SBR fed synthetic feed and 120 mg L^{-1} C as acetate. Lanes 5 – 14) Community profile from the aerated EBPR pilot plant. Lanes: 5) Run A removing P, 6) Run A failed, 7) Run B removing P, 8) Run B failed, 9) Run C start with no P removal, 10) Run C end no P removal, 11) Run D removing P, 12) Run D no P removal, 13) Run E start no P removal, and 14) Run E end no P removal.

Table 5-4 Sequences of the 16S rRNA targeted oligonucleotide FISH probes designed against OTU 1 and OTU 2 gammaproteobacterial clones.

Probe	Target	Probe Sequence (5' – 3')	Target site[1]	FA (%)[2]
DES 841	OTU 1 and 2	ACGACAGCGAGAAGTGAC	841 - 858	25
DES 448	OTU 2	AACCCCGCAACCCGTCCT	448 – 465	40
DES 447	OTU 1	GCCCCGCTTCCCGTCCTC	447 – 464	nd
DES H821	n/a	CCCCACCGTCTAGTTCTC	821 – 838	n/a
DES H865	n/a	GGCGGAGAACTTAACGCG	865 - 882	n/a

nd = not determined, n/a = not applicable
[1] *E. coli* numbering system, [2] Formamide concentration in hybridisation buffer

5.5.2 Probe evaluation

FISH hybridisation was carried out on a biomass sample taken from the pilot plant, in the absence of any pure culture controls. Hybridisation with the DES 841 probe in the absence of both helper probes yielded no visible fluorescent signal from the target cells. The addition of helper probe DES H865 alone did not enhance the fluorescent signal, while the addition of helper probe DES H821 increased the signal markedly. Thus, the application of the DES 841 in combination with the DES H821 helper probe is recommended. Increasing the formamide concentration in the hybridization buffer from 25% to 30% resulted in a drop in fluorescence intensity of the target cells with these helper probes, and 25% formamide was selected as the recommended level for the DES 841 probe. The DES 448 probe generated a strong fluorescence signal with the target cells up to a formamide concentration of 70%, although 35% formamide was used in this study.

5.6 Abundance of the large cocci in the aerated EBPR pilot plant

FISH analysis on biomass samples from the aerated EBPR pilot plant SBR revealed that the DES 448 probe hybridised with about half the large cocci (Figure 5.9a, b), while DES 841 appeared to hybridise with all of them (Figure 5.9c, d), confirming it targeted sequences of clones in both OTU clusters (Figure 5.7). On the basis of its distinctive coccal cell morphology and its habitat, it is proposed that this gammaproteobacterium targeted by these probes is named *Candidatus* 'Defluviisphaera carrumensis'. [De.flu.vi.sphae´ra GR. n. *defluvium* sewage; N. L. (Gr. derived) masc. n. *sphaera* sphere; M.L. fem. n. *Defluviisphaera* a sphere from sewage. ca.rrum.en´sis. L. nom. Fem. adj. *carrumensis* of Carrum, from where the bacteria originated]. FISH analyses on samples taken from the EBPR pilot plant over a six month period operating from summer through to mid winter 2008 showed the DES 841 targeted cells (Figure 5.10a, b) always dominated the biomass community at the end of each run when P removal had failed, shown in Figure 5.11. During Runs B – D their abundance increased significantly ($p < 0.05$) from the start to the end of the run. In some samples a second cell morphotype of long thick rods (Figure 5.9e, f) also fluoresced with this DES 841 probe but these never dominated the community as the large cocci did. Other populations present in the community during the six month period are shown in Table 5.3.

5.7 Presence of Candidatus *'Defluviisphaera carrumensis' in communities from the laboratory scale EBPR process*

FISH analyses were performed on biomass samples obtained from the communities of the aerated laboratory scale EBPR SBR run under all the different operational conditions, described in Sections 3 and 4. The DES 841 positive cells were never detected by FISH in the communities established in this reactor, even when run under conditions where EBPR had failed. The one

exception was when the process had failed at 15 °C, but visual assessment suggested their numbers in this community were < 1% of the total cell biovolume.

5.8 Presence of Candidatus *'Defluviisphaera carrumensis' in full scale plants*

FISH analyses were also performed on biomass samples taken from several full scale treatment plants from various locations around Australia, and analysed with the DES 841 probe. Data (Table 5.5) showed the Kyneton treatment plant biomass used to seed the operating pilot plant contained a few DES 841 positive cocci in clusters (Figure 5.10d), although these made up < 1% of the biovolume, a factor which may explain their absence from the communities in the laboratory scale reactor seeded with it. This relative abundance was much lower than the DES 841 positive cells seen in biomass samples from the Carrum ETP plant, which provided the secondary effluent feed for the pilot plant. Here they contributed as much as 5 ± 0.4% to the biovolume, appearing as loosely clustered cells (Figure 5.10c). Only one other anaerobic: aerobic EBPR plant biomass sample of those examined (from Nambour, Queensland) contained DES 841 FISH positive cells, which also appeared as clustered cocci, but visual assessment suggested they contributed < 1% to the total biovolume.

5.9 Candidatus *'Defluviisphaera carrumensis' in a laboratory scale EBPR SBR fed with ETP clarified effluent*

Results thus far showed that when the aerated EBPR pilot plant was operated with the Carrum ETP WWTP effluent its community was overrun by a previously unidentified gammaproteobacterial population, whose dominance always appeared to correspond to P removal failure. Cells hybridising with the DES 841 probe targeting this *Gammaproteobacteria* were present in high numbers in the biomass from the ETP treatment plant supplying effluent to this pilot plant. However, they did not appear to be present in biomass samples from most other full scale plants surveyed (Table 5.5). Earlier experiments (Sections 2 and 3) had shown that this aerated process removed P at laboratory scale in SBRs fed both a synthetic wastewater and clarified effluent from the Melton WWTP. It was hypothesis that these gammaproteobacterial cells were proliferating in the SBR pilot plant because of their high numbers in the ETP WWTP biomass, and were entering the pilot plant in this feed. Therefore, a laboratory scale SBR was operated under conditions which had previously successfully supported high EBPR capacity, and fed effluent from the ETP WWTP, to see whether this aerated EBPR process would fail when supplied this effluent.

5.9.1 SBR operation and P removal performance

A laboratory scale SBR was operated as previously described (Section 1.1) and initially fed synthetic wastewater to obtain reliable P removal. This wastewater was supplemented with 160 mg L^{-1} C as acetate, and the SBR operated at a pH of 7.5, with a sludge age of 15 d, at 20 °C. After it had successfully removed P for 26 d the feed was changed to the ETP clarified effluent (P = 13.8 mg L^{-1}), where it removed P for another 16 d before failing. The effluent P profile is shown in Figure 5.12. The chemical profile is given in Figure 5.13, and was obtained from samples taken on day 40, during P removal.

5.9.2 Community composition by FISH analysis

Biomass samples for FISH analysis were taken from the reactor after 20 and 41 days of successful P removal, and then after 48 d when P removal had failed. The community population compositions are detailed in Table 5.6. From these data it appears that the failure of the reactor fed ETP effluent was not from any excessive proliferation of *Candidatus* 'Defluviisphaera carrumensis', as had occurred in the pilot plant with the same feed. In fact, few or no cells hybridising with the DES 841 or DES 488 probes were detected in any of the biomass samples examined (data not shown). Instead failure of P removal in the reactor appeared to correspond to

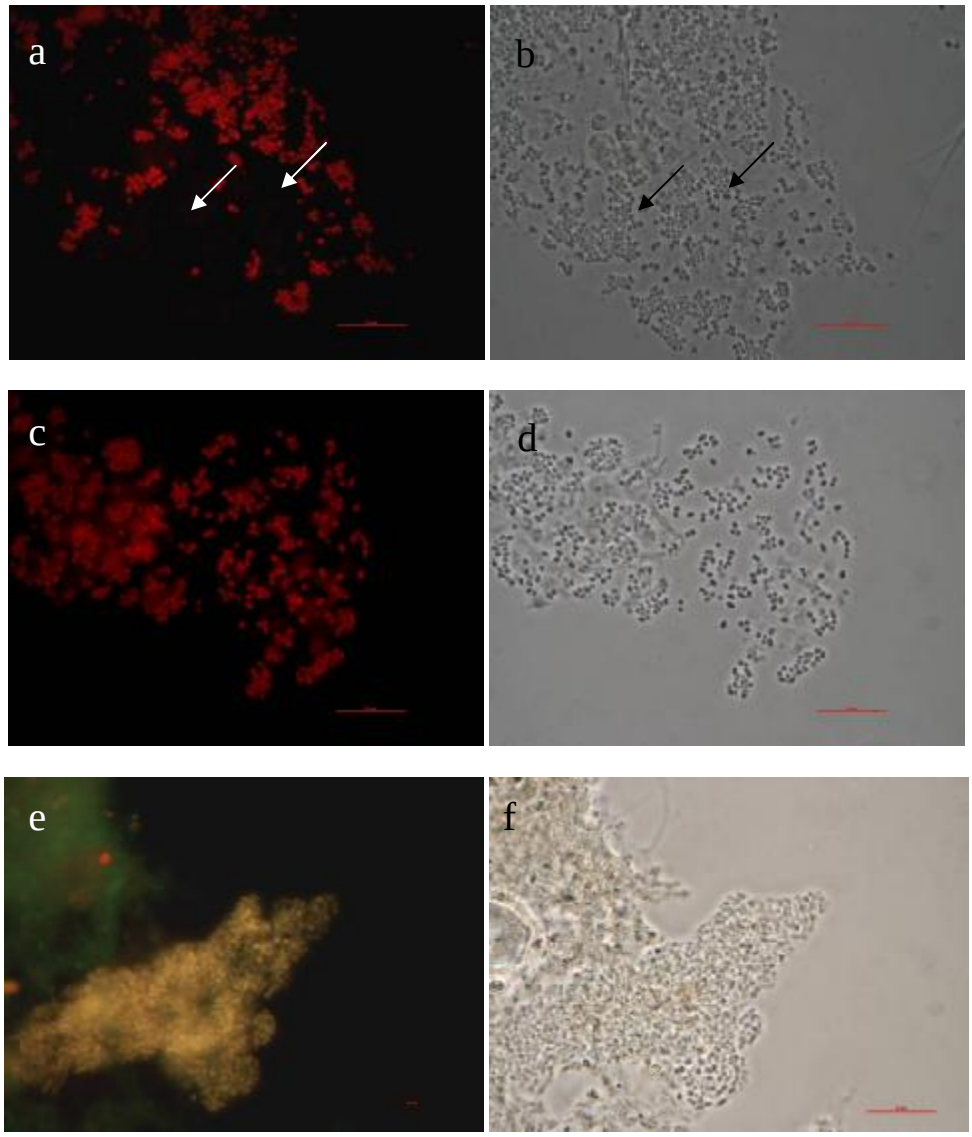


Figure 5.9 FISH micrographs and corresponding phase contrast images of cells from the aerated EBPR pilot plant. Community was fed ETP treatment plant effluent with 120 mg L^{-1} C as acetate. a) Cells hybridising with the DES 448 probe (Cy3), and b) same field of view showing not all large cocci hybridise with this probe (indicated by arrows). c) Cells hybridising with the DES 841 probe (Cy3), and d) same field of view showing all large cocci hybridise with this probe. e) Cells hybridising with the DES 841 probe (Cy3) and EUBmix probes (FLUOS), and f) same field of view, both showing the long rod morphology of some DES 841 positive cells. Cells hybridising with both Cy3 (red) and FLUOS (green) labelled probes appear yellow.

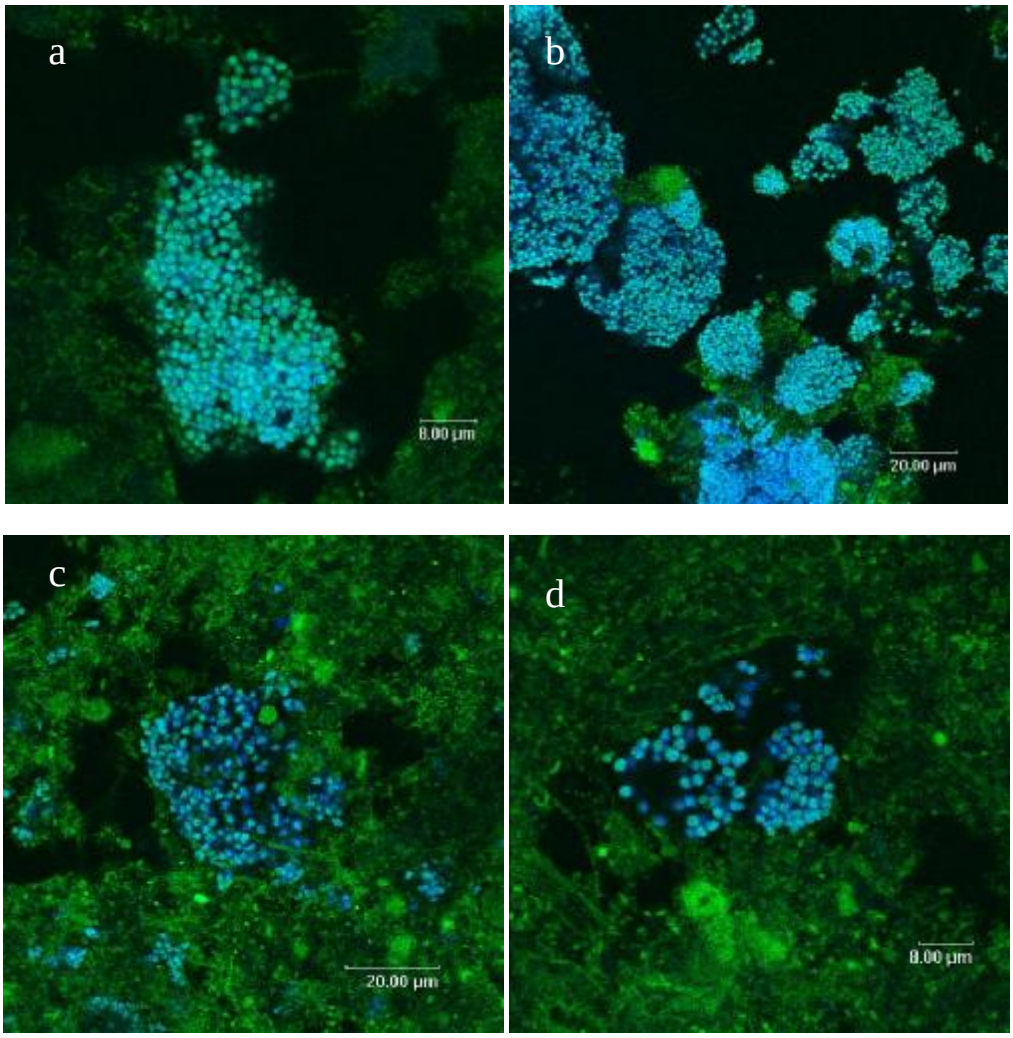


Figure 5.10 FISH micrographs of cells from the aerated EBPR SBR pilot plant. Community was fed ETP treatment plant effluent with 120 mg L^{-1} C as acetate. Cells are hybridising with the a and b) DES841 probe (Cy5) from the a) start of a run, and b) end of a run. c and d) Cells hybridising with the DES841 probe (Cy3) from c) ETP WWTP, and d) Kyneton WWTP. All cells are also hybridised with the EUBmix (FLUOS) probes and appear light blue when hybridising with both probes.

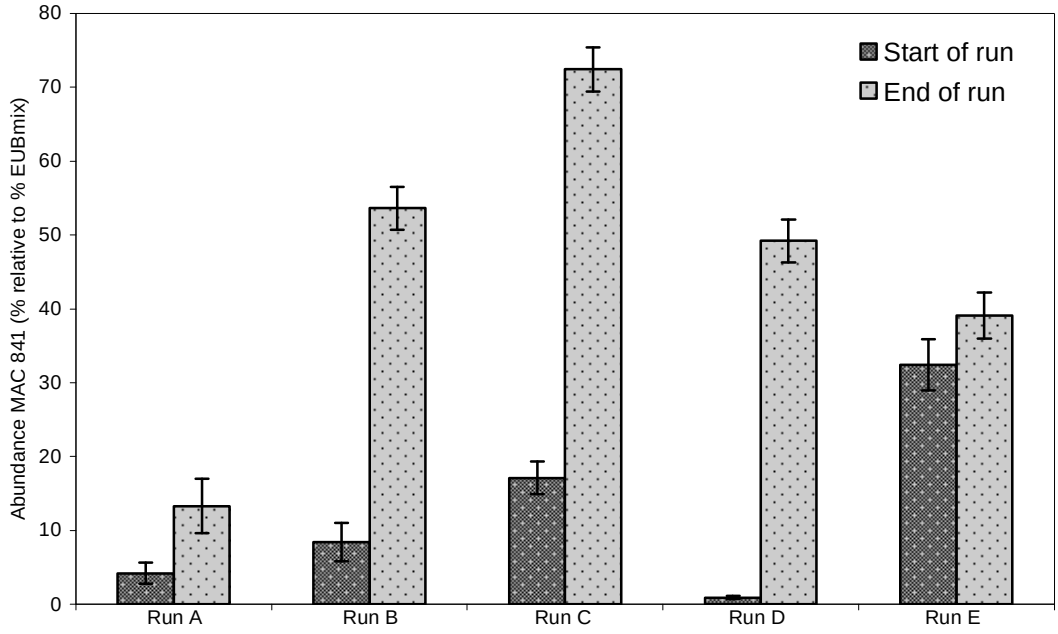


Figure 5.11 Abundance of DES 841 positive cells in the aerated EBPR pilot plant. The pilot plant was using ETP treatment plant effluent with 120 mg L^{-1} C as acetate operating over a six month period at the start and end of each run. Error bars show standard error.

Table 5-5 Occurrence of DES 841 targeted cells in various full scale WWTP in eastern Australia.

Plant	Configuration	Type of waste	P removal	ADWF[1] (ML day^{-1})	SRT[2] (days)	DES841
Kyneton	MUCT[3] Lutzig-Ettinger	Domestic	EBPR	1.1	34	+/++
ETP (Jan 08)	Lutzig- Ettinger with nitrification/denitrification	Domestic and industrial	No	370	6 – 10	++/+++
ETP (Aug 08)	Lutzig- Ettinger with nitrification/denitrification	Domestic and industrial	No	370	6 – 10	+/++
Coolum	Oxidation ditch	Domestic	EBPR	6.0	24	-
Nambour	Johannesburg	80% domestic, 20% industrial	EBPR	5.5	8	+
Merrimac	MUCT[1]	Domestic	EBPR	19.2	14	-
Bendigo	MUCT	Domestic	Chemical dosing	27.0	23 - 30	-
Morpeth	M-Johannesburg[4]	Domestic	EBPR	9.5	16	-
Castlemaine	MUCT	Domestic/meat processing plant	Chemical dosing	2.65	28 – 30	-
Maroochy	5 stage Bardenpho	95% domestic, 5% industrial	EBPR and alum	24.0	16	-
South Ballarat	Johannesburg	Domestic and industrial	EBPR	30 – 36	14	-
Dora Creek	Conventional extended aeration	Domestic	Chemical dosing	3.34	7.8	-
Branxton	Oxidation carousel/IDEA[5]	Domestic	Chemical dosing	0.89	21.3/41.3	-
Raymond Terrace	Modified Lutzig- Ettinger	Domestic	Chemical dosing	20.0	3.1	-
Karuah	IDEA	Domestic	No	70.0	0.28	-

nd = no data, - not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).
[1]ADWF = average dry weather flow, [2]SRT = sludge retention time (sludge age)[3]MUCT = modified University of Cape Town, [4]M-Johannesburg = modified Johannesburg, [5]IDEA = intermittently decanted extended aeration process

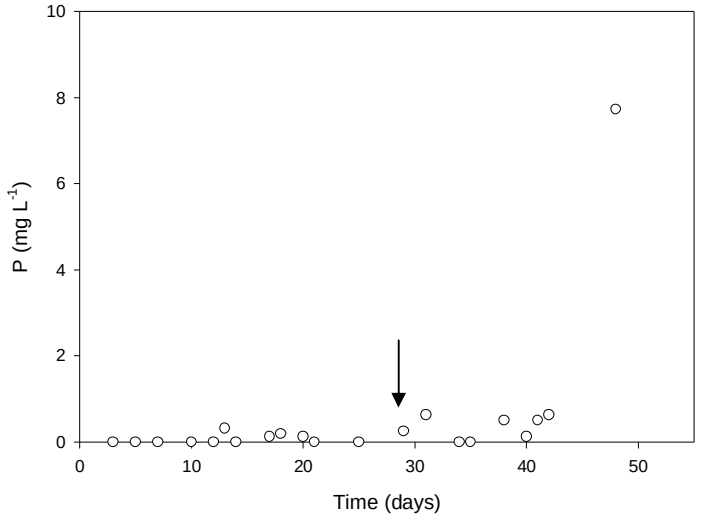


Figure 5.12 Time profile showing levels of P in the effluent for the aerated EBPR SBR. The SBR was operated using synthetic feed and secondary effluent from ETP treatment plant with 160 mg L^{-1} C as acetate. The arrow shows where the feed was changed from synthetic wastewater to clarified effluent (day 26). (Data provided by B Campbell).

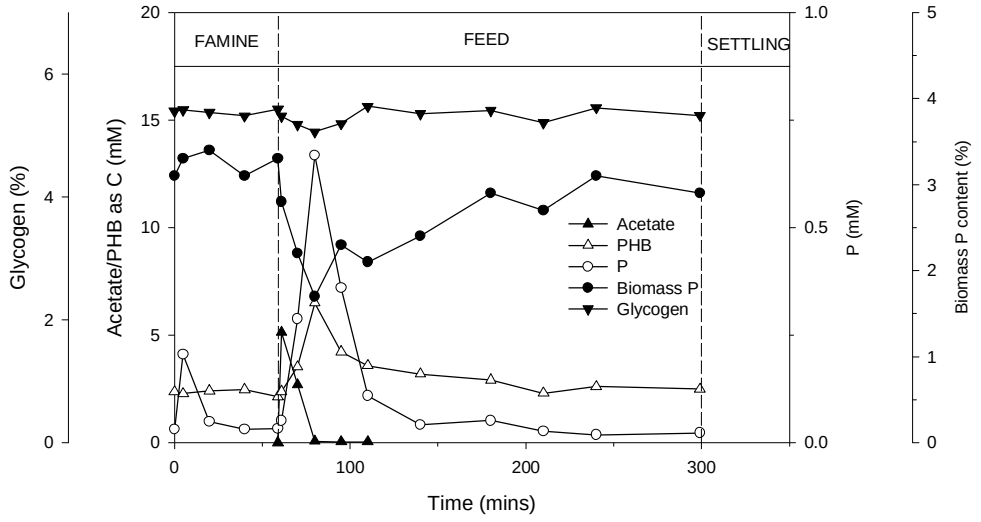


Figure 5.13 Chemical profile from the aerated EBPR SBR operated with effluent from ETP WWTP. Profile was taken on day 41, just before P removal failure. Changes in acetate levels (▲), P content of mixed liquor (○), P content of biomass (% w/w dry biomass) (●), PHB content of biomass (expressed as mole C L^{-1} mixed liquor) (△), and glycogen content of biomass (% w/w dry biomass) (▼) are shown over a FEED: FAMINE cycle. (Data provided by B Campbell).

a marked increase in the abundance of Cluster 2 *Defluviicoccus* related cells (Figure 5.14). However, when FISH analyses were performed of biomass samples from the ETP plant at the time when the effluent was used for these experiments, < 1% of the total cells biovolume were now hybridising with the DES 841 probe. This is quite different to the composition of the ETP communities examined earlier (Section 5.8) when the pilot plant was operating.

5.10 Ecophysiology of Candidatus *'Defluviisphaera carrumensis'*

MAR incubations were carried out as described in Section 1.7. For aerobic incubations, samples were taken from the pilot plant at the end of the FAMINE phase, just before addition of acetate. For anaerobic and anoxic incubations samples were taken from the ETP WWTP. Samples were incubated with acetate to a final concentration of 2 mM and radioactivity of 10 µCi $mgSS^{-1}$, and diluted to 1 gSS L^{-1} using filtered sludge water. Samples were incubated for 2 h (aerobic incubations only) and 4 h (anoxic and anaerobic incubations). Cells that gave a positive MAR signal after 2 days were considered to assimilate acetate.

FISH/MAR data revealed that DES 841 positive cells could assimilate acetate aerobically (Figure 5.15a – d), as revealed after 2 h incubations. However, not all these cells showed the same feature (indicated by arrows). This physiological feature did not appear to belong solely to one OTU (Figure 5.15e – h). By visual estimation based on silver grain deposition densities (Nielsen and Nielsen 2005), the *Accumulibacter* cells present in the same biomass sample appeared to assimilate acetate at a higher rate than the DES 841 positive cells (data not shown). On occasions after the emulsion and development steps of the MAR procedure (Section 1.7), no hybridisation signal from any of the DES 841 probed cells could be seen under the fluorescence microscope, even though FISH analysis of the biomass after MAR incubation, but before applying the emulsion and the subsequent development step revealed cells which had hybridised to the DES 841 probed cells (Figure 5.16c).

Thus, once these slides had been developed, the DES 841 probe no longer gave a detectable signal for any of the targeted cocci, although the EUBmix probes still imparted a strong fluorescent signal to them (Figure 5.16a, b). Furthermore, when FISH/MAR slides from samples incubated with acetate under anoxic and anaerobic conditions were examined, it was not possible to locate any DES 841 positive cells, even though these were present in the biomass samples chosen for the MAR incubations (Figure 5.16d, e). No explanation for these events is forthcoming.

FISH/DAPI staining showed the DES 841 positive cells in the pilot plant community never contained polyP during its operation, and FISH/PHB staining showed most of the DES 841 positive cells contained PHA in samples taken throughout the cycle (data not shown), agreeing with the chemical profile data (Figure 5.5 and 5.13).

5.11 Discussion

This section describes the presence and eventual dominance of a previously unidentified *Gammaproteobacteria* in the community of an aerated EBPR SBR pilot plant when fed acetate-supplemented clarified effluent from the ETP, conventional activated sludge system which was operating with a modified Lutzig-Ettinger configuration to remove N. The 16S rRNA clone library prepared from its community (Figure 5.7) revealed sequences distantly related to *Ectothiorhodospira* spp., and these clustered adjacent to but separately from those of *Competibacter*. In an effort to identify what these cells were, FISH probes were designed which targeted the distinct coccobacilli, which were provisionally named *Candidatus* 'Defluviisphaera carrumensis'.

Table 5-6 FISH analysis of populations present in the aerated EBPR SBR when removing and not removing P. SBR was operated with synthetic wastewater and clarified effluent from ETP WWTP with 160 mg L^{-1} C as acetate.

Target	Probe	Removing P (synthetic feed, 20 d)	Removing P (ETP effluent, 41 d)	Failed P removal (ETP effluent, 48 d)
Candidatus 'Accumulibacter phosphatis'	PAOmix	+++	+++	++
Cluster 2 *Defluviicoccus* related bacteria	DF2mix	+/++	+++	+++
Candidatus 'Defluviisphaera carrumensis'	DES 841	-	-	-
Candidatus 'Defluviisphaera carrumensis'	DES 448	-	-	-

nd = no data
– not detected, +/– very few, + few, ++ some, +++ common (Wong and Liu 2007).

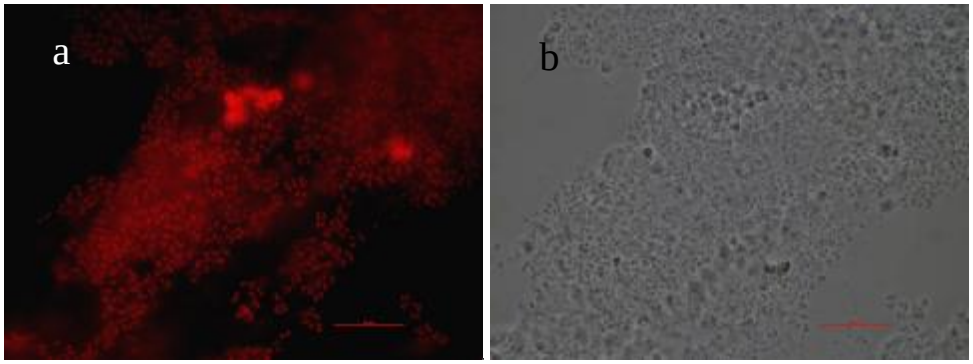


Figure 5.14 FISH and phase contrast micrographs of cells in the aerated EBPR SBR using ETP treatment plant effluent. SBR was operated with 120 mg L^{-1} C as acetate. a) Cluster 2 *Defluviicoccus* cells hybridising with the DF2mix probe (Cy3) in the aerated EBPR SBR after P removal had failed. b) Same field of view showing phase contrast image.

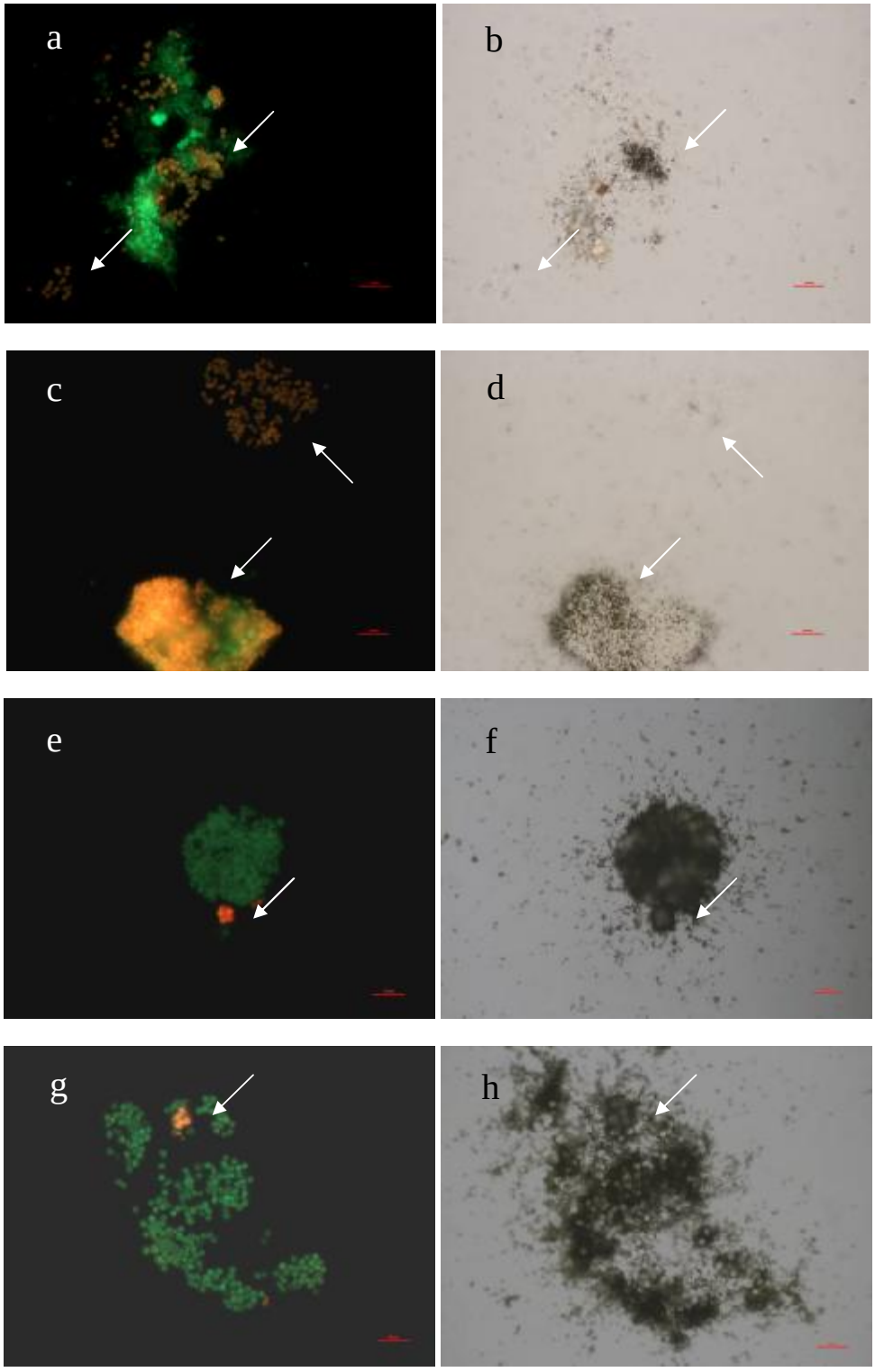


Figure 5.15 Micrographs of cells from the aerated EBPR SBR pilot plant after MAR incubations. Pilot plant was operated using ETP treatment plant effluent with 120 mg L^{-1} C as acetate. a and c) Cells hybridising with the DES 841 probe (Cy3) and EUBmix probes (FLUOS). b and d) Same field of view showing the ability of some cells to assimilate acetate (shown by arrows). e and g) Cells hybridising with the DES 448 probe (Cy3) targeting OTU 2 and EUBmix probes (FLUOS). f and h) Same field of view showing the DES 448 positive cells assimilating acetate. Cells hybridising with both Cy3 (red) and FLUOS (green) labelled probes appear yellow.

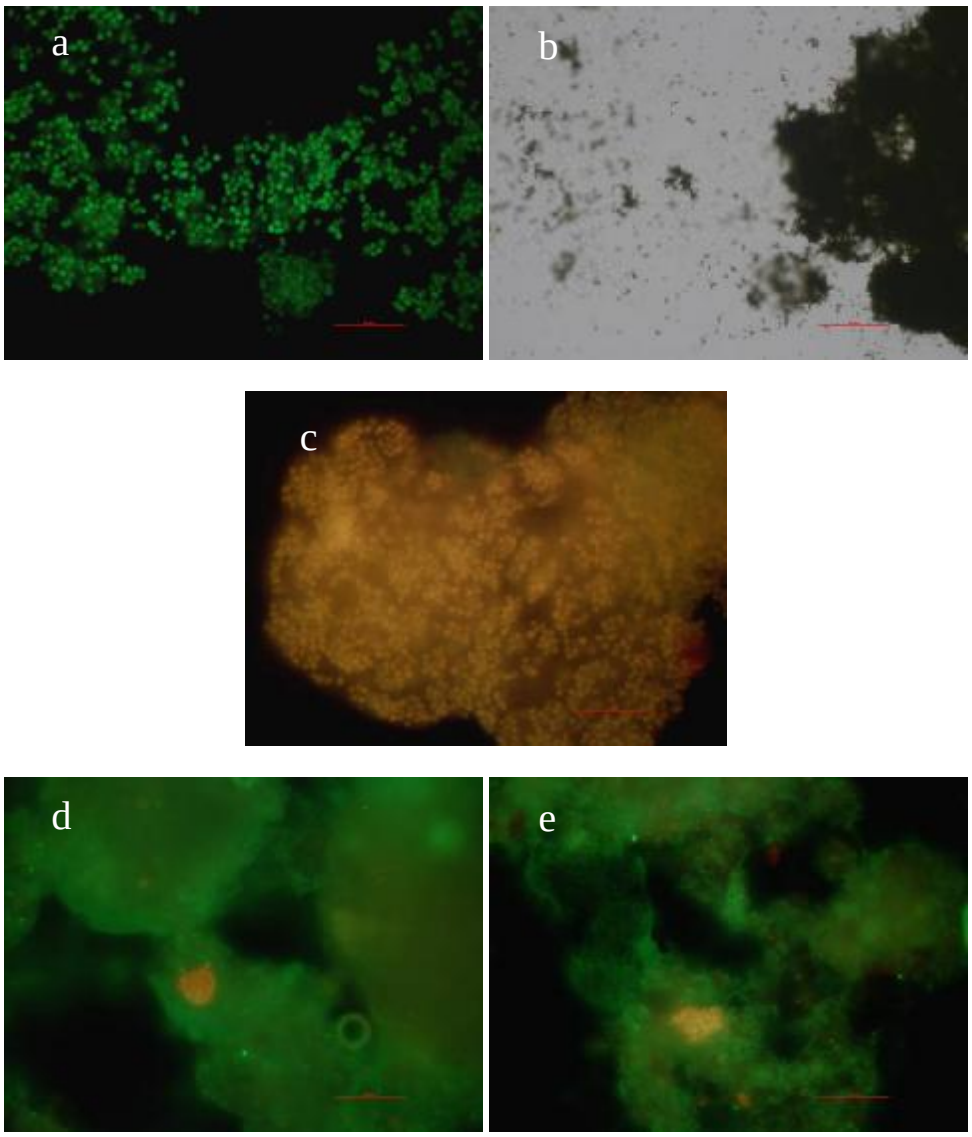


Figure 5.16 Micrographs of cells in the ETP WWTP and aerated EBPR SBR before and after MAR procedures. Cells hybridising with the a) EUBmix (FLUOS) probes, but not with the DES 841 probe (Cy3) in biomass from ETP treatment plant after aerobic MAR incubation, emulsion and development. b) Same field of view showing the ability of some of these cells to assimilate acetate. c) Cells hybridising with the DES 841 (Cy3) and EUBmix (FLUOS) probes in biomass from the aerated EBPR pilot plant after aerobic MAR incubation and before emulsion and development. Cells hybridising with the d) DES 448 probe (Cy3) and e) DES 841 probe and EUBmix probes (FLUOS) in biomass from the ETP treatment plant before anaerobic and anoxic MAR incubations. Cells hybridising with both Cy3 (red) and FLUOS (green) labelled probes appear yellow.

5.11.1 The role of *Candidatus* 'Defluviisphaera carrumensis' in the EBPR SBR pilot plant

Failure of this aerated EBPR SBR pilot plant process to remove P appeared to result always from their presence in large numbers in the communities. Their absence in communities from the aerated EBPR SBR process run at laboratory scale was probably because the reactors were not fed their P supply from the same source. FISH data suggest that these *Gammaproteobacteria* were entering the pilot plant in the ETP plant feed. Then, under the FEED: FAMINE conditions imposed, they eventually replaced the *Accumulibacter* PAO which were dominant initially, leading to EBPR failure.

Ecophysiological data showed these *Gammaproteobacteria* cells could assimilate acetate aerobically, the C source provided during the FEED stage of the process, and most of them stained positively with Nile blue A for intracellular PHA. DAPI staining showed they contained no polyP in samples taken during the SBR cycle. Therefore, they appear to display the same feed and famine phenotypic characters that the *Defluviicoccus* and *Dechloromonas* related cells showed when this process is run at laboratory scale, and which *Defluviicoccus* and *Competibacter* exhibit in anaerobic: aerobic EBPR processes (Crocetti et al. 2002; Wong et al. 2004; Meyer et al. 2006; Burow et al. 2007). This phenotype is considered to confer on a population an ability to compete with the PAO populations under alternate feed: famine conditions (Oehmen et al. 2007; Seviour and McIlroy 2008) and lead potentially to EBPR failure.

The 16S rRNA clone library revealed clone sequences from these *Gammaproteobacteria* clustering in OTU 2 were closely related to those recovered from a denitrifying sludge fed ^{13}C labelled acetate or methanol, with ^{13}C enriched DNA fractions obtained by stable isotope probing (Osaka et al. 2006). The clone 13C-A30 (AB205675) was recovered from the ^{13}C DNA fraction, suggesting it utilised acetate under the denitrifying conditions imposed. Consequently these novel gammaproteobacterial cocci may share the same physiological features, a view which is consistent with their presence in the ETP plant which operates to denitrify, and their demonstrated ability by MAR to assimilate acetate aerobically. Unfortunately acetate assimilation data from FISH/MAR studies with nitrate or nitrite as electron acceptors was unclear because of the problems in finding these cells in biomass samples, as discussed earlier (Section 5.10) (data not shown).

5.11.2 Do they have a role in full scale WWTP?

However, these *Gammaproteobacteria* cells were not found in any of the other denitrifying plants investigated. The community structure of denitrifiers can be affected by external C source availability (Hallin et al. 2006; Osaka et al. 2006), and their abundances and phylogenetic diversity may differ among different plants (Morgan-Sagastume et al. 2008). Therefore the operational conditions of other non EBPR full scale plants surveyed may have failed to support the growth of this gammaproteobacterium. A more comprehensive investigation of N removal plants with similar configurations to the ETP plant needs to be undertaken to determine whether these populations are in fact common members of denitrifying communities. From January to August the numbers of these cells in the ETP treatment plant appeared to decrease gradually. Whether they are favoured at the higher summer temperatures, or whether the chemical nature of the effluent in the summer provides them with an advantage, is not known.

Neither were these cells found in large numbers in any of the EBPR plants surveyed, although clones closely related to them, clustered in OTU 1 (Figure 6.7), have been recovered earlier from EBPR systems (Accession numbers AB231400, AB231402, AB231367, AF245331, EF015280). Their absence may suggest an inability to cope with the anaerobic: aerobic conditions found in these plants, and may reflect their inability to assimilate acetate anaerobically. Further investigation of their anaerobic ecophysiology may reveal more.

Whether these populations should also be referred to as GAO on the basis of their phenotype in the aerated system studied here, as they are in conventional anaerobic: aerobic systems, is open to doubt, as was already discussed in Section 2.9.3.3. It is not known if these cells accumulate glycogen, and the characteristic glycogen transformations distinctive of anaerobic: aerobic EBPR systems are not seen in this aerated process (Seviour et al. 2003; Oehmen et al. 2007). A similar lack of glycogen transformations in an EBPR system operated aerobically was reported previously (Pijuan et al. 2006). There it was suggested that because the *Accumulibacter* PAO could obtain energy and reducing power for growth by aerobically respiring the supplied acetate via the TCA cycle in the feed stage, they had no requirement to replenish their glycogen reserves from PHA metabolism during the famine stage. The same may apply to this *Gammaproteobacteria* is the aerated system.

5.12 Conclusion

Data presented in this section have described a previously unidentified *Gammaproteobacteria* dominating the pilot plant aerated EBPR SBR, as *Candidatus* 'Defluviisphaera carrumensis'. Further FISH/MAR studies are needed to elucidate whether these cells can assimilate substrates anaerobically and anoxically, and which substrates might support denitrification in these populations. Then a clearer picture of what determines their distribution in activated sludge systems might emerge. What the data presented here demonstrate is that they have the potential to behave as effective competitors with *Accumulibacter* PAO populations, being able to store PHA in the FEED stage. Their ability to assimilate acetate aerobically might suggest they may pose a threat in anaerobic: aerobic EBPR systems if dissolved oxygen levels in the anaerobic feed zone are high. The availability of the FISH probes described here will permit their distribution in activated sludge plants of varying configurations to be more precisely monitored.

CONCLUSIONS AND FUTURE WORK

Conclusions

- Work presented in this report investigated the microbial community compositions in a laboratory scale aerated EBPR SBR, designed as a P removal process to be added to non-EBPR activated sludge plants to remove P from their final effluents.

- This process could remove P reliably from both a synthetic wastewater feed and clarified effluent from a full scale conventional activated sludge plant under a range of operating conditions. However, in some cases, for example where acetate supplementation concentration in the feed stage was lowered, and the operating temperature was changed, P removal failed.

- Under all operating conditions *Candidatus* 'Accumulibacter phosphatis' were identified as the major PAO population. They displayed the typical feed: famine phenotype that the *Accumulibacter* PAO in anaerobic: aerobic EBPR processes have been shown to.

- Cluster 2 *Defluviicoccus* members and *Dechloromonas* related cells were also present, often in large numbers, in these communities under most operating conditions. Both appeared to possess a feed: famine phenotype similar to those described for GAO populations in anaerobic: aerobic EBPR systems. Hence both could assimilate acetate in the FEED stage to synthesize PHA, but not assimilate P for polyP production in the FAMINE stage of the process.

- The relative abundance of these main populations changed in response to operational conditions, which often reflected the P removal capacity of the process.

- In particular, failure of P removal often corresponded to an increase in relative abundances of *Defluviicoccus* related cells and a corresponding decrease in *Accumulibacter* relative abundances. This was thought to be the consequence of *Defluviicoccus* related cells out-competing the *Accumulibacter* PAO for acetate in the FEED stage.

- When attempts were made to find an alternative substrate for supplementation during the FEED stage, which only the *Accumulibacter* PAO assimilated, MAR/FISH data showed that none of those tested were taken up exclusively by *Accumulibacter* aerobically. In fact, the *Defluviicoccus* related cells assimilated a wider range of substrates under aerobic conditions.

- Failure of this process to remove P when scaled up to a 150 L pilot plant under conditions which were successful at laboratory scale, was attributed to the dominance of a previously unidentified *Gammaproteobacteria.* 16S rRNA clone library analysis allowed its identification, and it was tentatively named *Candidatus* 'Defluviisphaera carrumensis'. Subsequent FISH probe design from these sequences allowing its *in situ* identification showed it also possessed a phenotype in the FEED stage similar to that of the GAO in conventional anaerobic: aerobic EBPR systems.

- However, although it was present in high numbers in the full scale non-EBPR plant providing the feed to the pilot plant, FISH based surveys could not detect it in most other full scale EBPR and non-EBPR plants. Neither could it be detected in any of the aerated laboratory scale EBPR SBR communities.

Future Work

- Further work should be directed to elucidate why P removal failed in this aerated EBPR SBR process, and the role if any of *Defluviicoccus* in this. The basis for competition between the *Accumulibacter* and *Defluviicoccus* populations needs to be determined, especially during the period of acetate addition and assimilation in the FEED stage of the process.

- Varying other operating conditions, including running the process at different controlled dissolved oxygen levels and changing the acetate feed strategy from a dump feed might help explain under what conditions and why *Defluviicoccus* might be favoured.

- Further studies into the ecophysiology of *Defluviicoccus* and *Accumulibacter* related organisms under conditions of continuous aeration and the FEED: FAMINE regime used in this processes are needed. In particular, their metabolism in terms of glycogen transformations and PHA metabolism may help explain their relative competitive abilities. Applying a similar approach to that used so productively by Burow et al. (2008a; 2008b) with targeted metabolic inhibitors may elucidate how

carbon flux is allocated between them under the FEED conditions used in this process.

- Failure of the EBPR SBR at pilot plant stage was attributed to the dominance of a previously undescribed member of the *Gammaproteobacteria,* which was identified as *Candidatus* 'Defluviisphaera carrumensis'. The anaerobic and anoxic ecophysiology of this organism needs to be resolved satisfactorily to determine whether it is a denitrifying organism and/or can also assimilate substrates anaerobically, and hence pose as a potential competitor population to the PAO under some conditions. Its occurrence in other full scale EBPR and conventional plants needs to be more fully explored, which the FISH probes designed and validated in this study should facilitate.

- Attempts should be made to cultivate *Accumulibacter,* Cluster 2 *Defluviicoccus, Competibacter* and *Defluviisphaera,* possibly using an extinction culture method. While some attempts have been made to grow *Accumulibacter* in pure culture, none have yet been successful, and the only pure culture of GAO is *Defluviicoccus vanus,* belonging to Cluster 1 *Defluviicoccus.* The ability to grow these organisms in pure culture would provide valuable information by allowing whole genome sequencing and further examination of their metabolic features relative to EBPR.

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