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Biological phosphorus removal in a moving bed biofilm reactor

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Innovation and Creativity

Abstract

Phosphorus (P) and nitrogen (N) removal from municipal wastewater is performed to prevent or reduce eutrophication in the receiving water.

Both P and N can be removed physical/chemically as well as biologically. While biological processes have always dominated in N-removal, chemical P-removal is used in many cases. Biological P-removal using enhanced biological phosphorus removal (EBPR) is normally carried out in suspended culture (activated sludge) processes while biological N-removal (through nitrification and denitrification) can be done in biofilm processes as well. There are, however, potential advantages related to sludge separation and required reactor volumes, which suggest that a pure biofilm process can be an interesting alternative to activated sludge for biological phosphorus and nitrogen removal.

The scope of this study was to investigate use of the moving bed biofilm reactor (MBBR) process for biological phosphorus removal. The goal has been to describe the operating conditions required for biological phosphorus and nitrogen removal in a MBBR operated as a sequencing batch reactor (SBR), and determine dimensioning criteria for such a process.

Laboratory scale experiments were performed with constructed wastewater and verified in experiments with municipal wastewater. The results showed that biological phosphorus and nitrogen removal can be achieved in a moving bed biofilm reactor operated as a SBR. The required operating conditions will be dependant on the wastewater quality. Recommended first generation design criteria for a wastewater with a typical nutrient distribution: COD:N:P ~ 100:10:2 were found.

Oxalobacter sp. was probably the most common bacteria in the biomass and may be a phosphorus accumulating bacteria. This has not been reported previously, however, further studies are required to prove or disprove the possible involvement of *Oxalobacter* sp. in EBPR.

Acknowledgements

Summary

Phosphorus (P) and nitrogen (N) removal from municipal wastewater is performed to prevent or reduce eutrophication in the receiving water.

Both P and N can be removed physical/chemically as well as biologically. While biological processes have always dominated in N-removal, chemical P-removal is used in many cases. The main disadvantages of chemical phosphorus removal are the cost of chemicals and the relatively large sludge production. Biological phosphorus removal offers an alternative to chemical treatment methods that has a potential for reduced sludge production.

Biological P-removal using enhanced biological phosphorus removal (EBPR) is normally carried out in suspended culture (activated sludge) processes while biological N-removal (through nitrification and denitrification) can be done in biofilm processes as well.

There are potential advantages related to sludge separation and required reactor volumes, which suggest that a pure biofilm process can be an interesting alternative to activated sludge for biological phosphorus and nitrogen removal. In a biofilm process for EBPR the concentration of suspended solids in the influent to the sludge separation will be much lower than in an activated sludge process, which is an advantage with respect to sludge separation. In a biofilm process for EBPR simultaneous nitrification, denitrification and phosphate uptake can be achieved, which gives a potential for reduced hydraulic retention time because it can remove the need for a separate anoxic phase.

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Laboratory scale experiments were performed with constructed wastewater and thereafter the results were verified in experiments with municipal wastewater. The results showed that biological phosphorus and nitrogen

removal can be achieved in a moving bed biofilm reactor operated as a SBR. The operating conditions to achieve simultaneous nitrification – denitrification and phosphate uptake will be dependant on the wastewater quality, but the SBR cycle should be tuned to achieve near complete removal of easily biodegradable soluble COD in the anaerobic period and complete nitrification in the aerobic period. To achieve complete nitrification, the total COD-loading rate including the effect of particulate COD should be considered.

Hydrolysis of particulate COD increased the biodegradable soluble COD (BSCOD) available for the bacteria. However, the process is best suited for removal of soluble compounds and a process scheme including enhanced primary treatment and utilisation of primary sludge for production of carbon source should be considered.

A SBR cycle of 6 hours (1 hour and 40 minutes anaerobic phase followed by a 4 hour and 20 minutes aerobic phase) gave less than 10 mg soluble-N l^{-1} and 0.3 mg PO_4-P l^{-1} in the SBR effluent. A C/P-ratio of 15 mg BSCOD (mg PO_4-P)$^{-1}$ was required to achieve these effluent concentrations and required dosage of additional C-source with the wastewater used in this study.

The results indicated that the majority of the phosphorus accumulating bacteria was capable of utilising nitrate as electron acceptor. A high fraction of phosphate accumulating bacteria with denitrification capacity is an advantage with respect to carbon source requirement for P and N removal.

Efficient separation of effluent suspended solids was achieved by flotation without use of chemicals. Dosage of a polymer improved removal of total phosphorus probably due to coagulation and flocculation of sub micron particles.

Oxalobacter sp. was probably the most common bacteria in the biomass throughout the experimental period and may be a phosphorus accumulating bacteria. However, further studies would be required to prove or disprove the possible involvement of *Oxalobacter* sp. in aerobic and/or anoxic EBPR. *Acinetobacter* sp. was probably also a common bacteria in the biomass and may well have been partly responsible for P-removal.

Recommended first generation design criteria are given in the table below.

Table I. Recommended first generation design criteria for a wastewater with a typical nutrient distribution: COD:N:P ~ 100:10:2

Parameter	Design criteria
Total COD-loading rate, (g COD $m^{-2}d^{-1}$)	< 4
Anaerobic BSCOD-loading rate, (g BSCOD $m^{-2}d^{-1}$)	< 5
Aerobic ammonia loading rate, (g NH_4-N $m^{-2}d^{-1}$)	< 0.4
Required influent C/P-ratio, (mg BSCOD (mg PO_4-P)$^{-1}$)	20

BSCOD: Biodegradable soluble COD

Further work with biological nutrient removal in a sequencing batch moving bed biofilm reactor (SBMBBR) should address design of a full scale process and also a comparison between a continuous process based on activated sludge or a hybrid process and a SBMBBR.

Further work on characterisation of the bacteria involved in EBPR should include *Oxalobacter* sp. as one of the possible bacteria responsible for aerobic and/or anoxic P-removal.

1. Introduction

The main reason for removal of phosphorus (P) and nitrogen (N) from municipal wastewater is to prevent or reduce eutrophication in the receiving surface water. Untreated municipal wastewater contains nitrogen and phosphorus both as particulate species and soluble in the form of ammonia and phosphate. Especially the soluble compounds are readily used as nutrients by micro-organisms. Excessive discharge of nitrogen and phosphorus leads to increased growth of micro-organisms with consequently increased oxygen consumption. In extreme cases the concentration of dissolved oxygen (DO) can be reduced to zero in parts of the water column. In fresh water phosphorus is normally the limiting nutrient for growth of micro-organisms, while nitrogen is normally limiting for growth in marine waters. Although removal of phosphorus has been the only requirement for nutrient removal in some cases with discharge to fresh water recipients, removal of both phosphorus and nitrogen is normally necessary to meet treatment standards that require nutrient removal.

Phosphorus can be removed both chemically and biologically. Chemical phosphorus removal is done by precipitation of phosphate and coagulation – flocculation of particulate phosphorus using a metal salt of calcium, aluminium or iron. The main disadvantages of chemical phosphorus removal are the cost of chemicals and the relatively large sludge production that increases the cost of sludge treatment and the problems and cost of sludge disposal. Biological phosphorus removal offers an alternative to chemical treatment methods that has a potential for reduced sludge production.

Biological phosphorus removal is performed by phosphate accumulating micro-organisms (PAO) that have the ability to accumulate phosphate over and above what is required for growth. This biological process is referred to as bio-P or enhanced biological phosphate removal (EBPR). Although the biochemical mechanism was not understood at the time, EBPR in activated sludge processes was reported as early as 1965 (Levin and Shapiro, 1965). Since then research in the field has progressed to identify some of the bacteria which are involved and also to clarify the biochemical mechanisms behind EBPR. However, there are still unresolved questions

regarding both the bacteria responsible for EBPR and the biochemical mechanisms of EBPR.

In order to facilitate selection of the bacteria responsible for EBPR in a treatment plant, the biomass must be exposed to alternating anaerobic and aerobic or anoxic conditions. This can be done by alternating the conditions in a reactor, as in a sequencing batch reactor (SBR), or by moving the biomass from one reactor to another in a continuous process.

Nitrogen removal from wastewater is normally done biologically with nitrification of ammonia to nitrate under aerobic conditions, followed by denitrification of nitrate to nitrogen gas under anoxic conditions. Nitrification and denitrification are performed by separate groups of bacteria. To achieve nitrogen removal it is therefore not required to expose the biomass to both aerobic and anoxic conditions, but the wastewater must pass through aerobic and anoxic conditions. Nitrogen removal can therefore be achieved in both single sludge and two sludge activated sludge processes, and in biofilm processes with separate aerobic and anoxic reactors.

Currently all full scale processes that remove both phosphorus and nitrogen biologically are based on activated sludge. One of the reasons for this is that exposing the biomass to alternating anaerobic and anoxic or aerobic conditions required for EBPR, can be achieved by circulating the biomass through reactors with different conditions. EBPR can be combined with nitrogen removal by adding an anaerobic stage in front of the nitrogen removal process. Several process configurations have been developed to optimise the combined process, including hybrid processes with use of suspended biofilm carriers in the activated sludge to enhance nitrification and achieve a more compact process with a lower hydraulic retention time (HRT).

Since EBPR is achieved by incorporation of phosphorus in the biomass, a high concentration of phosphorus accumulating biomass in the process is an advantage. However, phosphorus is removed from the process by withdrawal of excess sludge. Efficient phosphorus removal will therefore depend on efficient separation of the biomass even if efficient selection of PAO and a high biomass concentration are achieved.

An activated sludge process for EBPR will be heavily dependant on effective separation of biomass to achieve a high biomass concentration in the process, and a low effluent total phosphorus (tot-P) concentration since this is correlated to the effluent suspended solids concentration. The normal sludge separation method in activated sludge is settling in clarifiers. Biological activity can deplete the oxygen concentration in the settled sludge and lead to anaerobic conditions in the sludge collection part of the clarifiers. As will be discussed later, anaerobic conditions lead to phosphorus release from an EBPR-sludge. Efficient phosphorus removal in an activated sludge process for EBPR will therefore also be dependant on avoiding secondary phosphorus release from the sludge in the clarifiers.

In a pure biofilm process for EBPR the concentration of suspended solids in the influent to the sludge separation will be much lower than in an activated sludge process. This is a potential advantage with respect to the problems stated above because a lower amount of settled sludge in the clarifier reduces the problem of secondary phosphorus release. Dissolved air flotation (DAF) is an alternative sludge separation method that can ensure aerobic conditions in separated sludge. The lower suspended solids concentration in the influent to the sludge separation with a biofilm process also makes use of flotation as separation method more attractive because the required amount of air for separation and thereby the cost is reduced compared to flotation with an activated sludge process. Also, in a biofilm process it can be easier to achieve a high biomass concentration than in an activated sludge process because there is no limitation due to sludge separation.

The main disadvantage with a pure biofilm process for EBPR is that submitting the biomass to alternating anaerobic and aerobic/anoxic conditions, essential for selection of the PAO, requires a sequencing operation. In a biofilm process, this can be done by operating the reactor(s) as a SBR with alternating anaerobic and aerobic/anoxic conditions in a time sequence or by having several biofilm reactors, i.e. fixed bed filters, in a series with a sequence that changes at set intervals. This type of operation may be more complex and require more piping and valves than a continuous activated sludge process. A SBR process may also require additional volume, compared to a continuous process, to compensate for the time for filling and drawing the reactors. However, in a biofilm SBR one can empty the reactor completely after each cycle and therefore achieve an efficient utilisation of the volume.

Previously, simultaneous nitrification, denitrification and phosphate uptake in the aerated phase of the operating sequence has been reported in laboratory experiments with fixed bed filters (Garzón-Zúñiga and González-Martínez, 1996). Simultaneous nitrification, denitrification and phosphate uptake gives a potential for reduced hydraulic retention time in a pure biofilm process for EBPR and nitrogen removal because it can remove the need for a separate anoxic phase.

There are therefore potential advantages that suggest that a pure biofilm process can be an interesting alternative to activated sludge processes for biological phosphorus and nitrogen removal.

2. Scope of study

The moving bed biofilm reactor (MBBR) was first developed for nitrogen removal from municipal wastewater (Ødegaard *et. al.*, 1994). Later, other applications of the process has been developed including treatment of industrial wastewaters, nitrification in water treatment for land based fish farming and removal of soluble organic matter in secondary treatment of municipal wastewater (Helness *et. al.*, 2005). Today the moving bed biofilm process is used in full scale applications in over 400 treatment plants world wide.

This study was initiated to investigate use of the moving bed biofilm process for biological phosphorus removal. The scope of the study was initially limited to investigate how EBPR could be achieved in a MBBR. However, as will be discussed, this is best done in combination with N-removal. The work in this study has therefore been focused on how combined biological N and P removal could be achieved in a MBBR through a process including simultaneous nitrification and denitrifying P-uptake.

The goal of this work has been to describe the operating conditions required for biological phosphorus and nitrogen removal in a MBBR with Kaldnes K1 biofilm media operated as a SBR, and determine dimensioning criteria for such a process.

3. Theory and literature review

Removal of phosphorus and nitrogen in a biological treatment plant is complex and involves a number of processes and removal mechanisms.

Nitrogen is mainly removed by nitrification and denitrification but assimilation of N due to growth and removal of N in particulate matter are also mechanisms that contribute to the total N removal.

Phosphorus is also removed by several mechanisms. Assimilation of phosphate due to growth, chemical precipitation and flocculation of particulate P are processes/mechanisms that can contribute to the P-removal in addition to EBPR depending on wastewater composition and process conditions.

The work in this study has been focused on P-removal by EBPR. Nitrogen removal has only been included due to the link with anoxic EBPR in a process with simultaneous nitrification and denitrifying P-uptake. Emphasis has therefore been placed on EBPR in the literature review, and only a brief presentation of nitrification and denitrification is included with some results from previous studies with MBBRs.

3.1 Nitrification

Nitrogen in raw sewage is found as ammonia and organic bound nitrogen in particulate matter. While nitrogen found in particles can be removed by particle removal processes, ammonia must be converted to nitrate as the first step of the nitrogen removal process. Ammonia is converted to nitrate by autotrophic nitrification. This is a two step process performed by autotrophic bacteria, where ammonia first is oxidised to nitrite and nitrite thereafter is oxidised to nitrate. The nitrification process is performed by a limited group of bacteria. The text book examples are the bacteria *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* perform the oxidation of ammonia to nitrite, while *Nitrobacter* oxidise nitrite to nitrate.

The stoichiometry of the two steps and the total reaction are given below in Equations 1, 2 and 3, respectively:

$$NH_4^+ + 1.5\ O_2 \rightarrow 2\ H^+ + H_2O + NO_2^- \tag{1}$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \tag{2}$$

$$NH_4^+ + 2\ O_2 \rightarrow 2\ H^+ + H_2O + NO_3^- \tag{3}$$
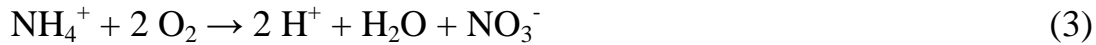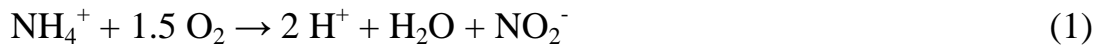
Nitrifiers are mesophilic bacteria and have an increasing growth rate up to about 35 – 40 ^{o}C, where the growth rate rapidly declines. The temperature dependency of the nitrification rate in the interval 10 – 22 ^{o}C can be described approximately by a simplified Arrhenius equation (Equation 4).

$$r_{N\text{-}T2} = r_{N\text{-}T1} \cdot \theta^{(T2 - T1)} \tag{4}$$

T1, T2:   temperatures, (^{o}C)
$r_{N\text{-}T1}$:   rate at temperature T1
$r_{N\text{-}T2}$:   rate at temperature T2
θ :   temperature coefficient

In previous studies with a MBBR, a value of 1.09 has been found for the temperature coefficient, (Rusten *et. al.* 1995a). Pastorelli *et. al* (1997a) reported a value of 1.124.

The optimal pH for the nitrification process is between 8 and 9, (Henze *et. al.* 1990). In wastewaters with low alkalinity, the alkalinity consumption and corresponding drop in pH due to nitrification can lead to lower nitrification rates.

The nitrification rate is strongly dependant on the oxygen concentration. In activated sludge systems a DO-concentration of at least 2 mg DO l^{-1} is normally used in nitrifying processes. In biofilm processes, the nitrification rate can show a dependency on the DO-concentration for much higher DO-concentrations.

In previous experiments in MBBRs with a constructed wastewater and DO-concentrations in the range 1 – 12 mg DO l^{-1}, it was found that the nitrification rate had a first order dependency on the DO-concentration (Hem 1991). Later experiments with primary and secondary effluent showed lower nitrification rates, but the same increasing trend with increasing DO-concentrations (Hem *et. al.*, 1994). Pastorelli *et. al.* (1997a,

1997b) reported that the nitrification rate was nearly first order with respect to DO concentration in pilot scale tests with MBBRs.

The organic loading rate on the process also has a significant effect on nitrification. Heterotrophic bacteria have higher growth rates and will win the competition for space and oxygen in a given process configuration. In activated sludge systems the nitrifiers will be washed out as the fraction of heterotrophs increase. In a biofilm system there is a limited amount of growth area available. Heterotrophic growth will also consume oxygen and reduce the oxygen available for nitrification. In a biofilm process this can have a pronounced effect as will be discussed further below in relation to nitrification kinetics in a MBBR.

Nitrifying bacteria can be inhibited by compounds in the wastewater. The nitrifiers are probably not more sensitive than other bacteria, but because nitrification is performed by a limited group of bacteria with slow growth rates compared to heterotrophic bacteria, the effect of inhibitory compounds can be more pronounced for nitrification activity than for degradation of organic matter.

Nitrification kinetics in a MBBR has been studied previously (Hem *et. al.* 1994, Rusten *et. al.* 1995a). The nitrification rate in a MBBR can be limited by the diffusion of ammonia or oxygen in to the biofilm. The ratio of oxygen concentration to ammonia concentration can be used to evaluate which substrate that is rate limiting. The nitrification rate is oxygen limited at DO/NH_4-ratios < 2 mg DO (mg NH_4-N)$^{-1}$ and ammonia limited at DO/NH_4-ratios > 5 mg DO (mg NH_4-N)$^{-1}$ (Hem *et. al.* 1994). For DO/NH_4-ratios between 2 and 5 there will be a transition from oxygen to ammonia limitation. A ratio of 3.2 mg DO (mg NH_4-N)$^{-1}$ has been reported as the transition ratio in the absence of organic matter (Rusten *et. al.* 1995a).

In situations where ammonia is the limiting substrate, the nitrification rate may be calculated by Equation 5.

$$r_N = k \cdot (S_N)^n \tag{5}$$

r_N : nitrification rate, (g NH_4-N m^{-2}d^{-1})
k : reaction rate constant (g NH_4-N m^{-3})$^{(1-n)}$ m^{-2}d^{-1}
S_N : concentration of ammonia in the reactor, (g NH_4-N m^{-3})
n : reaction order constant

Without limitation caused by liquid film diffusion, the reaction rate in a biofilm process changes from first order at low concentrations to half order at higher concentrations, with respect to the limiting substrate. An effect of liquid film diffusion is to increase the observed reaction order, which may then be higher than 0.5 at higher concentrations (Henze *et. al.* 1990). Liquid film diffusion has been found to be of importance in the MBBR process and Hem found a reaction order for Equation 5 of 0.7 (Hem *et. al.*, 1994).

The rate constant in Equation 5 in can be determined from nitrification rates measured under ammonia limiting conditions. Rate constants in the range 0.7 – 1.0 were found in experiments with a secondary effluent and oxygen concentrations of 4.5 – 5 mg DO l^{-1} (Hem *et. al.*, 1994).

The maximum nitrification rate at a given bulk DO concentration and temperature can be found from Equation 5, by using the ammonia concentration at the point where the oxygen concentration becomes rate limiting. When the transient ammonia concentration in the absence of organic matter is calculated, the heterotrophic oxygen consumption should be subtracted from the bulk oxygen concentration before calculation of the transient ammonia concentration.

An estimate of the heterotrophic oxygen consumption can be found from plots of nitrification rate versus oxygen concentration under conditions where the oxygen concentration is rate limiting. An oxygen consumption due to heterotrophic activity of 2.5 – 3 mg DO l^{-1} was found with a removal of soluble organic matter of 0.6 – 1.9 g SCOD $m^{-2}d^{-1}$. Nitrification rates of 1.01 g NH_4-N $m^{-2}d^{-1}$ and 1.24 g NH_4-N $m^{-2}d^{-1}$ were predicted for pre- and post-denitrifcation systems, respectively. The reported rates were for conditions with low organic loading rates, a temperature of 10 ^{o}C and an oxygen concentration of 10 mg DO l^{-1}, (Rusten *et. al.* 1995a).

The dependency of the nitrification rate in a MBBR on the organic loading rate, oxygen concentration and ammonia concentration is illustrated in Figure 1. Figure 1A shows the ammonia removal rate under oxygen limiting conditions at different organic loading rates and oxygen concentrations. In Figure 1B, the nitrification rate under ammonia limiting conditions and an organic loading rate of 0.4 g BOD $m^{-2}d^{-1}$ is shown. The

change from ammonia limitation to oxygen limitation at different oxygen concentrations is shown in the figure.
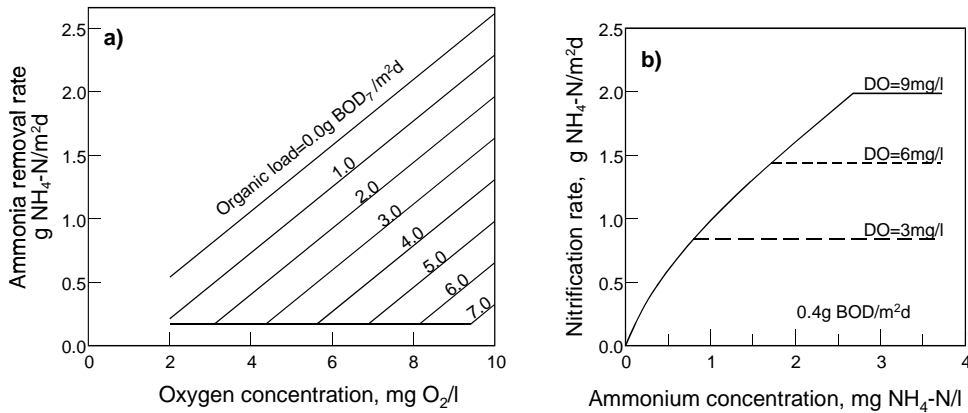
Figure 1. Influence on nitrification rate in a MBBR of organic loading rate and oxygen concentration (a) and ammonia concentration and oxygen concentration (b), (Ødegaard 2006).

3.2 Denitrification

Denitrification is performed by heterotrophic bacteria that use nitrate as electron acceptor when organic matter is oxidised. The denitrification process consists of several steps from nitrate to nitrogen gas via intermediate products (Equation 6).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \tag{6}$$
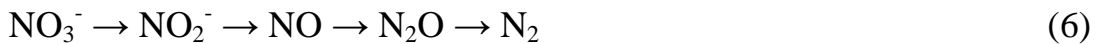
Denitrification is performed by a wide group of bacteria with different abilities. Some bacteria can perform all the steps from nitrate to nitrogen gas, while others only reduce nitrate to nitrite. Many denitrifiers are facultative and use oxygen as electron acceptor when this is available.

Nitrate and nitrite can also be used as nitrogen source for cell synthesis, but ammonia is preferred when available.

A variety of organic substrates can be oxidised during denitrification. With acetic acid as carbon source, the total reaction excluding growth of biomass can be written as in Equation 7.

$$0.2 \text{ NO}_3^- + 0.2 \text{ H}^+ + 0.125 \text{ CH}_3\text{COO}^- \rightarrow$$

$$0.1 \text{ N}_2 + 0.225 \text{ H}_2\text{O} + 0.125 \text{ CO}_2 + 0.125 \text{ HCO}_3^- \qquad (7)$$
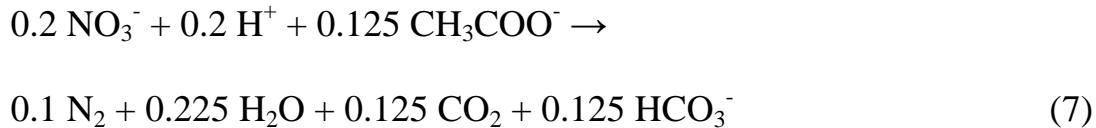
The pH increases during denitrification and reduces the pH reduction in the total nitrogen removal process. The optimal pH for denitrifying bacteria is in the same range as typical for other heterotrophic bacteria, pH: 7 – 9. A pH value lower than 7 increases the production of N_2O_2 (Ødegaard 1992).

The effect of other environmental factors such as temperature and nutrient requirements are in general about the same for denitrifying bacteria as for aerobic heterotrophic bacteria.

Oxygen will be detrimental for the denitrification process because aerobic growth will consume organic matter needed for denitrification and because many denitrifying bacteria are facultative and will use oxygen as electron acceptor when this is available.

A sufficient amount of a carbon source (C-source) that is easily biodegradable is a prerequisite for denitrification. The minimum theoretical COD consumption for denitrifying respiration with acetic acid as C-source is 2.87 mg COD_{HAc} (mg NO_3-N)$^{-1}$ based on the stoichiometry of Equation 7.

Volatile fatty acids (VFA) such as acetic acid give high denitrification rates, but a wide variety of C-sources can be used. In practice one tries to utilise the organic matter in the wastewater as C-source (internal C-source) as opposed to adding an external C-source. For this reason the denitrifying reactor(s) are often placed before the nitrifying reactors in a nitrogen removal plant. This process configuration is termed pre-denitrification and has a recycle of nitrified wastewater, as opposed to post-denitrification, where the denitrifying reactor(s) are placed after the nitrifying reactors. The wastewater composition and effluent standards in a given case will be deciding for which process configuration that will be most favourable.

Denitrification in a MBBR process has been investigated in previous studies. Rusten *et. al.* (1995b) studied nitrogen removal from municipal wastewater in a pilot plant operated in pre-denitrification mode and later in post-denitrification mode. In pre-denitrification mode, the denitrification rates were limited by the availability of C-source and the maximum

denitrification rates were as low as 0.4 g NO_x-N $m^{-2}d^{-1}$. In post-denitrification mode with pre-precipitated wastewater, maximum denitrification rates of up to 2.2 g NO_x-N $m^{-2}d^{-1}$ were measured with addition of additional C-source. Sodium acetate was used as additional C-source. The optimal C/N-ratio was fund to be 4 mg COD_{added} (mg NO_x-N_{eq})$^{-1}$. The reported C/N-ratio use nitrate equivalents (NO_x-N_{eq}) and therefore includes COD consumed by nitrite and oxygen as well as nitrate. Calculation of nitrate equivalents was done using the following conversion factors 1 mg DO l^{-1} = 0.35 mg NO_3-$N_{eq.}$ l^{-1} and 1 mg NO_2-N l^{-1} = 0.6 mg NO_3-$N_{eq.}$ l^{-1} (Rusten $et.$ $al.$ 1995b).

Aspegren $et.$ $al.$ (1998) reported a maximum denitrification rate of around 2.5 g NO_x-N $m^{-2}d^{-1}$ at 16 ^{o}C with ethanol ac C-source in a MBBR process for post-denitrification. The C/N-ratio required for complete denitrification was 4 – 5 COD_{added} (mg NO_x-N_{eq})$^{-1}$.

Pastorelli $et.$ $al.$ (1997) reported an average denitrification rate of 2.2 g NO_x-N $m^{-2}d^{-1}$ in batch tests with acetate as C-source and an average temperature of 20.1 ^{o}C.

Helness and Gisvold (2001) studied nitrogen removal in a MBBR operated with intermittent aeration to achieve simultaneous nitrification and denitrification. The results showed that a C/N-ratio of about 3.5 mg COD_{NaAc}-added (mg NO_3-$N_{eq.}$)$^{-1}$ was required for a nitrogen removal efficiency of 80 % or higher. A removal efficiency of 80 % could be achieved with an anoxic NO_x-N loading rate of about 1 g NO_x-N $m^{-2}d^{-1}$. Complete nitrification was achieved at least up to an aerobic ammonia loading rate of 0.5 g NH_4-N $m^{-2}d^{-1}$, indicating that the non-aerated phase should be about one third of the total cycle of non-aeration – aeration.

To achieve simultaneous nitrification – denitrification, a relatively thick biofilm is needed in order to maintain anoxic conditions in the deeper layers of the biofilm. The total COD loading rate on the process is therefore of importance as there will be a correlation between the steady state biomass concentration and the total COD loading rate. The results indicated that a total COD loading rate of about 5 g COD $m^{-2}d^{-1}$ was required to achieve 80 % removal efficiency for NO_x-N (Helness and Gisvold, 2001).

3.3 Enhanced biological phosphorus removal

3.3.1 EBPR process overview

EBPR is performed by PAO that are exposed to alternating anaerobic and aerobic or anoxic conditions. The process relies on several compounds: poly-β-hydroxy-alkanoates (PHA), glycogen and poly-phosphate (poly-P) that during the anaerobic – aerobic or anoxic cycle, are accumulated and stored in granules inside the bacterial cell and subsequently degraded, as well as on compounds in the wastewater.

Under anaerobic conditions the PAO take up easily biodegradable soluble organic matter (BSCOD) such as volatile fatty acids (VFA) from the wastewater and store this in intra cellular granules as PHA. The composition of PHA varies depending on the composition of the easily biodegradable compounds that are taken up. The energy required for the production of PHA under anaerobic conditions is supplied by degradation of previously stored intra cellular poly-P and degradation of previously stored intra cellular glycogen to PHB (poly-hydroxy-butyrate) (Smolders *et. al.* 1995). The phosphate produced in the degradation of poly-P is released to the wastewater. The ionic balance in the cell is preserved by release of metal cations (usually K^+, Mg^{2+}), (Christensson 1997).

Under aerobic conditions the internal storage of PHA is used for energy and as C-source for growth and for synthesis of glycogen that is stored in intra cellular granules. At the same time phosphate and counter ions are taken up from the wastewater. The phosphate is polymerised to poly-P and stored in intra cellular granules (Smolders *et. al.* 1995).

A schematic presentation of the release and uptake of phosphate and uptake and consumption of organic matter by PAO is shown in Figure 2 (Christensson, 1997).
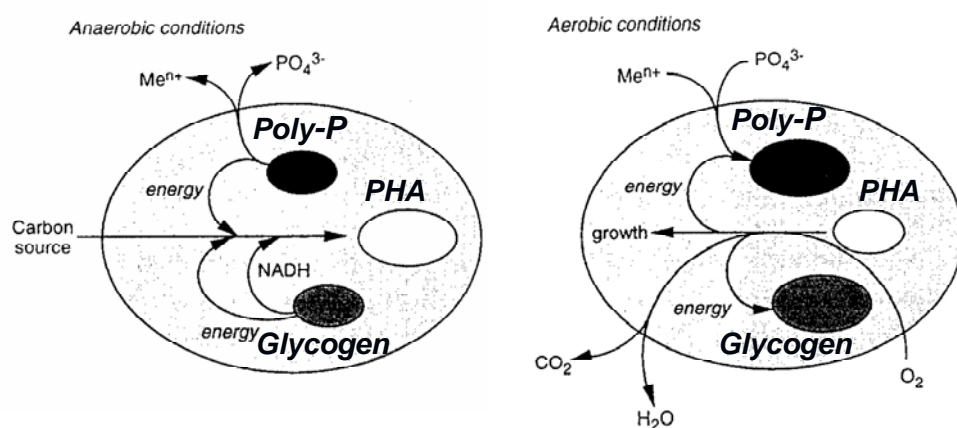
Figure 2.   Schematic presentation of EBPR. Uptake of C-source, production of PHA and release phosphate under anaerobic conditions (left). Uptake of phosphate and degradation of stored PHA (right). (Christensson, 1997).

Phosphate uptake also takes place under anoxic conditions, where nitrate is used as electron acceptor in stead of oxygen (Kerrn-Jespersen and Henze 1993). Degradation of PHA, accumulation of poly-P and synthesis of glycogen takes place during anoxic conditions, similarly to aerobic conditions. The nitrate is denitrified to nitrogen gas as in denitrification without phosphate uptake. One potential advantage with denitrifying phosphate uptake is efficient utilisation of C-source since phosphorus and nitrogen are removed simultaneously from the wastewater (Kuba *et. al.* 1996a).

Several authors have compared phosphate uptake under aerobic and anoxic conditions. Kerrn-Jespersen and Henze (1993) observed lower P-uptake rates under anoxic conditions compared to aerobic conditions and concluded that PAO could be divided in two groups: One group capable of utilising only oxygen as electron acceptor and another group capable of utilising oxygen or nitrate as electron acceptor. Wachtmeister *et. al.* (1997) found lower P-uptake rates in batch tests under anoxic conditions compared to aerobic conditions and that activated sludge not previously exposed to anoxic conditions also had a small anoxic P-uptake. Although the results could be explained by two different groups of PAO the authors tentatively concluded that it was more likely that the PAO were from one population with different induced denitrification activity. Meinhold *et. al.* (1999) determined the fraction of denitrifying PAO (DNPAO) using different batch tests. The authors recommended to use data from the initial part of

the P-uptake to avoid severe PHA limitation and concluded that the results strongly indicated two separate populations of PAO.

The ability of the PAO to accumulate substrate under anaerobic conditions and utilise this C-source under aerobic or anoxic conditions is thought to give the PAO an edge in the competition with other bacteria under changing anaerobic and aerobic or anoxic conditions.

Net growth of the PAO results in a net uptake of phosphate from the wastewater. The phosphorus is removed from the system with the excess sludge, which is removed from the end of the aerobic or anoxic part of the process where the phosphorus content of the sludge is highest.

3.3.2 The role of glycogen in the biochemistry of EBPR

The presentation of the EBPR process given above is in accordance with a metabolism for EBPR that includes three storage products: PHA, poly-P and glycogen. While PHA and poly-P have been commonly accepted, the role of glycogen in the EBPR process has been debated.

The EBPR biochemistry was reviewed by Mino *et. al.* (1998). Glycogen is thought to be involved in two aspects of the EBPR metabolism: Regulation of the redox balance in the bacterial cell and as a source of energy together with poly-P under anaerobic conditions.

Redox balance: Conversion of acetate to PHA under anaerobic conditions requires reducing power in the form of nicotinamide adenine dinucleotide (NADH). Production of NADH may be by degradation of intra cellular glycogen. This alternative for production of NADH in the anaerobic EBPR-metabolism is often referred to as "the Mino model". An other alternative, is that NADH is produced by oxidation of some acetate in the citric acid (TCA) cycle. This is often referred to as "the Comeau/Wentzel model". Results from several studies support the Mino model (Satoh *et. al.* 1992, 1996, Smolders *et. al.* 1994a, c, Bordacs and Chiesa 1989, Perieira 1996, cited by Mino *et. al.* 1998; Kong *et. al.* 2004), but does not exclude the possibility that part of the reducing power is produced by the TCA cycle (Perieira 1996, cited by Mino *et. al.* 1998).

Sodium acetate or acetic acid is often used as C-source in laboratory studies of EBPR. With acetate as C-source in an EBPR-process, degradation of glycogen is probably the source of reducing power required for PHA production as discussed above. However, since the PAO must be able to degrade a variety of organic substrates with different oxidation number, a mechanism for redox regulation in the bacteria is necessary. An integrated model for anaerobic uptake of different substrates has been developed where reducing power is produced by degradation of glycogen via pyruvate to acetyl-CoA, and consumed by degradation of glycogen via pyruvate to propionyl-CoA through the Succinate – Propionate pathway (Mino *et. al.* 1998).

Energy source: Pyruvate is an intermediate compound in the degradation of glycogen to acetyl-CoA and propionyl-CoA. The energy generated by degradation of glycogen is dependant on the pathway for production of pyruvate from glycogen, and this has also been debated. In the original Mino model the Emden-Meyerhof-Parnass (EMP) pathway was proposed for degradation of glycogen to pyruvate. The EMP pathway yields 3 moles of adenosine tri phosphate (ATP) per mole of glycogen monomer degraded. A modified Mino model with the Entner-Durhof (ED) pathway for degradation of glycogen to pyruvate has been proposed. The ED pathway yields 2 moles ATP per mole of glycogen monomer degraded. The lower energy yield of the ED pathway must therefore be compensated for by increased hydrolysis of poly-P. The different pathways therefore imply different ratios of P-release to C-source, typically acetate, uptake (Mino *et. al.* 1998).

The observed ratio of P-release to acetate uptake vary considerably between different studies, $0 - 1.52$ mol $P_{release}$ (mol $C_{acetate\ uptake}$)$^{-1}$ (Mino *et. al.* 1998) (see also section 3.3.4.3), and are therefore not conclusive regarding the pathway for degradation of glycogen. However, results using radioactive ^{13}C as tracer indicate that the ED pathway is used in the anaerobic degradation of glycogen by PAOs (Maurer 1997 cited by Mino *et. al.* 1998; Hesselmann *et. al.* 2000).

3.3.3 Carbon sources

The PAO require a carbon source for growth and also for synthesis of PHA and glycogen in the anaerobic phase. Studies with constructed wastewater

using different C-sources such as various VFAs, amino acids, glucose and alcohols, as well as studies with real wastewater in pilot scale or full scale treatment plants have shown that EBPR can be achieved with a variety of C-sources.

Acetic acid/acetate has been used as C-source in many studies (i.e. Smolders *et. al.*, 1995; Randall *et. al.*, 1997; Hood and Randall, 2001; Randall and Liu 2002; Chen *et. al.* 2005), and is generally accepted as an efficient C-source for EBPR. The first biochemical mechanisms for EBPR were based on acetic acid as C-source (the Comeau/Wentzel model and the Mino model), and mathematical models such as the metabolic model developed by Smolders *et. al.* (1995), often assume that the C-source is acetic acid/acetate. With acetic acid the main PHA produced under anaerobic conditions is poly-hydroxy-butyrate (PHB) (Randall and Liu 2002).

Propionic acid/propionate has also been investigated (i.e. Randall *et. al.*, 1997; Hood and Randall, 2001; Chen *et. al.* 2005), and found to be an efficient C-source for EBPR in some studies. However, studies with batch tests of different C-sources have reported that propionate was not as effective as acetate (Randall *et. al.*, 1997). Hood and Randall, (2001) found that propionate was effective in long term experiments with an SBR but inefficient for improving phosphorus removal in batch experiments, and concluded that the negative results from the batch tests could not be due to lack of acclimatisation. Chen *et. al.* (2005), however, found that the anaerobic P-release with propionic acid increased when the biomass was acclimatised to propionic acid, and also that the P-removal efficiency improved with acclimatisation. Lemos *et. al.* (2003) reported a metabolic pathway for utilisation of propionate by PAO based on ^{13}C-labeling and in vivo NMR (nuclear magnetic resonance) measurements. With propionic acid the main PHA produced under anaerobic conditions is poly-hydroxy-valerate (PHV) (Randall and Liu 2002; Lemos *et. al.*, 2003).

Glucose has been found to be detrimental for EBPR in some studies (Cech and Hartmann, 1990, 1993 cited by Mino *et. al.* 1998; Randall *et. al.*, 1997), while other studies have obtained good EBPR with glucose as the C-source (Jeon and Park, 2000; Wang *et. al.*, 2002; Kumar and Chaudhari, 2003). Poor results with glucose as C-source have been explained by proliferation of glycogen accumulating organisms (GAO) (see chapter 3.3.6.3) in the biomass and loss of EBPR (Cech and Hartmann, 1990, 1993

cited by Mino *et. al.* 1998). However, good phosphorus removal has also been achieved with glucose provided that operating conditions are optimised. Wang *et. al.* (2002) reported that EBPR with glucose was enhanced by a long anaerobic retention time and high initial glucose concentration. The authors proposed a biochemical model for the anaerobic utilisation of glucose in EBPR based on known biochemical pathways and the observed transformations of compounds in the SBR cycle. The model proposed by Wang *et. al.* (2002) only involved one type of bacteria (PAO). Jeon and Park, (2000) had previously proposed a mechanism for the utilisation of glucose in EBPR where glucose was accumulated and stored as glycogen by lactate producing organisms (LPO). The energy for glycogen storage was proposed to come from glycolysis of glucose to lactate. The lactate produced by the LPO was utilised by PAO for phosphorus removal. The proposed mechanism included the three storage products glycogen, PHA and poly-P, but glycogen was only stored by the LPO since PHA formation from lactate did not require degradation of glycogen by the PAO for production of NADH. The results with respect to transformation of the different compounds reported by Jeon and Park, (2000) are similar to those reported by Wang *et. al.* (2002). The proposed models from Jeon and Wang are therefore two alternatives that require verification, however, a long anaerobic retention time will favour selection of anaerobic bacteria that ferment glucose as proposed in the model by Jeon and Park, (2000). A type of mechanism where two bacteria groups interact may also require extended time to be established in a treatment plant or a laboratory system. The varying results reported with glucose as C-source may therefore be due to lack of acclimatisation or operating conditions that were detrimental for selection of a biomass with both LPO and PAO.

Other C-sources have also been studied. Randall *et. al.* (1997) compared different C-sources to a control with no C-source in batch tests with biomass from a SBR fed with VFAs produced by prefermentation of glucose. The study included glucose, fructose, starch, methanol, ethanol, propanol, isopropanol, butanol, n-amyl alcohol, formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, lactic acid, pyruvic acid and succinic acid. Isovaleric acid was identified as the most consistent and efficient C-source for inducing high P-removal, but all the $C_2 - C_5$ VFAs improved P-removal except for propionic acid. Branched isomers were found to be more effective for EBPR than linear compounds. Succinic acid was also an effective C-source for EBPR.

Butanol, propanol and isopropanol improved P-removal but were not as efficient as the VFAs with the same carbon chain length. Glucose had a negative effect on P-removal as referred to above. Fructose, starch, methanol, ethanol, n-amyl alcohol and formic acid did not have any significant effect on EBPR. Ethanol has also been investigated by others and found to be an effective C-source for EBPR, but that a long acclimatisation period (2 months) was required. The results indicated that ethanol was converted to acetate by other bacteria than the PAOs (Christensson, 1997). Hood and Randall (2001) compared the effects of different C-sources in batch tests with biomass from two SBRs that were acclimatised to acetic acid and propionic acid. The results for various C-sources were reported relative to a control where no C-source was added to the batch. The study concluded that besides acetic acid, isovaleric acid and succinic acid were also beneficial for EBPR. Glutamic acid gave higher P-release and higher P-uptake but no improvement of net P-removal. Cystine gave slightly lower P-release and P-uptake and consequently reduced net P-removal relative to the control.

Studies with different C-sources have shown that some substrates can be directly utilised in the EBPR process, while others probably require transformation before they are taken up in the anaerobic EBPR metabolism. Volatile fatty acids such as acetate and propionate can be used directly by the EBPR bacteria. Glucose and ethanol, however, must be fermented to VFA which are utilized in the EBPR metabolism, (Johansson, 1994).

In practise one will try to utilise the organic matter in the influent wastewater as a C-source. Since the concentration of VFA in influent wastewater can often be low, pre-fermentation of influent wastewater or primary sludge has been used to increase the amount of readily degradable C-source available for the PAO. VFA production by pre-fermentation and the effects of this on EBPR have been investigated in several studies (i.e. Jönsson *et. al.*, 1996; Christensson, 1997; Lie and Welander, 1997; Mavinic *et. al.*, 2000; Merzouki *et. al.*, 2005). In most cases increased VFA production by fermentation of primary sludge or influent wastewater have resulted in improved EBPR. However, increase in net P-removal may be limited. In a study with pre-fermentation in the primary settling tank, only an additional 0.5 mg P l^{-1} could be removed due to the increased VFA production in the primary settling tank (Christensson, 1997).

The ratio of COD consumed to phosphate removed varies in different studies. Abu-ghararah and Randall, (1991) concluded that at least 20 mg COD was required for removal of 1 mg P, but reported a range of 18.8 – 94 mg COD (mg P)$^{-1}$ depending on the C-source. Acetate gave the lowest value and valeric acid gave the highest value. Jönsson $et.\ al.$, 1996 found a COD-requirement of 10 – 20 mg COD (mg P)$^{-1}$ in a study of EBPR at a full scale UCT-plant. Henze $et.\ al.$, (1992), gives a value of 10 mg COD (mg P)$^{-1}$ with acetic acid as the C-source.

The observed $COD_{degraded}/P_{removed}$-ratio in a wastewater treatment plant will depend on the type of C-source since some COD will be lost if pre-fermentation is required before utilisation by PAOs, the fraction of PAOs in the mixed biomass since other heterotrophic bacteria may also degrade COD and if the COD requirement for P-removal can be clearly distinguished from the COD requirement for N-removal. These effects can to a certain extent be minimised in laboratory experiments with a constructed wastewater and a highly enriched PAO culture operated under well defined conditions. However, some variability will remain and reports of different values are therefore not surprising.

3.3.4 Environmental and operational factors affecting EBPR

3.3.4.1 Nitrate and nitrite

Several previous studies have reported that nitrate has a negative effect on the anaerobic phase of the EBPR process where phosphate release is reduced or turned to phosphate uptake if nitrate is fed to the anaerobic reactor, (i.e.; Hascoet $et.\ al.$, 1985; Wentzel $et.\ al.$, 1985). The reason for the observed effect may be that, as discussed earlier, nitrate can serve as electron acceptor for DNPAO. If nitrate is present in the phase of a SBR cycle or the reactor in an activated sludge system where the conditions are intended to be anaerobic, one will not have truly anaerobic conditions and the nitrate may cause some denitrifying P-uptake instead of P-release. This will reduce the uptake of readily biodegradable COD and production of PHA in the biomass. Another explanation is that nitrate is denitrified by non-PAO denitrifying bacteria and that this competition results in less C-source available for the PAO, and thus less production of PHA. The two explanations are not mutually exclusive. The net effect is that observed P-

release will be lower than without nitrate and that EBPR may deteriorate over time due to lack of PHA. In an EBPR treatment plant one therefore wishes to minimise the carry over of nitrate to the anaerobic stage with recirculation streams in an activated sludge plant or from the previous phase of the SBR cycle.

Nitrite is an intermediate species in both nitrification and denitrification, and is not detrimental to EBPR is the concentration is low. However, if nitrite accumulates in the system it may have a negative effect on EBPR (Kuba *et. al.*, 1996b; Meinhold *et. al.*, 1999, Saito *et. al.*, 2004)

3.3.4.2 Oxygen concentration

Aerobic phosphate uptake in EBPR depends on oxygen and therefore requires a certain level of oxygen concentration in the reactor. However, few studies have focused on the relationship between oxygen concentration and phosphate uptake. Levin and Shapiro (1965) showed that phosphate uptake depended on the aeration rate to a certain degree. At low aeration rates the phosphate uptake was low, and it increased with increased aeration up to a certain level, where the phosphate uptake reached a plateau. The dependency on oxygen concentration is typically modelled by a Monod term in mathematical models i.e. Activated Sludge Model no. 2D (Henze et. al. 1999). Aspegren (1995) operated an activated sludge pilot plant consisting of one anaerobic and two aerobic reactors. The dissolved oxygen, (DO), set point was 3 mg DO l^{-1} in the first aerobic reactor and 2 mg DO l^{-1} in the second. In a study with fixed film biofilters, Goncalves and Rogalla (1992) reported that the DO concentration in the aerobic filter quickly stabilized at 5 – 6 mg DO l^{-1} after start of aeration. Gonzalez-Martinez and Wilderer (1991) operated an anaerobic – aerobic biofilter with DO concentration increasing from above 4 mg DO l^{-1} at the start of the aerobic phase to about 8 mg DO l^{-1} at the end of the aerobic phase. The DO concentration dropped to zero within the first half hour of the anaerobic phase in most experiments, and within the first hour in all experiments. As with nitrate, one should avoid carry over of DO to the anaerobic phase of an EBPR process.

3.3.4.3 pH

Smolders *et. al.* (1994) showed that the ratio of P-release to acetic acid uptake varied with pH and explained this variation by the difference in energy required for the transport un-dissociated acetic acid (un-charged) and dissociated acetic acid (negative charge) across the cell membrane. The phosphate concentration can be quite high under anaerobic conditions. Depending on the conditions this can lead to precipitation of calcium phosphate. Maurer *et. al.* (1999) studied the kinetics of biologically induced phosphorus precipitation and found that at normal wastewater conditions with respect to pH and calcium, precipitation only occurred at high phosphate concentrations (PO_4-P concentration > 50 mg PO_4-P l^{-1} at pH 7.2 and 20 ^{o}C). At 10 ^{o}C the solubility of the calcium phosphate increased. The effect of pH on PAO is otherwise similar to other heterotrophic bacteria (Christensson 1997) and the pH-value is often controlled at pH ~7 in EBPR studies.

3.3.4.4 Temperature

Several studies have been conducted on the effect of temperature on EBPR. Brdjanovic *et. al.* (1997) studied the short-term (hours) effect of temperature in the range 5 – 30 ^{o}C and found a maximum anaerobic phosphate release at 20 ^{o}C. The aerobic conversion rates increased in the interval 5 – 30 ^{o}C. The overall anaerobic and aerobic temperature coefficients (θ) were 1.078 and 1.057, respectively. The stoichiometry under anaerobic conditions was insensitive to the temperature changes, but some effect on the aerobic stoichiometry was observed. The findings on temperature dependency and temperature coefficient were similar to previous studies (Mamais and Jenkins, 1992).

Brdjanovic *et. al.* (1998) also studied the long-term (weeks) effect of temperature in the range 5 – 30 ^{o}C, and found that the anaerobic stoichiometry was relatively insensitive to temperature changes, but that that the temperature had a marked influence on the anaerobic kinetics. The temperature coefficient in the long-term experiments was similar to the value found in the short-term experiments (θ = 1.085 versus 1.078, respectively). The long term effect of temperature on aerobic P-uptake was moderate although a marked temperature influence was found for some of the metabolic processes under aerobic conditions such as PHA

consumption and oxygen utilisation rate (OUR). The results from analysis of the microbial community using molecular ecological techniques showed that the microbial population changed with changing temperature and consisted of several (five to seven) different types of bacteria.

Optimum temperatures for phosphate uptake have been reported to be as high as 28 – 33 ^{o}C, (Mamais and Jenkins, 1992). However, good results have also been reported at lower temperatures.

Erdal *et. al.*, (2003) investigated the effect of a temperature change from 20 ^{o}C to 5 ^{o}C in a laboratory scale activated sludge process operated in a University of Cape Town (UCT) configuration (anaerobic – anoxic – aerobic series of reactors). The study concluded that the temperature effects on EBPR reaction rates were consistent with other biological and chemical reactions (i.e. decreased with decreasing temperature) and that wash out of PAO can occur at low temperatures if the sludge retention time (SRT) is too short. However, provided that the SRT was sufficient to avoid wash out, the PAO population increased at low temperatures resulting in an improved phosphorus removal. The authors concluded that the PAO are psycrophilic bacteria, and temperatures of 10 ^{o}C or lower give them an advantage relative to non-PAOs in activated sludge systems. The results are in accordance with previous findings for EBPR at low temperatures with respect to SRT (Daigger *et. al.*, (1987) and good P-removal Barnard *et. al.*, 1985).

Panswad *et. al.*, (2003) studied the effect of temperature on the microbial community of EBPR sludge and found that PAOs were dominant at 20 ^{o}C but that glycogen accumulating organisms (GAO) and ordinary heterotrophs became dominant at higher temperatures. At 30 ^{o}C the GAOs were the dominant group while ordinary heterotrophs dominated at 35 ^{o}C. The results indicated that PAOs were lower range mesophiles or psycrophiles. In an earlier study at Kelowna, Canada, based on four years of monitoring data, Vassos *et. al.*, (1987), found that an overall phosphorus removal of 68 % could be achieved at temperatures at or below 15 ^{o}C, while the treatment efficiency increased to 85 % with temperatures above 15 ^{o}C, indicating a temperature boundary at 15 ^{o}C.

The studies referred to above are all from activated sludge based systems. In a laboratory scale experiment with synthetic wastewater in a fixed film bio reactor Gonzales-Martines and Wilderer (1991) found that the

phosphate release increased with decreasing temperature in the temperature range of 15 – 25 ^{o}C. However, the phosphate concentration in the effluent at the end of the anaerobic – aerobic cycle was the same at the different temperatures. The over all removal of phosphate was thus not affected by the temperature.

3.3.4.5 Redox potential

Koch and Oldham, (1985), investigated use of redox potential, (ORP), as a parameter to control nutrient removal processes. They found that ORP could be used for this purpose provided that a suitable measurement method was chosen, and that there, at times, was a definite correlation between ORP and phosphate release in the anaerobic zone. However, the results were not reproducible when one compared data from different processes or data from different time periods from processes with the same basic configuration. According to Tracy and Flammino, (1987), a typical ORP in the anaerobic phase of a biological phosphorus removal process is under -200 mV and well over 0 mV in the aerobic phase.

3.3.4.6 Metals

Several studies conclude that potassium and magnesium are co-transported with phosphate in the EBPR mechanism, and that they are essential for the process. The molar ration between phosphate, potassium and magnesium are generally reported to be about 3:1:1 (Christensson, 1997). Tykesson and la Cour Jansen (2005) found average values for molar ratios of K/P and Mg/P in accordance with this with acetate as C-source but a higher molar ratio of K/P with propionate as C-source. Some researchers also report that magnesium may precipitate with phosphate (Gerber *et. al.*, 1987 and Rickard and McClintock, 1992).

The phosphate concentrations can reach high values under anaerobic conditions in the EBPR process and precipitation of metal phosphate may be possible if the pH is high enough. Iron, aluminium and especially calcium have been reported to precipitate phosphate in biological phosphorus removal processes, (Miya, Kitagawa and Tanaka, 1987; Arvin and Holm-Kristensen, 1985; Aspegren, 1995). However, there are also reports showing unchanged calcium concentration through the process

indicating that precipitation does not always take place, (Gerber, Mostert, Winter and de Villiers, 1987a and Aspegren, 1995).

Hascoet *et. al.*, (1985), studied the effect of copper, cadmium, nickel, zinc and lead. A slight increase in the anaerobic phosphate release was found for copper concentrations up to 10 mg Cu^{2+} l^{-1}. Phosphate uptake was slightly inhibited for copper concentrations up to 1 mg Cu^{2+} l^{-1}, and stopped completely for copper concentrations of 2 mg Cu^{2+} l^{-1} or higher. In tests including the effects of zinc, nickel, cadmium and lead, it was found that there was a threshold value beyond which there was a rapid inhibition for copper. The inhibition was proportional to the initial concentrations of zinc, nickel and cadmium. The maximum admissible initial doses were 1 mg Cu^{2+} l^{-1}, 10 mg Cd^{2+} l^{-1}, 1.5 mg Ni^{2+} l^{-1} and 5 mg Zn^{2+} l^{-1}. However a concentration of 100 mg Pb^{2+} l^{-1} had no effect on the phosphate uptake.

3.3.5 Stoichiomety and kinetics for PAO and DNPAO:

A metabolic model, based on acetic acid as C-source and including PHA, poly-P and glycogen as storage products was developed by Smolders *et. al.*, (1995) and formulated for denitrifying phosphate uptake by Kuba (Kuba *et. al.* 1996a). The conversion of compounds in moles after addition of 1 C-mole acetic acid is shown in Figure 3 (Kuba *et. al.*, 1996a).

The model was later revised to an integrated model including both aerobic and anoxic phosphate uptake to improve the description of the P-removing process under aerobic and anoxic conditions (Murnleitner *et. al.* 1997).
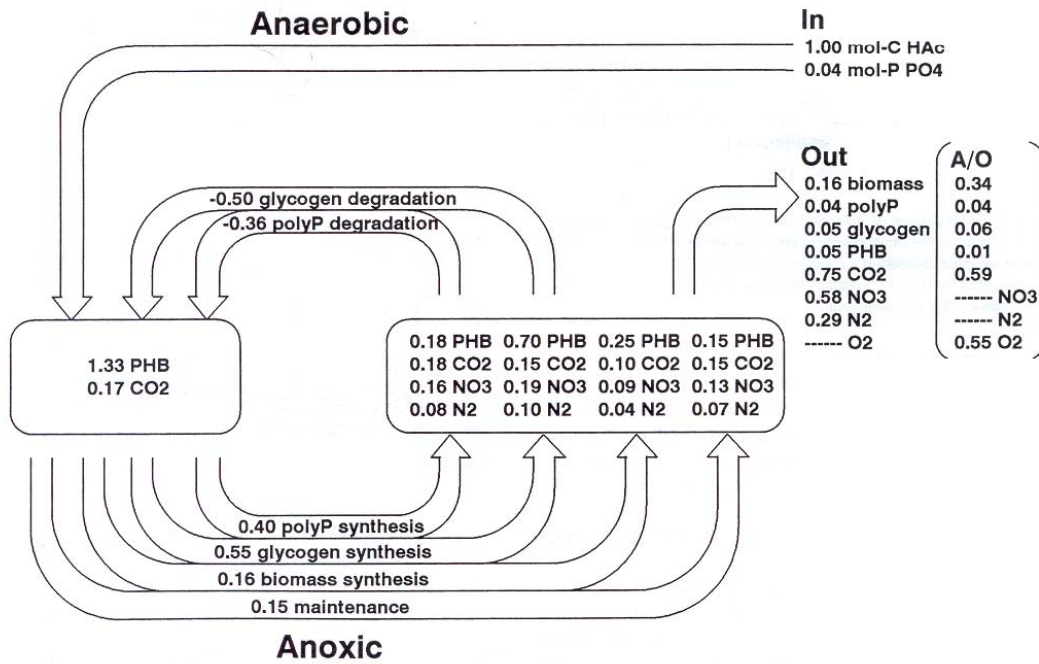
Figure 3. Conversions, expressed in moles, in the anaerobic and anoxic phase of EBPR with 8 days SRT. Output values for anaerobic – aerobic EBPR according to the model by Smolders *et. al.* with 8 days SRT shown in parentheses. (Kuba *et. al.* 1996a).

As discussed above, biological phosphorus removal depends not only on compounds in the wastewater and the biomass concentration, but also on poly-P, glycogen and PHA stored in intra cellular granules in the bacteria. In activated sludge systems the P-uptake rate, including the influence of the internal storage products, can be modelled by Equation 8, (Smolders *et. al.* 1995).

$$q_P = k_{PP} \cdot S_P/(K_P + S_P) \cdot (1-(f_{PP}/f_{PP\text{-}max})) \cdot f_{PHA} \qquad (8)$$

q_P: P-uptake rate, (g P g COD_{PAO}^{-1} h^{-1})
k_{PP}:   Rate constant, (g P g COD^{-1} h^{-1})
S_P: Phosphate concentration, (g PO_4-P m^{-3})
K_P: K_s value for phosphate, (g PO_4-P m^{-3})
f_{PP}:   PAO-biomass specific poly-P concentration, (g P g COD_{PAO}^{-1})
$f_{PP\text{-}max}$: Maximum storage of poly-P, (g P g COD_{PAO}^{-1})
f_{PHA}:   PAO-biomass specific PHA concentration, (g COD g COD_{PAO}^{-1})

In activated sludge systems the kinetic constants in Equation 8 have been found in experiments where the concentrations of poly-P and PHA in the biomass have been measured.

3.3.6 Bacteria responsible for EBPR

The microbiology of EBPR has been reviewed by several authors in the last decade (Mino *et. al.* 1998; Mino 2000; Kortstee *et. al.* 2000; Blackall *et. al.* 2002; de-Bashan and Bashan 2004). The field has developed from the common understanding that *Acinetobacter* was the bacteria primarily responsible for EBPR to the present recognition that EBPR-microbiology is complex and seems to involve a number of different bacteria. Much of this new knowledge is due to application of new of techniques for characterization of the biomass.

3.3.6.1 Characterization techniques

Chemical staining in EBPR is traditionally used to show inclusions of PHA (Sudan Black staining) and poly-P (methylene blue or toloudine blue staining) in the bacteria. Determination of whether bacteria are Gram positive or Gram negative is also based on colour staining.

Staining with the dye 4,6-diamidino-2-phenylindole (DAPI) is used to show metabolically active bacteria. DAPI-staining can also be used to show poly-P granules in bacteria since DAPI staining gives poly-P granules a yellow colour and stained biomass a blue colour under UV-excitation.

Staining can be applied to sludge samples (mixed bacterial culture) or after isolation of pure cultures (see below).

Culture based identification of bacteria is the traditional approach and depends on isolation of pure cultures by dilution of the sludge sample and spread plate inoculation on an artificial laboratory growth media. Identification of bacteria is based on the morphology and physiology of the isolated cultures that are determined according to standardized methodology and tests.

With methods developed in molecular biology, more detailed analysis of the biomass is possible and such methods are an improvement compared to the traditional culture based methods for characterization of the bacterial community. Below a short summary of the principles behind of some methods used in studies of EBPR is given. A more detailed discussion can be found in Wilderer *et. al.* (2002).

rRNA libraries are based on extraction of DNA from the bacteria and isolation of the DNA-sequences that code for the 16S rRNA and 23S rRNA molecules the bacteria use in protein synthesis. The 16S rDNA and 23S rDNA sequences are about 1500 and 3000 base pair long, respectively and contain both regions that are highly conserved (equal for all bacteria) and regions that are highly variable (specific for different bacteria). Comparison of rDNA sequences can therefore be used to differentiate between different bacteria and to determine their relation. To do this the rDNA sequences are amplified (copied) using polymerase chain reaction (PCR). PCR uses specific oligonucleotide primers that bind to conserved regions of the rDNA in two places and copies the rDNA sequences between the primers enzymatically using the enzyme DNA-polymerase. Multiple copies of rDNA strands from the total biomass are thereby obtained. Strands of rDNA with different sequences, i.e. from different bacteria, must be isolated and amplified before the sequences can be determined. This can be done by cloning where one single rDNA strand is inserted in a plasmid that is incorporated in to a bacterial cell (*E. Coli*). Different clones will therefore have different rDNA in the plasmids. After amplification in the growing *E. Coli*-cones the isolated rDNA can be extracted and sequenced. The sequences are then compared to known sequences from other bacteria and the composition of the bacterial community can be described in a phylogenetic tree where the relationship between the clones and known bacteria is shown. The method is not quantitative.

DNA or RNA fingerprinting is an approach that also starts with extraction of DNA from the bacteria in a sludge sample. However, instead of sequencing of rDNA from the total biomass as discussed above, the different rDNA strands are separated using different methods to obtain a fingerprint of the rDNA in the biomass.

Denaturating gradient gel electrophoresis (DGGE) is one such method and is suitable for analysis of microbial diversity in a sludge sample. The

first step in the analysis is extraction of rDNA from a biomass sample and amplification of the rDNA by PCR. This gives a PCR product with a number of different DNA fragments that are separated in a gel. With DGGE, separation of DNA fragments of the same length but with different base pair sequence can be achieved. This is because different base pair sequences give different melting temperatures and the partially melted DNA fragment has lowered mobility in the DGGE gel compared to the helical form (Muyzer *et. al.*, 1993). DGGE therefore gives increased resolution compared to neutral gel electrophoresis for separation of the PCR product. As for rDNA libraries, DGGE does not give quantitative results.

Restriction fragment length polymorphism (RFLP) is a method that is used in fingerprinting to classify rDNA strands that have been separated in a DGGE or by cloning. With RFLP, the rDNA strands are cut into fragments by enzymes that cut the rDNA stands at locations with specific sequences. The fragments are separated in a gel where the length of the fragment determines how far the fragment will migrate in the gel. Strands of rDNA with different sequences will therefore give different RFLP-patterns after separation of the fragments. As noted above, RFLP can be run on rDNA from clones and the results used to group clones with equal RFLP-patterns in to RFLP-types or operational taxonomy units (OTU). The method can also be used after initial separation of rDNA from the total biomass in a DGGE gel. Compared to sequencing, RFLP gives lower resolution, but is faster and can be used for screening and selection of clones or bands in a DGGE-gel for sequencing. RFLP does not give quantitative results and is not used directly on samples with rDNA strands from the total biomass. **Terminal restriction fragment length polymorphism (T-RFLP)** is a variation of the method where one end of the rDNA stand is marked. The analysis of the results after enzymatic cutting of the rDNA is based on comparing the length of the terminal marked fragment. An advantage of T-RFLP compared to RFLP is that the method can be used on samples with rDNA strands from the total biomass. The method is not quantitative.

Fluorescent in situ hybridisation (FISH) is a method that uses oligonucleotide probes that bond to rRNA inside whole bacterial cells. Attached to the gene probe is a fluorophore that gives a certain colour when exposed to laser light of a certain wave length. Probes are designed with different resolution i.e. specific for bacteria species, genus, group or all

bacteria. With FISH it is therefore possible to study the composition of a biomass sample by comparing microscope images of samples hybridized with different probes. In contrast to the gene methods discussed above, FISH can give quantitative results. FISH can also be combined with other techniques and used to link bacteria to a certain metabolism, i.e. FISH images showing certain bacteria can be combined with DAPI to identify bacteria that accumulate poly-P granules. FISH can also be combined with **microautoradiography (MAR)**, where radioactive labelled substrates (i.e. ^{13}P) are used to monitor the metabolism. However, FISH can only detect bacteria that are targeted by the probes used. To characterise the diversity of a bacterial community methods such as DGGE are better suited as an initial step. Also, FISH relies on penetration of the cell wall by the gene probe and optimisation of the procedure is required. This can include a pre-treatment of the sludge to ensure penetration of the cell wall of some bacteria.

As shown in the discussion above, a number of different DNA or RNA based techniques have been used to study the microbiology in EBPR systems. In addition some techniques that are not based on DNA or RNA have been used. **Quinone profiling** were quinones, which are part of the respiratory chain and are specific to different species, are analysed has been used by several researchers. Presence of **diaminopropane as a biomarker** for *Acinetobacter* has also been used.

3.3.6.2 Phosphate accumulating organisms – PAO

The common understanding that *Acinetobacter* was the bacteria primarily responsible for EBPR was based on microscopic studies of EBPR sludge, using chemical staining methods to show poly-P and PHA granules in the bacteria and culture based identification (Blackall *et. al.*, 2002).

With culture dependant methods for isolation of a pure species, only the bacteria that can grow on the artificial media can be selected. This is a limitation since it has been shown that probably only a small fraction of the bacteria in activated sludge can grow under these conditions (Mino *et. al.*, 1998). Uses of gene probe techniques have shown that culture dependant methods are strongly selective for *Acinetobacter* (Wagner *et. al.*, 1993). Identification of *Acinetobacter* as the main PAO may therefore have been biased by the isolation method in the early studies. Similar results were

obtained by Atkinson *et. al.* (2001) who studied the contribution of *Pseudomonas* to phosphorus uptake in a denitrifying activated sludge system. Results obtained by plating on solid growth media indicated that *Pseudomonas* was the dominating bacteria genus, but in situ identification showed that only 3 % of the bacteria were *Pseudomonas*.

Studies have been performed with pure cultures of different strains of *Acinetobacter* to investigate EBPR. In a study with *Acinetobacter Junii* (strain AS33) and sterilised mixed liquor medium based on mixed liquor from a full scale EBPR plant with addition of sodium acetate, batch tests to determine growth rates and phosphate release or uptake were performed (Momba and Cloete, 1996). The results showed phosphate release during the initial growth phase in tests with low initial biomass concentration followed by phosphate removal in the lag phase. In the tests with high initial biomass concentration, phosphate removal was observed throughout the experiment. The authors concluded that removal of phosphate was associated with biomass concentration and growth stage. However, the batch tests were performed without prior exposure of the biomass to cyclic anaerobic – aerobic conditions as used in full scale EBPR plants, and it is unclear to what extent *Acinetobacter Junii* would remove phosphate under such conditions.

Pauli and Kaitala (1996) also reported phosphate uptake with *Acinetobacter* isolates in batch tests under aerobic conditions. The four isolates were identified according to API 20NE as *A. lwoffii*, *A. baumannii* and *A. junii* (two isolates). The *Acinetobacter* isolates were obtained from aerobic activated sludge plants for pulp and paper mills. Prior to the batch tests the *Acinetobacter* isolates were pre-grown under phosphate limiting conditions in a synthetic medium with sodium acetate as carbon source. The experiments did not involve exposure to cyclic anaerobic – aerobic conditions. As noted above, it is therefore unclear to what extent the *Acinetobacter* isolates would remove phosphate under such conditions.

Tandoi *et. al.* (1998) tested *Acinetobacter* isolates from an EBPR treatment plant both under aerobic conditions and under cyclic anaerobic – aerobic conditions. The results showed phosphate accumulation under aerobic conditions but anaerobic phosphate release and acetate uptake was not observed. The authors concluded that the isolated *Acinetobacter* did not follow the metabolic models for EBPR, but that *Acinetobacter* could still

be of importance for biological phosphorus removal due to the ability to accumulate phosphate under aerobic conditions.

Zafiri *et. al.* (1999) used *Acinetobacter* (ATCC 11171) in batch experiments where conversions of phosphate, acetate and biomass were monitored under different conditions. The results showed phosphate uptake, acetate uptake and biomass growth under aerobic conditions, and a second period of growth under aerobic conditions after acetate in the water was completely consumed. Under such conditions, a release of acetate was observed during the initial part of the second growth period. This is not a typical characteristic of EBPR. In the experiments with an anaerobic phase after aerobic cultivation, the results showed phosphate release, acetate uptake and no biomass growth in the anaerobic phase. The results also showed acetate uptake and phosphorus release when acetate was added at the start of an anaerobic phase that followed a prolonged aerobic phase to give complete uptake of acetate from the water and reduce the carbon reserves stored in the bacteria. Experiments were also performed to investigate the transition from anaerobic to aerobic conditions and from aerobic to anaerobic conditions. The results from these batch experiments with several phases (anaerobic – aerobic, aerobic – anaerobic or aerobic – anaerobic – aerobic) showed the same responses as in the previous batch experiments with only one aerobic or anaerobic phase.

The results in this study showed many of the characteristics of EBPR. However, the study was limited to batch experiments performed within a time frame of some hours. Although acetate was added at the start of the anaerobic phase in at least one of the reported experiments and the batch experiment in some cases ended with a second aerobic phase, the biomass was not tested over time with repeated anaerobic – aerobic cycles according to the typical EBPR process scheme where the carbon source (wastewater) is added during anaerobic conditions.

The studies with pure cultures do not conclusively prove or disprove the involvement of *Acinetobacter* in EBPR. However, although failure to show characteristics of EBPR may be due to the experimental set up in some studies, none of the tested strains of *Acinetobacter* have shown all the characteristics of EBPR. In addition, several non-culture dependant methods have provided evidence that *Acinetobacter* are not the dominant PAOs in all cases.

Auling *et. al.* (1991) used diaminopropane as a biomarker for *Acinetobacter*. The results indicated that *Acinetobacter* was the dominant organism in treatment plants with low loading and nitrogen removal in addition to EBPR. However, the results from high loaded EBPR plants without nitrogen removal indicated that other bacteria than *Acinetobacter* were responsible for the phosphorus removal in such plants.

Wagner *et.al.* (1994) used oligonucleotide probes to study the bacterial community structure in a wastewater treatment plant with an anaerobic – anoxic – aerobic configuration (Phoredox without primary settling). The results showed that less than 10 % of the bacteria were *Acinetobacter*, and indicated that gram-positive bacteria with a high G + C DNA content were probably the most important bacteria with respect to phosphorus removal at the plant used in the study. One aspect that is included in the paper but not discussed in detail is, however, that the treatment plant in question only showed limited phosphorus release reaching a concentration of 5 – 8 mg P l^{-1} in the anaerobic stage. Although the results with respect to the fraction of *Acinetobacter* in the biomass are clear and the plant removed phosphorus from ~3 mg P l^{-1} in the influent down to ~0.5 mg P l^{-1} in the effluent, the limited P-release indicates that typical EBPR-activity was not the dominating biological process in the plant. It may therefore be questionable to correlate the dominating bacteria in the biomass, gram-positive bacteria with high G + C content, with EBPR or to rule out *Acinetobacter*.

Bond *et. al.* (1995) compared the bacterial community in activated sludge by phylogenetic analysis of 16S rDNA clone libraries from two laboratory scale SBRs, one with EBPR and one without EBPR. The results confirmed previous findings that *Acinetobacter* only constituted a small fraction of the bacteria. However, the study did not find representatives of gram-positive bacteria and thus did not confirm the findings of Wagner *et. al.* (1994) in this respect. The largest percentage of clones from both SBRs, were members of the proteobacterial beta subclass. The difference in bacterial community within this subclass between the two SBRs suggested that *Rhodocyclus* could have a specific role in EBPR. However, the authors concluded that this conclusion required further study and that: *"For the moment, bacterial phosphate removal in activated sludge remains an intriguing microbiological puzzle"*.

Later Bond *et. al.* (1999a) used FISH to characterise the biomass in laboratory scale SBRs fed with synthetic wastewater and operated to give good P-removal and later no P-removal. The results verified previous studies with respect to the limited role of *Acinetobacter*, and confirmed that beta proteobacteria were probably important for EBPR. The study also found that besides beta proteobacteria, Gram-positive bacteria with high G + C content were also a large group in the sludge with high P-removal activity. This is in accordance with Wagner *et. al.* (1994) but not in accordance with the previous study of Bond *et. al.* (1995).

Other studies have also provided evidence that *Acinetobacter* is not *the* EBPR bacteria (Cloete and Steyn, 1987; Hiraishi *et. al.*, 1989; Hiraishi and Morishima, 1990; Kämpfer *et. al.*, 1996; I Made *et. al.*, 1998, cited by Mino *et. al.*, 1998).

Other bacteria than *Acinetobacter* have also been tested as candidates for PAOs. Stante *et. al.* (1997) isolated *Lampropedia* from activated sludge and found acetate uptake, P-release and PHB formation under anaerobic conditions. Under aerobic conditions the results showed P-uptake and PHB consumption. The values of stoichiometric and kinetic parameters were lower than found in mixed cultures, but the authors concluded that *Lampropedia* could be classified as a PAO. As in many of the studies discussed above, the experiments were limited to batch experiments and did not include repeated anaerobic – aerobic cycles.

Ubukata and Takii (1994) investigated the need for repeated anaerobic – aerobic cycles. In this study the bacteria showing EBPR activity were Gram positive coccus. The results showed that organic substrate should be present in the anaerobic phase and absent in the aerobic phase to promote EBPR. The results indicated that casamino acids were efficient as substrates for EBPR. However, organic acids (acetic acid, propionic acid) were found to be inefficient as substrates for EBPR in contradiction to most other studies. The authors concluded that the enzyme system required for EBPR activity could be induced. However, the bacteria were isolated from a laboratory scale phosphorus removing activated sludge unit. It is therefore unclear if the EBPR activity was induced in the reported experiments or if this capability was already present in the bacteria isolated. The results showed that when anaerobic – aerobic cycles were introduced after aerobic incubation for one week, EBPR activity increased from the first to third cycle. This may be due to induction of the enzyme

system, but may also have been due to build up of the internal storage products that would have been depleted after the aerobic incubation.

Santos *et. al.* (1999) studied phosphorus and carbon metabolism in *Microlunatus phosphovorans* using nuclear magnetic resonance. The study was performed with a bioreactor operated under cyclic anaerobic – aerobic conditions with glucose ac C-source. The results showed glucose uptake and P-release under anaerobic conditions and P-uptake under aerobic conditions. However, the biomass did not seem to produce intra cellular storage products during the anaerobic phase, as the uptake of glucose was followed by release of acetate in the anaerobic phase. Acetate was thereafter consumed under aerobic conditions. The metabolism of the bacteria was therefore not in accordance with the metabolic models for EBPR and typical results obtained in studies with enrichment cultures. Phosphorus removal by *Microlunatus phosphovorus* has also been studied (Nakamura *et. al.*, 1991 and 1995, cited by Mino *et. al.* 1998) and was proposed as a candidate for the dominant PAO. Later studies with in situ identification of PAOs with oligonucleotide probes have, however, shown that although *Microlunatus phosphovorus* was a P-accumulating bacteria, it was present in small numbers (Kawaharasaki, *et. al.*, 1999). In this study, gram positive bacteria with high G + C content and bacteria belonging to the alpha subclass of *Proteobacteria* accumulated large amounts of poly-P. These bacteria, accounted for approximately 10 % and 7 % respectively of the total bacteria in the sludge (Kawaharasaki, *et. al.*, 1999).

Bond *et. al.*, (1999b) studied the microbial community in an activated sludge SBR operated to achieve very poor and very good phosphorus removal. The study showed differences in the bacterial community between P-removing sludge (P-sludge) and non P-removing sludge (Q-sludge) based on FISH with different probes. The dominant bacteria in the P-sludge were *β-2-Proteobacteria* and *Actinobacteria*. The Q-sludge had a less diverse bacterial community and was dominated by *β-Proteobacteria* from subgroups other than β-1 and β-2.

Crocetti *et. al.* (2000) verified the importance of *β-2-Proteobacteria* and *Actinobacteria* found previously, and designed 16S rRNA directed probes that were shown to be specific for phosphate accumulating bacteria in several EBPR sludges. The results demonstrated that bacteria closely related to *Rhodocyclus* and *Propionibacter pelehilus* were examples of PAOs. Hesselmann *et. al.* (1999, cited by Blackall *et. al.* 2002) had in a

similar study previously also concluded that a *Rhodocyclus* like bacteria, which was named *Accumulibacter phosphatis*, was a PAO.

Mino (2000) reviewed models for the carbon metabolism of PAO and candidates for PAO. The review concluded that PAO are not a few limited species but consist of phylogenetically and taxonomically diverse bacteria, and that the bacteria responsible for EBPR may vary in different situations.

Lee *et. al.* (2002) investigated population dynamics in two pilot scale activated sludge plants. One operated without nitrogen removal in the A/O configuration and the other operated with nitrogen removal in a UCT configuration. During a period of 2.5 years tests were performed without any dosage of substrates and during periods with dosage of additional phosphate, phosphate and acetate, and phosphate and glucose. The study showed that the general composition of the non-filamentous bacteria as characterised by FISH was relatively similar in the two plants and showed little change over time. The dominating bacteria in both systems were *Proteobacteria* and *Actinobacter*. In both systems bacteria related to *Rhodocyclus* were found. However, there was no significant correlation between the amount of different bacteria and the phosphorus content in the sludge indicating that no single group of bacteria could be responsible for the total EBPR in the systems.

Eschenhagen *et. al.*, (2003) analysed the microbial structure of two laboratory scale plants operated for EBPR. One plant was without nitrification (anaerobic – aerobic configuration) and the other was with nitrogen removal (Phoredox configuration). The study used FISH, T-RFLP and 16S rDNA libraries to study the bacterial community. The results varied according to the method used. The FISH-results showed a dominance of *β-Proteobacteria* and – surprisingly – no principal difference between the two plants. In contrast the results from the T-RFLP analysis showed higher bacterial diversity in the A/O-plant compared to the Phoredox plant. The results from analysis of the 16S rDNA sequences supported the diversity found by T-RFLP, but indicated a dominance of *α-Proteobacteria* in contrast to the FISH results. The differences in results between the two methods were probably because the frequencies of clones in clone libraries are not a quantitative measure of the respective bacteria in the biomass and because some bacteria are not detected by FISH without pre-treatment to make the cell wall permeable. The authors concluded that in their system *Tetrasphaera* was probably the dominant PAO, but other

PAOs such as *Microlunatus* and members of the *Rhodocyclus* group were also found in small quantities. The results support that EBPR is not performed by one type of bacteria, but is a quality found in many different bacteria.

Kong *et. al.* (2004) studied *Rhodocyclus*-related PAO in three full scale EBPR plants by FISH and microautoradiography. The results showed that *Rhodocyclus*-related bacteria accounted for between 5-10 % and 17-22% of the bacteria in the plants. The carbon and phosphate metabolism of these bacteria were in general consistent with the metabolic models for EBPR and the results indicated that glycolysis was the source of reducing power for PHA production in the anaerobic phase (Mino model).

Okunuki *et.al.* (2004) used quinine profiling, DAPI staining and FISH to monitor population changes in an activated sludge SBR. Although the reactor was operated at the same operating conditions, the EBPR activity changed significantly. In the first period good EBPR was observed, then followed a period with poor EBPR and finally a second period with good EBPR. The results form the biomass analysis showed that *β-Proteobacteria* and *Actinobacteria* were dominant in the first period with good EBPR. *Rhodocyclus* relatives were also found but the number as determined by FISH decreased during the first period. The authors did not link the decline in EBPR to the quantity of *Rhodocyclus* relatives, since the anaerobic P-release increased during the same period. During the period with low EBPR, *α-Proteobacteria* and *Actinobacteria* were the dominant bacteria based on the quinine profiles. The study concludes that the decrease in EBPR seemed to be caused by excessive P-release in the anaerobic period, but does not discuss the cause for this.

Kong *et. al.* (2005) also found that *Actinobacter* were involved in phosphorus removal in a full scale EBPR plant. The *Actinobacter* were cocci in clusters of tetrads and short rods in clumps. In contrast to the *Rhodocyclus* related PAOs found previously (Kong *et. al.*, 2004) and the EBPR mechanism described in current metabolic models, the *Actinobacter* found in this study did not take up acetate and did not produce PHA under anaerobic conditions. In stead, the bacteria utilized casamino acids and produced an unidentified storage product under anaerobic conditions. The *Actinobacter* were closely related to *Tetrasphaera*. The distribution of these *Actinobacter* PAO (APAO) and the *Rhodocyclus* related PAO (RPAO) was investigated in 10 full scale EBPR plant. The results showed

that the ratio between APAO and RPAO varied from low (0.2) to very high (9). The reason for this variation was not determined. The results show that APAO may also be important for EBPR and emphasise that metabolic models for EBPR based on acetate uptake with PHA production under anaerobic conditions may give an incomplete description of EBPR in full scale plants.

Liu *et. al.* (2005) used FISH and DAPI staining in combination with phylogenetic analysis of 16S rDNA sequences to analyse the microbial community in an SBR operated on fatty-acid rich wastewater. The phosphorus removal in the system was excellent with a 99.9 % removal of 15 mg P l^{-1} in the influent. The analysis of the 16S rDNA clone library showed that *Proteobacteria* and *Cytophaga Flavobacterium Bacteroides* (GFB) were the two main groups, and indicated that *Acinetobacter* was the predominant bacteria in the sludge (31.6 % of the 114 clones). Of the *Proteobacteria*, most clones were from the γ-subdivision followed by α- and β-subdivision. This is in contrast to some of the other studies cited were *β-Proteobacteria* have been found to be dominant (i.e. Bond *et. al.*, 1999b; Crocetti *et. al.*, 2000). However, this may be due to methodology. Eschenhagen *et. al.*, (2003) reported different results with respect to the dominating bacteria with FISH and 16S rDNA sequence analysis as discussed above. The FISH and DAPI staining was used to identify the PAO in the sludge by comparing FISH images with DAPI stain images of the same biomass. However, comparison of the corresponding FISH and DAPI images for *Acinetobacter* and for α-*Proteobacteria, β-Proteobacteria, γ-Proteobacteria* and CFB group did not show a correlation between the bacterial groups and the phosphate accumulation. Other FISH-probes including one for *Rhodocyclus* related PAO were used, but gave weak signals.

Tsuneda *et. al.* (2005) cloned nitrite reductase gene fragments obtained from two sludge samples from an anaerobic – aerobic SBR to characterise denitrifying PAO. The samples were taken shortly after start up (sample A) with low P-removal and no nitrification and after 28 days (sample B) with high P-removal (10-15 mg P (g MLSS)$^{-1}$) and nitrification. The clones were classified by RFLP analysis. The results showed that the fraction of clones in one of the RFLP patterns increased from 26 % of all clones in sample A to 67 % of the clones in sample B. Sequencing of the gene fragments and phylogenetic analysis showed that the clones from this RFLP pattern were closely related to bacteria in the *Rhodocyclus* group. Since both nitrate and

oxygen were present in the aerated phase and aerobic and anoxic P-uptake was demonstrated, the authors concluded that the gene fragment identified by the analysis was probably from a PAO that could utilise both oxygen and nitrate as electron acceptor.

Wong *et. al.* (2005) used FISH and DAPI staining to characterise the microbial community in full scale wastewater treatment plants (WWTP) in Japan. Of the 9 plants investigated, 2 were P and N removal plants, 5 were anaerobic – aerobic EBPR plants, 3 were pseudo anaerobic (anaerobic stage mixed by air) – aerobic plants and 3 were aerobic plants. The results from FISH with a number of probes specific for bacterial groups often found in EBPR processes and major bacterial divisions found in WWTP, showed that the probes could account for 30 – 60 % of the bacterial population in the plants. FISH probes specific for *Rhodocyclus* related PAOs indicated that 4 – 18 % of the bacteria were from this group. Another important group was a type of glycogen accumulating organisms (GAO) (see below) that accounted for 10 – 31 % of the bacteria detected by FISH. The number of cells with poly-P as determined by DAPI staining correlated with the total phosphorus content in sludge from aerobic conditions, but the correlation between the fraction of *Rhodocyclus* related PAOs in the biomass as determined by FISH and total phosphorus content in sludge was poor. The authors concluded that the microbial community in full scale EBPR plant is more complex than found in laboratory scale systems fed with synthetic wastewater.

It has been reported that the dominant quinines in PAO sludges are quinone-8 (Q-8) and mena-quinone-8(H_4). This has been an argument against *Acinetobacter* as a PAO since *Acinetobacter* has Q-9 (Mino *et. al.* 1998). In contrast, Lin *et. al.* (2000 and 2003) found a positive correlation between Q-9 and phosphorus removal activity measured as anaerobic P-release in several samples over a 14 week period. Although Q-8 was found to be the major quinone also in this study, there was a negative correlation between the content of Q-8 in the biomass and P-release. P-removal activity was also measured on isolated strains of bacteria. The bacteria strains with a high P-removal activity showed a clear correlation between Q-9 content and P-removal activity, while the bacteria with a low P-removal activity had much lower content of Q-9 Lin *et. al.* (2003).

Today it is commonly accepted that *Acinetobacter* is not the bacteria primarily responsible for EBPR. (Mino *et. al.*, 1998; Kortstee *et. al.*, 2000;

Blackall *et. al.*, 2002; de-Bashan and Bashan, 2004). However, some involvement of *Acinetobacter* in EBPR is still not excluded (de-Bashan and Bashan, 2004). There is an increasing understanding of the complexity of EBPR microbiology, and that EBPR is performed by a number of different bacteria that may not be the same in different places (Mino *et. al* 1998). Proteobacteria, especially belonging to the beta subclass, and Gram positive bacteria with high G + C content are commonly reported to constitute the largest biomass fraction in EBPR processes. However, other groups are also found in significant fractions. On a species level, *Accumulibacter phosphatis*, closely related to bacteria in the *Rhodocyclus* genus, bacteria in the *Actinobacter* genus closely related to *Tetrasphaera*, *Propionibacter pelehilus* and *Microlunatus phosphovorus* have been identified as PAOs with non cultivation based methods. However, the abundance of the different bacteria varies in different reports, and the microbiology of EBPR is still not understood in complete detail.

3.3.6.3 Glycogen accumulating non-poly-P organisms – GAO

Deterioration of EBPR has been reported in some laboratory studies and explained by proliferation of bacteria that can accumulate organic substrate in the anaerobic phase without P-release. However, the operating conditions that cause this are unclear and several causes have been reported including glucose in the wastewater (Cech and Hartmann, 1990, 1993, cited by Mino *et. al.* 1998), long SRT and HRT (Fucase *et. al.*, 1985, cited by Mino *et. al.* 1998) and improper seeding (Matsuo *et. al.*, 1982, cited by Mino *et. al.* 1998). Limiting the phosphate loading to the amount required for growth has been reported as a consistent method for enrichment of GAOs in laboratory processes (Mino *et. al.*, 1987; Liu *et. al.*, 1994, 1996a, cited by Mino *et. al.* 1998; Liu *et. al.* 1997).

The metabolism of GAOs is similar to PAOs except that the GAOs do not utilise poly-P to provide energy for anaerobic substrate uptake. Instead, glycogen is the sole energy source as well as the source of reducing power in the GAO metabolism (Cech and Hartmann, 1993; Mino *et. al.*, 1994, cited by Mino *et. al.* 1998). This can be possible if half of the pyruvate produced is metabolised to PHA through acetyl-CoA and half through propionyl-CoA and the glycogen is utilised through the ED pathway (Satoh *et. al.*, 1992, cited by Mino *et. al.* 1998). A model for the anaerobic conversion of acetate and propionate to PHA has been developed and can

explain the observed behaviour of GAOs when acetate or propionate is fed in the anaerobic phase (Mino *et. al.*, 1994; Liu *et. al.*, 1994; Satoh *et. al.*, 1994, cited by Mino *et. al.* 1998).

GAOs have been reported to exist in pairs or clusters, be Gram negative, stain Neisser negative or only positive on the cell walls and have no intracellular granules before anaerobic substrate uptake. (Cech and Hartmann, 1990, 1993; Liu, 1995; Liu *et. al.*, 1996a, cited by Mino *et. al.* 1998). Batch experiments with PAO enriched sludge that was poly-P exhausted but not glycogen exhausted could not take up acetate under anaerobic conditions, implying that the PAOs can not utilise glycogen as the sole energy source (Mino *et. al.*, 1998). The morphological and physiological evidence is limited, but implies that GAOs and PAOs are different organisms (Mino *et. al.*, 1998).

If GAOs and PAOs are different organisms, or even if they are the same organisms but run different metabolisms, there should be a competition for available substrate in the anaerobic phase. The PAOs utilise poly-P for energy production while the GAOs ferment glycogen to PHA and CO_2 for energy production. The GAOs metabolism is more complex and less efficient for energy production than the metabolism used by the PAOs, and the PAOs should therefore normally be able to compete with the GAOs. However, if the PAOs uptake of substrate in the anaerobic phase is disturbed for some reason, leaving excess C-source after the anaerobic phase, there may be a niece for the GAOs to grow (Liu *et. al.*, 1997). Some studies also indicate that glucose in the wastewater can replace internally stored glycogen, and this may be a factor that can induce proliferation of GAOs (Sathasivan *et. al.*, 1993; Liu *et. al.*, 1996a, cited by Mino *et. al.* 1998).

3.4 Process configurations for biological N and P removal

Traditionally full scale biological phosphorus removal has been performed with activated sludge processes but hybrid processes combining activated sludge and biofilm carriers have also been tested.

Several process configurations for activated sludge have been developed. The principle of some of these are shown in Figure 4, (Cooper *et. al.*, 1994).
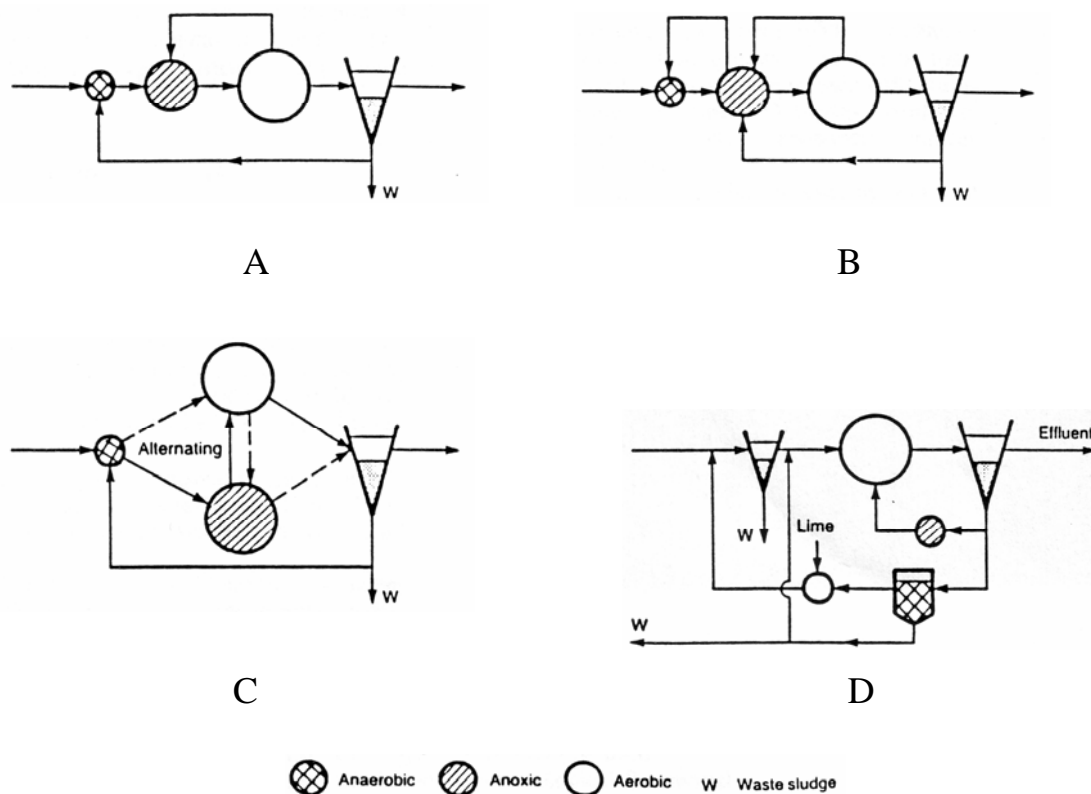
Figure 4. Principle of activated sludge biological phosphorus removal processes: Mainstream (A); UTC-process (B); Biodenipho process (C); Side stream (D), (Cooper *et. al.*, 1994).

There are two main categories of activated sludge processes for EBPR; main stream processes and side stream processes. In the main stream processes the whole wastewater stream is subjected to an anaerobic phase followed by an anoxic and/or aerobic phase. The simplest scheme is an anaerobic – aerobic (A/O) process. This is the basic process configuration required for EBPR. As noted in the introduction, nitrogen removal is normally also required and this requires an anaerobic – anoxic – aerobic (AA/O) process (Figure 4A).

Besides these basic configurations, there are several variations that seek to optimise the process or are based on special reactor types, for example: UTC (Figure 4B), Bardenpho, BB, Phoredox, Biodenipho (Figure 4C) and modified oxidation ditch and carousel process. The difference between these are the configuration of the aerobic, anoxic and anaerobic compartments and if they include nitrogen removal as well as phosphorus

removal. In a P and N removing configuration such as the UTC-process, the improvement compared to the basic configuration lies in reduced recycle of nitrate to the anaerobic stage.

In a side stream process (Figure 4D) a portion of the return sludge is subjected to anaerobic conditions in a stripper tank, where the phosphate is released from the sludge and easily biodegradable organic matter is taken up by the PAO. The sludge is returned to the aeration basin(s) from the phosphate stripper and the phosphorus is precipitated from the supernatant by addition of a suitable chemical, for example lime.

Biological N and P removal in a combined activated sludge and biofilm systems have also been reported. Nam *et. al.*, (2000) studied a laboratory scale A^2/O process where the reactors were filled with a net-type biofilm media. The experiments were performed with municipal wastewater and studied utilisation of the C-source in the wastewater. Efficient removal of nitrogen was achieved, but P removal was limited (68 %) probably due to C-source limitation. Kumar and Chaudhari (2003) compared a laboratory scale activated sludge SBR with sequencing batch biofilm reactors (SBBR) with different filling of biofilm media. The results showed equal P-removal in all three reactors, but the nitrogen removal was higher in the two SBBRs than in the activated sludge SBR.

Christensson and Welander (2004) tested an activated sludge/biofilm hybrid process in pilot scale experiments. EBPR was tested with a UCT configuration with biofilm carriers in the aerobic reactor. EBPR was achieved but depended on addition of acetic acid and sufficient anaerobic retention time (1.5 hours). The process concept with hybrid activated sludge and biofilm carriers is also known as integrated fixed film activated sludge (IFAS), (Figure 5).
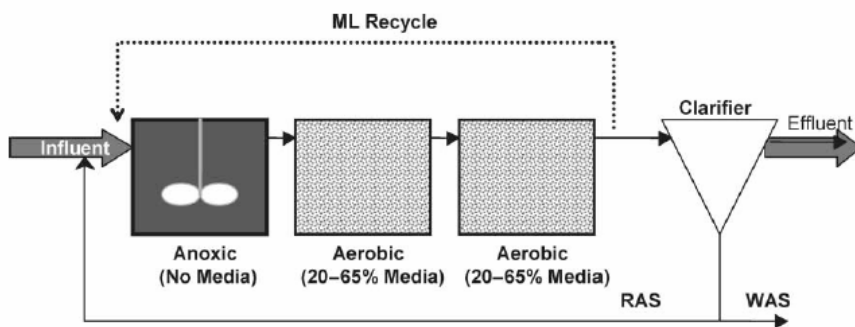

Figure 5. Illustration of the IFAS process, (Rogalla *et. al.* 2006).

The IFAS process been installed in full scale at Broomfield, USA in 2003 (Rogalla et. al. 2006). The authors conclude that the IFAS process has achieved low effluent concentrations of N and P and that the combination with carriers has reduced the aeration basin volume with about 50 % compared to conventional activated sludge.

3.5 EBPR in biofilm processes

Most of the research on EBPR discussed above has been conducted with activated sludge systems. However, there are several reports of EBPR in laboratory or pilot scale biofilm processes. In many of the early studies the focus was on the operating conditions for P- and in some cases N-removal with different biofilm reactor systems.

Gonzalez-Martinez and Wilderer, (1991), operated a fixed film SBR with cycle durations of 6, 8 and 12 hours with anaerobic periods of 25, 45 and 63 percent of the total cycle period. Shin and Park, (1991), operated a SBR with a moving bed of porous biofilm media with a total cycle time of 12 hours. The cycle consisted of a 2 hour anaerobic phase and a 5 hour aerobic phase. The rest of the time was for settling, decanting, filling and an idle period of 2.5 hours.

Goncalves and Rogalla, (1992), and Goncalves *et. al.*, (1994) reported laboratory and pilot scale experiments with several fixed film biofilters in series. In this process configuration the wastewater flow is such that the effluent from the anaerobic filter is directed to an anoxic or aerobic filter. Although the feed is continuous, the conditions in each filter must be changed after a period of time, to expose the biomass to alternating anaerobic and anoxic or aerobic conditions. Goncalves and Rogalla, (1992) reported that complete nitrification and 80 % P-removal could be achieved with a maximum HRT of 5 hours.

Goncalves *et. al.*, (1994), used five continuously fed bio filters in series with an anaerobic phase of 2.5 hours and an aerobic phase of 12 hours. This was later changed to 12 hours total cycle duration with an anaerobic phase of 1.75 hours and an aerobic phase of 9.6 hours. An illustration of the changing sequence of filters is shown in Figure 6.
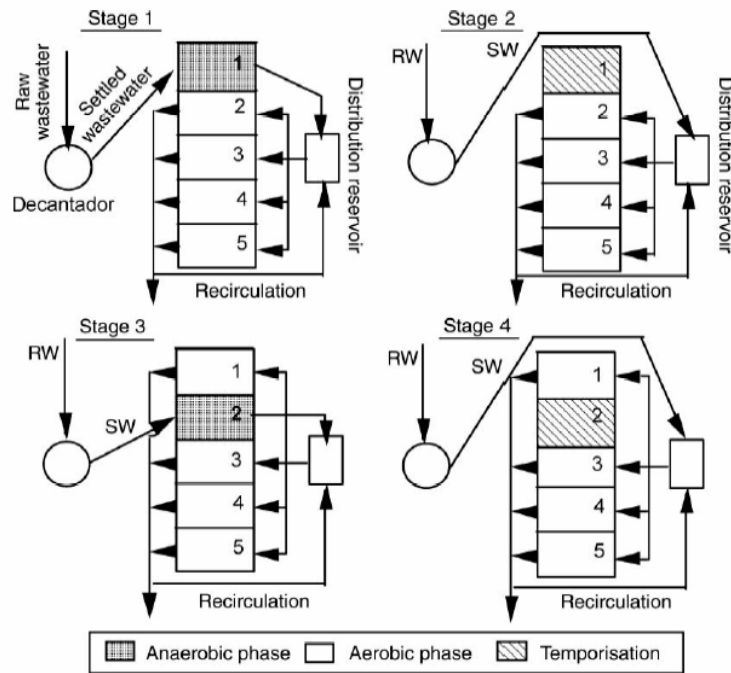
Figure 6. Illustration of the changing sequence of filters in experiments with EBPR, (Rogalla *et. al.* 2006).

Kerrn-Jespersen et. al., (1994), operated a fixed film reactor as a SBR and used a 2 hour anaerobic phase followed by a 4 hour anoxic phase. In this study the reactor was flushed with tap water between the phases to avoid acetate in the anoxic phase and nitrate in the anaerobic phase.

Rovatti *et. al.* (1995) reported on EBPR in a fluidised bed biological reactor operated as a SBR. The study concluded that this reactor type could achieve efficient P-removal but that several weeks were required for acclimatisation and selection of PAO.

Garzón-Zúñiga and González-Martínez, (1996) studied EBPR in fixed film filters operated as a SBR, and as discussed previously, reported simultaneous nitrification, denitrification and phosphate uptake in the aerated phase. Average removal efficiencies of COD, phosphate and ammonia of 89 %, 75 % and 87 %, respectively were obtained.

Morgenroth and Wilderer studied EBPR in a fixed bed filter operated as an SBR and reported on modelling of EBPR in such a system (Morgenroth and Wilderer, 1998). In a later paper they reported that mass transfer limitations by soluble compounds (oxygen and BSCOD) only had minor

effect on overall phosphorus removal and concluded that controlled removal of phosphorus rich biomass to ensure a thin biofilm was the key to successful operation (Morgenroth and Wilderer, 1999). A paper published in 2000 discussed the influence of detachment mechanisms on competition between different types of bacteria in biofilms. The study was based on the previously developed mathematical model and concluded that models with assumed constant biofilm thickness and laboratory experiments with constant erosion of biomass were not well suited to describing full-scale biofilm reactors with large variation in biofilm thickness over time (Morgenroth and Wilderer, 2000).

Falkentoft studied EBPR under denitrifying conditions in a fixed bed filter and reported on the effects of zones in the biofilm with different conditions with respect to electron acceptor and substrate. The discussion of results was based on biofilm theory (Jansen and Harremoës, 1984, cited by Falkentoft *et. al.* 1999) where a zero or half order reaction is observed in the bulk water phase depending on whether the biofilm is fully penetrated by the substrate or not. The first paper from the study reported that the results verified the theoretical half order and zero order kinetics and that the biofilm probably contained COD storing bacteria that did not remove P (Falkentoft *et. al.* 1999). Later the results were also evaluated using a mathematical model (Falkentoft *et. al.* 2000). In the same study, problems with the stability of the EBPR process were observed. The author concluded that the process could only be called quasi steady state for short periods of time and hypothesised that the observed decline could be caused by a change in the bacteria population of the biofilm (Falkentoft *et. al.*, 2001). This was further investigated in a system with nitrate as electron acceptor using FISH to characterise DNPAO (Falkentoft *et. al.*, 2002). The results showed a noticeable shift in bacteria population during the first two weeks after start up with sludge previously acclimatised to oxygen as electron acceptor. However, a decrease of EBPR activity after about one month could not be related to any measured change of the bacteria population.

The first reports on EBPR with the moving bed biofilm process were by Helness and Ødegaard, (1999), where the first results from the experiments with constructed wastewater in this study was reported. At the same time Pastorelli *et. al.*, (1999) reported on experiments with municipal wastewater where acetate was used as additional C-source. Both studies report good P-removal with simultaneous removal of nitrogen. Later a

paper covering all the experiments with constructed wastewater in this study has been published (Helness and Ødegaard 2000) and the experiments with municipal wastewater have been reported (Helness and Ødegaard, 2005).

Simultaneous N and P removal in a two-biofilter system has also been reported by Pak and Chang (2000a). In this study with municipal wastewater it was concluded that the deciding operating factors were the COD/N and COD/P ratios, nitrogen loading rate and hydraulic retention time. The process was also tested on wastewater from a car washing facility (Pak and Chang, 2000b).

Goncalves and Rogalla (2000) studied optimisation of EBPR in a submerged biofilter with continuous feed. The study concluded that a long anaerobic hydraulic retention time increased the selection of PAO in the biofilm, but that the presence of rapidly biodegradable COD in the influent to the anaerobic phase was more important than the anaerobic retention time. A phosphorus concentration in the waste sludge of 4 % (Tot-P/SST) was reported.

Castillo *et. al.*, (2000) studied start up of biofilm reactors for EBPR and compared a fixed bed filter with a biofilm membrane reactor. They reported that long anaerobic retention times during the start up were beneficial for selection of PAO because this enabled efficient competition with facultative bacteria. The authors concluded that organic loading rates higher than 5 g COD $m^{-2}d^{-1}$ were too high to guarantee the presence of PAO in the system.

Gieseke *et. al.*, (2002) studied simultaneous P and N removal in a biofilm SBR by online monitoring of concentrations in the wastewater, micro sensor measurements in the biofilm and FISH. The results showed that the nitrifying bacteria were limited to the oxic parts of the biofilm and that nitrification was delayed after the start of aeration, most probably due to competition for oxygen with PAO and other heterotrophic bacteria.

4. Biological P- and N-removal in a MBBR

4.1 The MBBR process

The idea behind the MBBR process was to combine the best features of activated sludge and biofilter processes without including the disadvantages (Ødegaard 2006). The MBBR process is based on biofilm carriers that move freely in the wastewater. The process therefore utilises the whole reactor volume. As with other biofilm processes, there is no need for a sludge recycle to maintain sufficient sludge concentration and only excess sludge need to be separated.

The MBBR can be operated with or without aeration. In an aerobic MBBR mixing is provided by the aeration while mixers are used for this purpose in anoxic or anaerobic reactors. The carriers are retained in the reactor by sieves on the outlet. Several versions of carriers have been developed. The original K1 carrier is a small cylinder with a cross inside and fins on the outside made from high density polyethylene and with density 0.95 g cm^{-3}. An illustration of an aerobic MBBR is given in Figure 7A and Figure 7B shows a full scale MBBR with an enlarged picture of a K1 carrier inserted.
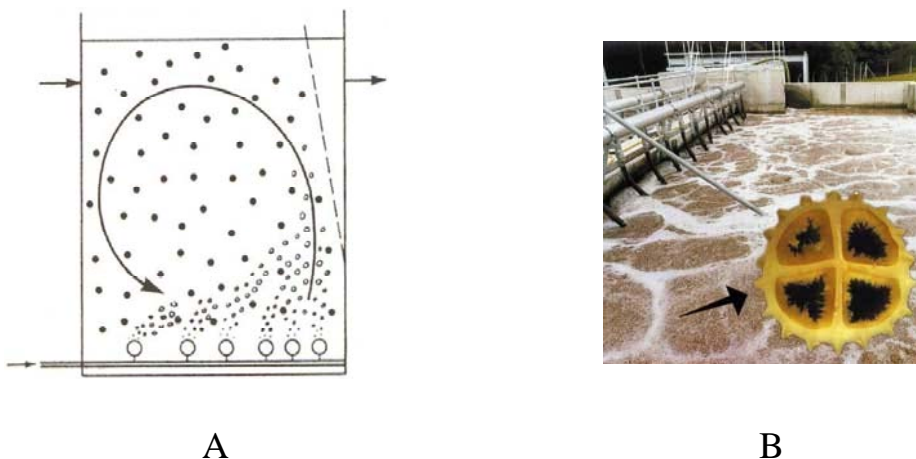
A   B

Figure 7. Principle of an aerobic MBBR (A) and full scale treatment plant with enlarged picture of K1 biofilm carrier inserted (B).

As noted previously, the MBBR was first developed for nitrogen removal but the process is used for a range of applications today. Figure 8 shows typical flow schemes for different applications.
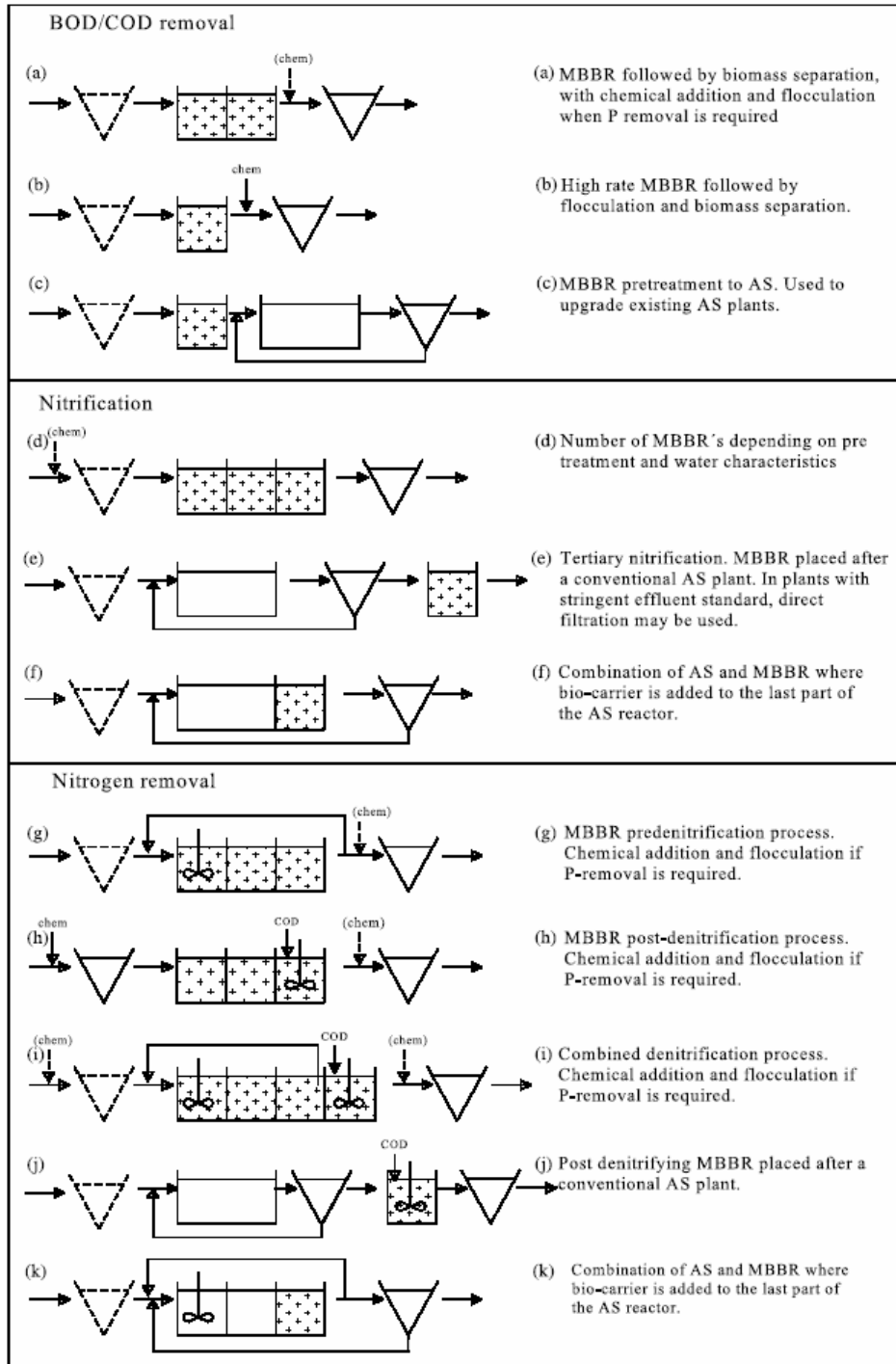

Figure 8. Typical flow schemes for various applications of the MBBR process (Ødegaard 2006).

As can be seen from the last example in Figure 8 (flow scheme k) the MBBR carriers can also be used in a hybrid configuration with activated sludge. However, as stated previously, the aim of this work was to study a pure biofilm process for EBPR. Below possible process configurations for EBPR in a pure biofilm MBBR process are discussed.

4.2 Process configurations for EBPR in a MBBR

With a moving bed there could be a possibility to achieve a process with continuous feed and exposure of the biomass to alternating conditions if the biofilm media could be moved through anaerobic and anoxic and/or aerobic zones in the reactor. Some options for this were discussed at an early stage in this study.

One way of moving the media from one zone to another could be to pump the media from one compartment to another by an air-lift pump. However, this would probably cause problems when moving the media to an anaerobic or anoxic compartment because of the resulting carry over of oxygen.

Another possibility could be to move the media hydraulically. In such a reactor the different zones could be separated by vertical walls with openings at the top and bottom. The media would then have to be moved in a vertical loop over and under the partitioning walls. This would probably be quite difficult to achieve in a satisfactory way, especially when one considers that the retention time should probably be different in the anaerobic and anoxic or aerobic zones.

One could also build the reactor as a carrousel and move the media in a horizontal loop in the same principle as carousel activated sludge plants. By placing aerators and mixers at appropriate intervals it could be possible to obtain both good vertical mixing and horizontal movement of the media.

Some ideas for possible reactor types in a continuous feed EBPR biofilm process with moving bed biofilm media are illustrated in Figure 9.
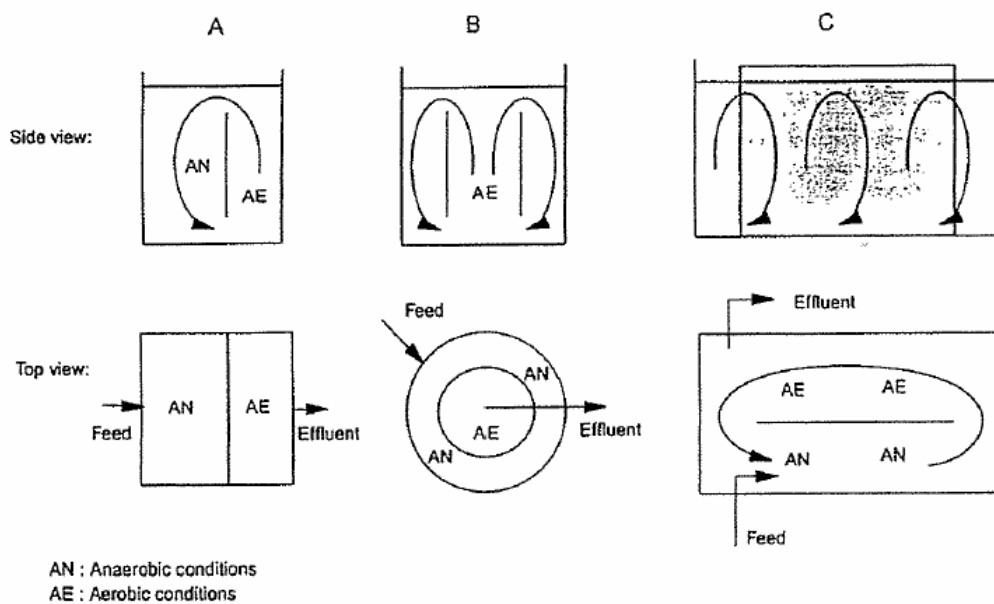
Figure 9. Possible reactor types with hydraulic movement of the KMT media. (A: vertical loop rectangular reactor, B: vertical loop circular reactor and C: horizontal carousel).

An alternative to a continuous process with a moving bed would be a sequencing process with the biofilm media in a moving bed SBR or in a stationary filter bed. A moving bed SBR would probably resemble an activated sludge SBR in many aspects. However, use of a biofilm process would facilitate simultaneous nitrification and denitrifying phosphate uptake, and an efficient utilisation of the reactor volume as discussed previously. In a process with filters one would remove biomass by back flushing of the filters and thus ensure that the biomass leaving the plant is phosphorus enriched. Also, one would probably not need a final clarifier after the biological reactors. However, a unit for separation of sludge and backflushing water would still be required.

A process based on a SBR or use of filters would need a more sophisticated control strategy than a moving bed carousel, and it would also probably be more expensive to build because of more piping and valves. However, a cost analysis would be required to conclude in this respect.

For the experiments in this study it was decided to use a SBR, since this process would be easy to implement in a laboratory scale. Operation in an anaerobic – aerated cycle would also facilitate simultaneous nitrification and anoxic P-uptake.

4.3 Stoichiometry and P-uptake rate equation

The metabolism of the bacteria performing EBPR and N-removal in a sequencing batch moving bed biofilm reactor (SBMBBR) was expected to be the same as in an activated sludge process. The stoichiometry for P and N removal with acetic acid as C-source should therefore be the same as in the metabolic models developed previously (Smolders *et. al.*, 1995; Kuba *et. al.* 1996a).

For an activated sludge process the P-uptake rate can be modelled as shown previously in Equation 8. For a biofilm system an equivalent equation can be formulated combining a power function for the effect of phosphate concentration with dimensionless expressions for poly-P and PHA (Equation 9).

$$q_{PA} = k_{nA} \cdot S_P^n \cdot (1-(f_{PP}/f_{PP\text{-}max})) \cdot f_{PHA}/f_{PHA\text{-}max} \tag{9}$$

q_{PA}:   Phosphate uptake rate, g PO_4-P $m^{-2}d^{-1}$
k_{nA}:   n-order rate constant, (g PO_4-P $m^{-3})^{(1-n)}$ $m^{-2}d^{-1}$
S_P: Phosphate concentration, (g PO4-P m^{-3})
n: Exponent giving apparent reaction order
f_{PP}:   Area specific poly-P concentration, (g P m^{-2})
$f_{PP\text{-}max}$:   Maximum storage of poly-P, (g P m^{-2})
f_{PHA}:   Area specific PHA concentration, (g COD m^{-2})
$f_{PHA\text{-}max}$:   Maximum storage of PHA, (g COD m^{-2})

The numerical values of the terms for poly-P and PHA will vary with the operating conditions and history of the biomass. The variations can be due to changes in the fraction of PAO in the biomass as well as variation in the concentration of PHA and poly-P in the biomass. In a biofilm system frequent sampling of the biomass during batch experiments is not easily done and frequent analysis of the storage compounds is not feasible. However, the terms giving the effect of the internal storage products will have a certain value at the end of a given anaerobic phase that combined with the true rate constant can be measured as an observed rate constant. The phosphate uptake kinetics can therefore be evaluated using the initial phosphate uptake rate in parallel batch experiments with different initial phosphate concentrations using a simplified form of Equation 9 with an

observed over all rate constant that is includes both the n-order rate constant and the effect of the storage compounds at the time of the batch experiments (Equation 10).

$$q_{PA} = k_{A\text{-obs}} \cdot S_P^{\ n} \hspace{4cm} (10)$$

q_{PA}:   Phosphate uptake rate, g PO_4-P $m^{-2}d^{-1}$

$k_{A\text{-obs}}$:   Observed rate constant, (g PO_4-P $m^{-3})^{(1-n)}$ $m^{-2}d^{-1}$

S_P: Phosphate concentration, (g PO4-P m^{-3})

n: Exponent giving apparent reaction order

4.4 Expected performance

In general the ratio of BSCOD (VFA) to phosphate in the influent to an EBPR process must be high enough for removal of all phosphate, and there must also be sufficient ammonium available for denitrification in a process based on denitrifying phosphate uptake. The criteria for the influent quality were expected to be the same for a SBMBBR as for an activated sludge process described in the metabolic models developed previously (Smolders *et. al.*, 1995; Kuba *et. al.* 1996a).

In an activated sludge system the solids retention time has a great influence and is used as a criteria for design. In a moving bed biofilm process the solids retention time is governed by sloughing of biomass. At steady state with a constant biomass concentration, the solids retention time (SRT) will correlate with the loading rate of the process. In a SBMBBR used for EBPR, one may define several loading rates referring to the anaerobic phase, the aerobic phase and the total cycle that can be used as design criteria.

The required values for such criteria were not clear from the literature study but qualitatively one can state that to avoid competition from non-phosphate accumulating aerobic heterotrophs, all influent BSCOD should be taken up by phosphate accumulating organisms (PAO) in the anaerobic phase. The length of the anaerobic phase in the SBR cycle will therefore depend on the loading of COD and on the composition of the COD. The length of the aerobic phase and/or anoxic phase must at the same time be sufficient for the bacteria to consume the released phosphate. This implies that even at the maximum anaerobic BSCOD-loading rate, one may have a

low total BSCOD-loading rate on the process. Based on the biochemical model for EBPR developed by Smolders (Smolders *et. al.*, 1995) and later formulated for denitrifying phosphate uptake by Kuba (Kuba *et. al.* 1996a), one may therefore expect the phosphate uptake to be controlled by the availability of PHA in the aerobic phase. Due to the low total BSCOD-loading rate, one will also get nitrification in such a process. However, the effect of heterotrophic activity may be important in a MBBR operated for simultaneous nitrification and denitrifying phosphate uptake, and the conditions in the aerated phase must allow for this to ensure complete nitrification.

One has therefore, a situation with potentially conflicting interests. On one hand the anaerobic BSCOD-loading rate should be kept low enough to avoid competition from non-phosphate accumulating aerobic heterotrophs and the aerobic ammonia loading rate low enough to achieve complete nitrification. On the other hand the total BSCOD-loading rate should be high enough to give sufficient PHA for phosphate uptake and a net growth of biomass.

4.5 Hypothesis

In summary our main expectations could be stated in the following hypothesis:

1. Biological phosphorus and nitrogen removal can be achieved in a SBMBBR with an anaerobic – aerobic SBR cycle.
    a. In the anaerobic phase uptake of carbon source and release of phosphate will take place as in an activated sludge process.
    b. In the aerated phase nitrification and denitrifying phosphate uptake will take place simultaneously, due to anoxic conditions in parts of the biofilm.

2. To achieve good removal of phosphate and nitrogen in a SBMBBR with simultaneous nitrification and denitrifying P-uptake, the process must be operated to avoid limitation caused by:
    a. Insufficient C-source.
    b. Competition between PAO and other heterotrophs
    c. Incomplete nitrification
    d. Low net phosphate removal due to limited growth

3. To avoid the limiting factors and achieve good removal of phosphate and nitrogen the process must be designed and operated within certain boundaries. These criteria can be defined by:
    a. C/P-ratio in the influent
    b. Anaerobic BSCOD-loading rate
    c. Aerobic NH_4-N-loading rate
    d. Total BSCOD-loading rate

5. Materials and methods

The experimental work was performed in two SBR reactors. The first set of experiments was performed with constructed wastewater in a laboratory scale SBR. Thereafter experiments were performed with municipal wastewater in a small scale pilot plant that included a pre-treatment unit.

5.1 SBR experiments with constructed wastewater

The studies were carried out in a laboratory scale SBR (10 l water volume, 53% filling of K1 KMT-media (Ødegaard *et. al.*, 1994)) with a constructed wastewater. A schematic representation of the laboratory apparatus is shown in Figure 10. Sodium acetate (NaCH$_3$COO x 3 H$_2$O) was used as carbon source and a phosphate buffer (NaH$_2$PO$_4$ and Na$_2$HPO$_4$) was used as P-source. The total loading rates of acetate measured as soluble COD (SCOD) and phosphorus were varied in the range 1.0 to 4.5 g SCOD m^{-2}d^{-1} and 0.04 to 0.49 g PO$_4$-P m^{-2}d^{-1}, respectively.
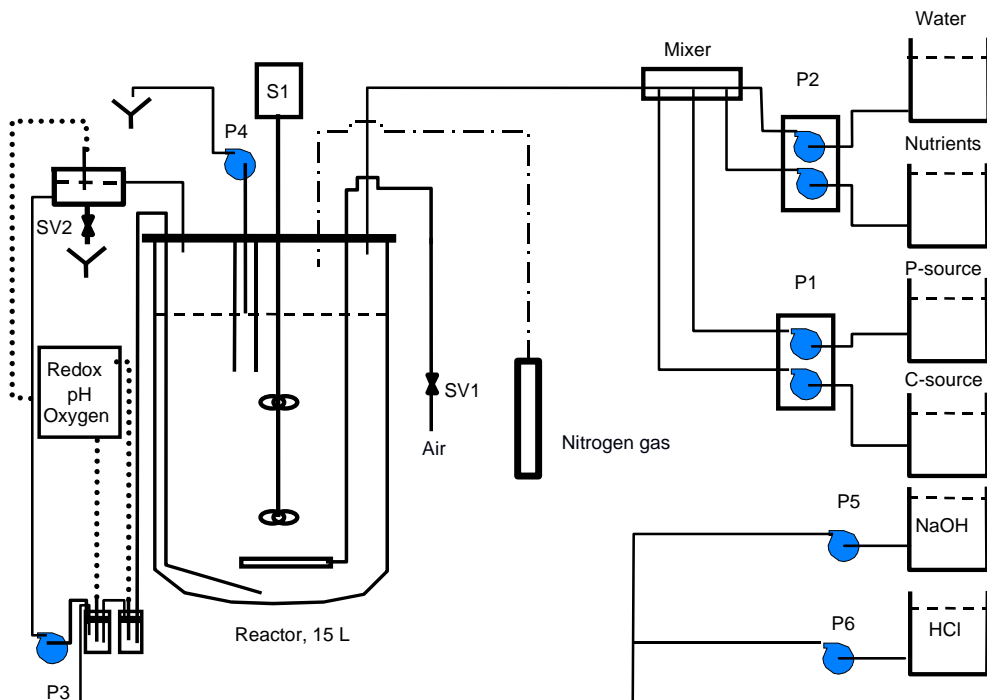

Figure 10. Laboratory SBR

The wastewater also contained ammonium that was added using a stock solution of $(NH_4)_2SO_4$. Due to variations in the concentration of the stock solution and capacity of the peristaltic pumps over time, the ammonia concentration at the start of the cycle varied in the range varied from $4.3 - 29.5$ mg NH_4 l^{-1} with an average of 21.3 mg NH_4 l^{-1}. The variation in the dosage of stock solution and cycle times gave an aerobic ammonia loading rate in the range of 0.11 to 1.1 g NH_4-N $m^{-2}d^{-1}$.

The wastewater was made with tap water and micro nutrients were not added since previous experience at our laboratory had determined that this was not required. However, $MgSO_4$ x 7 H_2O, KCl and $NaHCO_3$ was added to the wastewater giving concentrations in the feed of 7.7 mg Mg l^{-1}, 8.5 mg K l^{-1} and 65 mg l^{-1} respectively.

The pH was controlled at $7 - 7.5$ by addition of HCl (0.5 M) or NaOH (0.5 M). The headspace of the reactor was flushed with nitrogen gas to ensure anaerobic conditions in the anaerobic phase of the cycle.

The SBR was operated with a total cycle length of 4 to 6 hours and varying lengths of the anaerobic period (1 to 3 hours). Dissolved oxygen concentration (DO), redox potential (ORP) and pH were measured on-line and recorded by a data logger. The water volume in the reactor was emptied completely between each cycle. The SBR cycle is illustrated in Figure 11.

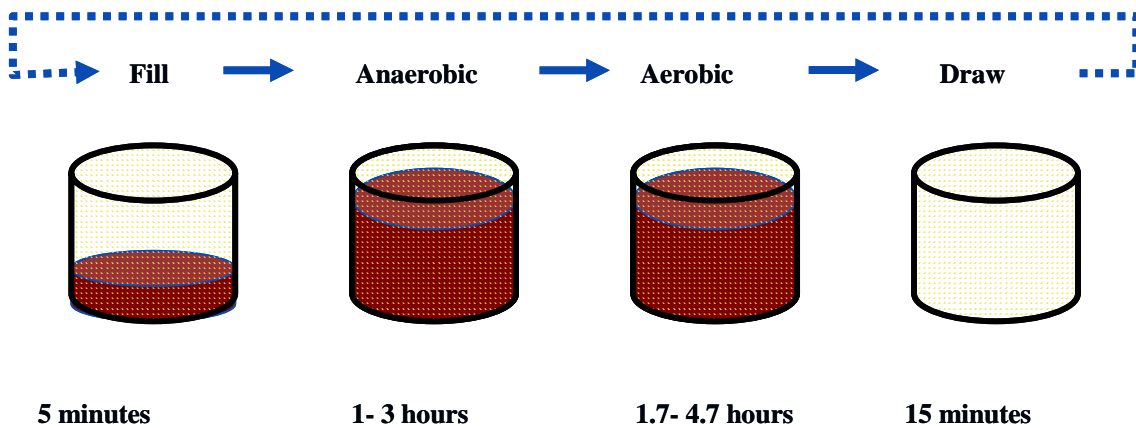| Fill	Anaerobic	Aerobic	Draw
5 minutes	1- 3 hours	1.7- 4.7 hours	15 minutes

Figure 11. Illustration of the SBR cycle used in the experiments with constructed wastewater.

All wastewater analyses were performed according to Norwegian Standard.

Batch experiments to characterise the aerobic and denitrifying phosphate uptake capacity of the biomass were performed as described in section 5.3 below.

5.2 SBR experiments with municipal wastewater

The experiments were performed in a small-scale pilot plant (Figure 12) (20 l water volume, 47 % filling of K1 KMT-media) operated in a sequencing batch mode with a total cycle length of 6 hours.

The reactor was fed with municipal wastewater pre-treated in a coarse media filter (CMF) to remove suspended solids. Between the CMF and the SBMBBR there was a buffer tank (20 l). Additional carbon source, Sodium Acetate (NaCH$_3$COO x 3 H$_2$O), was dosed at the start of each cycle in most experiments. Additional phosphate (NaH$_2$PO$_4$ and Na$_2$HPO$_4$ buffer solution) and ammonium ((NH$_4$)$_2$SO$_4$) was also dosed in some experiments as described below.
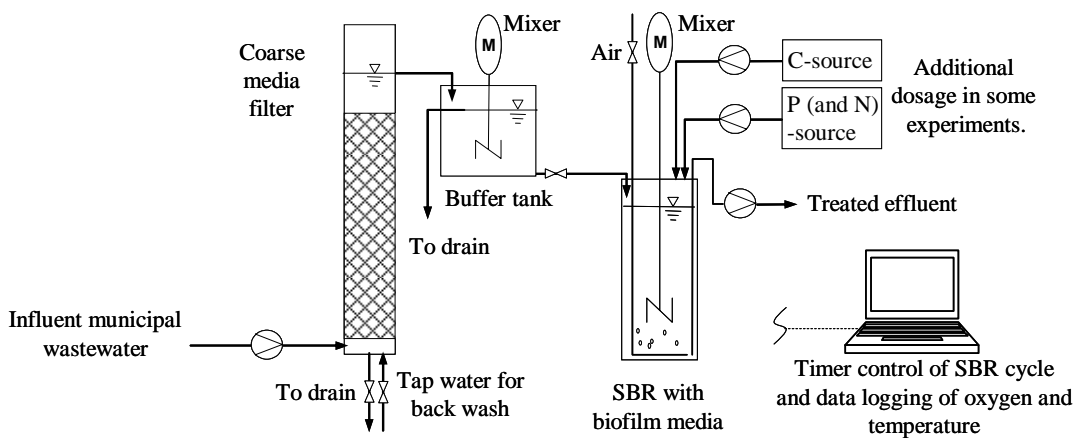

Figure 12. Illustration of the small-scale pilot plant used in the experiments.

The experiments were divided in two main parts each divided in several periods. In part 1, the focus was on maximum P-removal capacity. The SBR cycle had a 2 hour and 10 minutes anaerobic phase (including 10 minutes fill) followed by a 3 hour and 50 minutes aerobic phase (including 20 minutes draw). The CMF was back washed daily. A phosphate solution was dosed (additional 14 mg PO$_4$-P l^{-1}) to the influent wastewater to ensure that the system was not phosphate limited. In order to verify previous

results with respect to total and anaerobic loading rates of easily biodegradable soluble COD (BSCOD) the experiments were started without dosage of additional carbon source (C-source). Later the BSCOD-loading rate was varied by dosage of C-source at different levels.

Batch experiments to characterise the aerobic and denitrifying phosphate uptake capacity of the biomass were performed as described in section 5.3 below.

In part 2 of the experiments, the focus was on nitrification and utilisation of the influent COD. Thereafter the aim was to demonstrate stable N and P-removal over a period of time. The SBR cycle had a 1 hour and 40 minutes anaerobic phase (including 10 minutes fill) followed by a 4 hour and 20 minutes aerobic phase (including 20 minutes draw). The coarse media filter was back washed daily in the initial phase of period 2A. Thereafter the filter was not back washed to allow hydrolysis of particulate COD (PCOD) in the filter. In period 2A a phosphate solution was dosed as in part 1 and an ammonium solution was dosed (additional 5 mg NH_4-N l^{-1}) to increase the ammonium loading rate. In period 2B the ammonium dosage was stopped and the phosphate dosage was reduced to 2 mg PO_4-P l^{-1} giving an average phosphate concentration in the influent to the SBMBBR of 4.2 mg PO_4-P l^{-1}. Part 2 was started without dosage of additional C-source. Thereafter, in the last phase of period 2A and in period 2B, dosage of additional C-source was varied in small steps to optimise the performance. During period 2B, JAR flotation tests were performed as described by Melin *et. al.*, (2002) to evaluate separation of suspended solids in the SBMBBR effluent.

All analyses were performed according to Norwegian Standard.

5.3 Batch experiments

Batch experiments were performed with biomass from the laboratory scale SBR operated with constructed wastewater and with biomass from the small pilot scale SBR operated with municipal wastewater.

Batch experiments with constructed wastewater of the same type as used in the SBR experiments were performed, using beakers (1 l water volume) equipped with magnetic stirrers and diffusers for aeration. In anoxic

experiments the beakers had lids and were sparged with nitrogen gas. In each set of aerobic or anoxic experiments four beakers were used to obtain parallel experiments with different initial phosphate concentrations. The SBR reactor was stopped after the anaerobic phase. Thereafter, biofilm media with attached biomass was transferred from the SBR to a solution with the same constituents as the constructed wastewater, except for carbon source and phosphate, to rinse the biofilm media briefly before addition to the beakers. Each batch experiment was started by adding 0.5 l of K1 biofilm media to the beaker. In anoxic experiments nitrate ($30 \text{ mg } NO_3\text{-N l}^{-1}$) was added at the start of the experiments. Thereafter water samples (30 ml) were collected from the beakers at intervals using a syringe. The samples were immediately filtered through a Wathman GFC filter. Samples from anoxic beakers were collected through a sampling tube to avoid oxygen contamination.

Batch experiments with biomass from the SBR operated with municipal wastewater followed the same procedure except that previously collected centrifuged effluent from the SBR was used instead of constructed wastewater.

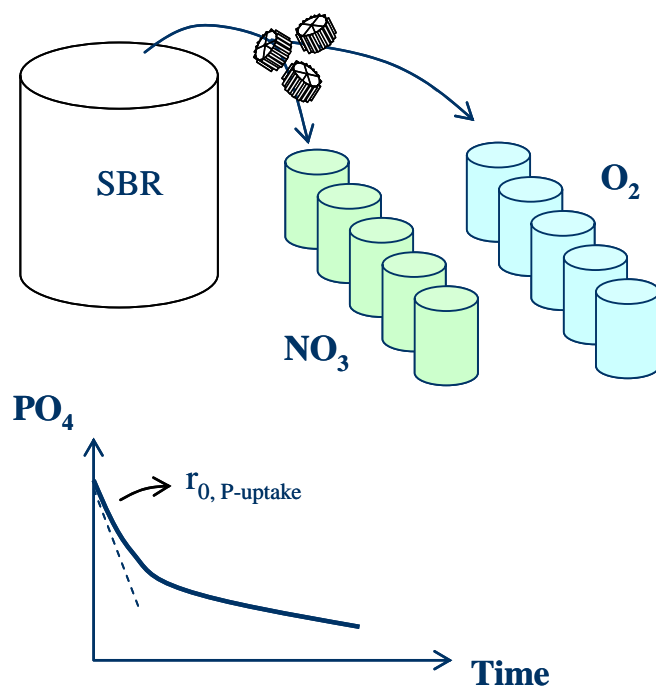All wastewater analyses were performed according to Norwegian Standard.

Figure 13. Illustration of parallel batch tests and determination of the phosphate uptake rates.

Phosphate uptake rates where determined by fitting a third order polynom to the experimental data. The choice of a third order polynom was empirical. However, this is an equation that allows curvature (the rate changes with time) and that the rate of change of the rate (second derivative of concentration with respect to time) changes with time. To avoid meaningless results, only data from the time interval before the inflection point in the third order equation have been used. An illustration of the batch tests and determination of the phosphate uptake rates is shown in Figure 13.

5.4 Biomass characterisation

During the experiments with municipal wastewater, biomass samples were collected approximately every fourth week at the end of the aerated phase by removing 20 pieces of biofilm carrier. The carriers with biomass were dried over night at 60 ^{o}C and weighted. Biomass was removed from the carriers by putting them in a flask with ultra pure water (20 ml) that was placed in an ultrasound bath for 60 minutes. After ultra sound treatment the carriers were rinsed with ultra pure water (2 x 10 ml) and the samples (40 ml) were frozen for later analysis as described below. The carriers were cleaned with detergent and additional ultra sound treatment to ensure complete removal of any residual biomass, dried at 60 ^{o}C and weighted for gravimetric determination of the biomass concentration on the carriers.

Analysis of the biomass samples was done at an external laboratory. Analysis of the biomass diversity was done by DGGE on DNA extracted from the biomass samples. The DNA in selected DGGE bands was purified by cloning and analysed by RFLP. Finally selected clones were sequenced to identify the closest related microorganisms. A detailed description of the procedures (Brakstad, 2003) is given in Appendix A.

6. Results and discussion

6.1 Experiments with constructed wastewater

6.1.1 Overview

The experiments with constructed wastewater were performed during a time period of nearly 4 years. During this time the bioreactor was kept in operation and checked daily. Samples were collected throughout the period with varying intensity depending on the experimental activity.

Figure 14 presents the aerobic phosphate uptake rate during the period of experiments with constructed wastewater. The dotted vertical lines mark changes in the SBR-cycle or average COD-loading rate as described in Table 1.
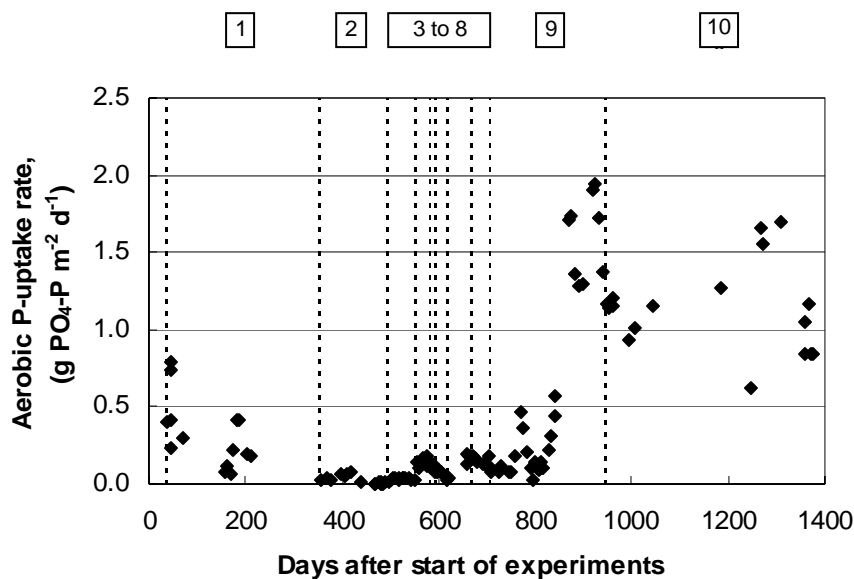

Figure 14. The aerobic phosphate uptake rate during the experiments with constructed wastewater. Vertical dotted lines mark changes in the operating conditions as described in Table 1 below.

The figure shows that limited EBPR activity was achieved after start up, but this declined during the first 200 – 300 days of operation in period 1. Thereafter followed about 500 days covering periods 2 through 8 where the EBPR activity was low or nonexistent. In these periods several strategies were followed with respect to combination of total cycle time, length of anaerobic period and COD-loading rate, and the boundaries for the limiting factors for EBPR in this system were investigated. Finally, after about 700 days of operation, EBPR activity was re-established in period 9 and maintained until the end of period 10 when the reactor was stopped after 1373 days of operation. During the last part of period 9 and in period 10 (day 869 – 1373) the EBPR activity was good with a phosphate release higher than 25 mg PO_4-P l^{-1}. This period is referred to as "good EBPR activity" in the following discussion of the results.

Table 1 gives an overview of operating conditions in the different experimental periods. The values for COD-loading rate and ratio of COD to phosphate in Table 1 are average values from analysis of the influent to the reactor, and the COD-loading rate refers to the total SBR-cycle (total COD-loading rate). In periods 9 and 10 the total COD-loading rate was varied by adjusting the concentration of sodium acetate in the feed to optimise performance. The total COD-loading rate and COD/P ratio therefore varied in the ranges 1.4 – 4.1 g SCOD $m^{-2}d^{-1}$ and 8 - 24 mg SCOD (mg PO_4-P)$^{-1}$, respectively.

Table 1. Operating conditions in experiments with constructed wastewater.

Period No.	Start samp-ling day	End of period	Aerobic period, (h)	Total cycle, (h)	Average COD-loading rate, (g SCOD $m^{-2}d^{-1}$)	Average influent COD/P, mg SCOD (mg PO_4-P)$^{-1}$
1	35	210	1	6	3.3	45
2	358	493	1	6	1.8	20
3	498	549	2	6	1.7	19
4	556	581	2	4	2.6	11
5	584	596	2	4.8	2.0	12
6	598	617	2	4.8	2.9	19
7	623	668	2	4.8	3.5	20
8	672	709	3	6	3.2	20
9	710	941	2.5	6	2.7	16
10	948	1373	2	6	2.6	16

In period 9, the aerobic phosphate uptake rate increased up to a maximum aerobic P-uptake rate of 1.95 g PO_4-P $m^{-2}d^{-1}$, but decreased again and typically varied in the interval 1 – 1.5 g PO_4-P $m^{-2}d^{-1}$ during the period with good EBPR activity. This variation can partly be explained by variations in the operating conditions, but also illustrates that the system is highly dynamic and requires optimisation for stable removal of phosphate.

Before the results are discussed with respect to removal of phosphate and nitrogen, the factors that set limits to the conditions for successful operation of the process shall therefore be discussed.

6.1.2 Limiting conditions

Figure 15 illustrates the importance of competition between PAO and other heterotrophs, and the effect of a too low total loading rate of COD. Figure 16 illustrates the effect of a low COD/P ratio in the feed and the effect of nitrite accumulation.
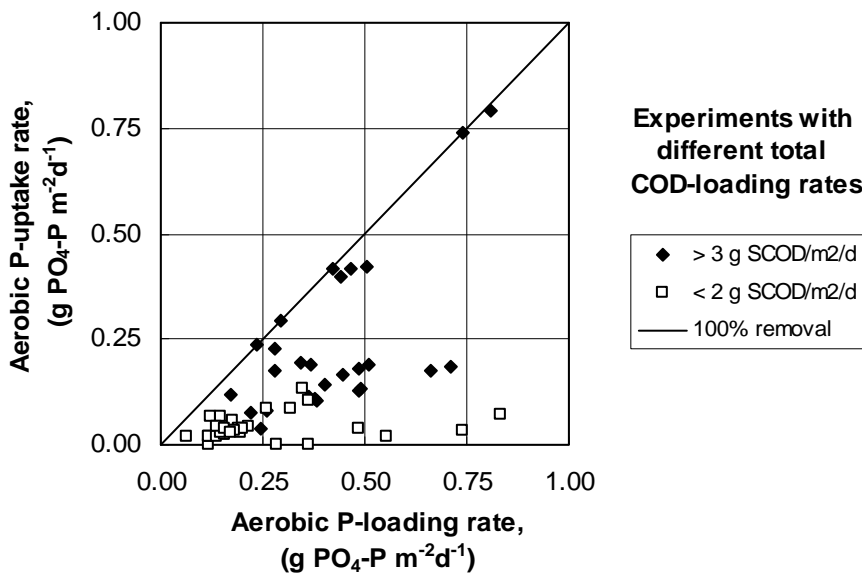

Figure 15. Aerobic phosphate uptake rate versus aerobic phosphate loading rate at different average total COD-loading rates.

In Figure 15 a plot of the aerobic phosphate uptake rate versus the aerobic phosphate loading rate is shown for experiments with average total COD-loading rates < 2 g SCOD $m^{-2}d^{-1}$ and > 3 g SCOD $m^{-2}d^{-1}$. The data for total

COD-loading rates < 2 g SCOD $m^{-2}d^{-1}$ are from periods 2, 3 and 5, and the data for total COD-loading rates > 3 g SCOD $m^{-2}d^{-1}$ are from periods 1, 7 and 8.

In period 1 the COD/P ratio (45 mg COD $(mg\ P)^{-1}$) was above what was required to achieve complete removal of phosphate, and the results show excellent phosphate removal in some samples and aerobic P-uptake rates higher than 0.24 g PO_4-P $m^{-2}d^{-1}$. However, some of the results from period 1 and all the results from periods 7 and 8 show phosphate uptake rates lower than 0.24 g PO_4-P $m^{-2}d^{-1}$ and incomplete P-removal (< 90 %). In these experiments the anaerobic COD removal was low (average 57 %) while the average anaerobic COD removal was on average 88 % in the experiments with phosphorus uptake rates of 0.24 g PO_4-P $m^{-2}d^{-1}$ or higher. The COD/P ratio was lower in periods 7 and 8, (20 mg COD $(mg\ P)^{-1}$), than in period 1. However, there was excess COD available at the end anaerobic period and EBPR was therefore not limited by availability of COD in the wastewater.

If the anaerobic period is shorter than necessary for consumption of all influent COD, the fraction of PAO in the biofilm may decrease due to growth of heterotrophs other than those responsible for EBPR in the aerobic period. This in turn will lead to a poor removal efficiency of phosphate. Also, this may cause the phosphate uptake in the aerobic phase to be limited by the amount of COD taken up by the PAO in the anaerobic period, even if the influent COD/P ratio is higher than required for complete removal of phosphate.

In the experiments with a total loading rate of COD < 2 g SCOD $m^{-2}d^{-1}$, the total COD-loading rate is too low to achieve sufficient net growth of biomass. Consequently the removal of phosphate is poor. The minimum total COD-loading rate will depend on the amount of biomass in the system. For the system used in these experiments this value was about 2 g SCOD $m^{-2}d^{-1}$.

In Figure 16, the aerobic phosphate uptake rate is shown for two sets of data from the experiments with an average total COD-loading rate in the range 2.6 – 2.9 g SCOD $m^{-2}d^{-1}$. The data are from periods 4, 6, 9 and 10 and the low COD/P ratio in the feed, especially in period 4, can be seen from the shift in results towards higher loading rates.

These experiments were, in most cases, run with an excess of phosphate relative to COD in the feed, and complete removal of phosphate was therefore not possible even if all influent COD was consumed in the anaerobic phase. However, in the experiments with a low average nitrite accumulation rate (0.02 g NO_2-N $m^{-2}d^{-1}$), the phosphate uptake rate increased with increasing phosphate loading rate, and the phosphate removal is close to the maximum possible considering the COD limitation in the feed. In the experiments with nitrite accumulation (average 0.14 g NO_2-N $m^{-2}d^{-1}$), the aerobic phosphate uptake rate showed a maximum value of 0.18 g PO_4-P $m^{-2}d^{-1}$. The reason for this is probably inhibition of the phosphate uptake by nitrite, which has also been reported in other studies, (Kuba *et. al.*, 1996b; Meinhold *et. al.*, 1999, Saito *et. al.*, 2004). In this set of experiments the aerobic phase was short (1.7 hours aeration time), giving a high ammonium-loading rate, which resulted in incomplete nitrification and accumulation of nitrite.
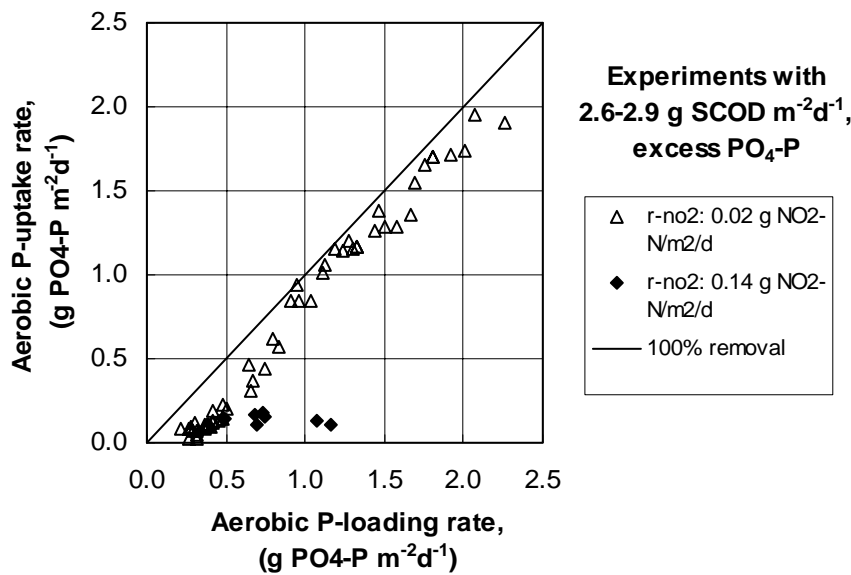

Figure 16. Aerobic phosphate uptake rate versus aerobic phosphate loading rate at different aerobic nitrite accumulation rates.

To achieve good phosphate removal the limiting conditions discussed above must be avoided. A low COD/P ratio in the feed can be dealt with by dosage of a carbon source (i.e. acetic acid), and maintaining a sufficient total COD-loading rate is a matter of controlling the total cycle length. To avoid competition from non P-accumulating aerobic heterotrophs, good anaerobic COD removal is essential.

Figure 17 shows the anaerobic COD-uptake rate versus the anaerobic COD-loading rate. The results indicate that the anaerobic COD-loading rate should be kept below 10 g SCOD $m^{-2}d^{-1}$ in order to have stable performance. However, much higher uptake rates were observed in the experiments with constructed wastewater and further studies were required to determine the limits with respect to the anaerobic COD-loading rate.
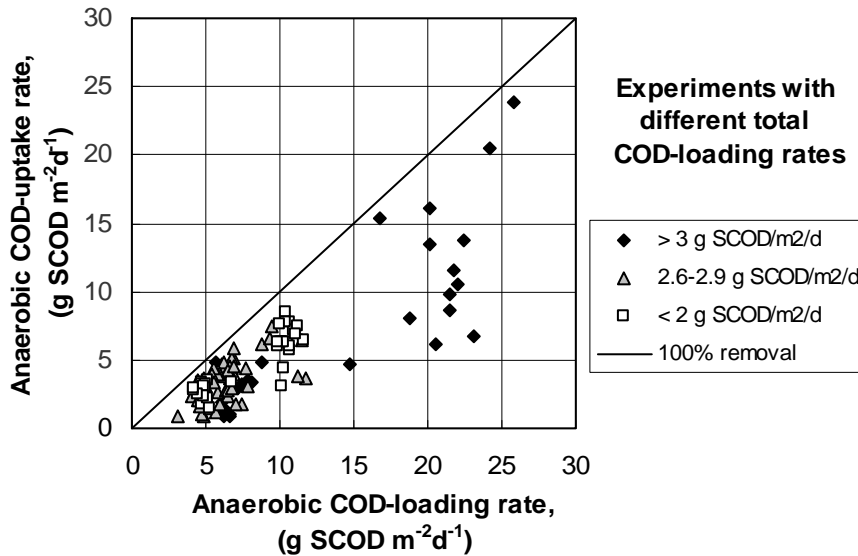

Figure 17. Anaerobic COD-uptake rate versus anaerobic COD-loading rate.

To avoid inhibition of the phosphate uptake by nitrite accumulation, the aerobic phase should be long enough to achieve complete nitrification. Complete nitrification is also a prerequisite for efficient nitrogen removal.

The aerobic ammonium removal rate is plotted versus the aerobic ammonia loading rate in Figure 18. The results showed that near complete removal of ammonia was achieved for aerobic ammonia loading rates up to about 0.6 g NH_4-N $m^{-2}d^{-1}$. However, the removal rate did not increase with higher ammonia loading rates, and there are also some data points at lower ammonia loading rates that show incomplete nitrification.

The data points in Figure 18 that are marked with red circles are from the experiments in Period 4 with nitrite accumulation discussed above. These experiments were performed with a short aerobic period (1.7 hours aeration), giving a high aerobic ammonia loading rate. The total cycle was

also 4 hours, and the relatively frequent changes in operating conditions may also have stressed the nitrifying bacteria and contributed to the nitrite accumulation. In the following period (Period 5) the average aerobic ammonium loading rate was reduced to below 0.6 g NH_4-N $m^{-2}d^{-1}$ by increasing the aeration time to 2.5 hours. The total cycle length was increased to 4.8 hours.
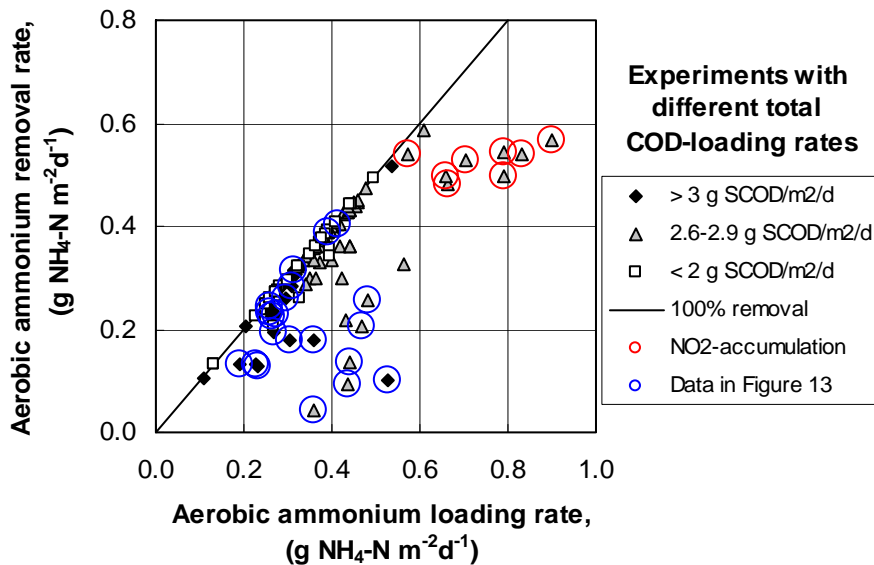

Figure 18. Aerobic ammonia removal rate versus aerobic ammonia loading rate. Data points marked by red circles show the experiments with nitrite accumulation. Data points marked by blue circles show the experiments from a period with high COD-loading rate.

The data points in Figure 18 that are marked with blue circles show data from a period where the influence of COD-loading rates and incomplete uptake of SCOD in the anaerobic phase was tested.

These data are also shown in Figure 19, where they are plotted as removal of ammonia versus day of operation. On day 618 (start of Period 7) the COD-loading rate was increased by increasing the concentration of SCOD in the influent. This caused a decline in ammonia removal due to excess COD at the end of the anaerobic period. The cycle time was adjusted by increasing the anaerobic period from 2 to 3 hours on day 672 (start of Period 8) to increase anaerobic COD-uptake and recover nitrification. Thereafter, on day 675 the influent SCOD concentration was further increased and caused another decline in the nitrification. The influent

SCOD-concentration was therefore reduced again on day 725 and the nitrification recovered again.
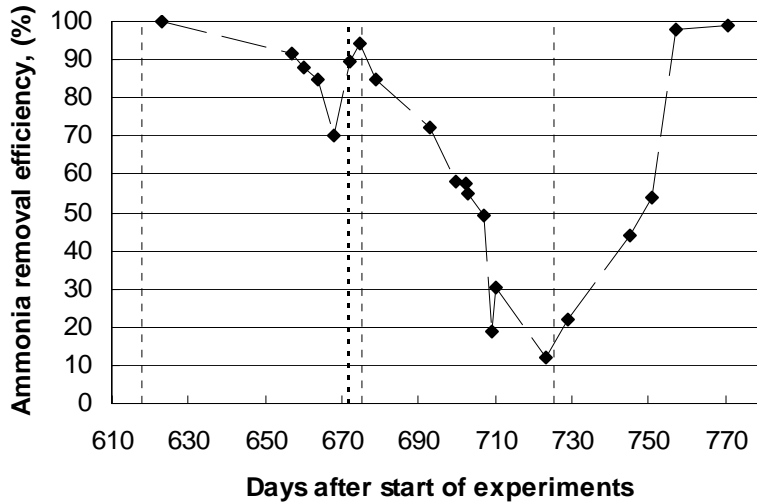

Figure 19. Aerobic ammonia removal efficiency during a period with manipulation of the COD-loading rate. Dotted vertical lines mark changes in operating conditions by changing the influent SCOD-concentration or cycle times as explained above.

There are also some other data points in Figure 18 that show an incomplete removal of ammonia caused by high COD-loading rate and incomplete removal of SCOD in the anaerobic phase.

A limit for the aerobic ammonia loading rate of 0.6 g NH_4-N $m^{-2}d^{-1}$ is low and higher rates have been observed in continuous nitrifying MBBR reactors (Rusten *et. al.* 1995a). However, as discussed previously nitrification is limited by diffusion of ammonia or DO into the biofilm and heterotrophic growth will reduce the oxygen available for nitrification. In the system used in this study, phosphate uptake also took place in the aerated phase of the SBR-cycle. Although some P-uptake was determined (see section 6.1.5) to be performed by denitrifying PAO, a fraction of the PAO performed P-uptake with oxygen as the electron acceptor.

In conclusion, the deciding factors with respect to nitrification in this system were to achieve complete removal of SCOD in the anaerobic phase and have an aerobic phase that was sufficient for complete nitrification.

The experiments with constructed wastewater indicated a limit for the anaerobic COD-loading rate of 10 g SCOD $m^{-2}d^{-1}$ and a limit for the aerobic ammonia loading rate of 0.6 g NH_4-N $m^{-2}d^{-1}$.

6.1.3 Phosphate and nitrogen removal

When the criteria discussed above were fulfilled, the process showed excellent phosphate removal, but there were some data with limited EBPR activity. The causes for this will be discussed further below. To do this, data where the EBPR activity was limited by low total COD-loading rate, incomplete anaerobic COD-uptake or nitrite accumulation in the periods prior to the period with good EBPR activity have been excluded from the figures presented below.

The aerobic phosphate uptake is plotted versus the anaerobic phosphate release in Figure 20. The strong correlation between the aerobic phosphate uptake and the anaerobic phosphate release is as expected.
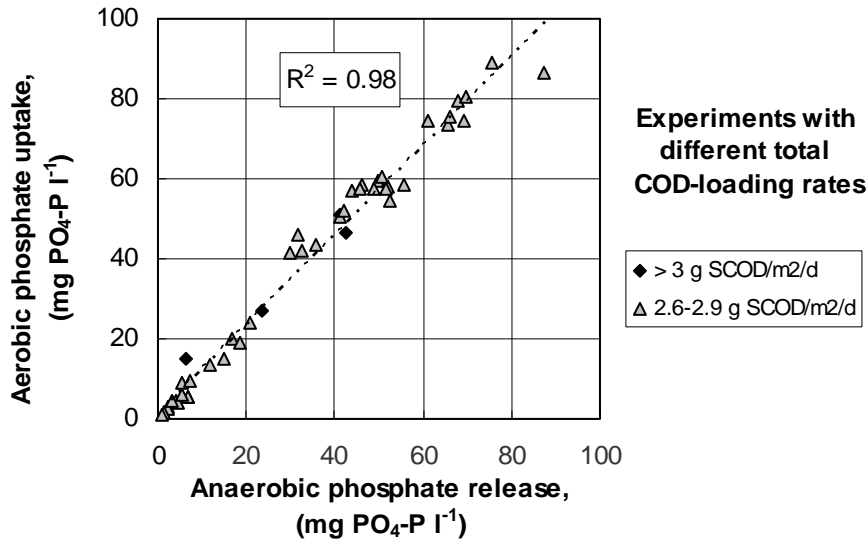

Figure 20. Aerobic phosphate uptake versus anaerobic phosphate release.

Since production of PHA necessary for phosphate uptake is linked to phosphate release, a high phosphate release is an advantage with respect to achieving a high net phosphate removal. The average net phosphate

removal in the period with good EBPR activity was 9 mg PO_4-P l^{-1}, demonstrating the phosphate removal capacity of the process. These results were obtained in experiments run with average total COD-loading rates in the range 2.6 – 2.9 g COD $m^{-2}d^{-1}$. The average ratio of COD removed in the anaerobic phase to phosphate removed in this period was 36 mg COD (mg PO_4-P)$^{-1}$. However, there were some extreme values and the median value of 21 mg COD (mg PO_4-P)$^{-1}$ is probably a better estimate for the required COD/P ratio.

However, Figure 20 also shows some data with limited EBPR activity under conditions where the limiting factors discussed above did not have an influence on the results. This will be discussed further, but first the nitrogen removal is presented.

Figure 21 shows the net nitrogen removal rate versus the aerobic nitrogen-loading rate. The results show nitrogen removal rates corresponding to removal efficiencies of 70 % – 90 % in most experiments.
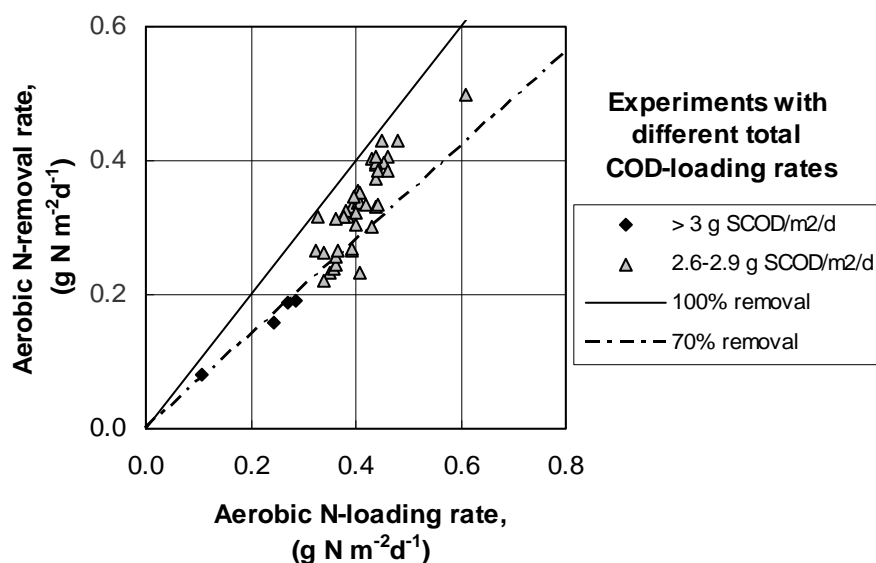

Figure 21. Net aerobic nitrogen removal rate versus aerobic nitrogen loading rate.

Since the only nitrogen fed to the system was ammonium, the results show that nitrification and denitrification can be achieved simultaneously in the aerated phase of such a process. The bulk DO concentration reached 2 mg DO l^{-1} shortly after start of aeration and increased up to typically

5 - 6 mg DO l^{-1} towards the end of the aerobic phase. Oxygen was therefore available as electron acceptor in the aerated phase.

6.1.4 Conceptual model

The observed nitrogen removal can be explained by a layered biofilm were the deeper layers are anoxic as illustrated in Figure 22. The layered biofilm in the figure is an idealised picture of reality. In practise the structure of the biofilm is more complex. However, zones with different conditions with respect to electron acceptors will exist also in a real biofilm with a complex structure and the simple conceptual model is useful for discussion of the transformations that take place in the non aerated and aerated phase of the SBR cycle.
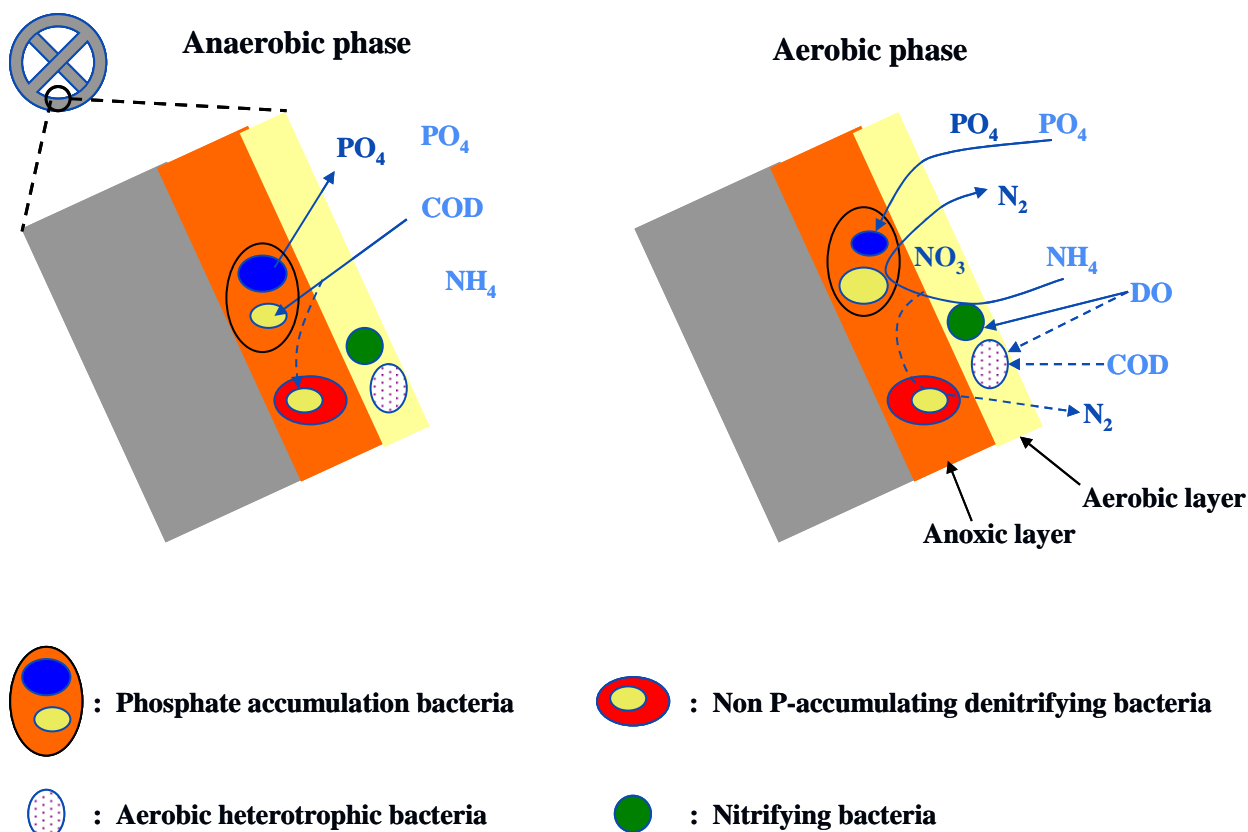

Figure 22. Illustration of the conceptual model of the biofilm.

The results showed that good nitrogen and phosphorus removal was only achieved in experiments with good anaerobic COD removal. Since the COD was removed in the anaerobic phase where there was no nitrate, it is

probable that phosphate was removed by bacteria using nitrate as electron acceptor. This is supported by a test were nitrate was added to the reactor at the start of the aerobic phase, resulting in an increased phosphate removal rate (data not shown), and verified in the batch tests to determine the aerobic and anoxic phosphate uptake capacity of the biofilm (Meinhold *et. al.*1999) that are discussed in section 6.1.5.

The results can be analysed based on the metabolic models for PAO and DNPAO developed by Smolders *et. al.* (1995) and Kuba *et. al.* (1996a), respectively. In the discussion below, the term DNPAO should be understood as bacteria accumulating phosphate with nitrate as electron acceptor and PAO should be understood as bacteria accumulating phosphate with oxygen as electron acceptor. DNPAO and PAO are only used to differentiate between the two types of P-uptake activity and no conclusion is drawn with respect to if the DNPAO and PAO are different bacteria.

According to the model for DNPAO the ratio of anaerobic COD-uptake to nitrogen removed is 3.9 mg COD_{uptake} (mg $N_{removed}$)$^{-1}$ However, the observed ratio of COD-uptake to nitrogen removed in the experiments during the period with good EBPR was on average 6.8 mg COD_{uptake} (mg $N_{removed}$)$^{-1}$. The results therefore indicated that other heterotrophic bacteria than DNPAO were active. This could be PAO but possibly also non P-accumulating bacteria.

Box 1. Finding the fraction of denitrifying phosphate uptake.

1. $\Delta P_{DNPAO} = X_{DNPAO} * \Delta P_{tot}$
   – X_{DNPAO} is the fraction of P-uptake performed by DNPAO.

2. $\Delta N_{tot}/\Delta P_{tot} = X_{DNPAO} *(\Delta N_{DNPAO}/\Delta P_{DNPAO})$
   – Assuming $\Delta N_{tot} = \Delta N_{DNPAO}$, and using equ. 1.

3. $X_{DNPAO} = (\Delta N_{tot}/\Delta P_{tot}) / (\Delta N_{DNPAO}/\Delta P_{DNPAO})$
   – X_{DNPAO} calculated from observed $\Delta N_{tot}/\Delta P_{tot}$ and $\Delta N_{DNPAO}/\Delta P_{DNPAO}$ from model.

4. $\Delta P_{tot} = \Delta P_{DNPAO} + \Delta P_{PAO}$
   – Total P-uptake in the aerated phase is the sum of P-uptake by DNPAO and PAO.

5. $\Delta P_{PAO} = (1 - X_{DNPAO}) * \Delta P_{tot}$
   – P-uptake from PAO can be found by combining equ. 1 and 4.

– ΔN_{tot} and ΔP_{tot}: **Measured values**
– $(\Delta N_{DNPAO}/\Delta P_{DNPAO}) = 0.655$, (Kuba *et. al*. 1996a)

The ratio of nitrogen removed to phosphorus uptake in the aerated phase can be used to assess the involvement of PAO (Box 1). In the period with good EBPR activity the ratio was on average 0.39 mg $N_{removed}$ (mg P_{uptake})$^{-1}$, which is lower than expected according to the DNPAO model (0.65 mg $N_{removed}$ (mg P_{uptake})$^{-1}$). Since the total P-uptake is the sum of P-uptake by DNPAO and P-uptake by PAO, the fraction of denitrifying P-uptake can be estimated by these ratios if one assumes that all denitrification is done by DNPAO. Based on the average observed N-removed to P-uptake ratio, the fraction of denitrifying P-uptake was 0.39/0.65 = 0.6, implying that 40 % of the phosphate uptake was performed by PAO.

If the fraction of denitrifying phosphate uptake is known, the COD consumption of the PAO and DNPAO can be assessed with the model. The maximum achievable P-content in the phosphate accumulating bacteria at steady state for given operating conditions is dependant on the active biomass yield (yield excluding the storage products poly-P, PHA and glycogen) and the maximum storage capacity for poly-P in the bacteria (Smolders, 1995). This implies that the minimum required anaerobic COD-uptake to phosphate removed ratio is also dependant on these factors. This ratio will be the minimum required COD/P ratio in the feed.

The ratio of nitrogen removed to phosphorus uptake used above is based on the conversions calculated with the model for a SRT of 8 days (Figure 3). The corresponding yields for DNPAO and PAO are 0.18 and 0.38 g $COD_{biomass}$ (g $COD_{HAc-degraded}$)$^{-1}$, respectively. Although the actual active biomass yield in the experiments discussed here may have been lower since the process was nitrifying, the yields based on conversions at 8 d SRT have been used below to have a consistent basis for the calculations.

The storage of poly-P in DNPAO and PAO according to the models with SRT of 8 days is 0.22 and 0.10 g P (g $COD_{biomass}$)$^{-1}$, respectively. The maximum storage capacity in the models is 0.29 g P (g $COD_{biomass}$)$^{-1}$ for both DNPAO and PAO. However, the actual maximum storage capacity for poly-P in phosphate accumulating bacteria is unclear (Smolders 1995). In the models it is assumed that all bacteria are DNPAO or PAO, and a low influent phosphate to COD ratio (excess COD) results in a low concentration of poly-P in the biomass. In a real system, excess COD will represent an ecological niche for other bacteria and excess COD may cause a lower fraction of PAO and DNPAO in the biomass, but the PAO and

DNPAO will probably accumulate as much P as possible. In the integrated model for PAO and DNPAO emphasis is placed on accumulation of the storage products as opposed to growth (Murnleitner *et. al.* 1997). In the calculations below a storing capacity of 0.22 g P (g $COD_{biomass}$)$^{-1}$ is assumed for both PAO and DNPAO.

The expected COD/P ratio for a situation with simultaneous denitrifying and aerobic P-uptake can be calculated as shown in Box 2.

Box 2. Calculation of the expected $COD_{uptake}/P_{removed}$-ratio.

1. $\Delta COD_{DNPAO}/\Delta P_{DNPAO} = 1/(Y_{sx\text{-}DNPAO} * f_{sx\text{-}DNPAO})$
   - COD/P ratio for DNPAO is dependant on active biomass yield (Y_{sx}) and storage capacity for poly-P.

2. $\Delta P_{DNPAO} = X_{DNPAO} * \Delta P_{tot}$
   - X_{DNPAO} is the fraction of P-uptake performed by DNPAO.

3. $\Delta COD_{DNPAO}/\Delta P_{tot} = X_{DNPAO}/(Y_{sx\text{-}DNPAO} * f_{sx\text{-}DNPAO})$
   - Combining equ 1 and 2.

4. $\Delta COD_{PAO}/\Delta P_{tot} = (1 - X_{DNPAO})/(Y_{sx\text{-}PAO} * f_{sx\text{-}PAO})$
   - Equivalent equation for PAO.

5. $\Delta COD_{PAO\&DNPAO}/\Delta P_{tot} = \Delta COD_{PAO}/\Delta P_{tot} + \Delta COD_{DNPAO}/\Delta P_{tot}$
   - Total COD uptake by PAO and DNPAO is the sum of COD uptake by PAO and DNPAO. Expected COD/P ratio is the sum of equ. 3 and 4.

6. $\Delta COD_{PAO\&DNPAO}/\Delta P_{tot} = \{[(1 - X_{DNPAO})/Y_{sx\text{-}PAO}] + [X_{DNPAO}/Y_{sx\text{-}DNPAO}]\}/ f_{sx\text{-}PAO\&DNPAO}$
   - Expected COD/P ratio assuming $f_{sx\text{-}PAO} = f_{sx\text{-}DNPAO}$.

- **$X_{DNPAO} = 0.6$, calculated as shown in Box 1.**
- **$Y_{sx\text{-}PAO} = 0.38$ g $COD_{Biomass}$/g $COD_{HAc\text{-}degraded}$, (Smolders *et. al.*, 1995)**
- **$Y_{sx\text{-}DNPAO} = 0.18$ g $COD_{Biomass}$/g $COD_{HAc\text{-}degraded}$, (Kuba *et. al.* 1996a)**
- **$f_{sx\text{-}PAO\&DNPAO} = 0.22$ g P/g $COD_{biomass}$ (based on Smolders *et. al.*, 1995 and Kuba *et. al.* 1996a)**

The result of the calculation with a fraction of 60 % DNPAO was an expected COD/P-ratio of 20 mg COD_{uptake} (mg $P_{removed}$)$^{-1}$ As discussed above, the results showed a required COD/P-ratio of 21 mg COD_{uptake} (mg $P_{removed}$)$^{-1}$ (median observed value in the experiments). The excellent agreement between the calculated and observed value supports that all COD-uptake in the anaerobic period was performed by DNPAO and PAO in the period with good EBPR. However, the result of the calculation is sensitive to the chosen value for the storage capacity and assumes steady state with respect to storage compounds.

For the experiments in Figure 20 showing a P-release < 25 mg P l^{-1}, the COD/P, COD/N and N/P ratios varied more showing some lower but

typically higher values than expected according to the models. The average values in these experiments that were not influenced by the limiting conditions discussed above, were 26 mg COD_{uptake} (mg $P_{removed}$)$^{-1}$, 10 mg COD_{uptake} (mg $N_{removed}$)$^{-1}$ and 2.5 mg $N_{removed}$ (mg P_{uptake})$^{-1}$, respectively. The average anaerobic COD removal was 201 mg COD l^{-1} (94 % removal). The expected COD/P ratio if all the P-uptake was performed by DNPAO would be 25 mg COD_{uptake} (mg $P_{removed}$)$^{-1}$, which is close to the measured value. However, the COD/N and N/P ratios are higher than the model for DNPAO, so the ratios indicate that there were non P-accumulating bacteria capable of storing COD in the anaerobic phase in the biomass, and that these included denitrifying bacteria. It has been reported that storage polymers might play an important role under conditions were the availability of substrate varies greatly (van Loosdrecht *et. al.* 1996).

Probably the biomass contained a mixed population of DNPAO and other denitrifying bacteria in the deeper anoxic layers as well as PAO and possibly other bacteria capable of anaerobic COD uptake in the outer aerobic layers (not shown in Figure 22). During the last part of period 9 and in period 10, DNPAO were probably the dominating denitrifying bacteria and PAO the dominating aerobic bacteria.

The COD consumption for simultaneous phosphate uptake and denitrification by DNPAO is less than the total COD consumption for aerobic phosphate uptake by PAO and separate denitrification by denitrifying bacteria that do not accumulate phosphate. In a process for combined nitrogen and phosphorus removal, simultaneous nitrification-denitrification and anoxic phosphate uptake is therefore an advantage with respect to COD requirement and plant volume, compared to aerobic phosphate uptake and denitrification in a separate anoxic phase. To achieve a high fraction of DNPAO in the biofilm one must create conditions where the DNPAO can out-compete other denitrifying bacteria with respect to uptake of easily biodegradable COD in the anaerobic phase. The conditions must also be such that the phosphate accumulating bacteria colonise the deeper anoxic layers in the biofilm in stead of the outer aerobic layers, in order to limit aerobic phosphate uptake by PAO.

After the decline of EBPR activity in period 1, the reactor was inoculated with raw sewage several times during period 2. The last inoculation was on day 485 and all the data with low EBPR activity in Figure 20 are from the

periods before the period with good EBPR activity. After the nitrification had fully recovered on day ~771 (Figure 19) it took about 60 days until the EBPR activity started to increase on day ~828. During this time the total COD loading rate was quite stable (average 2.9 g SCOD $m^{-2}d^{-1}$, standard deviation 0.3 g SCOD $m^{-2}d^{-1}$) and the SBR cycle had a total length of 6 hours with a 2.5 hour anaerobic period. The results therefore indicate that EBPR was induced in bacteria present in the reactor by the operating conditions rather than by introduction of new bacteria through inoculation. The question is therefore how to optimise the process to favour DNPAO.

The results from the experiments with constructed wastewater indicated that the total COD-loading rate should be in the range 2 – 3 g SCOD $m^{-2}d^{-1}$ with an anaerobic COD-loading rate below 10 g SCOD $m^{-2}d^{-1}$ and an aerobic ammonia loading rate below 0.6 g NH_4-N $m^{-2}d^{-1}$. In addition, the results from period 4 compared to period 9 and 10 indicated that the length of the anaerobic phase relative to the total cycle length is of importance for avoiding nitrite inhibition. A change from 2.5 to 2 hours anaerobic phase in a SBR cycle with total length of 6 hours during the period with good EBPR activity gave an increase of the N/P-ratio indicating a higher fraction of P-uptake by DNPAO. However, the increase was not significant.

The given above criteria can be met for a given influent quality by tuning of the SBR cycle and possibly addition of additional C-source. The results indicated that an influent COD/P ratio of 21 mg COD (mg PO_4-P)$^{-1}$ would be sufficient for complete phosphate removal and 70 % or higher nitrogen removal with such operating conditions.

However, other factors such as degradation of particulate organic matter, inoculation by bacteria in the influent wastewater may also play a role. Further experiments were therefore performed in order characterise the biomass in the reactor, with respect to the ratio of denitrifying phosphate uptake and aerobic phosphate uptake under different conditions, and to verify the operating criteria found in the experiments with constructed wastewater.

6.1.5 Aerobic and anoxic phosphate uptake capacity

The batch experiments during the experiments with constructed wastewater were performed to characterise the biomass with respect to the relative

proportion aerobic and anoxic P-uptake capacity. A total of five experiments were performed in period 9 and 10. The main difference in the operating conditions of the SBR during these two periods was the length of the anaerobic phase, 2.5 hours in period 9 and 2 hours in period 10.
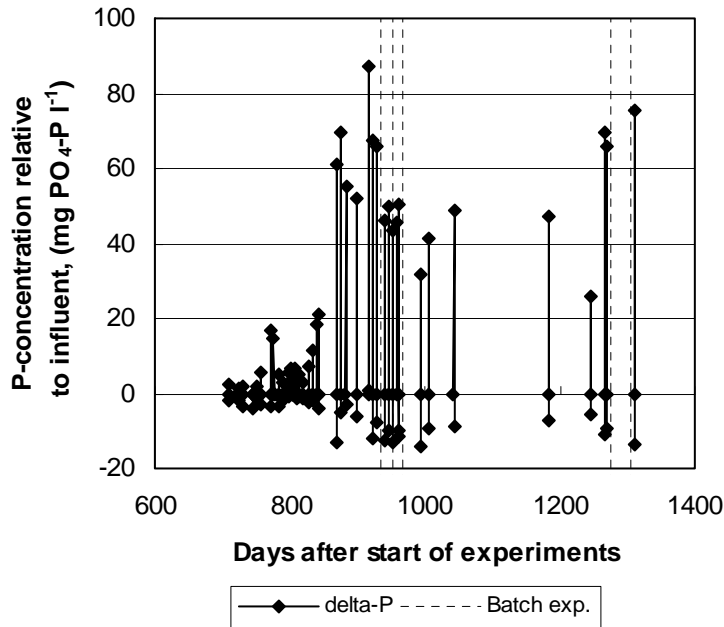

Figure 23. Phosphate concentrations in SBR cycle relative to influent concentration in the period before and during the batch experiments.
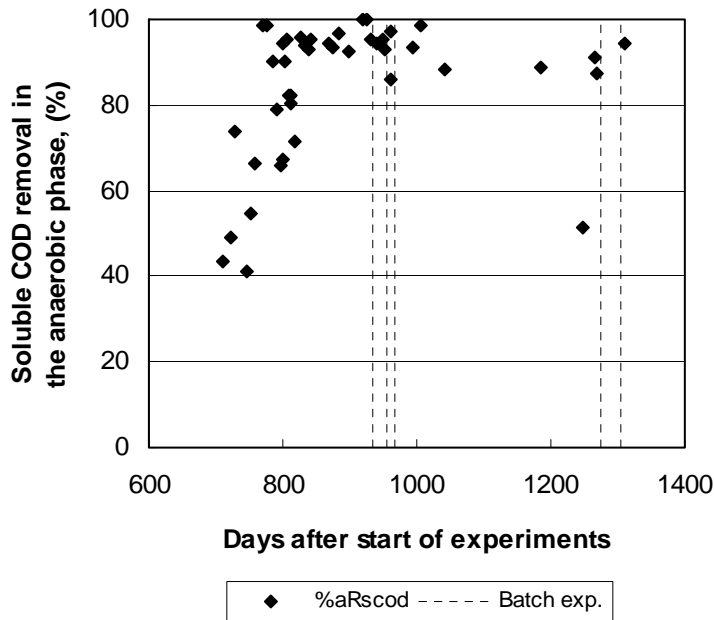

Figure 24. Removal of soluble COD in the anaerobic phase in the period before and during the batch experiments.

During period 9 and 10 the anaerobic P-release varied, but the net removal of phosphate was relatively stable (Figure 23) with an average of 9 mg PO_4-P l^{-1} as reported above. The anaerobic COD uptake was also good with a removal efficiency of > 85 % (Figure 24). In both figures the vertical dotted lines mark the time of the batch experiments.

Figures 25 and 26 show the phosphate concentration versus time in the aerobic and anoxic batch experiments, respectively.
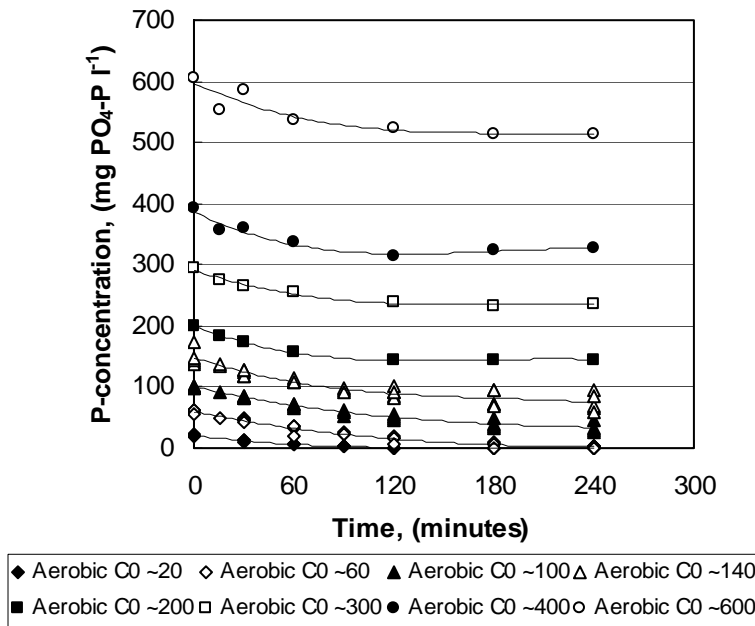
Figure 25. Concentration of phosphate versus time for aerobic batch experiments.

The phosphate concentration in the boxes with different initial P-concentration decreased with time during the batch experiments forming a curved line. In the aerobic batch experiments with initial phosphate concentrations of 200 mg PO_4-P l^{-1} or higher, the phosphate uptake stopped after a period of about 120 minutes (Figure 25). Qualitatively, this can be explained by limitation caused by depletion of PHA or near maximum storage of poly-P. In the anoxic batch experiments the phosphate uptake turned to phosphate release when the nitrate was consumed (Figure 26).
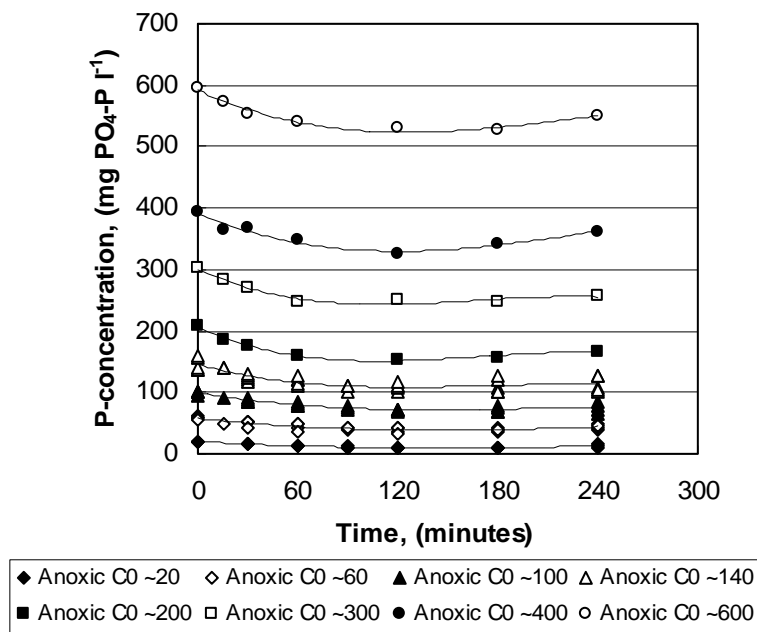
Figure 26. Concentration of phosphate versus time for anoxic batch experiments.

A total of five sets of batch experiments were performed over a period of one year. Initial phosphate uptake rates were calculated for each individual batch experiment and the average phosphate uptake rate for a given initial phosphate concentration was determined.

Half order kinetics with respect to the bulk concentration of phosphate in a biofilm EBPR process has been reported by Falkentoft, in experiments with a submerged biofilter (Falkentoft *et. al.* 2001). The average initial phosphate uptake rates where therefore plotted versus the square root of the initial phosphate concentration and against the phosphate concentration to evaluate reaction order.

The data from the aerobic batch experiments could be fitted to a straight line for initial phosphate concentrations from 20 – 200 mg PO_4-P l^{-1} when plotted versus the square root of the initial phosphate concentration (Figure 27), but data would not fit a straight line through the origin when the initial phosphate uptake rate was plotted against the initial phosphate concentrations as can be seen in Figure 28. The results therefore indicated ½ order kinetics for this range of initial phosphate concentrations.

Figure 27. Initial phosphate uptake rate versus square root of initial phosphate concentration in aerobic batch experiments.

Figure 28. Initial phosphate uptake rate versus initial phosphate concentration in aerobic batch experiments.

The curved line in Figure 28 shows calculated rates with the equation $r = 0.27C^{0.5}$, where C is the initial phosphate concentration and 0.27 is the rate constant found from the plot in Figure 27. The horizontal line in Figure

28 shows the average of the initial phosphate uptake rates in the batch experiments with initial phosphate concentrations from 300 – 600 mg PO_4-P l^{-1}. The dotted lines show the average (4.4 g PO_4-P $m^{-2}d^{-1}$) plus/minus the standard deviation (0.86 g PO_4-P $m^{-2}d^{-1}$).

The initial phosphate uptake rates in the anoxic batch experiments could not be fitted to a straight line when plotted against the square root of the initial phosphate concentration (Figure 29).
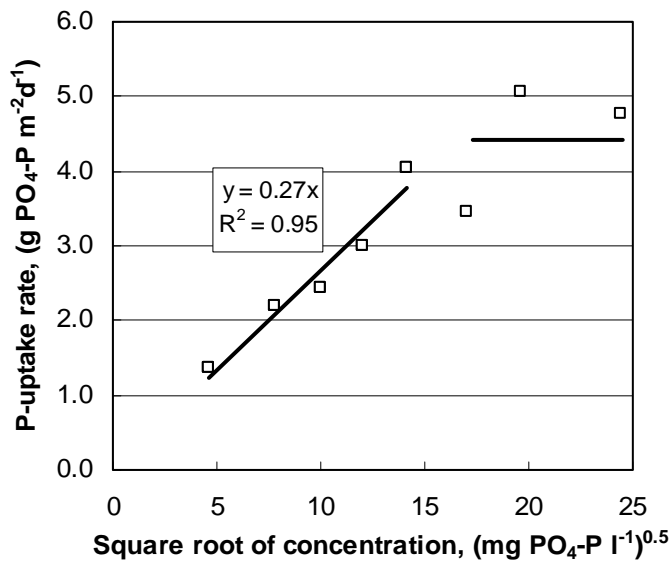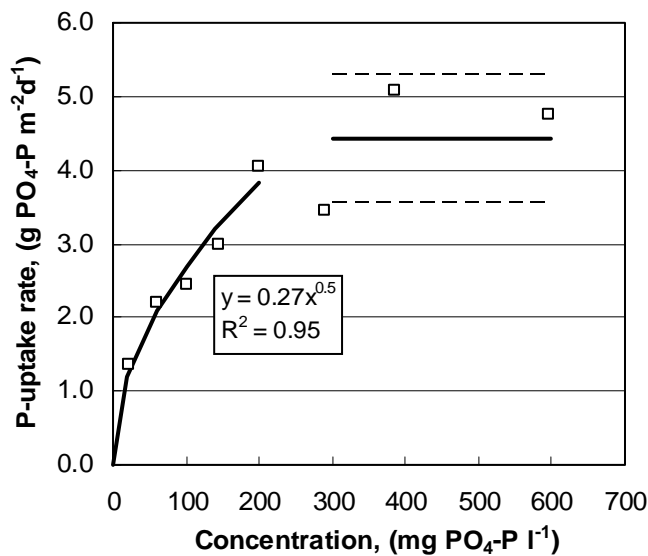

Figure 29. Initial phosphate uptake rate versus square root of initial phosphate concentration in anoxic batch experiments.

The best fit of a power function ($r = k \cdot C^n$) to the data from the anoxic batch experiments with initial phosphate concentrations in the range from 20 – 200 mg PO_4-P l^{-1} is shown in Figure 30. The result of the regression calculation was the equation $r = 0.04C^{0.85}$, where C is the initial phosphate concentration, 0.04 is the rate constant and 0.85 is the reaction order. The horizontal line in Figure 30 shows the average of the initial phosphate uptake rates in the anoxic batch experiments with initial phosphate concentrations from 300 – 600 mg PO_4-Pl^{-1}. The dotted lines show the average (4.6 g PO_4-P $m^{-2}d^{-1}$) plus/minus the standard deviation (0.23 g PO_4-P $m^{-2}d^{-1}$).
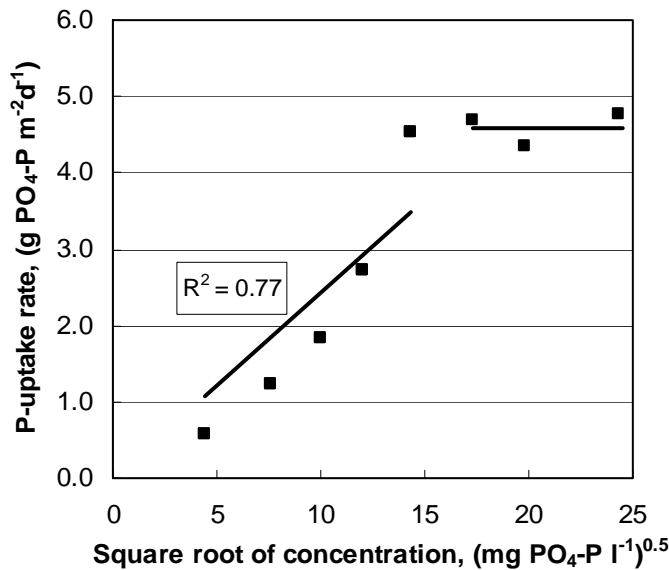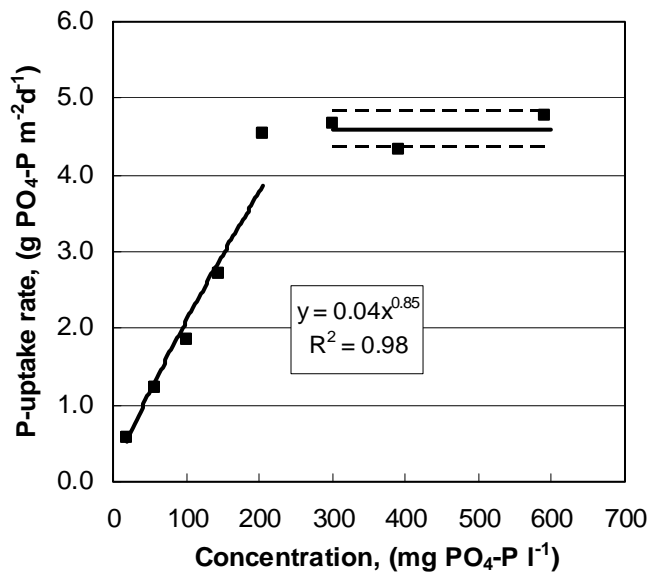
Figure 30. Initial phosphate uptake rate versus initial phosphate concentration in anoxic batch experiments.

The average value for the maximum phosphate uptake rate was higher for the anoxic batch experiments than for the aerobic batch experiments. However, considering the standard deviation the difference between the average values is not significant. The results therefore only show that the initial anoxic phosphate uptake rate can be as high as the initial aerobic phosphate uptake rate.

The ratio of the anoxic to the aerobic P-uptake rate increased with increasing initial concentration up to 200 mg PO_4-P l^{-1}. At higher concentrations ratios from 85% to 136% were observed with an average value of 106% (Figure 31).

In a biofilm system increasing bulk concentration will lead to deeper penetration of the biofilm. The increasing ratio between the anoxic and aerobic initial P-uptake rate demonstrate that the bacteria in the outer layers of the biofilm had limited capacity for anoxic P-uptake, and that this capacity increased with increasing depth of the biofilm.

The results can be explained by two groups of phosphate accumulating bacteria, one group only capable of P-uptake with oxygen as electron acceptor and one group capable of facultative aerobic or anoxic P-uptake

(Kerrn-Jespersen and Henze, 1993). The P-uptake under aerobic conditions would then be higher than under anoxic conditions.
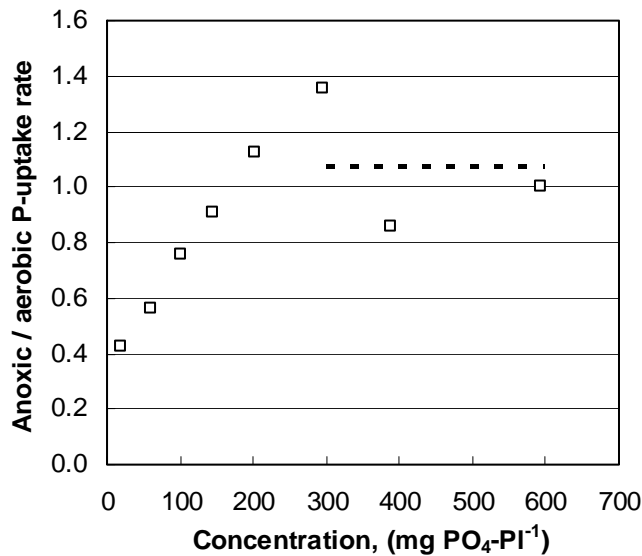
Figure 31. The ratio of anoxic to aerobic initial phosphate uptake rate versus the phosphate concentration.

However, the results in Figure 31 can also be explained by different induced denitrification activity in one population as concluded by Wachtmeister *et. al.* (1997). The bacteria in the outer layers of the biofilm would be most adapted to aerobic conditions, while the bacteria in the deeper layers would be more adapted to anoxic conditions. The increasing ratio and the fact that the anoxic P-uptake rate could be at least as high as the aerobic P-uptake rate supports that the phosphate accumulating bacteria were from one population with different induced denitrification ability. However, it can also be explained by different concentrations of strictly aerobic and facultative phosphate accumulating bacteria in different depths of the biofilm, so a definite conclusion can not be drawn.

The important result with respect to possible use of this process for biological N and P-removal is, however, that the anoxic P-uptake rate can be as high as the aerobic. There is therefore no incentive to find a design that could increase the fraction of aerobic P-uptake to reduce the required reactor volume since denitrifying P-uptake is as fast and gives a more efficient utilisation of the C-source required for removal of both N and P.

6.2 Experiments with municipal wastewater

The experiments with municipal wastewater were performed to verify the previous experiments with constructed wastewater. The experiments were divided in two main parts each divided in several periods. In part 1, the focus was on maximum P-removal capacity. In part 2, the focus was on nitrification and utilisation of the influent COD. Thereafter the aim was to demonstrate stable N and P-removal over a period of time.

6.2.1 Verification of operating conditions

Figure 32 shows the BSCOD-loading rate referred to the anaerobic part of the SBR-cycle and the total cycle length during the experiments with municipal wastewater. Figure 33 shows the aerobic phosphate uptake rate during the same time period. In both figures the different experimental periods are indicated by the vertical dotted lines. In part 1 the BSCOD-loading rate was varied by dosage of NaAc. In addition, the wastewater quality varied due to different weather conditions (Figure 32).

The experiments started without dosage of NaAc (average total BSCOD-loading rate: 0.47 g BSCOD m^{-2} d^{-1} in period 1A). There was good anaerobic soluble COD (SCOD) removal (average 49 mg SCOD l^{-1} at the end of the anaerobic phase), and relatively high nitrification efficiency (average 75%). The total removal (average 6%) of soluble nitrogen compounds (NH_4-N and NO_x-N) and the phosphate uptake rate were low (average 0.02 g PO_4-P m^{-2} d^{-1}) as expected due to the low total BSCOD-loading rate (Figure 33).

In period 1B the BSCOD-loading rate was increased (average 3.1 g BSCOD m^{-2} d^{-1} and 1.0 g BSCOD m^{-2} d^{-1} for anaerobic and total BSCOD-loading rate, respectively). The aerobic phosphate uptake rate increased to about 0.55 g PO_4-P m^{-2} d^{-1}. This was higher than expected from the experiments with constructed wastewater, probably due to hydrolysis of PCOD that increased the amount of BSCOD available for the phosphate accumulating bacteria. However, the rate was considerably lower than the maximum phosphate uptake rate of 1.95 g PO_4-P m^{-2} d^{-1} in the experiments with constructed wastewater due to the still relatively low total BSCOD-loading rate. The nitrification efficiency increased (average

84 %) and there was some denitrification (average 50 % removal of soluble nitrogen).
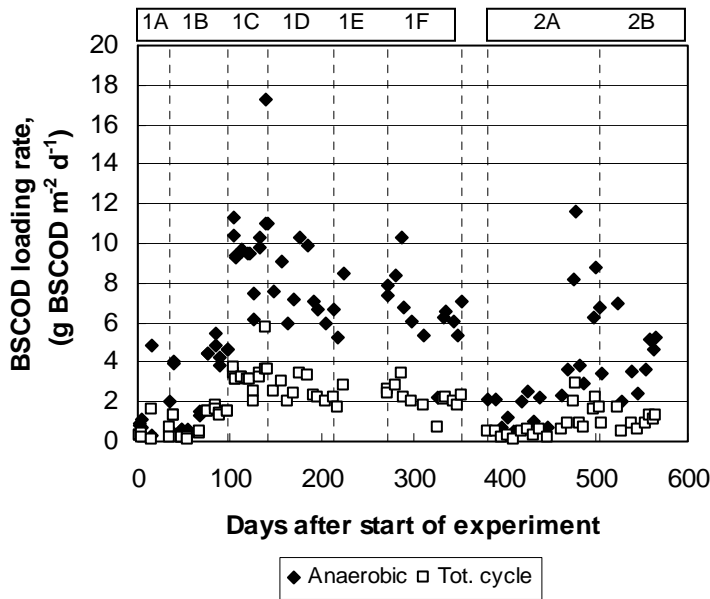

Figure 32. The anaerobic BSCOD-loading rate and the BSCOD-loading rate referred to the total cycle during the experimental period with municipal wastewater.
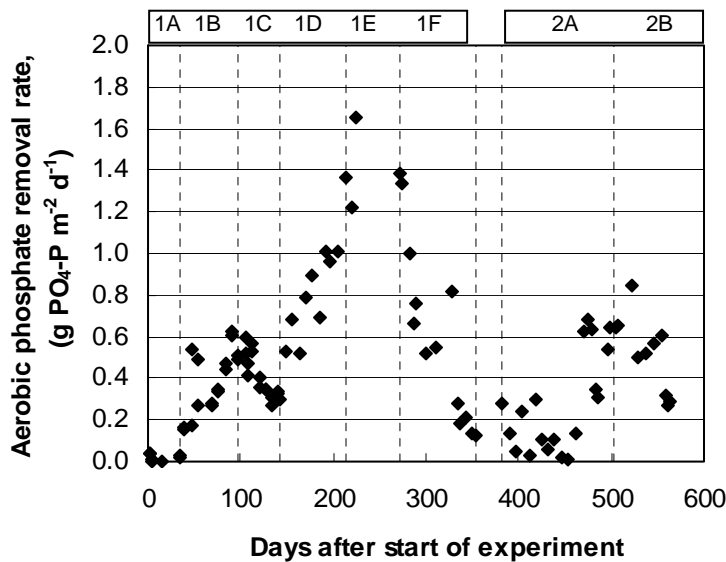

Figure 33. The aerobic phosphate uptake rate during the experimental period with municipal wastewater.

In period 1C the BSCOD-loading rate was increased further (average total BSCOD-loading rate 3.4 g BSCOD m^{-2} d^{-1}) to an anaerobic BSCOD-loading rate (average 10.1 g BSCOD m^{-2} d^{-1}) that gave incomplete anaerobic removal of BSCOD and the aerobic phosphate uptake rate started to decrease shortly after. The excess SCOD after the anaerobic phase (average 99 mg SCOD l^{-1}) gave an aerobic SCOD-loading rate of 2.6 g SCOD m^{-2} d^{-1} that probably resulted in growth of heterotrophic bacteria that did not accumulate phosphate and decreased the fraction of phosphate accumulating bacteria in the biomass. The nitrification efficiency and total removal of soluble nitrogen also decreased to average 52% and 46%, respectively. The results are in accordance with the conclusion from the previous experiments that the anaerobic BSCOD-loading rate should be below 10 g BSCOD m^{-2} d^{-1}.

In period 1D the average anaerobic BSCOD-loading rate was decreased to 7.6 g BSCOD m^{-2} d^{-1} (average total BSCOD-loading rate 2.5 g BSCOD m^{-2} d^{-1}) to give complete anaerobic removal of BSCOD. These lose loading rates corresponded to the conditions that gave the best performance in the experiments with constructed wastewater. The phosphate uptake rate increased sharply and continued to increase for a period of 70 days. The nitrification efficiency and total removal of soluble nitrogen compounds increased and thereafter decreased giving average values of 59% and 49%, respectively.

In period 1E the dosage of NaAc was first increased in small steps and thereafter reduced to optimise process performance. The anaerobic and total BSCOD-loading rates were 7.1 and 2.4 g BSCOD m^{-2} d^{-1} respectively. During a period of 60 days the phosphate removal rate varied between 1.2 and 1.7 g PO_4-P m^{-2} d^{-1} with an average of 1.4 g PO_4-P m^{-2} d^{-1}. The net phosphate removal in this period was on average 14 mg PO4-P l^{-1}. This is higher than the value (9 mg PO_4-P l^{-1}) found previously. However, the anaerobic and total BSCOD-loading rates were slightly higher than in the experiments with constructed wastewater where the corresponding average values were 6.1 and 2.3 g SCOD m^{-2} d^{-1}, respectively. The average ratio of BSCOD removed in the anaerobic phase to phosphate removed was 9.3 mg BSCOD (mg PO4-P)$^{-1}$. This value is lower than found previously. However, supply of BSCOD through hydrolysis of PCOD is not accounted for in this value and the nitrogen removal was also lower than in the previous experiments. The average values for nitrification efficiency and

total removal of soluble nitrogen compounds were 33 % and 28 %, respectively.

In period 1F an unexpected and irrecoverable decline in the phosphate uptake activity was observed. The cause was thought to be too high anaerobic BSCOD-loading rate (average 6.4 g BSCOD m^{-2} d^{-1}) and the dosage of NaAc was reduced but the decline continued. One possibility for the decline is proliferation of non-phosphate accumulating bacteria. However, investigation of the dominant bacteria groups in the biofilm during this study by molecular biological methods (DGGE, RFLP and sequencing of selected clones) did not reveal any such change in the bacteria population (see section 6.2.4).
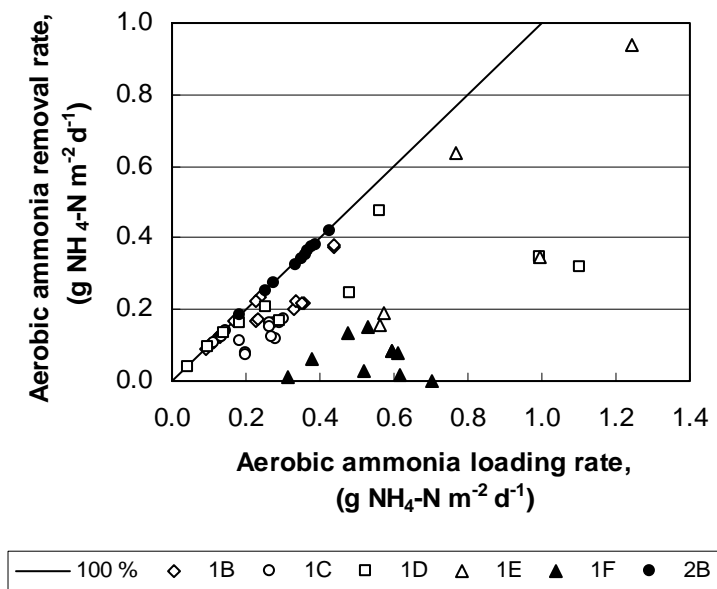
Figure 34. Aerobic ammonia removal rate versus aerobic ammonium loading rate. The start up periods 1A and 2A have been excluded for clarity.

The ammonium removal rates in periods 1B-D were always higher than in period 1F at comparable loading rates even with some variation in period 1D (Figure 34). In period 1E the ammonia removal rate was initially high, but declined towards the end of the period probably caused by to high total COD-loading rate. The anaerobic SCOD-removal rate in period 1F was initially lower than in the other periods at comparable BSCOD-loading rates (Figure 35). However, the phosphate uptake did not recover when the

anaerobic SCOD-removal efficiency recovered after reducing the dosage of additional carbon source.
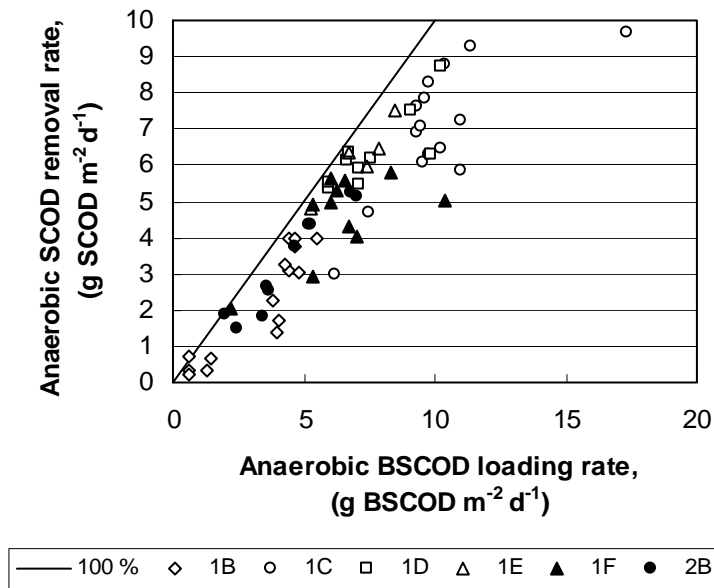
Figure 35. Anaerobic SCOD-removal rate versus anaerobic BSCOD-loading rate. The start up periods 1A and 2A have been excluded for clarity.

If a major part of the phosphate accumulating bacteria were localised in the deeper parts of the biofilm utilising nitrate produced by nitrification in the aerobic outer layers of the biofilm as electron acceptor, reduced nitrification would lead to decline in phosphate uptake. This would in turn lead to reduced anaerobic uptake of BSCOD. Incomplete anaerobic BSCOD-removal would contribute to oxygen consumption in the outer layers of the biofilm and further reduce oxygen available for nitrification. With a reduced dosage of additional C-source, there was no excess BSCOD after the anaerobic phase. However, nitrifying bacteria have slow growth rates and would not have recovered rapidly giving a situation with no electron acceptor available in the deeper oxygen depleted parts of the biofilm and less C-source for growth.

Morgenroth and Wilderer (1999) reported that mass transfer limitations by soluble compounds (oxygen and BSCOD) only had minor effect on overall phosphorus removal. They concluded that controlled removal of phosphorus rich biomass to ensure a thin biofilm was the key to successful operation. Lack of controlled removal of biomass may have contributed to

the observed decline. However, the process was not operated at a very high organic loading rate and the biomass concentration was not higher in period 1F than in the other periods (average 28.4 g DS m^{-2} in period 1F compared to average 28.5 g DS m^{-2} for all periods excluding start up). The most probable cause for the decline was therefore that nitrification could not be sustained and that phosphate uptake declined due to a lack of an electron acceptor in the oxygen depleted parts of the biofilm.

The controlling variables in a SBMBBR process will be the loading rates since controlled removal of biomass from a MBBR based process is complicated. A loading rate that can sustain nitrification must therefore be used. There was still some PCOD in the influent wastewater after the CMF (average 114 mg PCOD l^{-1}) that contributed to the total COD-loading rate. In order to enhance nitrification, the aerobic phase was made longer and the anaerobic phase shorter in part 2 of the experiments.
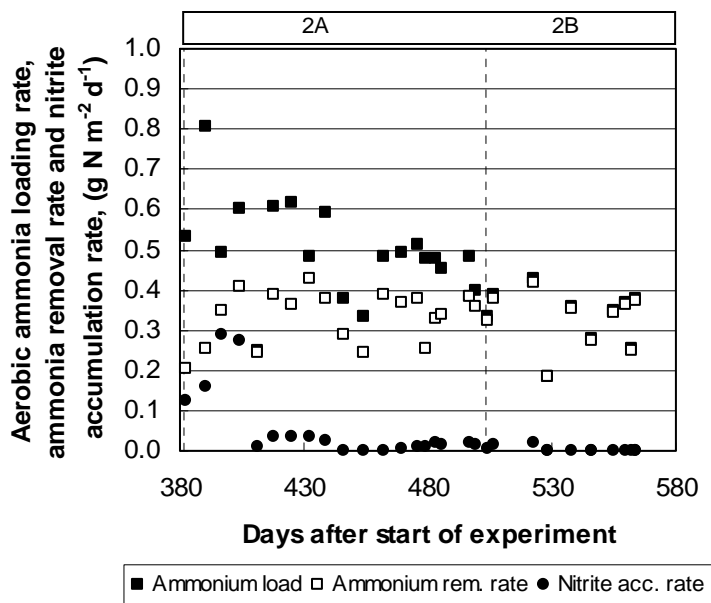
Figure 36. Ammonia loading rate, ammonia removal rate and nitrite accumulation rate during part 2 of the experiments.

The nitrification activity increased in period 2A and reached an ammonium removal rate of 0.4 g NH_4-N m^{-2} d^{-1} after 20 days (Figure 36). Thereafter the nitrification rate did not increase further even with good removal of SCOD in the anaerobic phase (average 54 mg SCOD l^{-1} at the end of the anaerobic phase) and there was still some nitrite accumulation. The aerobic ammonium loading rate (average 0.50 NH_4-N m^{-2} d^{-1} in 2A) was well

below the expected limit indicating that the limiting factor for nitrification was oxygen. The aeration was increased on day 446. This increased the dissolved oxygen level in the first part of the aerated phase. However, the oxygen concentration after 1 hour of aeration remained at about 6 mg DO l^{-1}. The nitrite accumulation stopped but the ammonia removal rate did not increase above 0.4 g NH_4-N m^{-2} d^{-1}.

In the second period (2B) the additional dosage of ammonia was stopped giving complete nitrification with a variation of the ammonia removal rate corresponding to the variation of the influent ammonia concentration.

Figure 37 shows the concentration profiles for phosphate, soluble COD, dissolved oxygen, ammonia and the soluble nitrogen compounds (sum of NH_4-N and NO_x-N) during one cycle in Period 2B. Figure 38 shows a plot of the ammonia removal rate during this cycle versus the ammonia concentration. The line in the figure shows the ammonia removal rate as calculated with the model (Equ. 5) using the parameters found previously (Hem *et. al.* 1991).
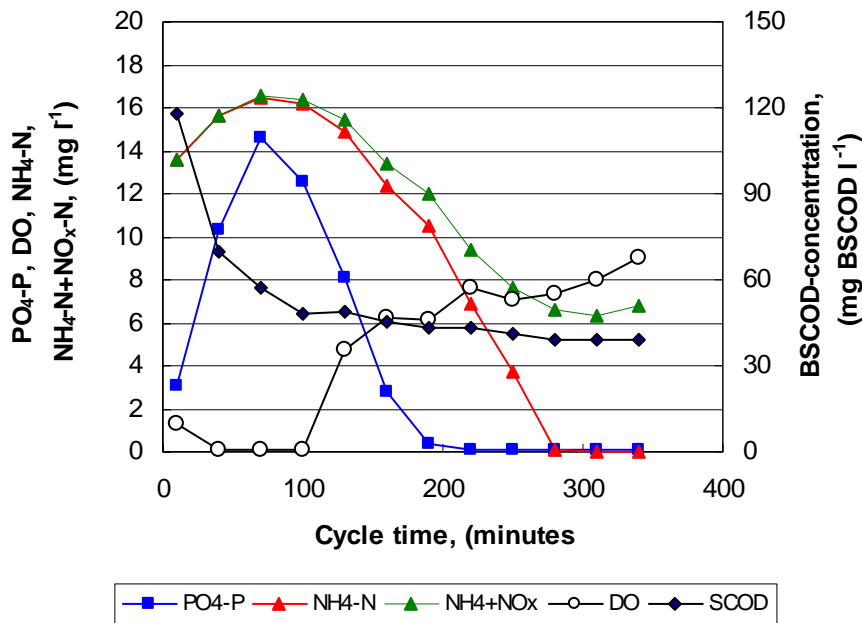
Figure 37. Concentration profiles of phosphate, ammonia, soluble nitrogen compounds, dissolved oxygen and soluble COD during one cycle in period 2B.

The results in Figure 38 show that the ammonia removal rate was limited by oxygen concentration during most of the cycle. This is supported by the

DO/NH$_4$-ratios that varied from 0.2 – 3.7 mg DO (mg NH$_4$-N)$^{-1}$ in the period from 100 to 280 minutes. Only during the final period of ammonia removal from 280 – 300 minutes in the cycle, was the removal rate controlled by the ammonia concentration. The increasing ammonia removal rate with lower ammonia concentrations in Figure 38 can be explained by the increasing DO concentration during the aerated part of the cycle. The aeration was constant during the aerated part of the SBR-cycle, so the increasing DO concentration is due to changes in the biological activity. I.e. the DO-concentration in Figure 37 increased from about 6 mg DO l^{-1} to an average of 7.4 mg DO l^{-1} when the phosphate uptake was complete.
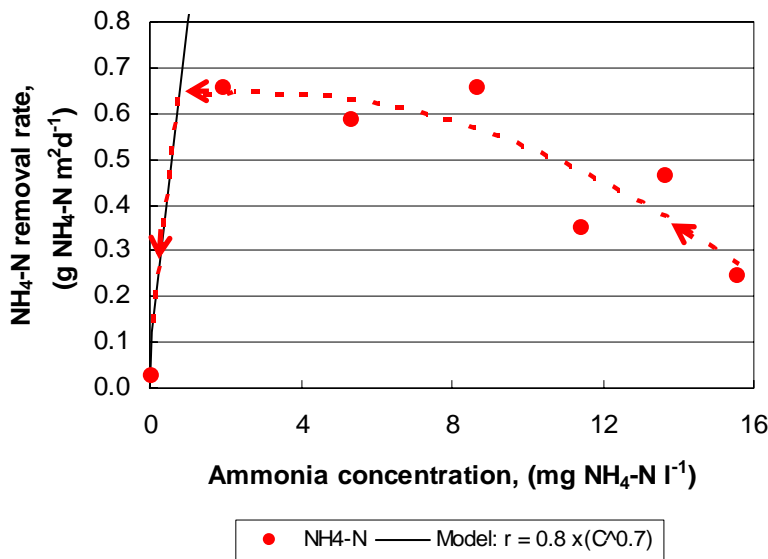
Figure 38. Ammonia removal rate versus ammonia concentration during the SBR-cycle shown in Figure 37. The broken red line indicates the change in ammonia removal during the aerated phase. Expected rate under ammonia limiting conditions is calculated with the model r = 0.8 x C$^{0.7}$.

In a continuous reactor one can determine the oxygen consumption due to heterotrophic activity by plotting the ammonia removal rate versus oxygen concentration. This assumes a steady state, and in a batch system this approach does not give reliable data because the activity chances through the cycle.

The average ammonia removal rate in the SBR-cycle shown in Figure 37 was 0.37 g NH$_4$-N m^{-2} d^{-1}, corresponding to the average rate for the second

part of the experiments. The low average is due to the severe oxygen limitation during the first part of the aerated phase where the oxygen consumption due to phosphate uptake and other heterotrophic activity was highest. Similar results have also been reported by Gieske *et. al.*, (2002), as discussed previously. The ammonia removal rate increased up to 0.63 g NH_4-N m^{-2} d^{-1} (average 220 – 280 minutes). Both the average and maximum values are low compared to MBBR processes operated for nitrification and also compared to the laboratory experiments. However, this was the maximum ammonium removal achieved in part 2 with the wastewater and process used in this study.
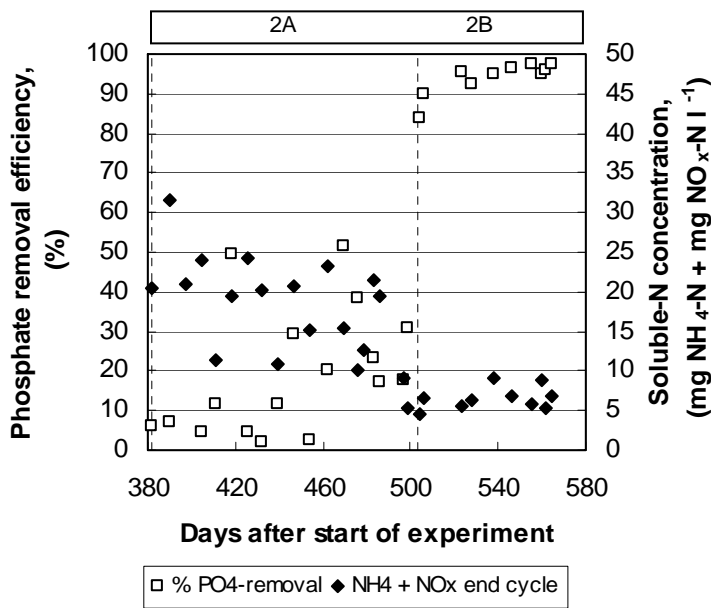
Figure 39. Phosphate removal efficiency and concentration of soluble nitrogen compounds in the effluent during part 2 of the experiments.

In the first phase of period 2A the phosphate removal was low. Although the difference in removal efficiency between period 2A and 2B (Figure 39) is exaggerated due to the difference in influent concentration (average 16 mg PO_4-P l^{-1} in 2A and 4.2 mg PO_4-P l^{-1} in 2B), the low phosphate uptake rate can be seen in Figure 33. There was also very little removal of soluble nitrogen compounds compared to the influent ammonium concentration (average 21 mg NH_4-N l^{-1} in 2A) as expected due to lack of C-source. On day 429 daily flushing of the CMF was stopped to allow hydrolysis of PCOD in the filter. This increased the SCOD in the influent by approximately 25 mg SCOD l^{-1} (average with daily back washing of CMF: 78 mg SCOD l^{-1} and average without back washing of CMF:

104 mg SCOD l^{-1}). However, considering that the inert soluble COD (ISCOD) in the wastewater at our laboratory is about 35 mg ISCOD l^{-1} the P-removal was still limited due to lack of C-source. The phosphate uptake rates were mostly < 0.2 mg PO_4-P m^{-2} d^{-1} (Figure 33) and the average net phosphate removal was 1.9 mg PO_4-P l^{-1}. There was some denitrification but in general there was low removal of soluble nitrogen compounds. Additional dosage of C-source was therefore started on day 464. The phosphate uptake rate increased immediately and the removal of soluble nitrogen compounds improved.

In period 2B there was no dosage of additional ammonium but a dosage of phosphate corresponding to an increase in the influent phosphate concentration of 2 mg PO_4-P l^{-1} was continued. The dosage of additional C-source was also continued. The phosphate removal efficiency was high during period 2B with an average effluent phosphate concentration of 0.3 mg PO_4-P l^{-1}. The variation in the phosphate uptake rate during period 2B seen in Figure 33 was therefore caused by natural variation in the raw wastewater quality. Removal of soluble nitrogen compounds was also good giving effluent concentrations below 10 mg N l^{-1} (average 6.5 mg N l^{-1}). The average influent ammonium concentration was 18 mg NH_4-N l^{-1} giving an average removal efficiency of 64%. After stable operation for 62 days the experiments were ended.

In period 2B, the ratio of nitrogen removed to phosphate uptake in the aerobic phase had an average value of 0.37 g $N_{removed}$ (g P_{uptake})$^{-1}$. This is lower than expected according to the metabolic model for DNPAO reported by Kuba (0.65 g $N_{removed}$ (g P_{uptake})$^{-1}$) (Kuba *et. al.* 1996a) if all phosphate uptake was performed by DNPAO. As discussed previously, this ratio can be used to assess the fraction of anoxic phosphate uptake. For period 2B the average observed ratio of nitrogen removed to phosphate uptake indicated that (0.37/0.65 = 0.57) the fraction of anoxic P-uptake was 57 %. Compared to the experiments with constructed wastewater this is a slight decrease. However, the ratio varied (range 0.08-0.76) showing that the PAO population could perform phosphate uptake under both aerobic and anoxic conditions and indicating that there were also denitrifying bacteria that did not perform phosphate uptake in the biomass. The results are therefore in accordance with the results obtained in the experiments with constructed wastewater.

The average ratio of BSCOD removed in the anaerobic phase to phosphate removed was 14.8 mg BSCOD (mg PO_4-P)$^{-1}$ in period 2B. This is lower than the expected value (19 mg BSCOD (mg PO_4-P)$^{-1}$) calculated as discussed previously, but higher than observed in period 1E. The COD/P-ratio is, however, dependant on the yield of active biomass and the yield of active biomass in the experiments may have been lower than assumed in the calculation as discussed previously. The increase compared to period 1E may partly be due to increased nitrogen removal. However, soluble COD produced by hydrolysis in the CMF was accounted for in the value above and this will also increase the observed value compared to period 1E.

The results show that PCOD in the wastewater can be utilised as carbon source in a SBMBBR. However, sufficient phosphate and nitrogen removal was dependent on dosage of additional carbon source with the wastewater quality in these experiments. To ensure nitrification and minimise the need for additional carbon source, the process scheme should include enhanced primary treatment and utilisation of primary sludge for production of carbon source.

In summary the experiments with municipal wastewater verified the qualitative conclusions from the experiments with constructed wastewater, but the numerical values for the operating conditions required to achieve efficient phosphate and nitrogen removal were in some cases lower. This was probably mainly due to the influence of particulate COD that has an influence on several parameters and values that should be chosen for design criteria.

Particulate COD will not be completely removed by hydrolysis and uptake of soluble COD in the anaerobic phase. The remaining PCOD will contribute to the oxygen consumption in the aerated phase and thereby reduce the permissible aerobic ammonia loading rate. The contribution from particulate COD to the total COD available for growth also makes the total COD loading rate the parameter to design for rather than the total BSCOD-loading rate. The average total COD-loading rate in period 2B was 3.3 g COD m^{-2} d^{-1}, and a maximum value of 4 g COD m^{-2}d^{-1} is chosen as design criteria. Particulate COD also reduced the observed $COD_{uptake}/P_{removed}$ ratio, but a value of 20 mg BSCOD (mg PO_4-P)$^{-1}$ is chosen for the suggested design criteria because a full scale process with enhanced primary treatment would probably achieve better removal of particulate COD than in the CMF used in the experiments with municipal

wastewater. Particulate COD will also have an influence on the permissible anaerobic BSCOD-loading rate due to hydrolysis. A maximum value of 5 g BSCOD $m^{-2}d^{-1}$ is chosen as design criteria. The values for total COD-loading rate and anaerobic BSCOD-loading rate are probably a conservative if enhanced primary treatment is used in the process scheme.

The average operating conditions during the final verification in period 2B are summarised in Table 2 together with criteria that should be taken as a recommendation for first generation design criteria.

Table 2. Average operating conditions in final verification period and recommended design criteria for a wastewater with COD:N:P ~ 100:10:2

Parameter	Average in 2B	Design criteria
Total COD-loading rate, (g COD $m^{-2}d^{-1}$)	3.3	< 4
Total BSCOD-loading rate, (g BSCOD $m^{-2}d^{-1}$)	1.1	- -
Anaerobic BSCOD-loading rate, (g BSCOD $m^{-2}d^{-1}$)	4.4	< 5
Aerobic ammonia loading rate, (g NH_4-N m^{-2} d^{-1})	0.33	< 0.4
Required influent C/P-ratio, (mg BSCOD (mg PO_4-P)$^{-1}$)	14.8	20

6.2.2 Aerobic and anoxic phosphate uptake capacity

During period 1E, batch experiments to characterise the biomass with respect to aerobic and anoxic phosphate uptake capacity were performed. The anoxic phosphate uptake rate was at least as high as the aerobic phosphate uptake rate. At the lowest two to three concentrations, the data points could be fitted to a straight line when plotted versus the square root of the phosphate concentration (Figure 40).

The line intersected the x-axis at a value > 0 and the results did therefore not follow half-order kinetics as reported by Falkentoft *et. al.* (2001). However, the results can be explained by a layered biofilm where the fraction of bacteria performing denitrifying phosphate uptake increases with depth as discussed previously.
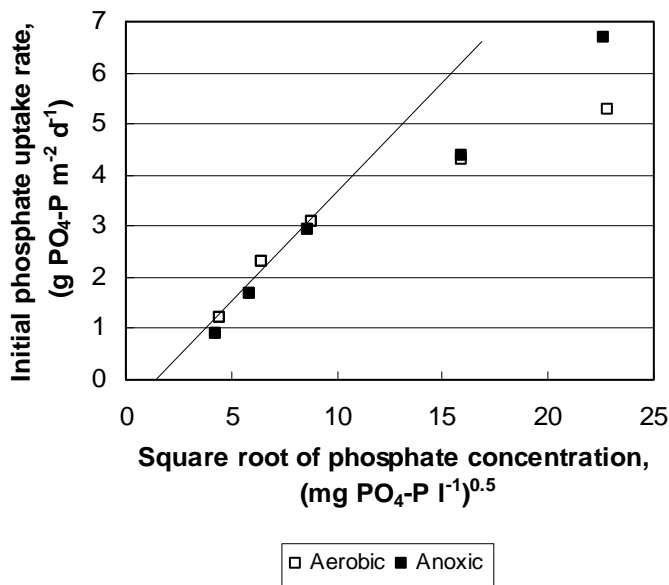
Figure 40. The initial phosphate uptake rate under aerobic and anoxic conditions versus the square root of the phosphate concentration.
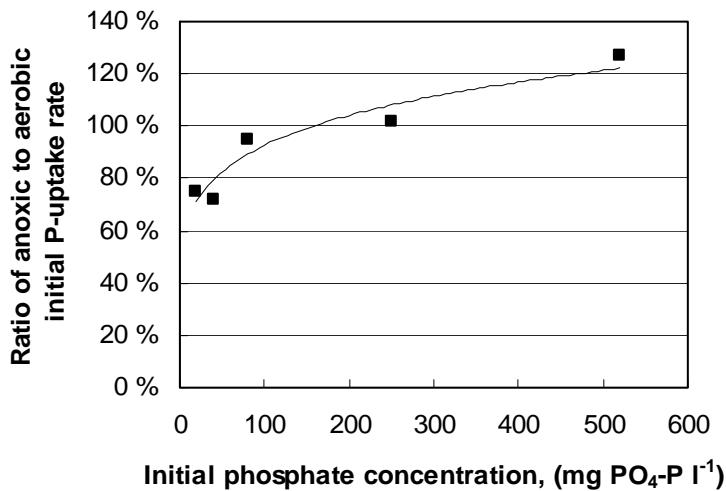
Figure 41. The ratio of aerobic to anoxic initial phosphate uptake rate versus the phosphate concentration.

At the highest concentration the anoxic phosphate uptake rate was higher than the aerobic phosphate uptake rate. This may seem to contradict that all PAO can utilise oxygen as reported by others (Kerrn-Jespersen and Henze 1993; Meinhold *et. al.*, 1999). However, this was also observed in the batch experiments with constructed wastewater and the results can be explained

by low oxygen levels in the deeper parts of the biofilm, due to oxygen consumption in the outer layers.

The results in Figure 41 support that the denitrifying phosphate uptake capacity increased with increasing biofilm depth and indicate that the majority of the PAO in the biofilm were acclimatised to use of nitrate as electron acceptor.

Compared to the batch experiments with biomass from the experiments with constructed wastewater the maximum P-uptake rates where higher in the batch experiments with biomass from the experiments with municipal wastewater. Another difference was that the ratio of anoxic to aerobic P-uptake was higher for the lowest initial phosphate concentrations as seen by comparing Figure 41 and 31. However, the results support the conclusion from the previous batch experiments that the anoxic P-uptake rate can be as high as the aerobic.

6.2.3 Separation of effluent suspended solids and sludge production

Flotation tests were performed with a JAR flotation apparatus comparing no dosage of chemicals with dosage of a polyDADMAC (C591) and a polyacrylamide (PAM, K1912). The effluent quality for suspended solids improved slightly with use of a polymer compared to no use of chemicals (Figure 42). However, the effluent quality based on total phosphorus improved with increasing dosage of the PAM (K1912) demonstrating that use of the polymer improved removal of particles smaller than ~1 μm due to coagulation and flocculation (Figure 43). The good results without use of chemicals indicated that the sludge had good separation properties.

In period 2B, the average sludge production was 0.10 kg DS $m^{-3}_{treated\ water}$, with minimum and maximum values of 0.07 and 0.15 kg DS $m^{-3}_{treated\ water}$, respectively. The phosphorus concentration (g P (g DS)$^{-1}$) of the biomass at the end of the cycle was on average 4 % and the COD content was 0.4 g COD (g DS)$^{-1}$. The average total yield including the storage products was 0.66 g SS (g COD$_{removed}$)$^{-1}$, based on sludge production and removal of COD during the cycles.
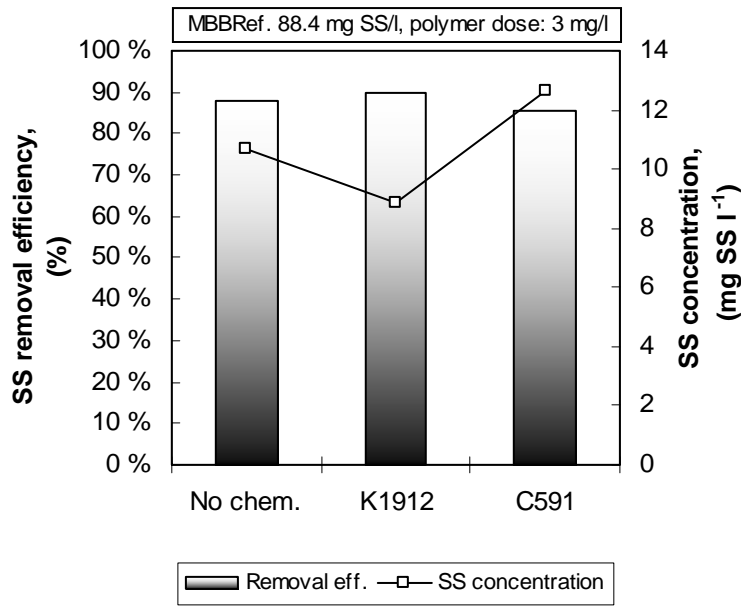
Figure 42. Suspended solids concentration in flotation effluent and removal efficiency in flotation test. Flotation tests without chemical dosage, with 3 mg l⁻¹ polyacrylamide (PAM) K1912 and 3 mg l⁻¹ polyDADMAC C591.
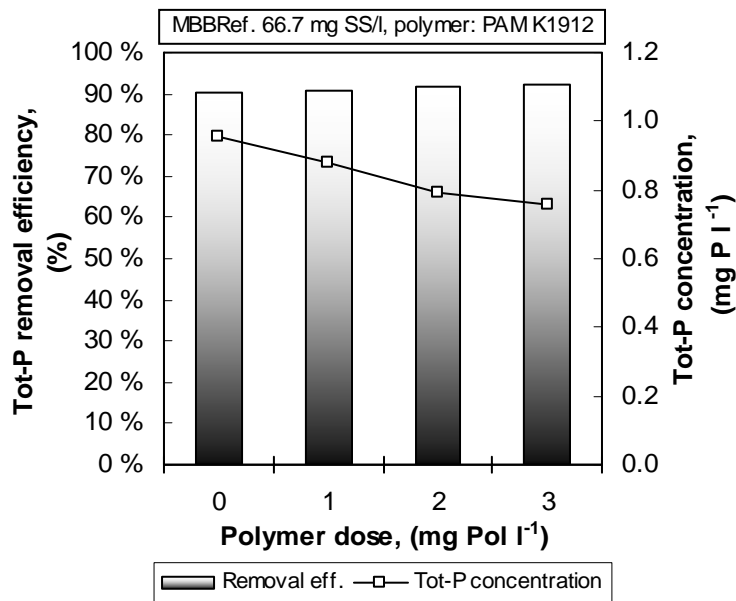
Figure 43. Tot-P concentration and tot-P removal efficiency in the total process (bio + flotation) for different doses of polyacrylamide K1912.

6.2.4 Characterisation of biomass diversity

Long term stability of the EBPR process requires that the operating conditions are favourable for PAO compared to other non-phosphate accumulating bacteria. This may seem straight forward if required operating conditions for EBPR are known. However, as discussed above there are reports from studies where the EBPR activity has declined after an initial period with good biological phosphorus removal (Falkentoft, 2001 and 2002). Loss of EBPR activity has also been reported in laboratory studies with activated sludge SBR processes and has been attributed to proliferation of glycogen accumulating bacteria (GAO) that can accumulate organic substrate in the anaerobic phase without P-release. Several causes for GAO proliferation have been reported including glucose in the wastewater (Cech and Hartmann, 1990, 1993, cited by Mino *et. al.* 1998), long SRT and HRT (Fucase *et. al.*, 1985, cited by Mino *et. al.* 1998) and improper seeding (Matsuo *et. al.*, 1982, cited by Mino *et. al.* 1998).

The research on biofilm EBPR systems is limited compared to the research conducted with activated sludge EBPR systems, and the key factors determining the performance and stability may be system specific. The objective of the biomass characterisation reported here was screening of the biomass with respect to the main bacteria groups and to assess if changes in EBPR activity could be correlated to changes in the bacteria population.

Figure 44 shows the net P-removal in the SBR-reactor during the experiments with municipal wastewater. The filled data points indicate collection of biomass samples in addition to the water samples taken throughout the cycle. Biomass samples were collected once or several times in each of the experimental periods described in previous sections.

Figure 45 shows the net P-removal in the SBR-cycles with collection of biomass samples. In the periods were several biomass samples were collected, the average net P-removal is shown.
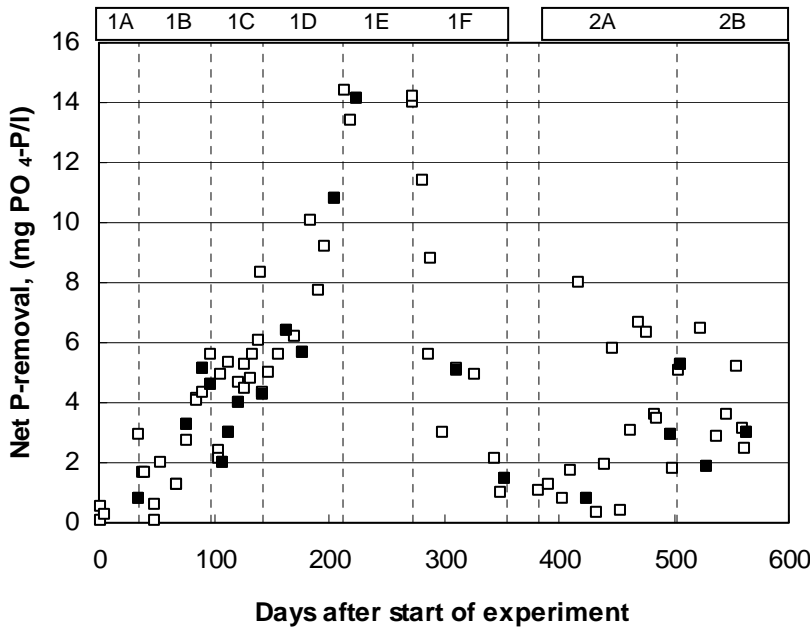
Figure 44. Net P-removal during the experiments with municipal wastewater. Filled data points indicate collection of biomass samples.
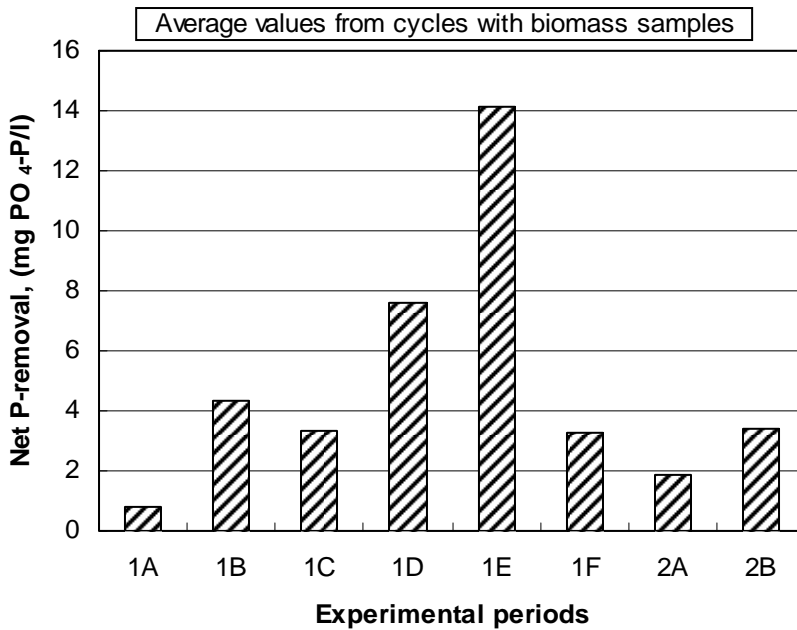
Figure 45. Net P-removal in cycles with collection of biomass samples. In periods with several biomass samples, the average net P-removal is shown.

The variation in the net P-removal in periods 1A through 1E has been discussed previously, and can be explained by variation in the total and anaerobic COD-loading rates. The same is the case for the increase from

period 2A to 2B. The decline in EBPR-activity and net P-removal observed in period 1F compared to 1E was also explained by operating conditions that caused loss of nitrification and thereby loss of electron acceptor for DNPAO. Although the variation in EBPR activity can be explained by the operating conditions, changes in the bacteria culture could not be ruled out.

Figure 46 shows the DGGE gel with the 19 biomass samples collected. The numbers at the top of the figure identify the biomass samples, and the numbers on the gel identify DGGE-bands that were cut out for further analysis. The different experimental periods are indicated at the bottom of the figure.
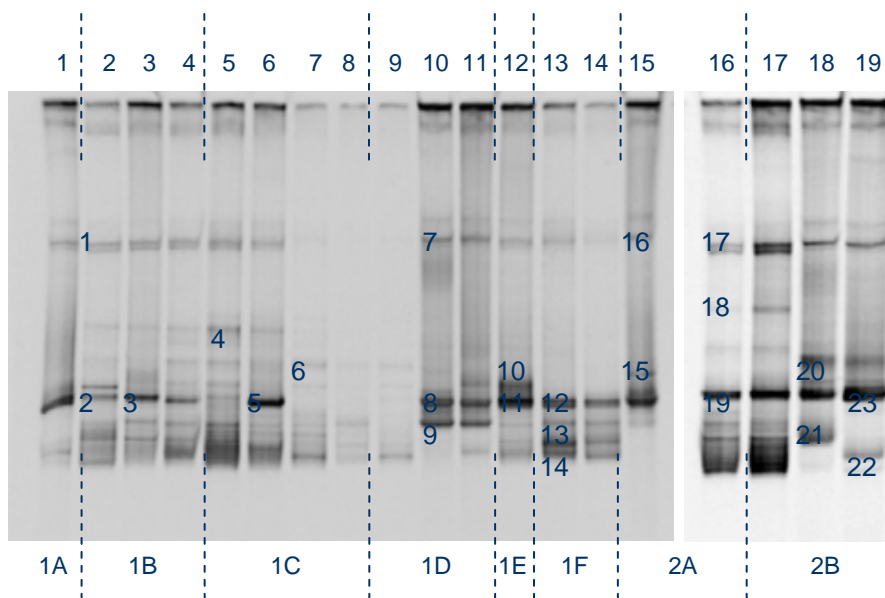
Figure 46. DGGE gel with biomass samples collected in periods 1A – 2B.

The DGGE gel showed several bands in all the experimental periods indicating a diverse microbial population in the bioreactor. The intensity of the bands varied both for a given sludge sample and between the sludge samples, with the lowest intensities in samples 7, 8 and 9. The low intensity in samples 7, 8 and 9 may be due to lower extraction efficiency of DNA from these sludge samples, but may also be due to lower sludge concentration in these samples than the other analysed samples. The DGGE results indicated that DNA-sequences with mobility corresponding to the position of band 7 and 8 in sludge sample 10 were common in all the experimental periods. Comparing the DGGE bands in period 1E with 1F, band 10 in sludge sample 12 (period 1E) is not found in period 1F and

bands 13 and 14 in sludge sample 13 (period 1F) have a higher intensity than in period 1E. Although DGGE is not a quantitative method, a higher intensity is caused by a higher DNA concentration in that band and indicates a higher concentration of the originating bacteria in the sludge sample. The DGGE patterns from period 1E and 1F therefore indicate a difference in microbial composition between these two periods. However, bands with mobility corresponding to band 10, 13 and 14 are also found in period 2B (band 20, 21 and 22) when the process showed good N and P removal (< 10 mg N l^{-1} in effluent and 98 % P-removal) as discussed previously. Although the DGGE patterns show a clear difference between period 1E and 1 F the results do therefore not support a conclusion that the decline in period 1F was caused by a change in the microbial population.

To investigate the microbial diversity further, DNA in 23 selected DGGE bands (Figure 46) was cloned and the clones analysed by RFLP. The results are summarised in Table 3.

In total there were 103 clones, and these were grouped in to 31 different RFLP types based on the combination of the patterns obtained with the two enzymes Rsa I and Hae III. The different RFLP types indicate bacteria with different DNA sequences and the 31 different types obtained for the DNA in 23 DGGE bands show that a DGGE band can contain DNA from different bacteria. This is not surprising since separation in the DGGE gel is based both on length and base sequences. DNA fragments with different length and different base sequence can therefore have the same mobility in the DGGE gel.

Of the 31 different RFLP types, most were found in only one clone. Only four RFLP-types (1, 2, 9 and 25) included 10 % or more of the clones and 3 RFLP types (6, 13 and 28) included from 5 – 10 % of the clones. The RFLP analysis indicates 4 – 7 main types of bacteria in a diverse biomass. All the main RFLP types were found in clones from several periods in both parts of the experiments with varying operating conditions and net P-removals. The results do not identify one or a few RFLP types that correlate with net P-removal.

A comparison of the RFLP types in period 1E and 1F shows that RFLP type 3, 14 and 18 were only found in period 1F, while RFLP type 11 and 27 were only found in period 1E. However, all these RFLP types include only 1 clone, corresponding to ~1 % of the clones in the library, and are

probably not from a dominating bacteria group in the biomass. RFLP type 9 was found both in period 1E and 1F. This was one of the most common types including 12 % of the clones in the library. Although the RFLP results indicate some difference in microbial composition between period 1E and 1F, they do not support a conclusion that this caused the decline in net P-removal.

Table 3. Summary of RFLP-results

RFLP type	# clones	% of clone library	Avg.P-rem.	Found in periods							Sequenced
				1B	1C	1D	1E	1F	2A	2B	
1	11	11 %	2.7	x				x		x	yes
2	13	13 %	3.7	x	x					x	yes
3	1	1 %	5.1					x			yes
4	3	3 %	4.5		x					x	yes
5	2	2 %	5.2							x	yes
6	8	8 %	4.4			x				x	yes
7	2	2 %	5.7			x					yes
8	1	1 %	3.3	x							no
9	12	12 %	6.5	x			x	x	x		yes
10	3	3 %	5.1	x				x			yes
11	1	1 %	14.1				x				no
12	1	1 %	5.2							x	yes
13	6	6 %	4.1	x						x	yes
14	1	1 %	5.1					x			no
15	1	1 %	3.3	x							no
16	3	3 %	3.0		x						yes
17	1	1 %	2.0		x						no
18	1	1 %	5.1					x			yes
19	1	1 %	3.0		x						no
20	1	1 %	5.2							x	no
21	1	1 %	2.0		x						no
22	1	1 %	2.0		x						no
23	4	4 %	3.5			x			x	x	yes
24	4	4 %	11.1				x			x	yes
25	10	10 %	3.9			x			x	x	yes
26	1	1 %	0.8						x		no
27	1	1 %	14.1				x				no
28	5	5 %	7.9				x		x	x	yes
29	1	1 %	5.2							x	yes
30	1	1 %	5.1	x							yes
31	1	1 %	3.0							x	no

From a total of 103 clones, 30 were selected for sequencing. The selection of clones covered all the main RFLP types and all the experimental periods. The results are shown in Figure 47, where the clones are placed in a phylogenetic tree. A summary of the sequenced clones is given in Table 4.
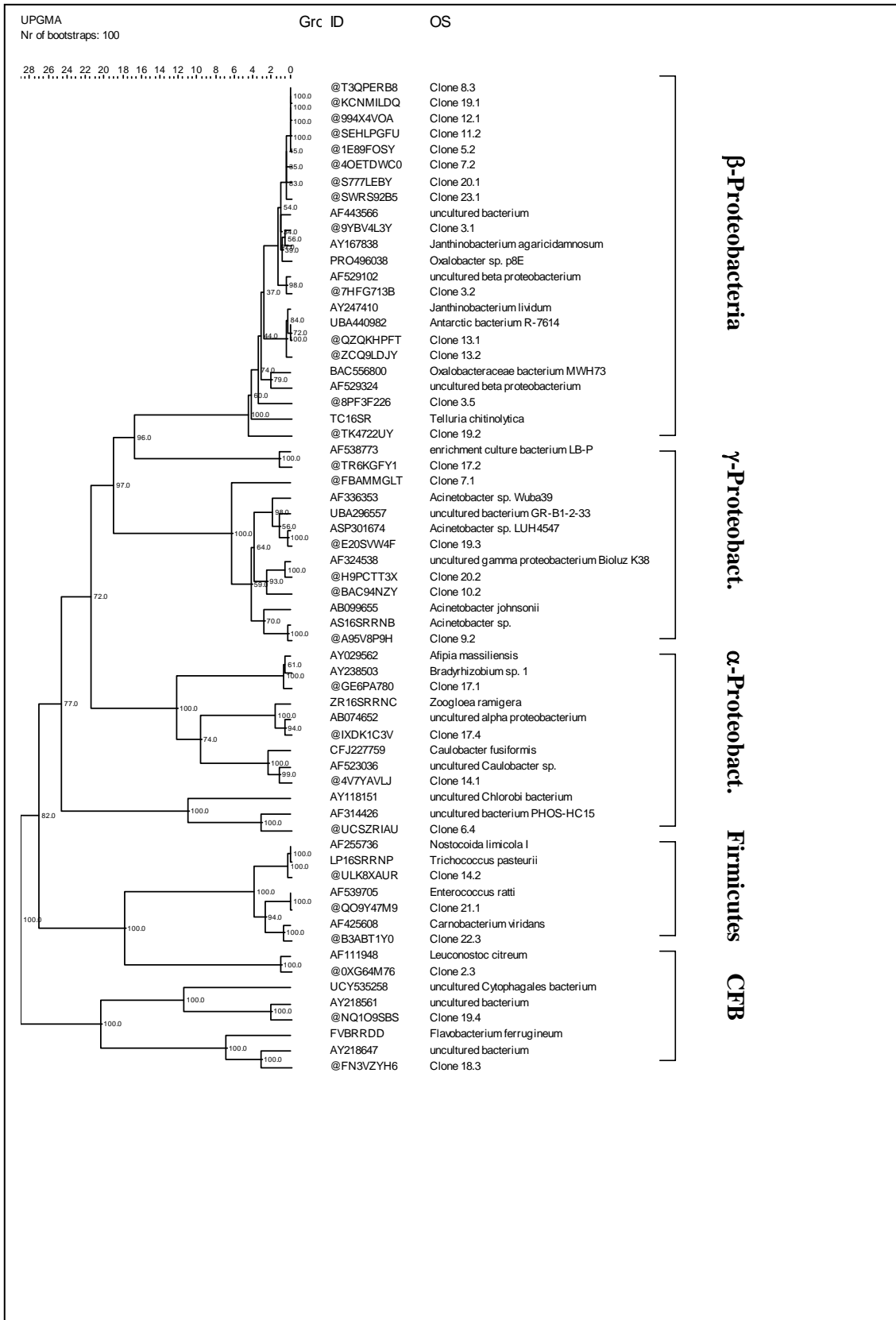
Figure 47. Phylogenetic tree

Of the 30 sequenced clones 10 clones were identified as *Oxalobacter* sp. *Oxalobacter* is a genus in the β-Proteobacteria group. The sequenced clones were from all the experimental periods except 2A and included clones classified as RFLP type 6, 9 and 13, which accounted for 8 %, 12 % and 6 %, respectively, of the clones in the RFLP-library. *Oxalobacter* sp. was therefore probably a major bacteria type in the biomass. Five clones were identified as *Acinetobacter* sp. *Acinetobacter* is a genus in the γ-Proteobacteria group. These clones were from period 1D, 1E and 2B and included clones classified as RFLP-type 25 and 28, which accounted for 10 % and 5 %, respectively of the clones in the RFLP-library. *Acinetobacter* sp. was therefore probably also a common bacteria type in the biomass.

Table 4. Summary of sequenced clones.

Clone	Period	RFLP-type	Colsest microorganism	% homology	Bacteria group
3.2	1B	13	*Oxalobacter* sp.	98	β-Proteobacteria
5.2	1C	16	*Oxalobacter* sp.	99	β-Proteobacteria
7.2	1D	7	*Oxalobacter* sp.	98	β-Proteobacteria
8.3	1D	6	*Oxalobacter* sp.	99	β-Proteobacteria
11.2	1E	9	*Oxalobacter* sp.	99	β-Proteobacteria
12.1	1F	9	*Oxalobacter* sp.	99	β-Proteobacteria
19.1	2B	12	*Oxalobacter* sp.	99	β-Proteobacteria
19.2	2B	13	*Oxalobacter* sp.	98	β-Proteobacteria
20.1	2B	13	*Oxalobacter* sp.	98	β-Proteobacteria
23.1	2B	6	*Oxalobacter* sp.	98	β-Proteobacteria
3.1	1B	10	*Janthinobacterium agaricidamnosum*	99	β-Proteobacteria
13.2	1F	10	*Janthinobacterium agaricidamnosum*	99	β-Proteobacteria
13.1	1F	9	*Janthinobacterium lividum*	99	β-Proteobacteria
3.5	1B	30	*Telluria chtinolytica*	95	β-Proteobacteria
7.1	1D	23	*Acinetobacter* sp.	99	γ-Proteobacteria
9.2	1D	25	*Acinetobacter* sp.	99	γ-Proteobacteria
10.2	1E	24	*Acinetobacter* sp.	96	γ-Proteobacteria
19.3	2B	28	*Acinetobacter* sp.	99	γ-Proteobacteria
20.2	2B	23	*Acinetobacter* sp.	96	γ-Proteobacteria
17.2	2B	4	*Lysobacter* sp.	96	γ-Proteobacteria
6.4	1C	2	Uncultured bacteria PHOS-HC-15	100	α-Proteobacteria
14.1	1F	18	*Caulobacter fusiformes*	97	α-Proteobacteria
17.1	2B	29	*Bradyrhizobium sp.*	99	α-Proteobacteria
17.4	2B	5	*Zoogloea ramigera*	98	α-Proteobacteria
2.3	1B	2	*Leuconostic lactis*	100	Firmicutes
14.2	1F	3	*Lactophaera pasteurii*	99	Firmicutes
21.1	2B	1	*Enterococcus rattus*	100	Firmicutes
22.3	2B	1	*Carnobacterium viridans*	99	Firmicutes
18.3	2B	2	*Flavobacterium ferrugineum*	93	CFB
19.4	2B	4	Uncultured *Cytophagales*	89	CFB

The remaining clones were identified as belonging to α-Proteobacteria, Firmicutes and CFB as well as some the β- and γ-Proteobacteria. The results are in accordance with previous studies in that Proteobacteria,

especially belonging to the β-subclass are dominating in EBPR systems (i.e. Bond *et. al.*, 1999; Crocetti *et. al.*, 2000).

Oxalobacter sp. has not previously been identified in EBPR systems and the results do not prove the involvement of *Oxalobacter* sp. in EBPR. However, the results indicate that this was the most common bacteria in the biomass throughout the experimental period and may be a phosphorus accumulating bacteria. Further studies would be required to prove or disprove the possible involvement of *Oxalobacter* sp. in aerobic and/or anoxic EBPR.

Acinetobacter sp. is well known from many studies but is currently thought not to be the bacteria primarily responsible for EBPR as discussed previously. However, *Acinetobacter* sp. was probably also a common bacteria in the biomass and may well have been partly responsible for the P-removal in our experiments.

7. Discussion of results in relation to the hypothesis

The results from the SBR experiments with both constructed and municipal wastewater were generally in accordance with hypothesis 1. However, aerobic phosphate uptake accounted for a significant fraction of the total P-uptake in both sets of experiments and a process with only anoxic P-uptake was never achieved. The results with constructed wastewater indicated that increasing the length of the anaerobic phase increased the fraction of anoxic P uptake. This was probably an effect of less time under aerated conditions relative to anaerobic conditions rather than due to the absolute length of the anaerobic phase, and the increase in anoxic P uptake was limited. Due to the need for complete nitrification and the limits this sets on minimum aeration time, one will probably always have a mix of aerobic and anoxic P-uptake in this process.

The importance of the limiting factors presented in hypothesis 2 was also verified in the experiments with constructed wastewater. However, their relative importance will differ in practice. Sufficient C-source is easy to ensure by dosage of additional C-source although this has an effect on the cost of the process. Low net P removal due to limited growth is will also be of little importance in practice because this can be controlled by the total length of the SBR cycle. Of the remaining two factors, incomplete nitrification is probably more important than competition from non P-accumulating heterotrophs. The reason for this is that to ensure complete nitrification the COD loading rate in the aerated phase must be low. This requires efficient anaerobic BSCOD uptake and probably a pre-treatment to remove particulate matter. Under such conditions there will be limited substrate for aerobic heterotrophs. Possible competition can then come from non P-accumulating bacteria that have the ability for anaerobic substrate uptake. However, the results from the last verification period in the experiments with municipal wastewater did not indicate that this was a problem.

With respect to hypothesis 3, the results support the choice of criteria with the exception of the total BSCOD loading rate. As discussed previously, the influence of particulate matter in the aerated phase makes the total COD loading rate a better choice for design criteria. It is of course the

biodegradable COD that is of importance and if information on the biodegradability of the COD is available this can be used.

With respect to particle separation and characterisation of the biomass, these issues were not included in the hypothesis at the start of the experiments with constructed wastewater. However, god separation properties of suspended solids after a low loaded MBBR process is in accordance with other studies with a MBBR (Ødegaard *et. al.*, 2000). As discussed previously, the characterisation results were in accordance with other studies with respect to Proteobacteria and *Acinetobacter*, but *Oxalobacter* has not previously been reported in EBPR systems and should be investigated as a possible PAO.

8. Further research

The results from the experiments in laboratory scale with constructed and municipal wastewater show that there is potential for a compact process. In order to illustrate this, a calculation of the necessary lengths of the anaerobic and aerobic phase is shown in an example below.

Given a wastewater with total-COD: 400 mg COD l^{-1}, BSCOD: 80 mg BSCOD l^{-1}, 3.5 mg PO4-P l^{-1} and 25 mg NH_4-N l^{-1}, a process scheme where particulate matter is efficiently removed by enhanced primary treatment (Ødegaard, 1998), so the objective in the biological step is mainly to remove soluble phosphate and nitrogen.

In this wastewater the influent BSCOD/P ratio (23 mg BSCOD (mg PO_4-P)$^{-1}$) would probably be sufficient for efficient P-removal. However, additional carbon source can be added for example as acetic acid, in cases where this would be required.

The length of the anaerobic phase should be chosen to ensure complete anaerobic uptake of easily biodegradable COD. Based on the results a choice of 5 g BSCOD $m^{-2}d^{-1}$ would be a conservative value. Assuming a specific biofilm growth area of 335 m^2 per m^3 reactor volume, a maximum anaerobic loading rate of 5 g BSCOD $m^{-2}d^{-1}$ gives a minimum anaerobic phase of ~1 hour and 10 minutes.

The results have shown that the length of aerated phase is determined by the conditions required for nitrification rather than P-uptake. A maximum ammonium loading rate of 0.4 g NH_4-N $m^{-2}d^{-1}$ gives a minimum aerobic phase of 4.5 hours. Thus the total cycle length would have to be set at 5.6 hours. The resulting total COD-loading rate assuming a COD concentration after pre-treatment of 240 mg COD l^{-1} (corresponding to a conservative estimate for removal of particulate COD of 50 % in the pre-treatment) would then be 3.1 g COD $m^{-2}d^{-1}$, which should be acceptable for nitrification

In addition there will be time needed for filling and emptying of the reactor, and for primary and final separation. However, a residence time of approximately 6 hours in the biological step illustrates the potential for

achieving a compact process for combined biological phosphorus and nitrogen removal.

The example, however, only indicates the potential of the process. Experiments in larger scale need to be run to verify this. Further work with biological nutrient removal in a SBMBBR should address design of a full scale process and also a comparison between a continuous process based on activated sludge or a hybrid process and a SBMBBR.

Further work on characterisation of the bacteria involved in EBPR should include *Oxalobacter* sp. as one of the possible bacteria responsible for aerobic and or anoxic P-removal.

9. Conclusions

➢ Biological phosphorus and nitrogen removal can be achieved in a moving bed biofilm reactor operated as a SBR. The operating conditions to achieve simultaneous nitrification – denitrification and phosphate uptake will be dependant on the wastewater quality, but the SBR cycle should be tuned to achieve near complete removal of easily biodegradable soluble COD in the anaerobic period and complete nitrification in the aerobic period. To achieve complete nitrification, the total COD-loading rate including the effect of particulate COD should be considered.

➢ A SBR cycle of 6 hours with 1 hour and 40 minutes anaerobic phase followed by a 4 hour and 20 minutes aerobic phase gave less than 10 mg soluble-N l^{-1} and 0.3 mg PO_4-P l^{-1} in the SBR effluent during the verification experiments with municipal wastewater (Influent after pre-treatment: 4.2 mg PO_4-P l^{-1}, 18 mg NH_4-N l^{-1}, 104 mg SCOD l^{-1} and 205 mg COD l^{-1}).

➢ Hydrolysis of particulate COD increased the biodegradable soluble COD. However, the process is best suited for removal of soluble compounds and a process scheme including enhanced primary treatment and utilisation of primary sludge for production of carbon source should be considered. Sufficient P and N removal could not be achieved without dosage of additional C-source with the wastewater used in this study. A ratio of 15 mg BSCOD (mg PO_4-P)$^{-1}$ was required to achieve the effluent concentration reported above.

➢ The results indicated that the majority of the phosphorus accumulating bacteria was capable of utilising nitrate as electron acceptor. This is an advantage with respect to carbon source requirement for P and N removal.

➢ Efficient separation of effluent suspended solids was achieved by flotation without use of chemicals. Dosage of a polymer improved removal of total phosphorus probably due to coagulation and flocculation of sub micron particles.

➢ *Oxalobacter* sp. was probably the most common bacteria in the biomass and may be a phosphorus accumulating bacteria. However, further studies would be required to prove or disprove the possible involvement of *Oxalobacter* sp. in aerobic and/or anoxic EBPR.

➢ *Acinetobacter* sp. was probably also a common bacteria in the biomass and may well have been partly responsible for P-removal.

➢ Recommended first generation design criteria for a wastewater with COD:N:P ~ 100:10:2 are given in Table 5 below.

Table 5. Recommended first generation design criteria for a wastewater with a typical nutrient distribution: COD:N:P ~ 100:10:2

Parameter	Design criteria
Total COD-loading rate, (g COD $m^{-2}d^{-1}$)	< 4
Anaerobic BSCOD-loading rate, (g BSCOD $m^{-2}d^{-1}$)	< 5
Aerobic ammonia loading rate, (g NH_4-N m^{-2} d^{-1})	< 0.4
Required influent C/P-ratio, (mg BSCOD (mg PO_4-P)$^{-1}$)	20

BSCOD: Biodegradable soluble COD

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Appendix A. Procedure for rDNA analysis methods used in characterisation of biomass

Nucleic acid extraction

BioP samples (40 ml) were centrifuged (5000 x g; 10 minutes), and nucleic acids extracted in pelleted materials by a bead-beating method (Ultraclean Soil DNA Kit; MoBio Laboratories, Solana Beach, Ca).

Solution S (60 µl) was added to each 2 ml Bead Solution tube 1 g of sediment and 60 µl of Solution S1. The tube was vortexed briefly, and 200 µl of Solution IRS (Inhibitor Removal Solution) was applied. Bead tubes were secured horizontally and extracted in a Mini-BeadBeater (BioSpec) at 2500 r.p.m. for 300 seconds, followed by centrifugation at 10,000 x *g* for 30 seconds. The supernatants were transferred to clean microcentrifuge tubes (provided with the kit). To each tube was added 250 µl of Solution S2, vortexed for 5 seconds, the tubes incubate 4°C for 5 minutes, and the then centrifuged at 10,000 x *g* for 1 minute. The supernatant volumes were transferred to clean microcentrifuge tubes (provided), 1.3 ml of Solution S3 carefully added to the supernatants, and the tubes vortexed for 5 seconds. Approximately 700 µl of the solution was loaded onto each spin filter and centrifuged at 10,000 x *g* for 1 minute, the flow-through solution discarded, the remaining supernatant added to the spin filter, and the tubes centrifuge at 10,000 x *g* for 1 minute. The process was repeated until all supernatant was passed through the spin filter. Solution S4 (300 µl) was added and centrifuged for 30 seconds at 10,000 x *g*. The flow through was discarded, and the tubes re-centrifuged for 1 minute. The spin filters were carefully placed in new clean tubes (provided), and 50µl of Solution S5 was added to the centre of the white filter membrane. The tubes were centrifuged for 30 seconds and the spin filters discarded. DNA extracts were stored at -20°C.

PCR amplification

Oligonucleotides and PCR reagents

Oligonucleotides for PCR amplification

Oligonucleotide primers were prepared specific for *Bacteria* (Teske *et al*., 1996):

Bacteria
341fBac: 5'-CCT-ACG-GGA-GGC-AGC-AG-3' (forward primer)
907rBac: 5'-CCC-CGT-CAA-TTC-CTT-TGA-GTT-3' (reverse primer)
Expected PCR product: 567 bp

The primers were synthesised by EuroGentec, Seraing, Belgium. The primers were diluted in sterile water as a stock solution of 50 μM distributed in 50 μl aliquots and stored at ÷20°C.

Deoxynucleotides
Stock solutions of deoxynucleotides (d'NTP) were prepared by diluting 100 mM of the d'NTPs 2'-deoxyadenosine 5'-triphosphate (d'ATP), 2'-deoxsythymidine 5'-triphosphate (d'TTP), 2'-deoxyguanosine 5'-triphosphate (d'GTP) and 2'-deoxycytidine 5'-triphosphate (d'CTP) (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A). Each d'NTP (100 μl) was diluted in sterile water (600 μl), resulting in final concentrations of 10 mM of each d'NTP. Solutions were distributed in 50 μl aliquots and stored at ÷20°C.

PCR amplification
A PCR mix of 100 μl mix consisted of 20 μl d'NTP (10 mM), 10 μl forward primer (50 μM), 10 μl reverse primer (50 μM), 50 μl bovine serum (0.1 % final concentration; Sigma, product no. B8667), 5 μl sterile water and 5 μl AmpliTaq DNA polymerase (Perkin Elmer Roche Molecular Systems, Branchburg, NJ, U.S.A).

DNA template (1-10 μl) was diluted in 10 μl [10x] PCR buffer with 15 mM MgCl$_2$ (Perkin Elmer Roche) and with sterile water to a final volume of 90 μl. A PCR mix of 10 μl was applied to each sample while the samples were still in the heating block (95°C) and the samples were immediately transferred to a DNA Thermal Cycler (iCycler, BioRad).

PCR was run with the following sequence cycles:

Denaturation: 95°C in 1 minute
Primer annealing: 55°C in 1 minute
DNA synthesis (primer extension): 72°C in 3 minutes
Number of cycles: 30

The PCR runs were terminated by incubation at 72°C in 15 minutes before cooling to 4°C.

Agarose gel electrophoresis

PCR products were analysed by horisontal agarose gel electrophoresis. Samples (27 µl) were mixed with [10x] gel-loading TBE buffer (3 µl) (0.9 M Tris, 0.9 M borate, 20 mM EDTA, pH 8.3, 50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue). A Low DNA Mass Ladder (Gibco BRL, Paisley, UK) was used as standard, 12 µl standard in 3 µl [10x] gel-loading TBE buffer.

Gels were prepared by heating agarose (2.0 g; Sigma) in 160 ml [0.5x] TBE buffer in a microwave oven (4 minutes), followed by cooling to 50°C in water bath. Ethidium bromide (10 µl) from a stock solution (10 mg/l ethidium bromide in sterile water) was applied to the agarose. The melted gel was cast horizontally in a plastic tray (the open ends of the tray were sealed) with a comb of 15-well or 20-wells in the electrophoresis apparatus (BioRad). The gel was set at room temperature for 20 minutes, submerged in [0.5x] TBE buffer, and the comb and seals carefully removed.

Prepared samples (20 µl) and standard (10 µl) were applied to the submerged gel wells and electrophoresis run with constant voltage (150 V) in 1.5-2 hours at room temperature. Gel documentation was performed over a UV-transilluminator table (BioRad). The gels were photographed with a digital camara (GelDoc, 2000, BioRad).

Denaturing gradient gel electrophoresis (DGGE)

Analytical DGGE

PCR products were generated with general primers defining *Bacteria* (341fBAC and 907rBAC) or *Archaea* (PARCH340f and PARCH519r) for DGGE analysis. To the 341f BAC-primer, or the 341PARCHf primer, a 40 mer GC-clamp was added to the 5'-end (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-G-3').

DGGE was performed with 6 % (w/v) polyacrylamide (PAA) gels in [0.5x] TAE buffer (20 mM Tris-acetat, pH 7.4; 10 mM acetat; 0.5 mM EDTA) using a 20-70 % gradient of the denaturing agents urea and formamide (100

% denaturing agents corresponded to 7 M urea and 40 % (v/v) deionised formamide) in a DCode Universal Mutation Detection system (BioRad).

A stock solutions of PAA/Bis-acrylamide (Bis) (40 %) was prepared of 38.93 g acrylamide and 1.07 g Bis dissolved in deionised water to 100 ml. A stock solution of [50x] TAE buffer was generated by mixing 242 g Tris, 57.1 g acetic acid, and 100 ml 0.5 M EDTA to a total volume of 1000 ml with deionised water. Linear gradient gels (thickness 1 mm) were prepared by mixing PAA/Bis-stock solution with denaturing agents to generate a 20 to 70 % linear gradient in a gradient delivery system (BioRad model 475). Solutions with 20 % or 70 % denaturing agents are described in Table A-1.

Table A-1. Composition of 20 % and 70 % denaturing solutions used in DGGE

	Denaturating solutions	
CHEMICALS	20 %	70 %
40 % acrylamide/Bis	15 ml	15 ml
[50x] TAE buffer	2 ml	2 ml
Formamide	8 ml	28 ml
Urea	8.4 g	29.4 g
Deionised water	to 100 ml	to 100 ml

For the preparation of one gel 18 ml of each solution was mixed with 200 μl ammonium persulphate (10 % (w/v) in deionised water) and 20 μl TEMED (BioRad), and the mixtures immediately transferred to each of two 30-ml syringes which were subsequently mounted in the gradient delivery system. The gel was cast as a parallel gradient gel (16 x 16 cm) with 1 mm thickness and allowed to polymerise for approximately 1 hour with a comb of 15 wells. The electrophoresis tank was filled with [1x] TAE buffer which was heated to 60°C in the tank, and 1-2 polymerised gels were placed vertically in the electrophoresis tank.

Each PCR product sample (10 μl) was mixed with 10 μl sample buffer (0.05 % bromophenol blue, 0.05 % xylene cyanol, 70 % glycerol, diluted in deionised water), and the complete volume (20 μl) applied to each well.

Vertical electrophoresis was performed with continuous temperature (60°C) and voltage (150 V) until both markers had migrated to the bottom of the gel (approximately 4.5 hours).

After electrophoresis the gels were stained in SYBR Gold (Molecular Probes, Leiden, The Netherlands), diluted 1:10,000 in [1x] TAE-buffer and incubated for 20-30 minutes. The gels were then photographed with the GelDoc system (BioRad).

The gel band patterns were compared for similarity and similarity indices generated by the Quantity One option of the GelDoc software program.

Cloning and sequencing

Preparative DGGE
DGGE was performed to separate bands for cloning and sequencing. DGGE was performed as described for analytical DGGE. After staining and documentation a picture was used to determine which bands to clone. While the gel was placed on a UV-table selected bands were carefully cut out with a sterile scalpel. Each band was transferred to a 1.5 ml Eppendorf tube and 50 µl sterile water added to each tube. The DNA was eluted overnight at 4°C, re-amplified in PCR for cloning (see below) without forward primer with GC-clamp.

Cloning of PCR products
Cloning was performed with the Qiagen PCR Cloning[Plus] Kit (Qiagen GmbH, Hilden, Germany) with the 3.85 Kbp pDrive Cloning Vector and QIAGEN EZ chemically competent *Escherichia coli* cells.

16S bacterial or archaeal rDNA was amplified in PCR as described above. For cloning PCR was performed as described above, except that the final termination at 72°C was prolonged to 15 minutes to generate 3'-adenine overhangs. The PCR products were purified in preparative agarose gel electrophoresis (see above).

A ligation-reaction mixture was prepared of 1 µl pDrive Cloning Vector (50 ng/µl), 2 µl purified PCR product, 5 µl Ligation Master Mix (2 x), and 2 µl sterile water. The mixture was gently mixed and incubated at 10°C for 30 minutes. Chemically competent Qiagen EZ cells were thawed on ice, 2 µl ligation-reaction mixture was added to each tube of competent cells, gently mixed, and incubated on ice for 5 minutes. Cells were heat-shocked at 42°C for 30 seconds without shaking and tubes incubated on ice for 2 minutes. SOC medium of the cloning kit (250 µl) at room temperature was

added to each tube and two different volumes (20 and 100 µl) of each culture spread directly onto Luria-Bertani (LB) agar plates (1.5 % agar, 1.0 % Tryptone, 0.5 % yeast extract, 1.0 % NaCl, pH 7.0; Sigma) supplemented with ampicillin (100 µg/ml agar medium). Before inoculation the plates were spread with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma), 40 µl of 40 mg/ml X-gal in dimethylformamide.

LB plates were incubated overnight at 37°, and then at 4°C for 3 hours. The plates were screened for blue and white colonies. Only transformants with inserted plasmid grew on the medium, due to the resistance gene of the plasmid. Colonies with PCR products ligated into the vector were observed as white colonies, opposed to light or dark blue colonies with vectors without PCR product inserts. The blue colony pigmentation was caused by the *lacZα* gene product of the vector which stains the chromogenic substrate X-gal. PCR products are inserted within the gene, thereby disrupting the gene function, resulting in white colonies. White transformant colonies were picked with sterile loops and inoculated into liquid LB medium, supplemented with ampicillin (100 µg/ml). The medium was distributed in 24-well sterile tissue culture plates (Corning Inc., Corning, NY, USA), with 2 ml medium/well. The clones were incubated at 37°C for 20-24 hours in the LB medium, and the plasmids purified.

Isolation of plasmids
The plasmids were isolated with a GenElute Plasmid Miniprep kit (Sigma), according to the instructions from the manufacturer. Transformant clones (2 ml) were harvested by centrifugation (Eppendorf microcentrifuge;) at 14 000 x g for 2 minutes. The pellets were resuspended in Resuspension Solution (200 µl) by vortexing. A Lysis Solution (200 µl) was added, the mixture gently inverted until clarification (8-10 times), and the lysis netralized within 3-5 minutes with 350 µl Neutralization/Binding Buffer. The cell debris was pelleted by centrifugation (14 000 x g for 10 minutes). The supernatants were transferred into GenElute Miniprep binding columns assembled in microcentrifuge tubes, centrifuged (14 000 x g for 2 minutes), and the flow-through liquid discarded. The binding columns were then washed with 750 µl Wash Solution and centrifuged (14 000 x g for 2 minutes), effluents discarded, and the columns re-centrifuged (14 000 x g for 2 minutes) to remove any additional solutions. The binding columns were then transferred to new microcentrifuge tubes, 100 µl sterile water

applied, and the tubes centrifuged (14 000 x g for 2 minutes). The isolated plasmid solutions were stored at ÷20°C.

Control of positive PCR insert

Positive PCR inserts in the transformant vectors were controlled by PCR with M13 primers defining vector sequences flanking the inserted.

The primer sequences were:

M13 Forward primer (-20): 5'-GTA-AAA-CGA-CGG-CCA-G-3'
M13 Reverse primer: 5'-CAG-GAA-ACA-GCT-ATG-AC-3'

The primer sites corresponded to the bases 391-406 (M13 Forward -20) and 205-221 (M13 Reverse) of the LacZα fragment of the vector. A plasmid without a positive PCR product insert would result in a 202 bp M13 PCR product, while a positive PCR product would result in a 769 bp PCR product.

A PCR mix was prepared as described above (see section 3.4.3) with the M13 primer set (50 μM stock solutions). Plasmid DNA template (2 μl) was diluted in PCR buffer and sterile water to a final volume of 90 μl as described above and mixed with PCR mix. The PCR was run according to the following sequence cycles:

Initial denaturation: 94°C in 2 minutes
Denaturation: 95°C in 1 minute
Primer annealing: 55°C in 1 minute
DNA synthesis (primer extension): 72°C in 1 minute
Number of cycles: 30

The PCR run was terminated at 72°C (7 minutes) and cooling at 4°C.

Restriction fragment length polymorphism (RFLP) analysis

M13 PCR products were analysed with the restriction endonucleases *Hae*III (Sigma) and *Rsa*I (Sigma). The restriction enzymes and corresponding enzymes (provided by the manufacturer; Sigma) is described in Table A-2.

Table A-2. Characteristics of restriction endonucleases and their buffers

ENZYME	ACTIVITY [A]	RECOGNITION SEQUENCE	DIGESTION BUFFER	BUFFER COMP. (1 X DILUTION)
*Hae*III	10 000 U/ml	5' GG/CC 3'	Buffer SM	10 mM Tris-HCl 50 mM NaCl 10 mM $MgCl_2$ 1 mM dithioerythritol pH 7.5
*Rsa*I	10 000 U/ml		Buffer SL	10 mM Tris-HCl 10 mM $MgCl_2$ 1 mM dithioerythritol pH 7.5

[A] *One unit of each enzyme cleaves 1 µg λDNA in 1 hour at 37 ℃*

Plasmid DNA amplified by M13 PCR (1, 5, or 10 µl) were mixed with restriction enzymes: 2.0 µl *Hae*III (20 U), or 2.0 µl *Rsa*I (20U). Each mixture was diluted to a total volume of 50 µl with the respective enzyme buffers [1x] concentration (see *Table A-2*). The reaction mixtures were incubated at 37°C for 2.5 hours and placed on ice to stop the reaction. The digested fragments were analysed on analytical agarose gel electrophoresis. Fragments < 100 bp were not considered for RFLP. Digest patterns were compared by performing matching analysis by the by the Quantity One option of the GelDoc software program. Matches were grouped within as RFLP types.

Sequencing of plasmid DNA
M13 PCR amplified the 6S rDNA PCR inserts on the purified plasmids, with M13 primers flanking the inserts. M13 PCR products were precipitated by 70 % ethanol and 0.1 M Na-acetate buffer (Teske *et al*., 1996). DNA was measured by the ethidium bromide method. The precipitated PCR-products were submitted for DNA sequencing (MedProbe, Oslo, Norway).

Partial 16S rRNA gene sequences were submitted to GenBank at the National Centre for Biotechnology Information (NCBI) for BLAST analysis in the NCBI database (Altschul *et al*., 1997). The BLAST program

conducts online alignments of submitted sequences to all relevant sequences within the database.

All 16S rRNA gene fragments from the seep communities, as well as closely related reference sequences from GenBank (NCBI alignments) were stored in our "Seep" database. The database was generated by the software program Kodon Total genome and sequence analysis (Kodon version 1.0; Applied Maths, Sint-Martens-Latem, Belgium). Phylogenetic trees were generated using the Kodon software: Alignment studies were performed by unweighted-pair group method using arithmetic averages (UPMGA) for cluster analysis with the "Multiple aligned distances" method. The Kimura two-parameter model was used for estimation of evolutionary distances, and 100 bootstraps were performed to assign confidence levels to the nodes of the trees.

Appendix B. List of papers

1. Helness, H and Ødegaard, H. (1999):"Biological phosphorous removal in a sequencing batch moving bed biofilm reactor" Wat. Sci. Tech. Vol 40, No 4-5, pp 161-168. Also in: Proc. 2'nd Int. Conf. on Advanced Wastewater Treatment, Recycling and Reuse, Milano, 14-16 Sept. 1998.

2. Helness, H and Ødegaard, H. (2001): "Biological phosphorous and nitrogen removal in a moving bed biofilm reactor" Wat. Sci. Tech. Vol 43, No 1, pp 233–240. Also in: Proc. from 1st. World Water Congress of the International Water Association (IWA) 3-7 July 2000, Paris.

3. Helness, H. and Ødegaard, H. (2005). "Biological phosphorus and nitrogen removal from municipal wastewater with a moving bed biofilm reactor". In Proc. IWA Specialized Conference, "Nutrient Management in Wastewater Treatment Processes and Recycle Streams", Krakow, Poland, 18-21 September 2005

4. Helness, H., Brakstad, O. G. and Ødegaard, H.: "Screening of biomass community in a moving bed biofilm reactor operated for biological phosphorus and nitrogen removal", Paper in preparation.