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Assessment of biomass functionalities in a biofilm membrane bioreactor (BF-MBR) targeting biological nutrient removal

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Preface

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Abstract

High concentrations of nutrients such as nitrogen, phosphorus and organic matter can cause serious eutrophication in receiving water bodies. In biological wastewater treatment microorganisms remove nutrients from the wastewater and ensure that these components in the final effluent stay at acceptable levels.

A biofilm membrane bioreactor (BF-MBR) removing nitrogen, phosphorus and carbon from municipal wastewater was operated for 85 days. An assessment of the process focusing on the biomass in the various reactors was carried out. The amount of ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), phosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO) was quantified. Fluorescence *in situ* hybridization (FISH), confocal laser scanning microscopy (CLSM) and digital image processing with the software *daime* were used for this purpose. Changes in the communities were compared to different operating conditions. The effect of a decrease in the hydraulic retention time (HRT) and diluted concentrations of nutrients in the influent were evaluated. The correlation between filamentous bacteria and the sludge volume index was studied. The viability of bacterial populations in a nitrifying biofilm was also investigated.

The BF-MBR produced a high quality effluent in terms of chemical oxygen demand (COD), nitrogen and total suspended solids (TSS). The system handled well the reduction in the HRT. The removal of phosphorus was not satisfactory.

The microbial communities investigated by FISH analysis were present in all samples, but their abundance varied. Substrate limitation due to reduced nutrient loading and the activity of denitrifying bacteria in the anaerobic reactor of the A/O bio-P process, affected the population of PAOs negatively. Results and observations from this study suggest that for the enrichment of PAOsin an A/O bio-P system it is crucial to have strictly anaerobic conditions in the reactor designated for this purpose and sufficient substrate available. When the abundance of PAOs increased, the phosphorus removal improved. A higher decay rate was observed for PAOsthan GAOs. It might be assumed that the high decay rate of PAOs was not caused by the substrate competition with GAOs, but rather with denitrifying bacteria.

In this study, the abundance of GAOs and AOB seemed solely limited by the available substrate being carbon and ammonium, respectively. The abundance of NOB and the total nitrifying community increased despite of periods with ammonium limitations. A live/dead analysis of the nitrifying biofilm suggested limited diffusion rates in the deeper layers of the biofilm, leading to cell decay. The entire microbial community investigated handled the reduction in the HRT well.

The microbial communities of the biomass were found to be more protected on the inside of a carrier, forming larger spherical clusters, than in the activated sludge.

The abundance of PAOs on a carrier was found to be superior of the amount of PAOs detected in the activated sludge surrounding the carrier in an IFAS system. A difference in the amount of PAOs and GAOs in the two reactors of the A/O bio-P process was also found.

Large amounts of filamentous bacteria were not observed in the A/O bio-P system despite the high sludge volume index.

Sammendrag

Høye konsentrasjoner av næringsstoffer som nitrogen, fosfor og organisk materiale kan føre til overgjødsling i innsjøer og vassdrag, fulgt av kraftig algeoppblomstring. I biologisk vannrensing fjerner mikroorganismer næringsstoffer fra avløpsvannet og sørger for at utslippsmengden av disse komponentene holder seg på lave nivåer.

En biofilm membran bioreaktor (BF-MBR) for fjerning av nitrogen, fosfor og organisk materiale fra kommunalt avløpsvann ble drevet i 85 dager. En evaluering av prosessen med fokus på biomassen i de forskjellige reaktorene ble gjort. Andelen av ammonium oksiderende bakterier (AOB), nitritt oksiderende bakterier (NOB), fosfor akkumulerende organismer (PAO) og glykogen akkumulerende organismer (GAO) ble undersøkt. Fluorescence *in situ* hybridization (FISH), Konfokal laser skanning mikroskop (KLSM) og programvaren *daime* ble brukt til kvantifisering av de ulike gruppene. Forandringer i det mikrobielle samfunnet ble så sammenlignet med ulike driftsbetingelser. Effekten av en lavere oppholdstid og uttynnet avløpsvann ble evaluert. Sammenhengen mellom filamentære bakterier og en høy slamvolumindeks ble studert. Andelen levende ogdøde bakterier i en biofilm ble også undersøkt.

Sluttproduktet fra renseprosessen var av god kvalitet med tanke på konsentrasjonene av nitrogen, organisk materiale og suspendert tørrstoff. Systemet håndterte bra en reduksjon i oppholdstiden. Fosfor-fjerningen var i denne studien ikke tilfredsstillende.

Bakteriegruppene som ble undersøkt ved hjelp av FISH ble påvist i alle prøver som ble analysert, men i varierende mengder. Substratbegrensning som følge av uttynnet avløpsvann og aktivitet av denitrifiserende bakterier i den vanligvis anaerobe reaktoren til biologisk fosforfjerning hadde en negativ innvirkning på PAOene i systemet. Resultater og observasjoner i denne studien tyder på at for å øke mengden PAO i systemet er det avgjørende at den anaerobe reaktoren holdes fullstendig anaerob, og at nok substrat er tilgjengelig for bakteriene. Da mengden av PAO økte i systemet forbedret fosforfjerningen seg. En høyere nedbrytningsrate ble observert for PAOene enn for GAOene. Grunnen til den høye nedbrytningsraten til PAO er antatt å være som følge av substratkonkurransemed denitrifiserende bakterier, og ikke GAOene.

I denne studien virket mengden av GAO og AOB kun avhengig av substrattilførselen som var henholdsvis organisk materiale og ammonium. Mengden av NOB og alle nitrifiserende bakterier (AOB og NOB tilsammen) økte til tross for perioder med ammoniumbegrensninger. En analyse av døde og levende bakterier i en biofilm viste en mulig begrensning i diffusjon av substrat og oksygen i de innerste lagene av biofilmen. Hele det mikrobielle samfunnet så ut til å håndtere reduksjonen i oppholdstiden bra.

Det mikrobielle samfunnet så ut til å være bedre beskyttet i en biofilm og dannet større og rundere kolonier her enn i slam.

En høyere andel av PAO ble funnet i en biofilm på et bæremedie, enn i det omkringliggende slammet i et IFAS system. En forskjell i mengdene av PAO og GAO i de to reaktorene for fosforfjerning ble også observert.

Det ble ikke funnet store mengder filamentære bakterier i slammet for fosforfjerning til tross for en høy slamvolumindeks.

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A FISH protocol **A1**

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Abbreviations

BF-MBR	Biofilm membrane bioreactor
FISH	Fluorescence *in situ* hybridization
CLSM	Confocal laser scanning microscopy
PAO	Phosphate accumulating organisms
GAO	Glycogen accumulating organisms
AOB	Ammonium oxidizing bacteria
NOB	Nitrite oxidizing bacteria
HRT	Hydraulic retention time
COD	Chemical oxygen demand
SCOD	Soluble chemical oxygen demand
TSS	Total suspended solids
A/O bio-P	Anaerobic/Aerobic biological phosphorus removal
AS-MBR	Activated sludge membrane bioreactor
MBBR	Moving bed biofilm reactor
SVI	Sludge volume index
VFA	Volatile fatty acid
PHA	Poly-hydroxy-alkanoate
PHB	Poly-hydroxy-butyrate
DO	Dissolved oxygen
IFAS	Fixed film activated sludge

Chapter 1

Introduction

1.1 Biological wastewater treatment

Wastewater is defined by Metcalf and Eddy (2003) as a combination of the liquid or water-carried wastes removed from residences, institutions, commercial and industrial establishments, together with such ground water, surface water, and storm water as may be present. The wastewater can contain pathogenic microorganisms that can cause diseases, as well as nutrients that can cause eutrophication in receiving water bodies. To protect the public health and the environment, the wastewater has to be subjected to treatment before being disposed into the environment.

Wastewater treatment can involve physical, chemical and biological processes. Compared to chemical treatment methods, biological nutrient removal uses less chemicals, produces less waste solids and has a lower energy consumption (Metcalf and Eddy, 2003). Biological nutrient removal is therefore a more desirable choice in wastewater treatment.

The main objectives of biological wastewater treatment are to oxidize soluble and particulate material, capture or incorporate suspended and non-settleable solids into flocs or biofilms, remove nutrients and in some cases remove trace organic constituents (Metcalf and Eddy, 2003).

There are two main types of biological treatment processes; suspended growth such as activated sludge, and attached growth such as biofilms in a MBBR system. Biological nutrient removal is a term applied to the re-

moval of phosphorus and nitrogen from the wastewater, but often includes the removal of carbonaceous organic matter.

The key to this process is the microorganisms responsible for the removal of carbon, nitrogen and phosphorus. Consequently it is important to have a fundamental understanding of the microbialcommunities present in the treatment systems and how they may respond to different operating conditions. Based on this knowledge the process can be optimized in order to produce a high quality effluent.

1.2 Phosphorus removal

Phosphorus (P) is an essential nutrient for all life forms, and also a limited resource on our planet. Untreated wastewater containing high amounts of phosphorus may lead to eutrophication of some freshwaters. Legislations have been implemented to avoid nutrient enrichment of water bodies, but phosphorus recovery from the wastewater is also being explored as an interesting option (Janssen et al., 2002).

In an activated sludge system, phosphorus can be found in three forms:

1. Organically bound phosphate. This form is important for the metabolism and growth of the microorganisms present in the sludge.
2. Polyphosphate which is formed and stored by the phosphate accumulating organisms.
3. Physically-chemically bound phosphate which can bind or precipitate with metal complexes.

The fraction of organically bound phosphate of the activated sludge in a wastewater treatment system with pre-settling is around 2%. The total P content in sludge of a domestic wastewater treatment plant is relatively low, around 3-5% (Janssen et al., 2002). The low content can be caused by several factors. Only a limited part of the organic matter in the influent can be used as a substrate for the bio-P process since the influent also contains inert, suspended matter not available for degradation. Other biological processes can also occur in the activated sludge, such as nitrification and denitrification, which can affect the uptake and release of phosphorus in the system.

To remove phosphorus from the wastewater, there are two techniques that are in use; chemical phosphorus removal and biological phosphorus removal. The biological phosphorus removal, hereafter referred to as the bio-P process, has been popular due to several factors. Recovery of the phosphorus is relatively easy due to concentrated levels in the activated sludge, and the effluent is lower in salinity. The bio-P process produces no chemical sludge and there is no deterioration of dewaterability of the excess sludge. The total nitrogen removal is less affected with a biological process, than with chemical removal and the quality of the sludge is better. Like every system, the bio-P process also has its drawbacks; the system performance depend on the wastewater composition and is therefore sensitive to changes such

as periods of heavy rainfall. Possible release of phosphorus during sludge treatment also has to be taken into consideration. Even though bio-P removal is one of the most complex challenges in the activated sludge process, this has not been an obstacle to its application in practice (Janssen et al., 2002).

1.2.1 The A/O bio-P process

The anaerobic/aerobic (A/O) bio-P process is based on some bacteria's capability to store soluble orthophosphate in the form of insoluble polyphosphate in their cells (Janssen et al., 2002). These bacteria are hereafter referred to as Phosphate Accumulating Organisms (PAOs). The process has two stages; one anaerobic and one aerobic stage. Figure 1.1 shows the schematic mechanism of the bio-P process.

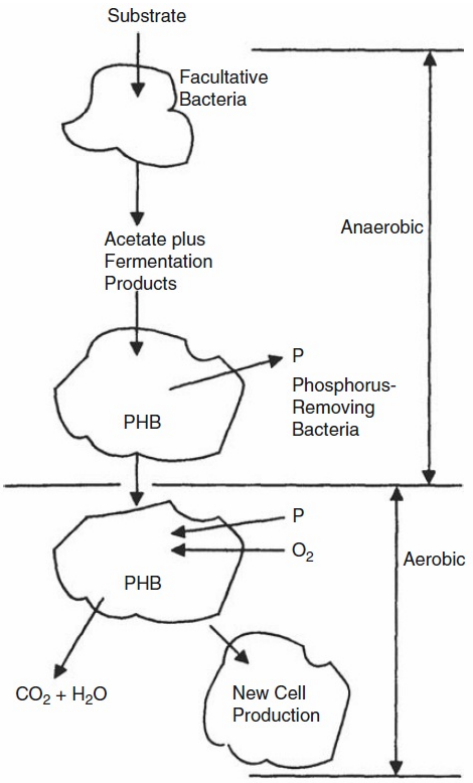


Figure 1.1 – Schematic mechanism of bio-P removal (Shammas and Wang, 2010).

Fermentation by facultative organisms in the anaerobic zone produces acetate, volatile fatty acids (VFAs) and other fermentation products from the influent substrate. The influent contains both soluble and particulate biochemical oxygen demand (BOD). The soluble portion is converted to fermentation products, while there is usually not enough time for the hydrolysis and conversion of the particulate BOD (Shammas and Wang, 2010). The fermentation products are then taken up by the PAOs and converted to a carbon-containing storage product, such as poly-hydroxy-butyrate (PHB). Splitting of the stored polyphosphate provides the energy needed for the substrate uptake (Janssen et al., 2002).This step therefore results in a release of orthophosphate in the bulk liquid, which is illustrated in Figure 1.2.

In the aerobic phase, the PHB is oxidized. The energy from this reaction is used by the PAOs to take up orthophosphate and store it as polyphosphate inside their cells. This step also contributes to cell growth. The stored polyphosphate is considered a energy or phosphorus backup, which can give these organisms an advantage over other bacteria in the activated sludge system (Janssen et al., 2002).

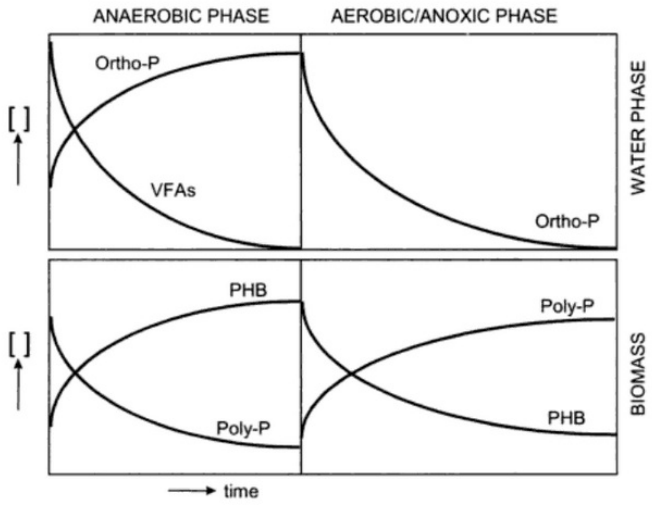


Figure 1.2 – The principle of the bio-P process (Janssen et al., 2002).

Removal of phosphorus from the system is achieved by taking out surplus sludge from the aerobic part of the system containing PAOs that have accumulated polyphosphate in their cells.

1.2.2 The microbiology of the A/O bio-P process

PAOs

The physiology of the bacteria involved in the bio-P process is complex. The bacteria have to be able to form and degrade polymers such as polyphosphate, and carbon compounds such as glycogen. According to available literature, there have been identified two types of bacteria able to take up larger amounts of phosphate; The Poly-P organisms and the Phosphate Accumulating Organisms (PAOs). Poly-P organisms such as *Acinetobacter* and *Microthrix parvicella* store phosphate in the form of Poly-P only for maintenance purposes. Influenced by the substrate storage in the anaerobic zone, the PAOs such as *Candidatus Accumulibacter phosphatis* (henceforth referred to as *Accumulibacter*) are capable of storing poly-P in the aerobic zone. These bacteria are considered the"real" phosphate bacteria contributing to the phosphorus uptake and removal from the wastewater (Janssen et al., 2002).

Due to their polyphosphate reserves both Poly-P organisms and PAOs can survive the anaerobic period. Because of their ability to take up substrate with the energy from the polyphosphate, PAOs will have an advantage in the anaerobic zone and are therefore considered to be in a dominant position compared to the Poly-P organisms (Janssen et al., 2002).

Accumulibacter is in the family of *Rhodocyclaceae* of subclass 2 of the *Betaproteobacteria* and has never been grown in a pure culture. *Accumulibacter* is Gram negative and the cells have a typical coccobacillus shape. In some bio-P communities they can be found as single cells, but they are almost always observed growing in microcolonies (Nielsen et al., 2009).

GAOs

A group of bacteria capable of competing with the PAOs for substrate in the anaerobic zone of the bio-P process is the Glycogen-accumulating organisms (GAOs) (Oehmen et al., 2005). Similar to the PAOs, the GAOs are able to convert VFAs to Polyhydroxy-alkanoates (PHAs) anaerobically. The most important difference is the GAOs preference for glycogen as the

sole or primary source of energy. Under anaerobic conditions, the GAOs degrade the stored glycogen via glycolysis to gain the energy needed for VFA uptake and the synthesis of PHAs. During the aerobic period, GAOs oxidize the PHAs to provide energy for glycogen uptake and cell growth (Zhou et al., 2008).

GAOs do not take up or release phosphorus and hence do not contribute to P-removal (Oehmen et al., 2005). *Candidatus Competibacter phosphatis* (henceforth referred to as *Competibacter*) has been identified as a GAO phenotype, and has been found both in lab-scale reactors fed with acetate and in full-scale wastewater treatment plants (López-Vázquez et al., 2008; Crocetti et al., 2002; Kong et al., 2002).

Competibacter belong within the *Gammaproteobacteria* and grow normally as coccobacilli or rods. When observed in the microscope, they tend to be relatively large and oval. Clusters of tetrads can be formed, but microcolonies can also appear (Nielsen et al., 2009).

The competition between PAOs and GAOs

Since the PAOs and GAOs are competing for the same substrate, and the GAOs are not contributing to P-removal, it is desirable to minimize the amount of GAOs in the system. This has been proven challenging (Lu et al., 2006), but some operational parameters can be controlled to favor the growth of PAOs over GAOs. Oehmen et al. (2006) found that *Competibacter* can take up propionate less efficient than acetate. They also found that under anaerobic conditions, *Accumulibacter* could take up both acetate and propionate. The carbon source is thus one parameter that can be used to control the population of PAOs and GAOs.

pH has also been found to affect the growth and activity of these two bacteria and a high pH has been proven to favor PAOs over GAOs (Oehmen et al., 2005).

Panswad et al. (2003) investigated the response of the microbial population to gradual temperature change in an enhanced biological phosphorus removal system. They found that PAOs are lower-range mesophiles or possibly psychrophiles, dominating the microbial community of the process at 20°C or lower. At an optimum temperature between 25°C and 32.5°C, the

GAOs were found to be mid-range mesophilic organisms.

Filamentous bacteria

Filamentous bacteria are present in all wastewater treatment plants. Their abundance are normally around 1-3 %, and are typically dominated by one or two filamentous morphotypes. In some cases they may proliferate to such an extent that they affect the system performance causing bulking or foaming in the treatment plant (Nielsen et al., 2009).

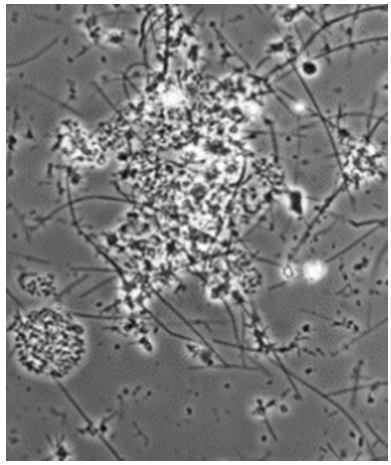


Figure 1.3 – The floc structure including filamentous bacteria in activated sludge (Witzig et al., 2002).

1.2.3 Parameters affecting biological phosphorus removal

Wastewater composition

Janssen et al. (2002) states that the composition of the influent wastewater is a crucial factor, and determines the operation and efficiency of the bio-P process. Janssen et al. (2002) also claims that 20 mg chemical oxygen demand (COD) is required to remove 1 mg P in this process. The COD refers to the fraction of readily and slowly biodegradable COD in the wastewater. The organic load is therefore an important factor for a stable operation of the system.

The load of phosphorus in the influent may also have an effect on the system. Sudiana et al. (1999) suggested that limited phosphorus loadings may favor the growth of GAOs, and at the same time suppress the development of PAOs. However, under rich phosphorus loadings the PAOs will be dominant.

Mulkerrins et al. (2004) recommend that the influent wastewater should be kept as constant as possible. Minimal fluctuations in the composition or sudden changes to the system should be avoided. Changes in the loading rate or other changes on the system should be done in small steps, increasing or decreasing over longer periods to avoid an upset in the system balance. The biological phosphorus removal (BPR) process is highly sensitive to disturbances, such as dilution of the influent due to heavy rainfall *etc.*, and prolonged disturbances can lead to a recovery time up to four weeks.

Temperature

The process temperature is an important factor in terms of the biological reaction rate constants and the efficiency of the process. The temperature not only has an effect on the settling characteristics of the biological solids, but also the metabolic activities of the microbial population such as the fraction of PAOs in the sludge (Metcalf and Eddy, 2003).

An increase in the process temperature generally gives an increase in the P-release and P-uptake rates of PAOs. The rates of nitrification, denitrification and acidification also increase due to a rise in the temperature (Janssen et al., 2002). It has been reported that temperatures below the optimum temperature have a lager impact on the growth rate than temperatures above the optimum. It has been observed that a 10°C increase corresponds

to a doubling of the growth rate until the optimum rate is reached (Metcalf and Eddy, 2003).

pH

The pH in the activated sludge can affect the bio-P process, and is especially crucial in the anaerobic stage (Janssen et al., 2002). The pH in the system will influence the transport of acetate into the cell, as well as the P-release and P-uptake. At a low pH, more acetate is needed per amount of released phosphorus. The rate of P-release and P-uptake will therefore decrease since the energy in the polyphosphate will be used for the transport of acetate through the cell membrane, and not the conversion of acetate to PHB (Janssen et al., 2002).

Janssen et al. (2002) also points out that the physical-chemical bounding of phosphate in the activated sludge can be affected by the pH. Higher pH values (>7.5) can result in the precipitation of metal-phosphates contributing to the total phosphorus removal. Stimulation of this spontaneous precipitation can be achieved in a bio-P process because of the higher cations and orthophosphate concentrations in the anaerobic phase.

High pH values can thus improve the phosphorus removal in two ways: by increasing the uptake of polyphosphate and by increasing the chemical precipitation. An optimum pH of 6.8 ± 0.7 for the anaerobic acetate metabolism was proposed by Liu et al. (1996).

Sludge "bulking" conditions in biological nutrient removal systems may also be caused by fluctuations in the pH (Metcalf and Eddy, 2003).

Oxygen

The level of dissolved oxygen in the system can affect the process both positively and negatively. The PAOs need oxygen to store the phosphate biologically in the aerobic zone of an activated sludge process. However, if the sludge return to the anaerobic stage contains high levels of dissolved oxygen, this can disturb the process. For each mg of oxygen, 2 mg of COD is oxidized (Janssen et al., 2002). This leaves less COD available for the PAOs, which will again affect the uptake of phosphorus in the aerobic zone.

An oxygen concentration between 3.0 and 4.0 mg/l in the aerobic zone has been recommended by Shehab et al. (1996). Concentrations of dissolved oxygen above 4 mg/l have been reported not to further stimulate the process, and are therefore considered a waste of energy for aeration purposes. Brdjanovic et al. (1998) reported that a negative effect on the bio-P process could be observed when the process was over-aerated. This could be linked to the decreasing rate of P-uptake due to depletion of PHB.

Nitrate

It has been reported that the presence of nitrate in the anaerobic zone can cause disturbances in the bio-P process (Shehab et al., 1996). Nitrate entering the anaerobic zone via the influent or recycling streams can cause anoxic conditions and favor the activity of denitrifying bacteria. The denitrifying bacteria utilize the substrate more efficiently than the PAOs, and will therefore compete more successfully for the available COD (Janssen et al., 2002).

During periods with low loadings caused by events such as heavy rainfall, snow melting or low wastewater influent during weekends, higher nitrate concentrations can be found due to over-aeration in the aerobic tank (Janssen et al., 2002).

Nitrous oxide (NO) is an intermediate formed during denitrification. NO can have a toxic effect on the PAOs. However, research has shown that the concentration of NO necessary to inhibit P-release is higher than the concentration normally recorded under practical conditions (Janssen et al., 2002).

1.3 Nitrogen removal

Nitrogen removal from wastewater is usually achieved by nitrification and denitrification. There are several set-ups available for nitrogen removal, two of them being post-denitrification and pre-denitrification. Both configurations are illustrated in Figure 1.4.

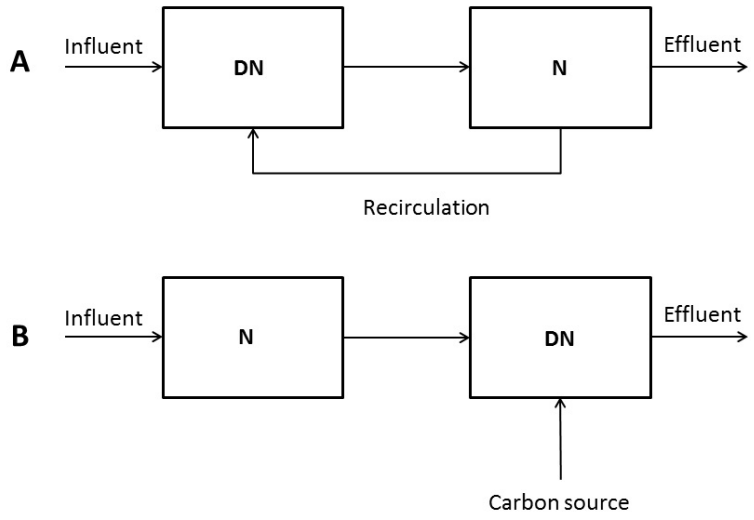


Figure 1.4 – Schematic set-up of a pre-denitrification (A) and post-denitrification (B) system. DN is the abbreviation for denitrification and N for nitrification.

In a pre-denitrification configuration the organic matter, which enters the denitrification reactor with the incoming wastewater, is utilized as a carbon source by the denitrifying bacteria. They use nitrate, which is recirculated from the nitrification reactor, as an electron acceptor. The lithoautrotrophic nitrifying bacteria use CO_2 as a carbon source, and O_2 as the terminal electron acceptor. Ideally, this system will not need any additional compounds other than the wastewater itself. One draw-back of this system is that no complete nitrogen removal can be achieved since only a part of the flow is recycled, and the rest of the effluent is discarded.

In a post-denitrification configuration the reactors switch places and nitrate is produced in the first step by the nitrifying bacteria. The nitrate then passes to the denitrification reactor where it serves as a electron ac-

ceptor for the denitrification process. An external carbon source has to be added to the denitrification reactor in order to achieve a complete nitrogen removal. The main draw-back of this configuration is the expense of adding an external carbon source.

The main difference between these two configurations is the order of the reactors for nitrification and denitrification, the origin of the carbon source and whether or not a complete nitrogen removal can be achieved.

1.3.1 Nitrification

In municipal wastewater, the nitrogen is mainly present in the form of ammonium (NH_4^+) and is organically bound. Most of the organic nitrogen in an ordinary biological treatment plant is converted to ammonium. In the two step nitrification process, the ammonium is first oxidized to nitrite (NO_2^-) which then is oxidized to nitrate (NO_3^-) (Ødegaard, 1993).

The first step of the process is where the ammonium oxidizing bacteria (AOB) such as *Nitrosomonas* is responsible for oxidizing the ammonium to nitrite, as shown in Equation (1.1).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \tag{1.1}$$

Under normal conditions, the first step is the velocity-limiting step.

The second step is where the nitrite oxidizing bacteria such as*Nitrobacter* oxidize the nitrite to nitrate, as shown in Equation (1.2).

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \tag{1.2}$$

The total overall process can be described with Equation (1.3):

$$NH_4^+ + 2.0O_2 \rightarrow NO_3^- + H_2O + 2H^+ \tag{1.3}$$

The nitrification process has a mesophilic temperature optimum at 30-37°C, and the concentration of dissolved oxygen in the bulk liquid should stay above 2 mg/l. The pH should be slightly alkalic and between 7.5 and 8.5 (Østgaard, 1995). Other limitations may also be the actual fraction of nitrifyers in the biomass, sludge age and toxic sensitivity (Østgaard, 1995).

The nitrifying bacteria

Research has shown that two of the bacteria responsible for the nitrification process belong to two separate linages within the *Proteobacteria* (Peng and Zhu, 2006). The two main groups to be assumed involved are *Nitrosomonas* (*Beta Proteobacteria*) and *Nitrobacter* (*Alpha Proteobacteria*), both of which are chemolithoautotrophic. Figure 1.5 shows the phylogenetic relationship between these two bacteria (Bock et al., 1992). The bacteria indicated in green belong to the AOB, while the red is NOB.

During the nitrification process, the AOB and NOB co-exist and benefit from their close physical association. One reason is the energetic demands of the process. The NOB are able to efficiently use the nitrite that is produced by the AOB, helping to cope with the poor energy yield of nitrite oxidation. At the same time this is beneficial for the AOB because the NOB relieve them from the toxic nitrite which can accumulate and form toxic by-products such as NO. NO can again interact with the bacterial enzymes (Peng and Zhu, 2006).

Compared to the heterotrophic bacteria, the yield and specific growth rate for the nitrifiers are low (Ødegaard, 1993). Henze et al. (2002)summarized the reaction rate constants for the nitrifying bacteria as shown in Table 1.1:

Table 1.1 – Reaction rate constants for nitrification at 20°C (Henze et al., 2002)

Parameter	Unit	AOB	NOB	Total process
Maximum specific growth rate	d^{-1}	0.6-0.8	0.6-1.0	0.6-0.8
Saturation constant	g NH_4-N/m^3	0.3-0.7	0.8-1.2	0.3-0.7
Saturation constant	g O_2/m^3	0.5-1.0	0.5-1.5	0.5-1.0
Maximum yield constant	g VSS/m^3	0.10-0.12	0.05-0.07	0.15-0.20
Decay constant	d^{-1}	0.03-0.06	0.03-0.06	0.03-0.06

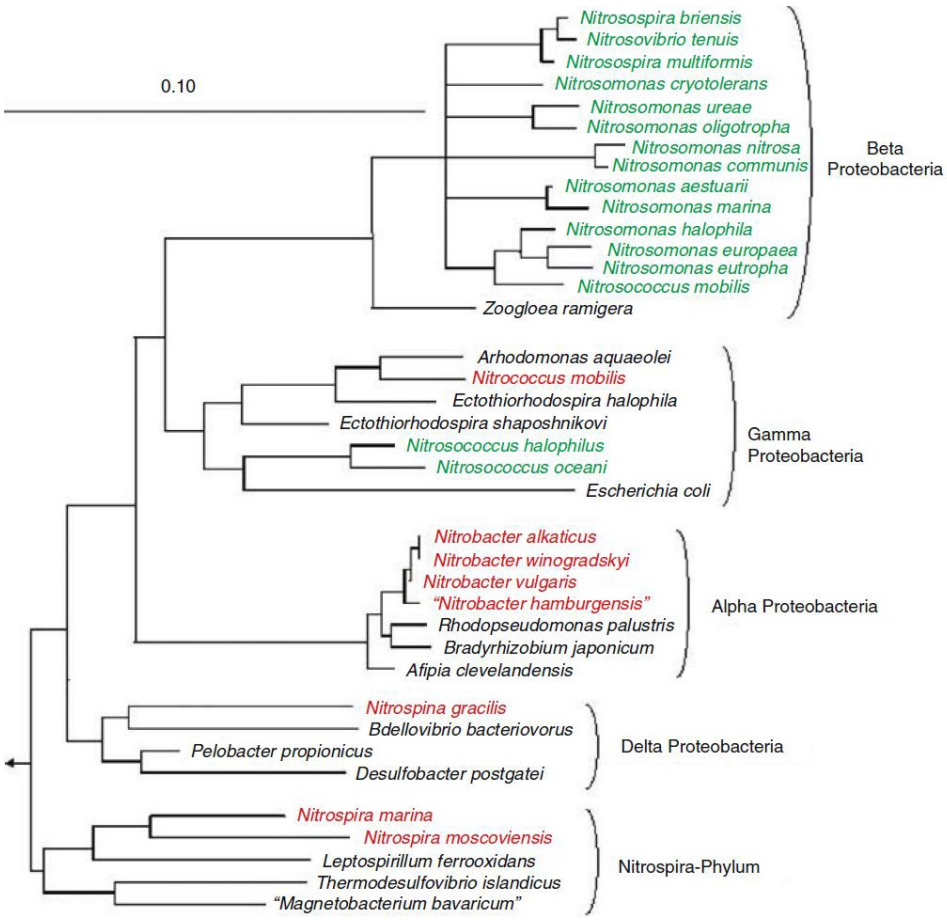


Figure 1.5 – Phylogenetic tree based on the 16S rRNA, reflecting the relationship of AOB (green) and NOB (red). The bar represents a 10 % estimated sequence divergence (Bock et al., 1992).

Under normal conditions, the velocity limiting step is the reaction of ammonium oxidation to nitrite. Nitrite is normally rapidly oxidized to nitrate (Peng and Zhu, 2006). However, when considering the difference in the oxygen saturation constant, the NOB are more sensitive towards low concentrations of dissolved oxygen (DO) than AOB. As a result, nitrite accumulation will occur when the oxidation of ammonium exceeds the velocity of nitrite oxidation, meaning the AOB work faster than the NOB (Hauser, 2011).

Figure 1.6 shows the spacial distribution, the concentration profiles and the activity zones in a biofilm studied by Okabe et al. (2004). They measured the steady-state concentration profiles of O_2, NH_4^+, NO_2^- , and NO_3^- in the oxic biofilm strata. Their research showed that the active NH_4^+-oxidizing zone is located in the outer part of the oxic biofilm, whereas the active NO_2^--oxidizing zone is located just below the NH_4^+-oxidizing zone. Since communities of NOB were found in the deeper layers of the oxic biofilm, oxygen diffusion becomes a more limiting factor than for the AOB which had a more even distribution in the outer layers of the biofilm.

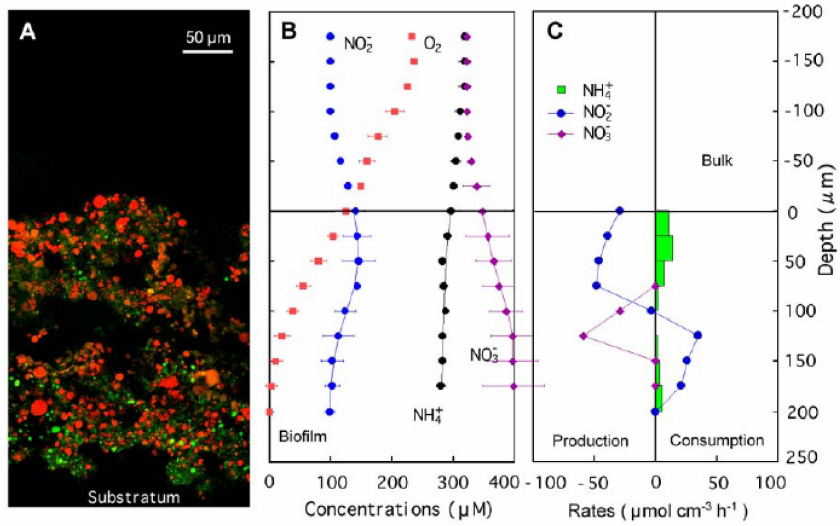


Figure 1.6 – Fluorescence *in situ* hybridization result combined with microsensor measurements. *In situ* hybridization of a thin vertical biofilm section with labeled probes specific for AOB of the betasubclass of the *Proteobacteria* (red stain clusters) and specific for *Nitrospira moscoviensis* and some environmental clones (green stain clusters) (A). Corresponding steady-state microprofiles in the autotrophic nitrifying biofilm (B). The distribution and magnitude of the estimated specific rates of net consumption and production (C). The solid lines are the best fits from the model to calculate the specific consumption and production rates of NH_4^+, NO_2^-, and NO_3^-. The biofilm surface was at a depth of zero (Okabe et al., 2004).

Effect of environmental factors

The bacterial growth rate and the nitrification rate are influenced by several environmental factors. pH, alkalinity, oxygen concentration, temperature, ammonium concentration, the concentration of organic matter, suspended solids and inhibitory factors all influence the nitrification process. Some of these factors may have a direct effect on the system, whereas others may have an indirect effect through biofilm structure, diffusion rates *etc.*

Oxygen and organic matter

Availability of oxygen, ammonium and organic matter are closely related factors of influence since the activity of a nitrification reactor often is determined by the competition between the heterotrophic and autotrophic nitrifiers. Heterotrophic bacteria consume oxygen and form a layer on the outer parts of the biofilms. The thickness of this layer depends on the amount of organic matter available, as well as the oxygen concentration. To achieve an efficient nitrification, oxygen and ammonium have to be able to diffuse through this layer of heterotrophic bacteria. Therefore, the nitrifying bacteria will not start to grow before the concentration of organic matter is reduced to a certain level in the biofilm. Since the heterotrophs will consume most of the oxygen in the system, the oxygen concentration will become the limiting factor since ammonium will normally manage to penetrate the total depth of the biofilm (Ødegaard, 1993).

pH and substrate inhibition

The optimum pH of nitrification is between 7.5 and 8. During the nitrification process acid is produced which can cause depletion of the alkalinity, resulting in reduced pH of the system and inhibition of the nitrifiers. An unstable pH may also cause substrate inhibition. Among the most relevant substrate inhibitions are the occurrence of ammonia (NH_3) and nitrous acid (HNO_2). Ammonia is known to inhibit both *Nitrosomonas* and *Nitrobacter*, while nitrous acid may inhibit the *Nitrobacter* (Ødegaard, 1993). The dissociation equilibra of $NH_3 \leftrightarrow NH_4^+$ ($pK_a = 9.3$) and $HNO_2 \leftrightarrow NO_2^-$ ($pK_a = 3.4$) are pH dependent and therefore also affect the nitrification activity (Hauser, 2011). The relationship between ammonia and nitrous acid inhibition of nitrifying bacteria is shown in Figure 1.7.

Temperature

The temperature can strongly influence the rate of nitrification, and affect

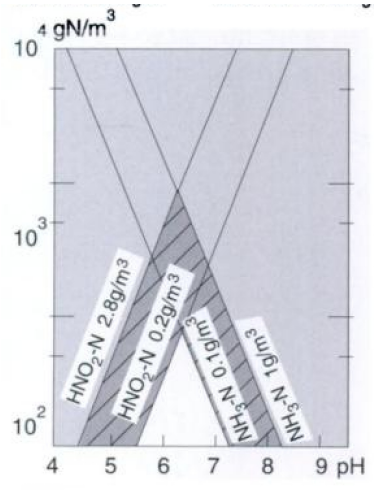


Figure 1.7 – The inhibition effect on the nitrification process as function of NH_3, HNO_2 and pH. Total inhibition is represented by grey areas, while the dashed area marks partial inhibition (Henze et al., 2002).

the enzyme activity, the diffusion rate of the substrate and the solubility of oxygen. The temperature optimum is approximately 30-37°C, but it has been demonstrated that nitrification in activated sludge systems can occur at temperatures below 5°C (Ødegaard, 1993; Østgaard, 1995).

Other factors

Other factors that may influence the nitrification activity are phosphorus limitation, suspended solids and some organic substances like sulfur, aniline, phenol and cyanide. Inhibitory compounds will individually affect the nitrifiers, but if the nitrifiers are simultaneously exposed this may lead to a stronger inhibition due to a synergetic effect (Ødegaard, 1993).

1.3.2 Denitrification

Denitrification is the process where nitrite and nitrate are reduced to gaseous nitrogen, mainly N_2, but N_2O may also be present. The denitrifying bacteria are often aerobic, but in lack of oxygen they can use nitrate as their electron acceptor and the denitrification process is therefore characterized as an anoxic process. Denitrifying bacteria are heterotrophic and can use organic compounds as carbon and energy source (Ødegaard, 1993).

A simplified description of the process is shown in Equation (1.4). OC stands for organic compound, and is displayed in its reduced and oxidized state.

$$OC_{reduced} + NO_3^- \rightarrow OC_{oxidized} + N_2 + CO_2 + H_2O \tag{1.4}$$

Heterotrophic bacteria use several enzymatic reactions in order to reduce NO_3^- to N_2 as shown in Equation (1.5).

$$NO_3^- \xrightarrow{a} NO_2^- \xrightarrow{b} NO \xrightarrow{c} N_2O \xrightarrow{d} N_2 \tag{1.5}$$

The enzymes for the sequential denitrification process are (a) Nitrate reductase, (b) Nitrite reductase, (c) NO reductase and (d) N_2O reductase (Hauser, 2011). All intermediate compounds are toxic, and should be avoided. Nitrite reductase is the key enzyme in the denitrification process, catalyzing the first step that leads to a gaseous intermediate. To achieve complete denitrification, all enzymes need to be present and the four modules have to be expressed at the same time, as illustrated in Figure 1.8.

The denitrifying bacteria

Most denitrifyers are aerobic organisms that oxidise a carbon source to an N oxide under anoxic conditions. Autotrophic denitrifyers utilize inorganic sulfur compounds, hydrogen, ammonia or nitrite (Zumft, 1997). The denitrifyers are hard to identify as a phylogenetic subclass since there are many facultative aerobic heterotrophs that may switch to nitrate in absence of oxygen (Østgaard, 1995). Their distribution does not follow a distinct pattern, but the reaction is carried out by a diversity of bacteria belonging taxonomically to various subclasses of the *Proteobacteria*. Denitrification

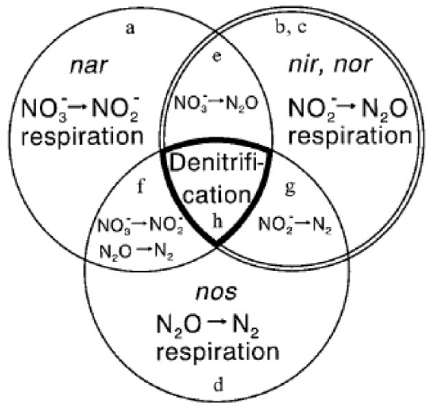


Figure 1.8 – Modular organization of denitrification. Four modules representing the respiratory systems utilizing nitrate (a), nitrite (b), NO (c) and N_2O (d) constitute the overall process. Complete denitrification (h) is achieved only when all four modules are activated. Pairwise overlaps (e to g) of the individual respiratory modules occur naturally in denitrifying or other N oxide-utilizing bacteria (Zumft, 1997).

also extends tothe archaea, where it is found among the halophilic and hyperthermophilic branches of this kingdom and may have evolutionary significance (Zumft, 1997).

The heterotrophic denitrifying bacteria have a significant higher yield and specific growth rate than autotrophic nitrifying bacteria (Ødegaard, 1993). Henze et al. (2002) summarized the reaction rate constants for the denitrifying bacteria as shown in Table 1.2.

Table 1.2 – Reaction rate constants for denitrification at 20°C (Henze et al., 2002).

Parameter	Unit	Denitrification
Maximum specific growth rate	d^{-1}	3-6
Half-Saturation constant	g NO_3-N/m^3	0.2-0.5
Half-Saturation constant	g O_2/m^3	0.1-0.5
Half-Saturation constant	g COD/m^3	10-20
Maximum yield constant	g COD/g COD	0.4-0.6
Maximum yield constant	g COD/g NO_3-N	1.6-1.8
Decay constant	d^{-1}	0.05-0.10

Effect of environmental factors

According to Ødegaard (1993), the factors that influence the denitrification rate the most are the concentration of organic matter, oxygen concentration, pH, alkalinity and temperature.

Organic matter
The most important factor in regulating the denitrification rate and the denitrifying capacity are probably the availability of organic matter. The denitrifyers can utilize a wide range of organic compounds, although low molecular weight and readily biodegradable substances are favored. Commonly used carbon sources are methanol, ethanol, acetic acid or wastewater.

Oxygen
The denitrification process is inhibited if oxygen is present. The denitrifyers will use oxygen as the electron acceptor instead of nitrate because this gives a higher energy yield. However, the activity is only temporarily inhibited while the oxygen is consumed. The presence of oxygen will lead to consumption of organic matter without obtaining denitrification in the outer layers of the biofilms. This aerobic layer may also prevent the nitrate diffusion into deeper layers. Therefore, the presence of oxygen should be kept at a minimum (Ødegaard, 1993).

Temperature
The denitrification process is less temperature sensitive than the nitrification process. This could be the result of the diverse group of denitrifying bacteria, and their high growth rate (Ødegaard, 1993).

pH
Denitrification also produces alkalinity (0.07 mequiv. per NO_3-N reduced to nitrogen gas). Depending on the diffusion efficiency and the biofilm thickness the pH inside the biofilm may be higher than in the bulk water phase. The pH also influences the formation of the end products (N_2 and N_2O). Low pH values favor N_2O production, while higher pH favor N_2 production and hence complete denitrification. The formation of N_2O should be avoided since this gas has a negative impact on the ozon layer, and is considered as one of the greenhouse gases that should be emitted to the air only in minimal amounts.

1.4 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) has become a popular technique for the visualization, identification, enumeration and localization of microbial cells (Moter and Göbel, 2000). FISH is based on the principle of hybridizing fluorescently labeled DNA Oligonucleotides (hereafter referred to as probes) to the ribosomal rRNA in the cell. The subunits 16S and 23S are typically used when identifying *Bacteria* (Nielsen et al., 2009). The 16S rRNA is the most commonly used gene because of its genetic stability, high copy number and its metabolically active cell. Even with monolabeled probes, single cells can be visualized (Moter and Göbel, 2000).

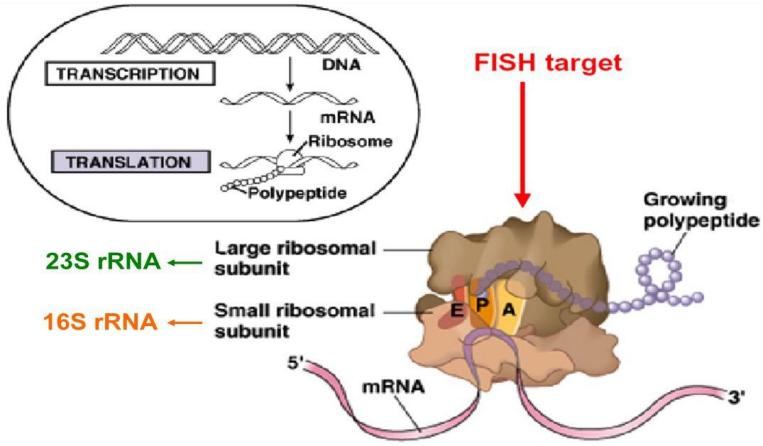


Figure 1.9 – The probe target on the ribosome (Rogne, 2010).

The probes are designed to bind to their complementary target sequence on the rRNA structure of the target cell. The probe is made on an automated synthesizer and is a short piece of DNA with a typical length of 15-30 base pairs (Moter and Göbel, 2000). A fluorescent dye molecule is either chemically or enzymatically bound to the probe. During synthesis the dye molecule can be chemically bound at the 5'-end of the probe through an aminolinker. Terminal transferase can be used to attach the dye enzymatically at the 3'-end of the nucleotide sequence (Moter and Göbel, 2000).

The probes can also be labeled indirectly by using digoxygenin (DIG), horseradish peroxidase (HRP), Tyramide Signal Amplification (TSA) or polyribonucleotide probes labeled with several fluorochrome molecules, de-

tailed by Zarda et al. (1991), Schönhuber et al. (1997) and DeLong et al. (1999).

To observe two or more microorganisms at the same time, fluorochromes with different excitation and emission maxima can be used. There are many fluorochromes available. Cy3 and Cy5 give a stronger signal than classical dyes, and are very stable to photobleaching (Moter and Göbel, 2000). Details of their wavelength and color are given in Table 1.3.

Table 1.3 – Details of the color, excitation and emission wavelength of the fluorochromes Cy3 and Cy5 (Moter and Göbel, 2000).

Fluorochrome	**Wavelength**		**Color**
	Excitation (nm)	**Emission (nm)**	
Cy3	550	570	Orange/red
Cy5	651	674	Infrared

Fixation of the samples is necessary prior to analyzing by FISH. The fixation enables the fluorescent probes to penetrate into the cells and stops the degradation of the RNA by endogenous RNAses. The composition of the fixative depends on the target cells. Most Gram-negative cells have to be fixated by a 3-4% formaldehyde or paraformaldehyde solution, while for Gram-positive cells ethanol can be used. An optimal fixation should maintain the cell integrity and morphologic details, as well as a good probe penetration and a high retention level of the target RNA (Moter and Göbel, 2000).

FISH has become a popular technique for monitoring the abundances and population dynamics of selected microbes in wastewater treatment plants. FISH can be used as a tool to understand the correlations between problems such as sludge bulking and the microbial community composition. The method is a rapid and cost effective and less prone to biases than some PCR approaches which can be sensitive to methodical errors and contaminants from the working environment (Nielsen et al., 2009).

The procedure for FISH analysis is summarized by Schramm and Amann (2008) in Figure 1.10.

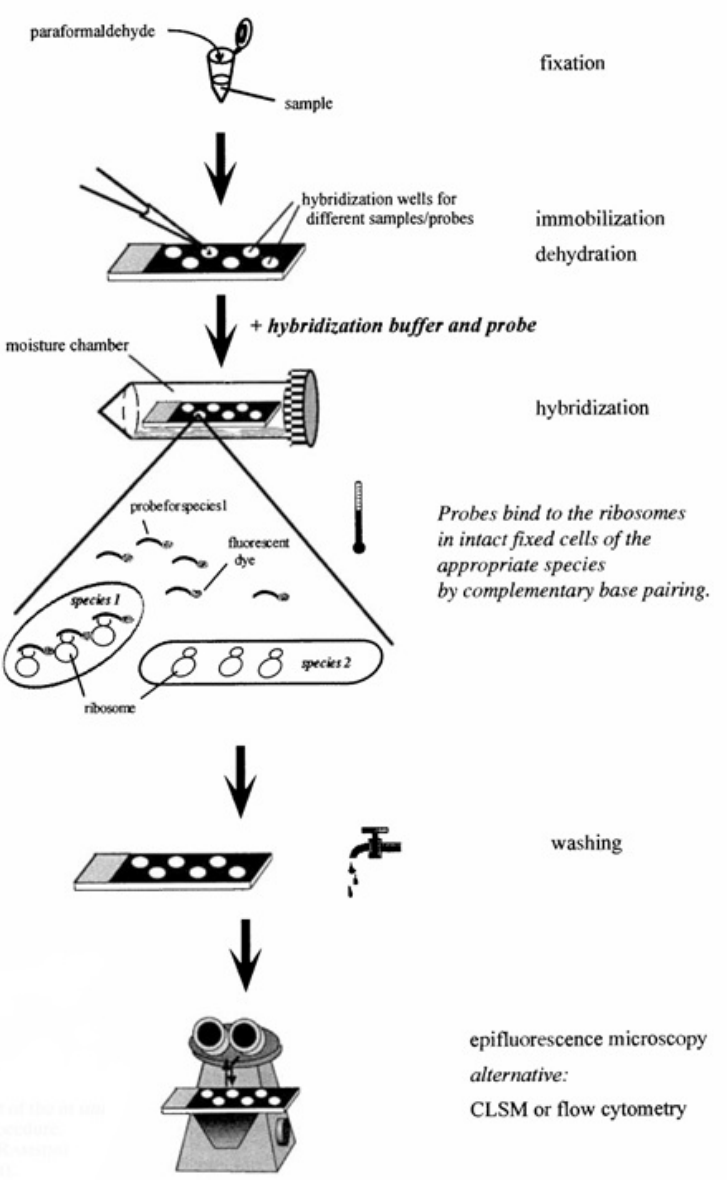


Figure 1.10 – Illustration of the FISH protocol (Schramm and Amann, 2008).

1.5 Reactor systems

1.5.1 Activated sludge membrane bioreactor (AS-MBR)

The separation of liquid and solids in biological wastewater treatment is challenging, and the increasing demand of high-quality effluent has promoted the use of membrane bioreactors (MBR). The MBR technology can be used in domestic and industrial wastewater treatment, and can remove organic and inorganic contaminants in addition to biological material (Naghizadeh et al., 2011). The use of a MBR system will replace the secondary clarifier normally used for the liquid/solids separation in treatment plants. The membrane will retain all sludge in the bioreactor which will lead to a more concentrated active biomass in the reactor.

The MBR process can be operated in two different configurations. The most common configuration is a submerged MBR where the membrane is immersed in the sludge in the bioreactor, as illustrated in Figure 1.11. In this configuration the permeate is extracted from the membrane by vacuum-driven filtration.

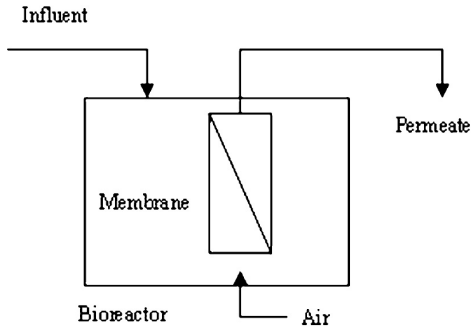


Figure 1.11 – Submerged MBR with internal vacuum-driven membrane filtration (Radjenović et al., 2008).

The other option is to have the membrane installed in a separate compartment downstream of the bioreactor, as illustrated in Figure 1.12. The filtration is then pressure-driven with a higher energy demand than a submerged membrane. This configuration is also more exposed to membrane fouling since the pressure can break up microbial flocs releasing foulant material (Radjenović et al., 2008).

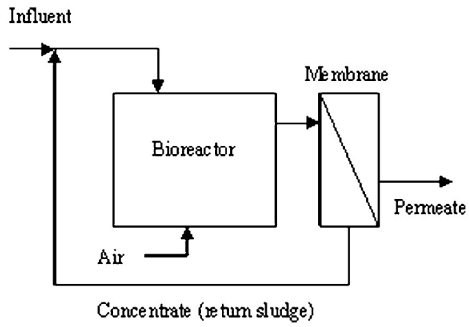


Figure 1.12 – Side-stream MBR with external pressure-driven membrane filtration (Radjenović et al., 2008).

The main advantages of a MBR system is the high quality of the effluent, the small footprint, a robust and reliable operation and the reduction of the disinfection requirements downstream (Naghizadeh et al., 2011). The main drawback is the need for membrane replacement or cleaning with or without chemicals.

1.5.2 Moving Bed Biofilm Reactor (MBBR)

Moving Bed Biofilm Reactors (MBBR) consist of small plastic carriers suspended within a liquid phase reactor, where biofilms are growing on the inner surfaces of the carriers (Gapes and Keller, 2009). MBBR are often favored because they are compact and volume-efficient and can tolerate high particulate loadings without having problems with clogging of the system (Ivanovic and Leiknes, 2012). It is also easier to control the biomass in the reactor in terms of a higher selectivity of the microorganisms in the biofilm attached to a carrier.

The biofilm is formed mainly on the protected surface, on the inside of the carriers. However, the total surface area is significantly larger than the effective biofilm surface area. According to available literature, it is recommended that the filling fraction of carriers in the reactor should not exceed 70% (Rusten et al., 2006), so that the carriers are able to move freely in the suspension. The carriers are kept within the reactor by a sieve or grill. This allows a simple separation of the treated water from the biomass-containing carriers. Excess biomass is sloughed off the biofilm, and leaves the reactor with the effluent (Gapes and Keller, 2009).

The movement of the carriers in an aerobic reactor is ensured by coarse bubble aeration, and by mechanical mixing for anaerobic or anoxic reactors (Ivanovic and Leiknes, 2012).

Drawbacks of the MBBR are the high cost of carriers, poor settleability of the particles in the effluent and the use of sieves to keep the carriers inside the reactor (Ivanovic and Leiknes, 2012).

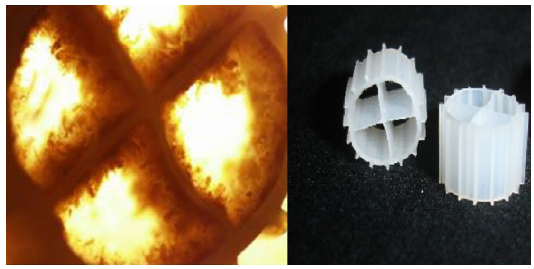


Figure 1.13 – Kaldnes K1 carriers with (left) and without (right) biofilm (Gapes and Keller, 2009)

The biofilm

A biofilm is a complex structure of microorganisms adhering to a surface such as a plastic carrier. In wastewater treatment the composition of such a biofilm will depend on the supply of biomass (Gapes and Keller, 2009). The biofilm develops a self-made polymeric matrix which gives the microorganisms attached better protection against environmental changes. Physical stress and improved mass transfer in terms of metabolic interactions between the populations present on the carriers are two factors that this matrix will protect the biofilm against. As a consequence of oxygen limitation in the inner depths of the biofilm, the thickness of a nitrifying biofilm is typically less than 100µm (Ødegaard, 1993).

Figure 1.14 shows the life cycle of a biofilm. The cycle may be divided into three phases: attachment, growth and dispersal. During the first phase, the free-floating bacteria are attached to a surface such as a carrier in a MBBR.

In the growth phase, the biofilm develop a complex three-dimensional structure due to bacterial growth. The structure is influenced by environmental factors.

In the last phase, the biofilm propagates through the detachment of clumps of cells, which also allows individual cells to detach. This enables the bacteria to reattach to another surface downstream of the original bacterial community (Rogne, 2010).

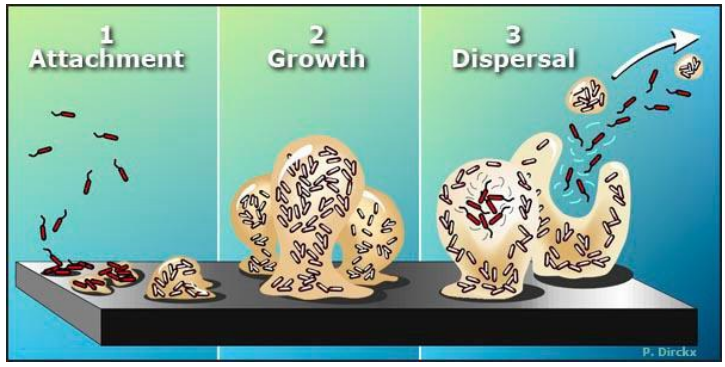


Figure 1.14 – The biofilm life cycle; 1) Attachment, 2) Growth, 3) Dispersal (Rogne, 2010)

1.5.3 The NTNU biofilm membrane bioreactor (BF-MBR)

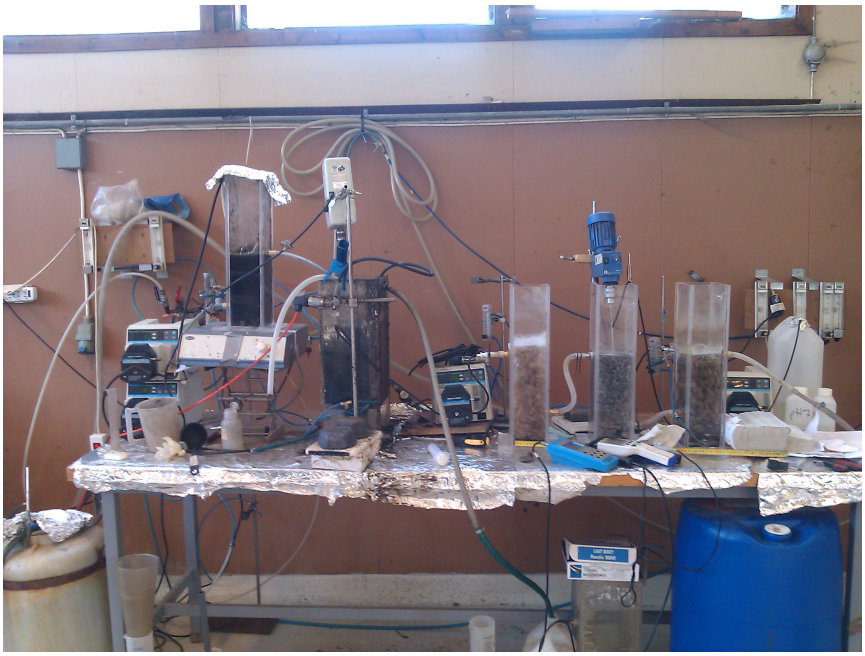


Figure 1.15 – The NTNU biofilm membrane bioreactor (BF-MBR) with phosphorus, nitrogen and COD removal.

The NTNU biofilm membrane bioreactor (BF-MBR) is a combination of MBBR and MBR technology. It consists of five reactors separated into two different processes linked together. The first two reactors form the A/O bio-P process (Shammas and Wang, 2010) for biological phosphorus removal with activated sludge. A membrane was integrated in the second reactor to filtrate the permeate passing on to the third reactor.

The third and fourth reactor constitute a post-denitrification process with nitrification followed by denitrification for the removal of nitrogen based on the MBBR technology (Rusten et al., 2006). The last reactor was implemented for carbon removal to meet the effluent requirements for COD. This reactor was also based on the MBBR technology.

1.6 The scope of this work

This master's thesis had a time-frame of 20 weeks. During this period an assessment of the functionalities of the biomass in a BF-MBR system was to be carried out. Changes in the microbial communities were to be compared to different operating conditions.

The work included the following tasks:

1. The joint responsibility of operating and maintaining the pilot plant, *i.e.* undertaking the daily chores, monitoring and checking the unit to ensure it was kept operating properly to fulfill the experimental plan.
2. Optimize a FISH protocol for this experiment.
3. Take samples from the biological reactors under varying operating conditions.
4. Investigate the characteristics and functionalities of the biomass in the system.
5. Analysis of the dominant biomass communities and how they may change due to different operating conditions.

Chapter 2

Materials and methods

2.1 Fluorescence *in situ* hybridization (FISH)

The main focus of this thesis was to analyze the microbial community in a BF-MBR reactor under different operating conditions. Several molecular techniques are available to identify and monitor the changes in the microbial community in wastewater treatment (Sanz and Köchling, 2007). Denaturant gradient gel electrophoresis (DGGE) can be used to monitor shifts in the microbial communities, but will notgive any quantitative information. Real time PCR can be used for the quantification of spesific microorganisms. FISH analysis provides a variety of information including visualization, identification, quantification and localization of individual microbial cells in a sample. In addition, FISH is time efficient compared to other molecular techniques such as DGGE and real time PCR. Therefore, FISH was used to analyze the biomass in this study.

It was decided to focus on the A/O bio-P and nitrification process when characterizing the biomass in this study. Many bacteria are able to denitrify, and therefore it would be difficult to get a precise evaluation of the bacteria involved in this process within the time-frame of this thesis.

8 samples from the anaerobic reactor of the A/O bio-P system (Reactor 1) and the nitrification reactor (Reactor 3) were collected to characterize the biomass by FISH. Samples from the aerobic reactor of the A/O bio-P process (Reactor 2) were also collected to analyze the difference between the anaerobic and aerobic reactor of the A/Obio-P process.

The protocol for FISH analysis was based on information provided by Gilda Carvalho at the Universidade NOVA de Lisboa, Amann et al. (1995) and Nielsen et al. (2009) and optimized for this study. The protocol is detailed in Appendix A. Reagents and equipment needed for the analysis are detailed in Appendix B.

The prepared samples were observed with a Zeiss LSM 700 confocal laser scanning microscopy (CLSM) through a 40x objective. Approximately 30 pictures were taken per sample analyzed.

The images obtained were analyzed using the software *daime* (Daims et al., 2006). *daime* was used to quantify the population of the target bacteria by counting FISH probe signals from the target probe and the general probe in the sample.

The brightness of the FISH images used in this report, with exception of section 3.1.2, was adjusted in the software ImageJ (Abramoff et al., 2004).

The standard deviation of the mean was calculated for each sample according to Equation 2.1. N is the number of images recorded per sample. The standard deviation was calculated using integrated formulas in Excel.

$$\text{Standard deviation of the mean} = \frac{\text{Standard deviation}}{\sqrt{\text{N}}} \tag{2.1}$$

The results obtained through FISH analysis were compared to data on operating conditions, collected in a parallel study.

2.1.1 Agarose coating of the biomass

As a part of the FISH analysis, Nielsen et al. (2009) suggested covering the biomass with agarose to avoid loss of biomass during the FISH procedure. The theory was tested with Agarose Type VII from Sigma-Aldrich.

2.1.2 Probes

Probes were selected based on recommendations from Nielsen et al. (2009) and discussions with Gilda Carvalho. The probes chosen for this study are listed in Table 2.1. The EUBmix was used for the detection of all bacteria, the PAOmix for *Accumulibacter*, the GAOmix for *Competibacter*, the AOB for *Nitrosomonas* and the NOB for *Nitrobacter*. Details of the probes and their coverage are illustrated in Appendix C.

Table 2.1 – Probes selected for this study, presented with their nucleotide sequence and scientific reference.

Probe name	Sequence 5' to 3'	Reference
EUBmix:		
EUB338	GCTGCCTCCCGTAGGAGT	Amann et al. (1995)
EUB338 II	GCAGCCACCCGTAGGTGT	Daims et al. (1999)
EUB338 III	GCTGCCACCCGTAGGTGT	Daims et al. (1999)
PAOmix:		
PAO462	CCGTCATCTACWCAGGGTATTAAC	Crocetti et al. (2000)
PAO651	CCCTCTGCCAAACTCCAG	Crocetti et al. (2000)
PAO846	GTTAGCTACGGCACTAAAAGG	Crocetti et al. (2000)
GAOmix:		
GB_G2	TTCCCCAGATGTCAAGGC	Kong et al. (2002)
cGB_G2	TTCCCCGGATGTCAAGGC	Kong et al. (2002)
GAOQ989	TTCCCCGGATGTCAAGGC	Crocetti et al. (2002)
AOB		
Nso1225	CGCCATTGTATTACGTGTGA	Mobarry et al. (1996)
NOB		
NIT3	CCTGTGCTCCATGCTCCG	Wagner et al. (1996)
cNIT3	CCTGTGCTCCAGGCTCCG	Wagner et al. (1996)

2.2 Chemical analysis

All chemical analysis were performed by Francesco Formisano and Igor Ivanovic at the Department of Hydraulic and Environmental Engineering at NTNU.

The methods used to measure COD, total phosphate, total nitrogen, ammonium and nitrate are given in Table 2.2. All cuvettes from Dr. Lange were read by a Dr. Lange Lasa 20 reader. The S::can ammo::lyser eco+pH and S::can ISE Probe V21 were used to measure the ammonium-nitrogen and COD concentrations from the 28.02.2013. Details of the data collected from the 11.02.2013 to 21.03.2013 can be found in Formisano's thesis.

Table 2.2 – Methods used to chemically measure the given compounds.

Parameter	Method
COD	Dr. Lange LCK 114
	Dr. Lange LCK 314
	S::can ISE Probe V21
Total phosphate (PO_4-P)	Dr. Lange LCK 348
Total Nitrogen (TN)	Dr. Lange LCK 338
	Dr. Lange LCK 238
Ammonium (NH_4-N)	Dr. Lange LCK 303
	Dr. Lange LCK 304
	S::can ammo::lyser eco + pH
Nitrate (NO_3-N)	Dr. Lange LCK 340
	Dr. Lange LCK 339

2.3 Analysis of live and dead cells in a nitrifying biofilm

At the 06.03.2013, a biofilm from the nitrification reactor was analyzed by Xin Jin at the Department of Hydraulic and Environmental Engineering at NTNU. A LIVE/DEAD BacLight^{TM} Bacterial Viability Kit from Invitro-

gen was used for the analysis. The cells with an intact membrane were, by this test, considered as live cells and those without an intact membrane were considered as dead cells. The sample was observed with a Zeiss LSM 700 confocal laser scanning microscopy (CLSM), and analyzed using the software ISA-2.

2.4 Total suspended solids and Sludge volume index

2.4.1 Total suspended solids

Total suspended solids (TSS) was measured by Francesco Formisano by filtering a sample through Whatman GF/C filters. The filters were then put in an oven at 105°C for 2 hours. The samples were analyzed in triplicates. From the 26.02.2013 all samples, except the sludge samples from the A/O bio-P system, were analyzed using a S::Can Spectrometer Probe V2 reader together with the software S::Can moni::tool v13. The TSS concentration was calculated according to Equation (2.2). Further details on the determination of TSS in this study can be found in Formisano's thesis.

$$\mathrm{TSS} = \frac{(\mathrm{M_{dry}} - \mathrm{M_{filter}})\ (\mathrm{mg})}{\text{Volume of filtered sample (l)}} = \frac{\mathrm{mg}}{\mathrm{l}} \tag{2.2}$$

2.4.2 Sludge volume index

The sludge volume index (SVI) was measured and calculated by Francesco Formisano at the Department of Hydraulic and Environmental Engineering at NTNU.

An Imhoff cone was filled with one liter of sludge from the aerobic reactor of the A/O bio-P system, and the the level of settled sludge was recorded after 30 minutes. Together with the TSS values the SVI was calculated according to Equation (2.3) provided by Metcalf and Eddy (2003).

$$\mathrm{SVI} = \frac{(\text{Settled volume of sludge (ml/l)}) \times (10^3\ (\mathrm{mg/g}))}{(\text{Total suspended solids (mg/l)})} = \frac{\mathrm{ml}}{\mathrm{g}} \tag{2.3}$$

2.5 Detection of filamentous bacteria

Samples from the 13.02.2013, 05.03.2013, 18.03.2013 and 15.04.2013, stained with the EUBmix were observed with a Zeiss LSM 700 confocal laser scanning microscopy (CLSM). A 20x objective was used to observe the presence of filamentous bacteria in the activated sludge.

2.6 The carriers

Two types of carriers were used in this study, the BWT S from Biowater Technology and the Kaldnes K1 developed by former AnoxKaldnes, now Krüger Kaldnes of Veolia Water Solutions & Technologies. The BWT S carriers were too large and caused technical problems with the mixer in the denitrification process. Therefore, Kaldnes K1 was used in this reactor. Specifications of the carriers are detailed in Table 2.3.

Table 2.3 – Details of carriers used in this study. [1] At 67 % filling fraction. (Ødegaard, 2006; Biowater Technology, 2013)

Carrier spesifications	
BWT S	
Dimensions, mm	14.5 x 14.5 x 8.2
Protected surface, m^2/m^3	650
Material	Polyethylene (PEHD)
Kaldnes K1	
Length, mm	7
Diameter, mm	10
Specific carrier area, m^2/m^3	465[1]
Effective specific surface area, m^2/m^3	335[1]
Density, g/cm^3	0.95
Material	Polyethylene (PEHD)

2.7 History of the biofilm and the activated sludge

The biomass in the A/O bio-P system was taken from existing reactor systems at the Department of Hydraulic and Environmental Engineering at NTNU. The initial sludge for start up was taken from an anoxic reactor with a sludge retention time (SRT) of more than 3 months. The 22.03.2013 new biomass had to be added due to a major spill accident by the tubing recycling biomass from the aerobic zone to the anaerobic zone. The new sludge was a mix of anaerobic sludge with a SRT of more then 6 months and sludge from the same anoxic reactor used in the initial start up.

After new biomass was added to the system a condition period followed. The first conditioning period was between the 21.01.2013 and 11.02.2013. The second period was between the 22.03.2013 and 27.03.2013.

The carriers for A/O bio-P removal, nitrification, denitrification and carbon removal were taken from previous experiments with aerobic reactors.

2.8 The wastewater

The wastewater used in this study was real wastewater from the local urban sewer network. The water was pumped into a buffer tank, serving as a primary clarifier. The wastewater was then directed to a second sedimentation tank before entering a storage tank of 60 liters supplying the pilot system with feed water.

Fluctuations in the wastewater composition were observed due to events such as heavy rainfall, snow melting *ect.* The Department had problems with the wastewater supply and the wastewater was therefore diluted with tap water between the 07.03.2013 and12.3.2013. These two factors affected the wastewater and the concentration of nutrients in the influent of the system, particularly between the 22.02.2013 and the 12.03.2013.

2.9 The reactors

The NTNU biofilm membrane bioreactor (BF-MBR) with phosphorus, nitrogen and COD removal is illustrated in Figure 2.1. In this report, the system is split in two parts and explained separately; Reactor 1 and 2 responsible for phosphorus and carbon removal by A/O bio-P combined with MBR technology. Reactor 3, 4 and 5 were responsible for nitrogen and carbon removal by post-denitrification with MBBR technology.

Stream 1 indicates the influent coming from the storage container. Stream 2 indicates the recycling stream from the aerobic reactor to the anaerobic reactor of the A/O bio-P process. Stream 3 indicates the permeate from the membrane passing into the post-denitrification configuration. Stream 4 supplied the external carbon source for denitrification. Stream 5 indicates the final effluent of the system.

The initial hydraulic retention time (HRT) of the system was 15 hours. At day 34 the HRT was decreased to 10 hours.

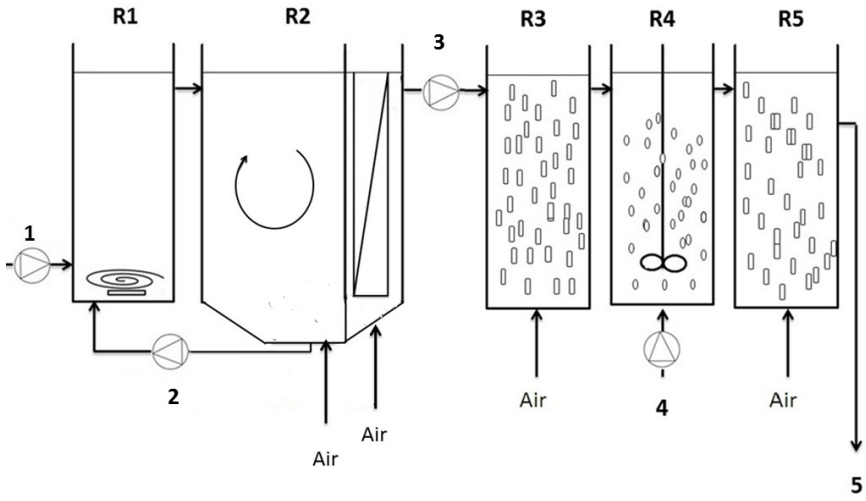


Figure 2.1 – The NTNU biofilm membrane bioreactor (BF-MBR) with phosphorus, nitrogen and COD removal.

2.9.1 A/O bio-P

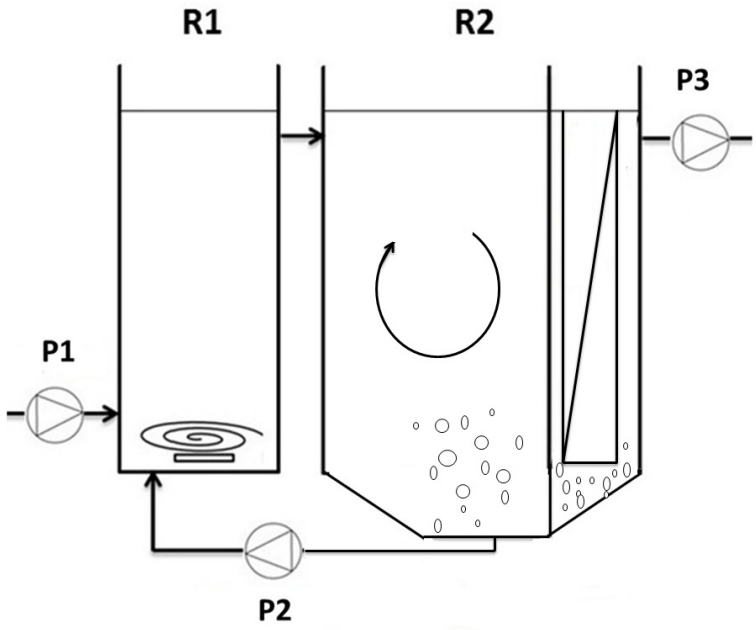


Figure 2.2 – Reactor 1 (anaerobic) and Reactor 2 (aerobic) for phosphorus and COD removal in the NTNU BF-MBR.

Reactor 1 (R1) and Reactor 2 (R2) were together operated as an A/O bio-P removal system with R1 as the anaerobic part and R2 as the aerobic part. During the start up of this experiment and until the 19.02.2013, Reactor 1 and 2 were operated as a Fixed Film Activated Sludge (IFAS) system with BWT S carriers in Reactor 2. This provided the system with a larger surface area for biofilm growth during the start up phase.

Reactor specifications

Both reactors were made of Plexiglas. Reactor 2 was used in previous studies by Sun et al. (2012) and consisted of two chambers, one for the bioreactor and one for the membrane. A vertical baffled wall with a coarse screen on the upper and lower parts separated the two chambers. This allowed suspended matter to pass into the chamber containing the membrane. Details of the reactors are presented in Table 2.4.

Table 2.4 – Details of Reactor 1 and 2.

Reactor specifications	
Reactor 1	
Total volume, l	5
Filling volume, l	2.35
Reactor 2	
Total volume, l	13
Volume bioreactor, l	11.4
Volume membrane chamber, l	1.6

Aeration

Air was supplied to both chambers in Reactor 2 through coarse air diffusers. The aeration of the two chambers was separated. The aeration rate of the membrane was operated at two different levels, depending on the flux of the membrane. A HRT of 15 hours gave a flux of 10 $Lm^{-2}h^{-1}$ and the aeration was set to 5 l/min. When the HRT was decreased to 10 hours, the flux was 15 $Lm^{-2}h^{-1}$ and the aeration was adjusted to 7 l/min.

The aeration rate of the bioreactor of Reactor 2 was initially set to 3 l/min. However, due to unwanted nitrification in Reactor 2, the aeration was reduced to 2 l/min at the 22.02.2013 and to 1 l/min at the 03.03.2013.

Mixer

When the rate of aeration in Reactor 2 was reduced, a mixer (Heidolph RZR) was introduced to ensure a complete mixing of the biomass in the reactor. In the beginning the biomass was mixed manually once a day, in addition to the mixing by aeration. Reactor 1 was equipped with a magnet stirrer from Stuart (SB301), and operated continuously at 300 rpm during the entire experiment.

Pumps

Three pumps were connected to Reactor 1 and 2. Pump 1 (P1) supplied the raw water from the storage container, Pump 2 (P2) was used for re-circulation of biomass from R2 to R1. The flow of recirculation was set to 100 % of the inflow, with exception of day 21 to 25when the recirculation

rate was turned up to 300 % of the inflow. This was done in an attempt to increase the P-removal efficiency. Pump 3 (P3) extracted the permeate from the membrane. All three pumps were supplied by Masterflex L/S with Easy-Load II pump heads (Model 77200-60).

The membrane

The membrane used in this study is illustrated in Figure 2.3. The membrane was supplied by Kubota. The specifications of this membrane are detailed in Table 2.5.

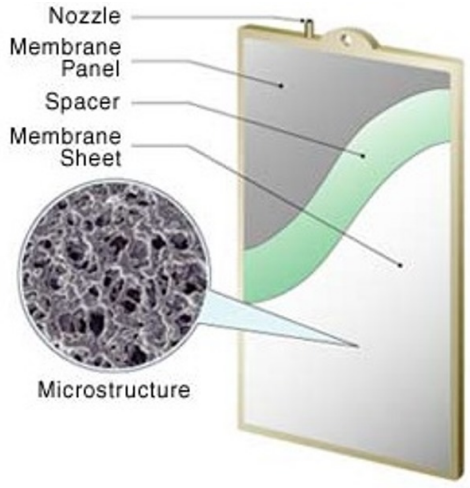


Figure 2.3 – The Kubota flat sheet microfiltration membrane.

The permeate from the membrane was withdrawn from a nozzel at the top of the membrane by Pump 3.

The membrane performance, expressed as transmembrane pressure (TMP), was recorded every minute by an online pressure meter (Genspec GP 4200) connected to a pressure transducer (Fieldpoint, FP100 FP-AI-110 analog input). The data were visualized in the data acquisition and analysis software LabVIEW. Further details can be found in Formisano's thesis.

The TMP data were also used to monitor the need for membrane cleaning. The membrane was cleaned with a sponge and cold water if the TMP

Table 2.5 – Spesifications of the Kubota flat sheet microfiltration membrane.

Spesifications	
Membrane type	Flat sheet
Membrane material	Chlorinated PE
Membrane area, cm^2	1160
Membrane pore size, µm	0.4
Membrane flux, initial, $lm^{-2}h^{-1}$	10
Membrane flux, reduced HRT, $lm^{-2}h^{-1}$	15
Aeration, initial, l/min	5
Aeration, reduced HRT, l/min	7

value was below -0.3 bar. During the experiment the membrane was changed once, the 16.02.2013.

2.9.2 Post-denitrification

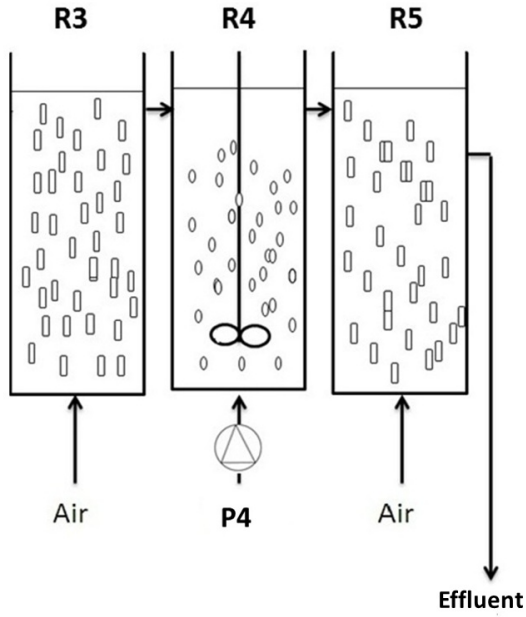


Figure 2.4 – Reactors 3, 4 and 5 for nitrogen and COD removal in the NTNU BF-MBR.

Reactors 3, 4 and 5 were based on the MBBR technology with carriers. The BWT S carriers from Biowater were used in Reactor 3 and 5, and Kaldnes K1 carriers were used in Reactor 4.

Reactor 3 (R3) and Reactor 4 (R4) were operated as a post-denitrification system with nitrification in R3 and denitrification in R4. Reactor 5 was added to remove excess carbon to ensure that the effluent met the regulation standards.

Reactor specifications

The reactors were made of Plexiglas. The geometry of all three reactors were the same and as specified in Table 2.6.

Table 2.6 – Reactor specifications for Reactors 3, 4 and 5.

Reactor specifications	
Total volume, l	5
Filling volume, l	2,35
Filling fraction of carriers, %	67

Aeration

The inlet of Reactor 3 was connected to Pump 3 which withdrew the permeate from the membrane of Reactor 2. Reactors 3 and 5 were supplied with air through a fine bubble diffuser at the bottom of the reactors. The aeration rate was kept constant at 5 l/min throughout the experiment. The air supply ensured a complete mixing of the reactor content.

Mixer

Reactor 4 was anoxic and equipped with a mixer from Ika Laboritechnik to obtain a complete mixing of the reactor content.

Pump

Ethanol was supplied to Reactor 4 by Pump 4 (Masterflex L/S with Easy-Load II pump heads (Model 77200-60)) as a carbon source for denitrification.

Carbon source

An external carbon source was added to the denitrification reactor since not enough carbon was available in the wastewater after the bio-P process and the membrane filtration. In this experiment ethanol (96 % VOL RECTA-PUR from VWR International AS) was used. The COD/NO_3-N ratio was initially 7 (4 ml ethanol /l), but was reduced to 3.5 (2 ml ethanol/l) the 19.02.2013 because of too high COD values in the effluent.

2.10 Monitoring of the system

pH, dissolved oxygen (DO), temperature and flow were recorded daily. The concentration of dissolved oxygen was recorded in mg/l by a HANNA HI 9146 Microprocessor Dissolved Oxygen Meter, which was calibrated daily. The temperature in degrees Celsius was also measured by the same device. The pH was recorded by a Mettler Toledo SG2 which was calibrated once a week. The flow was monitored after every pump in the system.

The storage tank was emptied for sludge and refilled with wastewater when needed. The mixing in Reactor 2 was not always satisfactory and before a mixer was introduced the biomass was mixed manually once a day.

When needed, the overflow tank was decanted and the biomass returned to Reactor 2. The exact amount of biomass removed daily from the A/O bio-P system was hard to control due to spill and leakage from Reactor 2.

Chapter 3

Results and discussions

The main focus of this chapter will be on the A/O bio-P process and the nitrification process. Details of the rector log for all reactors can be found in Appendix E. Days stands for days after first measurement, starting with day 1 the 11.02.2013, and ending with day 64 the 15.04.2013. Between day 12 and 29, the influent was subjected to periods of nutrient dilution due to heavy rain fall and problems with the wastewater supply. These periods are referred to as periods with substrate limitations.

3.1 Optimization of the FISH protocol

Four aspects of the FISH protocol was investigated and changes were made to optimize the procedure for this study. The FISH protocol with modifications based on the results presented in this section can be found in Appendix A.

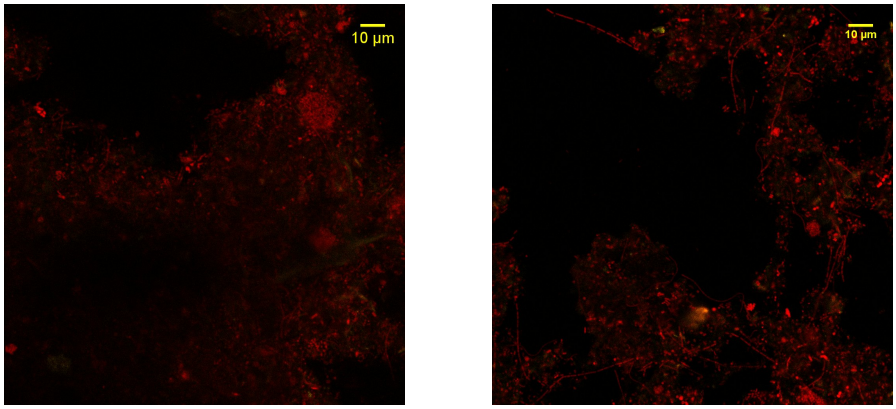
3.1.1 The amount of biomass

The amount of biomass applied in each well was determined based on recommendations from Gilda Carvalho and optimized for this study. The recommended amount was 5 µl applied in three layers. The result was satisfactory for the activated sludge samples, but the amount of biofilm sample per layer was increased to 10 µl. The biofilm sample was less dense and more sample had to be applied in order to have enough biomass for FISH quantification.

3.1.2 Agarose coating of the biomass

Nielsen et al. (2009) suggested covering the biomass applied to the slide with a thin layer of agarose. The agarose would act as a glue and avoid biomass loss due to washing.

The theory was tested with sludge from the A/O bio-P system, stained for the detection of PAOs. The result, illustrated in Figures 3.1, showed no big difference in the biomass retained on the slide. However, the slide coated with agarose (Figure 3.1(a)) gave a blur picture compared to the slide without coating (Figure 3.1(b)). Therefore, it was decided to prepare the samples for this study without a layer of agarose.



(a) With agarose coating (b) Without agarose coating

Figure 3.1 – Image of sludge sample obtained by FISH analysis. The sample was stained with the EUBmix (red) and the PAOmix (yellow).

3.1.3 Probe sensitivity to light

The probes containing fluorochromes may be subjected to photobleaching due to light exposure. Material provided by Gilda Carvalho recommended working with the probes in the dark to protect the probes. The protocol was followed once in daylight, and once with minimal light exposure of the probes. There was not observed a significant difference, but the probes were subjected to as little light as possible during this experiment to get the best results possible.

3.1.4 Number of images recorded per sample

The abundance of the target bacteria observed in the images of the same sample varied. Therefore, many images were required to decrease the standard deviation of the mean to obtain reliable results. Figures 3.2, 3.3, 3.4 and 3.5 demonstrate the differences that could be observed within the same sample by FISH analysis. It was decided to take approximately 30 images per sample to obtain a reliable result.

The amount of *Nitrosomonas* in each image of a sample varied, as illustrated in Figure 3.2. The sample collected was a biofilm from the nitrification reactor the 26.02.2013. The highest abundance of *Nitrosomonas* was recorded this day.

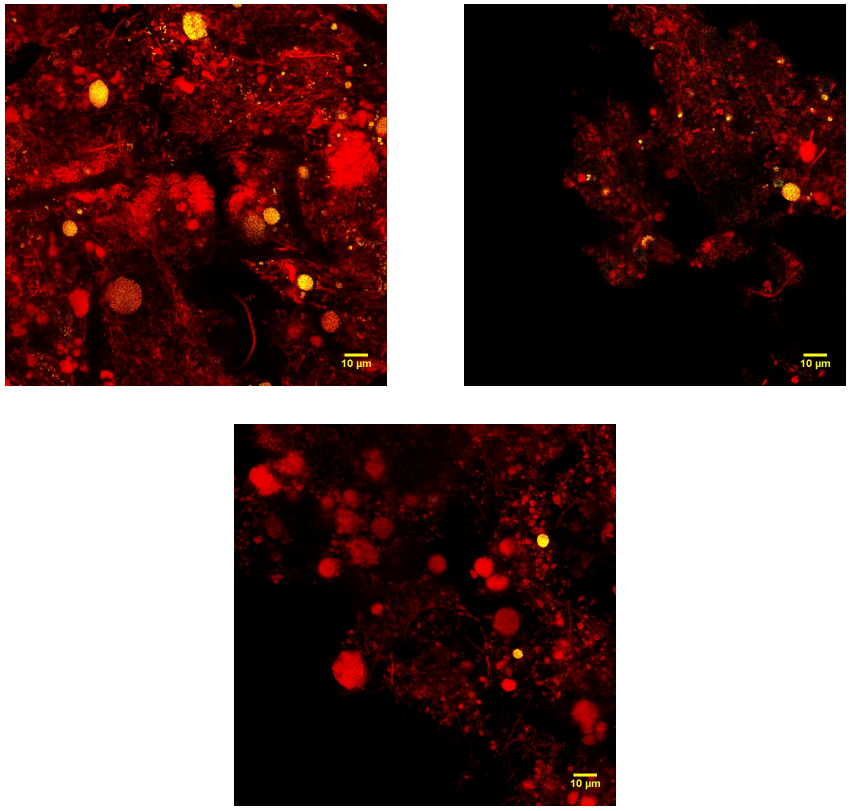


Figure 3.2 – Illustration of the variation of *Nitrosomonas* (yellow) compared to all bacteria (red) in one sample analyzed by FISH.

Figure 3.3 illustrates the variation of *Nitrobacter* within a sample. The sample collected the 15.04.2013 was a biofilm from the nitrification reactor. The highest abundance of *Nitrobacter* was recorded this day.

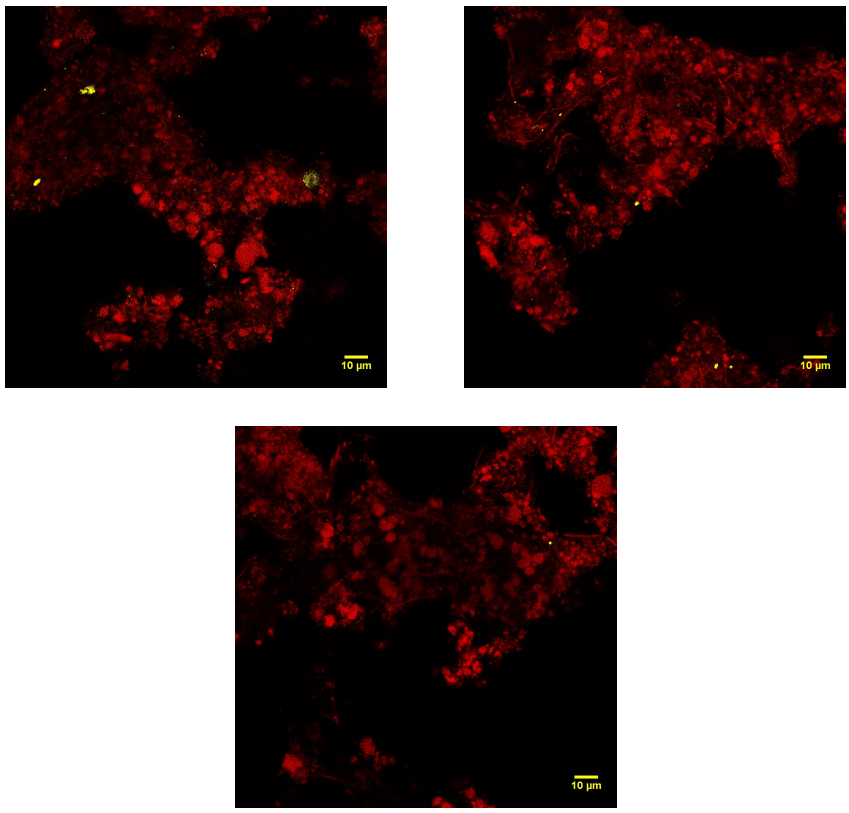


Figure 3.3 – Illustration of the variation of *Nitrobacter* (yellow) compared to all bacteria (red) in one sample analyzed by FISH.

The amount of *Accumulibacter* in each image of a sample varied, as illustrated in Figure 3.4. The sample was collected the 13.02.2013 from the activated sludge of the A/O bio-P process. The highest abundance of *Accumulibacter* was recorded this day.

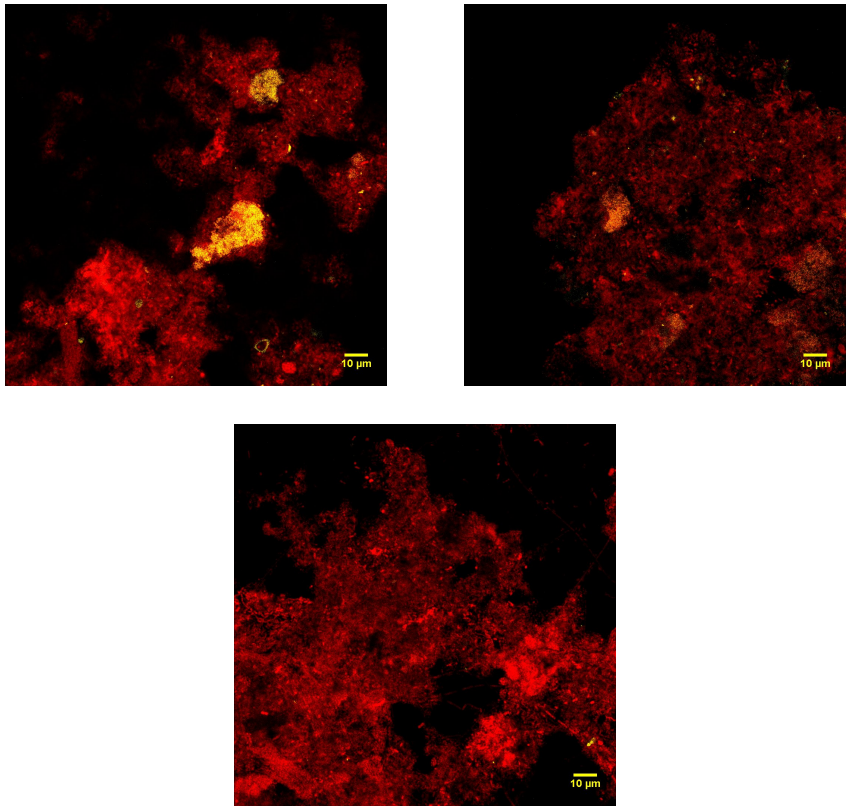


Figure 3.4 – Illustration of the variation of *Accumulibacter* (yellow) compared to all bacteria (red) in one sample analyzed by FISH.

Figure 3.5 illustrates the variation of *Competibacter* within a sample. The sample was collected the 04.04.2013 from the activated sludge of the A/O bio-P process. The highest abundance of *Competibacter* was recorded this day.

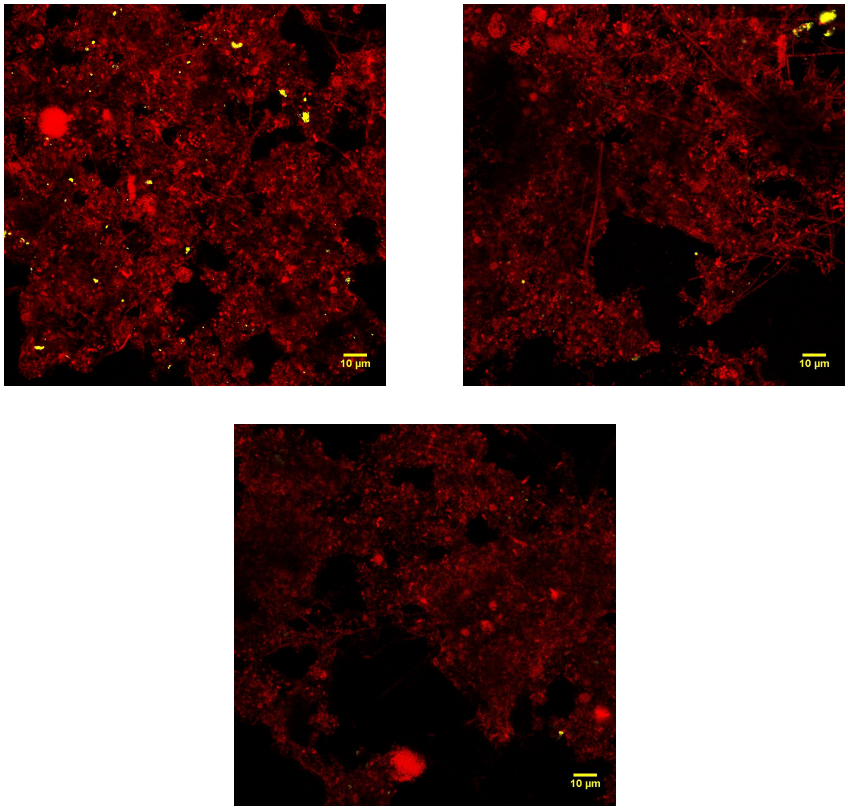


Figure 3.5 – Illustration of the variation of *Competibacter* (yellow) compared to all bacteria (red) in one sample analyzed by FISH.

3.2 System performance and operating conditions

The system performance is, in this report, defined as the system's ability to remove the components phosphorus, total nitrogen, ammonium, nitrate and carbon from the wastewater.

3.2.1 The A/O bio-P process

The recorded P-removal in the A/O bio-P system during the experimental period is presented in Figure 3.6.

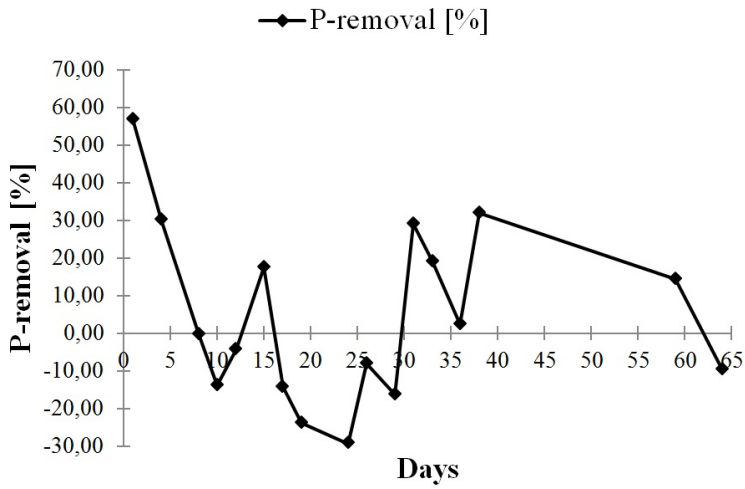
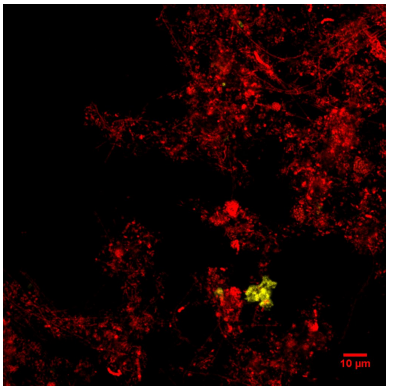


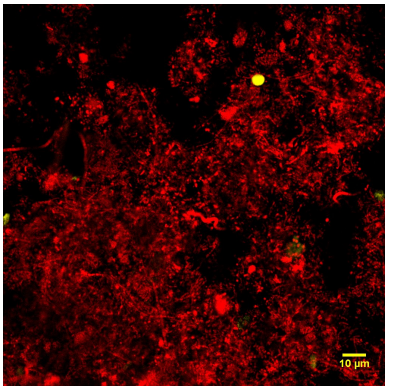
Figure 3.6 – The P-removal in the A/O bio-P system during the experimental period, presented in percent.

A positive P-removal efficiency was observed in 8 of the 17 measurements in Figure 3.6. During three periods the amount of orthophosphate released exceeded the amount taken up from the wastewater, resulting in a negative P-removal efficiency. These removal rates can be linked to periods of substrate limitations, in addition to the occurrence of nitrification in the aerobic bioreactor. Nitrification leads to increased levels of nitrate in the bulk liquid which, when recirculated, can cause anoxic conditions in the normally anaerobic reactor. Denitrifying bacteria will then compete with the PAOs for the available carbon (Mulkerrins et al., 2004). The presence of the nitrifying bacteria *Nitrosomonas* and *Nitrobacter* in the sludge was confirmed by FISH analysis, and the result is illustrated in Figure 3.7.

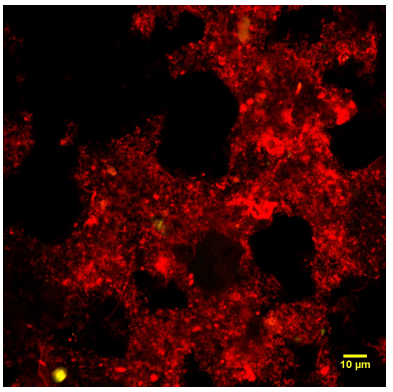
Chemical analysis also confirmed the production of nitrate in the aerobic reactor (Formisano, 2013). Carucci et al. (1999) reported similar observations due to periods of low organic carbon loads, where the level of phosphate in the effluent increased about 60 % in the following 1-2 days.



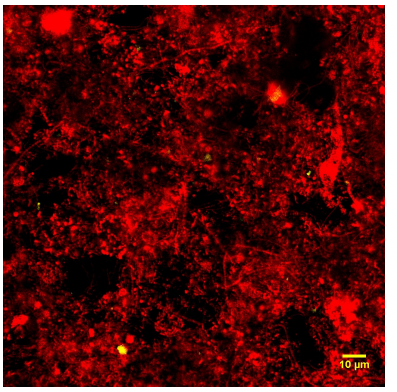
(a) *Nitrosomonas* (yellow) compared to all bacteria (red). The sample was collected on day 23 (05.03.2013).



(b) *Nitrobacter* (yellow) compared to all bacteria (red). The sample was collected on day 23 (05.03.2013).



(c) *Nitrosomonas* (yellow) compared to all bacteria (red). The sample was collected on day 64 (15.04.2013).



(d) *Nitrobacter* (yellow) compared to all bacteria (red). The sample was collected on day 64 (15.04.2013).

Figure 3.7 – Confirmation of nitrifying bacteria present in the A/O bio-P system.

The A/O bio-P process is also responsible for COD removal. The bacterial community in the biomass will mainly be able to consume the soluble part of the influent COD (SCOD). The removal efficiency of SCOD in the A/O bio-P system was fairly stable at 82 ± 6 %. Despite different events such as nitrification and low influent concentrations of COD, the A/O bio-P system was able to remove most of the SCOD.

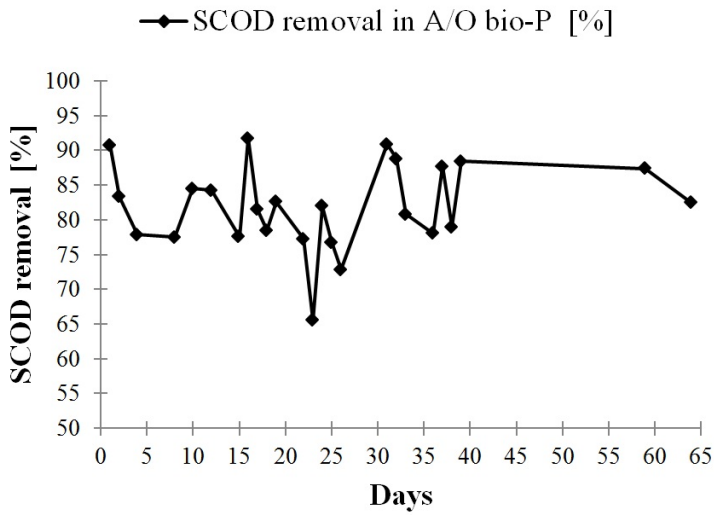


Figure 3.8 – The SCOD-removal in the A/O bio-P system during the experimental period, presented in percent.

pH

The average pH of the anaerobic and aerobic zone was 7.3 ± 0.3 and 7.2 ± 0.6, respectively. The recorded values are presented in Figure 3.9. Liu et al. (1996) reported an optimum pH of 6.8 ± 0.7 for the anaerobic acetate metabolism of PAOs. The average pH in this study were a little higher than the suggested optimum, but since Nielsen et al. (2009) pointed out that an increase in P-release and P-uptake was expected at higher pH, the system pH was not expected to be a limiting factor for the system performance.

The pH in the anaerobic reactor was fairly stable throughout the experiment. During two periods the pH was lower in the aerobic reactor than the anaerobic reactor. At this time nitrification was occurring in the aerobic reactor. The nitrification process results in the release of acid, which could explain the decrease in pH.

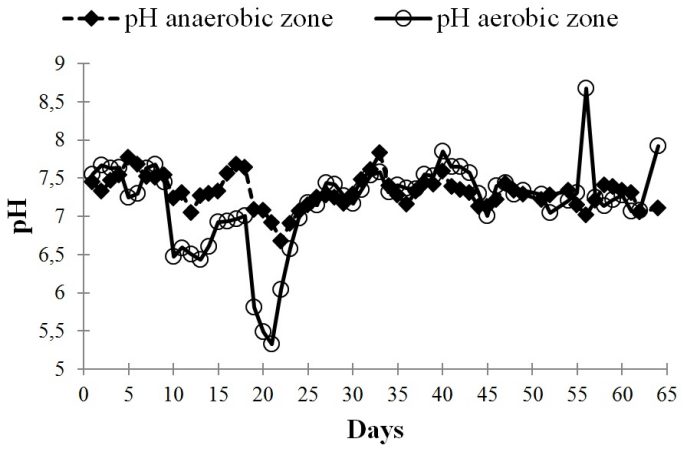


Figure 3.9 – The pH recorded in the A/O bio-P reactors during the experimental period.

Temperature

The temperature recorded in the A/O bio-P process is presented in Figure 3.10. The average temperature in the anaerobic and aerobic reactor was 20.7 ± 0.3 and 19.7 ± 1.5, respectively. Panswad et al. (2003) reported that the optimum temperature for the PAOs was at 20°C or possibly lower. The system temperature in this study is therefore considered to be acceptable and should not limit the PAOs.

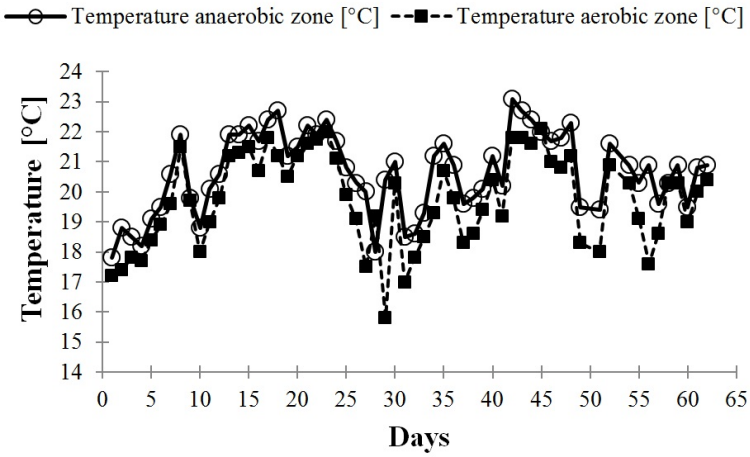


Figure 3.10 – The temperature recorded in the A/O bio-P reactors during the experimental period.

Dissolved oxygen

The concentration of dissolved oxygen (DO) during the experimental period is presented in Figure 3.11. The average DO concentration in the anaerobic and aerobic reactor was 0.07 ± 0.08 and 1.2 ± 1.0, respectively.

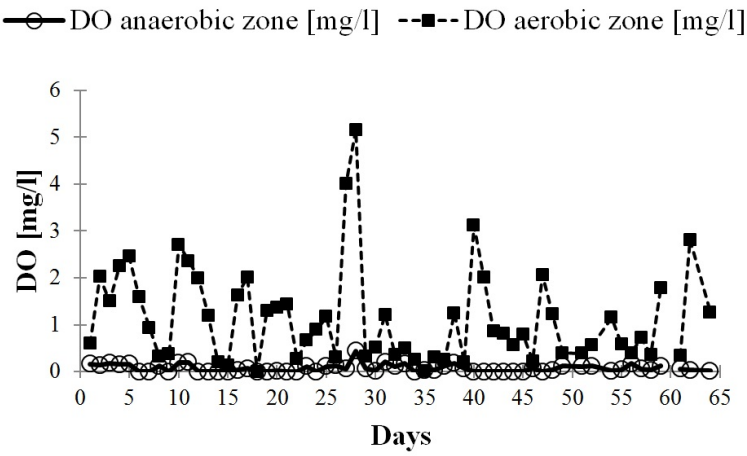


Figure 3.11 – The dissolved oxygen (DO) concentration recorded in the A/O bio-P reactors during the experimental period.

The DO concentration was hard to control in the aerobic reactor. The aeration of the membrane affected the DO concentration in the bioreactor, leading to higher DO concentrations in the area close to the membrane compartment. Due to unwanted nitrification in the aerobic reactor, the aeration rate was turned down in order to cease this process. Shehab et al. (1996) recommended a DO concentration of 3.0 - 4.0 mg/l. The average during this study was lower and unstable, and this could have had a negative effect on the A/O bio-P process.

Total suspended solids

The concentration of total suspended solids (TSS) in the A/O bio-P system between day 1 and 39 is presented in Figure 3.12. The general trend observed was the decrease of TSS concentration, followed by a relative stable concentration after day 25. During the period of substrate limitation, the TSS concentration was decreasing. Studies done by Vargas et al. (2013) and Lopez et al. (2006) describe a decrease in the TSS concentration in the A/O bio-P system when exposed to starvation conditions. They suggest that one reason for this decrease could be because of cell lysis.

The filling level of the aerobic reactor was hard to control, and a significant amount of biomass was lost due to spill from this reactor. This was probably the main reason for the reduction in TSS during this study.

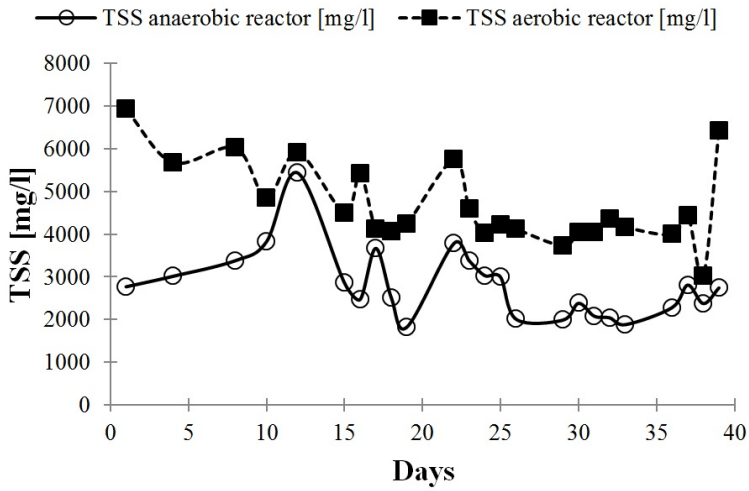


Figure 3.12 – The concentration of total suspended solids in the A/O bio-P process, presented in mg/l.

3.2.2 Nitrification

During the two step process of nitrification ammonium is oxidized to nitrite, which again is oxidized to nitrate. In this experiment it was assumed that if the first step was successful, full nitrification was occurring since the first part is considered the velocity limiting step.

The concentration of NH_4-N in the inlet and outlet of the nitrification reactor is illustrated in Figure 3.13.

Between day 10 and 30, and around day 64, nitrification was taking place in the A/O bio-P process. This event resulted in very low levels of ammonium entering the nitrification process leaving the nitrifying bacteria with no substrate. However, when the concentration of ammonium in the inlet of the nitrification process increased and after a few days of adaption, the nitrifying bacteria were able to oxidize almost all ammonium.

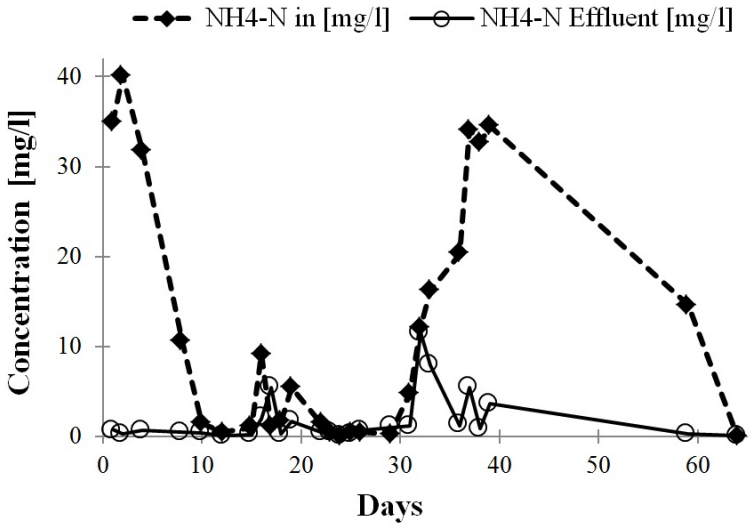


Figure 3.13 – Concentration of NH_4-N in the inlet of the nitrification reactor and the effluent of the system, presented in mg/l.

After the decrease in HRT at day 34, the nitrifying bacteria needed a few days to adapt, but successful nitrification was then observed until nitrification once more took place in the A/O bio-P process at the end of the experiment.

pH

The average pH of the nitrification reactor was 7.5 ± 0.8. The recorded values are presented in Figure 3.14. The optimum pH for nitrification lies between 7.5 and 8 (Østgaard, 1995). The average pH of this experiment corresponds well with the recommended value.

During periods with successful nitrification the pH dropped in the reactor, which coincides well with the theory of acid production by the nitrification process.

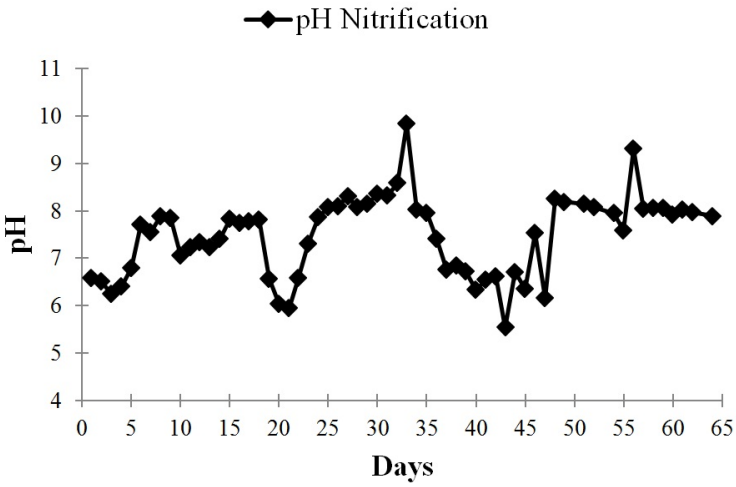


Figure 3.14 – The pH recorded in the nitrification reactor during the experimental period.

Temperature

The temperature recorded in the nitrification process is presented in Figure 3.15. The average temperature was 19.6 ± 1.7 °C.

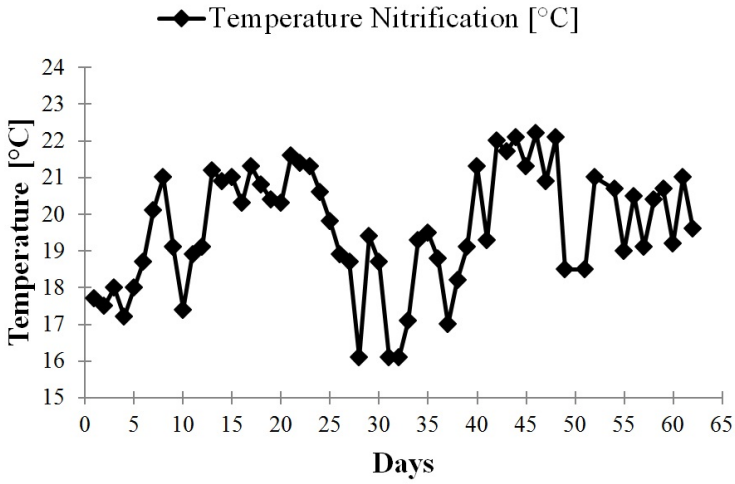


Figure 3.15 – The temperature recorded in the nitrification reactor during the experimental period.

The nitrifying bacteria are mesophilic and most effective around 30-37 °C. However, nitrification can occur at much lower temperatures (Ødegaard, 1993; Østgaard, 1995). The average process temperature in this study was lower than the optimum for nitrification, but full nitrification was observed. This suggest that the temperature was not limiting the process in this experiment. However, an optimization of the process temperature could lead to increased activity, which might have been necessary if the NH_4-N concentration in the inlet was higher.

Dissolved oxygen

The concentration of dissolved oxygen (DO) during the experimental period is presented in Figure 3.16. The average DO concentration in the nitrification reactor was 5.3 ± 0.9 mg/l.

The concentration of dissolved oxygen fluctuated during the experiment, but was always above the recommended concentration of 2 mg/l (Østgaard, 1995).

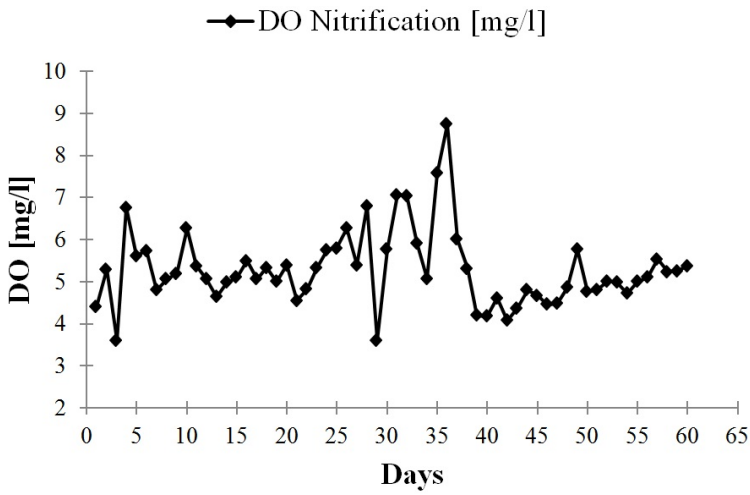


Figure 3.16 – The dissolved oxygen (DO) concentration recorded in the nitrification reactor during the experimental period.

3.2.3 Denitrification

Successful denitrification is achieved when the denitrifying bacteria oxidize carbon, using nitrate as the electron acceptor. The concentration of nitrate in the inlet and outlet of the denitrification process can thus give useful information about the activity of the denitrifying bacteria. The recorded levels of nitrate in the inlet of the denitrification process and the effluent are illustrated in Figure 3.17.

With the exception of four peaks, almost all nitrate was consumed, meaning denitrification took place in the system. The first two and the last peak were due to a dry ethanol supply. The third peak was just after the decrease in the HRT at day 34 and the bacteria probably needed a few days to adapt.

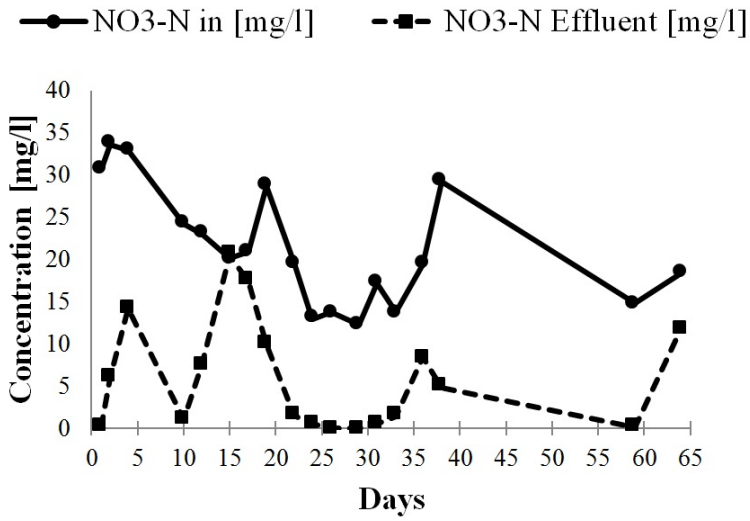


Figure 3.17 – Concentration of NO_3-N in the inlet of the denitrification reactor and the effluent of the system, presented in mg/l.

3.2.4 The NTNU biofilm membrane bioreactor (BF-MBR)

The removal efficiencies of COD, total phosphate and total nitrogen are illustrated in Figure 3.18. The efficiencies are calculated based on the concentrations measured in the influent and effluent of the BF-MBR system. Further details can be found in Formisano's thesis.

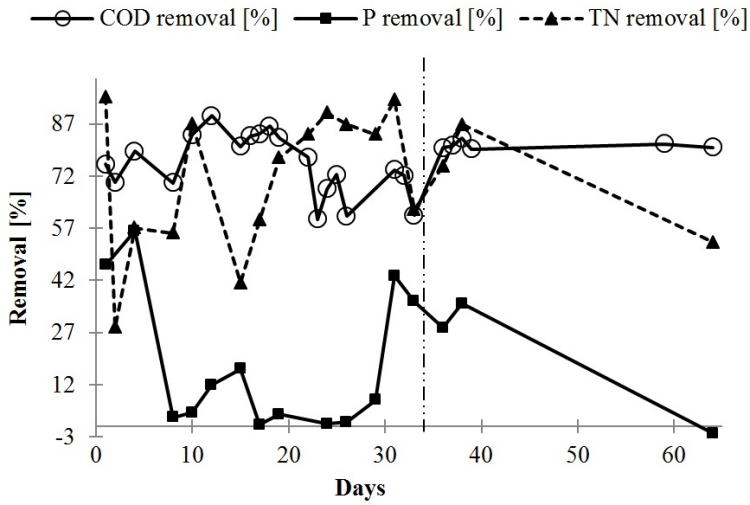


Figure 3.18 – The system performance in terms of COD removal, total phosphate removal and total nitrogen removal. The results are displayed in percent removal, and the vertical dashed line indicates the reduction in HRT.

The average of total COD removal from the wastewater was 77 ± 8 % and fairly stable throughout the experimental period. The average of total nitrogen removal was more unstable at 68 ± 23 %. At day 2, 15 and 63 the ethanol supply went dry and this can explain the drop in the total nitrogen removal efficiency, since there was no carbon source for denitrification.

The total phosphorus removal in the system was poor, meaning the effluent concentrations did not meet EU standards. A significant drop was observed between day 17 and 29, and at day 64. The average of total phosphorus removal was 23 ± 27 %. The unstable process can be explained by unwanted events such as nitrification and substrate limitations.

After the period of substrate limitation an increase in the concentration of nutrients in the influent was observed and the efficiencies improved.

The decrease in HRT at day 34 seemed to affect the COD, total nitrogen and phosphorus removal positively.

The TSS content of the effluent was expected to be within standard regulations because of the membrane filtration after the A/O bio-Pprocess. However, detachment of biomass from the biofilms due to hydraulic erosion in the post-denitrification system could cause higher TSS concentrations. The TSS concentration in the effluent was at 32 $\pm$ 30 mg/l and within the limit of EU regulations.

Effluent concentrations of COD, total nitrogen and TSS were in accordance with EU legislations, but the level of total phosphate was far from the accepted threshold. The Council Directive 91/271/EEC with current legislations regarding wastewater discharge is displayed in Appendix D.

3.3 Analysis of the microbial community

Samples from 8 different days during the operation of the reactors were analyzed by FISH. Results and observations are presented and discussed in this section. In total, 1105 images were recorded during this experiment to characterize the biomass. In the A/O bio-P process, the abundance of the phosphate accumulating bacterium *Accumulibacter* was quantified and is here referred to as PAO. The abundance of the glycogen accumulating bacterium *Competibacter* was quantified and is referred to as GAO. In the nitrification process, the abundance of two of the bacteria responsible for nitrification, *Nitrosomonas* and *Nitrobacter*, was quantified and is referred to as AOB and NOB, respectively.

3.3.1 The abundance of PAOs and GAOs

The population of PAOs and GAOs in the activated sludge was quantified by FISH analysis. The results with the respective standard deviations of the mean are presented in Figure 3.19. The GAOs seemed to be more homogeneously distributed in the sludge and the standard deviations were lower and more stable than for the PAOs. In the first four weeks of the experiment, the amount of GAOs in the system appeared to be quite stable. When the concentration of nutrients in the influent increased, the abundance of GAOs increased.

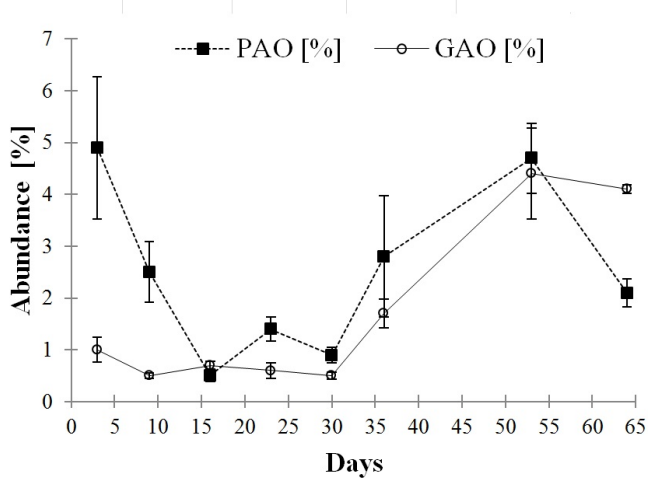


Figure 3.19 – The relative quantities of PAOs and GAOs in the A/O bio-P system as determined by FISH analysis. The error bars represent the standard deviation of the mean for each sample.

The decrease in the HRT at day 34 seemed to stimulate the growth of both PAOs and GAOs. The abundance of PAOs varied, and had a significant drop in the beginning and at the end of the experiment.

The amounts of PAOs varied between 0.5 ± 0.1 % and 4.9 ± 1.4 %, with an average of 2.5 ± 0.6 %. The abundance of GAOs ranged between 0.5 ± 0.2 % and 4.4 ± 0.9 %, with an average of 1.7 ± 0.2 %. A study by López-Vázquez et al. (2008) found the average population of PAOs and GAOs from seven different full scale wastewater treatment plants in the Netherlands to be 9.2 ± 2.1 % and 1.7 ± 0.4 %, respectively. The abundance of PAOs was lower in this study than found by López-Vázquez et al. (2008), but the abundance of GAOs was similar.

Figure 3.20 shows the abundance of PAOs in the system compared to the P-removal efficiency. The figure demonstrates that the P-removal was affected by the decreasing amount of PAOs in the system.

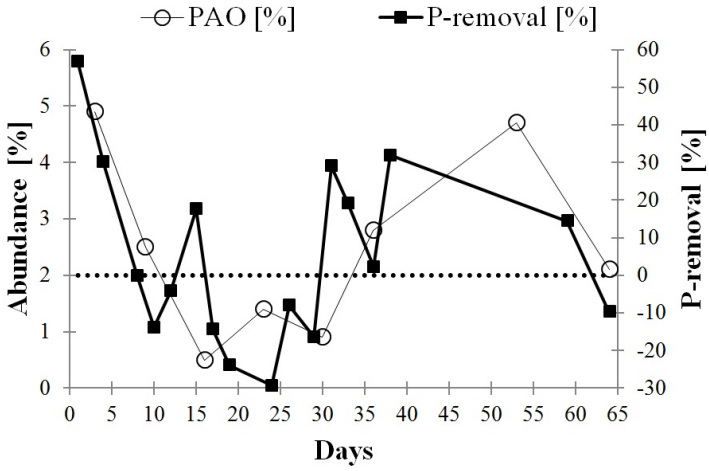


Figure 3.20 – The relative quantity of PAOs as determined by FISH analysis, compared to the P-removal in the A/O bio-P system. The horizontal dashed line represent 0 % P-removal.

A P-removal of less than 0 % indicates that more orthophosphate was released than taken up, causing the concentration of phosphate in the bulk liquid to increase. The reason for this can be explained by the combination of limited concentrations of soluble COD (SCOD) and the subsequent ni-

trification in the aerobic reactor of the bio-P process.

The PAOs and GAOs mainly take up the soluble part of the influent COD. Therefore the concentration of SCOD in the influent is a crucial factor for the PAOs and GAOs in the system. The amount of PAOs and GAOs in the biomass compared to the SCOD is presented in Figure 3.21. During low-load periods the population PAOs was more affected than the GAOs, decreasing as the concentration of SCOD decreased. Both PAOs and GAOs were positively affected by the increase of SCOD in the influent, as well as the decrease in the HRT at day 34. At the end the amount of GAOs exceeded the amount of PAOs in the system, possibly leading to substrate competition.

These observations agree with a study by Vargas et al. (2013) suggesting that PAOs are more affected by starvation conditions than GAOs. They also found a significant biomass decay for PAOs where 11 % of the activity decrease detected was caused by cell death of PAOs, while the biomass decay for GAOs was negligible. This can be supported by data obtained in this study where the population of GAOs never had a dramatic decrease, while the population of PAOs sharply decreased when SCOD was limited.

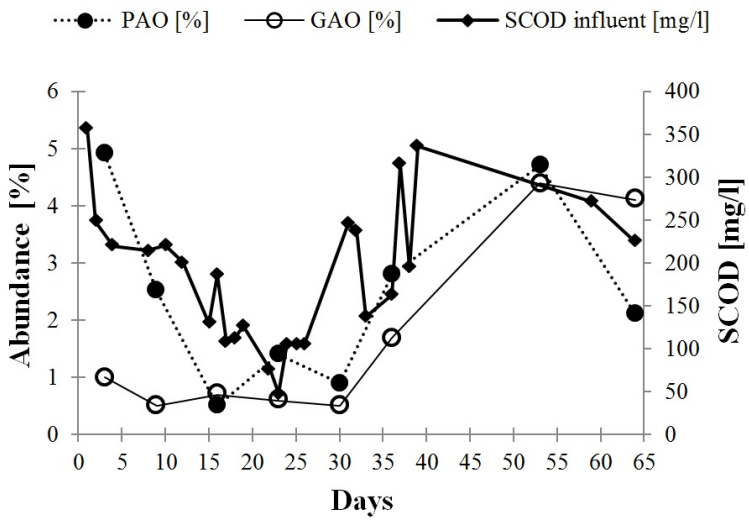


Figure 3.21 – The influent concentration of SCOD presented in mg/l and the abundance of PAOs and GAOs presented in percent.

Vargas et al. (2013) reported that as a result of substrate limitations, the PAOs can degrade the stored poly-phosphate for maintenance purposes causing the concentration of phosphate in the bulk liquid to increase. The energy of the poly-phosphate degradation is used for maintenance purposes, and not the uptake of VFAs and the subsequent conversion to PHBs. This will again affect the PAOs ability to take up the ortho-phosphate in the aerobic zone of the A/O bio-P process, thus increasing the effluent concentration of phosphate. During longer periods of starvation, cell lysis can also occur, releasing intracellular poly-phosphate into the bulk liquid.

After heavy rainfall or during low load periods the PAOs can suffer from over-aeration in the aerobic reactor since there is less organic material available for oxidation. This can again stimulate the growth of nitrifying bacteria. The problem of unwanted nitrification was also encountered by Lu et al. (2007) when studying the endogenous metabolism of *Accumulibacter* under starvation conditions. The final product of nitrification is nitrate. As a result of nitrification in the aerobic zone, nitrate will be recycled back to the anaerobic zone of the A/O bio-P process causing anoxic conditions. Many facultative aerobic heterotrophs are able to switch to nitrate respiration in the absence of oxygen by expressing the enzyme *nitrate reductase* (Østgaard, 1995). Denitrifying bacteria will then use nitrate as an electron acceptor to oxidize the available carbon. This leaves the PAOs with less substrate to feed on, which can lead to limited P-uptake and potentially cell death.

At day 9, the A/O bio-P system was changed from an IFAS configuration to solely activated sludge. FISH analysis showed that the population of PAOs on one carrier was 4.0 ± 0.9 %. The population of PAOs in the activated sludge at the same day was 2.5 ± 0.6 %. When the carriers were removed, a significant portion of the microbial community responsible for phosphorus removal was then also removed, leading to increased stress on the remaining PAOs. This could also have had a negative impact on the removal efficiency.

The difference in the microbial community of the anaerobic and aerobic reactor of the A/O bio-P system was investigated. Sludge samples from both reactors were collected at day 3, 9, 16 and 36 and analyzed by FISH. The biomass in the system was circulated between the two reactors, and in theory should not be significantly different. The result is presented in Figure 3.22. A difference wasobserved between the microbial community of PAOs and GAOs in the two reactors. However, no conclusive trend was observed and the differences were hard to link to operational parameters.

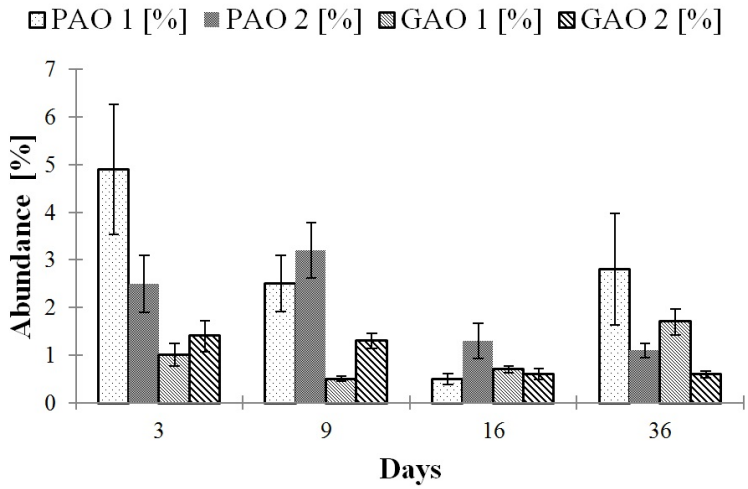


Figure 3.22 – The abundance of PAOs and GAOs in the anaerobic reactor (Reactor 1) and aerobic reactor (Reactor 2) of the A/O bio-P processpresented in percent with their respective standard deviations of the mean.

The PAOs and GAOs were mostly observed as single cells or small clusters, but did occasionally also form bigger clusters. Figure 3.23 and 3.24 show two images of PAO and GAO in the activated sludge, obtained by FISH analysis.

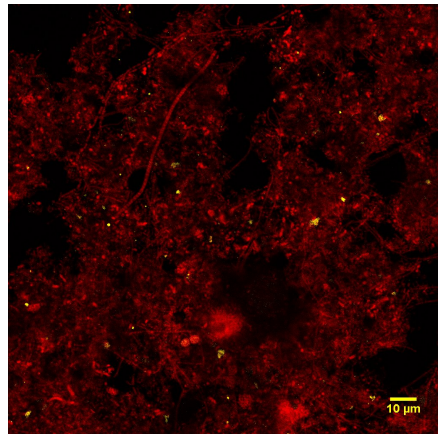


Figure 3.23 – Image obtained by FISH analysis of PAO (yellow) and all bacteria (red) present in the activated sludge. The sample was collected the 04.04.2013, at day 53

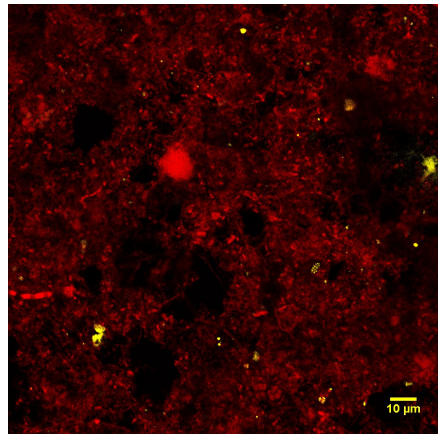


Figure 3.24 – Image obtained by FISH analysis of GAO (yellow) and all bacteria (red) present in the activated sludge. The sample was collected the 04.04.2013, at day 53

3.3.2 The abundance of AOB and NOB

The biofilm in the nitrification process was investigated by FISH analysis. The abundance of AOB appeared to vary with the influent composition during the experimental period. The amount of NOB in the biofilm increased during the experiment, and did not seem to be as affected by the influent composition as the AOB. The abundance of AOB and NOB in the biofilm during this experiment is illustrated in Figure 3.25 with their respective standard deviations of the mean. The NOB seemed more homogeneously distributed in the biofilm than the AOB, giving lower and more stable standard deviations for each sampling point.

The average population of AOB and NOB in this study was almost identical at 12.9 ± 1.8 % and 12.4 ± 1.6 %, respectively.

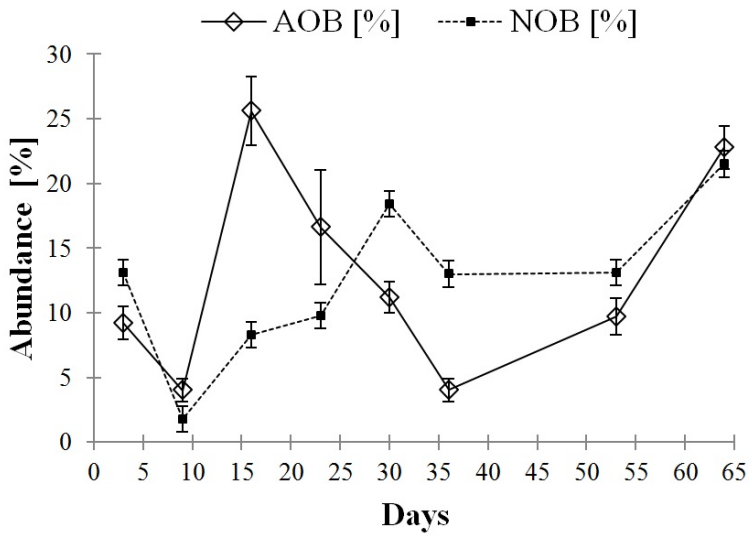


Figure 3.25 – The abundance of AOB and NOB in a biofilm from the nitrification reactor, presented in percent with their respective standard deviations of the mean.

The abundance of AOB seemed to respond to the changes in the concentration of ammonium in the inlet, as illustrated in Figure 3.26. A short period after an increase or decrease in the ammonium concentration, the population of AOB responded in accordance with this change. The reduction in the HRT at day 34 could have stimulated the growth of AOB, since more ammonium entered the reactor per hour.

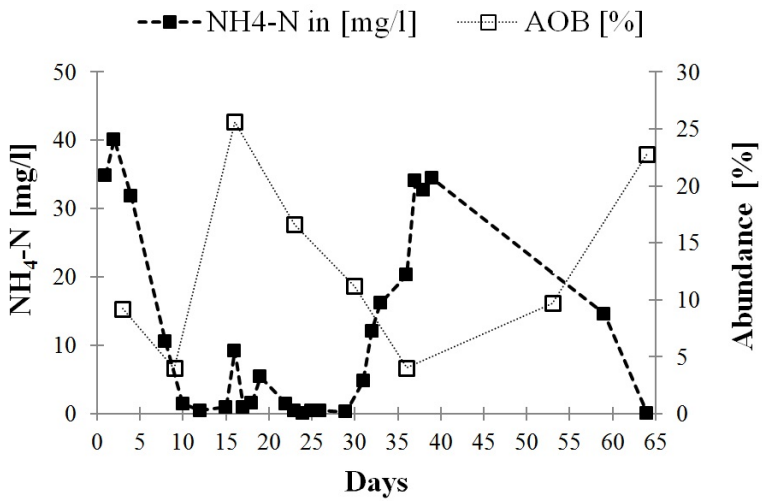


Figure 3.26 – The inlet concentration of NH_4-N presented in mg/l and the abundance of AOB presented in percent in the nitrification reactor throughout the experiment.

The amount of NOB grew steadily throughout the experiment, and did not seem gravely affected by the fluctuations in the concentration of ammonium. Nitrification is a two step process, and the NOB have to wait for the AOB to oxidize the ammonium to nitrite. This implies that when the AOB are exposed to substrate limitations, the NOB will be affected at a later stage than the AOB,as can be seen around day 25 to 35 in Figure 3.25.

The growth yield of AOB is higher than for NOB, and can explain the more prominent shifts in the abundance of AOB. The localization of the microbial communities in the biofilm may also render them more or less protected against environmental changes and predators such as *protozoa*. Huws et al. (2005) reported that bacterial growth in a biofilm may not give protectionagainst these grazing predators. Biomass loss due to erosion can also affect the microbial community. Okabe et al. (2004) suggested that

the AOB were localized in the outer layers of the biofilm, while the NOB were found deeper in the biofilm. The localization of AOB gives them an advantage of oxygen and substrate availability, but also makes them more exposed to predators and erosion than the NOB, which may also explain the continuous growth of NOB as opposed to the AOB.

The dissolved oxygen concentration in the reactor was on average 5.3 ± 0.9 mg/l and was not considered limiting in this study. Therefore it can be assumed that the substrate availability and diffusion into the biofilm were the crucial factors during this experiment.

Zhang et al. (2013) studied the responses of biofilm characteristics to variations in temperature and NH_4-N loading in a moving-bed biofilm reactor treating micro-polluted raw water. They found that when exposed to low levels of NH_4-N, the AOB and NOB accounted for 22.1 ± 2.6 % and 15.8 ± 2.0 % of the total biomass. When the NH_4-N loading increased, the populations increased to 31.6 ± 4.2 % and 20.8 ± 2.2 %. The quantified populations in this study were lower than found by Zhang et al. (2013), but a positive effect was observed when the concentration of NH_4-N increased.

High levels of COD in the influent can enhance the growth of heterotrophic bacteria and affect the nitrification process negatively. In this study, the A/O bio-P process prior to the nitrification reactor removed most of the soluble COD present in the wastewater. In addition, the membrane filtrated the wastewater before nitrification and therefore no particulate COD was transferred to the nitrification process. Inhibition of the nitrification process due to high loadings of COD was therefore considered negligiblein this study.

Despite the fact that the AOB was negatively affected by the reduction in NH_4-N concentration in the inlet, the total nitrifying community (AOB and NOB together) in the nitrification reactor increased during the experimental period, as illustrated in Figure 3.27. The average nitrifying community was 25.3 ± 1.7 %.

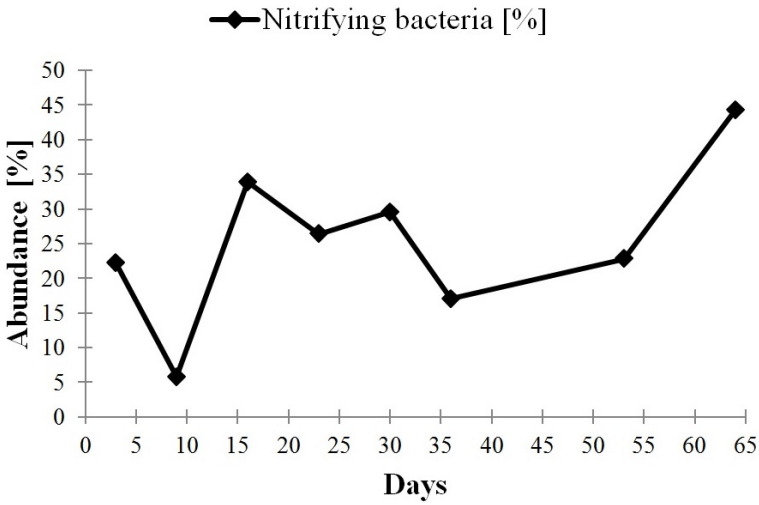


Figure 3.27 – The abundance of nitrifying bacteria in the biofilm, being the total of AOB and NOB together.

The AOB and NOB were often observed in well defined spherical clusters, but also occurred in smaller irregular microcolonies in this study. Figure 3.28 and 3.29 show two images of the AOB and NOB in the nitrifying biofilm, obtained by FISH analysis.

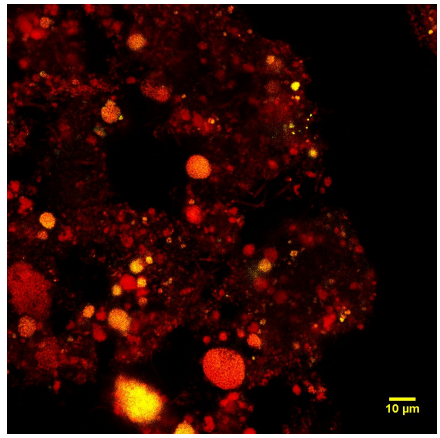


Figure 3.28 – Image obtained by FISH analysis of AOB (yellow) and all bacteria (red) present in the biofilm. The sample was collected the 18.03.2013, at day 36.

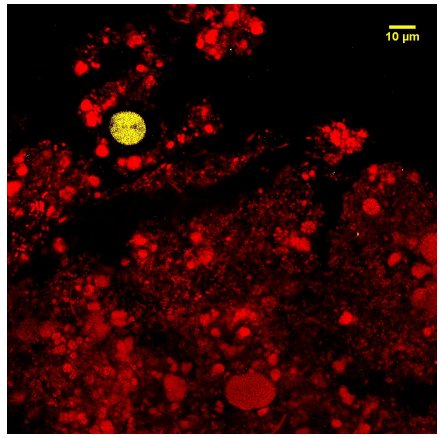


Figure 3.29 – Image obtained by FISH analysis of NOB (yellow) and all bacteria (red) present in the biofilm. The sample was collected the 13.02.2013, at day 3.

3.4 Live and dead cells in a nitrifying biofilm

The fraction of live and dead cells in a nitrifying biofilm from the 06.03.2013 was investigated. The sample was collected during a period where nitrification took place in the A/O bio-P process, and little ammonium entered the nitrification reactor.

The result, illustrated in Figure 3.30 and 3.31, revealed that 56,28 % of the nitrifying biofilm consisted of dead cells. Figure 3.30 illustrates the biofilm thickness as function of the live/dead ratio. The high live/dead ratio was most probably caused by substrate depletion in the reactor. Between day 10 and 29, the level of ammonium entering the reactor was close to zero, leaving the nitrifying bacteria with no substrate.

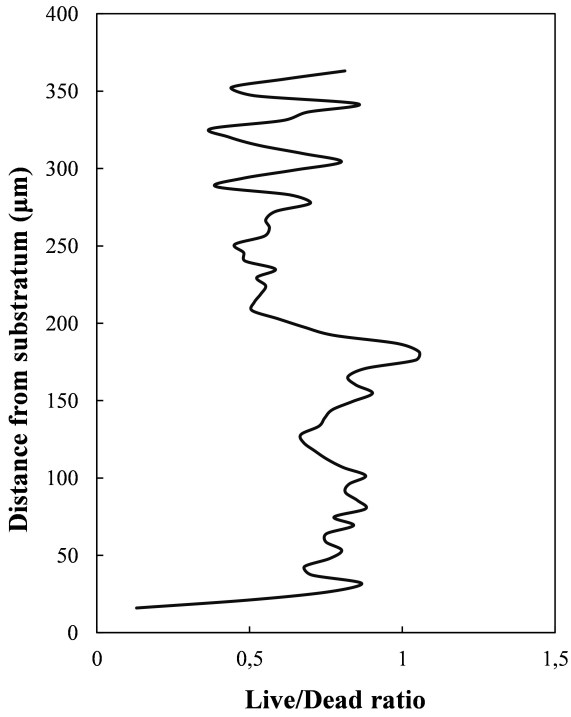


Figure 3.30 – The ratio of live and dead cells throughout the nitrifying biofilm from 06.03.2013. The substratum is the plastic carrier and the distance from the substratum illustrates the thickness of the biofilm.

The biofilm was measured to be about 360 µm thick. The largest fraction of dead cells was found in the outer layer of the biofilm. Ødegaard (2006) suggested that a low carbon loading rate resulted in "fluffy" biofilm dominated by stalked ciliates. The outer layers could also be *protozoa* or heterotrophic bacteria. The concentration of COD in the reactor was low and could explain the high amount of dead cells in this part of the biofilm.

On the inside of this layer the biofilm had a higher fraction of live cells, increasing as the distance from the carrier increased. This could be explained by the oxygen and substrate diffusion rates in the biofilm, meaning that in the inner layers of the biofilm had less oxygen and substrate available. Ødegaard (1993) reported a preferable thickness of a nitrifying biofilm around 100 µm. The biofilm in this study can therefore be considered thick with possibly poor conditions for growth in the inner parts of the biofilm.

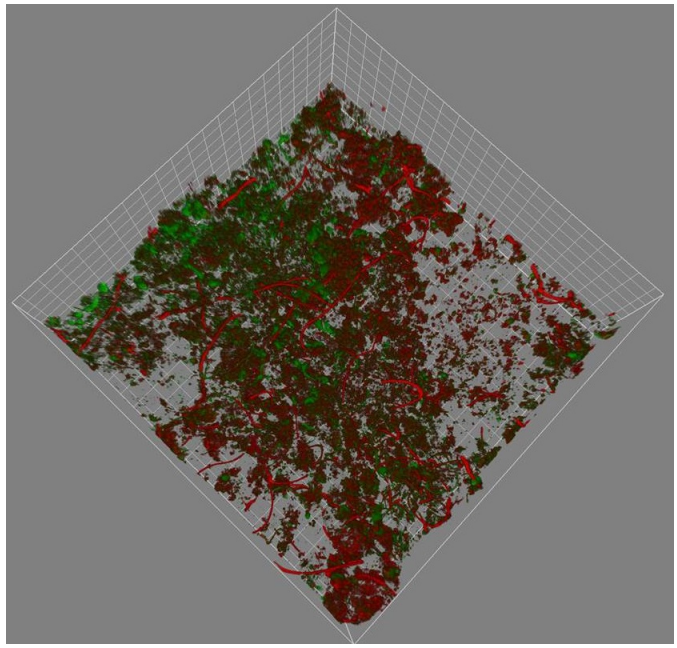


Figure 3.31 – Image of the nitrifying biofilm stained with the LIVE/DEAD BacLight^{TM} Bacterial Viability Kit for detection of live (green) and dead (red) cells. The image is taken looking down at the top of the biofilm.

3.5 Sludge volume index and filamentous bacteria

The sludge volume index (SVI) was calculated between the 25.02.2013 and 21.03.2013. The results showed a relatively stable SVI throughout the measured period, as illustrated in Figure 3.32. The average was at 207 ± 27 ml/g, which is high for an activated sludge system. This indicates poor settling of the sludge and potentially high concentrations of filamentous bacteria.

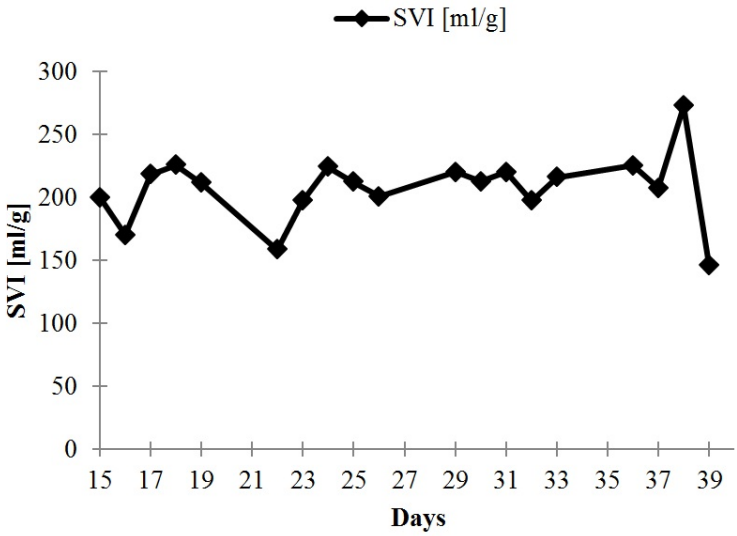
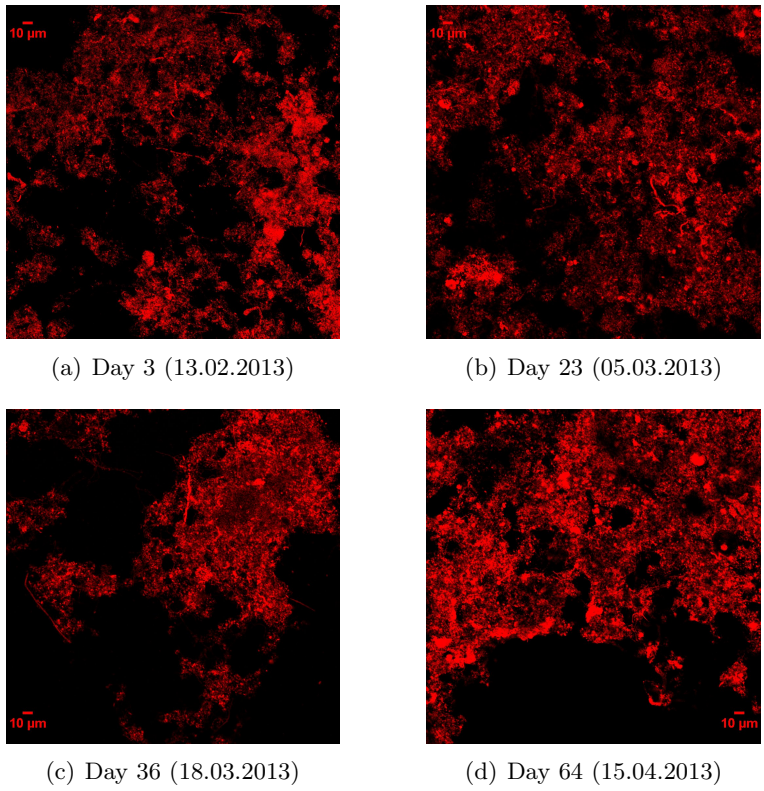


Figure 3.32 – The calculated SVI from the 25.02.2013 to 21.03.2013.

To determine whether or not filamentous bacteria were present in high concentrations, sludge from four sampling dates were examined through FISH analysis. The results revealed that filamentous bacteria were not dominant in these samples regardless of the high SVI. This result cannot be interpreted as a quantification of filamentous bacteria, but simply an observation and indication of the filamentous population present in the sludge. The images are presented in Figure 3.33.

The high SVI index may be explained by the size of the flocs present in the activated sludge. Large flocs will sink more easily than small flocs, giving a lower SVI. To reduce membrane fouling, the membrane aeration



(a) Day 3 (13.02.2013) (b) Day 23 (05.03.2013)

(c) Day 36 (18.03.2013) (d) Day 64 (15.04.2013)

Figure 3.33 – Detection of filamentous bacteria in four activated sludge samples. The samples were stained with the EUXmix and observed with a CLSM through a 20x objective.

was kept around 5 l/min. In this system the membrane was integrated in the bioreactor, and the aeration of the membrane affected the aeration of the bioreactor. The aeration could have teared the bigger flocs into smaller pieces, explaining the high SVI.

Chapter 4

Summary and future work

4.1 Summary

The overall system performance for nitrogen, carbon and TSS removal was successful, and in accordance with EU regulations. On the contrary, the removal of phosphorus was not satisfactory. The system handled the decrease in HRT well. The microbial communities investigated by FISH analysis were present in all samples, but their abundance varied.

The main reason for the unstable phosphorus removal was probably due to substrate limitation and nitrification in the aerobic reactorof the bio-P system. The following production of nitrate in the aerobic reactor lead to anoxic conditions in the first reactor. The carbon entering the reactor was then oxidized by denitrifying bacteria instead of being converted to PHB and stimulating the growth of PAOs in the system. The theory was confirmedby FISH analysis revealing a drop in the abundance of PAOs during this period. When the nitrification activity ceased, the amount of PAOs in the system started to increase followed by the P-removal efficiency. Nitrification in the A/O bio-P system could have been stimulated by the decrease in COD in the influent wastewater. Less carbon was available for oxidation, leading to over-aeration in the second reactor. The abundance of PAOs seemed positively influenced by the decrease in the HRT, giving them more substrate per hour.

The abundance of GAOs was not affected by the nitrification in the aerobic reactor in the same manner as the PAOs, confirming the theory of a

lower decay rate for GAOs. The amount of GAOs in the biomass was stable at first, but increased when the COD concentration in the influent increased and the HRT was reduced. At the last day of the experiment the amount of GAOs exceeded the amount of PAOs, maybe contributing to the decreasing P-removal efficiency because of substrate competition.

A difference, in terms of the abundance of PAOs and GAOs, was observed in the two reactors of the A/O bio-P process. Moreover, a difference between the fraction of PAOs in the sludge and a carrier in an IFAS system was also observed.

Despite the possibility of biomass loss due to erosion and predators the abundance of AOB in the nitrifying biofilm seemed mostly dependent on the concentration of ammonium in the inlet of the reactor in this study. A change in the substrate concentration was, after a few days, reflected in the amount of AOB in the biofilm. The NOB, possibly more protected in the deeper layers of the biofilmand affected by the substrate limitation at a later stage than the AOB, increased steadily during the experiment. Despite the sensibility of the AOB, the total nitrifying community in the biofilm increased during the experiment.

Oxygen and substrate diffusion can become a limiting factor in the deeper layers of the biofilm, causing cell death if the biofilm is too thick. An analysis of the biofilm showed an increase of dead cells close to the carrier, supporting this theory.

The PAOs and GAOs in the activated sludge formed small clusters evenly spread in the sample. In contrast, the AOB and NOB often formed larger spherical clusters. The investigation of AOB and NOB in the activated sludge revealed their presence, but in smaller clusters than found in the biofilm. These observations suggest that the geometry of the carrier protected the biofilm and the microbial communities from being teared into smaller clusters. This could strengthen their position compared to communities in suspended growth. The high sludge volume index coincide well with the theory of small flocs in the sludge investigated, since large amounts of filamentous bacteria were not detected.

The average pH, temperature and DO concentrations were at reasonable levels, and should not have affected the system negatively. However, sudden shifts from day to day could have an impact on the system performance.

FISH was used to monitor the shifts in the microbial communities in the different reactors. The method was a time efficient way of monitoring the abundance of different bacteria. The results obtained by FISH coincided well with the system behaviorand different events during the experiment. The analysis also allowed the observation of the spatial arrangement in the sample and the morphology of the cell clusters. The analysis can suffer from pitfalls such as autofluorescence and methodical errors, but is satisfactory as a rough estimate of the microbial community. FISH targets specific bacteria depending on the probes used, and is useful if the presence of some types of bacteria is to be clarified.

The combination of membrane and MBBR technology produced a high quality effluent, with exception of the phosphate content. The membrane had to be changed once and was only cleaned with water, indicating a good membrane performance with no major clogging of thepores. When substrate was available, the post-denitrification process was successful. When the nitrification activity ceased and the concentration of nutrients in the influent increased, an improvement in the P-removal efficiency was observed.

4.2 Future work

A more detailed study of the biomass in the two reactors of the A/O bio-P system could be interesting in future research in order to optimize this process. The difference between the microbial communities in suspended and attached biomass could also be investigated more throughly.

During this experiment, not enough data was collected to see how abrupt changes in operational parameters such as pH, DO and temperature could affect the biomass, but this could be an interesting topic for further research.

To improve the conditions for A/O bio-P removal and avoid nitrification, the geometry of the second reactor could be optimized. A complete mixing was hard to achieve, with sludge settling in the corners of the reactor. The nitrifying bacteria have a slow growth rate, and less time in the aerobic zone of the A/O bio-P system could reduce their proliferation. To avoid nitrification in the A/O bio-P process the system configuration could also be modified. An additional reactor could be added to the recirculating loop, which maybe would eliminate high concentrations of oxygen and ni-

trate entering the anaerobic reactor, since some carbon will be oxidized in this additional reactor. Placing the membrane in a separate compartment would facilitate the regulation of the DO concentration in the bioreactor if nitrification was already occurring, and at the same time not affect the membrane performance.

Replacing the post-denitrification configuration with pre-denitrification would also be an interesting topic for further research of this type of system. Since the system handled well the reduction in the HRT it could be interesting to further decrease the HRT to observe the limit of the system.

Real wastewater is subjected to fluctuations in the composition due to uncontrollable events such as heavy rainfall. The use of synthetic wastewater would make it easier to control the shifts in the influent composition and consequently the influence on the microbial community in the system. Establishing a stable microbial community can take time and depending on the time-frame of the project, the use of synthetic wastewater could be considered, at least in the beginning of the experiment.

Chapter 5

Conclusions

Characteristics of the biomass in a biofilm membrane bioreactor (BF-MBR) treating municipal wastewater were investigated. The microbial communities were monitored by the combination of Fluorescence *in situ* hybridization (FISH) analysis, confocal laser scanning microscopy (CLSM) and the software *daime*. The abundance of ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), phosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) was quantified and compared to operational conditions.

The FISH procedure was adapted and optimized for this study. FISH was found suitable for quantifying microbial populations as well astheir spatial arrangement and morphology.

The BF-MBR produced a high quality effluent in terms of chemical oxygen demand (COD), nitrogen and total suspended solids (TSS). The system handled well the reduction in the HRT. The removal of phosphorus was not satisfactory.

The microbial communities investigated by FISH analysis were detected in all samples, but their abundance varied. Substrate limitation due to reduced nutrient loading and the activity of denitrifying bacteria in the anaerobic reactor of the A/O bio-P process, affected the population of PAOs negatively. Results and observations from this study suggest that for the enrichment of PAOs in an A/O bio-P system it is crucial to have strictly anaerobic conditions in the rector designated for this purpose, and sufficient

substrate available. When the amount of PAOs increased, the phosphorus removal improved. A higher decay rate was observed for PAOs than GAOs. It might be assumed that the high decay rate of PAOs was not caused by the substrate competition with GAOs, but rather with denitrifying bacteria.

In this study, the abundance of GAOs and AOB seemed solely limited by the available substrate being carbon and ammonium, respectively. The amount of NOB and the total nitrifying community increased despite of periods with ammonium limitations. The entire microbial community investigated handled the reduction in the hydraulic retention time well.

The microbial communities of the biomass were found to be more protected on the inside of a carrier, forming larger spherical clusters than in the activated sludge.

A live/dead analysis of the nitrifying biofilm suggested limited diffusion rates in the deeper layers of the biofilm leading to cell decay.

The abundance of PAOs on a carrier in an IFAS system was found to be superior of the amount of PAOs detected in the activated sludge surrounding the carrier. A difference in the amount of PAOs and GAOs in the two reactors of the A/O bio-P process was also found.

Large amounts of filamentous bacteria were not observed in the A/O bio-P system, and the high sludge volume index was linked to sludge flocs being teared into smaller parts due to aeration, giving poor settleability of the sludge.

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Appendix A

FISH protocol

The protocol was based on information provided by Gilda Carvalho at the Universidade NOVA de Lisboa, Amann et al. (1995) and Nielsen et al. (2009). The protocol has been optimized to fit this study.

Step 1 - Sample preparation

In this study both biofilm from carriers, and activated sludge were collected from the wastewater pilot. One carrier from the nitrification reactor was collected for FISH analysis at each sampling point. The biofilm was scraped off the carrier using a sterile tool, *e.g* a pipette tip and suspended in 0.5 ml of 1x Phosphate Buffered Saline (1x PBS). The samples of activated sludge required no further preparation after they were collected from the reactors.

Step 2 - Sample fixation

1. Thaw frozen aliquots (1.5 ml) of 4 % Paraformaldehyde (PFA) in the fume hood.

2. Add 0.5 ml of sample to the PFA and mix.

3. Incubate sample at 4°Cfor 2 - 2.5 hours.

4. Centrifuge sample at 11 000 rpm for 3 minutes.

5. Decant the PFA and add 1 ml of 1x PBS to the pellet.
6. Centrifuge sample at 11 000 rpm for 3 minutes.
7. Decant the PBS
8. Repeat steps 6 and 7 again.
9. Add 0.5 ml of PBS and 0.5 ml of 96 % ethanol (-20°C) to the pellet.
10. Store at -20°C.

Step 3 - Sample application and dehydration

1. Identify slides with a pencil.
2. Apply 3 x 5 µl in 4 wells for activated sludge samples.
   Apply 3 x 10 µl in 4 wells for biofilm samples.
   Let the sample air dry between each layer.
3. Prepare ethanol solutions in 50 ml Falcon tubes; 50 %, 80 % and 96 %.
   The solutions can be used multiple times.
4. Dehydration of samples in the three different concentrations of ethanol, starting with 50 %, then 80 % and finally 96 %.
   Soak the sample for 3 minutes in each solution.
   2 slides can be dehydrated per tube, back to back.
5. Air dry

Step 4 - Probe hybridization

1. Make a "bed" of tissue paper inside a 50 ml Falcon tube. Use one tube for each slide.
2. Prepare the hybridization buffer in a 2 ml Eppendorf tube:
   a) 360 µl NaCl 5M
   b) 40 µl TRIS-HCl 1 M
   c) MilliQ water (see Table A.1 for amount)
   d) 2 µl SDS 10 % on the lid (SDS can interact with NaCl and precipitate. Applying the SDS on the lid ensures that the SDS is the last component to be mixed with the solution.)

e) Formamide (see Table A.1 for amount. Added in the fume hood.)

Prepare one Eppendorf tube for each slide.

3. Apply 8 µl of hybridization buffer in each well. Do not touch the slide with the tip of the pipette!

4. Apply the rest of the buffer to the tissue in the Falcon tube.
This will keep the inside of the Falcon tube at a constant hybridization atmosphere.

5. Apply 1 µl of the specific probe and 1 µl of the EUB mix to three wells.
Mix the probes with the hybridization buffer without touching the slide.
Keep one well with sample without any probe to check for autofluorescence at the microscope.

6. Place the slide into the Falcon tube in a horizontal position and seal the tube with the cap and parafilm.

7. Place the Falcon tube in the oven at 46 °C for 90 minutes.
A rack tipped on the side can be used to ensure that the tubes stay in a horizontal position.

Table A.1 – Amount of formamide and MilliQ water to prepare the hybridization buffer

Fomamide (µl)	**% Formamide**	H_2O **MilliQ (µl)**
0	0	1598
100	5	1498
200	10	1398
300	15	1298
400	20	1198
500	25	1098
600	30	998
700	35	898
800	40	798
900	45	698
1000	50	598

Step 5 - Washing

1. Prepare the washing buffer in 50 ml Falcon tubes. One tube for each slide.
   a) NaCl (see Table A.2 for amount)
   b) 1 ml TRIS-HCl 1M
   c) EDTA 0.5M (see Table A.2 for amount)
   d) Raise the volume with MilliQ water up to 50 ml
   e) 50 µl SDS 10 % (added at the end to avoid precipitation)
2. Place the buffer in a water bath at 48°C before washing the slides.
3. Wash each slide with a Pasteur pipette and let the excess go in a beaker. Wash the slides from the well without probe and downwards to make sure that there is no cross contamination.
4. Place the slide in the Falcon tube with the washing buffer and put the tube in a water bath at 48 °C for 10-15 minutes.
5. Take the slides out and wash front and back with MilliQ water at 4°C.
6. Dry the slides quickly to remove every single droplet from the slide to prevent probe dissociation.
   Use compressed air if available.

Step 6 - Mounting slides

1. Apply a few drops of Vectashield to the dried slides
2. Place the cover slip over the wells and gently press it to force the Vectashield to cover all wells
3. Clean the excess with a paper tissue
4. Apply nail polish to the edges of the cover slip to seal the slip to the slide and prevent the immersion oil from combining with the Vectashield
5. Store the slides at -20°C in the dark

Table A.2 – Amount of NaCl and EDTA to prepare the wash buffer

% Formamide	NaCl (µl)	EDTA (µl)
0	9000	-
5	6300	-
10	4500	-
15	31800	-
20	2150	500
25	1490	500
30	1020	500
35	700	500
40	460	500
45	300	500
50	180	500

Step 7 - Microscope
Zeiss LSM 700 confocal laser scanning microscopy (CLSM) and the software ZEN 2009 were used to observe the samples and obtain the images.

During this study, the following settings were applied:

- 40x Oil objective
- Filters for the Cy3 and Cy5 fluorochrome
- Scan speed: 7
- Data depht: 8 bits
- Mode: linear
- Pin hole: 1
- Scan average: 8
- Pixel resolution: 1024
- The well without probe was used to check for autofluorescence
- 30 pictures were taken with the same settings for quantification

Correcting for autofluorescence

The option "Auto Exposure" was used on a well containing biomass and probe to get the optimal settings. Afterwards the well with biomass and without probe was examined using the same settings as obtained in the previous step. If there was a signal, this meant that the sample was autofluorescent and this needed to be corrected to obtain a reliable result. The options Gain and Offset were adjusted so that the image was dark with little biomass lightning up.

Then the three wells with probe was used to acquire the images needed for the analysis.

Step 8 - Image analysis

Quantification was done by using the software *daime* (Daims et al., 2006). The software was downloaded from http://www.microbial-ecology.net/daime/.

ImageJ (Abramoff et al., 2004) was used to arbitrary adjust the brightness and contrast of the image.

Appendix B

FISH equipment and reagents

B.1 Equipment

- Nitrile gloves
- Hybridisation oven
- Microcentrifuge
- Water bath
- Teflon coated 8- or 10-well slides
- Cover slips
- Micropipettes from 0.5 µl to 1000 µl
- Micropipette tips
- Nail polish
- Vectashield Mounting Medium
- Falcon tubes 50ml
- Eppendorf tubes 2 ml
- Autoclave

B.2 Reagents

Phosphate Buffered Saline, pH 7.2
30x PBS: add 38.7 g Na_2HPO_2-$12H_2O$, 6.6 g NaH_2PO4-$2H_2O$ and 113.1 g NaCl to 500 mL of MilliQ water. Autoclave and store as stock.
Dilute 1:10 for 3x PBS and autoclave.
Dilute 1:30 for 1x PBS and autoclave.

4% Paraformaldehyde
Set up the balance and a heated stirrer in the fume hood.
Warm 65 mL of purified water to 60°C. Weight out 4 g of PFA powder (do not inhale). Add the PFA to the water to obtain a cloudy solution. Add 2 drops of 2 M NaOH and the PFA should be dissolved in 1-2 min. Cool to room temperature and add 33 ml of 3x PBS (safe to remove from the hood now). Adjust the pH to 7.2 with 1 M HCl. Filter throught 0.2 µm filter to remove any undissolved crystals. Aliquot to applicable volumes and freeze. In this study aliquots of 1.5 ml were frozen in 2 ml eppendorf tubes.

5 M NaCl
Add 58 g of NaCl to 200 mL of MilliQ water. After dilution, autoclave.

1 M Tris-HCl
Add 31.5g of Tris-HCl to 150 mL of MilliQ water, dissolve and adjust pH to 7.2 with 2 M NaOH.
Make up to 200 mL with MilliQ water and autoclave.

0.5 M EDTA
Add 18.6 g of EDTA disodium hydrate to 75 mL MilliQ water. Adjust to pH 7.2 with NaOH pellets. Make up to 100 mL with MilliQ water and autoclave.

10 % SDS
Dissolve 10 g of SDS in 100 mL of MilliQ water. Work in the fume wood.

Appendix C

FISH probes

DNA Oligonucleotides, hereafter referred to as probes, were ordered online from Sigma-Aldrich (http://www.sigmaaldrich.com/norway.html). Delivery time was approximately one week. Probes were selected based on information from Nielsen et al. (2009) and probeBase (Loy et al., 2007). Details of the probes ordered are found in Figure C.1.

The probes arrived lyophilised, and had to be diluted before being frozen into aliquots. Dilution was carried out according to instructions provided by Gilda Carvalho and information from the technical data sheet provided by Sigma-Aldrich:

- Autoclaved MilliQ water was added to each probe tube to obtain a concentration of approximately 500 ng/µl.
- The probes were then divided into aliquots of 5 µl needed for the experiment and frozen at -20°C.
- The probes were stored in boxes and protected from light.
- The stock solutions were stored in a dark container at -20°C.

Before usage, the probes had to be diluted once more. Three mixes of probes were used in this study; EUB mix, PAOmix and GAOmix. Eachprobe in these mixes could be hybridized at the same formamide concentration, and therefore could be mixed together prior to dilution. In addition, two probes had a competitor probe, which was included in the dilutions.

The probes were diluted to a final solution of 50 µl according to Table C.1.

Table C.1 – Details of probe dilution prior to usage.

Name of probes	**Amount of probe**	**Amount of MilliQ water**
EUBmix	3 x 5 µl	35 µl
PAOmix	3 x 5 µl	35 µl
GAOmix	3 x 5 µl	35 µl
NOB	2 x 5 µl	40 µl
AOB	1 x 5 µl	45 µl

Target bacteria	Oligo name	Sequence 5' to 3'	Modification	Reference
	EUBmix:			
Most Bacteria	EUB338	GCTGCCTCCCGTAGGAGT	Cy5	Amann R.I et al (1990)
	EUB338 II	GCAGCCACCCGTAGGTGT	Cy5	Daims H. et al (1999)
	EUB338 III	GCTGCCACCCGTAGGTGT	Cy5	Daims H. et al (1999)
	PAOmix:			
Candidatus Accumulibacter phosphatis	PAO462	CCGTCATCTACWCAGGGTATTAAC	Cy3	Crocetti G. R., et al (2000)
	PAO651	CCCTCTGCCAAACTCCAG	Cy3	Crocetti G. R., et al (2000)
	PAO846	GTTAGCTACGGCACTAAAAGG	Cy3	Crocetti G. R., et al (2000)
	GAOmix:			
Candidatus Competibacter phosphatis	GB_G2	TTCCCCAGATGTCAAGGC	Cy3	Kong et al., (2002)
	cGB_G2	TTCCCCGGATGTCAAGGC	-	Kong et al., (2002)
	GAOQ989	TTCCCCGGATGTCAAGGC	Cy3	Crocetti et al, (2002)
	AOB			
Nitrosomonas	Nso1225	CGCCATTGTATTACGTGTGA	Cy3	Mobarry B. K., et al (1996)
	NOB			
Nitrobacter	NIT3	CCTGTGCTCCATGCTCCG	Cy3	Wagner et al. (1996)
	cNIT3	CCTGTGCTCCAGGCTCCG	-	Wagner et al. (1996)

Figure C.1 – Details of the probes ordered for this study.

Appendix D

The Council Directive 91/271/EEC

An excerpt of The Council Directive 91/271/EEC concerning urban waste-water treatment is listed in Table D.1. Selected parameters are Chemical oxygen demand (COD), Total suspended solids (TSS), Total phosphate (TP) and Total nitrogen (TN).

Table D.1 – The Council Directive 91/271/EEC concerning urban waste-water treatment.

Parameter	**Threshold [mg/l]**	**Minimum reduction [%]**	**Comment**
COD	125	75	
TSS	35	90	p.e.> 10 000
	60	70	2 000 < p.e. < 10 000
TP	1	80	p.e. > 100 000
	2		10 000 < p.e. < 100 000
TN	10	70-80	p.e. > 100 000
	15		10 000 < p.e. < 100 000

Appendix E

Reactor log

E.1 A/O bio-P removal

Table E.1: Reactor log of the A/O bio-P system

Date	Days	Raw pH	P anaerobic Temp °C	DO mg/l	pH -	P aerobic Temp °C	DO mg/l	pH -
11.02.2013	1		17,8	0,16	7,45	17,2	0,6	7,55
12.02.2013	2		18,8	0,14	7,33	17,4	2,02	7,67
13.02.2013	3		18,5	0,18	7,47	17,8	1,5	7,63
14.02.2013	4		18,2	0,15	7,54	17,7	2,25	7,64
15.02.2013	5		19,1	0,16	7,77	18,4	2,46	7,25
16.02.2013	6		19,5	0	7,68	18,9	1,6	7,3
17.02.2013	7		20,6	0	7,52	19,6	0,94	7,63
18.02.2013	8		21,9	0,12	7,51	21,5	0,32	7,68
19.02.2013	9		19,8	0	7,54	19,7	0,37	7,45
20.02.2013	10		18,8	0,19	7,24	18	2,71	6,47
21.02.2013	11		20,1	0,2	7,31	19	2,36	6,59
22.02.2013	12		20,6	0	7,05	19,8	2	6,5
23.02.2013	13		21,9	0	7,27	21,2	1,2	6,43
24.02.2013	14		21,9	0	7,3	21,3	0,2	6,61
25.02.2013	15		22,2	0	7,33	21,5	0,13	6,93
26.02.2013	16		21,7	0,03	7,56	20,7	1,63	6,94
27.02.2013	17		22,4	0,07	7,68	21,8	2,01	6,97
28.02.2013	18		22,7	0	7,64	21,2	0,6	7,01
01.03.2013	19		21,2	0	7,09	20,5	1,3	5,81
02.03.2013	20		21,5	0,01	7,08	21,2	1,36	5,49
03.03.2013	21		22,2	0	6,92	21,6	1,44	5,33
04.03.2013	22		21,9	0	6,68	21,74	0,27	6,04
05.03.2013	23		22,4	0,11	6,91	22	0,67	6,58
06.03.2013	24		21,7	0	7,07	21,1	0,89	6,98
07.03.2013	25	7,15	20,8	0,11	7,15	19,9	1,17	7,18

To be continued on next page

Table E.1 – *Continued from previous page*

Date	Days	Raw	P anaerobic			P aerobic		
		pH	Temp	DO	pH	Temp	DO	pH
			°C	mg/l	-	°C	mg/l	-
08.03.2013	26	7,14	20,3	0,13	7,25	19,1	0,3	7,15
09.03.2013	27		20	0,06	7,28	17,5	4	7,44
10.03.2013	28	7,22	18	0,44	7,25	19,2	5,16	7,42
11.03.2013	29	7,13	20,4	0,07	7,17	15,8	0,32	7,27
12.03.2013	30		21	0,02	7,29	20,3	0,51	7,17
13.03.2013	31	7,34	18,5	0,21	7,48	17	1,21	7,35
14.03.2013	32	7,67	18,6	0,11	7,61	17,8	0,36	7,54
15.03.2013	33	8,99	19,3	0,17	7,83	18,5	0,5	7,58
16.03.2013	34	7,26	21,2	0	7,4	19,3	0,25	7,32
17.03.2013	35	7	21,6	0,03	7,28	20,7	0,02	7,41
18.03.2013	36	6,86	20,9	0,03	7,16	19,8	0,3	7,37
19.03.2013	37	6,79	19,6	0,12	7,33	18,3	0,25	7,36
20.03.2013	38	7,12	19,8	0,18	7,44	18,6	1,25	7,55
21.03.2013	39	7,12	20,1	0,07	7,42	19,4	0,21	7,53
22.03.2013	40	7,29	21,2	0	7,59	20,4	3,12	7,85
23.03.2013	41	7,01	20,2	0	7,39	19,2	2,01	7,65
24.03.2013	42	6,9	23,1	0	7,35	21,8	0,86	7,65
25.03.2013	43	6,84	22,7	0	7,31	21,8	0,81	7,57
26.03.2013	44	6,8	22,4	0	7,13	21,6	0,57	7,3
27.03.2013	45	7,17	22	0	7,13	22,1	0,8	7,01
28.03.2013	46	6,64	21,7	0,07	7,22	21	0,22	7,4
29.03.2013	47	6,81	21,8	0	7,43	20,8	2,07	7,44
30.03.2013	48		22,3	0,03	7,34	21,2	1,23	7,29
31.03.2013	49	6,61	19,5	0,12	7,29	18,3	0,4	7,34
02.04.2013	51	7,19	19,4	0,11	7,22	18	0,39	7,29
03.04.2013	52	6,7	21,6	0,12	7,28	20,9	0,56	7,05
05.04.2013	54	7,09	20,9	0,02	7,34	20,3	1,15	7,21
06.04.2013	55	6,92	20,3	0,04	7,15	19,1	0,58	7,31
07.04.2013	56	7,08	20,9	0,17	7,02	17,6	0,4	8,68
08.04.2013	57	6,89	19,6	0,06	7,21	18,6	0,72	7,25
09.04.2013	58	6,99	20,3	0,03	7,41	20,3	0,36	7,14
10.04.2013	59	7,05	20,9	0,12	7,39	20,3	1,78	7,22
11.04.2013	60	7,74			7,34			7,28
12.04.2013	61	7,18	19,5	0,06	7,31	19	0,34	7,07
13.04.2013	62	7,17	20,8	0,03	7,06	20	2,81	7,08
15.04.2013	64	7,26	20,9	0,02	7,11	20,4	1,27	7,92

E.2 Post-Denitrification

Table E.2: Reactor log of the post-denitrification system

Date	Days	N aerobic			DN anoxic			C aerobic		
		Temp	DO	pH	Temp	DO	pH	Temp	DO	pH
		°C	mg/l	-	°C	mg/l	-	°C	mg/l	-
11.02.2013	1	17,7	4,41	6,58	18,3	0,26	7,69	17,8	5,16	7,92
12.02.2013	2	17,5	5,29	6,51	18,2	0,68	8,1	17,1	10,14	7,78
13.02.2013	3	18	3,6	6,25	18,1	2,11	6,28	17,6	4,23	6,53
14.02.2013	4	17,2	6,76	6,4	17,6	0,86	7,94	17,1	6,34	7,79
15.02.2013	5	18	5,61	6,8	18,9	0,15	8,58	18,7	5,57	7,82
16.02.2013	6	18,7	5,73	7,7	19	2,4	7,33	18,6	7,9	5,92
17.02.2013	7	20,1	4,8	7,54	20,5	0,2	8,6	20,1	4,49	7,99
18.02.2013	8	21	5,06	7,89	21,2	1,85	7,72	20,7	5,3	7,97
19.02.2013	9	19,1	5,19	7,85	19,3	3,09	7,56	19,1	5,57	7,69
20.02.2013	10	17,4	6,27	7,05	17,8	0,57	7,44	17,3	6,13	7,9
21.02.2013	11	18,9	5,37	7,24	19,6	0	7,83	19,1	5,3	7,46
22.02.2013	12	19,1	5,06	7,34	20,4	0,19	8,46	20	4,94	7,28
23.02.2013	13	21,2	4,64	7,23	21,7	1,77	6,77	21,6	4,9	7,09
24.02.2013	14	20,9	4,98	7,4	21,4	0,83	6,86	21,4	5,12	7,29
25.02.2013	15	21	5,1	7,83	21,2	1,47	7,29	20,8	5,05	7,8
26.02.2013	16	20,3	5,49	7,75	20,6	0,14	7,47	19,9	5,6	7,97
27.02.2013	17	21,3	5,06	7,78	21,6	0,32	7,55	20,8	5,07	8,05
28.02.2013	18	20,8	5,34	7,82	21,4	0,47	8,18	20,6	5,06	8,37
01.03.2013	19	20,4	5	6,57	21	0,02	7,82	20,9	4,88	7,52
02.03.2013	20	20,3	5,39	6,03	20,7	0,39	8,42	19,9	5,3	8,28
03.03.2013	21	21,6	4,55	5,94	22	0,18	7,6	21,5	4,84	7,81
04.03.2013	22	21,4	4,82	6,58	21,9	0,33	8,58	21,4	4,45	8,04
05.03.2013	23	21,3	5,33	7,31	21,5	0,07	7,83	21,1	5,09	7,97
06.03.2013	24	20,6	5,75	7,86	21,2	0	8,22	20,4	5,7	8,25
07.03.2013	25	19,8	5,8	8,07	20,2	0,3	8,32	19,8	5,55	8,37
08.03.2013	26	18,9	6,27	8,1	19,4	0,58	8,4	18,8	6,14	8,35
09.03.2013	27	18,7	5,4	8,3	19,4	0	7,89	19,6	5,39	8,18
10.03.2013	28	16,1	6,8	8,07	17,1	0,97	7,91	16,3	5,89	8,31
11.03.2013	29	19,4	3,61	8,14	19,9	0,54	5,08	19,4	3,47	8,35
12.03.2013	30	18,7	5,77	8,35	19,6	0,5	8,54	19,4	5,27	8,47
13.03.2013	31	16,1	7,07	8,32	17,3	0,73	8,57	17,1	5,94	8,34
14.03.2013	32	16,1	7,05	8,58	17,2	0,72	8,85	16,9	5,81	8,66
15.03.2013	33	17,1	5,92	9,83	17,8	0,46	10,08	17,5	5,36	9,83
16.03.2013	34	19,3	5,07	8,02	20,3	0,05	8,23	19,9	4,97	7,84
17.03.2013	35	19,5	7,58	7,96	20,2	0	8,61	20	6,56	7,3
18.03.2013	36	18,8	8,74	7,4	19,7	0,81	8,67	19,6	7,6	7,616
19.03.2013	37	17	6,01	6,76	18,2	0,76	8,42	18	5,32	7,9
20.03.2013	38	18,2	5,31	6,85	18,5	0,32	8,34	18	5,88	8,05
21.03.2013	39	19,1	4,2	6,72	19,4	0,25	7,89	18,9	4,77	7,72
22.03.2013	40	21,3	4,18	6,33	22,2	0,04	7,22	21,4	4,66	7,82
23.03.2013	41	19,3	4,61	6,54	19,2	0,12	7,55	18,7	5,37	7,37
24.03.2013	42	22	4,08	6,61	22,4	0,05	8,19	22	4,88	7,86
25.03.2013	43	21,7	4,37	5,54	21,9	0	8,38	21,5	5,2	7,85
26.03.2013	44	22,1	4,8	6,71	22,6	0,03	8,3	22,1	5,35	7,63
27.03.2013	45	21,3	4,67	6,35	22	0,11	7,66	21,4	5,01	7,84
28.03.2013	46	22,2	4,46	7,53	23,4	0,84	7,64	22,6	4,51	8,25
29.03.2013	47	20,9	4,48	6,16	22,1	0,2	7,53	22,4	4,91	8,12
30.03.2013	48	22,1	4,86	8,25	22,6	0,19	7,59	23,3	4,94	8,23

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Table E.2 – *Continued from previous page*

Date	Days	N aerobic			DN anoxic			C aerobic		
		Temp	DO	pH	Temp	DO	pH	Temp	DO	pH
		°C	mg/l	-	°C	mg/l	-	°C	mg/l	-
31.03.2013	49	18,5	5,77	8,19	19,3	0,44	7,64	19	5,38	8,19
02.04.2013	51	18,5	4,76	8,15	19,2	0,34	7,83	18,9	4,85	8,3
03.04.2013	52	21	4,81	8,07	21,5	0,12	7,81	21	4,45	8,15
05.04.2013	54	20,7	5,01	7,95	21,3	0,12	7,76	20,9	5,01	8,12
06.04.2013	55	19	4,98	7,59	20,9	0,2	8,03	20,4	5,01	7,92
07.04.2013	56	20,5	4,72	9,31	21	0,3	9,39	20,4	4,58	9,74
08.04.2013	57	19,1	5	8,04	19,6	0,14	8,26	19,4	5,09	8,11
09.04.2013	58	20,4	5,1	8,05	20,9	0,16	8,53	21,7	4,91	8,08
10.04.2013	59	20,7	5,54	8,05	21,3	0,4	8,33	20,9	5,26	8,18
11.04.2013	60			7,91			8,01			7,91
12.04.2013	61	19,2	5,24	8,03	19,3	0,31	7,55	18,9	5,23	8,04
13.04.2013	62	21	5,25	7,97	20,9	0,28	7,47	21,6	5,07	7,96
15.04.2013	64	19,6	5,37	7,89	21,1	0,16	7,89	18,7	5,52	7,99