ON NITROGEN REMOVAL IN WASTEWATER TREATMENT

Bacterial growth and transformations of nitrogen and carbon

Marina Thörn

Göteborg 1997

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ABSTRACT

Nitrifying biofilm growth, production of N_2O and the role of exoenzymes in carbon utilization in denitrification was studied in wastewater treatment with biological nitrogen removal. Denitrification was the dominant nitrate removal process in the anoxic sludge, and the N_2O production as a fraction of denitrification was normally negligible. Occasions with high N_2O production could be connected to low pH. A pH below 6.5 increased the nitrous oxide production as a part of denitrification. The total N_2O production increased with decreasing pH down to pH 5.5, then decreasing again due to the decreasing denitrification.

The denitrification rate in the basin was limited by the amount of easy utilizable carbon source. The rate limiting step was the hydrolysis of polymeric compounds by extracellular enzymes released by the microorganisms. Based on extracellular enzyme activities, the importance of different substrates for denitrification was short chain esters > polysaccharides > peptides > long chain lipids. The exoenzyme activity found in the denitrification basin originated both from production in the basin and import from influent water and recirculating sludge, and no short-term correlation between exoenzyme activity and denitrification rate was found.

Castanospermine inhibited extracellular α-glucosidase activity from two of three bacteria, and in activated sludge. Respiration rate was inhibited by castanospermine in one of the bacterial species grown in starch, and by appromimately 10 % in activated sludge. No inhibition of the respiration rate was found in bacteria grown in complex medium. Castanospermine was concluded to not be unsuitable for measuring the importance of available saccharides in natural samples.

The nitrification process in a biofilm takes several months to initilize due to the slow growth of the nitrifying bacteria. Activities measured during the start-up period for a trickling filter showed that the nitrifying bacteria increased logaritmically at least from day eight. Irreversible attachment of the bacteria was not reached until day 20. Nitrification rate and bacterial count was correlated at least until day 80, indicating that nitrifiers were a constant fraction of the bacteria. The growth of the biofilm was patchy, and the bacteria was non-uniformly attached to the substratum.

Key words: Activated sludge, bacteria, biofilm, castanospermine, denitrification, exoenzyme, nitrification, nitrogen removal, wastewater treatment

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This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Thörn, M. & Sörensson, F.** (1996) Variation of nitrous oxide formation in the denitrification basin in a wastewater treatment plant with nitrogen removal. *Water Research* **30** (6): 1543-1547

II. **Thörn, M. & Sörensson, F.** (1997) Extracellular hydrolytic enzyme activity in denitrifying activated sludge. *Submitted*

III. **Thörn, M., Mustafa, N., Sörensson, F.** (1997) The effects of castanospermine on bacterial extracellular cell-surface associated α-glucosidase activity and respiration in pure cultures and activated sludge. *Manuscript*

IV. **Thörn, M., Mattsson, A., Sörensson, F.** (1996) Biofilm development in a nitrifying trickling filter. *Water Science & Technology* **34** (1-2): 83-89

Visst kan även "det meningslösa"
lämna betydande spår
spår som vi sällan ögnar
men som för evigt består

droppen som urholkar stenen
har inte stenen som mål
men i tidens längd kan den skapa
en formfulländad skål

Ur Bränningar och aforismer
Eric S. Alexandersson

CONTENTS

1. BACKGROUND

Eutrophication the marine environment is considered a problem today in many areas (10, 114). The eutrophication is due to an enhanced supply of nutrients, mainly nitrogen and phosphorus in the forms of ammonium (NH_4^-), nitrate (NO_3^-) and phosphate ions (PO_4^-). The increasing amount of anthropogenic nitrogen and phosphorus in the sea originates among others from combustion (leading to atmospheric nitrogen), fertilization of soils and forests (leaching to the rivers) and from sewage water.

Carbon and other nutrients like nitrogen and phosphorus supports the growth of algae and bacteria, which when reaching a high biomass can cause oxygen depletion in the deeper waters and sediments, and a subsequent death of higher organisms. Today, all the wastewater treatment plants (WWTP) in Sweden clean the wastewater from carbon and solid particles, and most of them also remove phosphorus by means of chemical precipitation or biological phosphorus removal. Nitrogen is up until now not removed to any large extent.

In the marine environments near Sweden the limiting nutrient is generally considered to be nitrogen rather than phosphorus (40, 117). It is thus important in WWTPs near the coasts to also reduce the effluent nitrogen. The present aim is that the anthropogenic sources of nitrogen should be reduced to at least 50 % of the amount in 1985 (102). This also applies to the effluent from sewage treatment plants, and thus all effluent nitrogen from Swedish WWTPs near the coasts will be reduced by 50%.

The nitrogen supply from wastewater in the Baltic sea and in Kattegatt is small compared to other sources, less than 10 % of the total load (78, 118). The nitrogen supply from Rya WWTP contributes to about 30 % the total inorganic nitrogen in the Göta river delta, effluent to the archipelago of Göteborg (126). The dominating nitrogenous species effluent from the WWTP is ammonium, and about 85 % of the ammonia in the Göta river after the outlet from the WWTP originated from the WWTP (126).

The bacteria utilizing ammonia and nitrate are crucial for the nitrogen removal process in wastewater treatment. Parameters affecting their growth and nutrient utilization also affect the nitrogen removal process, and could lead to insufficient nitrogen removal.

The introduction of nitrogen removal in the WWTPs could also lead to other, less favourable side effects. One could be the production of other end products than the desired (N_2), that is equally or more detrimental to the environment than the removed nutrients.

The work presented here was done to further increase the knowledge about the microbial processes in the nitrogen removal process, thereby increasing the possibility to achieve a generally functioning process.

2. AIMS

The aims of this thesis was to investigate possible factors affecting the performance of the nitrogen removal processes in a wastewater treatment plant. The ultimate aim was to provide knowledge for future modelling and operational control. The questions were centered around the microbial processes denitrification and nitrification.

Denitrification	✵ Unwanted end products, especially nitrous oxide. (paper I)
	✵ Access to carbon source for denitrification. (paper II and III)
Nitrification	✵ Initialization of the process in a biofilm. (paper IV)

The denitrification process is discussed in section 4 and the nitrification process in section 5.

3. WASTEWATER TREATMENT

3.1 Municipal wastewater

Municipal wastewater includes both water from households and industries, and sometimes also run-off water from urban areas. The wastewater consists mostly of biodegradable material. The organic material, approximately 70% of the solids, consists on average of carbohydrates (30 %), volatile and non-volatile acids (20 %) amino acids, peptides and proteins (10 %) and the rest is fats, grease and surfactants (131). Organic material, nitrogen, phosphorus and bacteria are the main parts of the wastewater from households. The aim for the treatment systems used today is primarily to reduce the levels of these compounds and suspended solids, in order to minimize eutrophication of the surrounding waters and also to prevent waterborne diseases. Besides these compounds other types of hazardous substances, mostly not removable in the WWTP, are also released into the sewers. These chemicals (predominantly heavy metals and toxic organic compounds) mostly originates from the chemicals used in households and industries, but also from land run-offs (102, 11). Some of these compounds can be reduced by the bacterial degradation and uptake in the activated sludge or by adhesion to particles and subsequent sedimentation in the WWTP.

3.1.1 *Nitrogen in wastewater*

The source of nitrogen in municipal wastewater are primarily human excretion, of which about 75% is urea and the rest is other organic nitrogen compounds (50). Both urea and the other organic nitrogen is to a large extent converted to ammonia by microbial processes, either during transport in the sewers or at the WWTP. Ammonia is thus the predominately soluble nitrogenous species present in the WWTP. Some ammonia is assimilated by the bacteria to support growth. Nitrate and nitrite can also be used, but is generally not preferred when ammonia is present since their utilization requires more energy (43). Ammonia assimilated by the bacteria in the aerated activated sludge are removed from the wastewater during the postsettling process .

3.2 Conventional activated sludge process

Today, all WWTPs in Sweden removes solid substances by some sort of mechanical process and carbon substances are removed with the help of microorganisms. Prior to the activated sludge process mostly larger particles are removed by lattices and precipitation. 40-60 % of the suspended solids and 25-35 % of the BOD is removed in this step (16). The organic material is removed in aerated activated sludge basins, and the sludge is separated from the water by postsedimentation of the activated sludge flocs (Fig. 1a). This the most widely used biological wastewater treatment system today, for both municipal and industrial wastewater.

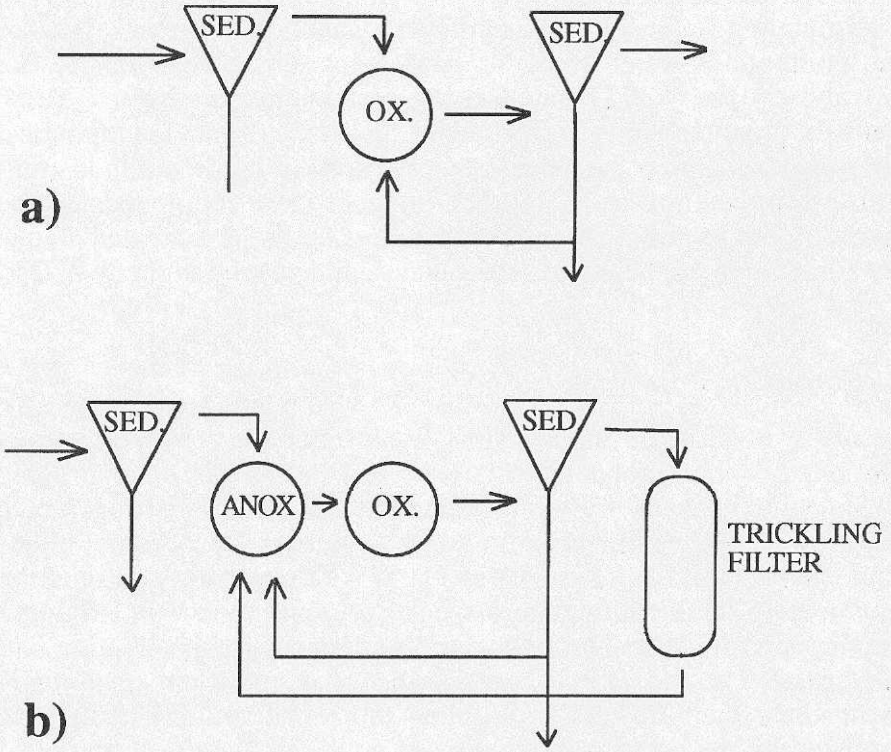

Figure 1. Schematic drawings of a) the conventional activated sludge process and b) the nitrogen removal process at Rya WWTP.
Sed. = settling basins, anox = anoxic basin, ox = aerated basin.

3.3 Activated sludge

Activated sludge is comprised of organic material originating from the influent water, growing bacteria and their extracellular polymer matrix, and of course, the water which is to be cleaned. Dissolved organic matter in the water is removed by microbial degradation and assimilation and by the subsequent precipitation. The tendency of WWTP bacteria to adhere to each other and to the suspended solids in the water leads to the aggregation into flocs in the sludge (33, 154). These flocs facilitates the sedimentation of the sludge in the last stage of the process. The presence of the flocs also creates many microenvironments and interfaces where different bacteria can exist. These interfaces can be difficult to reproduce in a laboratory environment, and many of bacteria may not easily be grown as pure cultures in the laboratory.

The environments inside and outside the flocs are often different. Limitations of oxygen/nitrate and other nutrients are likely to occur inside the flocs, and microbial waste products are more prevalent inside the flocs than outside (83, 95). Changing operational parameters like sludge ages can create flocs with different densities and sizes (7), thereby changing the microenvironments. The crude forces, existing in the treatment process, (stirring, pumping, sedimentation and subsequent suspension of the flocs) constantly breaks and rearranges the flocs. These processes makes an otherwise relatively stable environment inside the flocs also unstable over a long time.

The shifting activity from the beginning of the activated sludge process (more carbon leads to higher heterotrophic activity, which results in oxygen limitations in part of the sludge) to the end (less amount of easy accessible carbon source leads to more available oxygen) also leads to shifting environment during the transport through the WWTP. This is even more accentuated by the addition of a denitrification basin, which is a totally anoxic environment. In order to survive in this changing environment it is an advantage for the bacteria to be versatile in the choice of carbon source and electron acceptor.

There is a diverse microflora present in the sludge (92, 77). Denitrifying activated sludge is composed of both denitrifiers as well as non-denitrifying bacteria, yeasts and microfauna. These microorganisms live in a close ecological relationship, and one microorganisms end product can be another groups nutrient source (39). The exististence of these microbial consortia can also make it difficult to grow some bacteria in pure cultures.

3.4 Biofilm

A clean surface immersed in water in the nature does not stay clean for a long time. At first, it is covered by proteins and other molecules because of different adhesion mechanisms (ionic, dipolar or hydrogen bonds and hydrophobic interactions). These molecules function as nutrients for the bacteria. In the second step bacteria is adhering to the surface/substance layer, by the same mechanisms as the molecules previously adhered (93). These molecules functions as carbon and nutrient sources for the bacteria and supports the bacterial growth. Both the adhesion properties of a bacterium (that is, how well and in what way they adhere to the surface) and its to growth rate decides how well it can compete with other bacteria on the surface. When the bacteria adhere to the surface, they produce an exopolymeric matrix that permanents the adhesion to the substrate. The dominant component of this exopolymeric matrix is extracellular polysaccharide (EPS), which accounts for up to 90% (3). The type of EPS is dependent on the bacterial species present in the biofilm. The third step is that protozoans and other higher organisms establishes themselves on the bacterial film, grazing on it. The mature biofilm thus consists of bacteria and adhered organic material included in a exopolymeric matrix and a fauna grazing on this matrix. The crucial step in the biofilm development is the irreversible adhesion of the microorganisms. The forces between molecules (EPS) on the bacterial surface and those on the substratum are the key to long term adhesion (3). The mature biofilm reaches an eventual thickness, which is regulated by growth of the bacteria, grazing by predators and sloughing.

In the biofilm, as well as in the flocs of the activated sludge, the rates of diffusion, consumption and production of nutrients and waste products creates a wide variety of gradients.

3.4.1 *Activated sludge versus biofilms as microbial habitats*

The term biofilm is used to define microorganisms that are immobilized at a substratum surface and embedded in an organic polymer matrix of microbial origin (3). The tendency for bacteria to adhere is a key factor both in biofilm and the flocs in activated sludge. The components in biofilm, bacteria, extracellular polymers as "glue" and trapped organic material, are similar to activated sludge. The diffusional effects are also the same. Activated sludge can be seen as a submersed biofilm with other bacteria and solid substances present in the sludge as a substratum surface. The large difference between the habitats is that the biofilm

associated bacteria does not follow the sludge circulated through the WWTP. This creates a more stable environment for these bacteria and the water residence time, and thereby the space needed, can be minimized.

3.5 The nitrogen removal process

The nitrogen removal process is mostly an extension of the conventional activated sludge process. In the process at present built by Rya WWTP, the denitrification will be accomplished in anaerobic activated sludge in the beginning of the process, predenitrification, and nitrification will be carried out in a biofilm in a trickling filter at the end of the process (Fig. 1b). Extensive investigations, conducted at Rya, has foregone the decision for this solution (90, 91, 109). This configuration uses the organic carbon in the influent wastewater for denitrification and the need for a supply of external carbon is eliminated. Biological nitrogen removal without addition of external carbon source is practical both in terms of cost and administration. The water later passes through an aerated zone to remove residual carbon, and after sedimentation part of the water recirculates through a nitrifying trickling filter.

3.5.1 *Sampling site*

The studies in this thesis have been carried out on samples coming from pilot plants at the Rya WWTP in Göteborg. This is a rather big WWTP, receiving wastewater from the whole Göteborg area, about 750 000 personal equivalents, 550 000 thereof from households and the rest from industries. In addition Rya also receives rainwater from some parts of Göteborg, which causes variation of the dilution of nutrients in the water influent to the treatment plant. The influent water is relatively diluted, with an average COD of 200-300 mg O_2 l^{-1}, and influent NH_4 of 20 mg l^{-1}.

In the treatment process in paper I, nitrification was simulated by addition of nitrate to the anoxic basin (109). In paper II the process was run with nitrification in a rotating biological contactor (RBC). In the RBC nitrification is accomplished in a biofilm which alternatively is in air or submersed in the wastewater. The sampling was done in the denitrification basin and to some extent also in the other basins (paper II, Fig. 1). The sampling in paper III was not done in denitrifying activated sludge, but in aerated activated sludge from the ordinary wastewater

treatment process. In paper IV a pilot nitrifying trickling filter, separate from the pilot plant in paper I and II, was used (96).

4. DENITRIFICATION

Denitrification is a microbial process naturally occuring in almost all environments, in freshwater and marine water columns and sediments, wetlands, and soil (144, 115). In the denitrification process, the bacteria use oxidized nitrogenous compounds as the final electron acceptor in the electron transport chain instead of molecular oxygen. The compounds are reduced in the sequence (54):

$$\underset{\text{Na-R}}{NO_3} \Rightarrow \underset{\text{Ni-R}}{NO_2} \Rightarrow \underset{\text{NO-R}}{NO} \Rightarrow \underset{N_2O\text{-R}}{N_2O} \Rightarrow N_2$$

(Na=Nitrate, Ni=nitrite, R=reductase)

There has been a debate whether NO is a true intermediate, but this has now been shown (150, 133). The whole reduction from NO_3 to N_2 accepts five electrons per nitrogen atom. This reduction of the various nitrous compounds is less energetically favourable than the reduction of oxygen, i.e. gives less energy (134), but the difference is not large. For example the electron transport from N_2O to N_2 creates a membrane potential that is slightly lower than when oxygen is used, four H^+ ions are transported through the membrane versus six H^+ ions for O to H_2O (134). The reductases needed for this process are present in most denitrifying bacteria. Some of the bacteria can however be deficient in some of the reductases. For example, *Corynebacterium nephridii* do not have the N_2O reductase, and thus the end product for this bacterium's denitrification is nitrous oxide. Denitrification is controlled in two ways, through regulation of the gene expression and control of the electron transport chain (133). Some of the factors affecting the denitrification rate are:

Oxygen gas: All of the denitrification reductases are inhibited by the presence of molecular oxygen, both in synthesis and for already produced enzymes (72). The sensitivity for oxygen during synthesis varies for the denitrification reductases. Results in soil show that

synthesis of nitrate and nitrite reductases starts within hours whereas nitrous oxide reductase is delayed for more than one day, when the soil becomes anaerobic (23). In *Pseudomonas stutzeri*, nitrous oxide reductase is synthesized constitutively, even at air saturation, while nitrite reductase is only synthesized when O_2 falls below 2.5 mg l^{-1} (76).

For the already produced reductases, the reduction of nitrate to nitrite seems to be less sensitive for oxygen than the later reductases (14). An increase in oxygen concentration, up to a certain level, increases the fraction of nitrous oxide found relative to the total denitrification (71).

In a wastewater treatment plant it can be difficult know the amount of oxygen that reaches the denitrification bacteria. A certain concentration measured in the water does not imply the same concentration inside the flocs, because of diffusive and advective hindrance (95, 83). This can cause anaerobic or microaerophilic conditions inside the flocs, even in well aerated water. Denitrification in apparently oxic environment has also been proven to exist in soil and sediments (88). A few species of bacteria can also denitrify in quite high oxygen concentrations. Both *Alcaligenes faecalis* and Pseudomonas species has been reported to be able to denitrify at air saturation, but at a slower rate than under anoxic conditions (6, 142). *Thiosphaera pantotropha* (now *Paracoccus denitrificans* (89)) continued to denitrify at dissolved oxygen concentrations above 80% of air saturation (113, 112). A later paper from the same group reported a loss of the aerobic denitrifying ability by *T. pantotropha* to about ten percent of previously described (8). Denitrification enzymes have found to be synthesized under aerobic conditions in denitrifying bacteria isolated from activated sludge (74). Denitrification has also been reported in activated sludge when oxygen levels have been relatively high (2, 101). Simpkin and Boyle (127) found that the denitrification enzymes were present during the entire waste water treatment process when the bacteria were transported between the anoxic and aerob zone.

Carbon source access: The majority of the denitrifying bacteria are heterotrophs and thus needs organic carbon to be able to grow. Different carbon sources can give different denitrification rates in wastewater treatment (24). In soil, where the access to carbon is scarce, the denitrification increases when supplied with an external carbon source (71). Denitrification in activated sludge was increased when supplied with easy utilizable carbon source (51, paper II).

Temperature: The denitrification rate is normally sensitive for temperature changes in the interval between 10°C and 35°C. The rate is

almost doubled when the temperature is raised with 10°C (71). This was also the case in Rya denitrifying sludge (Fig. 3).

Nitrate concentration: The denitrification rate is dependent on the nitrate concentration at low concentrations (72).

pH: The denitrification rate has a pH optimum at about 7. At both higher and lower pH the denitrification rate decreases (71, 142).

The ability to denitrify is widespread among many taxonomically and physiologically different bacterial species. As opposed to the nitrification, a relatively large group of bacteria are able to denitrify. Denitrification occurs in almost 130 species belonging to 50 different genera (155), among others *Pseudomonas, Flavobacterium, Micrococcus, Achromobacter, Halobacterium* and *Bacillus* (143, 26). Most of the denitrifying bacteria use a large variation of different organic substances as electron supply. Some can grow autotrophically on hydrogen gas or reduced sulphur compounds. Another group is photosynthetic (143, 71). The denitrifying bacteria in the anoxic basin in WWTPs are assumed to be heterotrophic due to the slower growth of the autotrophs (wash-out) and a very low light penetration into the highly turbid activated sludge.

4.1 Other nitrate reducing processes

There are other pathways than denitrification in which nitrate can be reduced by bacteria: assimilative nitrate reduction and dissimilative ammonia production. The major end product at both these processes are ammonium. Assimilative nitrate reduction is accomplished by a majority of bacteria to incorporate nitrogen when the access to ammonium ions is limited. Dissimilative ammonium production (DAP) is a process to gain energy, but is not coupled to the electron transport chain as in denitrification. It has been found in bacteria that have a fermentative rather than oxidative metabolism, which is the opposite of denitrification (143). DAP is accomplished among others by many species of *Enterobacteriace*, *Bacillus* and*Clostridia*, and are reported in soil and marine sediments (73), accounting for 20-70 % of the total nitrate disappearance in long term anaerobic environments, and also in secondary digested sludge, where it accounted for 60-70% of the disappearing nitrate (68). At high access to carbon source and obligate anaerobic environment it seems that the DAP dominates over the denitrification (71). Studies have shown that up to a third of the DAP can give nitrous oxide as an end product (119).

To measure the nitrate removing processes, ^{15}N labelled nitrate was added to denitrifying sludge from Rya WWTP in Göteborg and the amount of ^{15}N label recovered in ammonia, nitrogen gas and particulate organic material was measured. The results presented below (Tab. 1.) are from two separate occasions.

Table 1. Denitrification rate, dissimilative ammonia production and assimilation of nitrate in Rya pilot denitrification basin (mg NO_3-N VSS^{-1} h^{-1}).

Date	Denitrification	DAP	Uptake	Total NO_3 disappeared
930721	3.3	0.26	0	4.2
930908	6.0	0.15	1.3	8.2
Mean	4.6 (75 %)	0.20 (3 %)	0.65 (10 %)	6.2 (100%)

Denitrification was the dominating process of the three. Neither DAP nor uptake were major sources of nitrate disappearance. No nitrite accumulation could be detected. These results indicates that the nitrous oxide production measured in the denitrification basin in this study did not come from DAP.

Studies in different environments indicate that the denitrification process increases and DAP decreases when the nitrate concentration increases (119). Since the nitrate concentration in the anoxic basin normally was high the findings that denitrification dominates in the Rya anoxic basin is consistent with these results.

Other nitrate reducing processes, generating nitrogen gas abiotically, are called chemodenitrification. The most significant of these processes is the acid-catalyzed degradation of nitrite, which can become significant at pH below 5.0. The predominant end product is NO. Since the denitrification rate in paper I and II was measured as evolved N_2O, and pH in the measured samples rarely was below 5.0, this process is not considered significant in this denitrification basin. The second most important is involves the oxidation of organic N by NO_2^-, forming N_2 gas. This mechanism has been shown to be of potential significance in frozen soil (143). This process is not considered significant either, since the temperature of the samples was way over the freezing point, and no

nitrite accumulation could be detected in the samples. Observations of enhanced reactions between trace amounts of NO and O_2 have also been made in aerobic soils, if acetylene is present in ratios over 0.1% (23). This would cause underestimation of the denitrification when measured with acetylene inhibition technique, since NO is consumed. Both the fact that the denitrification rate in Rya pilot plant was measured in anaerobic environment, and measurements of denitrification simultaneously done by acetylene inhibition and $^{15}NO_3$, suggests that this was not the case in paper I and II (see methods).

4.1.1 *Methods used*

The denitrification rate in these studies was measured with the acetylene inhibition method and head space gas analysis. The sampling was done from a gas phase, the concentration of nitrous oxide measured and the concentration in the water then calculated according to the gas solubility laws (147). Acetylene inhibits the nitrous oxide reductase, thereby causing the end product in denitrification to be nitrous oxide (152). This gas is present in minute amounts in the atmosphere, and a change in the concentration of nitrous oxide in the samples is thus much easier to detect than a change in nitrogen gas concentration. The measurement of nitrous oxide by gas chromatography is very sensitive.

At some occasions the denitrification rate was measured with ^{15}N technique (138). $^{15}NO_3$, 10-20% of the *in situ* nitrate concentration, was added to the samples before incubation. The concentration of ^{15}N-labelled N_2 was measured in the gas phase (consisting of non-nitrogen containing gas such as argon) during the incubation. This method is as sensitive as the acetylene inhibition method, and it does not overestimate the denitrification rate. It can however underestimate the denitrification rate if the nitrogen gas in the head space is not completely removed. It is also a more time consuming method at the sampling moment, and a lot of $^{15}NO_3$ needs to be added if there is a lot of natural nitrate present, as in a WWTP, to achieve sufficient labelling of the nitrate pool.

The acetylene inhibition method is suspected of sometimes giving slight overestimates of the true denitrification rate, since nitrate is only reduced in three steps rather than four when fully reduced to N_2. Measurements done with the acetylene inhibition technique or $^{15}NO_3$ addition on the same sludge showed that the $^{15}NO_3$ denitrification rate was somewhat lower (Tab. 2.), indicating that this was the case. This could mean that the fraction nitrous oxide in the denitrification may have been slightly

underestimated, but would in spite of this not affect the conclusions in paper I. A slightly lower denitrification rate at higher pH would only have minor affect on the relative nitrous oxide production, because of the low nitrous oxide production, and at a lower pH the measured denitrification is accurate since the end product in this case mainly is nitrous oxide.

Table 2. Denitrification in the Rya anoxic basin measured with acetylene inhibition or ^{15}N technique (mg N l^{-1} h^{-1}).

	Denitrification	
Date	Acetylene	^{15}N
920323	4.5	3.1
920504	2.4	1.7
920713	1.7	1.7
920715	1.4	1.4
Mean±SD	2.5±1.4	2.0±0.76

4.2 Nitrous oxide.

Nitrous oxide is produced in three of the biological nitrogen transforming processes, in denitrification as an intermediate, and in nitrification and DAP as a by-product. This gas is not toxic to humans, and it is naturally occurring in the troposphere. As an average, 0.031 % of the air we breath consist of nitrous oxide (23). Nitrous oxide is a hazard to the environment, since it reacts with ozone gas in the atmosphere. Nitrous oxide is relatively inert in the troposphere and diffuses up to the stratosphere and undergoes the photochemical reaction:

$N_2O + O \Rightarrow 2NO,$

with the oxygen released due to photolysis of ozone.

The nitric oxide produced catalyses the destruction of ozone according to (98):

$NO + O_3 \Rightarrow NO_2 + O_2, \quad NO_2 + O \Rightarrow NO + O_2$

Nitrous oxide also absorbs the infrared radiation that radiates from the earth and thus have an influence on the climate on the earth through the

greenhouse effect (6). The greenhouse effect of nitrous oxide is 290 times higher than CO_2 on weight basis (59). In addition it can react with the water in the atmosphere. The HNO_3 formed is transported to the ground with the rain, acidifies and brings fertilizing nitrogen to soil an sea.

Denitrification is, globally, believed to provide the dominant source for N_2O (98). It has been shown that up to 30-40% of the total denitrification in WWTPs can have nitrous oxide as the end product (120).

4.2.1 *Factors affecting nitrous oxide production in a denitrification basin.*

The factors affecting denitrification are, because nitrous oxide is an intermediate in denitrification, also suspected to affect the amount of nitrous oxide released into the atmosphere. All of the above factors were investigated in the Rya WWTP, and are discussed along with the other studies below.

Oxygen - The proportion of N_2O production of total denitrification has been found to increase when the oxygen concentration increases (35, 64). Nitrous oxide is also produced by nitrifying bacteria at low concentrations of oxygen. Goreau (38) showed in *Nitrosomonas* sp. that the nitrous oxide production reached a maximum at low (0.5 Pa) partial pressures of oxygen. Nitrous oxide in natural environments is produced by both denitrification and nitrification (64, 70). The largest formation of nitrous oxide should be in microaerophilic environment, when the nitrous oxide reduction in denitrification is inhibited by oxygen, and the nitrifying bacteria, limited in their use of oxygen, also produces nitrous oxide. However, in natural environments (lake and groundwater) denitrification has been found to be the dominating source for nitrous oxide production (151, 145). Jørgensen *et al.* (64) showed that in marine sediment the denitrifiers produced more nitrous oxide than nitrifiers in aerob environments, except in a narrow concentration range between 0.1-0.2 kPa O_2.

The denitrification rate is affected even at very low oxygen concentrations. The relation between the redox potential and the logarithm of dissolved oxygen has been found to be linear, and the denitrification has been shown to increase with decreasing redox potential in studies with activated sludge (84). The redox potential is however also affected by the nitrate concentration, which was constant in the above mentioned study. When nitrate was added, they found that the

redox potential increased (85). In the denitrification basin studied here, a decrease in nitrate concentration was correlated to a decrease in redox potential (Fig. 2). In Rya denitrification basin the redox potential thus seems to reflect the nitrate concentration rather than the oxygen concentration. A trend (not significant) where nitrous oxide increased when redox potential increased was also found in the anoxic basin (paper I). Since this correlation was not significant, it was concluded that changes in the redox potential was not the major cause of high nitrous oxide production in the denitrification basin.

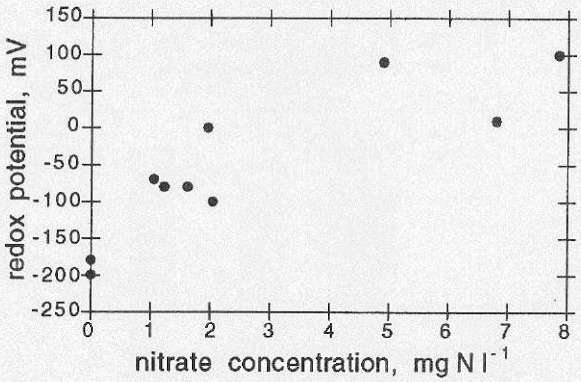

Figure 2. Redox potential versus nitrate concentration in the denitrification basin at Rya pilot plant. Correlation 0.75, p=0.01.

pH - The nitrous oxide reductase seems to be more affected by low pH values than the other reductases. The mole fraction of nitrous oxide to nitrogen gas increases a lower pH, and at pH 4-5 nitrous oxide is the major product (142, paper I). pH is shown to affect both the fraction of nitrous oxide in denitrification and the total amount of nitrous oxide as end product produced. At a pH lower than 6.5 the nitrous oxide production as a fraction of denitrification increased. The total nitrous oxide production increased with pH down to pH 5.5, and it then decreased again due to the decreasing denitrification (paper I). Hanaki *et al.* (44) also found in activated sludge, that the nitrous oxide production increased with lower pH. The N_2O production at pH 6.5 (up to 17% of nitrogen gas production) was significantly higher than that at pH 7.5, although the difference between pH 7.5 and 8.5. In situ measurements in

non-denitrifying activated sludge in Germany found N_2O production to be between zero and 6.2 mg m^{-3} h^{-1} with an average of 1.04 (137). In paper I an average of 9.1 mg is reported. However, the result in paper I includes two measurements in days with low pH. When calculating an average without these figures the average is -0.0015 mg N m^{-3} h^{-1}, which is lower than Sümer *et al.* (137). This indicates that no increase of N_2O production would occur with denitrification introduction in wastewater treatment, as long as the pH stays around 7 and above.

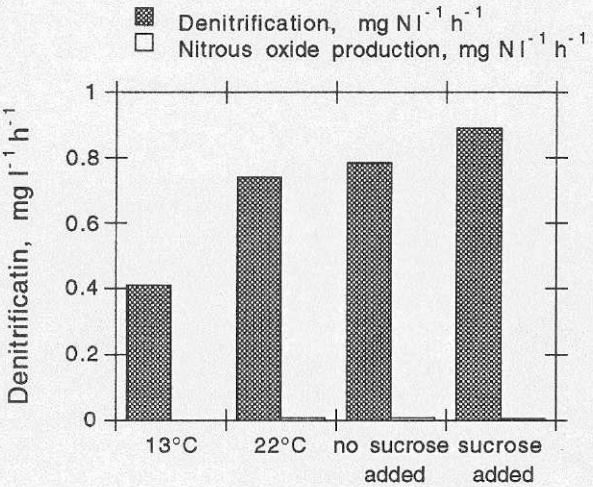

Figure 3. Denitrification and nitrous oxide production with or without carbon addition and after incubation at two different temperatures.

Other factors affecting nitrous oxide production during denitrification - High concentrations of NO_3 has been reported to cause higher nitrous oxide production in *Pseudomonas* sp. (44, 142). In a denitrifying activated sludge, Hanaki *et al.* (44) reported higher nitrous oxide formation at low COD/NO_3-N and short solids retention time, and also at nitrite concentrations over 10 mg NO_2-N l^{-1}. Sümer *et al.* (137) reported that the production of nitrous oxide in aerated activated sludge seemed to be related to high concentrations of NO_2 or NO_3. In the Rya denitrification basin, the nitrite concentration (under 10 mg N $l^{-1}h^{-1}$, paper I) did not affect the nitrous oxide production, which is consistent with Hanaki *et al.* (44). Addition of more nitrate, up to 18 mg N l^{-1}, to

denitrifying activated sludge from the Rya basin did not cause any elevated nitrous oxide production (data not shown).

It has been found that in soil and pure cultures of *Pseudomonas* sp. can different carbon sources that gives equal denitrification rate also give different amount of nitrous oxide as end product (71, 142). In the Rya denitrification basin, addition of more carbon source (sucrose) had no effect on the nitrous oxide production, and neither had a decrease in temperature from 22°C to 13°C (Fig. 3). Addition of more carbon source would increase the COD/NO_3-N ratio and a lack of N_2O production would thus agree with Hanaki *et al.* (44).

4.2.2 *Sewage treatment plants and total nitrous oxide production*

There are at present no indications of an extensive nitrous oxide production from WWTPs, neither with the present processes nor with the nitrogen removal extension. Reported measurements and calculations from Switzerland concludes that the planned introduction of nitrogen removal in Swiss sewage treatment plants will not lead to significant increases in N_2O emissions to the atmosphere, and that emissions from sewage treatment plants will account for less than 1% of the total N_2O emission in Switzerland (99). In Sweden it is reported that the emission of nitrous oxide from sewage treatment plants is negligible at present. The emission after full extension of the planned biological nitrogen reduction would be approximately 1% of the total emission in Sweden, when calculated using a value of 10% of the influent nitrogen transformed into nitrous oxide (111). Measurements of N_2O emissions from different sewage treatments plants in Sweden shows no extensive nitrous oxide emission. An average of 1.5 ‰ of the influent nitrogen ended up as N_2O (12). In aerated activated sludge N_2O has been shown to be produced mostly by nitrifiers in the nitrification process, rather than from heterotrophic denitrification (137). The finding in paper III that no excessive formation of N_2O takes place in the normally working denitrification process is consistent with these reports. It thus seems that the introduction of nitrogen removal in Swedish WWTPs not is going to increase the total outlet of N_2O to the atmosphere, as long as the nitrogen removal process is functioning correctly.

4.3 Carbon source and denitrification

It has been suggested that carbon may be the major factor controlling denitrifier population densities (144). Limitations in the access to carbon source also limits the denitrification rate in all systems (42). Many denitrification systems in WWTPs uses additions of external carbon source to the process in order to obtain maximum denitrification rate, both in pre- and postdenitrification processes (49, 156, 47). A post denitrification system can, since denitrification is occurring after the nitrification in the process, remove almost all of the influent nitrogen to the WWTP. This would be the best way to obtain an optimal removal of nitrate, but it also requires the addition of external carbon source since the influent carbon source is up used in the aerated activated sludge step. This creates a higher running cost for the process, and care must be taken not to add more carbon than necessary for the denitrification. Occasional addition of supplemental carbon can be used to enhance denitrification during peaks in nitrate concentration (60). If too much carbon is added, the excess would enter the natural aquatic systems and waste some of the former treatment. Predenitrification systems may also need extra carbon source addition, if not enough easy utilizable carbon is present in the influent water. With the predenitrification system used at Rya WWTP high denitrification rates have been achieved without external additions of carbon source (91).

Different carbon sources has proven to give different performance on denitrification in activated sludge (80). The type of carbon source had a significant influence on the denitrification rate, denitrification yield, sludge yield and composition of the microflora. With acetate and methanol higher denitrification yields, lower sludge yields and more true (end product N2) denitrifying bacteria were obtained than with crude syrup and hydrolyzed starch. Furthermore, with acetate a higher growth rate and a higher denitrification rate was obtained than with methanol (80). Ethanol was found to be a better carbon source for denitrification than methanol (18). Addition of different carbon sources (ethanol or methanol) also induced different bacterial populations in denitrifying activated sludge (41).

Characterization of wastewater and activated sludge is a tool that can be used for control and optimization of the wastewater treatment processes. The organic matter in wastewater treatment is usually divided into four parts, easily biodegradable, rapid hydrolyzable, slowly hydrolyzable and inert material. Measured as COD, 15 % of raw wastewater is easily biodegradable, 25 % rapid hydrolyzable, 28 % slowly hydrolyzable and the rest consists of bacteria and inert material (50). The values for the

water reaching Rya WWTP was 12 %, 14 % and 23 % for the three first parts respectively (148). The readily biodegradable material consists of small molecules such as volatile fatty acids, carbohydrates, alcohols and amino acids. These molecules can be taken up directly and utilized for catabolic or anabolic reactions in the microorganisms. The easily and the slowly degradable parts consist of polymeric molecules like fats, lipids, proteins and polysaccharides, the easy degradable part mainly present in the soluble part of the sewage and generally having shorter chains than the slowly degradable. The inert part consists of compounds that cannot be broken down, or are broken down by the microorganisms on a time scale significantly longer than the sludge residence time.

Different methods have been used to quantify these fractions in order to describe the available carbon source in WWTPs. These includes measurements of chemical oxygen demand (COD) in different fractions in the activated sludge, suspended solids (SS), volatile suspended solids (VSS), total organic carbon (TOC), biological oxygen demand (BOD), oxygen utilization rate (OUR) and nitrate utilization rate (NUR) (50). All of these measurements are crude and they often give little or no information about the actual substrate utilized.

Polymers cannot penetrate cell membrane. The microorganisms therefore, when easy degradable carbon sources are limiting, produce and excrete extracellular enzymes that hydrolyzes the polymeric substances into mono- or dimeric units (1), that are easily taken up by the bacteria by active transport (4). The enzymatic cleavage of the polymers is often the step limiting microbial production and substrate degrading in aquatic systems (50, 21, 58), and also in denitrifying activated sludge.

The monomeric substances originally present in the waste water are likely to be used by microorganisms in the sewer systems before the water reaches the WWTP (65). Extracellular enzymes are thus important in the breakdown of organic matter and the regeneration of nutrients in the activated sludge, and also for the denitrification rate in a biological nitrogen removal system without carbon addition.

4.3.1 *Extracellular enzymes*

Extracellular enzymes are generally defined as enzymes that have crossed the cytoplasmic membrane of the cell, including proteins attached to the outer surface of the membrane (108). The strategies of using free enzymes or enzymes attached to the surface of the bacteria are different. If the enzymes are attached to the surface, the products of the

hydrolyze is released in the vicinity of the cell. On the other hand, less substrate is available due to diffusive and steroid hindrance near the cell surface. Free enzymes are more likely to find their substrate, but the product is less likely to reach the cell that excreted the enzyme. The strategy of free enzymes would thus be an advantage when little organic matter is available, to increase the chances of finding the right substrate. Chrost (20) has suggested to call the adhered enzymes extracellular enzymes, and the free ectoenzymes. It is however difficult to tell them apart in practice, since the free enzymes can adhere to other surfaces. I therefore here use the terms extracellular enzymes and exoenzymes interchangeably meaning both free and adhered enzymes.

The production of the exoenzymes are either induced when the right substrate is available and no easy utilizable carbon source is present, or constitutively produced by the bacteria. Inducible systems saves energy and material because the enzymes are only produced when needed. The constant production is in favour when the substrate is likely to be present most of the time, or when it is an advantage due to competition to be able to utilize different substrates on a short notice. There are also evidences for microbial growth on many substrates simultaneously when the carbon sources are limiting (31). In natural environments, the exoenzyme production is often inducible (56, 100, 21).

In aquatic environments, extracellular enzymes are mainly produced by bacteria. Some may also originate from other organisms and from autolytic processes. The substrates for the hydrolysis are proteins, carbohydrates, fats and organic P- or S- compounds (57). Studies in natural environments have shown correlations between exoenzyme activity and bacterial activity or amount of carbon present (17) (river), (22) (lake). It is therefore possible that such connections also exists in activated sludge. A determination of exoenzyme activity could then give an estimate of the actual carbon sources present and available for the bacteria.

4.3.2 *Exoenzymes studied and target bonds*

The exoenzyme activity was measured with substrate analogues bound to methylumbelliferyl (MUF) with the special enzyme target bonds. Upon cleavage by exoenzymes, free MUF is released. The free MUF is highly fluorescent, whereas the bound compound is not, and the amount of substrate hydrolyzed can thus be measured. This is a very sensitive method and amounts of MUF down to about 100 nM can be measured.

Three classes of common molecules were chosen, carbohydrates (α- and ß-glucosidases), short and long chained lipids (esterase and lipase) and peptides (peptidases). Glucosidases hydrolyzes glucosides, which are carbohydrates with saccharide units linked by acetal bonds with hydroxyl groups of alcohols or phenols (O-glucosides) or with amino groups (N-glucosides) (125). The substrates used in paper II and III for α- and ß-glucosidase determination, MUF-glucosides, had O-glucosidic bonds. Glucosidic bonds can be found in a number of different substances, which may be present in activated sludge. α-glucosidic bonds exists in starch and glycogen, which are common products in microorganisms and function as reserve materials (132). ß-glucoside bonds are found in cellulose, in which glucose molecules are linked to one another by ß(1-4) linkages. Lipopolysaccharides and glucoproteins present among others in biological membranes also contains poly- and oligosaccharide chains. The exopolymers produced by bacteria for attachment can consist of up to 95 % of polysaccharides (3). Saponins is a large and widely distributed group of plant substances, named after their ability to form strongly foaming, soap-like solutions with water (37). They are all partly glucosides, and are used as detergents and foaming agents, and can thus also be present in wastewater.

Starch is used in paper III as an inducer of α-glucosidase activity in pure culture bacteria. It consists of D-glucose residues that are connected by α(1-4) linkages and at branch points by α(1-6) linkages. Starch is a storage material, and as such is structured to be degradable (1). It can be broken down in two ways. The most common is to first break the internal bonds by an endo-acting α-amylase, hydrolyzing amylose to oligosaccharides of five or six glucose residues. These oligosaccharides are subsequently hydrolyzed by an exo-acting α-glucosidase producing glucose, maltose and maltotriose. Glucoamylase (also called amyloglucosidase) also hydrolyzes the 1,4-α-linkages in amylopectin, amylose and glycogen, but it does so by attacking consecutive bonds starting at the nonreducing chain end, producing mono- and dimeric units directly. Many fungi and bacteria produce α-amylases and are capable of degrading starch.

It has been found that α-glucosidases are produced in a higher amount when bacteria are grown on starch, amylose and maltose than in bacteria grown on other carbon sources (97).

Ester bonds are carboxyl groups substituting the hydrogen atom for a carbohydrate chain. They exist for instance in fats and lipids. The esterases have a broad substrate specificity and they hydrolyze a very large number of different esters. The main factors influencing the

specificity are the lengths and shapes of the hydrophobic groups on either side of the ester link (28). The MUF-substrates used here had palmitate (long chain) or butyrate (short chain) bound to the MUF.

Proteins and peptides are polymers of amino acids, linked together with peptide bonds. The peptidases hydrolyzes the peptide bonds, and are usually very specific for one particular constellation. Most proteins are hydrolyzed to some extent by many proteolytic enzymes, since they contain a wide range of different peptide bonds which are likely to include those sensitive to a particular enzyme (28). The MUF-substrate used here is a substrate for thrombin and trypsin (62). These enzymes which have a specificity for bonds between arginine and glycine (thrombin) and lysine/arginine and one no specified amino acid (trypsin) (135).

The MUF-substrates used here primarily measured exo-acting enzyme activity, since only one molecule was attached to each MUF molecule.

4.3.3 *Exoenzyme activity in wastewater treatment*

Exoenzyme activity has been used previously to measure bacterial activity in wastewater treatment (45, 141, 110). Hankin and Sand (45) used it as a microbial population indicator, they measured bacterial groups and exoenzymes in different steps of the treatment process. Nybroe *et al.* (103) measured exoenzyme activity colometrically in denitrifying and oxygen respiring activated sludge and in raw wastewater. They found that variations in gross enzyme activities (dehydrogenase and esterase activity) in influent wastewater corresponded to variations in VSS and in number of cultivable bacteria, and that this suggested that enzyme activity reflects the microbial activity in wastewater. These findings supports the idea that an exoenzyme activity should reflect the microbial activity also in activated sludge. They found however no correlation in activated sludge (103). Lie (84) measured exoenzyme activity and oxygen respiration in activated sludge over a period of 75 days, and found a similar decrease in both parameters over the days measured. In paper II no connections between variations in VSS concentration and exoenzyme activity could be found, and neither between exoenzyme activity and denitrification potential in the denitrification basin. It was also concluded that the exoenzyme activity in the denitrification basin originated both from microbial production in the basin and from the influent sources of water and sludge. It was suggested that the additional exoenzyme activity coming from the

influent water and recirculating sludge was large enough to mask the inductional production of exoenzyme activity by the microorganisms in the basin, and thus no correlation could be measured.

Other studies has found that in WWTPs with different major carbon sources in the influent water, the carbon source available was reflected in the enzymatic patterns in the activated sludge. A high organic nitrogen load to the WWTP or an addition of hydrolyzed starch led to peptidase respective α-glucosidase as the major enzyme activity (103, 110). An occasional marked increase in COD gave a higher enzyme activity in all the measured enzymes (110). The authors called these enzymatic patterns "enzymatic fingerprints", and considered that these were unique for the individual treatment plant. These findings led to the assumption in paper II that a measured exoenzyme activity in the activated sludge reflected the amount of available carbon source in the denitrifying activated sludge. Paper III indicates that this assumption, at least when α-glucosidase was considered, was not necessarily correct. The presence of a species (*C. gleum*, paper III) in the sludge that constitutively produced α-glucosidase activity, and that this species produced the highest activity, leads to the conclusion that, in the case of α-glucosidase, the amount of exoenzyme does not have to reflect the amount of carbon source available for bacteria on a short time basis. This in addition to recirculation of the exoenzyme activity as mentioned before could explain why no correlation was found between exoenzyme activity and microbial parameters.

The enzymatic fingerprints seems to be due to long term adaptation of the sludge, thus depending on the composition of the microbial population. Large variations with time has however been found (paper II, 36). In paper II is also showed that exoenzyme activity could change on a short notice, in the order of hours. This indicates that apart from the long-term fingerprints, the bacteria also could respond to short term changes, without changing the microbial population. This shows that the bacteria in activated sludge are highly versatile in their carbon utilization.

Exoenzyme activity has also been used to localize and compare hydrolytic enzyme profiles in various fractions of activated sludge. In both intact and sonicated activated sludge peptidase activity dominated over esterase activity (13). Lemmer *et al.* (82) also found much higher peptidase than esterase activity in activated sludge. In the Rya denitrification basin esterase activity was by far the dominating exoenzyme activity, being about 10 times higher than the other enzymes measured (paper II). Esterase activity is often considered as a non-

specific measurement of bacterial activity, but connected to the fact that different enzymes dominated in sludges with different major carbon source it shows that in Rya WWTP the dominating carbon source available was substrates with ester bonds. Since the lipase activity was very low, the long-chained esters like fats does not seem to be important as substrate.

Between 75 % and 100 % of the enzyme activity in activated sludge is found in the sludge (141, 13). In paper II 68.5-95 % of the enzyme activity was found in the sedimented phase, and over 99.5 % was associated with bacteria. The exoenzyme activity in wastewater treatment has been localized to the exopolysaccharide (EPS) matrix (36). The existence of much higher enzyme activity per cell in the sludge matrix and high esterase activity in the EPS extracts led to that conclusion.

The studies in paper III are in part conducted in an aerated basin, and the discussion primarily concerns exoenzymes important for denitrification. The processes of denitrification and aerobic respiration show great similarity in that they both use the electron transport chain and share many of the involved components. The results obtained in the aerated basin can thus also be discussed in terms of denitrification. Despite this, there are differences in the enzyme pattern of the aerated and the anoxic basin. Henze and Mladenovsky (52) found that the hydrolysis rate of nitrogenous compounds was significantly affected by the electron donor available, and that the hydrolysis rate was higher under aerobic conditions. The present found that the order of potential enzyme rates in Rya WWTP aereted basin was: esterase > ß-glucosidase > α-glucosidase > peptidase > lipase, while it in the anoxic basin was: esterase > ß-glucosidase > peptidase > α-glucosidase > lipase (paper III and II). These measurements were however carried out at different times, and can therefore not be directly compared. The enzyme activities were in the same magnitude in the different basins, with esterase activity being much higher than the other enzymes measured.

4.3.4 *Exoenzyme activity compared with other habitats*

In comparision with other environments there are both differences and similarities with the exoenzyme activity of wastewater treatment. In many environments the main enzyme activity is found to be free. In marine water up to 100% of the enzymatic activity was found in the free phase (67). In soil, free enzymes can exist and accumulate for many years (128, 69). Lipase activity measured in mud and peat was due

mainly to extracellular enzyme activity (77). In a river 10-30% of the ß-glucosidase and 20-100 % of the peptidase activity was found free in the water (1). In an euthrophic lake only 0-7% of the ß-glucosidase was found free (19, 129), and 10-30% of the peptidase activity (61). In wastewater treatment most of the enzymatic activity was found connected to the sludge (see 4.3.2). It seems likely that in more eutrophic environments the bacteria makes enzymes attached to the cell surface rather than free enzymes. The higher concentration of available substrate closer to the cells increases the benefit of a cell-bound enzyme (see 4.3.1).

The enzyme activities found in activated sludge were higher than in any other environment. In marine water, α- and ß-glucosidase and peptidase activities of 0.5, 1 and 40 nM h^{-1} was found (67), and in marine snow 2.5, 6 and 3700 nM h^{-1} respectively (in July) (66). In a river Chappell and Goulder (17) found ß-glucosidase and peptidase activities of 20 and 400 nM h^{-1}. The only environment that comes near the activities in activated sludge is the peptidase activity in marine snow, which is still at least ten times less. The reason for this is not clear, but possible explanations are a higher bacterial density or that physical changes occur in the bacteria attached to the flocs, as discussed in paper III. In the paper about marine snow, they also found higher activity in the marine snow than in the free living bacteria, although the same number of bacteria were present (67). Other studies have also found that attachment to surfaces changes the performance of the bacteria, such that attached bacteria are often more active than free cells (146). The above mentioned (4.3.3) entrapment of exoenzymes in the LPS matrix could also concentrate the exoenzymes in activated sludge (36).

In marine environment the diurnal variation of α- and ß-glucosidases showed peaks at the same time (67). Covariation between α- and ß-glucosidases was also found in the Rya activated sludge, both when denitrifying and aerated (paper II and III).

Correlations between exoenzyme activity and microbial parameters has been found in several natural environments. In marine water ß-glucosidase was connected to nano- and picoflagellates, but neither α-glucosidase nor aminopeptidase were significantly correlated to any microbial biomass parameter (67). A connection between ß-glucosidase and particulate organic matter was found in a river (17). On the other hand, there was no correlation in marine snow between hydrolase activity, bacterial production and the concentration of dissolved organic nutrients found (66).

4.3.5 *Castanospermine*

Castanospermine is a known inhibitor of various glucosidases. The best documented one is the inhibition of α-glucosidase I in mammals, and that is why α-glucosidase activity in bacterial exoenzymes were chosen (paper III). The aim was to inhibit the enzyme activity in activated sludge and measure how this inhibition could affect the microbial activity, measured as respiration rate, thereby measuring the relative importance of this enzyme activity.

Castanospermine (1,6,7,8,-tetrahydroxioctahydroindolizine) is an alkaloid isolated from the seed of the australian tree *Castanospermum australe* (55). Stereochemical comparisions with natural glucosidase substrates such as maltose and methyl glucoside show great similarities in the positioning of functional groups (48). This alkaloid has been extensively studied because of its ability to inhibit growth of human viruses, such as HIV (140), human cytomegalovirus (CMV) (139) and influenza virus (106) by blocking the processing of certain glucoproteins necessary for the viruses. Castanospermine has also been used to inhibit N-linked oligosaccharide modification in various other cells (30,94). It inhibits α-glucosidase I in mammals, which is involved in the initial step of N-linked oligosaccharide processing of secretory and membrane glycoproteins.

Castanospermine is toxic to mammals after ingestion, causing severe gastrointestinal problems such as vomiting and diarrhoea. This effect is due to the inhibition of intestinal glucosidases. Cell free extracts of intestinal sucrase, maltase and trehalase from rats was inhibited more than 75 % when 300 μg ml^{-1} (appr. 1.5 mmol l^{-1}) of castanospermine was added to the extract (105).

Castanospermine was a better inhibitor at higher pH (122). They explained this effect by concluding that since that the pK_a for castanospermine is 6.09, the unprotonated form of castanospermine was more active than the protonated. It should be noted that the pH used in paper III was 7.2, and castanospermine should therefore be a potential inhibitor.

Besides mammals, castanospemine was effective against fungal ß-xylosidase but not yeast α-glucosidase (121). Castanospermine also inhibited the production of ß-hexosamidase in cells of *Aspergillus fumigatus* grown in castanospermine (32). The α-glucosidases from various plant was also inhibited by castanospermine (75, 29).

The pattern of inhibition of ß-glucosides and most α-glucosides (except trehalose) was different in the inhibition of hydrolysis in insect glucosides from those in mammals (124). This shows that inhibition of α-glucosidase by castanospermine could vary among species, and certainly between eukaryot cells and bacteria.

The effect of castanospermine on α-glucosidase activity and respiration of oxygen in three bacterial species (*Chryseobacterium gleum*, *Pseudomonas stutzeri* and strain 100g) and in activated sludge was studied in paper III. The bacteria were grown on starch to induce the α-glucosidase activity. *C. gleum* could not be grown solely with starch as carbon source, but exhibited α-glucosidase activity both in minimal media with starch and amino acids and in complex media (TSB). Extracellular α-glucosidase activity in two of the bacteria and in activated sludge was inhibited by castanospermine. The α-glucosidase activity in *C. gleum* was inhibited by castanospermine to 95% at 250 µM. The inhibition could in part be reversed by addition of starch. Castanospermine also inhibited α-glucosidase in *P. stutzeri* grown in starch and the activity in activated sludge to approximately 50 % at a concentration of 100 µM and higher. A denitrifying bacteria called 100g was however not affected at all up to a concentration of 1.3 mM.

The inhibition of respiration rate in the presence of castanospermine was lower than the inhibition of α-glucosidase activity. The inhibition of respiration rate in *C. gleum* grown in minimal medium reached 80% at a castanospermine addition of 900 µM. No significant inhibition was seen in *P. stutzeri* and in activated sludge. The respiration rate in strain 100g was not affected, and neither was the respiration rate in *C. gleum* grown in complex media, showing that no general inhibition by castanospermine could be found. This distribution of inhibition of both α-glucosidase and respiration by castanospermine led to the conclusion that castanospermine could not be used for general measurements of the α-glucosidase utilization natural environments. Maybe a species as *C. gleum* could be added and used as indicator organism for α-glucoside containing carbohydrate utilization.

5. NITRIFICATION

Nitrification is a microbial oxidation of ammonia to nitrate. The effect of pH on the process indicates that NH_3 rather than NH_4^+ is the substrate (136). Nitrification is accomplished in two steps by two groups of obligate aerobic bacteria. The first step is an oxidation of ammonium to nitrite. This is a cytochrome bound, energy demanding hydroxylation to form hydroxylamine (NH_2OH). The subsequent oxidation of hydroxylamine to nitrite is linked to a production of ATP via a membrane bound electron transport system. The sum of the reactions is:

$$2\ NH_4^+ + 3\ 0_2 \Rightarrow 2\ N0_2^- + 4\ H^+ + 2\ H_2O + 132\ kcal$$

This step is done by representatives from the genera *Nitrosococcus*, *Nitrosolobus*, *Nitrososomonas*, *Nitrospira* and *Nitrosovibrio*. The second step, oxidation of nitrite to nitrate is done by representatives of the genera *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*, and generates ATP via an electron transport chain (9) as in step one. The reaction is:

$$2\ NO_2^- + O_2 \Rightarrow\ 2\ NO_3 + 34\ kcal$$

The first step thus yields a higher energy gain, resulting in a faster growth rate for these bacteria. The growth of nitrifying bacteria is very slow due to the low energy yield from oxidation of ammonia or nitrate in comparision with organic substances. The ammonium or nitrate functions in nitrifying bacteria as an electron donor and the carbon source used is CO_2. The assimilation of CO_2 is very energy demanding, and this also contributes to the slow growth of nitrifying bacteria.

Since both steps are crucial for the whole nitrification reaction, disturbances in one of the bacterial steps would affect the whole process. Disturbances in the second step would in a WWTP cause a high concentration of nitrite in the treatment process. This could lead to lower nitrogen removal efficiency, and also a release of nitrite into the recipient. Nitrite can react with secondary amines to produce nitrosamines, some of them which are strongly carcinogenic (86). In most cases at the Rya WWTP, the concentration of nitrite leaving the nitrifying trickling filter low, indicating that the whole nitrification process was functioning within the filter. Nitrification in WWTPs can either be carried out in activated sludge basins (46) or in biofilm processes (5, 34).

5.1 Nitrification in biofilm

The advantages of using biofilms in a wastewater treatment is that the bacteria used are adhered to a solid, stationary material, and is thus retained in the treatment step.

The slow growth makes the nitrifiers susceptible for wash-out even at low water flow rates in an activated sludge system. In a biofilm the nitrifiers are attached to a surface and thereby prevented from being washed out from the system. Because of the slow growth, the volumes needed for nitrification are large, and biofilm processes are easier to extend vertically than activated sludge processes. These factors combined makes the need for area lesser for carrying material supported growth systems as compared to activated sludge processes.

Enhanced process rates have been found in adhered bacteria compared to free living (146). Also, because of diffusional hindrance, inhibiting substances cannot penetrate the biofilm and reach the bacteria as fast as they can with free-living cells, leading to less inhibition of microbial parameters such as nitrification in a biofilm than in free-living cells (107).

There is evidence that a lag phase occurs when nitrifiers are transferred from anoxic to aerobic environments before the bacteria starts to grow (27). They suggested that this lag phase was needed for the nitrifiers as a protective mechanism to ensure that nitrification only will take place if long term oxic conditions prevail. This would also favour a biofilm system, since the bacteria (nitrifiers) in such a system would not have to shift between oxic and anoxic conditions.

Another advantage is that the oxygen concentration in the water is high because of a large contact area between the water and the air. This increases the access of oxygen for the bacteria, decreasing the possibility of oxygen limitation and increases both bacterial growth and the process performance.

It is reported that contaminants can be accumulated in the exopolymer matrix, thereby increasing the susceptibility of these substances to the bacteria. The same study however also showed that grazing protozoa non-selectively ingested exopolymers, transferring the contaminants to the protozoa and removing them from the bacteria (149).

When high concentrations of organic material is present, the heterotrophs grow much faster than the nitrifiers and competes effectively for oxygen and space. The nitrification in a rotating biological contactor decreased

when the BOD in the influent water increased (34). In the nitrifying trickling filter studied here (paper IV) the water influent to the filter was coming from the effluent water from the existing WWTP, in which a lot of the organic material was already removed, thereby decreasing the growth rate of the heterotrophic bacteria and increasing the fraction of nitrifying bacteria in the biofilm. This is also a reason why a post nitrification design was chosen.

High organic material content in the influent water cases a thicker biofilm, both by supporting faster growth of heterotrophic bacteria and by entrapment of the organic material in the biofilm. Nitrifying bacteria present near the substratum in a thick film experience oxygen and ammonia limitation due to the long diffusion distance. On the other hand, they are protected from sloughing if existing in the inner part of the film. There exists an optimal film thickness, which protects the bacteria while still allowing reasonable diffusion rates. Because of this, the grazing fauna is believed to be of some advantage, since they consume the film and is thereby reducing the film thickness (see section 5.3).

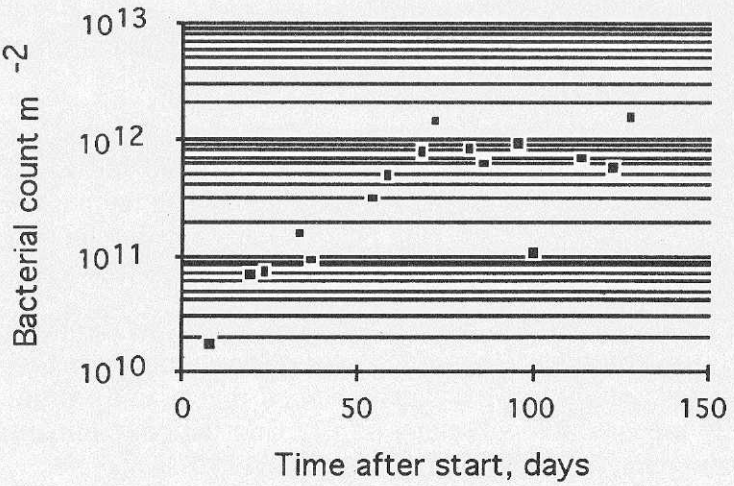

Figure 4. Bacterial growth in the Rya nitrifying trickling filter versus time.

5.2 Building up of a nitrifying biofilm

The time it takes for the biofilm to reach the mature stage is equal to the time needed for the nitrification process to reach its optimum rate in solid support systems. Several months are needed to start up a nitrifying biofilm (paper IV, 153), and during this time ammonia is still present in the effluent water.

Bacteria in the Rya trickling filter biofilm (paper IV) was counted and measured in an epifluorescence microscope after staining with acridine orange (53). The method has also been used on glass slides with pure cultures of *Nitrosomonas europea* (107). In the present study the black PVC material in the trickling filter was used. The black background of the PVC made it very suitable for epifluorescence studies.

The bacterial count as a function of time is seen in Fig. 4. This figure is plotted on a logarithmic scale instead of a normal which is exhibited in paper IV. This accentuates the fact that the growth of bacteria in the biofilm was logarithmic from the start, whereas both biofilm dry weight and area covered showed a lag phase before actually increasing (paper IV, Figs 2,3). The ammonium removal rate was also increasing logarithmically from the start, indicating that the nitrification rate was dependent on the bacterial count in the beginning of the biofilm formation. This is also concluded in paper IV, but more in connection with the biofilm dry weight than with the bacterial count. This shows that the nitrifying bacteria was present in the trickling filter from the beginning, and that the proportion of nitrifying bacteria to the total count was probably constant in the beginning of the biofilm build-up.

Later in the biofilm formation, after approximately 80 days (paper IV, Fig. 4), either the proportion of nitrifying bacteria in the biofilm started to fluctuate, or the nitrifying rate did. This is probably due to inhibiting factors for nitrification present in the biofilm (oxygen depletion or inhibiting substances) rather than fluctuating amount of nitrifying bacteria. In a trickling filter in a fish tank system with simultaneous removal of ammonia and organic matter, an increasing short term load of organic matter led to decreasing nitrification rates. The carrier material was plastic with a specific density of 200 m^2 m^{-3}, and the amount of NH_4-N removed was 0.65 g m^{-2} d^{-1} (15). This filter was thus comparable to the Rya trickling filter, and shows that the fluctuating nitrification rate could also be due to fluctuating loads of organic matter. This also indicates that the nitrification rate in the beginning of the build-up was due to the amount of nitrifying bacteria present. The film at this time was thin, less than one bacteria thick, so there could not have been

any oxygen limitations. Later, the thickness of the film probably was an influencing factor.

After an irreversible attachment has occurred, growth and division of the cells takes place at discrete sites on the surface, leading to microcolony formation (107). When these microcolonies grow, they form a layer of cells that cover the surface, but in a patchy way. As seen in paper IV, this was also the case in the biofilm in Rya trickling filter. The uneven distribution of bacteria was apparent on the microscale at least until about two month after the start-up of the trickling filter. Visual observations of the biofilm when the trickling filter was removed, about a year afterwards, revealed that the biofilm was still patchy.

The irreversible attachment of the bacteria to the surface is crucial for biofilm development. This process was fully developed after the 20th day of biofilm development (paper IV, Fig. 5). Some exopolymers surrounding bacteria could also be observed in the microscope at that time be. In general, bacteria can either have preformed extracellular polymer layers, and thus immediately adhere to the substratum surface, or adhere reversibly at first and later produce polymeric material for the irreversible attachment (116). In this case it is clear that the irreversible attachment was achieved after a while, showing that the bacteria produced the exopolymers after attachment. If a nitrifying bacterial strain produces exopolymers, it is more likely found adhered to surfaces in nature. The presence of such a strain can also help other strains, lacking of the polymer production, to adhere (130). Since the growth of bacteria in this biofilm was logarithmic from beginning, the amount of initially adhered bacteria seemed to have the largest effect on the start-up time in the trickling filter. An increased presence of such polymer producing, nitrifying bacteria in the beginning could maybe minimize the time for the start-up.

5.3 Biofilm predators and biofilm thickness.

The influence of predators on nitrification in aerobic biofilm has been investigated by Lee and Welander (81). They found that the presence of predators, mostly rotifers and nematodes, decreased the nitrification rate in continuous-flow suspended-carrier biofilm reactor by half of the rate without predators. Jeppsson *et al.* (63) describes a simplified modelling approach to include some possible effects of microfauna on nitrification. In this model, both the concentration of nitrate and total suspended solids in the bulk phase increased, when the microfauna was inhibited. In the

biofilm in the Rya trickling filter, red worms (*Annelida*) were found in the filter from approximately day 100. According to these studies the presence of these worms could have been negative for the nitrification rate found in the filter. One possible positive effect of these predators could be that their grazing of the biofilm reduces the film thickness, thereby increasing availability of oxygen and nutrients. This would have the largest effect if the nitrifying bacteria was stationed near the substratum. The nitrification rate was in the beginning of the building-up of the biofilm related to bacterial count (paper IV, Fig. 4). This indicates that the nitrification bacteria was present in the beginning of the building-up, and adhered near the substratum. If this was still the case when the biofilm matured the predators would have positive effect.

For a nitrifying reactor, there exists a minimum fixed biomass concentration that assures a maximum removal rate of ammonium, and once this minimum fixed biomass concentration is attained, the performance of the system is not improved with further increase in biomass (87). In that paper the calculated active thickness of the biofilm was 22 µm. This implies that the ammonium removal reaction takes place in a very thin portion of the biofilm, and can be considered a surface reaction (87). Shramm *et al.* (123) found by measuring nitrate with a microelectrode that nitrification in a trickling filter in aquaculture was restricted to a narrow zone of 50 µm on the very top of the film. Zhang *et al.* (153) showed a nitrification depth of 150 µm in a heterotrophic-autotrophic biofilm. These studies suggests that the nitrifying activity occurs in the top of the film. Predators grazing on the film would thus have negative effect.

Okabe *et al.* (104) showed that in biofilms fed with water containing different C:N ratio the nitrifying activity is found in different places of the biofilm. At a C:N ratio of 1.5, the nitrifiers was found in the innermost biofilm, near the substratum, whereas at C:N ratio of 0 (no carbon added) the nitrifiers was found in the outer biofilm. This study proves that nitrifiers could exist in the inner biofilm and contradicts the others. The C:N ratio in the influent water to the Rya trickling filter based upon COD and NH_4 measurements was on the average 1.2 in the days, and suggests that the nitrifying bacteria was in the interior of this film. On the other hand, the carbon source reaching the nitrifying trickling filter would not be easy degradable and thus decreasing the available C:N ratio.

Because of the growth of bacteria in microcolonies, there are open water channels between these colonies. Between 50 and 98 % of the space between these colonies are reported to consist of such open water

channels (25, 79). These channels could explain the presence of nitrifying activity in the bottom of the biofilm.

The discussion above indicates that the worms present in the Rya trickling filter did not have to be negative for the biofilm nitrification. This is however not conclusive, since actual determinations on where the nitrifying bacteria were situated.

6. CONCLUSIONS

The introduction of biological nitrogen removal in the present wastewater treatment systems does not seem not to enhance the emissions of nitrous oxide to the atmosphere. This is valid during normal conditions in the wastewater treatment system. In the denitrification process, unusual events such as low pH could disturb the process, resulting in a higher production of nitrous oxide, both totally and as a fraction of the denitrification rate.

In the denitrification basin the dominating nitrogen removal process was denitrification, about 75 % of the nitrogen disappeared could be found as nitrogen gas.

Polymeric substances are potential sources of carbon and are thus important for supporting the denitrification process. In the denitrification basin the availability of easy utilizable carbon limited the denitrification rate. No external carbon source was added, and the bacteria were thus relying on the hydrolysis of degradable organic matter. Based on exoenzyme activity in the denitrifying activated sludge, the carbon source used most was short chained esters, producing monomers such as fatty acids. Other poly/oligomers were also used, such as carbohydrates, peptides and long chain esters, but in much lower concentrations. The exoenzyme activity could not be correlated to denitrification rate. A large fraction of the enzyme activity originated from other sources than the microbes in the denitrification basin, and this could possible explain why no correlation was found.

Inhibiting α-glucosidase in activated sludge had very little effect on the respiration rate. The carbohydrate utilization (α-glucosides) thus seemed to contribute only a minor part to the total carbon utilization. The presence of species constantly producing α-glucosidase also indicated that present α-glucosidase activity did not have to reflect the momentarily carbohydrate utilization.

In the trickling filter nitrifying bacteria adhered and grew logarithmically almost from the beginning of the biofilm formation. Irreversible adhesion was achieved first after 20 days, and in the beginning of the process other types (larger) of bacteria were present. This indicates that the start-up process could be enhanced by increased amount nitrifying bacteria, capable of fast irreversible adhesion, in the beginning of biofilm formation.

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