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# Nitrifiers in constructed wetlands treating landfill leachates

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Nitrifiers in constructed wetlands treating landfill leachates

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Cover: The author in action at Korslöt overland flow area. Photo by Per-Eric Lindgren.

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“En del barn får åka till Spanien på semester och en del får åka till soptippen”

”Some kids go to Spain on their holiday and some kids go to the landfill”

Sara Jannes, Tvetå Recycling site 2004-07-06

.....it can depend on their mother.....



## Abstract

Landfill leachate is produced many years after a landfill site closes. Hence, treatment by “natural methods”, as e.g. constructed wetlands, with low management requirements is attractive. Constructed wetlands usually provide both shallow and deep areas with aerobic and anaerobic zones, which is suitable for nitrification followed by denitrification of the ammonium-rich leachates. Full-scale treatment systems are influenced by climatic variables that affect the microbial community. Also, the operational strategy can have a considerable impact on both activity and composition of the microorganisms. Many studies have measured inflow/outflow water quality in treatment systems. Such “black box” studies describe the treatment efficiency but add little to an increased understanding of the organisms performing the treatment or the spatial distribution of their activities and treatment processes. .

In this thesis we investigated seasonal and annual changes in potential nitrification and denitrification, and in the corresponding bacterial communities in constructed wetlands treating landfill leachates. Variations in the potential activity in full-scale systems were investigated over several years, using short-term incubation. The composition of the bacterial communities was investigated using a group-specific PCR primer pair targeting the 16S rRNA genes or a primer pair targeting the functional gene *nosZ*. The PCR products were analysed by denaturing gradient gelelectrophoresis and subsequent nucleotide sequencing and phylogenetic analysis.

A stable ammonia-oxidising bacterial (AOB) community composition and potential ammonia-oxidation (PAO) were detected in the system with a year-round operation. On the other hand, changes in the AOB community composition which followed the operational schedule were detected in the overland flow area (OFA) running seasonally. Furthermore, the influence of operational strategy was indicated by a low PAO in the wastewater overland flow area and compact constructed wetland receiving high hydraulic loads, indicating the value of aeration. Higher PAO was detected in the OFAs where the hydraulic load followed literature guidelines.

All systems supported diverse AOB communities, represented by several *Nitrosomonas* and *Nitrospira* populations. The number of different populations detected in these wetlands was much higher than reported in municipal wastewater treatment plants, and differed from those in a parallel OFA treating municipal wastewater. Furthermore, the large variation in both potential activity and sequences detected in replicate samples suggests that such systems are spatially heterogenic.

## LIST OF PAPERS

The following papers are included in the thesis. They are referred to in the text by their Roman numerals.

- I Sundberg, C., Stendahl, J.S.K., Tonderski, K., Lindgren, P-E. 2007. Overland flow systems for treatment of landfill leachates – potential nitrification and structure of the ammonia-oxidising bacterial community during a growing season. *Soil Biology and Biochemistry* 39, 127-138.
- II Sundberg, C., Tonderski, K., Lindgren, P-E. 2007. Potential nitrification & denitrification and the corresponding composition of the bacterial communities in a compact constructed wetland treating landfill leachates. *Water Science and Technology* 56(3), 159-166.
- III Sundberg, C., Tonderski, K., Lindgren, P-E. 2008. Development of the community structure and activity of ammonia-oxidising bacteria in overland flow systems used to treat landfill leachates. *Submitted to Journal of Applied Microbiology*.
- IV Sundberg, C., Tonderski, K., Lindgren, P-E. 2008. Ammonium oxidation and the corresponding bacterial communities in two overland flow areas treating wastewater or landfill leachates. *Manuscript*.

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## 1. Nitrogen as an environmental problem

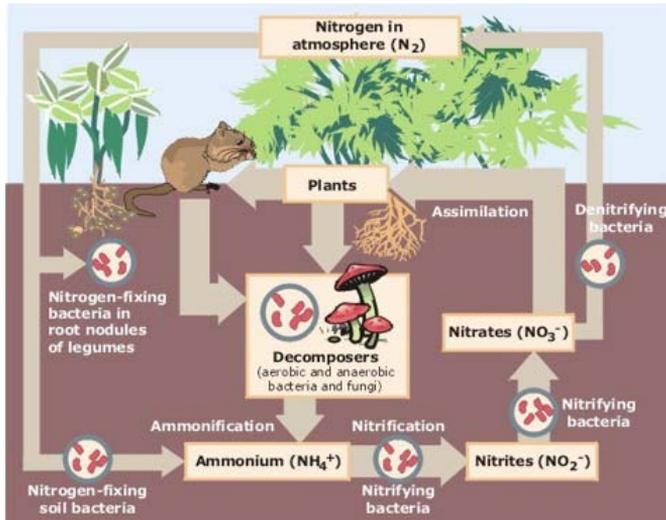
Nitrogen is common in the environment, for example the air contains 78% of nitrogen gas ( $N_2$ ), by volume. There is an opinion, however, that in some areas nitrogen levels are too low, e.g. the farmland, while in others they are too high e.g. wastewater and landfill leachate. Nitrogen is a common component in agriculture fertilizers and high application causes emissions to water in an agricultural landscape. Another nitrogen source is the outflow from wastewater treatment plants (WWTP) but there are regulations specifying maximum levels that may be released into the receiving water body. Of course, treatment in a WWTP is expensive and more cost-efficient methods are always attractive. Wetland treatment is becoming more common, partly because it increases cost-efficiency through reducing the volume of wastewater that needs to be treated at a WWTP.

Landfill leachate is often rich in ammonia. Nowadays, it is becoming more common to treat landfill leachate in on-site systems, close to the landfill (Mulamottil, 1999; SEPA, 1993). On-site systems can be exemplified by irrigation of energy crops or forests, infiltration arrangements or constructed wetlands.

Nitrogen compounds are mobile because they are changeable,  $NH_4^+$ ,  $NH_3$ ,  $NO_3^-$ ,  $NO_2^-$ ,  $NO$ ,  $N_2O$ ,  $N_2$  (Fig. 1). High concentrations of ammonia,  $>10 \text{ mg } NH_4^+-N \text{ l}^{-1}$  in the water can be toxic for living organisms (Rönnöls and Karlberg, 1996; Hammer and Hammer, 2001). The toxic form is ammonia, and the ratio between ammonium and ammonia ( $NH_3$ ) in water depends on pH and water temperature (Welander, 1998). In soil systems nitrogen compounds will change in response to pH, where  $NH_4^+-N$  dominates at neutral pH and  $NH_3$  dominates at pH at 11 (reaction 1).



Ammonia can be released to the air at high pH levels, which can be an easy way to get rid of the nitrogen. However, the problem is not solved, because the ammonia can still cause environmental problems. Gaseous ammonia will occur at pH higher than 8 (Reddy and Patrick, 1984). Ammonium can also be held in the soil by cat ion exchange (CEC) in soil, which is an important mechanism used in treatment wetlands. CEC causes ammonium ions to be attached to negative soil particles while other positive ions are released to the water phase (Rönnöls & Karlberg, 1996; Eriksson and Andersson, 1999). Ammonia concentrations can also be lowered by the microbial nitrification (Kadlec and Knight, 1996; Welander 1998). The transformation of ammonia to nitrate will render nitrogen-rich effluents less oxygen consuming and less toxic in the receiving water. Hence, nitrification is an important step towards the goal of good water quality (SEPA, 1993).



**Figure 1.** The Nitrogen cycle. Used by permission from USEPA.

Nitrogen emissions are involved in eutrophication and high levels of available ammonia will support a high growth of macrophytes and algae. Both ammonium ( $NH_4^+$ ) and nitrate ( $NO_3^-$ ) can be used as N-sources by macrophytes and algae. The high growth of macrophytes and especially algae produces a lot of material which will result in oxygen consumption when it is decomposed by microorganisms. This decomposition often takes place on the bottom of lakes or seas and can result in very low levels of oxygen close to the bottom with “bottom death” as a consequence.

This nitrogen-related environmental problem is not a recently detected problem and several studies have been done in the past. Studies have measured inflow/outflow water quality in treatment systems (Sundblad, 1998; Andersson, 2005). Inflow/outflow measurements describe the treatment efficiency but they do not tell us anything about the processes, the microorganisms or the spatial distribution of the treatment activity. The treatment system is seen as a “black box”.

Many studies have also investigated the corresponding nitrogen bacterial communities both with 16S rRNA genes 16S rDNA and functional genes. The community studies have resulted in detailed descriptions of the nitrogen cycling bacteria, both morphology (Belser and Schmidt, 1978; Madigan et al., 2003) and phylogeny (Utaaker et al., 1995; Stephen et al., 1996; Purkhold et al., 2000; Aakra et al., 2001; Purkhold et al., 2003). They have also thrown some light on in which habitat some groups are commonly detected (Table 1). Most of these studies have been done in conventional wastewater treatment systems. However, treatment in constructed wetlands and other semi-natural treatment systems is becoming increasingly common. Then the question arises if knowledge about bacterial communities in conventional treatment systems is valid also for the more complex semi-natural systems? Most microbial studies have been done in municipal wastewater treatment systems, but there are other point-sources discharging nitrogen, e.g. landfills. Is it possible to infer knowledge about the former to the function of the latter? To answer that question it is

time to look into the “black box” of treatment wetlands and link activity studies with studies of the communities.

## 2. Aim of this thesis

The overall aim was to look into the “black box” by investigating seasonal and annual changes in potential nitrification and denitrification, and in the corresponding bacterial communities in constructed wetlands treating landfill leachates. How the community composition in full-scale wetlands change over time and what will be the influence of one common operational strategy? It is also of interest to do comparisons with wastewater treatment systems to see the influence of water quality.

More specifically we wanted to:

- Investigate the seasonal variations in potential oxidation of ammonia and nitrite, and the composition of the ammonia-oxidising bacterial community in two overland flow areas (Paper I).
- Investigate the seasonal variations in potential ammonia oxidation and potential denitrification and the corresponding bacterial communities in a compact constructed wetland (Paper II).
- Investigate the development in two overland flow areas of different age and operational strategy by studying seasonal and annual changes in the potential ammonia oxidation and in the composition of the ammonia-oxidising bacterial community (Paper III).
- Investigate the development in two overland flow areas receiving dissimilar load treating landfill leachate or municipal wastewater by studying seasonal and annual changes in the potential ammonia oxidation and in the composition of the ammonia-oxidising bacterial community (Paper IV).

## 3. Nitrogen-rich emissions

### 3.1 Landfill leachate

The waste in a landfill will be decomposed by microbial processes in different steps which change the composition of the leachates. The first step is a short aerobic phase of several weeks which is followed by two anaerobic periods. The first one can go on for several years and it is called the “sour phase” followed by the very long “methanogenic phase” which can carry on for hundreds of years. The exact length of the different stages depends on the age of the landfill and also on the characteristics of the microbiological processes in each landfill (SEPA, 1993).

Landfill leachates defined by the Swedish environmental protection agency (SEPA) are “the precipitation, surface- or groundwater which is leaving the landfill after having passed through the landfill or on the surface of the landfill” (SEPA, 1996). Leachates contain high concentrations of ammonia: 800 mg l<sup>-1</sup> was observed in a Swedish study (Öman et al., 2000) and up to 1800 mg l<sup>-1</sup> in a South Korea study (Im et

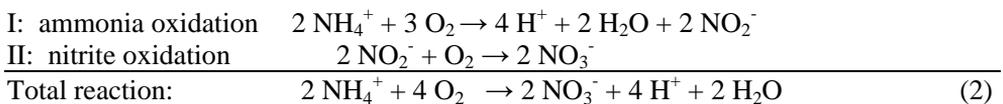
al., 2001). The volume and also the chemical composition of leachate depends on the amount of precipitation, surface and groundwater flows and also on the landfill itself e.g. the age, storage, water holding efficiency and methods of coverage (SEPA, 1993; Kadlec, 1999). In Sweden  $11 \cdot 10^6 \text{ m}^3$  of leachate was collected in 2001 and there is an intention to treat the leachates on-site at the landfills in order to reduce the loads on municipal wastewater treatment plants (RVF, 2003). Landfill leachate will be produced for hundreds of years, even after a landfill is closed, so treatment by low-cost and low maintenance methods as in constructed wetlands is attractive (SEPA, 1993; Rönnols & Karlberg, 1996; Welander 1998).

### 3.2 Wastewater

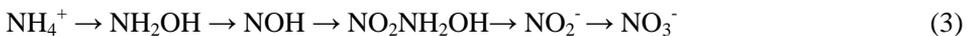
Wastewater usually being treated in municipal wastewater treatment plants and the effluent wastewater reaching a constructed wetland often have a high concentration of nitrate, rather than ammonia. The nitrate concentrations can be in the range of 10-20  $\text{mg l}^{-1}$  (Kallner et al., 2001; Kjellin et al., 2007), and ammonia in the range of 5-30  $\text{mg l}^{-1}$  (Eriksson and Andersson, 1999; Kallner et al., 2001; Hallin et al., 2005; Lydmark et. al., 2007). Wastewater also often contains organic carbon in different compounds, for example COD 210  $\text{mg l}^{-1}$  (Hallin et al., 2005), DOC 12  $\text{mg l}^{-1}$  (Bastviken et al., 2003). Another wastewater described by Sakano et al. (2002) contained higher ammonia concentrations, 150  $\text{mg l}^{-1}$  and a TOC level of 250  $\text{mg l}^{-1}$ . The latter wastewater is more like the Swedish level of a landfill leachate, in terms of ammonia concentrations.

### 4. Nitrification

Nitrification is an aerobic oxidation of ammonia in two steps. In the first step in which ammonium is oxidised to nitrite by ammonia oxidising bacteria and in the second step the nitrite is further oxidized to nitrate by nitrite oxidising bacteria (reaction 2)



In a treatment wetland nitrification can lower the soil pH since two hydrogen ions are released in the first part of the nitrification (reaction 2).



Optimal pH for nitrification is in the range of 8-9 (Ahlberg, 1998), or more exactly at pH 8.4 (Hammer and Hammer, 2001). Nitrification is one of the most pH sensitive processes in soil (Paul and Clark, 1989). Nitrification in acidic forests at pH 4 may depend on micro-niches which hold a higher pH or a nitrification activity by heterotrophic bacteria (Paul and Clark, 1989). In laboratory studies the lowest pH value for nitrification activity was at 6. Another pH study of nitrification in wastewater showed that nitrification is not carried out at pH lower than 6.45 and not at higher pH than 8.95 (Ruiz et al., 2003). Positive effects on nitrification were detected in both

liming and/or clear-cutting coniferous forests, because the higher pH and ammonia availability stimulated growth of ammonia oxidising bacteria (Bäckman et al., 2003).

Nitrification is an aerobic process and 4.6 kg oxygen is needed to oxidise 1 kg of  $\text{NH}_4^+\text{-N}$  to nitrate (Rönnöls & Karlberg 1996, Hammer & Hammer 2001). It is unclear where the oxygen limit for nitrification activity is placed and different concentrations have been observed in different studies. For example, a limiting value of 2-3  $\text{mg l}^{-1}$  was suggested by Ahlberg (1998), however, Hammer and Hammer (2001) reported 1.0  $\text{mg l}^{-1}$ . In another study, nitrite accumulation was detected below 1.4  $\text{mg l}^{-1}$  and reached a maximum at 0.7  $\text{mg O}_2 \text{l}^{-1}$  (Ruiz et al., 2003). Nitrite accumulation indicated that the ammonia-oxidising bacteria are limited at lower oxygen concentrations. At lower oxygen levels than 1.8  $\text{mg l}^{-1}$  the greenhouse gas  $\text{N}_2\text{O}$  is produced (Goreau et al., 1980; Lipschultz et al., 1981).

As with most microbial processes, nitrification will increase when the temperature increases. Growth of ammonia and nitrite oxidising bacteria is stimulated by higher temperatures (Painter 1986, Hammer & Hammer 2001). The optimal temperature for nitrifying bacteria is between 28 and 36°C (Ahlberg 1998) but nitrifying activity has been detected at temperatures as low as between 0 and 5°C (Sundblad & Wittgren 1991). In full-scale studies at low temperatures only nitrite production has been detected, indicating that ammonia oxidation worked at lower temperatures (Alleman, 1985).

Since nitrification is a microbial process, it is likely that the activity responds to everything in the environment not only one parameter, as usually investigated in laboratory studies. A combination of temperature and pH effects showed that the nitrite-oxidising bacteria *Nitrobacter* sp. was sensitive to changes while the ammonia-oxidising bacteria *Nitrosomonas* sp. was not (Paul & Clark 1989). *Nitrosomonas* sp. has a broad linear response to temperature at all pH values, whereas *Nitrobacter* sp. only has that response at pH 7.3, and otherwise is very sensitive to temperature changes. This may explain the observation of nitrite accumulation at low temperatures (Alleman, 1985). Painter (1986) summarized results from temperature and pH studies, and in some studies temperature was limiting nitrification and in other studies pH was the regulating factor.

There are other factors that can inhibit nitrification, for example, gaseous ammonia ( $\text{NH}_3$ ) or gaseous nitrous acid ( $\text{HNO}_2$ ) (Ahlberg 1998). Also organic compounds and metals such as copper, cadmium and nickel can inhibit the nitrification activity (Painter, 1986; Grunditz et al., 1998). A synergistic effect between low temperature and the toxicity of nickel was detected, where nickel was more toxic at low temperatures (Randall and Buth, 1984). Inhibiting chemicals can be used in laboratory studies which is an easy way to study parts of a microbial process, for example N-Allylthiourea,  $\text{C}_4\text{H}_8\text{N}_2\text{S}$  (ATU) inhibiting the first step between ammonium and hydroxylamine in nitrification, and nitrite-oxidation (Paper I) can be studied (Paul & Clark 1989).

There has been an opinion that organic material was toxic for the nitrifiers, but today there is another opinion (Paul & Clark 1989). The problem with organic matter may instead be about competition for available oxygen with the heterotrophs which are decaying the organic material.

#### 4.1. Ammonia- and nitrite-oxidising bacteria

Nitrifiers are aerobic chemoautotrophic bacteria, which means that they use inorganic carbon (CO<sub>2</sub>) or bicarbonate for cell synthesis, and use ammonium (NH<sub>4</sub><sup>+</sup>) or nitrite (NO<sub>2</sub><sup>-</sup>) as an energy source (Rönnöls & Karlberg 1996, Ahlberg 1998). The nitrifiers are slow growing (Prosser, 1986; Bock et al., 1995). The first step in nitrification, which is performed by the ammonia oxidising bacteria (AOB; reaction 1) is considered to be rate limiting. Therefore, they have been widely studied with the opportunity to optimize treatment systems for nitrogen-rich wastewater.

Ammonia oxidising bacteria belong to β-Proteobacteria, which includes the two genera *Nitrosospira* divided into five clusters, clusters 0-4, and *Nitrosomonas* divided into clusters 5-8, based on 16S rDNA gene sequences (Head et al., 1993; Stephen et al., 1996; Purkhold et al., 2000; Purkhold et al., 2003). These two groups are more or less present in typical habitat (Table 1), *Nitrosospira* sp. are more common in low ammonia environments (Hiorns et al., 1995) such as forests (Bäckman et al., 2003) and grasslands (Kowalchuk et al., 2000; Webster et al., 2002), while *Nitrosomonas* sp. are common in ammonia-rich environment (Hiorns et al., 1995; Princic et al., 1998) such as wastewater treatment plants (WWTP) (Mobarry et al., 1996; Purkhold et al., 2000; Lydmark et al., 2007) and activated sludge (Hallin et al., 2005).

**Table 1.** Characteristics of ammonia oxidising bacteria (Jiang and Bakken, 1999; Purkhold et al., 2000, Koops and Pommerening-Röser, 2001)

Species	Sequences derived from	Isolates/ Enrichments obtain from	Substrate affinity (K <sub>s</sub> )	Salt requirement	Urease activity
<i>Nitrosospira</i> cluster 0	soil	soil	11 μM		+
<i>Nitrosospira</i> cluster 2	sand dune, soil	sewage treatment, soil	8 μM		+
<i>Nitrosospira</i> cluster 3a	soil, sand dune, freshwater	concrete wall, soil, animal house, peatbog	6-9 μM		+
<i>Nitrosospira</i> cluster 3b	soil, sand dune, freshwater	concrete wall, soil, animal house, peatbog			
<i>Nitrosospira</i> cluster 4	soil, sand dune, freshwater	soil			
<i>Nitrosomonas</i> cluster 6a	Freshwater, soil, wastewater	aestuary, soil, activated sludge	1.9-4.2 μM	no salt requirement	+
<i>Nitrosomonas</i> cluster 6b	Seawater, freshwater, sand dune	seawater	50-52 μM	obligate halophilic	+
<i>Nitrosomonas</i> cluster 7	wastewater, seawater, freshwater	Activated sludge, biofilm, seawater, brackish water, concrete wall, animal house	30-61 μM	halotolerant Moderat halophilic	-

Some ammonia-oxidising bacteria have urease activity which means that they can grow on urea; they have enzymes to use urea as a source of ammonia (Table 1; Jiang

and Bakken, 1999). *Nitrosomonas* sp. is larger in size and grows more rapidly than *Nitrospira* sp. (Belser and Schmidt, 1978). When *Nitrosomonas* sp. and *Nitrospira* sp. grow together in laboratory cultures, the culture will be dominated by *Nitrosomonas* sp. almost every time. However, all ammonia oxidising bacteria are difficult to isolate or growth in the laboratory (Belser and Schmidt, 1978).

*Nitrospira* sp. and *Nitrosomonas* sp. have different substrate affinity ( $K_S$  value), where *Nitrospira* sp. has a lower  $K_S$  value and thereby lower substrate affinity (Koops and Pommerening-Röser, 2001). This suggests that *Nitrospira* sp. is more prevalent in an environment with less ammonia (Kowalchuk et al., 2000; Purkhold et al., 2000; Bäckman et al., 2003). This idea is strengthened by the findings that high-ammonia treatment systems are often were dominated by a single *Nitrosomonas* population (Schramm et al., 1996; Juretschko et al., 1998; Okabe et al., 1999). There is one *Nitrosomonas* species, *N. oligotropha* belonging to cluster 6a, which has  $K_S$  value 1.9-4.2  $\mu\text{M}$ , close to the  $K_S$  value of *Nitrospira* sp. (Koops and Pommerening-Röser, 2001). Other *Nitrosomonas* clusters have  $K_S$  values in the range 19-61  $\mu\text{M}$ , which may explain the high occurrence of them when ammonia is available. *Nitrosomonas* sp. has a higher maximum activity, which makes them able to dominate at high ammonia concentrations (Schramm et al., 1996; Juretschko et al., 1998; Okabe et al., 1999).

Sequences related to *Nitrosomonas europaea* and *Nitrosococcus mobilis* were more common in the manure, feces, and raw wastewater pond samples, while *Nitrospira* sequences, often belonging to cluster 0 and to cluster 3a, were more common in wetlands treating dairy waste (Ibekwe et al., 2003). *Nitrospira* cluster 0 is usually detected in unamended soils, for example *Nitrospira* sp. belonging to cluster 0 was only detected in older grassland (Dell et al., 2007).

AOB populations may diversify with different types of biofilm attachment sites. Greater numbers of nitrifying bacteria and higher activity were detected on macrophyte roots than in surrounding soil (Kyambadde et al., 2006) and differences were detected between AOB populations growing on living macrophyte tissue and detritus (Flood et al., 1999). AOB may also diversify within the biofilm itself and different organisms can be active in response to large micro-scale variations in the physiochemical environment as observed in unmanaged soil (Bruns et al., 1999; Ibekwe et al., 2002; Webster et al., 2002).

## 5. Denitrification

Denitrification is an anaerobic reduction of nitrate to nitrogen gas. Denitrification is a four-step process reducing  $\text{NO}_3^-$  to  $\text{N}_2$  via intermediates (3) (Myrøld, 1998).



Gaseous NO and  $\text{N}_2\text{O}$  may be released during denitrification, but the major product is  $\text{N}_2$  (Paul and Clark, 1989). High C:N ratios stimulate complete denitrification ending up with  $\text{N}_2$ , (Hunt et al., 2003). Emissions of  $\text{N}_2\text{O}$  from a treatment wetland were correlated with oxygen consumption in the rhizosphere regardless of season and plant growth (Inamori et al., 2007).

The large decrease in nitrate, typically observed in treatment wetlands, suggests that denitrification is an important process (Xue et al., 1999; Billore et al., 1999;

Braskerud et al., 2002; Lin et al., 2002; Edwards et al., 2006; Sirividhin and Grey, 2006). Denitrification in treatment wetlands was estimated to account for as much as 90% of the overall nitrogen removal (Xue et al., 1999; Lin et al., 2002).

Environmental factors that influence denitrification include the absence of oxygen, availability of organic matter and nitrate, appropriate redox potential, temperature, pH and indirectly soil type and the degree of moisture saturation which in turn influence oxygen concentration, pH, organic matter etc. (Focht and Verstrate, 1977; Vymazal, 1995).

The potential denitrification (when electron donors and acceptors are not limiting) in soil has been shown to increase significantly when the soil area is converted to a wetland, probably by the more anaerobic conditions. A common problem for high denitrification in treatment systems is the low availability of organic carbon. Therefore, in many wastewater treatment systems it is common to add of an easily degradable carbon source, for example methanol, ethanol etc. Probably, also wetland treatment systems for denitrification may supply too low levels of available carbon. Addition of organic carbon has been shown to increase the nitrate removal in constructed wetlands (Gersberg et al., 1983; Lin et al., 2002). However, at winter temperatures (7.5 °C) nitrate removal did not respond to added organic matter (Burchell II et al., 2007). When incubated, wetland sediment from an organic soil showed higher potential denitrification than sediment from a mineral soil (D'Angelo and Reddy, 1999; Davidsson and Ståhl, 2000). A linear relationship between denitrifying activity and sediment organic matter was detected, but only in the upper layer (Hernandez and Mitch, 2007). In another study, the addition of organic carbon had no effect on the denitrification activity, instead a positive effect was observed when nitrate was added (Toet et al., 2003). Furthermore, other activity studies noted correlations to the nitrate concentrations in the overlying water (Toet et al., 2003) and in the sediment itself (Toet et al., 2003; Hunt et al., 2003). Bastviken et al. (2003) detected highest potential denitrification in the sediments when comparing different surfaces for microbial biofilm in a treatment wetland, assuming the organic matter and not nitrate was the regulating factor.

An important contributor to organic matter in the wetlands is vegetation. Plants may supply organic carbon through rhizodeposition of C substrates and significantly more nitrate was removed in a planted versus unplanted microcosm and the effect was species-specific (Lin et al., 2002). However, higher nitrogen removal was detected in a free water surface wetland with 50% plant cover compared to one with 100% plant cover (Ibekwe et al., 2007). Potential denitrification varied significantly between vegetation communities (Toet et al., 2003), and higher potential denitrification was detected in emergent macrophytes than in the open water (Hernandez and Mitch, 2007). Higher denitrification was detected on perfoliate pondweed (*Potamogeton perfoliatus* L.) shoots with high periphyton abundance than on those with low abundance (Weisner et al., 1994).

Other regulating factors are temperature and pH which normally regulates microbial processes. Denitrification increases with increasing temperature and activity has been measured in temperatures up to 60-75°C (Toet et al., 2003; Burchell II et al., 2007). Whereas, denitrification rates were not only suppressed at low temperature in treatment wetlands (5°C) (Brodrick et al., 1988; Werker et al 2002; Burchell II et al.,

2007), but major low temperature products were the greenhouse gasses  $N_2O$  and  $NO$  (Bremner and Shaw, 1958; Broadbent and Clark, 1965).

Optimal pH is 6 – 8 (Paul and Clark, 1989) and the activity is low at pH 5 and absent in pH below 4 (Vymazal, 2007).

### 5.1 Denitrifying bacteria

Most denitrifying bacteria are facultative anaerobic chemoheterotrophs, using organic compounds as electron donors and as a source of cellular carbon and nitrogen oxides (in ion and gaseous form) as terminal electron acceptors (Hauck, 1984).

The genera *Bacillus*, *Micrococcus* and *Pseudomonas* are most common in soils, while *Pseudomonas*, *Aeromonas* and *Vibrio* are more common in aquatic environments (Grant and Long, 1981). The ammonia oxidising bacteria *Nitrosomonas eutropha* has been shown to be able to denitrify using hydrogen as electron donor and  $NO_2^-$  as electron acceptor and to produce  $NO$  and  $N_2O$  under anoxic conditions (Bock et al., 1995).

## 6. On-site treatment - Constructed wetlands

It has become common to treat landfill leachates and wastewater in constructed wetlands, both in Sweden and in the rest of the European Union (EU). In the last decades, several studies have demonstrated that constructed wetlands are effective for treatment of ammonia-rich wastewater (Sundblad, 1998; Andersson et al., 2005; Bastviken et al., 2003; Nurk et al., 2005) and landfill leachates (Renman and Kietlinska, 2000). The purpose of treatment of nitrogen-rich water in wetlands is to remove the nitrogen to the air *via* emissions of nitrogen gas ( $N_2$ ). Components of the N cycle (Fig. 1) in wetlands have been well studied, and there is a consensus that microbial processes dominate the transformations. Most studies have been done by indirect measurements such as inflow/outflow water quality and flow, but nowadays microbial studies have become more common.

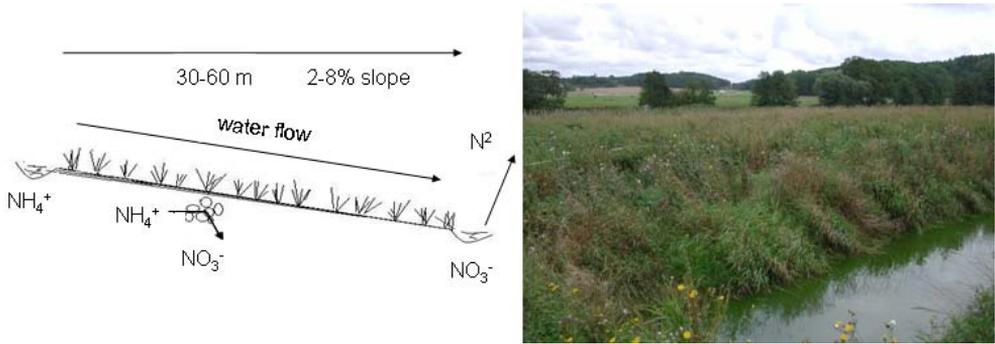
Constructed wetlands are designed to treat either ammonia-rich water by stimulating the nitrification process by aerobic conditions in the wetlands, e.g. low or fluctuating water levels, aerated periods and submersed macrophytes. Constructed wetlands can also be designed to treat nitrate-rich water by anaerobic conditions to stimulate denitrification, e.g. higher water levels and a high macrophytes production to provide carbon (Hume et al., 2002). Constructed wetlands usually provide both shallow and deep areas with aerobic and anaerobic zones. Overland flow areas can constitute one aerobic component of a constructed wetland, with surface areas for development of nitrifying biofilms. To increase the area for bacterial biofilm, the constructed pond can be filled with gravel material or slag in a filter bed (Silyn-Roberts and Lewis, 2001). These wetlands described as subsurface wetlands are commonly used especially when the land area is limited. Wastewater can be loaded in different ways either horizontally or vertically (Kadlec and Knight, 1996; Song et al., 2006; Vymazal, 2007). Filter bed systems are often planted with common reed (*Phragmites australis* Cav.) Filter bed systems can be constructed when the wetland area must be limited.

## 6.1 Overland flow areas

An overland flow area (OFA) is a treatment wetland where we control water levels (Fig. 2). Historically this technique was used to increase the yield of hay for fodder (Leonardson 1994, Rönnols and Karlberg, 1996). The volume of wastewater is reduced by plant uptake and by the evaporation from land and plants in the OFA (SEPA, 1993). The plants use both ammonia and nitrate for growth and the nitrifying bacteria use the plants as surfaces for their growth (Eriksson and Andersson, 1999; Bastviken et al., 2003).

Bacterial processes in soils are often stimulated by drying and rewetting procedures, intermittent application, and this can enhance the availability of oxygen (Leonardson, 1994; Sørensen 1974, Reddy & Patrick 1975, Garrett, 1991; Zirschky et al., 1989; Fierer and Schimel, 2002). Intermittent application during 8-12 h has been shown to dramatically increase the nitrogen removal even at higher loads whereas continuous flow resulted in lower nitrogen removal (Smith and Schroeder, 1985). Also, shorter application periods used together with longer drying periods significantly increased the nitrogen removal in comparison with continuous flow (Zirschky et al., 1989; Kruzic and Schroeder, 1990). Typical hydraulic application rates in overland flow areas range from 1 to 10 cm d<sup>-1</sup> (Kadlec and Knight, 1996). The highest ammonia removal from wastewater treated in overland flow areas was detected at the hydraulic load of 2.8-3.4 cm d<sup>-1</sup> (Zirschky et al., 1989) and of 1.9-2.6 cm d<sup>-1</sup> (Smith and Schroeder, 1985). Wastewater application of 5 cm in 18 h 5 d week<sup>-1</sup> was recommended by Peters et al. (1981), but they recommended a lower load during the winter i.e. 1 cm for 18 h 7 d week<sup>-1</sup>. A consultancy company designing overland flow areas in southeast Sweden often used 8 h application and 16 h drying period (Stråe 2000). The length of the wetland is also important, and higher nitrogen removal was observed in overland flow areas as long as 61 m and 91 m, than in those of 30-46 m (Zirsky et al., 1989).

Positive ammonium ions will adsorb to negatively charged soil particles and can be oxidised by nitrifying bacteria to the less harmful nitrate ion during an aerobic drying period. In the drying periods, the ammonia oxidising bacteria should have sufficient time to oxidise the attached ammonia. At the next application the negatively charged nitrate ion will be lost to the water. When that nitrate-rich water is collected in a deeper anaerobic pond, the nitrate will be reduced by denitrifying bacteria to nitrogen gas and be emitted to the air. The last step, denitrification, may be limited by available organic material but in the wetlands the macrophyte litter consists source of organic matter (Hume et al., 2002).



**Figure 2.** Design and principles of an overland flow area. Photo from Korslöt OFA showing the application channel.

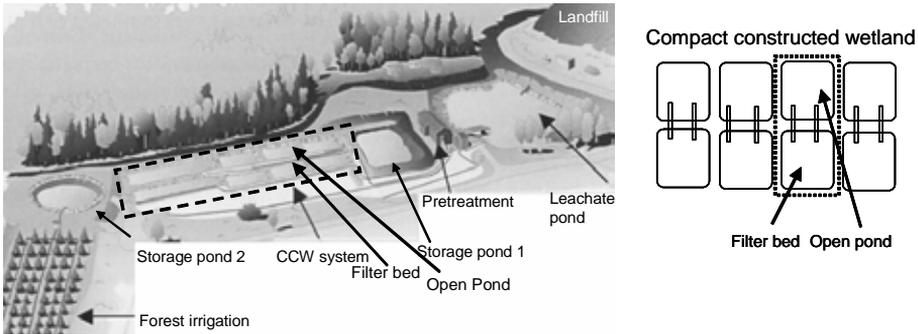
One of the most common planted grasses in overland flow areas is reed canary grass (*Phalaris arundinacea*, L.) (Reed et al., 1988). In Sweden *P. arundinacea* is also common, sometimes combined with creeping bent (*Agrostis stolonifera*, L.). When the overland flow area has received ammonia-rich water some years, common reed (*Pragmites australis*) usually invades the area (Stråe, 2000). Reed canary grass can tolerate a load of  $0.02 \text{ m d}^{-1}$  and both nutrients and temperature in the applied water will stimulate growth (Bernard, 1999). Plant uptake of nitrogen was higher in areas with reed canary grass than in areas with a mixture of different grasses (Leonardson, 1994). Wetland plant uptake can be at the level of  $0.1 \text{ g N m}^2 \text{ d}^{-1}$  (Lin et al., 2002). Most of the plant species can be harvested during the season without any harm if the height is over the water level (Bernard, 1999). Water sustainable plants have the possibility to transport oxygen to the roots/rhizomes and oxygen will also leak to the anoxic sediments (Brix and Schierup, 1990; Johnson et al., 1999). The commonly used water tolerant plants grow well in contaminated water, and they also store nutrients in rhizomes which is why they can start growing early in the spring (Bernard, 1999). The effect of the warm temperature in a landfill leachate (winter temperature  $5\text{-}8^\circ\text{C}$ ) was studied in constructed wetlands in New York. In the autumn the die back was two weeks delayed, and the growth in the spring was initiated two weeks earlier than in natural wetlands (Bernard, 1999).

## 6.2 Compact constructed wetlands

Wetland treatment is usually land consuming, which has led to the development of a compact constructed wetland (CCW) for use in areas with limited land availability (Renman and Kietlińska, 2000). A compact constructed wetland is one kind of a subsurface flow, SSF wetlands as described above. A CCW comprises a series of pairwise connected SSF wetlands and open ponds (Fig. 3). Each pair consists of one SSF wetland, and one open pond with no vegetation. The bottom of the pond is sealed with rubber membranes, and the paired ponds are interconnected by means of two pipes installed near the bottom. To promote denitrification, the filter bed consists of peat, wood debris and sand. It is also seeded with cattail (*Typha latifolia*, L.). As in subsurface flow wetlands, the purpose of the CCW filter bed is to increase the surface

area for the bacterial biofilms and also to create various microniches that can support different bacterial communities.

The leachate is batch feed to the surface of the filter bed via a perforated pipe, and it percolates through the material and then flows through the bottom pipes into the open pond. In pilot experiments, filter-beds and open ponds have been filled in various cycles to improve nitrogen removal and also to manage the leachate volume to be treated. The system can be run at temperatures above 0°C (Renman and Kietlińska, 2000; Kietlińska, 2003).



**Figure 3.** Compact constructed wetland treatment system for landfill leachates at Tveta Recycling Facilities, Sweden. (modified after Jannes, 2004). Investigated filter bed and connecting pond (Paper II) to the right.

## 7. Methods

### 7.1 Potential activity

Potential activity indicates the size of the actual bacteria community if the incubation is short enough, so the bacterial community does not have time to grow in size (Schmidt and Belser, 1994; Madigan et al., 2003). The interpretation is not that simple, as the activity per cell may be different in different environmental conditions. Potential activity of a bacterial community implies that there are optimal conditions without any limiting factors, e.g. substrate, oxygen/anoxic conditions, competition, temperature, pH, salinity, inhibiting substances, redox conditions etc. (Schmidt and Belser, 1994). Potential activity is measured by incubation where subsamples are taken at a decided schedule and the transformation of a substrate is measured directly or indirectly (Papers I, II, III, IV). Either the products or the consumed substrate can be measured, and the activity can be calculated per hour. It is common to incubate in a media which has a buffering capacity to maintain a stable pH. The media also contains micro- and macro-nutrients (Schmidt and Belser, 1994; Eriksson and Andersson, 1999).

#### *Potential ammonia oxidation – short-term slurry incubation*

For ammonia oxidising bacteria ammonium sulphate ( $\text{NH}_4\text{SO}_4$ ; in this thesis  $10 \text{ mg NH}_4^+\text{-N l}^{-1}$ ) is added and the samples aerated (Fig. 4; Schmidt and Belser, 1994). The sum of nitrite and nitrate ( $\text{NO}_2^- + \text{NO}_3^-$ )-N produced is analysed e.g. spectrophotometrically using an autonalyser (Fig. 4). A short-term incubation method is a commonly used method in treatment wetland studies (D'Ángelo and Reddy, 1999; Eriksson and Andersson, 1999; Kyambadde et al., 2004; Münch et al., 2005; Nurk et al., 2005; Truu et al., 2005; Edwards et al., 2006; Papers I, II, III and IV).

#### *Potential nitrite oxidation – short-term slurry incubation*

For nitrite-oxidising bacteria sodium nitrite ( $\text{NaNO}_2$ ; in this thesis  $10 \text{ mg NO}_2^-\text{-N l}^{-1}$ ) and N-allylthiourea (ATU; in this thesis  $10 \text{ mg l}^{-1}$ , Eriksson and Andersson, 1999), are added and the samples aerated (Fig. 4; Schmidt and Belser, 1994). ATU inhibits the first step in nitrification, the oxidation of ammonia to nitrite, and addition of ATU means that no nitrite will be produced during incubation. Therefore, the consumed nitrite is an effect of nitrite-oxidation. The consumed nitrite is analysed spectrophotometrically, for example, using an autonalyser (Fig. 4). (Paper I).

#### *Nitrification – isotope-dilution technique*

Isotope-dilution is a commonly used technique to measure the production of nitrate, e.g. nitrification (Koike and Hattori, 1978; Bastviken et al., 2003; Kyambadde et al., 2006).  $^{15}\text{N}$ -nitrate is added to the water phase and later on the amount of  $^{15}\text{NO}_3^-$  and  $^{14}\text{NO}_3^-$  is measured after a chemical conversion to  $\text{N}_2$ , which is analysed by a mass spectrometer. Otherwise, an enrichment culture of denitrifying bacteria can be added with the intention that they should convert  $\text{NO}_3^-$  to  $\text{N}_2$ , i.e. denitrification, which will be analysed (Rysgaard et al., 1993). The dilution of  $^{15}\text{NO}_3^-$  is proportional to the amount of nitrification.

### *Potential denitrification – stable isotope*

Actual nitrification (as isotope-dilution above) and potential denitrification can be measured using  $^{15}\text{NO}_3^-$  by analysing produced  $^{29}\text{N}_2$  or  $^{30}\text{N}_2$  which can be measured in a mass spectrometer (Nishio et al., 1983). The dilution of  $^{15}\text{NO}_3^-$  is proportional to actual nitrification. The amount of  $^{29}\text{N}_2$  or  $^{30}\text{N}_2$  may indicate if the availability of nitrate limits the denitrification. This technique is more expensive than acetylene inhibition (Xue et al., 1999; Bastviken, 2006)

### *Potential denitrification – acetylene inhibition method*

The acetylene inhibition method is a commonly used method for the detection of potential denitrification (Balderston et al., 1976). Acetylene inhibits the enzyme (nitrite oxidoreductase) activity in the last step in the denitrification process between  $\text{N}_2\text{O}$  and  $\text{N}_2$  (reaction 4), and the produced  $\text{N}_2\text{O}$  is analysed using a gas chromatograph.



The anaerobic conditions can be provided by incubation of the samples in gas tight bottles with nitrogen gas ( $\text{N}_2$ ) instead of oxygen. Excess nitrate ( $\text{NO}_3^-$ ) is added and easily degradable carbon can be supplied by addition of methanol or ethanol (organic matter) to create optimal conditions. The acetylene inhibition method cannot be used to study denitrification of the nitrate produced by nitrification in the sample, because acetylene also inhibits the nitrification activity (Klemmedtsson et al., 1988). The acetylene inhibition method has commonly been used in treatment wetland studies (Xue 1999; Bastviken et al., 2003; Toet et al., 2003; Hunt et al., 2003; Münch et al., 2005; Ishida et al., 2006; Sirivedhin and Grey, 2006; Smialek et al., 2006; Hernandez et al., 2007; Lin et al., 2007; Paper II)

## **7.2. Molecular methods used to investigate the “black box”**

Activity and potential activity studies give a picture of how the treatment wetlands work, but they do not show which bacteria are most important. Hence, the aim of the work in this thesis is to look in the “black box” and find out which bacteria are there “working” with the nitrogen removal molecular methods will be useful.

Total DNA isolated from various soil types has been successfully amplified in PCR with primers specific for bacteria. Misidentification of bacteria has been far less common with the gene coding for rRNA sequences than with morphology methods (Biovin-Jahns et al., 1995). Problems with misidentification using rDNA sequences usually depend on a lack of related sequences in the databases.

### *DNA extraction from the field samples*

Genomic DNA from all bacteria, fungi, plants, and animals in a soil community can be extracted as described by the manufacturer of one commercial kit (Bio 101, Inc., La Jolla, CA, USA). The resulting DNA ranges from ~6 to 25 kb. As described in common soil kit protocol the microorganisms are lysed without shearing the nucleic acids and without using enzymes, manual grinding or homogenisation. Different buffers are used to homogenize the samples and also for protein solubilisation. The

reagents enable extraction of genomic DNA with minimal RNA contamination. The included mixture of detergents and salts is there to contribute to the inactivation of the nucleases and to control the degree of shearing of the DNA. This new procedure is effective for removing PCR inhibitors from even the most difficult soil types e.g. methods/substances to remove humic substance and brown colour. Taken together, the commonly used commercial kit delivers a DNA of good quality and comparisons are easier when a universal kit is used (Papers I, II, III and IV).

### **7.3. Investigating bacterial communities using conventional PCR**

Polymerase chain reaction, PCR, is a method to multiply DNA segments by repeating cycles of high and low temperature, to separate the DNA strands and to synthesize new strands using Taq polymerase. Taq polymerase is commonly used in conventional PCR. However, other polymerases are available, for example Expand High Fidelity which has proof-reading functions and controls the bases in the synthesized new strands. Depending on which bacterial group that is the target, the primer pair may amplify either a 16S rDNA sequence or a functional gene sequence from that group. Some bacterial processes can be done by different bacterial groups, in which case the functional gene can be used for community analysis. One example is the denitrification process. When a mixed template was used, biases in the PCR amplification based on the primers and also on the amount of DNA have been detected (Farrelly et al., 1995; Suzuki and Giovannoni, 1996). DNA extracted from environmental samples can be a problem to amplify by the PCR-technique, since it may contain compounds, for example humus which inhibits the reaction. To reduce this problem it is common to add a protein, for example T4 Gene 32 Protein or BSA (Bovine Serum Albumin). The protein binds the inhibiting substances. Another simple method is to dilute the extracted DNA sample (Ferris and Ward, 1997). To avoid the establishment of unspecific amplified sequences in the PCR running has a decreasing of the amplification cycles in the PCR program been practiced with good result (Sakano and Kerkhof, 1998).

#### *16S rDNA PCR primer targeting ammonia oxidising bacteria*

It is common to study AOB communities by the 16S rDNA gene because of the taxonomic proximity in this bacterial group (Juretschko et al., 1998; Aakra et al., 2001; Purkhold et al., 2000; Purkhold et al., 2003). A commonly used ammonia oxidising bacterial primer pair is CTO189f/CTO654r targeting the V2-V4 region (Kowalchuk et al., 1997; Stephen et al., 1998; Kowalchuk et al., 2000; Boon et al., 2002; Bäckman et al., 2003; Lydmark et al., 2007). This primer pair is expected to detect all  $\beta$ -proteobacteria, and also some belonging to the  $\gamma$ -proteobacteria (Cébron et al., 2004). Purkhold et al. (2000) have reported that the CTO-primer pair had mismatches to the *Nitrosomonas* sp. belonging to cluster 7. However, when a slightly modified variant of the CTO-primer pair was used, additionally four new sequences were detected (Lydmark et al., 2007).

#### *Functional gene PCR primers*

All functional gene primers are designed to amplify fragments of the gene encoding enzymes involved in the function to be investigated, for example the *nosZ* genes.

Detection of denitrifying bacteria in the environment has generally targeted *nirK/nirS* genes, which encode the enzyme catalyzing reduction of  $\text{NO}_2^-$  to  $\text{NO}$  (Braker et al., 1998; Hallin and Lindgren, 1999; Angeloni et al., 2006) or the *nosZ* genes which encode the enzyme catalyzing reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  (Throbäck et al., 2004; Gomez-Villalba et al., 2006). The functional gene *amoA* can be targeted when studying the ammonia-oxidising bacterial group by the function e.g. the ammonium-monooxygenase enzyme (McTavish et al., 1993; Rotthauwe et al., 1997; Purkhold et al., 2000; Truu et al., 2005; Lydmark et al., 2007).

### *Analyses of the PCR products using gel electrophoresis*

#### DGGE as a method to study the bacterial community composition

The structure of DNA changes with temperature and these changes in structure affect the movement of DNA through the gel. Denaturing gradient gel electrophoresis (DGGE) is able to separate gene sequences of the same length, but with different base pair composition (Muyzer et al., 1993). A polyacrylamide gradient gel is composed with a range of denaturant in percent, with highest percent in the upper part of the gel. At the beginning of a run the DNA move through the gel. However, when the denaturing gradient increases the two strands of the DNA melt apart and finally the strand will partly separate and the moving slow down. The separation is based on variations in migration rates due to differences in conformation, where GC-rich regions hold together for longer than AT-rich components. To prevent a total denaturation of the double stranded DNA, a GC-clamp (i.e. a stretch of 30-40 base pairs of G and C to raise the melting temperature) is added to one of the primers (Kowalchuk et al., 1997). Some problems with co-migration of different strains to the same level in the gel have been detected (Kowalchuk et al., 1997; Bäckman et al., 2003). DGGE will give qualitative information, i.e. indicate the presence or not of a bacteria species, which can be analysed by presence-absence matrixes (Kjellin et al., 2007).

#### SSCP as a method to study the bacterial community composition

Single-strand conformation polymorphism (SSCP) has been developed, as DGGE, for detection of mutations. A single stranded DNA will fold into a secondary structure (conformations) according to its nucleotide sequence and physiochemical environment (Schweiger and Tebbe, 1998). Different conformations can be separated in a non-denaturing polyacrylamide gel. Some sequences made several conformations and resulted in more than one band, which is why reamplification of the cut out bands is necessary before sequencing (Schweiger and Tebbe, 1998; Bäckman et al., 2003).

#### DGGE as a method to study the active bacterial community composition

Functional diversity can be analysed using cDNA and DGGE, which gives an indication of the active bacterial community (Girvan et al., 2003). In some samples a more diverse composition has been detected with cDNA-DGGE than with rDNA-DGGE. Treatment differences were highlighted with the cDNA-DGGE method. For example short-term changes in land-use management were more obvious and easy to detect than with rDNA-DGGE (Girvan et al., 2003). If treatment wetlands were

disturbed and did not remove nitrogen in a satisfying manner, this method can be used to describe the composition of the active AOB community.

#### T-RFLP as a method to study bacterial community composition

Terminal restriction fragment length polymorphism (T-RFLP) is a PCR based method including two primers of which one or both can be fluorescently labelled (Liu et al., 1997). The PCR products are cleaved with restriction enzymes which give terminal restriction fragments (TRFs) of different sizes separated by gel electrophoreses. The relative abundance of TRFs gives a fingerprint of the dominant microbial community in the samples (Ishida et al., 2006). Land-use management's influence on the soil bacterial community was studied by T-RFLP and PCR-DGGE, and a greater variability between the replicates was detected in the T-RFLP assay (Girvan et al., 2003). In complex soil systems, the possibility to determine if a sequence is present or absent without the need to know the identity was used (Osborne et al., 2006), this comparison could also be done by PCR-DGGE.

#### *Sequence and phylogenetic analysis*

The cut out bands from the DGGE gels can be sequenced and analysed using sequence databases, for example GenBank (NCBI) (Bäckman et al., 2003; Hallin et al., 2005; Lydmark et al., 2007). Comparisons to determine similarity with known 16S rDNA sequences or functional gene sequences in the GenBank database will be done. At the comparisons the homology will be announced in percent. All sequences added in the database have an accession number which makes it possible to identify the originated study.

Phylogenetic trees can be made by several methods, for example neighbor joining with Jukes and Cantor correction (Jukes and Cantor, 1969). Neighbor joining is a quick non-parametric distance method which makes pair wise comparisons. Neighbor joining does not assume that all lineages evolve at the same rate (molecular clock hypothesis) and produces an unrooted tree. Rooted trees can be created by using an out-group and the root can then effectively be placed on the point in the tree where the edge from the outgroup connects. Bootstrap analysis can be done based on a selected number of replicates, a common number is 100 replicate (Papers I, II; III and IV not shown).

Maximum parsimony is used with most kinds of phylogenetic data; until recently, it was the only widely-used character-based tree estimation method used for morphological data.

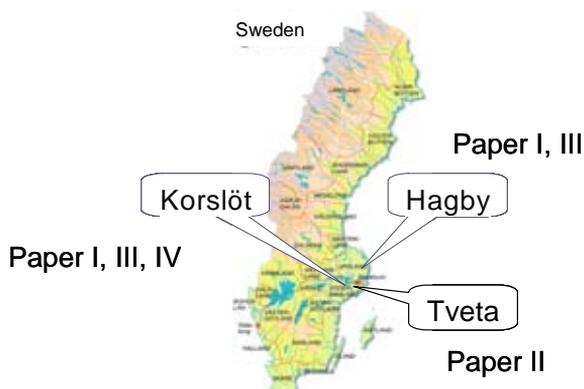
Maximum likelihood is a parametric statistical method, in that it employs an open model of character evolution. Such methods are potentially much more powerful than non-parametric statistical methods like parsimony, but only if the model used is a reasonable approximation of the processes that produced the data. Maximum likelihood has probably surpassed parsimony in popularity with nucleotide sequence data.

Relationship among rDNA sequences can be visualized using multidimensional scaling (MDS) analyses of the distance matrix, which is also used to calculate the phylogenetic tree (Bourneman et al., 1996).

## This work

### Site description

The study was carried out in southeast central Sweden at three landfills and one wastewater treatment system (Figs. 5 and 6). The landfills contain mainly household and construction/industrial waste. For the leachate water, each landfill has a settling pond.



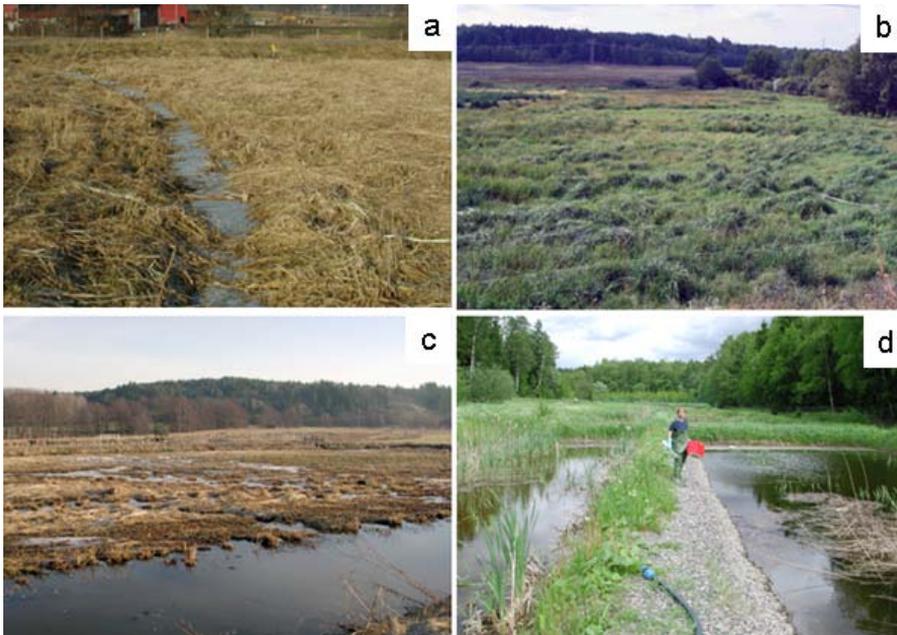
**Figure 5.** Map of the studied sites located in southeast Sweden, represented in the papers number.

Two of the landfills have a treatment system after the settling pond, consisting of an overland flow area with intermittent application (8 h watering and 16 h drying) to stimulate the nitrification, and finally a pond intended primarily for denitrification. The overland flow and wetland systems were designed by Water Revival Systems (WRS) Uppsala AB, Sweden, and they each have a well-defined inlet and outlet. Employees at the landfills collect water samples at designated times for nutrient analyses, approximately one per month. Approximate hydraulic load, nitrogen concentrations and pH are presented in Table 2.

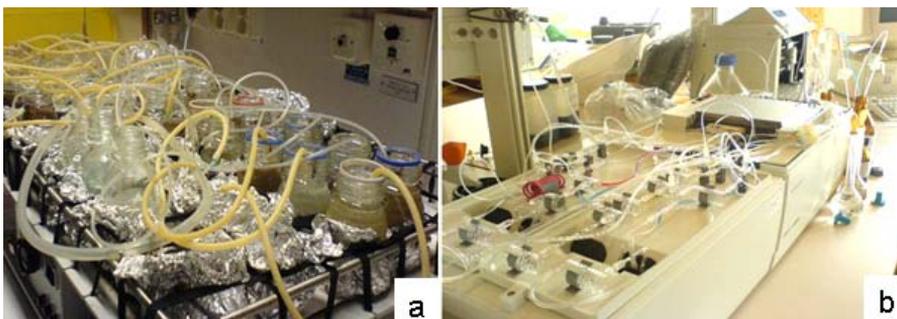
**Hagby recycling site** in Täby is run by Söderhalls Renhållningsverk AB (Fig. 6). The 11 600 m<sup>2</sup> overland flow area is situated on an organic soil. It was constructed in 2002 to treat all the landfill leachate, and was sown with a mixture of the reed canary-grass *Phalaris arundinacea* (Cav.) and creeping bent *Agrostis stolonifera* (L.). In 2003, the area was dominated by *P. arundinacea* with *A. stolonifera* growing in small patches between the *P. arundinacea* shoots. The water is applied through gated pipes, and the system is in operation only during the growing season from April to November.

**Korslöt wastewater treatment plant** in Vagnhärad is run by Trosabygdens Teknik AB (Fig. 6). The overland flow area was constructed in 2000 on old farmland and was sown with *P. arundinacea*. The OFA, segmented in six sections, is covered with *P. arundinacea* and common reed *Phragmites australis*. The wastewater is intermittently

applied via an open channel to the OFA. The system is in operation year round, the vegetation is harvested in October and the cut biomass left on site. Since January 2003, one section (800 m<sup>2</sup>) of the OFA is loaded with landfill leachates from **Korslöt recycling site** in the same manner as for the wastewater sections (the remaining 5 sections approximately 7000 m<sup>2</sup> is still used for applied with wastewater). Korslöt recycling site is also run by Trosabygdens Teknik AB (Fig. 6). The leachate is collected in a settling pond at the landfill and conveyed to the open channel at the OFA section for landfill leachate.



**Figure 6.** Korslöt landfill OFA (a), Hagby landfill OFA (b), Korslöt wastewater OFA (c) and Tveta CCW treating landfill leachates (d).



**Figure 4.** Short-time slurry incubation with addition of  $\text{NH}_4^+\text{-N}$  and oxygen (a). Analysis of produced  $(\text{NO}_2^- + \text{NO}_3^-)\text{-N}$  using an AutoAnalyzer 3 (b)

**Table 2.** Approximate hydraulic load and mean nitrogen concentrations in incoming landfill leachates and wastewater from the monitoring programs, 2003-2006. pH (mean, n=2-3).

	q (mm d <sup>-1</sup> )	Tot-N (mg l <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> -N (mg l <sup>-1</sup> )	(NO <sub>2</sub> +NO <sub>3</sub> )-N (mg l <sup>-1</sup> )	pH
<b>Hagby landfill</b>					
2003	44	104	78	4	7.4
2004	56	84	70	3.2	7.3
2005	62	99	82	10	7.2
2006	44	104	97	1.2	7.8
<b>Korslöt landfill</b>					
2003	16	204	166	1	7.4
2004	19	165	154	1.4	7.3
2005	40	110	99	2.7	7.5
2006	38	113	122	2.4	7.5
<b>Korslöt wastewater</b>					
2004	170	17	10	n.d.	6.9
2005	224	21	0.1	n.d.	6.8
2006	133	19	0.1	n.d.	7.0
<b>Tveta CCW Landfill</b>					
2004	n.d.*	178	128	1	7.6

\*The CCW was not loaded per day, total leachate volume 2004 was 100 000 m<sup>3</sup>

**Tveta Recycling Facility** in Södertälje is run by Telge Energi AB. Since 1998, the landfill leachate has been treated at temperatures above 0°C in a compact constructed wetland (CCW) consisting of four sets of pair-wise connected subsurface flow wetlands (SSF) and ponds (Figure 3; Renman and Kietlińska, 2000; Kietlińska, 2003). Each pair consists of one SSF (5 x 28 m) with a 0.75 m thick filter bed, and one open pond (8.5 x 28 m) with a clay bottom and no vegetation. To promote denitrification, the filter bed consists of peat, wood debris, and sand. The SSF was also seeded with cattail (*Typha latifolia*, L.). The bottoms of the ponds are sealed with a rubber membrane, and each pair of SSF and pond is interconnected by means of two pipes installed near the bottom. We investigated one of the four pairs of the CCW ponds (Figure 3). The CCW was loaded with landfill leachate containing on average 30 mg l<sup>-1</sup> BOD<sub>7</sub> (Jannes, 2004). The leachate was batch filled (Caselles-Osorio and Garcia, 2007) on the surface of the SSF via a perforated pipe, and it percolated through the material and then flowed through the bottom pipes into the open pond. The SSF and open ponds were fully filled at water levels of 0.50 and 1.25 m, respectively (Fig. 6). The schedule for filling and draining (it takes one day to fill and one day to empty the system) is shown in Table 3.

**Table 3.** Schedule of application of landfill leachates at Tveta Recycling Facility (Sweden) in 2004. \* Bold type indicates sampling dates.

Treatment periods	Leachate-filled period	Dry period
2004-05-18– <b>2004-07-06*</b>	1 week	1 week
2004-07-07– <b>2004-09-07*</b>	2 weeks	2 weeks
2004-09-08–2004-09-22	1 week	1 week
<b>2004-09-23–2004-11-03*</b>	Stagnant leachates the whole study period	

\*Samples were collected at 6<sup>th</sup> July, 7<sup>th</sup> September, and 3<sup>rd</sup> November 2004 and the system was emptied one day before.

### Environmental sampling

In the overland flow areas samples were taken in the macrophyte litter layer and in the rhizosphere (Front page picture). Seven samples were collected in May, August, and November on an area of 300 m<sup>2</sup> (Papers I, III and IV). The sampling points in the field were randomly chosen and there was a practical reason for the choice of seven samples, which was the maximum number that could be handled at the lab. To investigate the amount of litter in the OFA replicated samples were collected on an area of 0.23 m<sup>2</sup>, which were used to recount the activity from dry weight to wetland area (m<sup>2</sup>). In the rhizospheres samples for density determination were collected for the same reason (Papers I, II, III and IV). In the settling ponds, a sediment sampler (Ø 0.07 m) was used to collect sediment at seven sites (Papers I and III).

In the compact constructed wetland samples were collected at three depths in the filter bed using an auger (Ø 0.04 m), and at one depth in the pond sediment (Paper II). Samples for the potential activity incubations were stored at +4°C and used within 24 hours, except for the samples for the potential denitrification assay at Tveta CCW (Paper II) which were stored for 48 h. Samples for DNA extraction were stored at –20°C until used.

On all sampling occasions, dissolved oxygen and temperature in the surface water of the OFAs and the settling ponds were measured *in situ* (YSI Model 85, Brannum Lane Yellow Springs, Ohio, USA) (Papers I, III and IV), and one bottle (0.2 l) of leachate was collected from each of the settling ponds for measurement of pH (Radiometer 28 pH METER, Copenhagen, Denmark) (Papers I and III). Organic matter content was analysed as loss on ignition (550 °C, 6 h; W.C. Heraeus GmbH, Hanau, Germany) in three replicate samples from the rhizospheres at Korslöt and Hagby landfill OFA in May, August, and November 2003 (Paper I) and from the filter bed and the pond sediment at Tveta CCW in July, September and November 2004 (Paper II).

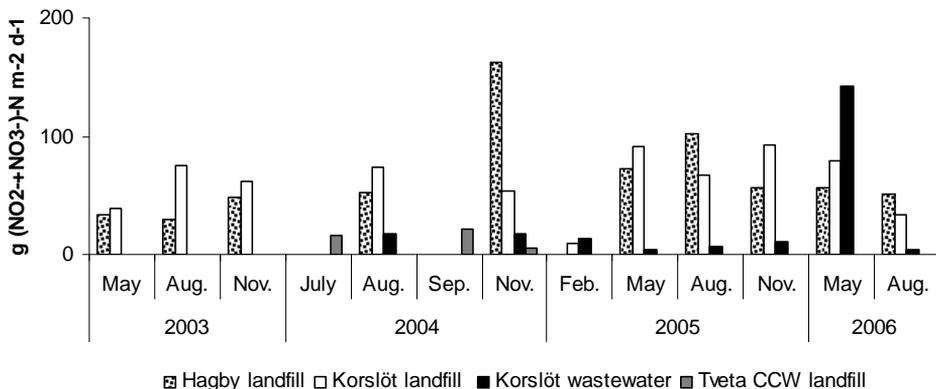
## Potential ammonia oxidation

Overall, the PAO was highest in the two overland flow areas which treat landfill leachates than in the one which treat municipal wastewater (Fig. 7). This may depend on different operational strategies as the hydraulic load was higher in the wastewater area. It might have been too high (Table 1; Fig. 6), which would have resulted in oxygen depletion in that area (Paper IV). The same thing may also have occurred in the Tveta CCW when the water level was 0.5 m above the filter bed (Paper II).

Another difference was the ammonia and oxygen concentration in the applied water (Papers I, III and IV). The low PAO in the wastewater OFA could have depended on the lower ammonia load in the wastewater OFA (Paper IV) which may have supported a smaller AOB community and therefore a lower PAO.

Abiotic factors as weather, precipitation and emissions may also affect the results. In our studies the treatment areas are located in the same region so we assumed that the small meteorological differences would have been of minor importance for the results obtained.

An inter-annual increase in the PAO was detected at Hagby OFA, especially in the litter layer (Paper III). The increasing nitrification indicated that there was a development in the OFA treating landfill leachates, which positively affected the nitrifiers. In the first year of landfill leachate application (2003), the PAO at Hagby was lower than at Korslöt (Fig. 7) but in subsequent years it was as high as or higher than in Korslöt. One reason for the difference in the first year could be that Korslöt had received wastewater during 2000-2002, and that this could have stimulated the growth and/or activity of the nitrifying bacteria more than in a natural soil (as in Hagby). However, it can not only be the number of received years that resulted in a higher PAO because the wastewater OFA had received ammonia-rich water several years and it showed a low PAO (Fig. 7).



**Figure 7.** Potential ammonia oxidation in constructed wetlands treating landfill leachates or wastewater in Sweden 2003-2006. Landfill leachate is treated in overland flow areas in Hagby and Korslöt and in a compact constructed wetland (CCW) at Tveta. Wastewater is treated in an overland flow area at Korslöt.

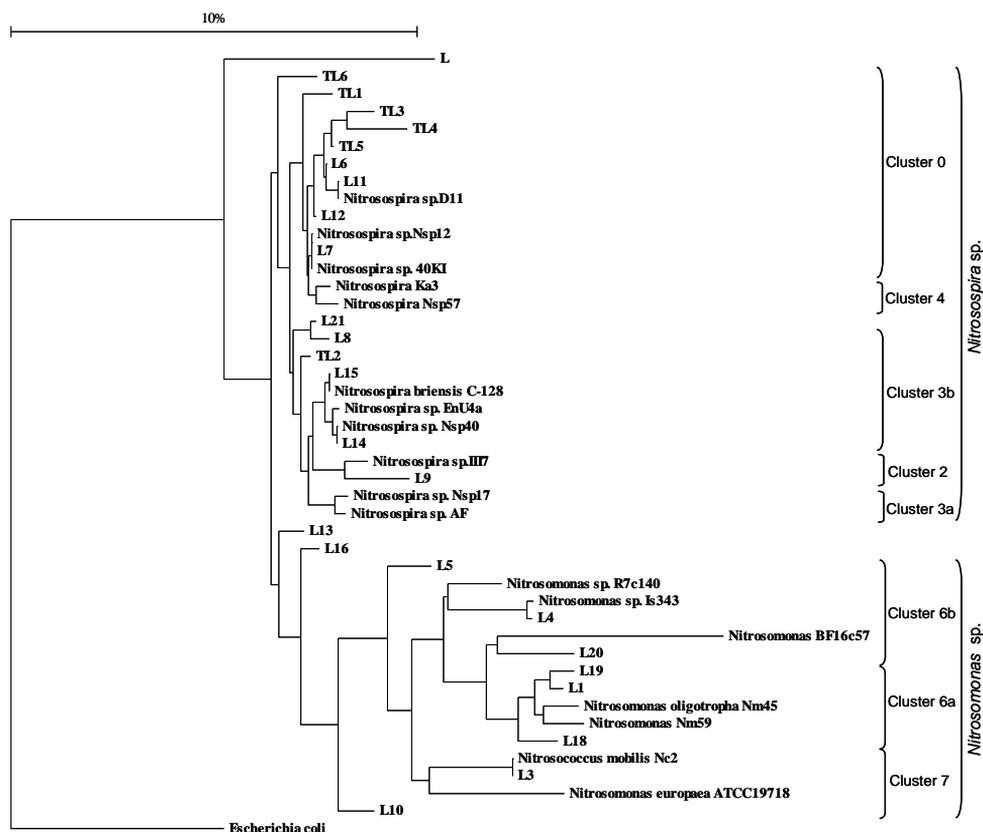
### *Seasonal variation*

We expected the potential ammonia oxidation to reach a peak in August when it is summer in Sweden, and the competition for available ammonia with growing macrophytes might have been lower than in May. Instead, a seasonal increase in PAO was detected during the studied season in 2003 (Paper I), except for the rhizosphere at Korslöt (Fig. 1 in Paper I). In that layer, the PAO in November was lower probably due to oxygen depletion caused by the fact that the area was cut and the biomass was left on the area hindering oxygen from reaching the rhizosphere possibly causing an oxygen competition with heterotrophic bacteria decaying the organic matter (Gilmore et al., 1999). However, the seasonal increase in the landfill overland flow areas at Korslöt and Hagby (Paper I) was not detected the following three years (Paper III; Fig 7). In November, the PAO was as high as the other months even if the air temperature had decreased, whereas in February a much lower PAO was observed (Fig. 7). Between November and February the AOB community in the OFA was affected by something which influenced the activity. The same irregular variations were observed in the OFA receiving wastewater in Korslöt (Paper IV). The lack of a seasonal trend indicated that air temperature was not a major regulating factor. Even if some years are warmer/colder than other years, seasonal trends would have been observed in this long-term studies. Instead a large variation between replicates, months and years indicated heterogeneity in the OFA. In this complex full-scale out-door treatment systems it may be impossible to find the regulating factor. To be noticed in the wastewater litter layer, the level of the PAO in February (Fig. 7) was in the same range as the PAO in May and in August, even though the low air temperature in February was expected to result in a lower community size and bacterial activity (Paper IV). It is possible that the AOB bacterial community in the OFA was more influenced by the water temperature than by the air temperature (Table 2 in Paper IV). The PAO in the area receiving wastewater was at the same level during the whole study except for May 2006 (Fig. 7).

The expected peak in PAO in August was detected at Tveta compact constructed wetland (CCW; Paper II). This might have been a response to the temperature and seasonality in plant ammonium uptake. Knowing the operational problems between September and November, with no aeration of the filter bed and no application of new leachates (Table 3), this would be too far reaching conclusions. It might just as well have been a depletion of both oxygen and ammonia in the CCW resulting in a low PAO in November (Fig. 7). Another explanation for the low PAO in November may have been the unusually cold period in October with two weeks of snow covering the CCW and a steady temperature below zero degrees (Fig. 8). Activity comparisons with Hagby and Korslöt OFAs in November 2004 which are located only 50 km north or south from Tveta. Hagby showed the highest PAO ever (Fig. 7). Korslöt landfill OFA showed a PAO in the same level as November 2003 and at Korslöt wastewater OFA the PAO was in the same level as almost every month (Fig. 7). Hence, the most likely explanation is that the low PAO at Tveta in November was related to the lack of aeration and leachate application more than to the low temperature.



**Figure 8.** Winter conditions at Tvetta compact constructed wetland in November 2004.



**Figure 9.** Neighbour-joining tree inferred from comparative analysis of partial 16S rDNA (AOB) sequences derived from Korslöt and Hagby landfill, Korslöt wastewater overland flow areas and from the Tveta compact constructed wetland treating landfill leachates (Sweden). AOB sequences were approximately 400 bp long (including gaps) and covered the V2–V4 region of the 16S rDNA. The sequences are denoted TL1–TL6. Estimated sequence divergence calculated using Jukes and Cantor correction. Cluster designations are those used by Purkhold et al. (2003). Bootstrap values are not given.

### The ammonia oxidising bacterial communities

In the one-season study (Paper I), a more diverse AOB community was detected in the litter layer at Korslöt than at Hagby. The structure of the AOB community was more similar in the rhizospheres. Year 2003 was the first year of landfill leachates application in both overland flow areas, but Korslöt have received wastewater during the three preceding years. It is possible that this influenced the structure of the AOB community more than we expected and that the AOB communities otherwise would have been more similar. At the end of the study in year 2006, AOB communities were

more similar each other in the landfills OFAs, including *Nitrospira* population belong to cluster 0 and *Nitrosomonas* population belong to cluster 7, and once a *Nitrosomonas* sequence belong to cluster 6a. This development to a more similar community composition strengthens the suggestion about the influence of the water applied.

Phylogenetic comparisons of the investigations (Papers II, III and IV) showed many different sequences in the areas, and they were represented in many clusters (Fig. 9). Sequences denoted L1-L21 (Fig. 9) represent sequences from that were found in both the OFAs and the pond sediments at Hagby and Korslöt. Seven sequences out of total 13 were found at both Hagby and Korslöt OFA landfills (Paper III) and three sequences out of a total 15 were detected both in Korslöt landfill and Korslöt wastewater OFA (Paper IV). In Figure 8 sequences from Tveta CCW are denoted TL1-TL6 (Paper II) and these were different from those detected in the OFAs.

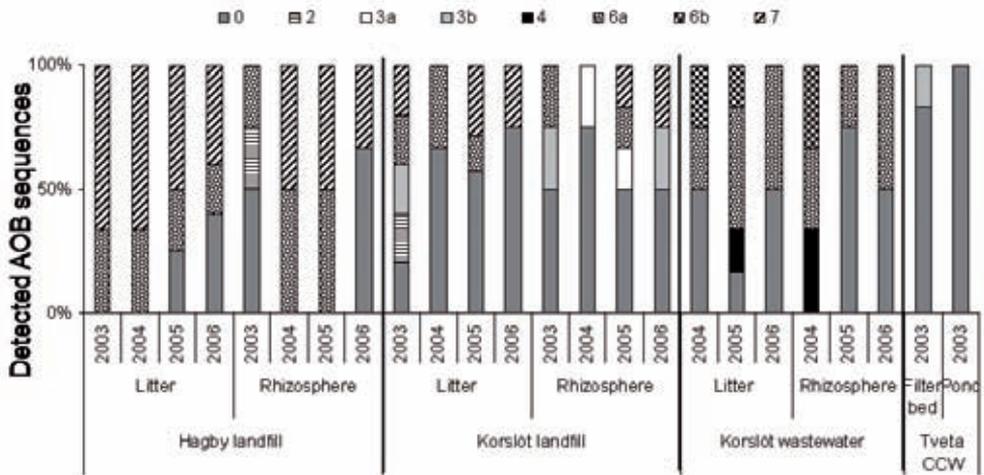
All investigated treatment systems were dominated by *Nitrospira*-like sequences (Fig. 10) despite the high ammonia load (Table 2). *Nitrospira* sp. has the possibility to hydrolyse urea, which is also true for the commonly detected *Nitrosomonas oligotropha* have (Table 1; Jiang and Bakken, 1999; Koops and Pommerening-Röser, 2001). Even if the oxidation of ammonia was sometimes inhibited in these environments *Nitrospira* sp. and *Nitrosomonas oligotropha* still had a way to grow. Maybe that is why they were most common.

#### *Seasonal changes*

The application schedule of landfill leachates at Hagby affected the AOB community composition, which showed an obvious seasonal change with fewer clusters represented at the beginning of the operation period (May; Paper III). Seasonal change, however, was not detected at the OFAs where landfill leachates or wastewater was applied year-round (Korslöt; Papers III and IV). Furthermore, seasonal change in the AOB community was not detected in the CCW at Tveta (Paper II), even though the system was closed during the winter. Operational strategies may not influence the batch feed CCW as obvious as an OFA, since the leachates is filled the bacteria may minimally be influenced by temperature and weather change.

#### *Annual changes*

Similarity to the PAO, the AOB community in the overland flow area at Hagby was also changed annually during the long-term study (Paper III). This change may depend on the way that the ammonia-rich leachate influenced the AOB community. In the litter layer, only *Nitrosomonas* populations were identified in the first two years (2003 and 2004; Fig. 10), whereas *Nitrospira* populations dominated in the rhizosphere (Paper III). However, in the following years (2004 and 2005) *Nitrosomonas* were dominating also in the rhizosphere (Paper III). This may indicate that the leachate has a profound influence on the composition of the AOB community, and that this takes longer time to show in the rhizosphere as infiltration is not a predominant water flow pathway in an OFA. A similar change in the dominating group was not detected at Korslöt landfill OFA (Fig. 10; Paper III). Maybe the previous application of ammonia-rich wastewater caused those changes earlier.



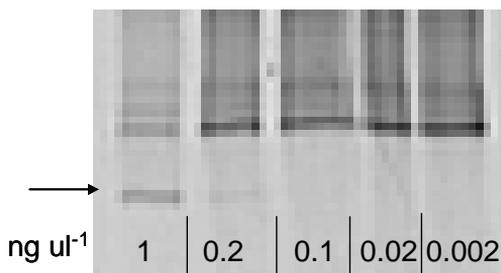
**Figure 10.** Clusters of ammonia-oxidising bacteria detected in Hagby and Korslöt overland flow areas and in Tveta compact constructed wetland during 2003-2006, Sweden. Clusters 0-4 belong to *Nitrosospira* sp. and clusters 6a-7 belong to *Nitrosomonas* sp.

As shown in Figure 10, there was no annual change of the dominating group, but there were changes at the cluster level at all sites. The first two studied years (2004 and 2005) more diverse AOB communities were detected in the wastewater litter layer, and in 2004 in the rhizosphere (Fig. 10). Overall, the wastewater OFA seemed to become dominated by *Nitrosomonas* population belonging to cluster 6a and *Nitrosospira* population belonging to cluster 0 (Paper IV). *Nitrosomonas* population included in cluster 6a is often detected in less ammonia-rich environments and also in less oxygen-rich environments (Gieseke et al., 2001). Population shifts at Korslöt landfill OFA were at the cluster level (Fig. 10) and dominated by *Nitrosospira* populations belonging to cluster 0 (Papers III and IV). However, *Nitrosomonas* populations belonging to clusters 6a and 7 were common at both Korslöt and Hagby landfills OFA. The latter was dominated by a *Nitrosomonas* population belonging to cluster 7, however, a *Nitrosomonas* population belonging to cluster 6a were nearly as common (Fig. 10; Paper III). *Nitrosomonas* populations belonging to cluster 7 were more common in ammonia-rich environments (Schramm et al., 1996; Juretschko et al., 1998; Okabe et al., 1990).

At Tveta CCW only *Nitrosospira* populations belonging to cluster 0 were detected. As well, and in the SSF also *Nitrosospira* populations belonging to cluster 3b were detected. *Nitrosospira* populations often detected under less favourable conditions for AOB, e.g. low ammonia or oxygen concentrations, may be common at Tveta because the high level of leachates (0.5 m above the SSF surface), which probably made the

system too anaerobic. Despite this high leachate level was the system filled during mostly one week without any aeration.

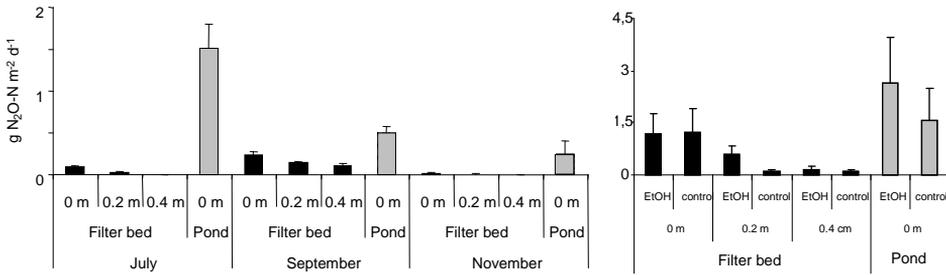
Purkhold et al. (2000) have reported that the CTO-primer pair had mismatches with the *Nitrosomonas* sp. belonging to cluster 7. To examine how the mismatches influenced the results in our study, we decided to perform a pilot investigation by adding five different concentrations (1.0, 0.2, 0.1, 0.02 and 0.002 ng  $\mu\text{l}^{-1}$ ) of DNA from *Nitrosomonas europaea* (NCIMB11850, Accession number AB070982) to one of the samples which gave rise to only one band in the DGGE analysis. We could only detect the *N. europaea* in the samples where the two highest concentrations (1.0 and 0.2 ng  $\mu\text{l}^{-1}$ ) were added. This indicates the possibility that they are undetected when they are present at low concentrations (Fig. 11)



**Figure 11.** DGGE photo from the pilot study with addition of *Nitrosomonas europaea* in different concentrations (noted in the picture) to an environmental sample from Hagby landfill overland flow area. *Nitrosomonas europaea* could be detected in the two highest concentrations (arrow).

### Potential denitrification

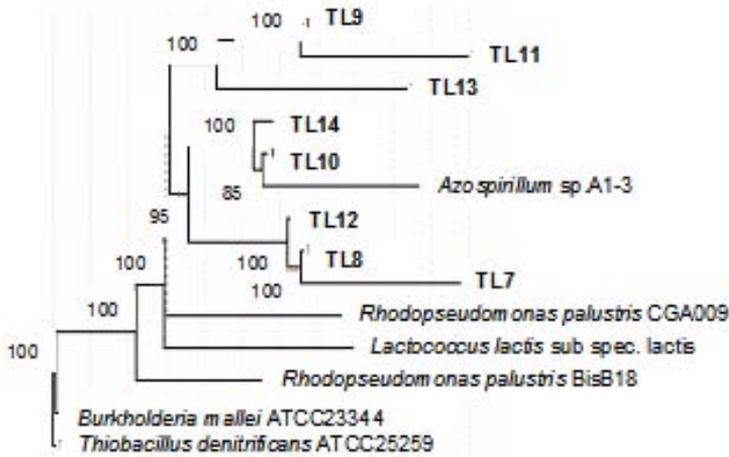
Potential denitrification (PD) was only studied in the CCW at Tveta. It was investigated during one growing season, 2004 (Paper II), using the acetylene inhibition method (Balderston et al., 1976). The potential denitrification was higher in the pond than in the SSF wetland at all sampling times, and it was decreased during the season (Fig. 12). The decrease may have been caused by the lack of new leachate between September and November, which may have caused a lack of ammonia with an accompanied lack of nitrate. There may also have been a lack of organic matter when no new leachate was applied. Normally the system was not limited by organic matter, shown by no obvious increase in PD when 30 mg  $\text{l}^{-1}$  ethanol was added during the incubation of the July samples (Fig. 12). The chosen amount was commonly added at Linköping's wastewater treatment plant (Eilertsson, communication Nov. 2001). The highest PD was detected in the pond sediment, and that was correlated with the highest amount of organic matter (Bastviken et al., 2003). At Tveta CCW the highest organic matter was detected in the sediment (Paper II).



**Figure 12.** Samples from Tvetå compact constructed wetland treating landfill leachates 2004, Sweden. Potential denitrification (left)  $n=7$ , SE, and potential denitrification with addition of 30 mg l<sup>-1</sup> ethanol (EtOH) (right) and without (control)  $n=4$ , SE

### Denitrifying bacterial communities

PCR amplification with the *nosZ* primer pair yielded one to eight bands on the DGGE gel (Paper II). When amplifying a bacterial community with primers targeting a functional gene, bacteria can be found from a lot of different groups (Fig.13, Table 4). The number of bands increased during the season in the upper layer of the SSF wetland, which also included the most diverse denitrifying community (Table 3; Paper II). The site for high diversity did not correlate with the site for the highest PD, which was detected in the pond sediment (Fig. 12). The pond sediment had more organic matter, and the pond was probably more anaerobic which may have favour a larger/or more active denitrifying population (Paper II). The band pattern from the pond sediment showed one strong band and some weaker ones. Avrahami et al. (2002) detected a shift in the denitrifying community which they thought depended on a higher ammonia load. However, they did not see this shift in the nitrifying community composition even if they detected an increased nitrification. They thought the question was about time, since the denitrifying bacteria grow faster than the AOB only the former shift was detected. In our study the increased number of bands was higher late in the season. This CCW treatment system is closed during the winter and probably the favoured conditions increased during the season together with the applied leachates.



**Figure 13.** Neighbour-joining tree inferred from comparative analysis of *nosZ* sequences derived from the Tveta Recycling Facility (Sweden). The *nosZ* sequences were 389 bp long (including gaps). The sequences are denoted TL7–TL14 (Table 4). Estimated sequence divergence calculated using Jukes and Cantor correction. Bootstrap values  $\geq 75\%$  are given at nodes (100 replicates).

**Table 4.** DNA sequencing results from samples collected at different depths (m) in the filter bed and sediment in the open pond at Tveta Recycling Facility, 2004.

\*Sequences designated TL7–TL14.

Seq.*	Filter Bed			Pond	Closest match	Similarity (%)	Strain
	(m)						
	0	0.2	0.4	0	Accession number		
<b>Denitrifying bacteria (<i>nosZ</i>)</b>							
TL7	x				CP000116.1	87	<i>T. denitrificans</i> ATCC25259
TL8	x				CP000116.1	81	<i>T. denitrificans</i> ATCC25259
TL9			x		BX572599.1	89	<i>R. palustris</i> CGA009
TL10				x	CP000301.1	88	<i>R. palustris</i> BisB18
TL11	x				CP000301.1	94	<i>R. palustris</i> BisB18
TL12	x				CP000010.1	84	<i>B. mallei</i> ATCC23344
TL13		x			AY074762.1	86	<i>Azospirillum</i> sp. A1-3
TL14	x				UO7640.1/LLU07640	98	<i>L. lactis</i> subsp. <i>lactis</i>

### Results from ponds

In Papers I and III samples were collected in the same way in the settling pond sediments at the landfills Hagby and Korslöt. In these papers comparisons were made between pond sediments, macrophyte litter layers and rhizospheres both with respect to PAO and the composition of the AOB communities. Summarizing these comparisons, we detected a low PAO in the sediments and this was probably due to low oxygen availability (Fig. 1 in Paper III). Furthermore, we detected diverse AOB communities without seasonal or annual changes (Figs. 2 and 3 in Paper III).

In Korslöt pond sediment, the PAO was approximately ten times higher than in the pond sediments at Tveta CCW and five times higher than the pond sediments at Hagby (Papers II and III). Since, the pond at Tveta CCW seemed to fit the anaerobic denitrifying bacteria, most likely the environment was not optimized for the aerobic nitrifying bacterium which was showed by only included the more tolerant *Nitrosospira* population (Paper II). Maybe this is the reason why both Korslöt and Hagby pond sediment showed a diverse AOB community, a less favourable environment may encourage several different populations.

About the results from the settling ponds, the energy-consuming mechanically aeration of the leachate in the settling pond at Hagby had no positive effect on the PAO (Paper III). A higher PAO was observed in Korslöt shown where the leachate levels in the settling pond changed and the sediments could have been aerated in that way. It is, however, impossible to draw any firm conclusion about the reason for the higher activity in the pond sediments at Korslöt, as the study was not designed to answer that question.

## Conclusions

- A high potential for treating ammonium-rich landfill leachates in constructed wetlands, especially in overland flow areas, was detected. Looking inside the “black box”, diverse AOB communities were detected as well as a large spatial heterogeneity..
- In the OFAs, the PAO was high without any seasonal or annual patterns. Despite the lower temperature in November, the PAO was high. Only in the winter month a lower PAO was detected. The low PAO in the wastewater OFA and in the CCW at Tveta suggested a strong influence of limited oxygen supply. Higher PAO was detected in the systems where the hydraulic load followed literature guidelines.
- Operational strategies were shown to influence both the PAO and the structure of the AOB community. More stable PAO and community compositions were detected in the systems with a year-round operation. In the other OFA, the composition of the AOB community changed in response to the operational schedule.
- All treatment systems supported diverse ammonia oxidising bacterial communities, represented by several *Nitrosomonas* and *Nitrosospira* populations. The number of different populations detected in these treatment wetlands was much higher than reported in municipal wastewater treatment plants.

## Future work

Treatment wetlands seem to support a more diverse AOB community than other investigated wastewater treatment systems. It is often suggested that more diverse systems would be less sensitive to disturbances, and this would be interesting to investigate further in treatment wetlands. This could be done as inhibition studies, where the activity changes are investigated and also which AOB populations dominate.

It would also be interesting to study the active AOB community using cDNA-DGGE. Another possibility is to quantify the AOB community using real-time PCR (Hermansson et al., 2004), together with a method that describes composition of the community.

Finally, I really want to investigate the effect of different retention times in the CCW at Tveta. I know they have an advanced timer system, even if it did not work during my study period. With a properly functioning loading system, it would be really interesting to measure both the treatment effect and the response of the microbial community to different loading regimes.

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