Structure and function of microbial communities in constructed wetlands - influence of environmental parameters and pesticides on denitrifying bacteria

Milenkovski, Susann

2009

Structure and Function of Microbial Communities in Constructed Wetlands

Influence of environmental parameters and pesticides on denitrifying bacteria

Susann Milenkovski

Doctoral thesis
Lund University, Sweden
2009

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in manuscript).

Cover photo by Henrik Nermar
Chapter photos by Susann Milenkovski, Geraldine Thiere or Henrik Nermar
Printed by Media-Tryck, Lund University, Lund, Sweden
ISBN: 978-91-7105-294-0

Table of Contents

Papers

This thesis is based on the following papers:

I Milenkovski, S., Thiere, G., Weisner, S.E.B., Berglund, O. and Lindgren, P-E. Variation of eubacterial and denitrifying bacterial biofilm communities among constructed wetlands. (Submitted, under revision).

II Milenkovski, S., Berglund, O., Thiere, G., Samuelsson, K., Weisner, S.E.B., and Lindgren, P-E. Composition of denitrifying bacterial enzyme genes *nir*S, *nir*K and *nos*Z in constructed wetlands. (Manuscript).

III Milenkovski, S., Svensson, J.M., Lindgren, P-E. and Berglund, O. Effects of environmental concentrations of pesticides on community structure and function of constructed wetland denitrifying bacteria. (Manuscript).

IV Milenkovski, S., Bååth, E., Lindgren, P-E. and Berglund, O. Leucine incorporation as a rapid, relevant and sensitive method to assess toxicity of fungicides to natural bacterial communities in aquatic environments. (Manuscript).

My contribution to the papers:

I I planned the study together with the co-authors. I and Geraldine Thiere conducted the field work. I conducted all laboratory analysis. G analysed the multivariate statistics. I wrote the manuscript with contributions from the co-authors.

II I planned the study together with my supervisors Olof Berglund and Per-Eric Lindgren. I conducted the field work, analysed the data and performed the statistical analyses. I wrote the manuscript with contributions from the co-authors.

III I planned the study with Olof Berglund and Jonas Svensson. I conducted the field work, analysed the data and performed the statistical analyses. I wrote the manuscript with contributions from the co-authors.

IV I planned the study with support from Olof Berglund. I conducted the laboratory analyses with support from Erland Bååth. I analysed the data. I wrote the manuscript with contributions from the co-authors.

Abstract

This thesis addresses the interactions and relationships between natural aquatic bacterial communities, environmental parameters, anthropogenic chemicals and the denitrification pathway in the habitat of agricultural constructed wetlands. The main aim was to gain fundamental knowledge of the drivers behind the processes of the denitrification (i.e. nitrogen removal) in constructed wetlands, hence, the structure and function of the denitrifying bacterial community as efficient nitrogen removal in wetlands will decrease the risk of eutrophication of freshwaters and oceans. Programmes for restoring and recreating wetlands in agricultural areas have been initiated throughout the world. Aquatic environments in these areas are also exposed to pollution from e.g. pesticides, in fact, wetland are also constructed with the purpose of reducing transport of pesticides. However, little is known whether the wetland may fulfil both purposes simultaneously. Hence, may constructed wetlands maintain a high denitrification efficiency even during pesticide exposure?

Both structure and function of the eubacterial and the denitrifying bacterial communities were analysed, but focus has been put on the denitrifying bacteria. Structural endpoints of the bacterial communities, as diversity and heterogeneity were analysed using molecular fingerprinting. Potential denitrification and leucine incorporation (i.e. bacterial growth) were measured as functional endpoints, when assessing the effects of pesticide exposure on constructed wetland bacterial communities. These structural and functional endpoints were measured without any treatments as well as measured after pesticide exposure.

The results showed that structural endpoints of eubacterial (16S rRNA gene) and denitrifying bacterial community (*nir*K, *nir*S and *nos*Z) varied between the studied constructed wetlands, and their communities were influenced by environmental parameters. The enzyme gene *nir*S showed higher community heterogeneity than both *nir*K and *nos*Z, while the enzyme gene *nir*K had the highest diversity based on structure and richness. Exposure to environmental concentrations of pesticides affected structure (16S rRNA gene but not *nos*Z) and function (potential denitrification rate) of the constructed wetland bacterial community, however there were few indications of direct toxic effects. Using leucine incorporation as an endpoint of bacterial activity and growth community was a quicker and more sensitive method to detect toxicity of fungicides exposure on bacterial communities than measuring potential denitrification, and clear concentration-response relationships were easily generated that could be standardized for community level risk assessments of pesticide exposure to aquatic environments.

1. Introduction

Intensive use of fertilizers and pesticides on agricultural fields may expose nearby water-resources and oceans to an excess of nutrients and pesticides from runoff and drift. This may cause an increased risk for algal blooms, oxygen depletion and/or toxic effects on non-target organisms, and alter the function of aquatic ecosystems. This problem is of global concern as agricultural land use has increased during the last century, and during the last two decades the agricultural production per square meter has also increased due to increasing use of fertilizers and pesticides.

One action to prevent further transport of nitrogen (N) and pesticides has been to restore and recreate wetlands (Stadmark and Leonardson, 2005; Thiere et al., 2009), most often close to agricultural practices, which are considered point sources of pollution. Large scale programmes have started throughout the world with the aims to reduce transport of redundant nutrients and pesticide leakage to freshwater systems and oceans. Sweden has started an ongoing project, to achieve a national environmental goal concerning N-removal, constructions of 12 000 ha wetlands (from year 2000 until 2010) (Swedish Board of Agriculture, 2000). Similar wetland projects have started in North America, where they are to construct about 28 000 ha of wetlands (Farm Service Agency, 2004).

Although, constructed wetlands in general reduce the nitrogen transport, the efficiency with which individual wetlands remove nitrogen (mainly through denitrification) varies greatly and has been associated with e.g. incoming nitrogen load and amount of available carbon (Fleischer et al., 1994; Weisner et al., 1994; Lin et al., 2002). However, the task of associating denitrification (i.e. the functional bacterial trait of N-removal in wetlands) with the structure of the denitrifying bacterial community (i.e. the key players in N-removal) is as of yet unresolved (Philippot and Hallin, 2005). Knowledge of the structure and function of the bacterial communities can be used to increase the efficiency of energy being transferred to higher trophic levels, decrease nutrient transport to eutrophied ecosystems and increase the efficiency of bioremediation in polluted ecosystems (Torsvik et al., 2002; Lovely, 2003: Bell et al., 2005). Hence, understanding the relationship between the structure and function of the denitrifying bacterial community may potentially be used to increase N-removal efficiency of constructed wetlands.

In addition, influence from environmental parameters and pesticide exposure on the structure and function of bacterial communities has to be considered when analysing agricultural constructed wetlands due to the high risk of pesticide exposure. Thus, the challenge is to construct efficient N-removal wetlands, which will maintain the same N-removal efficiency during pesticide exposure and/or changed environmental condition. The aim of my thesis was to provide a starting point to this challenge by describing variation and composition of denitrifying bacterial communities in constructed wetlands, and the influence of environmental parameters and pesticides on the structure and function of these communities.

1.1. Constructed wetlands

Surface waters in general, and natural wetlands (e.g. swamp, marsh and bog) in particular have frequently been converted into arable land through drainage measures, which has resulted in a loss of up to 90% of wetland areas in the intensively cultivated regions of Europe and North America (e.g. Hoffmann et al., 2000; Mitsch and Gosselink, 2000; Biggs et al., 2005). The loss of the natural wetlands, has led to a reduced ability of these habitats to function as efficient water cleaning systems. Construction of new wetlands is therefore an attempt to try to restore the natural balance within the agricultural ecosystems.

The constructed wetlands have, in contrast to the majority of natural wetlands, an open water surface. Their appearances are therefore identical to ponds or small lakes, but they are in general more shallow (mean depth of ≤ 1m), which enables plant growth. Plant growth generates available organic carbon, which is the energy source of the denitrifying bacteria. Except carbon, the denitrifying bacterial community requires nitrate (i.e. the starting product in denitrification), and an anaerobic environment, since the denitrifying bacterial community is facultative anaerobic, to be able to denitrify (Hallin and Lindgren, 1999).

It has been shown that constructed wetlands may sustain and promote heterogeneous communities of both plants and macroinvertebrates (Thiere et al., 2009). These results suggest that the environment of constructed wetlands should provide a variety of different carbon sources to the bacterial communities, but also a variety of predators. Bacterial communities in constructed wetlands have recently begun to receive attention (Sundberg et al., 2007) but have not as of yet been analysed to the same extent as in soils (e.g. Trobäck et al., 2004), marine systems (e.g. Braker et al., 2001) and lakes (e.g. Lindström, 2000). In order to link their structure to function, it has to be recognised what kind of bacterial communities, especially denitrifying bacteria, we may find in the habitats of constructed wetlands

1.2. Denitrification

Denitrification, which represents one part of the N-removal process, reduces nitrate (NO_3^-) to dinitrogen (N_2). Denitrification is the limiting process of nitrogen removal in the agricultural constructed wetlands, since most nitrogen enters the system in the form of NO_3^- (Bachand and Horne, 2000; Seitzinger et al., 2006). The N-cycle includes many more steps of N transformation, e.g. nitrification, nitrogen fixation, dissimilatory nitrate reduction and anammox (Fig 1), but in this thesis the focus will be put on denitrification. The overall aim of the work in this thesis is to gain such knowledge, which with added future research may be applied to reduce the excess of bioavailable NO_3^- and transform it to N_2 (i.e. increasing the N-removal efficiency) (Fig 1). N_2 is a stable gaseous

form and much less bioavailable in comparison to the other compounds of nitrogen.

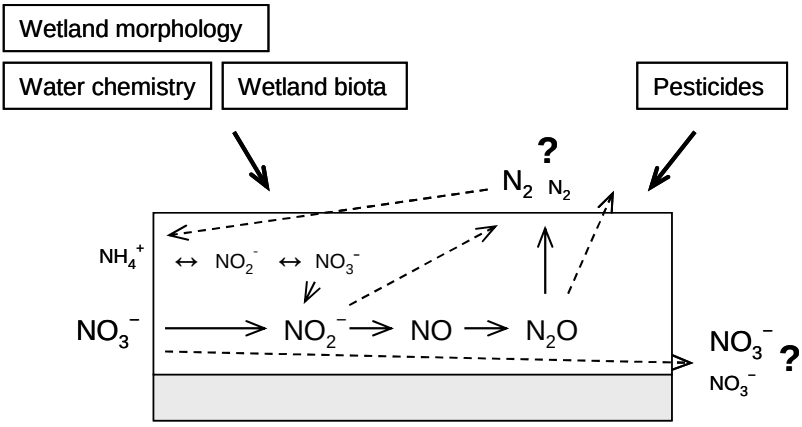

Figure 1. Constructed wetland with the different N-transformations. The white box represents the water column and the grey the sediment column of a constructed wetland. Wetland inflow of nitrate and ammonium is indicated from the left, and wetland outflow of nitrate is indicated to the right. Denitrification, NO_3^- to N_2, is the limiting process in these habitat systems, and the process this thesis will focus on. The question marks and the two different sizes of the NO_3^- and N_2 indicate that the amount varies between constructed wetlands, and if efficient denitrification may be achieved in these systems the transport of NO_3^- will decrease, and as a result, decrease the risk of eutrophication of other freshwaters and oceans. Many fundamental processes are addressed in this thesis, including influence from environmental parameters, regarding wetlands morphology, water chemistry and wetland biota (details in paper I), and agricultural pesticides on structure and function of the bacterial communities in the wetlands.

The ability to denitrify is a facultative trait spread among a wide variety of taxonomic groups (Zumft, 1997; Braker et al., 1998; Hallin and Lindgren, 1999). In this thesis the denitrifying bacterial community is analysed. The denitrifying bacterial community is a widespread functional bacterial group, which can be found in α-, β-, γ-, ε-*Proteobacteria*, gram-positive bacteria, *Bacteroides*, *Firmicutes* and *Actinobacteria* (Enwall, 2008). Through denitrification, the nitrogen is transported out of the wetlands as molecular nitrogen and this process is catalysed by different enzymes (Fig 2) encoded by the denitrifying bacteria (Bothe et al., 2007). The first step in denitrification is the nitrate reduction catalysed by nitrate reductase (encoded by *nar*/*nap*), which reduces nitrate (NO_3^-) to nitrite (NO_2^-). Then the nitrite reduction catalysed by nitrite reductase (encoded by *nir*) which reduces NO_2^- to nitric oxide (NO), followed by the nitric oxide reduction catalysed by the nitric reductase (encoded by *nor*/*qnor*) reduces NO to nitrous oxide (N_2O). The final step is the nitrous oxide reduction catalysed

by nitrous oxide reductase (encoded by *nos*), which reduces N_2O to dinitrogen (N_2) (Fig 2). The second step in the pathway where nitrite is reduced to nitric oxide by nitrite reductase (*nir*) is exclusively executed by the denitrifying bacterial community opposite the first step (*nar*/*nap*) in the pathway (Hallin and Lindgren, 1999). Two different nitrite reductase genes are known, the *nir*K product, which contains copper, and the *nir*S product, which contains cytochrome cd_1 (Braker et al., 1998). The two genes seem to occur mutually in a given strain, but both types have been found in different strains of the same species (Coyne et al., 1989). For example, Hallin and Lindgren (1999) found Cu-*nir* fragment in *Paracoccus denitrificans* Pd1222, which also can carry cd_1-*nir* fragment. The enzyme gene *nir*S has been found to be more widely distributed than *nir*K in aquatic and soil systems (Braker et al., 1998; Throbäck et al., 2004). Moreover, since we are interested in obtaining N_2 as the final denitrification product, rather than the green-house gas N_2O, the nitrous oxide reductase (*nos*Z) is of special importance. In general, denitrifying bacteria possesses some, but not all the functional enzyme genes (Zumft, 1997). Hence, individual denitrifying bacteria may catalyse only certain steps of the denitrification pathway. In addition, a denitrifying bacterial species, which possess one or several enzyme genes, may not express all the denitrifying bacterial enzyme genes simultaneously (Philippot and Hallin, 2005). Moreover, the community size of the denitrifying bacterial community (represented by the *nir*K and *nos*Z) has been shown to constitute around 5-6% of the total eubacterial 16S rRNA gene community (Henry et al., 2006). However, information of the denitrifying bacterial enzyme genes is limited and their structure and function remain to be discovered.

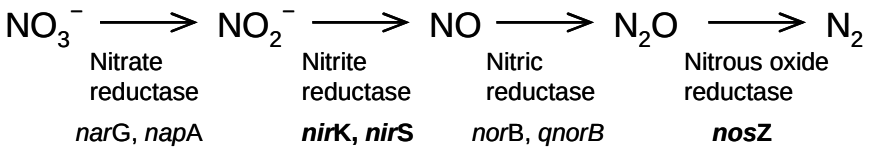

Figure 2. The denitrification pathway and the denitrifying bacterial enzyme genes. Different enzyme reductases catalyses each step and are encoded by different enzyme genes. In this thesis the enzyme genes *nir*K, *nir*S and *nos*Z will be analysed (marked in bold).

2. Biogeography of Bacterial Communities

So far, most studies on denitrification efficiency in constructed wetlands have focused on biotic and abiotic parameters in relation to functional measurements of denitrification (Weisner et al., 1994; Bachand and Horne, 2000; Torsvik et al., 2002; Reche et al., 2005; Beisner et al., 2006), and examination of the key players in the process, namely the denitrifying bacterial community, have been largely neglected. Therefore our knowledge regarding N-removal efficiency is limited, and fundamental research of the denitrifying bacterial community has to be established before we can link structure to function. The bacterial community, found in the domains of *Bacteria* and *Archaea* constitutes the most abundant species on earth (Kennedy, 1999; Torsvik et al., 2002), but the majority of their diversity and biogeography is not thoroughly investigated (Martiny et al., 2006). Biogeography aims to answer which bacterial species can be found in a certain environment, at what abundance, and why. Some first attempts should therefore be made to analyse the biogeography of denitrifying bacterial communities in agricultural constructed wetlands.

2.1. Theories of bacterial biogeography

World wide abundance of the bacteria and their microscopic size has given rise to the theory that 'everything is everywhere, but the environment selects' (Baas-Becking, 1934). This theory states that almost all the global microbial species are present at any local site (pool of species) and 'waits' for the right environmental conditions to increase their population size. Support for this theory has been given by Finlay and Clark (1999), who argued that flagellates (genus *Paraphysomonas*) probably are ubiquitous. However, it can also be argued that it takes a long time for rare or cryptic species to reach a detectable level using present molecular tools (Langenheder, 2005), and as a result the Baas-Becking (1934) theory cannot be falsified or rejected.

Recently, novel theories have started to be developed and tested, which are based on theories and empirical from the macroorganism community (Martiny et al., 2006). The theories include that there may be specific factors, which determine why, in which environment, and at what abundance we can find certain community composition of microbial communities, hence, their biogeography. The specific factors are divided in two major groups, environmental factors and geographical factors (Martiny et al., 2006; Logue and Lindström, 2008). The theories state that either environmental or geographical factors, or factors from both groups determine the biogeography of bacteria. The bacterial community composition has been shown to change with environmental parameters (Hewson et al., 2003; Hewson et al., 2007; Langenheder and Prosser, 2008), and with geographic distance (Lindström and Leskinen, 2002; Langenheder and Ragnarsson, 2007). The major difference between the theories is that the novel theories exclude the possibility that 'everything is everywhere' and focuses more on developing the last part 'selection driven exclusively by

environment'. Thus, knowledge of the bacterial biogeography may lead to the ability to predict bacterial responses to environmental changes and fluctuations (Green et al., 2008).

2.2. Community ecology of bacteria

During the last decade the use of cultivation independent molecular methods has improved, and allowed us to analyse the 'hidden' biodiversity of the bacterial communities. More important, the molecular methods have allowed us to analyse bacteria on community level as a complement to cultivation methods of single species (Brandt et al., 2004). Analysis of single bacterial species can provide important information on the direct response to different disturbances and pollution. However, bacterial single species analysis will not include realistic environmental stochastic effects such as, competition, predation, nutrient supply and many more food-web interactions, as analyses on a bacterial community level are capable of. Hence, it is therefore difficult to extrapolate results from analysis of single bacterial species to the scale of a bacterial community level, and further to the scale of an ecosystem level (Standing et al., 2007). It is important to emphasize that approaches of both single species and community composition analyses are needed to enhance the understanding of structure and function of bacterial communities (Dahllöf, 2002).

Another aspect that may be gained by community level analysis is the functional redundancy of bacterial species, which is a functional trait shared between different bacterial species. Resilience of bacterial communities can be estimated from how long time it takes for a bacterial community to recover and return to its original condition after being disturbed by e.g. pesticides. A long-lived theory states that increased biodiversity leads to a more stable ecosystem functioning due to an increased potential of resilience (McNaughton, 1977). It has also been suggested that the bacterial resilience is a useful indicator of ecosystem functioning, hence, with increasing quality of the examined habitat the functional redundancy of bacterial communities increased (Yin et al., 2000).

Recently, it has been shown that the recovery times for bacterial communities responsible for N turnover e.g. denitrification, after exposure to toxic substances may be long (Sundbäck et al., 2007). They argued that it was due to low functional redundancy and high sensitivity. However, a more heterogeneous denitrifying bacterial community may have a higher possibility to recover and sustain the denitrification rate than a more homogeneous community after disturbances. Nevertheless, it has been discussed that the potential of bacterial resilience may be lost after repeated exposures of disturbances (Sundbäck et al., 2007). Thus, a heterogeneous bacterial community may recover faster due to a higher capacity of resilience than a more homogeneous one. However, repeated disturbances, e.g. pesticide exposures, may lead to a decreased ability of bacterial communities to recover.

2.3. Variation in natural denitrifying bacterial community compositions

If a heterogeneous or homogeneous denitrifying bacterial community may support differently efficient denitrification in agricultural constructed wetlands is unknown. It is also unknown whether the composition of the denitrifying bacterial community varies between the wetland systems. Different environmental conditions and different bacterial species in the constructed wetlands may explain why there could be variation in the bacterial community composition and thereby a varied ecosystem functioning. Different species may use slightly different resources, and certain bacterial species may play a more important role (Loreau and Hector, 2001). Thus, if there is variation of the denitrifying bacterial community composition between the studied habitats that may be an essential factor that could explain the functional differences of constructed wetlands.

Previous studies on the variation of the denitrifying bacterial enzyme genes are difficult to compare with my studies because different molecular methods have been applied, and usually only one or two of the enzyme genes have been investigated. Most often the diversity has been calculated by using results from the molecular fingerprinting structure, richness and/or intensity analyses. In general, the diversity of *nir*K has shown to be marginally higher than *nir*S, and the diversity for *nos*Z has been lower than for both *nir*K and *nir*S (review by Wallenstein et al., 2006). More specifically, communities of *nir*K have previously been shown to be more diverse than the *nir*S and *nos*Z counterparts in soils (Avrahami et al., 2002; Throbäck et al., 2004; Bremer et al., 2007), whereas greater diversity has been noted for *nir*S than for *nir*K in marsh soil (Priemé et al., 2002) and marine sediments (Braker et al., 2000).

The heterogeneity of each denitrifying bacterial enzyme gene has been evaluated by constructing phylogenetic trees based on the similarities between investigated sequences and known bacterial sequences obtained from GeneBank (i.e. database with both known and unknown DNA sequences of bacterial species). The level of heterogeneity is evaluated by measuring the closeness of the investigated sequences to known partial bacterial nucleotide sequences from GeneBank. If the sequences cluster close to many different members of the known denitrifying bacteria the enzyme gene is evaluated as heterogeneous (see paper II for a more thorough description). Overall, members of the α-*Proteobacteria* showed to be dominant and represent the majority of clusters in phylogenetic trees of the denitrifying bacterial enzyme genes, followed by members of the β-*Proteobacteria*, and thereafter by members of the γ-*Proteobacteria* (Braker et al., 2000; Avrahami et al., 2002; Priemé et al., 2002; Throbäck et al., 2004; Bremer et al., 2007). In arable soil systems the enzyme gene *nir*S was shown to be heterogeneous by clustering close to members of α-, β- and γ- *Proteobacteria*, while *nir*K and *nos*Z only clustered close to α- and β-*Proteobacteria* (Throbäck et al., 2004). Similar studies of phylogenetic trees on the denitrifying bacterial enzyme genes from a municipal waste-water treatment plant showed that members of β-*Proteobacteria* represented the majority of the

clusters (Heylen et al., 2006). In a more recent investigation with the same waste-water treatment plant samples as in Heylen et al. (2006), the enzyme gene *nir*K clustered more often with members of α-*Proteobacteria*, while the *nir*S clustered more often to members of β-*Proteobacteria* (Heylen et al., 2007). These results suggest that heterogeneity of the denitrifying bacterial enzyme genes may differ depending on environmental conditions (e.g. closed system vs environmental systems). Hence, samples from waste-water treatment plant systems have more controlled conditions and less of stochastic effects from e.g. fluctuation of environmental parameters. Both the diversity and the heterogeneity of the denitrifying bacterial enzyme genes (community composition and DNA sequences) may be important to analyse to further link their community variation to denitrification efficiency.

3. Agricultural Pesticides

In areas of intensive agriculture practice, aquatic environments are exposed to agricultural pesticides, which are regularly detected in surface waters in these areas (Kreuger et al., 1999; Haarstad and Braskerud, 2005). Although many pesticides have designated target organisms, due to specific modes of action, they may have more general toxic effects on non-target organisms, including microorganisms (Pell et al., 1998; Johnsen et al., 2001; DeLorenzo et al., 2001). The three largest groups of pesticides that are used worldwide are insecticides, herbicides and fungicides. They have different modes of action depending on their target organism, but generally insecticides disturb or inhibit processes in the nervous system. Herbicides often disturb or inhibit respiration processes, while fungicides can disturb or inhibit processes in the cell membrane (Stenersen, 2004).

Environmental concentrations of different pesticides have previously been shown to affect the structure and/or function of bacterial communities in aquatic habitats (Widenfalk et al., 2004; Widenfalk et al., 2008). Thus, potential effects of pesticide exposure on bacterial communities in agricultural constructed wetlands should be analysed since they may alter wetland function (i.e. denitrification efficiency).

3.1. Single species test vs community level test

There is a lack of studies on the interactions between pesticide exposure and bacterial structure and function, as most aquatic toxicity assessments are performed on zooplankton, macroinvertebrates and/or fish. In addition, the majority of toxicity assessments on bacterial communities are performed by using pure cultures of single species, which is the ISO standard (e.g. 11348-3, Water quality—Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri*, luminescent bacteria test), or on extracted

bacteria without their natural matrixes (Christensen et al., 2006; Park and Choi, 2008), as recommended by authorities controlling pesticide registration. Although still not routinely performed, bacterial toxicity testing on a community level provides an attractive alternative to single-species bacterial toxicity tests, and the advantages of testing natural assemblages of microorganisms in microcosm experiments has been advocated (Brandt et al., 2004; Sundbäck et al., 2007; Widenfalk et al., 2008).

Consequently, there exists some inconsistency as to which methods to use and which matrix to analyse when performing toxicity assessment on bacterial communities in aquatic habitats. Test on the community level of bacteria should therefore serve as a complementary analysis since results using single bacterial species are difficult to extrapolate to potential effects on a bacterial community level. Thus, proper methods for environmental risk assessments of exposure from agricultural pesticide towards bacterial communities in aquatic environments should be determined and standardised. Hence, if insensitive or too sensitive methods are used for toxicity assessments there is a risk that the effects from pesticides on bacterial communities will be under- or overestimated, respectively.

3.2. Exposure and concentration-response relationships in assessments

Recommended pesticide application is chosen so that the active ingredient(s) of a pesticide will affect their target organisms but have negligible effects on non-target organisms (Crommentuijn et al., 2000). Several previous toxicity assessments have therefore analysed environmental concentrations of different pesticides (Widenfalk et al., 2004; Widenfalk et al., 2008; Van den Brink et al., 2009). The highest concentrations used in those studies have been 100 times or 1000 times the maximum permissible concentration (MPC), where MPC is a maximum pesticide concentration of what is permitted to apply onto a given area. It is important to emphasize that the MPC concentration of a pesticide should not be harmful to non-target organisms in the ecosystem (Crommentijun et al., 2000). Detection of pesticide concentration in the environment has often exceeded the MPC, but the concentrations have not surpassed 100 times the MPC (Kreuger, 1998; Kreuger et al., 1999). It has therefore been argued that concentration-response relationships of pesticides exposure on bacterial communities have little environmental relevance (Widenfalk et al., 2004).

Environmental concentrations of pesticides are useful when examining possible direct and indirect effects on non-target organisms, e.g. exposure on bacterial communities. In addition, environmental concentrations may be used to study the effects between organism interactions and from possible stochastic effects in a natural environment. However, to compare and evaluate different risk assessment methods by using few environmental concentrations of a pesticide would be difficult, due to failure in detecting a toxic effect (Fig 3), and establishing concentration-response relationships would also avoid spurious negative effects (Fig 3). To determine accurate effect concentration (EC) of pesticide on non-target organisms, a concentration-response dilution series is also

of fundamental importance (Schweiger and Jakobsen, 1998; Rousk et al., 2008). Consequently, studies of pesticide exposure using environmental concentrations and/or concentration-response may both be needed to understand all potential effects pesticides may have on aquatic bacterial communities.

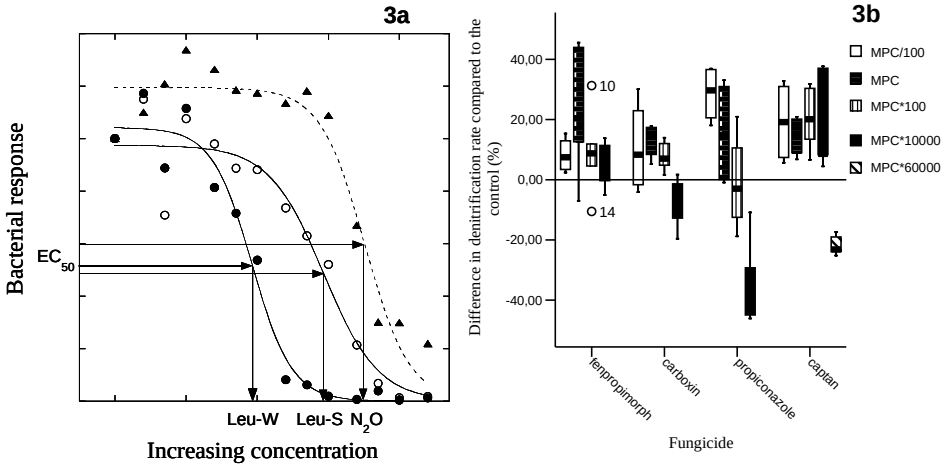

Figure 3. Figure 3a represents a complete concentration-response curve (for detailed information see paper IV, Fig 1J, Bronopol II), and the figure 3b represents an incomplete curve using mainly environmental concentrations of the tested fungicides (S. Milenkovski et al., unpublished data). A complete concentration-response curve will in general detect the effect concentration EC_{50} (i.e. the logarithm of the pesticide concentration resulting in 50% inhibition effect of the bacterial community), as presented by arrows in 3a. The first curve from the left characterise a bacterial community that is more affected by the pesticide due to a lower EC_{50} value, or it may represent a more sensitive method as stated in paper IV. The curve furthest to the right characterises the most tolerant bacterial community due to the higher EC_{50} value, or it may represent the least sensitive method as stated in paper IV. The horisontal line in figure 3b represents the mean value of the controls. The denitrification rate show both increasing and decreasing responses independent of concentrations, which is, no concentration-response curves (except the propiconazole treatment). Fig 3b shows responses on the denitrification rate (isotope ^{15}N pairing technique) after treatments with increasing fungicide concentrations.

3.3. Structural and functional responses, and sensitivity of bacterial communities

Direct and indirect effects from pesticide exposure have earlier been shown for bacterial communities (Engelen et al., 1998, Johnson et al., 2001, Sverdrup et al., 2002). Pesticides may directly affect bacterial communities by killing certain species, and indirectly as more tolerant bacterial species may benefit by the

ability to use the applied pesticide as a nutrient source (Engelen et al., 1998, Chen et al., 2001, Johnson et al., 2001, Sverdrup et al., 2002). Indirect effects of pesticides on bacterial communities may also involve any or all of the different trophic levels in the ecosystem, between species (Sigler and Turco, 2002), between predator and prey (Woin, 1998, Sverdrup et al., 2002) and between plants and animals (Bjørnlund et al., 2000). Beside food-web interactions, pesticides effects are also influenced by the stochastic effects as well as pesticide applications and pesticide properties (Relyea and Hoverman, 2006). The bacterial function has been suggested to be more resilient than structure towards disturbances because of potential functional redundancy. However, if the structure or function of the denitrifying bacterial community is more sensitive of pesticide exposure is unknown. Thus, the interactions and effects between bacterial communities, environmental parameters and pesticides are complex. Studies of both the structure and function of natural bacterial communities after pesticide exposure may increase the understanding of the relationship between the structure and function.

Furthermore, in aquatic systems bacterial communities can be exposed to pesticides both in the water column and in the sediment column. A pesticide will commonly first enter an aquatic system in the water body, and dependent on the properties of the pesticide, for example its octanol-water partition coefficient (K_{ow}) and water solubility, it will be more or less bioavailable for microorganisms in different habitats. The K_{ow} value indicates if the compound is more or less lipophilic, a more lipophilic compound may increase the risk of accumulation in the sediment. Increased water solubility of a pesticide will increase the risk of exposure in the water column. Thus, it may be valuable to apply methods of toxicity assessment that may analyse effects on bacterial communities in both the sediment and the water column, simultaneously.

In this thesis, I wanted to study potential community level effects from pesticides on the structure and function of natural bacteria in aquatic environments. To minimize biases from stochastic and indirect effects the analyses of pesticide exposure on natural aquatic bacterial communities were performed by using microcosms, in short-term studies. Toxicity assessments were performed by exposing bacterial communities to different pesticides in both environmental concentrations and by conducting concentrations-response analyses. Additionally, toxicity assessments were examined in the two aquatic matrixes, the water and sediment column, to recognize in which matrix each tested pesticide would have the largest effects on the bacterial communities.

4. Aims

The first two papers in the thesis examine the composition of denitrifying bacterial communities in agricultural constructed wetlands. The later two papers examine the effects of agricultural pesticides on the structure and function of the denitrifying bacterial community. More specifically I,

- examined the variation of bacterial community composition among agricultural constructed wetlands, and whether the community compositions were influenced by environmental parameters of the ecosystems.
- examined if the habitat of agricultural constructed wetlands sustained a heterogeneous or homogeneous composition of the denitrifying bacterial community.
- analysed if environmental concentrations of different pesticides affected the structure and/or function of constructed wetland bacterial communities.
- evaluated the sensitivity of two methods of toxicity assessments, measuring functional responses of constructed wetland bacterial communities after exposure of fungicides.

5. Methods

In this thesis both structural and functional endpoints have been measured by applying a variety of methods. The methods for analysing community compositions of the eubacteria and denitrifying bacteria have been the same in papers I, II and III. However, when measuring the functional endpoints of eubacterial and denitrifying bacterial communities, different methods have been used within or between these studies (papers III and IV).

5.1. Analyses of bacterial structure

The technique of using polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE), has been suggested to be a useful method when analysing changes in composition of bacterial communities (Trobäck et al., 2004; Larson et al., 2007; Petersen and Dahllöf, 2007; Sundberg et al., 2007; Jarvis et al., 2009). Different molecular methods have been used and evaluated to investigate composition and changes in the microbial community. Other molecular methods besides DGGE are, TGGE (temperature gradient gel electrophoresis) (Muyzer, 1999), T-RFLP (terminal restriction fragment length polymorphism) (Osborn et al., 2000), and RISA (ribosomal intergenic spacer region analysis) (Enwall et al., 2005). All these molecular methods are considered useful for estimation of the microbial community composition,

however, none can catch the entire diversity of a microbial community (Dabert et al., 2002; Dahllöf, 2002).

The advantage with PCR is that even low numbers of some bacterial strains can be revealed due to amplification of pre-chosen DNA sequences (Dahllöf, 2002) and thus, mapping the total bacterial community composition. In addition, the resolution in the DGGE gel is very good since sequences can be separated with one single different base pair (1/500 bp) (Kirk et al., 2004). On the other hand, the disadvantages with PCR are that it amplifies active as well as dormant DNA (Kirk et al., 2004) and it may amplify unspecific targets (Dahllöf, 2002). The number of DGGE bands from one sample is generally used as a measure of bacterial richness (Yin et al., 2000), and presence/absence of DGGE bands on different migration levels is used as a measure of bacterial structure. However, DGGE may underestimate the community diversity since one DGGE band could represent more than one population (Dabert et al., 2002), and one DGGE band is used in calculations to represent one denitrifying bacterial species, hence, a richness measure of the bacterial community. It has also been reported that variation in bacterial community composition can arise due to selection of primer pairs that do not offer suitable specificity for the studied habitat (Angeloni et al., 2006). Still, even with the potential flaws pointed out, all of the methods mentioned above are powerful tools in studies of microbial community composition (Crecchio et al., 2001; Boon et al., 2002).

The PCR-DGGE technique was applied in my thesis to study both the eubacterial 16S rRNA gene community and the denitrifying bacterial community (Fig 4). In particular, the structure of the denitrifying bacterial enzyme genes *nir*K, *nir*S and *nos*Z were analysed. These enzyme genes were chosen because they represent different steps in the denitrification pathway, and thus may provide knowledge of possible community variation between the denitrifying bacteria for the two separate steps. The first two enzyme genes catalyse the second step (*nir*) in the denitrification pathway, while the last enzyme gene catalyses the last step (*nos*) (Fig 2).

5.2. Analyses of bacterial function

Isotope (^{15}N) pairing technique and the acetylene inhibition method have both been shown to be useful denitrification measures (Knowles, 1982; Nielsen, 1992; Rysgaard et al., 1993; Svensson, 1998; Eriksson and Weisner, 1999; Widenfalk et al., 2004; Sirivedhin and Gray, 2006). The advantage with the acetylene inhibition method is that it is sensitive and quick (Kozub and Liehr, 1999; Bernot et al., 2003;). The disadvantage is that it is measuring the N_2O amount, and not the end-product, N_2, of the denitrification, which implies that it should not be used to measure the efficiency of denitrification in different habitats. The nitrogen isotope (^{15}N) pairing technique has not been used to the same extent as the acetylene inhibition method. However, the advantage is that it is a more accurate method than the acetylene inhibition and measures the amount of N_2, the end product of denitrification (Nielsen, 1992; Rysgaard et al., 1993;

Svensson, 1998). Hence, the method may be applied to both measures of denitrification efficiency, and comparison between different treatments. The disadvantage is that it is more cumbersome and more costly regarding both time and money. Thus, the latter method would be preferable when calculating wetland efficiency and trying to estimate absolute values of wetland function while the former may be applied when comparing relative differences between treatments on denitrification rate at micro- and mesocosm scale.

Leucine incorporation (indicating bacterial growth) was measured according to Smith and Azam (1992) with some modifications used for soil bacteria (Bååth et al., 2001). Leucine incorporation, has earlier been shown to be a sensitive, and also cost- and time-efficient, method to detect toxic effects in natural environments due to heavy metals (Díaz-Raviña and Bååth, 2001; Petersen et al., 2004; Sundbäck et al., 2007), surfactants (Brandt et al., 2004), phenols (Aldén Demoling and Bååth, 2008), pesticides (Widenfalk et al., 2004), antifouling biocides (Maraldo and Dahllöf, 2004) and antibiotics (Rousk et al., 2008). Leucine incorporation also has the advantage of being easily measured in different matrixes, e.g. soil, sediment and water (Bååth, 1994; Fischer and Pusch, 1999). Leucine incorporation has not been used to the same extent in toxicity assessments as measurements of bacterial respiration and/or biomass, but has proven to be more sensitive than bacterial respiration (Rousk et al., 2008). In my studies, all three methods, isotope (^{15}N) pairing technique, acetylene inhibition and leucine incorporation, were applied as measurements of the bacterial activity (Fig 4).

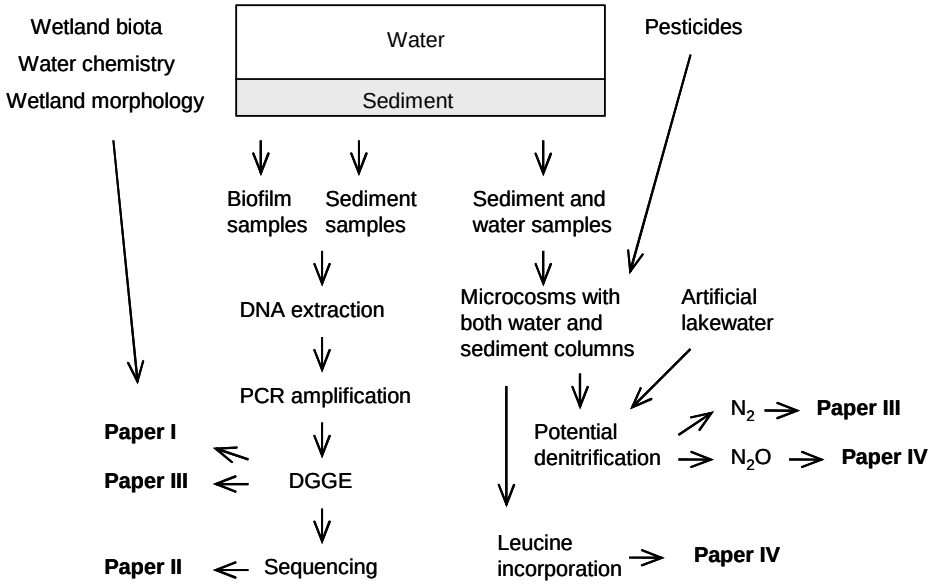

Figure 4. Overview of the different methods and techniques applied to analyse the structure and function of bacterial communities in agricultural constructed wetlands.

6. Performed Studies and Major Findings

My findings suggest that structure and function of the denitrifying bacterial community, responsible for the denitrification efficiency in agricultural constructed wetlands, may be affected by both natural environmental parameters and by pesticide pollution (papers I, III and IV). However, the explanation fractions from the studied factors were low (papers I and III). This may imply that there are additional drivers (Kent et al., 2004) or that the natural stochastic effects (Kadlec, 1997) are too large and as a result higher explanation fractions will not be possible to achieve. The fact that we found variation in the richness and structure of the wetland bacterial communities (papers I and II) opens up the possibility that there may be a linkage between structure and ecosystem functioning, as increasing bacterial species richness and structure have been shown to support an increased ecosystem functioning in e.g. semi-permanent rain pools (Bell et al., 2005). The enzyme gene *nir*K had the highest diversity, while the enzyme gene *nir*S had the highest heterogeneity among the studied wetlands. This suggests that the enzyme genes of *nir*S and *nir*K would have a higher probability than *nos*Z of maintaining their function when exposed to different disturbances due to functional redundancy (paper II), as it has been shown that a more diverse bacterial community may maintain their functional rate better than a less diverse community in soil systems (Girvan et al., 2005). Moreover, exposure to fungicide had a larger effect on the functional bacterial endpoint in comparison to exposures of herbicide or insecticide (paper III). The function of the denitrifying bacterial community was suggested to be more sensitive than their structure (paper III). Functional bacterial endpoints were suggested to be more useful and straight forward than structural bacterial endpoints in toxicity assessments, because structural endpoints may detect bacterial growth instead of toxicity (paper III). Finally, even though denitrification may be a more relevant functional endpoint to constructed wetland microbial communities, leucine incorporation was a quicker and more sensitive method than potential denitrification in toxicity assessments of pesticides (paper IV). The leucine incorporation was suggested to be applied as a standardized method for toxicity assessments of pesticides on microbial communities (paper IV).

6.1. Paper I

In paper I, I examine and describe variation of bacterial community compositions among agricultural constructed wetlands. In addition, I wanted to quantify influences of environmental parameters on the community composition. In a field survey we sampled 32 constructed wetlands for bacterial biofilm and environmental parameters (Fig 5). All investigated wetlands are included in the large-scale program to achieve the Swedish national environmental goal concerning N-removal.

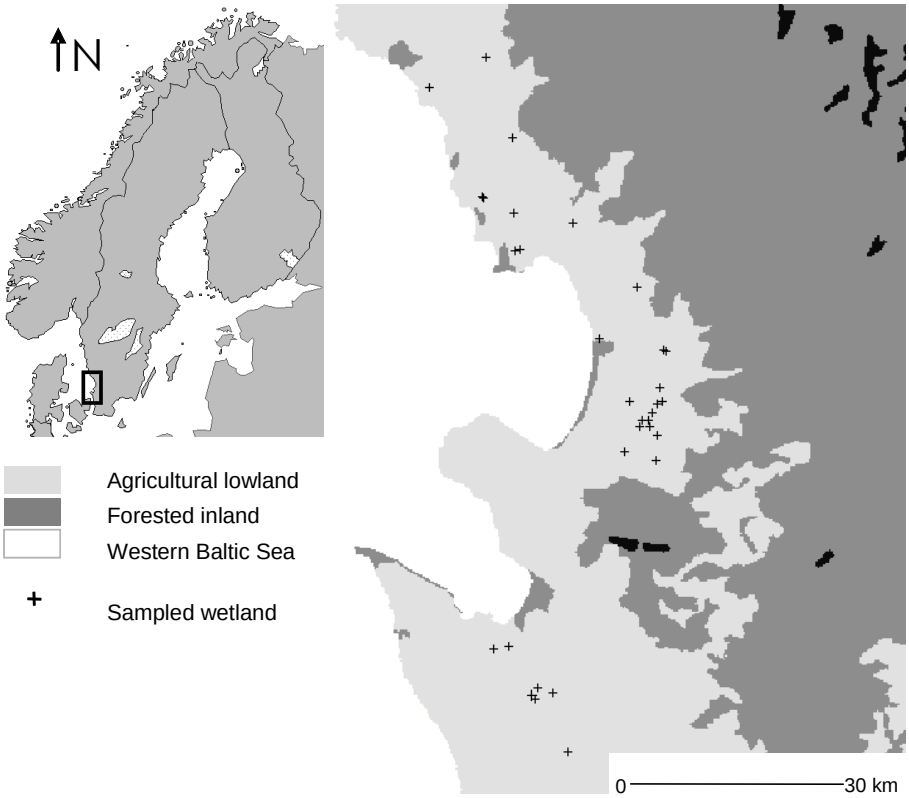

Figure 5. Geographical location of the studied agricultural constructed wetlands in the south of Sweden, papers I, II and III.

The variation of the bacterial communities from biofilm were analysed by applying PCR-DGGE (Fig 4). The denitrifying bacterial enzyme genes *nir*K, *nir*S and *nos*Z were analysed representing the denitrifying bacterial community. The eubacterial 16S rRNA gene community was also analysed in order to gain knowledge if the total bacterial community is influenced by the same environmental parameters as the denitrifying bacterial community. Hence, it may be sufficient to analyse the eubacterial community if the responses from environmental parameters is similar to those of the denitrifying bacterial community.

The results showed that the composition (structure and richness) of eubacterial and denitrifying bacterial communities varied between the investigated wetlands and was partly explained by different environmental parameters out of the 15 investigated (Fig 6). The enzyme gene *nir*K showed to have the most diverse DGGE banding pattern in comparison to *nir*S and *nos*Z. The structure of the eubacterial communities and the richness and structure of the denitrifying bacterial community were all related to different wetland environmental parameters. Succession stage parameters (e.g. plant community

composition) accounted for the largest impact on the eubacterial community, whereas water chemistry parameters (e.g. hydraulic load, inlet nitrogen concentration) accounted for the largest impact on the denitrifying bacterial community composition. The explanation fractions for DGGE structure and richness, ranged from 6 to 19% (Fig 6), thus, there may be additional drivers involved in bacterial community composition. The results also suggest that care should be taken when choosing denitrifying bacterial enzyme gene(s) in future studies, since using a single enzyme gene may not be sufficient to characterise denitrifying bacterial community composition in constructed agricultural wetlands. Thus, the composition of the denitrifying bacterial enzyme genes varied among the studied ones and could partly be explained by specific environmental parameters.

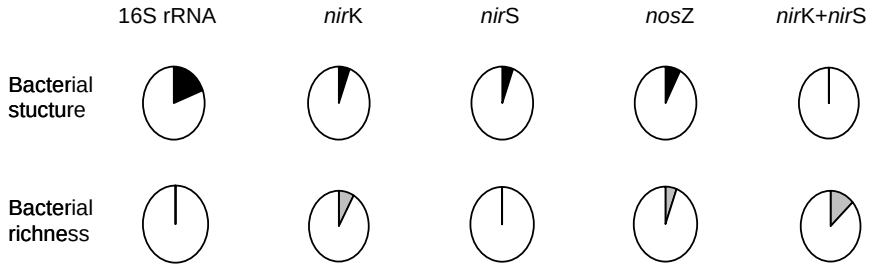

Figure 6. Illustrates the variation of either bacterial DGGE structure or richness that may be explained by specific wetland environmental parameters. The black and the grey areas in the respective circles indicate the fraction of either bacterial structure or richness of the eubacterial gene or the denitrifying bacterial enzyme genes. The white area represents the part which cannot be explained by the studied environmental parameters included in paper I (for detailed description of significant parameters see paper I).

6.2. Paper II

In paper II, I analyse the heterogeneity of denitrifying bacteria in constructed wetlands. I wanted to gain knowledge whether the habitat of agricultural constructed wetlands may sustain a heterogeneous or homogeneous denitrifying bacterial community in comparison to other habitats. The same DGGE banding pattern from paper I was used, but I continued to analyse the DGGE bands by DNA sequencing (Fig 4). The sequences were used to construct phylogenetic trees based on the sequences of each individual denitrifying bacterial enzyme gene. These methods were chosen to be able to distinguish and evaluate the heterogeneity of denitrifying bacterial enzyme genes at community level (see paper II for a detailed description). In papers I and II all samples were analysed without addition or exposure of any treatment to the samples. In

contrast, papers III and IV we analysed microcosms exposed to material from constructed wetlands to different pesticides (Fig 4).

My results showed that the highest heterogeneity was found for the enzyme gene *nir*S, while the least heterogeneity was found for the enzyme gene *nos*Z (Fig 7), corroborating a general relationship of lower diversity of *nos*Z in comparison to *nir*K and *nir*S (review by Wallenstein et al., 2006). Sequences from the enzyme gene *nir*S clustered close to members of α-, β-, γ-*Proteobacteria* and gram-positive bacteria. Sequences from studied denitrifying bacterial enzyme genes most often clustered together with members of α-*Proteobacteria*, followed by members of β-*Proteobacteria*, and then with members of γ-*Proteobacteria*. The results showed that the sequences of the denitrifying bacterial community was as heterogeneous among studied agricultural constructed wetland as it have been shown to be in arable soil (Throbäck et al., 2004).

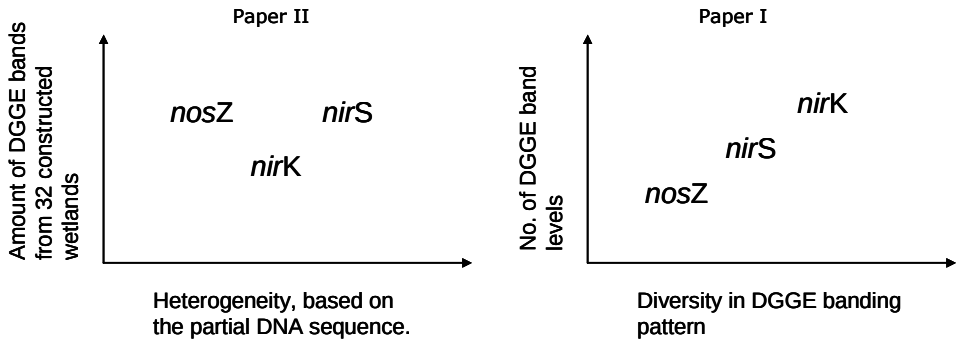

Figure 7. The figures illustrate how the different enzyme genes ranked in the measures of heterogeneity or diversity used in the studies. The left figure represents the relationship between heterogeneity and the studied denitrifying bacterial enzyme genes (details in paper II). The right figure represents the relationship of diversity and the studied denitrifying bacterial enzyme genes (details in paper I). The highest heterogeneity was found for the enzyme gene *nir*S, while the highest diversity was found for *nir*K among the agricultural constructed wetlands.

6.3. Paper III

In paper III, I examine if environmental concentrations of pesticides may influence the structure and/or function of constructed wetland bacterial communities. A field sampling was performed in one single constructed wetland (included in the wetlands in papers I and II) from which sediment samples were collected. Microcosms including both a sediment and water phase were set-up and the bacterial communities were incubated short-term with different pesticides. Structure and function of the denitrifying bacterial community were

analysed to compare which of those endpoints was more sensitive to pesticide exposure. The structure and function of the denitrifying bacterial community were analysed by using the isotope ^{15}N pairing technique for functional measures, and by using PCR-DGGE (as in papers I and II) for structural measures of the enzyme gene *nos*Z (Fig 4). We chose the methods because the purpose was to measure the last step in the denitrification pathway. Hence, the functional method measured potentially produced N_2, which is the end-product of the denitrification pathway, and *nos*Z catalyses the last step. In addition, the eubacterial 16S rRNA gene was analysed as a second structural measure to provide a wider perspective of pesticide effects on aquatic bacterial communities.

Our results showed that the composition of the eubacterial community was more sensitive, since the bacterial structure changed after exposure to all three pesticide groups (the fungicide propiconazole, the herbicide glyphosate and the insecticide pirimicarb) in contrast to the structure and function of the denitrifying bacterial community (Table 1). The structure of the eubacterial community was affected by all pesticide treatments at all tested concentrations when compared to the controls (Table 1). The function of the denitrifying bacterial community was partly negatively affected by the fungicide whereas their structure was unaffected. However, the toxic effects were small and the failure to detect effects in several treatments, and the lack of concentration-response effects severely restricts conclusions regarding the toxic effects of pesticide on structure and function in this mind of approach. In addition, structural endpoints of the eubacterial structure only showed increasing effect, which imply that it cannot be distinguished whether it measures toxic or positive effects of pesticides. Thus, functional endpoints should be included in toxicity assessments of pesticide exposure on aquatic bacterial communities and may be complemented with structural endpoints.

Table 1. Effects of three different pesticides, the fungicide (F) propiconazole, the herbicide (H) glyphosate, and the insecticide (I) pirimicarb, on the structure and/or function of denitrifying bacterial and eubacterial communities. x denotes observed effect, while gap denotes no effect.

Pesticide	**Denitrifying bacteria**		**Eubacteria**
Treatment	**structure**	**function**	**structure**
Propiconazole (F)		x	x
Glyphosate (H)			x
Pirimicarb (I)			x

6.4. Paper IV

Finally, in paper IV, I examine pesticide, in particular fungicide, effects on constructed wetland bacterial communities and compare to the sensitivity of two methods for toxicity assessment. The two methods were leucine incorporation (i.e. bacterial growth) and acetylene inhibition (i.e. potential denitrification). In addition, the effects from pesticide exposure were measured in both water and sediment column with the leucine incorporation method to compare toxic effects on the bacterial communities in the two matrixes. A field sampling was performed in one agricultural constructed wetland (not included in the previous studied wetlands) from which water and sediment samples were collected, and microcosms (as in paper III) were set-up. In contrast to paper III, a concentration-response approach was used, to facilitate quantification of the effect concentration (EC) values. The potential denitrification rate, as measured by amount of produced N_2O using the acetylene inhibition technique, and the bacterial growth as measured by the leucine incorporation were used (Fig 4). The acetylene inhibition was applied since the purpose was to compare denitrification efficiency between different treatments at microcosm scale, and since it is a quicker method than the one used in paper III. The leucine incorporation method was chosen because it has been valuated as a sensitive method in toxicity measurements in soil systems (Rousk et al., 2008). In addition, leucine incorporation method detects growth of the total bacterial community in contrast to acetylene inhibition, which detects denitrification in a functional group of the total bacterial community. These two endpoints were chosen based on results from papers I and III, which showed that the eubacterial and denitrifying bacterial community had different responses and relationships to environmental parameters and pesticides.

Our results showed that the leucine incorporation detected toxic effect for a higher number of fungicides, and produced lower EC_{50}-values for the tested compounds than the potential denitrification method, irrespective of being measured in the water or the sediment column (Table 2). Thus, leucine incorporation was suggested to be a more useful method when performing toxicity measures of pesticide exposure on aquatic bacterial communities than potential denitrification rate (N_2O).

Table 2. Effects of eight fungicides (F), one reference compound (R) and two bactericides (B) on bacterial communities activity. Results show that the leucine incorporation method, which measures bacterial growth, registered toxic effects of fungicides on aquatic bacterial communities with a higher sensitivity than measures of the potential denitrification using the acetylene inhibition method. Large X denotes a severe toxic effect, small x denotes minor toxic effect, and gap denotes no observed effect.

Pesticide	**Leucine incorporation**		**Potential denitrification**
Treatment	**water**	**sediment**	**water and sediment**
Benomyl (F)	X	x	
Captan (F)	X	X	X
Carbendazim (F)			
Carboxin (F)			
Cycloheximide (F)			
Fenpropimorph (F)	x		
Propiconazole (F)	x		
Thiram (F)	X	X	X
3,5-dichlorophenol (R)	X	x	x
Bronopol (B)	X	X	x
Chlortetracycline (B)	x	x	

7. Future Perspectives for Nitrogen Removal in Constructed Wetlands

The results of my studies provide partial answers to some of the fundamental questions concerning the structure and function of the denitrifying bacterial community, responsible for denitrification in agricultural constructed wetlands. Overall, my results show that the denitrifying bacterial enzyme genes vary between studied wetlands and that their structure and function may be affected by specific environmental parameters as well as by different pesticides. Papers I and II, suggests that the general denitrifying bacterial community is diverse and has the capacity of being heterogeneous, which open up the possibility of functional redundancy. The toxicity assessments of pesticide effects obtained in my papers III and IV, indicate that they will not affect the bacterial communities in constructed wetlands to a larger extent, because higher concentrations are needed than the concentrations detected in natural surface waters. Thus, my results suggest that constructed wetlands may maintain unaffected denitrification efficiency even during pesticide exposures.

However, many more fundamental issues, regarding the denitrifying bacterial communities in agricultural constructed wetlands, remain to be explored before we can understand the linkage between their structure and function. For instance, it remains to fully gain knowledge of the biogeography of bacterial communities in constructed wetlands. Similar biogeography approaches as the ones in my papers I and II should be of interest, but by applying complementary

methods, which can help broaden the perspective of the denitrifying bacterial community. For example, quantifying the presence of each denitrifying bacterial enzyme gene by using real-time PCR, will add information of the abundance of the enzyme genes. In addition, to analyse the mRNA of the denitrifying bacterial community to determine which enzyme genes that actually are active, as the PCR-DGGE do not separate between active and dormant DNA. For further studies, the potential variation between denitrifying bacterial community composition in efficient and less efficient agricultural constructed wetlands, would be a start to understand the structure and function linkage.

Many perspectives remain to be explored concerning pesticide exposure on aquatic bacterial communities. For instance, toxicity assessments on bacterial communities needs to be better developed, especially the knowledge of which method to use. A first approach would be to continue evaluating and comparing the sensitivity between methods that can be used in toxicity assessments of pesticides on aquatic bacterial communities. Moreover, toxicity assessment on both single species level and community level is needed to understand the mechanisms behind any effects. For instance, single species analysis may be used to analyse direct effects from pesticide exposure, while analysis at bacterial community level should be used to gain more environmentally relevant effect concentrations. Measures of leucine incorporation, has shown to be able to assess toxicity of different pesticides on bacterial communities in both water and sediment columns with high sensitivity, and has potential to become a standardized method when assessing toxicity on bacterial communities. Thus, many research areas in this field are left to explore until we will fully understand all mechanisms behind the structure and function of denitrifying bacterial community in agricultural constructed wetlands. However, with more cross-disciplinary studies involving molecular ecology and ecotoxicology, I am hopeful that new insights of N-removal efficiency in constructed wetlands can be obtained.

8. References

Aldén Demoling, L., Bååth, E., 2008. The use of leucine incorporation to determine the toxicity of phenols to bacterial communities extracted from soil. *Applied Soil Ecology*. 38, 34-41.

Angeloni, N.L., Jankowski, K-J., Tuchman, N.C., Kelly, J.J., 2006. Effects of an invasive cattail species (Typha x glauca) on sediment nitrogen and microbial community composition in a freshwater wetland. *FEMS Microbiology Letters*. 263, 86-92.

Avrahami, S., Conrad, R., Braker, G., 2002. Effect os soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. *Applied and Environmental Microbiology*. 68, 5685-5692.

Baas-Becking, L.G.M., 1934. Geologie of Inleidning tot de Milieukunde.The Hague, The Netherlands. W.P. Van Stockum.

Bachand, P.A.M., Horne, A.J., 2000. Denitrification in constructed free-water surface wetlands: I. Very high nitrate removal rates in a macrocosm study. *Ecological Engineering*. 14, 9-15.

Bell, T., Newman, J.A., Silverman, B.W., Turner, S.L., Lilley, A.K., 2005. The contribution of species richness and composition to bacterial services. *Nature*. 436, 1157-1160.

Beisner, B.E., Peres-Neto, P.R., Lindström, E.S., Barnett, A., Lorena Longhi, M., 2006. The role of environmental and spatial processes in structuring lake communities from bacteria to fish. *Ecology*. 87, 2985-2991.

Bernot, M.J., Dodds, W.K., Gardner, W.S., McCarthy, M.J., Sobolev, D., Tank, J.L., 2003. Comparing denitrification estimates for a Texas estuary by using acetylene inhibition and membrane inlet mass spectrometry. *Applied and Environmental Microbiology*. 69, 5950-5956.

Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Weatherby, A., 2005. 15 years of pond assessment in Britain: results and lessons learned from the work of pond conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 15, 693–714.

Bjørnlund, L., Ekelund, F., Christensen, S., Jacobsen, C.S., Krogh, P.H., Johnsen, K., 2000. Interactions between saprotrophic fungi, bacteria and protozoa on decomposing wheat roots in soil influenced by the fungicide fenpropimorph (Corbel®): a field study. *Soil Biology and Biochemistry*. 32: 967-975.

Braker, G., Fesefeldt, A., Witzel, K-P., 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology*. 64, 3769-3775.

Braker, G., Zhou, J., Wu, L., Devol, A.H., Tiedje, J.M., 2000. Nitrite reductase genes (*nir*K and *nir*S) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. *Applied and Environmental Microbiology*. 66, 2096-2104.

Braker, G., Ayala del Rio, H.L., Devol, A.H., Fesefeldt, A., Tiedje, J., 2001. Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nir*S) and 16S rRNA genes. *Applied and Environmental Microbiology*. 67, 1893-1901.

Brandt, K.K., Jørgensen, N.O.G., Nielsen, T.H., Winding, A., 2004. Microbial community-level toxicity testing of linear alkylbenzene sulfonates in aquatic microcosms. *FEMS Microbiology Ecology*. 49, 229-241.

Bremer, C., Braker, G., Matthies, D., Reuter, A. Engels, C., Conrad, R., 2007. Impact of plant functional group, plant species, and sampling time on the composition of nirK-type denitrifier communities in soil. *Applied and Environmental Microbiology*. 73, 6876-6884.

Boon, N., Windt, W.D., Verstraete, W., Top, E.M., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiology Ecology*. 39,101-112.

Bothe, H., Ferguson, S.J., Newton, W.E., 2007. Biology of the nitrogen cycle, First edition. The Netherlands. Amsterdam.

Bååth, E., 1994. Thymidine and leucine incorporation in soil bacteria with different cell size. *Microbial Ecology*. 27, 267-278.

Bååth, E., Petterson, M., Söderberg, K.H., 2001. Adaption of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. *Soil Biology and Biochemistry*. 33, 1571-1574.

Chen, S-K., Edwards, C.A., Subler, S., 2001. Effects of the fungicides benomyl, captan and chlorothalonil on soil microbial activity and nitrogen dynamics in laboratory incubations. *Soil Biology and Biochemistry*. 33, 1971-1980.

Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. *Environmental Toxicology and Chemistry*. 25, 2208-2215.

Coyne, M. S., Arunakumari, A., Averill, B. A., Tiedje, J. M., 1989. Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying Bacteria. *Applied and Environmental Microbiology*. 55, 2924-2931.

Crecchio, C., Curci, M., Pizzigallo, M.D.R., Ricciuti, P., Ruggerio, P., 2001. Molecular approaches to investigate herbicide-induced bacterial community changes in soil microcosms. *Biology and Fertility of Soils*. 33, 460-466.

Crommentuijn, T., Sijm, D., de Bruijn, J., van Leeuwen, K., van de Plassche, E., 2000. Maximum permissible and negligible concentrations for some organic substances and pesticides. *Journal of Environmental Management*. 58, 297-312.

Dabert, P., Delgenès, J-P., Moletta, R , Godon, J-J., 2002. Contribution of molecular microbiology to the study in water pollution removal of microbial community dynamics. *Environmental Science and Bio/Technology*. 1, 39-49.

Dahllöf, I., 2002. Molecular community analysis of microbial diversity. *Current Opinion in Biotechnology*. 13, 213-217.

DeLorenzo, M.E., Scott, G.I., Ross, P.E., 2001. Toxicity of pesticides to aquatic microorganisms: a review. *Environmental Toxiciology and Chemistry*. 20, 84-98.

Díaz-Raviña, M., Bååth, E., 2001. Response of soil bacterial communities pre-exposed to different metals and reinoculated in an unpolluted soil. *Soil Biology and Biochemistry*. 33, 241-248.

Engelen, B., Meinken, K., von Wintzingerode, F., Heuer, H., Malkomes, H-P., Backhaus, H., 1998. Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Applied and Environmental Microbiology*. 64, 2814-2821.

Enwall, K., Philippot, L., Hallin, S., 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Applied and Environmental Microbiology*. 71, 8335-8343.

Enwall, K., 2008. Community ecology of denitrifying bacteria in arable land. Doctoral Thesis no 2008:58. SLU Service. Uppsala. ISBN 978-91-85913-91-6.

Eriksson, P.G., Weisner, S.E.B., 1999. An experimental study on effects of submersed macrophytes on nitrification and denitrification in ammonium-rich aquatic systems. Limnology and Oceanography. 44, 1993-1999.

Farm Service Agency, 2004. United States Department of Agriculture. Farmable wetlands program, fact Sheet: 10. http://www.fsa.usda.gov (accessed 28/09/07).

Finlay, B.J., Clarke, K.J., 1999. Ubiquitous dispersal of microbial species. *Nature*. 400, 828.

Fischer, H., Pusch, M., 1999. Use of the [^{14}C]leucine incorporation technique to measure bacterial production in river sediments and the epiphyton. *Applied and Environmental Microbiology*. 65, 441-4418.

Fleischer, S., Gustafson, A., Joelsson A., Pansar, J., Stibe, L., 1994. Nitrogen removal in created ponds. *Ambio*. 23, 349-357.

Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology*. 7, 301-313.

Green, J.L., Bohannan, B.J.M., Whitaker, R.J., 2008. Microbial biogeography: from taxonomy to traits. *Science*. 320, 1039-1043.

Hallin, S., Lindgren P-E., 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Applied and Environmental Microbiology*. 65, 1652-1657.

Haarstad, K., Braskerud, B.C., 2005. Pesticide retention in the watershed and in a small constructed wetland treating diffuse pollution. *Water Science and Technology*. 51, 143-150.

Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 2006. Quantitative detection of the *nos*Z gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *nar*G, *nir*K, and *nos*Z genes in soils. *Applied and Environmental Microbiology*. 72, 5181-5189.

Heylen, K., Gevers, D., Vanparys, B., Wittebolle, L., Geets, J., Boon, N., De Vos, P., 2006. The indicies of *nir*S and *nir*K and their genetic heterogeneity in cultivated denitrifiers. *Environmental Microbiology*. 8, 2012-2021.

Heylen, K., Vanparys, B., Gevers, D., Wittebolle, L., Boon, N., De Vos, P., 2007. Nitric oxide reductase (*nor*B) gene sequence analysis reveals discrepancies with nitrite reductase (*nir*) gene phylogeny in cultivated denitrifiers. *Environmental Microbiology*. 9, 1072-1077.

Hewson, I., Vargo, G.A., Fuhrman, J.A., 2003. Bacterial diversity in shallow oligotrophic marine benthos and overlying waters: Effects of virus infection, containment, and nutrient enrichment. *Microbial Ecology*. 46, 322-336.

Hewson, I., Jacobson/Meyers, M.E., Fuhrman, J.A., 2007. Diversity and biogeography of bacterial assemblages in surface sediments across the San Pedro basin, Southern California borderlands. *Environmental Microbiology*. 9, 923-933.

Hoffmann, M., Johnsson, H., Gustafson, A., Grimvall, A., 2000. Leaching of nitrogen in Swedish agriculture – a historical perspective. *Agriculture, Ecosystems and Environment*. 80, 277-290.

Jarvis, Å., Sundberg, C., Milenkovski, S., Pell, M., Smårs, S., Lindgren, P-E., Hallin, S., 2009. Activity and composition of ammonia oxidizing bacterial communities and emission dynamics of NH_3 and N_2O in a compost reactor treating organic household waste. *Journal of Applied Microbiology*. In press.

Johnsen, K., Jacobsen, C.S., Torsvik, V., Sørensen, J., 2001. Pesticide effects on bacterial diversity in agricultural soils – a review. *Biology and Fertility of Soils*. 33, 443-453.

Kadlec, R.H., 1997 Deterministic and stochastic aspects of constructed wetland performance and design. *Water Science and Technology*. 35, 149-156.

Kennedy, A. C., 1999. Bacterial diversity in agroecosystems. *Agriculture, Ecosystems and Environment*. 74, 65-76.

Kent, A.D., Jones, S.E., Yannarell, A.C., Graham, J.M., Lauster, G.H., Kratz, T.K., Triplett, E.W., 2004. Annual patterns in bacterioplankton community variability in a humic lake. *Microbial Ecology*. 48, 550-560.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T., 2004. Methods of studying soil microbial diversity. *Journal of Microbiological Methods*. 58, 169-188.

Knowles, R., 1982. Denitrification. *Microbiological Reviews*. 46, 43-70.

Kozub, D.D., Liehr, S.K., 1999. Assessing denitrification rate limiting factors in a constructed wetland receiving landfill leachate. *Water Science and Technology*. 40, 75-82.

Kreuger, J., 1998. Pesticides in stream water within an agricultural catchment in Southern Sweden, 1990-1996. *The Science of the Total Environment*. 216, 227-251.

Kreuger, J., Peterson, M., Lundgren, E., 1999. Agricultural inputs of pesticide residues to stream and pond sediments in a small catchment in Southern Sweden. *Bulletin of Environmental Contamination and Toxicology*. 62, 55-62.

Langenheder, S., 2005. Links between structure and function of heterotrophic aquatic bacterial communities. Doctoral Thesis. Uppsala University.

Langenheder, S., Ragnarsson, H., 2007. The role of environmental and spatial factors for the composition of aquatic bacterial communities. *Ecology*. 88, 2154-2161.

Langenheder, S., Prosser, J.I., 2008. Resource availability influences the diversity of a functional group of heterotrophic soil bacteria. *Environmental Microbiology*. 10, 2245-2256.

Larson, F., Petersen, D.G., Dahllöf, I., Sundbäck, K., 2007. Combined effects of an antifouling biocide and nutrient status on a shallow-water microbenthic community. *Aquatic Microbial Ecology*. 48, 277-294.

Lin, Y-F., Jing, S-R., Wang, T-W., Lee, D-Y., 2002. Effects of macrophytes and external carbon sources on nitrate removal from groundwater in constructed wetlands. *Environmental Pollution*. 119, 413–420.

Lindström, E.S., 2000. Bacterioplankton community composition in five lakes differing in trophic status and humic content. *Microbial Ecology*. 40, 104-113.

Lindström, E.S., Leskinen, E., 2002. Do neighboring lakes share common taxa of bacterioplankton? Comparison of 16S rDNA fingerprints and sequences from three geogrphic regions. *Microbial Ecology*. 44, 1-9.

Logue, J.B., Lindström, E.S., 2008. Biogeography of bacterioplankton in inland waters. *Freshwater Reviews*. 1, 99-114.

Loreau, M., Hector, A., 2001. Partitioning selection and complementarity in biodiversity experiments. *Nature*. 412, 72-76.

Lovley, D., 2003. Cleaning up with genomics: applying molecular biology to bioremediation. *Nature Reviews*. 1, 35-44.

Maraldo, K., Dahllöf, I., 2004. Indirect estimation time for zinc pyrithione and copper pyrithione in seawater. *Marine Pollution Bulletin*. 48, 894-901.

Martiny, J.B.H., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., Horner-Devine, M.C., Kane, M., Adams Krumins, J., Kuske, C.R., Morin, P.J., Naeem, S., Øvreås, L., Reysenbach, A-L., Smith, V.H., Staley, J.T., 2006. Microbial biogeography: putting microorganisms on the map. *Nature Reviews*. 4, 102-112.

McNaughton, S.J., 1977. Diversity and stability of ecological communities: a comment on the role of empiricism in ecology. *The American Naturalist*. 111, 515-525.
Mitsch, W.J., Gosselink J.G., 2000. Wetlands. 3rd ed. New York, The United States of America. John Wiley & Sons, Inc.
Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Microbiology*. 2, 317-322.
Nielsen, L.P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. *FEMS Microbiology Ecology*. 86, 357-362.
Osborn, A.M., Moore, E.R.B., Timmis, K.N., 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RLFP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology*. 2, 39-50.
Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology*. 17, 526-538.
Pell, M., Stenberg, B., Torstensson, L., 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio*. 27, 24-28.
Petersen, D.G., Dahllöf, I., Nielsen, L.P., 2004. Effects of zinc pyrithione and copper pyrithione on microbial community function and structure in sediments. *Environmental Toxicology and Chemistry*. 23, 921-928.
Petersen, D.G., Dahllöf, I., 2007. Combined effects of pyrene and UV-light on algae and bacteria in an arctic sediment. *Ecotoxicology*. 16, 371-377.
Philippot, L., Hallin, S., 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. *Current Opinion in Microbiology*. 8, 234-239.
Priemé, A., Braker, G., Tiedje, J.M., 2002. Diversity of nitrite reductase (*nir*K and *nir*S) gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology*. 68, 1893-1900.
Reche, I., Pulido-Villena, E., Morales-Baquero, R., Casamayor, E.O., 2005. Does ecosystem size determine aquatic bacterial richness? *Ecology*. 86, 1715-1722.
Relyea, R., Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters*. 9, 1157-1171.
Rousk, J., Aldén Demoling, L., Bååth, E., 2008. Contrasting short-term antibiotic effects on respiration and bacterial growth compromises the validity of the selective respiratory inhibition technique to distinguish fungi and bacteria. *Microbial Ecology*. DOI 10.1007/s00248-008-9444-1.
Rysgaard, S., Risgaard-Petersen, N., Nielsen, L.P., Revsbech, N.P., 1993. Nitrification and denitrification in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing. *Applied and Environmental Microbiology*. 59, 2093-2098.
Schweiger, P.F., Jakobsen, I., 1998. Dose-response relationships between four pesticides and phosphorus uptake by hyphae of arbuscular mycorrhizas. *Soil Biology and Biochemistry*. 30, 1415-1422.
Seitzinger, S., Harrison, J.A., Böhlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., Tobias, C., Van Drecht, G., 2006. Denitrification across landscapes and waterscapes: a synthesis. *Ecological Applications*. 16, 2064-2090.
Sigler, W.V., Turco, R.F., 2002. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. *Applied Soil Ecology*. 21, 107-118.
Sirivedhin, T., Gray, K.A., 2006. Factors affecting denitrification rates in experimental wetlands: field and laboratory studies. *Ecological Engineering*. 26, 167-181.

Smith, D.C., Azam, F., 1992. A simple, economical method for measuring bacterial protein synthesis rates in seawater using tritiated-leucine. *Marine Microbial Food Webs*. 6, 107-114.

Stadmark, J., Leonardsson, L., 2005. Emissions of greenhouse gases from ponds constructed for nitrogen removal. *Ecological Engineering*. 25, 542-551.

Standing, D., Baggs, E.M., Wattenbach, M., Smith, P., Killham, K., 2007. Meeting the challenge of scaling up processes in the plant-soil-microbe system. *Biology and Fertility of Soils*. 44, 245-257.

Stenersen, J., 2004. Chemical pesticides, mode of action and toxicology. CRC press LLC. Florida. USA.

Sundberg, C., Tonderski, K., Lindgren, P-E., 2007. Potential nitrification and denitrification and the corresponding composition of the bacterial communities in a compact constructed wetland treating landfill leachates. *Water Science and Technology*. 56, 159-166.

Sundbäck, K., Groth Petersen, D., Dahllöf, I., Larson, F., 2007. Combined nutrient-toxicant effects on a shallow-water marine sediment system: sensitivity and resilience of ecosystem functions. *Marine Ecology Progress Series*. 330, 13-30.

Svensson, J. M., 1998. Emission of N_2O nitrification and denitrification in a eutrophic lake sediment bioturbated by *Chironomus plumosus*. *Aquatic Microbial Ecology*. 14, 289-299.

Sverdrup, L. E., Ekelund, F., Krogh, P.H., Nielsen, T., Johnsen, K., 2002. Soil microbial toxicity of eight polycyclic aromatic compounds: Effects on nitrification, the genetic diversity of bacteria, and the total number of protozoans. *Environmental Toxicology and Chemistry*. 21, 1644-1650.

Swedish Board of Agriculture, 2000. Jordbruksverket, Informationsmaterial: Miljö- och landsbygdsprogram för Sverige år 2000-2006, pp. 222. http://www.regeringen.se (accessed 28/09/2007).

Thiere, G., Milenkovski, S., Lindgren, P-E., Sahlén, G., Berglund, O., Weisner S.E.B., 2009. Wetland creation in agricultural landscapes: biodiversity benefits on local and regional scales. *Biological Conservation*. In press.

Torsvik, V., Øvreås, L., Thingstad, T.F., 2002. Prokaryotic diversity – magnitude, dynamics, and contolling factors. *Science*. 296, 1064-1066.

Throbäck I.N., Enwall, K., Jarvis, Å., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology*. 49, 401-417.

Van den Brink, P.J., Crum, S.J.H., Gylstra, R., Bransen, F., Cuppen, J.G.M., Brock, T.C.M., 2009. Effects of a herbicide-insecticide mixture in freshwater microcosms: Risk assessment and ecological effect chain. *Environmental Pollution*. 157, 237-249.

Wallenstein, M.D., Myrold, D.D., Firestone, M., Voytek, M., 2006. Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications*. 16, 2143-2152.

Weisner, S.E.B., Eriksson, P.G., Granéli, W., Leonardsson, L., 1994. Influence of macrophytes on nitrate removal in wetlands. *Ambio*. 23, 363-366.

Widenfalk, A., Svensson, M. J., Goedkoop, W., 2004. Effects of the pesticides captan, delthamethrin, isoproturon, and pirimicarb on the microbial community of a freshwater sediment. *Environmental Toxicology and Chemistry*. 23, 1920-1927.

Widenfalk, A., Bertilsson, S., Sundh, I., Goedkoop, W., 2008. Effects of pesticides on community composition and activity of sediment microbes – responses at various levels of microbial community organization. *Environmental Pollution*. 152, 576-584.

Woin, P., 1998. Short- and long-term effects of the pyrethroid insecticide fenvalerate on an invertebrate pnd community. *Ecotoxicology and Environmental Safety*. 41, 137-156.
Yin, B., Crowley, D., Sparovek, G., De Melo, W.J., Borneman, J., 2000. Bacterial functional redundancy along a soil reclamation gradient. *Applied and Environmental Microbiology*. 66, 4361-4365.
Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*. 61, 533-616.

Svensk Sammanfattning

Denitrifierarna i våtmarker – vem är de, varför är de där och hur känsliga är de för jordbruksgifter?

Under 1900-talet har jordbruksarealer runt om i världen expanderat för att kunna ge mat åt den växande befolkningen. Detta har resulterat i att stora arealer naturliga våtmarker, till exempel kärr och mossar, helt torrlagts och försvunnit. En direkt konsekvens från detta har blivit att vi gått miste om de naturliga våtmarkernas funktion, nämligen att fungera som naturliga reningsverk av näringsrikt och förorenat vatten. Näringsrikt och förorenat vatten kan orsaka övergödning och syrebrist om de når ut till sjöar och hav och de kan förändra organismernas födokedja. Föroreningar och gifter från jordbruksmark utgörs mestadels av olika sorters bekämpningsmedel som oavsiktligt kan läcka ut till närliggande vatten när bönderna besprutar sina arealer. Näringsämnen som också kan läcka ut från jordbruksmarken, från bland annat gödsel, består till stor del av kväve och fosfor. I min avhandling valde jag att fokusera på kvävet.

Ett försök till att minska överskottet av kväve och föroreningar har varit att anlägga våtmarker intill jordbruksmark. Ett problem med de anlagda våtmarkerna har dock varit att deras funktion som reningsverk varierar. Varför kvävereningen varierar vet man inte eftersom kunskapen inom detta område är begränsad. Kvävet kan minska i våtmarkerna genom att nitrat, som är mycket tillgängligt för organismer, reduceras till kvävgas som är mindre tillgängligt. Denna process kallas för denitrifikation och utförs via bakteriesamhället eller mer specifikt av denitrifierarna. Denitrifikation omvandlar nitrat till kvävgas i fyra olika steg och dessa steg utförs av olika enzymgener som produceras av denitrifierarna. Det är framförallt kunskapen om denitrifierarna som är mycket begränsad, trots att kännedom om deras samhällsstruktur och funktion kan leda till anläggande av effektiva våtmarker. Målet med denna avhandling var att försöka skapa en forskningsgrund genom att kartlägga denitrifierare i anlagda våtmarker vid jordbrukslandskap för att ta reda på vem de är, varför de är där och hur känsliga de är för jordbruksgifter.

Sverige har som mål att återskapa 12 000 ha våtmarker till år 2010 för att nå det nationella målet att minska näringstillförseln från jordbruksmark till sjöar och hav. Anlagda våtmarker har likadant utseende som dammar eller mindre sjöar men de är överlag grundare (medeldjup omkring en meter). Detta möjliggör för ökad växtlighet där växter utgör en kolkälla för bakterierna. Förutom tillgängligt kol behöver denitrifierarna nitrat och syrefattiga miljöer för att kunna producera enzymgenerna som katalyserar denitrifikationen. I mitt första projekt ville jag ta reda på om denitrifierarnas samhällsstruktur varierar mellan olika anlagda våtmarker och om det möjligtvis fanns andra faktorer förutom kol, nitrat och syrefattig miljö som påverkar/bestämmer deras förekomst. För att ta reda på detta undersökte jag denitrifierarnas samhällsstruktur i 32 stycken anlagda våtmarker. Jag mätte även 15 olika våtmarksfaktorer: bland annat undersöktes

växterna, våtmarkernas djup, kväve belastning i in och utflöde, vattnets uppehållstid, våtmarkernas area etc. Mina resultat visade att denitrifierarnas samhällsstruktur varierar mellan de undersökta våtmarkerna. Dessutom fann jag att ytterligare faktorer än de redan välkända kan påverka denitrifierarnas samhällsstruktur. Jag valde att fortsätta studera denitrifierarnas samhällsstruktur men istället undersöka om det fanns variation mellan de olika enzymgenerna som representerar de olika stegen i denitrifikations processen. Jag använde samma material från de 32 anlagda våtmarkerna från den första studien och genom kompletterande undersökning kunde jag granska förekomst av denitrifierarnas enzymgener. Mina resultat visade att enzymgenerna varierade och jag kunde dra slutsatsen att miljön i anlagda våtmarker intill jordbrukslandskap kan ge plats åt ett mycket varierande denitrifierarsamhälle.

Hänsyn måste också tas till att många konstruerade våtmarker med jämna mellanrum utsätts för olika bekämpningsmedel som kan påverka denitrifierarna. Bekämpningsmedel som används mot bland annat skadeinsekter, ogräs och svamp kan påverka bakteriesamhället i våtmarker. Påverkan kan vara både direkt eller indirekt, där direkt effekt kan döda vissa bakterier medan andra mer toleranta bakteriearter kan påverkas indirekt genom förmågan att utnyttja bekämpningsmedlet som en extra födokälla. Om denitrifierarna påverkas av bekämpningsmedel så kan detta orsaka att effektiviteten i denitrifikationen förändras, t.ex. genom att denitrifierarna slutar producera enzymgener. I min tredje studie utsatte jag bakteriesamhällen för olika bekämpningsmedel för att se om denitrifierarna i anlagda våtmarker kan utföra en effektiv denitrifikation även då de exponeras av olika bekämpningsmedel. Jag använde olika bekämpningsmedel i miljörelevanta koncentrationer och mätte deras effekt på denitrifierarnas samhällsstruktur och funktion (denitrifikationen). Mina resultat visade att det undersökta svampmedlet påverkade (positivt och negativt) både bakteriernas samhällsstruktur och deras funktion. Insekt- och ogräsmedlet påverkade endast samhällsstrukturen. Jag fortsatte därför att studera svampmedel i min sista studie där jag utökade antalet undersökta svampmedel och använde mig av många fler låga och höga koncentrationer för varje bekämpningsmedel. Jag valde dessutom att endast analysera bakteriesamhällets funktion genom att jämföra två olika metoder. Den första mätte denitrifikationshastighet och den andra bakteriernas tillväxthastighet. Mina resultat visade att vissa svampmedel kan påverka bakteriesamhällets funktion, både tillväxt och denitrifikation. Dessutom kunde jag dra slutsatsen att metoden som mätte bakteriernas tillväxthastighet var känsligare och därmed bättre på att mäta svampmedlens giftighet mot bakteriesamhällen än metoden som mätte denitrifikation.

Sammantaget visar mina studier att denitrifierarna finns i anlagda våtmarker, att deras samhällsstruktur varierar mellan våtmarker och att denna struktur kan påverkas av olika miljöfaktorer och gifter. Detta betyder att förutsättningen för att denitrifikation skall kunna utföras i anlagda våtmarker är stor, men problemet är mer komplicerat. En viktig kännedom är att även om olika denitrifierare finns i våtmarker så finns det ingen garanti för att de producerar de nödvändiga enzymgenerna för att denitrifikation skall kunna utföras. Därför

behövs mer forskning inom detta område som kan fortsätta studera hur denitrifierarnas samhällsstruktur är sammankopplad med deras funktion, denitrifikationen. Många våtmarker har anlagts och många fler är på gång och det är därför viktigt att se till att de kan fungera som effektiva reningsverk.

TACK!

Olof, det har varit en fantastisk rolig och givande tid och jag inser att jag haft tur som har haft dig som handledare. Jag har lärt mig oerhört mycket och jag är helt övertygad att det är tack vare din imponerande kunskap och pedagogiska förmåga. Samtidigt som du har gett mig konstruktiv kritik så har du fått mig att bli maximalt motiverad och uppmuntrad även om det inneburit att jag skall göra om (allt) och göra rätt. Dessutom har du tagit dig tid att komma på mina spinningpass, visat lika stort intresse för melodifestivalen som jag, och diskuterat färgval av datorer, vadarbyxor etc. Din hjälp, ditt stöd och tålamod under alla dessa år har varit ovärderlig! **TACK!**

Per-Eric, du har varit en fantastisk biträdande handledare. Det har varit omväxlande att med jämna perioder komma till Linköping och lära mig den molekylära biten som jag hade ganska begränsade kunskaper i från början. Din förmåga att hålla kontakt via telefon, mail och att alltid förespråka täta möten har gjort att mitt projekt flutit på och fungerat mycket bra. Tack för all din hjälp och uppmuntran!

Geraldine, det har varit fantastiskt att få samarbeta med dig och att ha ditt stöd under denna tid! Du har ställt upp så fort jag har behövt din hjälp och det har oftast (förutom när vi körde fast med bilen och när vi kom till våtmarken med alla mygg) blivit mycket lyckat! Tack för allt!

Stefan W, tack för all hjälp, alla WEB möten, med manus, och för din gästfrihet när vi hade våra möten i Halmstad! **Erland B**, tack för ett jättebra samarbete (paper IV), och för att jag insett att din inställning 'fast and dirty' är lysande! **Tina S**, **Carina S**, **Stefan B**, **Johan N**, **Christina S** och **Elisabeth**, tack för all hjälp med labbandet och för att jag med en gång kände mig välkommen till avdelningen i Linköping. **Nina Syde**, trots alla iglar, ungtjurar och sötvattenshajar som attackerade oss så jobbade du på och gjorde fältarbetet, (paper I) till en fantastisk tid, tack! **Johanna S**, tack för givande våtmarksdiskussioner, hjälp med GC:n, alla luncher och för alla kommentarer till kappan! **Mattias Mild** och **Dag Ahrén**, tack för att ni tog er tid för att hjälpa mig med de statistiska analyserna av de fylogenetiska träden, trots att ni under tiden (oberoende av varandra) hade överdrivet med jobb! **Jonas S**, tack för allt samarbete med lab och manus till paper III. **Per N**, tack för hjälp med att hitta en lämplig våtmark till paper IV och för hjälp ute i fält! **Marie S**, tack för din uppmuntran och hjälp när jag labbat hos limnologen eller bara hängt på ditt rum! **Thomas P**, tack för att hjälpte till ute i fält (paper IV) och ordnade en utsökt lunch! Tack till alla **markägare**, som låtit oss undersöka och hämta prover från deras våtmarker!

Thanks to my **colleagues** and **master students** (past and present) at **Chemical Ecology/Ecotoxicology** for making it a very pleasant workplace! Jag vill speciellt tacka **Helena B**, för all vänskap, stöd och hjälp under åren, och för kommentarer till min svenska sammanfattning. **Olof R**, för att du alltid tar dig tid att prata när jag kommer och hänger i ditt rum och för vår tävling som ännu inte är avgjord. **Anders Södergren**, min rumskamrat (på distans), för uppmuntran och visat intresse för mitt projekt! **Johanna Bergström**, för att du gjorde ett jättebra exjobb för mig som låg till grund för paper III. **Christer L**, för stöd och kommentarer till kappan. **Maria H**, för kommentarer till min svenska sammanfattning. **Erling J**, som fixar så att allting fungerar med datorer, lab, frukost och för att du tar hand om avdelningen!

Tack till alla övriga i **Ekologihuset** som gör det till en fantastisk arbetsplats! **Badmintongruppen**, badminton har aldrig varit roligare! Jag hoppas att någon annan tar över ansvaret att bjuda in duktiga (kines i världsklass) badmintonspelare som kan utveckla och inspirera oss andra! **Biologibiblioteket**, tack för att ni alltid har löst mina problem angående artikel- eller databassökningar och för att ni alltid är lika trevliga!

Tack till alla på **Medicinsk Mikrobiologi** i Linköping (mitt andra hem). Det har varit ett nöje att få lära känna er och få tillgång till ert, enligt mig, perfekta lab!

Tack till **Karin Tonderski** för bra och givande möten på TEMA Vatten i Linköping. Tack till alla i **Jönköpings- och Göteborgsgruppen** för alla inspirerande möten under åren, speciellt tack till **Sture L** och **Andreas M** som utan att tveka följde med på mitt spinningpass och för att ni sedan hjälpte mig och Olof att ”planka in” på en konferensmiddag.

Tack till alla mina **Vänner** som är de finaste vänner man kan ha!!!
Några vill jag tacka speciellt. **Lotta S**, **Anja C** och **Therese N**, tack för alla middagar och för att ni har varit fantastiska och omtänksamma vänner till mig! Tack till, **Malin D**, **Matilda B**, **Johan A**, **Anne-Lie** och **Veronica G,** de bästa barndomsvännerna som finns! **Pernilla W**, för en fantastisk vänskap och för att du är en av de bästa personerna jag känner! **Sara W** för att du bryr dig, alltid uppmuntrar och gör mig på gott humör! Jag vill även tacka alla mina fantastiska **arbetskollegor** på **Tjugonde** i Malmö!

Tack till min **släkt**! Framförallt vill jag tacka kusin **Annika** och **Mats** som stöttat mig och visat intresse för mitt projekt! Jag vill även tacka **Henriks familj**, speciellt Henriks syster **Sofie** och **Christian** för alla trevliga middagar och för allt som ni har hjälpt till att ordna under åren!

Till slut vill jag tacka min **familj** som är de viktigaste personerna i mitt liv!

Ett stort TACK till min **syster Diana** som är den bästa! Du ställer alltid upp och tror på mig, och det var en trygghet att du fanns nära till hands när jag flyttade ner till Skåne! Några av de allra bästa stunderna och händelserna i mitt liv (vilket det kommer att förbli) har jag upplevt tillsammans med dig! Vill även tacka min svåger **Johan** för all hjälp under åren och mina syskonbarn (ögonstenar) **Amanda**, **Philip** och **Alice**! Tack för allt!

Jag vill tacka min **Mamma** och **Pappa** (**Sonja** och **Strebe**) för att ni är världens bästa föräldrar! Tack för att ni ställer upp, stöttar, hjälper till och framförallt tycker att det viktigaste är att jag mår bra och är lycklig! Ett extra tack till min mamma som förutom att vara världens snällaste person, också bakat kakor i massor som jag sedan tagit med till jobbet och glänst med! TACK!

Henrik, mitt hjärta! Sedan du kom in i mitt liv så har det blivit mycket roligare och jag känner att jag blivit en bättre person tack vare dig! Tack för att du ställde upp och hjälpte till med fältarbete (trots din småkrypsfobi), och för att du tog många fina fotografier. Ditt omåttliga intresse för sladdar, sladdlöst och all elektronik börjar smitta av sig (framförallt det sladdlösa)! Tack för att du och våra katter **Kevin** och **Flisa** gör så att jag alltid längtar hem!

Paper I

Variation of eubacterial and denitrifying bacterial biofilm communities among constructed wetlands

Susann Milenkovski[1], Geraldine Thiere[2], Stefan E. B. Weisner[2], Olof Berglund[1] and Per-Eric Lindgren[3]

[1]Department of Chemical Ecology/Ecotoxicology, Ecology building, Lund University, SE-223 62 Lund, SWEDEN.
[2] Wetland Research Centre, School of Business and Engineering, Halmstad University, Box 823, SE-301 18 Halmstad, SWEDEN.
[3] Department of Clinical and Experimental Medicine, Division of Medical Microbiology, Linköping University, SE-581 85 Linköping, SWEDEN.

Submitted, under revision

Keywords: 16S rDNA, *nir*K, *nir*S, *nos*Z, bacterial community composition, environmental parameters, PCR-DGGE, denitrification.

Summary

Bacteria play important roles in the transformation of nutrients in wetlands, but few studies have examined parameters affecting variation in bacterial community composition between wetlands. We compared the composition of eubacterial and denitrifying bacterial biofilm communities in 32 agricultural constructed wetlands in southern Sweden, and the extent to which wetland environmental parameters could explain the observed variation. Structure and richness of the eubacterial 16S rRNA gene and three denitrifying bacterial enzyme genes (*nir*K, *nir*S and *nos*Z), analysed by molecular fingerprinting methods, varied among the constructed wetlands, which could be partly explained by different environmental parameters. Results from the enzyme gene analyses were also compared to determine whether the practice of using a single denitrifying bacterial gene could characterize the overall community composition of denitrifying bacteria. We found that *nir*K was more diverse than both *nir*S and the *nos*Z, and the band structure and richness of the three genes were not related to the same environmental parameters. This suggests that using a single enzyme gene may not suffice to characterize the community composition of denitrifying bacteria in constructed agricultural wetlands.

Introduction

The use of biological treatment methods driven by microbial communities is increasing worldwide as a means of remedying anthropogenic pollution (Lovely, 2003). Passive treatment in constructed wetlands is one such technique that is applied to handle a wide range of pollutants such as metals, organic substances, and nutrients. In particular, actions taken to decrease nitrogen transport to downstream recipients have led to increasing numbers of constructed wetlands in agricultural areas in Europe and North America. The efficiency with which individual wetlands remove nitrogen (mainly through denitrification) varies greatly and has been associated with e.g. incoming nitrogen load and amount of available carbon (Fleischer et al., 1994; Weisner et al., 1994; Lin et al., 2002). It is probable that the functioning of these ecosystems is influenced by the composition and diversity of bacteria communities that are present (Bell et al., 2005). Nevertheless, in that context, few studies of the performance of constructed wetlands have examined the denitrifying bacteria, which constitute an integral part of the nitrogen removal process. Information is lacking about the composition and activity of the denitryfing bacterial community (Philippot and Hallin, 2005), and its link to the denitrification rate in wetlands (Rich and Myrold, 2004), although there is evidence that bacterial community composition does affect the denitrification in both terrestrial and aquatic ecosystems (Wallenstein et al., 2006). Thus, knowledge regarding the composition of denitrifying bacterial communies and the parameters influencing them, may prove useful in improving nitrogen removal in constructed wetlands.

Theories about the distribution of bacterial communities include the idea that environmental parameters shape the community composition (Martiny et al., 2006; Shade et al., 2008). The denitrifying bacterial community requires the availability of organic carbon and an anaerobic environment in order to reduce nitrate, although there are also other parameters that may influence the community structure and thereby also the denitrification rate. In wetlands, it has been shown that the structure and/or function of microbial communities can be affected by several different environmental parameters, including the macrophyte community composition (Weisner et al., 1994), pH and hydraulic loads (Beisner et al., 2006), nitrate and nitrate loads (Horner-Devine et al., 2003; Hewson et al., 2003), and habitat size (Reche et al., 2005). Recently developed molecular methods have enabled characterization of bacterial communities and assessments of the relative importance of the multiple interacting drivers that structure these communities in different ecosystems. The analysis of the eubacterial 16S rRNA gene (rDNA) can provide a broad description of the bacterial community composition in a habitat (Dahllöf, 2002), and studies of several habitats are available in the literature for comparison (e.g. Langenheder and Prosser, 2008). The functional diversity represented by the denitrifying bacterial community phylogeny may not relate to the taxonomical diversity indicated by the 16S rDNA phylogeny (Song and Ward, 2003). Therefore, investigations of both types

of phylogeny will provide different perspectives and additional insights on the bacterial community composition. Denitrification is the stepwise reduction of nitrate to dinitrogen, in which each step is catalysed by different enzymes encoded by the genes *nar*/*nap*, *nir*, *nor*, and *nos*. In most cases, only one or two of the enzyme genes *nir*K, *nir*S, *nor*B or *nos*Z are investigated when studying the denitrifying bacterial community, because it is assumed that they are all adequately representive of the true community composition (Braker et al., 1998; Hallin and Lindgren, 1999; Braker and Tiedje, 2003; Hannig et al., 2006; Bremer et al., 2007). To date, to our knowledge no study has attempted to ascertain whether any individual enzyme genes can suffice to characterize the overall community composition of denitrifying bacteria. To do so it would be valuable to compare the occurrence of denitrifying bacterial enzyme genes in a large number of similar habitats.

The aim of this study was to examine the variation of eubacterial and denitrifying bacterial community composition among 32 constructed wetlands, and also to determine whether the community compositions were influenced by the environmental parameters of the ecosystems. The bacterial community composition (structure and richness) in wetland biofilm was assessed by PCR-DGGE analysis (Hallin and Lindgren, 1999), targeting 16S rDNA to describe the eubacterial community and the enzyme genes *nir*K, *nir*S, and *nos*Z to describe the denitrifying bacterial community. We determined whether the three denitrifying bacterial enzyme genes were evenly distributed among the wetlands, and if analysis of a single denitrifying bacterial enzyme gene could suffice to characterize the denitrifying bacterial community in agricultural constructed wetlands. Our results showed that the eubacterial and denitrifying bacterial community compositions in the wetlands varied, partly explained by wetland environmental parameters. The three denitrifying bacterial enzyme genes were not related to the same environmental parameters (nor to 16S rDNA), and *nir*K was more diverse than both *nir*S and *nos*Z. Thus, using a single denitrifying bacterial enzyme gene may not be sufficient to characterize the denitrifying bacterial community in constructed agricultural wetlands.

Results

Extraction and PCR amplification of DNA from biofilm samples

DNA was successfully extracted from biofilm samples from the 32 constructed wetlands. PCR amplification using the primer pairs targeting 16S rDNA, *nir*K, *nir*S, and *nos*Z yielded products of expected size from all samples: 550, 480, 450, and 420 bp, respectively.

Variation of eubacterial and denitrifying bacterial communities

The wetlands differed with regard to the DGGE band structure (i.e. presence/absence of band pattern) and band richness (i.e. the number of DGGE bands in a sample) observed for the eubacterial and denitrifying bacterial enzyme genes (Table 1 and Fig. 1). The majority of the wetlands shared two to three DGGE bands at the same migration level in the gels (Fig. 1). Jaccard similarity was lowest for *nir*K (indicating the least similarity in band structure between wetlands) and highest for *nos*Z (Table 1). There was no similarity in band richness between the denitrifying bacterial enzyme genes and the eubacterial community (16S rDNA). The band richness of *nir*K+*nir*S was included as an additional parameter in the analysis.

Table 1. Number of band levels and band pattern variation obtained by DGGE analysis of PCR products from different target genes in bacterial biofilm DNA from 32 constructed wetlands.

Band number	**16S rDNA**	***nir*K**	***nir*S**	***nos*Z**	***nir*K+*nir*S**
Total band levels	49	28	25	21	53
Present in at least 3 wetlands	46	22	19	17	41
Mean± SE per wetland	14 ± 0.5	5 ± 0.4	6 ± 0.3	6 ± 0.4	11 ± 0.4
Range per wetland	6–20	2–10	2–10	2–13	7–16
Variation in band pattern*	2.2	3.5	2.3	1.8	-
Jaccard similarity**	0.23	0.21	0.24	0.27	-

*Total inertia from canonical correspondence analysis (CCA). Increasing values indicate higher between-wetland differentiation of band patterns.

**The index sets the number of band levels shared by a pair of wetlands in relation to the total number of band levels found for the pair. The values given refer to the mean over all pairwise comparisons (n = 630) per primer. The index can range between zero (no bands in common) and one (all bands in common).

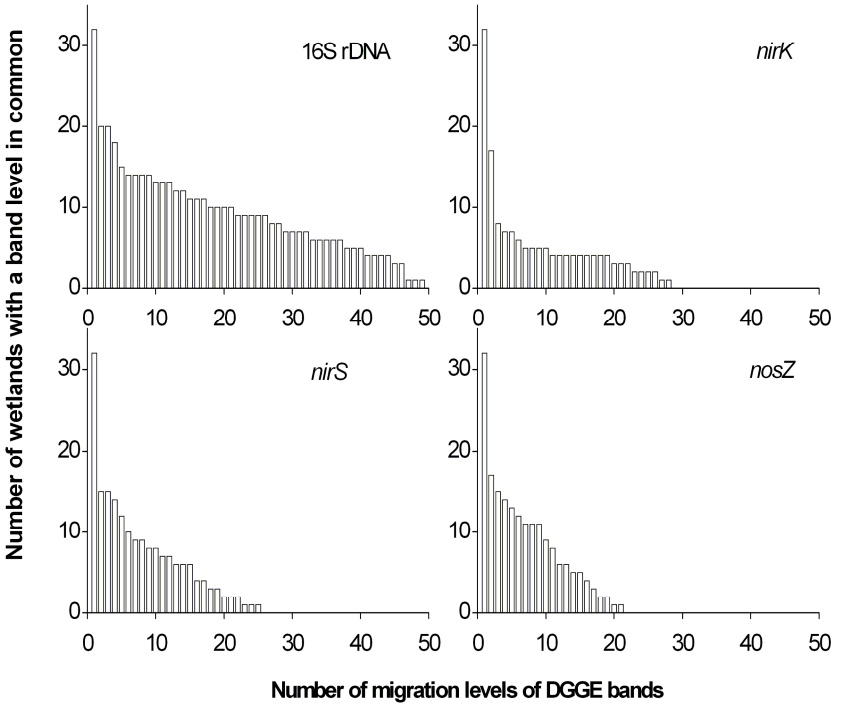

Figure 1. The diagrams illustrate the number of wetlands that shared bands at specific migration levels in DGGE gels used to analyse the eubacterial gene and each of the denitrifying bacterial enzyme genes. Steeper slope indicates greater similarity between the band patterns of the different wetlands.

Band structure of eubacterial and denitrifying bacteria in relation to environmental parameters

Canonical correspondence analysis showed that the band structures of both eubacterial and denitrifying bacterial communities were significantly related to environmental parameters. The eubacterial band structure was explained by parameters describing succession stage, water chemistry, and wetland morphology, whereas the denitrifying community band structure was explained only by water chemistry or wetland morphology parameters (Fig. 2). Four environmental parameters explained a total of 19% of the eubacterial band structure (eigenvalues 0.18 and 0.17): emergent plant richness (F-ratio 1.79, P = 0.004) and submerged plant richness (F-ratio 1.44, P = 0.042) together stood for 10%, nitrate concentration (F-ratio 1.59, P = 0.015) for 5%, and depth (F-ratio 1.40, P = 0.043) for 4%. Total phosphorous and pH explained 6% of the band structure of *nir*S (F-ratio 1.92, P = 0.011, eigenvalues 0.26 and 0.22) and *nir*K (F-ratio 2.05, P = 0.005, eigenvalues 0.31 and 0.28), respectively. Depth

explained 8% of the band structure of *nos*Z (F-ratio 2.49, $P = 0.003$, eigenvalues 0.21 and 0.16).

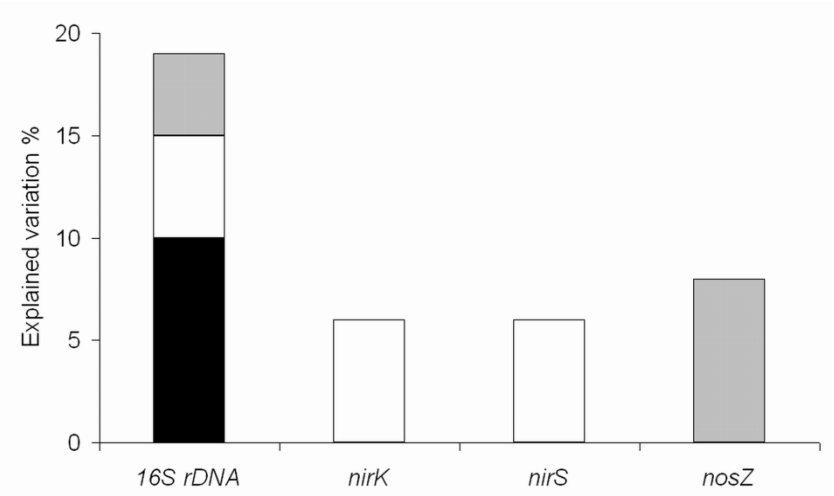

Figure 2. Explanatory power of the relationship between DGGE band structure and environmental parameters shown by canonical correspondence analysis. The environmental parameters considered here belonged to the three groups succession stage (black), water chemistry (white), and wetland morphology (grey).

Band richness of eubacterial and denitrifying bacteria in relation to environmental parameters

The ordination diagram in Figure 3 illustrates the relationship between eubacterial and denitrifying bacterial band richness and environmental parameters (axis eigenvalues 0.29 and 0.14). Three parameters were significantly correlated with the canonical axes and explained 7–12% of the total variation in denitrifying bacterial band richness: nitrate concentration (F-ratio 3.90, $P = 0.004$, 12%), richness of floating-leaved plants (F-ratio 3.45, $P = 0.017$, 9%), and hydraulic loading rate (F-ratio 2.65, $P = 0.028$, 7%). Subsequent regression analyses (Fig. 4) revealed significant linear relationships. Nitrate concentration was positively correlated with the band richness of *nos*Z and negatively correlated with the band richness of *nir*K+*nir*S. Hydraulic loading rate was negatively correlated with band richness of *nir*K+*nir*S, and presence of floating-leaved plants was positively correlated with the band richness of *nir*K. In all cases except *nir*K, the quadratic regression models (unimodal relationships) were also significant. However, the r^2 values for the linear and unimodal relationships differed only marginally, whereas the *P* values suggested relatively higher significance for the linear models in all cases. Eubacterial band richness was not significantly related to any of the tested environmental parameters.

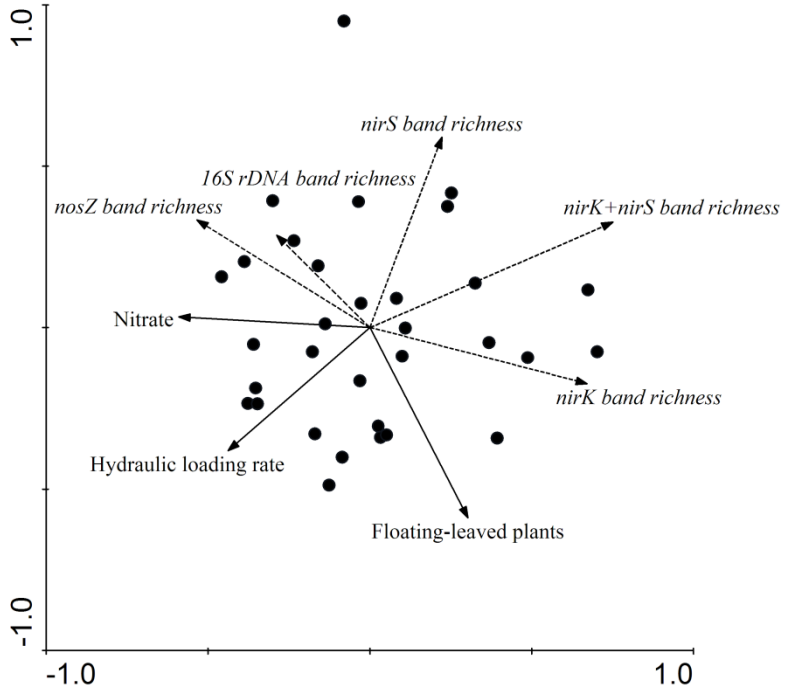

Figure 3. Ordination diagram obtained by redundancy analysis (RDA) of eubacterial and denitrifying bacterial DGGE band richness and environmental parameters. Symbols: closed circles represent wetlands; proximity between circles indicates similarity in band richness; dotted arrows denote the direction of increasing band richness; continuous arrows depict the direction of environmental parameters that were significantly related to the canonical axes (i.e. that explained variation in band richness).

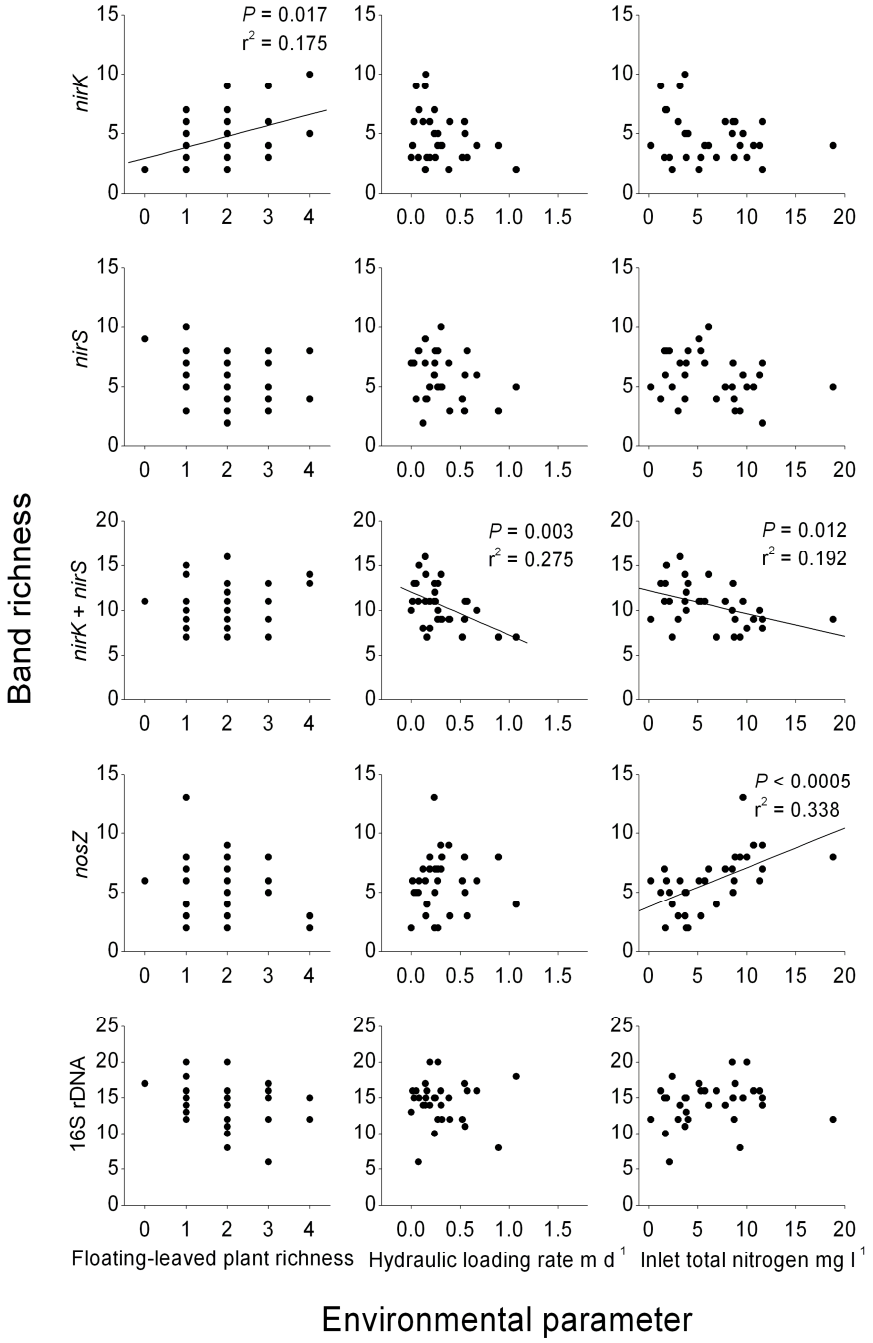

Figure 4. Linear regressions between three environmental parameters and DGGE band richness of eubacterial (16S rDNA) and denitrifying bacterial enzyme genes (*nir*K, *nir*S, *nir*K+*nir*S, and *nos*Z). Observe that values for hydraulic loading rates are square-root transformed. The lines indicate a significant linear relationship.

Discussion

We found that the composition (structure and richness) of eubacterial and denitrifying bacterial communities varied between the investigated wetlands and was partly explained by environmental parameters (Fig. 2 and 4), which implies that environmental parameters may shape bacterial community composition (Martiny et al., 2006). Furthermore, the observed compositional variation between our wetland sites, which represent equivalent habitats, is supported by previous findings regarding rock pools (Langenheder and Ragnarsson, 2007) and terrestrial soils (Throbäck et al., 2004). Similarly, studies of single aquatic systems have shown that temporal differences in the composition of denitrifying bacterial communities are related to changes in vegetation, nutrients, or season (Priemé et al., 2002; Angeloni et al., 2006; Kjellin et al., 2007; Sundberg et al., 2007). Aquatic environments are heterogeneous in nature (Simon et al., 2002; Franklin et al., 2002), and therefore, to reduce variation in analyses of bacterial community composition that can be caused by the sampling strategy, we used pooled samples from field-deployed polyethylene strips to obtain wetland biofilms (Araya et al., 2003). Accordingly, it can be assumed that the relative diversity of the bacterial communities in our study was probably not affected by the sampling strategy, and hence we argue that it was possible to compare this aspect of the investigated wetlands.

Specific environmental parameters of the wetlands, primarily those in the group succession stage, explained 19% of the total variation in the eubacterial community structure (Fig. 2). Eubacterial community structure may be influenced by parameters belonging to all three of the groups succession stage (Langenheder and Prosser, 2008), water chemistry (Hewson et al., 2003), and wetland morphology (Hewson et al., 2007), and similar magnitudes of influence have been reported in other aquatic habitats (Beisner et al., 2006; Langenheder and Ragnarsson, 2007). Langenheder and Ragnarsson (2007) noted that 14% of the eubacterial community composition in their study could be explained by environmental parameters and argued that this low explanation fraction may have been due to their failure to include all relevant parameters. However, we included a higher number of environmental parameters (15 compared to 8 considered by Langenheder and Ragnarsson, 2007) in our investigation, but the explanation fraction was not appreciably larger. Similarly low fractions have also been found when considering the impact of environmental factors on communities at higher trophic levels, such as fish in lakes (Beisner et al., 2006) and macroinvertebrates in constructed wetlands (G. Thiere et al., in press). Other parameters, describing food web interactions, for example predation or effects of fluctuations in the phytoplankton community, are also likely to have an impact on bacterial community composition (Kent et al., 2004). In addition, stochastic effects should be taken into consideration when analysing constructed wetlands (Kadlec, 1997). Such shallow aquatic systems have high hydraulic loading rates and short water retention times, and thus bacterial communities in those habitats can easily be

affected and altered by temporal changes in the environment, and they may rarely be in a stable state due to constant adaptations to environmental turbulence. Consequently, it may not be possible to achieve explanation fractions that are higher than those found in our study or other investigations simply by analysing more environmental parameters. Given that a large variation in community composition remain unexplained, possibly due to a lack of temporal (and spatial) resolution in field studies, the information added by the present results and the findings of other similar studies suggest that environmental parameters play a significant role in shaping the composition of bacterial communities (Langenheder and Ragnarsson, 2007).

The DGGE band structure and richness of the denitrifying bacterial enzyme genes were not related to the same environmental parameters (Fig. 2 and 4), nor to the band richness of the eubacterial 16S rDNA community. Band structure and richness indicated greater diversity of the denitrifying bacterial enzyme gene *nir*K compared to both *nir*S and *nos*Z (Table 1). This was indicated by the observation that, compared to both *nir*S and *nos*Z, *nir*K had a larger number of detected band levels in the DGGE gels (Table 1). The finding that diversity was greatest for *nir*K and lowest for *nos*Z is supported by a general relationship of lower diversity for *nos*Z compared to both *nir*K and *nir*S (Wallenstein et al., 2006). Communities of *nir*K have previously been shown to be more diverse than the *nir*S and *nos*Z counterparts in soils (Throbäck et al., 2004), whereas greater diversity has been noted for *nir*S than for *nir*K in marsh soil (Priemé et al., 2002) and marine sediments (Braker et al., 2000).

In our study, specific environmental parameters explained 6–8% of the variation in band structure (Fig. 2) and 7–12% of the differences in band richness (Figs. 3 and 4) of the denitrifying bacterial communities. All three of the parameter groups succession stage, water chemistry, and wetland morphology affected richness, whereas only the latter two influenced structure. The water chemistry parameters accounted for the largest impact on the total denitrifying bacterial community composition. Band structure and richness were not correlated with the same environmental parameters, except for nitrate concentration. The correlation between band richness and nitrate concentration was positive for *nos*Z but negative for *nir*K+*nir*S, indicating opposite effects on the richness of bacterial species encoding for the denitrifying bacterial enzyme genes. Very few comparable studies have examined several denitrifying bacterial enzyme genes, and only limited number of investigations have found differences in relationships between environmental parameters and community composition of single denitrifying bacterial enzyme genes (Bremer et al., 2007; Kjellin et al., 2007). Other studies have not separated the effects of individual environmental parameters on individual denitrifying bacterial enzyme genes (Cavigelli and Robertson, 2000; Hannig et al., 2006), and thus the results are not comparable. Differences of enzyme gene diversity among previous investigations may be partly owing to the use of different molecular techniques. It has also been

reported that variation in bacterial community composition can arise due to selection of primer pairs that do not offer suitable specificity for the studied habitat (Angeloni et al., 2006). Our molecular analyses produced satisfactory data for each of the enzyme genes, thus we suggest that use of single genes will not suffice to characterize the overall denitrifying bacterial community in constructed agricultural wetlands.

Interestingly, ecosystem functioning in semi-permanent rain pools has been shown to increase with increasing bacterial species richness, and, to a lesser degree, be affected by bacterial structure (Bell et al., 2005). There are two mechanisms that may explain the impact of biodiversity on ecosystem functioning: the 'complementary mechanism', which means that different species use slightly different resources, and the 'selection mechanism', implying that certain bacterial species play a more important role (Loreau and Hector, 2001). Since our results indicate that environmental parameters are partly responsible for the variation between wetlands with regard to both the structure and the richness of denitrifying bacterial communities, it may be possible to use that knowledge to develop testable hypotheses in future studies. More specifically, different structure and richness of the denitrifying bacterial enzyme genes may have different effects on the three separately occurring reactions of the denitrification process (Zumft, 1997) and thereby influence the rate of denitrification. Denitrification constitutes the main route of nitrogen removal in agricultural constructed wetlands because the incoming nitrogen is predominately in the form of nitrate (Bachand and Horne, 2000), and thus denitrification does not depend on nitrification (Seitzinger et al., 2006). If a skewed community composition of the enzyme genes (*nir* compared to *nos*) limits one of the denitrification reactions, it may result in a bottleneck in the denitrification process. It was recently suggested that expression of the genes that encode the enzymes that catalyse denitrification may occur at different rates in the various species of denitrifying bacteria (Kjellin et al., 2007; Bulow et al., 2008; Henry et al., 2008). Hence it is possible that the diversity of denitrifying bacteria is linked to wetland function because a high denitrification rate is dependent on specific denitrifying bacterial species for each enzyme gene (*nar*/*nap*, *nir*, *nor* and *nos*). Thus certain compositions of denitrifying bacterial phylotypes may be superior to others in catalysing denitrification, which would support increased activity of constructed wetlands.

In conclusion, we found that the structure and richness of the eubacterial and denitrifying bacterial communities varied among the investigated wetlands and could partly be explained by environmental parameters of the wetlands. The eubacterial and denitrifying community compositions were all related to different wetland environmental parameters, although the explanation fractions were low and thus there may be additional drivers involved. We recommend that care be taken when choosing denitrifying bacterial enzyme gene(s) in future studies, since our results suggest that using a single enzyme gene may not be sufficient to

characterize denitrifying bacterial community composition in constructed agricultural wetlands. More knowledge about the denitrifying bacterial community is needed before any causal relationships with the denitrification rate in constructed wetlands can be tested. For instance, the phylogenetic diversity of the denitrifying bacterial community and the relative contribution of different phylotypes to the denitrification rate should be determined. A promising approach to resolving the latter issue is the quantification of gene expression by different phylotypes (Bulow et al., 2008). Further research is also needed to elucidate the influence of physical-chemical ecosystem variability on community composition and continuous denitrification rates in constructed wetlands.

Experimental Procedures

Wetland ecosystems

Thirty-two wetlands that had been constructed for the purpose of nutrient (nitrogen and phosphorous) retention in southwestern Sweden were surveyed from March to October 2004. The wetlands represented nutrient-rich, flow-through freshwater systems with a permanent water body, and both the wetlands and their catchments were located in an agriculturally dominated lowland area within 15 km of the water shore (57º02'N, 12º23'E to 55º59'N, 13º06'E). Nutrient concentrations in the incoming water ranged from 3.3 to 18.9 mg l^{-1} for total nitrogen (2.5 to 18.8 mg l^{-1} nitrate) and from 5 to 318 µg l^{-1} for total phosphorous. The wetlands were between one and six years old, and they ranged in size from 0.1 to 2.1 ha (mean area 0.4 ha) and had average water depths of 0.2 to 3.9 m (mean 1.0 m). The environmental parameters investigated were divided into three groups designated as follows (Table 2): succession stage (wetland age, wetland plants, richness of submerged, floating-leaved and emergent plants), water chemistry (total nitrogen, nitrate, total phosphorous, phosphate, N:P ratio, organic suspended solids, pH, conductivity, and oxygen saturation), and wetland morphology (wetland area, depth and hydraulic loading rate). These parameters were measured on three separate occasions from March to September 2004 (publication of sampling procedures and detailed values in; G. Thiere et al., in press).

Table 2. Environmental parameters used to characterize the succession stage, water chemistry, and morphology of the constructed wetlands.

	Environmental parameter	Unit	Description
Succesion stage			
1	Wetland plants	species	As defined by Biggs et al. (1998)
2	Marginal plants	species	Emergent and herbaceous wetland plants
3	Submersed plants	species	Plants with all parts submerged below the water surface
4	Floating-leaved plants	species	Plants (free or rooted) with leaves floating on the surface
5	Plant cover	scale	0, up to 5% cover; 1, up to 33% cover; 2, up to 67% cover; 3, up to 100% cover
6	Wetland age	months	Time between construction and investigation (colonization time)
7	16S rDNA	bands	As detected in the present study; treated as a parameter only in relation to denitrifying genes
Water chemistry			
8	Total nitrogen	g m^{-3}	Mean inlet concentration
9	Nitrate	g m^{-3}	Mean inlet concentration
10	Total phosphorous	g m^{-3}	Mean inlet concentration
11	N:P ratio		Mean inlet mass ratio (total N: total P)
12	Nitrate load	g m^{-2} d^{-1}	Nitrate concentration times daily flow per wetland area
13	Organic suspended solids	g m^{-3}	Mean inlet concentration
14	Oxygen saturation	%	
15	Conductivity	µS cm^{-1}	
16	pH		
Wetland morphology			
17	Wetland area	m^2	Water body size
18	Depth	m	Average water depth
19	Hyraulic loading rate	m d^{-1}	Discharge per wetland area

Experimental setup and biofilm sampling strategy

We sampled and analysed biofilm from each wetland to compare the systems with regard to the community composition of both eubacteria and denitrifying bacteria. Pre-washed polyethylene strips (3.2 m long, 15 mm wide, 0.1 mm thick) were used to cover both the wetland sediment surface and the water column in sites along the main flow channel between the inlet and outlet of each system. The horizontal strip (1.6 m) was prepared with weights in each end to keep it

integrated in the upper part of the sediment. The vertical strip (1.6 m) continued from one of the horizontal ends and was kept afloat at the water surface by use of a cork. Five strips were placed at regular intervals between the inlet and outlet of each wetland. The strips were left *in situ* for four weeks (September to October 2004) to allow biofilm development. Thereafter, they were collected in pre-washed polyethylene bags and kept on ice (for 8 h) during transport to the laboratory. Upon arrival at that facility, the strips were immediately placed in a refrigerator (4°C) and were kept there overnight. To obtain samples of biofilm for analysis, a mean total area of 600 cm^2 was scraped off from the polyethylene strips with a pre-washed razor blade. The samples from each wetland were pooled, transferred to sterile Eppendorf vials, and centrifuged (1500 rpm for 2 min), after which excess water was discarded. The vials were then stored at –20°C pending DNA extraction.

DNA extraction

Total DNA from the biofilm was extracted using a FastDNA® Spin Kit for Soil (Bio 101 Inc., La Jolla, CA, USA). Approximately 0.65 g (wet weight) of biofilm sample was diluted with 2 x 978 µl of sodium phosphate buffer (SPB; buffer supplemented in the kit). The mixture was homogenized with a blender (DIAX 900 Homogeniser Tool G6, Heidolph, Kelheim, Germany) for 5 min while kept on ice. A 500-µl aliquot of the homogenized suspension was diluted with 478 µl of SPB and thereafter the manufacturer's protocol was followed. The 50-µl DNA extracts were stored at –20°C until subjected to PCR analysis.

PCR

The primer pairs selected for *nirK* and *nirS* were FlaCu:R3Cu and cd3aF:R3cd, respectively (Throbäck et al., 2004). The primer pair *nos*ZF:*nos*Z1622R used for *nos*Z was modified from Throbäck et al. (2004) to *nos*Z11F: 5´- CGY TGT TCM TCG ACA GCC AG - 3´ and *nos*Z611R: 5´- CGS ACC TTS TTG CCS TYG CG - 3´. A 33-bp GC-clamp, 5´ - GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC - 3´, was attached to the 5´ end of each of the primers R3cd, R3cu and nosZ1611R. All primers were purchased from Invitrogen (Stockholm, Sweden). PCR amplification of the *nir*S and *nir*K primer pairs, respectively, was performed according to Throbäck et al. (2004). The PCR mixture for *nos*Z analysis (total volume 50 µl) contained 1.33 U Taq polymerase, 5 µl of 10 x buffer (including 1.5 mM $MgCl_2$; Roche Diagnostic GmbH, Mannheim, Germany), 200 µM dNTPs, each primer at 0.125 µM, 600 ng μl^{1} BSA, and 2 µl of template DNA. The PCR amplification for *nosZ* was done as follows: an initial denaturing step for 15 min at 95°C; then 10 cycles of 30 s at 94°C, 1 min at 66°C (decreased 1°C per cycle) and 1 min at 72°C; thereafter 30 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C; finally, a primer extension step of 10 min at 72°C. All PCR reactions were performed on a PTC-100™ thermal cycler (MJ

Research Inc., San Francisco, CA, USA), and agarose gel electrophoresis analysis was subsequently conducted to confirm the size of the products.

To analyse the total bacterial community, we used the primer pair GM5F-GC and DS907R, which targets the 16S rDNA (Teske et al., 1996). The 50 µl of PCR mixture consisted of 1.33 U Taq polymerase, 5 µl of 10 x buffer (including 1.5 mM $MgCl_2$; Roche Diagnostic GmbH, Mannheim, Germany), 200 µM dNTPs, each primer at 0.25 µM, and 5 µl of template DNA. The PCR amplification was done as follows: an initial denaturing step for 2 min at 94°C; then 9 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C; thereafter 19 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C (increased 5 s each cycle); finally, a primer extension step of 7 min at 72°C. The products were run on an agarose gels to confirm the size of the products before DGGE analysis.

DGGE

The polyacrylamide gels (160 x 160 x 1 mm) used were composed of 37.5:1 acrylamide:bisacrylamide (6.5% for *nir* and *nos*, and 10% for 16S rDNA) and 1 x TAE **(**40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA; pH 7.4). 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide (Muyzer et al., 1993). A gradient maker (GM-40, C.B.S. Scientific Company Inc., Del Mar, CA, USA) was employed to prepare the different denaturing gradients: 50–80% for *nir*K, 60–80% for *nir*S, 40–80% for *nos*Z, and 25–65% for 16S rDNA. To polymerize the gel, 6.9 µl of 100% TEMED and 110.5 µl of 10% ammonium persulphate were added to 25.6 ml of the gel mix. PCR product (15 µl) was loaded on the gel and run in 1 x TAE buffer at 62°C and 130 V. The DGGE (Bio-Rad Laboratories Inc., Hercules, CA, USA) was always run for 13.5 h. The bands were visualized by staining for 1 h with SYBR Gold (Roche Diagnostic GmbH, Mannheim Germany) diluted 3000-fold in 1 x TAE buffer.

Analysis of DGGE banding patterns

The DGGE banding patterns were analysed using the software Quantity One® (Bio-Rad Laboratories Inc., Hercules, CA, USA), which has been used by other investigators for similar analyses (Martinez et al., 2001; Throbäck et al., 2004). This software allowed us to detect discrete bands on the DGGE gels. The band migration level on each gel was normalized with PCR products after amplification of a reference strain (*Escherichia coli* ATCC25922). Thereafter, the migration levels of DGGE bands from the eubacterial (16S rDNA) and the denitrifying bacterial (*nir*K, *nir*S and *nos*Z) communities were normalized, respectively. To relate the bacterial communities to environmental parameters, and to be able to compare the denitrifying bacterial enzyme genes, we chose to study the band structure and band richness of the bacteria. Band structure refers to the pattern of bands in a gel and was measured by creating similarity matrices that illustrated the presence/absence of DGGE bands at all defined migration

levels. Richness was measured by counting the number of DGGE bands for each wetland and gene. The statistical analyses were applied separately to the band structure and band richness findings for all the different genes. We also chose to analyse the pooled band richness results for *nir*K and *nir*S (*nir*K+*nir*S), which we assumed would represent the major part of the nitrite reduction step in the denitrification process.

Statistical analysis

We performed four different types of statistical analyses to assess (i) the composition of bacterial communities, (ii) the variation in environmental parameters, (iii) band structure in relation to environmental parameters, and (iv) band richness in relation to environmental parameters.

i. The distribution frequency of band levels was used to compare the commonness and uniqueness of genes. To measure the similarity of band patterns among wetlands, we calculated Jaccard similarity (Jaccard, 1912) for all pairwise combinations of wetlands and compared the means per gene.
ii. Environmental parameters were log(x+1)-transformed, and Z-scores were calculated (Langenheder and Ragnarsson, 2007) before further assessment. Principal component analysis (PCA) was applied to show ordination of environmental parameters (Lepš and Šmilauer, 2003). If the two parameters in a pair were highly co-linear (inflation factor > 10), one of them was excluded from analysis. The following four environmental parameters were excluded because of detected co-linearity: number of total wetland plants (co-linear with number of submerged, floating-leaved, and emergent plants), total nitrogen and N:P ratio (both co-linear with nitrate concentration), and nitrate load (co-linear with hydraulic loading rate).
iii. Band structure was set in relation to environmental parameters by applying canonical correspondence analysis (CCA) (Lepš and Šmilauer, 2003), which was restricted to band levels that were encountered in at least three wetlands in order to serve the unimodal model assumptions of CCA. Two subsequent steps (detection and final model) were carried out for structural analysis of the eubacterial and denitrifying bacterial genes, respectively. In the first step, we detected the wetland parameters in the three groups considered (succession, water chemistry, and morphology) that were significantly related to band structure; all other environmental parameters were excluded. In the second step, the significant parameters from the three groups were entered simultaneously in the final CCA model. All CCAs were computed using CANOCO 4.5 (ter Braak and Šmilauer, 2002) and based on interspecies distances and biplot scaling with 999 Monte Carlo permutation tests done under the reduced model.
iv. Redundancy analysis (RDA; Lepš and Šmilauer, 2003) was applied to assess the relationship between band richness and environmental

parameters. RDA was computed using CANOCO 4.5 (ter Braak and Šmilauer, 2002) and based on centered and standardized ecosystem parameters (reduced model with 999 Monte Carlo permutation tests). The environmental parameters that were significant in the RDA analysis were tested for the type of relationship (linear or unimodal) with band richness by linear and quadratic regressions.

For both multivariate statistics (iii, iv), the eigenvalues of the first two canonical axes are given as a measurement of their explanatory power (Lepš and Šmilauer, 2003).

Acknowledgements

This study was partly funded by FORMAS, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (grant nos. 25/2-2001-2677, 214-2004-1678, 21.5/2003-1111, and 215/2005-507), and the Crafoord Foundation (grant no. 20050957). The authors thank Nina Syde for assistance in the field and Kristina Samuelsson and Carina Sundberg for support with molecular analysis.

References

Angeloni, N.L., Jankowski, K-J., Tuchman, N.C. and Kelly, J.J. (2006) Effects of an invasive cattail species (Typha x glauca) on sediment nitrogen and microbial community composition in a freshwater wetland. *FEMS Microbiology Letters* **263**: 86-92.

Araya, R., Tani, K., Takagi, T., Yamaguchi, N. and Nasu, M. (2003) Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent in situ hybridization and DGGE analysis. *FEMS Microbiology Ecology* **43**: 111-119.

Bachand, P.A.M., and Horne, A.J. (2000) Denitrification in constructed free-water surface wetlands: II. Very high nitrate removal rates in a macrocosm study. *Ecological Engineering* **14**: 17-32.

Braker, G., Fesefeldt, A. and Witzel, K-P. (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology* **64**: 3769-3775.

Braker, G., Zhou, J., Wu, L., Devol, A.H. and Tiedje, J.M. (2000) Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. *Applied and Environmental Microbiology* **66**: 2096-2104.

Braker, G. and Tiedje, J.M. (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Applied and Environmental Microbiology* **69**: 3476-3483.

Bell, T., Newman, J.A., Silverman, B.W., Turner, S.L. and Lilley, A.K. (2005) The contribution of species richness and composition to bacterial services. *Nature* **436**: 1157-1160.

Beisner, B.E., Peres-Neto, P.R., Lindström, E.S., Barnett, A., and Lorena Longhi, M. (2006) The role of environmental and spatial processes in structuring lake communities from bacteria to fish. *Ecology* **87**: 2985-2991.

Biggs, J., Fox, G., Nicolet, P., Walker, D., Whitfield, M. and Williams, P. (1998) A Guide to the Methods of the National Pond. Pond Action. Oxford.

Bremer, C., Braker, G., Matthies, D., Reuter, A. Engels, C. and Conrad, R. (2007) Impact of plant functional group, plant species, and sampling time on the composition of nirK-type denitrifier communities in soil. *Applied and Environmental Microbiology* **73**: 6876-6884.

Bulow, S.E., Francis, C.A., Jackson, G.A. and Ward, B.B. (2008) Sediment denitrifier community composition and *nir*S gene expression investigated with functional gene microarrays. *Environmental Microbiology* **10**: 3057-3069.

Cavigelli, M.A. and Robertson, G.P. (2000) The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology* **81**: 1402-1414.

Dahllöf, I. (2002) Molecular community analysis of microbial diversity. *Current Opinion in Biotechnology* **13**: 213-217.

Fleischer, S., Gustafson, A., Joelsson A., Pansar, J. & Stibe, L. (1994) Nitrogen removal in created ponds. *Ambio* **23**: 349-357.

Franklin, R.B., Blum, L.K., McComb, A.C. and Mills, A.L. (2002) A geostatistical analysis of small-scale variability in bacterial abundance and community structure in salt marsh creek bank sediments. *FEMS Microbiology Ecology* **42**: 71-80.

Hallin, S. and Lindgren P-E. (1999) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Applied and Environmental Microbiology* **65**: 1652-1657.

Hannig, M., Braker, G., Dippner, J. and Jürgens, K. (2006) Linking denitri¢er community structure and prevalent biogeochemical parameters in the pelagial of the central Baltic Proper (Baltic Sea). *FEMS Microbiology Ecology* **57**: 260-271.

Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Chèneby, D., et al. (2008) Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight inte the role of root exudates. *Environmental Microbiology* **10**: 3082-3092.

Hewson, I., Vargo, G.A. and Fuhrman, J.A. (2003) Bacterial diversity in shallow oligotrophic marine benthos and overlying waters: Effects of virus infection, containment, and nutrient enrichment. *Microbial Ecology* **46**: 322-336.

Hewson, I., Jacobson/Meyers, M.E. and Fuhrman, J.A. (2007) Diversity and biogeography of bacterial assemblages in surface sediments across the San

Pedro basin, Southern California borderlands. *Environmental Microbiology* **9**: 923-933.

Horner-Devine, C.M., Leibold, M.A., Smith, V.H. and Bohannan, B.J.M. (2003) Bacterial diversity patterns along a gradient of primary productivity. *Ecology Letters* **6**: 613-622.

Jaccard, P. (1912) The distribution of the flora in the alpine zone. *New Phytologist* **11**: 37–50.

Kadlec, R.H. (1997) Deterministic and stochastic aspects of constructed wetland performance and design. *Water Science and Technology* **35**: 149-156.

Kent, A.D., Jones, S.E., Yannarell, A.C., Graham, J.M., Lauster, G.H., Kratz, T.K., et al. (2004) Annual patterns in bacterioplankton community variability in a humic lake. *Microbial Ecology* **48**: 550-560.

Kjellin, J., Hallin, S. and Wörman, A. (2007) Spatial variations in denitrification activity in wetland sediments explained by hydrology and denitrifying community structure. *Water Research* **41**: 4710-4720.

Langenheder, S. and Prosser, J.I. (2008) Resource availability influences the diversity of a functional group of heterotrophic soil bacteria. *Environmental Microbiology* doi:10.1111/j.1462-2920.2008.01647.x.

Langenheder, S. and Ragnarsson, H. (2007) The role of environmental and spatial factors for the composition of aquatic bacterial communities. *Ecology* **88**: 2154-2161.

Lepš, J., and Šmilauer, P. (2003) Multivariate analysis of ecological data using canoco. Cambridge. Cambridge University Press. United Kingdom.

Lin, Y-F., Jing, S-R., Wang, T-W., and Lee, D-Y. (2002) Effects of macrophytes and external carbon sources on nitrate removal from groundwater in constructed wetlands. *Environmental Pollution* **119**: 413–420.

Loreau, M. and Hector, A. (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature* **412**: 72-76.

Lovley, D. (2003) Cleaning up with genomics: applying molecular biology to bioremediation. *Nature Reviews* **1**: 35-44.

Martinez, J., Perez-Serrano, J., Bernadina, W.E. and Rodriguez-Caabeiro, F. (2001) Stress response to cold in *Trichinella* species. *Cryobiology* **43**: 293-302.

Martiny, J.B.H., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., et al. (2006) Microbial biogeography: putting microorganisms on the map. *Nature Reviews* **4**:102-112.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.

Philippot, L. and Hallin, S. (2005) Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. *Current Opinion in Microbiology* **8**: 234-239.

Priemé, A., Braker, G. and Tiedje, J.M. (2002) Diversity of nitrite reductase (*nir*K and *nir*S) gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology* **68**: 1893-1900.

Reche, I., Pulido-Villena, E., Morales-Baquero, R. and Casamayor, E.O. (2005) Does ecosystem size determine aquatic bacterial richness? *Ecology* **86**: 1715-1722.

Rich, J.J. and Myrold, D.D. (2004) Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biology and Biochemistry* **36**: 1431-1441.

Seitzinger, S., Harrison, J.A., Böhlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., et al. (2006) Denitrification across landscapes and waterscapes: a synthesis. *Ecological Applications* **16**: 2064-2090.

Shade, A., Jones, S.E. and McMahon, K. (2008) The influence of habitat heterogeneity on freshwater bacterial community composition and dynamics. *Environmental Microbiology* doi:10.1111/j.1462-2920.2007.01527.x.

Simon, M., Grossart, H-P., Schweitzer, B. and Ploug, H. (2002) Microbial ecology of organic aggregates in aquatic ecosystems. *Aquatic Microbial Ecology* **28**: 175-211.

Song, B. and Ward, B.B. (2003) Nitrite reductase genes in halobenzoate degrading denitrifying bacteria. *FEMS Microbiology Ecology* **43**: 349-357.

Sundberg, C., Tonderski, K. and Lindgren, P-E. (2007) Potential nitrification and denitrification and the corresponding composition of the bacterial communities in a compact constructed wetland treating landfill leachates. *Water Science and Technology* **56**: 159-166.

ter Braak, C. J. F. and Šmilauer, P. (2002) CANOCO 4.5. Biometrics. Wageningen University and Research Centre, Wageningen, The Netherlands.

Teske, A., Sigalevich, P., Cohen, Y. and Muyzer, G. (1996) Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology* **62**: 4210-4215.

Thiere, G., Milenkovski, S., Lindgren, P-E., Sahlén, G., Berglund, O. and Weisner S.E.B. (2009) Wetland creation in agricultural landscapes: biodiversity benefits on local and regional scales. *Biological Conservation*. In press.

Throbäck I.N., Enwall, K., Jarvis, Å. and Hallin, S. (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology* **49**: 401-417.

Wallenstein, M.D., Myrold, D.D., Firestone, M. and Voytek, M. (2006) Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications* **16**: 2143-2152.

Weisner, S.E.B., Eriksson, P.G., Granéli, W. and Leonardsson, L. (1994) Influence of macrophytes on nitrate removal in wetlands. *Ambio* **23**: 363-366.

Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61**: 533-616.

Paper II

Composition of denitrifying bacterial enzyme genes *nir*S, *nir*K and *nos*Z in constructed wetlands

Milenkovski, S[1]., Berglund, O[1]., Thiere, G[3]., Samuelsson, K[2]., Weisner, S[3]. and Lindgren, P-E[4].

[1]Department of Chemical Ecology/Ecotoxicology, Ecology building, Lund University, SE-223 62 Lund, SWEDEN
[2]Swedish County Administrative Board, Länsstyrelsen Kalmar län, SE-391 86 Kalmar, SWEDEN
[3]Wetland Research Centre, Halmstad University, Box 823, SE-301 18 Halmstad, SWEDEN
[4]Department of Clinical and Experimental Medicine, Division of Medical Microbiology, Linköping University, SE-581 85 Linköping, SWEDEN

Keywords: denitrification, phylogenetic tree, PCR-DGGE, sequencing.

Abstract

In this study the composition of the denitrifying bacterial community among constructed wetlands in agricultural areas was investigated. Thirty-two constructed wetlands located in Southern Sweden were surveyed, and biofilm samples from each were analyzed by applying denaturing gradient gel electrophoresis, to investigate the community composition of the three denitrifying bacterial enzyme genes *nir*K, *nir*S and *nos*Z. The DNA sequences of the enzyme genes were compared to known DNA sequences in GeneBank using BLAST. The results of the denitrifying bacterial enzyme genes indicated that these habitats may harbour a heterogeneous denitrifying bacterial community. Individual analysis of the enzyme genes revealed that *nir*S was more heterogeneous than both *nir*K and *nos*Z. Most sequences from the present study clustered with known sequences from species belonging to the group of α-*Proteobacteria*, and to a lesser extent with β- *Proteobacteria* and γ-*Proteobacteria*, and only *nir*S clustered with a member of gram-positive bacteria.

Introduction

Denitrification is the stepwise reduction of nitrate (NO_3^-) to dinitrogen gas (N_2), which leads to a reduction of the more bioavailable NO_3^- in the environment. Increased leaking of NO_3^- from agricultural practices increases the risk of eutrophication, and concomitant oxygen depletion in freshwater systems and oceans. One action taken to prevent the transport of bioavailable nitrogen (N) from agricultural practices has been to restore or recreate wetlands (Biggs et al., 2005, Stadmark and Leonardson, 2005). Denitrification is the limiting process in wetlands constructed on agricultural land as the majority of the N enters as NO_3^- (Bachand and Horne, 2000; Thiere et al., in press). The efficiency with which each wetland reduces N-transport varies and has previously been associated with several biotic and abiotic parameters (Fleischer et al., 1994; Weisner et al., 1994; Lin et al., 2002). However, the denitrifying bacterial community, which is the key player in denitrification, has only recently started to be examined in, for example in marine sediments (Braker et al., 2000) and in soil systems (Throbäck et al., 2004). If and how the community composition of denitrifying bacteria may affect its function (i.e. denitrification) is not established. Thus, to understand the role of denitrifying bacterial communities for N-removal in constructed wetlands, firstly their composition has to be characterised, before the relationship with denitrification may be understood (Braker et al., 2000; Braker and Tiedje, 2003; Wallenstein et al., 2006).

The ability to denitrify is a widespread functional trait that can be carried out by organisms from different taxa. Denitrification is a series of reductions beginning with nitrate (NO_3^-) catalysed by nitrate reductase (encoded by *nar* and *nap*), to nitrite (NO_2^-), which is then catalysed by nitrite reductase (encoded by *nir*) nitric oxide (NO). Nitric reductase (encoded by *nor* and *qnor*) catalyses the reduction of NO to nitrous oxide (N_2O), and the final step is when nitrous reductase (encoded by *nos*) catalyses the reduction of N_2O to dinitrogen gas (N_2). The denitrifying bacterial community is distinguished from other denitrifiers by the capacity to catalyse the reduction of nitrite to nitric oxide (Hallin and Lindgren, 1999). One method for analysis of the bacterial community composition is PCR-DGGE, for both the 16S rRNA gene and functional gene analyses (Hallin et al., 2005; Ibekwe et al., 2006; Langenheder and Prosser, 2008). Because the ability to denitrify is sporadically distributed among different bacterial phyla, which is not congruent with the general 16S rRNA gene community (Song and Ward, 2003) the use of functional genes is a more appropriate approach than using the 16S rRNA gene, when investigating the denitrifying bacteria. Commonly, only one or two of the enzyme genes *nir*K, *nir*S, *nor*B or *nos*Z are investigated when studying the denitrifying bacterial community and assumed to represent the community composition (Braker et al., 1998; Hallin and Lindgren, 1999; Braker and Tiedje, 2003; Hannig et al., 2006; Bremer et al., 2007). However, it has been shown that simultaneous analyses of several denitrifying bacterial enzyme genes

may result in as better or more complete characterization of the community composition, than analysis of a single individual gene (S. Milenkovski et al., unpublished)

Trait-based approaches using functional genes to characterise microbial community composition offer the promise of advancing ecological theory and predicting responses to environmental change (Green et al., 2008). However, as of yet, knowledge regarding how diversity of a bacterial community may affect the ecosystem efficiency in a changing environment is limited. A long-lived theory is that an increased biodiversity leads to a more stable ecosystem functioning (McNaughton, 1977). Functional properties of a community may be overlapped by different species (i.e. functional redundancy), and the ecosystem function will have a higher probability of remaining stable. Studies have shown that increased richness of bacterial species in semi-permanent rainpools increase ecosystem functioning (Bell et al., 2005), and a diverse soil bacterial community (16S rRNA gene community) may maintain functional activity better than a less diverse, after toxicant exposure (Girvan et al., 2005). Thus, a more diverse and heterogeneous bacterial community may have a higher probabilty to maintain its function when being disturbed, for example by agricultural pesticides.

The main part of the denitrifying bacterial community belongs to the subclasses of *Proteobacteria*, mainly to the three groups, α, β, and γ (Heylen et al., 2007), but they may also belong to gram-positive bacteria, *Bacteroides*, *Firmicutes* and *Actinobacteria*. A more heterogeneous denitrifying bacterial community may have bacterial species belonging to several or all above mentioned groups, while a less heterogeneous community may have bacterial species belonging only to a single group (Braker et al., 2000). Soil systems in arable fields have earlier been shown to harbour diverse communities of *nir*K, *nir*S and *nos*Z (Throbäck et al., 2004). Thus, since agricultural wetlands are constructed onto similar arable soil fields, the environmental conditions should be in favour for these constructed wetlands harbouring a diverse denitrifying bacterial community.

Consequently, before the relationship between structure and function of the denitrifying bacterial community can be understood, fundamental knowledge of their composition has to be investigated. The aim of present study was to characterise the composition of the denitrifying bacterial community, responsible for N-removal, in agricultural constructed wetlands. We analysed, (1) the composition of three denitrifying bacterial enzyme genes, and (2) the heterogeneity of each individual denitrifying bacterial enzyme gene, in 32 agricultural constructed wetlands located in southern Sweden. The denitrifying bacterial community was analysed by targeting the enzyme genes, *nir*K, *nir*S and *nos*Z. The bacterial community was analysed with PCR-DGGE technique (Hallin and Lindgren, 1999), and DGGE bands were sequenced and compared to known DNA sequences in GeneBank.

Material and Methods

Wetland ecosystems

Thirty-two wetlands, constructed for the purpose of nutrient retention (nitrogen and phosphorus) in southwest of Sweden, were surveyed during March-October 2004. The wetlands represented nutrient-rich, flow-through freshwater systems with a permanent water body, and had their location and catchments in an agriculturally dominated lowland area within 15 km off the coast (57º02'N, 12º23'O to 55º59'N, 13º06'O). Nutrient concentrations of incoming water ranged from 3.3 to 18.9 mg L^{-1} for total nitrogen (2.5 to 18.8 mg L^{-1} nitrate), and from 5 to 318 µg L^{-1} for total phosphorus. The wetlands were between one and six years old, covering a size range of 0.1 to 2.1 ha (mean wetland area 0.4 ha), and average water depth of 0.2 to 3.9 m (mean water depth 1.0 m) at the time of sampling (for details see Thiere et al., in press).

Experimental setup and biofilm sampling strategy

Heterogeneous constructed wetland environments were sampled, using polyethylene strips for biofilm development in a similar way in all wetlands, to minimize variation in the bacterial community composition due to sampling bias. Five pre-washed polyethylene strips (3.2 m long, 15 mm wide and 0.1 mm thick) covering both the wetland sediment surface and the water column, were placed in each wetland, regularly spaced in sites along the main flow channel between wetland in- and outlet. The horizontal strip (1.6 m) was prepared with weights at the ends, to keep it integrated in the upper part of the sediment. The vertical strip (1.6 m) continued from one of the horizontal ends and was afloat at the water surface by a cork. The strips were left *in situ* for four weeks (September to October 2004) for biofilm development. A mean area of 600 cm^2 was scraped off with a pre-washed razor blade from each wetland set of strips, in order to collect biofilm from each wetland. The biofilm was then collected in sterile eppendorf vials and excess water was discarded after careful (1500 rpm for 2 min) centrifugation. The biofilm samples were then stored at 20°C until DNA extraction.

DNA extraction

Total DNA from biofilm samples from all wetlands was extracted using the FastDNA® Spin Kit for Soil (Bio 101, Inc., La Jolla, CA, USA). Approximately 0.65 g (wet weight) biofilm sample was diluted with 2*978 µl sodium phosphate buffer (SPB-buffer supplemented in the kit). The mixture was homogenised with a blender (DIAX 900 Homogeniser Tool G6, Heidolph, Kelheim, Germany) for 5 min, while kept on ice. 500 µl of the homogenised suspension was diluted with

478 µl SPB-buffer, and thereafter the manufacturer's protocol was followed. The 50 µl DNA extracts were stored at – 20°C until PCR analysis.

PCR

The primer pairs selected for *nirK* and *nirS* were FlaCu:R3Cu and cd3aF:R3cd, respectively (Throbäck et al., 2004). The primer pair *nos*ZF:*nos*Z1622R from Throbäck et al. (2004), used for *nos*Z, was modified to *nos*Z11F, 5´- CGY TGT TCM TCG ACA GCC AG -3´ and *nos*Z611R, 5´- CGS ACC TTS TTG CCS TYG CG -3´. A 33-bp GC-clamp, 5´ - GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC - 3´, was attached to the 5´ end of each of the primers R3cd, R3cu and nosZ1611R. All primers were purchased from Invitrogen (Stockholm, Sweden). PCR amplification, using the respective *nir*S and *nir*K primer pairs, was performed according to Throbäck et al. (2004). For the *nos*Z primer pair we modified the PCR amplification from Throbäck et al. (2004). The PCR mixture for *nos*Z analysis contained 1.33 U Taq polymerase, 5 µl of 10 x buffer (including 1.5 mM $MgCl_2$; Roche Diagnostic GmbH, Mannheim, Germany), 200 µM dNTPs, 0.125 µM of each primer, 600 ng μl^{1} BSA, and 2 µl template DNA, in a 50 µl mixture. The PCR amplification for *nosZ* included an initial denaturing step for 15 min at 95°C; followed by 10 cycles of 30 s at 94°C, 1 min at 66°C (decreased 1°C per cycle) and 1 min at 72°C, thereafter 30 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C, and finally, a primer extension step of 10 min at 72°C. All PCR-reactions were performed on a PTC-100^{TM} thermal cycler (MJ Research Inc., San Fransisco, CA, USA), followed by agarose gel electrophoresis analysis, in order to confirm the size of the products.

Denaturing gradient gel electrophoresis (DGGE) and nucleotide sequencing

Polyacrylamide gels (160x160x1) mm were composed of 37.5:1 acrylamide:bisacrylamide (6.5% acrylamide) and 1 x TAE (40 mM Tris-HCl; 20 mM sodium acetate; 1 mM EDTA, pH 7.4). 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide (Muyzer et al. 1993). The denaturing gradients, 50-80% for *nir*K, 60-80% for *nir*S, and 40-80% for *nos*Z, were prepared with a gradient maker (GM-40, C.B.S. Scientific Company Inc., Del Mar, CA, USA). To polymerise the gel, 6.9 µl 100% TEMED, and 110.5 µl 10% ammonium persulphate, were added to 25.6 ml gelmix. 15 µl PCR-product was loaded on the gel and run in 1 x TAE buffer at 62°C and 130 V. The DGGE (Bio-Rad Laboratories Inc., Hercules, CA, USA) was always run for 13.5 h. The bands were visualised by staining for 1 h with SYBR Gold (Roche Diagnostic GmbH, Mannheim Germany), diluted 3000-fold in 1 x TAE buffer.

All visible DGGE bands in the UV transillumination were cut out from each gel. The bands were placed in 160 ml of sterile dH_2O and stored at (-70°C). DNA was

eluted from the samples by a freeze and thawing procedure (1 h at 20°C, 1 h at 70°C, and 4 h at 4°C), and was reamplified using 5 ml of the eluted DNA as a template in PCR amplification with CTO primers without the GC-clamp. Afterwards, the PCR products were vacuum dried and diluted with 25µl 1 X TBE buffer and sequenced based on Big Dye chemistry by Macrogen Inc. (Seoul, South Korea).

Analysis of DGGE banding pattern

The DGGE banding patterns were analysed using the software Quantity One® (Bio-Rad Laboratories Inc., Hercules, CA, USA), which have earlier been used for similar analyses (e.g. Throbäck et al. 2004). DGGE band migration levels on each gel were normalised with PCR-products after amplification of the reference strain *Escherichia coli* ATCC25922. Thereafter, migration level of DGGE bands from the three denitrifying bacterial communities (*nir*K, *nir*S and *nos*Z) was normalised. To compare DGGE band structure we created similarity matrices, which illustrate absence/presence of DGGE bands at all defined migration levels. To measure the number of bands detected for each enzyme gene, DGGE bands were counted in each lane in the gels.

Statistical analysis and phylogenic analysis of the nucleotide sequences

Nucleotide sequences were aligned using the CLUSTAL W software (www.ebi.ac.uk/clustalw/). The sequences were compared to denitrifying bacterial gene sequences of equivalent length from the GeneBank (NCBI) database using the BLAST (Basic Local Alignment Search Tool; NCBI) (www.ncbi.nlm.nih.gov/BLAST/). A model test (Posada and Crandall, 1998) was used to recognize which maximum-likelihood (ML) model fitted the sequences best. A phylogenetic tree was constructed using heuristic search with PAUP* 4.0 (Sinauer Associates, Inc. Publishers, USA). To validate the trees, 100 bootstrap replicates were performed using the LUNARC computer cluster (http://www.lunarc.lu.se) at Lund University, Sweden. We also constructed trees using maximum-parsimony PAUP* 4.0 for each denitrifying bacterial enzyme gene, which illustrated similar branching as the ML trees (data not shown). The phylogenetic trees were divided into three to nine clusters, which are marked by roman numerals in the phylogenetic trees. The clusters were divided based on the distance measured in the branch length, because several clusters were not supported by the bootstrap analyses. The maximum distance between two nucleotide sequences in the phylogenetic trees within the clusters is less than 45% nucleotide substitutions, which is approximately eight times the indicated scale bar. Similar cluster divisions have been shown in earlier studies on denitrifying bacterial enzyme genes (Braker et al., 2000; Throbäck et al., 2004).

Nucleotide sequence accession number

The partial nucleotide sequences obtained in this study are available in the GeneBank database under accession numbers XXXX to XXXX.

Results & Discussion

DNA extraction and PCR amplification

DNA from biofilm samples was successfully extracted from each of the 32 constructed wetlands. PCR amplification, using the primer pairs encoding parts of *nir*K, *nir*S and *nos*Z yielded products of expected size from all samples; 480 bp, 450 bp, and 420 bp, respectively (data not shown).

Analysis of nirK, nirS and nosZ sequences obtained after amplification with group specific PCR-primers by DGGE and nucleotide sequencing

The DGGE analysis of the PCR products resulted in 28, 25, 21 and 53 band migration levels of the denitrifying enzyme genes *nir*K, *nir*S, *nos*Z and (*nir*K+*nir*S), respectively. The total number of DGGE bands detected from all wetlands for each of the enzyme genes was slightly higher for both *nir*S and *nos*Z than for *nir*K. In the phylogenetic tree, 38 sequences from our study together with 17 sequences obtained from Genebank represented the *nir*K community. The *nir*S community was represented by 58 sequences from our study together with 8 sequences obtained from GeneBank, while the *nos*Z community was represented by 37 sequences from our study together with 12 sequences obtained from GeneBank. Sequences of *nir*K were more similar to bacterial sequences obtained from the GeneBank than *nir*S and *nos*Z sequences (Fig 1). Our results suggest higher heterogeneity of the total (*nir*K+*nir*S) nitrite reductase community composition, than for the *nos*Z community. Hence, the *nir*K+*nir*S had higher number of DGGE bands, higher number of band migration levels, and more diverse matches from the GeneBank results. A higher bacterial diversity (richness and structure) increases the probability of supporting a functional trait after disturbances (Bell et al., 2005; Girvan et al., 2005), implying that the nitrite reductase step (*nir*) may be less sensitive to disturbances than the nitrous reductase step (*nos*) in agricultural constructed wetlands.

Composition of denitrifying bacterial enzyme genes in constructed wetlands

The results from the three denitrifying bacterial enzyme gene sequences showed that agricultural constructed wetlands harbour a heterogeneous community, corroborating previous results from soil systems (Priemé et al., 2002; Throbäck et al., 2004). The enzyme gene *nir*S had the most heterogeneous community, while the enzyme gene *nos*Z had the least heterogeneous community. The three denitrifying bacterial enzyme genes in our study together with sequences retrieved from GeneBank showed clustered phylogenetic trees, where our sequences could be found in the majority of the clusters (Figs 1, 2 and 3). Overall, members of the α-*Proteobacteria* were dominant with representatives in several clusters in the three phylogenetic trees, followed by members of the β-*Proteobacteria*, and thereafter by members of the γ-*Proteobacteria* (Figs 1, 2 and 3). The same order, α, β, and then γ, of *Proteobacteria* have been suggested in earlier studies investigating the denitrifying bacterial enzyme gene(s) (Braker et al., 2000; Avrahami et al., 2002; Priemé et al., 2002; Throbäck et al, 2004; Bremer et al., 2007). However, not all sequences obtained in the present study clustered close to members of *Proteobacteria*. Therefore, some clusters in the phylogenetic trees are exclusively represented by sequences obtained in the present study (hereafter referred to as unspecific *Proteobacteria* clusters). Overall, our study suggests that the denitrifying bacterial community in agricultural constructed wetlands is as heterogeneous as previous communities described from soil systems, when comparing the clustering of the phylogenetic trees (Throbäck et al., 2004).

Heterogeneous communities of each individual denitrifying bacterial enzyme gene

When we studied the separate denitrifying bacterial enzyme genes, sequences representing *nir*S exhibited a more heterogeneous community than *nir*K and *nos*Z (Figs 1, 2 and 3). The enzyme gene *nir*S clustered close to members of all three groups of α-, β-, γ-*Proteobacteria*, but also close to a member of gram-positive bacteria (Fig 2). The enzyme genes *nir*K and *nos*Z only clustered close to members of α-*Proteobacteria* (Figs 1 and 3). However, the unspecific *Proteobacteria* clusters of each phylogenetic tree are disregarded when analysing this result, because more similar known sequences, in comparison to the sequences in the presents study, were not obtained through GeneBank. Both *nir*K and *nos*Z exhibited more unspecific *Proteobacteria* clusters in their phylogenetic trees than *nir*S. More specifically, the phylogenetic tree of *nir*K had seven clusters that could be distinguished (Fig 1). The *nir*K sequences, clustered close to members of α-*Proteobacteria* (Fig 1, clusters I, II, III, IV). The known denitrifying bacterial species belonging to α-*Proteobacteria*, which clustered close to the *nir*K sequences in this study were, *Rhodobacter sphaeroides forma sp. denitrificans* (α), *Mesorhizobium sp*. (α), *Rhizobium sp*. (α), *Blastobacter denitrificans* (α) and *Bradyrhizobium japonicum* (α).

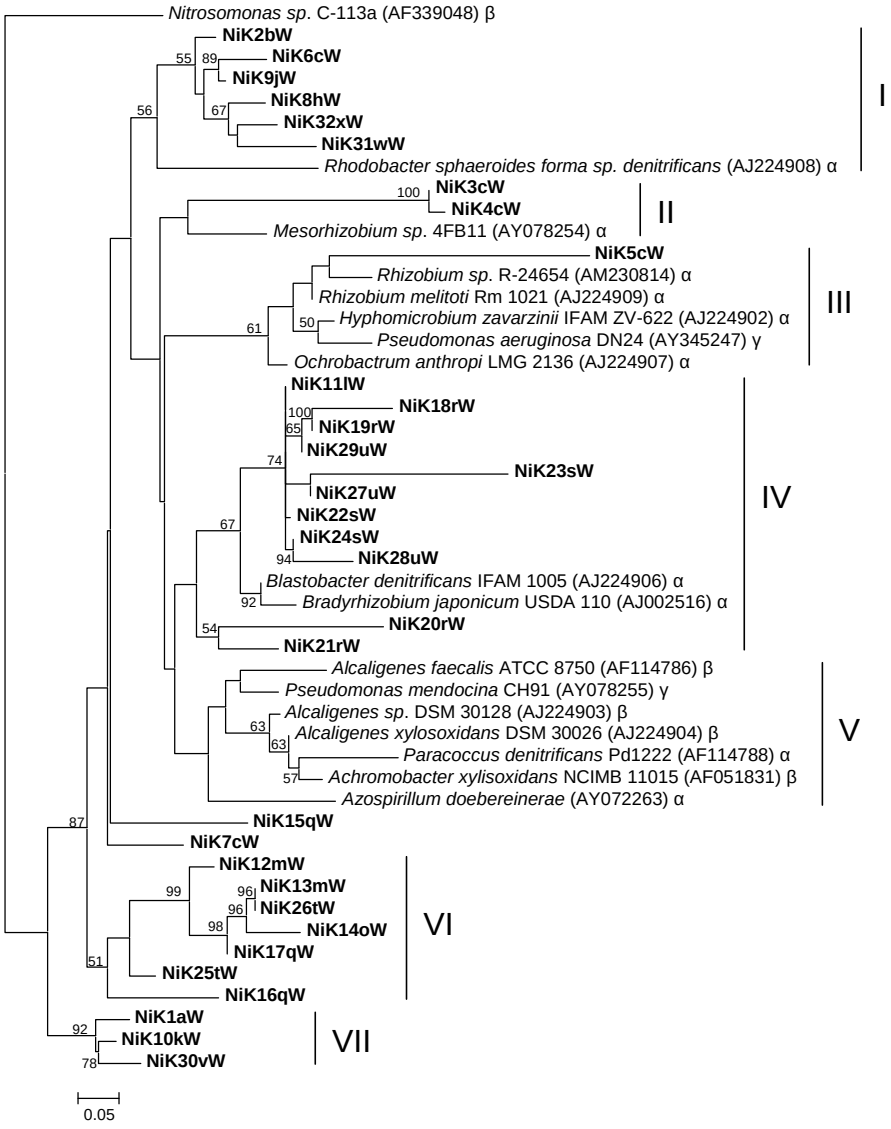

Figure 1. Phylogenetic tree of the partial *nir*K gene sequences (marked in bold) (based on a partial DNA fragment of 315 bp) analysed with maximum likelihood statistical method performed with 100 bootstraps. Bootstrap values higher than 50 are shown. The scale bar indicates 5% nucleotide substitutions. Denitrifying bacterial strains from the GeneBank are indicated by α, β, γ for *Proteobacteria* affiliation. Seven clusters were identified I – VII.

The phylogenetic tree of *nir*S had four distinguished clusters (Fig 2). Not only known bacterial species belonging to α-, β-, and γ-*Proteobacteria* clustered close to the sequences of the present study, but also a gram-positive bacterial species (Fig 2, cluster II). The known *Proteobacteria* denitrifying bacterial species which clustered close to the *nir*S sequences in this study were, *Paracoccus denitrificans* (α), *Paracoccus pantotrophus* (α), *Roseobacter denitrificans* (α), *Ralstonia eutropha* (β), *Kocuria varians* (gram+), *Pseudomonas fluorescens* (γ), *Pseudomonas lini* (γ) (Fig 2).

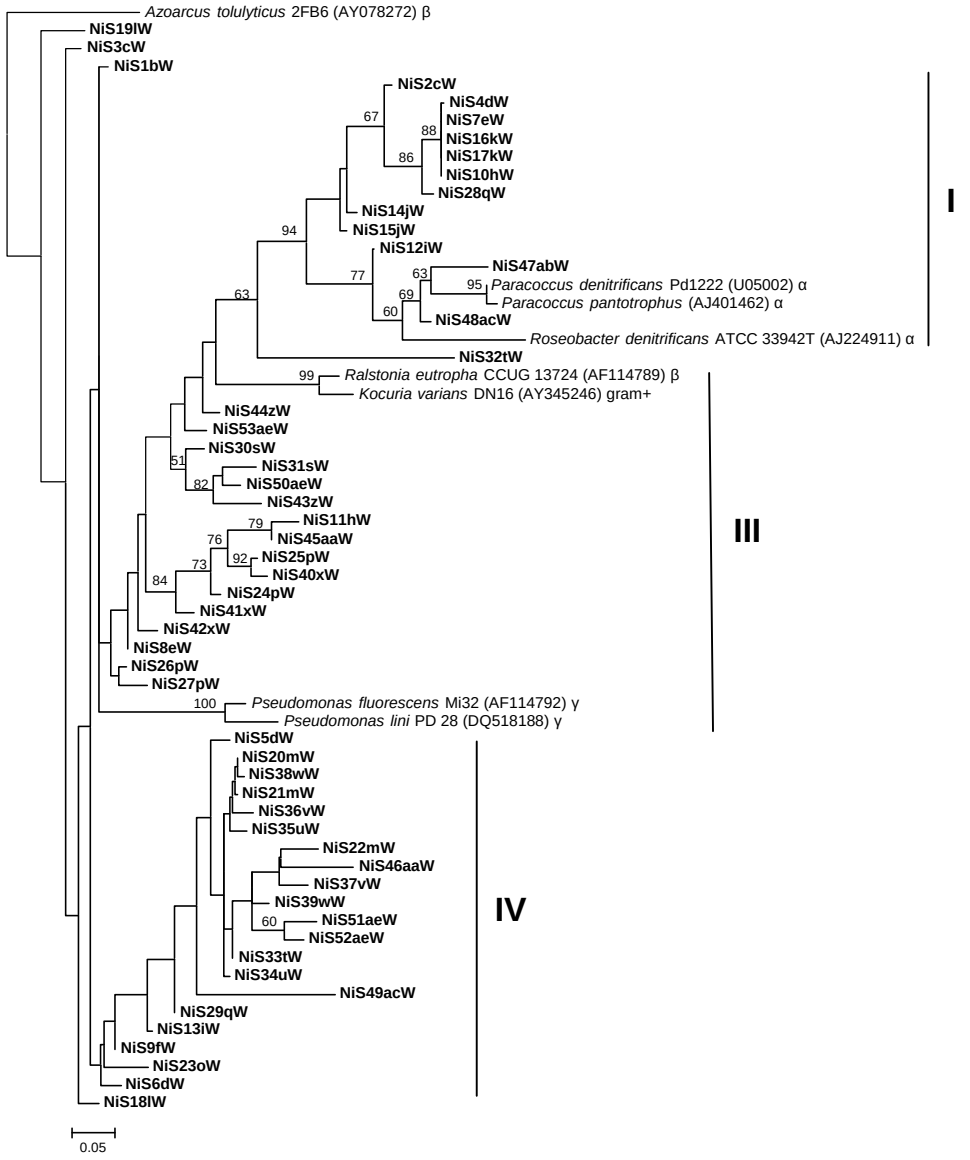

Figure 2. Phylogenetic tree of the partial *nir*S gene sequences (marked in bold) (based on a partial DNA fragment of 276 bp) analysed with maximum likelihood statistical method performed with 100 bootstraps. Bootstrap values higher than 50 are shown. The scale bar indicates 5% nucleotide substitutions. Denitrifying bacterial strains from the GeneBank are indicated by α, β, γ for *Proteobacteria* affiliation, and by gram+ for gram-positive bacteria affiliation. Three clusters were identified I – III.

Nine clusters are representing the phylogenetic tree of *nos*Z (Fig 3). However, few *nos*Z sequences from this study clustered with known bacterial sequences (Fig 3, clusters I and II). The known bacterial sequences in clusters I and II, which clustered close to the *nos*Z sequences in present study were, *Ochrobactrum anthropi* (α), *Rhizobium meliloti* (α), *Azospirillum lipoferum* (α), *Azospirillum sp*. (α), *Azospirillum halopraeferens* (α) (Fig 3).

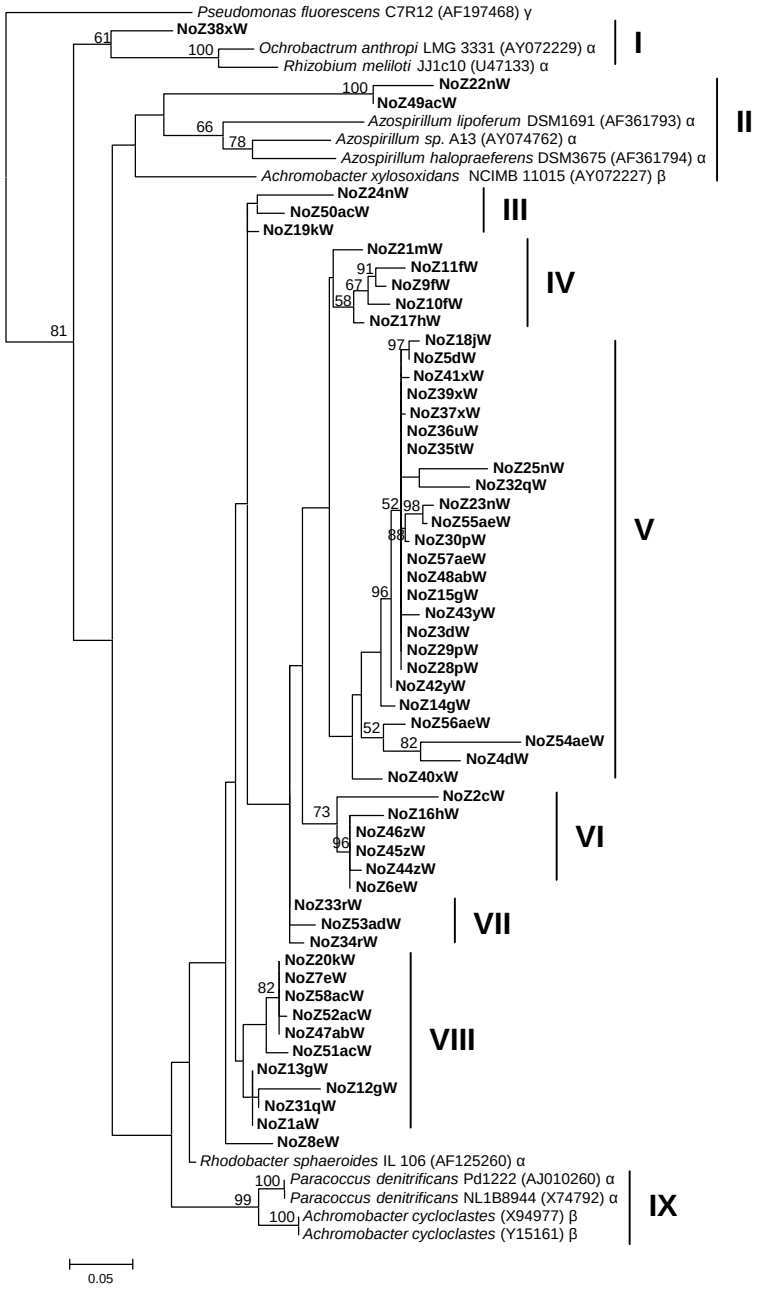

Figure 3. Phylogenetic tree of the partial *nos*Z gene sequences (marked in bold) (based on a partial DNA fragment of 309 bp) analysed with maximum likelihood statistical method performed with 100 bootstraps. Bootstrap values higher than 50 are shown. The scale bar indicates 5% nucleotide substitutions. Denitrifying bacterial strains from the GeneBank are indicated by α, β, γ for *Proteobacteria* affiliation. Nine clusters were identified I – IX.

Previous studies have concluded that the enzyme genes *nir*S and/or *nir*K and/or *nos*Z represent heterogeneous bacterial communities in terrestrial soil systems (Avrahami et al., 2002; Prieme et al., 2002; Throbäck et al, 2004; Bremer et al., 2007), and in a marine sediment systems (Braker et al., 2001), but with a less heterogeneous composition of the enzyme gene *nir*K (Braker et al., 2000). Independent of habitat, the sequences representing the denitrifying bacterial community more often clustered close to members of α-*Proteobacteria*. In contrast, when analysing denitrifying bacteria from a municipal waste-water treatment plant, the majority of the isolate clustered close to members of β-*Proteobacteria* (Heylen et al., 2006a). Studies made with isolates from Heylen et al. (2006a) showed that the enzyme genes *nir*K and *cnor*B (encoding for nitric oxide reductase) more often clustered to members of α-*Proteobacteria*, while the *nir*S more often clustered to members of β-*Proteobacteria* (Heylen et al., 2006b; Heylen et al., 2007). Samples from waste-water treatment plant systems may differ from environmental samples due to the controlled conditions of the surrounding environment in the former system. A controlled system will be less influenced by stochastic effects, which have a large impact on shallow freshwater systems (Kadlec, 1997). The results from studies on waste-water treatment systems may therefore be difficult to extrapolate to environmental samples. Hence, it may be that bacterial communities in environmental ecosystems are driven to a more heterogeneous composition by adapting to disturbances, than bacterial communities in controlled systems. Our result suggests that the denitrifying bacterial enzyme gene *nir*S was the most heterogeneous of the three studied enzyme genes in agricultural constructed wetlands. However, this result may change if more knowledge would be available for the unspecific *Proteobacteria* clusters. To reduce this potential bias, more studies of the denitrifying bacterial enzyme genes are needed in order to develop and increase the number of sequences and information found in GeneBank.

Dominant denitrifying bacterial species in constructed wetlands

Our study showed that within the DGGE gels, one dominant band at the same migration level was found for each of the denitrifying bacterial enzyme genes, indicating common bacterial species that may catalyse each of the studied N transformations represented by the enzyme genes among the constructed wetlands (data not shown). The sequences of the dominant band in the *nir*K community were suggested to belong to α-*Proteobacteria* in the groups I, IV or in the unspecific *Proteobacteria* group VI (Fig 1). In the *nir*S community the sequences of the dominant band was suggested to belong to unspecific *Protoebacteria* group IV (Fig 2), while the sequences were suggested to belong to the unspecific *Proteobacteria* groups IV, V and VIII for the *nos*Z community (Fig 3). Further studies are needed to determine the origin of the detected dominant bands and to gain knowledge of which common bacterial species they

represent in the habitats. With such knowledge, the efficiency in actively expressing the enzyme genes may be determined for the common denitrifying bacterial species, and as a result, we may come a step closer to understanding the relationship between bacterial structure and function in constructed wetlands.

Conclusions

Our study shows that the community composition of the denitrifying bacterial enzyme genes was as heterogeneous among studied agricultural constructed wetlands as it has been shown to be in arable soil (Throbäck et al., 2004). The enzyme gene *nir*S exhibited the most heterogeneous community, whereas the enzyme gene nosZ exhibited the least heterogeneous community. Partial nucleotide sequences from the present study often clustered close to members of α-*Proteobacteria*, thereafter to members of β-*Proteobacteria*, members of γ-*Proteobacteria*, and finally to gram-positive bacteria. The enzyme genes, representing the nitrite reduction (*nir*K+*nir*S) step, showed a higher number of DGGE bands, higher number of band migration levels, and more heterogeneous communities, than the nitrous oxide reduction step (*nos*Z). Dominant DGGE bands were found for each denitrifying bacterial enzyme gene, suggesting some bacterial species prevalent in the habitat of agricultural constructed wetlands. Our results suggest that agricultural constructed wetlands may harbour a diverse and heterogeneous community of denitrifying bacteria. Understanding of the variation (i.e. diversity and heterogeneity) of the denitrifying bacterial community is essential for future efforts to link structure to ecosystem functioning of the constructed wetlands.

Acknowledgement

This study was partly funded by FORMAS, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (grant nos. 25/2-2001-2677, 214-2004-1678, 21.5/2003-1111, and 215/2005-507), and the Crafoord Foundation (grant no. 20050957). The authors would like to thank Mattias Mild and Dag Ahrén for valuable assistence with statistical analysis of the phylogenetic trees.

References

Avrahami, S., Conrad, R., Braker, G., 2002. Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. *Applied and Environmental Microbiology*. 68, 5685-5692.

Bachand, P.A.M., Horne, A.J., 2000. Denitrification in constructed free-water surface wetlands: II. Very high nitrate removal rates in a macrocosm study. *Ecological Engineering*. 14, 17-32.

Bell, T., Newman, J.A., Silverman, B.W., Turner, S.L., Lilley, A.K., 2005. The contribution of species richness and composition to bacterial services. *Nature*. 436, 1157-1160.

Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Weatherby, A., 2005. 15 years of pond assessment in Britain: results and lessons learned from the work of pond conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 15, 693–714.

Braker, G., Fesefeldt, A., Witzel, K-P., 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology*. 64, 3769-3775.

Braker, G., Zhou, J., Wu, L., Devol, A.H., Tiedje, J.M., 2000. Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. *Applied and Environmental Microbiology*. 66, 2096-2104.

Braker, G., Ayala del Rio, H.L., Devol, A.H., Fesefeldt, A., Tiedje, J., 2001. Community structure of denitrifiers, Bacteria, and Archae along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nir*S) and 16S rRNA genes. *Applied and Environmental Microbiology*. 67, 1893-1901.

Braker, G., Tiedje, J.M., 2003. Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Applied and Environmental Microbiology*. 69, 3476-3483.

Bremer, C., Braker, G., Matthies, D., Reuter, A. Engels, C., Conrad, R., 2007. Impact of plant functional group, plant species, and sampling time on the composition of nirK-type denitrifier communities in soil. *Applied and Environmental Microbiology*. 73, 6876-6884.

Fleischer, S., Gustafson, A., Joelsson A., Pansar, J., Stibe, L., 1994. Nitrogen removal in created ponds. *Ambio*. 23, 349-357.

Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology*. 7, 301-313.

Green, J.L., Bohannan, B.J.M., Whitaker, R.J., 2008. Microbial biogeography: from taxonomy to traits. *Science*. 320, 1039-1043.

Hallin, S., Lindgren P-E., 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Applied and Environmental Microbiology*. 65, 1652-1657.

Hallin, S., Lydmark, P., Kokalj, S., Hermansson, M., Sörensson, F., Jarvis, Å., Lindgren, P-E., 2005. Community survey of ammonia-oxidizing bacteria in full-scale activated sludge processes with different solids retention time. *Journal od Applied Microbiology*. 99, 629-640.

Hannig, M., Braker, G., Dippner, J., Jürgens, K., 2006. Linking denitri¢er community structure and prevalent biogeochemical parameters in the pelagial of the central Baltic Proper (Baltic Sea). *FEMS Microbiology Ecology*. 57, 260-271.

Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N., De Vos, P., 2006a. Cultivation of denitrifying bacteria: optimization of isolates conditions and diversity study. *Applied and Environmental Microbiology*. 72, 2637-2643.

Heylen, K., Gevers, D., Vanparys, B., Wittebolle, L., Geets, J., Boon, N., De Vos, P., 2006b. The indicies of *nir*S and *nir*K and their genetic heterogeneity in cultivated denitrifiers. *Environmental Microbiology*. 8, 2012-2021.

Heylen, K., Vanparys, B., Gevers, D., Wittebolle, L., Boon, N., De Vos, P., 2007. Nitric oxide reductase (*nor*B) gene sequence analysis reveals discrepancies with nitrite reductase (nir) gene phylogeny in cultivated denitrifiers. *Environmental Microbiology*. 9, 1072-1077.

Ibekwe, A.M., Lyon, S.R., Leddy, M., Jacobson-Meyers, M., 2006. Impact of plant density and microbial composition on water quality from a free water surface constructed wetland. *Journal od Applied Microbiology*. 102, 921-936.

Kadlec, R.H., 1997. Deterministic and stochastic aspects of constructed wetland performance and design. *Water Science and Technology*. 35, 149-156.

Langenheder, S., Prosser, J.I., 2008. Resource availability influences the diversity of a functional group of heterotrophic soil bacteria. *Environmental Microbiology*. 10, 2245-2256.

Lin, Y-F., Jing, S-R., Wang, T-W., Lee, D-Y., 2002. Effects of macrophytes and external carbon sources on nitrate removal from groundwater in constructed wetlands. *Environmental Pollution*. 119, 413–420.

Martinez, J., Perez-Serrano, J., Bernadina, W.E., Rodriguez-Caabeiro, F., 2001. Stress response to cold in *Trichinella* species. *Cryobiology*. 43, 293-302.

McNaughton, S.J., 1977. Diversity and stability of ecological communities: a comment on the role of empiricism in ecology. *The American Naturalist*. 111, 515-525.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*. 59, 695-700.

Posada, D., Crandall, K.A., 1998. MODEL TEST: testing the model of DNA substitution. *Bioinformatics*. 14, 817– 818.

Priemé, A., Braker, G., Tiedje, J.M., 2002. Diversity of nitrite reductase (*nir*K and *nir*S) gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology*. 68, 1893-1900.

Song, B., Ward, B.B., 2003. Nitrite reductase genes in halobenzoate degrading denitrifying bacteria. *FEMS Microbiology Ecology*. 43, 349-357.

Stadmark, J., Leonardsson, L., 2005. Emissions of greenhouse gases from ponds constructed for nitrogen removal. *Ecological Engineering*. 25, 542-551.

Thiere, G., Milenkovski, S., Lindgren, P-E., Sahlén, G., Berglund, O., Weisner, S.E.B., 2009. Wetland creation in agricultural landscapes: biodiversity benefits on local and regional scales. *Biological Conservation*. In press.

Throbäck I.N., Enwall, K., Jarvis, Å., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology*. 49, 401-417.

Wallenstein, M.D., Myrold, D.D., Firestone, M., Voytek, M., 2006. Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications*. 16, 2143-2152.

Weisner, S.E.B., Eriksson, P.G., Granéli, W., Leonardsson, L., 1994. Influence of macrophytes on nitrate removal in wetlands. *Ambio*. 23, 363-366.

Paper III

Effects of environmental concentrations of pesticides on community structure and function of constructed wetland denitrifying bacteria

Milenkovski, S[1]., Svensson, J.M[2]., Lindgren, P-E[3]. and Berglund, O[1].

[1]Department of Chemical Ecology/Ecotoxicology, Ecology building, Lund University, SE-223 62 Lund, SWEDEN
[2]Swedish County Administrative Board, Länsstyrelsen Halland län, SE-301 86 Halmstad, SWEDEN
[3]Department of Medical Microbiology, Linköping University, SE-581 85 Linköping, SWEDEN

Keywords: denitrification, eubacterial community, *nos*Z, DGGE, fungicide, insecticide, herbicide.

Abstract

The aim with present study was to assess whether environmentally relevant concentrations of different pesticides may affect the structure and/or function of bacterial communities in constructed wetlands (CWs). Effects of the insecticide pirimicarb, the herbicide glyphosate, and the fungicide propiconazole, on eubacterial and denitrifying bacterial community composition, and potential denitrification rates, were tested in microcosm set-ups with natural sediment from a CW situated in an agricultural area in the south of Sweden. PCR-DGGE, targeting the eubacterial 16S rRNA gene, and the functional enzyme gene *nos*Z, were used to measure changes in eubacterial, and denitrifying bacterial community structure, respectively, and potential denitrification rates were measured using the isotope ^{15}N pairing technique. Pesticide-induced changes were observed in the eubacterial community structure, while no changes were observed in the denitrifying community structure. Denitrification was inhibited in the propiconazole treatments, however, no concentration-response relationship could be observed. Glyphosate and pirimicarb did not affect denitrification at environmentally relevant concentrations. Our study showed no consistent, negative effects of these, commonly used agricultural pesticides on the microbial community involved in the nitrogen removal in CWs.

1. Introduction

During the last decades, programmes for restoring and recreating wetlands have independently been initiated world wide, in order to reduce further nutrient and pollutant transport to freshwater systems, coastal areas, and oceans (Zedler, 2003; 2004; Moreno-Mateos et al., 2008). In agricultural areas, constructed wetlands (CWs) have been extensively developed with the main purpose of removing excess nitrogen, thus reducing eutrophication of associated aquatic environments (Biggs et al., 2005; Stadmark and Leonardsson, 2005). Nitrogen removal in wetlands occurs through direct assimilation by plants, and through microbial nitrification and denitrification. Denitrification, a stepwise reduction of nitrate (NO_3^-) to dinitrogen gas (N_2), mainly performed by bacteria (Hallin and Lindgren, 1999), is the limiting process of nitrogen removal in agricultural CWs, due to the typically high loads of NO_3^- (Bachand and Horne, 2000; Seitzinger et al., 2006). Thus, the efficiency of nitrogen removal in these CWs is highly dependent on the structure and function of the denitrifying bacterial community (Enwall et al., 2005; 2007).

Aquatic environments in the agricultural landscape are also exposed to several pesticides of different kinds, and active ingredients of herbicides, insecticides and fungicides are regularly detected in surface waters in these areas (Kreuger et al., 1999; Haarstad and Braskerud, 2005). Although many pesticides have designated target organisms, due to specific modes of action, several may have more general toxic effects on non-target organisms, like bacteria (Engelen et al., 1998; Johnsen et al., 2001) and, may affect aquatic bacterial communities at environmentally relevant concentrations (Wang et al., 2004; Knapp et al., 2005). Thus, there is a need for assessing potential impairment of the intended nitrogen removal in CWs, by pesticide exposure, or specifically, if environmental concentrations of different groups of pesticides may affect CW denitrification rates, through toxic effects on the denitrifying bacterial community.

Microbial toxicity testing on a community level provides an attractive alternative to the more routinely performed single-species toxicity tests, and the advantages of testing natural microbial assemblages in microcosm experiments have been advocated (Brandt et al., 2004; Rohr et al., 2006; Sundbäck et al., 2007; Widenfalk et al., 2008). However, there are associated difficulties with the interpretation of results, concomitant with the increased complexity and relevance of these toxicity tests. Particularly for longer incubation times, stochastic effects may decrease the predictive ability, and recorded changes in community compositions may not necessarily be related to toxic effects. Ideally, the effects of exposure on a combination of functional and structural endpoints of the microbial community should be assessed. Structural endpoints may reveal changes in community composition, obscured in single species tests or functional endpoint analyses due to the inherent functional redundancy of bacteria, i.e., the

ability of tolerant species to compensate for loss of functions associated with more sensitive ones (Girvan et al., 2005). The denitrifying bacterial community is very diverse (Braker et al., 1998), hence, functional redundancy very likely, and functional endpoints may therefore be less sensitive than structural for this group.

Cultivation-independent molecular techniques that enable assessment of microbial community composition have been made available during the last decade. One regularly used method for analyses of the bacterial community composition is PCR-DGGE, both for 16S rRNA gene, and functional gene analyses (Hallin et al., 2005; Ibekwe et al., 2006; Langenheder and Prosser, 2008). As the ability to denitrify is sporadically distributed among different bacterial phyla, which is not congruent with the general 16S rRNA gene community (Song and Ward, 2003) the use of functional genes may be a more suitable approach when assessing effects on the denitrification community in CWs. However, complementary analysis of the 16S rRNA gene, characterizing the bacterial community on a different scale, may provide a broader overview (Dahllöf, 2002). Denitrifying bacterial community composition has successfully been analysed in environmental samples by targeting functional genes encoding for enzymes involved in different steps of the denitrification pathway, such as *nirS* and *nirK*, encoding nitrite reductases, catalyzing the reduction of nitrite to nitric oxide, and *nosZ*, encoding nitrous oxide reductase, catalyzing the last step in the pathway, reduction of nitrous oxide to dinitrogen gas (e.g. Throbäck et al 2004).

Recently, studies have addressed structure-function relationships in the denitrifying bacterial community using molecular techniques targeting functional genes (Cavigelli and Robertson, 2000; Rich and Myrold, 2004; Enwall et al., 2005; Kjellin et al., 2007). However, some studies have related diverging structural and functional traits of the denitrifying bacterial community (Cavigelli and Robertson, 2000; Rich and Myrold, 2004; Enwall et al., 2005). Studies of the relationship between function and structure should, ideally, characterize both traits with measures on the matching bacterial community. Studies of denitrification rates have commonly analyzed the potential amount of produced nitrous oxide (e.g. Kozub and Liehr, 1999; Sirivedhin and Gray, 2006, Kjellin et al., 2007), or, to a lesser extent, dinitrogen gas (Nielsen, 1992, Rysgaard et al., 1993, Svensson, 1998). However, since an unknown amount of the denitrifying bacterial community is lacking the code to transcript the enzyme gene *nos*Z, a minor part of the nitrous oxide will not be reduced to dinitrogen gas. This has to be considered as a bias when estimating the potentially produced nitrous oxide in comparison to dinitrogen gas. In this study we chose to target the functional gene *nosZ* as a structural endpoint representing the denitrifying bacterial community, and to measure potential production of dinitrogen gas as a functional endpoint representing CW efficiency, to avoid mismatches between structure and function measurements.

The aim with present study was to examine if environmentally relevant concentrations of active ingredients from three pesticides, targeting different organisms (the insecticide pirimicarb, the herbicide glyphosate, and the fungicide propiconazole), may affect the structure and/or function of bacterial communities in constructed wetlands. Natural CW sediment microbial communities were exposed to pesticides in a sediment/water microcosm experimental set-up. We assessed whether pesticide exposure affected (1) the potential denitrification rate, (2) part of the denitrifying bacterial community composition, and in addition, (3) the eubacterial community composition. The isotope (^{15}N) pairing technique (Svensson, 1998) was used to analyse potential production of dinitrogen gas, as the functional endpoint of the denitrifying bacterial community. We used PCR-DGGE technique (Hallin and Lindgren., 1999) targeting the enzyme gene *nos*Z as the structural endpoint of the denitrifying bacterial community, and the eubacterial 16S rRNA gene as a structural endpoint of the total bacterial community.

2. Materials and methods

2.1. Site description and sampling

Sediment was collected from a constructed wetland outside Vinberg, in the southwest of Sweden (56°55′N, 12°33′E) in April 2006. The wetland was constructed 2002 in an agricultural landscape, and has an area of 6500 m^2 and an average depth of 1 m. Surface water temperature was 13°C, and pH was 7.3 during the day of sampling. Sediment was sampled from the main flow path in the center of the wetland. Sediment cores, of 2-10 cm depth plus 5 cm of overlaying water, were sampled with pre-washed polyethylene cylinder core samplers, and pooled in a larger, pre washed polyethylene container. Sediment and water were kept dark, and on ice during transportation (6 hrs) to the laboratory. Upon arrival to the laboratory, the sediment slurry was sieved through a 1 mm mesh to remove larger materials. Subsamples were taken to determine, water content of the sediment, 58.8 ± 0.6% (n=6, 105°C, over night) and organic matter content, measured as loss on ignition, 9.3 ± 0.5% (n=6, 550°C for 2 h). The sediment was kept dark at 13°C until the start of the microcosm experiments.

2.2. Pesticides

Three active ingredients of pesticides, pirimicarb (Cas no. 23103-98-2), glyphosate (Cas no. 38641-94-0) and propiconazole (Cas no. 60 207-90-1) (Ehrenstorfer Reference standards, determined purity ≥ 97%) were used in the microcosm experiments. These pesticides were chosen as they are commonly applied, in large quantities to agricultural fields worldwide. Three concentrations, low, intermediate, and high, of each pesticide were used in the experiments,

except for propiconazole where we also used an additional, very high concentration, intended as a positive control. The intermediate concentration of each pesticide represented the maximum permissible concentration (MPC) (Crommentuijun et al., 2000) according to assessments by the Swedish Chemical Agency (www.kemi.se). The low concentration was 100 times lower than the MPC, and the high 100 times higher than the MPC. The additional very high concentration of propiconazole was 15 000 times the MPC. The nominal concentrations were, for pirimicarb 0.006, 0.06, and 6 µg/L, for glyphosate 0.1, 10, and 1000 µg/L, and for propiconazole 0.7, 7, and 700 µg/L with the additional concentration of 105 mg/L. Microcosms were filled with sediment and water, with a mean sediment volume of 0.6 mL. Nominal pesticide concentrations were calculated by $C_{nom} = (C_{stock} * V_{stock})/(V_{sed} + V_{water} + V_{stock})$, where C_{stock} is the concentration of the stock solution of the active ingredient, V_{stock} is the volume of added stock solution, V_{sed} and V_{water} are the volumes of the added sediment and water to the microcosms, respectively. Artificial lakewater was added to give the total volume of 12 mL in each microcosm. Stock solutions of all pesticides were prepared in milliQ-water and stored dark for two days at 4°C.

2.3. Experimental setup

The microcosms, 12 mL glass vials (Labco Limited, UK), were filled with 0.9 g wet weight of sediment using a pipette. Before addition, the sediment slurry was gently homogenized by stirring. Then, the microcosms were completely filled (avoiding gas bubbles) with artificial lake water (Lehman, 1980), prepared with respective pesticide and enriched with nitrogen (N) as KNO^3 and carbon (C) as CH_3COONa, to achieve a 1:5 ratio of N:C (Ingersoll and Baker., 1998, Xia et al., 2008). To avoid limitation of C and N during incubation, 3 mg L^{-1} of N, and 15 mg L^{-1} of C, were added to each microcosm (S. Milenkovski et al., unpublished data). All liquids were bubbled with nitrogen gas for 20 min before they were added to the microcosms to generate an anaerobic environment resembling natural wetland conditions. Controls without added pesticides and microcosm with only milliQ-water were also prepared. The values of the controls were used to compare with the values of the pesticide treatments, and the values of the milliQ-water microcosms were used to estimate the background value of nitrous oxide. For the toxicity tests, two separate sets of microcosms were prepared for each pesticide and concentration, n=5 for potential denitrification measurements, and n=3 for molecular analysis. Potential denitrification was measured after 10h, and after 70h, using two separate microcosm sets, while molecular analysis was performed after 70h incubation on a separate microcosm set. Microcosms incubated for 70h were enriched a second time, after 35h, with N and C, with the same amounts as described earlier. The microcosms were kept dark at 13°C during incubation.

2.4. DNA extraction

Total DNA from the sediment slurry was extracted using the FastDNA® Spin Kit for Soil (Bio 101, Inc., La Jolla, CA, USA). Approximately 0.2 g (wet weight) sediment sample was diluted with 978 µl sodium phosphate buffer (SPB-buffer supplemented in the kit). The mixture was homogenised with a blender (DIAX 900 Homogeniser Tool G6, Heidolph, Kelheim, Germany) for 5 min, while kept on ice. 500 µl of the homogenised suspension was diluted with 478 µl SPB-buffer, and thereafter the manufacturer's protocol was followed. The 50 µl DNA extracts were stored at – 20°C until PCR analysis.

2.5. PCR

The primer pair *nos*ZF:*nos*Z1622R from Throbäck et al. (2004), used to detect *nos*Z sequences, was modified to, *nos*Z11F: 5´- CGY TGT TCM TCG ACA GCC AG - 3´ and *nos*Z611R: 5´- CGS ACC TTS TTG CCS TYG CG - 3´. A 33-bp GC-clamp, 5´ - GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC - 3´, was attached to the 5´ end of nosZ1611R. The primers were purchased from Invitrogen (Stockholm, Sweden). PCR amplification for the *nos*Z primer pair were modified from the PCR amplification from Throbäck et al., (2004). The PCR mixture for *nos*Z analysis contained 1.33 U Taq polymerase, 5 µl of 10 x buffer (including 1.5 mM $MgCl_2$; Roche Diagnostic GmbH, Mannheim, Germany), 200 µM dNTPs, 0.125 µM of each primer, 600 ng μl^{-1} BSA, and 2 µl template DNA, in a 50 µl mixture. The PCR amplification for *nosZ* included an initial denaturing step for 15 min at 95°C; followed by 10 cycles of 30 s at 94°C, 1 min at 66°C (decreased 1°C per cycle) and 1 min at 72°C, thereafter 30 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C, and finally, a primer extension step of 10 min at 72°C. All PCR-reactions were performed on a PTC-100™ thermal cycler (MJ Research Inc., San Fransisco, CA, USA), followed by agarose gel electrophoresis analysis in order to confirm the size of the products.

To analyse the eubacterial community, the primer pair GM5F and DS907R, targeting the 16S rDNA, was used (Teske et al., 1996). Same 33-bp GC-clamp as described above was attached to the 5´ end of GM5F. The 50 µl PCR-mixture consisted of 1.33 U Taq polymerase, 5 µl of 10 x buffer (including 1.5 mM $MgCl_2$; Roche Diagnostic GmbH, Mannheim, Germany), 200 µM dNTPs, 0.25 µM of each primer and 5 µl template DNA. The PCR amplification started with an initial denaturing step for 2 min at 94°C; followed by 9 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C; thereafter 19 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C (increased 5 s each cycle); and finally, a primer extension step of 7 min at 72°C. The products were run on an agarose gels to confirm the size of the products before DGGE analysis.

2.6. DGGE

Polyacrylamide gels (160x160x1) mm were composed of 37.5:1 acrylamide:bisacrylamide (6.5% for *nos*, and 10% for 16S rDNA) and 1 x TAE (40 mM Tris-HCl; 20 mM sodium acetate; 1 mM EDTA, pH 7.4). 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide (Muyzer et al., 1993). The different denaturing gradients, 40-80% for *nos*Z and 25-65% for 16S rDNA, were prepared with a gradient maker (GM-40, C.B.S. Scientific Company Inc., Del Mar, CA, USA). To polymerise the gel 6.9 µl of 100% TEMED, and 110.5 µl of 10% ammonium persulphate, were added to 25.6 ml gelmix. 15 µl PCR-product was loaded on the gel and run in 1 x TAE buffer at 62°C and 130 V. The DGGE (Bio-Rad Laboratories Inc., Hercules, CA, USA) was always run for 13.5 h. The bands were visualised by staining for 1 h with SYBR Gold (Roche Diagnostic GmbH, Mannheim Germany), diluted 3000-fold in 1 x TAE buffer.

2.7. Analysis of DGGE banding patterns

DGGE banding patterns were analysed using the software Quantity One® (Bio-Rad Laboratories Inc., Hercules, CA, USA), which has been used for similar analyses (Throbäck et al., 2004). This software allowed us to detect discrete bands on the DGGE gels. The band migration level on each gel was normalized with PCR products after amplification of a reference strain (*Escherichia coli* ATCC25922). Thereafter, the migration levels of DGGE bands from the eubacterial (16S rDNA) and the denitrifying bacterial (*nos*Z) communities were normalized, respectively. To detect responses of the structural endpoint of the bacterial communities after pesticide treatment, we chose to study band structure, richness and intensity. Band structure refers to the pattern of bands in a gel and was measured by creating similarity matrices illustrating presence/absence of DGGE bands at defined migration levels. Band richness was determined by counting all detected bands in each DGGE lane. The DGGE band structure and intensity, of the 16S rRNA gene, were only analysed in the gel part where actual community change could be detected, while band richness were counted of the entire lanes. Band intensities were analysed using the software Quantity One®.

2.8. Denitrification analysis

The microcosms, including both the sediment and water column, were sampled for denitrification measurements according to the isotope pairing technique (Nielsen, 1992). After 10h and 70h of microcosm incubation, 2 ml of the microcosms water phase were replaced with helium. Thereafter the samples were vigorously shaken and 100 µl of gas sample was injected in a gas chromatograph in line with an isotope-ratio mass spectrometer (Hewlett-Packard 4100, GCMS), for ^{15}N-labelled dinitrogen pairs, $^{14}N^{15}N$ or $^{15}N^{15}N$, formed by denitrification (Nielsen, 1992).

The rates of denitrification per cm^3 and h were estimated using the ^{15}N isotope pairing technique (Nielsen, 1992). The production of the single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) nitrogen pairs represents the net fluxes of denitrification. These are used to calculate d15 and d14, which are the rates of denitrification of $^{15}NO_3$ and $^{14}NO_3$, respectively (Nielsen, 1992).

$$d15 = (^{14}N^{15}N) + 2(^{15}N^{15}N)$$

$$d14 = [d15(^{14}N^{15}N)] / 2(^{15}N^{15}N)$$

potential denitrification = (d14 + d15) – mean background value

2.9. Statistical analysis

To analyse if the pesticide treatments affected the denitrification rate (used as dependent variable) after 10 h and/or 70h, we conducted a two-way ANOVA (SPSS version 14.0) to test differences between pesticide treatment and controls.

3. Results

DNA from the sediment samples was successfully extracted from all treatments. The PCR amplification using the primer pairs encoding parts of 16S rRNA and *nos*Z yielded products of expected size from all samples; 550 bp for 16S rRNA gene and 420 bp for *nos*Z. The values of the band intensities could not be estimated by using the Quantity One due to failure when comparing different gels of the same gene, 16S rRNA and nosZ, respectively. The only intensity measures that were estimated in present study were then increased, decreased or similar intensity between the DGGE bands of the controls and the pesticide treatments (Figs 2 and 3).

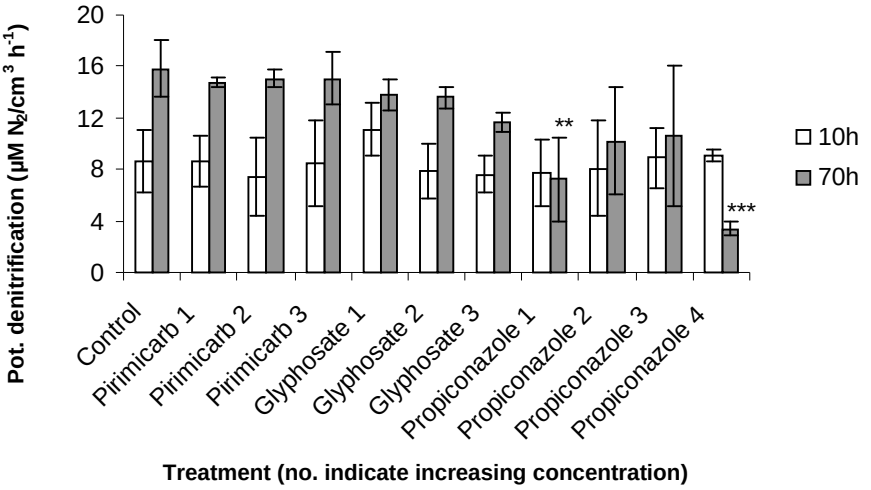

Figure 1. Effect of pesticide exposure on potential denitrification (N_2) rate. Nutrient solution (C and N) was added to the microcosms. Potential denitrification rate was measured after 10h (white bars) and 70h (grey bars) incubation. The tested pesticides were the insecticide pirimicarb, the herbicide glyphosate, and the fungicide propiconazole. Numbers 1-4 indicate increasing conentration, where 1 is MPC/100, 2 is MPC, 3 is MPC*100, and 4 is MPC*15 000. Two pesticide treatments were significantly lower than the controls after 70 h incubation, propiconazole 1 and 4, indicated by asteriks.

All three pesticides affected the eubacterial 16S rRNA community composition compared to the controls (Fig 2). For all treatments, band richness, of the selected part of the gel, of the 16S rRNA gene, varied between 22 and 24. In all pesticide treatments, new DGGE bands appeared, and a few bands increased in intensity. All the different pesticide treatments, irrespective of concentrations, showed similar changes in comparison to the controls (Fig 2).

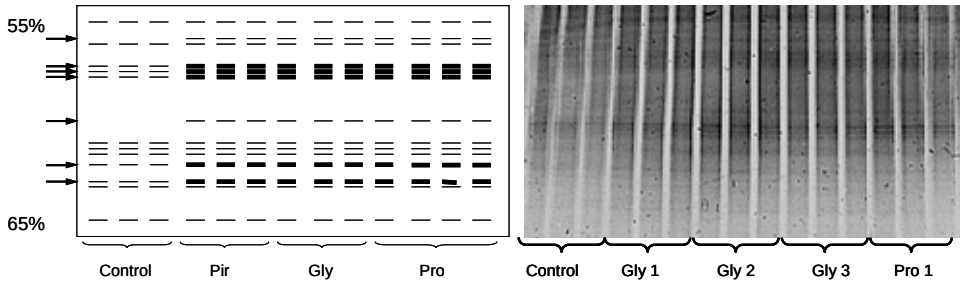

Figure 2. The DGGE gel picture (right) and illustration of original DGGE banding pattern (left) of the eubacterial 16S rRNA gene community after 70h incubation. Only a smaller part of the whole DGGE banding pattern is showed in this illustration. The excluded DGGE part showed no difference in banding pattern between controls and pesticide treatments. Each treatment had three replicates but since the replicates showed similar results, only one replicate is shown for each treatment. All the replicates are illustrated for the control. For all three pesticide treatments the vertical slots represents (from left to right) MPC/100, MPC, and MPC*100 concentrations. The last vertical lane for propiconazole represents the 15 000*MPC concentration. The first and fifth arrows, from top, indicate new DGGE bands that have appeared in all pesticide treatments and not in the controls. The other arrows indicate increased intensity of DGGE band for all pesticide treatments compared to the controls. In the DGGE gel picture the replicates for the complete glyphosate treatment is shown as well as the lowest concentration of propiconazole.

The pesticide treatments did not affect the community composition of the denitrifying bacterial enzyme gene *nos*Z (Fig 3). The richness of the enzyme gene *nosZ* was 7 bands in each lane (Fig 3). No changes between treatments were observed in band richness, structure or intensity.

Only the fungicide propiconazole, after 70 hours incubation, had a significant negative effect on the potential denitrification rate (Fig 1). The additional very high concentration, 15,000 times MPC ($p<0.001$), and the low concentration, MPC/100 ($p<0.01$), of propiconazole had significantly lower rates than the control. No other treatment differed from the controls (Fig 1).

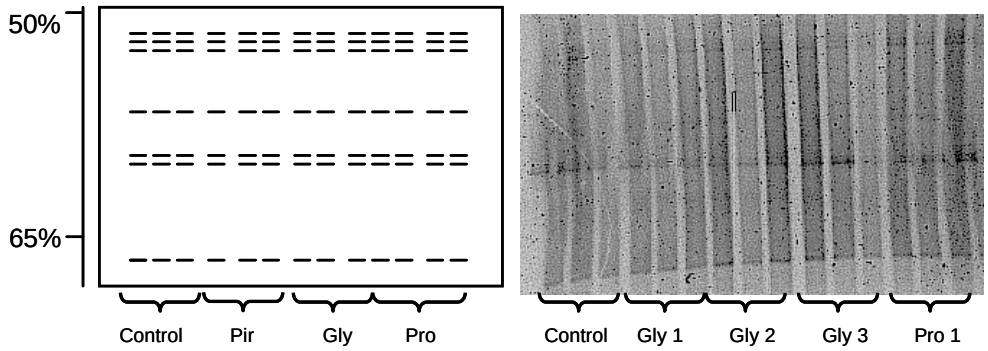

Figure 3. The DGGE gel picture (right) and illustration of original DGGE banding pattern (left) of the denitrifying bacterial enzyme gene *nos*Z community after 70h incubation. No difference in the banding pattern was detected when comparing the controls with the pesticide treatments. Only a smaller part of the whole DGGE banding pattern is showed for both illustration and DGGE picture. The excluded DGGE part showed no difference in banding pattern between controls and pesticide treatments. Each treatment had three replicates but since the replicates showed similar results, only one replicate is shown in the illustration for each treatment. All the replicates are illustrated for the control. For all three pesticide treatments the vertical slots represents (from left to right) MPC/100, MPC, and MPC*100 concentrations. The last vertical lane for propiconazole represents the 15 000*MPC concentration. In the DGGE gel picture the replicates for the complete glyphosate treatment is shown as well as the lowest concentration of propiconazole.

4. Discussion

Our study suggests that, we cannot exclude that environmentally relevant concentrations of commonly used pesticides may affect the structure and function on non-target microbial communities in constructed wetland sediments, although we could only observe very few direct toxic effects of the pesticides tested in this study. Only the fungicide propiconazole had an inhibiting effect on denitrification rates, at the low concentration and the very high concentration after 70 hours incubation, while the herbicide glyphosate and the insecticide pirimicarb both had no affect at concentrations at or around the MPC. Denitrification rate has earlier been shown to be affected by different pesticides at environmental concentrations in both bacterial community analysis (Pell et al., 1998) and in single bacterial species tests (Sáez et al., 2006), while others have failed to detect effects (Widenfalk et al., 2004). The observed lack of community-level effects on function or activity after exposure to environmental concentrations of pesticides have been reported elsewhere (Widenfalk et al., 2008; Van den Brink et al., 2009; Daam et al., 2009), and have been attributed to the time frames over which activity has been measured. A potential immediate response of the community may be overcome, and recovery due to adaptations, community shifts or decreased bioavailability may be rapid. However, in this

study the first measurements of denitrification rates were made after 10 hours incubation, thus, the probability for recovery after this short time should be low. Our study indicated that environmental concentrations of pesticides may induce bacterial community shifts in CW sediments, as observed in other habitats (Rousseaux et al., 2003; Enwall et al., 2005). The compositions of the eubacterial 16S rRNA gene community differed between the pesticide treatments and controls, while the composition of the enzyme gene *nosZ* was similar in all treatments and controls. The functional redundancy in the bacterial community may obscure functionally important effects from pesticide exposure on community composition. Tolerant species may compensate for the loss of function associated with sensitive ones, thus conserving overall community function. However, this resilience of the system may be exhausted e.g. after prolonged, or repeated exposure to different stressors like pesticides, further reducing the functional diversity. Structural endpoints, describing changes in the bacterial community composition, may be able to detect these effects.

The observed change in 16S rRNA gene composition in this study was the appearance of new bands, and an increased intensity of some bands in all the pesticide treatments, indicating that certain groups of bacteria were stimulated by pesticide addition. Stimulatory effects on the diversity of bacterial communities from low concentrations of pollutants have previously been reported (Brandt et al., 2004; Throbäck et al., 2007; Widenfalk et al., 2008). The intermediate disturbance hypothesis have been used to explain increased diversity after e.g. heavy metal contamination (Giller et al., 1998), while increased diversity after exposure to organic contaminants may also be due to the ability of some bacterial groups to utilize them as carbon substrates or nutrient sources (Widenfalk et al., 2008). Thus, the observed changes in PCR-DGGE patterns after pesticide exposure does not necessarily substantiate toxicity of the compounds, and as we also failed to detect any dose-response relationships in our pesticide treatments, these results should be cautiously interpreted as indications of disturbance rather than toxicity.

We did not observe any pesticide-induced changes in the composition of the functional enzyme gene *nosZ* in our study. The compositions were similar to the controls, even for the very high propiconazole treatment, were we did observe a significant inhibition of denitrification rates, measured by the production of dinitrogen gas, the product of the last reduction step, catalyzed by nitrous oxide reductase encoded by *nos*. Thus, we could not link the reduced function of the CW sediment denitrifying bacterial community to any changes in the structure of the community after 70 hours. However, structural changes cannot be completely excluded as some of the detected DGGE bands may represent several bacterial species, thus underestimating the true diversity and obscuring subtle changes, and as some of the bands may originate from PCR amplification of nonviable cells still present in the sediment, due to insufficient time for degradation.

Incubation times of 10 and 70 hours for effects on denitrification rates, and 70 hours for effects on eubacterial and denitrifying bacterial community composition were chosen in our study. A common feature for several microbial community microcosm experiments with longer incubation times are increasing stochastic effects associated with community succession (Thirup et al., 2001; Brandt et al., 2004), which may complicate interpretation, and restrict the ability of prediction and extrapolation of the toxicity data. Decreasing the incubation time may reduce the influence of these stochastic effects, however, incubation time must be sufficient for toxicant-induced community succession to occur, and for the methods of structural analysis to detect it. In our study only stimulatory, or no effects, were recorded on the eubacterial and denitrifying bacterial community structure, new PCR-DGGE bands appeared and some increased in intensity. The failure to record disappearance of bands after pesticide exposure, that have been reported in other, longer-term studies (Engelen et al., 1998; Thirup et al., 2001), may be due to a shorter incubation time used in our study.

In our study we did not record any dose-response relationships between pesticides and the functional and structural endpoints, similar to other studies investigating effects of environmental concentrations of contaminants (Widenfalk et al., 2004; 2008, Van den Brink et al., 2009). The failure of detecting effects and dose-response relationships will limit the general use of toxicity information derived from these assays in risk assessments as no effect concentrations, EC_{50}, LOEC, or NOEC, can be calculated. However, they may provide site-specific estimates of risks under given environmental conditions, e.g. risks of environmentally relevant concentrations of pesticides affecting function of natural microbial communities in constructed wetlands. The results of our study did not imply any substantial direct effects of the tested pesticides on CW denitrifying bacterial communities at or around MPCs, that are in the same order of magnitude, low $\mu g\ L^{-1}$, as maximum concentrations detected in surface waters in these areas (Kreuger et al., 1999). However, environmental concentrations of these pesticides at, or exceeding MPCs, set at levels that should protect all species in an ecosystem (Crommentuijn et al., 2000), may affect the function of microbial wetland communities indirectly, through trait- or density-mediated effects on other, more sensitive non-target organisms, e.g. in the macrophyte or grazer community (Wendt-Rasch et al., 2003).

Acknowledgements

This study was partly funded by FORMAS, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (grant nos. 25/2-2001-2677 and 214-2004-1678), and the Crafoord Foundation (grant no. 20050957).

References

Bachand, P.A.M., Horne, A.J., 2000. Denitrification in constructed free-water surface wetlands: II. Very high nitrate removal rates in a macrocosm study. *Ecological Engineering*. 14, 17-32.

Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Weatherby, A., 2005. 15 years of pond assessment in Britain: results and lessons learned from the work of pond conservation. Aquatic Conservation: Marine *and Freshwater Ecosystems*. 15, 693–714.

Braker, G., Fesefeldt, A., Witzel, K-P., 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology*. 64, 3769-3775.

Brandt, K.K., Jørgensen, N.O.G., Nielsen, T.H., Winding, A., 2004. Microbial community-level toxicity testing of linear alkylbenzene sulfonates in aquatic microcosms. *FEMS Microbiology Ecology*. 49, 229-241.

Cavigelli, M.A., Robertson, G.P., 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology*. 81, 1402-1414.

Crommentuijn, T., Sijm, D., de Bruijn, J., van Leeuwen, K., van de Plassche, E., 2000. Maximum permissible and negligible concentrations for some organic substances and pesticides. *Journal of Environmental Management*. 58, 297-312.

Daam, M.A., Rodriguez, A.M.F., Van den Brink, P.J., Nogueira, A.J.A., 2009. Ecological effects of the herbicide linuron in tropical freshwater microcosms. *Ecotoxicology and Environmental Safety*. 72, 410-423.

Dahllöf, I., 2002. Molecular community analysis of microbial diversity. *Current Opinion in Biotechnology*. 13, 213-217.

Engelen, B., Meinken, K., von Wintzingerode, F., Heuer, H., Malkomes, H-P., Backhaus, H., 1998. Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Applied and Environmental Microbiology*. 64, 2814-2821.

Enwall, K., Philippot, L., Hallin, S., 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Applied and Environmental Microbiology*. 71, 8335-8343.

Enwall, K., Nyberg, K., Bertilsson, S., Cederlund, H., Stenstrom, J., Hallin, S., 2007. Long-term impact of fertilization on activity and composition of bacterial

communities and metabolic guilds in agricultural soil. *Soil Biology and Biochemistry*. 39: 106-115.
Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biology and Biochemistry*. 30, 1389-1414.
Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology*. 7, 301-313.
Haarstad, K., Braskerud, B.C., 2005. Pesticide retention in the watershed and in a small constructed wetland treating diffuse pollution. *Water Science and Technology*. 51, 143-150.
Hallin, S., Lindgren P-E., 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Applied and Environmental Microbiology*. 65, 1652-1657.
Hallin, S., Lydmark, P., Kokalj, S., Hermansson, M., Sörensson, F., Jarvis, Å., Lindgren, P-E., 2005. Community survey of ammonia-oxidizing bacteria in full-scale activated sludge processes with different solids retention time. *Journal of Applied Microbiology*. 99, 629-640.
Ibekwe, A.M., Lyon, S.R., Leddy, M., Jacobson-Meyers, M., 2006. Impact of plant density and microbial composition on water quality from a free water surface constructed wetland. *Journal of Applied Microbiology*. 102, 921-936.
Ingersoll, T. L., Baker, L. A., 1998. Nitrate removal in wetland microcosms. *Water Research*. 32, 677-684.
Johnsen, K., Jacobsen, C.S., Torsvik, V., Sørensen, J., 2001. Pesticide effects on bacterial diversity in agricultural soils – a review. *Biology and Fertility of Soils*. 33, 443-453.
Kjellin, J., Hallin, S., Wörman, A., 2007. Spatial variations in denitrification activity in wetland sediments explained by hydrology and denitrifying community structure. *Water Research*. 41, 4710-4720.
Knapp, C.W., Caquet, T., Hanson, M.L., Lagadic, L., Graham, D.W., 2005. Response of water column microbial communities to sudden exposure to deltamethrin in aquatic mesocosms. *FEMS Microbiology Ecology*. 54, 157-165.
Kozub, D.D., Liehr, S.K., 1999. Assessing denitrification rate limiting factors in a constructed wetland receiving landfill leachate. *Water Science and Technology*. 40, 75-82.
Kreuger, J., Peterson, M., Lundgren, E., 1999. Agricultural inputs of pesticide residues to stream and pond sediments in a small catchment in Southern Sweden. *Bulletin of Environmental Contamination and Toxicology*. 62, 55-62.
Langenheder, S., Prosser, J.I., 2008. Resource availability influences the diversity of a functional group of heterotrophic soil bacteria. *Environmental Microbiology*. 10, 2245-2256.
Lehman, J.T., 1980. Release and cycling of nutrients between planktonic algae and herbivores. *Limnology and Oceanography*. 25, 620–632.

Moreno-Mateos, D., Comín, F.A., Pedrocchi, C., Rodríguez-Ochoa, R., 2008. Effects of wetland construction on nutrient, SOM and salt content in semi-arid zones degraded by intensive agricultural use. *Applied Soil Ecology*. 40, 57-66.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*. 59, 695-700.

Nielsen, L.P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. *FEMS Microbiology Ecology*. 86, 357-362.

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology*. 17, 526-538.

Pell, M., Stenberg, B., Torstensson, L., 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio*. 27, 24-28.

Ratcliff, A.W., Busse, M.D., Shestak, C.J., 2006. Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. *Applied Soil Ecology*. 34, 114-124.

Rich, J.J., Myrold, D.D., 2004. Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biology and Biochemistry*. 36, 1431-1441.

Rohr, J.R., Kerby, J.L., Sih, A., 2006. Community ecology as a framework for predicting contaminant effects. *TRENDS in Ecology and Evolution*. 21, 606-613.

Rousseaux, S., Hartmann, A., Rouard, N., Soulas, G., 2003. A simplified procedure for terminal restriction fragment length polymorphism analysis of the soil bacterial community to study the effects of pesticides on the soil microflora using 4,6-dinitroorthocresol as a test case. *Biology and Fertility of Soils*. 37, 250-254.

Rysgaard, S., Risgaard-Petersen, N., Nielsen, L.P., Revsbech, N.P., 1993. Nitrification and denitrification in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing. *Applied and Environmental Microbiology*. 59, 2093-2098.

Sáez, F., Pozo, C., Gómez, M.A., Martínez-Toledo, M.V., Rodelas, B., Gónzalez-López, J., 2006. Growth and denitrifying activity of Xanthobacter autotrophicus CECT 7064 in the presence of selected pesticides. *Applied Microbiology and Biotechnology*. 71, 563-567.

Seitzinger, S., Harrison, J.A., Böhlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., Tobias, C., Van Drecht, G., 2006. Denitrification across landscapes and waterscapes: a synthesis. *Ecological Applications*. 16, 2064-2090.

Song, B., Ward, B.B., 2003. Nitrite reductase genes in halobenzoate degrading denitrifying bacteria. *FEMS Microbiology Ecology*. 43, 349-357.

Sirivedhin, T., Gray, K.A., 2006. Factors affecting denitrification rates in experimental wetlands: field and laboratory studies. *Ecological Engineering*. 26, 167-181.

Stadmark, J., Leonardsson, L., 2005. Emissions of greenhouse gases from ponds constructed for nitrogen removal. *Ecological Engineering*. 25, 542-551.

Sundbäck, K., Groth Petersen, D., Dahllöf, I., Larson, F., 2007. Combined nutrient-toxicant effects on a shallow-water marine sediment system: sensitivity and resilience of ecosystem functions. *Marine Ecology Progress Series*. 330, 13-30.

Svensson, J. M., 1998. Emission of N_2O nitrification and denitrification in a eutrophic lake sediment bioturbated by *Chironomus plumosus*. *Aquatic Microbial Ecology*. 14, 289-299.

Swedish Chemical Agency. KEMI. (http://www.kemi.se, accessed 01/02/2006)

Teske, A., Sigalevich, P., Cohen, Y., Muyzer, G., 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology*. 62, 4210-4215.

Thirup, L., Johnsen, K., Torsvik, V., Spliid, N.H., Jacobsen, C.S., 2001. Effects of fenpropimorph on bacteria and fungi during decomposition of barley roots. *Soil Biology and Biochemistry*. 33, 1517-1524.

Throbäck I.N., Enwall, K., Jarvis, Å., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology*. 49, 401-417.

Throbäck, I.N., Johansson, M., Rosenquist, M., Pell, M., Hallin, S., 2007. Silver (Ag^+) reduces denitrification and induces enrichment of novel nirK genotypes in soil. *FEMS Microbiology Letters*. 270, 189-194.

Van den Brink, P.J., Crum, S.J.H., Gylstra, R., Bransen, F., Cuppen, J.G.M., Brock, T.C.M., 2009. Effects of a herbicide-insecticide mixture in freshwater microcosms: Risk assessment and ecological effect chain. *Environmental Pollution*. 157, 237-249.

Wang, Y-S., Wen, C-Y., Chiu, T-C., Yen, J-H., 2004. Effect of fungicide iprodione on soil bacterial community. *Ecotoxicology and Environmental Safety*. 59, 127-132.

Wendt-Rasch, L., Friberg-Jensen, U., Woin, P., Christoffersen, K., 2003. Effects of the pyrethroid insecticide cypermethrin on a freshwater community studied under field conditions. II. Direct and indirect effects on the species composition. *Aquatic Toxicology*. 63, 373-389.

Widenfalk, A., Svensson, M. J., Goedkoop, W., 2004. Effects of the pesticides captan, delthamethrin, isoproturon, and pirimicarb on the microbial community of a freshwater sediment. *Environmental Toxicology and Chemistry*. 23, 1920-1927.

Widenfalk, A., Bertilsson, S., Sundh, I., Goedkoop, W., 2008. Effects of pesticides on community composition and activity of sediment microbes – responses at various levels of microbial community organization. *Environmental Pollution*. 152, 576-584.

Xia, S., Li, J., Wang, R., 2008. Nitrogen removal performance and microbial community structure dynamics response to carbon nitrogen ratio in a compact suspended carrier biofilm reactor. *Ecological Engineering*. 32, 256-262.

Zedler, J.B., 2003. Wetlands at your service: reducing impacts of agriculture at the watershed scale. *Frontiers in Ecology and the Environment*. 1, 65-72.
Zedler, J.B., 2004. Compensating for wetland losses in the United States. *Ibis*. 146, 92-100.

Paper IV

Leucine incorporation as a rapid, relevant and sensitive method to assess toxicity of fungicides to natural bacterial communities in aquatic environments

Susann Milenkovski[1], Erland Bååth[2], Per-Eric Lindgren[3] and Olof Berglund[1]

[1]Chemical Ecology/Ecotoxicology, Dept of Ecology, Ecology Building, Lund University, SE-223 62 Lund, SWEDEN
[2]Microbial Ecology, Dept of Ecology, Ecology Building, Lund University, SE-223 62 Lund, SWEDEN
[3]Department of Clinical and Experimental Medicine, Division of Medical Microbiology, Linköping University, SE-581 85 Linköping, SWEDEN

Abstract

We compared the sensitivity of two endpoints indicating bacterial activity, leucine incorporation and potential denitrification, in detecting effects of fungicides on natural bacterial communities from constructed wetlands. The effects of eight fungicides (benomyl, carbendazim, carboxin, captan, cycloheximide, fenpropimorph, propiconazole and thiram), two bactericides (bronopol and chlortetracycline) as controls, and one reference compound (3,5-dichlorophenol), in the concentration ranges 0.005-75, 0.010-100 or 0.025-250 mg l^{-1}, were tested in a water-sediment, microcosm set-up. Leucine incorporation was measured in both the water and sediment column, while potential denitrification was measured for the entire microcosm. With one exception, the bactericides and the reference compound all gave sigmoid concentration-response curves on both endpoints. The fungicides thiram, captan and benomyl, and to a lesser extent fenpropimorph and propiconazole had quantifiable toxic effects on leucine incorporation, while carbendazim, carboxin and cycloheximide had little effect at the investigated concentrations. Only thiram and captan inhibited potential denitrification, the other fungicides showed no quantifiable effect. Additionally, a higher toxic effect on leucine incorporation was recorded for bacterial communities associated with the water column compared to the sediment column for all tested compounds. Thus, leucine incorporation was a more sensitive method for toxicity assessment of bacterial communities, and also allowed for a rapid and simple way of comparing exposure in the sediment and water column, making it an attractive standard method for community based toxicological assays in aquatic environments.

1. Introduction

During the last decade, increased attention has been directed towards the usefulness of wetlands for ameliorating several environmental problems of concern, e.g. pollution of aquatic environments and decreased biodiversity in the agricultural landscape (Lovely, 2003, Biggs et al., 2005; Thiere et al., in press). One particular area of interest is their capacity for reducing downstream nutrient transport from agricultural land to coastal areas and oceans, where nitrogen pollution has been of special concern (Stadmark and Leonardsson, 2005). Consequently, large-scale programmes for restoring and recreating wetlands in agricultural areas have been initiated in e.g. Sweden and North America to reduce nitrogen transport (Swedish Board of Agriculture, 2000; Farm Service Agency, 2004). Bacterial communities, responsible for the bulk of the organic matter transformation and nutrient recycling in aquatic environments, are considered as key players in the nitrogen removal processes in wetlands. In areas of intensive agriculture, aquatic environments are, however, also exposed to agricultural pesticides, which are regularly detected in surface waters in these areas (Kreuger et al., 1999; Haarstad and Braskerud, 2005). Although many pesticides have designated target organisms, due to specific modes of action, they may have more general toxic effects on non-target organisms, including microorganisms (Pell et al., 1998; Johnsen et al., 2001; DeLorenzo et al., 2001). If wetland bacterial communities are affected by pesticides, the removal of nutrients may be altered and, thus, wetland function may be impaired. Thus, proper environmental risk assessments of agricultural pesticides in surrounding aquatic environments should include studies of bacterial community responses. It is therefore of importance that direct and sensitive methods for toxicity assessment of potential pesticide effects on bacterial communities are evaluated and made available for routine analysis.

Although still not routinely performed, microbial toxicity testing on a community level provides an attractive alternative to single-species microbial toxicity tests and the advantages of testing natural assemblages of microorganisms in microcosm experiments has been advocated (Brandt et al., 2004; Petersen et al; 2004; Sundbäck et al., 2007; Widenfalk et al., 2008). However, there are several unresolved issues, of both scientific and practical nature, regarding choice of endpoints and experimental design. Structural endpoints using modern techniques (e.g. DNA fingerprinting approaches) may be more sensitive than functional endpoints, as the latter may fail to detect changes in composition due to the inherent functional redundancy of the bacterial community. However, structural changes does not imply whether a chemical is toxic or not, and data interpretation of structural changes in community level tests is not always straight forward. Hence, extrapolation to natural environments is uncertain and the predictive ability may be compromised. Also, these methods are both costly and time-consuming compared to some functional endpoints. Previous studies investigating environmentally relevant concentrations have sometimes failed to

detect concentration-response relationships (Widenfalk et al., 2004, 2008; Van den Brink et al., 2009). It has been argued that concentration-response relationships of pesticides exposure, including high concentrations, on bacterial communities have little environmental relevancy (Widenfalk et al., 2004), and are therefore often disregarded. However, to compare and evaluate different toxicity assays using only few concentrations of a pesticide is difficult due to the failure of detecting a toxic effect, and, deriving clear concentration-response relationships would also avoid spurious negative effects. Also, concentration-response studies can provide understanding of the toxic mechanism on the non-target organisms and enable general predictions or extrapolations to natural environments, and to determine accurate effect concentrations (EC) of pesticides on non-target organisms, a concentration-response dilution series is of fundamental importance (Schweiger and Jakobsen, 1998; De Zwart and Posthuma, 2005; Rousk et al., 2008). Thus, suitable endpoint measurements should not only be sensitive in detecting toxic effects from pesticide exposure, but also allow for extensive replication in order to achieve proper concentration-response curves.

The nitrogen removal in agricultural wetlands is dependent on the denitrification rate, the transformation of nitrate to dinitrogen gas, mainly performed by the denitrifying bacterial community in the sediment/water interface (Seitzinger et al., 2006; Wallenstein et al., 2006). A relevant test system for pesticide toxicity assessment in wetlands would, thus, be natural sediment assemblages measuring potential denitrification as an endpoint. Denitrification has earlier been evaluated as an endpoint of intermediate sensitivity to toxic compounds (Domsch et al., 1983) but has later been suggested as a valuable method for toxicity screening (Pell et al., 1998; Kreuger et al., 1999). However, it is possible that over short-term, community denitrification, similar to respiration, will be insensitive to toxic compounds (Broos et al., 2005), partly because it is a common trait among bacteria, but also due to bacteriostatic conditions, when bacterial function will proceed even though bacterial growth may be inhibited (Rousk et al., 2008). These obstacles could be over-come if growth is directly measured. Leucine incorporation, which measures a proxy of bacterial growth, has earlier been shown to be a sensitive, and also cost- and time-efficient, method to detect toxic effects in natural environments. Although less commonly used than bacterial respiration and/or biomass, the leucine incorporation has been successfully employed to assess effects of heavy metals (Díaz-Raviña and Bååth, 2001; Shi et al., 2002; Almeida et al., 2007), surfactants (Brandt et al., 2004), phenols (Aldén Demoling and Bååth, 2008), antifouling biocides (Petersen et al., 2004) and antibiotics (Rousk et al., 2008). Leucine incorporation also has the advantage of being easily measured in different matrixes e.g. soil, sediment and water (Kirchman et al., 1985; Bååth, 1994; Fischer and Pusch, 1999; Kirschner and Velimirov, 1999).
The aim of the present study was to evaluate the potential of microbial community level test systems, to assess toxicity of pesticides in wetland systems.

We assessed the sensitivity of the functional endpoints potential denitrification and leucine incorporation in detecting pesticide toxicity on natural wetland bacterial communities in sediment/water microcosms. We tested eight different fungicides, including also two bacterial antibiotics and a reference compound, 3,5-dichlorophenol under 18h of incubation of the microcosms. Concentration-response relationships were tested for all substances, and toxicity was also compared between the sediment and the water matrix using the leucine incorporation method.

2. Materials and methods

2.1. Wetland sediment

Sediment and surface water were collected from a constructed wetland in Frihult, in the south of Sweden (55°33′N, 13°39′E) in June 2008. The wetland has an area of 2800 m^2 has a mean depth of < 1.0m. Surface water temperature at the time of sampling was 21°C and pH was 9.1. The wetland is > 60 yrs old and is situated in an agricultural landscape. The agricultural fields are organically farmed, hence no pesticides have been applied since 2001.

The sediment was collected with pre-washed polyethylene core samplers, 10 cm in diameter and a height of 60 cm. Sediment cores, 10-25 cm from the sediment surface, plus 5 cm overlaying water, was collected from sites in the centre of the wetland. Additionally, wetland surface water was collected. Upon arrival to the laboratory, the sediment was sieved through a 1 mm mesh to remove large materials, and the water was filtered through 1.2 µm glass fibre filters (GF/C, Whatman, England). Water content of the sediment was determined to 81.7 ± 0.1% (n=5, 105°C, over night) and organic matter content, measured as loss on ignition, was 29.6 ± 0.2% (n=4, 550°C for 2 h). The sediment and water were kept dark at 4°C until onset of the microcosm experiments.

2.2. Pesticides

Eight fungicides (benomyl, carbendazim, carboxin, captan, cycloheximide, fenpropimorph, propiconazole and thiram, Ehrenstorfer Reference standards, determined purity ≥ 97%), two bactericides (bronopol and chlortetracycline) as controls, and 3,5-dichlorophenol (Sigma-Aldrich) as reference compound were used. The fungicides were chosen because they are or have been used world wide and represent different modes of action.

Different concentration ranges of each compound were selected based on water solubility (Table 1) of the compounds. The bactericide bronopol was analysed at two separate times and concentration ranges (bronopol I and II) to test if repeated

measures would reproduce similar results. A lower dilution series, 0.005-75 mg l^{-1} was used for propiconazole and bronopol I, an intermediate, 0.010-100 mg l^{-1} for benomyl, carbendazim, captan, fenpropimorph and thiram, and a higher, 0.025-250 mg l^{-1} for bronopol II, carboxin, cycloheximide, chlortetracycline and 3,5-dichlorophenol. Stock solutions of all pesticides were prepared in dimethylsulfoxide (DMSO), resulting in final microcosm concentrations of 0.1 %, earlier shown to have no effect on bacteria (Kahru et al., 1996; Widenfalk et al., 2008).

Table 1. Properties of the tested pesticides.

Pesticide	Cas number	Classification	log K_{ow}	Water solubility (mg l^{-1})
Benomyl	17804-35-2	fungicide	2.42[a]	3.8 at 25°C[a]
Carbendazim	10605-21-7	fungicide	1.56[a]	8.0 at 25°C[a]
Carboxin	5234-68-4	fungicide	2.18[a]	170 at 25°C[a]
Captan	133-06-2	fungicide	2.35[a]	8.7 at 25°C[a]
Cycloheximide	66-81-9	fungicide	0.55[c]	21g l^{-1} at 2°C[c]
Fenpropimorph	67564-91-4	fungicide	4.20[a]	4.3 at 20°C[b]
Propiconazole	60207-90-1	fungicide	3.50[a]	110 at 20°C[a]
Thiram	137-26-8	fungicide	1.73[a]	17.4 at 22°C[a]
Bronopol	52-51-7	bactericide	−0.64[c]	250 g l^{-1} at 22°C[c]
Chlortetracycline	57-62-5	bactericide	−0.62[c]	630 at 25°C[c]
3,5-dichlorophenol	591-35-5	reference compound	3.62[c]	5380 at 25°C[c]

a=Mackay et al., 2006
b=Tadeo et al., 2007
c=National Library of Medicine, US, ChemlDplus, 2003

2.3. Experimental setup

The microcosms consisted of 12 ml glass vials, filled with 1.2 ± 0.1 g wet weight of sediment, representing the sediment column. Before the sediment was added it was homogenized by gentle stirring. Then 8 ml of filtered wetland water, containing the respective pesticide, was added, representing the water column. A head space of 3 ml was left in each microcosm. The nominal pesticide concentrations were calculated as: nominal pesticide concentration in microcosm = diluted stock solution concentration / (added volume water + added volume sediment). Controls with and without DMSO (n = 3, for each control) were prepared for all pesticides. All microcosms were vigorously shaken twice for 30 s and then bubbled with nitrogen gas for 5 min to generate an anaerobic environment resembling natural wetland conditions.

Two separate sets of microcosms were prepared with a pesticide dilution series of 13 concentrations: one set for the leucine incorporation assay and one set for the potential denitrification assay. Measurements of leucine incorporation were performed on both the water and the sediment column, which will hereafter be referred to as leucine-water and leucine-sediment. Measurements of the potential denitrification assay did not separate between the two columns and represent both the water and sediment column. The microcosms were shaken once during the 18 h of incubation time, and kept dark during the whole incubation at room temperature (21°C) before analysis.

2.4. Leucine incorporation

We measured leucine incorporation (indicating bacterial growth) according to Smith and Azam (1992) with some modifications used for soil bacteria (Bååth et al., 2001). Before analysis, water and sediment columns were separated. A 1.5 ml water sample was taken at a predetermined depth of 5 cm from the top of the microcosm and transferred into a 2 ml microcentrifuge vial (used for leucine-water). The remaining water column in the microcosm was discharged. The sediment was then shaken with 20 ml distilled water in 50 ml centrifuge tubes for 3 min on a vortex at full speed, followed by low-speed centrifugation (1000 g for 8 min). Thereafter 1.5 ml of the supernatant was taken from a predetermined depth of 7 cm from the top of the centrifuge tube and put into a 2 ml microcentrifuge vial (used for leucine-sediment).

To the 1.5 ml bacterial suspensions 2 µl [^{3}H]leucine (37 MBq ml^{-1}. 5.74 TBq mmol^{-1}, Amersham) was added together with non-labelled leucine, resulting in 275 nM leucine in the bacterial suspension. The samples were incubated for 1 h at room temperature (21°C). Then 75 µl ice-cold 100% trichloroacetic acid (TCA) was added to stop bacterial growth. Washing and subsequent measurement of incorporated radioactivity were performed as described by Bååth et al. (2001).

2.5. Potential denitrification

We used the acetylene inhibition method to measure potential denitrification (Knowles, 1982; Bernot et al., 2003) in our microcosms. We added a nutrient solution, with nitrogen (N) as KNO^{3} and carbon (C) as CH_3COONa, to achieve a 1:5 ratio of N:C (Ingersoll and Baker, 1998; Xia et al., 2008). The amount of N required to avoid nutrient limitation of the denitrifying community in the 12 ml microcosms during incubation was estimated to 3 mg l^{-1} (S. Milenkovski, unpublished data). After 18 hrs incubation of the microcosms, acetylene gas was first bubbled through concentrated sulphur acid and distilled water, with two empty bottles between the solutions, to remove acetone. Then the nutrient solution was bubbled with acetylene (C_2H_2) gas for 10 min, and 1 ml of the acetylene saturated nutrient solution was added to each microcosms. Acetylene

inhibits the last step in the denitrification pathway (nitrous oxide to nitrogen gas), resulting in nitrous oxide (N_2O) as the final step. Measurements of produced N_2O indicating potential denitrification were performed at 1 h and 6 hrs after adding nutrients. Before measurements the microcosms were shaken for 1 min and thereafter 0.5 ml gas sample was taken with a syringe and analysed on a GC (Varian GC 3400 with an electron capture detector). Standards with fixed N_2O amounts were used to calculate the amount of N_2O in each microcosm.

2.6 Statistical analysis

To assess the toxic effect of pesticides we calculated the logarithm of the concentrations resulting in 50% and 10% inhibitory effect of the bacterial community (EC_{50} and EC_{10} indicating standardized toxicity and threshold toxicity, respectively). The log EC_{50} and log EC_{10} values were calculated using the logistic model $Y = c/[1 + e^{b(X-a)}]$, where Y is the leucine incorporation rate or the N_2O production rate, X the logarithm of toxicant concentration, a the log EC_{50}, c the rate in the control, and b a slope parameter indicating the inhibition rate. The program Statistica® (StatSoft, Tulsa, OK, USA) was used to fit the data using nonlinear regression. To analyse reproducibility, we conducted a paired t-test to compare the mean values of the two separate tests of bronopol I and II. To analyse if the pesticides log K_{ow} and water solubility values may be used to predict the effects on bacterial communities in the water and sediment column, respectively, a simple regression was used to find possible linear relationships between the ratio of leucine-water (EC_{50}) and leucine-sediment (EC_{50}) with each pesticide property.

3. Results

Leucine incorporation rates or potential denitrification rates did not differ between the controls with and without DMSO. The mean and the standard deviation values of the controls without and with DMSO were, 1.8 ± 3.6, and 1.7 ± 3.4 mol N_2O/h in the potential denitrification assay, 106000 ± 27000, and 92000 ± 27000 dpm in the leucine-water assay, and 21000 ± 6000, and 23000 ± 13000 dpm in the leucine-sediment assay, respectively. Control data were pooled, and standard errors indicated in Fig. 1 are based on 6 control microcosms. The bactericide bronopol was tested twice at separate occasions using different concentration ranges (Bronopol I and II) with no significant differences (Paired t-tests, $p > 0.1$) in EC_{50}-values between the assays with any of the endpoints used (Table 2). Hereafter, we will only refer to results based on bronopol II. The EC_{50} values, in the results and discussion, hereafter represents the mean values from the calculated nonlinear regressions curves.

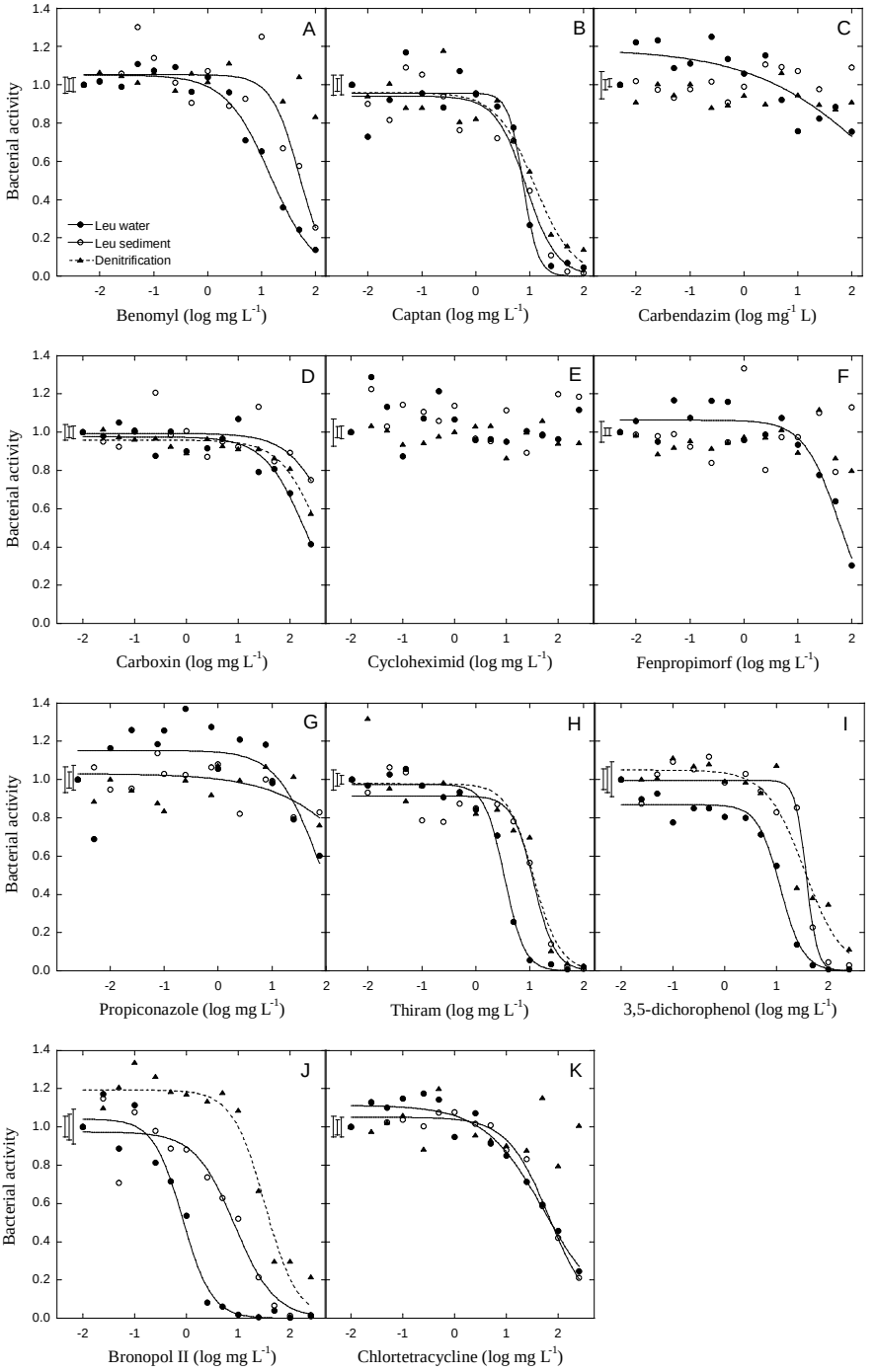

Figure 1. Concentration-response relationships of the studied pesticides on the activity of the aquatic bacterial communities. Leucine-water and leucine-sediment indicates toxicity measured using leucine incorporation in water and sediment, respectively, while

denitrification denotes toxicity measured using potential denitrification in the whole microcosm including both water and sediment. The error bars from left to right correspond to SE for controls of leucine-water (filled dots), leucine-sediment (open dots) and potential denitrification (filled triangles) (n=6). Lack of fitted line indicates no concentration-response effect.

The bactericides included as positive controls showed clear concentration-response curves for all three assays using bronopol (Fig. 1J) and for leucine-water and leucine-sediment using chlortetracycline (Fig. 1K). Concentration-response curves for all three assays were also obtained for the tests with the reference compound 3,5-dichlorophenol (Fig. 1I). The bactericides and the reference compound gave similar results, having the largest effect in the leucine-water assay, followed by the leucine-sediment assay, and the least effect on potential denitrification (Table 2). Bronopol had the lowest EC_{50} for the leucine-water assay, 1.18 mg l^{-1}, followed by the leucine-sediment assay, 8.6 mg l^{-1}, and EC_{50} on potential denitrification was highest 32.6 mg l^{-1} (Table 2). Chlortetracycline was less toxic with EC_{50} values of 55.2 mg l^{-1} in the leucine-water assay, 68.1 mg l^{-1} in the leucine-sediment assay, and no quantifiable effect on potential denitrification (Table 2). The reference compound, 3,5-dichlorophenol had an EC_{50} value of 11.9 mg l^{-1} in the leucine-water assay, 37.7 mg l^{-1} in the leucine-sediment assay, and 34.8 mg l^{-1} on potential denitrification (Table 2).

The fungicides thiram, captan and benomyl strongly inhibited leucine incorporation both in the water and in the sediment column. EC_{50} values of the three fungicides were 3.4, 7.6 and 14.6 mg l^{-1} in the leucine-water assay, and 12.0, 8.6 and 50.3 mg l^{-1} in the leucine-sediment assay. Fenpropimorph and propiconazole had moderate inhibitory effects, only were detected in the leucine-water assay (EC_{50} values of 57.5 mg l^{-1} and 71.7 mg l^{-1}, respectively). Carbendazim, carboxin and cycloheximide had little effect in the leucine-water or leucine-sediment assays with EC_{50} values >100 mg l^{-1} (Table 2, Fig. 1). In all leucine incorporation assays the toxicity was higher in the water phase than in the sediment, with consistently lower EC_{50} values in the leucine-water assays compared to the leucine-sediment assays. Ratios of EC_{50} values for leucine-water (EC_{50}) and leucine-sediment (EC_{50}) varied between 0.11 and 0.94. The ratios were not related to the log K_{ow} or the water solubility of the tested compounds (Simple regression, $p>0.5$).

Table 2. Toxicity assessments by three separate assays. Leucine-water and leucine-sediment indicates toxicity measured using leucine incorporation in water and sediment, respectively, while denitrification denotes toxicity measured using potential denitrification in the whole microcosm including both water and sediment. r^2 indicates the fit of the logistic equation used to calculate EC_{50} and EC_{10}. The range of EC_{50} ± SE is given within parenthesis.

Pesticid	Phase	EC_{50} ± SE (mg l^{-1})	EC_{10} (mg l^{-1})	r^2
Thiram	Leucin water	3.4 (3.2-3.7)	1,4	0,99
	Leucin sediment	12.0 (10.3-14.0)	4,4	0,95
	Denitrification	11.8 (9.3-14.9)	3,4	0,91
Captan	Leucin water	7.6 (6.6-8.7)	3,8	0,94
	Leucin sediment	8.6 (7.1-10.4)	2,0	0,95
	Denitrification	12.2 (9.7-15.4)	2,0	0,92
Benomyl	Leucin water	14.6 (12.9-16.5)	1,8	0,98
	Leucin sediment	50.3 (39.9-63.5)	13,5	0,78
	Denitrification	>100		
Fenpropimorph	Leucin water	57.5 (49.2-67.2)	11,0	0,90
	Leucin sediment	>100		
	Denitrification	>100		
Propiconazole	Leucin water	71.7 (42.0-122.1)	10,0	0,48
	Leucin sediment	>100		
	Denitrification	>100		
Carbendazim	Leucin water	>100		
	Leucin sediment	>100		
	Denitrification	>100		
Carboxin	Leucin water	>100		
	Leucin sediment	>100		
	Denitrification	>100		
Cycloheximide	Leucin water	>100		
	Leucin sediment	>100		
	Denitrification	>100		
Bronopol I	Leucin water	0.59 (0.48-0.72)	0,2	0,95
	Leucin sediment	11.9 (9.2-15.3)	1,4	0,90
	Denitrification	39.2 (26.0-59.2)	13,2	0,54
Bronopol II	Leucin water	1.18 (1.00-1.38)	0,2	0,97
	Leucin sediment	8.6 (6.6-11.1)	1,2	0,94
	Denitrification	32.6 (27.1-39.1)	6,7	0,93
Chlortetracycline	Leucin water	55.2 (46.2-65.9)	2,9	0,96
	Leucin sediment	68.1 (61.9-74.9)	8,8	0,98
	Denitrification	>100		
3,5-dichlorophenol	Leucin water	11.9 (10.7-13.4)	3,9	0,98
	Leucin sediment	37.7 (34.2-41.4)	21,9	0,96
	Denitrification	34.8 (28.1-43.1)	5,4	0,92

Only two of the fungicides, thiram and captan, strongly inhibited potential denitrification, with EC_{50} values of 11.8 mg l^{-1} and 12.2 mg l^{-1}, respectively. The other six fungicides had little or no detectable effect on potential denitrification in the concentration ranges used, with EC_{50} values >100 mg l^{-1} (Table 2). Thus, the toxic effect in the potential denitrification assays was always less than, or similar to, that in the leucine incorporation assays, irrespective whether the latter was measured in the sediment or in the water phase of the microcosms.

4. Discussion

The importance of assessing functional bacterial groups, especially the nitrogen transforming bacterial communities, in toxicity testing has previously been emphasized (Pell et al., 1998; Kreuger et al., 1999; Johnsen et al., 2001; Petersen et al., 2004; Larson et al., 2007). Authorities controlling pesticide registration have also required assessments over effects of pesticides on functional bacterial groups for pesticide approval. Our study showed, however, that leucine incorporation was a more sensitive method than potential denitrification in detecting effects of pesticides on constructed wetland bacterial communities. The leucine incorporation assay detected toxic effects for a higher number of fungicides, and produced lower EC_{50}-values for the tested compounds than the potential denitrification assay, irrespective of being measured in the water or the sediment column in the microcosms. Leucine incorporation has previous been shown to be a more sensitive method than respiration measurements in arable soil, even when respiration was measured after adding substrate (Rousk et al., 2008). Thus, leucine incorporation seems to be more sensitive than both potential denitrification and respiration, two methods that are often applied in environmental toxicological tests (Pell et al., 1998; Bailey et al., 2003; Sáez et al., 2006), although, leucine incorporation being less sensitive than other endpoints have also been reported (Petersen et al., 2004; Sundbäck et al., 2007). Given that leucine incorporation is a rapid and easily standardized method, ours and added results suggest that measurement of bacterial growth using leucine incorporation should be an attractive standard endpoint to assess toxicity of pesticides both in soil and aquatic systems using natural microbial communities.

The reference compound 3,5-dichlorophenol had an EC_{50} value of 11.9 mg l^{-1} for the leucine-water assay, fulfilling the criteria in the ISO standard of an EC_{50} between 4 and 12 mg l^{-1} (ISO 15522), albeit in the higher end of the range. For the leucine-sediment assay and the potential denitrification assay, EC_{50} values were higher (>30 mg l^{-1}) than the ISO criterion. However, our microcosm test systems consisted of natural, environmental samples containing high amounts of organic matter, especially the sediment. Such samples may not be directly comparable to pure culture systems, where the ISO standard most often is used, or to extracted bacteria without the natural matrixes (Christensen et al., 2006). The EC_{50}–values derived for bronopol did not differ between the two tests

differing in dates and concentration ranges used, and the variation in controls were low. Thus, it seems possible to obtain reproducibility and repeatability in these microcosm systems, using natural wetland bacterial communities with functional endpoints, particularly by using the leucine incorporation technique. For compounds inducing toxic effects in the concentration ranges tested, EC_{50} values could successfully be derived from a logistic model with high degree of explanation, r^2 was generally in the range of 0.80 - 0.99. Thus, concentrations replicates are probably not required for these test systems, which will facilitate range finding in the toxicity testing procedure. Stochastic effects involved in the succession of natural microbial communities may reduce the ability to perform controlled, replicated toxicity tests, especially in longer-term tests (Brandt et al., 2004; Widenfalk et al., 2008). In this study, the analysis of our microcosm systems did not seem to suffer greatly from stochastic effects during this relatively shorter test-duration, so this problem may be avoided using these methods.

Generally, a close correlation is expected between bacterial growth and activity, measured as respiration (during aerobic conditions) and denitrification (during anaerobic conditions) (Miller et al., 2008). However, no apparent correlation is found in shorter-term assays, measuring endpoints within a short period of time (< 30 h) after adding toxicants (present study, Rousk et al., 2008), indicating an uncoupling between growth and activity during this time. This phenomenon has also been observed earlier during short term incubations at high temperatures (Pietikäinen et al., 2005). The most likely mechanism is that respiration and potential denitrification will proceed also under situations of no growth, due to the enzymes still being active, or to the toxicant having a bacteriostatic rather than a bacteriocidal effect (Welp and Brümmer, 1997; Loffhagen et al., 2003). The latter means that the bacteria will still metabolize, but growth will be restricted. Another explanation could be that the functional redundancy of the denitrifying bacterial community supports an unchanged function even though parts of the community may be inhibited or killed (Girvan et al., 2005). For the purpose of exploring the mechanisms of functional redundancy, complementary toxicity tests with structural endpoints would be to recommend. However, the shorter incubation time used here to avoid stochastic effects may be rather short to detect changes in structural endpoints. Thus, assessment of bacterial growth (leucine incorporation) will always be an equally, or more sensitive endpoint than methods based on general process measures, like respiration and potential denitrification, although a possible functional redundancy of bacteria may lend support to a combination of structural and functional endpoints in assessments.

Previous studies have assessed toxicity on bacterial communities from sediment samples Widenfalk et al., 2004), soils (see review by Johnsen et al., 2001), activated sludge (Christensen et al., 2006), and pure cultures (Christensen et al., 2006; Park and Choi, 2008), while toxicity studies on bacterial communities in water samples are less common (Maraldo and Dahllöf, 2004), and few have

measured toxicity by simultaneously using the same methodology in both water and sediment (Mankiewicz-Boczek et al., 2008). Furthermore, ambient pesticide concentrations in aquatic systems are most often reported on surface water samples, while studies of pesticide concentration in the sediment are scarce (Warren et al., 2003). As a result, there is some inconsistency in which methods to use and which matrix to analyse when performing toxicity assessment on bacterial communities in aquatic habitats. Our result imply that consideration should be made regarding choosing the most sensitive group of organism in the ecosystem, but also regarding the most sensitive method and which matrix to test in toxicity assessments. Structural bacterial endpoints using molecular fingerprinting have been shown useful and in some cases more sensitive than leucine incorporation in assessing toxicity (Brandt et al., 2004). However, it cannot be distinguished whether structural endpoints reflect changes due to direct toxic effects or if they reflect differences in growth of bacterial groups with differing ability to degrade the tested compound. The leucine incorporation technique has the advantage of being a direct method that measures integrated toxic effects. Thus, leucine incorporation, which appears to work equally well under aerobic and anaerobic conditions (Bastviken and Tranvik, 2001) may be used to directly assess toxicity on bacterial communities in both the water column and the sediment matrix in aquatic environments such as wetlands.

Our study suggests that the added pesticides were more toxic to the bacterial community in the water column than in the sediment column. One explanation may be that the tested pesticides in general were less bioavailable to the bacterial communities in the sediment column due to association with organic matter in the sediment (Welp and Brümmer, 1999), although we found no correlation between neither the log K_{ow}, nor the water solubility of compounds, and the ratio between EC_{50}-values in water and sediment. Hence, lipophilicity or hydrophibicity of a substance could not be directly used to predict differences in toxicity. However, more studies using toxicants with a wider range of K_{ow} and water solubility and chemical analysis of the partitioning between the two phases are needed to test whether the differences in toxicity are due to bioavailability or differences in sensitivity between water-, and sediment-associated bacterial communities.

By using the leucine incorporation we detected a strong inhibitory effect after exposure to three fungicides, thiram, captan and benomyl, while fenpropimorph and propiconazole only showed minor inhibitory effects. The other fungicides did not have any detectable effect on the bacterial community at the tested concentrations. Leucine incorporation was also used by Rousk et al. (2008), who studied three of the fungicides used in the present study, recording the same relative toxicity on soil microorganisms as on leucine-sediment assay, being highest for captan, followed by benomyl and with negligible effects of cycloheximide. Inhibitory effects of captan on leucine incorporation in freshwater sediment were also found by Widenfalk et al. (2004), although only at

intermediate concentrations (that is, no concentration-response effect), and in a later study no effect of captan on leucine incorporation was found (Widenfalk et al., 2008). Thus, the risk of failing to detect toxic effects on bacterial communities increases, by using few, environmental concentrations of the tested pesticides. Therefore, concentration-response studies are better suited for general toxicity assessments than using environmental concentrations. Unexpectedly, thiram and captan had lower EC_{50} values than both the bactericide chlortetracycline and the reference compound, 3,5-dichlorophenol. This suggests that these two fungicides have stronger inhibitory effects on non-target bacterial organisms than the bactericide on its target organisms. Effects on non-target organisms from fungicide exposure have been detected in previous studies (Schmidt et al., 2000). Thiram and captan have been found to have stronger inhibitory effects on bacteria when compared to benomyl and carboxin (Cameron and Julian, 1984), corroborating our results. These two fungicides, thus, are hazardous to bacterial communities and may constitute a risk since they are commonly used worldwide with a broad application field (Chen et al., 2001; Ekundayo, 2002). Interestingly, the most toxic fungicides to bacteria detected in this study, thiram, captan and benomyl, are prohibited from agricultural use in Sweden as they are thought to have an excessively high risk of general toxicity. However, their high EC_{50} and EC_{10} values derived in this study, suggest only minor environmental risks towards constructed wetland bacterial communities. All the tested fungicides in the present study represent active ingredients (a.i.), included in commercially used pesticide mixtures with different concentration fractions. The recommended application dose on agricultural fields of an a.i. determined by the authorities are much lower than the dose required to reach the concentrations of the calculated EC_{10} values of the fungicides in the present study. Thus, by following the recommended a.i. application, the fungicides tested in present study should constitute minor risks for the natural non-target bacterial communities in aquatic environments.

In conclusion, our study showed that leucine incorporation was more sensitive in detecting toxic effects from pesticides on bacterial communities than potential denitrification. In addition, this study showed higher toxicities to bacterial communities in the microcosm water column than in the sediment column. Our study suggests that sediment-water microcosms with leucine incorporation, as a bacterial community endpoint, is a rapid, relevant and sensitive method to detect toxicity to microbial communities in aquatic systems. Leucine incorporation could be used as endpoint in both water and sediment assays, allowing for simple, rapid and low-cost test-systems comparing effects in different habitats of wetland ecosystems.

Acknowledgements

This study was partly funded by FORMAS, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (grant nos. 25/2-2001-

2677 and 214-2004-1678), and the Crafoord Foundation (grant no. 20050957). The authors would like to thank Johanna Stadmark and Lars Leonardson for the use and support of the GC.

References

Aldén Demoling, L., Bååth, E., 2008. The use of leucine incorporation to determine the toxicity of phenols to bacterial communities extracted from soil. *Applied Soil Ecology*. 38, 34-41.

Almeida, A., Cunha, Â., Fernandes, S., Sobral, P., Alcântara, F., 2007. Copper effects on bacterial activity of eustarine silty sediments. *Eustarine Coastal and Shelf Science*. 73, 743-752.

Bastviken, D., Tranvik, L., 2001. The leucine incorporation method estimates bacterial growth equally well in both oxic and anoxic lake water. *Applied and Environmental Microbiology*. 67, 2916-2921.

Bernot, M.J., Dodds, W.K., Gardner, W.S., McCarthy, M.J., Sobolev, D., Tank, J.L., 2003. Comparing denitrification estimates for a Texas estuary by using acetylene inhibition and membrane inlet mass spectrometry. *Applied and Environmental Microbiology*. 69, 5950-5956.

Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Weatherby, A., 2005. 15 years of pond assessment in Britain: results and lessons learned from the work of pond conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems*. 15, 693–714.

Brandt, K.K., Jørgensen, N.O.G., Nielsen, T.H., Winding, A., 2004. Microbial community-level toxicity testing of linear alkylbenzene sulfonates in aquatic microcosms. *FEMS Microbiology Ecology*. 49, 229-241.

Broos, K., Mertens, J., Smolders, E., 2005. Toxicity of heavy metals in soil assessed with various soil microbial and plant growth assays: As comparative study. *Environmental Toxicology and Chemistry*. 24, 634-640.

Bååth, E., 1994. Measurement of protein synthesis by soil bacterial assemblages with the leucine incorporation technique. *Biology and Fertility of Soils*. 17, 147-153.

Bååth, E., Petterson, M., Söderberg, K.H., 2001. Adaption of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. *Soil Biology and Biochenistry*. 33, 1571-1574.

Cameron, H.J., Julian, G.R., 1984. The effects of four commonly used fungicides on the growth of Cyanobacteria. *Plant and Soil*. 78, 409-415.

Chen, S-K., Edwards, C.A., Subler, S., 2001. Effects of the fungicides benomyl, captan and chlorothalonil on soil microbial activity and nitrogen dynamics in laboratory incubations. *Soil Biology and Biochemistry*. 33, 1971-1980.

Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. *Environmental Toxicology and Chemistry*. 25, 2208-2215.

DeLorenzo, M.E., Scott, G.I., Ross, P.E., 2001. Toxicity of pesticides to aquatic microorganisms: a review. *Environmental Toxicology and Chemistry*. 20, 84-98.

De Zwart, D., Posthuma, L., 2005. Complex mixture for single and multiple species: proposed methodologies. *Environmental Toxicology and Chemistry*. 24, 2665-2676.

Díaz-Raviña, M., Bååth, E., 2001. Response of soil bacterial communities pre-exposed to different metals and reinoculated in an unpolluted soil. *Soil Biology and Biochemistry*. 33, 241-248.

Domsch, K.H., Jagnow, G., Andersson, T-H., 1983. An ecological concept for assessment of side-effects of agrochemicals on soil microorganisms. *Residue Reviews*. 86, 65-105.

Ekundayo, E.O., 2002. Effect of common pesticides used in the niger delta basin of Southern Nigeria on soil microbial populations. *Environmental Monitoring and Assessment*. 89, 35-41.

Farm Service Agency, 2004. United States Department of Agriculture. Farmable wetlands program, fact Sheet: 10. http://www.fsa.usda.gov (accessed 28/09/07).

Fischer, H., Pusch, M., 1999. Use of the [^{14}C]leucine incorporation technique to measure bacterial production in river sediments and the epiphyton. *Applied and Environmental Microbiology*. 65, 441-4418.

Haarstad, K., Braskerud, B.C., 2005. Pesticide retention in the watershed and in a small constructed wetland treating diffuse pollution. *Water Science and Technology*. 51, 143-150.

Ingersoll, T. L., Baker, L. A., 1998. Nitrate removal in wetland microcosms. *Water Research*. 32, 677-684.

ISO 15522, International Organization of Standardization., 1999. Water quality – determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms. Geneva. Switzerland.

Johnsen, K., Jacobsen, C.S., Torsvik, V., Sørensen, J., 2001. Pesticide effects on bacterial diversity in agricultural soils – a review. *Biology and Fertility of Soils*. 33, 443-453.

Kahru, A., Tomson, K., Pall, T., Külm, I., 1996. Study of toxicity of pesticides using luminiscent bacteria *Photobacterium phosphoreum*. *Water Science and Technology*. 33, 147-154.

Kirchman, D., K´nees, E., Hodson, R., 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. *Applied and Environmental Microbiology*. 49, 599-607.

Kirschner, A.K.T., Velimiron, B., 1999. Benthic bacterial secondary production measured via simultaneous 3H-thymidine and 14C-leucine incorporation, and its implication for the carbon cycle of a shallow macrophyte-dominated backwater system. *Limnology and Oceanography*. 44, 1871-1881.

Knowles, R., 1982. Denitrification. *Microbiological Reviews*. 46, 43-70.

Kreuger, J., Peterson, M., Lundgren, E., 1999. Agricultural inputs of pesticide residues to stream and pond sediments in a small catchment in Southern Sweden. *Bulletin of Environmental Contamination and Toxicology*. 62, 55-62.

Larson, F., Petersen, D.G., Dahllöf, I., Sundbäck, K., 2007. Combined effects of an antifouling biocide and nutrient status on a shallow-water microbenthic community. *Aquatic Microbial Ecology*. 48, 277-294.

Lovley, D., 2003. Cleaning up with genomics: applying molecular biology to bioremediation. *Nature Reviews*. 1, 35-44.

Mackay, D., Shiu, W.Y., Ma, K-C., Lee, S.C., 2006. Handbook of physical-chemical properties and environmental fate for organic chemicals, second edition. Volume, I-IV. CRC press. New York, US.

Mankiewicz-Boczek, J., Nałęcz-Jawecki, G., Drobniewska, A., Kaza, M., Sumorok, B., Izydorczyk, K., Zalewski, M. and Sawicki, J., 2008. Application of a microbiotests battery for complete toxicity assessment of rivers. *Ecotoxicology and Environmental Safety*. 71, 830-836.

Maraldo, K. and Dahllöf, I., 2004. Indirect estimation time for zinc pyrithione and copper pyrithione in seawater. *Marine Pollution Bulletin*. 48, 894-901.

Martínez-Toledo, M.V., Salmerón, V., Rodelas, B., Pozo, C., González-López, J., 1998. Effects of the fungicide captan on some functional groups of soil microflora. *Applied Soil Ecology*. 7, 245-255.

Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L. Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N2O emissions and denitrifier community abundance in soil. *Soil Biology and Biochemistry*. 40, 2553-2562.

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology*. 17, 526-538.

Pell, M., Stenberg, B., Torstensson, L., 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio*. 27, 24-28.

Petersen, D.G., Dahllöf, I., Nielsen, L.P., 2004. Effects of zinc pyrithione and copper pyrithione on microbial community function and structure in sediments. *Environmental Toxicology and Chemistry*. 23, 921-928.

Pietikäinen, J., Pettersson, M., Bååth, E., 2005. Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. *FEMS Microbiology Ecology*. 52, 49-58.

Rousk, J., Aldén Demoling, L., Bååth, E., 2008. Contrasting short-term antibiotic effects on respiration and bacterial growth compromises the validity of the selective respiratory inhibition technique to distinguish fungi and bacteria. *Microbial Ecology*. DOI 10.1007/s00248-008-9444-1.

Schmidt, I.K., Ruess, L., Bååth, E., Michelsen, A., Ekelund, F., Jonasson, S., 2000. Long-term manipulation of the microbes and microfauna of two subarctic heaths by addition of fungicide, bactericide, carbon and fertilizer. *Soil Biology and Biochemistry*. 32, 707-720.

Schweiger, P.F., Jakobsen, I., 1998. Dose-response relationships between four pesticides and phosphorus uptake by hyphae of arbuscular mycorrhizas. *Soil Biology and Biochemistry*. 30, 1415-1422.

Seitzinger, S., Harrison, J.A., Böhlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., et al., .2006. Denitrification across landscapes and waterscapes: a synthesis. *Ecological Applications*. 16, 2064-2090.

Shi, W., Bischoff, M., Turco, R., Konopka, A., 2002. Long-term effects of chromium and lead upon the activity of soil microbial communities. *Applied Soil Ecology*. 21, 169-177.

Smith, D.C., Azam, F., 1992. A simple, economical method for measuring bacterial protein synthesis rates in seawater using tritiated-leucine. *Marine Microbial Food Webs*. 6, 107-114.

Stadmark, J., Leonardsson, L., 2005. Emissions of greenhouse gases from ponds constructed for nitrogen removal. *Ecological Engineering*. 25, 542-551.

Sundbäck, K., Groth Petersen, D., Dahllöf, I., Larson, F., 2007. Combined nutrient-toxicant effects on a shallow-water marine sediment system: sensitivity and resilience of ecosystem functions. *Marine Ecology Progress Series*. 330, 13-30.

Swedish Board of Agriculture, 2000. Jordbruksverket, Informationsmaterial: Miljö- och landsbygdsprogram för Sverige år 2000-2006, pp. 222. http://www.regeringen.se (accessed 28/09/2007).

Tadeo, J.L, Sánchez-Brunete, C., González, L., 2007. Pesticides: classification and properties. In: Tadeo, J.L., Analysis of pesticides in food and environmental samples. CRC press. New York, US.

Thiere, G., Milenkovski, S., Lindgren, P-E., Sahlén, G., Berglund, O., Weisner, S.E.B., 2009. Wetland creation in agricultural landscapes: biodiversity benefits on local and regional scales. *Biological Conservation*. In press.

Van den Brink, P.J., Crum, S.J.H., Gylstra, R., Bransen, F., Cuppen, J.G.M., Brock, T.C.M., 2008. Effects of a herbicide-insecticide mixture in freshwater microcosms: Risk assessment and ecological effect chain. *Environmental Pollution*. 157, 237-249.

Wallenstein, M.D., Myrold, D.D., Firestone, M. and Voytek, M., 2006. Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications*. 16, 2143-2152.

Warren, N., Allan, I.J., Carter, J.E., House, W.A., Parker, A., 2003. Pesticides and other micro-organic contaminants in freshwater sedimentary environments – a review. *Applied Geochemistry*. 18, 159-194.

Widenfalk, A., Bertilsson, S., Sundh, I., Goedkoop, W., 2008. Effects of pesticides on community composition and activity of sediment microbes – responses at various levels of microbial community organization. *Environmental Pollution*. 152, 576-584.

Widenfalk, A., Svensson, M. J., Goedkoop, W., 2004. Effects of the pesticides captan, delthamethrin, isoproturon, and pirimicarb on the microbial community of a freshwater sediment. *Environmental Toxicology and Chemistry*. 23, 1920-1927.

Xia, S., Li, J., Wang, R., 2008. Nitrogen removal performance and microbial community structure dynamics response to carbon nitrogen ratio in a compact suspended carrier biofilm reactor. *Ecological Engineering*. 32, 256-262.

The following is a list of Doctoral theses from the Section of Chemical Ecology and Ecotoxicology, Department of Ecology, University of Lund, Sweden

1. ANDERS TUNLID, Chemical signatures in studies of bacterial communities. Highly sensitive analyses by gas chromatography and mass spectrometry. October 3, 1986

2. ANDERS THURÉN, Phthalate esters in the environment: analytical methods, occurrence, distribution and biological effects. November 4, 1988.

3. PETER SUNDIN, Plant root exudates in interactions between plants and soil microorganisms. A gnobiotic approach. March 16, 1990.

4. ANDERS VALEUR, Utilization of chromatography and mass spectrometry for the estimation of microbial dynamics. October 16, 1992.

5. HANS EK, Nitrogen acquisition, transport and metabolisation in intact ectomycorrhizial assocoations studied by 15N stable isotope techniques. May 14, 1993

6. ROLAND LINDQUIST, Dispersal of bacteria in ground water-mechanism, kinetics and consequences for facilitated transport. December 3, 1993.

7. ALMUT GERHARDT, Effect of metals on stream invertebrates. February 17, 1995.

8. OLOF REGNELL, Methyl mercury in lakes: factors affecting its production and partitioning between water and sediment. April 21, 1995.

9. PER WOIN, Xenobiotics in aquatic ecosystems: effects at different levels of organization. December 15, 1995.

10. GÖRAN EWALD, Role of lipids in the fate of organochlorine compounds in aquatic ecosystems. October 18, 1996.

11. JOHAN KNULST, Interfaces in aquatic ecosystems: Implications for transport and impact of anthropogenic compounds. December 13, 1996

12. GUDRUN BREMLE, Polychlorinated biphenyls (PCB) in a river ecosystem. April 25, 1997.

13. CHRISTER BERGWALL, Denitrification as an adaptive trait in soil and groundwater bacteria. November 14, 1997

14. ANNA WALLSTEDT, Temporal variation and phytotoxicity of Batatasin-III produced by Empetrum hermaphrodytum. November 27, 1998

15. DARIUS SABALIUNAS, Semipermeable membrane devices in monitoring of organic pollutants in the aquatic environment. April 28, 1999.

16. CECILIA AGRELL, Atmospheric transport of persistent organic pollutants to aquatic ecosystems. May 21, 1999

17. OLOF BERGLUND, The influence of ecological processes on the accumulation of persistent organochlorines in aquatic ecosystems. September 17, 1999

18. HELENA BJÖRN, Uptake, turn-over and distribution of chlorinated fatty acids in aquatic biota. October 1, 1999

19. RUEY-JANE FAN, Learning and memory in moths Plasticity in behaviour and neurophysiology. December 1, 2000.

20. FREDRIK ÖSTRAND, Behaviour of pine sawflies in relation to pheromone-based pest management. January 19, 2001.

21. MATTIAS LARSSON, Neural interfaces to the odour world of scarab beetles. March 2, 2001

22. CECILIA BACKE, Persistent organic pollutants in the atmosphere-spatial and temporal variations. May 4, 2001.

23. RICKARD IGNELL, Olfaction in desert locusts — Anatomy, function and plasticity of the central olfactory system. May 11, 2001.

24. CAMILLA RYNE, Pyralid moth reproduction: Communication, constraints & control. November 2, 2001.

25. DAINIUS PLEPYS, Odour-mediated nectar foraging in the silver Y moth, *Autographa gamma*. November 30, 2001.

26. DAVID ABRAHAM, Molecular aspects of pheromone evolution in moths. May 7, 2002.

27. GLENN SVENSSON, Disruption of moth mating behaviour by olfactory and acoustic cues. October 25, 2002.

28. LINA WENDT-RASCH, Ecological effects of pesticides in freshwater model ecosystems. February 28, 2003.

29. ARNOUT TER SCHURE, Polybrominated diphenylethers in the environment. October 3, 2003.

30. ELNA NILSSON, Movement patterns and displacement of a soil insect. May 28, 2004.

31. PER BENGTSON, Microbial mobilization and immobilization of soil nitrogen. June 4, 2004.

32. BETINA KOZLOWSKY SUZUKI, Effects of toxin-producing phytoplancton on copepods: feeding, reproduction and implications to the fate of toxins. June 7, 2004, Campus Helsingborg.

33. NIKLAS TÖRNEMAN, Spatial variability linking carbon resource heterogeneity and microorganisms; causes and consequences. May 13, 2005.

34. NIKLAS HOLMQVIST, Persistent organic pollutants in benthic foodwebs. June 10, 2005.

35. P.-O. CHRISTIAN OLSSON, Semiochemical-mediated attraction and oviposition in pyralid moths. November 11, 2005.

36. PARDIS PIRZADEH, Ecotoxicological assessment by microcosm tests on plankton communities. April 7, 2006.

37. CHRISTEL CARLSSON, Limitations and possibilities for microbial degradation of organic contaminants in aquifers. April 21, 2006.

38. MARIA PERSSON, The threat to the Baltic Salmon: a combination of persistent pollutants, parasites and oxidative stress. Nov 17, 2006.

39. GERMUND VON WOWERN, Circadian rhytms in moth sex pheromone communication. November 24, 2006.

40. LINA KRISTOFFERSEN, Getting to know *Trioza apicalis* (Homoptera: Psylloidea) – a specialist host-alternating insect with a tiny olfactory system December 1, 2006.

41. SÉVERINE JANSEN, Structure and function of odorant binding proteins and chemosensory proteins in moths. March 6, 2007.

42. MARIA STRANDH, Pheromones, genes & transcriptomes – a molecular analysis of moth sex pheromone production. November 30, 2007.

43. KATIA MONTENEGRO, Hierarchical responses to organic contaminants in aquatic ecotoxicological bioassays: from microsystins to biodegradation. September 23, 2008.

44. FRANCISCO J. PICADO PAVÒN, Fluvial transport and risk of mercury in a gold mining area. September 30, 2008.

45. NATALIA LADYGINA, Indirect biotic interactions in the rhizosphere. February 27, 2009.

46. SUSANN MILENKOVSKI, Structure and function of microbial communities in constructed wetlands – influence of environmental parameters and pesticides on denitrifying bacteria. April 3, 2009.