Structure and Function of Microbial Communities in Constructed Wetlands

Influence of environmental parameters and pesticides on denitrifying bacteria

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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.

Paper II

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

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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).

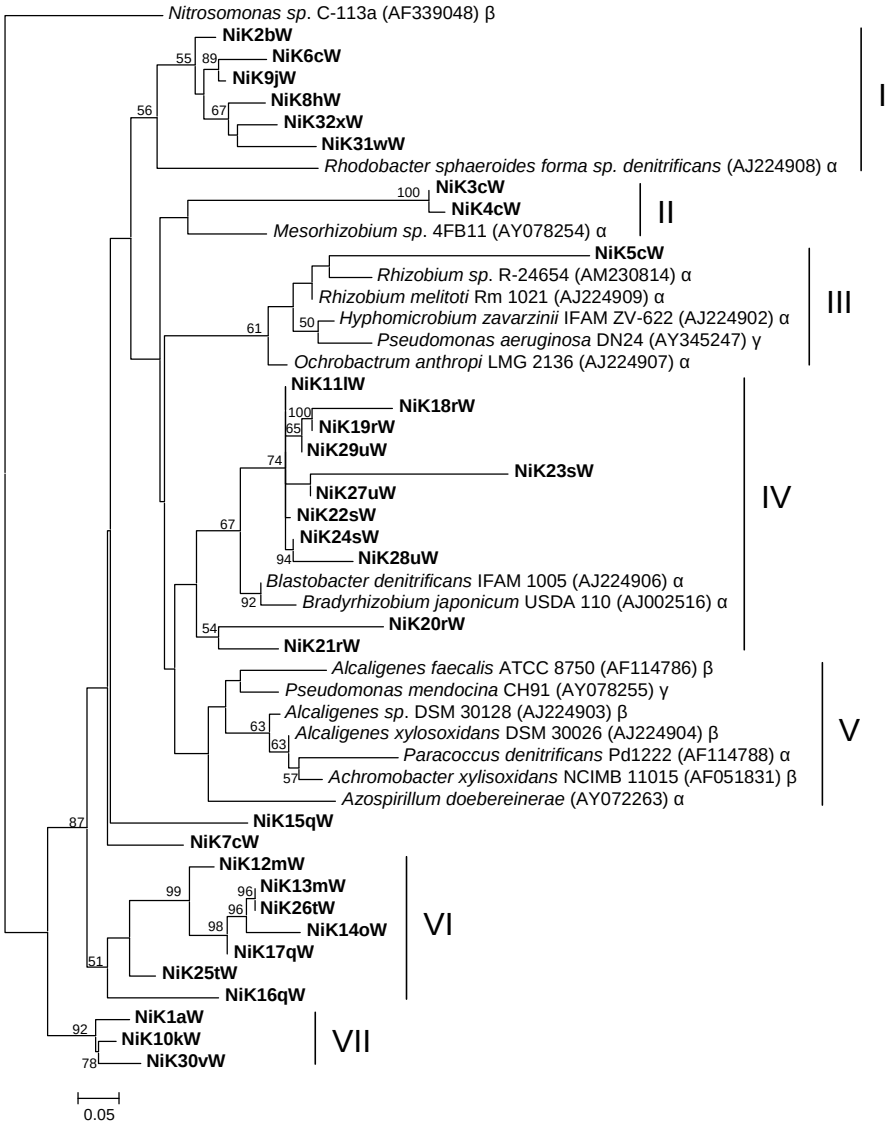

Figure 1. Phylogenetic tree of the partial *nir*K gene sequences (marked in bold) (based on a partial DNA fragment of 315 bp) analysed with maximum likelihood statistical method performed with 100 bootstraps. Bootstrap values higher than 50 are shown. The scale bar indicates 5% nucleotide substitutions. 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).

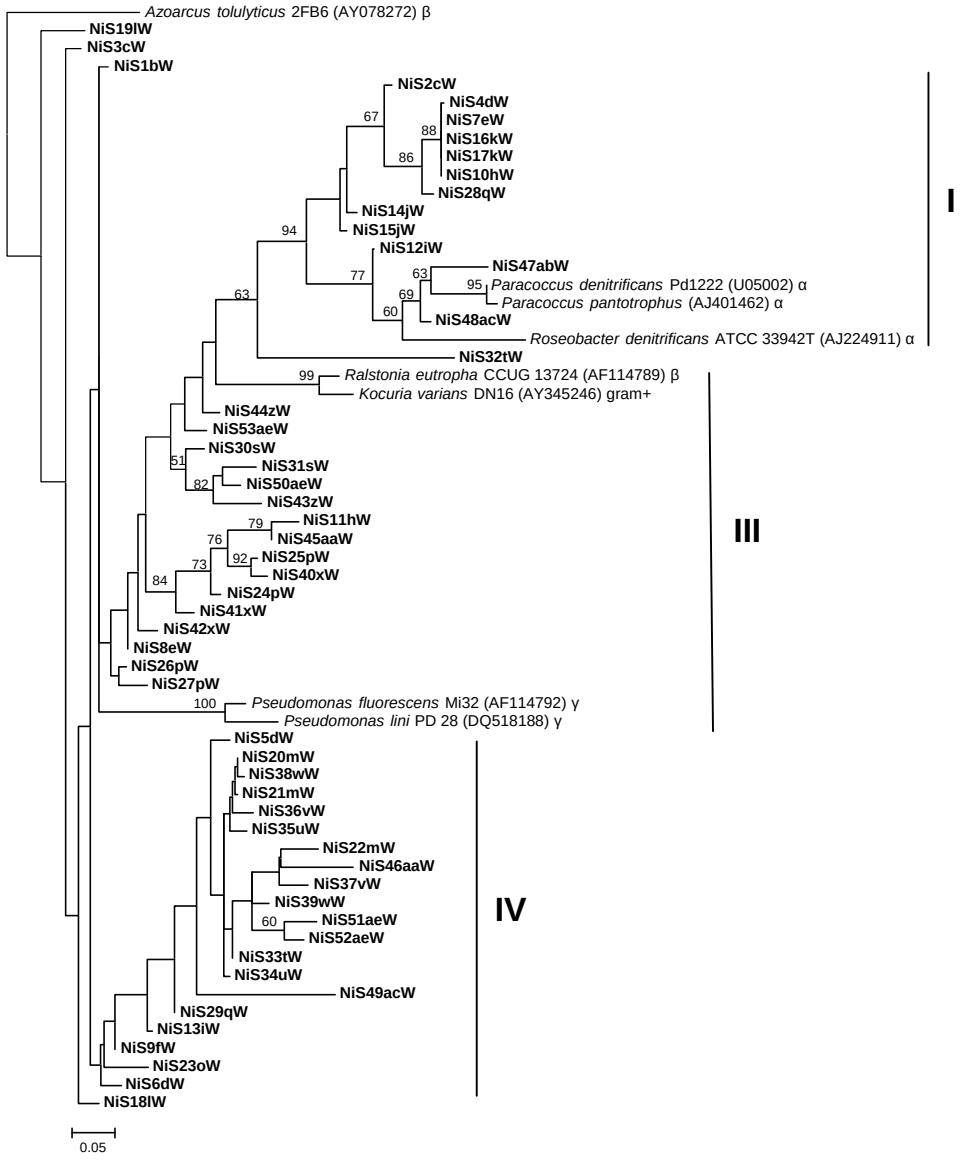

Figure 2. Phylogenetic tree of the partial *nir*S gene sequences (marked in bold) (based on a partial DNA fragment of 276 bp) analysed with maximum likelihood statistical method performed with 100 bootstraps. Bootstrap values higher than 50 are shown. The scale bar indicates 5% nucleotide substitutions. 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).

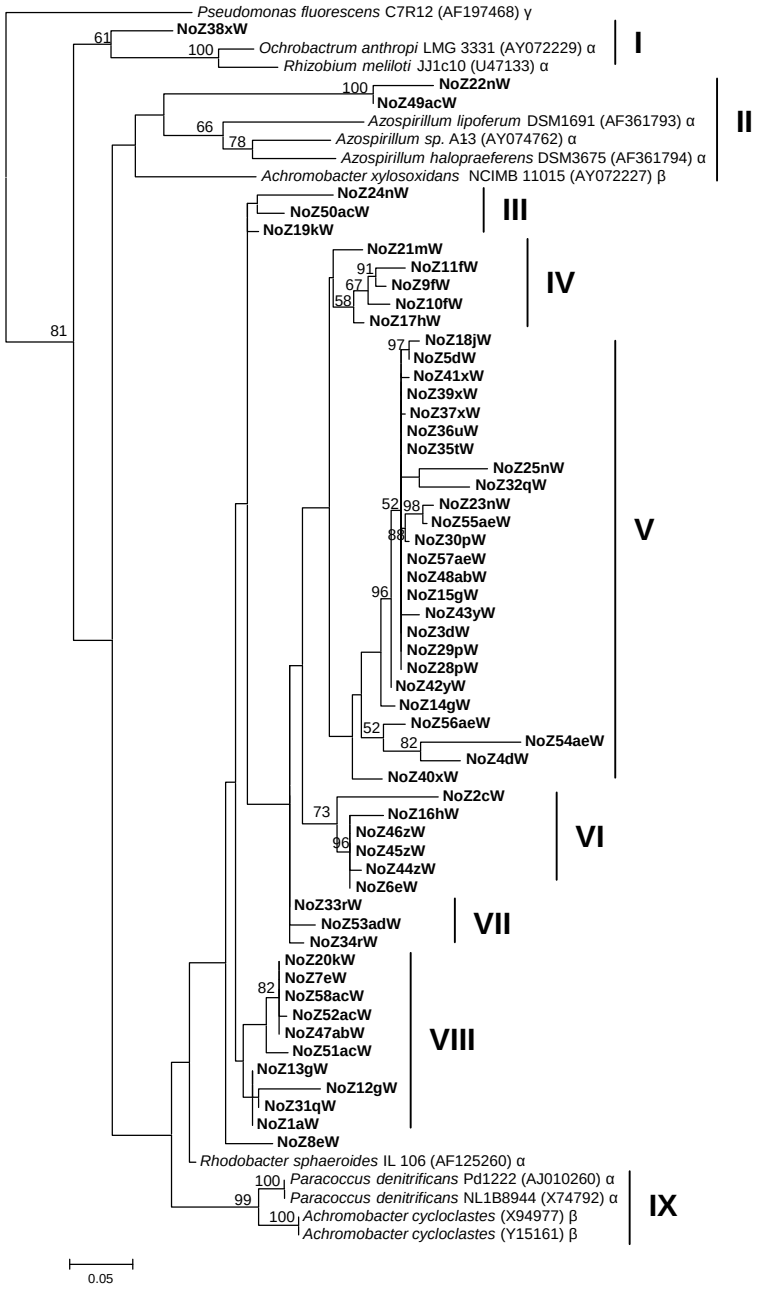

Figure 3. Phylogenetic tree of the partial *nos*Z gene sequences (marked in bold) (based on a partial DNA fragment of 309 bp) analysed with maximum likelihood statistical method performed with 100 bootstraps. Bootstrap values higher than 50 are shown. The scale bar indicates 5% nucleotide substitutions. 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.

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