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

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 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).

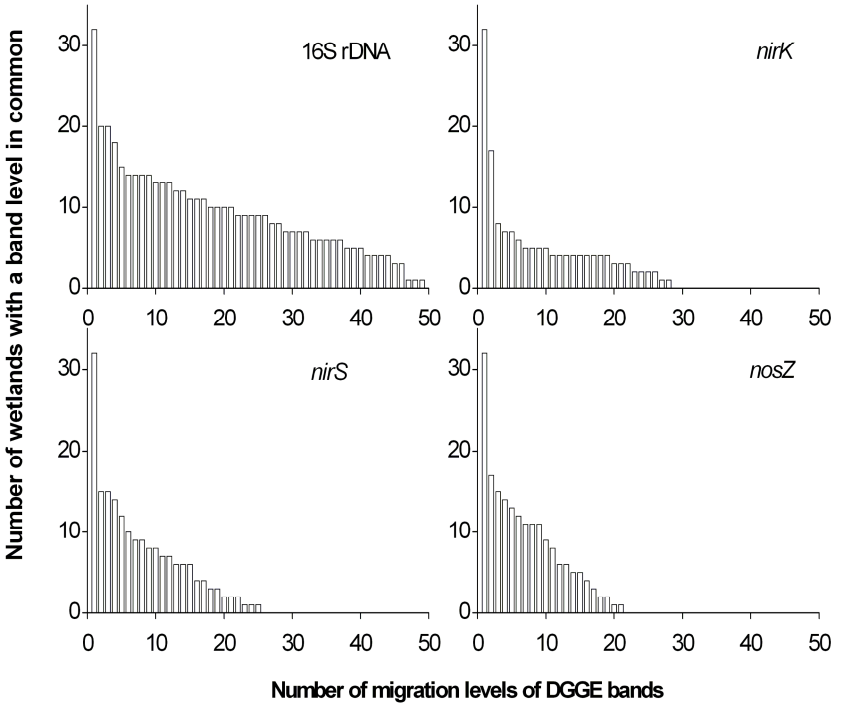

Figure 1. The diagrams illustrate the number of wetlands that shared bands at specific migration levels in DGGE gels used to analyse the eubacterial gene and each of the denitrifying bacterial enzyme genes. Steeper slope indicates greater similarity between the band patterns of the different wetlands.

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).

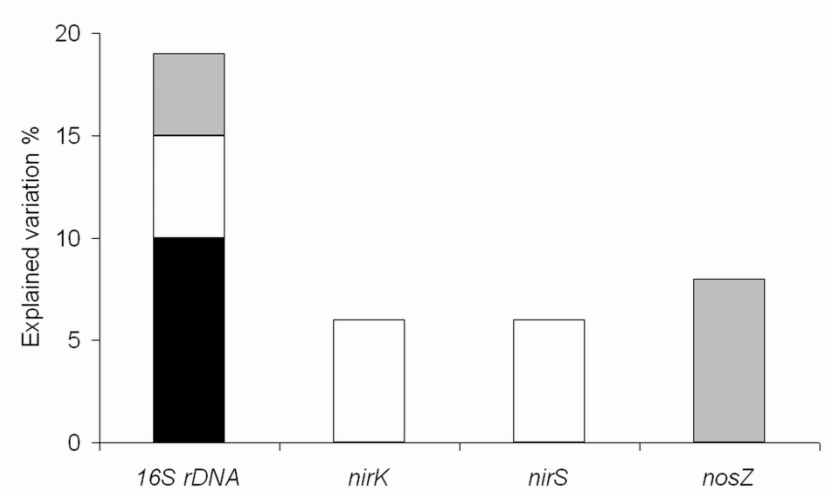

Figure 2. Explanatory power of the relationship between DGGE band structure and environmental parameters shown by canonical correspondence analysis. The environmental parameters considered here belonged to the three groups succession stage (black), water chemistry (white), and wetland morphology (grey).

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.

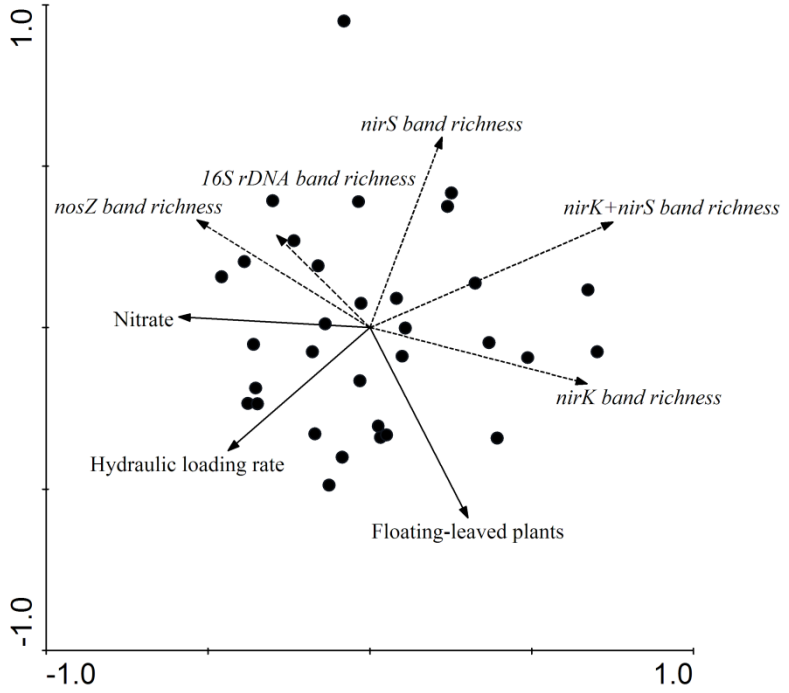

Figure 3. Ordination diagram obtained by redundancy analysis (RDA) of eubacterial and denitrifying bacterial DGGE band richness and environmental parameters. Symbols: closed circles represent wetlands; proximity between circles indicates similarity in band richness; dotted arrows denote the direction 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).

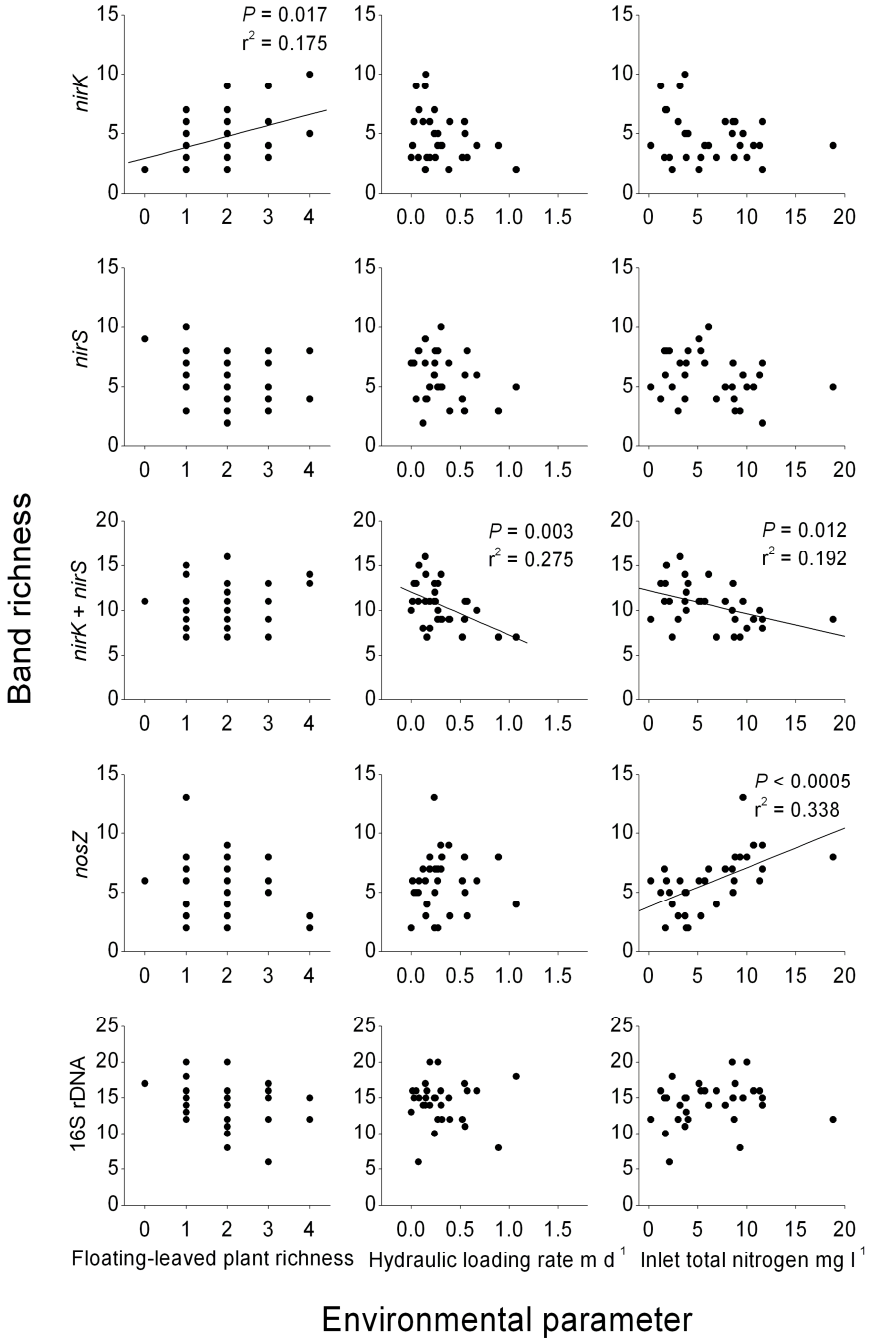

Figure 4. Linear regressions between three environmental parameters and DGGE band richness of eubacterial (16S rDNA) and denitrifying bacterial enzyme genes (*nir*K, *nir*S, *nir*K+*nir*S, and *nos*Z). Observe that values for hydraulic loading rates are square-root transformed. The lines indicate a significant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