RESEARCH ARTICLE

Structure and function of denitrifying and nitrifying bacterial communities in relation to the plant species in a constructed wetland

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Abstract

The community structure and potential activities of nitrifying and denitrifying bacteria were studied in the rhizosphere of *Typha latifolia* and *Phragmites australis* present in a free water system constructed wetland (CW). Potential nitrate reduction and nitrification activities were shown to be significantly higher in the rhizosphere when compared with the nonvegetated sediment. Higher rates were generally obtained for *P. australis*. The community structure of denitrifying bacteria in the rhizosphere differed from that found at the bulk sediment, as revealed by PCR-denaturing gradient gel electrophoresis (DGGE) of the nitrous oxide reductase encoding gene *nosZ*. Results also show a greater *nosZ* genotype diversification and suggest a plant species effect in rhizosphere samples obtained during events of low hydraulic retention times. Ammonia-oxidizing communities were less complex on the basis of PCR-DGGE analysis of the 16S rRNA gene. Retrieved sequences were all related to *Nitrosomonas marina* and *Nitrosomonas ureae*, being both present in rhizosphere and bulk sediment regardless of environmental changes. The results demonstrate the effect of vegetation on the functioning and structure of bacterial communities involved in the removal of nitrogen in the treatment cells of a CW and point to the use of vegetation coverage to promote nitrification or denitrification in particular areas.

Introduction

Constructed wetlands (CWs) have been extensively developed in the last decades as alternatives to on-site treatment methods for diffuse or nonpoint nitrogen pollution of water, and are used for biological treatment of wastewater from industries or medium size municipalities (Leonard & Swanson, 2001). CWs are particularly suited to promoting water reuse in areas of reduced freshwater resources, such as the Mediterranean region (Mandi *et al.*, 1998; Angelakis *et al.*, 1999). Nitrogen removal in wetlands occurs, apart from direct assimilation by plants, through microbial nitrification and denitrification activities. Nitrification includes two aerobic processes; oxidation of ammonia to nitrite, which is further oxidized to nitrate. The first process is performed by a phylogenetically constrained group of ammonia-oxidizing bacteria in addition to crenarchaea, whereas the last step is carried out by the nitrite-oxidizing bacteria. Denitrification is an anaerobic respiration process, in which nitrate is reduced stepwise to dinitrogen gas by a diverse range of bacteria and archaea. In wetlands, these processes are mainly associated with the sediment or other submersed surfaces (Kallner Bastviken *et al.*, 2003).

Vegetation coverage is a key factor controlling nitrogen removal in wetlands both directly and indirectly. It slows down the water flow rate, which prolongs the residence time and thereby the reaction time, provides organic matter and promotes growth of microorganisms in the sediment (Cooper *et al.*, 1996; Ibekwe *et al.*, 2007). Submerged and emergent plants may also represent an additional oxygen

source for bacteria growing as biofilms on plant surfaces, in the rhizosphere or the adjacent sediment, explaining differences in coupled nitrification and denitrification in the rhizosphere compared with the bulk sediment (Risgaard-Petersen & Jensen, 1997; Ottosen *et al*., 1999). Despite the importance of vegetation, plant and plant-species effects on nitrification and denitrification and the microbial communities involved have not been well studied in CWs. In soil ecosystems, plants have been shown to induce and stimulate the growth of specific bacterial groups and create well-defined bacterial communities around the rhizosphere due to multiple and interwoven factors, for example root exudation or rhizodeposition at the root surface (Sørensen, 1997; Jaeger *et al*., 1999; Duineveld *et al*., 2001; Smalla *et al*., 2001; Philippot *et al*., 2002; Sharma *et al*., 2005).

A comparative study of functional groups involved in nitrogen removal in the rhizosphere of wetland vegetation may help to better understand the distribution of these microorganisms and to infer their activity in distinct areas of treatment wetlands, especially when compared with potential sediment metabolic capabilities (Kallner Bastviken *et al*., 2003; Dollhopf *et al*., 2005). Cattail (*Typha latifolia* and *Typha angustifolia*), reed (*Phragmites australis* and *Phragmites communis*) and bulrush (*Scirpus* sp. and *Schoenoplectus lacustris*) are among the most commonly used species for artificial vegetation coverage of CWs worldwide. *Typha* sp. and *Phragmites* sp. dominate in the Spanish Mediterranean area and are often planted in single or mixed stands. Knowledge about plant species effects on nitrogen removal is crucial when designing CWs to optimize and maybe compartmentalize the nitrification and denitrification processes.

The objective was to determine the impact of *Typha* sp. and *Phragmites* sp. on community structure and activity of nitrifying and denitrifying bacteria in the sediment of a free water-surface CW (FWS-CW) at two different operational conditions. The FWS-CW is located in the Costa Brava area in Spain and has to cope with highly varying influent loadings due to the many tourists during summer vacation compared with other periods of the year. The two sampling occasions were chosen to evaluate plant effects under low and high water flows and nitrogen loadings.

Materials and methods

Site description and sampling of sediment and rhizosphere

The FWS-CW system is located downstream the wastewater treatment plant of EDAR-Empuriabrava, which is situated outside Girona in the Costa Brava area in Spain. The wetland consists of three parallel cells with a total surface area of 8000 m^2 and an average depth of 0.5 m. The aquatic vegetation is composed of *P. australis* and *Typha* sp., which mainly form separate stands. Large seasonal variations in the influent wastewater flow are common due to tourism in the region in summer. To assure that effluent ammonium concentrations never exceed 7 mg L^{-1}, the load is restricted by bypassing the water flow to the nearby located Muga River. This action is regularly performed to prevent the development of severe anoxic conditions in the CW, which have been related to the development of avian botulism outbreaks in the natural reserve Els Aiguamolls de l'Empordà located downstream of the CW.

Sediment and rhizosphere were sampled June 16 and September 28, 2006. The June samples were representative for a restricted water-flow period and the CW was almost drained (Table 1). The September period was characterized by short water residence times in the wetland and a high nitrogen load, mainly in the form of ammonia. Three to six replicate rhizosphere samples of *P. australis* (P1–P8) and *T. latifolia* (T1–T8), as well as sediment devoid of vegetation (S1–S12) were collected in the third cell of the Empuriabrava CW. Replicates from each sample type were collected randomly in a 1 m^2 area. Reed and cattail stands were located 40 m apart at a central point in the wetland. Because of difficulties accessing the CW, bulk sediment was not sampled from exactly the same site in June and September.

Table 1. Concentration of ammonia and nitrate from the effluents of Empuriabrava wastewater treatment plant (WWTP) and the FWS-CW during the sampling period

	WWTP effluent		FWS-CW effluent				
	$N\text{-}NH_4^+$ (mg L^{-1})	$N\text{-}NO_3^-$ (mg L^{-1})	$N\text{-}NH_4^+$ (mg L^{-1})	$N\text{-}NO_3^-$ (mg L^{-1})	Flow (m^3 day^{-1})	Total N load FWS-CW (kg)	N removal FWS-CW (%)
16th June	15.1	2.3	1.5	1.9	0.0*	NA	NA
June mean (SE)	18.43 (1.92)	1.36 (0.41)	2.65 (0.88)	0.48 (0.13)	54.8 (44.9)	39	99
28th September	5.3	2.2	1.7	3.1	2714.0	21.63	72
September mean (SE)	9.83 (4.24)	4.91 (1.54)	1.26 (0.30)	2.55 (0.29)	1074.1 (288.5)	591	94

*Flow to the FWS-CW stopped due to excess ammonia.
Mean monthly values and actual data corresponding to sampling date are indicated. The total volume of the three treatment cells is 12 311 m^3.
NA, not applicable.

Sampling was done using a 7-cm-diameter plexiglass tube mounted in a manual core sampler. Roots and root fragments were carefully removed from sediments by hand. Rhizosphere samples were considered as the portion of sediment that remained attached to plant roots after washing twice with sterile isotonic solution. Sediments were manually homogenized using a sterile spatula and roots were cut into *c.* 0.5-cm pieces and homogenized. Aliquots of all sediment replicates were maintained at $-20\,^{\circ}C$ for further analysis.

Potential activity assays

Potential nitrate+nitrite reduction rates were determined as an indication of denitrification activity in all samples. For the September samples, potential denitrification activity was also measured using the acetylene inhibition technique, according to Pell *et al.* (1996) with some modifications. Preparation of sediment slurries was similar for both methods. Approximately 10 g of fresh sediment or roots were placed in 250-mL flasks and diluted in 49 mL of sterile isotonic solution. The atmosphere in the flasks was exchanged after five evacuation and filling cycles with N_2. After 30-min agitation, incubations were supplemented with KNO_3 ($28\,mg\,L^{-1}\,NO_3^-$-N, final concentration) and ammonium acetate ($0.5\,g\,L^{-1}$), ethanol ($0.2\,g\,L^{-1}$) and propionic acid ($0.3\,g\,L^{-1}$). When measuring denitrification with the acetylene method, 30 mL acetylene were added to reach a 0.1 atm partial pressure before substrate addition. Samples of the liquid phase for nitrite and nitrate determination were taken every 30–45 min for the first 2 h of incubation and every hour for the next 4 h. Gas samples were taken every 12 min during the first hour and then every half hour for 5 h.

Potential nitrification activity measurements were determined as nitrate+nitrite production rates. Incubations were performed in a similar way as those for nitrate+nitrite reduction, but under aerobic conditions. The slurries were amended with $(NH_4)_2SO_2$ to a final concentration of $210\,mg\,L^{-1}\,NH_4^+$-N and liquid samples were collected over a 24-h period. All activity measurements were done at $25\,^{\circ}C$ with continuous agitation (150 r.p.m.). Controls with sterilized sediments and roots were included.

Samples for the analysis of nitrate and nitrite concentrations (1 mL) were collected directly from slurries, centrifuged for 2 min at 12 000 ***g*** and filtered (0.2 μM). Samples were kept at $-20\,^{\circ}C$ until analyzed by HPLC (Waters Corporation, Barcelona, Spain) using a 4.6×200 mm Waters Spherisorb® 5 μM SAX as analytical column (Smith *et al.*, 2002). Nitrous oxide in gas vials was analyzed on a gas chromatograph (model CP 9000, Chrompack, Rotterdam, the Netherlands) equipped with a ^{63}Ni electron capture detector. Initial and final ammonium concentrations in the nitrate+nitrite reduction activity experiments were determined in a destillator BÜCHI B-324, using an automatic titrator (Titrino 719S Metrohm). Five milliliter samples of either sediment or rhizosphere slurries were centrifuged at 3000 ***g*** for 5 min and the supernatant was collected in a sterile tube and acidified with 1N HCl. Samples were kept at $-20\,^{\circ}C$ until analyzed.

DNA extraction and PCR conditions

DNA was extracted using the Ultraclean Soil DNA kit (MO BIO Laboratories Inc.) following the manufacturer's instructions and the concentration was measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). PCR for the amplification of *nosZ* gene fragments were performed with primers nosZ-F (5′-CGY TGT TCM TCG ACA GCC AG-3′) (Kloos *et al.*, 2001) and nosZ 1622R (5′-CGS ACC TTS TTG CCS TYG CG-3′), or nosZ1622R-GC before denaturing gradient gel electrophoresis (DGGE) (Throbäck *et al.*, 2004). A touchdown PCR was performed in a minicycler (MJ Research) according to PCR conditions presented by Enwall *et al.* (2005).

Partial 16S rRNA genes of the β-subgroup of ammonia oxidizers were obtained by PCR amplification using the primer pair CTO189F AB/C-GC and CTO654R (5′-CTA GCY TTG TAG TTT CAA ACG C-3′) (Kowalchuk *et al.*, 1997). Amplification reactions were run in a Geneamp 9700 thermal cycler according to PCR conditions described by (Kowalchuk *et al.*, 1997).

DGGE analysis of *nosZ* and 16S rRNA genes

Three hundred nanograms of *nosZ* PCR products were loaded on 7% (v/v) acrylamide–bis-acrylamide gels with a 40–70% urea–formamide denaturing gradient. DGGE was performed in a DCode system from Bio-Rad Laboratories Inc. according to Enwall *et al.* (2005). For DGGE analysis of the 16S rRNA gene of the ammonia-oxidising bacteria (AOB), DGGE was carried out in an Ingeny phorU system (Ingeny, the Netherlands) using 6% acrylamide–bis-acrylamide gels and a 35–70% urea–formamide denaturing gradient (Bäckman *et al.*, 2003). In both DGGE assays, gels were run for 17 h at 120 V and stained for 30 min with Sybr® Gold (Molecular Probes Europe, Invitrogen Corporation), for visualization under UV excitation. Images were captured with GelDoc 2000 system. For AOB gels, representative bands of every position in the gel were excised using a sterile scalpel. The DNA fragment was recovered by elution in Tris/HCl at $65\,^{\circ}C$ during 1 h and reamplified as described above.

Clone libraries

Based on the DGGE fingerprinting results, rhizosphere samples from June and September were selected for

construction of four *nosZ* clone libraries. PCR products from sample triplicates were pooled, separated by electrophoresis on agarose gels, and purified with the MiniElute Gel Extraction Kit (Qiagen). Amplicons were then cloned with the TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's specifications. Transformants were checked for correct insert size by transferring cells with a toothpick into 25 μL PCR reactions prepared as described above. Restriction fragment length polymorphism (RFLP) was used to screen 90, 87, 63, and 84 positive clones for *Typha* sp. September (TS), *Typha* sp. June (TJ), *Phragmites* sp. September (PS) and *Phragmites* sp. June (PJ) samples. PCR products were digested overnight with AluI at 37 °C, and then loaded onto 2% agarose gels, electrophoresed for 2 h at 90 V. Gel image analysis was carried out using Gelcompar II (Applied Maths, NV) to identify different RFLP patterns and group clones accordingly. Coverage was calculated according to $C = 1 - nN^{-1}$, where n is the number of different RFLP types encountered only once, and N is the total number of clones analyzed in the library. Clones from RFLP pattern types represented by at least two clones were selected from each of the four libraries and grown overnight in Luria–Bertani tubes at 37 °C before plasmid isolation with the QIAprep Spin Miniprep kit (Qiagen).

Sequencing

Sequencing in both directions of cloned partial *nosZ* gene fragments and AOB 16S rRNA gene fragments obtained from reamplification of DGGE excised bands, was performed by the Macrogen service (Macrogen, Korea). Sequences were manually checked and aligned using the CLUSTALW software (European Bioinformatics Institute, http://www.ebi.ac.uk).

Statistical and phylogenetic analyses

Differences in potential activity rates were tested for the effects of planted vs. nonplanted areas, plant species, sampling time and the corresponding interactions by ANOVA using the SPSS software (SPSS Inc.). Differences between means of either potential nitrification or nitrate+nitrite reduction were analyzed using Tukey's honestly significant difference (HSD) *post hoc* test at $P \leq 0.05$. The analyses were performed with the General Linear Model (GLM) procedure of the SPSS software (SPSS Inc.).

DGGE patterns of *nosZ* genes were analyzed using Gelcompar II (Applied Maths). Known samples were loaded in all DGGE gels and used as internal standards for the comparison between fingerprints. For sample comparison, a presence–absence matrix was used to calculate similarities between patterns and statistical analysis based on hierarchical cluster analysis was performed with the Jaccard distance and the unweighted pair group method with arithmetic mean grouping algorithm. The sequences were compared with those deposited in the GeneBank (NCBI) database using the BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analyses of the deduced amino acid sequences were conducted using the software MEGA version 4 (Tamura *et al.*, 2007). Neighbour-joining trees were reconstructed by Amino Poisson correction and pairwise deletion. Tree topology was evaluated by bootstrap analysis using 1000 replicates.

Nucleotide sequence accession numbers

The *nosZ* and AOB 16S rRNA gene sequences have been deposited in GenBank under accession numbers EU271684–EU271752 and EU281631–EU281658, respectively.

Results

Potential activities

The nitrate+nitrite reduction rates were significantly ($P < 0.05$) higher in the *P. australis* and *Typha* sp. rhizosphere samples compared with the sediment samples on both sampling occasions (Fig. 1a). Not only the plant, but

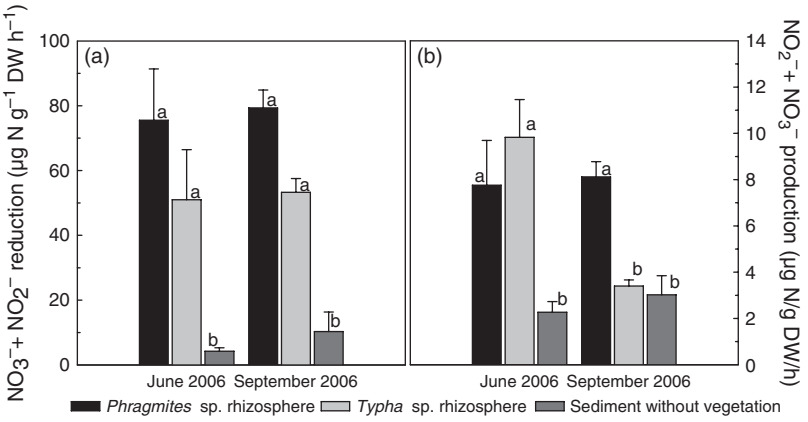

Fig. 1. Potential nitrite+nitrate reduction (a) and potential nitrification (b) rates in rhizosphere and sediment sampled in June and September. Rates are expressed as means of three field replicates and SEs. Different letters above the bars indicate treatments with significant differences ($P < 0.05$) between samples within each sampling date according to Tukey's HSD test.

also the plant species affected the reduction rate. Potential rates measured in the *P. australis* rhizosphere were slightly higher than in that of *Typha* sp., although differences were not significant, due to the variation within replicates at each rhizosphere location. No significant differences in nitrite + nitrate reduction potential rates between any of the locations were observed at both sampling occasions. Ammonium was not detected ($< 0.3\ mg\ NH_4^+\text{-}N\ L^{-1}$) during the incubation, which indicates neglectable dissimilatory nitrate reduction to ammonia under these conditions. In the September samples, potential denitrification rates were also determined from N_2O production after acetylene inhibition. Denitrification rates were 19.9 ± 3.6 and 14.1 ± 1.1 in the *P. australis* and *Typha* sp. rhizosphere, respectively, whereas the rate in the sediment was $2.9 \pm 0.4\ \mu g\ N_2O\text{-}N\ g^{-1}\ DW\ h^{-1}$ (data not shown). Thus, the plant effect on denitrification rates were in agreement with those determined as nitrate + nitrite reduction, and the two methods correlated ($R^2 = 0.8$). Nevertheless, the denitrification rates were two to three times lower compared with the nitrate + nitrite reduction rates. A similar relationship between the two techniques has been reported previously when applied to CW sediments and related to an incomplete blockage of the reduction of nitrous oxide (Seitzinger *et al.*, 1993; Kallner Bastviken *et al.*, 2003).

The potential nitrification rates were significantly higher in the planted areas compared with nonplanted sediments ($P < 0.01$). Sampling time was not a significant variable for nitrification potential in the bulk sediment or the *P. australis* rhizosphere according to the results of a one-way ANOVA test, but a more detailed analysis using Tukey's HSD test indicated a significant shift in the potential rate found in *Typha* sp. rhizosphere. Here, the rate was similar to the rates in the rhizosphere of *P. australis* during June, but comparable to that obtained for the bulk sediment during September (Fig. 1b). However, the interaction between plant species and time was not significant.

nosZ community structure

The DGGE patterns of *nosZ* genotypes from the rhizosphere samples were different from the sediment samples, although some bands were shared between most DGGE profiles (Fig. 2). Thus, vegetation was an important factor controlling the denitrifier community structure in the wetland. Besides the rhizosphere effect, a temporal effect was detected as the June and September samples separated from each other within the sediment and rhizosphere cluster, respectively. A plant species-specific separation was seen in the September samples, but this was not as obvious in the June

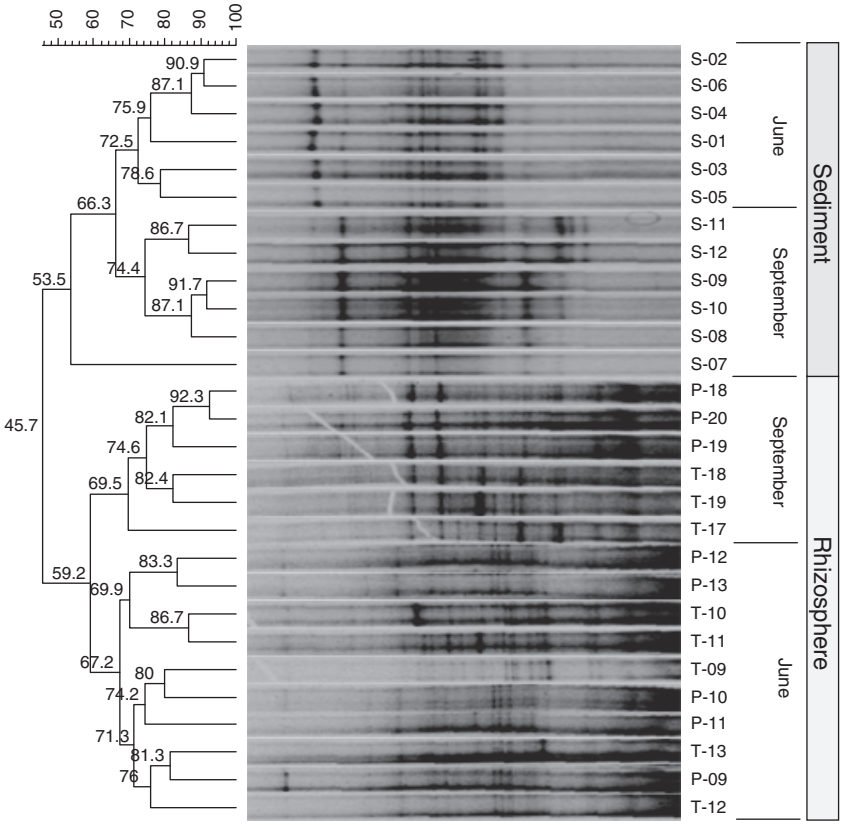

Fig. 2. DGGE profiles of PCR-amplified *nosZ* gene fragments from sediments (S) and rhizospheres of *Typha* sp. (T) and *Phragmites australis* (P) communities sampled in June and September. Scale bar indicates percent similarities in the unweighted pair group method with arithmetic mean dendrogram, based on Jaccard distances.

samples of the two rhizospheres. In addition, DGGE fingerprints corresponding to rhizospheres obtained from *Typha* sp. showed higher small-scale variation between replicates whereas the *P. australis* rhizosphere fingerprints were more similar between replicates, indicating a less heterogeneous environment in the proximity of the roots of this plant species.

From the clone libraries of the rhizosphere samples, in total 37 RFLP types could be assigned and 31 of them were found in more than one clone. Based on the RFLP types, the coverage was estimated to be 93%, 93%, 85% and 97% in the TJ, TS, PJ and PS samples, respectively. A few types dominated and seven RFLP types comprised almost 70% of the clones. Whereas some types were detected in all libraries, the majority of RFLP types, including the most abundant, seemed to be site or time specific (Fig. 3). As an example, types 25 and 19 as well as the less abundant types 16 and 27, were only found in clones obtained from *Typha* sp. rhizosphere, while types 32, 28 and 24 appeared only in clone libraries from *P. australis*. In general, September libraries contained a higher number of different RFLP types.

A number of clones from all RFLP types represented by at least two clones were selected for sequencing. The sequences showed similarities with previously reported *nosZ* sequences from environmental clones retrieved from natural river sediments, agricultural soils and activated sludge. The phylogram of the deduced amino acid sequences separated the clones into two main groups (Fig. 4). Sequences present in cluster I corresponded to the RFLP types frequent in samples from June, whereas cluster II was more heterogeneous and dominated by clones retrieved from the September samples. Because the dominant RFLP types were represented by all four clone libraries, sequences obtained from either *Typha* sp. or *P. australis* libraries appeared randomly distributed over the phylogram.

AOB community structure

The AOB community pattern had a rather low complexity (Fig. 5). Sediment and rhizosphere AOB community fingerprints were similar in June, but the September samples showed higher variation between sample types. In addition, the DGGE patterns indicate a season-related change for all sample types. Sequencing confirmed that all bands were AOB, except bands 1, 2 and 18. These were related to *Methylobacillus* sp. and only detected in the September profiles. All AOB sequences were phylogenetically affiliated to *Nitrosomonas* species, and showed at least 98% homology with sequences from environmental clones retrieved from wastewater treatment systems. The majority of sequenced AOB bands showed high similarities to each other and the closest cultured match was *Nitrosomonas marina*, while the fuzzier bands (nos 7, 19 and 30) were related to *Nitrosomonas ureae* (Fig. 6).

Discussion

Both potential nitrification and nitrate reduction rates in the sediment were in general higher in the rhizosphere compared with bulk samples, showing that vegetation was an important factor for the nitrogen removal capacity in the CW. Plant surfaces in wetlands have been pointed out as significant sites for nitrifying bacteria (Eriksson & Weisner, 1999; Körner, 1999; Kallner Bastviken *et al.*, 2003) and this could be part of the explanation for the higher nitrification rates observed in the rhizosphere. Emergent plants also control dissolved oxygen concentrations in the rhizosphere,

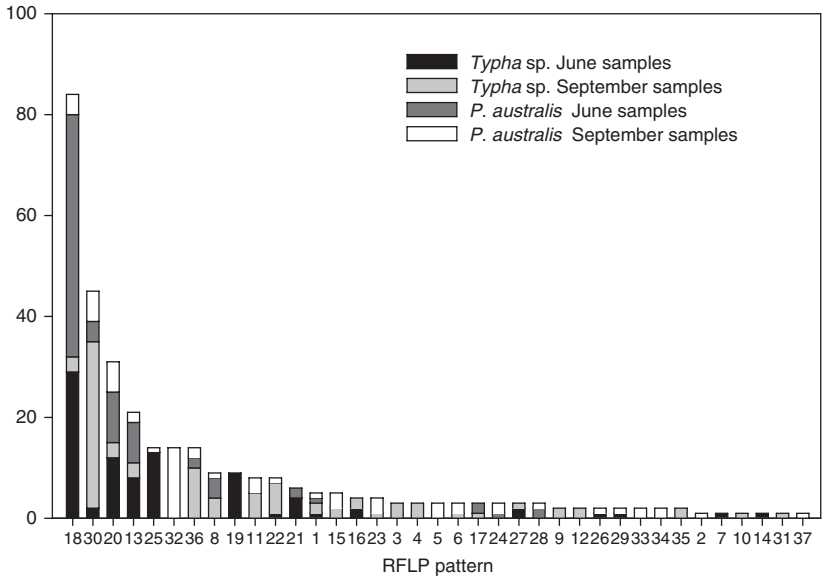

Fig. 3. Frequency distribution of *nosZ* gene RFLP types from clone libraries obtained from *Typha* sp. and *Phragmites australis* rhizosphere sampled in June and September. Abundance is represented by absolute frequency and distribution of RFLP types within each clone library is indicated.

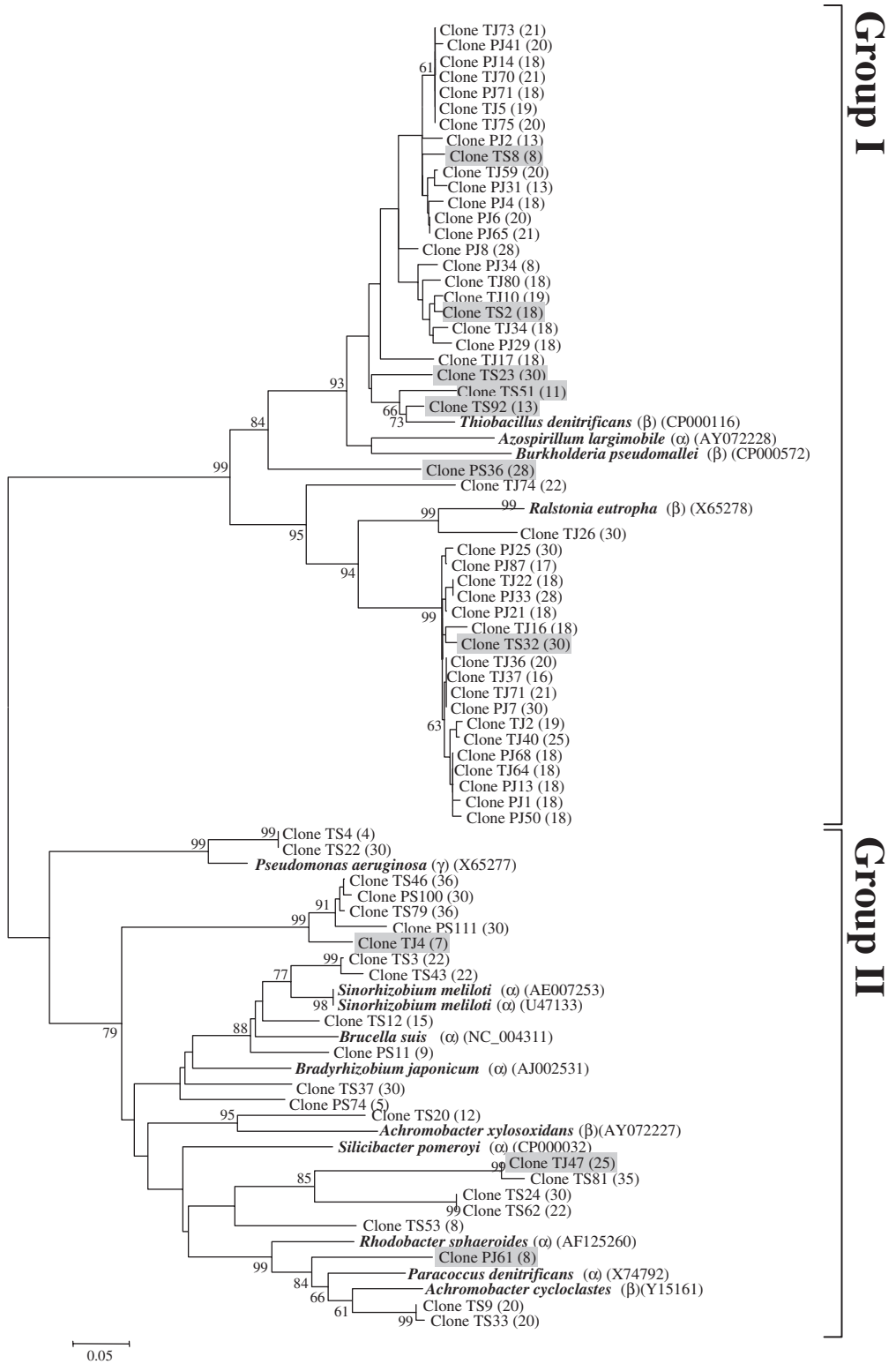

Fig. 4. Neighbour-joining phylogenetic tree of partial *nosZ*-deduced amino acid sequences (149 residues). Bootstrap values above 60% are shown. Accession numbers of selected sequences from cultivated bacteria (in bold) are shown in parentheses, together with the RFLP pattern-type numbers. Sequences from June are mainly found in cluster I, and cluster II is dominated by September samples. Exceptions are shaded in gray.

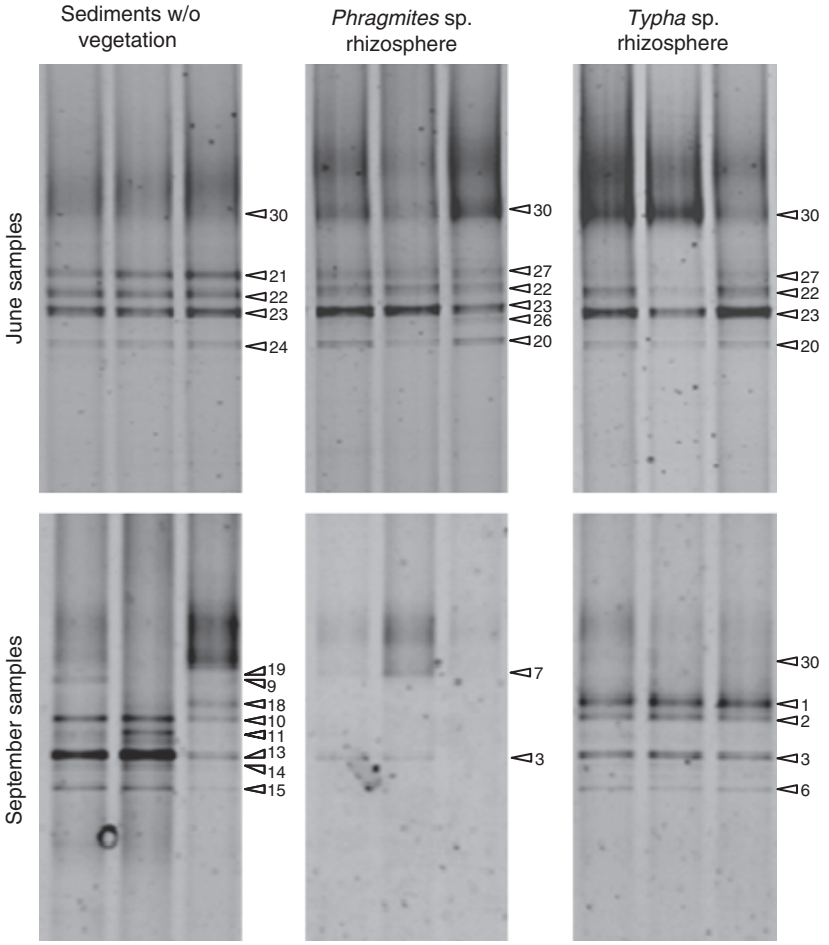

Fig. 5. DGGE profiles of PCR-amplified ammonia-oxidizing bacterial 16S rRNA gene fragments from sediments and rhizospheres of *Typha* sp. and *Phragmites australis* rhizospheres sampled in June and September. Numbers indicate the identity of excised bands.

by root respiration and transport of oxygen from the air down through the aerenchyma to the water-saturated sediment. The plant effect on nitrification rates was probably attributed to the oxygen supplied by the plants (Jespersen *et al.*, 1998; Armstrong & Armstrong, 2001). In contrast, effects on nitrate reduction can be a mix of various factors. It has been argued that the ability to respire anaerobically using nitrogenous compounds could be a selective advantage for denitrifiers in the rhizosphere. Mutants unable to synthesize the membrane-bound nitrate reductase, the cd_1 nitrite reductase or the copper nitrite reductase, were outcompeted by the wild-type strains in maize rhizosphere, which demonstrates that denitrification itself could be an advantage for root colonization (Philippot *et al.*, 1995; Ghiglione *et al.*, 2000). In agreement, von Berg & Bothe (1992) found that the ratio of denitrifiers to other heterotrophic bacteria increased near the roots.

The results indicated that *P. australis* supported nitrification and nitrate reduction in the sediment better than *T. latifolia* did, which points toward a plant species dependence of the measured potential activities. The greater development of the *P. australis* root zone in deep sediment layers would increase the oxygen supply, which favors the activity of AOB. Ammonium removal in CW have been shown to be higher in *Phragmites* sp. stands than in those planted with *Typha* sp. (Gersberg *et al.*, 1986), but it remains unclear whether this is mainly due to higher nitrification rates in *Phragmites* sp. rhizosphere, or due to a more efficient denitrification in sediments covered with this plant species. The finding that potential denitrification activities in intact cores with *Phragmites* sp. or *Typha* sp. shoots were not significantly different, as reported by Kallner Bastviken *et al.* (2003), supports the theory that *Phragmites* sp. mainly plays a role in nitrogen removal by enhancing nitrification activity. In our study, potential nitrification rates were low and accounted for $< 10\%$ of the potential nitrate reduction rates. Because nitrogen removal in treatment wetlands predominantly loaded with ammonia depend on an efficient nitrification step, management practices to favor nitrification are important. Single-stage CWs seldom achieve high removal of total nitrogen due to their inability to provide both aerobic and anaerobic conditions at the same time (Vymazal, 2007). The results obtained in the present work indicate the potential use of *Phragmites* sp. as the best

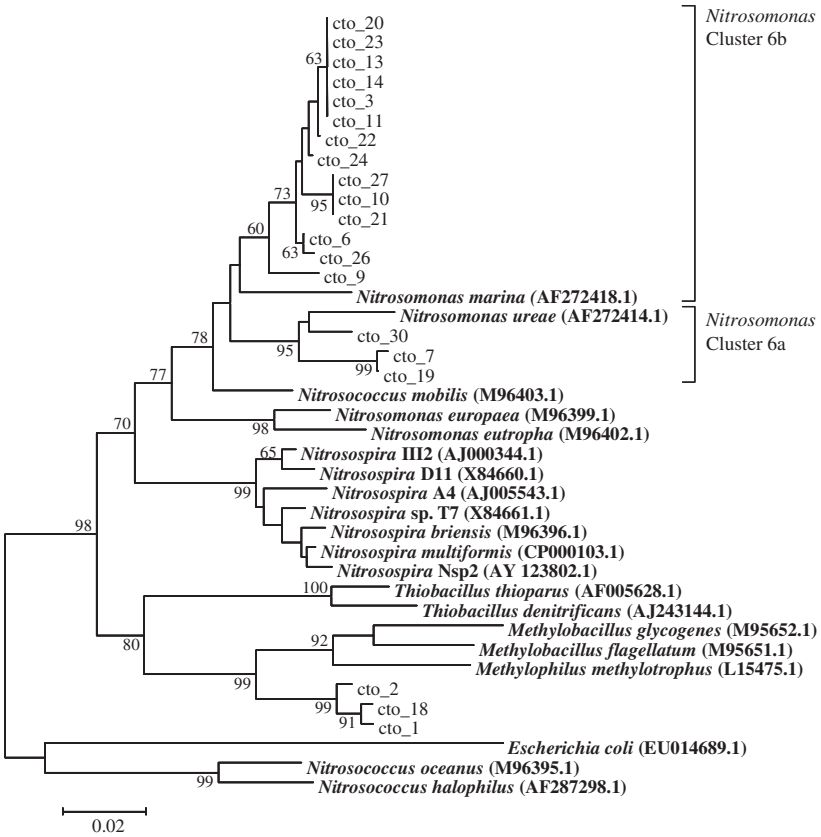

Fig. 6. Neighbour-joining phylogenetic tree of partial ammonia-oxidizing bacterial 16S rRNA gene sequences (367 nucleotides). Bootstrap values above 60% are shown. Accession numbers of selected sequences from cultivated bacteria (in bold) are shown in parentheses.

candidate plant species to support nitrogen removal along the water flow in CWs.

Because the *Phragmites* sp. effect on nitrification and nitrate reduction potential was only significant in the September samples, we suggest that the observed differences might depend on changes in environmental conditions over time in addition to changes in plant physiology. The total nitrogen load and the ammonium to nitrate ratio in the influent to the CWs changed over the studied period, mainly because of fluctuations in the mean water residence time. The wastewater loading to the CW during June was very low due to the bypass flow used when ammonium concentration in the effluent from the wastewater treatment plant is higher than 7 mg L^{-1} This situation caused a volume loss of *c.* 30% in the CW, which limited the nutrient load for a long period. The condition in September was the opposite and sampling coincided with high nutrient loads and low water retention time.

The number of AOB genotypes detected by DGGE showed only minor differences among samples. In June, both rhizosphere fingerprints were comparable to those from the bulk sediment, but, in September, the bulk sediment was more complex than the rhizosphere samples. However, no clear difference either between sample type or occasion for the AOB was observed when analyzing the sequences derived from the DGGE. All retrieved AOB-like sequences revealed homologies to yet uncultivated bacteria related to *N. ureae* and *Nitrosomonas oligotropha* (cluster 6a, three sequences) and to *N. marina* (cluster 6b, 14 sequences). The former group has been shown to dominate in some activated sludge plants treating wastewater (Dionisi *et al.*, 2002; Hallin *et al.*, 2005) and in soil and freshwater sediments contaminated with wastewater (Purkhold *et al.*, 2000; Cebron *et al.*, 2003). Cluster 6b-related sequences appear to be dominating the AOB community in the wetland and this cluster is also numerically important in activated sludge processes (Purkhold *et al.*, 2000; Wagner *et al.*, 2002). In our study, it may also be related to seawater infiltration to the wastewater pipes in the area. In fact, relatively high conductivity values are routinely measured in the water (1400–4 500 μS cm^{-1}; L. Sala, Consorci de la Costa Brava, Girona, pers. commun.). A few sequences obtained from the bulk sediment in September clustered with *Methylobacillus* sp. and *Methylophilus* sp. It has previously been shown that the CTO primers can amplify non-AOB sequences (Purkhold *et al.*, 2000; Bäckman *et al.*, 2003), which implies that misleading conclusions can be drawn if only based on DGGE fingerprints.

In contrast to the AOB community, a clear plant effect on the structure of the denitrifier community was detected by the *nosZ* PCR-DGGE fingerprints. Similarly, Philippot *et al.* (2002), focusing on *narG* genes coding for nitrate reductase, found that the community structure of nitrate-reducing bacteria in unplanted and maize-planted soils was linked to plant distribution, supposedly due to selection for some strains, which might possess better competitive traits. In our study, the DGGE fingerprints of *nosZ* furthermore suggested plant species-specific effects on the composition of *nosZ* genotypes in the CW, especially in the September samples. A similar effect was observed in another wetland, where the denitrifying community structure, targeted by *nirS* genes coding for the heme cd_1-nitrite reductase, differed in the sediment with an invasive cattail hybrid *Typha* × *glauca* compared with the sediment with native plant species, for example *Scirpus* sp. (Angeloni *et al.*, 2006). Also in soil ecosystems, plant species have been shown to influence the composition of denitrifiers (Patra *et al.*, 2006; Bremer *et al.*, 2007), although when investigating the effect of *Lolium perenne* and *Trifolium repens* on the nitrate reducer community, plant species had no effect (Deiglmayr *et al.*, 2004). In studies assessing the total bacterial community, specific community patterns were associated with different plants in agricultural soils (Smalla *et al.*, 2001; Costa *et al.*, 2006), but Ravit *et al.* (2006) suggested that individual plant species have a less pronounced effect in wetlands, especially if subject to disturbances.

In addition to the plant effects, we found clear seasonal variations in the *nosZ* genotype community structure in the DGGE patterns, both within bulk sediment and rhizosphere sites. The cloned *nosZ* fragments from rhizosphere samples revealed differences in the community structure mainly according to sampling time and only partly according to plant species. Seasonal variations in denitrifying communities are most likely explained by a combination of plant effects and wetland management-related factors. These include water residence time and nutrient load, which have been identified as key regulators of denitrifier community activity and composition in treatment wetlands (Kjellin *et al.*, 2007). In agreement, ammonium and nitrate loadings have previously been shown to correlate to changes in the community structure of *nirS*-type denitrifiers in the bulk sediment of the Empuriabrava FWS-CW in areas devoid of vegetation (Ruiz-Rueda *et al.*, 2007), but the effect on the rhizosphere community was not investigated. Sequences retrieved from June showed fewer polymorphisms and mainly related to *nosZ* in *Betaproteobacteria*, which contrasted with the greater heterogeneity encountered in sequences obtained from the September rhizosphere samples. Most of the *nosZ* sequences showed homologies to those from uncultivated bacteria obtained from environmental samples, such as estuarine sediments, agricultural soil and activated sludge.

In conclusion, vegetation influenced potential nitrification and nitrate reduction as well as the community structure of ammonia-oxidizing and denitrifying bacteria in the sediment of the CW. *T. latifolia* and *P. australis* rhizospheres exhibited significantly different nitrification and nitrate reduction rates, which may be of particular interest in the management of CWs at high nutrient loadings. A plant species effect on the microbial community was only seen for denitrifying bacteria and the differences between *Typha* sp. and *Phragmites* sp. associated denitrifiers were more evident in periods of high nutrient load, indicating that environmental conditions, plant development and plant species together changed the bacterial community in the rhizosphere of emergent plants in CWs.

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