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Diversity of the nitrite reductase gene *nirS* in the sediment of a free-water surface constructed wetland

Olaya Ruiz-Rueda,[1] Rosalia Trias,[1] Librado J. Garcia-Gil,[2] Lluís Bañeras[1]*

[1]Institute of Aquatic Ecology, Faculty of Sciences, University of Girona, Girona, Spain.
[2]Department of Biology, University of Girona, Girona, Spain

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Summary. The diversity of the nitrite reductase gene *nirS* was studied in the bulk sediment of a free-water surface constructed wetland (FWS-CW) located next to the Empuriabrava wastewater treatment plant (WWTP), in Castelló d'Empúries (Girona, NE Spain). The study period extended from the inception of the treatment wetland, in June 1998, until March 1999 and comprised periods of relatively high nitrate and ammonium concentrations at the influent and low nitrate-removal efficiencies. To evaluate *nirS* diversity, partial gene sequences were obtained by cloning of the respective PCR products. Rarefaction curves based on DOTUR analyses of the deduced amino-acid sequences predicted a greater diversity of *nirS* genes in samples containing higher ammonium concentrations. Estimated Shannon-Weaver indices of the four cloned samples showed a positive relationship with the $N\text{-}NH_4^+/N\text{-}NO_3^-$ ratios measured at the FWS-CW inlet. Identities between the deduced amino-acid sequences and those previously deposited in public databases ranged from 72 to 97%. Phylogenetic analysis based on these deduced sequences grouped 165 *nirS* clones in seven main clusters according to high similarity indices. Up to 60% of the clones clustered together in a highly homogeneous group with little homologies to any sequence retrieved from cultured representatives. Moreover, prevailing environmental conditions appeared to select for particular denitrifying populations, e.g., with respect to ammonium load and nitrogen removal efficiencies. This observation is of particular interest for the management of treatment wetlands, in which only slight variations in the theoretical denitrification potential of the system can occur. [**Int Microbiol** 2007; 10(4): 253-260]

Key words: *nirS* gene · denitrification · free water surface constructed wetland · sediments

Introduction

Constructed wetlands are engineered ecosystems designed for the unconventional on-site treatment of diffuse or non-point polluted water, such as often derived from industrial and rural management or as wastewater from small settlements. The two main types of constructed wetlands are free-water surface and subsurface flow systems [18]. Free-water surface constructed wetlands (FWS-CWs) can be used to treat the wastewater of large municipalities, especially those located in temperate climates. Treatment processes within a FWS-CW mainly involve the use of vegetation, which promotes the massive growth of bacteria through the production of organic-matter exudates and by oxygen diffusion to the sediments surrounding the root system [24]. In addition, emergent plants slow down the rate of water flow and thus are able to trap many pollutants, which may be further transformed and removed from the water by microbial communities [5,7,16].

Organic-matter reduction and nitrification/denitrification processes are among the major purposes of FWS-CW. Denitrification, in which there is a net loss of nitrogen from water, takes place ubiquitously in soils and sediments. It consists of the stepwise biological reduction of nitrogen oxides to

***Corresponding author:** L. Bañeras
Institut d'Ecologia Aquàtica
Universitat de Girona
Campus de Montilivi s/n
17071 Girona, Spain
Tel. +34-972418177. Fax +34-972418150
E-mail: lluis.banyeras@udg.es

molecular nitrogen, coupled to the phosphorylation of elements in the electron-transport chain. Denitrification has become an issue of global concern, since some of the produced intermediates, i.e., nitric and nitrous oxides, may contribute to the degradation of the ozone layer and to act as greenhouse gases [14].

Denitrifying organisms are present in a broad range of taxa, including *Bacteria* and *Archaea*; in addition, denitrification is carried out in the mitochondria of several species of fungi [30]. True denitrifiers differ from nitrate-reducing organisms in that the former can reduce NO_2^- to NO and finally to nitrogen gas, thus removing nitrogen from water. Nitrite reductases are responsible for this step and can be found among denitrifiers in two structurally different but physiologically and functionally equivalent enzymes: Cu-containing nitrite reductases and heme-containing cd_1 nitrite reductases, encoded by the *nirK* and *nirS* genes, respectively [9,30]. The screening of functional genes involved in denitrification as molecular markers of this process has proven useful to study the diversity of denitrifying bacteria in different environments. Specific PCR primers for *nirK*, *nirS*, and *nos*Z (the latter encoding nitrous oxide reductase) have been used to survey the composition of the denitrifying community in samples from aquatic and soil habitats and to establish correlations with environmental variables [3,4,6,12,13,25]. Moreover, the information gained from the cloning and sequencing of *nirS* and *nirK* genes has provided a comprehensive measure of the community diversity of environmental samples and has revealed the existence of novel denitrifying bacteria that have diverged completely from previously cultured bacteria [3,8,11].

Constructed wetlands are systems designed to improve water quality and yield parallel benefits such as increasing the availability of food and habitats for wildlife as well as for aesthetic recreation. Therefore, intensive research on the denitrifier communities in FWS-CWs and the changes in community composition that occur due to natural or human impacts on wastewater treatment systems is essential to better understand the role of denitrifiers in nitrogen cycling. The aim of this study was to assess the structure of the denitrifying community within a model FWS-CW and to monitor seasonal and human-derived changes in the community through the analysis of *nirS* genes as functional molecular markers.

Materials and methods

Site description and sampling. The FWS-CW of Empuriabrava was set up in the summer of 1998 as a tertiary treatment of a wastewater treatment plant (WWTP). It is located in the vicinity of a highly developed tourist resort in Castelló d'Empúries (Girona, NE Spain) and was originally designed to provide water of improved quality for flooding the nearby marsh area of the natural reserve Els Aiguamolls de l'Empordà. The system covers a surface area of 64,802 m^2 with an average depth of 0.5 m and it comprises three parallel cells plus a receiving lagoon. The treatment cells are sparsely covered with vegetation, mainly consisting of reeds (*Phragmites australis*) and cattails (*Typha latifolia*) grouped in independent communities. The wetland receives an annual mean flow of 1600 m^3/day, with strong seasonal variations ranging from 4984 m^3/day (August) to 650 m^3/day (February).

Physical and chemical monitoring of the FWS-CWs were carried out routinely every 15 days. Water samples for chemical analyses (100 ml) were collected directly from the effluent of the WWTP and at the pumping station in the receiving lagoon (see Fig. 1 for locations of sampling points). Conventional standard methods employed in wastewater analysis were used to

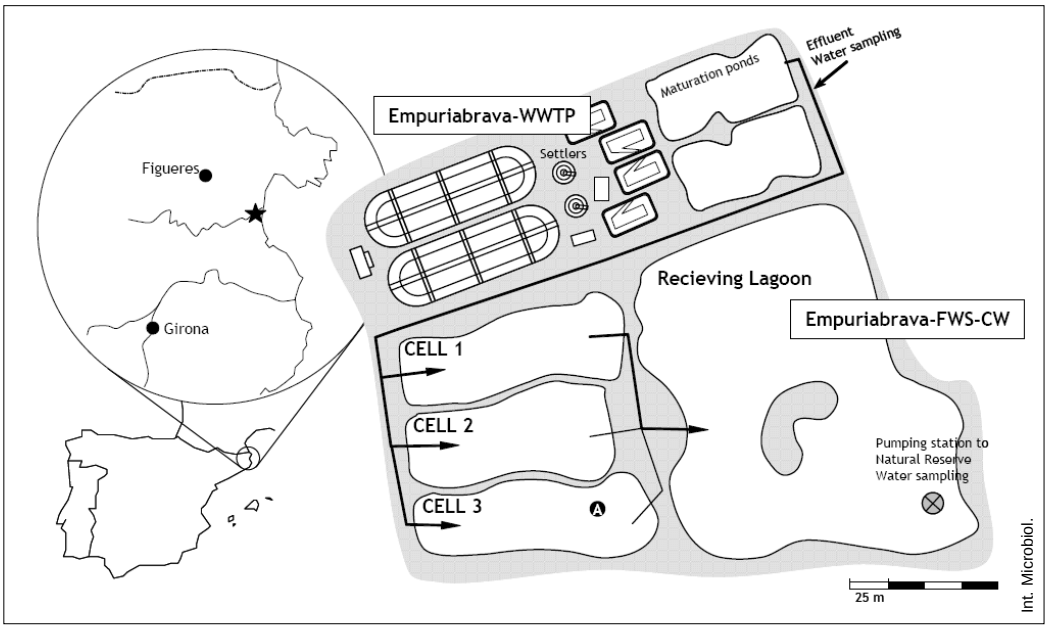

Fig. 1. Schematic map of the Empuriabrava wastewater treatment plant (WWTP) and the adjacent free water surface constructed wetlands system (FWS-CW). Sediment samples were taken at station A. Points for water sampling are marked as Effluent and Pumping station. Solid line shows the preferential water flow.

measure pH and N-NO_3^-, N-NO_2^- and N-NH_4^+ concentrations [1]. Total inorganic nitrogen (TIN) was calculated from the sum of the amounts of nitrate, nitrite, and ammonia.

The sampling station was located 30 m away from the outlet of the third cell of the constructed wetland (Fig. 1). Samples were obtained regularly from the inception of the FWS-CW. All data refer uniquely to four sampling dates, June 1998, October 1998, December 1998, and March 1999. Sediment samples were collected as single undisturbed sediment cores by using a manual sampler mounted with a 9-cm diameter Plexiglas tube, and were transported to the laboratory in a portable ice box in the dark. Smaller sediment cores (2.5 cm diameter) were obtained aseptically and the upper 3 cm were completely homogenized by means of manual stirring. Triplicate 1-g aliquots were placed in sterile 2-ml Eppendorf tubes and stored at –20ºC until processed.

Nucleic acid extraction. Nucleic acids were extracted according to a previously described phenol-chloroform method for the isolation of DNA from wetland sediment samples [20], with the following modifications: 200–400 mg of sediment was added to 2-ml screw-capped vials containing 1.5 g of a mixture of 0.1-mm (diameter) glass and 0.5-mm (diameter) silica beads (BioSpec Products, Bartlesville, OK). The beads were mill-homogenized at maximum intensity (2800 rpm) for two consecutive periods of 45 s in a MiniBeadBeater-8 mill (BioSpec Products). Both chemical and enzymatic treatments and DNA purification and concentration were done following the methods described by Miller et al. for freshwater sediments [20].

PCR amplification of *nirS* genes. The *nirS* gene was amplified in a Geneamp PCR system 9700 (Applied Biosystems, Foster City, CA) with the primer pair nirS1F:nirS6R [2]. PCR reaction mixtures contained 1 × PCR reaction buffer, 2 mM $MgCl_2$, 200 μM total dNTPs, 0.8 mg bovine serum albumin (New England Biolabs, Beverly, MA)/ml, 1 μM of each primer, 10–100 ng of the DNA extracts, and 0.025 U *Taq* polymerase (AmpliTaq DNA Polymerase, Applied Biosystems) in a total volume of 50 μl. After an initial denaturation step of 5 min at 94ºC, amplification reactions were carried out with 10 touchdown cycles of denaturation (30 s at 94ºC), primer annealing (1 min, 60ºC, decreasing 0.5ºC at each cycle), and primer extension (1 min, 72ºC), followed by 30 cycles at an annealing temperature of 57ºC and a final extension step of 10 min at 72ºC. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized after staining with ethidium bromide (0.5 mg/l).

Clone libraries of *nirS*. Four sediment samples were selected and used for an extensive analysis of *nirS* gene diversity as determined through cloning experiments. The four samples were obtained from station B at four different phases of the flooding and colonization of the FWS-CW: June, October, and December 1998, and March 1999. The *nirS* gene was cloned using the pGEM-T Easy vector (Promega, Madison, WI) following the instructions provided by the manufacturer. Ligated products were transformed into *Escherichia coli* DH5 competent cells, and positive transformants were color-screened on LB plates supplemented with ampicillin (100 μg/ml), X-Gal (80 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 20 mM). Clones were selected for positive *nirS* fragments by PCR using the primer set nirS1F:nirS6R in 20-ml reactions as described above but without the addition of BSA and using a 35-cycle PCR program at a constant annealing temperature of 57ºC. Positive clones were sequenced using vector-specific primers M13F and M13R (Macrogen, Seoul, South Korea). Sequences were manually refined using the BioEdit package [10] and then aligned using ClustalW [28].

Construction of rarefaction curves and phylogeny. DNA alignments of partial *nirS* gene sequences retrieved from clones were used to construct a DNAdist (DOTUR package) DNA distance matrix. Rarefaction matrices and Shannon-diversity and evenness indices were also calculated with the DOTUR package [26]. Cut-off values of 20% sequence divergence were selected, following the recommendations for protein-coding genes. Phylogenetic and molecular evolutionary analyses were carried out with deduced amino-acid sequences and by using the MEGA version 3.0 software. Phylogeny was reconstructed according to the Amino Poisson correction method and by pair-wise deletion using a bootstrap value of 1000. *Pseudomonas aeruginosa* gene sequences *nir*N (D84475) and *nir*F (D50473) were used for comparison.

Nucleotide sequence accession numbers. The partial *nirS* sequences were deposited in GenBank under accession numbers EF558372 through EF558536.

Results

Chemical characterization of the Empuriabrava-FWS-CW. The amount of water flowing through the treatment wetland strongly depended on the sizes of the summer tourist populations in the nearby tourist resorts of Empuriabrava and Roses. Calculated mean residence times varied from approximately 6.3 days in August to almost 33 days in February. The differences in water mean residence time were mainly due to changes in the amount of water flowing through the Empuriabrava WWTP, since there was little influence due to evaporation or changes in the water level at the treatment wetlands. Significant positive and negative correlations ($P \leq 0.05$) were found between wastewater flow and ammonium or nitrate concentration at the influent, respectively (Table 1). Generally, high ammonium concentrations occurred during summer, with maximum point values of 26.5 mg N-NH_4^+/l recorded during July 1998. Nitrate concentration at the influent showed the opposite behavior, with maximum values recorded at low flow rates and during high residence times in the winter and early spring. Nitrite showed little variation throughout the study period. Concentrations of less than 0.3 mg N-NO_2^-/l were invariably recorded at the inlet and outlet sampling stations of the FWS-CW (results not shown). The removal of TIN, calculated in the FWS-CW according to mass-balance equations, varied from 118 to 2737 kg N/month, which corresponded to efficiencies of 25.9 and 91.7% of the nitrogen load. Greater removal efficiencies were measured during the summer.

In contrast to the wide variations in nitrogen concentration, the pH of the water in the Empuriabrava-WWTP remained almost constant, with values around 7.4 (Table 1). However, a measurable increase in pH over the entire study period was measured at the constructed wetland. At the sampling point located at the pumping station, pH records reached mean values of 10.0 during March 1999. The higher pH values recorded at the outlet may have been indicative of high-level metabolic activity.

Table 1. Mean monthly values and deviation of the mean of relevant environmental variables at the Empuriabrava free water surface constructed wetland (FWS-CW) during the study period

	Residence time (days)	pH inlet	pH outlet	NH_4^+ inlet (mg N/l)	NO_3^- inlet (mg N/l)	TIN removal (%)[b]
Year 1998						
June[a]	17.4	7.4 ± 0.2	9.1 ± 0.3	7.2 ± 3.8	6.2 ± 0.6	56.7
July	9.8	7.6 ± 0.1	8.5 ± 0.5	16.1 ± 6.9	2.3 ± 2.4	78.0
August	6.3	7.3 ± 0.1	8.2 ± 0.4	17.2 ± 6.0	1.8 ± 1.1	91.7
September	10.4	7.5 ± 0.2	8.8 ± 0.2	11.2 ± 3.8	3.1 ± 2.4	50.5
October	16.0	7.5 ± 0.3	8.8 ± 0.0	10.5 ± 4.5	3.3 ± 4.0	83.7
November	28.2	7.2 ± 0.1	8.4 ± 0.7	4.8 ± 0.9	7.6 ± 2.0	49.0
December	21.4	7.7 ± 0.2	8.0 ± 0.4	4.6 ± 0.3	9.8 ± 0.2	25.9
Year 1999						
January	19.8	7.3 ± 0.1	8.6 ± 0.0	7.6 ± 2.6	9.35 ± 0.3	55.2
February	33.5	7.9 ± 0.1	9.1 ± 0.5	8.0 ± 2.8	9.43 ± 0.4	49.0
March	28.6	7.6 ± 0.1	10.0 ± 0.3	2.8 ± 1.0	9.30 ± 0.1	68.7
April	19.7	7.6 ± 0.1	9.1 ± 0.1	5.6 ± 0.6	2.63 ± 1.1	75.2

[a]Data collected beginning June 15. [b]Mean monthly removal efficiencies (%) of total inorganic nitrogen.

Analysis of the nitrite reductase (*nirS*) gene clone library. *nir*S sequences were obtained from four sediment samples taken at different times from the sampling station of the FWS-CW treatment cells: the inception of the FWS-CW, June 1998 (JUN98, CloneD library); October 1998, a period of high ammonium content and highly efficient removal rates (OCT98, CloneA library); and December 1998 (DEC98, CloneC library) and March 1999 (MAR99, CloneB library), two periods of relatively high nitrate concentrations. Positive *nir*S clones from each library were randomly selected and completely sequenced. There were few positive clones from the JUN98 sample, although cloning was carried out twice, slightly changing the PCR conditions. Nonetheless, only 15 positive clones could be obtained and sequenced. The effective coverage with respect to overall diversity and number of species was evaluated from rarefaction curve plots (Fig. 2). Sample OCT98 contained the highest richness, resulting in 18 operational taxonomic units (OTUs) out of 68 clones screened. In contrast, although the rarefaction curves could not be saturated for the sample JUN98 and, consequently, fewer OTUs were detected, the estimated Shannon-Weaver (SW) diversity index was comparable to that determined in October 1998 (Table 2).

Samples DEC98 and MAR99, representing conditions of higher nitrate concentration at the inlet of the FWS-CW and TIN removal efficiencies of 26 and 69%, respectively, had lower diversity indices.

Deduced NirS amino-acid sequences revealed at least 72% identity to previously deposited sequences in public databases. Phylogenetic analyses produced a relatively high number of groups with low similarity to sequences from previously isolated and cultured microorganisms (Fig. 3). This clustering agrees with previous topologies found for the *nirS* gene in marine environments and marsh soils [22]. Group I (130 clones, 79% of the total) consisted of *nirS* sequences with the lowest identities to known sequences, and thus little relatedness to identified bacteria. In general, a BLAST search for these sequences revealed their high similarity to *nirS* clones retrieved from marine environments. Sequences in subgroup I.a had 78–92% identity to marine clones but showed little homology with any cultured representative. Subgroup I.b comprised clones with similarities to previous sequences of 76–83%, *Azoarcus tolulyticus* (AY078272.1) being the closest cultured reference. Six clone sequences were assigned to subgroup I.c, together with *nirS* sequences of *Ralstonia eutropha* (AF114789), and were 79–97% identical to cloned *nirS* sequences retrieved from activated sludge treatment reactors.

Group II (30 clones) comprised several subgroups supported by high bootstrap values. Members of subgroup II.a were 85–90% related to sequences present in activated sludge reactors and showed little similarity with reported sequences derived from cultured representatives. CloneB46,

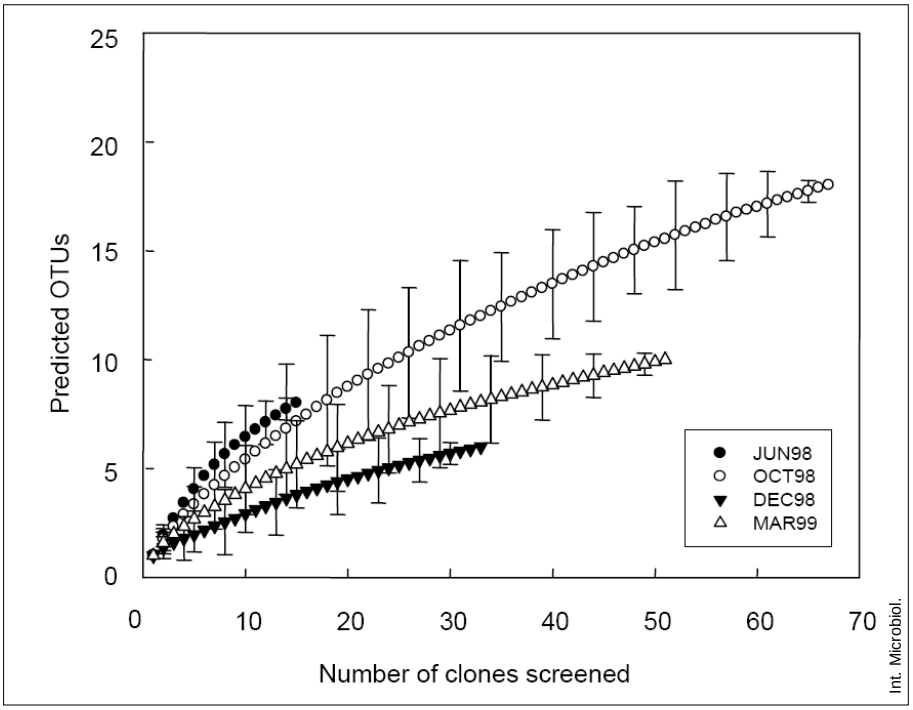

Fig. 2. DOTUR rarefaction curves indicating the diversity of *nirS* gene sequences of individual cloned samples from June 1998, October 1998, December 1998, and March 1999 of the Empuriabrava FWS-CW. Error bars represent the 95% confidence interval.

obtained in March 1999, clustered with *Azospirillum brasilense* and was considered as a single group. Most reference sequences from cultivated microorganisms clustered together with CloneA38, CloneA44, and CloneA68 in group IIb. All environmental sequences derived from the October 1998 sample. Clones included in subgroup II.c shared more than 90% identity with previously known *nirS* gene sequences, mostly retrieved from nitrate- and uranium-contaminated aquifers.

Finally, CloneC8, isolated in December 1998, branched individually in Group III. This clone was found to be closely related to *nirS* sequences retrieved from marine samples, which were previously shown to cluster apart from other *nirS* sequences [3,22].

Table 2. Diversity indices calculated from DOTUR rarefaction analysis using a distance similarity cut-off of 20% of the deduced amino acid sequences of partial *nirS* genes. *S*: number of operational taxonomic units (OTUs) determined based on rarefaction analysis. The number of clones screened is shown in parentheses. *H*, Shannon-Weaver diversity index; *E*, evenness calculated as $E = H/Hmax$, where $Hmax = ln(S)$

	S	*H*	*E*
JUN98	8 (15)	1.933	0.929
OCT98	18 (67)	2.015	0.697
DEC98	6 (33)	0.845	0.471
MAR99	10 (51)	1.360	0.590

Discussion

A cloning-based analysis was used to estimate the composition and diversity of NirS-containing denitrifying bacteria in sediments of the Empuriabrava FWS-CW. The study of denitrifying communities in wastewater treatment systems such as this one is of major importance, since nitrogen removal from water depends mostly on denitrification rates. Although our results are limited to the analysis of *nirS* gene sequences, we also attempted a similar study on *nirK*. Unfortunately, despite the use of different primer sets designed for the analysis of *nirK* genes and reactions carried out at several annealing temperatures, no positive PCR products were obtained from the samples studied. Other reports in the literature have highlighted the difficulties in amplifying either kind of nitrite reductase from certain ecosystems, including upland soils and marine sediments. For example, *nirS* genes could not be detected in a sample obtained from a forested upland soil in Michigan, USA, nor in the rhizosphere of cultivated grain legumes [22,27]. However, *nirK* was detected, albeit not readily, in marine sediments from Puget Sound, Washington, USA, and from the River Colne estuary, in the UK, even though high denitrification rates were assumed to occur in the latter [3,21]. Yan et al. [29] observed different trends in *nirS*- or *nirK*-harboring denitrifier communities retrieved from five contaminated groundwater sites. These authors also estimated inversely related diversity indices for *nirK* and

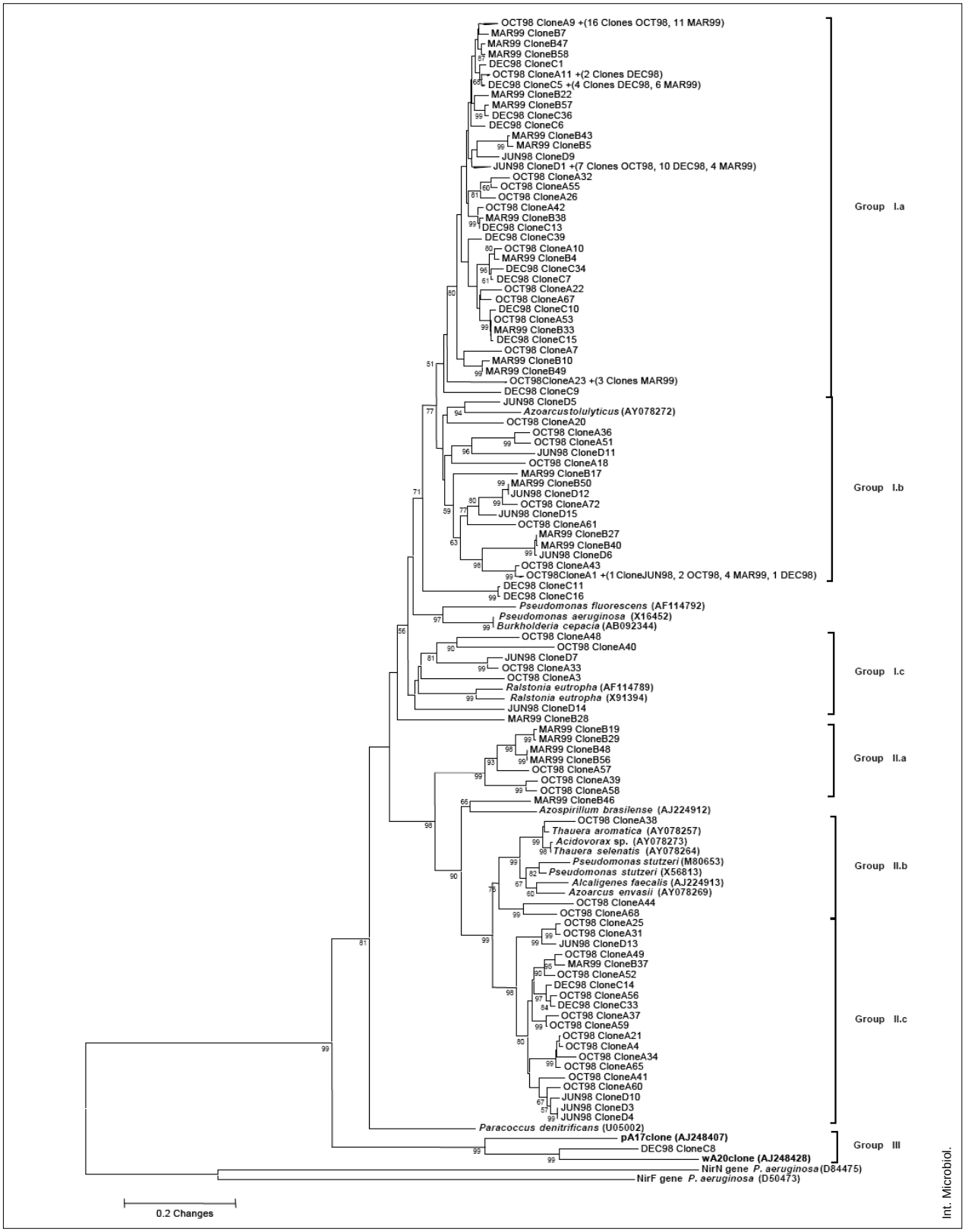

Fig. 3. Neighbor-joining phylogenetic diagram of translated *nirS* partial sequences (ca. 290 residues) from bulk sediment of the Empuriabrava FWS-CW. Amino Poison correction and pairwise deletion were used. Percentage values supporting bootstrap values higher than 50% are indicated at branch points. *nirF* (D50473) and *nirN* (D84475) gene products served as outgroups. Clusters containing >99% identical sequences are indicated by a representative sequence followed by the amount and origins of the other sequences. Selected database sequences and accession numbers are highlighted in bold type.

Table 3. Relative frequencies (%) of partial *nirS* sequences obtained from cloned samples in every group defined from the phylogenetic analysis

	Group I			Group II				
	I.a	I.b	I.c	II.a	II.b	II.c	Group III	Others
JUN98 (*n* = 14)	14	43	14			29		
OCT98 (*n* = 67)	51	15	6	4	4	19		
DEC98 (*n* = 33)	82	3				6	3	6
MAR99 (*n* = 51)	71	16		8		2		4

nirS genes. This apparent preference of microorganisms harboring either type of nitrite reductase for certain environments might be due to the selection of denitrifying bacteria better adapted to particular environmental conditions. However, the presence of *nirK*-harboring organisms in the Empuriabrava FWS-CW cannot be completely ruled out from the analyses reported here, and other studies, such as selective isolation of denitrifying bacteria, should be conducted in those sediments.

The existence of a widely occurring group of *nirS* sequences that lack any cultured representatives (approximately 80% of retrieved sequences) stresses the need to intensify efforts aimed at the isolation of new species of denitrifiers. The phylogenetic analysis clustered all clones into three main groups. Group I, especially subgroup I.a, contained most of the clones and comprised sequences less closely related to those found in the databases, which suggests that the diversity of *nirS* will increase as new environments and conditions are investigated. The greatest homologies found in this group corresponded to partial *nirS* fragments obtained from either marine sediments or the marine water column [3,17]. This could be due to the scarce studies on denitrifying organism from non-marine environments, which reduces the number of sequences available for comparison. Some of the clones included in Group I, in particular those in subgroup I.c, showed closest homology with *nirS* sequences retrieved from activated sludge treatment reactors and cultured representatives such as *Ralstonia eutropha*. Sequences in Group II represented only 20% of all analyzed sequences, branched together with sequences from well-characterized denitrifying microorganisms, and resembled *nirS* sequences obtained from wastewater treatment reactors [29].

The prevailing environmental conditions, such as the ammonium concentration in the influent entering the constructed wetland or the removal capacity, determine the diversity of the *nirS*-containing denitrifying community. Relative abundances of clones clustering in groups I.a–III varied according to the sampling date (Table 3). Sample JUN98, obtained at the inception and during flooding of the FWS-CW, contained the highest relative number of clones belonging to Groups I.b and II.c, which were poorly represented in the other cloned sequences. This suggests that bacterial diversity is mainly affected by the water of the WWTP itself or the original microbial diversity of the impermeabilization bed covering the bottom of the wetland. The highest diversity indices were detected in the June and October 1998 samples, when the FWS-CW influent was characterized by a high ammonium/nitrate ratio. Although this condition was less favorable for high specific denitrification rates, the removal efficiencies of TIN under other conditions have been reported to fall in the same range. By contrast, in the winter, nitrate supplies and retention times in the wetland were higher. As shown by the diversity of the *nirS* gene, the high nitrate supply and the retention times favor the onset of a few defined populations, such that more than 85% of the retrieved sequences clustered in groups I.a and I.b. Using similar molecular techniques, other authors previously reported variations in the community composition of denitrifying bacteria as a response to environmental factors [15]. Nitrate and oxygen were shown to have a decisive effect on the structure of denitrifier communities in the continental margin sediments of the oxygen-deficient zone of the Pacific Coast of Mexico [19]. Moreover, Castro-González et al. were able to demonstrate variations in the relative abundances of *nirS* terminal restriction fragments along O_2, NO_3^-, NO_2^- and depth gradients in the water column of the oxygen minimum zone in the Eastern South Pacific [6].

The study of shifts in the diversity and composition of denitrifying and other microbial communities [23] is of particular interest in engineered systems, such as the constructed wetland studied here, that face high seasonal variations. In treatment systems implemented mostly for the removal of nitrogen, the complete potential of the denitrification activity must be expressed regardless of changing conditions, including lower nitrification rates in the WWTP and a high ammonium concentration at the inlet of the constructed wetland.

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