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ORIGINAL ARTICLE

Impact of plant density and microbial composition on water quality from a free water surface constructed wetland

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Abstract

Aims: To correlate microbial community composition and water quality changes within wetland cells containing varying plant densities and composition in a free water surface (FWS) constructed wetland.

Methods and Results: Water chemistry was monitored weekly for nitrate, orthophosphate, and suspended solids, at various sites throughout the wetland for 6 months. Treatment ponds with 50% plant cover had about a 96·3% nitrate removal. The average change between the influent and effluent was 50–60% nitrate removal and 40–50% orthophosphate removal. Community profile of total DNA, generated by using denaturing gradient gel electrophoresis (DGGE), was used to determine the major microbial composition associated with the wetland sediment, rhizosphere, and surface water. Bacterial cloned libraries were constructed, and 300 clones were analysed by amplified ribosomal DNA restriction analysis (ARDRA) and grouped into operational taxonomic units (OTUs). A total of 35, 31, and 36 different OTU were obtained from sediment, rhizosphere, and surface water, respectively. The bacterial members within the dominant group of our clone library belonged to unclassified taxa, while the second predominant group consisted of members of the phylum *Proteobacteria*. The dominant organisms within the class were in the γ, β, and δ classes.

Conclusion: Microbial diversity as determined by Shannon-Weaver index (H) was higher in the wetland cells with 50% plant density than the 100%. This was in agreement with the most efficient wetland contaminant removal units.

Significance and Impact of the Study: This study provides evidence that wetlands with 50% plant cover may promote the growth of diverse microbial communities that facilitate decomposition of chemical pollutants in surface water, and improve water quality.

Introduction

The Prado Wetlands have been operating for the removal of nitrate-nitrogen (NO_3-N), to provide habitats to various aquatic invertebrates, as well as provide valuable wildlife habitat to endangered species (e.g. the Least Bell's Vireo, *Vereo bellii pusillus* Couse) since 1992. Water sources feeding the wetland include: the Santa Ana River, an effluent-dominated body of water that receives natural mountain runoff and tertiary wastewater from various municipalities in San Bernardino and Riverside Counties, and water from various creeks in the area that passes through a 10 000 ha area with about 350 000 cattle. This area has the highest concentration of cattle in the United States and was once designated as a Dairy Preserve. During the summer, influent NO_3-N from the Santa Ana

River averages 10 mg l^{-1} and can be reduced to 1 mg l^{-1} at the outflow of the wetlands (Orange County Water District 2001). The wetlands operate through a combination of microbial and plant processes to reduce nutrients from the river before the recharge basin. Approximately, half of the daily flow of the Santa Ana River is diverted into the 186-ha Prado Wetlands. The residence time is 5–7 days, depending on the time of year and level of plant cover. Individual cells in the wetlands can have removal efficiencies between 10% and 95% of nitrate in the water column (Orange County Water District 2001).

Constructed wetlands can offer significant benefits to human populations in both developed and developing countries (Kadlec and Knight 1996; Kivaisi 2001). Benefits include water quality improvement, water reclamation, habitat for species conservation, flood control, recreational and education activities. Our goal is to increase the understanding of the interaction of nutrient cycling rates with microbial population structure, wetland plant cover, and water/sediment interaction. We therefore, hope to appraise the interaction of wetland plant, bacterial community, and efficiency of removal of nitrate in this wetland.

One of the major concerns of constructed wetland has been the issue of vegetation management. Those opposed to vegetation management believe that the regular harvest of treatment systems is too costly, unsustainable, impractical (Reed *et al.* 1995; Kadlec and Knight 1996; USEPA 1999), does little to improve water treatment (Tchobanoglous 1987; USEPA 2000; Wetzel 2000), and it reduces the readily available carbon source necessary for denitrification (USEPA 1999). On the other hand, it has strongly been argued that not only is vegetation management used as an effective way to remove the accumulated nutrients within the plants (Reddy and DeBusk 1987; Breen 1990; Asaeda *et al.* 2000), but by opening up dense vegetated areas it reduces mosquito habitat (Tchobanoglous 1987; Russell 1999) as well as promotes the photosynthetic periphyton in the system (Grimshaw *et al.* 1997; Wetzel 2000). Aquatic plants in treatment wetland act as filters (Hammer 1989; Brix 1994), take up nutrients (Liu *et al.* 2000), provide a substrate for microbiota (algae, bacteria, fungi, protists) (USEPA 2000; Wetzel 2000), and provide a carbon source for denitrification (Ingersoll and Baker 1998).

While the advantages and disadvantages of vegetation management are highly documented, and general features such as influent/effluent nutrient concentrations are well known, changes in microbial processes and activity governed by wetland per cent cover of plants is still poorly understood. It is well established that constructed wetlands with clean water systems, may be nitrogen-limited causing competition for nutrients among bacteria, fungi, and algae (Rheinheimer 1980). Constructed treatment wetlands with nitrogenous compounds are likely to support large populations of nitrifying and denitrifying bacteria, fungi, and phytoplankton that use inorganic nitrogen. Ammonium nitrogen reduction is often a major factor in the design of constructed treatment wetlands (Kadlec and Knight 1996).

In this study, the role of plant density in the development of microbial composition in surface water, sediment, and on the roots of wetland plants was examined. Most of the work done in association with microbial composition in subsurface open wetlands has been at the descriptive or anecdotal level using plate counts. Here we used classical techniques of microbial ecology including plate counts and direct measurements of nutrients and in addition we used molecular methods specifically, the polymerase chain reaction (PCR), a powerful tool for amplifying and detecting specific nucleic acid molecules present at low levels in the environment coupled with analysing 16S ribosomal RNA (rRNA) profiles generated by denaturing gradient gel electrophoresis (DGGE). The dynamics of the dominant bacterial communities inhabiting the wetland cells were examined using the PCR approach for analysing 16S rRNA profiles generated by DGGE. The resulting DNA band pattern provided a fingerprint of the microbial community structure, in which each band represented a group of bacteria having 16S rRNA sequences with a similar melting temperature (Muyzer *et al.* 1993). This approach provided evidence of changes in microbial composition with different plant species and density in a way that the culture-dependent method lacked. This approach has resulted in a better understanding of the distribution, diversity, and composition of microbial communities associated with a large wetland system under different plant densities.

Materials and methods

Experimental site

The Prado Wetlands (Fig. 1; 33·9°N, 117·9°W) in Chino, California is approximately 186 ha free water surface (FWS) constructed wetland consisting of 50 interconnecting shallow ponds that treats approximately 50% of Santa Ana River water prior to its passage to Orange County, California where it is used for groundwater recharge. The primary functions of the wetlands are to reduce nitrate levels in Santa Ana River water destined for groundwater storage basins in Orange County, California (Mills *et al.* 1998) as well as to provide valuable wildlife habitat to endangered species (e.g. the Least Bell's Vireo, *Vireo bellii pusillus* Coues). We carried out this study in six replicate wetlands cells or ponds plus the inflow and outflow

Figure 1 Prado wetlands showing the sampling sights for June–November 2004. The arrows indicate the direction of flow. The wetlands treat 50% of Santa Ana River water. The sampling sites were chosen based on their percentage of plant cover: (1) inflow; (2) outflow; (3) 25% cover; (4) 50% cover; (5) 75% cover; (6) 50% cover; (7) 100% cover. Letters A and B after the numbers indicate influent water to that cell and effluent water out of the cell as seen by the direction of water flow. Two cells were used for each treatment.

sections of the wetland from June to November 2004. The research cells measuring approx. 80 m by 12 m were filled from a common inflow marsh to a depth of approx. 0·5 m (Fig. 1). A more detailed description of the research cells can be found in Walton and Jiannino (2005). Emergent vegetation was dominated at equal ratio by cattail (*Typha* sp.) and bulrush [*Schoenoplectus californicus* (Meyer) Soják] in all the wetland cells with plants.

Chemical and microbiological analysis of wetland samples

Water chemistry was determined weekly from June to November 2004 in five replicate wetland ponds (Fig. 1) plus the wetland inflow and outflow. The five wetland cells (Fig. 1) are 7 A&B (100% plant cover), 6 and 4 A&B (50% plant cover), 5 A&B (75% plant cover), and 3 A&B (25% plant cover). Samples were analysed for NO_3-N, biochemical oxygen demand (BOD), total suspended solids (TSS), orthophosphate (PO_4-P), and pH (APHA 1995). Bacterial analysis was performed on surface water samples filtered in phosphate-buffered saline (PBS) (0·0425 g l^{-1} KH_2PO_4 and 0·4055 g l^{-1} $MgCl_2$). Ten- and 100-fold dilutions were also prepared in PBS, vortexed, and 1 ml of each dilution was filtered in duplicate. One, 10, and 100 ml volumes (via membrane filtration on agar) were plated onto Tryptic soy agar (TSA) for heterotrophic plate counts (HPC); enterococci were enumerated using Enterococcosel media and incubated for 22–24 h at 41°C (USEPA 2000); Sorbitol MacConkey agar (SMAC) was used for general *Escherichia coli* and incubated at 37°C for 24 h and colonies were enumerated. All media were purchased from Difco (Detroit, MI, USA) as dehydrated powders. Samples were also collected once from sediment, rhizosphere, and two times from water column and transported to the laboratory on ice for DNA extraction.

Water samples were collected with three 1-l grab samples taken from the middle of each wetland cells (Fig. 1) and from inflow to outflow and immediately placed on ice, transported to the laboratory, and processed within 8 h. Rhizosphere samples were separated by cutting the below ground part of the plant into different sterile ziplock bags. Bacteria were recovered from the rhizosphere (volume of soil adjacent to and tightly held by plant roots and influenced by the plant roots) by homogenization with 100 ml of PBS for 2 min at 260 rev min^{-1} in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK). Sediment samples (10–20 g) were collected (0–20 cm) below the sediment–water interface and transferred to sterile ziplock bags. Sediment samples were transported to the laboratory as described earlier and processed within 48 h for DNA extraction.

DNA extraction from wetland samples and PCR-DGGE analysis

Total bacterial DNA was extracted from 500 mg of the sediment and rhizosphere samples and from 250 mg pellet of concentrated water sample (1 l) centrifuged at 3000 ***g*** for 10 min. DNA was extracted using UltraClean soil and Water DNA kits (MO BIO, Inc., Solana Beach, CA, USA), according to the manufacturer's protocol with slight modifications and stored at −20°C. A 236-bp DNA fragment in the V3 region of the small subunit ribosomal RNA genes of bacteria was amplified by using primer set PRBA338f and PRUN518r (Øvreås *et al.* 1997). For the purpose of separating the 16S rRNA bacterial communities in a DGGE gel, a GC clamp was added at the end of primer PRBA338f. Ready-To-Go PCR beads from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and 5 pmol of primers in a total volume of 25 ml were used in the PCR reaction. PCR amplifications were performed under the following conditions: 92°C for 2 min; 30 cycles of 92°C for 1 min, 55°C for 30 s, 72°C for 1 min followed by a final extension at 72°C for 6 min.

DGGE was performed with 8% (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30% to 70% denaturant with 100% defined as 7 mol l^{-1} of urea and 40% formamide. Gels were run for 3 h at 200 V with the Dcode^{TM} Universal Mutation System (Bio-Rad Laboratories, Hercules, CA, USA). DNA was visualized after ethidium bromide staining by UV transillumination and photographed with a polaroid camera. Major bands were excised for identification of bacterial species. Bands were placed into sterilized vials with 20 μl of sterilized, distilled water and stored overnight at 4°C to allow the DNA to passively diffuse out of the gel strips. Ten microlitre of eluted DNA was used as the DNA template with the bacteria primers. DNA was cloned into the TOPO TA cloning kit (Invitrogen, Carlbad, CA, USA). Isolation of plasmids from *E. coli* was performed using the Qiagen plasmid mini kit (Valencia, CA, USA). The purified plasmids were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA, USA).

PCR amplification of 16S rRNA sequences and library construction

The PCR amplification of 16S rRNA sequences was carried out using the bacterial specific-primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTAC CTT GTT ACG ACT T-3′). Amplification of the 16S rRNA was performed using the following steps: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension step of 72°C for 7 min. The resulting PCR products were purified from a 1% agarose gel and cloned into the TOPO TA cloning kit (Invitrogen). After PCR amplification of cloned rRNA genes using the 27f-1492r primer set, 5 μl of each amplicon (300 amplicons in total) were digested for 3 h at 37°C with 2·5 U of *Hinf*I and *Hha*I (New England Biolabs, Beverly, MA, USA) in 20-μl reaction mixtures. Restriction patterns were visualized on a 3% agarose gel in 1× TAE buffer and stained with ethidium bromide [1% (wt/vol)]. Gel images were digitalized, and restriction patterns were analysed using the Image master as previously stated (Ibekwe *et al.* 2001). Each different restriction pattern was defined as an operational taxonomical unit (OTU). DNA plasmids from one of each different representative OTU was commercially sequenced with an Applied Biosystems Prism 377 DNA sequencer using universal M13 forward and reverse primers.

DNA sequence and statistical analyses

DNA fingerprints obtained from the 16S rRNA banding patterns on the DGGE gels were photographed and digitized using ImageMaster Labscan (Amersham-Pharmacia Biotech, Uppsala, Sweden). The lanes were normalized to contain the same amount of total signal after background subtraction. The gel images were straightened and aligned using ImageMaster 1D Elite 3·01 (Amersham-Pharmacia Biotech) and analysed to give a densitometric curve for each gel. Band positions were converted to R_f values between 0 and 1 and profile similarity was calculated by determining Pearson's coefficient for the total number of lane patterns from two gels (Ibekwe *et al.* 2001). 16S rRNA libraries were constructed from the wetland samples containing a total of 300 sequences from samples collected in July 2004. The sequences of these clones were BLAST analysed using a 16S rRNA database containing over 33 000 sequences (Cole *et al.* 2003). Based on different restriction patterns, a clone which represents a restriction pattern was selected for sequencing. DNA sequences were edited manually to correct falsely called bases and trimmed at both the 5′ and 3′ ends using the Chromas software (Technelysium Pty. Ltd, Helensvale, Australia). Only sequences with unambiguous reads of about 600 bp were used for further analysis. The predicted 16S rRNA sequences from this study were compared with 16S rRNA sequences from the BLAST gene bank (ftp://ftp.ncbi/nih.gov/blast/executables/LATEST) and a FASTA-formatted file containing the predicted 16S rRNA sequences. Matrices of evolutionary distances were computed using the Phylip program with the Jukes-Cantor model (Jukes and Cantor 1969). Phylogenetic trees were constructed and checked by bootstrap analysis (1000 data sets) using the

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program SEQBOOT. Bootstrap values represented the frequency of resampling that supported a specific branching pattern. OTU were defined as clones with >97% sequence identity. For dendrogram construction, 35, 31, and 36 partial 16S rRNA sequences representing the most prevalent OTU from each environment (soil, rhizosphere, and water) were aligned using CLUSTALX version 1.8 for Windows (Thompson *et al.* 1997). Also included in this alignment were the most similar 16S rRNA sequences to each OTU from the NCBI databases. A second approach was used to determine community structure based on peak height from the different bacterial groups (16S rRNA bands) and was analysed to generate diversity indices (H). The peak height values generated from the sampling points were integrated and analysed. Data obtained were used to integrate the area under each peak for each lane in every treatment. For this analysis, each band was presumed to represent the ability of that bacterial species to be amplified. The Shannon index of diversity (H) was used to compare changes in diversity of microbial communities within all treatments at each time (Shannon and Weaver 1963) by using the following function:

$$H = -\sum P_i \log P_i$$

when $P_i = n_i/N$, n_i is the height of the peak, and N is the sum of all peak heights in the curve. Comparisons of bacterial (HPC, *E. coli*, and enterococci) plate counts of treatment means at any date were accomplished with the Tukey's test ($P = 0{\cdot}05$). All calculations were performed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS 1991).

Results

Wetland water and sediment characteristics

There was a substantial fluctuation in nitrate and orthophosphate levels throughout the summer and fall (Fig. 2a,b). NO_3-N concentration in the wetland water ranged from about 3·5 mg l^{-1} in early August to a maximum of about 10 mg l^{-1} in early October in the inflow water. The outflow nitrate concentrations followed the same pattern. The highest concentrations of inflow phosphate were found during the last week of September and early October and the high concentrations were maintained during the rest of the autumn sampling period (Fig. 2b). The cells with 50% plant cover had as high as 96·3% nitrate removal (Table 1), whereas, in the 100% plant cover nitrate removal was about 11·4%. These reduction levels were based on the differences between the nitrate concentration of the inflow to the wetland system and the effluent from the particular cell with 50% or 100% plant cover. Between 50–60% of nitrate and 40–50% orthophosphate was removed for the total wetland system. The TSS levels vary greatly from cell to cell. The highest variation was seen in the shallow cells at the end of the wetland system that have the least plant cover and therefore subject to wave action that stirs up the clay bottoms of the ponds (data not shown).

Bacterial counts

While there was little change in the HPC within the wetland, there were substantial drop both in *E. coli* and enterococci numbers (Fig. 3a,b) from inflow to outflow. The changes for *E. coli* and enterococci were more pronounced in wetland inflow where river water comes in contact with the wetland plants. In cells that had 50% plant density, the numbers of *E. coli* and enterococci in the water were higher in the effluent than the influent (Fig. 3a). The same effect was observed with the cells with 100% plant density. When sediment samples were analysed, higher numbers of *E. coli* and enterococci were found in sediment samples than water samples (Fig. 3a). There were no changes in the numbers of *E. coli* and enterococci throughout the wetland sediment; however, the numbers of *E. coli* in the sediment was consistently higher than that of enterococci in all the wetland cells examined (Fig. 3b).

Bacterial community composition in wetland water, rhizosphere, and sediment

Banding patterns from water, sediment, and rhizosphere samples were analysed using 16S rRNA DGGE-PCR amplicons and are presented in Fig. 4a,b. The number of bands (species) per lane varied on the average from four in water samples retrieved from 100% plant density and inflow water to ten in the outflow water. Some differences were noted in band position, intensity, and number of bands present in the sediment and rhizosphere (Fig. 4a). The sediment and the rhizosphere had their own unique profiles, indicating variation in microbial community composition between bulk sediment and rhizosphere. DGGE analyses from water samples demonstrated relatively stable banding patterns throughout the collection period (Fig. 4b). The Shannon-Weaver index of diversity (H) was used to estimate microbial diversity in the water, sediment, and rhizosphere samples at various points throughout the wetland. Diversity in water, sediment, or rhizospere was consistently higher in the wetland cells with 50% plant density than in cells with 100% plant density (Table 1). Diversity was also higher in the outflow water than in the inflow water. Bacterial diversity was higher in the sediment and rhizosphere samples than the water samples. Furthermore,

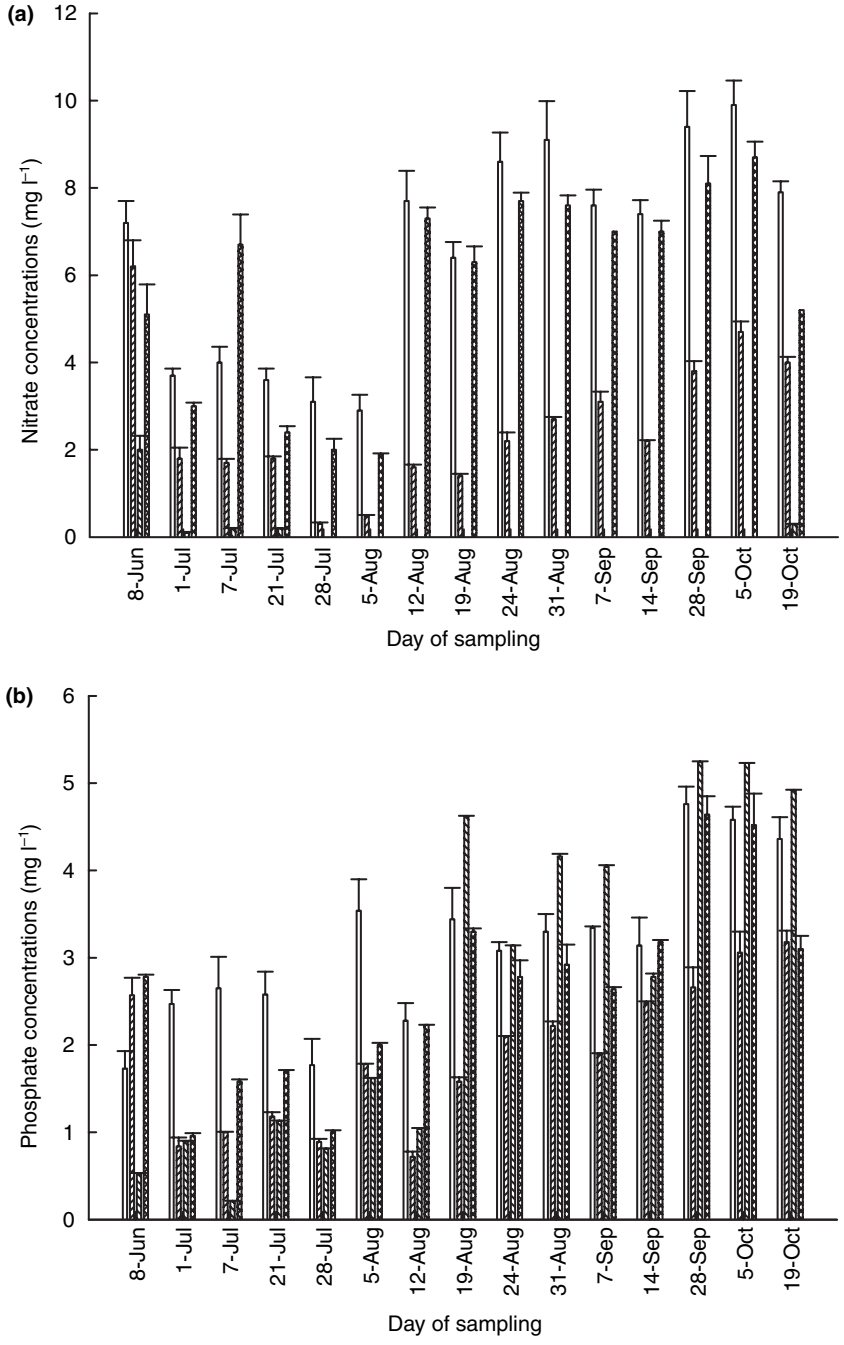

Figure 2 Concentrations of nitrate-nitrogen (a) and orthophosphate (b) removed in Prado wetland. □, inflow; ▨, outflow; ▧, 50% plant cover; ▩, 100% plant cover. Analyses were done on weekly samples. Results are also presented as an average removal rate in Table 1.

Table 1 Prado wetland nutrient and microbiological characteristics

Treatment	Nitrate removal (%)	Phosphate removal (%)	water (*H*)+ (June)*	Water (*H*) (July)	Sediment (*H*)	Rhizosphere (*H*)
Inflow	NA	NA	0·82 ± 0·12 (7·5)	0·49 ± 0·15 (4·9)	0·70 ± 0·15 (5·5)	1·24 ± 0·21 (20)
Outflow	64·3	39·3	1·07 ± 0·23 (12·5)	0·91 ± 0·21 (10)	1·13 ± 0·23 (16)	NA
50% cover	96·3	22·3	0·71 ± 0·06 (6·5)	0·91 ± 0·11 (8·5)	1·09 ± 0·11 (15)	1·21 ± 0·18 (19·5)
100% cover	11·4	16·1	0·42 ± 0·05 (4)	0·74 ± 0·08 (5·5)	1·06 ± 0·13 (14)	0·96 ± 0·14 (11)

H, Shannon-Weaver diversity index; NA, not applicable because samples were not taken from the sites.
*Numbers in parentheses represent species richness from each sample.

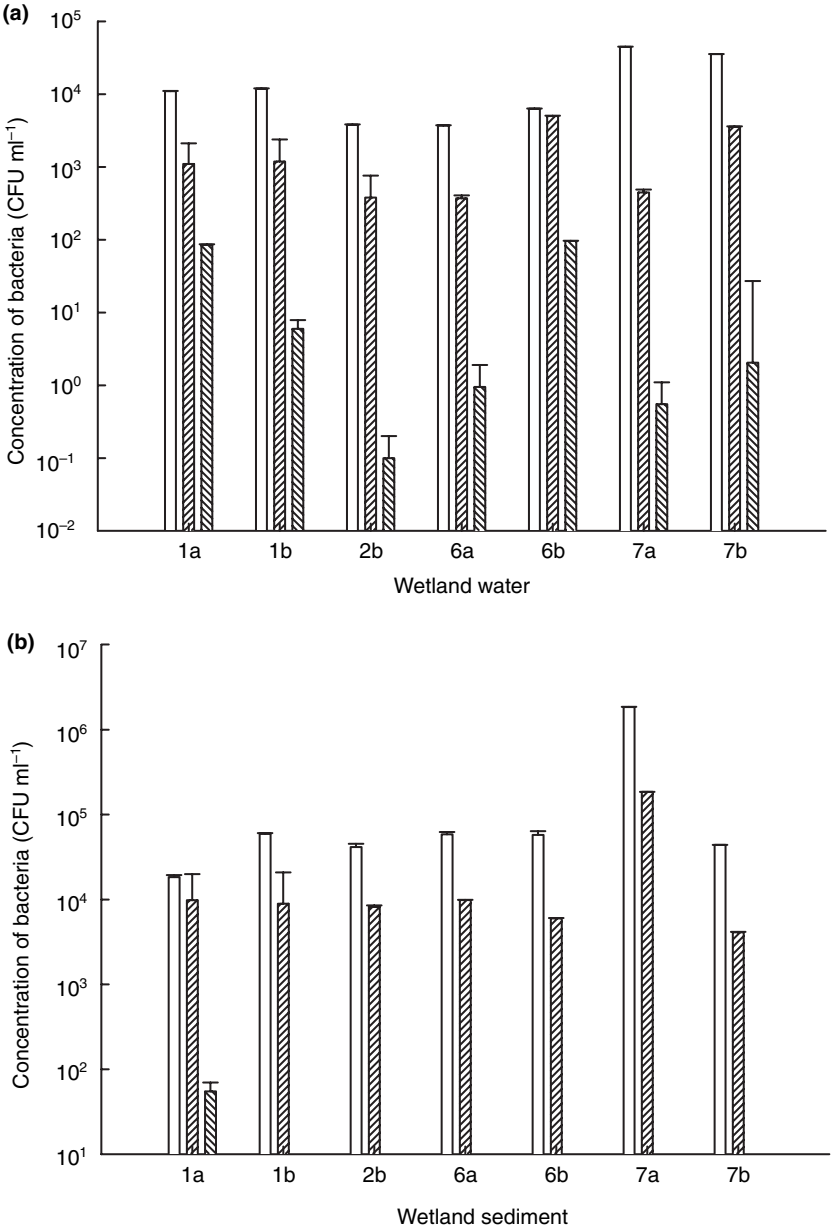

Figure 3 Changes in: □, heterotrophic plate count; ▨, *Escherichia coli*; ▧, enterococci concentrations in: (1) inflow; (2) outflow; (3) 25% cover; (4) 50% cover; (5) 75% cover; (6) 50% cover; (7) 100% cover. Values shown here are means from four samples averaged from each of the sampling points. Concentration of bacteria was determined from 100 ml of samples taken at different locations in the wetlands: (a) water samples and (b) sediment samples. Numbers on the *x*-axis indicates: (1) inflow; (2) outflow; (3) 25% cover; (4) 50% cover; (5) 75% cover; (6) 50% cover; (7) 100% cover. Letters A and B after the numbers indicate influent water to that cell and effluent water out of the cell as seen by the direction of water flow. Two cells were used for each treatment.

our data showed that the rhizosphere had the most diverse group of bacteria based on the diversity index. The Image Master ID was used to create a database set for these samples with different band positions. The bands were found in different combinations among the DGGE banding patterns. Occurrence of different bands from the two gels showed that 38% of the bands occurred in >75% of the samples. Pattern comparisons were made between the two gels by using Pearson coefficient (Fig. 4c). From the database analysis, the Pearson values indicated that all samples shared a large portion of the band set (data not shown), while a few had unique bands as indicated by the banding patterns in Fig. 4a,b.

As distinct banding patterns were observed within the three samples, a cluster analysis was performed using UPGMA algorithm. Figure 4c is an example of the cluster pattern formed with samples collected in the summer of 2004. The results showed that pattern similarities were associated more strongly with sample type and to some extent with plant cover (Fig. 4c). The samples separated into two main clusters with the sediment and rhizosphere samples were grouped together and the water samples in another cluster. The sediment and rhizosphere samples were approximately 80% similar, indicating that microbial community compositions from the sediment and rhizosphere samples were closely related. This can be seen with

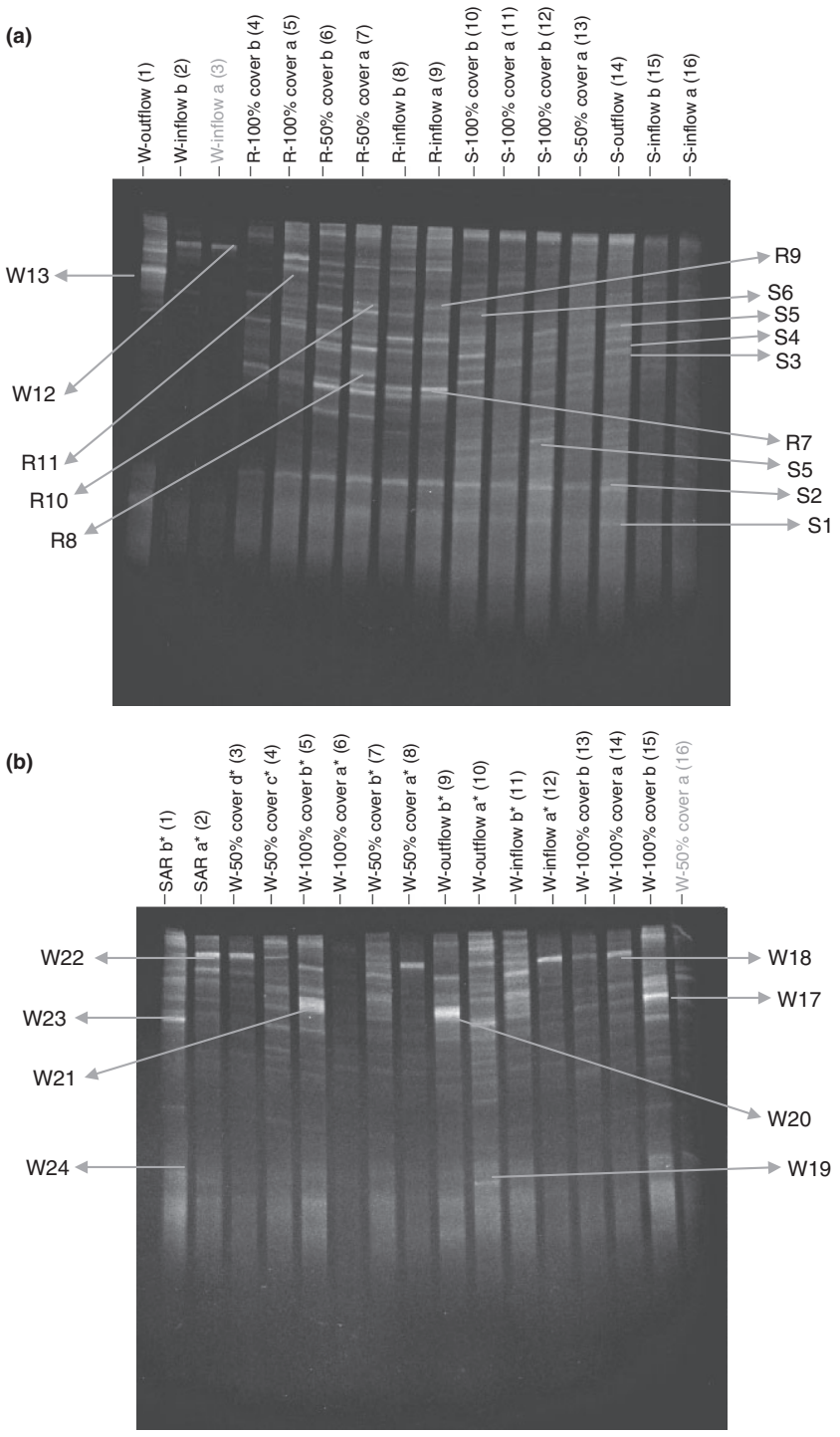

Figure 4 Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA fragments of total bacterial population from rhizosphere, sediment, and water samples. (a) Gel image from samples collected from rhizosphere (R) and sediment (S) samples in July 2004. All samples were collected in duplicates from each pond. (b) Gel image from samples collected from water (W) samples in June* and July 2004. The 100% or 50% cover indicates ratio of plants to water. SAR indicates Santa Ana River water near the wetland. Amplified products were separated on a gradient gel of 30–70% denaturant. All labelled bands were excised from the gel, reamplified, and subjected to sequence analysis. These reamplification products were cloned and screened as described in the text and as shown in Table 2. (c) Cluster analysis of microbial communities generated by the analysis of DGGE 16S rRNA polymerase chain reaction (PCR) patterns representing the genetic similarity of the microbial community profiles obtained by PCR-DGGE from a and b. Symbols are as shown in gels.

the horizontal line of increasing similarity of branches from the dendrogram nodes (Fig. 4c). However, microbial compositions from water samples were about 50% similar to those of the sediment and rhizosphere.

Further analyses of the predominant bacterial species were carried out with water, sediment, and rhizosphere samples. Bands selected for analysis are shown in Fig. 4a,b. Table 2 shows the prominent bands recovered from the DGGE gel. A total of 24 bands were excised from the gel and sequenced. There were two prominent bands (S1 and S2) present in the sediment samples. In addition to the two bands, three other bands (S3, S4, and S5) were also dominant in most of the sediment and rhizosphere samples. The derived sequences from these bands confirmed S1 and S2

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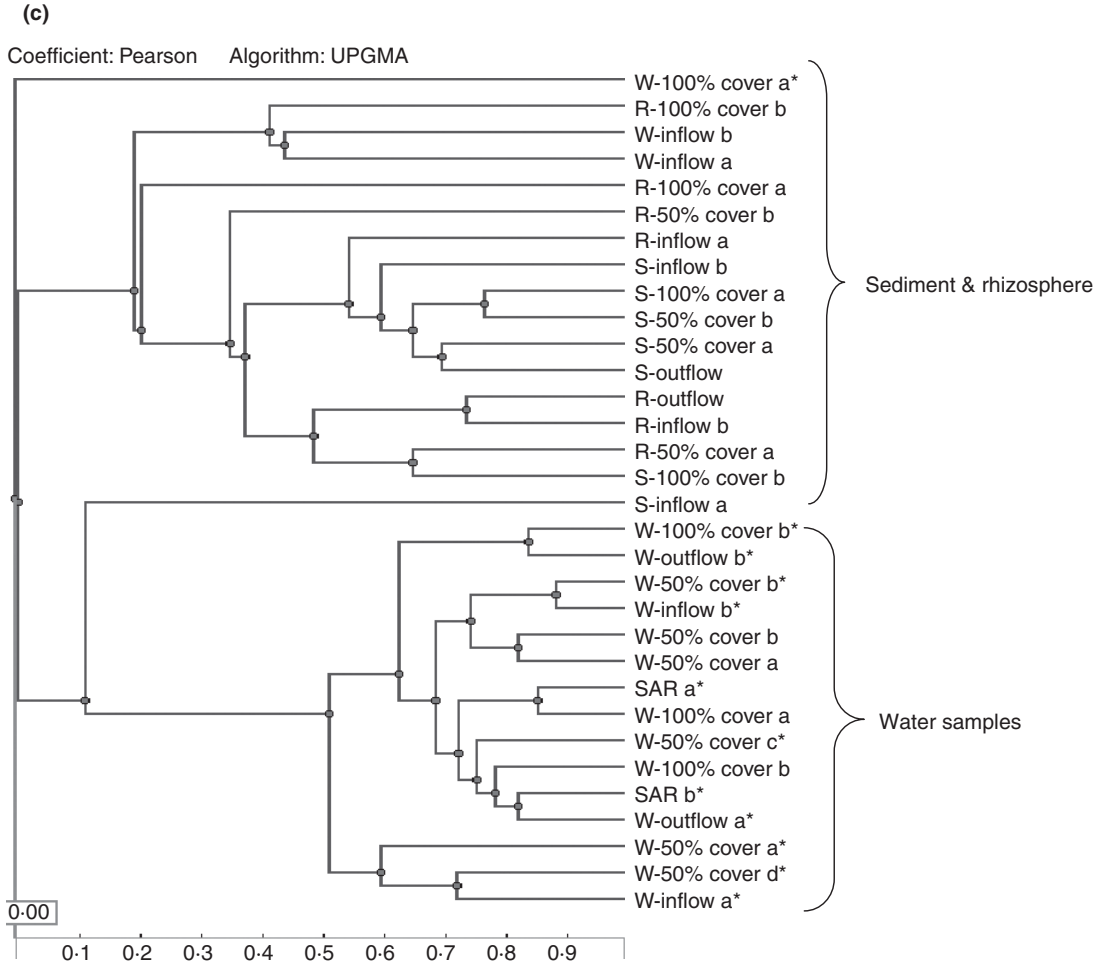

Figure 4 Continued.

to be 98% and 95% similar to unidentified bacterium and uncultured bacterium derived from sediments reservoirs of different trophic states (Wobus *et al.* 2003), and from a forested wetland sediment impacted by reject coal (Broffit *et al.* 2002), respectively. A total of ten bands were classified as unidentified or uncultured bacterium while six were in the *Proteobacteria* phylum and these were derived mainly from the sediment and rhizosphere samples. Most of the strains identified in this study had 93–100% similarity levels with sequences from the database (Table 1). Most of the prominent bands retrieved from the water samples (W14, W16, W20) were closely related to sequences previously isolated from fresh water cyanobacterial blooms (Eiler and Bertilsson 2004).

Analysis of 16S rRNA libraries derived from wetland samples

A total of 102 clones from the 16S rRNA gene library were selected for sequencing and analysed to estimate the bacterial diversity in sediment, rhizosphere, and water samples (Table 3; Fig. 5a–c). Pattern analysis grouped clones into 35, 31, and 36 OTU from the sediment, rhizosphere and water samples, respectively. On the basis of the phylogenetic analysis, the clonal sequences were affiliated with at least ten classes of the domain *Bacteria*, including unidentified bacterium. Clone library was dominated by unidentified bacterium followed by the phylum *Proteobacteria.* The dominant organisms within the class were in the γ, β, and δ classes (Table 3), with the β and the γ groups being more abundant in the rhizosphere while the δ group was more abundant in the sediment. The third most dominant group of the clone library was classified into the phylum *Cyanobacteria.* The other groups in the library were *Acidobacteria*, *Bacteroidetes*, and others as shown in Table 2. Some clones affiliated with the candidate phyla OP10 and TM7 were also observed. In our study, sequences allocated to the β and γ groups of the phylum *Proteobacteria* were retrieved in higher numbers than the other groups within the phyla

Table 2 Sequence analysis of bands excised from denaturing gradient gel electrophoresis (DGGE) gels derived from bacterial 16S rRNA extracted from wetland sediment (S), rhizosphere plants (R), and surface water (W)

Bands origin	Related bacterial sequences	Per cent similarity	Accession #
S1	Unidentified bacterium	98	AJ518382
S2	Uncultured bacterium	95	AF523957
S3	Uncultured bacterium	97	AB200300
S4	Uncultured *Proteobacterium*	100	AY193046
S5	Uncultured *γ-Proteobacterium*	99	AY221611
S6	Uncultured soil bacterium	95	AY289463
R7	Uncultured *Chromatiales* bacterium	92	AY711225
R8	*δ-Proteobacterium* LacK9	96	AY771933/4
R9	Uncultured *Nitrospira* sp.	99	AF351231
R10	*γ-Proteobacterium* JB	100	AF542077
R11	*Aeromonas* sp. Tf 228	98	AY461686
W12	Bacterium Mn113133	97	AY928241
W13	Unidentified bacterium	98	AJ518307
W14	Uncultured *Spirochete*	90	AY082470
W15	Uncultured bacterium	98	AB174868
W16	Uncultured *Bacteroidetes* bacterium	93	AY509337
W17	Uncultured bacterium	95	AY662000
W18	Uncultured bacterium	95	AJ617866
W19	Uncultured bacterium	95	AJ617866
W20	Uncultured *Cyanobacteria*	98	AY858030
W21	Uncultured *Crater Lake* bacterium CL120-102	98	AF316709
W22	Uncultured bacterium		AY662000
W23	Uncultured soil bacterium	100	AB080334
W24	Uncultured bacterium	98	AB194345

Table 3 Phylogenetic affiliations of 16S rRNA gene clones belonging to the domain *Bacteria*

Phylum	Sediment OTU	Rhizosphere OTU	Water OTU	Per cent similarity
Alphaproteobacteria	0	1	0	92–100
β-Proteobacteria	2	4	3	91–99
γ-Proteobacteria	1	14	1	96–100
δ-Proteobacteria	5	1	0	95–100
Epsilonproteobacteria	0	2	2	96–100
Acidobacteria	2	0	1	96–98
Nitrospirae	1	0	0	96
Gemmatimonades	1	0	0	96
Chlorobi	1	0	0	97
Firmicute	2	0	0	96–99
Cyanobacteria	0	0	6	97–100
Bacteroides	0	1	0	93
Fusobacteria	0	1	0	97
Unidentified	20	7	23	92–100
Total OTU per sample	35	31	36	

OTU, operational taxonomic units.

and these were dominated by samples from the rhizosphere (Fig. 5b).

Discussion

Our investigations showed that a 50% vegetated to a 50% open water ratio in the wetlands facilitated nitrate removal efficiency of about 96·3%. This suggests that managing wetland vegetation by keeping it interspersed with open water significantly improves the NO_3-N removal capabilities of a FWS constructed wetland. Our data indicate that this interspersion effect was also effective in removing orthophosphate and other wastewater constituents.

Our data showed that as stands of California [*Schoenoplectus* (formerly *Scirpus*) *californicus* (C.A. Meyer)] or hardstem [*Schoenoplectus acutus* (G.H.E. Muhlenberg ex J. Bigelow)] bulrushes reached maximum stand at the 100% plant density, they can contribute significantly to the internal loading of nitrogen in the system and the development of less microbial community diversity compared to the ponds with 50% plant cover. One likely explanation for the reduced efficiency in the 100% plant cover may be because of low dissolved oxygen levels due to excessive plant materials acting as both a detrital source and a diffusion barrier. This may contribute to short-circuiting of the water flow which can encourage stagnant water areas with the pond, resulting in the poor efficacy of ponds with 100% plant cover.

The short circuiting of water flow can create anaerobic conditions or can cause a reduction in microbial community diversity resulting in fewer species. Results from this study have shown consistent higher diversity indices in the samples with 50% plant cover than in the 100% (Table 1). Maintenance of 50% plant cover has previously been shown to be the most effective way of keeping high efficiency of nutrient removal in constructed wetlands (Thullen *et al.* 2002). These authors showed that with a 50% plant cover at the beginning of wetland growth, the vegetation voids were large, effectively interspacing the planted zones with open water, but 16 months later the wetland vegetation stands became denser, and an average of only 4% NH_4-N was detected. They also showed that with denser plant cover, anaerobic zones were created limiting dissolved oxygen in the water column as well as algal photosynthesis. They concluded that as vegetations reached maximum density and biomass (which contributed to internal nutrient loading and large anaerobic zones) and were no longer interspersed with open water (further limiting dissolved oxygen in the water column by restricting mixing and

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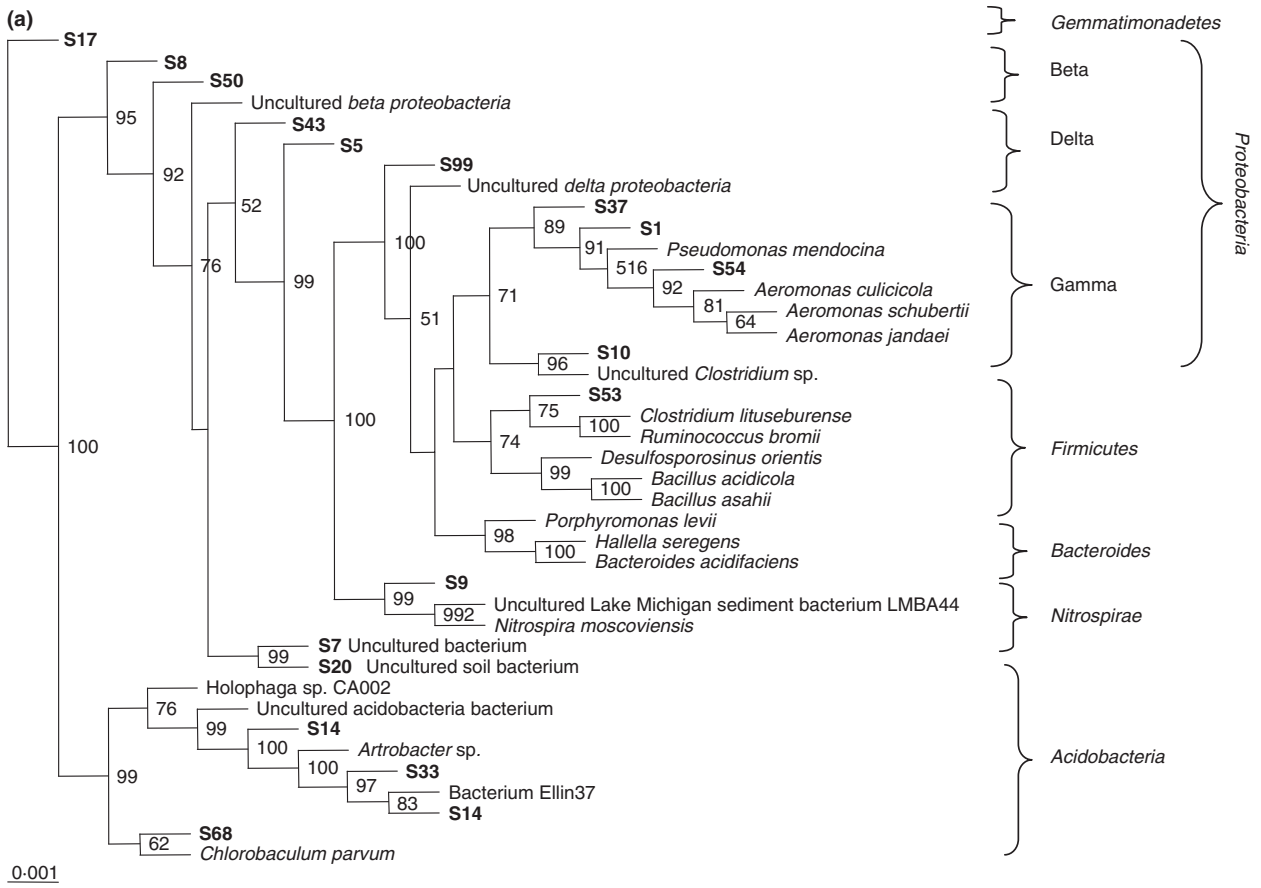

Figure 5 Phylogenetic relationship of the operational taxonomic units (OTU) isolated from the wetland samples. Matrices of evolutionary distances were computed using the Phylip program with the Jukes-Cantor model (Jukes and Cantor 1969). 16S rRNA sequences representing the most prevalent OTU from each environment: sediment (A), rhizosphere (B), and water (C) were aligned using CLUSTALX version 1.8 for Windows (Thompson *et al.* 1997). Phylogenetic trees were constructed and checked by bootstrap analysis (1000 data sets) using the program SEQBOOT. Bootstrap values (>50%) generated from 1000 replicates are shown at the nodes. The scale bar represents substitution per site. Phylum and class designations are indicated on the right.

algal photosynthesis), lower NH_4-N reduction efficiency resulted. The role of 50% plant cover for maximum efficiency of a free surface wetland has been well documented for newly established wetlands that are 2–5 years old and this conclusion has been extended to a free surface wetland that is more than 12 years old as it is shown in this study. Therefore, for a free surface wetland to operate at maximum capacity, best management practices involving seasonal clearing of plants for enhanced interspacing of plants density with water at a 1 : 1 ratio needs to be considered strongly by wetland managers. This has additional advantage for pest control, because decreasing vegetation biomass reduced mosquito refuge areas while increasing mosquito predator habitats (Thullen *et al.* 2002).

In this study, removal efficiency of nitrate was related to plant density relative to open water. Next, we explored the nature of the microbial community associated with roots, sediment, and open water to quantify members of the community performing these functions. High densities of heterotrophs are maintained to remove BOD, suspended solids, and toxic organic compounds such as resin acids and organochlorines prior to discharge. Based on DGGE analysis of cloned sequences, we have shown that the 50% plant cover has greater diversity of microorganisms than the 100% plant cover. Two bands (R8 and R10) were unique to rhizosphere plants with 50% plant cover while R11 was unique to the rhizosphere of plants with 100% plant cover. Bands R8 and R10 are *δ-Proteobacteria* and *γ-Proteobacteria*, respectively (Table 2), and had been previously reported to be recovered from intertidal mud flats of the Wadden Sea (Mussmann *et al.* 2005), while R11 was 98% similar to *Aeromonas* sp. Tf 228 recovered from tropical sediments

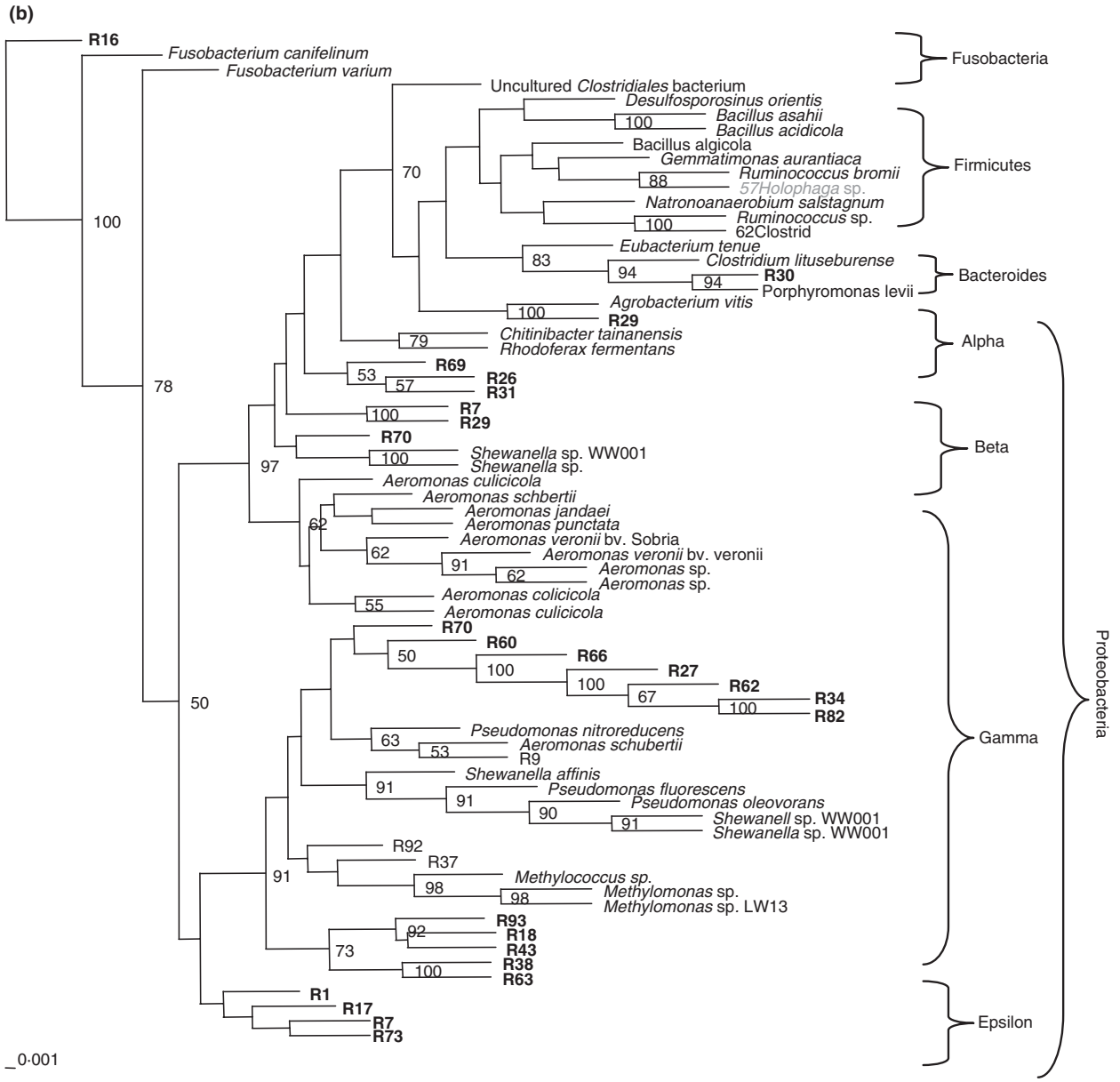

Figure 5 Continued.

(Tiradoet *et al.* unpublished). Band S9 (uncultured *Nitrospira*) derived from the rhizosphere is known for it role as nitrite oxidizers. The occurrence of uncultured *Bacteroidetes* (Band W16 in Table 2) has been reported to be associated with fresh water bacterial communities with *Cyanobacterial* bloom in four Swedish lakes (Kirchman 2001; Eiler and Bertilsson 2004).

Comparison of 16S rRNA sequences in the sediment, rhizosphere, and surface water, showed that the rhizosphere was dominated by *Proteobacteria* and the water column was dominated by *Cyanobacteria* while the sediment showed no dominant group. *Cyanobacteria* may be key players for the biogeochemistry of photosynthetic surface water materials in the wetland system as they are the predominant primary producers in other systems such as hot spring microbial mats (Steunou *et al.* 2006). They play a role in nitrogen fixation, therefore maybe important contributors in this ecosystem in increasing wetland overlying water nitrogen. Cyanobacterial exudates also may provide endogenous source of growth substrates to bacteria (Kirkwood *et al.* 2006). These authors showed that diverse communities of *Cyanobacteria* would likely

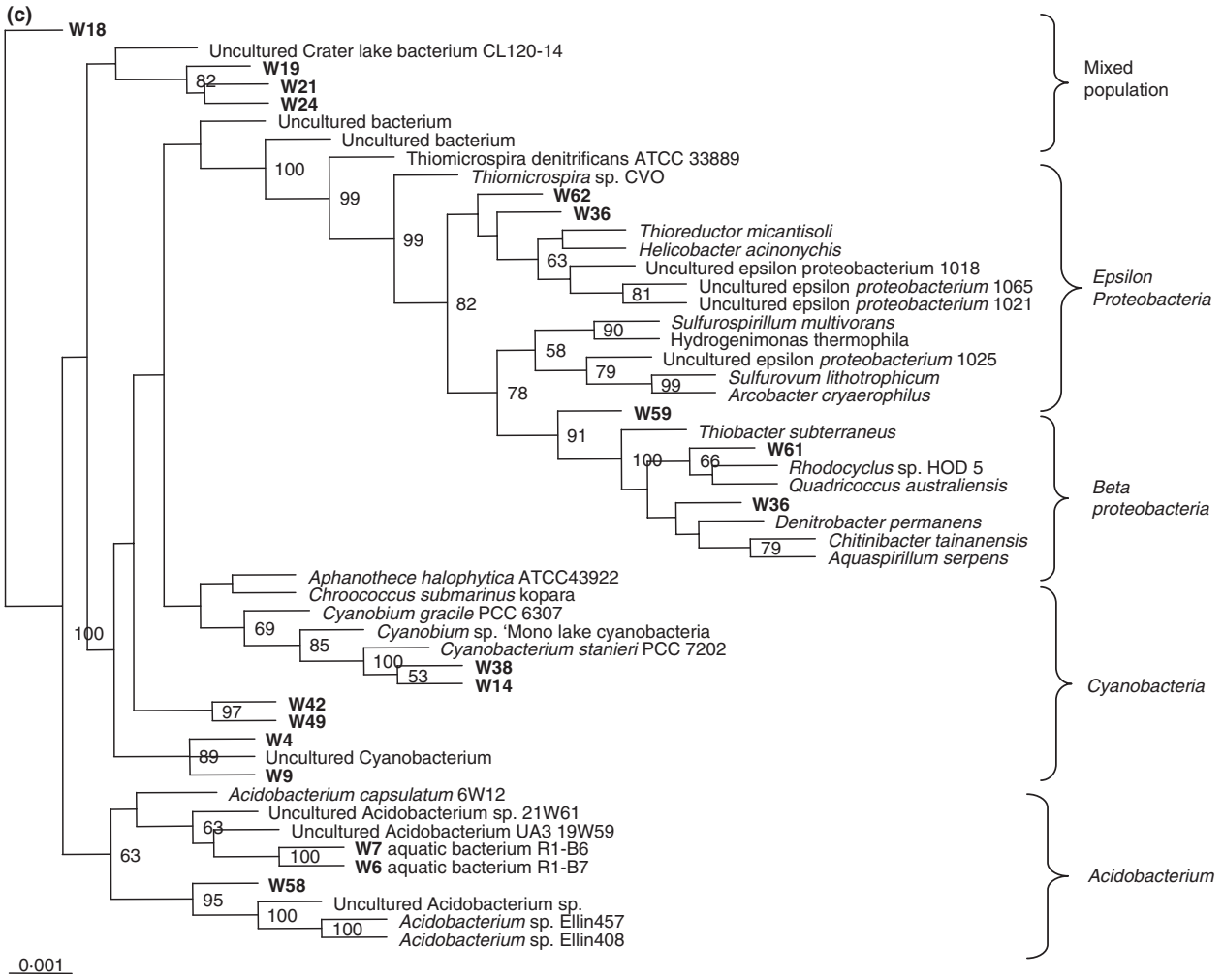

Figure 5 Continued.

contribute variable quantities of dissolved organic carbon to wastewater, but also exudates of variable quality for bacterial utilization.

Apart from the *Bacteroidetes* and the *Cyanobacteria*, *Proteobacteria* was another major component of our wetland system. All studied libraries contained cloned sequences from the subdivision α, *β*, and *γ-Proteobacteria* except sediment and water samples that did not contain *α-Proteobacteria* (Fig. 4a–c). This is in agreement with Glöckner *et al.* (1999) who showed that the abundance of bacterioplankton affiliated with *α-Proteobacteria* differs largely between freshwater lakes and makes up between 0% and 20% of the total number of bacterial cells. Our data showed that *α-Proteobacteria* retrieved from this study were 97% similar to *Agrobacterium vitus* (*Rhizobium vitis*), which is associated with nitrogen fixation in the terrestrial environment. *β-Proteobacteria* were abundant in all of our wetland samples supporting the findings that most freshwater environments harbour large numbers of this group (Bosshard *et al.* 1999; Glöckner *et al.* 1999; Brümmer *et al.* 2003). They are believed to be of particular importance during early biofilms succession (Davey and O'Toole 2000; Jackson *et al.* 2001). The presence of a large fraction of *β-Proteobacteria* implies a ready source of oxidizable ammonia (Araya *et al.* 2003) as well as degradation of complex organic molecules (Kirchman 2001), which are very critical for the maximum efficiency of the wetland system. The *γ-Proteobacteria* subdivision represented the highest proportion of clones in the rhizosphere of the wetland plants, but very few clones from the sediment and water samples. Four of these clones could be grouped into distinct taxonomic groups, e.g. the *Pseudomonadales*, *Methylococcales*, *Aeromonadaceae*, and *Shewanellaceae* (Fig. 5b). The low frequency in the water column supports previous observations that *γ-Proteobacteria* are less

common members of bacterial communities in pelagic freshwater habitats (Glöckner *et al.* 1999).

In our previous study (Ibekwe *et al.* 2003), using a subsurface-constructed wetland, the wetland effluent was found to be more suitable for on-site reuse and the amount of contaminants entering groundwater supplies as a result of percolation of washwater stored in ponds and sprayed on disposal lands were significantly reduced. It was possible from the previous study that on-site reuse of the wetlands-treated washwater through spray irrigation on disposal lands was optimized by the reduction of organic loads and solids in washwater, allowing more water of higher quality to be used for the irrigation of pasture. The direct effect of that study was the removal of the main pollutants from the dairy washwater with beneficial impacts on the surface and groundwater in the Chino Basin, which resulted in better quality of water leading into the Santa Ana River and the Orange County groundwater basin. Others have reported the use of other methods for treating wastewater such as the use of circulatory treatment lagoons (McGarvey *et al.* 2004, 2005), and the use of plants and different filtered sand materials in constructed wetland for water quality improvement (Vacca *et al.* 2005).

In this study, the 50% plant cover was shown to be the most effective ratio of plant to water for maximum efficiency of free water constructed wetland. The high efficiency is derived from the availability of energy sources required by diverse microbial population that have the potential to act synergistically for the removal of contaminants such as NO_3-N from the wetland effluent. Future studies might thus be aimed at determining specific classes of microbial populations responsible for specific functions in the wetland by combining *in situ* cell identification with activity measurements.

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