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BACTERIAL COMMUNITY ANALYSIS OF THE WATER SURFACE LAYER FROM A RICE-PLANTED AND AN UNPLANTED FLOODED FIELD

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ABSTRACT

The bacterial communities in floodwater, from a rice-planted and an unplanted field were characterized at the beginning (flooding stage) and at the end (harvest stage) of the rice cropping cycle. Most probable number estimations and plate counts of aerobic and anaerobic heterotrophic bacteria and of several metabolic bacterial groups (methanogens, sulfate-reducers, anaerobic sulfur and nonsulfur phototrophs, denitrifiers and ammonifiers) were similar in rice and unplanted floodwater at both sampling times. The analysis of denitrifiers and methanogens by fluorescent in situ hybridization revealed a shift in the phylogenetic affiliation only of the former group in the rice-planted floodwater. Terminal restriction fragment length polymorphism of 16S rRNA gene amplicons indicated that the bacterial communities of the rice-planted and unplanted soils were consistently diverse and strongly influenced by the season.

Key words: floodwater; rice; microbial community; T-RFLP.

INTRODUCTION

Rice is an important crop with 75 percent of the world production managed as irrigated rice. Freshwater is a major component of this agricultural activity. A high percentage of rice-planted fields in Uruguay were formerly wetlands that represented a considerable reserve of biodiversity. Floodwater harbors part of this diversity and constitutes a habitat that sustains essential biochemical process for the cropping.

Microbial communities contribute to the sustainability of the crop by regulating the bio-geochemical cycles and supplying plant nutrients. Anaerobic conditions, caused by microbial respiration and accumulation of free hydrogen sulfide and short-chain fatty acids generated by sulfate-reducing and fermentative bacteria respectively, reduce nutrient uptake by paddy rice (37). Agricultural practices like soil amendments, irrigation, or tillage produce different bacterial communities (4, 5, 6) indicating that microbial communities are impacted by soil disturbance. Most studies on microbial communities in rice cropping are focused on bulk soil populations developed in experimentally defined laboratory microcosms (7, 13, 16, 21). Although floodwater and its interface with soil play a relevant role in plant nutrition and are rather impacted by the cropping, i.e., gas exchange with the atmosphere and fertilization, microbial communities from this habitat have been studied only in long-term fertilized

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Japanese (19, 35) and in Brazilian (22) paddy fields. Further, Asakawa and Kimura (3) observed that floodwater were distinct and different in the diversity from other habitats (as layer soil, rice roots or percolating water) of the Japanese paddy field ecosystem.

The analysis of bacterial communities by culturing methods -although valuable to characterize metabolic activities of their members- allows the characterization of only 1 to 3% of the microscopically-detectable cells in soils (2). Molecular methods are an indispensable tool to provide a more comprehensive description of the community structure. In this work, the floodwater community structure of planted and unplanted rice fields was analyzed at flooding and at rice harvesting stage by culturing and molecular methods to investigate the effect of rice cropping in the floodwater biodiversity.

MATERIALS AND METHODS

Site description and sampling

Rice is produced in Uruguay in a temperate climate (mean temperature 25 °C) with an annual cropping regime under permanent irrigation in alternate crop-pasture cycles of 3 or 4 years. The region selected for this study is located in eastern Uruguay and covers an area of 3,850,000 hectares including permanent and temporary wetlands. It has been designated as biosphere reserve area by UNESCO in 1976 and since 1993 the area is under the supervision of PROBIDES (Conservation of Biodiversity and Sustainable Development Program for the Eastern Wetlands). Floodwater samples were taken from a rice field that had had two successive years of cropping and from a neighboring field with similar soil characteristics. The unplanted field had never been used for agricultural purposes. Samples were collected in November 1999 -Rice Flooding Stage (RFS), Unplanted Flooding Stage (UFS) - and February 2000 – Rice Harvest Stage (RHS), Unplanted Harvest Stage (UHS) - corresponding to 7 and 110 days after flooding, respectively (21 and 124 days after plant sprouting). For each field a composite sample was made by anaerobically pooling 5 randomly collected samples spaced 15 m apart from the upper (10 ml) and lower (10 ml) floodwater layers. The lower layer included 1 cm of muddy soil-water interface.

Physicochemical parameters of the floodwater

The pH and dissolved oxygen were measured “in situ” by appropriate electrodes in the upper and lower floodwater layers for each sampling site. The chemical composition of composite samples was analyzed as follows, nitrate and nitrite by HPLC (11), volatile fatty acids by HPLC (27) and organic matter as the volatile fraction (determined after heating at 550°C for one hour) expressed as the percentage of the dry weight.

Microbial counts

For viable heterotrophic aerobic bacterial counts, dilution series were prepared in sterile saline (0.9% NaCl) and plated onto Plate Count Agar (Difco). For anaerobic heterotrophic bacterial counts, dilution series were prepared in anaerobic basal BCYT medium (27), plated in PY agar (36) supplemented with 20 mM glucose and incubated in an anaerobic chamber. Metabolic groups of bacteria were counted by the three-tubes most probable number (MPN) method. Methanogenic bacteria were enumerated in anaerobic BCYT supplemented with 20 mM potassium acetate and 140 kPa of H_2/CO_2 and dispensed in serum bottles (120 ml). Methane production was measured as previously described (37). Sulfate-reducing bacteria were counted as previously reported (12), whereas counts of denitrifying and ammonifying bacteria were performed according to Guynot *et al.* (14). Enumeration of anoxygenic photosynthetic bacteria was performed in medium containing 0.6 g/l of NaCl, 0.37 g/l of KCl, 0.33 g/l of $(NH_4)_2SO_4$, 0.4 g/l of KH_2PO_4, 1.2 g/l of K_2HPO_4, 0.5 g/l of yeast extract, 10 ml/l of minerals trace solution (27), 5 mM of sodium malate, 5 mM of sodium succinate and 5 mM of sodium acetate. The medium was anaerobically prepared as the BCYT medium (27) under $N_2:CO_2$ atmosphere (70:30, v/v); $KHCO_3$ (3.6 g/l) was added and the pH was adjusted to 7.0. The medium (10 ml) was anaerobically dispensed into Hungate tubes and sealed with butyl-rubber stoppers. After sterilization,

vitamins (27), and salts were added from sterile concentrated stock solutions to reach final concentrations of 0.5 mM $CaCl_2$ and 1.1 mM $MgCl_2$. The medium was supplemented with 0.1 mM Na_2S or 1.0 mM Na_2S and 0.1 mg/l B_{12} vitamin for nonsulfur and sulfur bacteria, respectively. After anaerobic incubation under a 25-W tungsten lamp, positive tubes turned purple, red, or brown. Pigments were analyzed spectrophotometrically as previously described (20). All incubations were performed at 30°C for two weeks except for methanogens that were incubated for five weeks.

The significance of the difference between two estimated population sizes counted by MPN was tested as previously described (8). Two population sizes were considered significantly different if there was no overlap in the values for the 95% confidence limits.

To determine total direct microscopic counts, samples were concentrated by centrifugation (5000 X g, 10 min), resuspended in PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2), fixed in 3% paraformaldehyde at 4°C for 2 h and stained with 100 mg/l acridine orange (final concentration) as described elsewhere (18).

FISH analysis of MPN cultures

The most abundant methanogens and denitrifiers were analyzed by FISH (fluorescence *in situ* hybridization). To reduce the relative proportion of putative scavengers, the last three positive tubes of MPN counts were sub-cultured in the respective medium and methane production or nitrate consumption were confirmed. Aliquots of each tube were centrifuged (5000 X g, 10 min), the pellet resuspended in PBS (1 vol) and fixed with 4% paraformaldehyde in PBS (3 vol) for 18 h at 4°C. After the pellet was washed in the same buffer, the cells were resuspended in PBS (1 vol) and absolute ethanol (1 vol) was added. The samples were maintained at –20 °C until further processing. For *in situ* hybridization, 5 to 10 µl of sample was spotted onto slides and dried for 30 min at room temperature followed by dehydration with increasing ethanol concentrations (3 min each in 50, 80 and 100% ethanol). A 10 µl-sample of hybridization buffer (Tris HCl [pH 8] 20 mM, NaCl 0.9 M, SDS 0.01% and formamide 20 % in Milli-Q water) was dropped onto each well and 50 ng of each probe was added. Incubation was performed in humid chambers for 1.5 h at 46 °C. The slides were rinsed with 2 ml of washing buffer (Tris HCl [pH 8] 20 mM, NaCl 0.215 M, SDS 0.01% in Milli-Q water), previously warmed at 48°C, and immediately incubated for 10 min at 48°C. After the hybridization was performed, the washing buffer was removed with cold distilled water and the slide was dried and embedded with Citifluor (Canterbury, United Kindom). Samples were observed in an Olympus BX41 microscope (filters U MWIG for Cy3). *In situ* hybridizations were performed with the following Cy3 labeled oligonucleotide probes, EUB338, specific for *Bacteria*; BET42a specific for the beta subdivision of the class *Proteobacteria*; ALF968 specific for the alpha subdivision of *Proteobacteria*; GAM42a specific for the gamma subdivision of *Proteobacteria*; ARC915 specific for *Archaea*; MB1174 specific for the Family *Methanobacteriaceae*; MSMX860 specific for the Family *Methanosarcinaceae* and MG1200 specific for the Families *Methanomicrobiaceae*, *Methanocorpusculaceae* and *Methanoplanaceae* (1, 25, 31).

DNA extraction; 16S rRNA PCR amplification and T-RFLP

Composite samples (30 ml corresponding to 0.22 to 0.50 g of pellet) were centrifuged (5000 X g, 10 min) and used for DNA extraction with UltraClean Soil DNA Purification Kit (Mo Bio Laboratories, California, USA). Bacterial 16S rDNA was amplified by PCR from 4 ng of extracted DNA. PCR was carried out using primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-ACGGTTACCTTGTTACGACTT-3'). Primer 27f was labeled at the 5' end with hexachlorofluorescein (Integrated DNA Technologies Inc., Iowa, USA). 100 µl of the reaction mixture contained 0.1 µM of each primer, 200 µM of each deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$, 0.2 mg/ml of bovine serum albumin, 10 µl of *Taq* buffer (0.1 M Tris-HCl [pH 8.3], 0.5 M KCl) and 4 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) (26). PCR amplification

was performed in an automated thermal cycler (Gene Amp 2400, Perkin-Elmer Cetus, Norwalk, Conn.) with an initial denaturation (94°C for 5 min) followed by 25 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 3 min) and a single final extension (72°C for 7 min). The 16S rDNA products were purified and concentrated with Microcon-100 columns (Amicon. Inc., MA, USA) and quantified in a 0.8% agarose gel stained with ethidium bromide. 300 ng of purified PCR products were digested overnight at 37ºC with 5U of *Hae*III or 4U of *Hha*I (Gibco BRL) in the reaction buffers recommended by the manufacturer.

Aliquots (4 µl) of the digest were mixed with a master mix (16 µl) containing deionized formamide, loading buffer (Applied Biosystems Instruments [ABI], Foster City, CA, USA), and 0.5 µl of a DNA fragment length standard (TAMRA GS 2500, ABI). After denaturing of the DNA at 94°C for 5 min and immediate chilling on ice, they were loaded onto a capillary automated DNA sequencer (3100 Genetic Analyzer). After electrophoresis, the lengths of fluorescently labeled terminal restriction fragments (T-RFs) were analyzed by the GeneScan 3.1 software (ABI).

Terminal restriction fragment (T-RF) sizes between 50 and 381 bp with peak heights larger than 100 and 50 (for *Hae*III and *Hha*I, respectively) fluorescence units were considered for the analysis to obtain reproducible T-RF profiles (29). All profiles were aligned considering as identical the T-RFs that differed by less than 1.5 bp. DNA quantity analyzed for each of the four samples was compared and standardized to the lowest quantity as previously described (10). The data of relative abundance of each fragment were utilized to calculate the Shannon-Weaver diversity index (34) and to perform the cluster analysis with the Morisita similarity index using the Past 1.42 software (http://folk.uio.no/ohammer/past/).

RESULTS AND DISCUSSION

Physicochemical parameters of the floodwater

At the harvest stage, organic matter was 0.099 and 0.152 g (g dry wt sediment)$^{-1}$ in unplanted and rice fields, respectively. Very low levels of nitrate and nitrite (< 1 mg l^{-1}) were verified in all the samples. Volatile fatty acids were detected only at the harvest stage in the rice floodwater (9 mg lactate l^{-1} and 6 mg acetate l^{-1}).

The pH and dissolved oxygen measurements indicate that each of the four floodwaters constituted homogeneous environments according to the standard deviations observed for the five sampling points (Table 1). Likewise, nutrients and microorganisms may also be more uniformly distributed in floodwater than in bulk soil. pH remained relatively constant but dissolved oxygen was reduced at the harvest stage, indicating that the crop season influenced oxygen availability. Also at this stage, the oxygen gradient between surface and bottom floodwater decreased. Thus, physicochemical properties of floodwater fed with water coming from natural events (unplanted fields) or from irrigation (rice fields) converged at the end of the crop season producing an anoxic and neutral pH habitat.

Table 1. pH and dissolved oxygen of the upper and lower floodwater layers from rice-planted and unplanted fields at flooding and harvest stages.

		pH[c]		Dissolved oxygen[c] (mg l^{-1})	
Samples[a]	Depth[b,c] (cm)	surface	bottom	surface	bottom
RFS	17.4 (5.9)	7.6 (0.8)	6.7 (0.2)	10.2 (1.7)	5.5 (3.6)
RHS	9.8 (1.3)	7.2 (0.7)	7.2 (0.4)	4.1 (2.4)	1.9 (1.0)
UFS	19.0 (6.6)	8.1 (0.5)	6.7 (0.2)	13.8 (3.0)	6.8 (1.7)
UHS	18.2 (4.2)	6.7 (0.1)	6.6 (0.2)	5.4 (4.4)	0.7 (1.3)

[a]RFS, rice flooding stage; RHS, rice harvest stage; UFS, unplanted flooding stage; UHS, unplanted harvest stage.
[b]Depth of the floodwater column.
[c]Values are means of five sampling points (standard deviations in parentheses).

Cultivable community size

High numbers (10^4 - 10^6 cfu ml^{-1}) of aerobic and anaerobic heterotrophic bacteria were detected in rice and unplanted floodwater at the flooding and harvest stages (Table 2).

The fraction of culturable microorganisms was higher in the unplanted field representing 57% of the total counts, whereas in rice floodwater only 4% of the total counts were recovered as aerobic heterotrophic bacteria. Table 2 shows that specific groups of anaerobic and facultative anaerobic bacteria involved in carbon, nitrogen and sulfur cycles were quantitatively similar in both fields since the confidence intervals of MPN counts overlapped. These results indicate that the abundance of bacteria involved in nutrients recycling was not strongly impacted by rice cropping and suggest that rice and natural flooded fields sustain comparable conditions for the biological transformation of carbon, nitrogen and sulfur.

Table 2. Microbial counts in composite samples from floodwaters of rice-planted and unplanted fields at flooding and harvest stages (Log number of bacteria ml $^{-1}$ water).

	Samples[a]			
Counts	**RFS**	**RHS**	**UFS**	**UHS**
Total direct[b]	7.7	ND[e]	6.6	ND[e]
Heterotrophic aerobic[c]	6.3	5.0	6.4	5.8
Heterotrophic anaerobic[c]	5.5	4.3	5.0	5.3
Methanogenic[d]	2.0	2.6	3.0	3.3
Sulfate-reducing[d]	3.3	3.9	4.0	4.3
Anaerobic phototrophic sulfur[d]	2.9	3.7	3.1	4.3
Anaerobic phototrophic nonsulfur[d]	3.9	4.0	3.9	5.4
Denitrifying[d]	4.7	3.3	4.3	3.9
Ammonifying[d]	4.4	2.6	3.3	2.9

Differences of up to two logs in counts between the beginning and the end of the crop season were measured in floodwater from both fields. Similar variations over time have been observed in culturable guilds in rice soils from continuously cropped experimental fields (33) and from greenhouse experiments under different water regimes (32).

Anaerobic phototrophic nonsulfur bacteria (APNB) showed the highest numbers among the specific groups of bacteria counted in both sites at harvest. Their persistence or predominance is likely due to their ability to grow phototrophically in anaerobic conditions, such as those observed in both floodwaters at harvest. The activity of APNB may be relevant for crop productivity since most of these bacteria have the ability to fix nitrogen (24). In addition, the outcompetition of APNB over methanogens for substrates like acetate (15) can contribute to the rice cropping sustainability by reducing methane emissions.

Molecular analysis of MPN cultures

The phylogenetic affiliation of the dominant culturable bacteria was investigated by performing FISH on the populations recovered from the terminal MPN positive tubes. Denitrifying and methanogenic microorganisms, which are key organisms in the nitrogen and carbon losses, were chosen as representatives of the *Bacteria* and *Archaea* domains, respectively. The most abundant methanogenic microorganism in both fields and seasons belonged to the family *Methanobacteriaceae* (100% of the Arc915- hybridized cells),

indicating that H_2 and formate were main substrates for methanogenesis in floodwater, like in rice paddy soils (17). The stability of the methanogenic population over time has also been reported for rice soil after flooding (23).

In contrast, the culturable denitrifying population was more complex and dynamic. Denitrifiers from rice floodwater shifted from exclusively γ-*Proteobacteria* (100% of the EUB338-hybridized cells) at flooding to a phylogenetically more heterogeneous population at harvest, when only 50% of the total Bacteria could be classified (10% γ-*Proteobacteria* and 40% β-*Proteobacteria*). Unplanted floodwater denitrifiers were constituted by approximately equal proportions of β- and γ-*Proteobacteria* (50% and 50% at flooding and 43% and 43% at harvest, respectively). The shift of rice floodwater denitrifiers to phylogenetically less related populations at harvest suggests that this metabolic group is more susceptible to cropping activities than methanogens.

Community analysis by T-RFLP

Dominant ribotypes present in pristine and rice floodwaters were determined by T-RFLP. This method has proven to be consistent with other molecular methods and suitable for rapid screening of bacterial communities (9, 28). The number of different T-RFs obtained in the four samples after the normalization procedure was 30 and 20 with *Hae*III and *Hha*I, respectively. All treatments shared only one T-RF when digestion was performed with *Hae*III and a high percentage (40%) of unique fragments was observed (Table 3). Although with *Hha*I the number of fragments was lower (between 8 and 10), a similar trend was observed: two T-RFs were common to all samples and 55% were unique (data not shown). These results indicate that uncultivable bacteria contributed to differentiate these communities.

The Shannon-Weaver indices were similar in the four samples (Table 3 for *Hae*III; data not shown for *Hha*I) suggesting that the bacterial diversity was not affected by the rice cropping. In contrast with the reduced diversity of microbial communities observed by Pett-Ridge and Firestone (30) after anoxic incubation of soil, the diversity of the floodwater communities from both sites endured the more anoxic conditions of the harvest season. No significant differences in diversity of culturable bacteria during the cultivation cycle have also been observed by Reche and Fiuza (22).

The cluster analysis based on T-RFLP profiles showed that season strongly influenced the bacterial community (Fig. 1). The higher similarities were observed between communities from different sites at the same season. Kimura *et al*. (19) reported that in Japanese paddy fields fertilizer application affected the structure of the community more than the season. On the contrary, we observed that communities from planted (fertilized) and unplanted (non fertilized) sites were more similar. This similarity among pristine and rice-planted bacterial communities is probably due to the soil management applied in our country, where the alternate rice-pasture cycle may have lower impact on the diversity than the long-term fertilization in Japanese fields.

The dual approach used in this work shows that floodwater from a rice-planted field sustains a quite diverse and active bacterial community with most of the main metabolic groups represented. Although some members of the community changed, the diversity and community structure of rice-planted floodwater were similar to those of the unplanted field.

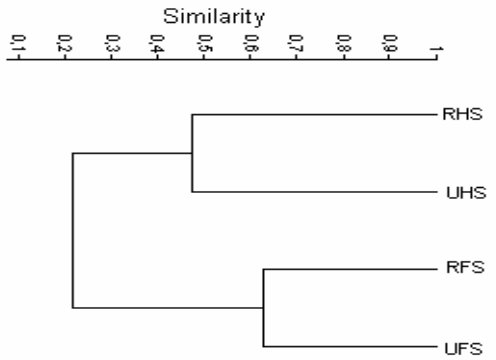

Figure 1. Cluster analysis of the T-RFLP patterns from the four bacterial communities. Similarity calculated according to the Morisita index is shown. The data obtained with the two restriction enzymes were combined. RFS, rice flooding stage; RHS, rice harvest stage; UFS, unplanted flooding stage; UHS, unplanted harvest stage.

Table 3. T-RFLP fragments obtained after *Hae*III digestion of 16S rRNA PCR products and Shannon-Weaver diversity index from samples in rice-planted and unplanted fields at flooding and harvest stages.

	Samples[a]			
T-RF (bp)	**RFS**	**RHS**	**UFS**	**UHS**
53			+	+
62		+	+	+
67	+	+		+
72			+	
77	+		+	
80				+
86				+
93				+
122				+
124				+
144				+
190		+		
193		+		+
195	+	+		
198	+	+	+	
201			+	
213	+	+	+	
217	+	+	+	+
224	+	+	+	
227		+		
229	+		+	
233	+			
238	+		+	
243	+		+	
259	+	+		+
261		+	+	+
263	+		+	
292		+		+
295	+			
381		+		+
H[b]	**2,5**	**2,3**	**2,5**	**2,4**

[a]RFS, rice flooding stage; RHS, rice harvest stage; UFS, unplanted flooding stage; UHS, unplanted harvest stage.
[b] Shannon-Weaver diversity index.

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