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ORIGINAL PAPER

Bacterial diversity and bioaugmentation in floodwater of a paddy field in the presence of the herbicide molinate

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Abstract This work aimed at studying variations on the diversity and composition of the bacterial community of a rice paddy field floodwater, subjected to conventional management, namely by using the herbicide molinate. The promotion of the herbicide biodegradation either by the autochthonous microbiota or by a bioaugmentation process was also assessed. This study comprehended four sampling campaigns at key dates of the farming procedures (seeding, immediately and 6 days after application of the herbicide molinate, and after synthetic fertilization) and the subsequent physic-chemical and microbiological characterization (pH, DOC and molinate contents, total cells, cultivable bacteria and DGGE profiling) of the samples. Multivariate analysis of the DGGE profiles showed temporal variations in the bacterial community structure and the Shannon's index values indicated that the bacterial diversity reached its minimum at the molinate application day. The highest bacterial diversity coincided with the periods with undetectable concentrations of the herbicide, although microcosm assays suggested that other factors than molinate may have been responsible for the decrease of the bacterial diversity. The ability of autochthonous microorganisms to degrade molinate and the influence of the herbicide on the bacterial community composition were assessed in microcosm assays using floodwater collected at the same dates. Given molinate was not degraded by autochthonous microorganisms, and considering it represents an environmental contaminant, bioaugmentation microcosms were assayed aiming the assessment of the feasibility of a bioremediation process to clean contaminated floodwater. A molinate-mineralizing culture, previously isolated, promoted molinate removal, induced alterations in the autochthonous bacterial community structure and diversity, and was undetected after 7 days of incubation, suggesting the feasibility of the process.

Keywords Floodwater · Molinate · Bacterial community structure and composition · Bacterial diversity · Biodegradation

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Introduction

Rice is one of the most cultivated crops worldwide, frequently protected by the use of pesticides and other phyto-pharmaceuticals in order to increase crop

yields (Singh et al. 2008; Wang et al. 2007). Despite the benefits that may arise from the use of pesticides, it is widely recognized that the environmental (air, soils, surface and ground waters) contamination with these compounds may have negative impacts on public health and on biological diversity, including on microbial communities. Bacteria are important members of the microbial communities in agriculture-related habitats, contributing to sustain the metabolic and physiological equilibrium of the ecosystem (Blackwood and Paul 2003; Gelsomino and Cacco 2006; Normander and Prosser 2000; Wakelin et al. 2008). Perturbations of the microbial diversity and activity due to pesticide contamination have been reported in experimental studies of aquatic bacterial communities in paddy field microcosms, in aquatic mesocosms and in riverine populations (Knapp et al. 2005; Pesce et al. 2006; Saeki and Toyota 2004). Also in natural lotic ecosystems, the discharge of organic contaminants has been considered responsible for similar perturbations (Dorigo et al. 2002, 2004; Pesce et al. 2008; Valle et al. 2006).

In rice paddy fields, floodwater overlays the field surface during rice cultivation. Floodwater is a complex habitat where organic matter is produced by algae and phytoplankton, with the contribution of floating weeds, and where microorganisms promote different catabolic processes (Asari et al. 2007; Kimura et al. 2002; Nakayama et al. 2006). When compared with other natural aquatic environments, the floodwater ecosystem is unstable, being presumably affected by an interrelated set of factors (Shibagaki-Shimizu et al. 2006). For instance, besides the climate and geological conditions, also the type of field management, such as phyto-pharmaceutical treatment, top-dressing of fertilizer and drainage, may influence the characteristics and perturbations of the ecosystem. In fact, different types of alterations in the floodwater microbial, phytoplankton and zooplankton communities have been attributed to the use of fertilizers, the growth stage of rice plants or the midseason drainage (Kimura et al. 2002; Okabe et al. 2000; Yamazaki et al. 2001). The lack of information on the floodwater bacterial diversity and composition of conventionally farmed rice fields motivated the present study. Molinate is one of the main pesticides used for weed control in rice culture worldwide. It is a selective and systemic thiocarbamate herbicide, interfering in lipid biosynthesis and inhibiting cell division during mitosis (Tomlin 2000). Molinate is generally applied in rice paddies once a year. It has been described as one of the most recalcitrant thiocarbamates (Nagy et al. 1995), and may have toxic effects in some bacteria (Köck et al. 2010; Phyu et al. 2005). Thus, with this study it was aimed both at assessing the effect of molinate on the floodwater bacterial diversity and at inferring about the potential of the autochthonous microbiota to degrade the herbicide. Floodwater bacterial diversity was screened using a cultivation-independent approach and the possible effects of molinate and its biodegradation by autochthonous microorganisms were assessed in microcosm assays. Given autochthonous microorganisms were not capable of molinate degradation, a fact that may explain previous studies reporting the herbicide detection in natural water streams receiving rice field tail waters (Castro et al. 2005; Claver et al. 2006; Kuivila and Jennings 2007; Sudo et al. 2005), we decided to test a bioaugmentation approach to remediate the floodwaters. The feasibility of environmental pesticide removal through bioaugmentation processes has been addressed in several studies (Benimeli et al. 2008; Hong et al. 2007; Lima et al. 2009; Silva et al. 2004a). One of the most important factors for a successful implementation of a bioaugmentation process is the availability of an efficient degrading culture. In our study, this was not a limitation, as in previous work we established a five-member bacterial mixed culture (named DC) with constitutive capacity to mineralize molinate (Barreiros et al. 2003, 2008). Culture DC is able to promote molinate degradation under a wide array of abiotic conditions (Carvalho et al. 2010; Coelho et al. 2006; Silva et al. 2004b), without the accumulation of any toxic metabolites (Correia et al. 2006). Thus, in the current study we aimed at assessing the ability of culture DC to remove the pesticide from floodwaters, and inferring at the possible perturbations this culture could induce in the autochthonous community. The addressing of these issues seemed relevant as previous studies have demonstrated that competition between exogenous and autochthonous microbiota may hamper the successful implementation of bioaugmentation processes (Bouchez et al. 2000; Chapalamadugu and Chaudhry 1992; Das and Mukherjee 2007; Mohanty and Mukherji 2008; Wenderoth et al. 2003).

Materials and methods

Site identification and sampling

This study was conducted in the experimental farm "Bico da Barca", from Direcção Regional de Agricultura e Pescas do Centro (DRAP Centro), located in the Mondego river valley, Montemor-o-Velho, central Portugal. The studied rice paddy field, with an area of 7200 m^2, is used in conventional agriculture and has a background of molinate application of six consecutive years. Samples of 5 l of floodwater were collected during 2007 according to the schedule and phyto-pharmaceutical regimen indicated in Table 1.

Chemical and microbiological characterization of the floodwater

Each sample was characterized for pH and dissolved organic carbon content (DOC). The pH was measured directly in the floodwater samples with an electrode. For DOC determination, samples were filtered through 1.2 μm-pore-size glass microfiber filters (Albet, Barcelona, Spain) and the filtrates were subsequently analysed as described before (Barreiros et al. 2003). Samples collected after Ordram application were also characterized for molinate content, as described below.

Total cells were enumerated using the 4′-6-diamidino-2-phenylindole (DAPI) staining method, as described before (Manuel et al. 2007). Briefly, a ten-fold dilution of each water sample was sonicated (35 kHz, 160 W, 10 min), filtered through a 0.2 μm-pore-size black polycarbonate membrane (Whatman, Kent, UK) and stained with 100 μg ml^{-1} DAPI (Sigma-Aldrich, Steinheim, Germany). The total number of cells was determined by direct counting under epifluorescence microscopy (Leica, Wetzlar, Germany). On average, 12 microscopic fields, corresponding to a minimum number of 400 cells, were counted per preparation. Fast growing heterotrophic bacteria were enumerated by the membrane filtration method, using 0.45 μm-pore-size cellulose nitrate membranes (Albet, Barcelona, Spain) on Plate Count Agar (Merck, Darmstadt, Germany), incubated for 48 h at 30°C. Bacterial community composition of floodwater was assessed by Denaturing Gradient Gel Electrophoresis (DGGE), as described below.

Microcosm assays and bioaugmentation

The bioaugmentation microcosm assays were prepared with floodwater collected at three different stages of rice culture—3 days before (seeding day, S), 5 h (O5h) and 6 days (O6d) after Ordram application (Table 1). To assess its performance, culture DC, composed by five bacterial strains (*Pseudomonas chlororaphis* ON1, *Stenotrophomonas maltophilia* ON2, *Pseudomonas nitroreducens* ON3, *Gulosibacter molinativorax* $ON4^T$ and *Achromobacter xylosoxidans* ON5) (Barreiros et al. 2003; Manaia et al. 2004), was used as inoculum. Given the highest molinate concentration found in floodwaters is described to be around 1.5 mg l^{-1} (Castro et al. 2005; Quayle et al. 2006; Son et al. 2006; present work), the water

Table 1 Rice culture procedures and dates of sampling

Date	Rice agriculture procedures	Samples
19th April	Flood (±10 cm)	
23rd April	Seeding	S
26th April	Ordram[a], 50 kg ha^{-1} (molinate)	O5h
2nd May		O6d
1st June	Midseason drainage	
4th June	Stam, 18 l ha^{-1} (propanil)	
	Basagran, 4 l ha^{-1} (bentazone)	
6th June	Flood (±10 cm)	
8th June	Fertilizers: $(NH_4)_2SO_4$,102 kg ha^{-1}; P_2O_5, 63 kg ha^{-1}; K_2O, 35 kg ha^{-1}	
13th June		Ad5d
27th September	Harvesting	

[a] Ordram is a commercial formulation containing 7.5% (w/w) of molinate

Table 2 Characterization of rice paddy floodwater over the phyto-pharmaceutical treatment

Parameter	Paddy field floodwater			
	S	O5h	O6d	Ad5d
pH	7.3 ± 0.2^{a}	7.2 ± 0.2^{a}	7.1 ± 0.2^{a}	6.8 ± 0.2^{b}
DOC (mg l^{-1})	6.3 ± 0.1^{a}	10.5 ± 0.2^{b}	7.8 ± 0.2^{c}	6.7 ± 0.1^{d}
Molinate (mg l^{-1})	ND	1.7^{e}	0.3	$<0.04^{f}$
Heterotrophs (CFU ml^{-1}) ($\times 10^5$)	2.9 ± 0.21^{a}	2.5 ± 0.30^{a}	3.1 ± 0.18^{a}	15 ± 1.3^{b}
Total cells (cells ml^{-1}) ($\times 10^7$)	2.5 ± 0.02^{a}	3.4 ± 0.18^{b}	2.6 ± 0.14^{a}	1.6 ± 0.07^{c}
% Cultivable population	1.2	0.7	1.2	9.3

The values are the average of three independent determinations ± standard deviation

ND not determined

[a,b,c,d] Homogeneous subsets, as determined by the Tukey test. Coherent homogeneous subsets were formed using both CFU ml^{-1} and cells ml^{-1} or the respective logarithms

[e] The DOC content of an Ordram aqueous solution with 1.7 mg l^{-1} molinate (determined by HPLC) was 3.7 mg l^{-1}, from which 2.1 mg l^{-1} corresponded to the DOC content of distilled water

[f] Detection limit of 0.04 mg l^{-1}

samples collected before and 6 days after Ordram application (concentrations indicated in Table 2) were spiked with molinate (Herbex, Estoril, Portugal) to reach a similar final concentration of ~1.5 mg l^{-1} (S M^m and O6d M^m). The sample collected 5 h after pesticide application (O5h^m) was not supplemented with the herbicide, because, in this case, after the inoculum addition, the molinate concentration reached ~1.5 mg l^{-1} (Table 3). Additionally, molinate degradation under conditions of reduced bio-availability (low molinate concentration, Table 2) was monitored in microcosms using floodwater collected 6 days after Ordram application without any molinate supplement (O6d^m). Mixed culture DC, pregrown in mineral medium B containing 750 mg l^{-1} molinate as the only source of carbon, nitrogen and energy (Barreiros et al. 2003), was inoculated into floodwater at a cell density of 1×10^7 cells ml^{-1}, not exceeding the total cell numbers of autochthonous microbiota (Table 2). Non-inoculated paddy field floodwater microcosms were tested simultaneously in order to assess the intrinsic depletion of molinate, due both to abiotic losses and to the autochthonous microbiota biodegradation. In these intrinsic remediation microcosm assays, sterile distilled water was added (~20 ml) to reproduce the dilution effect due to culture DC inoculation. As positive controls, culture DC was grown in mineral medium B (Barreiros et al. 2003) supplemented with molinate as the only source of carbon, nitrogen and energy at a similar concentration (0.3 or 1.5 mg l^{-1}), and at the same initial cell density (1×10^7 cells ml^{-1}). Assays and controls were performed, respectively, in 200 ml of floodwater, without any other nutrient, and of medium B, in 1 l screw-capped Erlenmeyer flasks with Teflon-lined caps, incubated at 30°C and 120 rpm for a period of 7 days. Bioaugmentation assays were performed in duplicate. Molinate content, heterotrophic bacteria, total cells and bacterial community composition were determined for each bioaugmentation and intrinsic remediation microcosm, as well as for the positive controls, at the initial (t0 = 0 days) and final (t7 = 7 days) time of incubation.

Molinate quantification

Molinate was quantified in 20 ml aliquots of floodwater samples or microcosm supernatants after two successive extractions with one volume of *n*-hexane. The hexane extracts were dried under vacuum, reconstituted in 1 ml of methanol and analysed by high performance liquid chromatography (HPLC) (Barreiros et al. 2003). Cycloate (5 mg l^{-1}) (Riedel-de Haën, Seelze, Germany) was used as internal standard for molinate extraction and quantification.

PCR-DGGE analysis

Bacterial cells were collected from 150 ml samples after filtration through 0.2 μm-pore-size sterile

Table 3 Molinate and microbial monitoring in microcosm assays (*m*) and in positive controls, grown in mineral medium with molinate at the same concentration and inoculated with culture DC at the same initial cell density (MM + DC)

	Intrinsic		Biaugmentation (+DC) (1)		Biaugmentation (+DC) (2)		Control (MM + DC)	
	0 d	7 d	0 d	7 d	0 d	7 d	0 d	7 d
S M^m								
Heterotrophs (CFU ml^{-1}) ($\times 10^5$)	2.5	0.7	265.0	4.9	390.0	3.7	255.0	300.0
Total cells (cells ml^{-1}) ($\times 10^7$)	2.6	2.7	3.1	2.5	3.0	2.6	2.7	2.5
Molinate (mg l^{-1})	1.2	1.2	1.5	0.4	1.5	0.3	1.5	0.2
Molinate degradation (%)		0		73		80		87
O5h^m								
Heterotrophs (CFU ml^{-1}) ($\times 10^5$)	5.5	1.0	280.0	4.1	240.0	3.3	255.0	300.0
Total cells (cells ml^{-1}) ($\times 10^7$)	2.5	2.9	3.4	2.6	3.4	2.5	2.7	2.5
Molinate (mg l^{-1})	1.4	1.3	1.5	0.4	1.5	0.3	1.5	0.2
Molinate degradation (%)		7		73		80		87
O6 d^m								
Heterotrophs (CFU ml^{-1}) ($\times 10^5$)	1.2	0.8	174.0	2.0	159.0	2.2	250.0	300.0
Total cells (cells ml^{-1}) ($\times 10^7$)	3.3	2.6	4.1	2.9	3.1	2.6	3.6	3.0
Molinate (mg l^{-1})	0.3	0.3	0.2	0.1	0.3	0.1	0.2	0.1
Molinate degradation (%)		0		50		67		50
O6d M^m								
Heterotrophs (CFU ml^{-1}) ($\times 10^5$)	2.4	0.9	250.0	3.7	215.0	5.6	240.0	280.0
Total cells (cells ml^{-1}) ($\times 10^7$)	2.7	2.6	3.5	2.7	3.3	2.6	3.3	2.4
Molinate (mg l^{-1})	1.5	1.4	1.5	0.4	1.5	0.5	1.5	0.3
Molinate degradation (%)		6		73		67		80

Numbers 1 and 2 correspond to two independent assays

polycarbonate membranes (Whatman, Kent, UK). Total DNA extraction was performed using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions with slight modifications. After filtration, each membrane was inserted in a supplied PowerBead tube and cell lysis was followed by an additional incubation period of 15 min at 65°C. A 200 bp fragment of the 16S rRNA gene was amplified from 1.5 to 4 μg ml^{-1} DNA template (Qubit™ Fluorometer, Invitrogen, United States) using the bacterial 16S rRNA gene primers forward 338F_GC, containing a GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′), and reverse 518R (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al. 1993). The obtained PCR products had a DNA concentration of approximately 30 μg ml^{-1} (Qubit™ Fluorometer). Aliquots of ~1.2 μg DNA were loaded and run in a 8% (w/v) poly-acrylamide gel with a denaturing gradient ranging from 28 to 57% (where 100% denaturant contained 7 M urea and 40% formamide), as previously described (Barreiros et al. 2008). Major DGGE bands were excised from the gels, eluted with 20 μl of ultra pure water and re-amplified by PCR with the same primers. For different samples, bands with identical electrophoretic mobility were excised and the respective nucleotide sequences were compared to confirm that co-migration was due to the correspondence to the same phylotype. The purity of each excised band was confirmed after DGGE under the same conditions. The majority of the bands (74 out of the 103 excised bands) required two steps of purification. After cleaning (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Biosciences, New Jersey, USA), the bands corresponding to pure DNA segments were sequenced with the primer 518R. Seventeen bands, excised from the lower part of the gels, could not be purified as subsequent DGGE runs yielded always more than one band, and were excluded

of this study. In other cases (bands 5, 7, 10, 13, 14, 15, 16, 17, 18, 20, 21, 22, 25, 26, and 28), bands yielding a single DNA fragment after a subsequent DGGE run originated sequences with ambiguous nucleotide identification. These bands were re-amplified by PCR and the respective PCR products were cloned using the pGEM-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. DNA inserts of four different clones matching the original band in the respective DGGE pattern were subsequently sequenced with the primer M13F (pGEM-T Easy Vector System). Nucleotide sequences were determined using a model ABI 3700 DNA Analyser (Applied Biosystems, California, USA) and their quality was checked manually using the BioEdit software (Hall 1999). Sequences were compared to the GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) in order to infer about their phylogenetic affiliation. The authenticity of culture DC DGGE patterns was confirmed by comparing the nucleotide sequences of the corresponding DGGE excised bands with those of the 16S rRNA gene of each isolate (Table 5) (Barreiros et al. 2003).

Data analyses

DGGE profiles were compared using the Bionumerics software (version 5.10, Applied Maths, Belgium), after the visual examination of the patterns. Reference samples, loaded in the first and last lane of each DGGE gel, were used to normalize and allow the comparison between different gels. DGGE banding data were used to estimate two indices of biological diversity, the Shannon index of diversity ($H = -\sum(n_i/N)\log(n_i/N)$; Shannon and Weaver 1963) and the evenness index ($E = H/\log S$; Pielou 1966). Each band was treated as an individual operational taxonomic unit (OTU). The number of DGGE bands was used to indicate the number of species (S). The relative surface intensity of each band, expressed as peak height in the densitometric curve (n_i), and the sum of all peak heights in the curve of a given sample (N) were used as estimates of species abundance (Fromin et al. 2002).

The statistical significance of differences in chemical (pH, DOC) and microbiological (heterotrophs, total cells) parameters data and in diversity indices was evaluated with one-way analysis of variance (ANOVA) and, whenever applicable, the Post Hoc test of Tukey. The comparison of diversity indices within and between bioaugmentation and intrinsic remediation microcosm assays was performed using the two-sample t-test (Analysis ToolPak package of Microsoft Excel 2003 software for Windows).

Ordination techniques based on DGGE fingerprints were used to analyse the differences in bacterial community composition (band intensity) and its possible linkage with abiotic and biotic environmental factors monitored during the rice crop cycle. To test whether unimodal or linear methods were appropriate, Detrended Correspondence Analysis (DCA) was performed using the PC-ORD software (version 5.0, MJM Software, Gleneden, USA). The longest gradients resulting from DCA of floodwater samples and microcosms were 3.3 and 3.6, respectively. These values did not indicate a clear linear or unimodal relationship (Leps and Smilauer 2003; ter Braak 1994; ter Braak and Smilauer 2002), so a linear method (Principal Components Analysis, PCA) and a unimodal method (DCA) were simultaneously performed and compared. For both community ordination analyses, biplot scaling was used.

Results

Chemical and microbiological characterization of the rice field floodwater

The floodwater of the studied paddy field was analysed at four stages over the rice culture cycle, during the year 2007 (Tables 1, 2). Throughout this period, the DOC content varied significantly ($P < 0.01$) between 6.3 and 10.5 mg l^{-1}, and the pH decreased from 7.3 to 6.8 ($P < 0.01$). Given Ordram co-adjuvants have a low organic content (Table 2 footnote), the highest DOC values registered in the floodwater 5 h after the application of the commercial phyto-pharmaceutical formulation may have been due, in part, to the pesticide input, corresponding to 1.7 mg l^{-1} of molinate. Six days after Ordram application, molinate dissipated to a lower concentration (0.3 mg l^{-1}) and the DOC value also decreased (Table 2). As expected, due to midseason drainage and herbicide dissipation, molinate was

below the detection limit (0.04 mg l^{-1}) in the sample collected in June.

Although in the same order of magnitude (10^7 cells ml^{-1}), the number of total cells varied significantly ($P < 0.01$) over the rice crop cycle, with the highest value being observed 5 h after Ordram application and the lowest in June. In contrast, the highest ($P < 0.01$) counts of heterotrophs were observed in June (Table 2), after fertilization and when the rice plants were actively growing towards maturation.

Floodwater bacterial community structure

The DGGE profiling evidenced modifications in the floodwater bacterial community over the rice crop cycle. The bacterial diversity of floodwater was compared on basis of the Shannon (H) and the evenness (E) diversity indices. The Shannon diversity index ranged between 0.95 and 1.18, corresponding to the samples with the lowest (O5h, 11 bands) and highest (Ad5d, 19 bands) average number of detectable bands. Despite the variation observed in the bacterial diversity (H) ($P < 0.01$), the evenness index did not vary significantly over the rice crop cycle (Table 4).

The relationship between community patterns, chemical and microbiological variables was assessed through an ordination analysis, comprising the different parameters measured in each rice culture stage. Because of the intermediate gradient length, both a PCA and a DCA were used. The results obtained with both ordination methods were similar. Thus, for a matter of simplicity, only the results of the linear method PCA are shown (Fig. 1).

PCA results showed that the first two principal components (axis 1 and 2 in the PCA biplot) accounted, respectively, for 33.8 and 22.2% of the bacterial community variation. The variables pH (0.937, $P < 0.001$), total cells (0.830, $P < 0.001$) and heterotrophs (−0.898, $P < 0.001$) strongly contributed to the variation explained by axis 1, while DOC (0.734, $P < 0.001$) and molinate contents (0.650, $P < 0.001$) influenced the variation explained by axis 2. PCA biplot defined four distinct groups of DGGE profiles, corresponding to each rice culture sampling stage (S, O5h, O6d, Ad5d). The group constituted by DGGE profiles of the seeding day (S) presented the highest dispersion. The DGGE patterns of floodwater after fertilization (Ad5d) were separated from the others along axis 1 by pH and microbial parameters (total cells and heterotrophs). This principal component also

Table 4 Diversity indices of floodwater and the corresponding microcosms microbial communities, over the rice phyto-pharmaceutical treatment and at beginning (t0) and end (t7) of incubation, respectively

Floodwater	Microcosm	Shannon index (H)		Evenness index (E)	
		Floodwater (= Microcosm, t0)	Microcosm (t7)	Floodwater (= Microcosm, t0)	Microcosm (t7)
S	S M^m	$1.14 \pm 0.02^{a,I}$	$1.11 \pm 0.01^{a,I}$	$0.93 \pm 0.01^{a,I}$	$0.93 \pm 0.04^{a,I}$
	S M^m + DC	$1.13 \pm 0.03^{a,I}$	$1.12 \pm 0.01^{a,I}$	$0.94 \pm 0.01^{a,I}$	$0.94 \pm 0.005^{a,I}$
O5 h	O5h^m	$0.95 \pm 0.0002^{b,I}$	$0.85 \pm 0.01^{b,II}$	$0.94 \pm 0.003^{a,I}$	$0.93 \pm 0.04^{a,I}$
	O5h^m + DC	$0.95 \pm 0.007^{b,I}$	$1.04 \pm 0.01^{c,II}$	$0.94 \pm 0.02^{a,I}$	$0.93 \pm 0.03^{a,I}$
O6d	O6d^m	$1.06 \pm 0.004^{c,I}$	$1.07 \pm 0.01^{c,I}$	$0.90 \pm 0.02^{a,I}$	$0.91 \pm 0.01^{a,I}$
	O6d^m + DC	$1.06 \pm 0.01^{c,I}$	$1.07 \pm 0.01^{c,I}$	$0.91 \pm 0.01^{a,I}$	$0.90 \pm 0.01^{a,I}$
	O6d M^m	$1.06 \pm 0.002^{c,I}$	$1.06 \pm 0.02^{c,I}$	$0.91 \pm 0.01^{a,I}$	$0.90 \pm 0.02^{a,I}$
	O6d M^m + DC	$1.06 \pm 0.003^{c,I}$	$1.17 \pm 0.01^{d,II}$	$0.91 \pm 0.01^{a,I}$	$0.92 \pm 0.01^{a,I}$
Ad5d	NA	1.18 ± 0.02^{a}	NA	0.93 ± 0.02^{a}	NA

See text for details

NA not applicable

[a,b,c,d] Homogeneous subsets, as determined by the Tukey test comparing diversity indices between the different floodwater/microcosms, at the same time of incubation (t0 or t7)

[I,II] Homogeneous subsets, as determined by the two-sample t-test comparing diversity indices within each microcosm assay (t0 and t7)

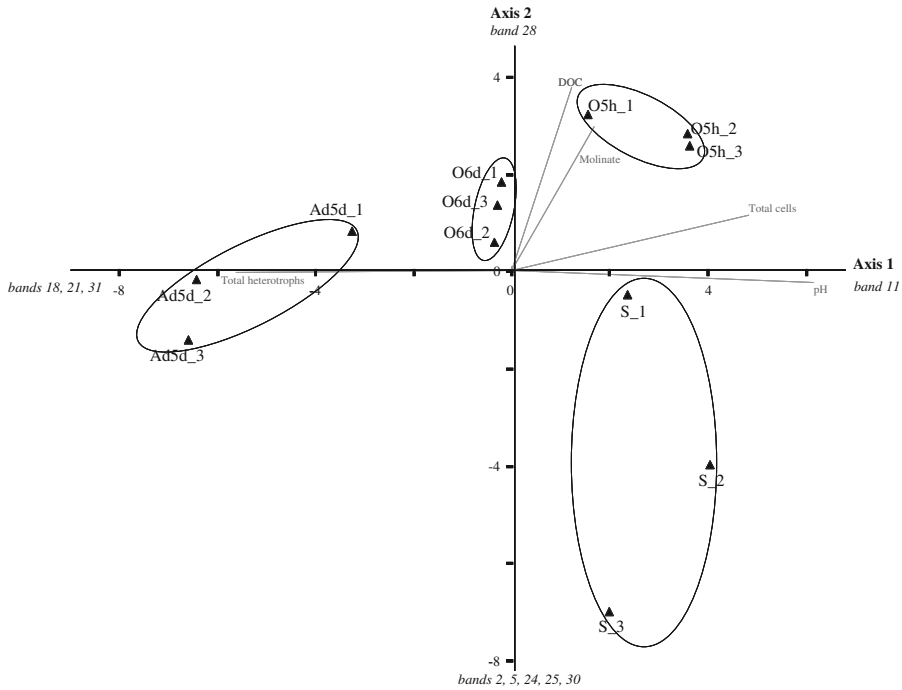

Fig. 1 PCA biplot of floodwater microbial community and environmental parameters (represented by *arrows*) over the rice phyto-pharmaceutical treatment

separated the DGGE profiles of floodwater collected after Ordram application (O5h and O6d). Furthermore, these two last groups were separated from the DGGE patterns of floodwater of the seeding day (S) along the second axis, influenced by DOC and molinate contents.

Composition of floodwater bacterial community

The phylogenetic diversity of the paddy field floodwater was assessed on basis of 16S rRNA gene DGGE profiling (Table 5). The closest neighbours of most of the nucleotide sequences of the DGGE bands analysed in floodwater samples corresponded to clones and/or bacterial strains of aquatic environments or agricultural soils. According to BLAST search, the majority of the bands detected in floodwater corresponded to organisms affiliated to the phyla *Bacteroidetes* (seven different bands), *Verrucomicrobia* (seven different bands), *Actinobacteria* (three different bands), and the class *Betaproteobacteria* (four different bands) (Table 5).

Considering the totality of floodwater DGGE patterns, among the 37 bands detected, only three were present in all samples, although with higher intensity in the seeding day. Two of these bands were related to *Actinobacteria* (band 11) and *Verrucomicrobia* (band 24). Given their variation (presence/absence or intensity) over the different patterns, the highest Eigenvalues and significant correlation values either with axis 1 or 2 corresponded to bands 2, 5, 11, 18, 21, 24, 25, 30 and 31. Most of these bands were observed exclusively in the samples with the highest Shannon diversity values (S, Ad5d) and were those which contributed most to differentiate the floodwater microbiota community over the rice crop cycle. Such differentiating bands were assigned to the taxa prevailing in floodwater, mainly *Verrucomicrobia* (5, 24, 25, 30, 31) and in a lesser extent, *Proteobacteria* (18), *Firmicutes* (21), *Actinobacteria* (11) and *Bacteroidetes* (2). Bands corresponding to *Bacteroidetes* were either observed exclusively in the day of Ordram application (band 28) or in every sample except in this (band 2). In the same way, for the differentiation of floodwater without molinate, contributed *Verrucomicrobia*-related bands (25 and 30), detected simultaneously in S and Ad5d samples. The period after fertilization, June, was distinguished by the presence of three bands corresponding to *Proteobacteria, Firmicutes* and *Verrucomicrobia* (18, 21 and 31). Additionally, band 5, corresponding to an organism related to the phylum *Verrucomicrobia* and which intensity decreased from the seeding day to become undetectable after fertilization, also contributed to differentiate the samples of June.

Table 5 Phylogenetic affiliation of major DGGE bands shown in Fig. 2 and distribution of these organisms in the different floodwater samples or microcosm assays (*m*)

				Paddy field floodwater				Microcosm								
Closest Relative	% Similarity	Accession Number	Origin	S	O5h	O6d	Ad5d	S M^m	S M^m + DC	$O5h^m$	$O5h^m$ + DC	$O6d^m$	$O6d^m$ + DC	O6d M^m	O6d M^m + DC	DGGE Band
Actinobacteria																
Uncultured actinobacterium	96	EF446337	Lake							t0		t7	t7	t7	t7	11
Uncultured actinobacterium	91	EF135052	Amended soils							t0						9
Uncultured actinobacterium	92	EF650869	Australian Vertisol						t7		t7		t7	t7	t7	26
Uncultured actinobacterium	96	DQ316355	Lakes-Germany					t7		t7				t0		8
Bacteroidetes																
Uncultured *Flavobacteria*	100	EF520552	Acid-impacted lakes									t7	t7		t7	2
Flavobacterium terrigena	91	DQ889724	Soil						t7				t0	t0		6
Uncultured *Bacteroidetes*	91	EF219724	Antarctic habitats													28
Uncultured *Sphingobacteria*	90	EF520595	Acid-impacted lakes									t7		t7	t0	3
Spirosoma linguale	93	AM000023	Water steam generator					t0	t0		t7	t0		t0	t0	17
Uncultured *Flavobacteria*	90	EF061064	Freshwater										t7		t7	29
Uncultured *Sphingobacteria*	92	EF072475	Soil-USA										t7			23
Cyanobacteria																
Uncultured cyanobacterium	93	EF632989	Saline wetland-Chile									t0	t0			1
Firmicutes																
Uncultured *Firmicutes*	99	EU297454	Agricultural soil-USA						t7		t7					13
Uncultured *Firmicutes*	93	EU297454	Agricultural soil-USA						t7							21
Alphaproteobacteria																
Sphingopyxis sp.	90	EF424406	Agricultural field						t7				t7		t7	12

				Paddy Field				Microcosm								
Closest Relative	% Similarity	Accession number	Origin	S	O5h	O6d	Ad5d	S M^m	S M^m + DC	$O5h^m$	$O5h^m$ + DC	$O6d^m$	$O6d^m$ + DC	O6d M^m	O6d M^m + DC	DGGE Band
Betaproteobacteria																
Uncultured proteobacterium	100	AY947907	Bacterioplankton-rivers					t7	t7		t7			t0		22
Uncultured *Rhodocyclaceae*	94	EF018674	Soil					t0			t7					20
Proteobacterium	100	AJ964892	Freshwater						t7		t0		t7	t7	t7	7
Aquaspirillum sp.	98	AF321032	Northern Baltic Sea							t0				t0		15
Gammaproteobacteria																
Uncultured *Chromatiaceae*	98	AY360644	Oxic rice field soil								t7		t7			18
Xanthomonas sp.	97	AM490086	River sediment						t7	t0						14
Uncultured *Legionella* sp.	99	AY924163	Treated drinking water						t7	t7			t0		t0	16
Deltaproteobacteria																
Uncultured proteobacterium	90	EU300326	Agricultural soil-USA						t7		t7		t7			19
Verrucomicrobia																
Uncultured *Verrucomicrobia*	91	EU043654	Soils					t0			t7	t7	t0	t7	t7	24
Uncultured *Verrucomicrobia*	91	AF141391	Estuary-Columbia river					t0	t7						t7	4
Uncultured *Verrucomicrobia*	93	AY214834	Soil									t0		t0		27
Uncultured *Verrucomicrobia*	91	DQ829316	Soil						t0		t7			t7		30
Uncultured *Verrucomicrobia*	92	EU298281	Agricultural soil-USA					t7	t7	t7	t7	t7	t7	t7	t7	10
Uncultured *Verrucomicrobia*	95	AY509518	Lakes-Sweden									t0				5
Uncultured *Verrucomicrobia*	97	EF220751	Antarctic habitat						t7		t7					31
Uncultured *Opitutaceae*	97	EU298661	Agricultural soil-USA						t7	t0		t7	t7		t7	25
Mixed culture DC																
Gammaproteobacteria																
Pseudomonas chlororaphis	99	AJ306832	Strain ON1						t0		t0		t0		t0	a
Stenotrophomonas maltophilia	100	AJ306833	Strain ON2						t0		t0		t0		t0	b
Pseudomonas nitroreducens	100	AJ306834	Strain ON3						t0		t0		t0		t0	c
Actinobacteria																
Gulosibacter molinativorax	99	AJ306835	Strain ON4^T						t0		t0		t0		t0	d
Betaproteobacteria																
Achromobacter xylosoxidans	99	AJ306836	Strain ON5						t0		t0		t0		t0	e

Black shadowing represents organisms detected in the respective floodwater sample or both at initial and final time of incubation of the microcosm assay, while t0 and t7, organisms detected only in the initial or final stages of the microcosm assay, respectively. See text for details

Degradation of molinate in paddy field floodwater microcosms

In intrinsic remediation microcosm assays, molinate concentration did not decrease after 7 days of incubation, indicating the inability of the autochthonous microbiota to degrade the herbicide. In contrast, in bioaugmentation microcosm assays, molinate removal yields of ~75–80% of the initial 1.5 mg l^{-1} were observed (Table 3). Slightly lower removal

yields (~55%) were observed in the presence of lower initial molinate concentrations (~0.3 mg l^{-1}). This was probably due to the lower bio-availability of the herbicide in these conditions, as similar results were observed in the positive control (Table 3). Molinate degradation in the bioaugmentation microcosm assays was accompanied by the decrease of the cultivable population, in contrast to what was observed in the positive controls. In positive controls the counts of heterotrophic bacteria were kept approximately constant at ~10^7 CFU ml^{-1}, whereas in the bioaugmentation microcosm assays the initial 10^7 CFU ml^{-1} heterotrophs declined approximately 100 times at the end of the incubation period (Table 3).

Microcosm bacterial community composition

To assess the fate of culture DC members in the bioaugmentation microcosm assays, the DGGE profiles of the different microcosms were analysed. In all bioaugmentation microcosm assays, culture DC members yielded intense DGGE bands at the beginning of the incubation period (Fig. 2, lanes 3 and 5—t0) and pale bands at the end of the assay (after 7 days of incubation) (Fig. 2, lanes 4 and 6—t7). To evaluate the effect of mixed culture DC in the floodwater bacterial community, the DGGE patterns of the inoculated and non-inoculated microcosms were compared (bands corresponding to culture DC were excluded of this analysis). The values of Shannon and evenness indices revealed that in most of the microcosm assays the diversity and evenness of the bacterial community did not vary over the incubation period (Table 4). The exceptions were the microcosms O5h (O5h^m and O5h^m + DC) and O6dM^m + DC. While in the intrinsic remediation microcosm assay O5h^m the diversity *H* index value decreased ($P < 0.01$) over the incubation period, in the bioaugmentation microcosms O5h^m + DC and O6dM^m + DC it increased ($P < 0.01$).

The PCA analysis of the DGGE microcosms profiles allowed the definition of four groups, with axis 1 and 2 explaining, respectively, 21.3 and 13.2% of the total variation (Fig. 3). Groups I and IV comprised the DGGE profiles at the end of the incubation period of the bioaugmentation microcosm assays of the seeding day (S M^m + DC-t7) and 6 days after Ordram application (O6d^m + DC-t7 and O6d M^m + DC-t7), respectively. Group II included the DGGE patterns of the remaining S M^m microcosms and bioaugmentation microcosms of 5 h after Ordram application at the end of the incubation period (O5h^m + DC-t7). The DGGE profiles of the remaining microcosms O5h^m, O6d^m and O6d M^m grouped together in group III.

As expected, most of the DGGE bands were observed simultaneously in the floodwater samples and in microcosms (Table 5). Among the microcosms DGGE patterns, bands with higher Eigenvalues and therefore with the highest contribution to the observed variation in floodwater microcosms communities corresponded to members of the phyla *Proteobacteria* (divisions *Alpha-*, 12; *Beta-*, 7 and 22; *Delta-*, 19), *Bacteroidetes* (29) and *Verrucomicrobia* (30). All these bands were more intense in bioaugmentation microcosm assays at the final time of incubation. Among the differentiating bands, those corresponding to *Beta-* and *Deltaproteobacteria* were more intense in the DGGE patterns of the inoculated microcosms produced with floodwater of the seeding day (group I) (Fig. 3). Band 30, which corresponds to a member of the phylum *Verrucomicrobia*, and which presence was related with the absence of molinate in floodwater as mentioned above, was, in the microcosms, more intense in the DGGE patterns of either non-inoculated assays with seeding day floodwater or where molinate was degraded (inoculated assays O5h^m + DC-t7, group II). Finally, the two other bands, corresponding to organisms related to the class *Alphaproteobacteria* (band 12) and the phylum *Bacteroidetes* (band 29), were detected exclusively in DGGE patterns of inoculated microcosms produced with floodwater collected 6 days after Ordram application (group IV).

These results pointed out that, irrespectively of the floodwater used in the microcosms assays, the initial and final DGGE patterns of the non-inoculated microcosms grouped together. In contrast, the effect of the inoculation with a molinate biodegrading culture led to the separation of the DGGE profiles at the end of the incubation period from those of the beginning of the assays.

Discussion

Characterization of the rice field floodwater over the rice cycle

The multivariate analysis of floodwater DGGE profiles revealed that the bacterial community structure

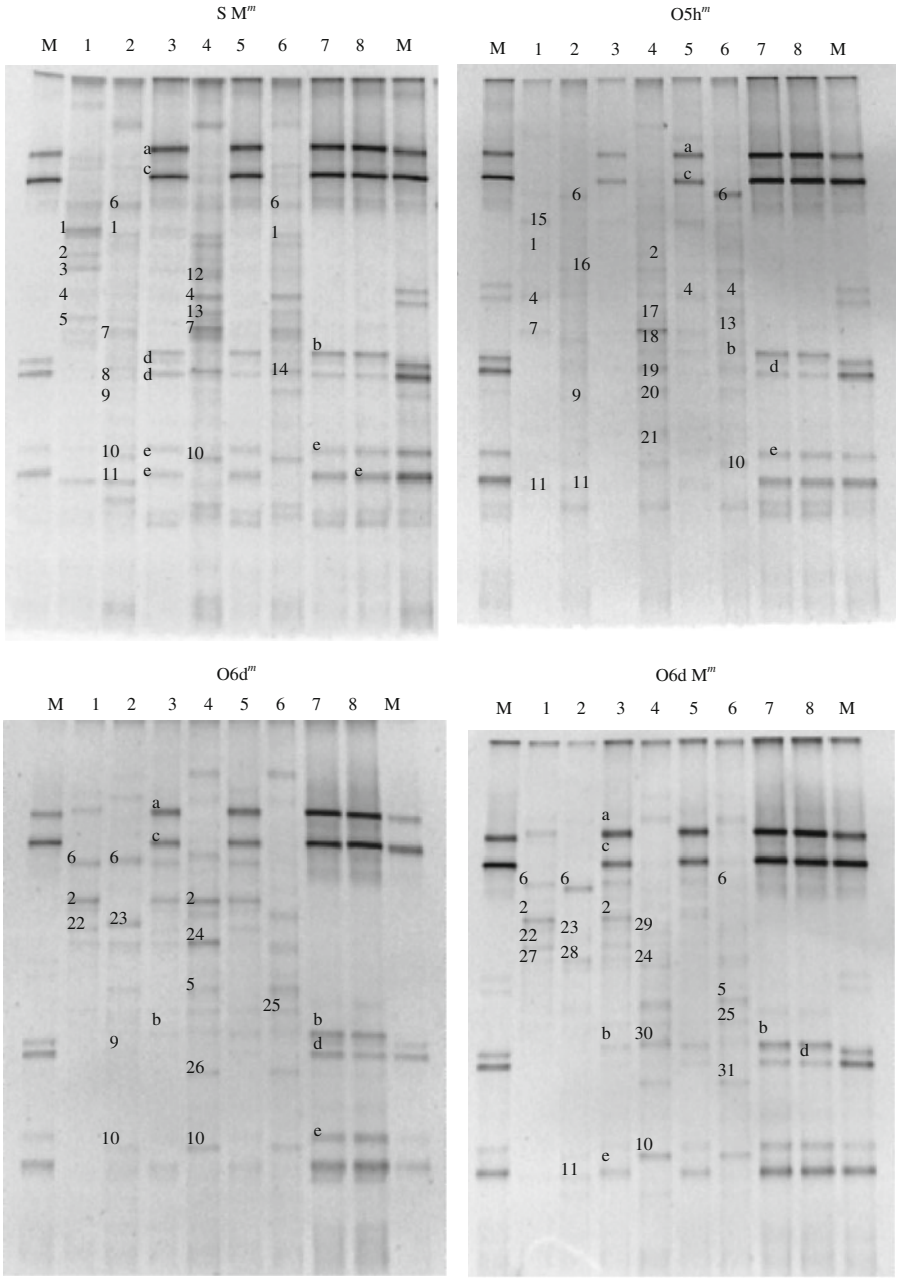

Fig. 2 DGGE profiles of microcosm assays with rice paddy floodwaters. Intrinsic (non-inoculated) remediation microcosms at t0 (*lanes 1*) and t7 (*lanes 2*). Bioaugmentation (inoculated with culture DC) microcosms at t0 (*lanes 3, 5*) and t7 (*lanes 4, 6*). Positive control, culture DC grown in mineral medium with the same concentration of molinate (MM + DC) at t0 (*lanes 7*) and t7 (*lanes 8*). *M* marker, culture DC grown in mineral medium with 4 mM molinate. Bands that were excised for sequence analysis are numbered as indicated in Table 5

changed over time, as the samples formed four distinct groups coherent with the sampling dates (Fig. 1). According to the Shannon diversity index values (Table 4), the sample collected 5 h after Ordram application presented the lowest bacterial diversity. The values of the evenness diversity index indicated an even distribution of species present in floodwater over time.

The majority of the nucleotide sequences analysed after the DGGE fingerprinting (79–87% of the sequences retrieved from samples S, O5h, O6d and Ad5d) presented the highest identity scores with 16S rRNA gene sequences of uncultured bacteria. Similar findings were reported for other DGGE based studies on rice paddies (Kikuchi et al. 2007; Shibagaki-Shimizu et al. 2006). Although this result is, in part,

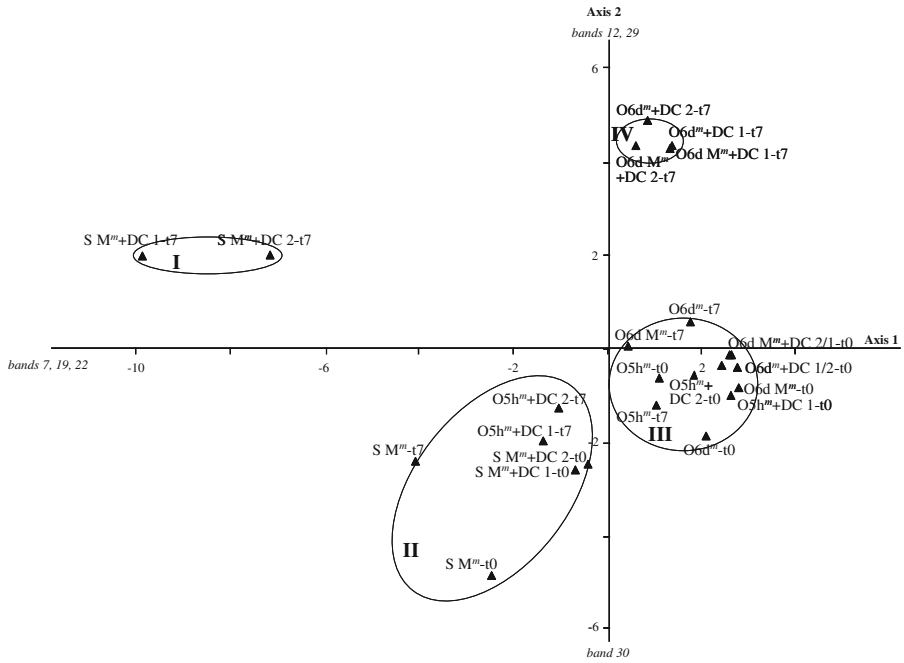

Fig. 3 PCA biplot of DGGE band patterns from microcosm assays. The bands of culture DC members were excluded in the bioaugmentation patterns

due to the fact that uncultured organisms represent nowadays an important slice of the nucleotide sequences deposited in public databases, those authors attributed the scarcity of sequences presenting high identity scores with cultured organisms to the oligotrophic character of the rice field ecosystem. The closest neighbours of most of the analysed DGGE bands nucleotide sequences corresponded to organisms of the phyla *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, and of the class *Betaproteobacteria*, *taxa* previously reported as the dominant members of bacterial communities of rice paddy fields (Hengstmann et al. 1999; Kikuchi et al. 2007; Liesack et al. 2000; Murase et al. 2005; Nakayama et al. 2006; Shibagaki-Shimizu et al. 2006).

According to our results, Ordram application may have imposed stressful conditions in the floodwater community as the lowest *H* diversity value was found for this bacterial community (O5h), which also contained the lowest proportion of cultivable heterotrophs. As EC_{50} values of 1–10 mg l^{-1} molinate have been reported for the model organism *Vibrio fischeri* (Köck et al. 2010; Phyu et al. 2005), in our case, Ordram application could have induced an immediate toxicity effect due to the presence of molinate at a concentration of 1.7 mg l^{-1}. Nevertheless, some organisms, affiliated to the phylum *Bacteroidetes*, were detected (band 28) or not (band 2), exclusively on the day of Ordram application. Moreover, such alterations were apparently transient, as the highest diversity values were observed before Ordram application (S) and in June (Ad5d), when the herbicide was at trace levels in floodwaters. Thus, it is possible to conclude that after phyto-pharmaceutical treatment, the floodwater community had the ability to recover its diversity.

The highest cultivability proportion was observed in June (Ad5d). This result may be attributed to several factors, such as the release of possible Ordram negative effects, the beneficial influence of nitrogen and phosphorus compounds supplied in the fertilizers, the growth stage of rice plants, and/or the increase of temperature, characteristics of this season. These factors, independently or in combination, might have favoured the development of a wide range of organisms, increasing the ratio of cultivable heterotrophs. In fact, the effect of fertilizers input and seasonal variations were indicated as possible inducers of modifications in the microbial community structure and dynamics of paddy field soil and water

(Kim et al. 2008; Kimura et al. 2002). Particularly, it is suggested that the growth stage of rice plants may have a strong influence on the microbiota diversity and abundance (Bai et al. 2000; Chen et al. 2009; Hoque et al. 2001; Inubushi et al. 2001; Lu et al. 2002). For instance, in a study on rice paddy soil Bai et al. (2000) observed that rice plants growth towards maturity was accompanied by an increase of Gram-negative bacteria and methanotrophs. Similarly, Chen et al. (2009) reported an increase in the complexity of the DGGE profiles of a paddy soil with the growth of rice plants. Such an effect is presumably due to the nutrients supply maintained by root exudates, where diverse plant metabolites represent carbon, nitrogen and energy sources for the surrounding microbiota (Bai et al. 2000; Curl and Truelove 1986; Inubushi and Watanabe 1986; Kong et al. 2008; Lu et al. 2002; Lynch and Whipps 1990).

In general, it was observed that pH, cultivability percentage, DOC and molinate contents were underlying factors for the variation on the type and number of species present in floodwater over time. According to the ordination analysis, the highest cultivability and lowest pH had high contribution to distinguish the community of June (Ad5d) from those of April (S, O5h) and May (O6d). On the other hand, the lowest DOC and molinate contents contributed to explain the separation of the seeding day community (S) from those of late April and May (O5h and O6d).

Effect and biodegradation of molinate in microcosms with paddy field floodwater

The data gathered in this study suggested that bacterial diversity and cultivability in floodwater could be affected by the presence of molinate. The apparent recovery of these parameters in June supported the hypothesis that molinate dissipation allowed the reestablishment of the diversity. An aspect that was still to be clarified was whether bacterial diversity reestablishment resulted from the molinate biodegradation, leading to its dissipation, or if other factors were involved. Thus, the settling of microcosm assays was fundamental to assess indigenous molinate biodegradation. In fact, given Ordram is being used for several years in the rice paddy field under study, it could be expected that these waters contained microbial communities adapted to metabolize molinate. The inability of the autochthonous microbiota to degrade the herbicide was an interesting and somehow unexpected finding. Biodegradation has been pointed out before as a mode of molinate dissipation in floodwater under field conditions (Deuel et al. 1978). In the present study, the apparent inability of the autochthonous microbiota to degrade molinate may hint the physico-chemical degradation of the herbicide in the studied paddy field water over time. For instance, volatilization has been reported as the major route of dissipation of this thiocarbamate (Quayle et al. 2006; Ross and Sava 1986; Soderquist et al. 1977).

In order to test the hypothesis that molinate application could have lead to a reduction of the bacterial diversity, additional comparative studies in microcosms assays were necessary. These assays permitted to observe that molinate seems to have little effect on the floodwater bacterial community structure and diversity. In fact, the DGGE profiles of non-inoculated microcosms at the beginning and final time of incubation grouped together and the diversity H index values of S M^m, $O6d^m$ and O6d M^m microcosms did not vary over the incubation period. In turn, the decrease of the H value in the $O5h^m$ microcosm suggests that co-adjuvants present in the commercial herbicide Ordram, or other prevailing condition on that day, may have been responsible for the decrease in the microbial diversity observed in the floodwater. Further studies using Ordram as nutrient will be needed to clarify its effects on the floodwater bacterial community.

Molinate has been widely used in conventional rice crop protection and the field concentrations found in our study are in the range of values found in paddy waters worldwide (Portugal, Castro et al. 2005; USA, Deuel et al. 1978; Soderquist et al. 1977; Australia, Quayle et al. 2006; and Japan, Son et al. 2006) and are consistent with half-life values of 3–10 days, reported for this herbicide (Deuel et al. 1978; Johnson and Lavy 1995; Mabury et al. 1996; Ross and Sava 1986). The drainage of paddy fields, taking place about 1 month after Ordram application, has been leading to the contamination of rivers and aquifers with molinate, frequently at concentrations above the values legally recommended (Albanis et al. 1998; Castro et al. 2005; Cerejeira et al. 2003; Son et al. 2006). Therefore, the removal of molinate from floodwaters, by biological methods as bioaugmentation, may represent a valuable preventive measure to avoid environmental contamination after water

drainage. The feasibility of such a process was assessed in bioaugmentation microcosm assays.

The bioaugmentation assays confirmed the ability of culture DC to degrade the herbicide in floodwater, in the absence of any additional nutrients or co-factors and in the presence of the autochthonous microbiota. Although these results suggested that culture DC may be used as a bioremediation tool, it was also important to assess the possible perturbations induced in the autochthonous community. With this objective, the DGGE patterns of the bacterial communities of the different microcosm assays were analysed. One of the first conclusions retrieved from this study was the fact that culture DC was not able to proliferate in floodwater. In fact, the intensity of the DGGE bands of culture DC in bioaugmentation assays decreased after 7 days of incubation (Fig. 2, t0—lanes 3, 5; t7—lanes 4, 6). These observations are in accordance with the number of cultivable heterotrophs observed in these assays, which decreased from densities around 10^7 to 10^5 CFU ml^{-1} over the incubation period. Moreover, we could confirm that the decrease of intensity of the culture DC DGGE bands in paddy water microcosms was not due to the lack of nutrients, as after 7 days of incubation in mineral medium, with molinate at the same initial concentration constituting the only organic nutrient, all of its five members were detected (Fig. 2, lanes 8) and the number of cultivable cells was maintained at 10^7 CFU ml^{-1} (Table 3). If elimination of mixed culture DC was due to the competition of the autochthonous microbiota or to the presence of growth inhibitors in paddy water, acting independently or in combination, it is not known; nevertheless, herbicide degradation was not hampered.

The multivariate analysis of the DGGE profiles of the microcosms, excluding the bands corresponding to culture DC members, revealed that the bacterial community of the bioaugmentation assays changed after the incubation period. The distribution of DGGE profiles in the PCA biplot (Fig. 3) showed the separation of bacterial communities of bioaugmentation microcosm assays after 7 days of incubation from their non-inoculated and initial time counterparts. The S M^m DGGE profiles, included in group II at the beginning of the incubation period, were, due to the presence of culture DC, integrated in group I (S M^m + DC, 7 days incubation). Similarly, microcosms $O5h^m$, $O6d^m$ and O6d M^m, in which the presence of Ordram seems to impose community rearrangements capable of including these profiles in group III, were allocated to groups II and IV, respectively, when incubated in the presence of culture DC ($O5h^m$ + DC, group II; $O6d^m$ + DC and O6d M^m + DC, group IV). A possible contribute for the community rearrangement can be the nutrient input due to culture DC inoculation, which might have stimulated the growth of autochthonous bacteria. In fact, some DGGE bands (12 and 19) were detected only in the bioaugmentation microcosms after 7 days of incubation, and included organisms affiliated to different classes of *Proteobacteria* not detected in the field samples. In the same way, other bands such as band 30, were more intense in microcosm or field samples with low molinate concentrations, i.e., at the end of incubation of bioaugmentation microcosm assays or in S and Ad5d samples. Nevertheless, the introduction of the exogenous culture did not favour the development of a certain type of organism, as among the bioaugmentation assays different DGGE bands were more intense at the final time of incubation, although also observed at the beginning of the assay. Besides, culture DC seems to have improved the floodwater bacterial diversity in microcosm assays. In fact, after the incubation period, $O5h^m$ + DC and O6d M^m + DC bioaugmentation microcosm assays presented significantly higher H diversity values ($P < 0.05$) than their non-inoculated counterparts (Table 4).

Conclusions

Alterations in the floodwater bacterial community were observed over the rice production cycle. These alterations could have been due either to temporal changes or to agricultural management procedures. The data obtained suggested that Ordram application may have contributed to a transient reduction in the bacterial diversity and cultivability, although microcosm assays suggest that such effects can be attributed to other factors than the herbicide molinate.

In spite of the history of molinate use in the studied paddy, floodwater microorganisms could not degrade this herbicide, suggesting that other dissipation modes may have taken place in the field. Possible bioaugmentation strategies, using culture DC, may contribute to avoid environmental contamination by floodwater. Culture DC was able to remove up to 55–80% of the herbicide. Despite not being detected

at the final time of incubation, its presence induced the modification of autochthonous bacterial community structure, sometimes contributing to the increase of floodwater bacterial diversity.

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