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Microbial Communities of Continuously Cropped, Irrigated Rice Fields

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In continuously cropped, irrigated rice fields, soil microbial biomass as measured by total phospholipid fatty acid concentrations declined during the second half of the crop cycle. This decline was also observed in other components of the microbial community assessed by viable counts, including denitrifiers and sporeformers. Simultaneous with total biomass decline was the increase in potential indicators of nutrient stress—such as ratios of cyclopropanol (Σ[cy/ω7c]) and *trans* (Σ[ω7t/ω7c]) phospholipid fatty acids—in plain crop soil but not in the rhizosphere. Polyhydroxyalkanoate levels were enhanced in the root environment of mature rice. Polyunsaturated eukaryotic biomarkers accounted for only 13 to 16 mol% of the total phospholipids, including 2 mol% of 18:2ω6, which is considered a fungal biomarker. Single biomarkers for defined physiological groups of bacteria did not follow the declining trend of total microbial biomass. Signature compounds for gram-positive and gram-negative fermenters (plasmalogen phospholipids), methanogenic bacteria (diether lipids), and methanotrophs (18:1ω8c) increased as the crop approached maturity. Methanotrophs were not particularly enriched in the rhizosphere. Methanogenic biomarkers were, however, most abundant in root extracts from mature rice plants. Assuming that soil microbial biomass plays a significant role as a passive nutrient pool, its reduction during the second half of the cropping season suggests a mechanism that may ultimately contribute to declining productivity in irrigated, continuous rice cropping systems.

Microbial biomass serves the nutrient supply of rice crops in two ways: as a mixture of microbial catalysts governing nutrient availability and as a passive source of nutrients. Productivity of irrigated rice crops is declining over years of continuous, intensive cultivation and appears to be associated with a decreasing N supply capacity of the soil (5, 6). It has further been hypothesized that soil microbial biomass is turned over rapidly enough to be able to constitute a major source of nitrogen for rice crops (18, 19). Hence, rice soil microbial biomass may be considered as key to the better understanding of a decreasing nutrient supply capacity.

As intensively farmed agroecosystems that stay flooded for most of the crop season, irrigated rice soils are unique in microbial ecology. Yet a satisfactory inventory of the microbial biomass in rice fields is still lacking. The effects of submergence, crop stage, and root environment on the rice soil microbial community with its impact on nutrient cycling have hardly been studied.

This investigation is a first attempt to answer the question whether continuous intensive cultivation of rice in the tropics can reduce or alter the soil microbial biomass with the possible result of a decreased nutrient-supplying capacity. An experimental field site at the International Rice Research Institute (IRRI; Los Baños, Philippines) with a particular history of continuous intensive cropping (9) was chosen to detect any cropping stage-dependent shifts of the microbial biomass and certain population segments. Analyses of phospholipid biomarkers (11, 13, 48) were carried out at cardinal stages of rice growth (52) and supplemented by a number of selective viable counts.

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MATERIALS AND METHODS

Sampling. Samples of soil were taken at different stages of crop growth (52) from a continuous rice cropping experimental field with *Oryza sativa* L. var. IR72 at the International Rice Research Institute (IRRI) during the dry season of 1995. Additional samples beyond the flowering stage to study rhizosphere effects at physiological maturity were collected from the preceding (wet season) crop of 1994. The investigated rice crops were grown without fertilizer N. Four replicate samples represented four blocks each with an area of 8 by 8 m which received identical treatment. Each of the replicate samples was made up of 12 randomly collected, pooled, and thoroughly mixed core samples (15-cm length and 2.7-cm diameter).

To obtain soil samples from the rhizosphere, the roots of freshly harvested rice plants were aseptically agitated to strip off loosely adhering soil and then immersed in a 250-ml measuring cylinder containing 100 ml of sterile distilled water. After the roots were dragged through the fluid for 2 min, the increase in volume was recorded. The bulk suspension of rhizosphere soil was lyophilized for lipid extraction and dry weight determination, while 10-ml portions were processed immediately for viable count determinations.

To obtain rhizoplane samples for lipid extraction, 10-g (wet weight) aliquots of rhizosphere-stripped roots were reimmersed in dilution bottles containing 100 ml of ice-cold distilled water and sonicated three times for 5 s with a Branson 450 sonifier with a microtip at 50 W. The sonicated roots were lyophilized for lipid analysis.

Viable counts. Serial dilutions obtained by dispersing 10 cm^3 of an aliquot of sample in 100-ml dilution flasks with 90 ml of 10 mM Tris-HCl buffer, pH 7.0, and 10 mg of Tween 80 per liter were put on a reciprocal shaker for 10 min, before agar plates were inoculated with 0.1-ml aliquots by the spread plate method with presterilized glass spatulas.

Most probable numbers (MPN) were based on five replicates and obtained from growth in serial dilutions of the respective culture media with 10-ml tubes with 9.0 ml of the medium and 1.0 ml of the inoculum. Plates, with the exception of gelatin plates (18°C), and MPN tubes were incubated at 30°C. Total heterotrophic bacteria were enumerated after 2 weeks of incubation under both aerobic and anaerobic (N_2 atmosphere) conditions on a casein-peptone–starch–glycerol agar (8).

To detect sporeformers, 10-ml tubes containing 5 ml of a sample suspension were pasteurized by immersion (25 min) in a water bath at 80°C, before 0.1-ml aliquots were spread on casein-peptone–starch–glycerol agar and incubated for 2 weeks under both aerobic and anaerobic (N_2 atmosphere) conditions.

To detect protein liquefiers, a gelatin-agar medium (36) was used.

The MPN of denitrifying bacteria were determined after 1 to 2 weeks of growth in nutrient broth (8.0 g/liter) containing 0.5 of KNO_3 per liter (44). Gas formation (Durham tubes) and nitrate disappearance scored positive.

Chemoautotrophic ammonium oxidizers were detected with an inorganic MPN medium that contained 0.5 g of $(NH_4)_2 SO_4$ per liter, 1.0 g of $CaCO_3$ per liter, 0.25 g of K_2HPO_4 per liter, 0.13 g of NaCl per liter, 2.5 mg of $FeSO_4$ per liter, and 2.5 mg of $MnSO_4$, pH 7.2, per liter. After 3 weeks of incubation, spot tests were run for nitrite and nitrate (42).

Phospholipids. Phospholipid extraction for capillary gas chromatography followed established procedures (4, 11, 51). Up to 60 g of a fresh sample was frozen below −60°C and lyophilized, and subsamples (10 ± 5 g) were extracted by a chloroform-methanol two-phase extraction technique (3). Phospholipids in dried chloroform extracts were separated on a Unisil silica gel column (100/200 mesh).

The fatty acids of the phospholipid fraction were methylated by mild alkaline methanolysis and purified (11).

Gas chromatographic identification and quantification of the fatty acid methyl esters were carried out with a Varian 3700 capillary gas chromatograph with flame ionization detector (4). Final peak confirmation was accomplished with a Hewlett-Packard 5996A gas chromatography mass spectrometry combination with a RTE-6/VM data system (30, 31). Molar phospholipid fatty acid (PLFA) data were based on the assumption of equimolar responses with a C19 standard.

Diether lipid biomarkers of methanogenic bacteria were detected by using high-pressure liquid chromatography of diethers and tetraethers after acid hydrolysis with chloroform-methanol-HCl (29, 31).

Plasmalogen phospholipids were measured as fatty acid dimethyl acetals (15, 35).

Poly-β-hydroxyalkanoates (including poly-β-hydroxybutyric acid) were determined by gas-liquid chromatography as ethyl esters originating from the polyhydroxy alkanoates (PHA) in the glycolipid (13).

Statistical treatment of the data included Student's *t* test, analysis of variance, and MPN specific tests according to the work of Cochran (7), using software of the SAS Institute, Inc. (41).

RESULTS AND DISCUSSION

In the long-term continuous cropping system investigated, crop residues were plowed under 2 months before the rice was transplanted and the field was flooded. The plots were fertilized before being transplanted with 60 kg each of P_2O_5 and K_2O per ha but received no nitrogen fertilizer. Hence, in addition to oxygen deficiency in the flooded, waterlogged soil, the main environmental variables likely to govern the microbial community during a cropping cycle corresponded with the indigenous energy and nutrient resources of the soil-floodwater system. Furthermore, with advancing crop growth, the plant roots were expected to contribute increasing amounts to the pool of potential organic substrates.

Microbial community changes up to the flowering stage. Since root growth of rice reaches its peak near the flowering stage (43), this was considered a key point of time for crop-induced changes in the soil microbial community. Variation of total PLFA concentrations at three preceding growth stages (viz., 13 days after transplanting, maximum tillering, and panicle initiation) was extremely low. Compared with the pooled data of PLFA before flowering, concentrations of these biomarkers of total microbial biomass in bulk soil had decreased to half of that level at the flowering stage (significant at $P = 0.10$, *t* test) (Table 1, column 1).

As PLFA analyses have only recently been applied in soil microbiology (2, 34, 47), there are no comparable investigations of PLFA in rice soils. A widely used chloroform fumigation incubation method to detect soil microbial biomass seems to show no correlation with and to be inferior to phospholipid analyses (35). On the other hand, data from recent greenhouse experiments reveal a similar decline of microscopic total bacterial counts during the second part of the growth cycle, whereas viable counts of different bacterial groups follow an opposite trend (38). Although using again different conditions and methods, Goshal and Singh (16) also found sharp decreases in soil microbial biomass during the period from seedling stage to flowering stage of rice.

The declining trend of microbial biomass was not reflected by the group of aerobic and anaerobic copiotrophic heterotrophs, but rather by subgroups of these heterotrophs such as spore-forming and denitrifying bacteria. Densities of these

TABLE 1. Phospholipid signature compounds in flooded rice soil before and at flowering stage of dry season crop in 1995[a]

Stage and sample	Total PLFA (nmol/cm³)	Mol% of compounds indicating:											
		Eukaryotes				Anaerobic prokaryotes					Methane oxidizers		
											Group I		Group II
		Total poly-unsaturated	18:2ω6	20:4ω6	20:5ω3	Diether lipids	10 me 16:0	cy 17:0	i17:1ω7c	Plasmalogens	16:1ω7t	16:1ω5c	18:1ω8c	18:1ω7t
Before flowering (bulk soil)[b]	9.56 (0.32)	14.30 (1.10)	1.80 (0.36)	1.83 (0.15)	1.63 (0.12)	5.30 (0.50)	7.07 (0.15)	2.97 (0.15)	1.30 (0.00)	1.97 (0.48)	0.53 (0.05)	1.83 (0.15)	2.17 (0.06)	0.90 (0.10)
Flowering														
Bulk soil	5.27 (0.43)	13.10 (1.70)	1.53 (0.45)	1.03 (0.12)	0.50 (0.10)	11.70 (2.60)	7.27 (0.45)	2.70 (0.20)	1.07 (0.06)	3.54 (0.88)	0.47 (0.06)	1.77 (0.25)	3.87 (0.32)	1.33 (0.12)
Rhizosphere	6.29 (2.11)	15.40 (1.10)	2.03 (0.21)	1.67 (0.31)	0.90 (0.17)	7.30 (0.90)	5.70 (0.26)	2.60 (0.00)	1.27 (0.06)	3.91 (0.18)	0.47 (0.06)	1.77 (0.32)	2.93 (0.29)	1.13 (0.12)

[a] Parentheses indicate standard deviation ($n = 3$).
[b] Pooled data from sampling at 13 days after transplanting, maximum tillering, and panicle initiation.

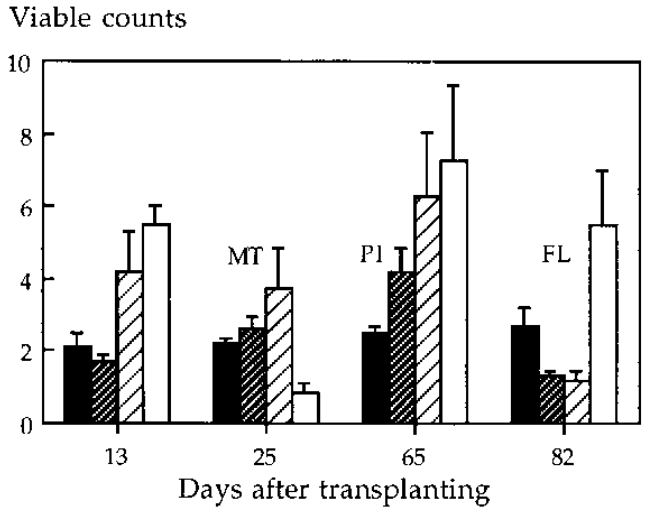

FIG. 1. Viable counts of total copiotrophic heterotrophs, spore-forming bacteria, denitrifiers, and ammonium oxidizers in dry season irrigated rice soil. Samples are from unfertilized intensively cultivated crop soil at different cropping stages: maximum tillering (MT), panicle initiation (PI), and flowering (FL). Standard errors of the mean are indicated ($n = 4$). Bars: ■, copiotrophic heterotrophs (10^7); ▨, spore-forming bacteria (10^6); ▨, denitrifiers (10^4); □, ammonium oxidizers (10^3).

populations reached their minimum at the flowering stage (Fig. 1). No phospholipid biomarkers are known to match the heterogeneous group of denitrifying bacteria (51) as well as other prokaryotes with major roles in soil nitrogen cycling.

Given the high proportion of dormant or unculturable microorganisms to be expected in soils and sediments (33, 45), a close correlation between viable counts and phospholipid biomarkers in rice soils is not very likely. Since densities of total heterotrophic copiotrophs remained unchanged during the total PLFA biomass decline (Fig. 1 and Table 1, column 1), it can be inferred that biomass losses occurred at the expense of the less rapidly growing populations.

Indicators of community status. Increasing proportions of cyclopropyl fatty acids and of *trans* isomers (ratios of cy/ω7c and ω7t/ω7c fatty acids) are known as potential indicators of environmental stress such as starvation (11, 14). Bulk soil at flowering showed a significantly higher Σcy/ω7c ratio ($P = 0.05$, t test) than both the corresponding rhizosphere and bulk soil before flowering (Table 2). This suggests a coincidence of the observed total biomass decline with increasing nutrient stress on the microbial community, after the rice plants were entering their reproductive crop stage. Lower cyclopropyl- and *trans*-fatty acid ratios in the rhizosphere of the flowering crop than in its bulk soil indicate a reduction of environmental stress in the vicinity of the roots.

Infrastructural changes as indicated by single signature compounds. Only a limited number of functional groups within a microbial community can be detected by PLFA or diether lipid biomarkers (11, 14, 28, 31, 50). However, important segments of the prokaryotic community with a major role in nutrient cycling in oxygen-deficient environments possess rather specific signature compounds for physiologically defined groups, sulfate reducers and methane cycle bacteria being the main examples. For analyzing the community structure of largely anoxic flooded rice soils, this was deemed an advantage. Single biomarkers listed in Table 1, columns 6 to 10, indicate that certain, mainly anaerobic, populations maintained or even increased their abundance levels as the submerged crop approached maturity.

Polyunsaturated fatty acid biomarkers of eukaryotic microorganisms accounted for 13 to 16 mol% of the total phospholipids (Table 1, columns 2 to 5). Among these, a statistically insignificant peak of 18:2:ω6 suggests that fungal biomarkers (34) amounted to not more than 2 mol% (rhizosphere at flowering). Concentrations of the 18:2ω6 fatty acid biomarker can be reduced as a result of physical disturbance (34). Therefore, the observed low level could be explained by pertinent practices of irrigated rice farming such as pudding, which causes an intensive disruption of the soil texture. In contrast to the irrigated rice system investigated, rain-fed rice fields harbor a substantially greater amount of fungal biomass, although their microbial biomass shows a similar tendency to decline as the crop approaches maturity (16).

Plasmalogen phospholipids as fingerprints of fermentative anaerobes such as *Clostridium* spp. (51) had obviously responded to the oxygen-deficient conditions by doubling their relative contribution to the total PLFA pool in both plain crop soil and rhizosphere (Table 1, columns 6 to 10).

In a similar way, diether lipid concentrations suggest a significant ($P = 0.05$, t test) doubling of methanogenic populations before the flowering stage was reached in the bulk soil (Table 1, columns 6 to 10). This contrasts with results obtained with conventional selective enrichment techniques: in Japanese rice fields, population densities of methanogenic bacteria remained almost unchanged with soil depth, submergence, or fertilizer inputs (1). On the other hand, cultivation-based techniques will scarcely lead to representative accounts of the total methanogenic populations. Only recently, a novel group of methanogenic, acidoclastic bacteria was first detected as close associates of *japonica* rice roots by using 16S ribosomal DNA as molecular marker genes (26). No comparable trend was noted for methanogenic biomarkers in the rhizosphere of the investigated field crop.

Signature compounds of sulfate-reducing bacteria (10Me16:O, cy17:O, i17:1ω7c) did not reveal any significant changes over time, nor were they associated with the roots (Table 1, columns 6 to 10). Non-spore-forming incomplete oxidizers are known to predominate among the sulfate-reducing bacteria in tropical rice soils (37).

We found no indication of a competition between sulfate-reducing and methanogenic populations (23). Assuming that rice-crop-associated methanogens are mainly acetoclastic (26), there remains no reason why these two groups of obligate anaerobes should not coexist. Furthermore, acetate is one of the major organic acids in rice root exudates (27).

As indicated by significant increases ($P = 0.05$, t test) of 18:1ω8c fatty acid in bulk soil of the flowering crop, group II methanotrophic bacteria (17) followed the same trend as the meth-

TABLE 2. Ratios of PLFA groups indicating stress in flooded rice soil before and at flowering stage of dry season crop in 1995[a]

Stage and sample	Ratio	
	Σcy/ω7c	Σω7t/ω7c
Before flowering (bulk soil)[b]	1.15 (0.01)	0.19 (0.01)
Flowering		
Bulk soil	1.66 (0.16)	0.28 (0.02)
Rhizosphere	1.21 (0.10)	0.21 (0.01)

[a] Parentheses indicate standard deviation ($n = 3$).
[b] Pooled data from sampling at 13 days after transplanting, maximum tillering, and panicle initiation.

anogens (Table 1, columns 11 to 14). Although oxygen excretion from rice roots is likely to turn its rhizosphere into a niche for microaerophiles (39, 46, 49), group II methanotrophs failed to enrich in this microenvironment. Group II methanotrophs are known as major methane consumers in the rhizosphere of certain aquatic grasses (24), and it seems that many methanotrophs survive better under anaerobic than under aerobic conditions (40). Given the opportunity of a close metabolic coupling, even some kind of symbiotic relationship between methanotrophs and methanogens cannot be ruled out.

Effects of the root environment. Concentrations of total phospholipids were slightly (though not significantly at $P = 0.1$) higher in the rhizosphere than in bulk soil at the flowering stage (Table 1, column 1). Compared with microorganisms in bulk soil that are not reached by organic matter released from the roots, root-associated microflora is likely to benefit from more favorable growth conditions. Reduced levels of stress indicators (Σcy/ω7c [11]) in the rhizosphere at flowering stage would corroborate this assumption (Table 2).

A selective influence of the root environment on certain signature compounds was noted only in the case of diether lipids (significant at $P = 0.05$, analysis of variance [Table 1, columns 6 to 10]). Based on these biomarkers, methanogenic bacteria had become predominant members of the anaerobic microbial community at the flowering stage. Finally, high concentrations of diether lipids were associated with rhizoplane samples at the stage of physiological maturity (Table 3). This indicated that aging roots that had been stripped of their loosely attached rhizosphere microflora were favoring the enrichment of methanogens. By means of their autofluorescence (10), methanogens were also directly detectable in microscopic preparations of macerated root tissue (38).

Summary profiles of PLFA classes (functional groups of PLFA) have been considered helpful in assessing the contribution of large microbial taxa such as heterotrophic gram-negative prokaryotes (through monoenoic PLFA) and obligately anaerobic gram-negative or *Arthrobacter*-like organisms (through terminally branched saturated *iso*- or *anteiso*-configurated PLFA, respectively [25]). A comparison between bulk soil and rhizosphere of a mature crop, however, revealed no gnificant differences (Table 3). Simultaneously taken rhizoplane samples, on the other hand, showed strong increases in moles percent diether lipids, polyenoics, and normal saturates, most of the latter two classes originating most likely from plant material rather than from microorganisms (20).

The root environment of mature rice plants and their rhizoplane, in particular, contained also higher levels of PHA than bulk soil (Table 3). An elevated level of these indicators of unbalanced microbial growth (12, 32) suggests some kind of nutrient limitation of the root-adhering microflora. This contrasts with distribution patterns of PHA in the rhizosphere of terrestrial plants (*Brassica napus* [47]) but could possibly be explained by anatomical and physiological changes. These occur in aging roots of mature aquatic plants (22, 39, 52) and could make structural biopolymers more accessible to heterotrophic root-associated microorganisms.

Also, potential indicators of environmental stress such as the proportion of cyclopropyl and *trans* fatty acids (as expressed by the ratios of Σcy/ω7 and Σω7t/ω7c [11, 21]) were extremely low in the rhizoplane of mature rice plants (Table 3). This microhabitat can thus be characterized as an enrichment site with unbalanced microbial growth.

It has yet to be investigated whether the observed crop-stage-dependent decline of total microbial biomass implies also a loss of functional biodiversity, and to what extent the microbial community is able to recover before the next crop-

TABLE 3. Microbial community structure and status according to groups and ratios of PLFA signature compounds in different compartments of irrigated rice soil at physiological maturity (wet season crop in 1994)[a]

Compartment	Total PLFA[b]	Mol% of PLFA for group:							Ratio		
		Polyenoics	Branched monoenoics	Mid-chain branched saturates	Terminally branched saturates	Normal saturates	Monoenoics	Diether lipids[c]	PHA/PLFA (pmol)	Σcy/ω7c	Σω7t/ω7c
Bulk soil	8.09 (3.91)	4.38 (1.03)	3.38 (0.19)	15.68 (1.70)	15.94 (2.37)	25.44 (0.60)	35.24 (1.86)	9.06 (0.88)	0.07 (0.04)	1.25 (0.23)	0.16 (0.01)
Rhizosphere	12.02 (2.49)	3.72 (0.89)	3.36 (0.17)	14.98 (0.60)	20.92 (0.88)	24.48 (0.30)	32.62 (0.90)	12.28 (2.15)	0.08 (0.05)	1.14 (0.06)	0.18 (0.01)
Rhizoplane	419.29 (7.29)	40.60 (1.00)	0.20 (0.00)	0.30 (0.00)	2.40 (0.00)	42.30 (0.42)	14.30 (0.28)	43.55 (5.61)	0.25 (0.06)	0.29 (0.01)	0.06 (0.00)

[a] Parentheses indicate standard deviation ($n = 5$).
[b] For bulk soil and rhizosphere, in nanomoles per cubic centimeter; for rhizoplane, in nanomoles per gram.
[c] Values are 0.01 moles percent.

ping season. In tropical irrigated soils, time intervals between rice cropping seasons can vary from more than half a year to less than 1 month. The latter applies also to the site of the continuous cropping field experiment in our investigation.

Inubushi and Watanabe (18) have suggested that microbial biomass in rice soils is turned over rapidly enough to serve directly as an effective, major source of nitrogen. Apparently, population dynamic shifts in the microbial community during a cropping season favor the obligate anaerobes at the expense of aerobes in the bulk soil. Periods of aerobic recovery may be too short under the conditions of continuous rice monocultures. Therefore, quantitative and qualitative changes of the microbial communities during one crop cycle may already be considered as a potential mechanistic element of long-term productivity decline in intensive irrigated rice cropping systems.

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