

Characterization of Microbial Consortia in Paddy Rice Soil by Phospholipid Analysis

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ABSTRACT

Microbial biomass and community structure in paddy rice soil during the vegetation period of rice were estimated by analysis of their phospholipid fatty acids (PLFA), hydroxy fatty acids of lipopolysaccharides (LPS-HYFA), and phospholipid ether lipids (PLEL) directly extracted from the soil. A clear change in the composition of the community structure at different sampling periods was observed, indicated by the principal component analysis of the PLFA. A dramatic decline of ester-linked PLFA was observed in the soil samples taken at the second sampling time. In contrast to the ester-linked PLFA, the non-ester-linked PLFA composition did not change. The hydroxy fatty acids of lipopolysaccharides as well as ether lipids decreased consecutively during the observation period. Total microbial abundance was estimated to be $(4.1\text{--}7.3) \times 10^9$ cells g⁻¹ soil (dry weight). About 44% account for aerobic and 32% for facultative anaerobic bacteria, and 24% for archaea, on average. According to the profile and patterns of PLFA in the soil sample, it may be suggested that the paddy soil at the August sampling period contained more abundant facultative anaerobic bacteria (ca. 36%) and archaea (ca. 37%), but the total microbial biomass was significantly lower than in the remaining sampling periods. As the plant approached maturity, the microbial community structure in the soil changed to contain more abundant Gram-negative bacteria and methanotrophs.

Introduction

Rice paddy soils are anoxic during the period of plant development and are characterized by an oxic environment during the period between the harvest of the mature crop

and the planting of seedlings. These soils ecosystems are complex because of their gradients of oxygen and alternative electron acceptors in both oxic and waterlogged conditions [9]. The complexity of this ecosystem requires the application of phospholipid ether-linked lipids (PLEL) as indicators for methanogens additionally to the phospholipid fatty acids (PLFA) [5], which is generally accepted as an indicator for microbial community (excepted Archaea) and microbial biomass.

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Various trends were described in the literature concerning the microbial biomass during rice growth. Bossio and Scow [6] reported a large set of data varying from no change up to slight increase of the concentration of PLFA during rice vegetation period. Reichardt et al. [23] observed a significant decline of soil microbial biomass expressed as total phospholipid fatty acids during the second half of the crop cycle. They found also at comparable growth stage that the total microbial biomass decreased to the half of that level at flowering stage. Goshal and Singh [10] observed also that the levels of microbial biomass decreased sharply from the seedling to the flowering stage and then increased slightly with crop maturity.

The advantages of biochemical procedures over classical enumeration procedures have been reviewed [26]. These methods are based upon the determination of membrane phospholipids in microbial cells [2, 4, 29, 31, 35]. The chemical character of the linkages of the side chains with the glycerol backbone in the phospholipid molecule can give important information about the taxonomical grouping of microorganisms. Ester linkages predominate in aerobic organisms, and amino and ether linkages in anaerobic organisms (monoalkyl), while diether and tetraether compounds were mainly found in domain Archaea, including the methanogens [12]. Analyses of fatty acids and ether lipids derived from phospholipids of microorganisms in soil columns enriched with natural gas [18] and in wetlands [5] provide a reproducible and quantitative measure of the biomass and community structure of microbial assemblages. This assay can serve as a complement to molecular approaches of members of all three major domains of life: Bacteria, Eukarya (especially fungi and plants), and Archaea.

In the present study we analyzed the phospholipid fatty acid and ether lipid patterns, as well as the composition of hydroxy fatty acids of lipopolysaccharides (LPS-HYFA) in soils derived from different time periods of crop growth. We addressed the following special questions: (1) Does the community structure differ significantly in samples taken from soils being in oxic or in waterlogged conditions? (2) Can a typical condition be correlated to a specific microbial community structure? (3) What is the adaptation strategy of microbial communities in oxic and in waterlogged soil?

Materials and Methods

Paddy soil and Vegetation

The field trial was conducted in a net-house of the National Agro-environment Protection Institute, Tianjin, China (39°05' N,

117°10' E). The test plots were in waterlogged condition only during the rice growth period for 3 successive years. The experiments were carried out in the third year. The soil used in this experiment was of alluvial origin with pH 7.4. The soil contained 0.098% N, 0.25% P₂O₅, 0.84% K₂O, and 1.25% organic carbon. The size of each plot was 3 m × 4 m. Each plot received approximately 60 kg of treated municipal sludge as manure for the soil. This kind of organic manuring is a very common agricultural practice for rice production in China. Seedlings of rice plants (21-day-old) were transplanted to the plots at spacing of 30 × 30 cm, with 3–4 seedlings per hill, on the 1st of June 1997, just one day after the plots were flooded by irrigation. The investigated rice crop were grown without artificial fertilizer. Diseases, insects, and weeds were controlled as required to avoid yield loss. The water level of the flooded plots was maintained at about 4 cm above soil surface until drainage on September 2, just after the third sampling. Samples of soils were taken at different stages of crop growth [36]: first sampling at transplanting of the seedlings (July 1); second sampling at the growth stage of panicle initiation (August 1); third sampling at the end phase of flowering (September 1); and the fourth sampling was carried out 14 days before harvest of the mature rice (October 1). In the first and the fourth samplings, oxic soil prevailed, whereas in the second and third samplings the soil was waterlogged and rather anoxic. Soil moisture contents in plots were on average 58%, 52.6%, and 49.8% of fresh soil in the first three samplings during the flooded period. About 1 month after the draining, moisture content was 23.2%.

Sampling

Three replicate sample represented three plots that received identical treatments. Each of the replicate samples was made up of eight randomly collected, pooled, and thoroughly mixed surface soil (0 to 15 cm) cores. An aliquot approximately equivalent to 200 g dry weight of soil slurry (105°C overnight) was transferred to a 2.5-L bottle containing 500 ml methanol, 250 ml chloroform, and 125 ml 0.1 M phosphate buffer with pH 7.4 (the water content of the used slurry was subtracted from the buffer).

Lipid Extraction of Soil Samples

The bottle was shaken by hand and sonicated for 5 min, then deposited for 6 h; 250 ml chloroform and 250 ml water were added, shaken evenly, and let stand overnight. After separation of the two phases, the lower phase was carefully removed and dried by passing a funnel containing 2 cm anhydrous Na₂SO₄. The chloroform solvent was evaporated nearly to dryness in a rotary evaporator with a water bath at approx. 35°C. The separation of total lipids into neutral, glyco-, and phospholipids by a silica-gel SPE column was described elsewhere [29, 32].

Determination of Phospholipid Fatty Acids and Ether Lipids

Aliquots of the phospholipid fraction were used for fatty acid and ether lipid analyses. The different steps of phospholipid fatty acid

analysis followed the procedures described elsewhere [29, 32]. Liberation of hydroxy fatty acids from the lipopolysaccharides (LPS-HYFA) was carried out by the extraction technique of Parker et al. [20]. For the determination of ether lipids, the procedure of Ohtsubo et al. [19] was applied. Their separation was carried out by high-performance liquid chromatography, following the procedures described by Bai and Zelles [3].

The following designations and classifications for selected fatty acids were used: ester-linked phospholipid fatty acids (EL-PLFA) composed of saturated fatty acids (SATFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and hydroxy substituted fatty acids (PLOH). The subunits of the non-ester-linked phospholipid fatty acids (NEL-PLFA) are the unsubstituted (UNSAFA) and hydroxy-substituted fatty acids (UNOH). The subdivisions of hydroxy-substituted fatty acids (PLOH, UNOH, and LPS-HYFA) are characterized according to the position of the hydroxy group in the fatty acid molecule. EL-SATFA were composed of the subunits straight-chain or "normal" and branched-chain fatty acids. The position of methyl branching in the molecule is either initial-branched, e.g., on the second or fourth C atom, or mid-chain branched and terminal-branched, represented by *iso* and *anteiso* fatty acids. The ether lipids were designated as archaeol (Ar), caldarchaeol (Ca), β -hydroxyarchaeol (β -OH-Ar), and caldarchaeol with cyclization rings (cyc-Ca).

Microbial Abundance Estimates

Conversion of PLFA and PLEL concentration data into number of cells per gram of soil can be performed using the following approximations [5, 18]: average bacteria the size of *Escherichia coli* contain 100 $\mu\text{mol PLFA g}^{-1}$ dry weight, whereas average archaea contain 2.5 $\mu\text{mol PLEL per gram dry weight}$; 1 g (dry weight) of bacteria/archaea is equivalent to 5.9×10^{12} cells. This conversion assumes that bacteria contain a constant portion of their biomass as phospholipids under natural conditions [25]; the approach has been verified by Balkwill et al. [4].

Statistical Analysis

The data were subjected to analyses of variance (ANOVA). Concentrations of all the individual phospholipid fatty acids ($\log_{10}$ mol%) were subjected to principal component analysis (PCA) to elucidate major variation patterns. The multivariate calculations were performed using the computer program SPSS for Windows.

Results

Biomass, Quantitative Determination of the main PLFA

We determined the amounts of individual phospholipid fatty acids in different groups based on their bindings within the lipid molecules. Figure 1A shows variation of the total PLFA concentrations and two major components ester-linked (EL-PLFA) and non-ester-linked (NEL-PLFA) at the

four sampling periods. Microbial biomass expressed as total PLFA concentration decreased sharply from the largest value in July to the lowest value in August and increased again with prolonged vegetation. The larger part of the PLFA consisted of EL-PLFA, and consequently, the distribution of these fatty acids showed clear similarities to those of total PLFA. The NEL-PLFA represented a smaller part of the PLFA and their content did not change significantly throughout the study period.

The total amounts of hydroxy-substituted fatty acids derived from lipopolysaccharides (LPS-HYFA) (Fig. 1B) as well as the total amounts of ether lipids (Fig. 1C) exhibited the highest concentration at the July sampling period and then decreased continuously with prolonged vegetation.

Community Structure

The principal component analysis was applied to the $\log_{10}$ mol% values for all single PLFA of paddy soils to classify samples into groups based on overall similarity (Fig. 2). The component weights were plotted for the first three variables, which accounted for 79%. This analysis indicated that there were clear differences in PLFA compositions at each sampling time. Differences were obtained between samples derived from soils of oxic conditions (July and October) and those of waterlogged conditions (August and September), and also between the early phase of vegetation (July and August) and the latter phase of crop growth (September and October). These results provide evidence for a shift in composition of the microbial community at different stages of the plant growth and the change of aeration status.

Shifts in Microbial Community

Phospholipid fatty acids. To find the micro-organisms that are responsible for the shift in the microbial community, the change in pattern percent distribution of various groups and subgroups of PLFA were considered. The main effect was that the EL-PLFA (except the PLOH subgroup) have a lower proportion in August samples than in the remaining sampling periods, while the distribution of NEL-PLFA was reversed (Table 1). This suggests that the proportion of anaerobic organisms in August samples were clearly larger than in the remaining samples. The percentage of NEL-PLFA increased and reached the value of the EL-PLFA in August samples (50%), while in the remaining samples they accounted for 26–34% of the total.

A second clear difference was found in the percent dis-

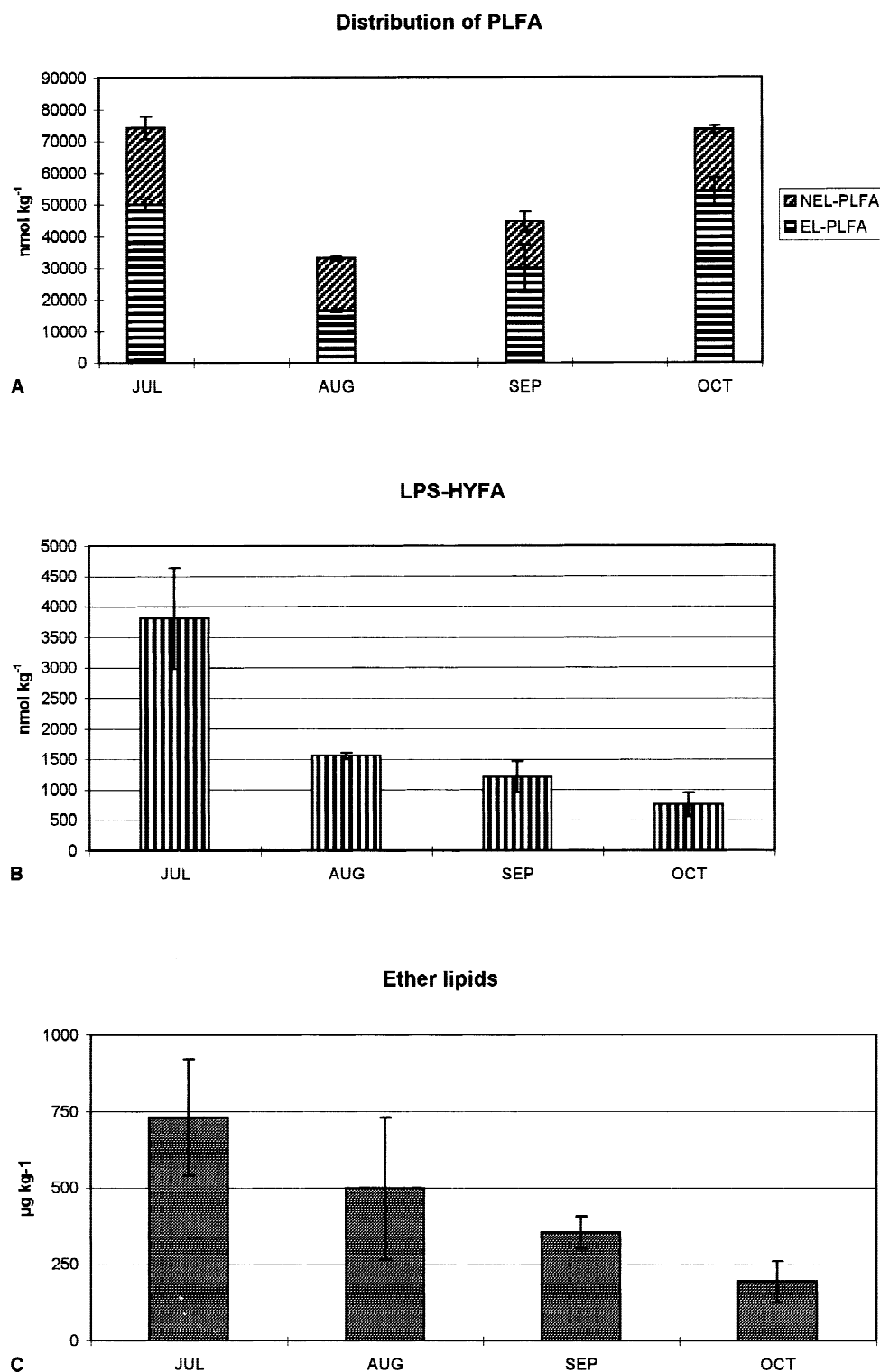


Fig. 1. The total amounts of different lipid components of measured soils. (A) The amounts of PLFA and their main groups: ester-linked PLFA (EL-PLFA) and non-ester-linked PLFA (NEL-PLFA); (B) the amounts of hydroxy-substituted fatty acids derived from lipopolysaccharides; (C) the total amounts of ether lipids in four sampling periods: July (JUL), August (AUG), September (SEP), and October (OCT).

tribution of branched-chain fatty acids in relation to the monounsaturated fatty acids in samples derived from flooded (July, August, and September) and unflooded soil (October). The ratios in the former samples were between 0.90 and 0.94, whereas in the latter sample it was 0.77. This

is a sensitive indicator between the proportion of Gram-positive and Gram-negative bacteria and suggests that the unflooded soil contains a remarkably higher proportion of Gram-negative bacteria than the flooded one.

Further, it should be taken into account that ratios of

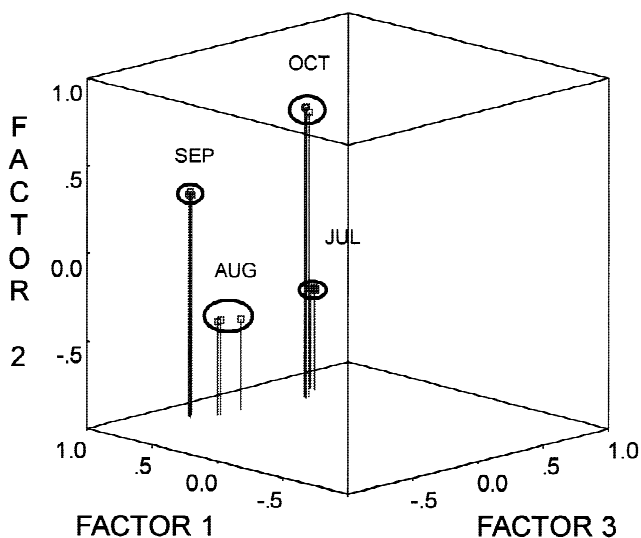


Fig. 2. Principal component analysis of all individual phospholipid fatty acid (PLFA), expressed as $\log_{10}$ mol% in paddy soils sampled in different periods.

EL-MUFA to EL-SATFA fractions from the four soil samples were calculated to be 0.69, 0.58, 0.69, and 0.88, respectively (Table 1), which clearly discriminated from the patterns of the PLFA in aerobic soils, which deliver values between 0.89 and 1.40 [28, 30]. Only three monounsaturated fatty acids (16:1 ω 5c and 16:1 ω 5t, and 16:1 ω 7) representing the group I methanotrophs [7] were found (data not shown). Their concentrations were ranging between 1 and 2% of the total MUFA. Except 16:1 ω 5c, all MUFA were obtained from samples when the soil was flooded. No true signature fatty acid of group II methanotrophs was found in any of the samples. Group II methanotrophs are known as major methane consumers in the rhizosphere of certain aquatic grasses [13].

The branched-chain fatty acids may be further subdivided into a few subgroups determined by the position or character of methyl branching in the molecules: for example, initial-branched (at the second or fourth C atom), mid-chain branched (i.e., 10 ME), or terminal-branched (iso, anteiso), or the cyclopropyl branching. Figure 3 shows the distribution of the concentrations for some of the important branched subgroup of SATFA from the four soils. The main characters for the soil were that the sample in August had trace amount of anteiso branched chain fatty acids, in contrast to the remaining periods of sampling, which contained higher amounts of these fatty acids.

Finally, clear differences were observed in the composition of branched-chain fatty acids in samples taken in July

and in September. The 10 ME branched fatty acids (characteristic for actinomycetes) were present in the July samples with $3.5 \pm 0.6\%$ and in the September samples only with $0.8 \pm 0.1\%$; the remaining mid-chain branched fatty acids showed reversed distribution, accounting for $2.6 \pm 0.3\%$ in July samples and $4.6 \pm 0.3\%$ in September samples, respectively.

Two of the main groups of LPS-HYFA, the beta and the omega hydroxy fatty acids, show a contradictory percent distribution (Table 1): the beta hydroxy fatty acids decrease continuously, while the omega hydroxy fatty acids increase and reach at October about twice the percent contribution of July.

Ether lipids. The presence of archaea can be estimated directly from the concentration of phospholipid-derived ether lipids (PLEL). Generally, the PLEL and their components decreased gradually during the vegetation period. Throughout this period archaeol (Ar) showed the highest and β -hydroxyarchaeol (β -OH-Ar) the lowest concentration of all identified ether lipids (Fig. 4). The archaeol reached the highest proportion in August and decreased continuously until October.

A change in the archaeol/caldarchaeol ratio from 1.9 in July, to 4.6 in August, to 2.5 in October may indicate a shift in the methanogenic community. A shift in the archaeal community can also be suggested on a higher taxonomic level: In August the percentage of cyclic caldarchaeol of the total PLEL concentration was 13% and increased to 32% in October. This might be indicative for an increase of the Crenarchaeota/Euryarchaeota ratio under more oxic conditions. The archaeol concentration was higher in August than in July samples, but decreased in the following periods. The caldarchaeol and cyclic caldarchaeol showed reversed course.

Microbial Abundance Estimates

PLFA and PLEL together represent the cell membrane components of all the microorganisms inhabiting paddy rice soil. Based upon conversion factors as mentioned above cell numbers were calculated (Table 2). The cell numbers of bacteria and eukarya are varying between 1.1 and 3.4×10^9 g⁻¹ dry matter when ester-linked fatty acids are considered and are in the range of 1.3 – 2.2×10^9 cells in the case of non-ester-linked fatty acids, and they are between 2.6 and 5.3×10^9 when total PLFA are considered. Archaeal cell numbers are ranging between 5.2×10^8 and 2.0×10^9 .

The proportion of archaeal cells based on PLEL concentrations of cell numbers calculated from total phospholipid

Table 1. Percentage distribution of groups and subgroups of phospholipid fatty acids and lipopolysaccharides (LPS-HYFA) in paddy soil samples

Divisions of PLFA	Sampling periods			
	July	August	September	October
PLFA total	100.0 ± 2.6	100.0 ± 1.2	100.0 ± 23.5	100.0 ± 6.7
EL-PLFA	67.5 ± 4.0	49.5 ± 1.3	66.2 ± 1.7	73.5 ± 1.4
SATFA	37.4 ± 2.0	26.5 ± 2.5	36.6 ± 1.2	36.9 ± 1.6
BRANC	23.9 ± 1.2	14.0 ± 2.0	23.5 ± 1.1	25.2 ± 0.9
MUFA	25.7 ± 2.7	15.1 ± 1.5	25.1 ± 1.4	32.5 ± 0.7
PUFA	2.1 ± 0.4	1.6 ± 0.2	2.0 ± 0.2	1.8 ± 0.3
PLOH	2.2 ± 0.7	5.9 ± 0.3	2.5 ± 0.5	2.2 ± 0.2
NEL-PLFA	32.5 ± 4.0	50.5 ± 1.3	33.8 ± 1.8	26.6 ± 1.4
UNSFA	15.9 ± 2.6	25.3 ± 1.5	17.8 ± 0.5	11.8 ± 1.0
UNOH	16.6 ± 2.7	25.2 ± 2.1	16.0 ± 1.2	14.8 ± 0.5
LPS-HYFA	100.0 ± 21.7	100.0 ± 3.2	100.0 ± 21.0	100.0 ± 25.3
Beta OH	57.8 ± 2.3	52.7 ± 17.9	51.8 ± 9.2	33.2 ± 9.9
Omega OH	5.7 ± 0.5	7.2 ± 0.6	8.6 ± 1.3	12.6 ± 1.0

concentration was in average 23.9%. The percent contribution of EL-PLFA was the highest in all sampling periods except in August (Table 2). The NEL-PLFA and also the PLEL in the August sampling were higher than that of EL-PLFA, indicating a dominance of anaerobic organisms.

Discussion

Measurement of microbial biomass using the phospholipid approach indicated a good correlation with measurements

by other methods, e.g., muramic acid levels [26], total adenosine triphosphate [16, 26] substrate induced respiration, fumigation extraction, and various enzymatic activities [33].

The EL-SATFA can be roughly divided into straight-chain and branched-chain fatty acids. The straight-chain fatty acids are widely distributed among the organisms, delivered just superficial information, and possess only low biomarker capacity, whereas the different types of branched-chain fatty acids are biomarkers for Gram-positive bacteria and anaerobic Gram-negative sulfate-reducing bacteria and in the gen-

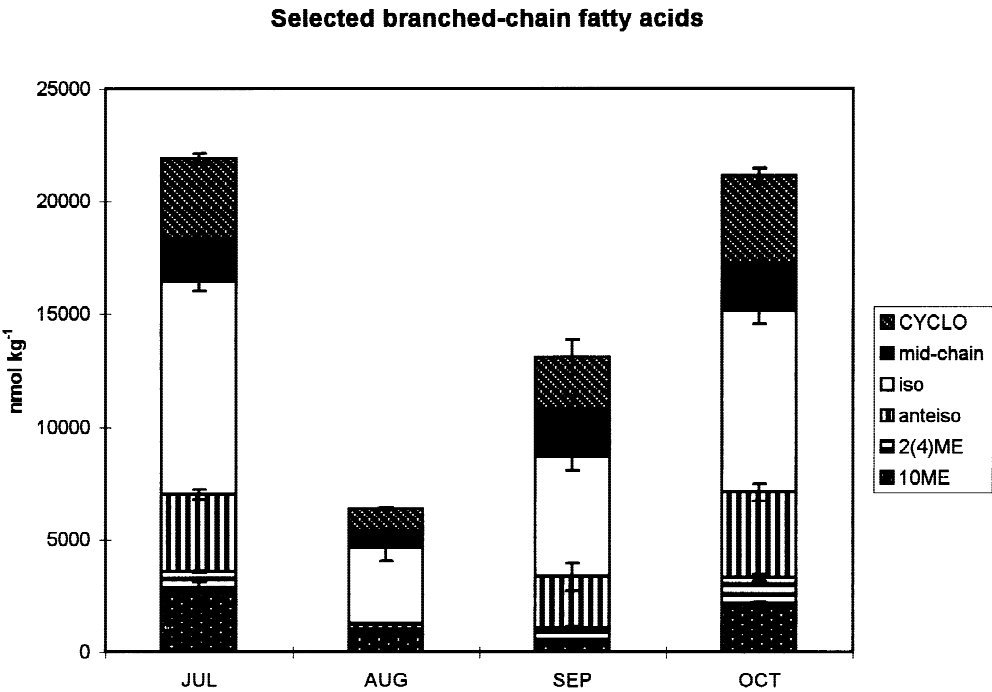


Fig. 3. Distribution of selected branched-chain phospholipid fatty acids: cyclopropyl ring in carbon chain (CYCLO), the position of the methyl branching not identified (mid-chain), methyl branching in iso (iso) or anteiso (anteiso) position, methyl branching on second or fourth position, methyl branching on 10th C-atom, (10ME).

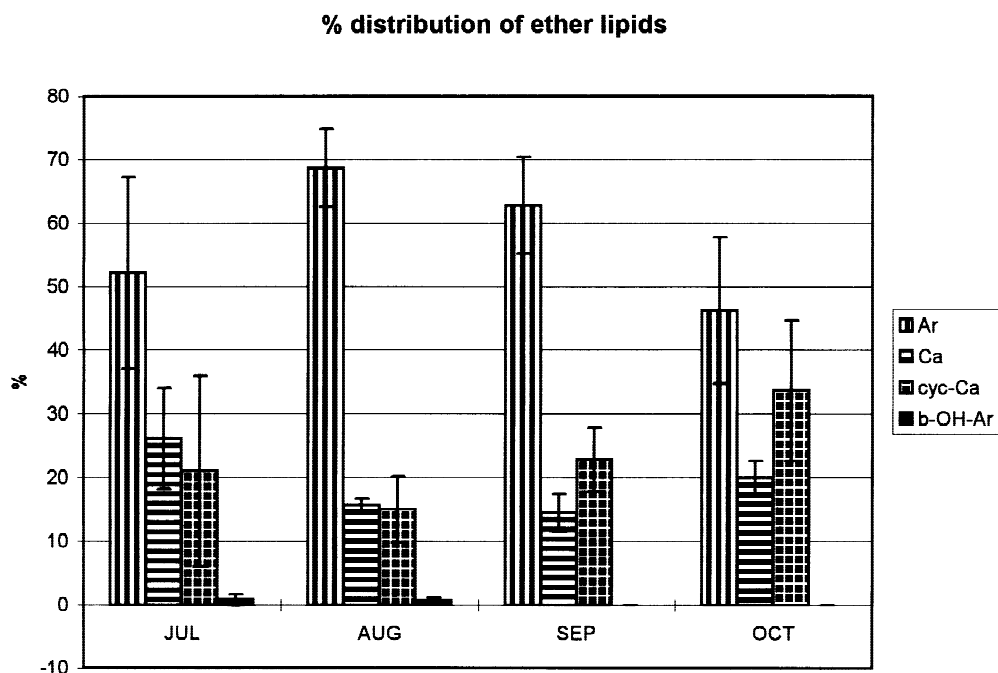


Fig. 4. The percent distribution of ether lipids and their subcomponents: Archaeol (Ar), caldarchaeol (Ca), cyclic caldarchaeol (cyc-Ca), β -hydroxyarchaeol (b-OH-Ar) in four sampling periods: July (JUL), August (AUG), September (SEP), and October (OCT).

era *Cytophaga* and *Flavobacterium*, whereas cyclopropyl fatty acids are common in some Gram-negative strains, as well as in some anaerobic strains of Gram-positive bacteria [11, 22]. Methyl branching on the 10th carbon atom (10 ME) in the molecule is specific for actinomycetes [15]. Although EL-MUFA can occur in both Gram-negative and Gram-positive bacteria, their relative contribution to total PLFA content in Gram-positive bacteria is typically very small (e.g., <20%). Thus, EL-MUFA can be roughly used as biomarkers for Gram-negative bacteria [22].

The LPS-HYFA are generally regarded as signature fatty acids for Gram-negative bacteria [24, 27]. However, this

statement should be taken with caution because it has been shown that, in addition to Gram-negative bacteria, fungi can also contain extremely large amounts of hydroxy fatty acids after saponification of whole cells [34]. Generally, the omega hydroxy fatty acid is regarded as an indicator of fungi [34].

Methanogens that belong to the order Euryarchaeota are characterized by archaeol (Ar), caldarchaeol (Ca), and β -hydroxyarchaeol (β -OH-Ar) [1, 14], whereas cyclic caldarchaeol (cyc-Ca) is significant for Crenarchaeota, which also include nonthermophilic Archaea [8]. Our results are in contrast to the findings of Asakawa et al. [1], who estimated β -hydroxyarchaeol at higher concentrations than caldar-

Table 2. Cell numbers (g^{-1} dry weight) of organisms calculated from the relevant phospholipid fractions, and their portion (%) in the community^a

	Bacteria and Eukarya			Archaea					
	EL-PLFA g^{-1} dw.	NEL-PLFA g^{-1} dw.	tot-PLFA g^{-1} dw.	NEL:tot (PLFA) %	PLEL g^{-1} dw.	PL (tot) g^{-1} dw.	EL-PLFA:PL(tot) %	NEL-PLFA:PL(tot) %	PLEL:PL(tot) %
July	3.15×10^9	2.16×10^9	5.31×10^9	40.7	2.00×10^9	7.31×10^9	43.0	29.6	27.4
August	1.11×10^9	1.48×10^9	2.59×10^9	57.1	1.54×10^9	4.14×10^9	26.9	35.8	37.3
September	1.86×10^9	1.30×10^9	3.16×10^9	41.1	1.05×10^9	4.21×10^9	44.2	30.9	24.9
October	3.36×10^9	1.79×10^9	5.15×10^9	34.7	5.17×10^8	5.67×10^9	59.3	31.6	9.1
Average	2.47×10^9	1.69×10^9	4.06×10^9	41.6	1.28×10^9	5.33×10^9	44.4	31.6	23.9

^a EL-PLFA, ester-linked phospholipid fatty acids; NEL-PLFA, non-ester-linked phospholipid fatty acids; tot PLFA, total phospholipid fatty acids; NEL:tot (PLFA), % portion of NEL-PLFA of the total PLFA; PLEL, ether-linked phospholipids; PL (tot), total phospholipids; EL-PLFA:PL(tot), % portion of EL-PLFA of the total phospholipids; NEL-PLFA:PL(tot), % portion of NEL-PLFA of the total phospholipids; PLEL:PL(tot), % portion of PLEL of the total phospholipids.

chaeol and supported the predominance of the family Methanosarcinaceae in paddy rice soils. We detected hydroxyarchaeol at very low concentrations and only on the first two sampling dates. The existence of Methanobacteriaceae in this study can be strongly suggested, as this family contains significant amounts of caldarchaeol [14]. Rajagopal et al. [21] isolated Methanobacterium-like and Methanosarcina-like strains from rice fields.

Boon et al. [5] estimated in wetland sediment the total prokaryotic abundance (i.e., bacteria plus archaea), which was between 7 and 17×10^9 cells g^{-1} sediment. In our measurement we found between 4.1 and 7.3×10^9 cells g^{-1} dry paddy soil. Methanogenic archaea were estimated by Boon et al. [5] to number $1\text{--}5.4 \times 10^9$ cells g^{-1} using the same conversion factors. In our study archaeal cell numbers were between 10^8 and 10^9 g^{-1} dry soil, in contrast to Asakawa et al. [1], who estimated methanogenic cell numbers by lipid analysis between 10^7 and 10^8 g^{-1} in dry paddy field soils. However, they used a different conversion factor. Asakawa et al. [1] compared the cell number estimation carried out by MPN and lipid analysis. The values obtained by lipid extraction were 10–100 times higher than the values estimated by the MPN method. The lipid analysis overestimates bacterial (methanogenic) cell numbers because the quantification by this method can include lipids from non-viable cells. On the other hand, the MPN method underestimates cell numbers owing to medium selectivity, cell aggregates, etc.

The sample taken in July contained more abundant Gram-positive bacteria, and their composition is also completely different from the soil taken at the second sampling time. Until September and later, the total microbial biomass in the soil was gradually recovering, and in the mean time, the microbial community structure in the paddy soil was switched to facultative and even rather aerobic microorganisms. For microeukarya, no evidence could be used to indicate any change occurring during the plant growth, although small fluctuation of the quantity of EL-PUFA was present.

In comparison to the agriculturally cultivated soils, which contain around 11% [33], the proportion of NEL-PLFA to total PLFA in paddy soil is considerably enlarged. This is also supported by the fact that the samples taken from drained soil (October) contained a lower proportion of NEL-PLFA than the samples derived from flooded soils. NEL-PLFA are mainly derived from sphingolipids, ornithine lipids, and aminolipids, all of which are generally present in phospholipid fractions [17].

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