Impact of Protists on the Activity and Structure of the Bacterial Community in a Rice Field Soil†

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Flooded rice fields have become a model system for the study of soil microbial ecology. In Italian rice fields, in particular, aspects from biogeochemistry to molecular ecology have been studied, but the impact of protistan grazing on the structure and function of the prokaryotic community has not been examined yet. We compared an untreated control soil with a γ-radiation-sterilized soil that had been reinoculated with a natural bacterial assemblage. In order to verify that the observed effects were due to protistan grazing and did not result from sterilization, we set up a third set of microcosms containing sterilized soil that had been reinoculated with natural assemblage bacteria plus protists. The spatial and temporal changes in the protistan and prokaryotic communities were examined by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analysis, respectively, both based on the small-subunit gene. Sequences retrieved from DGGE bands were preferentially affiliated with Cercozoa and other bacteriovorous flagellates. Without protists, the level of total DNA increased with incubation time, indicating that the level of the microbial biomass was elevated. Betaproteobacteria were preferentially preyed upon, while low-G+C-content gram-positive bacteria became more dominant under grazing pressure. The bacterial diversity detectable by T-RFLP analysis was greater in the presence of protists. The level of extractable NH4+ was lower and the level of extractable SO42− was higher without protists, indicating that nitrogen mineralization and SO42− reduction were stimulated by protists. Most of these effects were more obvious in the partially oxic surface layer (0 to 3 mm), but they could also be detected in the anoxic subsurface layer (10 to 13 mm). Our observations fit well into the overall framework developed for protistan grazing, but with some modifications pertinent to the wetland situation: O2 was a major control, and O2 availability may have limited directly and indirectly the development of protists. Although detectable in the lower anoxic layer, grazing effects were much more obvious in the partially oxic surface layer.

Wetland soils are characterized by unique biogeochemical cycles and by unique microbial communities compared to upland soils (16, 38, 49). The most important controls for microbial activity are organic matter input and the restricted availability of O2. Both of these factors depend on the dominant vegetation, which may supply the soil not only with organic matter but also to a significant but varying extent with O2 (28, 34). The same controls act on natural and man-made wetlands, and among the latter rice fields are by far the best-studied ecosystems (16, 37). Significant progress has been made in understanding the interaction between rice plants, soil biogeochemistry, and microbes (7, 8, 16, 37, 43), and thus rice fields are one of the best-studied model systems in soil microbial ecology. However, most work published so far has ignored the role of microbial mortality. From recent work on ciliates it became evident that grazing may have an effect on soil bacteria in rice fields (57, 58). However, ciliates were outnumbered by flagellates, which might have a much greater effect on microbes (58).

† Supplemental material for this article may be found at http://aem.asm.org/.

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microorganisms, and the inhibitors may be degraded during prolonged incubation (4). Therefore, we compared an untreated control soil with a γ-radiation-sterilized soil that had been reincoculated with a natural bacterial assemblage. In order to verify that the observed effects were due to protistan grazing and did not result from sterilization, we set up a third set of microcosms containing sterilized soil that had been reincoculated with bacteria plus protists. In order to determine the effect of oxygen gradients on both the microbial (51) and protistan communities, we analyzed two layers, a partially oxic upper layer (0 to 3 mm) and a totally anoxic lower layer (10 to 13 mm). Eukaryotic and bacterial communities were analyzed by molecular methods. Respiration, carbon and nitrogen mineralization, and porewater chemistry were used to study the gross effect of protists on biogeochemical functions.

**MATERIALS AND METHODS**

**Soil and field site.** Soil was taken from a rice field of the Istituto Sperimentale della Risicoltura (Vercelli, Italy) in spring 2000 before flooding. The field site and soil properties have described previously (33, 39). The soil was air dried and stored as dry lumps at room temperature. Prior to use, the soil was ground with a jaw crusher (Retsch, Hahn, Germany) to obtain particles that were <2 mm in diameter.

**Microcosms and inocula.** Soil was sterilized by γ irradiation (25 kGy; 60Co; Zentrale Strahlenschutzgruppe der Justus-Liebig-Universität, Giessen, Germany). When slurried and inoculated anaerobically for 2 weeks, γ-irradiated soil showed neither CO2 production nor CH4 production. Microcosms were prepared by reinoculating the sterilized soil with indigenous protistan and bacterial assemblages prepared as described below. Twenty grams of the sterilized soil was added to a serum bottle (inside diameter, 3.4 cm; volume, 60 cm3), forming a soil layer 15 mm deep. The soil was inoculated with protists and bacteria (treatment P + B) or with only bacteria (treatment B) while the headspace was flushed with N2 to minimize the detrimental effect of O2 on the anaerobic microorganisms. Aerobic microorganisms were assumed to tolerate the anoxia during preparation of inocula and microcosms, which was completed in ≤1 h. For comparison, unsterilized soil was inoculated with a filter-sterilized inoculum and served as a control.

The inoculum (8 ml) waterlogged the soil completely. However, the soil was not flooded to exclude protists that might have grown in the overlying water. The bottles were plugged with butyl rubber stoppers and aluminum caps. Bubbles entrapped in the soil were removed, and the soil surface was flattened by gently knocking the bottom of the bottle on a table. Altogether, 45 bottles were prepared. The headspace was flushed using hypodermic needles with N2 and filter-sterilized air for 5 min, and the bottles were incubated at 25°C in the dark. Flushing was done at the beginning and after 2, 6, 13, and 20 days. For preparation of protistan and inocula, 2 kg of soil was mixed with 1 liter of distilled water and incubated for 30 days at 25°C in the dark. Autoclaved O2-free water was used to extract microorganisms from the soil by using the following procedures. The top 15 mm of the incubated soil was mixed with water at ratios of 1:1 (wt/wt) (protists) and 1:2 (bacteria). The soil slurry for preparation of the protistan inoculum was shaken manually and allowed to settle for 15 min before the supernatant was collected (treatment S) (Table 1). The soil slurry used for preparation of the bacterial mixture was homogenized with a Waring blender for 1 min. In a pilot experiment this treatment gave the best recovery of bacteria (data not shown). The supernatant was obtained after centrifugation at 1,500 × g for 10 min (treatment C) (Table 1). The supernatants were subjected to the following successive filtration steps: (i) a 200-μm mesh sieve to exclude coarse soil particles, which most protists and detached bacteria could pass through; (ii) a 3-μm Nuclepore filter to exclude protists; and (iii) a 0.2-μm membrane filter to exclude protists and bacteria. Finally, three different inocula were prepared from the appropriate size fractions, as summarized in Table 1. The mixing ratio of the preparations obtained from treatments S and C was 1:1 (vol/vol). All steps were carried out with N2 flushing, and the inocula were stored at 4°C under N2, until use.

**Biogeochemistry.** Gas samples (50 μl) were taken before and 2 to 3 days after the headspace was flushed. CO2 was measured with a gas chromatograph equipped with a methanizer and a flame ionization detector after conversion to methane, and O2 was measured with a gas chromatograph equipped with a thermal conductivity detector. The rates of CO2 accumulation and O2 consumption were calculated from the changes in gas concentrations.

**TABLE 1. Preparation of inocula**

<table>
<thead>
<tr>
<th>Soil</th>
<th>Inoculum for treatment</th>
<th>Previous treatment</th>
<th>Fractions</th>
</tr>
</thead>
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<tr>
<td>γ-Irradiated</td>
<td>P + B</td>
<td>S</td>
<td>&lt;200 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;3 μm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.2 μm</td>
</tr>
<tr>
<td>γ-Irradiated</td>
<td>B</td>
<td>S</td>
<td></td>
</tr>
<tr>
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<tr>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

* For further details see the text.

**Soil Inoculum for preparation**

- γ-Irradiated P + B
- γ-Irradiated B
- Untreated Control

**Molecular analyses.** DNA was extracted from 0.3 g (wt/g) of soil with a Fast DNA SPIN kit (Bio 101, La Jolla, CA) according to the manufacturer’s instructions but with an additional washing step with guanidine isothiocyanate. DNA was eluted from the binding matrix with 100 μl of DNase-free water and stored at −20°C. DNA concentrations were determined fluorometrically using a PicoGreen double-stranded DNA (d(dNA) quantitation kit (Molecular Probes, Leiden, The Netherlands) in 96-well microtiter plates with a microplate reader.

The eukaryotic community was analyzed by denaturing gradient gel electrophoresis (DGGE) as described by Díez et al. (20). A fragment of the 18S rRNA gene (approximately 560 bp) was amplified from 2 μl of environmental DNA with primers Euk1A and Euk516r-GC (20). The reaction mixture (100 μl) contained 50 pmol of each primer, each deoxyribonucleoside triphosphate at a concentration of 200 μM, 400 ng μl−1 of bovine serum albumin (Roche Diagnostik, Mannheim, Germany), 1.5 mM MgCl2 (Promega, Madison, WI), 2.5 U Taq polymerase (Promega), and 0.1 volume of a 10% β-mercaptoethanol solution with respect to the soil surface. The O2 profiles were determined by ion chromatography (5).

**DGGE of the amplified 18S rRNA gene fragments was performed with a DCode system (Bio-Rad, California) by using 1-mm-thick 6% polyacrylamide gels and a 20 to 50% denaturant gradient (100% denaturant contained 7 M urea and 40% [vol/vol] formamide). Ten microliters of PCR products was loaded onto
the gels, and electrophoresis was carried out in 1× Tris-acetate-EDTA buffer at 100 V for 16 h at a constant temperature (60°C). The gels were stained with 1:10,000 (vol/vol) SYBR Green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min and scanned with a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

DGGE bands were excised from the DGGE gel and reamplified, the correct mobility on a DGGE gel was verified, and the bands were sequenced. Sequencing reactions were performed with an ABI PRISM BigDye terminator version 1.1 cycle sequencing kit (Applied Biosystems, California) used according to the manufacturer’s instruction, using the same primer set but without the GC clamp. The cycle sequencing products were analyzed with an ABI 377 DNA sequencer.

The bacterial community was analyzed by the terminal restriction fragment length polymorphism (T-RFLP) method. The 16S rRNA genes were amplified from 0.1 μl of the environmental DNA extract using primers 8-27F (21) and 907R (42). The forward primer was 5’ labeled with 6-carboxyfluorescein.

The reaction mixture (50 μl) contained 10 pmol of each primer, each dNTP, 1.5 mM MgCl₂ (Promega), 0.5 U of Taq polymerase (Promega), and 0.1 volume of a 10× PCR buffer provided with the enzyme. The PCR program included an initial denaturation step of 5 min at 95°C, followed by 30 cycles of denaturation (1 min, 95°C), primer annealing (1 min, 57°C), and primer extension (3 min, 72°C) and a final extension step of 7 min at 72°C. The PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The concentration of the purified products was determined photometrically.

Aliquots of the ampiclons (50 ng) were digested with 3 U of MspI (CCGG; Promega) for 2 h at 37°C in a 10× μl (total volume) reaction mixture containing 1 μl of the 10× incubation buffer (Promega) and 1 μl of bovine serum albumin. Fluorescently labeled terminal restriction fragments (T-RFs) were size separated with an ABI 373A automated sequencer (PE Applied Biosystems). T-RFLP electropherograms were analyzed by determining peak height (GeneScan 2.1 software; PE Applied Biosystems). The percent abundance (Ap) of a T-RF was calculated by comparison with the total fluorescence intensity of all T-RFs in the sample. Only T-RFs that were between 50 and 900 bp long and had an Ap of >1% in any sample were included in further calculations. The phylogenetic affiliation of the T-RFs was based on a library containing 190 clones of the eubacterial 16S rRNA and its gene obtained previously from the same soil (51).

Nucleotide sequence accession numbers. The 18S rRNA gene sequences of the DGGE bands have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB222279 to AB222351.

RESULTS

Biogeochemistry. As soon as 10 h after flooding, the porewater O₂ saturation in the control microcosms dropped to nearly zero at a depth of 1.5 mm (Fig. 1). In the reinoculated microcosms (treatments B and P+B), however, the O₂ saturation at the same depth was >40%, corresponding to >100 μM (Fig. 1A). During the first 2 days, the treatment B microcosms respired as much O₂ as the control, while the levels of respiration in the treatment P+B microcosms were significantly lower (Fig. 2A). After 7 days the oxic zone was not deeper than 0.9 mm, and the profiles were very similar for all microcosms, with the standard errors overlapping (Fig. 1B). O₂ uptake remained constant with time for treatment P+B but decreased for the other treatments (Fig. 2A). CO₂ emission was highest during the first 2 days and decreased later to about 50% (Fig. 2B). In addition to this common trend, the treatment B microcosms emitted less CO₂.

The level of exchangeable NH₄⁺ was low (0.6 to 1.1 μmol · g [dry weight] of soil⁻¹) at day 0, but it increased with time (Fig. 3). The NH₄⁺ levels in the upper and lower layers were not significantly different within the treatments. The levels of NH₄⁺ were not different for different treatments at day 0, but
at days 7, 14, and 21 soil from treatment B microcosms contained significantly less exchangeable NH$_4^+$ than soil from the control and treatment P/B microcosms contained (Fig. 3). Soil from the control microcosms contained 0.01 mol NO$_3^-$·g (dry weight) of soil at day 0 (detection limit), while soil from the treatment B and P/B microcosms contained 0.7 to 1.1 mol NO$_3^-$·g (dry weight) of soil (data not shown). From day 7 onward, the NO$_3^-$ contents in soil from the control microcosms and microcosms that received both treatments were <0.01 mol · g (dry weight) of soil$^{-1}$. The amount of extractable SO$_4^{2-}$ decreased with time in both the upper and lower layers (Fig. 4). The soil from treatment B microcosms contained significantly more SO$_4^{2-}$ than the soil from the microcosms that received the other treatments (14 and 21 days).

Molecular ecology. The initial amount of DNA ranged from 5.6 to 8.0 µg · g (dry weight) of soil$^{-1}$ (Fig. 5). In the lower soil layer, the value stayed nearly constant with time. However, the amount of DNA in the upper layer increased to 10 to 16 µg · g (dry weight) of soil$^{-1}$ in the microcosms with protists (treatment P/B and control) and to up to 30 µg · g (dry weight) of soil$^{-1}$ in the treatment B microcosms.

At day 0, the DGGE banding patterns of the 18S rRNA gene amplicons were very similar for all layers and treatments and were also similar to the patterns for the air-dried and sterilized soils (Fig. 6A). All lanes contained bands B, F, G, H, and P (Fig. 6A). No time- or treatment-specific pattern was observed for the other bands. In the upper layer, the number of DGGE bands remained low for treatment B, but the number increased with time for treatment P+B and the control (Fig. 6B). The following three groups of bands could be identified, although some were not observed for all three replicates: (i) bands that cooccurred in treatment P+B and the control (i.e., in all microcosms with protists), including bands D, F, H, J2, K, P, and Q; (ii) bands specific to treatment P+B, including bands E, I, J1, J3, M1, N, O, and S; and (iii) bands specific to the control, including bands A, B, C, G, L, M2, and R. The banding pattern for the lower layer was less diverse, but one band was observed for both treatment P+B and the control (band B on days 14 and 21).
and 21), and a few bands occurred in only the control (bands A, E, and H) (Fig. 6C).

Table 2 summarizes the tentative phylogenetic affiliations of the sequences retrieved from different DGGE bands. Of 40 different sequences, 13 could be affiliated with flagellates, 7 could be affiliated with fungi, 5 could be affiliated with metazoans (including microcrustaceans), 5 could be affiliated with green algae, 4 could be affiliated with higher plants, 3 could be affiliated with ciliates, and 2 could be affiliated with amoebae. Cercozoa was the dominant taxon among the flagellates (11 of 13 sequences). Sixteen of 22 sequences from the upper layer could be assigned to protists. In the lower layer, the nearest match for the sequence retrieved from band B (obtained from treatment P+B and the control) (Fig. 6C) was the match with an anaerobic Cercamonas sp. strain (ATCC 50367).

T-RFLP patterns were determined on day 21. In the upper layer, the most dominant T-RFs could be assigned to Dechloromonas (430 to 433 bp), Betaproteobacteria (489 bp), Bacillus (150 to 152 bp), Clostridium (270 to 271 and 515 to 518 bp), and Clostridium cluster I (507 to 510 bp). Betaproteobacteria, including Dechloromonas, had a higher Ap in treatment P+B microcosms than in treatment P+B and control microcosms, while bacilli and clostridia were more important in the presence of protists. T-RFs assigned to Chloroflexi (120 bp) and

FIG. 6. DGGE banding patterns of 18S rRNA gene partial sequences. (A) Comparison of all treatments and layers at day 0. (B and C) Comparison of days 7 to 21 for the upper layer (0 to 3 mm) (B) and the lower layer (10 to 13 mm) (C). Three replicate microcosms were analyzed per day and treatment. P+B, sterilized soil inoculated with protists and bacteria; B, sterilized soil inoculated with bacteria; Cont., control; S, γ-irradiation-sterilized soil; C, intact soil before sterilization. Bands with different mobilities are indicated by different designations. For the phylogenetic affiliation see Table 2. Note that the same designation may indicate different bands in the different gels. Only bands that appeared after day 0 are indicated in panels B and C.
Bacillus/Geobacter (128 bp) had also a higher Ap in treatment P+B microcosms but did not occur in all three replicates. Summarizing the patterns for the upper layer, treatment P+B and control microcosms showed greater complexity than treatment B microcosms. For details see the supplemental material.

In the lower layer, the dominant T-RFs were 270- and 510-bp T-RFs, followed by 515- and 145-bp T-RFs (assigned to Bacillus), and 152-bp T-RFs. The patterns for the lower layer changed between days 0 and 7, but they stayed quite constant later (see the supplemental material). The most obvious difference between treatments was the difference in the Ap of T-RF 270 (clostridia), which was the dominant T-RF in treatment P+B. A correspondence analysis of the T-RFLP patterns showed that there was clear separation of samples according to layer, treatment, and time of incubation (Fig. 7). As soon as after 10 h (day 0) the upper and lower layers were clearly separated. In the upper layer, the T-RFLP patterns had changed remarkably after 7 days, and all the treatments were well separated from each other. Treatment P+B was located halfway between treatment B and the control (see the supplemental material). In the lower layer, only minor temporal changes were

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### Table 2. Similarities of sequences obtained from the excised DGGE bands to sequences in the NCBI database

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<th>Band</th>
<th>Presence in treatment microcosms</th>
<th>No. of lanes sequenced</th>
<th>Closest relative</th>
<th>Accession no.</th>
<th>% Similarity</th>
<th>Phylogenetic group</th>
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<td></td>
<td>P+B</td>
<td>B</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>B</td>
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<td></td>
<td>3 Spongomonas minima</td>
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Upper layer

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<th>% Similarity</th>
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<td>93.9</td>
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Lower layer

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<th>Band</th>
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<th>No. of lanes sequenced</th>
<th>Closest relative</th>
<th>Accession no.</th>
<th>% Similarity</th>
<th>Phylogenetic group</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>E</td>
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<td></td>
<td>Lephestheria compleximanus</td>
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</table>

---

a See Figure 6 for the designations of DGGE bands.
b Number of lanes from which the same band was sequenced. Note that for the sake of clarity the band is labeled in Fig. 6 in only one lane.
c Accession number of the closest relative.
d A range is given if the sequences retrieved from different lanes were very similar but not identical.

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### legend

- **P**: Presence in the treated microcosms
- **B**: Presence in the control microcosms
- **Control**: Presence in the control microcosms
- **No. of lanes sequenced**: Number of lanes from which the same band was sequenced.
- **Closest relative**: Accession number of the closest relative.
- **% Similarity**: % Similarity of T-RF bands.
- **Phylogenetic group**: Phylogenetic group of the closest relative.
observed after day 7. We also compared the T-RFLP patterns of the archaeal 16S rRNA gene but found no difference among the treatments (primer Ar 109f and primer Ar 915r labeled with 6-carboxyfluorescein [13] (data not shown).

**DISCUSSION**

In spite of the intense biogeochemical, microbiological, and molecular work done on rice fields, the impact of protistan grazing on the structure and function of the prokaryotic community was largely unknown previously. Our results demonstrate effects on different levels, including biomass, population structure, and activities. Without protists, the biomass (total DNA) increased (Fig. 5), *Beataproteobacteria* instead of bacilli and clostridia dominated the bacterial community (see the supplemental material), the level of extractable NH$_4^+$ was lower, and the level of extractable SO$_4^{2-}$ was higher (Fig. 3 and 4). These effects of protists were most obvious in the partially oxic surface layer (0 to 3 mm) but could also be detected in the totally anoxic subsurface layer (10 to 13 mm).

**Experimental design.** Microcosms and microbial model systems in particular allow a degree of control, replication, and reproducibility that is unsurpassed in field experiments (35). Nevertheless, microcosms have been criticized as systems that are too simplistic for certain questions in community or ecosystem ecology (10). However, in a previous study we observed very similar dynamics of and controls on methane-oxidizing bacteria in microcosms and in the field (23). Even if methanotrophs are only a subset of the microbial community, the degree of agreement allowed us to suggest that the microcosms used here are a reliable model for the interaction between protists and bacteria at the soil surface.

Our conclusions depend on effective sterilization of the soil used for the microcosms manipulated. In a recent meta-analysis a γ-radiation dose of 25 kGy was found to be sufficient to kill nearly everything except some radioresistant bacteria (48). The latter are not to be expected to play any role in this rice field soil. Indeed, the sterilized soil produced neither CO$_2$ nor CH$_4$ when it was slurried and incubated anaerobically (data not shown). This indicates that the activities measured for treatments P + B and B were catalyzed by microorganisms from the inocula. Nevertheless, DNA extracted from the sterilized soil could be amplified with eukaryotic primers (Fig. 6, lanes S). The bands could be affiliated with green algae (Fig. 6A and Table 2). However, green algae are sensitive to γ-radiation (48). In addition, the microcosms were incubated in the dark, which prevented growth of phototrophic organisms. Hence, the sequences were thought not to represent live organisms. Because we prepared bacterial inocula by filtering them through a 3-μm filter, some small protists may have passed through. However, only the ubiquitous DGGE bands affiliated with algae were detected in treatment B (Fig. 6A and Table 2), indicating that there was successful exclusion of living protists.

The soil microcosms were not flooded but were water saturated. Using this technique, we could ascertain that the redox gradients typical for flooded soils developed without the growth of free-swimming fauna and flora in the overlying water that might have compromised the analysis of soil-specific interactions (57). Indeed, the oxygen profiles measured in the control microcosms were quite similar to those reported for previous experiments with flooded soil microcosms (44, 51). This suggests that redox conditions typically found in a flooded rice field soil were successfully established in the microcosms.

**Biogeochemistry.** After 10 h (day 0), the O$_2$ gradients in the control microcosms were much steeper than those in the treatments B and P + B microcosms (Fig. 1). Similarly, the NO$_3^-$ content in the control microcosms was below the detection limit (0.01 μmol · g [dry weight] soil$^{-1}$) on day 0, while the treatment B and P + B microcosms contained 0.7 to 1.1 μmol NO$_3^-$ · g (dry weight) soil$^{-1}$. This indicates that the sequential reduction of electron acceptors (45, 54) was retarded in the reinoculated microcosms. Until day 7 the differences became less pronounced, and at day 14 CO$_2$ emission and O$_2$ uptake were highest in treatment P + B microcosms (Fig. 1). This may have been a side effect of γ-radiation, which is reported to facilitate substrate availability in sterilized soils (2). The measured rates were consistent with the depth of penetration of O$_2$ (Fig. 1 and 2). Altogether, this indicates that protists have a stimulating effect on carbon and nitrogen mineralization in water-saturated soils, as found previously for upland soils (1, 14, 22, 27, 53).

The high SO$_4^{2-}$ concentration in treatment B microcosms (Fig. 4) suggests that protists may also stimulate SO$_4^{2-}$ reduction. To our knowledge, this is the first evidence for an effect of protists on the sulfur cycle. It is not clear, however, if the protists affect SO$_4^{2-}$ reduction directly by grazing on SO$_4^{2-}$-reducing bacteria or indirectly via creating more reduced soil conditions by stimulating the overall microbial activities.

The level of soil dsDNA correlates well with soil microbial biomass (6, 46). In the upper layer, the total level of dsDNA was lower in the presence of protists than in the absence of protists (Fig. 5), suggesting that protists controlled the microbial population. Different studies have demonstrated that protistan grazing may reduce the number (30, 31, 53) and biomass...
of bacteria (1). One may argue that part of the dsDNA was extracted from eukaryotes or even from dead biomass. However, the dsDNA content increased with time in all treatments and was highest without protists (Fig. 5). In summary, these findings provide evidence that the difference was due to prokaryotes alone. Based on these assumptions, the microbial biomass in the upper layer in treatment P+B microcosms was one-half that in treatment B microcosms at day 21 (Fig. 5). A similar effect was reported for experiments performed with upland soil microcosms (41). The real impact of protists on microbial biomass may have been even more significant in theoxic surface soil, because (i) the microbial biomass in the lower anoxic layer was apparently not affected by protists (Fig. 5B) and (ii) about one-half of the upper layer was anoxic (Fig. 2) on days 7 to 21.

This estimate was based on the standing stock. However, the smaller biomass in treatment P+B microcosms may have had higher gross production, as indicated by higher activities. Grazing may accelerate nutrient cycling in general (9, 15), and elevated specific metabolic activities are typical for a bacterial population under grazing pressure (1, 3, 41). This effect could also be detected in the anoxic lower layer, even if a eukaryotic community was barely detectable by PCR-DGGE analysis (see below). While the total dsDNA content was not affected, the level of nitrogen mineralization was higher and the level of sulfate reduction was lower in the presence of protists, as indicated by the extractable soil NH$_4^+$ and SO$_4^{2-}$ contents, respectively (Fig. 3 and 4).

**Molecular ecology.** T-RFLP and DGGE offer the best compromise between processing time and information gained if a large number of samples has to be processed. For the bacteria, we could rely on a clone library generated previously from the same soil (51). However, in previous work on protists from the soil we focused on ciliates and relied on morphology-based identification (57, 58). To cover the full diversity of protists, we had to use a molecular approach. We opted for an 18S rRNA gene-based assay in combination with DGGE that covered a very wide range of eukaryotic phylotypes (20).

**Eukaryotic community.** The majority of the DGGE bands could be affiliated with flagellates. Most of the flagellate-related sequences were assigned to the Cercomonadida in the phylum Cercozoa and to the Cercomonadidae and Heteromitiidae in particular (11, 50). Members of the Cercomonadida have been known for a long time as the most abundant and widespread soil flagellates (50), and Cercomonas and Heteromita are common soil bacterivores that graze on attached bacteria (25, 47). They have been shown to affect the bacterial community in vitro (53) and to excrete ammonia as a main form of nitrogen when they are grazing on bacteria (18).

In contrast to the upper layer, the lower layer contained a much less diverse eukaryotic community, with only one sequence assigned to protists (Cercomonas) (Table 2). In a previous experiment, we quantified anaerobic ciliates (direct counts, up to 60 cells · g [dry weight] soil$^{-1}$) and flagellates (most probable number, up to 700 cells · g [dry weight] soil$^{-1}$) in the same soil (58), but the resulting number of targets was obviously too low to be detected in the presence of the background algal DNA (Table 2 and Fig. 6C). Less microbial biomass and the low cell yield of anoxic protists (24) may be the factors limiting the development of anaerobic eukaryotes. The only DGGE band affiliated with Cercomonas was obtained from both the control soil and treatment P+B microcosms (Table 2 and Fig. 6C), suggesting that this genus is the dominant genus in anoxic soil also.

**Prokaryotic community.** As soon as 10 h after flooding (day 0) we detected similar bacterial communities in the upper soil layers of the microcosms that received different treatments, indicating that bacterial diversity had been successfully reestablished (Fig. 7). The bacterial communities in upper and lower layers were different (Fig. 7). This finding is in accordance with previous work (51) and correlates well with the rapid evolution of oxygen gradients (Fig. 2). Hence, the availability of e$^-$ acceptors is the most probable control during early development of the microbial community. There were no major differences in the microbial succession (days 7 to 21) in the lower layer between treatments (Fig. 7). In the upper layer, the bacterial community changed much more with time. The bacterial communities in the treatment P+B and control microcosms were not identical, but they were more similar to each other than to the bacterial communities in the treatment B microcosms, at least at day 21 (Fig. 7). The archaeal community, however, stayed constant. The most conspicuous change in the bacterial community was the high abundance of T-RFs affiliated with Betaproteobacteria (430 and 489 bp) in treatment B. In the presence of protists, low-G+C-content gram-positive bacteria (Bacilllus and clostridia) were more dominant. This may suggest that Betaproteobacteria were grazed upon preferably (see the supplemental material).

The upper layer as defined and sampled in this experiment covers the oxic-anoxic boundary zone. Many important redox reactions take place at this highly active interface, including methane oxidation, oxidation of reduced metal ions, coupled nitrification-denitrification, sulfurlation, and other reactions. In this paper we show the gross effects of grazing. It will be challenging to become more specific and to study the effect of protistan grazing on particular processes in detail, but this is a promising area of research.

**ACKNOWLEDGMENTS**

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