CHARACTERISTICS AND DISTRIBUTIONS OF NITROUS OXIDE-PRODUCING DENITRIFYING FUNGI IN NATURAL ENVIRONMENTS

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Abstract Tea field soils, and sediments of an irrigation pond and a tidal river, in which a variety of organic matter was supplied as energy sources, were collected. The activities of bacterial and fungal denitrifications in these samples were determined. Denitrifying fungi in all of these samples produced N_2O from nitrate and nitrite as a final product, whereas denitrifying bacteria produced N_2 . Nitrous oxide produced by fungi was reduced to N_2 by bacteria. The fungal denitrification potentials were the highest in the submerged litter on the pond sediment, followed by the farmyard manure-amended soil, the inorganic fertilizer-amended soil, the litter-free pond sediment, and the tidal river sediment. The enrichments of denitrifying fungi in natural environments were related with the distributions of the organic material such as straws and litter. The contributions of fungal denitrification to total denitrification were large in soil environments, especially in the farmyard manure-amended soil, and were small in aquatic environments such as the sediments of pond and river. The pH *in situ* was not related with the fungal denitrification potentials.

Keywords fungal denitrification; bacterial denitrification; nitrous oxide; organic matter; sediments; soils

Introduction

Fungi generally are found in lakes, ponds, rivers, estuaries, marine, wastewater, and soils. Despite their wide occurrence, little attention has been given to the presence and ecological significance of fungi. Especially, characteristics and contributions of fungal denitrification in natural environments are poorly understood. Denitrification is a process in which nitrite and/or nitrate are reduced to N_2 gas through N_2O . The process has been considered to be mainly caused by bacteria. However, pure culture experiments have shown that fungi such as *Fusarium sp.*, *Trichoderma hamatum*, *Chaetomium sp.*, *Gibberella fujikuroi* etc., can reduce nitrite and several strains can reduce nitrate as well, but the final product is mainly N_2O rather than N_2 (Bleakley and Tiedje, 1982; Burth and Ottow, 1983; Shoun and Tanimoto, 1991; Shoun *et al.*, 1992). The distributions of denitrifying fungi, which produce N_2O as a final product, would be ecologically important to understand the contribution of natural systems to the atmospheric concentration of N_2O . Previous studies have been conducted with pure cultures of fungi. However, the characteristics and distributions of denitrifying fungi in natural environments are unknown.

In this study, tea soils, and the sediments of an irrigation pond and a tidal river, to which different types of organic matter were supplied, were collected. The final products of fungal denitrification in these soils and sediments were determined, and the distribution of denitrifying fungi in natural system was estimated.

Materials

Tea field soils

Soils were collected at the surface (0-10cm) in two tea fields. One was mainly amended with a farmyard manure (organic soil), and the other with inorganic fertilizer only (inorganic soil). The farmyard manure was a compost of rice straws, grasses and cattle waste. The inorganic fertilizer was a mixture of ammonium phosphate, ammonium sulfate, potassium sulfate, fish meal, and rapeseed oil meal. Each soil was passed through a 2mm mesh. The soils were freeze-dried and then an ignition loss at 600 °C, and TC and TN contents by a Yanaco CHN analyzer MT-700 were measured (Table 1). Soil pH was determined in a soil-water suspension (1:2.5, at dry wt basis) (Tokuda and Hayatsu, 2000).

Sediment of an irrigation pond

The irrigation pond is constructed in the tea plantation noted above. The water quality of the pond originates

Table1 Characteristics of tea soils, the submerged litter and sediment of an irrigation pond, and the sediment of a tidal river used in this study

	pH (-)	organic matter contents* (%)	TC (%)	TN (%)	C/N (-)
Tea soils					
farmyard manure amended	4.8	27.4	11.3	0.96	11.8
chemical fertilizer amended	5.5	7.5	3.0	0.37	8.1
Irrigation pond					
submerged litter	6.9**	75.7	41.5	1.98	21.0
sediment	6.9	8.8	5.51	0.45	12.2
Tidal river					
sediment	7.6	4.8	1.06	0.08	13.2

* Organic matter contents(%) were shown as ignition loss at 600°C for 2 hours.

** This value is that of the irrigation pond water.

from the tea soils. As the pond is surrounded by small deciduous trees, their leaves (litter) fall into the pond in every autumn, and the surface sediment in the pond becomes covered with them. The litter and surface sediments of the pond were collected in June. The litter on the surface sediment which had fallen in the last autumn retained the original form when collected. It was crushed with a blender. The residues, rigid and lignified stem buried in the sediment were partly degraded. They were removed from the sediments by sieve of 2mm. The contents of organic matter, TC and TN in the litter and the sediment were measured by the same procedures as those of soils. Sediment pH was measured in the supernatant after centrifuged at 1500xG for 10 minutes.

Sediments of a tidal river

Sediments were collected from the surface layer (0-1cm) in a tidal zone and centrifuged at 1500xG for 10 minutes. A pH was measured in the supernatant. Then the solid was rinsed twice with a 0.1M phosphate buffer (*in situ* pH). The contents of organic matter, TC and TN were measured by the same procedures used for the soils (Table 1).

Methods

Preparation of samples

Chloramphenicol and cycloheximide were used as specific growth inhibitors of bacteria and fungi, respectively (Anderson and Domsch, 1975; Newell *et al.*, 1983; Fuhrman and McManus, 1984; Bjornsen, 1986). The samples were cultured in 0.1M phosphate buffer (*in situ* pH of each sample) containing the inhibitors (chloramphenicol and/or cycloheximide; 1mg/50g sample each) at 30 °C for 24 hours. Then the contamination of bacteria or fungi was tested using microscopy, and examined on heterotrophic agar plates and potato-dextrose agar plates using a spread plate technique.

Measurements of bacterial, fungal, and the total denitrification potentials

A suspension was prepared in a vial of 70ml volume. It contained a 3g (as dry wt) of each sample pretreated as described above, 3ml of 0.1M phosphate buffer (*in situ* pH of each sample), containing potassium nitrate or sodium nitrite and sodium fumarate at various concentrations, and 0.1mg of the same antibiotic as used for the pretreatment to continue the inhibition effect. Sodium fumarate was added as a supplement to avoid carbon limitation for denitrification rates (Sato *et al.*, 1989; Sato and Matsumoto, 1990). The ratio of the fumarate to nitrate or nitrite was given at C/N=2. The vials were sealed with rubber septa and purged with N₂ gas. These vials were divided into two groups. Acetylene (C₂H₂) gas was added at 10kPa to one of them as a control (Yoshinari and Knowles, 1976; Yoshinari *et al.*, 1977; Hynes and Knowles, 1984). All experiments were carried out with at least five replicates. The vials were incubated by agitating gently to maintain the homoge-

Figure 1 Behaviors of N_2O produced from nitrate and nitrite by denitrification of a farmyard manure-amended soil at *in situ* pH in the absence and presence of C_2H_2 .

(a) the intact soil, (b) the soil modified with cycloheximide, (c) the soil modified with chloramphenicol

The soil pretreated with chloramphenicol or cycloheximide and intact soil (3g as dry wt) was incubated at 30°C with 3ml of 0.1mole phosphate buffer (pH=5.0) containing 1mgN of nitrate or nitrite and sodium fumarate. Open and solid symbols show N_2O produced in the absence and presence of C_2H_2 , respectively.

neity of the suspensions at 30°C in the dark. The N_2O concentrations in the vials were determined and denitrification rates were calculated from the N_2O concentration in the control vials.

Finally, the suspensions were centrifuged at $1500\times\text{G}$ for 10 minutes and the supernatants were filtered through $0.45\mu\text{m}$ pore size membranes. The filtrates were analyzed with a ChemLab autoanalyzer for nitrite and nitrate concentrations. The pHs were measured at the beginning and end of the experiment. The contamination of bacteria or fungi was tested by the same procedures as the beginning of the incubation.

N_2O analysis

The N_2O concentrations were determined with a Shimadzu gas chromatograph GC8A, equipped with an ECD, and molecular sieves 60/80 mesh-column (length 2m). Detector/injector and column temperatures were 330°C and 200°C , respectively. The flow of carrier gas (N_2) was 30ml min^{-1} . The solubility of N_2O in water at 30°C was taken into account in the calculation of the resulting N_2O concentration (Weiss and Price, 1980; Fogg and Gerrand, 1991).

Results

Characteristics of fungal denitrification in sediments and soils

Figure 1 shows the behaviors of N_2O produced from nitrite or nitrate by the intact soil and the soils modified with chloramphenicol or cycloheximide. In the absence of C_2H_2 , the N_2O produced by bacteria ((b) in Figure 1) disappeared completely at the earlier stage of the denitrification process, whereas the N_2O produced by fungi ((c) in Figure 1) was approximately the same concentration as that in the presence of C_2H_2 . The denitrifying fungi predominantly produced N_2O as the final product as well as those of pure strains. The denitrifying bacteria produced N_2O as a transient intermediate and the final product was N_2 . The denitrification rates of nitrite by denitrifying bacteria were slightly higher than those of nitrate. On the other hand, there is no difference in N_2O production rates from nitrate and nitrite by fungi. The results from the organic soil were similar to those of all samples examined in this study.

Contribution of fungal denitrification in natural environment

The mass balance of nitrogen was calculated from the amounts of N_2O and the residual nitrogen compounds (nitrate and nitrite) in the control vials in which N_2O reduction was inhibited by C_2H_2 . In all samples, the total recoveries of nitrogen compounds were more than 80%. The sums of bacterial and fungal denitrifications nearly corresponded to the total denitrification. In addition, the samples pretreated with both chloramphenicol and cycloheximide neither utilized the substrates, nor produced N_2O (not shown). Accordingly, the N_2O was mainly produced by denitrifying bacteria and fungi.

Table 2 shows the bacterial, fungal and the total denitrification potentials at *in situ* pH and at 30 °C, and the ratios of fungal denitrification to the total denitrification. The total denitrification potentials were the highest in the submerged litter (670-720 $\mu\text{gN g}^{-1} \text{d}^{-1}$) followed by the tidal river sediment (150-158 $\mu\text{gN g}^{-1} \text{d}^{-1}$), the organic soil (70-100 $\mu\text{gN g}^{-1} \text{d}^{-1}$), the sediment of the pond (56-67 $\mu\text{gN g}^{-1} \text{d}^{-1}$), and the inorganic soil (18-21 $\mu\text{gN g}^{-1} \text{d}^{-1}$). The fungal denitrification potentials were the highest in the submerged litter of the pond (56-68 $\mu\text{gN g}^{-1} \text{d}^{-1}$) followed by the organic soil (22-27 $\mu\text{gN g}^{-1} \text{d}^{-1}$), the inorganic soil (2.2-2.8 $\mu\text{gN g}^{-1} \text{d}^{-1}$), the pond sediment (2-3 $\mu\text{gN g}^{-1} \text{d}^{-1}$), and the tidal river sediment (<1 $\mu\text{gN g}^{-1} \text{d}^{-1}$). The ratios of fungal denitrification to the total denitrification were the largest in the soils, especially in the organic soil (23-38%), and were small in the pond sediments (3-5%) and the river sediments (<0.6%). The submerged litter were the largest potentials in fungal denitrification, but the ratios to the total denitrification were 7.8-10% and the values were smaller than those in the soils.

Discussion

The determination of contributions of individual microorganism to total metabolism in an ecosystem has remained difficult because of methodological problems. The individual biomass and metabolic activity in the

Table 2 Potentials and contributions of bacterial and fungal denitrifications in tea soils, the submerged litter and sediment of an irrigation pond, and the sediment of a tidal river at *in situ* pH.

	Denitrification potential* ($\mu\text{gN} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$)			Fungal/Total denitrification (%)
	Total	Bacterial	Fungal	
Tea soils				
farmyard manure amended	70 ~ 100	48 ~ 62	22 ~ 27	23 ~ 38
chemical fertilizer amended	18 ~ 21	14 ~ 16	2.2 ~ 2.8	10 ~ 15
Irrigation pond				
litter	670 ~ 720	610 ~ 710	56 ~ 68	7.8 ~ 10
sediment	56 ~ 67	48 ~ 65	2 ~ 3	3 ~ 5
Tidal river				
sediment	150 ~ 158	148 ~ 156	< 1	< 0.6

*Denitrification potentials were calculated from N_2O production rates in the vials which N_2O reduction was inhibited by C_2H_2 at 30 °C and *in situ* pH of each sample.

complex ecosystems such as soils and aquatic environments can not be estimated without some destruction of the systems. The contributions of bacterial and fungal denitrifications were studied by using selective inhibitors in this study. Problems in this technique are also indicated by Newell *et al.* (1983) and Wu and Knowles (1995). Wu and Knowles (1995) reported that chloramphenicol affects denitrification activities of *Flexibacter canadensis* and *Pseudomonas denitrificans*. Furthermore, the sensitivities to inhibitors depended on the concentration, culture condition, and species of microorganisms. The results obtained by pure culture experiments couldn't be directly applied to natural samples. In this study, the bacterial and fungal denitrifications were measured after pretreatment with antibiotics. Accordingly, antibiotics might inhibit in part denitrification activities of some bacteria and fungi, but the degree of inhibition would be negligibly small. The denitrification potentials and the contributions shown in Table 2 do not always represent those *in situ* because of pretreatment, but would show relative rates and contributions of bacterial and fungal denitrifications.

It is normal for natural environments to be supplied with a variety of organic matter as energy sources. The predominant organisms *in situ* depend on the characteristics of organic matters. Samples collected in this study contained organic matters such as farmyard manure, litter, and detritus *etc.*. Generally, plant materials of terrestrial origin are rich in lignocellulose and lignin, which are hard to degrade biologically. Fungi are a major lignin degrader in the ecosystem, although some bacterial strains, the genera *Nocardia*, *Streptomyces* and *Bacillus*, can degrade lignin to CO₂ (Crawford and Crawford, 1980). In this study, the fungal denitrification potentials were relatively high in the submerged litter of the pond and the organic soil, and were low in the inorganic soil, and the sediments of the pond and the tidal river (Table 2). The farmyard manure supplied to the tea soil was mainly composed of rice straws, grasses and cattle waste. Sakamoto and Oba (1994,1995) reported that amendments such as straws and their composts selectively enriched fungi in soils, in comparison with amendments such as cattle feces and sewage sludge composts. Burdige and Martens (1988) reported that amino acids played an important role as available organic matters in coastal marine sediments. These organic matters, however, will not enrich fungi. The data suggest that the component of organic matters is one of the important factors involved in enrichment of denitrifying fungi. The inorganic fertilizer contains some organic matters such as fish meal and rapeseed oil meal, but these organics do not enrich denitrifying fungi. All bacteria and fungi do not have denitrification activities. Consequently, species and strains of denitrifying fungi were not identified, and the biomass was not determined, either.

Shoun *et al.* (1992) reported that most of denitrifying fungi could not reduce nitrate. However, the reduction of nitrate by fungi was the same degree as that of nitrite (Figure 1). The results suggest that a variety of denitrifying fungi, which can utilize nitrate and nitrite, is distributed in natural environments. Nitrous oxide produced by fungi seems to be reduced to N₂ by bacteria (Figure 1). Denitrifying bacteria work as "sink" and "source" of N₂O, and fungi only work as "source". Therefore, the relative potentials and ratios of bacterial and fungal denitrifications are the important factors in controlling N₂O emission in natural systems.

Most fungi are aerobic or microaerobic, although a few species show anaerobic metabolism (Tabak and Cooke, 1968). Therefore, it seems that the contributions of fungal denitrification to total denitrification are large in the soil environments (Table 2). Fungal denitrification also showed high potential at both the neutral pH (*i.e.* the sediments) and the low pH (*i.e.* the soils). A low pH value affected bacterial denitrification, but not fungal denitrification (Oishi and Kusuda, 1997). Anderson and Domsch (1975) also suggested that the degree of fungal dominance was not dependent on the pH of the soil. Therefore enrichment of denitrifying fungi would depend on the components of available organic matter rather than pH.

Conclusions

A variety of denitrifying fungi was distributed in natural environments, and can utilize not only nitrite but also nitrate. The denitrifying fungi were enriched in the environment which the organic material such as straws and litter was supplied. Nitrous oxide produced by fungi was reduced to N₂ by bacteria in natural environments. The contributions of fungal denitrification to total denitrification were larger in soils than those in aquatic environments.

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