Visualization and Direct Counting of Individual Denitrifying Bacterial Cells in Soil by nirK-Targeted Direct in situ PCR

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The abundance of denitrifying bacteria in soil has been determined primarily by the conventional most probable number (MPN) method. We have developed a single-cell identification technique that is culture-independent, direct in situ PCR, to enumerate denitrifying bacteria in soils. The specificity of this method was evaluated with six species of denitrifying bacteria using nirK as the target gene; Escherichia coli was used as a negative control. Almost all (97.3%–100%) of the nirK-type denitrifying bacteria (Agromonas oligotrophica, Alcaligenes faecalis, Achromobacter denitrificans, Bradyrhizobium japonicum, and Pseudomonas chlororaphis) were detected by direct in situ PCR, whereas no E. coli cells and only a few cells (2.4%) of nirS-type denitrifying bacteria (Pseudomonas aeruginosa) were detected. Numbers of denitrifying bacteria in upland and paddy soil samples quantified by this method were 3.3 × 10^8 to 2.6 × 10^9 cells g^-1 dry soil. These values are approximately 1,000 to 300,000 times higher than those estimated by the MPN method. These results suggest that direct in situ PCR is a better tool for quantifying denitrifying bacteria in soil than the conventional MPN method.

Key words: denitrifying bacteria, direct in situ PCR, MPN method, nirK, soil

Denitrification, the final step of the nitrogen cycle, is a type of bacterial respiration that utilizes oxidized forms of nitrogen as electron acceptors under oxygen-restricted conditions. This process is involved in global warming (27), ozone layer destruction (42), fertilizer loss from agricultural soil (20), and removal of nitrogen compounds from wastewater (25), and thus deserves careful attention from various environmental perspectives.

Denitrifying bacteria are the microorganisms that convert nitrates to free atmospheric nitrogen. An understanding of the nitrogen cycle requires quantification of denitrifying bacteria, which provides insight into their ecology. The most probable number (MPN) method has been most commonly used for this purpose (7, 38); however, this method is time-consuming (typically requiring a 1-week incubation) and may underestimate populations because most soil bacteria are difficult to cultivate.

To overcome these problems, culture-independent molecular biological techniques have been developed to quantify denitrifying bacteria in the environment (34). Denitrifying bacteria are phylogenetically diverse (33); thus, probes and primers based on rRNA sequences are not appropriate for their detection and quantification.

Denitrification consists of four reduction steps, in which nitrate is ultimately reduced to nitrogen gas by nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (47). The reduction of nitrite to nitric oxide distinguishes denitrifying bacteria from other nitrate-respiring bacteria; therefore, nitrite reductase genes (nirK and nirS) have been recently used to detect denitrifying bacteria (8) in various environments including an inland sea (30), wastewater treatment plants and activated sludge (13), forest soil (28, 29), and forested upland and wetland soils (36). In addition, quantitative PCR methods targeting nirK or nirS, such as the real-time PCR and competitive PCR methods, have been developed to quantify denitrifying bacteria in lake and marine environments (12), stream sediment (37), activated sludge (11), foreland soil (23), and arable soil (16).

Although quantitative PCR methods can quantify denitrifying bacteria in the environment, problems with the evaluation of soil samples have been reported. In particular, DNA extraction from soils can be difficult, probably due to the presence of organic matter. PCR amplification can also be inhibited by humic acid (29). Furthermore, 6% or less of the indigenous bacterial DNA can be recovered from some soils because the soil’s clay content strongly influences DNA recovery (10). Therefore, analyzing soil samples with quantitative PCR methods is still problematic.

Fluorescence in situ hybridization (FISH) using rRNA-targeting oligonucleotide probes has been widely applied to the specific detection and enumeration of the individual cells of targeted bacteria in natural environments (3, 32). However, the conventional FISH technique is unable to detect individual bacterial cells when the copy number of a target gene is only one or two per cell, such as a functional gene in the chromosomes, because many target sequences are required to detect a fluorescent signal by microscopy. Only recently, advanced FISH techniques were introduced for the detection of single cells that have a targeted functional gene (32). They include recognition of individual genes (RING)-FISH (35), in situ rolling circle amplification (in situ RCA)-FISH (21), and two-pass tyramide signal

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amplification (two-pass TSA)-FISH (24). RING-FISH was successfully used to detect nirK of denitrifying bacteria in activated sludge by employing a polynucleotide probe (35). In situ RCA-FISH was introduced for the detection of nirS and nosZ of denitrifying bacteria in activated sludge (21). Two-pass TSA-FISH was used for visualizing individual cells of a methanogen by detecting the methyl coenzyme M reductase gene, though its application to environmental samples remains restricted (24). These FISH techniques are, however, still limited to qualitative purposes and need to be refined for quantitative applications (35).

As an alternative approach, in situ PCR with the 2-hydroxy-3-naphthoic acid-2'-phenylalanilide phosphate (HNPP) -Fast Red TR system has been applied to the visualization and quantification of single bacterial cells that have only one copy or a few copies of a functional gene target in the chromosomes (19). This approach has successfully detected Escherichia coli O157 in river water (26) and ammonia-oxidizing bacteria in biofilm (22). However, no fluorescence microscopic technique including in situ PCR and FISH has been applied to the single cell detection of denitrifying bacteria in soil. This is probably due to the presence of organic matter and clay minerals that disrupt the reaction with the probe and the targeted site of the cells (4, 5).

In the present study, we developed a culture-independent direct in situ PCR technique for single-cell enumeration of denitrifying bacteria in soil. To reduce the unfavorable effects of organic matter and clay minerals, bacterial cells were extracted from soil and used. In addition, we compared this technique with the conventional MPN method. As the target gene in the present study, nirK was used because it has been reported to be more abundant than nirS in soils (28, 45, 46).

Materials and Methods

Bacterial strains and soil samples

Bacterial strains used in this study are listed in Table 1. These strains are often found in soil, and Agromonas oligotropha is the predominant species among culturable oligotrophic bacteria in paddy soil (31). Other than A. oligotropha and Bradyrhizobium japonicum, these strains were grown in nutrient broth (Eiken, Tokyo, Japan) at 30°C for 9 to 12 h and harvested in the stationary phase. A. oligotropha was grown in 1/100 strength nutrient broth at 30°C for 3 d, and B. japonicum was grown in YM medium (1.0 g yeast extract, 5.0 g mannitol, 0.7 g K₂HPO₄, 0.1 g KH₂PO₄, and 1.0 g MgSO₄·7H₂O, pH 7.2) in 1 L distilled water [DW]) at 30°C for 6 d.

Upland and paddy soil samples were collected from experimental fields of Saga University, Japan (Table 2). These soils were grey lowland soils classified as clay loam. The upland field was amended with 100 kg ha⁻¹ of chemical fertilizers (10% N-NH₄, 31% P-phosphate, and 16% K) twice a year and used for soybean-barley corn rotation for over 25 years. The paddy field was amended with 50 kg ha⁻¹ of chemical fertilizers (9% N-NH₄, 12% P-phosphate, and 15% K) before transplanting and used for over 30 years. In each field, five soil samples were collected 5 to 15 cm below the soil surface and mixed well. About 500 g of the soil mixture was sealed in a plastic bag, which was stored at 4°C prior to use. The water content of the soil was determined by weighing soil samples before and after drying at 105°C for 10 h. The soil pH was determined in a water suspension (1:2.5, w/v) with a pH meter (F-12, Horiba, Kyoto, Japan). Electrical conductivity of the soil was determined in a water suspension (1:5, w/v) with an electrical conductivity meter (ES-14, Horiba). We used the Kjeldahl method and the Turin method to determine total nitrogen and total carbon, respectively.

Direct in situ PCR

Direct in situ PCR was carried out according to the procedure of Tani et al. (41), who used it to detect E. coli in river water, with some modifications for denitrifying bacteria in soil samples. We selected a nirK2F/3R primer set for direct in situ PCR, because it showed the highest specificity among all the primer sets reported by Braker et al. (6) for PCR amplification of several oligotrophic denitrifying bacteria, including A. oligotropha, isolated from soil (unpublished data). Bacterial cells harvested in the stationary phase, or bacterial fractions obtained by extraction from the soil samples (described below), were used. The specimen was fixed with paraformaldehyde and 30 µL of suspension (about 10⁷ cells mL⁻¹) was spotted onto a silane-coated slide (Muto Pure Chemicals, Tokyo, Japan). To increase cell membrane permeability, the specimen was incubated with 30 µL of lysozyme (0.5 mg mL⁻¹ lysozyme [Sigma, St Louis, MO, USA], 100 mM Tris-HCl [pH 8.2], and 50 mM EDTA [pH 8.0]) at room temperature (RT) for 30 min and rinsed with 1 mL of DW, after which it was dehydrated in an ethanol series (30 µL each; 50%, 80%, and 100% [v/v] ethanol for 3 min each). Then, the specimen was treated with 30 µL of proteinase K (Sigma) (0.1 µg mL⁻¹ in PBS) at RT for 10 min to further increase membrane permeability.

The specimen was mixed with 50 µL of PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM MgCl₂) containing 0.2 mM (each) deoxynucleoside triphosphates, 0.035 mM DIG-dUTP (Roche Diagnostics, Manheim, Germany), 0.4 µM nirK2F and nirK2R, 0.2 µM 3R, and 0.2 µM 2F, and 0.2 µM 2R. The PCR was carried out according to the procedure of Braker et al. (6) for PCR amplification of several oligotrophic denitrifying bacteria, including A. oligotropha, isolated from soil (unpublished data).

Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Nir type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agromonas oligotropha</td>
<td>JCM1494</td>
<td>nirK</td>
<td>HQ5259691*</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
<td>NBRC13111</td>
<td>nirK</td>
<td>(37)</td>
</tr>
<tr>
<td>Achromobacter denitrificans</td>
<td>NBRC15125</td>
<td>nirK</td>
<td>(18)</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>NBRC14792</td>
<td>nirK</td>
<td>(39)</td>
</tr>
<tr>
<td>Pseudomonas chlororaphis</td>
<td>NBRC3521</td>
<td>nirK</td>
<td>(37)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>NBRC12689</td>
<td>nirS</td>
<td>(18)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>NBRC3301</td>
<td>None</td>
<td>(37)</td>
</tr>
</tbody>
</table>

* GenBank accession number.

Table 2. Characteristics of soil samples

<table>
<thead>
<tr>
<th>Soil sample (date of sampling)</th>
<th>Water content (%)</th>
<th>pH</th>
<th>EC (µS cm⁻¹)</th>
<th>T-N (%)</th>
<th>T-C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upland soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (April 27, 2005)</td>
<td>20.0</td>
<td>7.2</td>
<td>43.7</td>
<td>0.25</td>
<td>2.46</td>
</tr>
<tr>
<td>B (April 21, 2008)</td>
<td>23.7</td>
<td>7.2</td>
<td>65.0</td>
<td>0.20</td>
<td>2.56</td>
</tr>
<tr>
<td>Paddy soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Drained soil (April 27, 2005)</td>
<td>34.0</td>
<td>6.0</td>
<td>25.7</td>
<td>0.20</td>
<td>1.77</td>
</tr>
<tr>
<td>D. Submerged soil (July 22, 2005)</td>
<td>40.8</td>
<td>6.6</td>
<td>63.3</td>
<td>0.20</td>
<td>1.52</td>
</tr>
<tr>
<td>E. Drained soil (August 30, 2005)</td>
<td>34.1</td>
<td>6.3</td>
<td>32.3</td>
<td>0.20</td>
<td>2.45</td>
</tr>
</tbody>
</table>

EC, electrical conductivity; T-C, total carbon; T-N, total nitrogen.
Microscopic observation and quantification by direct PCR

Optimum conditions for direct in situ PCR

The final annealing temperature and number of PCR cycles were determined for A. oligotrophica cells collected in the stationary phase (Fig. 1). Ten cycles of direct in situ PCR were performed using 60°C, 62°C, or 64°C as the optimal annealing temperature (Fig. 1A). Detection was highest at 62°C. The optimal number of PCR cycles (10, 15, 20, and 30) was then determined using the optimal annealing temperature of 62°C (Fig. 1B). Detection was highest (ca. 100%) with 30 cycles. Based on these results, an annealing temperature of 62°C and amplification cycle number of 30 were adopted as the optimal conditions for direct in situ PCR in subsequent experiments.

Evaluation of specificity

The specificity of direct in situ PCR was evaluated with six species of denitrifying bacteria (five nirK-type species and one nirS-type species) and a non-denitrifying species (Table 3). Almost all cells (97.3%–100%) of the five nirK-type denitrifying species (A. oligotrophica, A. faecalis, A. denitrificans, B. japonicum, and P. chlororaphis) were positive for nirK, demonstrating the high specificity of direct in situ PCR. Almost all nirK-negative species (2.4%) of the nirS-type denitrifying P. aeruginosa and no cells of E. coli were positive, demonstrating the high specificity of this technique.

Visualization of denitrifying bacteria in soil

The direct in situ PCR method was then applied to soil samples. Denitrifying bacteria in the bacterial fraction from soil produced sufficient fluorescence intensity to obtain clear images (Fig. 3). Non-specific HNP/TR signals were also observed (Fig. 3 NF); however, these particles were easily distinguished from denitrifying bacteria (Fig. 3 DB) because they were not accompanied by the blue fluorescence of...
**Results expressed as the mean at 62°C and 30 cycles of amplification.**

Direct denitrifying bacteria was 3.2 × 10^8 cells (4 × 10^8 to 1.4 × 10^9 cells g^−1 dry soil) in the upland soil samples and 1.1 × 10^6 to 1.4 × 10^6 cells g^−1 dry soil in the paddy soil. The number of denitrifying bacteria estimated by DAPI. Without bacterial extraction from soil, no clear image was obtained by direct in situ PCR due to considerable nonspecific fluorescence and strong autofluorescence (data not shown).

**Cell recovery of denitrifying bacteria from soil by direct in situ PCR**

To determine the accuracy of direct in situ PCR for enumerating denitrifying bacteria in soil, a cell suspension of *A. oligotrophica* was added to a sample of upland soil. The bacterial cells were then extracted from the soil and quantified by direct in situ PCR. Cell recovery of indigenous bacteria (with no addition of *A. oligotrophica*) was 99.3% as determined by EB staining before and after the bacterial extraction and that of soil bacteria with the addition of *A. oligotrophica* was 81.4% as a mean. A good linear correlation (R^2=0.975) was observed between the number of added cells (4 × 10^8 to 3 × 10^9 cells g^−1 dry soil) and the direct in situ PCR cell counts (Fig. 4), with a consistent cell recovery of 52% (slope of the regression line), which shows the efficiency of direct in situ PCR. The number of indigenous denitrifying bacteria was 3.2 × 10^8 cells g^−1 dry soil as determined from the y-intercept of the regression line.

**Enumeration of nirK-type denitrifying bacteria in soil**

To quantify denitrifying bacteria in soil, direct in situ PCR targeting nirK and the conventional MPN method were used and compared (Table 4). The total number of bacteria was 6.8 × 10^9 and 6.5 × 10^9 cells g^−1 dry soil in the upland soil samples and 1.1 × 10^6 to 1.4 × 10^6 cells g^−1 dry soil in the paddy soil. The number of denitrifying bacteria estimated by direct in situ PCR was 6.1 × 10^5 and 9.1 × 10^5 cells g^−1 dry soil in the upland soil samples and 1.1 × 10^7 to 2.5 × 10^7 cells g^−1 dry soil in the paddy soil. The ratio of denitrifying bacteria detected by in situ PCR to the total number of bacteria was 8.9 to 18.5% in these soil samples (Table 4, b/a). In contrast, the numbers estimated by the MPN method were 6.8 × 10^9 and 7.1 × 10^9 MPN g^−1 dry soil in the upland soil samples and 5.3 × 10^7 to 2.2 × 10^8 MPN g^−1 dry soil in the paddy soil. Thus, the cell counts by direct in situ PCR were approximately 1,000 to 300,000 times higher than those produced by the

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**Table 3. Detection of pure culture cells by direct in situ PCR targeting nirK**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Nir type</th>
<th>Detection rate (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. oligotrophica</em> JCM1494</td>
<td>nirK</td>
<td>100.0±0.0**</td>
<td></td>
</tr>
<tr>
<td><em>A. faecalis</em> NBRC13111</td>
<td>nirK</td>
<td>100.0±0.0</td>
<td></td>
</tr>
<tr>
<td><em>A. denitrificans</em> NBRC15125</td>
<td>nirK</td>
<td>99.3±0.8</td>
<td></td>
</tr>
<tr>
<td><em>B. japonicum</em> NBRC14792</td>
<td>nirK</td>
<td>97.3±1.2</td>
<td></td>
</tr>
<tr>
<td><em>P. chlororaphis</em> NBRC3521</td>
<td>nirK</td>
<td>97.6±0.9</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NBRC12689</td>
<td>nirS</td>
<td>2.4±0.3</td>
<td></td>
</tr>
<tr>
<td>E. coli NBRC3301</td>
<td>None</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Direct in situ PCR was performed at the optimum conditions (annealing at 62°C and 30 cycles of amplification).

* Ratio of bacterial cells detected by direct in situ PCR to total number of bacteria, as assessed by DAPI staining.

**Results expressed as the mean ± standard deviation of triplicate determinations.**

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**Table 4. Detection of pure culture cells by conventional MPN and direct in situ PCR targeting nirK.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>MPN (MPN g^−1 dry soil)</th>
<th>Direct in situ PCR (10^6 cells g^−1 dry soil)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Upland soil</td>
<td>6.8 × 10^9</td>
<td>6.1 × 10^5</td>
<td></td>
</tr>
<tr>
<td>Paddy soil</td>
<td>1.1 × 10^7</td>
<td>9.1 × 10^5</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2.** Fluorescence images of pure bacterial cultures quantified by direct in situ PCR targeting nirK. A: Almost all *A. oligotrophica* cells were positive for both DAPI (blue fluorescence) and HNP/TR (red fluorescence) under UV excitation. B: All *E. coli* cells were negative for HNP/TR but positive for DAPI staining.

**Fig. 3.** Fluorescence image of denitrifying bacteria in upland soil quantified by direct in situ PCR. Arrows (DB) indicate denitrifying bacteria exhibiting both red and blue fluorescence derived from HNP/TR and DAPI, respectively. Arrows (NF) indicate nonspecific fluorescence.

**Fig. 4.** Cell recovery of denitrifying bacteria added to upland soil. Cell suspensions of *A. oligotrophica* were added to upland soil sample A (see Table 2). Cell counts obtained by direct in situ PCR showed a good correlation with the number of added cells (R^2=0.975).
MPN method (Table 4, b/c). In the paddy field samples, the number of denitrifying bacteria in the submerged soil was higher than that of the two drained soils according to both methods. It is also noteworthy that cell counts obtained by direct in situ PCR were higher in paddy soil than in upland soil, whereas the counts obtained by the MPN method showed the opposite pattern.

Discussion

In the present study, we developed a direct in situ PCR method for the visualization and specific enumeration of denitrifying bacteria in soil. To the best of our knowledge, this study is the first to demonstrate that denitrifying bacteria in soil can be enumerated successfully by fluorescence microscopy. Direct in situ PCR has some advantages compared with other culture-independent techniques such as quantitative PCR. This method allows the visualization of denitrifying bacterial cells in their microhabitat. It also allows direct counts of denitrifying bacteria, which provide basic information for population analyses.

The successful application of direct in situ PCR to soil samples is thought to be due primarily to the technique of bacterial extraction from soil, enzyme treatments to increase cell permeability, and optimization of PCR conditions (annealing temperature and number of amplification cycles). Organic matter and clay minerals from soil often interfere with fluorescence microscopy because of their autofluorescence and nonspecific absorbability of fluorochromes and other chemicals used in microscopy. Autofluorescence intensifies under excitation by shorter wavelengths such as the UV light used for DAPI, which is often used as a counter stain for FISH and direct in situ PCR. Hence, we used the bacterial extraction procedure introduced by Wu et al. (44), who successfully applied the direct viable count FISH method to the specific enumeration of E. coli in cow manure. As a result, clear images of denitrifying bacteria with adequate fluorescence intensity were obtained (Fig. 3) due to the bacterial extraction technique. Enhancement of cell permeability is also important for good results. Hodson et al. (19) suggested altering cell membrane permeability to allow easy passage of enzymes and reagents into bacterial cells and to retain the amplified PCR products inside the cell. During the optimization of PCR conditions, the optimum annealing temperature was revealed to be 62°C, and the optimum number of amplification cycles was 30 or more (Fig. 1). However, more than 30 amplification cycles can result in significant amounts of PCR artifacts that produce nonspecific extracellular fluorescence (22); therefore, we adopted 30 PCR cycles. The optimization was performed by using A. oligotrophica, whose GC content (ca. 50%) is similar to that of other nirK-type denitrifying bacteria (45–52%) used in this study. Thus conditions fit for A. oligotrophica would also be applicable to other bacterial species.

Counts of denitrifying bacteria in soil obtained by direct in situ PCR were 10⁸–10⁹ cells g⁻¹ dry soil. These results were approximately 1,000 to 300,000 times higher than those obtained by conventional MPN (Table 4), demonstrating that the MPN method would markedly underestimate the number of denitrifying bacteria in soil. Similar low counts (10⁵–10⁶ MPN g⁻¹ dry soil) by the MPN method were reported in the analysis of upland soil (7). A MPN method using microtiter plates and an anaerobic culture system has been developed; this method produces higher counts of denitrifying bacteria in soil than the conventional MPN method, but the counts were still relatively low (10⁵–10⁶ MPN g⁻¹ dry soil) (38).

The markedly high counts obtained by direct in situ PCR may be due to overestimation to some extent. Some nirS-type denitrifying bacteria can be detected by the method as shown in the experiment with pure cultures (Table 3), though the contribution would be very small considering the low detection rate for such bacteria. The misreading of non-specific HNPR signals as denitrifying bacteria would also cause overestimation. However, the effect would be not great, since the cell recovery rate for denitrifying bacteria added to the soil was less than 100% (52%) as shown in Fig. 4. Counts of dead cells could also be involved in the estimation by in situ PCR. This may cause extensive overestimation, since low viability (4–11%) of indigenous bacteria in soil has been demonstrated by micro-colony counting (43), though the viability of denitrifying bacteria in soil is still unknown. Combinations of in situ PCR and vital staining such as the direct viable count (DVC) technique may provide information on the viability of denitrifying bacteria in soil, for example, the DVC-FISH of E. coli in cow manure (44). In contrast, the MPN method gives counts of cultivable denitrifying bacteria. It is well recognized that only a small percentage of the bacteria in soil can be cultivated. Further-

### Table 4. Number of denitrifying bacteria in soil as determined by the direct in situ PCR and MPN methods

<table>
<thead>
<tr>
<th>Soil samples*</th>
<th>TDC*** (cells g⁻¹ dry soil)</th>
<th>Direct in situ PCR**** (cells g⁻¹ dry soil)</th>
<th>MPN***** (MPN g⁻¹ dry soil)</th>
<th>b/a (%)</th>
<th>b/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upland soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>(6.8 ± 0.7) × 10⁹</td>
<td>(6.1 ± 0.1) × 10⁹</td>
<td>6.8 × 10⁹</td>
<td>8.9</td>
<td>9.0 × 10⁷</td>
</tr>
<tr>
<td>B</td>
<td>(6.5 ± 0.6) × 10⁹</td>
<td>(9.1 ± 3.1) × 10⁹</td>
<td>7.1 × 10⁹</td>
<td>14.0</td>
<td>1.3 × 10⁷</td>
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<tr>
<td>Paddy soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C, drained</td>
<td>(1.1 ± 0.9) × 10⁸</td>
<td>(1.1 ± 1.0) × 10⁸</td>
<td>7.1 × 10⁹</td>
<td>10.3</td>
<td>1.5 × 10⁴</td>
</tr>
<tr>
<td>D, submerged</td>
<td>(1.4 ± 0.3) × 10⁸</td>
<td>(2.5 ± 0.6) × 10⁸</td>
<td>2.2 × 10⁹</td>
<td>18.5</td>
<td>1.1 × 10⁴</td>
</tr>
<tr>
<td>E, drained</td>
<td>(1.1 ± 0.4) × 10⁹</td>
<td>(1.6 ± 0.7) × 10⁹</td>
<td>5.3 × 10⁹</td>
<td>15.0</td>
<td>3.0 × 10⁵</td>
</tr>
</tbody>
</table>

* see Table 2.
** Total direct count of bacteria in soil determined by EB staining.
*** Expressed as the mean ± standard deviation of triplicate determinations.
**** 95% confidence limits are 0.33–3.3 MPN.
more, the MPN method with an eutrophic medium such as Giltay’s medium may underestimate numbers of denitrifying bacteria since oligotrophs are predominant in denitrifying bacterial populations (15).

Counts of denitrifying bacteria by direct in situ PCR were higher in paddy soil than in upland soil, whereas the counts obtained by the MPN method produced the opposite pattern (Table 4). Paddy soil is expected to harbor more denitrifying bacteria than upland soil because denitrification becomes more active in an anaerobic environment such as submerged soil than an aerobic environment such as well-drained soil (1). Therefore, the MPN technique not only underestimates bacterial counts but inaccurately reflects the behavior of denitrifying bacteria in soil, although it is still useful for bacterial isolation.

As assessed by quantitative PCR methods, the abundance of denitrifying bacteria was reported to be $10^5$ to $10^9$ g$^{-1}$ upland soil (9, 14, 16, 17, 37) and $10^6$ to $10^7$ g$^{-1}$ paddy soil (45, 46). These counts appear to be lower than the results obtained in the present study by direct in situ PCR. This difference may indicate the superiority of direct in situ PCR, which is free of the problems associated with quantitative PCR procedures such as low DNA recovery from soil (10) and interference with PCR amplification by humic acid and clay minerals (29). The advantages of in situ PCR would mostly be ascribed to the introduction of cell extraction procedures with high recovery as mentioned above. Furthermore, with direct in situ PCR, the amplification is conducted intracellularly, where there would be less interference from soil organic matter and clay minerals, rather than in a solution of DNA extracted from soil. Mergel et al. (28) reported that the concentration of denitrifying bacteria would need to be at least $10^8$ cells g$^{-1}$ dry soil to produce the emission rates ($15–20$ nmol N$\text{g}^{-1}$ dry soil) observed in forest soil samples, based on pure culture studies. Therefore, our findings of $10^8$–$10^9$ cells g$^{-1}$ dry soil appear to be reasonable.

Several limitations of the present study should be noted. Our in situ PCR analysis targeted nirK but not nirS. However, it is preferable to target both nirK and nirS, even though nirK has been reported to be more abundant than nirS in both upland and paddy soils (28, 45, 46). In addition, the detection limit of direct in situ PCR should be considered. In the present study, the detection limit was approximately $10^7$ cells g$^{-1}$ dry soil. Although this is much higher than the detection limit of quantitative PCR methods, the abundance of denitrifying bacteria in soil is typically higher (Table 4).

In the present study, high counts of denitrifying bacteria in soil were obtained. Even though, numbers may have been overestimated to some extent, denitrifying bacteria may comprise a larger functional group in the soil bacterial community than previously thought.

References


