Nitrate and Nitrite Microgradients in Barley Rhizosphere as Detected by a Highly Sensitive Denitrification Bioassay

SVEND JØRGEN BINNERUP* AND JAN SØRENSEN

Microbiology Section, Department of Ecology and Molecular Biology, The Royal Veterinary and Agricultural University, Rolighedsvej 21, DK-1958 Frederiksberg C, Denmark

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A highly sensitive denitrification bioassay was developed for detection of NO$_3^-$ and NO$_2^-$ in rhizosphere soil samples. Denitrifying *Pseudomonas aeruginosa* ON12 was grown anaerobically in citrate (30 mM) minimal medium with KClO$_3$ (10 mM) and NaNO$_3$ (3 mM), which gave cells capable of NO$_2^-$ reduction to N$_2$O but incapable of NO$_3^-$ reduction to NO$_2^-$. Growth on citrate minimal medium further resulted in the absence of N$_2$O. When added to small soil samples in O$_2$-free vials, such cells could be used to convert the indigenous NO$_3^-$ pool to N$_2$O, which was subsequently quantified by gas chromatography. Cells grown in KClO$_3$-free citrate medium with 10 mM NaNO$_3$ as the electron acceptor were capable of reducing both NO$_3^-$ and NO$_2^-$, and these cells could subsequently be added to the sample to convert the indigenous NO$_3^-$ pool to N$_2$O. Concentrations of both NO$_3^-$ and NO$_2^-$ were thus determined as N$_2$O with a detection limit of approximately 10 pmol of N. The bioassay could be used to determine NO$_3^-$ and NO$_2^-$ pools in 10-mg soil samples taken along a microgradient in the rhizosphere of field-grown barley plants. At both low (10%, wt/wt) and high (18%, wt/wt) water content, relatively high levels of NO$_3^-$ were found in the rhizosphere compared with bulk soil. Under dry conditions, NO$_3^-$ was also more abundant in the rhizosphere than in the bulk soil, whereas such a difference was not observed at the high water content. The roles of plant metabolism and bacterial nitrification and denitrification processes for NO$_3^-$ and NO$_2^-$ availability in the rhizosphere are discussed.

Nitrification and denitrification processes in soil are strongly influenced by the availability of O$_2$ since denitrification is an anaerobic process, whereas nitrification only proceeds under aerobic conditions. Root respiration, microbial respiration, and restricted diffusion of O$_2$ (especially in wet soils) may all contribute to low O$_2$ concentrations in the rhizosphere, and O$_2$ microgradients between the root and the bulk soil may develop. Thus, while NO$_3^-$ is produced by nitrification activity in theoxic part of the microenvironment, it is in turn the substrate of denitrification when O$_2$ availability is low. NO$_3^-$ is usually only present in small concentrations in natural soils, probably because it is an intermediate in both nitrification and denitrification. Nevertheless, NO$_2^-$ has been suggested to stimulate root growth by a phytohormonal effect (16). Detailed studies of NO$_3^-$ and NO$_2^-$ turnover in rhizosphere microgradients may provide new information about NO$_3^-$ and NO$_2^-$ effects on root growth and about nitrification and denitrification activities, including the coupling between the two processes, in the rhizosphere.

When studying the nitrogen dynamics in such microgradients, it is necessary to determine the nitrogen pools in very small samples taken close to the root surface. NO$_3^-$ and NO$_2^-$ are usually determined by colorimetry (1) or by high-performance liquid chromatography with UV detection (3), but these methods may not be sensitive enough when analyzing small samples of soil. Alternatively, NO$_3^-$ or NO$_2^-$ can be converted to N$_2$O in the samples, e.g., by denitrifying bacteria blocked in reduction of N$_2$O to N$_2$, and subsequently determined by gas chromatography. Such a bioassay has been used to determine NO$_3^-$/NO$_2^-$ in natural water samples with the addition of denitrifying *Pseudomonas chlororaphis* ATCC 43928 (3) and in soil samples by C$_2$H$_2$ inhibition of N$_2$O reductase activity (9).

In this report, we present a method to determine both NO$_3^-$ and NO$_2^-$ pools in 10-mg soil samples by a sensitive denitrification bioassay. We used a ClO$_3^-$-resistant mutant of denitrifying *Pseudomonas aeruginosa* ON12 (12), which was unable to reduce NO$_3^-$, to convert the indigenous NO$_2^-$ pool into N$_2$O for gas chromatographic analysis. Subsequently, other *P. aeruginosa* cells capable of reducing NO$_3^-$ were used to convert the NO$_3^-$ pool into N$_2$O. Both the wild-type and mutant strains of *P. aeruginosa* ON12 have a functional N$_2$O reductase when grown in complex medium with NO$_3^-$ or NO$_2^-$ as the electron acceptor. In contrast, the N$_2$O reductase produced during growth in citrate minimal medium on NO$_3^-$ or NO$_2^-$ remained inactive in the stationary phase and when the cells were subsequently used in the bioassay. This strain could thus be used in the bioassay without the addition of acetylene to prevent N$_2$O reduction; the possible problem of incomplete acetylene inhibition of N$_2$O reductase at low concentrations of NO$_3^-$ or NO$_2^-$ (11) was therefore avoided. The present bioassay easily separates both NO$_3^-$ and NO$_2^-$ pools from other N compounds in the sample and is therefore also a useful tool when preparing samples for $^{15}$N isotope analysis by mass or emission spectrometry (3).

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** *P. aeruginosa* isolate ON12 (12) was chosen for the bioassay because of its high growth rate in O$_2$-free tryptic soy broth (Difco) with 3 mM NaNO$_2$. One milliliter of a stationary-phase culture was used to inoculate a flask with tryptic soy broth medium also containing 3 mM NaNO$_2$ and 10 mM KClO$_3$. A chlorate-resistant culture which was unable to reduce NO$_3^-$ grew up

* Corresponding author.
within 1 week. Two milliliters of this culture was used to inoculate a 500-ml infusion bottle containing 400 ml of a citrate minimal medium with 3 mM NaNO₃ and 10 mM KClO₃. The citrate medium had the following composition (grams per liter): trisodium citrate, 7; NH₄Cl, 1; K₂HPO₄, 2; KH₂PO₄, 2; MgSO₄, 0.2; Fe₃(PO₄), 0.02; 1 ml of a 1 mM trace mineral solution (10). When the first NO₂⁻ pool was exhausted after about 1 week, the NaNO₂ concentration was again adjusted to 3 mM to obtain a high cell density while avoiding toxic levels of NaNO₂. These cells were then used to inoculate another batch of citrate minimal medium with NO₂⁻ and ClO₃⁻. From this culture, cells were streaked on King’s B (Difco) and grown for 2 days at 30°C under aerobic conditions. Several colonies were screened for KClO₃ resistance by inoculation on tryptic soy broth with 3 mM NaNO₂ and 10 mM KClO₃ under anaerobic conditions. Among the ClO₃⁻-resistant colonies, one colony was picked out for the NO₂⁻ bioassay because of its lack of NO₃⁻ reductase activity when tested for growth in tryptic soy broth with 10 mM NaNO₃ and 10 mM ClO₃⁻.

For the NO₃⁻ determinations, cells of the ON12 wild-type isolate were grown in the citrate minimal medium as above except that KClO₃ was omitted and a single addition of 10 mM NaNO₃ was used as the oxidant rather than NaNO₂. All cultures were grown at 30°C in a shaking water bath.

**Determination of NO₃⁻ and NO₂⁻.** Cells from the early-stationary-phase cultures grown on NaNO₃ or NaNO₂, were harvested by centrifugation (9,600 × g, 20 min, 5°C) and resuspended in their growth media to optical densities of approximately 1.0 and 0.5 (660 nm), respectively. To obtain an NO₂⁻-free medium for the NO₂⁻ bioassay, 5 ml of NO₃⁻-grown cells was added to 50 ml of the citrate minimal medium. After anaerobic incubation for 30 min, the NO₂⁻-reducing cells were subsequently removed by centrifugation and the medium was filtered through a 0.2-μm-pore-size polycarbonate filter (Sartorius, Göttingen, Germany). The NO₂⁻-grown cells were first washed once in 15 ml and then resuspended in 35 ml of this NO₂⁻-free medium. The suspensions of NO₂⁻- or NO₃⁻-grown cells were purged with N₂ for complete removal of N₂O and kept in 120-ml serum bottles. If kept in a refrigerator, the cultures could be used for up to 1 week after preparation.

NO₃⁻ and NO₂⁻ measurements in liquid standards (0.1 ml) or soil samples (10 to 500 mg of soil in 0.1 ml of membrane-filtered water) were performed at room temperature in 3.5-ml blood collection vials (Venoject; Terumo, Leuven, Belgium) unless otherwise indicated. Standards were prepared by serial dilutions of a stock solution containing 1 mM NaNO₃ and 1 mM NaNO₂ in deionized membrane-filtered water (Milli-Q; Millipore). An N₂ purge of the samples in the Venoject vials was performed to obtain a low background level of O₂ and N₂O and then 0.2 ml of the NO₂⁻-grown culture was injected into the vials with a 1-ml syringe and the N₂ purge was removed. The N₂O concentration was analyzed at time zero, at about 10 min, and again at about 20 min to ensure that a constant level of N₂O was achieved corresponding to a complete conversion of NO₂⁻. After stabilization of N₂O concentrations, 0.2 ml of the NO₃⁻-grown culture was added and the gas analysis was repeated. Blank samples with 0.1 ml of water lacking NO₃⁻ or NO₂⁻ always produced insignificant amounts of N₂O.

**Collection and treatment of soil samples.** Barley plants (*Hordeum vulgare*) were collected from a field site (loamy sand) immediately before harvest in late August. The plants were carefully brought to the laboratory without disturbing the root system in the soil. Samples were taken immediately from one-half of the soil samples, which were relatively dry at the sampling time (10% [wt/wt] of water). The other half was wetted (up to approximately 18% [wt/wt] of water) and incubated for 10 days loosely covered by aluminum foil at in situ temperatures.

Samples from the root zone were fractionated into (i) bulk soil, not associated with the roots; (ii) rhizosphere soil, adhering to the roots after gentle shaking (rhizosphere soil was separated from the root by vortexing in 0.1 ml of cold water); and (iii) rhizoplane, representing root surface-associated material after the adhering soil was removed. All treatments were done on ice to avoid any changes of the indigenous NO₃⁻ and NO₂⁻ pools. All Venoject vials were immediately closed by rubber stoppers; before injection of cell suspensions for bioassay, the vials were placed in a 70°C water bath for 10 s to prevent activity of indigenous nitrifying or denitrifying bacteria during the assay.

Soil water content was calculated by drying ca. 200 g of bulk soil overnight at 105°C. Fresh weight of the root was determined at the end of the fractionation procedure after adherent water had been removed by blotting on filter paper.

**Analyses and calculations.** N₂O was measured on a gas chromatograph (Packard model 428) equipped with an electron capture detector and a Porapak Q column. The analysis was performed on 0.3-ml gas samples withdrawn from the Venoject vials after injection of 0.3 ml of N₂ with the gastight syringe. The Bunsen solubility coefficient (15) was used to calculate the fraction of N₂O dissolved in the water phase. Details of calculations can be found in Christensen and Tiedje (3).

For comparison with the bioassay, colorimetric determinations of NO₃⁻ and NO₂⁻ (1) were made on 1.5-g soil samples extracted in 2 ml of 2 M KCl for 2 h.

**RESULTS**

**Bioassays.** Figures 1 and 2 show the general outline of the bioassay. A rapid and complete conversion of both NO₃⁻ and NO₂⁻ pools into N₂O occurred after the denitrifying cells were added to the O₂-free vials.

A sample with 10 nmol of NO₃⁻ was used to test whether the NO₂⁻-reducing mutant cells could reduce NO₃⁻ if KClO₃ was omitted in the assay suspension; there was no production of N₂O from NO₃⁻ by the NO₂⁻-reducing culture (Fig. 1). When KClO₃ was included in the assay suspension, KClO₃ concentrations up to 8 mM had no influence on the NO₃⁻ reduction by wild-type cells grown without KClO₃.
Only when such cells were exposed to 10 mM KClO₃ did NO₃⁻ reduction decline, and it finally ceased when only about 40% of NO₂⁻ was recovered as N₂O.

Standard curves obtained by adding the NO₂⁻-reducing and then the NO₃⁻ (and NO₂⁻)-reducing culture of P. aeruginosa ON12 to 0.1-ml water samples with equal amounts of NO₃⁻ and NO₂⁻ are shown in Fig. 3. The double-logarithmic plots show that all NO₃⁻ and NO₂⁻ were completely reduced to N₂O by the bacteria. Controls with the NO₂⁻-reducing mutant in 1 mM NO₃⁻ (NO₂⁻-free) standards gave insignificant N₂O production, as did the blank samples with cell suspensions lacking NO₃⁻ or NO₂⁻. These controls confirmed that NO₃⁻ was not reduced by the NO₂⁻-reducing cells and that no NO₂⁻ was left in the suspensions with NO₂⁻-reducing cells. Ten picomoles of N in a 0.1-ml sample (100 nM) represented the lower detection limit of the bioassay since N₂O levels in the blanks (without NO₃⁻ or NO₂⁻) at this concentration yielded up to 60% of the total peak height obtained with 10 pmol of NO₃⁻ or NO₂⁻. The standards were not extended to higher amounts than 10 nmol of NO₃⁻ or NO₂⁻ because the standard curve based on N₂O peak heights became nonlinear on our electron-capture-equipped gas chromatograph when the gas samples contained more than 40 ppm N₂O (corresponding to approximately 12 nmol of N).

Figure 2 shows the N measured as N₂O in a 0.1-ml water sample containing NO₃⁻ and NO₂⁻ standards and in a 100-ng soil sample after the addition of 0.2 ml of NO₂⁻-reducing cell suspension (arrow 1) and 0.2 ml of NO₃⁻-reducing cell suspension (arrow 2). In both cases, the conversion into N₂O was rapid and complete conversions were obtained within 5 to 15 min depending on the amount added. In the water sample with 5 nmol of NO₂⁻ and 60 nmol of NO₃⁻, a total of 4.9 ± 0.1 and 59.5 ± 0.2 nmol, respectively, were recovered as N₂O-N. The soil sample was found to contain 0.13 ± 0.01 nmol of NO₂⁻ and 4.49 ± 0.01 nmol of NO₃⁻. The standard deviations were calculated from three to four measurements.

When the bioassay is used in soil samples, it is important to avoid interference from concomitant NO₂⁻ or NO₃⁻ production by indigenous nitrification or denitrification activity. Figure 4 shows results from control experiments to identify such interferences. Sieved (2-mm mesh) soil samples of 0.5 g were placed in vials. One sample was incubated aerobically at room temperature for 1 h to stimulate nitrification activity. After this, the sample was analyzed for NO₂⁻ as in the standard assay, except that the initial heat treatment to denature the soil enzymes was omitted. The other sample received 0.2 ml of water without preincubation and was heat denatured as in the standard assay. The preincubated sample produced NO₂⁻ both during the 1 h in air and during the assay. In the standard bioassay, 10 min of incubation is adequate time to determine the indigenous NO₂⁻ pools in soil samples. However, two to three times more NO₂⁻ was reduced to N₂O during the first 10 min of the
TABLE 1. Determination of NO₃⁻ and NO₂⁻ concentrations in field soils by using the denitrification bioassay or colorimetric method

<table>
<thead>
<tr>
<th>Soil</th>
<th>NO₃⁻</th>
<th>NO₂⁻</th>
<th>Bioassay</th>
<th>Colorimetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clay</td>
<td>334 ± 8</td>
<td>308 ± 5</td>
<td>14.3 ± 1.0</td>
<td>14.6 ± 2.0</td>
</tr>
<tr>
<td>Sand</td>
<td>265 ± 68</td>
<td>229 ± 14</td>
<td>0.9 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Sand</td>
<td>1,590 ± 250</td>
<td>1,329 ± 75</td>
<td>0.13 ± 0.05</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
</table>

* Standard deviations (n = 4) are indicated.
* 1- to 3-g samples extracted for 12 h (clay) or 2 h (sand) in 2 ml of 2 M KCl.
* Freshly collected in field (April 1992).
* Stored for 6 months in plastic bags at 5°C (collected in October 1991).

When the bioassay was compared with standard colorimetry, the two techniques gave similar results for both NO₃⁻ and NO₂⁻ concentrations. Table 1 shows results from this comparison in three different soils, representing both a clay and two different sandy soils.

NO₂⁻ and NO₂⁻ pools in the barley rhizosphere. Samples were taken from two different depth horizons in the root zone: one from the proximal part of the root system where each root was surrounded by a well-developed layer of root hairs imbedded in mucus, and a second horizon approximately 10 cm below the soil surface, where thinner roots with a less developed rhizosheet were typical.

The NO₃⁻ and NO₂⁻ pools measured in the rhizoplane (mucous layer, etc.), the rhizosphere (soil adhering to the root), and the bulk soil from the two horizons are shown in Fig. 5. The left panel shows results from samples taken at in situ water content (10%, wt/wt) immediately after the samples were brought to the laboratory. The right panel contains results from the soil which was wetted to 18% (wt/wt) of water and preincubated in pots for 1 week at in situ temperature.

The NO₃⁻ and NO₂⁻ data from the rhizoplane (upper part of Fig. 5) were expressed per unit weight of fresh root and, therefore, are not directly comparable to the data from the rhizosphere and bulk soil (bottom of Fig. 5), which were expressed per unit weight of dry soil. Nevertheless, the

Fig. 5. NO₃⁻ (□) and NO₂⁻ (□□) in the rhizosphere of barley immediately after sampling (late August; 10% water, wt/wt) and after incubation for 1 week after wetting (18% water, wt/wt). Samples were taken near the soil surface (0 to 1 cm) and from a deeper layer (8 to 10 cm). NO₃⁻ and NO₂⁻ were measured in the rhizoplane, rhizosphere, and bulk soil (see text). Bars indicate standard errors of the mean (n = 4).
relative sizes of the NO$_3$\(^-\) and NO$_2$\(^-\) pools in the rhizoplane and rhizosphere layers could still be compared, and NO$_2$\(^-\) was always found in higher proportion in the rhizoplane (the NO$_3$\(^-\) pool was from 0.7 to 5.9\% of the NO$_3$\(^-\) pool) than in the rhizosphere soil (the NO$_3$\(^-\) pool was from 0.1 to 1.4\% of the NO$_3$\(^-\) pool). This was particularly pronounced in the upper depth horizon of the wet soil.

Higher amounts of NO$_3$\(^-\)$ were generally observed in the rhizosphere soil than in the bulk soil, in both the upper and lower horizons. The difference was most pronounced at the in situ (low) water content, at which NO$_3$\(^-\)$ was much higher in the rhizosphere soil (2.9 and 1.9 \(\mu\)mol of N per g of dry soil in the upper and lower depth horizons, respectively) than in the bulk soil (0.6 and 0.9 \(\mu\)mol of N per g of dry soil). In the preincubated wet soil, the NO$_3$\(^-\)$ content in the rhizosphere was significantly lower (1.0 and 1.1 \(\mu\)mol of N per g of dry soil), but still slightly above that in the bulk soil (0.7 and 0.6 \(\mu\)mol of N per g of dry soil). The NO$_2$\(^-\)$ content was also significantly higher in the rhizosphere soil than in the bulk soil, particularly in the upper horizon.

**DISCUSSION**

Denitrifying bacteria have previously been used in bioassays to measure low concentrations of NO$_3$\(^-\)$ in natural water (3) or soil samples (9). However, the present study is the first to include the NO$_2$\(^-\)$ pool in such a bioassay. Since both NO$_3$\(^-\)$ and NO$_2$\(^-\)$ are converted to N$_2$O and subsequently measured by gas chromatography, it is first of all important that the two compounds can be discriminated in the bioassay and that further reduction of N$_2$O to N$_2$ is excluded. In preliminary experiments with *Pseudomonas* species unable to synthesize N$_2$O reductase, we observed that both *P. chlororaphis* (ATCC 43928) used by Christensen and Tiedje (3) and *P. aureofaciens* (ATCC 13985) grew very slowly with NO$_2$\(^-\)$ as the only electron acceptor. In the present study, we therefore used either wild-type or mutant (NO$_2$\(^-\)$ reductase deficient) cells of our *P. aeruginosa* ON12 isolate (12), which both grew rapidly on citrate minimal medium with NO$_3$\(^-\)$ or NO$_2$\(^-\)$ as the electron acceptor. This isolate further synthesized an N$_2$O reductase, which was inactive in both the logarithmic and stationary phases in the culture medium (1a) and in the bioassay cell suspensions.

Determination of NO$_3$\(^-\)$-pools by conversion into N$_2$O required NO$_2$\(^-\)$-reducing cells that were unable to reduce NO$_3$\(^-\)$. To obtain such cells, we amended the NO$_2$\(^-\)$-containing citrate medium with 10 mM KClO$_4$ and subsequently isolated a mutant which grew rapidly with NO$_2$\(^-\)$ as the electron acceptor but was unable to reduce NO$_3$\(^-\)$. The cells were unaffected by the presence of KClO$_4$ and grew at a high rate similar to that of the wild-type cells in medium without KClO$_4$. This indicated that we had obtained a mutant resembling the ClO$_3$\(^-\)$-resistant mutants (6, 13), which synthesize subunits of NO$_2$\(^-\)$ reductase during growth on KClO$_4$, but are unable to insert a functional molybdenum cofactor into the enzyme. By comparison, growth of the wild-type cells soon ceased completely when the cells were exposed to KClO$_4$ (data not shown), probably because of the toxic ClO$_3$\(^-\)$ being produced from ClO$_2$\(^-\)$ reductase by the functional NO$_3$\(^-\)$ reductase.

A combined denitrification assay for determination of both NO$_3$\(^-\)$ and NO$_2$\(^-\)$ pools in water and soil samples has several advantages compared with conventional techniques. One is the low detection limit for N$_2$O produced by the denitrifying bacteria, using electron capture detection in gas chromatography. We thus found a detection limit of 10 pmol of N for the combined NO$_3$\(^-\)$ and NO$_2$\(^-\)$ bioassay and were able to measure both NO$_3$\(^-\)$ and NO$_2$\(^-\)$ in 10-mg nonfertilized soil samples. Christensen and Tiedje (3) in their NO$_3$\(^-\)$ bioassay measured down to about 10 pmol of N from NO$_3$\(^-\)$ in 10-mI water samples.

The validity of the NO$_3$\(^-\)$ and NO$_2$\(^-\)$ bioassay for use in soils was checked. (i) Comparison with a conventional colorimetric assay was performed in different soil types. No significant differences were observed between NO$_3$\(^-\)$ and NO$_2$\(^-\)$ concentrations determined by the two techniques (Table 1). (ii) Indigenous soil bacteria would interfere with the denitrification bioassay if precautions were not taken to exclude their activity (Fig. 4). This problem was eliminated by briefly heating the soil sample to 70°C before the denitrifying cell suspension was added. (iii) Determination of inorganic N pools in soil often involves extraction with KCl, e.g., for exchangeable NH$_4^+$. The present bioassay was not affected by 2 M KCl concentrations in the extracts (data not shown), and extraction of the exchangeable NH$_4^+$ pool in 2 M KCl will therefore not exclude determination of NO$_3$\(^-\)$ and NO$_2$\(^-\)$ in the sample by the bioassay.

We used the bioassay to determine NO$_3$\(^-\)$ and NO$_2$\(^-\)$-concentration gradients over short distances in the rhizosphere of barley plants. The highest concentrations of both NO$_3$\(^-\)$ and NO$_2$\(^-\)$ were generally observed in the rhizosphere soil close to the root, indicating a net accumulation of the two compounds from nitrification activity at the root surface. Nitrification activity is the only source of NO$_3$\(^-\)$ production in the soil after NO$_3$\(^-\)$ fertilizer added in the spring is consumed. When comparing the results from soils with 10 and 18% (wt/wt) of water, relatively less NO$_3$\(^-\)$ but more NO$_2$\(^-\)$ was observed in the rhizosphere (and rhizoplane) at the higher water content. This could be a consequence of either decreasing nitrification or increasing denitrification activities. Wetting of the soil probably resulted in decreased O$_2$ concentrations near the root, where O$_2$ consumption exerted by both the root tissue and the microbes is likely to be higher than that in the bulk soil (2). With anaerobic conditions appearing in the rhizosphere of such a wetted soil, denitrification activity may result in higher NO$_3$\(^-\)$ consumption and thus a lower NO$_3$\(^-\)$ concentration than in the dry soil. Alternatively, nitrification activity could be impeded at the decreased O$_2$ concentrations in the wet soil. Furthermore, since NH$_4^+$-oxidizing nitrifiers may be more active at the low O$_2$ concentrations than NO$_2$\(^-\)$-oxidizing nitrifiers (5, 8), the wet soil conditions could favor accumulation of NO$_2$\(^-\)$ rather than NO$_3$\(^-\)$. However, we cannot in detail discern the relative importance of nitrifiers and denitrifiers in the NO$_3$\(^-\)$ and NO$_2$\(^-\)$ accumulations in the rhizosphere.

NO$_2$\(^-\)$ turnover is usually overlooked when studying nitrogen transformations in soil or sediment systems. Because it is an intermediate in both nitrification and denitrification, the NO$_2$\(^-\)$ turnover studied by the $^{15}$N technique may reveal the coupling between nitrification and denitrification and thus the influence of O$_2$ levels on these processes. The present bioassay offers a new approach to determine $^{15}$N contents in small NO$_3$\(^-\)$ and NO$_2$\(^-\)$ pools by converting them into N$_2$O (or N$_2$ if the cells were grown and suspended in a medium supporting an active N$_2$O reductase) as a preparative step for $^{15}$N mass or emission spectrometry. Most chemical techniques to convert nitrogen compounds into N$_2$ for $^{15}$N studies are hampered by the risk of interference from other nitrogen compounds (7). The bioassay approach holds further promise in analyzing very small amounts of NO$_3$\(^-\)$.
and NO$_3^-$ because the risk of contamination from atmospheric N$_2$ may be virtually eliminated.

When the bioassay method is used to measure NO$_3^-$ and NO$_2^-$ in soil samples, the resolution of sampling under the microscope becomes limiting, rather than sensitivity of detection (sample size). In comparison, the newly developed NO$_3^-$ microsensors (4, 14) combine excellent resolution and high sensitivity but are not easily applied because of the physical restraints, air-filled pores, and possible interference from other ions in the heterogeneous soils.

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