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Conversion of Ammonia to Dinitrogen in Wastewater by *Nitrosomonas europaea*

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Abstract

Because *Nitrosomonas europaea* contains ammonia-oxidizing enzyme, nitrite reductase, and nitrous oxide reductase, the conversion of ammonia to dinitrogen was tried with different reaction conditions. In aerobic reaction conditions, ammonium was converted to nitrite (NO_2^-), while under oxygen-limiting or oxygen-free conditions, NO_2^--N formed from ammonia oxidation by *N. europaea* was reduced to N_2O and dinitrogen with 22% conversion. During denitrification, optimal pH for the production of N_2O and dinitrogen was found to be 7.0–8.0. Dinitrogen was not produced in acidic pH <7.0. A low partial oxygen pressure as well as oxygen-free conditions are favorable for high production of dinitrogen.

Index Entries: Denitrification; dinitrogen; nitrous oxide; nitrification; *Nitrosomonas europaea*.

Introduction

Nitrogen removal is an important process in present-day wastewater treatment. Recently, a range of new microbial processes that might occur in wastewater treatment plants (e.g., aerobic denitrification and heterotrophic nitrification *[1,2]*, anaerobic ammonium oxidation *[2]*, and denitrification by lithoautotrophic nitrifying bacteria *[3,4]*) has been reported. In wastewater treatment, lithoautotrophic ammonia-oxidizing bacteria are critical

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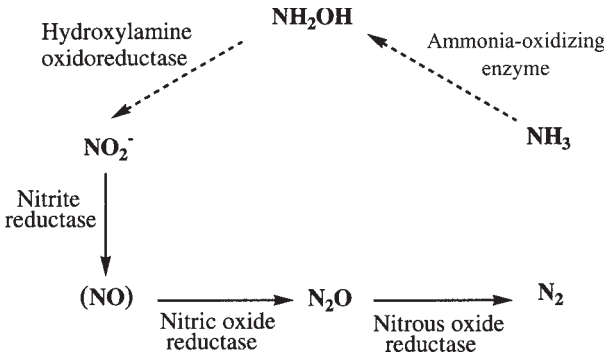

Scheme 1. Proposed pathway of conversion of ammonia to dinitrogen by *N. europaea*. – – – – – –→, Nitrification; ————→, denitrification.

for the oxidation of ammonium to nitrite. Ammonia-oxidizing bacteria of the genus *Nitrosomonas* are lithoautotrophic nitrifying bacteria that can be utilized for both nitrification and denitrification in removing ammonia nitrogen from wastewater. The bacterial cells gain their energy by oxidizing ammonia to nitrite in a two-step reaction *(5)*. The initial oxidation of ammonia yields hydroxylamine. This initial reaction is O_2 dependent and catalyzed by ammonia monooxygenase. Subsequently, hydroxylamine is oxidized to nitrite catalyzed by hydroxylamine oxidoreductase. Two of the four electrons generated from hydroxylamine oxidation are used to support the oxidation of additional ammonia molecules, while the other two electrons enter the electron transfer chain and are used to support adenosine triphosphate biosynthesis and CO_2 reduction *(5)*.

Recently, *Nitrosomonas* has also been shown to be a denitrifying organism *(6,7)* that uses nitrite reductase *(8)* and nitrous oxide reductase *(9,10)* to form nitrous oxide (N_2O) and dinitrogen, respectively. By using nitrite as an electron acceptor, cells can save oxygen under O_2-limiting conditions by employing the nitrite in only monooxygenase reactions *(11)*. When *Nitrosomonas* cells are adapted to oxygen limitation, nitrification levels are low, and the little nitrite formed is reduced to gaseous N compounds *(10)*. Dinitrogen is the main end product under these conditions *(4)*.

However, denitrifying capabilities in the *Nitrosomonas* genus have not been used in the treatment of wastewater, since denitrification is usually regarded as an anoxic process performed by chemoorganotrophic bacteria. However, when using chemoorganotrophic bacteria for the denitrification process, strong efforts are needed to separate oxic lithoautotrophic nitrification from anoxic heterotrophic denitrification *(12)*. Moreover, since chemoorganotrophic denitrifiers need an organic electron donor, additional substrates are often necessary to achieve complete denitrification *(13)*. By contrast, ammonia oxidizers of the genus *Nitrosomonas*, such as those found in sewage sludge, seem to be capable of nitrification and simultaneous denitrification by using ammonia as an electron donor (Scheme 1).

If the optimal conditions of this process are defined and these nitrifiers/denitrifiers can be employed in controlled conditions, there may be no need for large-scale separation of nitrification and denitrification.

The aim of the present study was to investigate whether it is possible to achieve significant dinitrogen conversion of ammonia using a pure culture of *N. europaea* in a laboratory-scale reactor. The effect of oxygen in the denitrification process performed by *N. europaea* was investigated.

Materials and Methods

Organism

The experiments were performed with *N. europaea* (ATCC 25978).

Medium

N. europaea were grown lithoautotrophically in ATCC medium 1573 of the following composition: 1.7 g of $(NH_4)_2SO_4$, 20 mg of $CaCl_2{\cdot}2H_2O$, 200 mg of $MgSO_4{\cdot}7H_2O$, 1 mg of FeEDTA, 15 mg of K_2HPO_4, 1 mL of trace elements (10 mg of $Na_2MoO_4{\cdot}2H_2O$, 20 mg of $MnCl_2{\cdot}4H_2O$, 10 mg of $ZnSO_4{\cdot}7H_2O$, 0.2 mg of $CoCl_2{\cdot}6H_2O$, 2 mg of $CuSO_4{\cdot}5H_2O$, and 100 mL of distilled water) and 1 L of distilled water. The medium was sterilized at 120°C for 20 min. Ammonium nitrogen (NH_4^+-N) contained in the growth medium was 360 mg/L.

Growth

Batch cultures of *N. europaea* (ATCC 25978) were grown in 300-mL flasks (200 mL of medium) while being stirred at room temperature. Flasks were aerated by an air pump continuously. The oxygen concentration in the culture medium was 7.3 mg/L. Air was sterilized through a 0.45-µm pore size air filter (FG50; Millipore). Throughout bacterial growth, the pH of the culture solution was adjusted to 7.5–7.8 with 10% K_2CO_3 using a peristaltic pump. After 3 d, 200-mL cultures were used to inoculate 550 mL of new medium. Cells were harvested during late logarithmic growth by centrifugation (6762*g* for 10 min) 7 d after inoculation. The resulting cell paste was washed with an ammonium-free medium. The ammonium-free medium was then used to generate a 1:1 ratio dilution of the cell paste and the diluted cells were stored at –80°C.

Oxidation of Ammonium and Formation of Nitrite

The oxidation of ammonia and formation of nitrite by *N. europaea* was observed in 300-mL capacity flask (200 mL of medium) batch cultures prepared as described above. The oxidation of ammonia and formation of nitrite was monitored over time from the beginning of the incubation to the stationary phase growth of cells. The growing cultures were withdrawn by syringes, filtered with a Millex-GP filter unit (0.22-µm pore size), and analyzed for ammonium and nitrite.

N_2 and N_2O Formation from Ammonia

The ability of *N. europaea* under O_2-limiting or O_2-free conditions to produce N_2O and dinitrogen was tested using batch-type reactors. The batch reactors employed 160-mL serum bottles with white rubber septums. The serum bottles were filled with 25 mL of 25.7 m*M* ammonium containing the ATCC 1573 medium and were then sterilized at 120°C for 20 min. Twenty-five milliliters of 3-d-old cultures of *N. europaea* was added aseptically to the serum bottles such that the total volume of the reaction mixture was 50 mL and the cell density was 1.05 mg of wet cell/mL. The pH of the solution was adjusted to 6.0–8.0 according to treatment conditions. Fifty millimolar MES-KOH buffer was used for pH values between 6.0 and 6.5 and 50 m*M* HEPES-HCl buffer was used for pH values between 7.0 and 8.0. Anaerobic conditions were made by the substitution of O_2 with helium gas (99.99% pure) inside the serum bottles. In aerobic conditions, oxygen concentration in the reaction solution was maintained at 0.6, 1.2, and 1.4 mg/L, respectively. Serum bottles were then incubated in a thermostatic water bath incubator at 30°C. Gas samples were taken from the headspace with a gastight syringe and analyzed by gas chromatography.

In another experiment, N_2O and dinitrogen production from externally added nitrite under anoxic conditions were determined as follows. Serum bottles (160 mL) were filled with 50 mL of ammonium-free ATCC 1573 medium containing 2–20 m*M* nitrite and 50 m*M* MES-KOH buffer (pH 6.0–6.5) or HEPES-HCl buffer (pH 7.0–8.0) according to reaction conditions. After sterilization at 120°C for 20 min, cell paste was used to inoculate the medium using a syringe. The serum bottles were sealed gastight with white rubber septums. Anaerobic conditions are generated as described above. Serum bottles were then incubated in a thermostatic water bath at 30°C. Gas samples from the headspaces were analyzed by gas chromatography.

Analytical Procedures

Nitrite and nitrate concentrations were determined colorimetrically *(14)* or by means of high-performance liquid chromatography techniques (4.6 × 250 mm; Inertsil ODS-3) in 5 m*M* tetrabutyl-ammoniumhydrogensulfate in 10% (v/v) methanol at pH 6.4 *(15)* and flow rate of 1 mL/min. The samples were visualized by UV-VIS detection (wavelength 225 nm; UV-VIS detector SPD-10AV Shimadzu; Kyoto, Japan).

Ammonium was determined colorimetrically *(14)*. N_2O and nitrogen gas levels were determined by gas chromatography using a Shimadzu gas chromatograph Model GC-8AIT equipped with a thermal conductivity detector. Helium was used as the carrier gas (99.99% pure). The chromatographic column was stainless steel (3 m × 2 mm) packed with a 13X molecular sieve. Biomass (*N. europaea*) was determined by a UV-VIS spectrophotometer (Hitachi U-2000) with the absorbance at 660 nm.

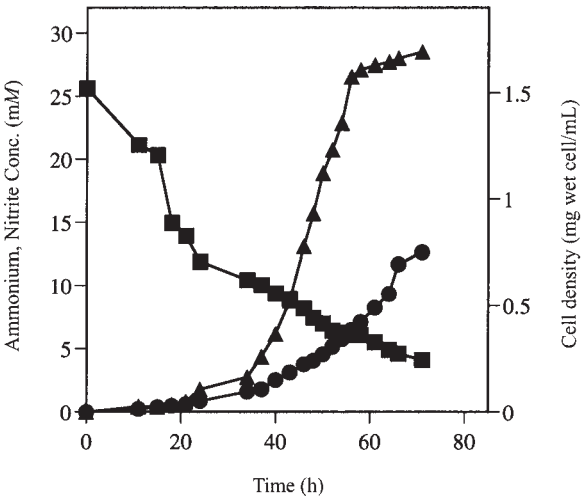

Fig. 1. Ammonium oxidation and nitrite formation during the growth of *N. europaea* over time. ■, Ammonium; ●, nitrite; ▲, cell density.

Results and Discussion

When the flask was stirred without aeration or pH adjustment, no cell growth was detected and hardly any ammonium was utilized (data not shown). The optimal pH range for the growth of *N. europaea* in pure culture is reported to be 5.8–8.5 *(16)*. Below pH 5.8, ammonia oxidation ceases almost completely. Ammonia oxidation is not favorable at acidic pH because the NH_3-NH_4^+ equilibrium shifts toward NH_4^+ and away from the NH_3 substrate for ammonia monooxygenase *(17)*. Therefore, when the flasks were stirred with aeration and the pH (7.5–7.8) was adjusted with 10% K_2CO_3, ammonia oxidation was detected (Fig. 1). The consumption of ammonium and formation of nitrite did not occur at a 1:1 ratio. This is likely because some ammonium is utilized by the cells for their internal metabolism, whereas other ammonium changes into hydroxylamine. The ammonium consumption was coupled to an exponential increase in cell density until a maximum value was reached. After 50 h of incubation, ammonia oxidation and the formation of nitrite continued to occur further biomass production. The growth of *N. europaea* did not stop completely and gradually increased, and the ammonium oxidation and nitrite formation proceeded also after 50 h of incubation. Figure 1 shows that when the production of biomass increased gradually, ammonium oxidation and nitrite formation continued to occur. Some nitrate was also seen in the stationary phase of growth (data not shown). The generation time and growth rate constant of *N. europaea* were found to be 9.03 h and 7.67×10^{-2}/h, respectively. The ammonium oxidation was also observed without *N. europaea* at the pH and aeration used in the *N. europaea*. No ammonium oxidation was observed without *N. europaea*. Thus, ammonium stripping did not occur at the pH and aeration used.

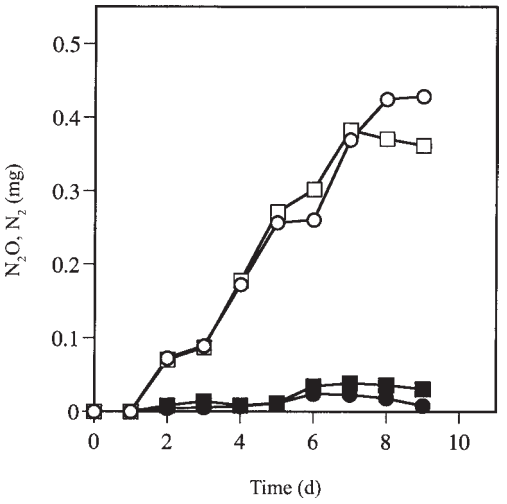

Fig. 2. Production of N_2O and N_2 from ammonium by *N. europaea* during denitrification. The serum bottles contained 50 mL of 3-d-old culture and 11.16 mg of NO_2^--N produced by ammonium oxidation. Solid symbols represent the production of N_2O: ■, anaerobic; ●, aerobic (0.6 mg of O_2/L). Open symbols represent the production of N_2: □, anaerobic; ○, aerobic (0.6 mg of O_2/L). The reactions were carried out in anaerobic conditions at 30°C.

The capacity of *N. europaea* for denitrification was observed. As shown in Fig. 2, when 50-mL samples of the 3-d-old cultures (2.12 mg of wet cell/mL) were transferred to the gastight serum bottles and anaerobic conditions generated by helium gas (99.99% pure) substitution, the NO_2^--N from ammonium oxidation in the 50-mL culture formed was reduced to dinitrogen and N_2O-N at 3.4 and 2.2% conversion efficiencies, respectively. However, when the oxygen concentration on the reaction solution was maintained at 0.6 mg/L, conversion efficiencies for dinitrogen and N_2O-N were found to be 3.85 and 1.35%, respectively. In the anaerobic denitrification, dinitrogen was detected as an end product. Ammonium apparently served as an electron donor, and N_2O was observed as an intermediate. These results indicate that denitrification was indeed carried out by *N. europaea*.

The effect of oxygen on the production of dinitrogen and N_2O in the denitrification process by *N. europaea* was observed. When 25-mL samples of 3-d-old cultures (2.12 mg of wet cell/mL) were transferred into the gastight serum bottles containing 25 mL of fresh medium with 50 m*M* HEPES-HCl buffer (pH 7.5), NO_2^--N in 50 mL of anaerobic reaction solution was reduced to dinitrogen and N_2O-N at 21.11 and 1.90% conversion efficiencies, respectively. When the oxygen concentration in the reaction solution was maintained to 0.6, 1.2, and 1.4 mg/L, respectively, dinitrogen and N_2O conversion levels decreased with increasing oxygen concentration (Table 1). These results show that low partial oxygen pressure or oxygen-free conditions are suitable for the production of dinitrogen

Table 1
Effect of Oxygen on Production
of Dinitrogen and N_2O by *N. europaea* During Denitrification

Reaction condition[a]	NO_2^--N in 50-mL solution (mg)[b]	N_2O-N conversion (%)[c]	N_2 conversion (%)[c]
Anaerobic	5.78	1.90	21.11
Aerobic (0.6 mg O_2/L)	5.78	2.08	21.80
Aerobic (1.2 mg O_2/L)	5.78	1.04	6.23
Aerobic (1.4 mg O_2/L)	5.78	0.00	5.36

[a]Each 50-mL solution contained 25 mL of 3-d-old culture and 25 mL of fresh medium in 50 m*M* HEPES-HCl buffer, pH 7.5, and the reaction was carried out for 9 d.
[b]NO_2^--N in the reaction solution was obtained by oxidation of ammonium.
[c]Percentage of conversion was calculated from the N_2 and N_2O-N from production levels converted from the NO_2^--N in the 50-mL solutions.

and N_2O during denitrification by *N. europaea*. While the production of dinitrogen and N_2O in aerobic (0.6 mg of O_2/L) and anaerobic conditions was found to be almost identical (Table 1), it was inhibited at higher O_2 concentrations. A more common denitrifying activity associated with ammonia oxidizers is the production of NO, N_2O, and N_2, mostly from the reduction of nitrite under low O_2 pressure. The enzymes responsible for this activity in *N. europaea* are a copper-containing nitrite reductase having cytochrome oxidase activity *(7,11,18)* and an N_2O reductase *(9,10)*. When the saturated oxygen concentration was present in the reaction mixture, no dinitrogen and N_2O formation occurred (data not shown), whereas it was observed under low partial oxygen pressure as well as in oxygen-free conditions. In support of the notion that *N. europaea* was indeed responsible for the conversions, it has been found that the reducing enzyme of *N. europaea* is active and able to reduce nitrite under low partial oxygen pressure *(9,15)*.

A change in pH showed a significant effect on the production of dinitrogen and N_2O. When 25-mL samples of the 3-d-old cultures were transferred to the gastight serum bottles containing 25 mL of fresh medium at different pH values ranging from 6.0 to 8.0, NO_2^--N in the 50-mL reaction solution was reduced to dinitrogen and N_2O (Fig. 3). Optimal pH for the production of dinitrogen and N_2O was found to be between 7.0 and 8.0. There were similar levels of dinitrogen and N_2O production at these pH values. No dinitrogen or N_2O was detected at acidic pH values <7.0. At lower pH values, nitrite spontaneously yields nitrosonium cation (NO^+) by the reaction of nitrous acid (HNO_2) with free protons *(19)*. Nitrosonium cations are highly reactive with metals, such as iron and copper, and readily form metal-nitrosyl complexes to prevent bacterial growth and functions *(20)*. Therefore, the lack of dinitrogen and N_2O production at lower pH values may be owing to NO^+ production followed by inactivation of the bacterial enzymes.

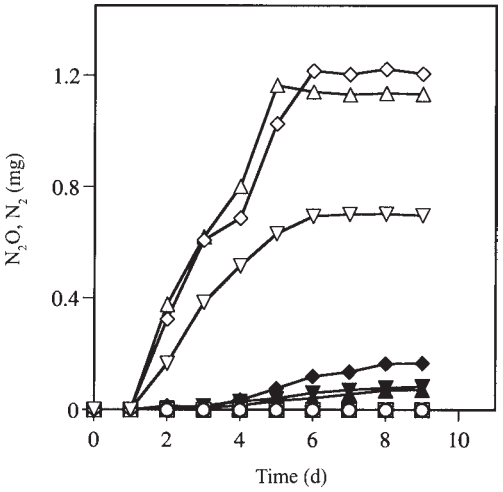

Fig. 3. Effects of pH on conversion of ammonium to N_2O and N_2 by *N. europaea* in denitrification. Fifty millimolar MES-KOH buffer (pH 6.0–6.5) and 50 m*M* HEPES-HCl buffer (pH 7.0–8.0) were used. Solid symbols represent the production of N_2O: ■, pH 6.0; ●, pH 6.5; ▲, pH 7.0; ◆, pH 7.5; ▼, pH 8.0. Open symbols represent the production of N_2: □, pH 6.0; ○, pH 6.5; △, pH 7.0; ◇, pH 7.5; ▽, pH 8.0. The reactions were carried out in anoxic conditions at 30°C.

When the bacteria were transferred to fresh medium with final ammonium concentrations of 12.86, 25, and 50 m*M*, respectively, NO_2^--N in all sample solutions was reduced to dinitrogen and N_2O (Fig. 4). However, samples with starting concentrations of ammonium >25 m*M* had lower dinitrogen and N_2O production. The dinitrogen conversion at 12.86, 25, and 50 m*M* was found to be 21.11, 22.49, and 6.47%, respectively, and N_2O-N conversion was 1.90, 2.08, and 0.35%, respectively. This indicates that a high amount of ammonium in the reaction solution is not suitable for high production of dinitrogen and N_2O.

Maximum reduction of NO_2^--N to dinitrogen was found to be 22%. When aerobically grown cells were not transferred into new media for the denitrification process, the conversion of NO_2^--N to dinitrogen was much lower, with a conversion level of only 4%. This indicates the necessity of fresh medium wherein bacteria can carry out denitrification after being transferred into serum bottles. The better response of the culture may be caused by some unknown final product, or owing to an exhausted unbalance culture.

Nitric oxide (NO) and N_2O are both well-known gaseous products of lithotropic ammonia oxidation. However, the mechanism of the formation of these compounds is still unclear. On the one hand, the production of NO and N_2O by ammonia oxidizer is attributed to reduction of nitrite by enzyme nitrite reductase using hydroxylamine as the electron donor *(8,11,18,21)*. On the other hand, it was assumed that NO is a direct product of the hydroxylamine oxidoreductase reaction *(22)*. The hypothetical intermedi-

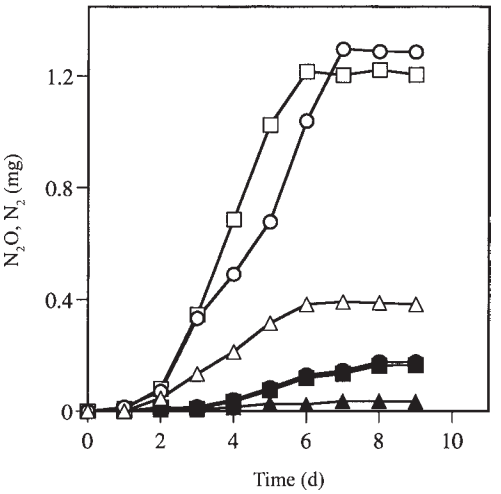

Fig. 4. Effects of ammonium on the production of N_2O and N_2 by *N. europaea* during denitrification. The 50 mL of solution in the serum bottles contained 12.86, 25, and 50 m*M* ammonium, respectively. Fifty millimolar HEPES-HCl buffer (pH 7.5) was used in the reaction mixture. Solid symbols represent the production of N_2O: ■, 12.85 m*M*; ●, 25 m*M*; ▲, 50 m*M* ammonium. Open symbols represent the production of N_2: □, 12.85 m*M*; ○, 25 m*M*; △, 50 m*M* ammonium. The reactions were carried out in anoxic conditions at 30°C.

ate of this pathway [NOH] is assumed to be unstable and NO might be formed directly from enzyme complex. Thus, in this experiment, the dinitrogen and N_2O produced by *Nitrosomonas* is proposed to be similar to a sequential pathway, as it is speculated for other denitrifiers: $NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ *(23,24)*. The proposed pathway by *Nitrosomonas* is shown in Scheme 1.

In another experiment, dinitrogen and N_2O produced from externally added nitrite and *N. europaea* in ammonium-free media was observed. Note that in this denitrification process, the source of the nitrite was not ammonium oxidation and the stored cells were added externally. The pH was adjusted in the range of 6.0–8.0 with MES and HEPES buffer. When 50-mL samples of ammonium-free medium containing 7 mg of NO_2^--N were transferred to the gastight serum bottles with 2.2 mg of wet cell/mL, NO_2^--N in the medium was reduced to dinitrogen and N_2O in samples in the pH range of 7.0–8.0. Favorable pH values for dinitrogen and N_2O production were found to be similar to that whereby bacteria-denitrified nitrite was produced via ammonium oxidation. In favorable pH values, dinitrogen conversion was 10% and N_2O-N conversion was 12–19% (Table 2). When the starting concentration of NO_2^--N was high in the reaction solution, dinitrogen and N_2O production was remarkably lower relative to production levels in samples with a lower starting concentration. Dinitrogen production was higher in samples with low starting concentrations whereas similar levels were produced in other samples (Table 3). When nitrite con-

Table 2
Effect of pH on Production
of Dinitrogen and N_2O *N. europaea* from Externally Added Nitrite
During Denitrification

pH of reaction solution[a]	NO_2^--N in 50-mL solution (mg)[b]	N_2O-N conversion (%)[c]	N_2 conversion (%)[c]
6.0	7.00	0.28	0.00
6.5	7.00	0.14	0.00
7.0	7.00	19.43	10.00
7.5	7.00	19.71	10.71
8.0	7.00	12.57	10.86

[a]Each 50-mL solution contained ammonium-free medium in 50 m*M* MES-KOH buffer, pH 6.0–6.5, and 50 m*M* HEPES-HCl buffer, pH 7.0–8.0, and cell density of 2.2 mg of wet cell/mL. The reaction was carried out in anaerobic conditions for 9 d.

[b]NO_2^--N in the reaction solution was added externally.

[c]Percentage of conversion was calculated from the N_2 and N_2O-N from production levels converted from the NO_2^--N in the 50-mL solutions.

Table 3
Effects of Externally Added Nitrite Quantity on Production
of Dinitrogen and N_2O by *N. europaea* During Denitrification

NO_2^--N in 50-mL solution (mg)[a,b]	N_2O-N conversion (%)[c]	N_2 conversion (%)[c]
1.40	15.00	78.57
3.50	28.86	19.71
7.00	19.71	10.71
14.00	4.57	5.57

[a]Each 50-mL solution contained ammonium-free medium in 50 m*M* HEPES-HCl buffer, pH 7.5, and a cell density of 2.2 mg of wet cell/mL. The reaction was carried out in anaerobic conditions for 9 d.

[b]NO_2^--N in the reaction solution was added externally.

[c]Percentage of conversion was calculated from the N_2 and N_2O-N from production levels converted from the NO_2^--N in the 50-mL solutions.

centration increases, N_2 production decreases. Thus, nitrite inhibited N_2 production. From this point, the pathway as mentioned in Scheme 1 is proposed.

When starting bacterial cell density was varied in the reaction mixture, dinitrogen and N_2O production differed drastically between samples. Table 4 reveals a trend of increasing dinitrogen conversion efficiency with increasing starting bacterial quantity. Conversion to dinitrogen was highest at 49% when 6.6 mg of wet cell/mL was used. By contrast, there was no similar trend found for the effect of bacterial concentration on the production of N_2O.

Table 4
Effect of Wet Cell Quantity (*N. europaea*)
on Dinitrogen and N_2O Production from Externally Added Nitrite
During Denitrification

Cell density in reaction solution (mg of wet cell/mL)[a]	NO_2^--N in 50-mL solution (mg)[b]	N_2O-N conversion (%)[c]	N_2 conversion (%)[c]
1.20	7.00	4.43	2.43
2.20	7.00	19.71	10.71
3.30	7.00	12.00	22.28
6.60	7.00	1.71	48.86

[a]Each 50-mL solution contained ammonium-free medium in 50 m*M* HEPES-HCl buffer, pH 7.5, and the reaction was carried out in anaerobic conditions for 9 d.
[b]NO_2^--N in the reaction solution was added externally.
[c]Percentage of conversion was calculated from the N_2 and N_2O-N from production levels converted from the NO_2^--N in the 50-mL solutions.

A cell quantity, an NO_2^--N concentration, and a pH were used (2.2 mg of wet cell/mL, 7 mg/50 mL, and 7.5 [50 m*M* HEPES-HCl buffer], respectively), that were otherwise indicated for the denitrification by *N. europaea* with externally added NO_2^--N. Denitrification levels by *N. europaea* from externally added nitrite and nitrite derived from ammonia oxidation were found to be similar. When the starting concentration of cells in the reaction solution was high, a higher conversion to dinitrogen was observed (Table 4). Therefore, to achieve high conversion of dinitrogen from NH_4^+-N in wastewater, the number of cells should be high. Recall that when aerobically grown cells were not transferred to fresh medium, low dinitrogen conversion efficiency was observed (Fig. 2), suggesting that aerobically grown cells should be transferred into fresh medium.

The results of this study indicate that *N. europaea* cells are capable of simultaneous nitrification and denitrification at low partial oxygen pressure as well as in oxygen-free conditions. If this process is carried out under controlled conditions, NH_4^+-N from wastewater can be converted to dinitrogen with high conversion efficiency without employing large-scale separation of nitrification and denitrification.

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