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Nitrous Oxide Production by *Alcaligenes faecalis* under Transient and Dynamic Aerobic and Anaerobic Conditions

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Nitrous oxide can be a harmful by-product in nitrogen removal from wastewater. Since wastewater treatment systems operate under different aeration regimens, the influence of different oxygen concentrations and oxygen fluctuations on denitrification was studied. Continuous cultures of *Alcaligenes faecalis* TUD produced N_2O under anaerobic as well as aerobic conditions. Below a dissolved oxygen concentration of 5% air saturation, the relatively highest N_2O production was observed. Under these conditions, significant activities of nitrite reductase could be measured. After transition from aerobic to anaerobic conditions, there was insufficient nitrite reductase present to sustain growth and the culture began to wash out. After 20 h, nitrite reductase became detectable and the culture started to recover. Nitrous oxide reductase became measurable only after 27 h, suggesting sequential induction of the denitrification reductases, causing the transient accumulation of N_2O. After transition from anaerobic conditions to aerobic conditions, nitrite reduction continued (at a lower rate) for several hours. N_2O reduction appeared to stop immediately after the switch, indicating inhibition of nitrous oxide reductase, resulting in high N_2O emissions (maximum, 1.4 mmol liter^{-1} h^{-1}). The nitrite reductase was not inactivated by oxygen, but its synthesis was repressed. A half-life of 16 to 22 h for nitrite reductase under these conditions was calculated. In a dynamic aerobic-anaerobic culture of *A. faecalis*, a semisteady state in which most of the N_2O production took place after the transition from anaerobic to aerobic conditions was obtained. The nitrite consumption rate in this culture was equal to that in an anaerobic culture (0.95 and 0.92 mmol liter^{-1} h^{-1}, respectively), but the production of N_2O was higher in the dynamic culture (28 and 26% of nitrite consumption, respectively).

Nitrous oxide (N_2O) production is undesirable, because it contributes to various environmental problems, including the greenhouse effect (33) and ozone depletion in the stratosphere (26). A significant part of the global N_2O emission can be attributed to microbial processes, especially nitrification and denitrification (19), which are used in biological wastewater treatment systems. Recently, it has been reported that a significant amount of biogenic N_2O production comes from suboptimally functioning wastewater treatment systems (17, 28). Studies with activated sludge from these systems indicated that the denitrification process might be the largest contributor to N_2O emission (12). In order to prevent such N_2O emission, it is essential that the underlying mechanisms are fully understood. Many studies suggest that the key parameters involved in N_2O production during nitrification and denitrification are related to environmental stress. Treatment of industrial wastes with toxic compounds or an unbalanced C/N ratio could be a cause of N_2O emissions (35). Continually changing environmental conditions, such as oxygen fluctuations, could also contribute to N_2O emission. For a long time, it was assumed that denitrification was restricted to anaerobic and oxygen-limited environments (9). The reason for this need of anoxia could be that oxygen competes for electrons with the reductases involved (7), but oxygen may also inhibit gene expression or enzyme activity. The possibility of aerobic denitrification, as shown for *Thiosphaera pantotropha* (25) and *Alcaligenes faecalis* (23), implied that inhibition of denitrification by oxygen does not always occur and hence that nitrification and denitrification may take place simultaneously under aerobic conditions. The inhibitory effect of oxygen differs for every reductase and may not be the same in all denitrifying bacteria. In wastewater treatment plants, both the oxygen concentration and oxygen fluctuations could therefore influence the amount of emitted N_2O. Few studies, however, have reported on the effect of transient and dynamic oxic and anoxic conditions on N_2O emission from denitrifying continuous cultures (31, 32). Unbalanced induction, repression, or inhibition of the reductases involved would possibly result in accumulation of intermediates including N_2O emission.

A. faecalis, commonly found in soil, water, and wastewater treatment systems (11, 20, 27), was chosen as a model denitrifier. *A. faecalis* TUD is a heterotrophic nitrifier and has been shown to produce N_2O in aerobic batch cultures (22, 24). This strain has no nitrate reductases but is able to reduce nitrite to dinitrogen gas (29). The aim of this investigation was to establish the efficiency of denitrification and the enzymes involved, with the emphasis on N_2O production, during the transient phase from aerobic to anaerobic conditions and vice versa in continuous cultures. The effect of repetitive changing aerobic-anaerobic conditions on N_2O production was also studied.

MATERIALS AND METHODS

Organisms and cultivation. *A. faecalis* TUD (LMD 89.147) was kindly provided by D. Castignetti, Loyala University, Chicago, Ill. A stock culture was stored at −70°C in 30% glycerol. Continuous cultures were made in Applikon fermentors, provided with autoclavable O_2 and pH electrodes. The temperature and pH were maintained at 30°C and 8.0, respectively. Cultures were sparged with nitrogen, helium (0.15 liter min^{-1}), or air and stirred at 800 rpm. The dilution rate was 0.1 or 0.05 h^{-1}, and the volume was kept constant at 2 liters. The cultures were grown under acetate limitation. The medium was supplied in two equivolumetric parts. Medium A contained, per liter, the following: 2 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4 ml of trace element solution (30) (but with 2.2 g of $ZnSO_4 \cdot 7H_2O$), 0.025 ml of silicon antifoam

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TABLE 1. Steady-state measurements of acetate-limited continuous cultures of *A. faecalis*

Expt no.	% Air	D (h^{-1})	NO_2^- added (mM)	Yield[a] (g [dry wt] mol^{-1})	NO_2^-[b] (mM)	N_2O[c] production (μmol $liter^{-1}$ h^{-1})	% N_2O[d]
1	86	0.1	0	13.6	+0.14[e,f]	ND[g]	ND
2	76	0.1	0	13.6	+0.12[e,f]	10	9
3	27	0.1	0	14.0	+0.05[f]	9	10
4	27	0.1	5	14.0	−0.3	6	9
5	5	0.06	25	12.3	−2.3	65	90
6	0.1	0.05	25	12.7	−7.2	93	45
7	0	0.05	25	11.3	−18.4	102	26

[a] Experiments were performed twice; standard deviation, approximately 2 to 9%.
[b] Experiments were performed twice; standard deviation, approximately 17 to 30%. +, produced; −, consumed.
[c] Experiments were performed twice; standard deviation, approximately 20 to 23%.
[d] Percent N as N_2O, as percentage of nitrogen used (sum of NH_4^+ nitrified and NO_2^- consumed); standard deviation, approximately 2 to 27%.
[e] 0.1 mM nitrate produced.
[f] 0.25 to 0.29 mM hydroxylamine produced.
[g] ND, not determined.

agent, and 0.5 ml of concentrated H_2SO_4. After sterilization, 0.1 ml of a vitamin solution (per liter, 20 mg of biotin, 200 mg of nicotinamide acid, 100 mg of thiamine, 100 mg of *p*-aminobenzoic acid, 50 mg of Ca-pantothenate, 500 mg of pyridoxine-HCl, 10 mg of riboflavin, and 10 mg of vitamin B_{12}) $liter^{-1}$ was added. Medium B contained, per liter, 20 mM sodium acetate as a sole carbon source, 0.2 g of NaOH, and 10 or 50 mM $NaNO_2$ when required.

Analytical procedures. Biomass was determined either by measuring the optical density at 450 nm, by total organic carbon determination with a TOCA master 915-B, or by dry weight determinations with 0.2-μm-pore-size nitrocellulose filters. Protein was measured spectrophotometrically (13). Acetate was determined as total organic carbon. The nitrite, ammonia, and hydroxylamine concentrations were measured colorimetrically as described by Griess-Romein-van Eck (14), Fawcett and Scott (8), and Frear and Burrell (10), respectively. Nitrate was measured colorimetrically (5), after removing nitrite with excess sulfaminic acid. An elemental composition of *A. faecalis* biomass of $CH_2O_{0.5}N_{0.25}$ was used, assuming that it is the same as that for *T. pantotropha* and *Pseudomonas* species, as determined by L. A. Robertson (22a).

Gas chromatography and mass spectrometry. Off-gas analysis was performed by on-line gas chromatography using a GC 8340 model (Fisons Instruments, Interscience, Breda, The Netherlands), equipped with a thermal conductivity detector and an electron capture detector. Helium was used as a carrier gas. N_2 and O_2 were separated on a molecular sieve 5 Å (0.5 nm) column, and N_2O and CO_2 were separated on a Hayesep Q column (Chrompack, Middelburg, The Netherlands) at 55°C. The injector, electron capture detector, and thermal conductivity detector temperatures were 110, 350, and 180°C, respectively. Peak areas were integrated by means of a computer running GC-Assist software (Chromcard; Fisons Instruments). Gas flow rates were determined with a Schlumberger gas flow meter (model 25750).

Mass spectrometry was performed with a Prima 600 mass spectrometer (VG Instruments). Because of either high background levels ($^{14,14}N_2$) or overlapping cracking patterns (e.g., mass 30), some readings are less reliable than others. Therefore, only masses 29, 45, and 46 were used for determination (2).

Activity measurements. The activities of nitrite reductase (NiR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR) were determined anaerobically at 30°C. Cells were washed in 25 mM HEPES buffer (*N*-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], pH 8.0). A total of 0.2 mM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine [Sigma]) and 1.25 mM ascorbate (Sigma) were used as the electron donor couple. EDTA (1.0 mM) was added to prevent possible chemical reduction (35). The amount of chemical reduction was estimated by control experiments using pasteurized (2 min, 95°C) cells. Nitrite disappearance (0.5 mM) was followed by taking samples at 2-min intervals from a reaction chamber (5 ml). The reaction was stopped by adding the sample to the acid reagents for nitrite determination. NOR was determined by measuring NO (71 μM) disappearance polarographically, with a Clark-type oxygen electrode, after the addition of an anaerobic saturated solution of NO (2.1 mM at 20°C). The polarizing voltage used was 0.95 V. As NO inhibits its own reduction ($K_i \approx$ 30 μM [7a]), the maximum reduction rate, rather than the initial reduction, was determined. N_2OR was also determined polarographically (1) with a polarizing voltage of 1.2 V and 100 mM KCl plus 1 M NaOH as the electrolyte.

RESULTS

Steady-state characteristics at different dissolved oxygen concentrations. *A. faecalis* was grown under acetate limitation at different oxygen concentrations in the presence and absence of nitrite. Table 1 shows the results of these steady states. During aerobic growth with ammonia as the nitrogen source, products of heterotrophic nitrification (hydroxylamine, nitrite, and nitrate) were formed, as has been shown previously (29). In cultures provided with nitrite, little or no nitrite consumption and no NiR, N_2OR, and NOR activities were measurable in cells grown above 25% air saturation, even though N_2O was detected. This N_2O production was not affected by the nitrite concentration in the medium (Table 1, row 4). At a dissolved oxygen concentration of 5% air saturation (Table 1, row 5), 2.3 mM nitrite was reduced, equivalent to a denitrification rate of 12.5% of the activity in complete anaerobic cultures. However, a large proportion of the reduced nitrite appeared as N_2O (90%), indicating that either the amount or the activities of NiR and NOR were higher than that of N_2OR under these circumstances. NiR (0.15 μmol min^{-1} mg of $protein^{-1}$) and N_2OR (0.1 μmol min^{-1} mg of $protein^{-1}$) activities were detectable at 5% air saturation, which is in the same order of magnitude as during anaerobic growth (0.22 μmol min^{-1} mg of $protein^{-1}$ and 0.15 μmol min^{-1} mg of $protein^{-1}$, respectively). The anaerobic NOR activity (1.1 μmol min^{-1} mg of $protein^{-1}$) was much higher than the NiR activity. Under aerobic conditions, the yield of *A. faecalis* was marginally higher than the anaerobic yield (Table 1).

Transition from aerobic to anaerobic conditions with acetate-limited continuous cultures. *A. faecalis* was grown to steady state at a dissolved oxygen concentration of 46% air saturation, at a growth rate of 0.05 h^{-1}, and in the presence of 5 mM nitrite. Heterotrophic nitrification was obviously taking place, because more NH_4^+ disappeared (0.17 mmol $liter^{-1}$ h^{-1}) than was needed for biomass production (0.09 mmol $liter^{-1}$ h^{-1}). Nitrite also disappeared (0.11 mmol $liter^{-1}$ h^{-1}) and some N_2O and N_2 were produced (0.025 and 0.096 mmol $liter^{-1}$ h^{-1}, respectively). If present, NiR or N_2OR activity could not be detected, since the activities, required to account for the observed in vivo rates, would be below the detection level of the method used. Figure 1 shows the transient response to a change from aerobic to anaerobic conditions. Gas analysis (Fig. 1A) revealed that the N_2O production increased steadily after the transition, indicating that a very low NiR activity had been present at the start. N_2 production remained at the same level, but the CO_2 production and the optical density at 450 nm decreased, indicating that the culture was washing out. Acetate accumulated in the medium (up to 5 mM), and nitrite was not significantly consumed, suggesting that the initial NiR activity was not sufficient to support anaerobic growth. After approximately 20 h, the culture stabilized, nitrite began to disappear from the medium, and N_2 production increased. This coincided with the moment that

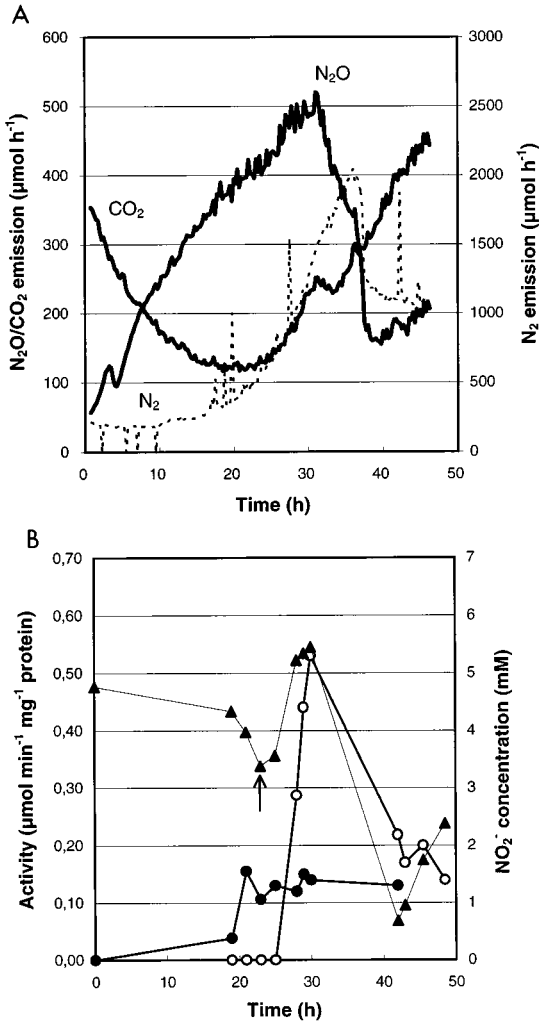

FIG. 1. Transition from aerobic to anaerobic conditions with an acetate-limited continuous culture of *A. faecalis*. (A) Off-gas analysis measured on-line by gas chromatography. (B) Enzyme activity measurements and nitrite concentration in the growth medium. The arrow indicates the moment that the inlet nitrite concentration was changed from 10 to 50 mM. Symbols: ○, N_2OR; ●, NiR; ▲, nitrite concentration.

NiR activity became detectable (Fig. 1B). After 27 h, N_2OR activity became significant, suggesting a stepwise induction of the denitrification enzymes. This transition experiment was repeated with ^{15}N-labelled nitrite in combination with mass spectrometry, to confirm that the gas production was indeed coming from denitrification (results not shown). Five micromolars $^{15}NO_2^-$ was added to acetate-limited cultures, grown in the presence of 5 mM $^{14}NO_2^-$, and gas production was monitored for 8 h by mass spectrometry. Immediately after addition, there was a slight increase of both $^{14,15}N$- and $^{15,15}N$-labelled N_2, indicating that there was N_2 production under aerobic conditions. $^{14,15}N_2O$ was also detected, as was some $^{15,15}N_2O$. After the switch to anaerobic conditions, there was a decrease in $^{14,15}N_2$ and $^{15,15}N_2$ production and an increase in $^{14,15}N_2O$ and $^{15,15}N_2O$. These results are in agreement with the previously described experiment, although the decrease in N_2 production was not observed before.

A continuous culture, grown at a dissolved oxygen concentration of 5% air saturation (under these conditions, *A. faecalis* actively denitrifies [Table 1, row 5]), adapted rapidly to anaerobic conditions. Immediately after the transition to complete anaerobiosis, N_2O production increased, as in the previous experiment. However, N_2 production also increased immediately after the transition, indicating that N_2OR was active. NiR and N_2OR reached their maximum activities within 2 and 7 h, respectively.

Transition from anaerobic to aerobic conditions with acetate-limited continuous cultures. The effect of oxygen on denitrification was examined during a switch from anaerobic to aerobic (25% air saturation) conditions. An acetate-limited continuous culture was grown anaerobically in the presence of 25 mM nitrite at a dilution rate of 0.05 h^{-1}. The specific nitrite consumption rate was approximately 1 mmol $liter^{-1}$ h^{-1} in steady state. This rate agrees reasonably with the measured NiR activity of 0.16 µmol min^{-1} mg of $protein^{-1}$ (approximately 0.65 mmol $liter^{-1}$ h^{-1}). After oxygen was supplied to the culture, gas analysis (Fig. 2A) showed an immediate, strong reduction of N_2 production (fluctuations are due to disturbances in the measurements by gas chromatography). The simultaneous increase in N_2O production suggests inhibition of N_2OR. NiR and NOR appeared to retain their activity during

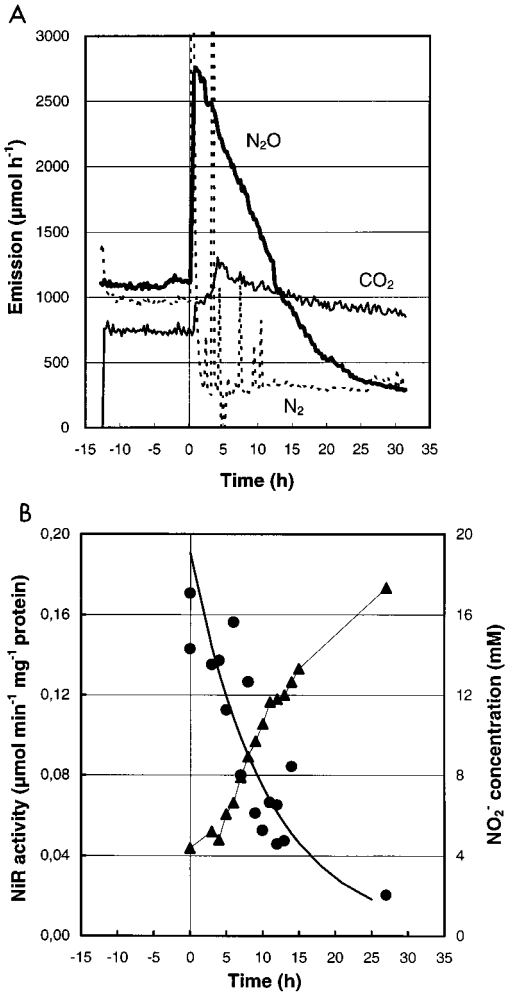

FIG. 2. Transition from anaerobic to aerobic conditions with an acetate-limited continuous culture of *A. faecalis*. (A) Off-gas analysis measured on-line by gas chromatography. (B) NiR activity measurements and nitrite concentration in the growth medium. Symbols: ●, NiR; ▲, nitrite concentration.

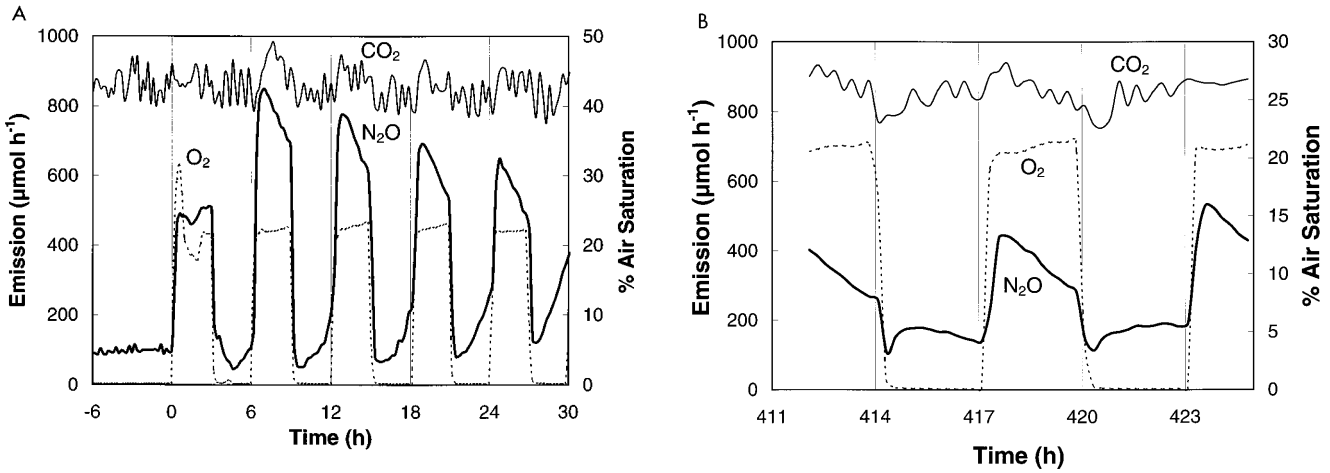

FIG. 3. Repetitive changing aerobic-anaerobic conditions with an acetate-limited continuous culture of *A. faecalis*. (A) Off-gas analysis during the initial repetitions. (B) Off-gas analysis during the semisteady state.

the first hours after the transition. Indeed, nitrite was still consumed some hours after the transition (Fig. 2B). Hydroxylamine accumulated transiently in the medium (up to 0.2 mM), indicating that heterotrophic nitrification had been initiated. The NiR activity decreased in a pattern, similar to that of the wash-out curve (Fig. 2B), suggesting that synthesis of the enzyme had ceased. N_2O production decreased after the first 2 h. From these results and the dilution rate, a half-life of 16 to 22 h could be calculated for NiR. This value, however, is an underestimation, since some N_2 was still produced, indicating that N_2OR was active.

Repetitively changing aerobic-anaerobic conditions with acetate-limited continuous cultures. To analyze the effect of repetitively changing aerobic-anaerobic conditions (dynamic cultures), an anaerobic steady-state continuous culture was alternately sparged with helium and a mixture of helium and oxygen (dissolved O_2 concentration of 20% air saturation). The dilution rate of the culture was kept at 0.059 h^{-1}. The periods of aerobic and anaerobic conditions were the same; the total time of each cycle was 6 h.

During the first cycle, the culture responded to the switch to aerobic conditions in the same way as the previously described experiment. The N_2O production rate increased from 50 to 225 μmol of N_2O $liter^{-1}$ h^{-1} (Fig. 3A), nitrite consumption continued at a lower level (0.6 mmol $liter^{-1}$ h^{-1}), and hydroxylamine accumulated (1.5 mM). After the culture was switched back to anaerobic conditions, nitrite consumption increased (1.1 mmol $liter^{-1}$ h^{-1}) and hydroxylamine accumulation ceased. Gas analysis showed that N_2O production decreased rapidly to the level expected of an anaerobic steady-state culture. This fast adaptation of the culture to anaerobic conditions is clearly related to its growth history (i.e., anaerobic steady state), and it suggests that oxygen inhibition of the reductases is reversible. After the second switch to aerobic conditions, the N_2O concentration increased to over 400 μmol $liter^{-1}$ h^{-1}, after which it decreased again. A maximum concentration of hydroxylamine of 3 mM was measured during the aerobic phase of the fifth cycle. A semisteady state was measured after 67 cycles, in which the response of the culture to switching from aerobic to anaerobic conditions and vice versa was similar in each cycle (Fig. 3B). In this semisteady state, the level of N_2O emission in each cycle fluctuated less than 10%. The average N_2O production during one cycle decreased from 1.2 mmol $liter^{-1}$ 6 h^{-1} (40% of nitrite consumption), during the first cycles, to 0.8 mmol $liter^{-1}$ 6 h^{-1} (28% of nitrite consumption) in the semisteady state. The nitrite consumption decreased from 6.4 mmol $liter^{-1}$ 6 h^{-1} to 5.7 mmol $liter^{-1}$ 6 h^{-1}, and there was no more accumulation of hydroxylamine during aerobic periods. The cell yield in this semisteady state (12 g mol of $acetate^{-1}$) was comparable to the yield obtained at very low oxygen concentrations (Table 1) but was lower than the aerobic yield. The dynamic semisteady-state culture had, during a single cycle, a nitrite consumption rate of 103% of that of an anaerobic steady-state culture (Table 1), even though the culture was only anaerobic 50% of the time. In the anaerobic steady-state culture, 26% of the nitrite reduced appeared as N_2O. In the dynamic culture, this was higher, being 40% during the first transition and 28% in the semisteady state.

DISCUSSION

The influence of oxygen on denitrification, especially on N_2O emission, has been shown to be very important. The mechanism of oxygen inhibition or repression of denitrification is very complex and appears to differ for each reductase and denitrifying organism. In this investigation, *A. faecalis* TUD produced N_2O under all conditions, from fully anaerobic to fully aerobic. Relatively, the highest N_2O emissions were observed under oxygen-limiting conditions, although the NiR activity observed under these conditions (5% air saturation) was only 12.5% of that under fully anaerobic conditions. During a transition from anaerobic to aerobic conditions, an immediate accumulation of N_2O and a decrease in N_2 production were observed, suggesting that N_2OR is more sensitive to oxygen than NiR. NiR was not irreversibly inhibited by oxygen, as shown by activity measurements, but its synthesis seemed to have ceased. A half-life could be calculated for NiR of approximately 16 to 22 h, although this is an underestimation. In *Pseudomonas nautica* (4) and *Paracoccus denitrificans* (32), more or less the same pattern of inhibition was found. In *P. nautica*, it was shown that NaR was reversibly inhibited by oxygen, in contrast to NiR and N_2OR, which were inhibited irreversibly. In *P. denitrificans*, the decrease in NaR activity followed wash-out kinetics, indicating that oxygen repressed its synthesis. In comparison with these results, the NiR of *A. faecalis* appears to be less sensitive to inhibition by oxygen than those of *P. denitrificans* and *P. nautica*. This is rather remarkable, as the latter two bacteria have a cytochrome cd_1-type NiR

and *A. faecalis* has a copper-type NiR (21, 29). It is the general view that the Cu-type NiR cannot be active in intact cells in the presence of oxygen (6, 9). The NOR was not measured in this transition experiment, but from steady-state measurements, it was observed that NOR activity was approximately 10 times higher than NiR activity, thus ensuring that the very toxic compound NO does not accumulate. It is generally assumed that NiR and NOR are controlled interdependently at the activity and transcriptional level (34). After the switch, there was also a transient accumulation of hydroxylamine, suggesting that the enzymes of the nitrification pathway were also induced sequentially.

During the transition from aerobic to anaerobic conditions, there seemed to be NiR present in that immediately after the switch N_2O was produced. Experiments using ^{15}N-labelled nitrite in combination with mass spectrometry showed that this N_2O was indeed produced by NiR. However, this activity was not enough to sustain growth, since the culture was washing out. It took approximately 20 h for the NiR to become sufficiently induced to permit growth again. At this time, the N_2 production increased, but it was not until 27 h after the switch that N_2OR activity became detectable. These results suggest that NiR may be constitutively present in small amounts and that under anaerobiosis the enzymes of dentrification are induced sequentially. This stepwise induction has also been found in *Pseudomonas stutzeri* (16) and in *P. denitrificans* (3, 18, 32), although the induction of the reductases in *P. stutzeri* was not dependent on oxygen alone; it also needed the presence of a nitrogen oxide. The induction was much faster in *P. denitrificans* than observed for *A. faecalis*, and according to mRNA analysis (3), the sequence of induction differed in that N_2OR induction preceded that of NiR. During a transition of *A. faecalis* from low-oxygen conditions (5% air saturation) to anaerobiosis, it took only 2 and 7 h for the NiR and the N_2OR to reach their maximal activities, respectively. This indicates that, at a low oxygen concentration, the reductases are already induced.

In order to mimic conditions in wastewater treatment plants, the effect of repetitive changing aerobic-anaerobic conditions was investigated. During the initial transitions, the same pattern was observed as that described for the single transitions. After approximately 67 cycles (400 h), a semisteady state was reached, in which the response to a shift from aerobic conditions to anaerobic conditions and vice versa was similar in each cycle. In this semisteady state, N_2O production occurred mainly after the transition from anaerobic to aerobic conditions. The nitrite consumption rate was approximately 103% of the consumption in an anaerobic culture. The total N_2O production was generally higher in the dynamic culture than in an anaerobic culture. Little is known about N_2O production by denitrifying pure cultures under dynamic conditions. Waki et al. (31) carried out studies on dynamic oxic and anoxic continuous cultures of *P. denitrificans*. One of their findings was that the rate of nitrate reduction in the anaerobic period was unchanged by the length of the aerobic periods; however, the nitrite reduction rate was decreased by longer aerobic periods. Unfortunately, they did not measure gaseous nitrogen oxides during these transitions.

The observation that NiR is active under aerobic conditions in *A. faecalis*, together with the observations from, among others, Hochstein et al. (15) and Robertson and Kuenen (25), confirmed that denitrification can occur in the presence of oxygen. It is, however, remarkable that the Cu-type NiR of *A. faecalis* is active under aerobic conditions. Further research is needed in this area. It would also be very interesting to study the effect of various lengths and different ratios between the aerobic and anaerobic periods on the total denitrification activity and N_2O production by *A. faecalis.*

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