Autotrophic Biological Transformations of Nitrogen under Oxygen-stressed and Anaerobic Conditions

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Abstract

Microorganisms have demonstrated considerable versatility in cycling nitrogenous compounds in natural and engineered environments. Experiments were conducted to study the controllability and potential applications of these less commonly considered phenomena. Stressed biological systems, lacking oxygen and labile organic compounds, with reduced and oxidized nitrogen substrates were investigated. Results demonstrated conversions of soluble nitrogenous compounds that could not be fully explained by traditional nitrification or denitrification. Comparisons are made to reports of others and discussed in terms of environmental engineering significance and items of interest that merit further inquiry are identified.

Key words : ammonium, hydroxylamine, nitrite, nitrate, nitrification, denitrification, nitrogenous compounds

INTRODUCTION

Literature Review Exceptions to the traditionally held routes of aerobic autotrophic nitrification and anoxic heterotrophic denitrification as explanations for nitrogen transformations in aqueous environments have become more and more common in recent years. Such phenomenon as heterotrophic nitrification and aerobic denitrification cannot be ruled out as potentially significant factors in the environmental cycling of nitrogen.^{1, 2)} Also of significance are the apparent simultaneous conversions of reduced and oxidized forms of nitrogen to gaseous products by pure cultures (referenced examples following).

Nitrifying microorganisms in particular have proven to be more versatile than previously thought and much remains to be understood concerning their metabolic capabilities.³⁾

Remde and Conrad⁴⁾ observed that *Nitrosomonas europaea* produced nitric oxide (NO) and nitrous oxide (N₂O) while simultaneously utilizing ammonium (NH₄⁺) and nitrite (NO₂⁻). Chlorite, a denitrification inhibitor, stopped the production of these nitrogen gasses. Furthermore, when hydrazine (N₂H₄), from which NO is not produced, was used in place of NH₄⁺, production of NO and N₂O was not reduced. Thus it was demonstrated that only NO₂⁻ was donating nitrogen to gas production and intermediates in the nitrification pathway

were not contributing. Production of N_2O was greatest under anaerobic and oxygen-limiting conditions and was only a minor product under aerobic conditions. NO production, however, was relatively insensitive to dioxygen (O_2) levels; NO reductase activity thus appeared to be the more sensitive to O_2 .

Brock *et al.*⁵⁾ observed that *N. europaea* under O_2 limitation (< 0.4 mg/l) could oxidize NH_4^+ and reduce NO_2^- at the same time evidently with both O_2 and NO_2^- serving as electron acceptors. Also, in suffocated anaerobic culture, equimolar losses of NH_4^+ and NO_2^- occurred when O_2 was considered absent. Furthermore, under low redox conditions *N. eutropha* demonstrated cell growth and denitrification activity utilizing hydrogen as an electron donor with NO_2^- as the only electron acceptor while NH_4^+ , though present, was not used. Without molecular hydrogen, NH_4^+ served as a suitable electron donor for NO_2^- reduction for *N. eutropha* both in pure culture and in mixed culture with *Enterobacter aerogenes* under anoxic conditions in an organic media. Interestingly, with *E. aerogenes* present the equimolar reductions of NH_4^+ and NO_2^- were enhanced over that of the pure *N. eutropha* culture. *E. aerogenes* (an chemoorganotroph) is not known to use NH_4^+ and NO_2^- , thus it appears that it somehow stimulated the use of these substrates by *N. eutropha*. *Escherichia coli* and *Serratia marcescens* when used in place of *E. aerogenes* demonstrated no such benefit. Electron micrographs revealed differences in the compositions of intra-cytoplasmic membranes of NH_4^+ oxidizing bacteria adapted to O_2 -limitation versus those from well aerated environments possibly reflecting higher enzyme counts in the O_2 -stressed organisms.

Poth⁶⁾ isolated a *Nitrosomonas* sp. capable

of producing dinitrogen (N_2) while converting NH_4^+ and NO_2^- under O_2 -stressed conditions for the first time. CO_2 provided assimilatory carbon and energy was obtained from oxidation of NH_4^+ . Labeled nitrogen and kinetic considerations were used to demonstrate that N_2 production was only from denitrification of NO_2^- and not a side reaction in the nitrification path. They emphasized the commonly held view that strict anaerobic conditions cannot support growth of nitrifying organisms because O_2 is required for the initial conversion of NH_4^+ to hydroxylamine (NH_2OH). de Bruijn *et al.*,⁷⁾ however, reported production of N_2O by *N. europaea* under anaerobic conditions with NO_2^- serving as an electron acceptor and either NH_4^+ or NH_2OH serving as the donor. While no growth was observed under anaerobic conditions, aerobic growth of *N. europaea* while oxidizing NH_2OH (mixotrophically with NH_4^+) was demonstrated for the first time. Under these aerobic growth conditions, nitrogen loss to gaseous forms, evidently as a nitrification side product, were increased nearly an order of magnitude by use of the NH_2OH substrate (perhaps involving a chemical reaction with NO_2^-).

Mulder *et al.*⁸⁾ observed the disappearance of NH_4^+ in a denitrifying fluidized bed reactor where both NH_4^+ and nitrate (NO_3^-) consumption were correlated with an increase in gas production. This phenomena was further characterized by van de Graaf *et al.*⁹⁾ and confirmed to be a biological conversion. Anaerobic testing conditions were used to demonstrated that O_2 was not involved. Labeled nitrogen was used to show that both NH_4^+ and NO_3^- were donating to N_2 formation in essentially equimolar amounts. These factors demonstrated the unlikelihood that known nitrifying bacteria were involved. Stoichiometric considerations suggested that

NO_2^- was the direct oxidizing agent of NH_4^+ in an energetically favorable exergonic reaction as per the following equation:¹⁰⁾

The anaerobic ammonium-oxidizing (Anammox) organisms were further studied in an autotrophic synthetic medium.¹¹⁾ With NH_4^+ and NO_2^- supplied as the electron donor and acceptor, respectively, N_2 was the main reaction product and incorporation of inorganic carbon was demonstrated. It is interesting that organic compounds were shown to be inhibitory to the process considering that the phenomena was first discovered in a denitrifying reactor with a substantial organic loading. Electron micrographs showed unusual, irregular shapes for the dominant organisms. Furthermore, an increase in the unique metabolic activity was correlated with an increase in ether lipids, cytochromes, and a red culture tint. Though preliminary, the observed lipid composition opens the possibility that archaeal microorganisms are involved. Strous *et al.*¹²⁾ further elucidated that the Anammox process is reversibly inhibited by O_2 and discussed its potential use in a nitrogen abatement process. An alternating aerobic-anaerobic treatment scheme could conceivably find a balance between generation of NO_2^- by conventional aerobic nitrification and its subsequent conversion to N_2 via an interaction with NH_4^+ in an induced anaerobic phase—both phases occurring in a mutually autotrophic system.

Objectives The objective of this work was to investigate biological nitrogen transformations in environments where O_2 and labile organic compounds are limiting or missing. These constraints were considered in order to probe less commonly considered phenomena for possible beneficial

applications. Where cycling nitrogen from a reduced form (NH_4^+) via an oxidized state (NO_2^- or NO_3^-) to a gaseous product (e.g., N_2) is considered, environments lacking in O_2 or organic compounds are at an obvious disadvantage. Pulsed chemical and energy inputs would be required. Such inputs are common for nitrogenous wastewater treatment facilities; however, where treatment is required for a drinking water source, addition of an organic substrate / contaminant is problematic if not risky. This investigation is envisioned to be a step in improving our understanding of nitrogen cycling capabilities and methods to enhance these phenomena in a remedial capacity.

These objectives were met by conducting batch assays with cultures acclimated to the proposed testing environment. O_2 -limiting / stressed conditions were initially used where cultivation of facultative organisms would be favorable. Oxidized forms of nitrogen (NO_2^- or NO_3^-) were provided as potential electron acceptors and reduced nitrogen (NH_4^+), as the intended electron donor. Inorganic carbon was supplied as to be non-limiting. Organic carbon was not supplied but its presence in trace quantities as cellular storage material or via endogenous decay is not excluded as a possibility. Frequent mixing was employed to reduce testing variables that can be contributed to gradients. Performance was evaluated by quantification of key aqueous components— NH_4^+ , NO_2^- , NO_3^- , pH, and oxidation reduction potential (ORP).

MATERIALS AND METHODS

Initial batch assays were conducted in 1-liter or 0.5-liter glass flasks (1.0 l or 0.4 l initial sample volumes) closed with rubber stoppers. Plastic syringe (24 ml) attachments were used for pressure relief. For attachment connections either silicon or polyvinyl (PV) tubing (ca. 20 cm with 0.6 cm

diameter) was used as noted. Subsequent jar assays were conducted in 250 ml or 100 ml amber glass jars (200 ml or 80 ml samples) with tight fitting plastic screw caps (with inner rubber cushion) or rubber stoppers. No attachments were used with jar assays.

Sludge used as culture seed came from two sources: an ongoing, in-house anoxic denitrifying batch reactor utilizing methanol as an organic substrate (ca. 0.2 mm diameter granular sludge) and an ongoing, in-house aerobic activated sludge reactor (floculant sludge). Sludges were washed with tap and deionized water prior to use. Prepared culture solutions were purged in their respective containers (flasks or jars) with nitrogen gas for about 15 minutes. Jars were then evacuated and sealed in a positive pressure nitrogen glove-box. Sealed flasks and jars were incubated in the dark at 25 °C (outside of glove-box). Containers were opened for sampling only in the glove-box.

The reduced nitrogenous substrate was added as $(\text{NH}_4)_2\text{SO}_4$ or NH_4Cl ; in oxidized form, NaNO_3 or NaNO_2 was used. An activated sludge nutrient solution was used for one initial assay (as noted in Results) and consisted of (per liter): 105 mg NaHCO_3 , 2.7 mg KH_2PO_4 , 5.0 mg NaCl , 7.0 mg KCl , 7.0 mg CaCl_2 , and 5.0 mg MgSO_4 . The nutrient solution for most assays (referred to herein as the standard type nutrient solution) consisted of (per liter): 500 mg KHCO_3 , 27 mg KH_2PO_4 , 50 mg NaCl , 70 mg KCl , 70 mg CaCl_2 , 50 mg MgSO_4 , 9.0 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0 ml of micro-nutrient solution. The micro-nutrient solution consisted of (per liter): 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.43 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.99 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.22 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.19 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.11 g Na_2SeO_4 , and 0.014 g H_3BO_3 (after van de Graaf *et al.*,¹¹⁾). Adjustments to pH were made with 1 N H_2SO_4 .

NO_3^- was determined by the UV spectrophotometric screening method and NO_2^- by the colorimetric method.¹³⁾ NO_2^- was found to give an absorbance response in the NO_3^- screening method equal to 25 % of the NO_3^- response on an equal weight basis and corrections were appropriately made. NH_4^+ was determined by the phenate method (JIS, K0102 42). Oxidation reduction potential (ORP) was measured with a UK 2030 meter using a UC-702E (Pt) electrode (Central Kagaku, Kyoto), dissolved oxygen (DO) with a UC-12 Digital DO meter using a standard probe (Central Kagaku), and pH with a Mettler Toledo 320 pH meter. For jar assays with rubber stoppers only, ORP (as above) and pH (with Duotest pH indicator paper, 0.3 unit accuracy; Macherey-Nagel GmbH & Co., Duren, Germany) were measured *in situ* in the nitrogen glove-box. Total sludge concentrations were estimated as mixed liquor suspended solids (MLSS) and are reported as averages of duplicate assays. Sludge samples were washed twice (centrifuging at 3,000 g for 10 minutes) and dried at 105 °C (2 hours) to determine the MLSS component. Standard deviations (SD) were determined with respect to n-1 data.

Scanning electron microscopy (SEM) micrographs were taken with an electron microscope (JEOL JSM-5310LV, Nihon Denshi Co.). The sample was prepared as follows: A suspension containing approximately 25 mg (dry weight) of sludge was centrifuged (3,000 g for 5 minutes, typical) and decanted, washed five times (with vortex mixing, centrifuging and decanting) in 0.1 M phosphate buffer (pH 7.4), hardened for 60 minutes in 2.5 % glutaraldehyde (prepared in phosphate buffer), washed five times (as above), fixed for 30 minutes in 1.0 % OsO_4 (in phosphate buffer), washed three times (as above), dewatered in 50 % and 70 % ethanol for 10

minutes each (with centrifuging and decanting), stored in 90 % ethanol overnight (4 °C), then washed in t-butyl alcohol three times, dried on a critical point dryer (JEOL JFD-300), and coated with gold on an ion sputtering device (JEOL JFC-1100E).

RESEARCH PROTOCOL

Experiments were conducted in three phases. In Phase I, preliminary work focused on assessing responses of seed sludges from differing origins under O_2 limiting or stressed conditions with no attempt to assure strict anaerobiosis (defined herein as O_2 -stressed conditions). Phase II focused on employing stringency in O_2 exclusion (anaerobic conditions). While NO_2^- was produced in Phase I assays and introduced in one Phase II test, its use as a substrate in place of NO_3^- was the focus of research in Phase III under anaerobic conditions.

Testing conditions used in Phase I are outlined in Table 1. Sludges of differing oxidation state origins (i.e., aerobic activated sludge and anoxic denitrifying sludge) were used in flasks with silicon tubing (not excluding limited transfusion of O_2). The transition from A.1 to A.2 consisted of

washing the sludge under ambient air and restocking with fresh substrates and nutrients as for the initiation of a new assay (as per Materials and Methods). The transition from B.1 to B.2 constituted of addition of supplemental components to the existing culture in the glove-box.

Testing conditions used in Phase II under anaerobic conditions are outlined in Table 2. B.3 contained sludge of denitrifying origins that had been used in Phase I and was thus conditioned without an organic substrate for about four months. C.1, also containing sludge of denitrifying origins, had been cultured under autotrophic, O_2 -stressed conditions (flask with silicon tubing) for about two months prior to use in Phase II.

Testing conditions used in Phase III are outlined in Table 3. Anaerobic conditions (as in Phase II) were continued with NO_2^- being supplied as the only intended electron acceptor. Assay C.2 contained sludge that had been used in Phase II. C.2 then served as the sludge source for all subsequent assays with C.2, C.3, and C.5 differing only in culture age. C.4 and C.6 served as parallel inhibitory controls to C.3 and C.5, respectively.

Table 1 Conditions of assays in Phase I. All assays conducted in flask with silicon tubes. Solids and ORP data shown in Figure 1.

Assay	Sludge Source	Nitrogen Substrates	Supplement Nutrients	pH
A.1	Activated sludge	$(NH_4)_2SO_4$ & $NaNO_3$	Activated sludge type	8.2 unadjusted (SD, 0.37; n, 21)
A.2	A.1	$(NH_4)_2SO_4$ & $NaNO_3$	Standard type	7.1 adjusted ^{a)} (SD, 0.28; n, 15)
B.1	Denitri. sludge	$(NH_4)_2SO_4$ & $NaNO_3$	Standard type ^{b)}	8.2 unadjusted (SD, 0.18; n, 17)
B.2	B.1 ^{c)}	NH_4Cl & $NaNO_3$	Standard type	8.2 unadjusted (SD, 0.22; n, 8)

^{a)}Adjusted only once at beginning of assay. ^{b)}Standard type nutrients without micro-nutrient addition (elsewhere micro-nutrients included with standard type). ^{c)}Existing culture supplemented with substrates and micro nutrients; SO_4^{2-} from $(NH_4)_2SO_4$ of B.1 remaining in solution.

Table 2 Conditions of assays in Phase II. All assays conducted with NH_4Cl , standard nutrients, and unadjusted pH.

Assay	Sludge source	Approx. MLSS	Container	Oxidized N-substrate
B.3	B.2 ^{a)}	5 g l ⁻¹	Jar with screw cap	NaNO_2 & NaNO_3 ^{b)}
C.1	Denitri. sludge	3 g l ⁻¹	Flask with PV tubing	NaNO_3

^{a)}Sludge from B.2 washed and stored under nitrogen gas (room temperature) for 19 days prior to use in B.3. ^{b)}Initially seeded with NaNO_2 with subsequent additions of NaNO_3 .

Table 3 Conditions of assays in Phase III. All assays conducted with NH_4Cl and NaNO_2 substrates and standard nutrients. Inhibitors, in C.4 and C.6, were used at 250 mg l⁻¹.

Assay (inhibitor)	Sludge source	Approx. MLSS	pH	ORP (mV)
C.2 ^{a)}	C.1	1.5 gl ⁻¹	7.2 unadjusted (end of assay)	260 (end of assay)
C.3 ^{b)}	C.2	5 gl ⁻¹	7.3 adjusted ^{c)} (SD, 0.1; n, 15)	266 (SD, 54; n, 15)
C.4 ^{b)} (HgCl_2)	C.2	5 gl ⁻¹	7.4 adjusted ^{c)} (SD, 0; n, 5)	130 (SD, 16; n, 3)
C.5 ^{b)}	C.3	5 gl ⁻¹	7.4 adjusted ^{c)} (SD, 0; n, 5)	201 (SD, 69; n, 6)
C.6 ^{b)} (TCMP) ^{d)}	C.3	5 gl ⁻¹	7.4 adjusted ^{c)} (SD, 0; n, 5)	226 (SD, 22; n, 5)

^{a)}Flask with PV tubing. ^{b)}Jar with rubber stopper. ^{c)}pH adjusted at beginning of assay only. ^{d)}2-chloro-6-(trichloromethyl)-pyridine.

RESULTS

Phase I For assay A.1, an initial decline in sludge solids (MLSS) and NO_3^- with an increase in NH_4^+ occurred over the first 20 days (Figures 1 and 2). Evidently denitrification was occurring with organic compounds from storage or endogenous decay serving as electron donors with concurrent ammonification. Over the first 50 days, ORP gradually increased to a plateau of about 250 mV (Figure 1). Also at about day 50, the rate of NO_3^- disappearance

declined and NH_4^+ disappearance began with a accumulation of NO_2^- (Figure 2). Total nitrogen (total-N) disappearance rate was about 5 mg N l⁻¹ d⁻¹ during the course of A.1 when denitrification activity was dominant. During A.2, when NO_3^- reduction was greatly reduced and NH_4^+ disappearance (ca. 2 mg N l⁻¹ d⁻¹) dominated, total-N disappeared at a rate of 3 mg N l⁻¹ d⁻¹.

B.1 also initially demonstrated apparent denitrification (Figure 3). Ammonification, however, was not evident and MLSS

Fig.1 Time course of MLSS and ORP for A and B series of Phase I (see Table 1). Vertical line at day 75 indicates separation of parts 1 and 2 of both series.

Fig.2 Time course of nitrogen transformations for A.1 and A.2 assays of Phase I (see Table 1). Sludge washed and fresh substrates and nutrients introduced at transition from A.1 to A.2 (day 75).

concentrations were widely scattered (the grainy textured sludge was difficult to sample in an even suspension due to rapid settling), thus no trends could be discerned (Figure 1). After only 20 days, B.1 experienced a significant reduction in NO_3^- disappearance and an onset of NH_4^+ oxidation with accumulation of NO_2^- . This response was demonstrated repeatedly in B. 2 (Figure 3). Over the course of B.1 and B. 2 during periods when NH_4^+ disappearance (3 to $6 \text{ mg N l}^{-1} \text{ d}^{-1}$) was the dominant form of nitrogen loss, total-N disappearance varied from about 2.5 to $5 \text{ mg N l}^{-1} \text{ d}^{-1}$.

Sulfate concentrations demonstrated no significant trends over the course of testing in all cases where used (data not shown). The use of sulfate in conjunction with a substrate was discontinued in subsequent assays (i.e., switch from $(\text{NH}_4)_2\text{SO}_4$ to NH_4Cl), though still included as a supplemental nutrient.

Transfer of O_2 through silicon tubing was assessed by rigging a flask with 1.0 l of tap water and otherwise treating the flask as per an ordinary assay. O_2 intrusion was monitored by drawing samples and measuring DO in the glove-box. Over a 7-day period, a gradual declining rate of increase was observed (data not shown). The highest rate of transfer was estimated to be about $0.1 \text{ mg O}_2 \text{ l}^{-1} \text{ hr}^{-1}$ over the first 5 hours. Subsequently, two tests were conducted with PV tubing. In the first test, an increase of about $0.02 \text{ mg O}_2 \text{ l}^{-1} \text{ hr}^{-1}$ was observed over the first 24 hours and $0.01 \text{ mg O}_2 \text{ l}^{-1} \text{ hr}^{-1}$ over the second 24 hours. In the second test, no intrusion was observed over 20 hours. Evidently, the rate of O_2 intrusion can be reduced by an order of magnitude if not eliminated by use of PV material. The syringe apparatuses continually demonstrated pressure integrity precluding intrusion by channels other than the silicon matrix. O_2

Fig. 3 Time course of nitrogen transformations for B.1 and B.2 assays of Phase I (see Table 1). Transition from B.1 to B.2 (day 75) consisted of chemical additions to the existing culture. Total-N not shown for B.2 (for clarity of other components).

intrusion with a screw cap jar was also assessed. With storage in the glove-box, no O_2 intrusion was detected over 48 hours (as expected). Furthermore, after transfer to storage in ambient air, no leakage was detected over 5 days.

Phase II Assay B.3 differed in its response from B.2 of which it originated. Over the first month when relatively low concentrations of NO_2^- and NO_3^- were added, a gradual but consistent loss of NH_4^+ ($0.6 \text{ mg N l}^{-1} \text{ d}^{-1}$) occurred (Figure 4). The introduction of a relatively high concentration of NO_3^- (day 37) resulted in immediate, rapid NO_3^- loss with a gradual accumulation of NH_4^+ . During the latter half of B.3 when NH_4^+ concentration demonstrated no significant changes, total-N disappearance rate was stable at about $1 \text{ mg N l}^{-1} \text{ d}^{-1}$. The rapid climb of NO_2^- with loss of NH_4^+ following day 98 is attributed to traditional nitrification

due to O_2 intrusion during sampling (glove-box leak).

During assay C.1 (consisting of sludge of the same origins as the B series) a gradual accumulation of NH_4^+ occurred over the first 40 days (Figure 5). Total-N losses were about $2 \text{ mg N l}^{-1} \text{ d}^{-1}$ and disappearance of NH_4^+ was not evidenced (not precluding the possibility of NH_4^+ generation offsetting loss).

Phase III Apparently some NO_3^- had been carried over from C.1 into C.2 which was quickly reduced with the applied NO_2^- (Figure 6). Subsequent additions of NO_2^- were reduced at a lower rate (ca. $0.2 \text{ mg N l}^{-1} \text{ d}^{-1}$) which was about half that of the overall rate of NH_4^+ loss ($0.55 \text{ mg N l}^{-1} \text{ d}^{-1}$). Following the transfer to C.3, an initial increase in NO_2^- with a loss of NH_4^+ occurred (Figure 7). Evidently some O_2 introduced during assay preparation had

Fig. 4 Time course of nitrogen transformations for B.3 assay of Phase II (see Table 2). NO_2^- substrate used at beginning (days 0 and 10) and NO_3^- subsequently added on days 14, 33, and 37. O_2 intrusion occurred following day 98 (see text).

Fig. 5 Time course of nitrogen transformations for C.1 assay of Phase II (see Table 2).

Fig. 6 Time course of nitrogen transformations for C.2 assay of Phase III (see Table 3). NO₂⁻ substrate used from beginning and subsequently added on days 10 and 42.

Fig. 7 Time course of nitrogen transformations for C.3 and C.4 (HgCl_2 control) assays of Phase III (see Table 3). For C.3, subsequent additions of NH_4^+ and NO_2^- substrates were made on days 34 and 36, respectively.

persisted (inadequate purging) resulted in nitrification. Subsequent additions of NH_4^+ and NO_2^- were followed by disappearance rates of 1.7 and 0.8 $\text{mg N l}^{-1} \text{d}^{-1}$, respectively. In assays C.3, C.4, C.5, and C.6, NO_3^- with few exceptions was not detected (maximum occurrence of ca. 0.5 mg N l^{-1}).

In assay C.4, NO_2^- transformations were arrested by the HgCl_2 addition (Figure 7). NH_4^+ , however, increased. The HgCl_2 addition (250 mg l^{-1}), which was intended to terminate all microbial activity, may have been inadequate for the 5 g l^{-1} sludge concentration allowing for some heterotrophs to persist. This or a chemical reaction may have lead to an oxidative release of NH_4^+ .

In assay C.5 there was an immediate, steady disappearance of NO_2^- from the onset (Figure 8). Over the final nine-days, the disappearance rate of NH_4^+ (1.4 $\text{mg N l}^{-1} \text{d}^{-1}$) was about half that of NO_2^- (2.7 mg N l^{-1}

d^{-1}). TCMP, used in C.6, is a nitrification inhibitor that prevents the oxidation of NH_4^+ to hydroxylamine (NH_2OH). In C.6, NO_2^- disappearance matched that of C.5 (without TCMP) but NH_4^+ gradually accumulated (Figure 8).

An SEM micrograph of organisms from the C.5 assay is shown in Figure 9. Most organisms were elongated and less than 1.0 μm in length. Unusual morphologies, i.e., crescent or jagged bends in cell surfaces, were common. The culture was extremely filamentous and attachment of long tail-like appendages to cells was frequently evident. A few tightly packed clusters of cocci organisms (ca. 0.5 μm diameter) were also evident (not shown).

DISCUSSION

The source of seed sludge clearly made a difference in the initial acclimation periods

Fig. 8 Time course of nitrogen transformations for C.5 and C.6 (TCMP control) assays of Phase III (see Table 3). Subsequent additions of NO_2^- substrate were made on days 6 and 7 for C.6 and C.5, respectively.

Fig. 9 Scanning electron micrograph of sample from C.5 at end of assay. Bar is $1.0\ \mu\text{m}$.

when denitrification was apparent. Ammonification was evident in A.1 (of activated sludge origins, Figure 2) but not in B.1 (of denitrifying origins, Figure 3). Furthermore, the initial period of rapid NO_3^- decline in B.1 was about half as long as that of A.1 (20 versus 40 days). That this initial reduction of NO_3^- in A.1 was traditional denitrification is supported by the concurrent decline in MLSS (Figure 1) and the increase in NH_4^+ (ammonification), both indicative of an endogenous source of organic substrate. In B.1, carry over trace/storage organic materials with the source sludge may have been utilized.

In both the A and B series of Phase I, a decline in the rate of NO_3^- reduction was associated with an onset of NH_4^+ disappearance and the appearance of NO_2^- . Furthermore, in B.1 the subsequent reduction in the NH_4^+ disappearance rate and concentration (to near depletion) was followed by a restoration of the previous NO_3^- disappearance rate (Figure 3). While the use of storage or trace organic materials to support denitrification during the first 20 days of B.1 is plausible, after 60 days of incubation this explanation becomes tenuous and the possibility of alternate electron donors merits consideration.

The observed pattern of NO_2^- appearance continued in B.2 even after NO_3^- was allowed to expire (Figure 3). If the observed NO_2^- production was due to traditional nitrification, it is interesting that the organisms (or conditions) for NO_2^- oxidation to NO_3^- were missing (or inadequate). Thus the previously noted reduction in the rate of NO_3^- disappearance was probably not due to an offset from NO_2^- oxidation to NO_3^- . The occurrence of nitrification terminating in NO_2^- was seen by Strous *et al.*¹²⁾ when O_2 was introduced to an anaerobic NH_4^+ -oxidizing culture. Gas

transfusion through silicon tubing (used in Phase I) could have provided an O_2 source for nitrification. However, the maximum intrusion rate of $0.1 \text{ mg O}_2 \text{ l}^{-1} \text{ hr}^{-1}$ is nearly an order of magnitude lower than the theoretical O_2 demand for conversion of NH_4^+ to NO_2^- at the highest rate observed in the B series (i.e., $0.86 \text{ mg O}_2 \text{ l}^{-1} \text{ hr}^{-1}$ required for oxidation $0.25 \text{ mg NH}_4^+ \text{ N l}^{-1} \text{ hr}^{-1}$). Pure cultures of *N. europaea* have been observed to simultaneously utilize both NH_4^+ and NO_2^- under various redox conditions.^{4, 5, 14)} Production of NO_2^- from oxidation of NH_4^+ and its reduction, again utilizing NH_4^+ ⁴⁾ or hydrogen⁵⁾ as a reductant could be a contributing factor to transformations in B.2 (Figure 3) under O_2 -stressed conditions. Similar transformations were observed with the activated sludge culture though at lower rates (A.2, Figure 2).

Expanding on the idea that NO_2^- may serve to oxidize NH_4^+ to NO_2^- , NO_2^- was supplied in place of NO_3^- as a substrate at the beginning of assay B.3 under anaerobic conditions (Phase II). Though containing sludge from B.2 (Phase I), the previous pronounced NH_4^+ disappearance with NO_2^- accumulation was not reproduced (Figure 4). Over the first month, though, when relatively low concentrations of NO_2^- and, subsequently, NO_3^- were added a gradual but consistent loss of NH_4^+ occurred; however, the subsequent addition of a high concentration of NO_3^- (over 250 mg l^{-1}) resulted in a gradual accumulation of NH_4^+ . Though speculative, NH_4^+ oxidizing populations may have been overtaken and dissimilatory reduction of oxidized forms of nitrogen to NH_4^+ may have contributed to the observed accumulation.¹⁵⁾ The immediate drop in NH_4^+ with production of NO_2^- in response to O_2 intrusion following day 98 demonstrated the versatility of the NH_4^+

-oxidizing organisms. It appears these organisms do not require an extended lag period to adapt or switch enzyme systems as observed by Bodelier *et al.*¹⁶⁾ for nitrifying organisms of an anoxic environment.

The transformations in C.1 utilizing NH_4^+ and NO_3^- substrates (Figure 5) follow similar patterns to those of the latter half of B.3 (both of Phase II). The sludge in C.1 (of denitrifying origins) was carried over into C.2 (Phase III) and used to more closely access transformations associated with the use of NO_2^- as the oxidized nitrogen substrate. The mutual reductions of NH_4^+ and NO_2^- observed over the first month of B.3 were again observed (at similar rates) in C.2 (Figure 6). The continuation into C.3 and ultimately C.5 demonstrated the reproducibility of these simultaneous conversions of NH_4^+ and NO_2^- (latter parts of Figures 7 and 8, respectively). TCMP stopped the disappearance of NH_4^+ and had no effect on NO_2^- conversion (C.6, Figure 8). Thus it appears that NH_4^+ was not serving as an electron donor for NO_2^- reduction under anaerobic conditions. These results differ from those of the anaerobic NH_4^+ -oxidizing culture of Mulder *et al.*;⁸⁾ however, the SEM micrographs (Figure 9) of organisms in the final assay bear a resemblance to the irregularly shaped organisms in that work¹¹⁾ yet differing in their filamentous nature.

In this screening effort, a gradual disappearance rate for NH_4^+ ($0.55 \text{ mg N l}^{-1} \text{ d}^{-1}$) concurrent with NO_2^- reduction ($0.2 \text{ mg N l}^{-1} \text{ d}^{-1}$) occurred after about 5 months of incubation. An additional two months were required to achieve the maximum observed NH_4^+ removal rate of $1.7 \text{ mg N l}^{-1} \text{ d}^{-1}$ (NO_2^- reduction rate, $0.8 \text{ mg N l}^{-1} \text{ d}^{-1}$). Further inquiry is needed to determine if these phenomena can be optimized for inclusion in a remediation system. O_2 levels

and ORP control are key factors. Strict anaerobic conditions would be difficult to sustain in a commercial scale unit process without an organic substrate. Creating the conditions that allow for combining (or overlapping) nitrification and denitrification processes appear feasible and has been of interest in recent years.^{1,12)}

In natural environments the stressed conditions that could support the unique nitrogen transformations hereto discussed may be common. In this arena, however, our inquiry should focus not only on *in situ* remedial benefits but also on the potential environmental dangers. In both natural and engineered environments, stressed conditions are associated with nitrogen conversions terminating in N_2O , contributing to acid rain, greenhouse effect, and ozone depletion.^{1,17)} Accordingly, nitrogen remedial schemes cannot stop at unique solutions to water treatment problems—accountability of ultimate conversion products to a safe form such as N_2 is essential.

SUMMARY

Biological transformation assays of soluble forms of inorganic nitrogen in autotrophic media under anaerobic and O_2 -stressed conditions revealed the following:

*During initial acclimation periods, cultures with seed sludge of denitrifying origins adapted quicker to the O_2 -stressed, autotrophic testing environment than those of activated sludge origins.

*Under O_2 -stressed conditions a decline in the rate of NO_3^- reduction was associated with an onset of NH_4^+ disappearance. The estimated rate of O_2 intrusion, however, was not sufficient to account for the NH_4^+ transformation and NO_2^- was not being oxidized to NO_3^- . Near depletion of NH_4^+ resulted in a restoration of NO_3^- reduction where an organic electron donor was not

considered present.

*Under anaerobic conditions (O_2 exclusion) use of low levels of NO_2^- and NO_3^- ($< 15 \text{ mg l}^{-1}$) as substrates were associated with a gradual disappearance of NH_4^+ which was inhibited by relatively high concentrations of NO_3^- .

*Using NO_2^- as a potential electron acceptor, concurrent disappearances of NO_2^- and NH_4^+ occurred. These reproducible conversions, however, were not mutual; i.e., termination of NH_4^+ oxidation did not stop NO_2^- reduction. SEM micrographs showed a dominance of irregularly shaped microorganisms.

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