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ORIGINAL PAPER

Nitrate and nitrite inhibition of methanogenesis during denitrification in granular biofilms and digested domestic sludges

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Abstract Anaerobic bioreactors that can support simultaneous microbial processes of denitrification and methanogenesis are of interest to nutrient nitrogen removal. However, an important concern is the potential toxicity of nitrate (NO_3^-) and nitrite (NO_2^-) to methanogenesis. The methanogenic toxicity of the NO_x^- compounds to anaerobic granular biofilms and municipal anaerobic digested sludge with two types of substrates, acetate and hydrogen, was studied. The inhibition was the severest when the NO_x^- compounds were still present in the media (exposure period). During this period, 95% or greater inhibition of methanogenesis was evident at the lowest concentrations of added NO_2^- tested (7.6–10.2 mg NO_2^--N l^{-1}) or 8.3–121 mg NO_3^--N l^{-1} of added NO_3^-, depending on substrate and inoculum source. The inhibition imparted by NO_3^- was not due directly to NO_3^- itself, but instead due to reduced intermediates (e.g., NO_2^-) formed during the denitrification process. The toxicity of NO_x^- was found to be reversible after the exposure period. The recovery of activity was nearly complete at low added NO_x^- concentrations; whereas the recovery was only partial at high added NO_x^- concentrations. The recovery is attributed to the metabolism of the NO_x^- compounds. The assay substrate had a large impact on the rate of NO_2^- metabolism. Hydrogen reduced NO_2^- slowly such that NO_2^- accumulated more and as a result, the toxicity was greater compared to acetate as a substrate. The final methane yield was inversely proportional to the amount of NO_x^- compounds added indicating that they were the preferred electron acceptors compared to methanogenesis.

Keywords Upflow anaerobic sludge blanket (UASB) · Denitrification · Methanogenic inhibition · Dissimilatory nitrate reduction to ammonium (DNRA) · Biological nutrient removal

Abbreviations

Anammox	Anaerobic ammonium oxidation
AOB	Ammonia oxidizing bacteria
AS	Activated sludge
BNNR	Biological nutrient nitrogen removal
DNRA	Dissimilatory nitrate reduction to ammonium
EGSB	Expanded granular sludge bed reactor
NPB	Nitrite oxidizing bacteria
VFA	Volatile fatty acids
UASB	Upflow anaerobic sludge blanket reactor
WWTPs	Wastewater treatment plants

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Introduction

Ammonium nitrogen, formed from the decomposition of organic matter, is an important contaminant in a large variety of wastewaters. Some of the better known examples include municipal domestic wastewater (Henze and Comeau 2008; Tchobanoglous et al. 2003), animal wastes (Knight et al. 2000; Mallin and Cahoon 2003) and landfill leachate (Kjeldsen et al. 2002). Currently environmental regulations on nutrient nitrogen discharges are becoming stricter, and consequently biological nutrient nitrogen removal (BNNR) is progressively becoming an integral part of wastewater treatment. The overwhelming majority of nutrient nitrogen removal options are based on the microbial processes of nitrification and denitrification (Tchobanoglous et al. 2003; Ekama and Wentzel 2008). Nitrification (Eq. 1) is carried out by two sequential reactions of obligate aerobic chemolithoautotrophic bacteria, the ammonia oxidizing bacteria (AOB); and the nitrite oxidizing bacteria (NOB).

$$NH_4^+ + 2.0O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (1)$$

Denitrification is carried out by a broad diversity of mostly heterotrophic as well as a few autotrophic bacteria that utilize many types of organic and reduced inorganic compounds as their energy source in the absence of elemental oxygen. Nitrate is reduced to inert environmentally benign dinitrogen gas. Examples of heterotrophic and autotrophic denitrification reactions are shown below in Eqs. 2 and 3 with acetic acid and hydrogen gas as the electron donating substrates, respectively:

$$NO_3^- + H^+ + 0.625CH_3COOH \rightarrow 0.5N_2 + 1.25CO_2 + 1.75H_2O \quad (2)$$

$$NO_3^- + H^+ + 2.5H_2 \rightarrow 0.5N_2 + 3H_2O \quad (3)$$

BNNR is achieved by sequential nitrification in the presence of O_2 followed by denitrification driven by organic substrates (or reduced inorganic substrates) in the absence of O_2. As a technology, this can be staged in sequential reactors, a nitrification reactor followed by a denitrification reactor. Or alternatively, the processes can be separated temporally in sequencing batch reactors. Both processes can also occur simultaneously in a single oxygen limited aerobic reactor (Tchobanoglous et al. 2003; Ekama and Wentzel 2008; Rittman and McCarty 2001).

The microbial process of anaerobic ammonium oxidation (Anammox) is the missing link of the nitrogen cycle discovered in The Netherlands in the early 1990s (Mulder et al. 1995; Vandegraaf et al. 1995). Anammox involves the reaction of ammonium (NH_4^+) with nitrite (NO_2^-) to produce dinitrogen gas (N_2) (Jetten et al. 2005; Kuenen 2008; van der Star et al. 2007) as summarized in the simplified equation shown below:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (4)$$

The thermodynamically favorable reaction ($\Delta G'^\circ = -257$ kJ) utilizes NH_4^+ as the electron donor and NO_2^- as the electron acceptor. Anammox is catalyzed by chemolithoautotrophic aquatic bacteria belonging to the order planctomycetes (Strous et al. 1999). Anammox adds to the repertoire of possible microbial reactions that can be utilized in BNNR during wastewater treatment. To realize anammox with a waste stream rich in NH_4^+, it is necessary to oxidize approximately half of the NH_4^+ to NO_2^-. This is achieved by partial nitifrication (nitritation) with AOB followed by anammox (van Dongen et al. 2001) or partial denitrification to nitrite followed by anammox (Kalyuzhnyi et al. 2008; Sumino et al. 2006).

All the processes of BNNR share a common feature; nitrogen oxides (NO_x^-), NO_3^- and/or NO_2^-, are brought into contact with microbial active consortia under anaerobic conditions to promote their conversion to N_2 gas. Since anaerobic reactors relying on methanogenesis to convert organic carbon to methane are utilized at many wastewater treatment plants (WWTPs), the question arises as to whether they can serve the dual function of NO_x^- removal. At municipal WWTPs, anaerobic reactors are commonplace for sludge stabilization (anaerobic digesters) of waste activated sludge (Appels et al. 2008). Additionally, high rate anaerobic biofilm bioreactors are applied for the treatment of industrial effluents (Kassam et al. 2003; Frankin 2001) and domestic wastewater (van Haandel et al. 2006).

Several studies have evaluated combined denitrification and methanogenesis in anaerobic biofilm reactors, where nitrate is introduced into the anaerobic bioreactor. Denitrification occurred in upflow anaerobic sludge blanket (UASB) reactors fed with nitrates and synthetic feeds composed of volatile fatty acids (VFA; Hendriksen and Ahring 1996; Lee et al. 2004).

Steady state denitrification rates up to a of loading of 336–600 mg NO_3-N l^{-1} d^{-1} and 3,300–6,600 mg chemical oxygen demand (COD) l^{-1} d^{-1} with more than 99% removal of both nitrate and carbon was achieved in these studies. COD that was not utilized for denitrification was converted to methane. The specific denitrification activity of the biofilms reached values as high as 1.1–4.3 g NO_3^--N g^{-1} VSS d^{-1}.

During wastewater treatment, a common strategy proposed for combined C and N removal involves a sequential treatment of the wastewater in an anaerobic reactor followed by an aerobic post-treatment with up-front recycle of the final effluent to the anaerobic reactor to promote denitrification of NO_x^- formed during post-treatment as shown in Fig. 1. This approach has been tested in several types of systems. In one study, a synthetic wastewater containing sucrose and peptone was treated with a UASB followed by an airlift reactor (Tai et al. 2006). The

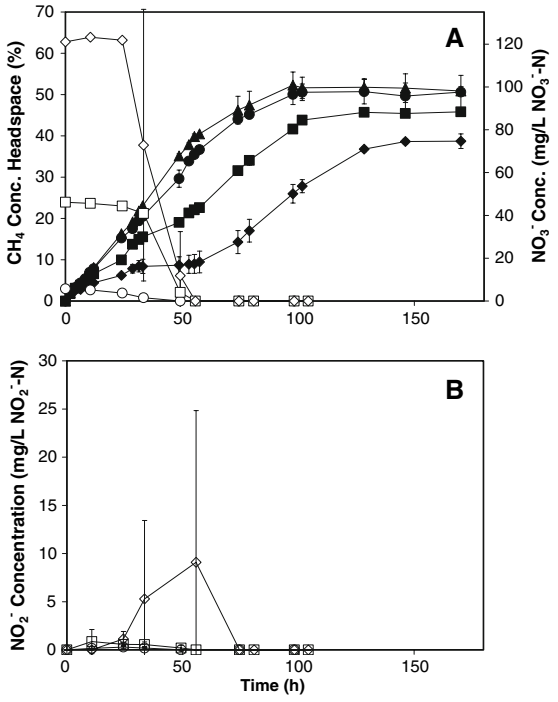

Fig. 1 Impact of NO_3^--N concentration on methane formation and denitrification by Mahou granular sludge with acetate as the electron donor. *Panel A*. Headspace methane concentrations with initial NO_3^--N concentrations of: (▲), 0; (●), 16; (■), 46; (◆), 121 mg N l^{-1}. NO_3^--N concentrations with initial NO_3^--N concentrations of: (○), 16; (□), 46; (◇), 121 mg N l^{-1}. *Panel B*. NO_2^--N concentrations with initial NO_3^--N concentrations of: (○), 16; (□), 46; (◇), 121 mg N l^{-1}

denitrification efficiency of the integrated system was 86%, when the recycle ratio was 400%. A brewery wastewater was treated in a baffled granular sludge bed reactor with five compartments followed by a nitrification unit. With an effluent recycle of 200%, the inlet NO_x^- of the anaerobic reactor was 70 mg l^{-1} and the NO_x^- concentration dropped to zero in the second compartment of the baffle reactor (Baloch et al. 2006). A pre-settled piggery wastewater was treated in a UASB—activated sludge (AS) system in which nitrified effluent of the AS reactor was recycled to the UASB at a recycle ratio of 300% (Huang et al. 2007). The combined reactor system achieved efficient removal of COD (96–97%) and total nitrogen removal of (54–77%). Methanogenesis occurred with nearly-complete denitrification in the UASB reactor (Huang et al. 2007). The up-front recycle approach has also been employed with post-treatment nitritation, where NO_2^- is the predominant NO_x^- recycled back to the anaerobic reactor. Landfill leachate was treated in a two-stage UASB reactor followed by post treatment in an anoxic/oxic baffled reactor for nitritation. Simultaneous methanogenesis and denitrification of NO_2^- (denitritation) were observed in the first UASB reactor; where a maximum NO_2^--N removal rate of 3 g N l^{-1} d^{-1} was obtained (Peng et al. 2008). A combined UASB and aerobic membrane reactor for nitritation was utilized with a synthetic wastewater (glucose, VFA, meat extract and peptone) and an effluent recycle of up to 800% was employed (An et al. 2008). In the UASB nitrite was converted to N_2 gas and excess organic carbon was simultaneously converted to methane. Organic carbon and total nitrogen removal achieved in the combined system was 98 and 83%, respectively.

Recycling of NO_2^- back to the anaerobic reactor can also result in the anaerobic reactor becoming enriched with anammox bacteria since the NO_2^- will occur simultaneously with NH_4^+-N. In this regard an expanded granular sludge bed (EGSB) reactor was evaluated for simultaneous anammox, denitrification and methanogenesis (Zhang 2003). NO_2^- removal efficiencies of 97–100% were achieved with removal rates as high as 800 mg N l^{-1} d^{-1}. NO_2^- formed by partial denitrification of NO_3^- in a UASB reactor can also favor the co-occurrence of anammox bacteria and methanogens (Sumino et al. 2006).

The reports of simultaneous methanogenesis with denitrification and anammox are intriguing because

there is also evidence that NO_x^- compounds are inhibitory to methanogenesis. As early as 1941, Barker reported that high concentrations of nitrate inhibited a pure culture of the methanogen, *Methanobacterium omelianskii* (Barker 1941). Complete growth inhibition was observed at 140 mg NO_3^--N l^{-1} and higher. Autotrophic methanogenesis by pure cultures of *Methanobacterium thermoautotrophicum* and *Methanobacterium formicicum* were partially inhibited by 130 mg l^{-1} NO_3^--N and severely to completely inhibited in the presence of 13 mg l^{-1} NO_2^--N (Balderston and Payne 1976). Autotrophic methanogenesis by *Methanosarcina barkeri* was inhibited 50% by 42 mg NO_3^--N l^{-1} and 0.07 mg NO_2^--N l^{-1} (Kluber and Conrad 1998). Acetoclastic methanogenesis by *Methanosarcina mazei* was only partially inhibited by high concentrations of NO_3^-, ranging in concentrations from 200 to 1,000 mg l^{-1} NO_3^--N but severely inhibited by only 2.5 mg l^{-1} NO_2^--N (Clarens et al. 1998). The results taken as a whole suggest that NO_3^- inhibition of methanogenesis is low to moderate whereas the inhibition caused by NO_2^- is very severe.

The objective of this study is to determine how toxic NO_x^- compounds are in mixed microbial consortia found in anaerobic bioreactors. The methanogenic inhibition and denitrification of NO_3^- and NO_2^- were studied with anaerobic granular biofilms from a UASB reactor and a municipal digested sludge from a digester. The impact of two substrates, H_2/CO_2 and acetate on NO_x^- inhibition and denitrification was also evaluated for each microbial consortium.

Materials and methods

Chemicals

Potassium nitrate (>99% purity) was obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Sodium nitrite (>99%) and sodium acetate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were used as received.

Sludge sources

Two different methanogenic inocula were used in this investigation including granular sludge (Mahou) and anaerobically digested sludge (Ina road sludge). Mahou sludge was obtained from a full-scale UASB treating wastewater at Mahou beer brewery in Guadalajara, Spain. The sludge was washed and sieved to remove fine particles before use in the toxicity assays. Ina road sludge was obtained from a municipal anaerobic digester at a local municipal wastewater treatment plant (Ina road wastewater treatment plant, Tucson, Arizona). The content of volatile suspended solids (VSS) in Mahou and Ina road sludge was 8.13 and 1.23%, respectively, of the wet weight. The maximum specific activity of the Mahou sludge was 409 and 207 mg chemical oxygen demand (COD) as CH_4 g^{-1} VSS d^{-1}, respectively with acetate and hydrogen as the substrates. The maximum specific activity of the Ina sludge was 185 and 214 mg CH_4-COD as CH_4 g^{-1} VSS d^{-1}, respectively with acetate and hydrogen as the substrates.

Basal media

The basal mineral medium was prepared using ultrapure water (Milli-Q system; Millipore) and contained (in mg l^{-1}): KH_2PO_4 (37); $CaCl_2{\cdot}2H_2O$ (10); $MgSO_4{\cdot}7H_2O$ (10); $MgCl_2{\cdot}6H_2O$ (78.1); NH_4HCO_3 (987.9); $NaHCO_3$ (3,003); Yeast extract (20), and trace element solution (1.1 ml l^{-1}). The trace element solution contained (in mg l^{-1}): H_3BO_3 (50); $FeCl_2{\cdot}4H_2O$ (2,000); $ZnCl_2$ (50); $MnCl_2{\cdot}4H_2O$ (50); $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (50); $AlCl_3{\cdot}6H_2O$ (90); $CoCl_2{\cdot}6H_2O$ (2,000); $NiCl_2{\cdot}6H_20$ (50); $CuCl_2{\cdot}2H_2O$ (30); $NaSeO_3{\cdot}5H_2O$ (100); EDTA (1,000); resazurin (200) and 36% HCl (1 ml l^{-1}). The final pH of the basal medium was adjusted to 7.2 with HCl.

Methanogenic toxicity assays

Methanogenic toxicity assays were conducted in glass serum flasks (160 ml) at 30 ± 2°C in an orbital shaker (75 rpm). Mahou granular sludge and Ina road sludge were added at a concentration of 1.5 and 1.21 g VSS l^{-1}; respectively, to serum flasks containing 70 ml basal medium. All flasks were sealed with butyl rubber stoppers and aluminum crimp seals and their headspace was flushed with N_2/CO_2 (80:20, v/v) for 5 min to create an anaerobic condition. Both acetate and hydrogen were used as an electron donor. For the acetoclastic assays, the acetate was supplemented at a final concentration of 2 g COD l^{-1}. For hydrogenotrophic assays, the H_2/CO_2 was supplied

by pressurizing the flasks to 1.5 atm (80:20, v/v). The flasks were pre-incubated overnight to ensure that the sludge was adapted to medium conditions. On the following day, either nitrite or nitrate was added at different concentrations then the headspace of the flasks for acetoclastic assays was flushed again with N_2/CO_2 (80:20, v/v). For the hydrogenotrophic assays, the headspace of the flasks was first flushed with N_2/CO_2 (80:20, v/v) and then pressurized with H_2/CO_2 (80:20, v/v) to 1.5 atm, the final partial pressure of H_2 was 1.2 atm, corresponding to 1 g COD l_{liq}^{-1} Then, the flasks were incubated at least 2 h prior to determination of methane content in the headspace of each flask. The methane content of the headspace of each flask was measured several times during the first day of the experiment and periodically during the subsequent days until all the substrate (acetate or H_2) was depleted. In addition, several liquid samples were taken from each flask over the course of the experiment for the determination of nitrite and nitrate. Triplicate controls were included in each assay where no toxicant was added.

The specific methanogenic activities (mg CH_4-COD g^{-1} VSS d^{-1}) were calculated from the slope of the cumulative methane production versus time and the initial biomass concentration as the mean value of the triplicate assays. The activities of treatments spiked with NO_3^- or NO_2^- were normalized to the activity in a control lacking NO_x^- compounds.

Analytical methods

The methane concentration from the headspace of the serum flasks was determined by gas chromatography (GC) using an HP5290 series II system (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (GC-FID). The GC was fitted with a DB-FFAP column (J&W Scientific, Palo Alto, CA) capillary column. The temperature of the column, the injector port and the detector was 140, 180, and 250°C, respectively. The carrier gas was helium at a flow rate of 9.3 ml min^{-1} and a split flow of 32.4 ml min^{-1}. Samples for measuring methane content (100 µl) in the headspace were collected using a pressure-lock gas syringe.

Nitrite and nitrate were analyzed by suppressed conductivity ion chromatography using a Dionex 3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS18 analytical column (4 mm × 250 mm) and AG18 guard column (4 mm × 50 mm). The column was maintained at 35°C. The eluent used was 10 mM KOH at a flow rate of 1.0 ml min^{-1}. The injection volume was 25 µl. Before measurement, all samples were centrifuged at 10,000 rpm for 10 min.

Volatile suspended solids and other analytical parameters were determined according to standard methods for examination of water and wastewater (APHA 2005).

Results

Methanogenic inhibition

The impact of NO_3^- on methanogenesis by granular anaerobic sludge (Mahou) is shown in Figs. 1 and 2 in assays with acetate and hydrogen. In the assay with both acetate and hydrogen, the denitrification lag phase lasted for approximately 1 day, during which time there was no large impacts on methanogenesis. As soon as denitrification started, inhibition of methanogenesis was evident, being more severe at the higher initial NO_3^- concentrations. The inhibition was markedly higher in assays with hydrogen. The initiation of denitrification was paralleled by an accumulation of NO_2^-. This accumulation was low in the case of acetate and high in the case of nitrite. At the peak, of the accumulation, NO_2^- was recovered with an 8 and 88% molar yield of the added NO_3^- in the assay with acetate and hydrogen, respectively. An additional consequence was the sludge was exposed to NO_2^- for a longer period in the hydrogen assay. The methanogenesis resumed after all the NO_x^- species were consumed. During the period when NO_x^- species were present, the degree of inhibition was markedly less in the acetate assay compared to the hydrogen assay. At the two highest NO_3^- concentrations tested, the inhibition was close to 100% in the hydrogen assay. The methanogenic activity was completely to partially reversible depending on the initial NO_3^- concentration and electron-donor used. The final yield of methane was inversely proportional to the NO_3^- initially added.

The inhibition of methanogenesis by NO_2^- was also evaluated with the Mahou sludge using acetate and hydrogen as the electron donating substrate (Figs. 3, 4). In both assays, the methanogenesis was inhibited nearly completely from time 0 as long as the

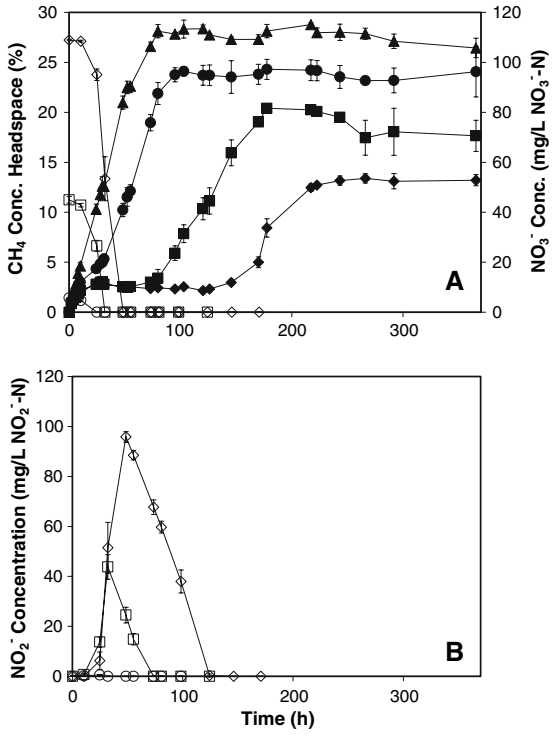

Fig. 2 Impact of NO_3^--N concentration on methane formation and denitrification by Mahou granular sludge with hydrogen gas as the electron donor. *Panel A*. Headspace methane concentrations with initial NO_3^--N concentrations of: (▲), 0; (●), 6; (■), 45; (◆), 109 mg N l^{-1}. NO_3^--N concentrations with initial NO_3^--N concentrations of: (○), 6; (□), 45; (◇), 109 mg N l^{-1}. *Panel B*. NO_2^--N concentrations with initial NO_3^--N concentrations of: (○), 6; (□), 45; (◇), 109 mg N l^{-1}

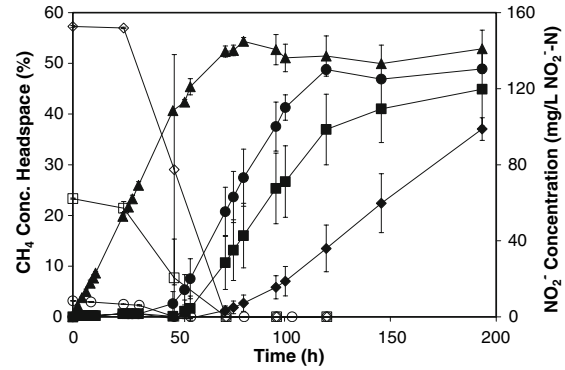

Fig. 3 Impact of NO_2^--N concentration on methane formation and denitrification by Mahou granular sludge with acetate as the electron donor. Headspace methane concentrations with initial NO_2^--N concentrations of: (▲), 0; (●), 8; (■), 62; (◆), 153 mg N l^{-1}. NO_2^--N concentrations with initial NO_2^--N concentrations of: (○), 8; (□), 62; (◇), 153 mg N l^{-1}

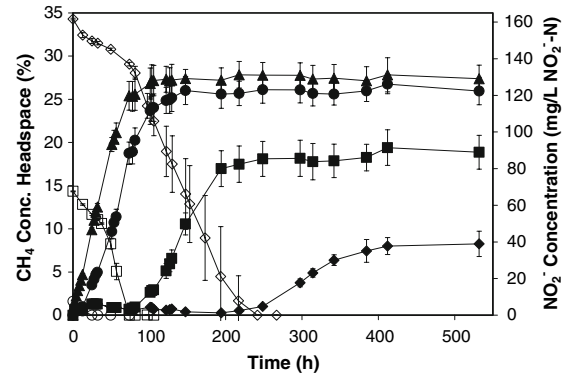

Fig. 4 Impact of NO_2^--N concentration on methane formation and denitrification by Mahou granular sludge with hydrogen gas as the electron donor. Headspace methane concentrations with initial NO_2^--N concentrations of: (▲), 0; (●), 8; (■), 68; (◆), 162 mg N l^{-1}. NO_2^--N concentrations with initial NO_2^--N concentrations of: (○), 8; (□), 68; (◇), 162 mg N l^{-1}

NO_2^- was present. The methanogenesis started once the NO_2^- was consumed. This occurred earlier in the acetate assay because the NO_2^- was consumed faster compared to the hydrogen assay. The methanogenic activity was more severely impacted by the highest NO_2^- concentration in the hydrogen assay, most likely because it was exposed to NO_2^- for a longer period. As was observed with NO_3^-, the final yield of methane was inversely proportional to the addition of NO_2^-.

Anaerobic digester sludge (Ina) was also tested to determine if the trends observed were consistent with a completely different source of inoculum. Ina sludge was assayed with NO_3^- and hydrogen as electron acceptor as shown in Fig. 5. Unlike, Mahou sludge, denitrification started without a lag phase. This coincided with evidence for nearly complete inhibition of methanogenesis at the start of the experiment. Denitrification was rapid with only a low peak NO_2^- concentration (molar yield of 14% compared to added NO_3^-). When all the NO_x^- species were consumed after 2 days methanogenesis started and the activity was only partuially inhibited compared to the untreated control. As was observed in the Mahou sludge, the final yield of methane was inversely proportional to the addition of NO_3^-.

The relative activity of all the experiments are summarized in Fig. 6, including additional experiments

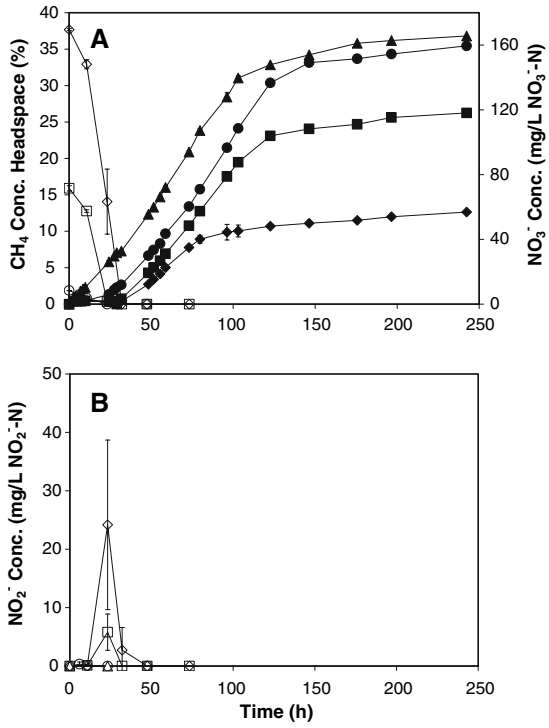

Fig. 5 Impact of NO_3^--N concentration on methane formation and denitrification by Ina municipal digester sludge with hydrogen gas as the electron donor. *Panel A*. Headspace methane concentrations with initial NO_3^--N concentrations of: (▲), 0; (●), 8; (■), 72; (◆), 170 mg N l^{-1}. NO_3^--N concentrations with initial NO_3^--N concentrations of: (○), 8; (□), 72; (◇), 170 mg N l^{-1}. *Panel B*. NO_2^--N concentrations with initial NO_3^--N concentrations of: (○), 8; (□), 72; (◇), 170 mg N l^{-1}

conducted with Ina sludge. For the most part, the methanogenic inhibition was nearly complete during exposure to NO_x^- compounds. An exception was the lower NO_3^- concentration treatments in the Mahou sludge assay with acetate. However, after metabolism of the NO_x^-, methanogenic activity was partially restored. The degree of restoration was greater at lower NO_x^- concentrations. The restoration of methanogenic activity was greater when acetate was the electron donor compared to hydrogen.

Electron balance

A balance of the electron equivalents is shown in Fig. 7 for the assay with Mahou sludge spiked with NO_3^- and acetate. The balance shows that as the NO_3^--N concentrations were progressively increased, the decrease in methane yield coincided with electrons required to reduce NO_3^- to N_2. All the other assays had a different pattern as shown in a typical example with Ina sludge spiked with NO_3^- in Fig. 8. This example shows that as the NO_3^--N concentrations were progressively increased, the decrease in methane yield could not be totally accounted for by the electrons required to reduce NO_3^- to N_2. Instead, the hole in the balance could be accounted for if the NO_3^- were assumed to have undergone dissimilatory nitrate reduction to ammonium (DNRA).

Nitrate and nitrite metabolism

Figures 1, 2, 3, 4, and 5 indicate that both NO_3^- and NO_2^- were readily metabolized during the methanogenic inhibition assays. The denitrification or DNRA was initiated in most cases with a lag phase of 1 d or less. The results indicate that anaerobic sludges are abound with bacteria capable of denitrification or DNRA. The maximum specific NO_x^- removal activity is summarized in Table 1. The highest specific rates of NO_x^- metabolism were observed with NO_3^- and hydrogen in Ina sludge as well as NO_3^- and acetate in Mahou sludge. The lowest specific rates were observed with NO_2^- and H_2 regardless of the inoculum source. The low rate probably accounts for the high accumulation of NO_2^- in the experiment with Mahou sludge and hydrogen. The slow metabolism of NO_2^- in the experiment with Ina sludge and H_2 is due to inhibition of NO_2^- metabolism by high NO_2^- concentrations. Compared to the rates recorded at the lower concentrations, the NO_2^- removal activity at 180 mg NO_2^--N l^{-1} was 40% inhibited (results not shown).

Discussion

The results of this study indicate that NO_x^- compounds are highly toxic to methanogenesis and they are also readily metabolized by the anaerobic sludges. The inhibition was the severest when denitrification was active and the NO_x^- compounds were still present in the media (exposure period). During this period, 95% or greater inhibition of methanogenesis was already evident at the lowest NO_2^- concentrations tested (7.6–10.2 mg NO_2^--N l^{-1}) in all assays of this study. In the case of NO_3^-, 95% or greater inhibition of methanogenesis during the exposure period was

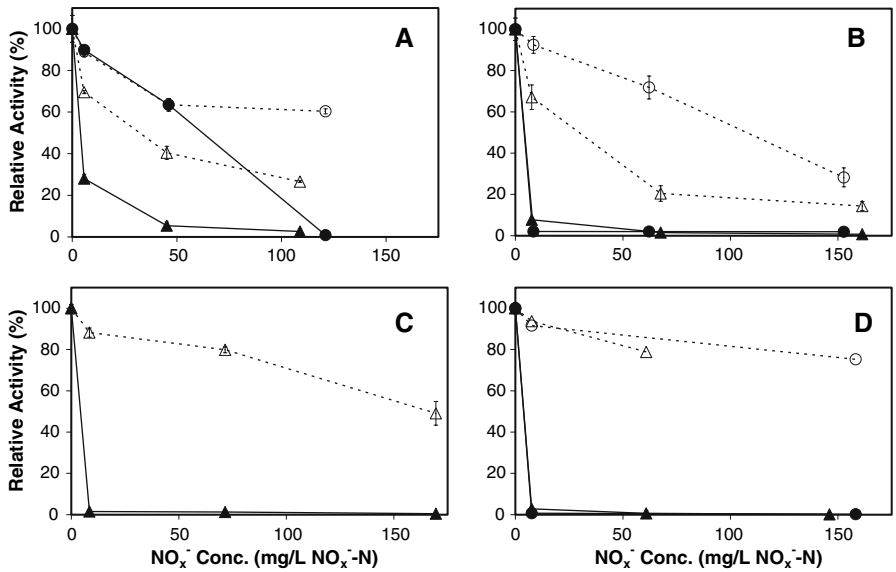

Fig. 6 Relative methanogenic activity as a function of NO_x^--N concentration. *Panel A*. Mahou granular sludge with added NO_3^-. *Panel B*. Mahou granular sludge with added NO_2^-. *Panel C*. Ina municipal digester sludge with added NO_3^-. *Panel D*. Ina municipal digester sludge with added NO_2^-. Relative activities during the period of NO_x^- exposure: (●), acetate as electron-donor; (▲), hydrogen as electron donor. Relative activity after metabolism of NO_x^-: (○), acetate as electron-donor; (Δ), hydrogen as electron donor. The activities are normalized to a parallel incubated control with no added NO_x^-. The highest NO_2^- treatment in *Panel D* for Ina sludge with hydrogen could not be plotted for the period after metabolism, because NO_2^- was not completely metabolized

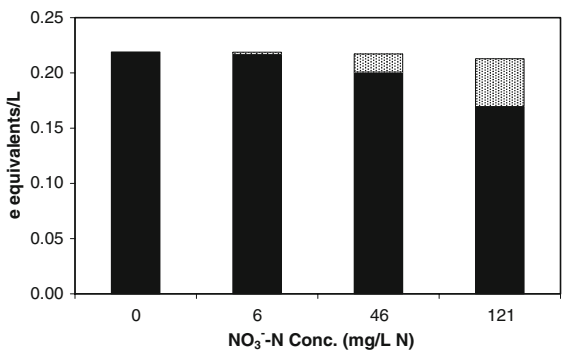

Fig. 7 The balance of electron equivalents used for methanogenesis and NO_3^- removal as a function of increasing NO_3^- addition in the assay with Mahou sludge and acetate. The *black bars* indicate methane production, the *spotted bars* indicate loss of NO_3^-, assuming conversion to N_2

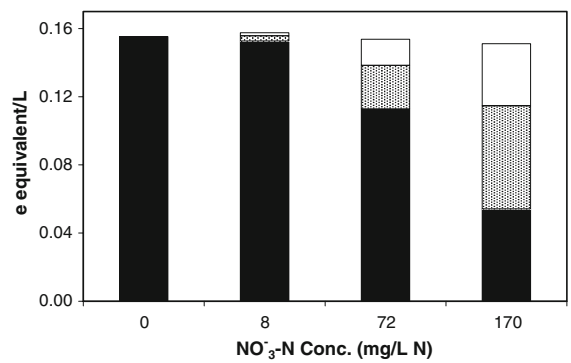

Fig. 8 The balance of electron equivalents used for methanogenesis and NO_3^- removal as a function of increasing NO_3^- addition in the assay with Ina sludge and hydrogen. The *black bars* indicate methane production, the *spotted bars* indicate loss of NO_3^-, assuming conversion to N_2, the *white bars* indicate the additional electron equivalents beyond denitrification if the NO_3^- were reduced by dissimilatory nitrate reduction to ammonia (DNRA)

evident at concentrations of 8.3–121 mg NO_3^--N l^{-1}, depending on substrate and inoculum source.

The inhibition imparted by NO_3^- does not appear to be directly due to NO_3^- itself, but instead due to reduced intermediates formed during the denitrification process. There are multiple lines of evidence to support this hypothesis. Firstly, during the first day of the experiments with the granular biofilm sludge (Mahou), there is a lag phase prior to the initiation of

Table 1 Specific rates of NO_x^- metabolism in anaerobic sludge with no prior enrichment

Sludge	NO_x^-	Electron donor	Specific activity (mg NO_x^--N g^{-1} VSS d^{-1})
Mahou	NO_2^-	Acetate	717
Mahou	NO_2^-	H_2	250
Mahou	NO_3^-	Acetate	1,036
Mahou	NO_3^-	H_2	680
Ina	NO_2^-	Acetate	302
Ina	NO_2^-	H_2	77
Ina	NO_3^-	H_2	1,938

denitrification, and during this period there is no noteworthy inhibition of methanogenesis. Secondly, the lag phase was followed by a temporal accumulation of NO_2^-, a known intermediate of denitrification and during this period, there was a complete cessation of methane production. Thirdly, when NO_2^- was completely consumed, the methane production resumed again. These findings are in agreement with previous studies exploring the inhibition NO_2^- and NO_3^- to methanogenesis where it has been consistently observed that NO_2^- is markedly more toxic to methanogenesis. This has been evident in pure cultures (Clarens et al. 1998; Balderston and Payne 1976; Kluber and Conrad 1998) as well as natural mixed and enrichment cultures (Balderston and Payne 1976; Tugtas and Pavlostathis 2007a). Likewise in this study, the inhibitory impact of NO_2^- to Mahou during the period of NO_x^- exposure was greater than that of NO_3^-. Fourthly, the process of denitrification itself has been associated with a greater toxicity of NO_3^- to methanogenesis. Co-culture experiments showed that the methanogen, *Methanosarcina mazei*, growing with 210 mg NO_3^--N l^{-1}, produced methane from acetate until the denitrifying bacterium *Pseudomonas stutzeri* was introduced into the culture and NO_3^- denitrification began Clarens et al. (1998), suggesting that reduced products of NO_3^- were responsible for the toxicity. While exploring different electron donor substrates for denitrification in a methanogenic enrichment culture, it was observed that the substrates causing a greater accumulation of reduced denitrification intermediates [e.g., NO_2^- and nitric oxide (NO)] were associated with a greater toxicity caused by added NO_3^- (Tugtas and Pavlostathis 2008).

The pattern of methane formation with the Mahou sludge and added NO_3^- followed a staircase pattern with an initial upward slope—followed by a plateau—followed by a resumption of the upward slope—followed by a final plateau. The first plateau corresponded to the period of NO_2^- accumulation; the second plateau corresponded to the exhaustion of substrate. This pattern was distinct with the Ina sludge and added NO_3^-, which lacked the initial period of methane production and the methane production only started after intermediate NO_2^- had disappeared. The difference in behavior may possibly be attributed to the more rapid initiation of denitrification in the Ina sludge experiments as evidenced from an early start of the NO_3^- removal without any lag phase. The lowest NO_3^- concentration tested was inhibitory initially even though hardly any measurable NO_2^- was formed in that case (Fig. 5). Other denitrification products aside from NO_2^-, such as NO may have accumulated as well. Nitric oxide is known to be one of the most toxic intermediates of denitrification to methanogenesis (Balderston and Payne 1976; Tugtas and Pavlostathis 2007a).

Figure 9 summarizes the inhibition data of this study as a function of the maximum NO_2^- concentration, irregardless of whether the NO_2^- was added initially or formed form the biotransformation of added NO_3^-. The figure illustrates a relationship between the observed inhibition during the exposure period and the maximum NO_2^- concentration in that period. The NO_2^- was very toxic, such that at most concentrations nearly complete inhibition was observed. A few points in the range of 0.3–0.6 mg l^{-1} NO_2^--N provided partial inhibition data enabling estimates of the 50% inhibiting concentration (50%IC). The 50%IC of acetoclastic and hydrogenotrophic methanogenesis in the Mahou sludge was estimated to be 0.83 and 0.38 mg l^{-1} NO_2^--N, respectively; indicating that the hydrogenotrophic methanogens were more sensitive to NO_2^- toxicity. The hydrogenotrophic methanogensis of the Ina sludge was inhibited by 98.5% at 0.38 mg l^{-1} NO_2^--N, corresponding to an estimated 50%IC of 0.19 mg l^{-1} NO_2^--N. The higher toxicity of the latter is most likely attributable to dispersed biomass in the Ina sludge which was more exposed to NO_2^- toxicity compared to methanogens in biofilm granules of the Mahou sludge.

The toxicity of NO_x^- was found to be reversible after the exposure period. The reversal is attributed to

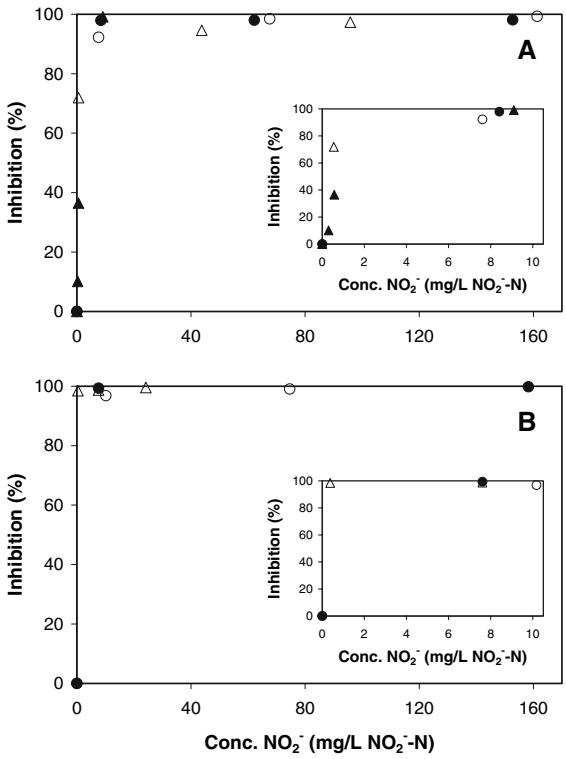

Fig. 9 Inhibition of methanogenic activity during the exposure as a function of the maximum NO_2^--N concentration measured. *Panel A*. Mahou granular sludge. *Panel B*. Ina municipal digester sludge. Legend: (▲), acetate as electron-donor in assays with added NO_3^-; (●), acetate as electron-donor in assays with added NO_2^-; (Δ), hydrogen as electron donor in assays with added NO_3^-; (○), hydrogen as electron donor in assays with added NO_2^-

the metabolism of the NO_x^- compounds and intermediates. The recovery of activity was complete at low NO_x^- concentrations; whereas the recovery was only partial at high NO_x^- concentrations. The pattern of inhibition recovery to NO_x^- exposure has been observed in other studies. In a methanogenic enrichment culture, with added NO_2^--N additions ranging from 17 to 500 mg l^{-1}, the recovery at 17–50 mg NO_2^--N l^{-1} was nearly complete; whereas, the activity recoveries at 250–500 mg NO_2^--N l^{-1} were only a fraction of the original activity (Tugtas and Pavlostathis 2007a). In a mesophilic sulfate adapted sludge, complete recovery from NO_2^- exposure was observed at added concentrations ranging from 5 to 150 mg NO_2^--N l^{-1}, the only difference was that the lag phase for the recovery increased with increasing concentrations (O'Reilly and Colleran 2005). The results taken as a whole seem to suggest that exposure to very high NO_2^- concentrations (and possibly other reduced denitrification intermediates) are damaging to the methanogens; whereas low concentrations only cause a largely reversible inhibition.

The electron donor of the experiment also impacted the toxicity. When H_2 was used as the substrate, reduction of NO_2^- was slower compared to acetate. In the Mahou sludge 160 mg NO_2^--N l^{-1} was completely denitritated in 70 h; with acetate; whereas it took 240 h to completely denitritate the same concentration with H_2 as electron donor. The resulting impact on the residual methanogenic activity was noticeable. The recovery of activity with acetate was 28%; whereas the longer exposure to NO_2^- with H_2 as an electron donor, resulted in a recovery of only 14% of the methanogenic activity. The electron donor also impacted the temporal accumulation of NO_2^- from NO_3^-. With acetate and Mahou sludge, the maximum accumulation of NO_2^--N from 120 mg NO_3^--N added l^{-1} was 10 mg l^{-1}; whereas with H_2, the accumulation was 96 mg l^{-1}, which was almost a stoichiometric conversion. The difference on the recovery of the methanogenic activity following NO_x^- exposure was noteworthy with the 60 and 27% recovery in the acetate and hydrogen experiment, respectively. The greater accumulation of NO_2^- with H_2 versus acetate as an electron donor was also noted in a methanogenic enrichment culture, and that difference also corresponded to a markedly enhanced toxicity as well (Tugtas and Pavlostathis 2008).

The electron balances in this study indicate that denitrification to N_2 gas occurred in Mahou sludge with added NO_3^- and acetate as electron donor. However, most of the other assays could only be properly balanced if the NO_x^- compounds were assumed to have undergone DNRA. DNRA is known to occur in very reduced environments with either sulfide or high chemical oxygen demand to NO_3^- ratios (Tugtas and Pavlostathis 2007b; Akunna et al. 1992). Injection of nitrate into an anaerobic digester treating municipal solid waste was associated with ammonium accumulation and DNRA was suspected (Vigneron et al. 2007). In this study the most noteworthy distinguishing feature between cultures suspected of carrying out DNRA compared to denitrification, is that the latter was less inhibited by NO_x^- compounds. Therefore when methanogenesis is not functioning properly there is a greater need for a sink of electrons and DNRA may be that sink.

The NO_x^- compounds were the preferred electron acceptors compared to methanogenesis. In most cases, once denitrification commenced, the electron equivalents were exclusively directed towards denitrification or DNRA. The sludges used were not previously enriched for denitrification, yet in all cases the NO_x^- removal was fully underway after 1 day. Specific rates of nitrate removal ranged up to 1,938 mg NO_3^--N g^{-1} VSS d^{-1}; and specific rates of denitritation ranged up to 717 mg NO_2^--N g^{-1} VSS d^{-1}. These rates are comparable to rates obtained in highly enriched biofilms granules (Hendriksen and Ahring 1996; Lee et al. 2004).

While this study confirms that NO_x^- compounds are highly toxic to methanogenesis this observation is not necessarily inconsistent with the numerous reports demonstrating simultaneous denitrification and methanogenesis in anaerobic biofilms reactors (Hendriksen and Ahring 1996; Lee et al. 2004; Huang et al. 2007; Peng et al. 2008; An et al. 2008). The lessons learned from this study indicate that denitrification intermediate concentrations in the anaerobic bioreactors have to be maintained at very low concentrations. Additionally, biofilms may develop zones where methanogens are protected from denitrification products by spatial separation from denitrifiers.

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