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Quantitative evaluation of inhibitory effect of various substances on anaerobic ammonia oxidation (anammox)

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The inhibitory effect of 20 substances of various chemical species on the anaerobic ammonia oxidation (anammox) activity of an enrichment culture, predominated by *Candidatus* Brocadia, was determined systematically by using a ^{15}N tracer technique. The initial anammox rate was determined during first 25 min with a small-scale anaerobic batch incubation supplemented with possible inhibitors. Although Cu^{2+} and Mn^{2+} did not inhibit anammox, the remaining 18 substances [Ni^{2+}, Zn^{2+}, Co^{2+}, MoO_4^{2-}, Fe^{2+}, 4 amines, ethylenediaminetetraacetic acid (EDTA), ethylenediamine-*N,N'*-bis (2-hydroxyphenylacetic acid) (EDDHA), citric acid, nitrilotriacetic acid (NTA), *N,N*-dimethylacetamide (DMA), 1,4-dioxane, dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF) and tetrahydrofuran (THF)] were inhibitory. Inhibitory effect of NTA, EDDHA, THF, DMF, DMA and amines on anammox was first determined in this study. Inhibitory effects of metals were re-evaluated because chelators, which may interfere inhibitory effect, have been used to dissolve metal salts into assay solution. The relative anammox activities as a function of concentration of each substance were described successfully (R^2 > 0.91) either with a linear inhibition model or with a Michaelis–Menten-based inhibition model. IC_{50} values were estimated based on either model, and were compared. The IC_{50} values of the 4 chelators (0.06–2.7 mM) and 5 metal ions (0.02–1.09 mM) were significantly lower than those of the 4 amines (10.6–29.1 mM) and 5 organic solvents (3.5–82 mM). Although it did not show any inhibition within 25 min, 0.1 mM Cu^{2+} completely inhibited anammox activity in 240 min, suggesting that the inhibitory effect caused by Cu^{2+} is time-dependent.

[**Key words:** Anaerobic ammonia oxidation; ^{15}N-tracer; IC_{50}; Inhibition models; Amines; Metals; Organic solvents; Chelators]

Anaerobic ammonia oxidation (anammox) is an anoxic reaction carried out by chemolithoautotrophic bacteria, in which the electron donor is ammonium and the electron acceptor is nitrite (1–4). The anammox process has been considered a novel cost-effective wastewater treatment process (5) because it requires neither external electron donor nor aeration, which are major expenses in conventional nitrification–denitrification processes. It has been applied to various wastewater sources, including various industrial wastewaters (6–8), landfill leachates, sludge concentrates, semiconductor effluents, and livestock wastewaters, among others. When applying the anammox process to treat various types of wastewaters, it is necessary to consider the influence of substances coexisting with NH_4^+ on anammox activity. Wastewater may occasionally contain substances that could adversely affect to wastewater treatment performance. For example, it has been reported that heavy metals are inhibitory to anammox, and, in fact, Cu^{2+}, Zn^{2+} and other heavy metals have been detected in the effluent of an anaerobic digester, in anaerobic piggery wastewater, and in slurries from the dairy products industry (9,10); similarly, Cu^{2+} has been detected in industrial wastewater from the semiconductor industry (11,12).

The adverse effects of heavy metals have been previously studied (8,13–20). Various metal species are necessary nutrients for anammox microorganisms. Copper and molybdenum are key cofactors in nitrite reductase (21) and nitrite oxidoreductase (22), which are responsible for anammox catabolism, and nickel and zinc are required for hydrogenase and dehydrogenase reactions, respectively (23). Thus, while some metal species are necessary nutrients for anammox microorganisms, they may also inhibit anammox activity when present in excess. The effects of other substances, such as monoethanolamine (MEA) (24), flocculants, allylthiourea, chloramphenicol (25), and anions (chloride, acetate, phosphate and sulfide), on the anammox process have also been examined (2,25). Information on the inhibitory effects of various substances on the anammox process has obtained recently, but systematic and quantitative data for a variety of chemical substances are still lacking.

In previous studies, inhibitory effects have been examined by monitoring the nitrogen removal performance of a bench- or pilot-scale anammox reactor, or by using a batch test where the rates of overall substrate consumption, N_2 gas emission, or increases in headspace gas pressure were determined as an index of anammox activity (26). These procedures are all indirect, in terms of

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anammox activity determination, and time-consuming. They also require, and sometimes sacrifice, a large amount of anammox biomass. Thus, these procedures are not really adequate to obtain a series of data for a variety of potential inhibitory substances. This technical obstacle is one reason for the deficiency of systematic data on the inhibitory effects of suspect substances. In order to overcome these obstacles, a high-throughput method based on a quick and direct anammox activity determination using a ^{15}N tracer technique with a simple batch test was designed by modifying a previously described method (27,28). In the present study, the inhibitory effects of 20 substances on anammox activity were quantitatively determined and compared using this method. These substances, which can be classified with respect to chemical species of each substance, included 7 metals, 4 amines, 5 chelators and 3 organic solvents. So far, it has not been reported inhibitory effect of such chelators as nitrilotriacetic acid (NTA), ethylenediamine-*N,N'*-bis (2-hydroxyphenylacetic acid) (EDDHA), tetrahydrofuran (THF), *N,N*-dimethylacetamide (DMA) and amines on anammox. To compare the efficacy of their inhibitory effects, IC_{50} values were calculated using kinetic data obtained in the presence and absence of each putative inhibitory substance. We also identified a few sources that may cause underestimation of the inhibitory efficacy and discussed appropriate procedures for fair comparison of the inhibitory effects.

MATERIALS AND METHODS

Basal solution The basal solution for determining anammox activity contained 25 mM HEPES, 0.50 mM $NaHCO_3$, 0.20 mM KH_2PO_4, 0.02 mM $MgSO_4 \cdot 7H_2O$, and 0.02 mM $CaCl_2 \cdot 2H_2O$. The pH of the basal solution was maintained at 7.4 ± 0.1, even if either amines or metals were added at the highest concentration examined in the present study. It was confirmed that 25 mM HEPES showed no adverse effect on anammox activity (data not shown). Amounts of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ in the basal solution were minimized, as cations may form chelate complexes with chelators, which would decrease the free chelator concentration.

It is notable that the composition of the basal solution used in the test appears key for the unbiased comparison of substances of different chemical species. Careful attention to the basal solution helps avoid conditions that may hamper the fair evaluation of inhibitory efficacy, such as degradation of the substance by co-existing microbes, massive adsorption onto biomass and formation of chelate complexes.

Stock solutions for substances examined Stock solutions of metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, MoO_4^{2-} and Fe^{2+}), amines [MEA, diethylamine (DEA), triethylamine (TEA) and 1,6-hexamethylenediamine (HMDA)], chelators [ethylenediaminetetraacetic acid (EDTA), EDDHA, NTA and citric acid] and the organic solvent DMA were prepared at 5–500 mM (pH 7.4). These stock solutions and 4 other organic solvents: 1,4-dioxane, dimethyl sulfoxide (DMSO), THF and *N,N*-dimethylformamide (DMF), were transferred to 12.5 mL serum vials that were closed with butyl rubber stoppers and aluminum seals, and the headspace gas was replaced with ultrapure (>99.9999%) He at 1.5 atm. The 5 organic solvents examined are all categorized as polar aprotic solvents. The vials were stored in the dark at 25°C for no longer than 2 weeks.

Anammox population The anammox enrichment culture, the inoculum of which was sewage sludge, was cultivated at 30°C in an up-flow reactor (41 mm diameter × 330 mm length; working volume, 410 mL) filled with non-woven fabric carriers. A synthetic medium comprising basal medium (6.0 mM $NaHCO_3$, 0.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$) and substrates for anammox microorganisms (1.9–9.0 mM NH_4Cl, 1.4–7.4 mM $NaNO_2$) was continuously fed into the reactor. An anammox enrichment culture at steady state, in which loading rate was 0.702 ± 0.09 kg-N m^{-3} d^{-1} (fed with 9.0 mM NH_4Cl and 7.4 mM $NaNO_2$) and the nitrogen removal efficiency was $73.3 \pm 5.7\%$, was sampled for use in inhibitory experiments. As the biomass sample was largely aggregated and planktonic cells were much less abundant, it was immediately sieved through a stainless steel screen (opening size, 300 μm unless otherwise stated), and suspended in the basal solution at a density at 6 mg wet weight mL^{-1}. The size of maximum frequency in the particle size distribution of this suspension, which was determined with a laser particle size distribution analyzer (HELOS/KR; Sympatec GmbH, Clausthal-Zellerfeld, Germany), was 388 μm. The suspension was stored at 4°C for no longer than 2 h before being used to acquire anammox activity measurements. Clone analysis of the 16S rRNA genes of the anammox microorganisms, performed with PCR amplification using primer sets described previously (27), revealed the predominance of *Candidatus* Brocadia sp. in the examined biomass. This organism is most closely related to *Ca.* Brocadia fulgida and *Ca.* Brocadia caroliniensis (Fig. S1).

Small-scale batch test for inhibition assay on anammox Anammox activity was determined using a previously described method (28), with modifications. The basal solution was newly designed as described above and the assay volume was minimized. The anammox population was washed with the basal solution three times with centrifugation. The washed biomass was suspended in basal solution, purged with pure Ar until the dissolved oxygen concentration reached 0 mg L^{-1} and 2 mL aliquots of this suspension were anaerobically transferred to 12.5 mL serum vials in an anaerobic box filled with 95% Ar + 5% H_2. After adding 3 mL of anaerobic basal solution and a small glass boiling stone, the vial was closed with a butyl rubber stopper and an aluminum seal. The wet weight of the anammox biomass in this inhibition assay suspension was 2.4 mg mL^{-1}. Cell protein concentrations were analyzed by the lowry method (29), using *DC* Protein Assay (Bio Rad, Hercules, CA, USA). The protein content was 56.4 μg mL^{-1}. The headspace gas was replaced with ultrapure (>99.9999%) He (at 1.5 atm) by repeated vacuum purging with a gas-exchange manifold (Sanshin Kogyo, Yokohama, Japan). After equilibrating the anaerobic suspension of anammox biomass at 30°C, the substrates ($^{15}NH_4^+$ and non-labeled NO_2^- at final concentrations of 1 mM) and a test substance were added to the suspension through a butyl rubber stopper using a gas-tight syringe at various concentrations. This anammox activity assay system was incubated at 30°C, and amount of $^{29}N_2$ derived from anammox was determined using GC/MS (GCMS-QP2010 plus; Shimadzu Co. Ltd., Kyoto, Japan) as a function of time. The anammox activity was determined as the rate of $^{29}N_2$ production during the first 25 min of incubation. During this time period, the decrease in substrate concentrations was thought to be negligible, therefore this rate represents the initial rate of the reaction, *v*.

It was supposed that various effects could become significant if the anammox population were exposed to the inhibitory substance for a long period of time. It was also supposed that these assays would not be simple and interpretation of the experimental data would not be easy. Therefore, the initial rate of the reaction responsible for the anammox activity was determined in order to minimize the effects resulting from longer exposure to the inhibitory substance, as well as to establish a rapid and simple assay system.

Determining K_i values using the Michaelis–Menten-based inhibition model We determined anammox activity as the initial rate of the anammox reaction in the presence and absence of added inhibitory substance, using the batch test for inhibition assay described earlier, and the ratio of the two rates is presented as the relative anammox activity (%). The relative anammox activity was plotted as a function of the inhibitory substance concentration, and the curve was either analyzed using linear regression or fitted to a simplified Michaelis–Menten equation using KaleidaGraph version 4.0 software (Synergy Software, Reading, PA, USA). We call the former the linear inhibition model, and the latter the Michaelis–Menten-based inhibition model.

Noncompetitive and uncompetitive inhibition models in Michaelis–Menten enzyme kinetics are described by Eqs. 1 and 2, respectively.

$$v = \frac{V \cdot S}{\left(1 + \frac{i}{K_i}\right)S + \left(1 + \frac{i}{K_i}\right) \cdot K_s} \quad (1)$$

$$v = \frac{V \cdot S}{\left(1 + \frac{i}{K_i}\right)S + K_s} \quad (2)$$

If the substrate (NH_4^+) concentration, *S*, exceeds the saturation constant, K_s, both Eqs. 1 and 2 can be reduced to Eq. 3:

$$v = \frac{V}{1 + \frac{i}{K_i}} = \frac{V \cdot K_i}{i + K_i} \quad (3)$$

where *V* is maximum anammox activity, *i* is the concentration of the inhibitory substance, and *v* is the relative anammox activity at *i*. In the inhibitory assay employed in this study, NH_4^+ (*S*) was supplied at 1000 μM, and the experimentally determined K_s for anammox was 26.8 μM (Fig. S2). Therefore, the assumption that $S >> K_s$ was justified. Because *V* is constant (*V* = 100% relative anammox activity), Eq. 3 can be characterized solely by K_i. Therefore, K_i can work as an index of inhibitory efficacy for the substance examined. This equation (Eq. 3) is identical to the Monod-based inhibition model previously reported (13,24,30,31), which basically reduces anammox activity to a first-order reaction. On the other hand, because anammox activity is determined as the rate constant of a zero-order reaction in this study, it would be appropriate to call the model the Michaelis–Menten-based inhibition model.

The half-maximal inhibitory concentration (IC_{50}), which has been commonly used as an indicator of inhibition efficacy, was estimated as the concentration of inhibitory substance required to provide 50% relative anammox activity in the linear inhibition model. If the efficacy of an inhibitory substance is expressed using the Michaelis–Menten-based inhibition model, and the substance is presumed to completely inhibit anammox activity, the concentration of the inhibitor that gives *V*/2 is the IC_{50}. Therefore,

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