



NOTE

# Effect of nitrification inhibitors on the Baltic Sea ammonia-oxidizing community and precision of the denitrifier method

Helena Jäntti<sup>1,\*</sup>, Simo Jokinen<sup>2</sup>, Susanna Hietanen<sup>1</sup>

<sup>1</sup>University of Helsinki, Department of Environmental Sciences, Aquatic Sciences, 00014 University of Helsinki, Finland

<sup>2</sup>University of Eastern Finland, Department of Environmental Science, Biogeochemistry Research Group, 70211 Kuopio, Finland

**ABSTRACT:** Nitrification is a key process in nitrogen cycling. Several nitrification rate measurement techniques rely on the use of chemical inhibitors that effectively and specifically inhibit nitrifying bacteria. The discovery of ammonia-oxidizing archaea has raised the question of whether these inhibitors are also effective for archaea. The efficiency of 2 commonly used chemicals—allylthiourea (ATU) and nitrapyrin (commercial name N-serve)—in inhibiting nitrification was tested in natural seawater samples containing archaea. In addition, the effect of these inhibitors was tested in a pure culture of *Pseudomonas chlororaphis*, a bacterium that is used in the denitrifier method to reduce nitrate to nitrous oxide, whose isotopic composition can be analyzed with isotopic ratio mass spectrometers. This method is commonly used in stable isotope based nitrification rate measurements. ATU inhibited nitrification by 80% at a 100 μM concentration, whereas N-serve inhibited nitrification completely at a 150 μM concentration. Although the inhibition of nitrification by ATU was not complete for the natural seawater ammonia-oxidizing community, <100 μM concentration has been shown to be efficient. For N-serve, the previously found effective concentration (150 μM) for the natural ammonia-oxidizing community inhibited nitrification completely. Both ATU and N-serve reduced nitrous oxide production in the denitrifier method, but the <sup>15</sup>N atom% values were not affected. The nitrification rates measured with inhibitors should be interpreted with caution because inhibitors can alter the functioning of bacterial methods used to analyze the nitrification rates.

**KEY WORDS:** Nitrification · ATU · N-serve · <sup>15</sup>N · Denitrifier method · Archaea

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## INTRODUCTION

Nitrification proceeds in 2 steps, in which ammonia (dissolved form ammonium, NH<sub>4</sub><sup>+</sup>) is oxidized to nitrite (NO<sub>2</sub><sup>-</sup>) in the first step and NO<sub>2</sub><sup>-</sup> is oxidized to nitrate (NO<sub>3</sub><sup>-</sup>) in the second. The capacity for ammonia oxidation was originally believed to be restricted to autotrophic bacteria that produce ammonia monooxygenase, the enzyme that is required to convert NH<sub>4</sub><sup>+</sup> to NO<sub>2</sub><sup>-</sup>. Later, ammonia-oxidizing archaea (AOA) were discovered (Könneke et al. 2005), and

once discovered, an increasing number of publications have reported that they are omnipresent in the environment (Erguder et al. 2009). Based on the information gathered using molecular tools, AOA thrive particularly in oligotrophic environments that were previously believed to be unsuitable for nitrification (Agogue et al. 2008). This may be because AOA have higher substrate affinities for NH<sub>4</sub><sup>+</sup> than do ammonia-oxidizing bacteria (AOB; Martens-Habbena et al. 2009). However, the presence of functional genes does not indicate whether nitrification

\*Email: helena.jantti@helsinki.fi

occurs or not, and consequently the functional role of AOA in most ecosystems is still unknown.

To assess the functional role of AOA, precise nitrification rate measurement techniques are required. Nitrification rates can be estimated by adding a substance that inhibits the nitrification process and then comparing the accumulation rate of  $\text{NO}_3^-$  or  $\text{NH}_4^+$  in treatments with and without the inhibitor. The difference in  $\text{NO}_3^-$  or  $\text{NH}_4^+$  accumulation is the nitrification rate. Some of the most common nitrification inhibitors are allylthiourea (ATU; Hall 1984) and nitrapyrin (commercial name N-serve; Goring 1962). Alternatively, the nitrification rates can be assessed using the  $^{15}\text{NH}_4^+$  tracer technique, in which  $^{15}\text{NH}_4^+$  is added to the sample and the production of  $^{15}\text{NO}_3^-$  is measured. The precision of the  $^{15}\text{NH}_4^+$  tracer technique ( $\sim 0.8$  pmol N) is approximately 100 times better than the precision of standard  $\text{NO}_3^-$  or  $\text{NH}_4^+$  concentration measurements ( $\sim 50$  to  $150$  pmol N; Grasshoff et al. 1983). The use of  $^{15}\text{NH}_4^+$  and inhibitors can also be combined (e.g. Sloth et al. 1992, Santoro et al. 2010). In such cases, the difference in the  $^{15}\text{NO}_3^-$  concentration with and without the inhibitor reflects the nitrification rate. In addition to nitrification rate measurements, nitrification inhibitors can also be used in studies in which the production of  $\text{NH}_4^+$  is measured (i.e. measurements of dissimilatory nitrate reduction to ammonium, DNRA) to ensure that the  $\text{NH}_4^+$  produced is not immediately taken up by nitrification, which would cause underestimation of the  $\text{NH}_4^+$  production rates (Lam et al. 2009).

If the nitrification rate is determined using the  $^{15}\text{NH}_4^+$  tracer technique, the  $^{15}\text{N}$  contents of the  $\text{NO}_3^-$  produced must be measured precisely. One common way to measure the  $^{15}\text{N}$  in  $\text{NO}_3^-$  is the denitrifier method (Sigman et al. 2001), in which cultured *Pseudomonas chlororaphis* lacking a nitrous oxide ( $\text{N}_2\text{O}$ ) reductase, convert the  $\text{NO}_3^-$  and  $\text{NO}_2^-$  (noted as  $\text{NO}_x$  from here on) present in the sample to  $\text{N}_2\text{O}$ . The isotopic composition of  $\text{N}_2\text{O}$  is then analyzed, using a gas chromatographic isotopic ratio mass spectrometer (GC-IRMS).

This study aimed to test 2 important factors in the nitrification measurements. First, we tested the effectiveness of ATU and N-serve at inhibiting nitrification in natural seawater samples containing both AOA (Labrenz et al. 2010) and AOB. Second, we evaluated the applicability of the denitrifier method for samples that were treated with ATU and N-serve, as the substances used to inhibit nitrification may also have a negative effect on the *Pseudomonas chlororaphis* utilized in the denitrifier method. Both N-serve (Henninger & Bollag 1976), and, to a lesser

extent, ATU (Jensen. et al. 2007) inhibit denitrification, but the effect of these inhibitors specifically on the functioning of *P. chlororaphis* has not yet been investigated.

## MATERIALS AND METHODS

### Preparation of the standard samples

The suitability of the denitrifier method (Sigman et al. 2001) for samples treated with ATU and N-serve (both Sigma-Aldrich) was first tested with standard samples prepared from ultrapure MilliQ water (Millipore), potassium nitrate ( $\text{KNO}_3$ ), ATU, and N-serve (Table 1). The inhibitor concentrations used, viz. 100 and 150  $\mu\text{M}$ , respectively, inhibit nitrification effectively (Ginestet et al. 1998, Southwell et al. 2008). The water-insoluble N-serve was first dissolved in acetone, and the final acetone concentration in the standard solution was 4% (vol/vol).

### Sampling and incubation of the seawater samples

The samples for the nitrification measurements were collected on the RV 'Pelagia' in May 2011 from the Baltic Proper, at the Gotland Deep station ( $57^\circ 19.21' \text{N}$ ,  $20^\circ 02.99' \text{E}$ ; depth 244 m), where Labrenz et al. (2010) showed dominance of archaea in the ammonia-oxidizing community in samples gathered in 2006. The sampling depth (128 m) was chosen as the depth at which  $\text{O}_2$  was below the detection

Table 1. Different treatments used in the study. For standard samples, the solution was ultrapure water ( $n = 3$  per treatment). For seawater samples, the solution was water collected from the Baltic Sea redoxcline ( $n = 9$  per treatment). In all samples,  $^{15}\text{NO}_x$  content was analyzed after incubation. ATU: allylthiourea, N-serve: commercial name for nitrapyrin

Sample	$^{14}\text{NH}_4^+$ ( $\mu\text{M}$ )	$^{15}\text{NH}_4^+$ ( $\mu\text{M}$ )	$\text{NO}_x$ ( $\mu\text{M}$ )	Inhibitor
<b>Standard</b>				
	0	0	10	None (control)
	0	0	10	100 $\mu\text{M}$ ATU
	0	0	10	150 $\mu\text{M}$ N-serve
	0	0	10	100 $\mu\text{M}$ ATU + 150 $\mu\text{M}$ N-serve
<b>Seawater</b>				
	0.03	5	4.54	None (control)
	0.03	5	4.54	100 $\mu\text{M}$ ATU
	0.03	5	4.54	150 $\mu\text{M}$ N-serve
	0.03	5	4.54	100 $\mu\text{M}$ ATU + 150 $\mu\text{M}$ N-serve

limit (precision of the sensor:  $\pm 0.23 \text{ mg l}^{-1}$ ;  $7.19 \text{ } \mu\text{mol l}^{-1}$ ) but measurable 1 m above, since these are the conditions under which the highest nitrification rates were previously found in this area (Hietanen et al. 2012). The  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and  $\text{NH}_4^+$  concentrations were measured at the sampling depth according to Grasshoff et al. (1983) and were 4.50, 0.04, and  $0.03 \text{ } \mu\text{M}$ , respectively. The water samples were collected using Niskin bottles that were attached to the conductivity-temperature-depth (CTD)-rosette system. Once the Niskin bottles were on deck, the sample water was carefully transferred into glass bottles with a 3-fold overflow, and  $^{15}\text{NH}_4^+$  (final concentration  $5 \text{ } \mu\text{M}$ ;  $^{15}\text{NH}_4\text{Cl}$ , 99%  $^{15}\text{N}$ , Sigma Aldrich) and the inhibitors were added to the samples (Table 1) under an  $\text{N}_2$  atmosphere. The acetone concentration in the seawater samples with N-serve was 0.4% (vol/vol). The samples were then divided into 20 ml glass vials ( $n = 9$  per treatment) that were sealed gas tight with butyl rubber stoppers and aluminum crimps and incubated in the dark at near *in situ* ( $\sim 7^\circ\text{C}$ ) temperature. For each treatment, 3 replicate samples were filtered approximately every 3 to 4 h through pre-washed  $0.2 \text{ } \mu\text{M}$  syringe filters (polyethylsulfone membrane, VWR International) to terminate the incubation. The maximum incubation time of the samples was approximately 12 h.

### $^{15}\text{NO}_3^-$ analyses

The samples were analyzed using the denitrifier method (Sigman et al. 2001) with small modifications. *Pseudomonas chlororaphis* (American Type Culture Collection 13985) was grown in an 800 ml liquid culture (containing tryptic soy broth [Fluka Analytical; Sigma-Aldrich Chemie], 10 mM  $\text{KNO}_3$ , 1 mM ammonium sulfate  $[(\text{NH}_4)_2\text{SO}_4]$ , and 1 ml  $\text{l}^{-1}$  antifoaming agent [Dow Corning Antifoam RD emulsion, VWR International]) on a shaker table ( $150 \text{ rotations min}^{-1}$ ) for 8 d in the dark at room temperature. Thereafter, the bacterial culture was concentrated 10-fold, and the concentrated culture was divided into 2 ml aliquots in 12 ml gastight glass vials (Exetainer, Labco). The vials were closed and purged with  $\text{N}_2$  for 5 h. A sample amount corresponding to 8 nmol  $\text{NO}_x$  ( $0.8 \text{ ml}$  for standard samples,  $1.8 \text{ ml}$  for the seawater samples) was injected into each vial, and after overnight incubation in the dark,  $0.1 \text{ ml}$  of 10 N NaOH was injected into each vial to lyse the bacteria and strip the carbon dioxide ( $\text{CO}_2$ ) from the headspace to the liquid. The  $^{15}\text{N}$  label in the  $\text{N}_2\text{O}$  produced was analyzed with a GC-IRMS system

(Thermo Finnigan Delta V plus with ConFlo IV; Thermo Fisher Scientific) with a trace gas pre-concentrator (PreCon, Thermo Fisher Scientific) in the Department of Environmental Science, University of Eastern Finland, Kuopio.

### Calculations and statistical analyses

The nitrification rate was calculated by plotting the increase in  $\text{NO}_x$  concentration against incubation time (see Fig. 2):

$$\Delta\text{NO}_x \text{ concentration} = \frac{[\text{NO}_x] \times \left( \frac{\Delta\text{atom}\% \text{ } ^{15}\text{NO}_x}{100} \right)}{\frac{(^{15}\text{NH}_4^+_{\text{added}} + \text{NH}_4^+_{\text{ambient}})}{^{15}\text{NH}_4^+_{\text{added}}}} \quad (1)$$

where  $\Delta\text{atom}\% \text{ } ^{15}\text{NO}_x$  is the change in the atom% of  $^{15}\text{N}$ - $\text{NO}_x$  over incubation and  $[\text{NO}_x]$  is the  $\text{NO}_x$  concentration in the sample. The slope of the linear regression equation is the nitrification rate (including both  $^{14}\text{NH}_4^+$  and  $^{15}\text{NH}_4^+$  oxidation). During the seawater sample IRMS run, there were problems in some samples with the sample gas syringe in the continuous flow, and consequently some of the peak areas measured were very low. These outlier values (<50% of the average value in that treatment) were omitted from further calculations (total of 3 values, 1 from each inhibitor treatment).

The effect of the inhibitors on the results of the denitrifier method was determined in 2 ways. First, the peak area values (masses of  $^{44}\text{N}_2\text{O}$  and  $^{45}\text{N}_2\text{O}$  in voltage seconds), which reflect the amount of  $\text{N}_2\text{O}$  produced by the denitrifying culture, were compared between the inhibitor and control treatments within the seawater and standard samples. The atom% values were then compared between the control and inhibitor treatments in the standard samples. The significant differences of the peak areas and atom% values between the various treatments were tested using analysis of variance (ANOVA) with a post hoc Dunnett's test with PASW statistical software (IBM). The differences were deemed significant at  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Sensitivity of *Pseudomonas chlororaphis* to ATU and N-serve

The ANOVA indicated significant differences in the peak areas between the various treatments in both the seawater and standard samples. In the standard samples, the peak area of the control samples

was significantly higher than in samples treated with ATU, N-serve, and ATU + N-serve combined. In seawater samples, there was a significant difference between the control samples and the samples treated with N-serve and ATU+N-serve combined (Fig. 1). It is unclear why ATU did not have a negative effect on *P. chlororaphis* in seawater samples. It may be that compounds present in the seawater react with ATU,

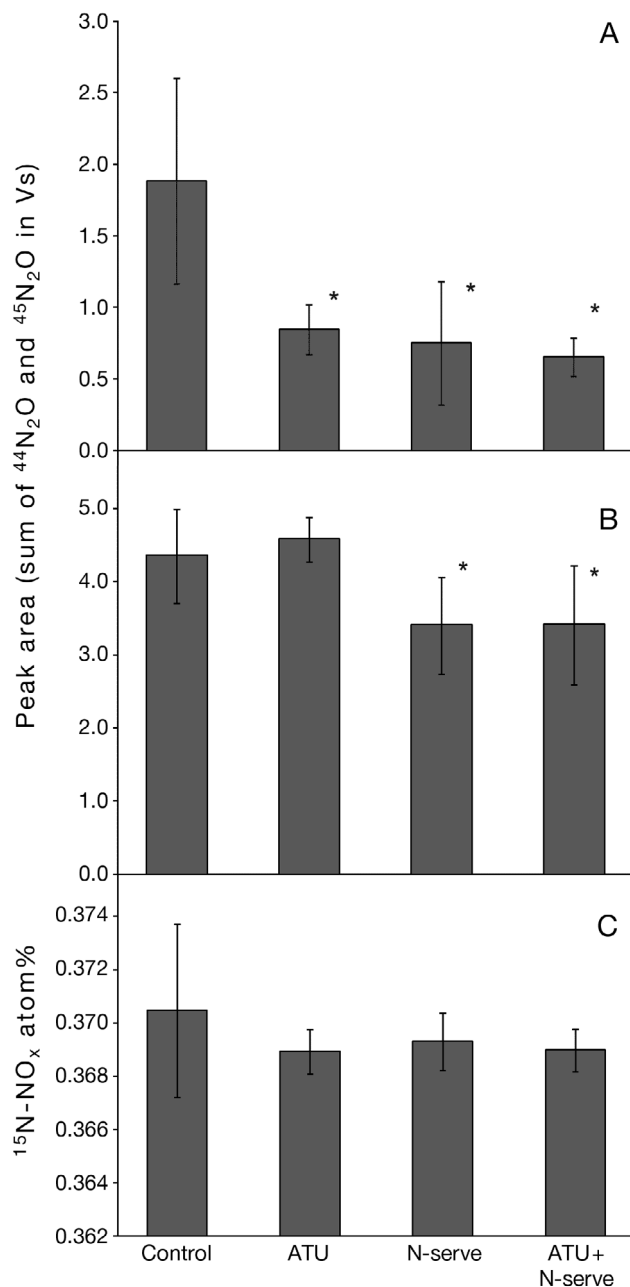


Fig. 1. Peak areas (mean  $\pm$  SD) of (A) the standard samples ( $n = 3$  per treatment) and (B) seawater samples ( $n = 9$  per treatment), and (C) the atom% values (mean  $\pm$  SD) of the standard samples. Vs: voltage second. \*Significantly lower than the control ( $p < 0.05$ )

decreasing its inhibitory effect on *P. chlororaphis*. However, to date no such reactions between ATU and seawater have been reported.

Denitrifying bacteria are inhibited at slightly higher N-serve concentrations ( $\sim 215 \mu\text{M}$ ; Henninger & Bollag 1976) and therefore our results are not surprising, although the effect of N-serve has never, to our knowledge, been tested specifically for *Pseudomonas chlororaphis* before. The acetone in which N-serve was dissolved can also affect denitrification but is stimulatory rather than inhibitory, since denitrifying bacteria can use acetone as their organic carbon source (Gross & Bremner 1992). ATU also inhibits denitrification, but less than N-serve (Jensen et al. 2007), which was also seen in our results.

Incomplete conversion of  $\text{NO}_x$  to  $\text{N}_2\text{O}$  in the N-serve-treated samples can lead to isotopic fractionation (kinetic preference of  $^{14}\text{N}$  over  $^{15}\text{N}$ ) causing lower  $^{15}\text{N}$  content in the  $\text{N}_2\text{O}$  than in complete conversion. Since the fractionation occurs at the per mill scale, it is a problem in those samples in which the  $^{15}\text{N}$  enrichment is at a similar scale (close to natural abundance). However, in samples that have been enriched above 0.6 atom%, any possible fractionation caused by incomplete conversion of  $\text{NO}_x$  to  $\text{N}_2\text{O}$  is insignificant (Mathieu et al. 2007). Although the standard samples analyzed in the present study had  $^{15}\text{N}$  enrichment below 0.6 atom% and the peak areas indicated incomplete conversion of  $\text{NO}_3^-$  to  $\text{N}_2\text{O}$ , it did not seem to cause noticeable fractionation since there was no significant difference between the atom% values between the different treatments (Fig. 1C).

The effect of fractionation in the atom% values measured in the seawater samples could not be determined because the fractionation factor could not be calculated from the standard samples, and consequently nitrification and fractionation cannot be separated from the measured  $^{15}\text{NO}_3^-$  values. However, the effect of possible fractionation due to incomplete conversion of  $\text{NO}_x$  to  $\text{N}_2\text{O}$  is likely to be small, particularly in the ATU-treated samples in which the peak areas did not significantly differ from the control samples. The theoretical underestimation of  $\text{NO}_x$  production, assuming the maximum fractionation factor for incomplete conversion of  $\text{NO}_3^-$  to  $\text{N}_2\text{O}$  ( $\alpha = 1.024$ ; Mathieu et al. 2007), would be approximately  $\pm 0.65 \text{ nmol N}$  per sampling point. Had fractionation this high occurred, it would have distorted the nitrification rate 1.27 to 3.13  $\text{nmol N l}^{-1} \text{ d}^{-1}$ , which is low compared to the nitrification rate and standard deviation measured in the control samples ( $27.4 \pm 12.4 \text{ nmol N l}^{-1} \text{ d}^{-1}$ ; Fig. 2).

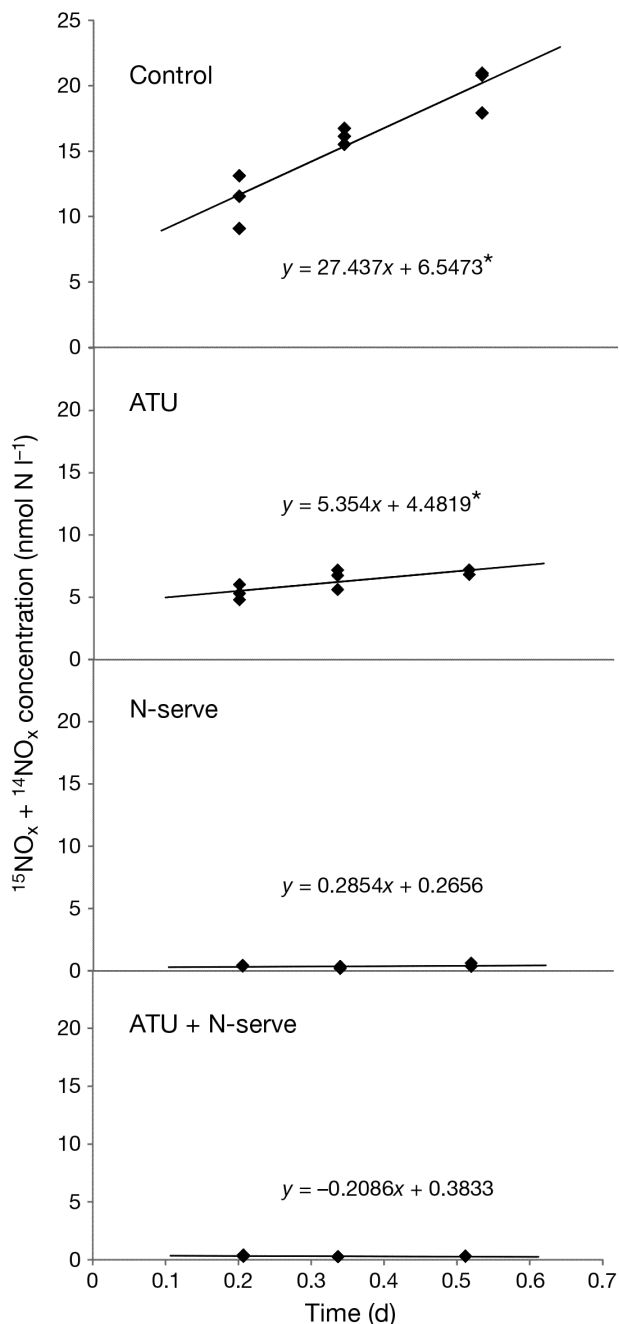


Fig. 2. Increase in the total  $\text{NO}_x$  concentration at different incubation time points in the seawater samples treated with different inhibitors. The slope of the equation represents the nitrification rate in  $\text{nmol NO}_x \text{ l}^{-1} \text{ d}^{-1}$ . \*Slope is significant ( $p < 0.05$ )

### Nitrification rate measurements

We observed a significant linear increase in the  $\text{NO}_3^-$  concentration in the control samples and in the ATU-treated samples, but not in the samples that

contained N-serve or ATU+N-serve (Fig. 2). The nitrification rate in ATU-treated samples was substantially lower (by 80%) when compared to the nitrification rate in the control samples (Fig. 2), although the ATU concentration used was higher (100  $\mu\text{M}$ ) than that effectively inhibiting nitrification in a previous study (86  $\mu\text{M}$  ATU; Ginestet et al. 1998). The effective concentration in that study was determined using nitrifying culture mainly containing AOB, and apparently, the active nitrifying community in the Baltic Proper has a higher tolerance towards ATU than the culture used by Ginestet et al. (1998). A previous study has shown an almost complete dominance of a single archaeal clade, GD2, in the ammonia-oxidizing community in the sampling area (Labrenz et al. 2010), which probably contributes to the higher tolerance, since AOB and AOA have different hydroxylamine oxidoreductase complexes (Walker et al. 2010) and may therefore also have different sensitivities to inhibitory compounds. It may, however, be that higher ATU concentrations would inhibit nitrification completely, which should be investigated in future studies.

Unlike ATU, N-serve effectively inhibited nitrification of the seawater ammonia-oxidizing community. Acetone, as well as alternative solvents for N-serve, such as dimethyl sulfoxide, also has inhibitory effects on nitrification (Hall 1984, Southwell et al. 2008). Therefore, the effect of N-serve in inhibiting nitrification is partially caused by the solvent that is used to dissolve N-serve.

Nitrification inhibitors may also affect other nitrogen transformation processes, such as DNRA, but no data are available on such inhibitory effects. However, the negative effect of N-serve on the functioning of *Pseudomonas chlororaphis*, among other denitrifying bacteria (Henninger & Bollag 1976), suggests that  $\text{NO}_x$  reduction processes are also inhibited by some nitrification inhibitors. This should be taken into account in determining linkages between nitrogen-cycling processes.

### CONCLUSIONS

The results of this experiment clearly show that ATU should not be used as a nitrification inhibitor in communities that may contain AOA. The inhibition of nitrate reduction bacteria that are used in the method to measure nitrification rates was negligible when ATU was used, particularly in seawater samples. N-serve effectively inhibits nitrification of both AOA and AOB, but it can also have an inhibiting

effect on  $\text{NO}_3^-$ -reducing bacteria. However, the atom% values measured with the denitrifer method did not seem to be affected by either of the inhibitors. While N-serve inhibition is a cost-efficient method for measuring nitrification, the results should be carefully interpreted because N-serve and the N-serve solvent used may affect the functioning of the entire microbial community. Alteration of the feedback mechanisms in the nitrogen cycle may, in the end, also bias the nitrification estimates.

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