A Proposed Improvement for Measuring Hydroxylamine in Seawater

By: Lily Sandler
School of Earth and Atmospheric Sciences
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PhD Candidate Mentor: Amanda Cavazos

Faculty Member 1: Dr. Jennifer Glass

Faculty Member 2: Dr. Yuanzhi Tang

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Abstract

Hydroxylamine is a chemical intermediate in the nitrogen cycle that can quickly react via biotic and abiotic processes to yield nitrous oxide, a potent greenhouse gas. Because of its high reactivity, hydroxylamine tends to be present at low concentrations in aquatic ecosystems. High reactivity and low concentrations also make hydroxylamine difficult to measure. Our goal was to improve the method for measuring environmental concentrations of hydroxylamine. The current method involves converting hydroxylamine to nitrous oxide with ferric ammonium sulfate at low pH and analyzing the nitrous oxide produced by gas chromatography. This method requires a recovery curve because the conversion of hydroxylamine to nitrous oxide does not always go to completion. Here, we propose a new method using a manganese oxide mineral pyrolusite, which rapidly oxidizes hydroxylamine and completely converts it to nitrous oxide, thus eliminating the need for a recovery curve and sample acidification. The method involves (1) crushing and sieving commercial pyrolusite to increase reactive surface area; (2) adding crushed pyrolusite to airtight bottles containing hydroxylamine in artificial seawater at neutral pH; (3) incubating for two hours; and (4) analyzing of the oxidized product, nitrous oxide, by gas chromatography. Hydroxylamine concentrations are calculated from the concentration of nitrous oxide in headspace before and after pyrolusite addition. Addition of crushed pyrolusite resulted in complete conversion of hydroxylamine to nitrous oxide within two hours, whereas minimal conversion occurred without pyrolusite. This method has a shorter reaction time and goes to completion, allowing for more rapid and accurate measurements of hydroxylamine in aquatic environments.

Introduction/ Literature Review

Nitrous oxide (N_2O) is a greenhouse gas with 250 times the warming potential of carbon dioxide over a 100-year period [1]. Approximately one-quarter of global atmospheric N_2O is emitted from the oceans [2]. Nitrification, the microbial oxidation of ammonium to nitrite and nitrate, can produce N_2O as a by-product. A key intermediate in the first step of nitrification is hydroxylamine (NH_2OH) [3]. Because NH_2OH is highly reactive, it is typically undetectable in natural waters, though it has been detected in coastal seawater during periods of intense nitrification [4-7]. In natural waters, NH_2OH is typically present in the range of 1 to 250 nM and is thought to play a role in N_2O production via biotic and/or abiotic processes [3]. The production of N_2O could occur as a side reaction of nitrification if NH_2OH leaks out of cells and reacts with other species before conversion to nitric oxide. This pathway could potentially serve as a significant source of N_2O production from oceans [8].

Abiotic transformation of NH_2OH to N_2O has been shown to occur in soils [9-11] and seawater [3]. Additionally, recent studies have shown that NH_2OH, a strong reductant, can react quickly and to completion with manganese oxides, a group of strong oxidants, to produce N_2O [9, 12].

Manganese oxides are ubiquitous in nature and tend to accumulate at oxic-anoxic interfaces where soluble dissolved Mn^{2+} meets oxygen-rich waters [13]. Nitrification also peaks at oxic-anoxic boundaries, where NH_2OH can reach concentrations upwards of 200 nM [4, 5]. Even in low quantities, NH_2OH and manganese oxides can react rapidly to produce N_2O to near completion, even at circumneutral pH [9, 12] according to the following reaction:

$$2NH_2OH + 2Mn(IV)O_2 + 4H^+ \rightarrow N_2O + 2Mn^{2+} + 5\ H_2O$$

This reaction has been identified in soil [9], but it has not been verified in the ocean due to the difficulties involved with measuring low concentrations of NH_2OH in seawater.

NH_2OH is a chemical relevant to many fields of science, including, but not limited to, geochemistry, soil chemistry, and pharmaceuticals. Thus, many methods have been developed to quantify NH_2OH (**Table 1**). These methods use gas chromatography and spectrophotometry. Methods developed for natural samples (e.g. soil and natural waters) are optimized for nanomolar concentrations typical for natural environments. The most commonly used method for measuring NH_2OH in natural waters at nanomolar concentrations involves collecting samples in airtight bottles, injecting glacial acetic acid and then ferric ammonium sulfate solution to convert NH_2OH to N_2O, and analyzing the sample headspace by gas chromatography with an Electron Capture Detector (GC-ECD). Because the reaction of acidic ferric ammonium sulfate and NH_2OH does not always go to completion, with yields ranging from 20 to 80% [14], a recovery curve must be created to deduce the actual NH_2OH concentration in the samples [14]. The recovery curve requires additional sample volume and introduces extra steps, creating more room for error. These difficulties in these methods have made widespread NH_2OH measurements elusive and hindered a deeper understanding of the biogeochemical role of NH_2OH.

Table 1. Established methods for quantifying NH_2OH. These methods use gas chromatography or spectrophotometry. Only the ferric ammonium sulfate method is capable of detecting low nanomolar concentrations.

Instrument	Oxidant	Chemical Measured	Absorption Peak (nm)	Sample Stability (hours)	Sample Type	Range of quantification (μM)	Reference
Spectrophotometer	8-Quinolinol	Indooxxine (5,8-quinolinequinone -5-(8-hydroxy-5-quinolylimide)	705	0.5	Varied	50,000	[15]
	Sodium Arsenate	Nitrite	545	3	Pharmaceutical	NA	[16]
	Iodate		530	N.A.	Soil	3-27	[17]
	Iodine		543	2-3	Seawater	0.5-91	[18], [19]
	Bromide	Bromine	520	N.A.	Pharmaceutical	6	[20]
Gas chromatograph with ECD detector	Ferric Ammonium Sulfate	Nitrous oxide	N/A	384	Aqueous	0.0012 to 0.560	[21], [22] **
	Iron (III) Chloride Hexahydrate			N.A.	Soil	>0.3 μg N kg^{-1} dry soil	[23]

**improvement of adding sulfanilamide to decrease nitrite interference
N.A.: not provided in manuscripts

A more effective and streamlined method for NH_2OH quantification could expand our understanding of the role of NH_2OH in ocean biogeochemistry by enabling NH_2OH to be more commonly measured in addition to standard nitrogen species (nitrate, nitrite, ammonia, etc.). The current method for NH_2OH quantification has been the standard since its development and publication in 1982 by Von Breymann et al. [22] and has been optimized for various environments, including sediments and seawater, but remains relatively unchanged. Because NH_2OH can be rapidly oxidized to completion by Mn oxides at neutral pH [9, 12], we propose to improve the current method by using manganese oxides in place of iron. This change would eliminate the need for a recovery curve, prevent the need to acidify the sample, and reduce the amount of inherent error in the method by reducing the number of steps. Here, we provide an improvement to the GC method by replacing iron with a commercially available Mn oxide. Complete conversion of NH_2OH to N_2O occurs within two hours without the need for sample acidification.

Methods

Preparation of Synthetic Ocean Water and Experimental Bottles

All serum bottles, stoppers, and Falcon tubes were cleaned with dilute oxalic acid and rinsed thoroughly with MilliQ 18.2 M ohm-cm water. Synthetic Ocean Water (SOW, pH 7.8) was prepared according to the protocol of Morel et al. (1979) [24] **(Appendix A)** using MilliQ 18.2 M ohm-cm water. No trace metals were added to SOW. The pH of the SOW was adjusted to 7.8 using KOH.

Preparation of Pyrolusite

Pyrolusite (MnO_2; Millipore Sigma, catalog # 805958) was crushed to a fine powder using a mortar and pestle that were cleaned with dilute oxalic acid, rinsed thoroughly with 18.2 M ohm-cm water, and dried prior to use. After thorough crushing, the pyrolusite was sieved (106 μm; Fischer Scientific USA Standard Testing Sieve) to increase the reactive surface area. The <106 μm pyrolusite fraction was stored in a 10-mL Falcon tube prior to use. On the day before the experiment, a pyrolusite suspension (200 mM) was prepared in a serum bottle containing 25 mL SOW for later injection into experimental bottles. The pyrolusite suspension was placed in the shaker at 115 rotations per minute to allow the surface of pyrolusite to equilibrate with the ions in SOW.

Experimental Procedure

On the day of the experiment, a 1 M solution of $NH_2OH{\cdot}HCl$ (Acros Organics) was prepared in SOW and diluted to 10 or 100 μM working stocks. In 37 mL serum bottles, final concentrations

of 50, 100, 500, or 1000 nM NH_2OH were prepared from the working stocks. The final volume in each bottle was 25 mL with 12 mL headspace. Bottles were immediately sealed with thick butyl rubber stoppers and aluminum crimps.

To initiate the incubation, 0.25 mL of 200 mM pyrolusite solution via a plastic syringe was injected into select incubation bottles for a final concentration of 20 mM pyrolusite. The pyrolusite solution was shaken immediately prior to each extraction to obtain a consistent concentration of pyrolusite with each injection. Larger gauge needles (≤20 gauge) are necessary for pyrolusite extraction; >23-gauge needles caused larger particles to get lodged in the needle. All samples were stored upside down in the dark in a shaker (New Brunswick Scientific, Excella E24 Incubator Shaker) at 30°C at 115 rotations per minute for two hours.

Gas Chromatography

The samples were analyzed for headspace N_2O with a GC-ECD (SRI 8610C). Glass airtight syringes were used for all injections. A calibration curve was created with 6 to 7 points using ultra-high purity (UHP) N_2O calibration gas (Scotty Brand Transportable 17L). For each sample, the volume of air to be analyzed was injected into the bottle and then extracted from the bottle to conserve atmospheric pressure in the bottle. All injections and extractions were performed using airtight syringes. The N_2O peak areas of each of these samples were recorded at 2.6 minutes of a 3.5 minute total run-time.

Calculations

For the calibration curve, moles of gas (based on volume injected) and peak area were plotted against each other, and the slope of the line was obtained to calculate the nanomoles of N_2O per

1 mL gas injected. Then, **Equation 1** was used to calculate the concentration of N_2O per 12 mL of headspace:

$$(nmols\ per\ 12\ mL\ headspace) = (nmols\ per\ 1\ mL\ gas\ injected) \times \frac{(12\ mL\ headspace)}{(x\ mL\ gas\ injected)} \quad (1)$$

The Bunsen coefficient, β, of 0.01818 was used based on a salinity of 35 ppt and a temperature of 30˚C from Weiss and Price 1980 [25].

The total number of moles of N_2O in the bottle, n_{N_2O}, is equal to the number of moles of N_2O in the water ($n_{N_2O_W}$) plus the number of moles of N_2O in the headspace ($n_{N_2O_{HS}}$) (**Equation 2**).

$$n_{N_2O} = n_{N_2O_W} + n_{N_2O_{HS}} \quad (2)$$

The concentration of N_2O in the bottle (mol L^{-1}) was obtained by dividing n_{N_2O} by the volume of water in the bottle (V_w) (**Equation 3**).

$$[N_2O] = \frac{n_{N_2O}}{V_w} \quad (3)$$

The number of moles of N_2O in the headspace was obtained by multiplying the partial pressure of N_2O in the headspace ($P_{N_2O_{headspace}}$) by the volume of the headspace (V_{HS}) in liters (L), and dividing by the ideal gas constant (0.08206 L atm mol^{-1} K^{-1}) and the temperature of the solution in Kelvin (**Equation 4**).

$$n_{N_2O_{HS}} = \frac{P_{N_2O_{headspace}} V_{HS}}{R(298.15\ K)} \quad (4)$$

The number of moles of N_2O in the water was obtained by multiplying the partial pressure of N_2O in the headspace ($P_{N_2O_{headspace}}$) by the ratio of the water to headspace volumes ($\frac{V_W}{V_{HS}}$) in L and the Bunsen coefficient for N_2O, and dividing by the ideal gas constant (0.08206 L atm mol^{-1} K^{-1}) and the temperature of the solution in Kelvin (**Equation 5**).

$$n_{N_2O_W} = \beta \frac{P_{N_2O_{headspace}} \times \frac{V_W}{V_{HS}}}{R \times (298.15\ K)} \qquad (5)$$

Conversion

Bottles with just SOW were analyzed via GC to provide the amount of background N_2O in the bottles ($n_{background}$). A background concentration of 80 nM was obtained. Conversion of NH_2OH to N_2O for each bottle ($n_{conversion}$) was calculated by subtracting the background N_2O from the total N_2O (n_{N_2O}) obtained from each sample **(Equation 6).**

$$n_{conversion} = n_{N_2O} - n_{background} \quad (6)$$

Results

Complete conversion of NH_2OH to N_2O occurred within two hours in all bottles with pyrolusite. Bottles without pyrolusite had little to no conversion to N_2O (<1%). Bottles with pyrolusite had significant conversion (161 ± 76%). Complete conversion occurred in all trials, from 50 nM to 1 μM NH_2OH (**Figs. 1, 2**), suggesting that this method is as sensitive as the Von Breymann method (**Table 1**). Any conversion in bottles without pyrolusite can be attributed to autooxidation of NH_2OH.

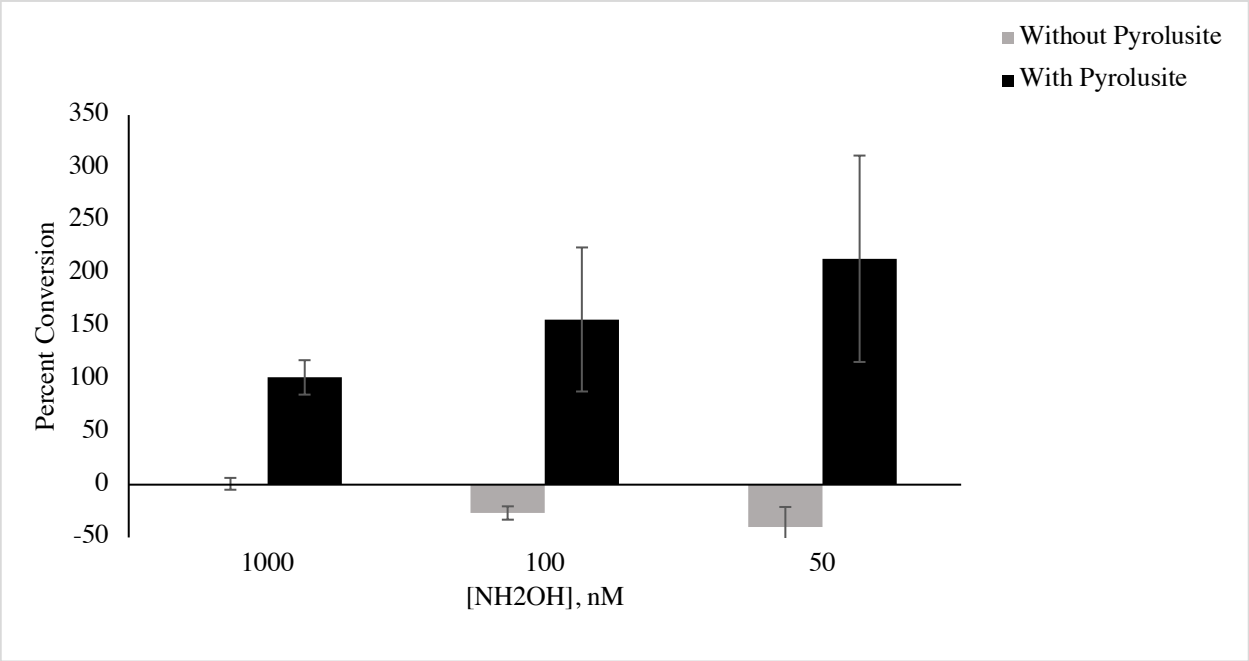

Figure 1. Percent N_2O conversion from varied concentrations of NH_2OH. The black bars display the percent recovery of N_2O from solutions containing hydroxylamine and pyrolusite. The gray bars display the percent recovery of N_2O from solutions containing hydroxylamine but no pyrolusite. When these values were calculated, background N_2O was subtracted out of total detected N_2O by the GC.

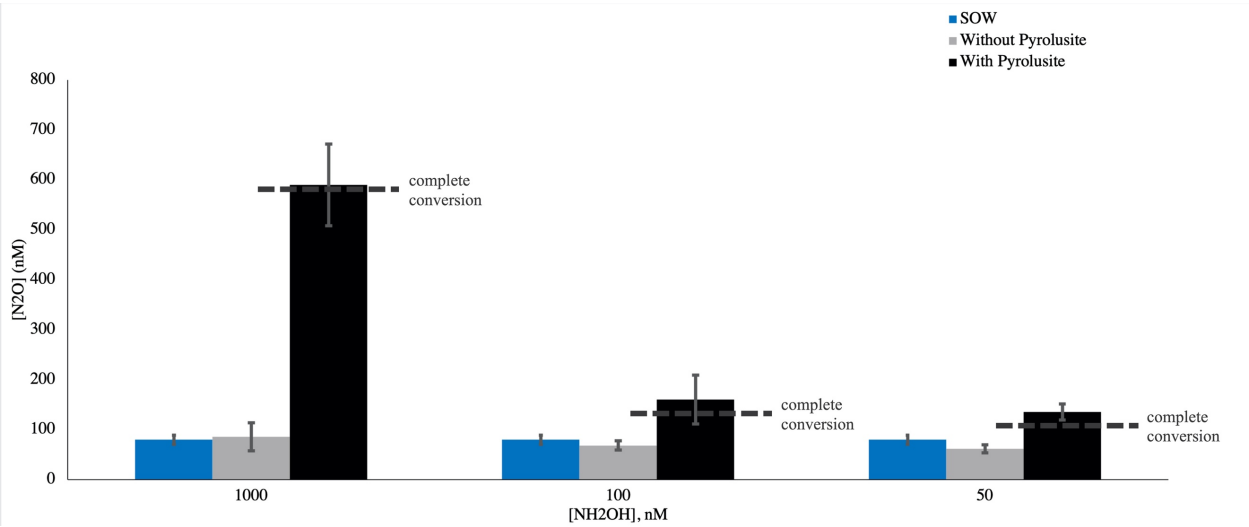

Figure 2. Absolute N_2O conversion from varied concentrations of NH_2OH. The black bars display the total recovery of N_2O in nM from solutions containing hydroxylamine and pyrolusite. The gray bars display the total recovery of N_2O from solutions containing hydroxylamine but no pyrolusite. Blue bars represent the background nanomolar concentration of N_2O in bottles containing just synthetic ocean water. Dashed gray lines represent theoretical exact conversion, the sum of the SOW background N_2O concentration and the theoretical yield of N_2O due to hydroxylamine conversion.

Discussion

Any N_2O conversion in the negative controls beyond the calculated background N_2O is the result of autooxidation of NH_2OH, as NH_2OH is known to autooxidize to form N_2O over time [26]. Up to 35% of NH_2OH has been found to autooxidize in oxic environments [27]. Any conversion in the bottles with pyrolusite added that had N_2O headspace concentrations exceeding the autooxidation values detected in the bottles with no pyrolusite added must be due to conversion by pyrolusite.

The sensitive detection limit of this method is essential to understanding NH_2OH in marine environments, as it is typically present in nanomolar concentrations. This simplified method for quantifying NH_2OH will allow for more detailed investigation into global aqueous concentrations of NH_2OH and its potential contribution to abiotic production of N_2O. While the role of NH_2OH in N_2O production has been shown in terrestrial environments [7, 10], NH_2OH in oceanic environments is scarcely studied [3]. A simplified, more time-efficient method should allow for more widespread measurements of NH_2OH in marine systems and allow elucidation of the role NH_2OH plays in oceanic N_2O emissions. A better understanding of this pathway could help scientists to develop improved models for both marine and global N_2O production.

This method is a significant improvement when compared to other methods. Unlike the current von Breymann method for NH_2OH conversion via ferric ammonium sulfate, this manganese oxide-based method requires no recovery curve, does not require sample acidification, and greatly reduces reaction time for conversion to N_2O. These factors should allow for greater ease in analysis and application to natural samples.

Our method used pyrolusite because it is shelf-stable, and preparation of the crushed and sieved powder only takes a matter of minutes. Other forms of manganese oxides might be suitable as oxidants, as long as they are stable and equilibrated with the solution. It should be

noted that different manganese oxide minerals are known to change structure over time. For example, birnessite would be an ideal candidate to use for this method if not for its limitations of stability and preparation. It is known to react rapidly and to completion with NH_2OH within minutes [12]. However, birnessite must be synthesized and is only reactive for a matter of days, making it inconvenient for everyday laboratory use or prolonged sampling trips. Because of the variability in reactivity and storage life of different Mn minerals, any Mn mineral of interest should be tested using the proposed method with known concentrations of NH_2OH before application to environmental samples.

Conclusions

We present an improved method to measure NH_2OH in aqueous samples using GC-ECD that replaces ferric ammonium sulfate with pyrolusite. Using pyrolusite reduces reaction time, results in complete conversion of NH_2OH to N_2O, and removes the need for sample acidification and the construction of a recovery curve. These improvements reduce the overall time of analysis and reduce the amount of sample volume required. The most challenging aspect of this proposed improvement is ensuring that the injected pyrolusite remains suspended in solution so that concentrations remain consistent in each bottle.

Our method makes NH_2OH analysis easier and more efficient, which should allow for more widespread measurements of oceanic NH_2OH. As the role of NH_2OH in N_2O production is being studied in terrestrial environments [7, 10], studies in marine systems are limited [3]. It has been suggested that NH_2OH reacting rapidly with Mn oxides could be a significant source of oceanic N_2O [12]. More thorough measurements of marine NH_2OH are needed to better estimate the contribution of oceanic N_2O from NH_2OH interactions.

Future Directions

While this method has proven successful in laboratory conditions and solutions, future work is needed to test this method's applicability to environmental samples. The true value of this method is in its ability to facilitate a deeper understanding of where NH_2OH exists in aqueous bodies and in what concentrations. In the case of using environmental samples, they would need to be injected with mercury or zinc chloride to ensure complete eradication of biotic activity within the bottles. Complications could arise from possible adsorption of the mercury or zinc chloride to the pyrolusite surface [28], which could lead to error in N_2O measurements if microbes capable of producing N_2O are not killed due to the adsorption of mercury or zinc chloride onto the pyrolusite.

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Appendix

Making 10 L Aquil synthetic ocean water

1) Put on latex gloves (not nitrile!)
2) Acid wash a 15 L carboy for at least 24 hours
3) Thoroughly rinse with Millipore water (18.2 M ohm)
4) Add 9L of Millipore 18.2 M ohm water to 15 L carboy
5) Add the following salts directly to water in carboy, recording the exact weight added of each in notebook. Allow each to dissolve before adding next.
    a. NaCl: 245.4 grams
    b. Na_2SO_4: 40.9 grams
    c. $MgCl_2*6H_2O$: 111 grams
6) Obtain acid washed 1 L graduated cylinder
7) Add 600 mL Millipore 18.2 M ohm water to graduated cylinder and stir bar to cylinder (clean the stir bar by soaking in 1.2 N HCl for a minute and washing thoroughly with 18.2 M ohm Millipore water before adding to cylinder).
8) Add the following **anhydrous** salts to 600 mL water in cylinder, recording the exact weight added of each in notebook. Allow each to dissolve before adding next:
    a. KCl: 7 grams
    b. $NaHCO_3$: 2 grams
    c. KBr: 1 grams
    d. H_3BO_3: 0.03 grams
    e. NaF: 0.03 grams
9) After all salts are dissolved, pour the contents of the graduated cylinder into the carboy.
10) Add 400 mL more Millipore 18.2 M ohm water to graduated cylinder and stir bar to cylinder (clean the stir bar by soaking in 1.2 N HCl for a minute and washing thoroughly with 18.2 M ohm Millipore water before adding to cylinder).
11) Add the following **hydrous** salts to 400 mL water in cylinder, recording the exact weight added of each in notebook. Allow each to dissolve before adding next:
    a. $CaCl_2*2H_2O$: 15.4 grams
    b. $SrCl_2*6H_2O$: 0.17 grams
12) After all salts are dissolved, pour the contents of the graduated cylinder into the carboy.
13) Nutrients and trace metals must be added separately.
14) pH artificial seawater to 7.8.