We developed a method for quantifying trace NH$_2$OH in brackish- and sea-water samples. Previously reported methods applicable to fresh water cannot be applied to such samples. We determined that interference in seawater owing to the bromide ion can be removed by the addition of phenol. In our procedure, phenol and hypochlorite solutions were added to a sample solution to oxidize NH$_2$OH to N$_2$O. N$_2$O in the sample was then quantified by headspace analysis. The method is not affected by the salt content or ammonia, nitrate, or nitrite at concentrations of 300 μgN L$^{-1}$ or less. It has a limit of detection of 0.2 μgN L$^{-1}$, and can quantify NH$_2$OH in natural water samples with a wide range of salinity. It was applied to samples from Lake Nakaumi, a brackish lake located in the eastern part of Shimane Prefecture, Japan.

**Keywords** Brackish water, environmental water, estuary, gas chromatography, nitrous oxide, hydroxylamine, *in situ* method

**Introduction**

We have developed simple and/or sensitive methods for the determination of chemical species, such as dissolved sulfide, phosphate, ammonia, nitrite, and hydrazine in environmental water. Dissolved sulfide in environmental water is produced from microbial sulfate reduction. It is highly toxic to most organism, and stable only in anoxic aqueous environments, which are linked with water pollution. Therefore the dissolved sulfide is important for understanding the oxidation-reduction level and water quality in various environmental water locations. On the other hand, nitrogen and phosphorus compounds (nutrients) cause eutrophication in lakes and marshes. Because of problems, such as blue-green algal blooms and red tide due to eutrophication, environmental quality standards have been established for nitrogen and phosphorus compounds. The major naturally occurring inorganic nitrogen species are N$_2$, NH$_3$, NO$_2$, and NO$_3$. These species, utilized and recycled by microorganisms, are intimately involved in the ecosystem, and are important to the environment. In addition, hydrazine has attracted attention in recent years as an intermediate in anaerobic ammonia oxidation (ANAMMOX). Nitrous oxide (N$_2$O) is a greenhouse gas that is targeted for emission cuts under the Kyoto Protocol. Its global-warming potential is about 300-times greater than that of carbon dioxide (CO$_2$). In addition, N$_2$O can deplete the ozone layer. Hydroxylamine (NH$_2$OH) is an important substance for understanding N$_2$O formation. NH$_2$OH is generated by both nitrification and nitrate reduction. Thus, understanding NH$_2$OH generation in the hydrosphere, which encompasses the vast majority of the Earth’s surface, would clarify the mechanism of N$_2$O generation. NH$_2$OH is also an intermediate in anaerobic ammonia oxidation (ANAMMOX). By quantifying NH$_2$OH, we can understand the ANAMMOX reaction in more detail.

In air, an aqueous NH$_2$OH solution (with a concentration of up to several mM) is stable at pH 4 for several days. However, the same solution at pH 7.8 is stable for 60 min at most, which indicates the ease of NH$_2$OH oxidation with dissolved oxygen. Because NH$_2$OH is unstable, and is therefore present only in trace amounts, there are no good quantitative methods applicable to aqueous environmental samples.

Spectrophotometry and titration are typical quantitative methods for NH$_2$OH determination. A procedure based on the oxidation of NH$_2$OH to NO$_2$ with iodine and colorimetry of NO$_2$ has been developed. However, a drawback of the method is its complexity in detection sensitivity and quantitative operation. Seike *et al.* developed a quantitative procedure that uses sodium hypochlorite (NaClO) to oxidize NH$_2$OH to N$_2$O and a gas chromatograph with an electron-capture detector (ECD) to conduct the measurement. However, the method can be applied to fresh water only, and is not applicable to brackish water and seawater. In this study, we identified the interfering substance in brackish water and seawater and developed a quantitative
method capable of dealing with such samples. We also demonstrated the applicability of the method in Lake Nakaumi, a brackish lake located in the eastern part of Shimane Prefecture, Japan.

Experimental

Reagents

High-purity reagents (Wako, Special Class) were used in all cases, except for the sodium hypochlorite solution. Milli-Q water (Millipore) was used for all reagent preparation.

A NH2OH standard solution (500 mgN L–1) was prepared by dissolving 0.2481 g of hydroxylamine hydrochloride in deoxygenated water and diluting to 100 mL. Suitable dilutions were made at the time of use. Fresh reagent was prepared for each experiment.

A NaClO solution (3.5 mM) was prepared by adding 2.5 mL of a sodium hypochlorite solution (5% active chlorine, Kanto Chemical, Shika Class 1) in water and diluting to 50 mL; 5 mL of this solution was diluted to 100 mL with water.

A phenol solution (63.6 mM) was prepared by adding 10 mL of ethanol (to dissolve phenol) to 10 g of phenol followed by dilution to 100 mL with water; 6 mL of this solution was diluted to 100 mL with water.

A NH4+ standard solution (100 mgN L–1) was prepared by dissolving 0.3819 g of ammonium chloride in 1 L of water. Suitable concentrations were obtained by dilution at the time of use.

A NO2 standard solution (100 mgN L–1) was prepared by dissolving 0.1231 g of sodium nitrite in 250 mL of water. Suitable concentrations were obtained by dilution at the time of use.

A NO3 standard solution (100 mgN L–1) was prepared by dissolving 0.1805 g of potassium nitrate in 250 mL of water. Suitable concentrations were obtained by dilution at the time of use.

Artificial seawater was prepared according to the Lyman and Flaming-method.25

Apparatus

A Shimadzu GC-14B-type gas chromatograph with an electron-capture detector (ECD) was used to determine N2O. Each pH value in solution was measured with a Horiba F-23 type pH meter.

Condition of gas chromatography

A 2-m long stainless-steel column of 2.6 mm i.d. packed with Unibeads C (60/80 mesh) was used at an oven temperature of 130°C. Each temperature of injector and detector (ECD) was 200 and 300°C, respectively. A carrier gas, 99.999995% grade nitrogen (N2), was used at a rate of 50 mL min–1.

Standard procedure

Each sample water was transferred into a 70-mL brown glass vial, which was capped with butyl rubber and an aluminum seal without any head-space to prevent intrusion of air. Then, 1.0 mL of 63.6 mM phenol solution and 1.5 mL of 3.5 mM hypochlorite solution were injected (Fig. 1) to oxidize NH2OH to N2O. Glass beads were placed in the vial beforehand to mix the solution. A headspace technique was used to quantify the N2O generated. Then, 40 mL of nitrogen gas (99.9% purity) was admitted into the vial using a magnus syringe for the headspace analysis. After shaking the vial for several minutes, 0.2 mL of the headspace gas was injected to a gas chromatograph with ECD to measure the N2O in the headspace. The N2O concentration in the liquid phase was calculated from the Weiss and Price formula.26 For the N2O blank, we performed each measurement without any addition of the phenol and hypochlorite solutions. The NH2OH concentration was obtained by subtracting the N2O blank from the N2O signal generated by the phenol and hypochlorite solutions. The limit of detection of N2O by gas chromatography was 0.2 μgN L–1.

In field studies, formaldehyde (1% final concentration) was injected into the sample water to stop the biological activity for the original N2O analysis. This gave the original amount of dissolved N2O in the samples. For another sample (NH2OH), 1 mL of phenol solution and 1.5 mL of hypochlorite solution were injected to oxidize NH2OH to N2O. The resulting solutions were brought back to the laboratory for gas-chromatographic measurements.

Results and Discussion

Interfering substances in brackish water and seawater samples

We previously discovered that the reaction of NH2OH with hypochlorite quantitatively generates N2O as shown by the following equation (Eq. (1)):24

\[
2\text{NH}_2\text{OH} + 2\text{NaClO} \rightarrow \text{N}_2\text{O} + 2\text{NaCl} + 3\text{H}_2\text{O}
\]  

(1)

However, while this reaction occurs quantitatively for freshwater samples, interferences were encountered for brackish- and sea-water samples. We investigated the effect of salinity on
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NH₂OH to N₂O generation by this method using artificial seawater (0 – 35‰ salinity). After the addition of NaClO to 75 μM into each NH₂OH sample (50 μgN L⁻¹) in the range of 0 – 35‰ salinity, the generated N₂O was measured by a gas chromatograph with ECD. The recovery of N₂O from NH₂OH declined as salinity increased (Fig. 2(A)). We next investigated the effect of salinity when NH₄⁺ (1 mgN L⁻¹) was present under the same conditions. In the presence of NH₃, N₂O recovery increased with increasing salinity at 0 – 17‰, and then declined at 17 – 35‰ (Fig. 2(B)).

To find out the interfering substances in brackish- and sea-water samples, we investigated each influence of the individual seawater component (10 major components). Each component was prepared to its concentration at 17‰ salinity, and sodium bicarbonate (NaHCO₃) was added to each sample to adjust the pH of seawater. Standard NH₂OH solution was added to achieve 50 μgN L⁻¹. Only KBr showed a remarkable decrease in N₂O recovery with increasing salinity at 0 – 17‰, and then declined at 17 – 35‰ (Fig. 2(B)).

Fig. 2  Effect of salinity (artificial seawater) on the oxidation of NH₂OH to N₂O using NaClO in the absence (A) and presence (B) of 1 mgN L⁻¹ NH₄⁺. NH₂OH: ○, 50 μgN L⁻¹; □, no addition.

Fig. 3  Effect of individual seawater components (at 17‰ salinity) on the oxidation of NH₂OH to N₂O using NaClO in the absence (A) and presence (B) of 1 mgN L⁻¹ NH₄⁺. All samples contain NaHCO₃ for pH adjustment. NH₂OH concentration: 50 μgN L⁻¹. pH: □, N₂O.

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From these results (Figs. 2 and 3), we learned that the interfering component in the conventional method¹⁵ is Br, which is present in brackish- and sea-water samples. Bromide ion reacts with hypochlorite to generate Br₂ (Eq. (2)) and then the generated Br₂ reacts with NH₂OH to form HNO₃ (Eq. (3)). This mechanism is responsible for interference of N₂O production by the conventional method:

$$2Br^- + HClO^- + H_2O \rightarrow Br_2 + Cl^- + 2OH^- \quad (2)$$

$$NH_2OH + 3Br_2 + 2H_2O \rightarrow HNO_3 + 6HBr \quad (3)$$

On the other hand, it was suggested that in the presence of NH₃, it reacts with Br₂ to form NH₂Br (Eq. (4)), and then its NH₂Br reacts with Br₂ to form N₂O (Eq. (5)). In addition, in the case of a high concentration of Br₂, Br₂ reacts with NH₂Br to form NHBr₂ (Eq. (6)), and then its NHBr₂ reacts with NH₂Br to form N₂ (Eq. (7)). This hypothesis is explicable based on the result shown in Fig. 2(B), because the amount of Br₂ generated increases with increasing salinity (include Br⁻).

$$NH_3 + Br_2 \rightarrow NH_2Br + HBr \quad (4)$$

$$2NH_2Br + 2Br_2 + H_2O \rightarrow N_2O + 6HBr \quad (5)$$

$$NH_2Br + Br_2 \rightarrow NHBr_2 + HBr \quad (6)$$

$$NH_2Br + NHBr_2 \rightarrow N_2 + 3HBr \quad (7)$$

Exclusion of the Interfering Substance (Br₂)

As shown at Eq. (8), three phenolic H atoms are replaced in the reaction by Br₂ to generate tribromophenol. We therefore investigated a method for removing the interfering substance (Br₂) using phenol. We added phenol solutions (0.12.2 mM) to samples containing 0.82 mM Br⁻ (equivalent to seawater) and examined the recovery of N₂O from NH₂OH. We found that the interference can be removed using 0.45 mM or greater phenol (data not shown). A phenol concentration of 0.90 mM was used in subsequent experiments as a precaution.

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We next added sodium hypochlorite at concentrations of 1994 μM and observed the recovery of N₂O from NH₂OH. A high N₂O recovery was observed at concentrations of 47 - 94 μM (Fig. 4). Thus, solutions were prepared with 75 μM sodium hypochlorite.

**Effect of pH**

We investigated the effect of pH on the oxidation of NH₂OH to N₂O. Diluted sodium hydroxide and sulfuric acid solutions were added to artificial seawater (excluding sodium bicarbonate) to prepare samples of pH 4 - 10. As shown in Fig. 5, we were able to quantitatively recover N₂O from NH₂OH in the range of pH 6 to 8.5.

**Effects of other substances**

The nitrogen species NH₄⁺, NO₂⁻, and NO₃⁻ are present in environmental water in addition to NH₂OH. We investigated the effects of these substances on the proposed method. No interference by NH₄⁺ and NO₃⁻ was observed in the range of 0 to 2000 μgN L⁻¹ (data not shown), and we were able to quantitatively recover N₂O from NH₂OH. No interference due to NO₂⁻ was found from 0 to 300 μgN L⁻¹, and N₂O was quantitatively recovered. However, the N₂O recovery declined at 500 μgN L⁻¹ or greater NO₂⁻ (data not shown). Because the NO₂⁻ concentration is normally low in ordinary environmental water, and rarely surpasses 300 μgN L⁻¹ in Lake Nakaumi (a brackish lake located in the eastern part of Shimane Prefecture, Japan), we surmise that there is practically no interference from this substance.

**Effects of salinity**

Because we learned in the quantitative application of this proposed method that Br⁻ interference can be removed, we examined the possible effects of salinity using artificial seawater. A NH₂OH standard solution (50 μgN L⁻¹) was prepared using artificial seawater (0 - 35 psu). The concentrations of phenol and NaClO were adjusted to 0.90 mM and 75 μM NaClO, respectively. Gas chromatograph with ECD was used to determine N₂O. We repeated the measurement in the presence of NH₄⁺ (1 mgN L⁻¹) and investigated the effect of salinity. Regardless of the presence or absence of NH₄⁺, we observed no salinity effects in the range from fresh water (0%) to seawater (35%) (data not shown).

**Recovery and reproducibility**

To further study the NH₂OH to N₂O recovery of the proposed method, we performed experiments in which NH₂OH (20 and 50 μgN L⁻¹) was added to brackish water samples (2.2 to 26.0% salinity). As shown in Table 1, NH₂OH was quantitatively recovered from the sample solutions with a standard deviation (RSD) of 1.6 to 3.8%. NH₂OH recovery ranged from 101 to 105%.

**Application to environmental water**

The proposed method was applied to a water sample from Lake Nakaumi (a brackish lake) on August 21, 2014. A sample taken with a Kitahara-type water sampler was collected and sealed in a brown glass vial (70 mL). Then, 1 mL of phenol solution (63.6 mM) and 1.5 mL NaClO solution (3.5 mM) were added to determine the NH₂OH, and 1% formaldehyde was added to stop biological activity. These procedures were performed in situ and carried back to a laboratory. The headspace technique and gas chromatograph with ECD were used to measure N₂O. We also collected and filtered water samples in polyethylene bottles for measurements of ammonia, nitrite, and nitrate.

The vertical distributions of NH₂OH, N₂O, ammonia, nitrite, and nitrate are shown in Fig. 6. The NH₂OH concentration began to increase at a depth of 5 m, and attained a maximum value of 1.6 μgN L⁻¹ at a depth of 6 m. The N₂O concentration began to increase at a depth of 4 m and reached a maximum value of 1.0 μgN L⁻¹ at a depth of 6.5 m. The ammonia and nitrite levels increased with increasing depth. Ammonia is reported to be released from sediments at the lake bottom, whereas nitrite accumulation is attributed to light inhibition by
ammonia- and nitrite-oxidizing bacteria, which use the oxygen supplied by seawater intrusion from the Sea of Japan\(^2\). Nitrite at the bottom layer of Lake Nakaumi is thought to be strongly associated with the formation of N\(_2\)O and NH\(_2\)OH.

**Conclusions**

We previously demonstrated\(^24\) that the substance interfering in the determination of NH\(_2\)OH in brackish- and sea-water samples by the hypochlorite method is a component in seawater. We have shown that this interference by Br\(_2\) generated from Br\(^-\) can be removed by the addition of phenol prior to adding hypochlorite, and have developed a new quantitative method for NH\(_2\)OH that is applicable to brackish- and sea-water samples. The proposed method has a 0.2 \(\mu\)gN L\(^{-1}\) limit of detection and a-water samples with a wide range of salinity. When applied to brackish water samples from Lake Nakaumi, high concentrations of NH\(_2\)OH, N\(_2\)O, ammonia, and nitrite were observed. The presence of nitrite is thought to be strongly associated with the generation of N\(_2\)O and NH\(_2\)OH in the bottom layer of Lake Nakaumi.

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