

Current Status of Homogeneity and Stability of the Reference Materials for Nutrients in Seawater

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The development of Reference Materials for Nutrients in Seawater (RMNS) has been in progress since 1993. When RMNS were produced for nitrate, silicate, and phosphate, their initial homogeneities were as low as 0.1 to 0.2% in samples of high-nutrient seawater, such as deep water from the Pacific Ocean. The relative standard uncertainties associated with instability during long-term (4.8 years) storage were approximately 0.2, 0.2, and 0.4% for nitrate, silicate, and phosphate, respectively. No instability was observed for 1.9 to 6.4 years based on the ISO Guide 35:2006 criteria; however, the relative standard uncertainties associated with instability during long-term storage were larger than the initial homogeneities of RMNS. RMNS produced by state-of-the-art techniques are currently available for global use to improve the comparability of nutrients data in the open ocean and, as discussed here, are reliable candidates to be used for certified reference materials.

(Received December 2, 2011; Accepted July 17, 2012; Published September 10, 2012)

Introduction

Reference Materials for Nutrients in Seawater (RMNS) are produced from natural seawater, and since 2003 have been available as standards to ensure the comparability and traceability of nutrient data concerning the open ocean. Although the importance of RMNS has been well recognized since the 1990s,^{1,2} this importance has increased because of their relevance to studies of climate research and biogeochemical cycling in the ocean.

The high reproducibility and precision of nutrient measurements required by the World Ocean Circulation Experiment (WOCE) Hydrographic Program (WHP) one-time survey³ has underscored the fact that prior to the 1990s no standards were available to facilitate the measurement of nutrient concentrations in seawater. The WOCE requirements were quantified in terms of coefficients of variation (CVs), standard deviations expressed as percentages of means. The required CVs were 1, 1–2, and 1–3% for nitrate, phosphate, and silicate, respectively. Although nutrient data from the WOCE one-time survey were of unprecedented quality and coverage because of the care given to sampling and measurements, differences in the nutrient concentrations were still found at crossover points, where different expeditions made measurements at the same place, but at different times.^{4–6} For example, the mean discrepancy in the nitrate concentrations measured in deep ocean water containing about 100 $\mu\text{mol kg}^{-1}$

nitrate was 0.5 $\mu\text{mol kg}^{-1}$ at 345 crossover points, and the maximum discrepancy was 1.7 $\mu\text{mol kg}^{-1}$.⁶ At 31 crossover points along the Pacific WHP one-time lines, the required CV was 1% for nitrate; this requirement was fulfilled at only about half of the crossover points. Among these crossover points the maximum observed difference in nitrate concentrations was as high as 7% of the mean value, even in deep waters where the potential temperature was lower than 1.6°C.⁴

The International Council for the Exploration of the Sea (ICES) conducted an intercomparison exercise for nutrients in seawater (NUTS I/C) in 1989/1990 (NUTS I/C 4) and 1992/1993 (NUTS I/C 5). Autoclaving, which has been studied for the production of RMNS,^{7,8} was used to stabilize samples for the 5th intercomparison exercise conducted in 1992/1993.⁹ Aminot and Kerouel⁸ concluded that nitrate and nitrite in autoclaved seawater were extremely stable throughout the 27-month storage experiment, with overall CVs lower than 0.3 and 0.8% for means of 5–50 $\mu\text{mol L}^{-1}$ and 0.5–5 $\mu\text{mol L}^{-1}$, respectively. For phosphate, leaching from the glass container caused a slight increase of 0.02–0.07 $\mu\text{mol L}^{-1}$ per year. The main cause of nutrient variation in seawater samples was believed to be microbial activity; hence, the production of RMNS depended on the inactivation of biological activity in the samples. A previous study showed that autoclaving to inactivate biological activity was acceptable for RMNS preparation.⁷

The European Union (EU) supported the Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME) project between 1993 and 1995. The aim of this project was to develop a holistic quality-assurance program for marine environmental monitoring information in Europe. As a result of this pioneering project, a marine network and

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laboratory performance protocols have been established for most of the determinants measured in the EU marine environmental programs for both monitoring and research purposes. The nutrient portion of QUASIMEME was based entirely on groundbreaking work of ICES experts. The project demonstrated that laboratories that regularly undertook training programs and carried out quality control tests improved the quality of their data. After the EU funding ended in 1995, the QUASIMEME scheme continued on a subscription basis. It is now possible for any laboratory in the world to participate in the QUASIMEME scheme. The QUASIMEME results have been used to assess the quality of data submitted to marine conventions.¹⁰

Studies have also been carried out on the addition of toxic chemical preservatives, particularly mercuric chloride, to samples of natural seawater and laboratory-prepared standard seawater.^{11,12} Kirkwood¹¹ concluded that mercuric chloride is an effective preservative for storing standard nutrient solutions when added at a concentration of 20 $\mu\text{g mL}^{-1}$. Although the use of toxic chemicals, especially mercury, may effectively inactivate biological activity, the subsequent release of waste solutions containing such chemicals may lead to chemical pollution of the environment. We therefore excluded the use of toxic chemicals as preservatives of seawater in this study.

We also reviewed other techniques for stabilizing nutrient concentrations in preserved seawater samples, including gamma-ray exposure and storage at temperatures ranging from -20 to 5°C . However, these techniques had disadvantages, including changes in the sample matrix, analytical interference, shipping challenges, silicate polymerization, nitrate generation, and nitrite generation.⁸

Pasteurization has recently been studied as a method for the preservation of nitrate and nitrite in seawater samples.^{13,14} Pasteurization allows samples to be stored at room temperature, without any special equipment, for a long period of time, and has the potential to produce RMNS in a seawater matrix.

The Meteorological Research Institute, Japan began a series of studies with the RMNS that we prepared in 2003. Testing of the materials started with inter-laboratory comparisons in a study that included 18 laboratories.¹⁵⁻¹⁷ The tests expanded to 55, 56 and 69 different laboratories worldwide in 2006, 2008 and 2012, respectively.^{17,18} The studies clearly indicated that the universal use of RMNS will make it possible to greatly improve the comparability of nutrient data from the world's oceans.¹⁹ In addition to the international inter-laboratory comparisons, the RMNS have already been used in eight Climate Variability and Predictability project cruises from 2003 to 2010. Therefore, the next stage was to use the RMNS to produce a globally accepted mechanism for achieving comparability of nutrient data in the world's ocean. The IOC-UNESCO 25th assembly accepted a proposal for establishing a joint ICES-IOC study group.²⁰ One of the principal objectives of the Study Group on Nutrient Standards was to establish global comparability and traceability of seawater nutrient data from the world's oceans through the development of appropriate certified reference materials (CRMs). To guarantee comparability of data from different laboratories and different cruises, a single international "scale" for nutrients is to be developed and used throughout the marine chemistry community. An example of what can be achieved is already provided by the work of marine chemists on the carbonate chemistry of seawater. For measurements of carbonate system parameters, these researchers used CRMs that were certified for total dissolved inorganic carbon with a Ruska electronic constant-volume manometer.^{21,22} The equivalent indicators for nutrients are the RMNS that display acceptable

homogeneity and stability. Although the homogeneity and stability of the RMNS can be improved more in the future, the currently available RMNS, as reported in this paper, are reliable candidates for certification and use, and their use in nutrient analyses would be analogous to the use of CRMs for measurements of carbonate system parameters.

In this paper, we present and discuss the current status of the homogeneity and stability of our RMNS.

Experimental

Source seawater for the RMNS and onboard pasteurization

The source seawater samples were collected for the RMNS from the surface to 1500 m in the open ocean, or at a coastal station in Japan.¹⁵ The source water samples to produce high-concentration RMNS were collected at a depth of approximately 1500 m at 160 to 180°E longitude and 40 to 55°N latitude in the North Pacific Ocean by using 12-L Niskin bottles on the R/V Mirai's Carousel/CTD system over a period from 2005 to 2010. In 2010 we also used the R/V Mirai to collect source seawater at a depth of 690 m at 73.6°N , 160.0°W in the Arctic Ocean. To obtain nutrient-depleted water characteristic of surface seawater for the production of the RMNS, we used an *in situ* pump or bucket to collect source seawater in the North Pacific Ocean, e.g., at 30.0°N , 145.0°E .

Because seawater samples collected by research vessels in the open ocean were stored for a minimum of a few months before the research vessels returned to Japan to offload the seawater samples, we pasteurized the seawater at 85°C for a minimum of 5 h to preserve the original nutrient concentrations during storage when the source seawater had been collected from below the nutricline in the North Pacific Ocean and the Arctic Ocean. We pasteurized the source seawater samples collected from below the nutricline in a sauna room aboard the R/V Mirai, where we put the source seawater in 20-L polyethylene containers, the caps of which were then tightened. Previously when we froze seawater samples, we observed a decrease of a few percent or more in silicate and phosphate concentrations, and we therefore did not freeze the source seawater. Nitrate, phosphate, and silicate concentrations of the source seawater collected from the surface layer were already depleted; therefore, we did not need to pasteurize the surface seawater samples.

The RMNS production facility

The RMNS production facility included a filtration room, a large autoclave (W, 140 cm; H, 165 cm; D, 280 cm), a cooling room (clean level of 2.00×10^4 particle counts m^{-3}), and a bottling room (2.00×10^3 particle counts m^{-3}). We installed a Class 100 clean bench (CCV-1311; Hitachi Appliances, Tokyo, Japan) as a bottling bay in the bottling room. Ota *et al.*²³ describe more details of this facility.

To produce the RMNS at several concentration levels of the nutrients, we developed a method to achieve the target nutrient concentrations by mixing source seawater with different concentrations in various ratios. This method was patented in Japan²⁴ and described in detail by Ota *et al.*²³ A 230-L reaction chamber was made of SUS316, a type of stainless-steel that is highly resistant to corrosion from seawater.²⁵ We inserted stainless-steel tubes through sanitary seals into the reaction chamber before autoclaving, so that we could withdraw the seawater from the reaction chamber after autoclaving without breaking the seal. We mounted a stopcock on the outer end of each tube to maintain the hermetic seal during autoclaving. Autoclaving was performed at 120°C for 2 h on two occasions

about two days apart. This treatment denatured the proteins that compose microorganisms, and halted microbiological activity.

Long-term storage of the RMNS

We used polypropylene bottles with a volume of 100 mL to store the RMNS. We selected polypropylene bottles so as to avoid possible leaching of phosphate and silicate. The bottles were cleaned with surfactant and ultrapure water, dried, and sealed in double bags in a Class 100 clean room by a bottle manufacturer. The bottles were then sterilized with ultraviolet (UV) lamps in the preparation room of the RMNS production facility to inactivate mold and bacteria that might be on the inside walls of the polypropylene bottles. We set the total UV exposure on the inside walls of the polypropylene bottles to $8800 \mu\text{W min cm}^{-2}$. This amount of total UV exposure corresponds to twice the total amount of UV exposure required to inactivate *Aspergillus niger*, which shows the highest UV resistance among mold and bacteria.²⁶ We expected that this treatment would inactivate 99.99% of *Aspergillus niger* spores. After the seawater had been autoclaved in the reaction chamber, we transferred an aliquot of 90 mL into each sterilized polypropylene bottle through a $0.22\text{-}\mu\text{m}$ pore size filter in the clean room of the RMNS production facility.

For old lots, we used a polyethylene film bag to pack the RMNS bottles. The salinity increase and mass of the liquid contents decrease of approximately 0.1% per year were noted to occur in such bags,²³ because of gas exchange across the polyethylene film bag. We therefore used an aluminum film bag (MAL-1320S) manufactured by Meiwa Sanshou Co.²⁷ to pack each RMNS bottle after August 2006 onwards. We hermetically sealed the bottles in MAL-1320S bags with a vacuum heat-sealer in a clean room of the RMNS production facility.

Although the polypropylene bottles used for the RMNS did not have inner caps, the capability of the bottle to retain the mass of the liquid contents was confirmed both by the bottle manufacturer and by a long-term test of salinity and mass changes of the liquid contents under severe conditions in our laboratory. The producer of the polypropylene bottle confirmed that no leak was detected when the cap was closed with a torque of 147 N cm and stored upside down for 12 h. We also performed a long-term severe conditions test from 24 August 2009 to 15 February 2010. During the test, the ambient temperature was changed from 10 to 40°C in a 4-h cycle. We controlled the cycle of the ambient temperature by following the four steps described below:

- Step 1 Maintain the temperature at 10°C for 1 h
- Step 2 Linearly increase the temperature from 10 to 40°C in 1 h
- Step 3 Maintain the temperature at 40°C for 1 h
- Step 4 Linearly decrease the temperature from 40 to 10°C in 1 h

As a result of the severe conditions test, the mass change of the liquid contents in bottles with no aluminum film bag changed by 0.2%, whereas the mass change of the liquid contents in bottles with the aluminum film bag, MAL-1320S, changed only by 0.02%. A long-term test was also performed at room temperature for three years, and very small changes in mass of the liquid contents in the bottles, less than 0.01%, were detected.²³ We therefore concluded that the MAL-1320S bag was an excellent long-term barrier to water evaporation, and its use led to minimal changes in the nutrient concentrations.

Analytical methods

We used a Technicon TRAACS 800 Chemistry Analyzer and BLTEC-SEAL QuAAtro 2-HR Analyzer to measure the nutrient

Table 1 Mass change during autoclaving for lots AO, BR, BS, and BT

Lot name	Before/kg	After/kg	Difference/kg
AO ^a	230.00	229.99	0.01
BR	407.52	407.50	0.02
BS	401.52	401.38	0.14
BT	487.70	487.66	0.04

a. The mass change for lot AO was estimated from the salinity change. A direct mass measurement could not be carried out, because a balance to measure the mass after autoclaving was not installed at that time.

concentrations in this study. The phosphate analysis method was a modification of the procedure of Murphy and Riley.²⁸ The nitrate and nitrite concentrations were analyzed according to the modified cadmium-reduction method of Grasshoff.²⁹ We computed the nitrate concentrations from the difference between the concentrations of nitrate plus nitrite and nitrite.²¹ The silicate method was analogous to that described for phosphate, and was essentially a procedure described by Grasshoff *et al.*³⁰ The practical details and modifications of the measurements are described by Aoyama *et al.*³¹ and Hydes *et al.*³²

To maintain comparability of the nutrient measurements during the long-term stability test, we used the same chemicals to prepare the in-house standard solutions. For the nitrate standard solution, we used “potassium nitrate 99.995 Suprapur[®]”, provided by Merck KGaA. For the phosphate standard solution, we used “potassium dihydrogen phosphate anhydrous 99.995 Suprapur[®]”, provided by Merck KGaA. For the nitrite standard solution, “sodium nitrite, super grade”, provided by Wako Pure Chemical Industries, Ltd., was used and prepared with JIS K8019:1992.³³ For the silicate standard solution, we used the “silicon standard solution SiO₂ in 0.5 mol/L NaOH CertiPUR[®]” provided by Merck KGaA. The silicate concentration of this solution is associated with a relative standard uncertainty of 0.5% and is traceable to Silicon Standard Solution, Standard Reference Materials 3150, provided by the National Institute of Standards and Technology, USA.^{34,35}

Results and Discussion

It is important to monitor any changes of the mass, salinity and nutrient concentrations of seawater that may occur as a result of autoclaving. Table 1 gives an example of a mass change of source seawater during autoclaving. The magnitude of the changes in mass of the source seawater ranged from 0.004 to 0.03% for four lots of RMNS. These changes in mass of the source seawater are small enough rather than homogeneity and stability of the RMNS. Tables 2(a) and 2(b) show changes of the nutrient concentrations in seawater samples as a result of autoclaving. These comparisons were made for the lot AO produced in 2003 and for lots BR, BS, and BT produced in 2010. We detected only minor changes in the nutrient concentrations after two autoclaving treatments at 120°C. As shown in Tables 2(a) and 2(b), the nutrient concentrations of the source seawater samples were well maintained, and these results were consistent with the results of mass monitoring the source seawater samples, as shown in Table 1.

A relatively large phosphate concentration decrease by 2.5% (*i.e.*, by $0.07 \mu\text{mol kg}^{-1}$ from an initial concentration of $2.7 \mu\text{mol kg}^{-1}$) was observed when the inner wall of the stainless-steel reaction chamber was cleaned with only an acid

Table 2 (a) Nutrient concentrations before and after autoclaving for lot AO

	Salinity/ —	Nitrate/ $\mu\text{mol kg}^{-1}$	Nitrite/ $\mu\text{mol kg}^{-1}$	Silicate/ $\mu\text{mol kg}^{-1}$	Phosphate/ $\mu\text{mol kg}^{-1}$
Before autoclaving					
Average	34.3374	36.05	0.04	100.3	2.72
SD	0.0001	0.01	0.01	0.43	0.01
CV, %	0	0	15.7	0.4	0.5
n	3	3	3	3	3
After autoclaving					
Average	34.3386	36.19	0.04	99.69	2.73
SD	0.0004	0.07	0	0.12	0.01
CV, %	0.001	0.2	11.0	0.1	0.4
n	20	10	10	10	10
Difference ("After" minus "Before")					
Average	0.0012	0.14	0	-0.61	0.01
%	0.003	0.388	0.000	-0.608	0.368

Note: This table is reproduced from Ota *et al.*²³

(b) Nutrient concentrations before and after autoclaving for lots BR, BS, and BT

	Salinity/ —	Nitrate/ $\mu\text{mol kg}^{-1}$	Nitrite/ $\mu\text{mol kg}^{-1}$	Silicate/ $\mu\text{mol kg}^{-1}$	Phosphate/ $\mu\text{mol kg}^{-1}$
Lot BR					
Before	34.5855	15.50	0.394	29.95	1.105
After	34.5886	15.33	0.406	29.61	1.124
Difference	0.0031	-0.17	0.012	-0.34	0.019
Lot BS					
Before	34.6357	0.060	0.010	2.434	0.089
After	34.6631	0.058	0.017	2.411	0.054
Difference	0.0274	-0.002	0.007	-0.023	-0.035
Lot BT					
Before	34.4123	18.30	0.479	42.66	1.302
After	34.4131	18.15	0.471	42.02	1.296
Difference	0.0007	-0.15	-0.008	-0.6	-0.006

solution, or was buff polished with cotton. This 2.5% decrease of the phosphate concentration was larger than the changes of nitrate and silicate concentrations, and also larger than that derived from a change of mass of the source seawater and salinity. However, the change in the phosphate concentration became small after electropolishing was used to clean the inner surface of the reaction chamber²⁵ for lot AO, as shown in Table 2(a).²⁵ The magnitude of electropolishing of the inner surface of the reaction chamber in terms of surface roughness ranged from 0.03 to 0.5 μm .

The homogeneity of the RMNS discussed in this paper for lots K, AH, BF, and BG was expressed as CVs of 60 concentrations, obtained from duplicate measurements of 30 bottles of each lot of the RMNS. The homogeneity of the RMNS has been improved over the last several years, as shown in Table 3 and Fig. 1, especially for phosphate. The homogeneity of the RMNS for lot K produced in 2001 were 0.3, 1.0, and 0.2% for nitrate, silicate, and phosphate, respectively. Recently the homogeneity of the RMNS for lots BF and BG both produced in 2007 were approximately 0.1, 0.2, and 0.1 for nitrate, silicate, and phosphate, respectively. These RMNS lots BF and BG were produced by the reaction chamber, of which the inner surface was electropolished.

Although the effects of electropolishing diminished after the chamber was used several times for autoclaving, subsequent electropolishing restored the improved homogeneity of the

Table 3 History of the homogeneity of RMNS and a sample used in ICES I/C 5

Year	Lot name	Nitrate, %	Phosphate, %	Silicate, %	Reference
1995	ICES I/C 5	0.31	0.3	N.A.	9
2001	K	0.3	1.0	0.2	31
2003	AH	0.44	0.8	0.15	31
2007	BF	0.11	0.21	0.10	31
2007	BG	0.14	0.17	0.08	31

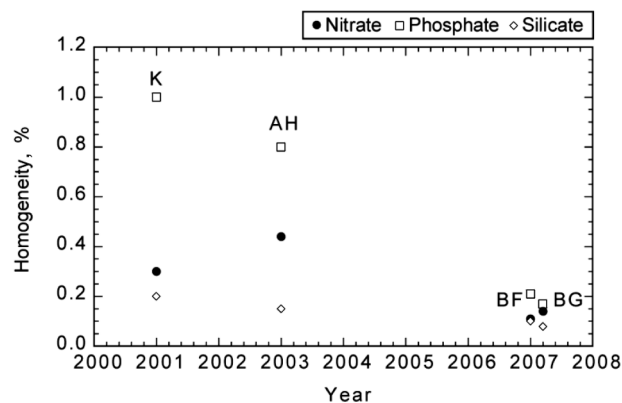


Fig. 1 History of the homogeneity of RMNS. Open square, phosphate; solid circle, nitrate; open diamond, silicate. K, AH, BF, and BG: RMNS lot names.

RMNS, and reduced the change in the phosphate concentration associated with autoclaving. Therefore, we can conclude that less concentration change of phosphate associated with autoclaving and improved homogeneity of the RMNS were primarily the result by electropolishing the inner surface of the reaction chamber.

We performed a long-term stability study of the RMNS in accordance with clause 8.3 of the ISO Guide 35:2006,³⁶ and based our conclusions on the recorded measurements of nitrate, phosphate, and silicate concentrations for lots AH, AV, AX, and BF, as shown in Fig. 2. The calculated slopes of linear regressions of nitrate, phosphate, and silicate concentrations *versus* time for lots AH, AV, AX, and BF did not differ significantly from zero ($p < 0.05$), except for the nitrate concentration slope of lot AV. We therefore observed no instability for study periods of 1.9–6.4 years for nitrate, phosphate, and silicate concentrations for lots AH, AV, AX, and BF, with the exception of the nitrate concentration of lot AV, as shown in Table 4.

According to clause 8.5 of ISO Guide 35:2006, the estimated relative standard uncertainty associated with long-term stability can be equated to the relative standard uncertainty associated with the slope of the measured data derived from clause 8.3, with time in years as the independent variable. The relative standards uncertainties associated with long-term stability during a shelf life of 4.8 years were approximately 0.2, 0.2, and 0.4% for nitrate, silicate, and phosphate, respectively (Table 4). As stated previously, we observed no instability for study periods of 1.9–6.4 years for nitrate, phosphate, and silicate concentrations for lots AH, AV, AX, and BF, with the exception of the nitrate concentration of lot AV according to the criterion of ISO Guide 35:2006; however, the relative standard

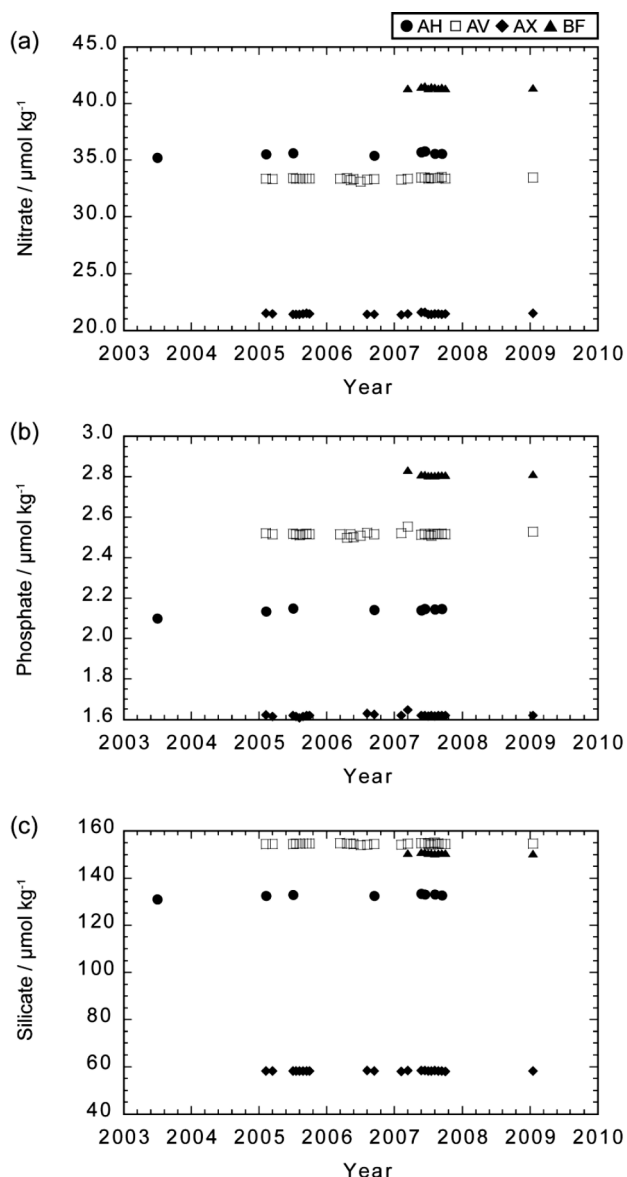


Fig. 2 Time series of nutrient concentrations of RMNS. (a) Nitrate, (b) phosphate, (c) silicate.

uncertainties associated with long-term stability during shelf lives of 1.9–6.4 years were larger in value than the initial homogeneities of the RMNS.

The improved homogeneity and stability we achieved in this study resulted especially from electropolishing the surface of the inner wall of the reaction chamber, as discussed previously. Although we do not fully understand the mechanisms responsible for the improved homogeneity of the RMNS evidenced in Fig. 1 and the decrease of the change in the phosphate concentration, both improvements occurred simultaneously. The surface reaction between the inner surface of the steel reaction chamber and the source seawater in the steel reaction chamber could be largely suppressed by electropolishing the inner surface of the reaction chamber. As a consequence, the currently available state-of-the-art RMNS presented and discussed in this paper are reliable candidates for global use to improve the comparability of nutrients data in the open ocean and for use as CRMs.

Table 4 Results of stability study following ISO Guide 35:2006

Lot name	Conc./ $\mu\text{mol kg}^{-1}$	Time/year	ISO Guide 35 stability ^a	Initial homogeneity, ^b %	Relative standard uncertainty due to long-term stability to the time, %
Nitrate					
AH	35.54	6.4	yes	0.44	0.7
AV	33.88	4.8	no	N.A.	0.2
AX	21.45	4.8	yes	N.A.	0.2
BF	41.41	1.9	yes	0.11	0.20
Phosphate					
AH	2.13	6.4	yes	0.8	1.0
AV	2.52	4.8	yes	N.A.	0.3
AX	1.62	4.8	yes	N.A.	0.4
BF	2.81	1.9	yes	0.21	0.38
Silicate					
AH	132.6	6.4	yes	0.15	0.6
AV	154.4	4.8	yes	N.A.	0.1
AX	58.21	4.8	yes	N.A.	0.2
BF	150.58	1.9	yes	0.08	0.19

a. “yes” means that calculated slopes for nitrate, phosphate, and silicate concentrations did not differ significantly from zero at 95% level of confidence by the Student’s *t*-test for *n*-2 degree freedom.

b. The initial homogeneity of RMNS was CVs of concentrations of 60 values obtained by duplicate measurements of 30 bottles of the lot when it was produced.

Conclusions

Electropolishing the inner surface of the stainless-steel reaction chamber greatly suppressed any surface reactions with the source seawater. Therefore, state-of-the-art RMNS can now be produced and used effectively to improve the comparability of nutrient data in the world’s oceans. The initial homogeneity of the RMNS for nitrate, silicate, and phosphate were as low as 0.1–0.2% in high-nutrient seawater, such as subnitracine water from the Pacific Ocean. We were able to confirm a shelf life of as long as 1.9–6.4 years. The relative standard uncertainties associated with long-term stability during a shelf life of 4.8 years were approximately 0.2, 0.2, and 0.4% for nitrate, silicate, and phosphate, respectively. By the criterion of the ISO Guide 35:2006 there was no instability of the RMNS nutrient concentrations; however, the relative standard uncertainties associated with long-term stability were larger than the initial homogeneities of RMNS.

Acknowledgements

The authors thank Aoi Mori and Yukiko Yoshimura for preparing the tables and figures included in this article.

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