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Practical Considerations for the Segmented-flow Analysis of Nitrate and Ammonium in Seawater and the Avoidance of Matrix Effects

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Abstract – In this study we describe measures taken in our laboratory to improve the long-term precision of nitrate and ammonia analysis in seawater using a microflow segmented-flow analyzer. To improve the nitrate reduction efficiency using a flow-through open tube cadmium reactor (OTCR), we compared alternative buffer formulations and regeneration procedures for an OTCR. We improved long-term stability for nitrate with a modified flow scheme and color reagent formulation and for ammonia by isolating samples from the ambient air and purifying the air used for bubble segmentation. We demonstrate the importance of taking into consideration the residual nutrient content of the artificial seawater used for the preparation of calibration standards. We describe how an operating procedure to eliminate errors from that source as well as from the refractive index of the matrix itself can be modified to include the minimization of dynamic refractive index effects resulting from differences between the matrix of the samples, the calibrants, and the wash solution. We compare the data for long-term measurements of certified reference material under two different conditions, using ultrapure water (UPW) and artificial seawater (ASW) for the sampler wash.

Key words – segmented-flow analysis (SFA), open tube cadmium reactor (OTCR), nitrate, ammonium, matrix effect, refractive index

1. Introduction

Dissolved nutrients in seawater are an essential factor for the photosynthesis of phytoplankton within the surface mixed layer (SML) and they control the total amount of primary production as well as the composition and distribution of primary producers within the ocean. Dissolved nutrients are influenced by mixing process in the SML (i.e. reduced surface mixing, caused by surface warming and freshening, leading to a decrease in surface nutrient concentration) and by global scale meridional overturning circulation (MOC) in the deep ocean (i.e. a slowdown of MOC may result in the increase of deep nutrient reservoirs). Dissolved nutrients are regarded as important biogeochemical properties that provide useful information for tracing the changes in global physical and biological processes in the ocean. For reliable long-term understanding of nutrient distribution, accurate and precise measurements are needed. Several attempts at detecting coherent basin scale changes in nutrient concentrations have been hindered by a lack of comparability between laboratories at different times (Bindoff et al. 2007).

During the last three decades, numerous efforts have been made to establish the comparability and traceability of nutrient data measured in different laboratories around the world such as inter-comparison exercises for nutrient analysis, and the development of nutrient reference materials (Aminot and Kirkwood 1995; Aminot et al. 1997; MRI 2008, 2010). In these inter-comparison exercises, notable data discrepancies among participants were identified as having their origins in the accuracy of calibration standards and ammonia contamination from the environment (Aminot and Kirkwood 1995) and the lack of certified reference material in early studies. A particular

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*Corresponding author. E-mail: tkrho@kiost.ac source of error was identified as the failure to satisfactorily determine or correct for residual nutrients in the low nutrient seawater (LNSW) or artificial seawater (ASW) used as the basis for calibration standards. Several suggestions have been made to solve this problem, either by accurately determining and correcting for the residual nutrient content (Hydes et al. 2010) or by eliminating it from calculations with a special calibration procedure (Aminot et al. 2009; Coverly et al. 2012). In the rest of this paper the terms ASW and LNSW are interchangeable unless otherwise specified.

Further errors arise from salinity (the so-called "salt effect"), which may influence reaction kinetics and light transmission through the photometer flowcell. Four categories of "salt effect" for a gas-segmented flow analysis (SFA) system were described by Mantoura and Woodward (1983). Coverly et al. (2012) reclassified matrix effects into two groups: (1) sample-specific and chemistry-related effects such as matrix absorbance, scattering or fluorescence, and salt influence on the reaction rate or end point; and (2) hardware-related effects affected by instrument design including static salt influences on the optical signal, such as the refractive index blank (RIB) and dynamic salt influences on the optical signal, such as Schlieren and lens effects. Different approaches to reduce the dynamic salt effect have been described, such as either use of ASW or LNSW for the inter-sample wash and the baseline (Gordon et al. 1993) or use of a bubble-through-flowcell, preventing direct contact between sample and wash water within the flowcell by the introduction of air segmentation within the flowcell (Coverly et al. 2012).

As part of our work to develop reference standard material for nutrients in seawater with proven stability, we required highly accurate and precise analytical methods. We encountered some of the problems reported in early studies, particularly in regards to ammonia contamination and the fluctuation of nitrate reduction efficiency described as a possible error source for nitrate analysis in natural seawater by Garside (1993). In the text below, we describe the procedures for analysis and for OTCR regeneration, precautions against ammonia contamination, manifold optimization and calibration procedure that contributed to improved precision in our laboratory.

2. Materials and Methods

The gas-segmented flow analyzer (SFA) used for this study is the QuAAtro system of SEAL Analytical, a "microflow" instrument fitted with manifold components of 1 mm internal diameter, 10 × 1 mm flow cells without a de-bubbler and a flow-through Cd coil for nitrate analysis. The principle of the analytical method for each nutrient (except for ammonium) is similar to those described by Aminot et al. (2009) with modifications for a microflow analyzer and slightly different reagent formulations. According to Aminot et al. (2009), ammonium concentration was determined by the fluorometric method (Kérouel and Aminot 1997), but the Berthelot reaction, in which ammonium ions react with salicylate and free chlorine to form a blue-green colored complex, was used in this study, following the manufacture's manual. Our instrument was fitted with two different manifold configurations having reference numbers MT3A and MT3B. Each can be used for several different nutrients, depending on the reagents used. The major differences between MT3A and MT3B are the length of reaction coils, the flow rate and the number of reagents. The factory setting of our system was to use MT3A manifold for nitrite, nitrate+nitrite (including OTCR), and phosphate determination, and was to use MT3B for ammonium and silicate determination.

All chemicals were of analytical reagent grade or equivalent (e.g. ACS grade, Analar, Pro Analysi) from Merck, Sigma-Aldrich and Wako Pure Chemical Industries. Calibration standard solutions were prepared from Suprapur® (potassium nitrate, potassium dihydrogen phosphate, ammonium sulfate) and Pro Analysi (sodium nitrite) grade reagents from Merck. As a silicate standard solution we used the CertiPUR® silicon standard solution SiO_2 in 0.5 mol/L NaOH from Merck. Nutrient reference materials for seawater, prepared by KANSO Techno and the Korea Institute of Ocean Science and Technology (Rho et al. 2015), were measured in each test to assess the accuracy and reproducibility of measurements made under different conditions.

3. Results and Discussion

Improving the precision of nitrate analysis

Long-term monitoring of the calibration curve slope and the absorbance of the first peak in each run, usually the highest standard solution, showed fluctuations of up to 10% during the period of 2012–2013 (Fig. 1, first seven data points), and we carried out a series of tests to systematically optimize different parts of the analytical manifold.

Reduction of nitrate to nitrite in the Cd coil

Nitrate in seawater can be accurately determined by the

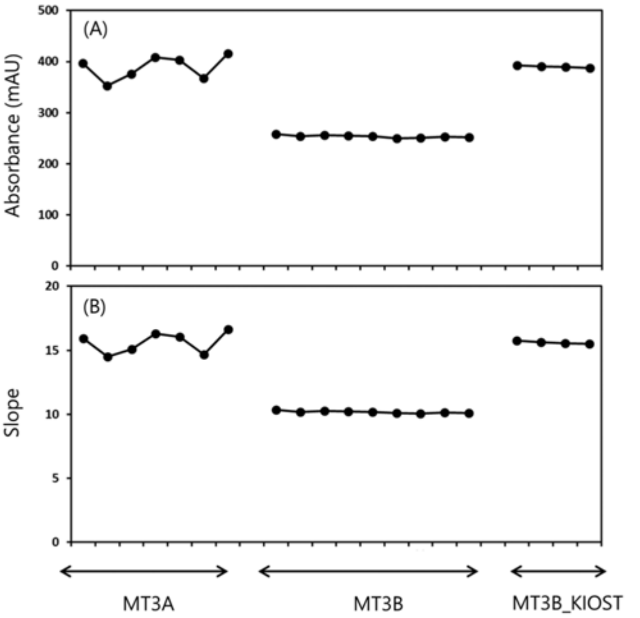

Fig. 1. Changes in Primer absorbance (Nitrate, 25 μM) and calibration curve slope by the modification from MT3A manifold (Q-047-04 method) to MT3B and MT3B_KIOST manifolds (Q-035 method). MT3A manifold is a short reaction configuration using two of 20 turn reaction coil with less reagent input ports. MT3B manifold is long reaction configuration using three of 20 turn reaction coil with more reagent input port. MT3B_KIOST manifold has the same reaction configuration as the MT3B but different flow rate for sample and imidazole buffer

quantitative reduction of nitrate to nitrite in a copperized cadmium reductor (e.g. Wood et al. 1967), followed by Benschneider and Robinson's (1952) diazotization colorimetric method, based on Griess (1952). Two types of Cd reductor are in widespread use: a column packed with Cd granules (Cd packed column) and the Open Tube Cadmium Reactor (OTCR), first described by Patton (1982) and commonly referred to as a Cd coil. In both cases the reduction efficiency is enhanced by coating the Cd with Cu using a solution of $CuSO_4$. The OTCR reduces carry-over by allowing gas-segmented passage through the Cd reductor, so avoiding the dispersion inherent in a packed column. A comparison of the OTCR and a packed column was made by Zhang et al. (2000).

The microflow SFA system used in this study was fitted with an OTCR made from a length of Cd tubing about 36 cm long, wound into a coil and painted on the outside, with an internal diameter of about 1.2 mm that increases as Cd is slowly dissolved during use and regeneration. The retention time within the OTCR was 20–22 seconds. Finally, the Cd tubing was adjusted to a length of 45 cm for better performance with the retention time of 26–28 seconds. Nitrogen gas was used for segmentation to eliminate the adverse effect of oxygen within the OTCR (Gordon et al. 1993; Zhang et al. 2000).

Factors affecting recovery: buffer for nitrate reduction

Previous workers have shown that the efficiency of a Cd reductor, determined by comparing a nitrate standard to a nitrite standard of the same concentration, is dependent on the surface activity of the Cd, pH, and the residence time within the reductor (Wood et al. 1967; Nydahl 1976; Grasshoff et al. 1999). Optimum reduction occurs at pH 8.0 (Nydahl 1976) and is maintained by a buffer solution. Ammonium chloride is commonly used with a Cd packed column. Patton (1986) proposed imidazole for the OTCR so as to reduce the retarding effect of chloride ions on the reduction rate of nitrate to nitrite. Imidazole also improves a multi-channel analyzer by dispensing with the need for using a reagent containing large amounts of ammonium in an instrument that is measuring ammonium in micromole concentrations. Various workers have recommended different buffer formulations, pH, and in some cases the addition of Cu to extend the reductor efficiency (Table 1).

Table 1. Composition of Cd reduction buffer solutions for nitrate analysis

Buffer Types	Concentration (g/L)	pH	$CuSO_4$ added	Reference
Ammonium chloride	10	8.5	No	Grasshoff et al. (1999)
Ammonium chloride	11	8.5	No	Aminot et al. (2009)
Imidazole	3.4	7.8	No	Zhang (1997)*
Imidazole	3.4	7.8–7.85	Yes	Gordon et al. (1993)
Imidazole	17	7.5	No	Q-030-04 (rev. 4)
Imidazole	4	-	Yes	Q-035-04 (rev. 7), Q-047-04 (rev. 5)
Imidazole	6.8	7.8	Yes$^+$	KIOST method

*Zhang (1997): similar to EPA method 353.4.

+$CuSO_4$ and surfactant added to stock buffer shortly before use.

We investigated the effects of pH, reductor preparation, and regeneration. Our starting point was the manufacturer's recommended imidazole reagent, described here as the Q-035-04 method, prepared with the addition of 0.5 ml stock copper sulfate (0.01 M) and 2 ml conc. HCl per liter. No pH value is stated, but we found that this ranged from 7.2–7.4 depending on the strength of the hydrochloric acid. Second, we also prepared a similar buffer with the pH adjusted to 7.4, described hereafter as the Q-035-K method, following Zhang (1997) and Zhang et al. (2000). Third, we prepared a buffer based on the one described by Gordon et al. (1993), in which a stock imidazole solution is prepared with 6.81 g imidazole per liter of UPW. We adjusted the pH to 7.8, following Zhang (1997) and Zhang et al. (2000). Shortly before use, to avoid the possible growth of mold or bacteria in weak surfactant solutions, we prepared a working buffer from the stock solution with the addition of 0.5 ml 2% $CuSO_4$ (0.01 M) and 1 ml of Triton-X (50%) per liter, depending on the volume needed (KIOST method for imidazole buffer solution).

There was no measured difference in the reduction efficiency of OTCR among various imidazole buffer solutions, but the KIOST method showed slightly lower relative standard deviation (RSD) compared to the others (Table 2). For this reason and because it is convenient to prepare the working buffer from a stock imidazole buffer solution, which may also be more suitable for onboard use, we used the KIOST buffer for further tests.

Comparison of coil regeneration procedures

There are several descriptions of the preparation of a Cd packed column although the procedure and reagents differ (e.g. Grasshoff et al. 1999; Aminot et al. 2009). However, there are few detailed explanations about the OTCR preparation. Gordon et al. (1993) briefly describe the procedure and suggest following the instructions for preparing and maintaining the OTCR that are found in the instrument manual. Zhang et al. (2000) give a detailed description of how to activate the OTCR and explain the reason for each step. We compared Zhang's procedure (Zhang et al. 2000) with the procedures suggested by two manufacturers, OI Analytical and SEAL Analytical. Both instruments are used in our laboratory, and both use Cd coils made from the same type of tubing. Different recommendations are made for the (1) the concentration of hydrochloric acid used for OTCR wash (0.5 N HCl vs. conc. HCl), (2) direction of reagent through the OTCR (flushing vs. sucking), (3) introduction of air during the activation procedure (no air vs. sucked air at the end of each stroke), and (4) the use of an initial cleaning reagent for old coils that are being regenerated (2N HNO_3 for SEAL Analytical). Zhang et al. (2000) recommend using 10 ml of 0.5 N HCl for initial cleaning followed by slow and continuous flushing of 2% $CuSO_4$ solution until large amounts of black precipitate elute as an indication of complete copper coating. Their newly activated OTCR was then filled with imidazole buffer and conditioned with 40 µM nitrate solution for 10–15 minutes before sample analysis to accomplish constant reduction efficiency. Regeneration of a used OTCR when the reduction efficiency fell below 95% was conducted by pumping $CuSO_4$ solution through the column for 10 minutes followed by conditioning with 40 µM nitrate solution.

When we activated an OTCR following this procedure, after rapid flushing with 10 ml of 0.5 N HCl acid, the coil was flushed with water. 2% $CuSO_4$ was then introduced into the coil at a rate of 10 ml/min until the elution of large amounts of black precipitate. This occurred after about 30 ml $CuSO_4$ had been introduced. The newly activated OTCR was then filled with working imidazole buffer until the analysis. When the newly activated OTCR was attached to our QuAAtro SFA system for the conditioning with 40 µM nitrate solution, the bubbles leaving the OTCR were broken into smaller bubbles and fine black particles were eluted out of the OTCR. We concluded that the copperization was excessive and that a flushing step is needed to remove excess copper. We propose a modified procedure that incorporates these changes.

Prepare reagents (0.5 N HCl, 2% $CuSO_4$, daily imidazole buffer, UPW) and 10 ml and 50 ml syringes.

1) Place ca. 200 ml of ultrapure water (UPW) and ca. 50 ml of 0.5 N HCl in separate beakers.

Table 2. Mean absorbance values and relative standard deviation of nitrate (25 µM) and calibration curve slope in different imidazole buffer for nitrate reductor. RSD (%) = standard deviation / average × 100

Method	pH	Primer Absorbance		Slope of Calibration curve	
		Average (mAU)	RSD (%)	Average	RSD (%)
Q-035-04	7.2~7.4	456.6	1.82	15.3	1.79
Q-035-K	7.4	456.8	1.69	15.3	1.84
KIOST	7.8	456.5	1.55	15.3	1.65

2) Fill a 10 ml syringe with UPW and connect the syringe to one end of the OTCR, with a transparent drain tube at the other end. Flush the OTCR with the 10 ml of UPW. If black particles are observed in the drain tube, repeat until the effluent is clear.

3) Draw 40 ml of air into a 50 ml syringe, followed by 10 ml of 0.5 N HCl. Connect the syringe to the Cd coil and completely discharge the syringe so that the HCl is immediately followed by an air flush. This limits the time of exposure to HCl and prevents excessive loss of Cd.

4) Immediately repeat the above procedure using UPW.

5) Repeat step 4 until no black particles are observed in the drain tube. This usually takes 2-3 times, but may take longer as the OTCR gets older.

6) Wash three times with 10 ml of daily working imidazole buffer.

7) Introduce 10 ml of 2% $CuSO_4$ into the OTCR for 2 minutes and wait 10 minutes for complete copperization.

8) After 10 minutes, remove unattached colloidal copper particles within the OTCR by washing with 30 ml daily working imidazole buffer in a 50 ml syringe at the rate of 10 ml per minute. This process should be carefully performed, or excessive copper will be washed out.

9) Fill the OTCR with working imidazole buffer, and connect both ends of the OTCR to close it. Wait for at least 12 hours before use.

Zhang et al. (2000) recommend exposing the newly activated OTCR to a 40 μM nitrate solution because the newly activated surface has a tendency to over-reduce. We conducted tests with a newly activated or regenerated OTCR and one that had been stored for 12 hours filled with working imidazole buffer. Figure 2 shows the response from both coils after being installed in the manifold with all reagents pumping. A 40 μM nitrate solution was pumped through the sample line for 16 minutes and the response measured at regular intervals. Whereas the newly prepared OTCR shows an increase in response of about 4% during the initial five minutes of exposure to nitrate solution, the OTCR stored with working imidazole buffer for 12 hours shows very little increase. We found that storage filled with working imidazole buffer for at least 12 hours eliminated the need for prior conditioning with nitrate. After the 10 day storage with working imidazole buffer, the reduction efficiency of regenerated OTCRn also was greater than 97%.

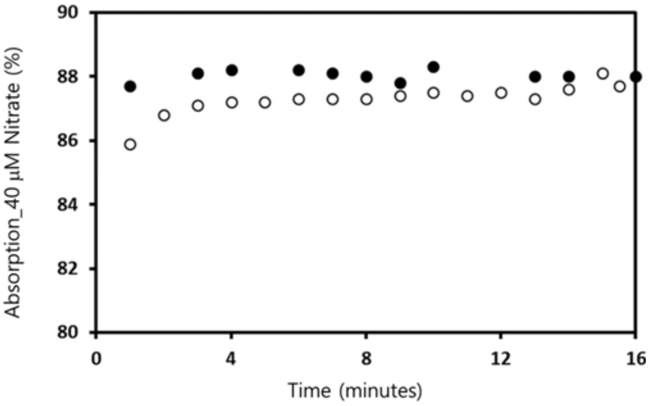

Fig. 2. Change of absorption (%) during the elution of 40 μM nitrate solution as an indication of Cd reductor reduction efficiency. Open circles indicate newly regenerated OTCR. Filled circles are OTCR stored in working imidazole for more than 12 hours after regeneration

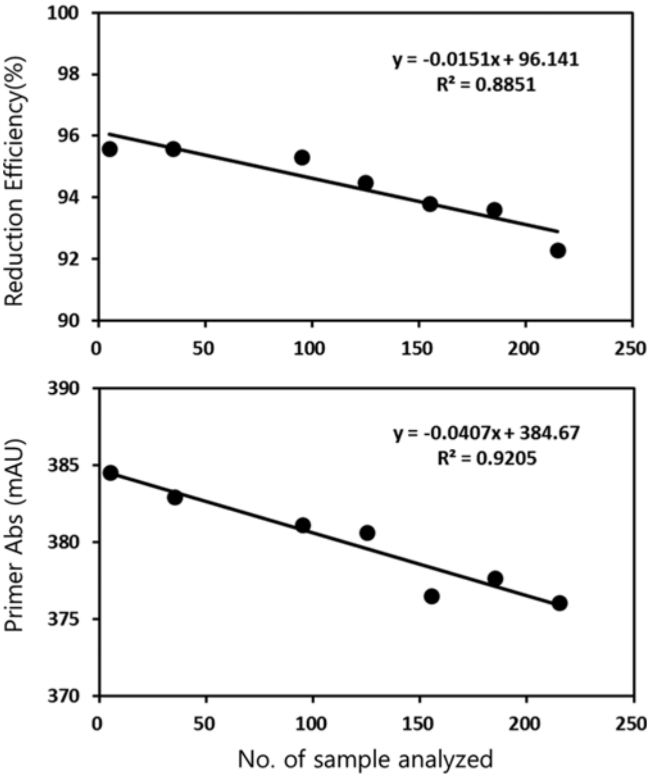

Fig. 3. Decreasing trend of Cd reductor reduction efficiency with continuous use after the regeneration of Cd reduction column

We observed a gradual decrease in reduction efficiency during the day. Figure 3 shows the primer absorbance and slope of a series of runs with a total of 215 samples from the Ulleung Basin of East Sea. Such an effect was not commonly reported by other researchers, some of whom maintain recovery > 95% for more than one week. As we were careful

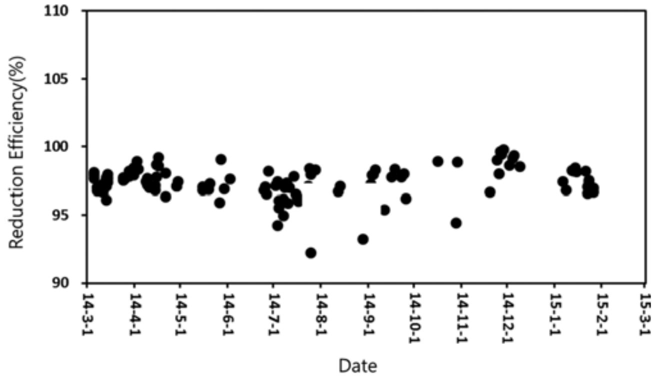

Fig. 4. Long-term trend of Cd reductor reduction efficiency after daily regeneration and 12 hour storage in working imidazole buffer

to exclude oxygen, to use freshly made surfactant solutions from material less than 1 year old, and to avoid excessive surfactant, all of which can cause a loss of reduction activity, we attribute the drop to samples from the Ulleung Basin of East Sea, which is known to have a high concentration of transparent exopolymer particles (TEP) in the euphotic layer (Jeon et al. 2012).

We regenerated the OTCR daily to maintain high reduction efficiency. The reduction efficiency was consistently restored to 97% after daily regeneration followed by storing with imidazole buffer for at least 12 hours. Figure 4 shows the daily reduction efficiency measured over a period of several months. We found that a Cd coil could be regenerated about 60 times before it became impossible to maintain the reduction efficiency or the increase in internal diameter because repeated acid washing resulted in irregular segmentation, causing misshapen peaks and errors in peak timing.

Flow rate and reagent optimization for improved stability and sensitivity

The MT3A manifold used the Q-047-04 method for nitrate analysis, which employed a single pump tube for a color reagent containing a mixture of sulfanilamide and N-1-Naphthylethylenediamine di-HCl (NEDD). This differs from the configurations described by Gordon et al. (1993) and Aminot et al. (2009), which pumped sulfanilamide and NEDD reagents separately through two pump tubes. After the change of MT3A to MT3B manifold for nitrate analysis, we used SEAL Analytical method no. Q-035, that separately delivers sulfanilamide (0.111 ml/min) and NEDD (0.111 ml/min) into the analytical system. This reduced the relative standard deviation (RSD) of primer absorbance signals and slope of calibration curve substantially, suggesting that the separate reagents are more stable (Fig. 1). We subsequently modified the flow rates of sample and imidazole buffer to increase the proportion of sample in the reaction mixture while maintaining a similar flow rate within the reduction coil. We refer to this manifold as MT3B KIOST. Table 3 shows the effect of these changes on reproducibility and sensitivity.

Stabilization of ammonium analysis

For the Fifth ICES Intercomparison Exercise for nutrient determination in seawater (NUTS I/C 5), ammonium was included in such a large-scale exercise for the first time. However, the RSD (%) for medium/high levels and low level of ammonium was about 22–23% and 56%, respectively. These were large errors compared with other nutrients (Aminot and Kirkwood 1995). Responses from a questionnaire on NUTS I/C 5 participants' procedures for the determination of ammonium were analyzed by Aminot et al. (1997), who made suggestions for improving performance. These include (1) the type of reagents, order of reagent addition, mixing and reaction conditions, (2) the use of freshly prepared ultra-pure deionized water, (3) the use of low-nutrient seawater (LNSW) for calibrants or a salt-effect correction within the salinity range of samples and calibrants, (4) consideration of refractive index effects in the measurement of the blank, and (5) identification of contamination sources and precautions for their avoidance. Several sources of contamination and preventative measures were addressed, but these did not include atmospheric contamination in the laboratory because this was not commonly reported. However, in a later report, Aminot et al. (2009) clearly showed the influence of air-borne ammonium on samples waiting for analysis in various

Table 3. Mean absorbance values and relative standard deviation of nitrate (25 μM) and calibration curve slope in different analytical manifold. RSD (%) = standard deviation / average × 100

Manifold type	Sample / buffer flow ratio	%sample through reductor	Primer Absorbance (mAU)		Slope of Calibration curve	
			mean	RSD (%)	mean	RSD (%)
MT3A	0.635 / 0.381	62.5	388.7	6.1	15.6	5.4
MT3B	0.323 / 0.635	33.7	254.0	1.1	10.2	1.0
MT3B_KIOST	0.484 / 0.484	50	390.2	0.6	15.6	0.7

types of sample receptacle and the level of sample within them. It is clear that air-borne contamination is significant and should be removed by appropriate measures. Although the use of acid-scrubbed air for the segmentation of sample stream within the ammonium manifold to avoid air-borne ammonium contamination was described by Mantoura and Woodward (1983), it is omitted in several recommended methods in the literature as well as in some instrument manufacturers' recommended procedures.

In this study we took several precautions to avoid atmospheric contamination. We (1) used acid-scrubbed air, passed through 10% HCl solution, for the air segmentation within the ammonium manifold as well as for the displaced air drawn into reagent bottles and the sampler wash water. Since HCl fumes quickly cause stainless steel to rust, we now recommend a weak acid such as acetic acid (0.1 M) in place of 10% HCl. We also (2) separated the sample from direct contact with the atmosphere with a simple cover of plastic film, as used for food wrapping, over the tubes in the sample tray. The tip of the sample probe is sharpened at 45° so that it easily pierces the film. Finally (3) we set the sampler wash pump to introduce wash water continuously into the sampler wash reservoir instead of interrupting the flow during sample intake. With these precautions, long-term monitoring of nutrient reference material by KIOST (Rho et al. 2015) showed consistent results over several months (Fig. 5). The RSD (%) was 7–11% at ammonium levels between 0.5 and 6.6 μM (Table 4), which is much lower than the values reported in Aminot et al. (1997). These optimizations allowed us to produce stable onboard ammonium measurements using our SFA system.

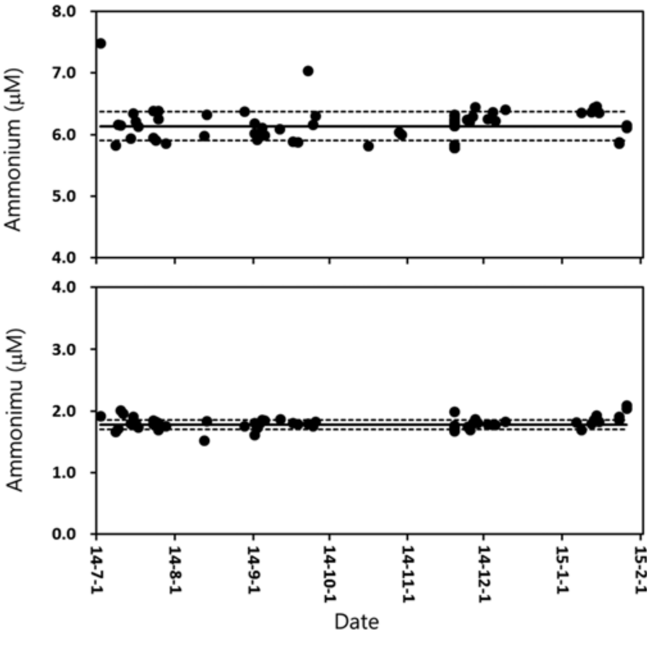

Fig. 5. Long-term trend of ammonium concentration in KIOST nutrient reference materials for 7 months (modified from Rho et al. 2015)

Reducing static and dynamic refractive index effects

Baseline stability

A stable and noise-free baseline is a pre-condition for accurate low-level segmented-flow analysis. The baseline noise is directly related to the detection limit. In routine analysis we establish a stable baseline with freshly drawn UPW, degassed if necessary by stirring for ten minutes under reduced pressure, with surfactant corresponding to the method being tested. Daily, we perform a visual baseline check by setting the chart window to 0.1 AUFS and expanding it to full screen. With this UPW baseline we assess the baseline noise in terms of the number of pixels variation seen in ten minutes. We regularly achieve a target value of ± 1 pixel, which corresponds to about ± 100 mAU. At least once per week we perform a quantitative test of instrument parameters that influence baseline noise - detector energy, flowcell transmission, bubble pattern stability, baseline noise and drift - using the diagnostic program provided by the instrument manufacturer. This UPW baseline is taken as the zero reference point for absorbance measurements.

Peak components and matrix effects

Figure 6 shows peak components of saline calibration standards and samples as measured from a UPW baseline, which are: (1) the absorbance signal due to a static refractive

Table 4. Mean values, standard deviation, and relative standard deviation of ammonium concentration in various nutrient reference materials. RSD (%) = standard deviation / average × 100

	KIOST-NAD	KIOST-NAE	KIOST-NAF	KIOST-NAG	KANSO-BF	MOOS-3
Mean (μM)	6.047	1.715	1.171	1.216	0.502	6.592
Std dev (μM)	0.589	0.120	0.105	0.094	0.054	0.515
RSD (%)	10	7	9	8	11	8
n	279	141	30	30	25	165

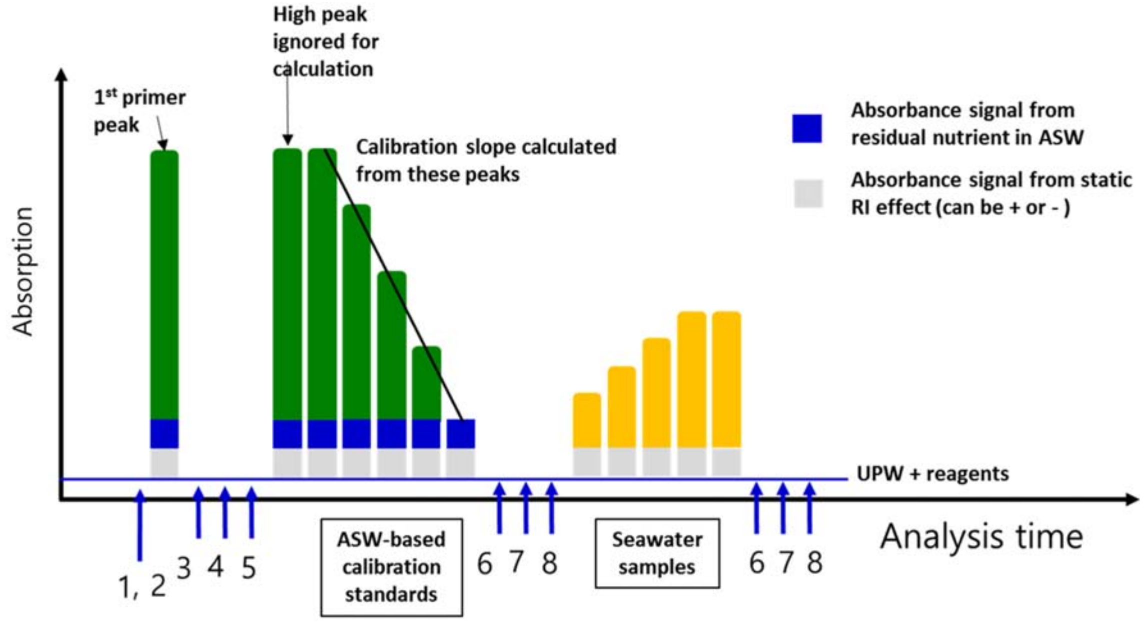

Fig. 6. Diagram showing the initial ultrapure water (UPW) baseline and peak heights determined from the UPW baseline (shown as absorption). Numbers indicate events during analysis. 1: Software finds an initial UPW baseline. 2: Primer peak, from which sensitivity is calculated relative to UPW. 3, 4, 5: Null peaks during which UPW-ASW wash water transition occurs. 6, 7, 8: Baseline drift correction: three samples from fresh UPW in sample cups in the sample tray; baseline measured on the third peak (8)

index (RI) effect, which is caused by the refractive index difference between UPW and seawater. This signal primarily depends on the flowcell and photometer design. It may be positive, negative or zero; (2) the absorbance due to residual nutrient in the ASW or LNSW used for the calibrants; (3) the absorbance from the added nutrient in calibrants and from samples. It is essential either to determine the nutrients concentration in the standards matrix or to eliminate it by calculation because it is very difficult to obtain a perfectly nutrient-free natural or synthetic solution. Failure to perform this correction can result in large errors, as shown in our measurements with different batches of NaCl used to prepare ASW when using a conventional linear calibration (Fig. 7, hollow data points).

A detailed procedure for identifying and compensating for potential errors from residual nutrient and refractive index effects in the LNSW used for calibrants is described by Hydes et al. (2010). We describe here our daily procedure which eliminates both static and dynamic matrix effects. The baseline reference level is reagents + UPW drawn from the sampler wash. After the initial baseline and first peak have been read, the sampler wash is changed to ASW that is similar or identical to the calibrants matrix. Baseline drift correction during the run, and the final baseline, are taken from freshly drawn UPW

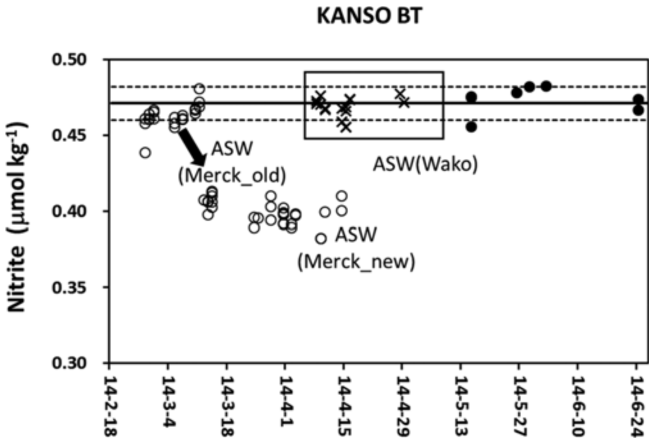

Fig. 7. Time series measurements of KANSO nutrient reference material (BT, 0.471 ± 0.011 μmol kg^{-1}) show the nitrite impurity in the NaCl used for artificial seawater (ASW). The solid line indicates the certified value and two broken lines indicate ± standard deviation. The sudden decrease coincides with a change of NaCl used for the ASW used as the standard matrix, from the same manufacture (Merck) with a different purchasing date (open circle, Merk_old vs. Merk_new). Simultaneous analysis with ASW prepared from another manufacture (denoted as x within the rectangle box) confirmed that the residual nitrite in new stock of NaCl may be responsible for the sudden change of nitrite concentration in nutrient reference material. Closed circle represent the nitrite concentration after the modification of baseline determination

contained in sample cups.

The baseline is not included in the calibration: it is only used for baseline drift correction. A slope-only calibration (Coverly et al. 2012) offsets the origin of the calibration curve to the UPW + reagent baseline and so eliminates any residual nutrient or refractive index contribution from the calibrant matrix. The zero calibrant consists of the ASW or LNSW used as the matrix for the other calibrants. The arrows in Figure 6 show the sequence of events. With a calibration of this kind it is necessary to correct for any static RI contribution from samples. We found that static RI contributions for nitrite, nitrate + nitrite, ammonium, phosphate, and silicate were 0.006 ± 0.005, 0.003 ± 0.004, –0.237 ± 0.096, –0.011 ± 0.004, and –0.163 ± 0.079 µM, respectively.

The operating procedure is:

1) Adjust the tubing length of the sample wash water so that the time taken from the wash water bottle to the sampler wash reservoir is greater than the time from the sample probe to the manifold by a time equivalent to between one and two sample and wash periods. The transit time is easily measured by introducing a bubble into the feed line. Prepare two sampler wash water bottles (UPW wash water and ASW wash water).

2) Prepare calibration standards and the primer (first peak in the run, normally the top standard) in ASW or LNSW. Include after the primer a series of null samples taken from the same solution. The wash solution transition takes place during these peaks. The sample tray used in the run in Figure 8 can be abbreviated as P, 4N, B, 5C, B, 5S, B, E, where P =

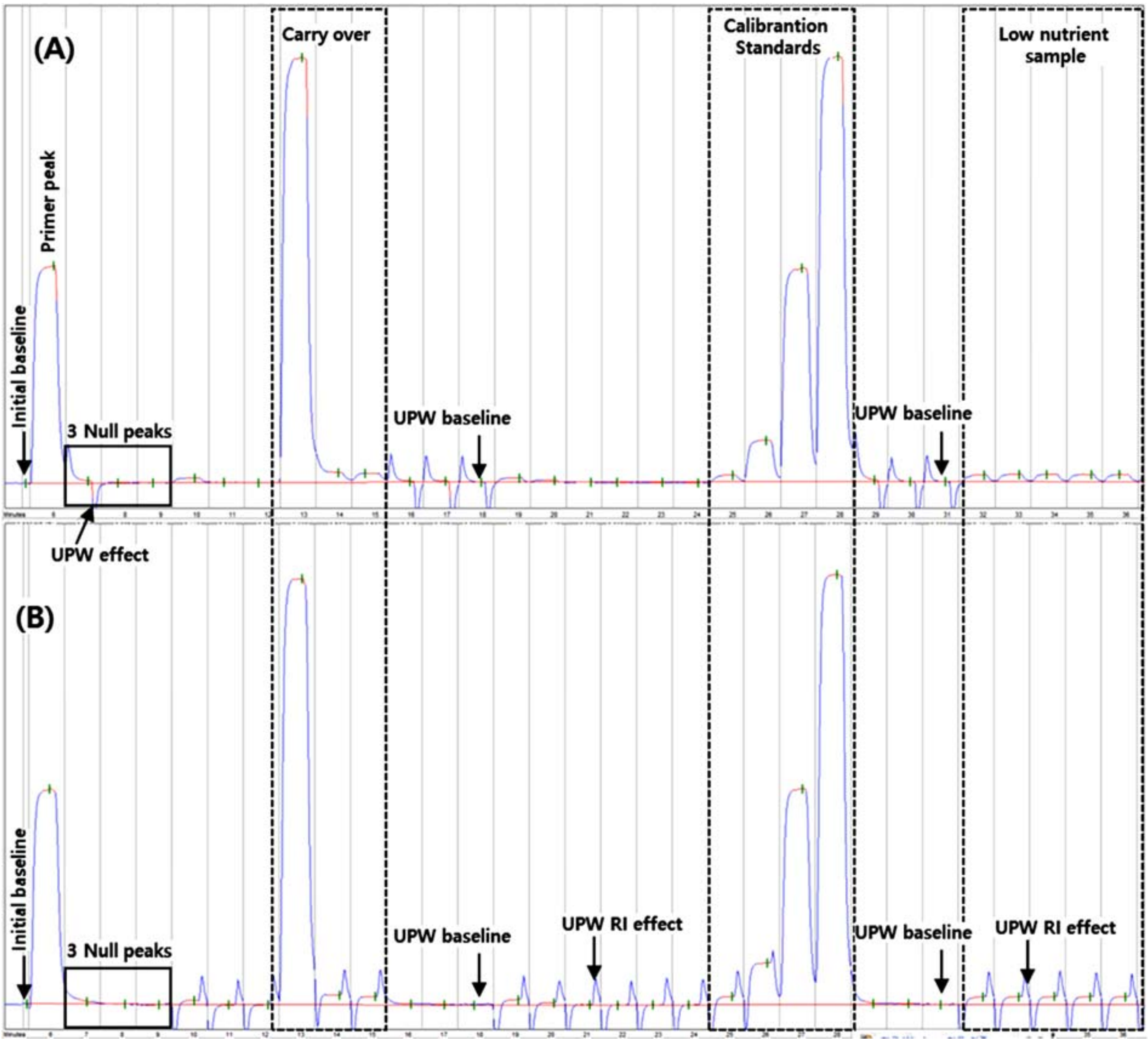

Fig. 8. Phosphate charts ASW wash (A) and UPW wash (B). Calibration standards were prepared with ASW in both analysis. Dynamic UPW RIB effects are evident at the beginning and end of low nutrient saline sample peaks but the results are affected

primer, C = calibration standard, S = samples, N = null peak ignored in calculations, B = baseline correction consisting of three UPW peaks, E = end. The effect of the wash change occurs during the N peaks.

3) Start the analysis with the UPW sampler wash connected. When the sample probe moves to the first cup in the run, switch the wash water from UPW to saline solution. In our laboratory the switch is performed with a 3-way valve. The initial baseline, read just before the first peak, is read with UPW. About 2 minutes later, during the Null peaks, the saline wash arrives in the system, and is present for the remainder of the run. Figure 8 shows the chart from a run where the wash changeover occurs at the end of the second peak.

4) Intermediate and final baseline correction points are read from sample tubes containing fresh UPW. This is usually sampled three times in succession, and the baseline determination taken on the third peak, to eliminate any carryover effect from a previous high concentration peak.

5) Results are calculated using a “slope only” calibration, which uses only the slope of a linear fit by setting the b factor of the equation y = ax + b to zero so that the origin becomes the UPW + reagent baseline. This calibration function is available in the operating software of our analyzer (AACE 6.06, SEAL Analytical): alternatively it can be performed post-run with Excel.

We checked the accuracy of this approach by using calibrants made up in nutrient-containing ASW and additional measurements of the nitrite concentration of reference material as shown in Figure 7. The software-corrected values are shown in the filled circles, and all lie within ± 1 SD of reference material (0.471 ± 0.011 μmol kg^{-1}). Figure 8 shows the chart from such a run with another run using a UPW wash for comparison.

We also performed similar runs using UPW as wash water throughout the run instead of changing to ASW. The RMS (%) of differences between UPW for baseline and wash water and UPW baseline/ASW wash water ranged from -1.1 to 0.4 (Table 5), which is comparable to the homogeneity value of nutrient reference material (Aoyama et al. 2012; Rho et al. 2015). According to the T-test at 5% significance the results of nitrite, silicate, and ammonium were statistically different while those of nitrate+nitrite and phosphate were the same (Table 5). If the difference is really significant, it may be related to a static RI effect in the UPW baseline and wash water, because no additional RIB correction procedure was conducted in this study.

The peak shapes of low nutrient levels of nitrite, nitrate +nitrite, and silicate showed no apparent dynamic RI effect at the beginning and end of peaks in both UPW baseline and wash water and the combined procedure of UPW baseline/ ASW wash water. In phosphate analysis, the dynamic RI effect was evident using UPW for baseline and wash water compared with the combined procedure (Fig. 8). For ammonium analysis, the dynamic RI effect was apparent at the beginning and end of sample peaks in both procedures (not shown).

4. Summary

The measures to optimize the cadmium reduction and the color formation steps for nitrate analysis resulted in an improvement in day-to-day precision from 6.1% RSD to 0.6% RSD in primer absorbance. The careful exclusion of environmental contamination by introducing acid-cleaned air bubbles for air segmentation in the ammonium manifold and ammonium analysis reagent bottles and by blocking direct contact between ambient air and sample with plastic wrap at the top of sample tubes in the sample tray during analysis resulted in a day-to-day standard deviation of 0.035 μM in the range of 0.5–6.6 μM ammonium concentration. The lowest detection limits, obtained from various optimization procedures, are 0.003 μM, 0.003 μM, 0.02 μM, 0.009 μM, and 0.02 μM for nitrite, nitrate+nitrite, ammonium, phosphate, and silicate, respectively.

Table 5. Measurements of KIOST reference material using UPW wash water (A) and a combined procedure of UPW baseline + ASW wash (B). Percent difference (PD, %) was calculated as (PD = (B – A) / B × 100). P-values were calculated from a T-test at 5% significance

	UPW (A)		UPW+ASW combined (B)		Statistics	
	Mean (n = 10)	Std (n = 10)	Mean (n = 10)	Std (n = 10)	PD (%)	p-value
Nitrite (μM)	2.910	0.0120	2.919	0.0070	0.31	0.047
Nitrate+Nitrite (μM)	14.07	0.0710	14.09	0.0520	0.11	0.581
Ammonium (μM)	4.567	0.0320	4.516	0.0320	-1.1	0.002
Phosphate (μM)	3.585	0.0110	3.588	0.0200	0.10	0.638
Silicate (μM)	103.8	0.2920	104.2	0.1160	0.40	0.001

The combined procedure of a UPW baseline and ASW wash water that adopts the advantages of UPW in providing an "absolute zero" baseline with ASW for the sampler wash, together with a calibration procedure to eliminate interference from the matrix of the calibrants, was effective in removing the influence of residual nutrient in the calibration standards and wash water as well as avoiding the adverse effects of dynamic RI changes on peak shape.

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