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Optimization of a colorimetric method to determine trace urea in seawater

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Abstract

This study optimized the experimental conditions for the analysis of dissolved urea in seawater. The kinetics of the colorimetric reaction of urea with diacetyl monoxime were studied under different conditions: reagent stability, pH, and reaction temperature and time, based on which robust procedures were developed for both normal (> 100 nmol L^{-1}, using a five centimeter cuvette) and trace (<100 nmol L^{-1}, using a one meter path length capillary cell) levels of dissolved urea. Our trace level method showed very high sensitivity (detection limit of 1.2 nmol L^{-1}) and high reproducibility (relative standard deviations were 0.29–1.09%), which was a significant improvement as compared to what was reported previously. The current method afforded a more stable colorimetric product, which provided better reproducibility when a large number of samples were analyzed. The normal-level method was applied to studying the urea distribution in estuarine surface water, and the trace-level method was applied to studying the vertical distribution of urea in the South China Sea basin (> 4000 m).

Introduction

As one of the most abundant small dissolved organic nitrogen molecules in the marine environment, urea is a major nitrogen source for planktonic communities, with similar or higher uptake rates than ammonium in the euphotic zone (Painter et al. 2008; Solomon et al. 2010). In the polar and deep ocean, the utilization of urea was also recognized as an essential chemoautotrophic pathway nurturing microorganisms (e.g., Archaea; Alonso-Sáez et al. 2012). Thus, urea is involved in diverse metabolic pathways of transport, production, and decomposition by different microorganisms in the ocean and plays an important role in the marine carbon and nitrogen cycles (Solomon et al. 2010). However, obtaining reliable measurements of urea in the ocean is not trivial due to the fact that dissolved urea is typically at trace levels, generally < 0.5 μmol L^{-1} in the surface ocean and even lower in the deep ocean. The dynamic nature of urea production and removal makes accurate measurements even more difficult in the ocean. As such, very limited data on urea concentration has been reported for the ocean, and what exist shows tremendous variations but no obvious geographic pattern (Painter et al. 2008; Alonso-Sáez et al. 2012). It is clear, therefore, that methods with better sensitivity and reproducibility for measuring urea in seawater are needed to better understanding this important component of the marine nitrogen cycle.

The determination of urea in different matrices has been extensively reviewed (Butler and Walsh 1982; Francis et al. 2002; Lambert et al. 2004; Dhawan et al. 2009). Currently, two main methods are used for the analysis of dissolved urea in seawater. The enzymatic method reported by McCarthy (McCarthy 1970) uses urease to indirectly quantify urea from the amount of ammonia obtained after its enzymatic hydrolysis. The direct method produces a red colored product when urea reacts with diacetyl monoxime (DAM) in an acid solution (Newell et al. 1967). Several manual (Koroleff 1983; Mulvenna and Savidge 1992; Goeyens et al. 1998; Revilla et al. 2005) and automatic (Demanche et al. 1973; Price and Harrison 1987; Cozzi 2004) techniques have been reported for the reaction of urea with DAM at room or higher temperatures.

Several studies used either the urease or DAM method for measuring urea concentrations. Two studies (Price and Harrison 1987; Revilla et al. 2005) compared these two methods comprehensively. Price and Harrison (1987) confirmed that the urease method was affected by the pH, seawater matrix, Ca^{2+} and Mg^{2+} inhibition, and incomplete hydrolysis,

Additional Supporting Information may be found in the online version of this article.

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Table 1. Comparison of the previous reported methods with the analytical methods in this research.

Reference	DAM (mg L^{-1})	TSC (mg L^{-1})	SCHC (mg L^{-1})	H_2SO_4 (v/v)	Fe^{3+} (mg L^{-1})	Cl^- (g L^{-1})	Other reagents	Temperature (°C)	Reaction time	Range (nmol L^{-1} urea)	ε (mol^{-1} L urea-N cm^{-1})	Detection limit (nmol L^{-1})
Newell et al. (1967)	710	—	8.57	11%	—	2.13	KNO_3, Mn^{2+},PO_4^{3-}	70	90 min	35–700	18,000	—
Koroleff F. (1983)	770	—	8.57	12.0%	—	1.85	KNO_3, Mn^{2+}, PO_4^{3-}	75	120 min	>10,000	11,000	91
Mulvenna and Savidge (1992)	1796	20.1	—	9.37%	0.85	1.60	—	85	20 min	0–15,00	16,000	70
Goeyens et al. (1998)	1800	20.1	—	9.37%	0.85	1.60	—	Room temp.	3 days	—	21,000	100
								85	20 min	0–5,000	19,000	140
Revilla et al. (2005)	1800	20.0	—	9.37%	0.85	1.60	—	Room temp.	3 days	—	19,000	20
This research-normal level	1500	10.0	—	20%	0.84	—	—	85	30 min	0–5,000	29,700	20
This research-trace level	1000	3.0	—	20%	0.84	—	—	85	30 min	0–100	24,000	1.2

Note: SCHC represents for semicarbazide hydrochloride and ε represents for molar absorption coefficient.

causing the underestimation of dissolved urea in seawater. Therefore, the obtained values and recoveries varied in different sampling locations and times of collection, and internal standards must be used for each sample analysis. The enzyme used in the urease method can catalyze the hydrolysis of at least eight other compounds, with ammonium as the end product. The loss of ammonium during heating also contributes to the underestimation of urea by the urease method. Moreover, enzymes purchased from different manufacturers may vary in activity. Another challenge of using the urease method is that the determination of ammonium, particularly the trace analysis of ammonium, is difficult. Therefore, the DAM method, which is more accurate and less salinity dependent than the urease method, is presently being more widely used in determining the urea concentration in a seawater matrix (Revilla et al. 2005).

Since the development of the DAM chemistry by Fearon (1939), efforts have been made to optimize the experimental parameters for biological samples (Coulombe and Favreau 1963; Lugosi et al. 1972; Rahmatullah and Boyde 1980; Butler and Walsh 1982). However, contradictory conclusions have been reported by different studies. For example, Butler et al. (1981) found that the addition of phosphate did not intensify the color of the product using pure reagents; however, another study concluded that the presence of phosphate ions enables reasonable reproducibility (Koroleff 1983). The operation parameters of the previously reported methods for the analysis of dissolved urea in seawater are listed in Table 1, indicating diverse conditions for spiking reagents, reaction time, and detection limit (DL). Previously reported methods require very careful operations and strict timing during the sample treatment which makes the quality control very challenging, particularly for trace-level analysis. Moreover, a method with higher sensitivity is needed to measure trace levels of urea in oceanic samples to achieve a consistent distribution pattern. In recent years, liquid waveguide capillary cells (LWCC) made of Teflon AF with a small diameter and long path length have been widely used to increase the sensitivity of spectrophotometric systems, for example, in the determination of nanomolar levels of nutrients in seawater (Ma et al. 2014). However, LWCC has not been applied to the trace analysis of dissolved urea.

The aims of this research are to: (1) study the kinetics of the urea–DAM reaction and optimize the parameters for urea analysis in a seawater matrix, including the concentrations of DAM, thiosemicarbazide (TSC), H_2SO_4, $Fe_2(SO_4)_3$, temperature, and reaction time; (2) develop a standard operating protocol for normal-level urea analysis (>100 nmol L^{-1}) and trace-level urea analysis (<100 nmol L^{-1}) using an LWCC; (3) evaluate the quality of developed methods including the effect of salinity, sample storage strategies, and comparison with previously reported methods; and (4) recommend an optimized protocol for the determination of dissolved urea in seawater.

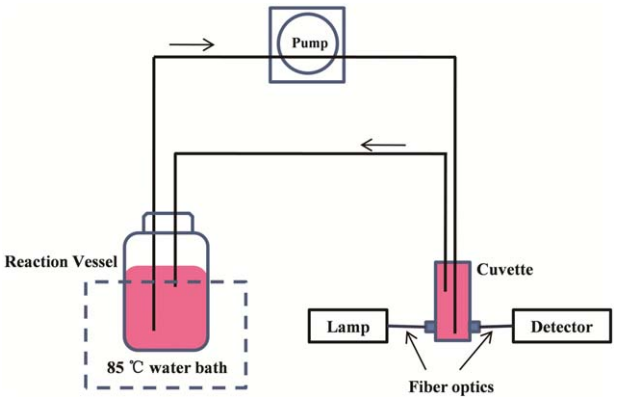

Fig. 1. Diagram of the continuous flow manifold configuration for studying the colorimetric reaction.

Materials and methods

Reagents and solutions

A urea stock standard solution (100 mmol L^{-1}) was prepared by dissolving a urea standard (Sigma-Aldrich) in purified water (Millipore) and was stored in a brown glass bottle at 4°C; this solution was stable for months. The working standard solutions were prepared daily by the stepwise dilution of the stock standard solution with purified water. The DAM (50.0 g L^{-1}) and TSC (2.0 g L^{-1}) solutions were prepared by dissolving solid DAM and TSC (Fluka) in purified water, and stored in amber glass bottles at 4°C; these solution were stable for one week. Concentrated H_2SO_4 (Guaranteed Reagent, Sinopharm Chemical Reagent, China) was used to prepare 50% (v/v) H_2SO_4. The $Fe_2(SO_4)_3$ solution (600 mg L^{-1}) was prepared by dissolving an appropriate amount of $Fe_2(SO_4)_3$ (Sinopharm Chemical Reagent, China) in a H_2SO_4 solution (5%, v/v) to prevent hydrolytic decomposition. The deep seawater DOC reference standard was obtained from Dr. Dennis Hansell's laboratory, University of Miami.

Apparatus

Figure 1 shows a schematic diagram of the continuous flow setup for studying the dynamics of the colorimetric reaction. The aqueous standard solution (2.0 μmol L^{-1}) was mixed with the reagents in a brown glass bottle to obtain a colored complex. A tungsten halogen lamp and a miniature multichannel USB 2000+ spectrophotometer (Ocean Optics) were connected to a two centimeter quartz flow cell via two fiber optic cables at the opposite sides of the cell (Fig. 1). A thermostatted water bath (Jintan Shunhua Instrument, China) was used for temperature control. A peristaltic pump (Baoding Longer Precision Pump, China) was used to circulate the liquid. Absorbance data from the detector were recorded every 0.3 s using SpectraSuite® software (Ocean Optics).

Procedure for sample analysis

Normal-level (> 100 nmol L^{-1}) seawater samples were analyzed following the optimized procedure. The water sample (18 mL) was added to a brown reaction bottle, and then 0.9 mL DAM, 0.15 mL TSC, 0.15 mL $Fe_2(SO_4)_3$, and 12 mL H_2SO_4 were added to the solution sequentially. The resulting mixture was heated on a water bath at 85°C for 30 min and cooled by immersing in tap water for 10 min. The obtained red complex was stable for at least 1 h (*vide infra*). The absorbance of the complex was measured using a five centimeter cuvette at 520 nm using a UV–visible spectrophotometer (UV-2450, Shimadzu, Japan).

Trace-level seawater (< 100 nmol L^{-1}) samples were analyzed using the same reaction procedure with a different recipe of reagents for the reaction. To the sample (18 mL) in a reaction bottle, 0.6 mL DAM, 0.045 mL TSC, 0.15 mL $Fe_2(SO_4)_3$, and 12 mL H_2SO_4 were added in sequence. The resulting reaction mixture was pumped into a one meter LWCC at a speed of < 1.0 mL min^{-1}, and the absorbance of the complex was measured using a USB 2000+ detector as described above.

Sampling

To test the applicability of this method, both estuarine and oceanic samples were collected for the analysis of dissolved urea. Surface water samples were collected from the Pearl River Estuary (PRE), China, along the salinity gradient in November 2013, and one high-resolution depth profile was collected in June 2014 at the South-East Asian Time-series Study (SEATS) station located at 116.04°E, 18.04°N in the northern South China Sea (SCS). The sample locations are shown in the Supporting Information (Fig. S1). The temperature and salinity were determined shipboard using an SBE 911 plus conductivity-temperature-depth (CTD) recorder (Sea-Bird) attached to a Rosette sampler. Seawater samples were collected using Niskin bottles attached to the Rosette sampler. The estuarine samples were gravity filtered using an online combusted GF/F filter (47 mm, Whatman™) connected to the Niskin bottles and immediately frozen at −20°C until the analysis within one month. The oceanic samples were stored using the same method without filtration because of a low particle level. All the glassware used in this study was precleaned by soaking in 1–2 mol L^{-1} HCl solution overnight, followed by thoroughly rinsing with purified water, and combustion for 5 h at 450°C.

Assessment

Optimization of reaction parameters

To optimize the experimental parameters, the reaction dynamics of the urea standard were investigated with different levels of reactant (normal and trace levels), stabilizing reagents, and reaction times and temperatures using the circular setup shown in Fig. 1.

The reaction of urea with DAM results in a substituted six-membered complex after a condensation reaction under acidic conditions in the presence of TSC and ferric ions to stabilize the red product (Beale 1961; Mulvenna and Savidge

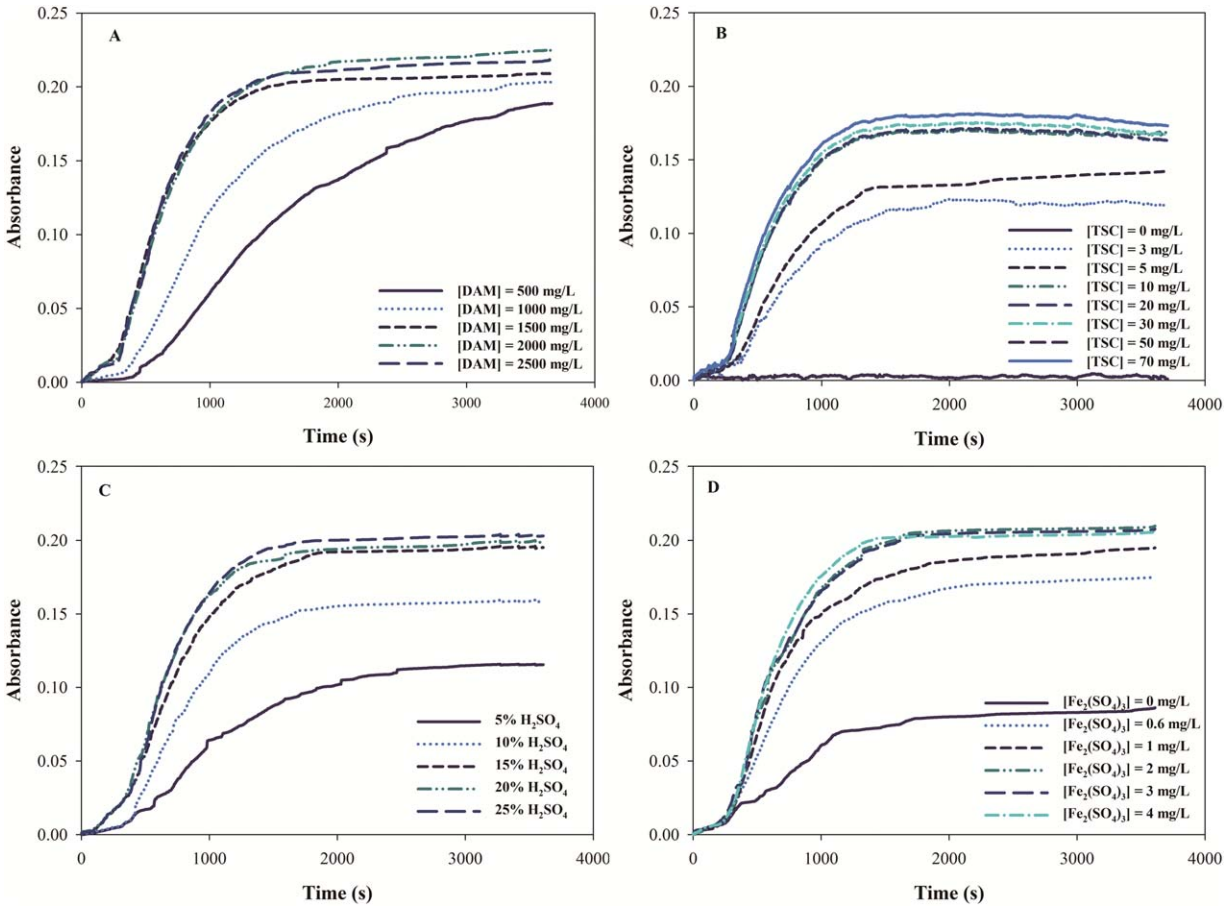

Fig. 2. Effects of the reagent concentrations of (A) DAM, (B) TSC, (C) H_2SO_4, and (D) $Fe_2(SO_4)_3$ on the kinetics of the colorimetric reaction.

1992). This reaction product has a maximum absorbance at 520 nm as shown in Fig. S2 of the Supporting Information. The kinetic curves of the reaction for different levels of reagents added to the reaction system are represented in Fig. 2 by the variance of the absorbance vs. time. The variation in the initial DAM concentrations (500–2500 mg L^{-1}) showed the effect of the equilibration time of the reaction, which reached equilibrium in 30 min when the DAM concentration was > 1500 mg L^{-1} (Fig. 2A).

The presence of TSC increased the production of the red product as shown in Fig. 2B, consistent with previous studies (Sullivan and Havlin 1991; Francis et al. 2002). Only a slightly red product was obtained when TSC was not used in the reaction. The kinetic curves indicate that the amount of the red product increased with increasing TSC concentrations until approximately 10 mg L^{-1}.

The effect of the final H_2SO_4 concentration in solution was investigated in the range 5–25% (v/v) for this reaction (Fig. 2C). The increase in acidity effectively increased the reaction rate and decreased the equilibration time required for color development, but no difference was observed when the acid concentration was > 20%.

Previously, the color enhancement was achieved by adding Fe^{3+} (Sullivan and Havlin 1991; Francis et al. 2002). The kinetics results indicate that the absorbance significantly increased with increasing concentration of $Fe_2(SO_4)_3$, but no difference was observed when the concentration was > 2 mg L^{-1} (Fig. 2D). The optimized chemical concentrations for urea analysis are listed in Table 1.

Optimization of reaction temperature and time

Using the optimized conditions described above, the effect of temperature on the reaction kinetics was evaluated in the range 55–95°C using a water bath as shown in Fig. 3. The formation of the red product was temperature dependent; an increase in temperature decreased the equilibration time and increased the yield of the red product. The reaction did not reach equilibrium at either 55°C or 65°C within 1 h, and similar amounts of the red product were obtained at 75°C and 85°C but with different equilibration times, that is, approximately 60 min and 25 min, respectively. The temperature and time were optimized when the reaction equilibrium was achieved within the least time as shown in Table 1. The reaction at 95°C produced more red product in a

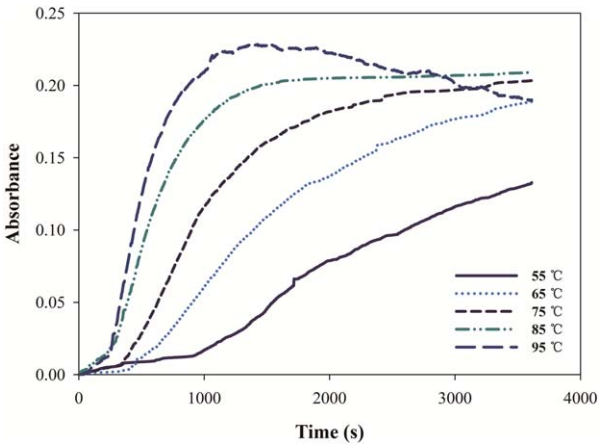

Fig. 3. Effect of temperature on the kinetics of the colorimetric reaction.

shorter time, but the reaction equilibrium could not be reached because of the decomposition of the red product. Poor reproducibility was observed when the reaction temperature was set at 95°C. Thus, the direct observation of these results may explain the conclusion reported in a previous study, that is, the nonlinearity in the recorded calibration curve when the reaction temperature was > 85°C (Mulvenna and Savidge 1992). Considering the analysis of a large number of samples on board or in land-based laboratories, the stabilization of the red product within a certain time before the analysis was a key factor in achieving better linearity and reproducibility. Therefore, 85°C and 30 min reaction time were selected for further studies.

Validation of the method

Linearity, reproducibility, and DL

Normal-level urea analysis (> 100 nmol L^{-1})

Standard solutions were prepared using the aged deep seawater (3000 m) collected from the SCS. The reagent blank prepared in aged SCS deep seawater processed with same steps described above had the lowest absorbance compared to the purified water and deep seawater reference (*see* Supporting Information Table S1). Using the optimized manual method described above, a calibration curve ranging from 0.0 μmol L^{-1} to 5.0 μmol L^{-1} urea was obtained (Fig. 4A). An excellent linear regression ($r^2 = 0.9999$) was achieved. The molar absorbance coefficient calculated from the slope was 297,000 mol^{-1} L urea-N cm^{-1}, higher than those obtained in the previous reports (Table 1). Relative standard deviations (RSDs) for the triplicate standards ranged between 0.16% and 1.38%. The DL, estimated as three times the standard deviations of the average of blanks ($n = 10$) divided by the calibration slope, was 0.02 μmol L^{-1}. The reproducibility of the method was evaluated by repetitive determinations of 2.0 μmol L^{-1} spiked seawater samples, and the RSD was 1.15% ($n = 9$).

Trace-level urea analysis (< 100 nmol L^{-1})

The red product obtained from the trace-level urea was analyzed using an LWCC to enhance the sensitivity under conditions similar to those shown in Table 1. Notably for the trace-level analysis, less DAM (1000 mg L^{-1}) and TSC (3 mg L^{-1}) were used for the reaction to obtain an acceptable molar absorbance coefficient (Table 1) and a lower reagent blank. As shown in Fig. 4B, good linearity was obtained below 100 nmol L^{-1} with an r^2 of 0.9973. The RSDs for the triplicate standards ranged from 0.29% to 1.09%. The DL of the trace-level analysis, calculated in the same manner as in the normal level method, was 1.2 nmol L^{-1}. To the best of our knowledge, this is the lowest DL ever reported for the analysis of dissolved urea in seawater.

Salinity effect

To investigate the effect of salinity, an assay was conducted to determine the absorbance of the colorimetric product in seawaters of different salinity values from 0 to 35. These samples were prepared by diluting aged seawater collected from the surface of the SCS with pure water. No significant difference ($p < 0.0002$) was observed in the samples diluted to different salinities. Therefore, this method can be applied to seawater samples from estuarine to oceanic salinities without any further calibration. More detailed data are shown in Supporting Information Figs. S3 and S4.

Recovery

Seawater samples collected from the PRE and SCS were spiked with the urea standard solution in both high and low concentrations and analyzed using this method. The recoveries (as shown in Table 2) for normal-level spiking (0.5 μmol L^{-1} and 2.0 μmol L^{-1}) were 98.5% and 99.4%, respectively. The recoveries for trace-level spiking (20 nmol L^{-1}, 40 nmol L^{-1}, and 70 nmol L^{-1}) were between 95.6% and 118.0%. Excellent recoveries were observed for both high and low-level spikings, indicating that this method has little matrix effect.

Comparison with previously reported methods

The methods used in this study were compared to the previously reported methods in terms of the variation in the slopes of the calibration and in the field measurements. As shown in Fig. 5A,B, the standard curves obtained using the Mulvenna and Savidge (1992) method (M-S 92) and methods developed here were compared. After the reactions were stopped, the slopes of calibration curves obtained from the M-S 92 method and the normal-level method were 0.1356 and 0.1485 absorbance/μmol L^{-1} respectively. However, the slope of the M-S 92 method standard series decreased by approximately 20% within 60 min (Fig. 5A), this may be due to the decomposition of the reaction product. This is one of the reasons that sample preparation and measurement timing need to be controlled very precisely as reported previously (Mulvenna and Savidge 1992; Revilla et al. 2005). And

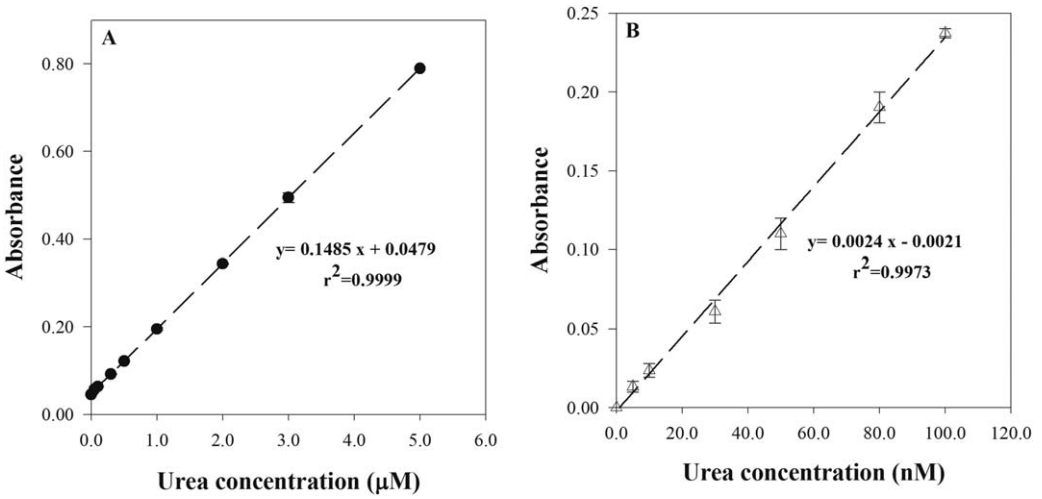

Fig. 4. Calibration curves of (A) normal-level urea ranging from 0 μmol L^{-1} to 5.0 μmol L^{-1} and (B) trace-level urea ranging from 0 nmol L^{-1} to 100 nmol L^{-1}. The standard deviations for triplicate standards are shown in the figure.

for the standard series obtained by the method of this research, there was no significant difference for the change of the slopes ($p < 0.0001$) within 1 h (Fig. 5B) because that the reaction product produced by the optimized reaction conditions was more stable.

The field estuarine samples were measured using both the current and M-S 92 methods. All samples were measured within 15 min after the red product was obtained. As shown in Fig. 5C, the urea concentrations of the estuarine samples showed no significant difference ($p < 0.0001$) between the two methods.

Storage methods for seawater samples

A suitable sample storage strategy is very important for seawater analysis, particularly when the samples cannot be measured on board immediately. Thus, the samples should be preserved in a way that successfully maintains the initial concentration until analysis. Different sample storage methods were compared for seawater samples with an initial concentration of 0.33 μmol L^{-1} urea. The samples were collected and filtered through precombusted GF/F filters, and triplicate samples were stored under three specified conditions, that is, at room temperature, refrigerated at 4°C, and frozen at −20°C. At room temperature, the urea concentration decreased steadily to approximately 90% of the initial concentration within 24 h (Fig. 6A). The presence of bacteria in the samples might cause the decomposition of urea to inorganic nitrogen. The urea samples remained stable for a day when stored at 4°C, and decreased by 12% after 30 d (Fig. 6B). The urea concentration did not change appreciably for two months when stored at −20°C (Fig. 6C). Based on these data, it is recommended that filtered seawater should be analyzed within one day if stored at 4°C and within two months if stored frozen, consistent with previous studies (Gardolinski et al. 2001; Fellman et al. 2008).

Detection of dissolved urea in estuarine and oceanic samples

Using the optimized conditions as described in the Procedure for sample analysis, the estuarine and oceanic samples were analyzed using the normal and trace-level methods, respectively. The distributions of urea in the surface water of the PRE along the salinity gradient collected in November 2013 are shown in Fig. 7. The urea concentrations ranged

Table 2. Recovery of spiking urea in different seawater samples. Standard deviations were listed in the table depending on triplicate samples measured by the trace-level method (SW1) and normal-level method (SW2 and SW3).

	Concentration (nmol L^{-1})			
Sample ID	Spiked amount	Original amount	Found amount	Recovery
SW1	20	57.7±0.7	76.8±0.8	95.6%
	40	57.7±0.7	104.9±3.4	118.0%
	70	57.7±0.7	126.6±2.3	98.5%
SW2	500	98.9±1.0	591.6±11.9	98.5%
SW3	2000	1212.14±3.3	3207.1±13.6	99.7%

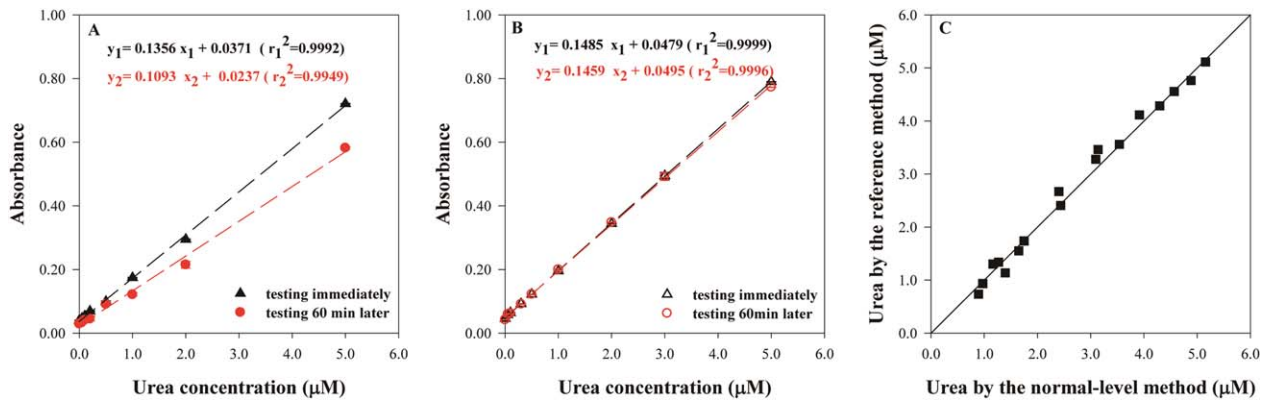

Fig. 5. Calibration curves of urea standard solutions ranging from 0 μmol L^{-1} to 5.0 μmol L^{-1} (A) by testing immediately (filled triangles) and 60 min later (filled circles) using the M-S 92 method (1992), (B) by testing immediately (open triangles) and 60 min later (open circles) using the normal-level method optimized here, and (C) comparison of the normal-level and M-S 92 methods using samples collected from the Jiulong River, Xiamen.

from 0.08 μmol L^{-1} to 4.12 μmol L^{-1} with overall higher levels in the upstream PRE, which was heavily affected by the discharge from municipal sewage plants (Dai et al. 2006; He et al. 2014), and low levels in the downstream PRE. Similar levels were detected in coastal waters (Gulf of Trieste, Italy) where anthropogenic loads were significant (median = 0.55 μmol L^{-1}, maximum = 9.9 μmol L^{-1}) using the automated colorimetric method based on the same colorimetric reaction (Cozzi et al. 2014). Inputs from the East River, a major branch of the Pearl River flowing through an extensively urbanized area, were observed around stations P05, P06, and P07 (*see* Supporting Information, Table S2). Urea showed a nonconservative pattern in the middle and lower PRE where the fresh water mixed with seawater, and removal of urea was observed in samples with salinity lower than 15 (Fig. 7).

A high-resolution vertical profile at the SEATS station in the SCS is shown in Fig. 8. The urea concentration varied significantly in the upper 300 m, with a maximum of 125 nmol L^{-1} at 70 m, corresponding to the bottom of the seasonal thermocline and the chlorophyll maximum depth (Fig. 8). The urea distribution below the euphotic zone was relatively uniform ranging from 62 nmol L^{-1} to 81 nmol L^{-1}. Further investigation is needed to fully interpret the distribution profile.

Comments and recommendations

In previous studies, the analysis of dissolved urea in seawater by the direct colorimetric method required very strict adherence to the methods and the recipe of the reagents varied in different studies. Our careful examination of the kinetics of the urea–DAM reaction under different conditions resulted in a stable red complex within an appropriate time. For the manual method, it is important that the absorbance be recorded when the reaction reaches the state of equilibrium. Therefore, after the optimization of the operating procedure and sample storage method, the dissolved urea in seawater can be determined conveniently and reproducibly.

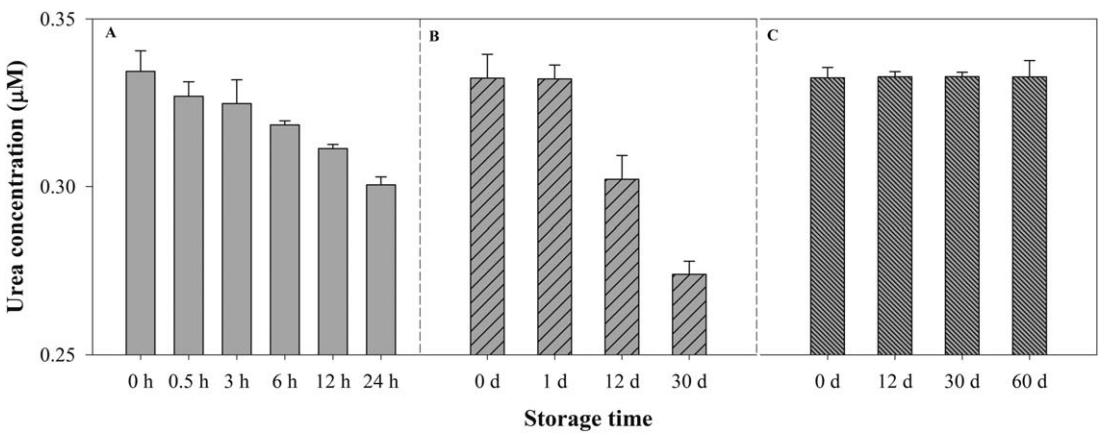

Fig. 6. Measured urea concentrations in the control samples stored at (A) room temperature for a day, (B) 4 °C for a month, and (C) −20 °C for 2 months. Standard deviations of triplicate samples were shown as error bars.

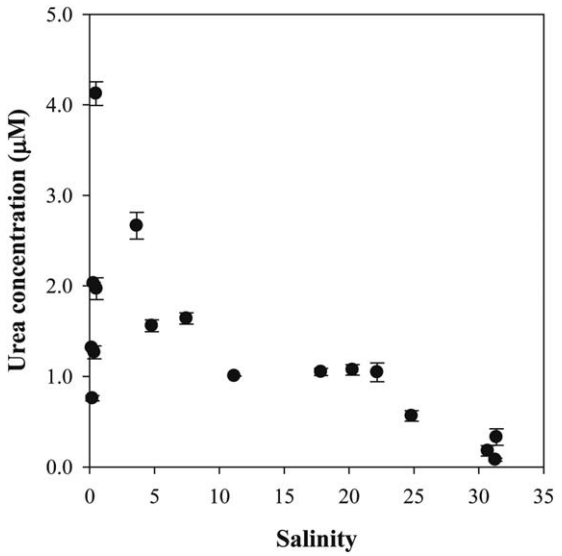

Fig. 7. Distribution of the urea concentrations vs. salinity in the surface waters collected from the Pearl River Estuary in November, 2013. Standard deviations of analyzed triplicate samples are shown as error bars.

Furthermore, the reaction kinetics results obtained in this study may provide a starting point for the experimental design of the flow analysis of dissolved urea in seawater. The concentrations of dissolved urea in oceanic samples were only a few times higher than the DL of the previously reported methods, which is challenging for the experimental operator, and large errors may be unavoidable. Therefore, the use of the LWCC method is recommended for determining dissolved urea at trace levels in future studies.

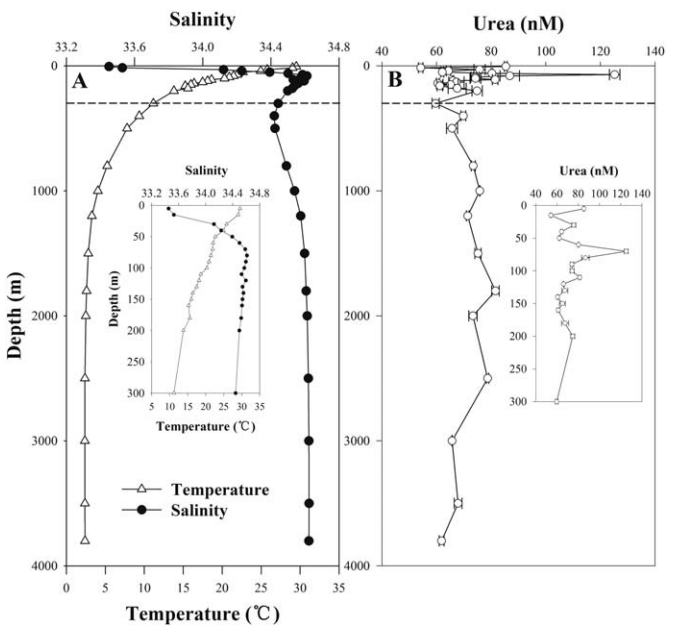

Fig. 8. Vertical profiles of (A) temperature (open triangles) and salinity (filled circles), (B) trace-level urea concentration at SEATS (116.0365°E, 18.04185°N) in the SCS in June 2014 (inset shows the enlargement of < 300 m depth). Standard deviations of duplicate samples were showed as error bars.

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