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ORIGINAL ARTICLE

Optimization of dissolved urea measurements in coastal waters with the combination of a single reagent and high temperature

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Abstract Urea is an unstable and intermediate organic nitrogenous compound present in coastal environments and is derived from the excretion of some aquatic organisms and wastewater discharges. Urea plays an important role in the nitrogen cycle, where it is utilized by algae, including diatoms. However, there are very limited relevant data on the production, consumption, and degradation of urea because of the lack of appropriate measurement techniques. The conventional method is based on the formation of a colored product when urea reacts with diacetyl monoxime in a sulfuric acid solution. We examined the optimal conditions for the formation of the colored product; specifically, we evaluated different temperatures (22–80 °C), reaction times, mixing ratios of color reagents, and sample storage times. Application of the single mixed color-developing reagent (COLDER) at 70 °C resulted in the optimal formation of the colored product within a short reaction time of 60 min. This method was then used to measure dissolved urea in different coastal environments. The concentrations detected were as follows: 0.65–0.72, 0.49–0.58, and 1.09–2.28 μM urea-N at coral reef, seagrass, and mangrove sites, respectively. Our results showed high precision (SD = 0.02, CV = 1.2%), a low detection limit (0.03 μM urea-N), and a high recovery rate (94–99%). In summary, this high-temperature procedure for urea measurements should be valuable for obtaining high-precision data that can further the understanding of urea dynamics and its role in coastal ecosystems.

Keywords Dissolved urea · Direct method · High temperature · Manual procedure · High precision · Coastal ecosystems

1 Introduction

Urea is an organic nitrogenous compound that plays an important role in the nitrogen biogeochemical cycle of marine environments (Antia et al. 1991; Bronk 2002; Cozzi et al. 2014; Solomon et al. 2010). Urea is a product that is excreted by some terrestrial and aquatic animals, and marine organisms known to produce urea include micro- and macro-zooplankton, molluscs, copepods, teleost fishes, spot prawns, and blue sharks (Antia et al. 1991; Cozzi et al. 2014; L'Helguen et al. 2005; Solomon et al. 2010); it is also regenerated by microheterotrophs like protozoans and bacteria (L'Helguen et al. 2005). Urea is an important source of nitrogen for phytoplankton communities. However, macrozooplankton provide only a minor fraction of the total amount of urea N that is assimilated by phytoplankton (Båmstedt 1985; Conover and Gustavson 1999; L'Helguen et al. 2005; Miller and Glibert 1998). Several studies have shown that regeneration by microheterotrophs is responsible for a substantial fraction of the urea that is used to meet the requirements of phytoplankton communities (Bronk et al. 1998; Cho et al. 1996; L'Helguen et al. 2005; Price et al. 1985; Slawyk et al. 1990). Heterotrophic bacteria produce urea as a result of cellular metabolic processes that take place during the regeneration of particulate/

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dissolved organic nitrogen (Antia et al. 1991; Cozzi et al. 2014; Solomon et al. 2010). Considering the N assimilation forms, inorganic N-ammonium is typically preferred (NH_4^+ > urea > NO_3^-) by phytoplankton communities (Cozzi et al. 2014), but preferences for urea have been demonstrated in *Phaeodactylum tricornutum* (Antia et al. 1991). Moreover, urea can make up 20–50% of the total nitrogen demand for phytoplankton species (Cozzi et al. 2014; Goeyens et al. 1998; Solomon et al. 2010). In the benthic environment, urea can be formed by bacteria and macrofauna and released from the sediments to the water column (Antia et al. 1991; Cozzi et al. 2014; Solomon et al. 2010). The concentrations of dissolved urea in oceanic environments are generally less than 0.3 μM, whereas higher values (0–13 μM) are often observed in coastal and estuarine environments (Table 1; Cozzi et al. 2014; McCarthy 1980).

Coastal monitoring programs have revealed that external nutrient inputs through sources such as urban and agricultural sewage system discharges are enriching the organic dissolved nitrogenous pool in coastal and estuarine systems (Cozzi et al. 2014; Glibert et al. 2005; Sultana et al. 2016). Furthermore, in the past several decades, worldwide use of urea as a fertilizer has increased by more than 100-fold and now amounts to >50% of global nitrogenous fertilizer usage (Glibert et al. 2006); consequently, runoff from agricultural fields treated with urea is also contributing to enrichments in the organic dissolved nitrogenous pool in coastal areas. Inputs of urea to coastal systems have significantly increased over recent years according to several studies (Bronk 2002; Revilla et al. 2005; Smil 2001; Solomon et al. 2010). This current global trend of increasing urea concentrations in coastal environments might be producing harmful effects on marine organisms and human health (Glibert et al. 2006; Revilla et al. 2005). For example, high rates of urea uptake by some phytoplankton are directly related to brown and red tides, paralytic shellfish poisoning events, and cyanobacterial blooms (Fan et al. 2003; Glibert et al. 2004; Kana et al. 2004; Price and Harrisson 1987; Revilla et al. 2005). Additionally, changes in the structure of plankton communities, whereby the communities become mostly dominated by dinoflagellates and cyanobacteria, have been reported in several coastal zones (Cozzi et al. 2014; Glibert et al. 2006); this can be a key trigger for harmful algal blooms (HABs). Urea also plays an important role in food webs by affecting low trophic level communities in coastal ecosystems. Therefore, accurate measurements of urea concentrations in coastal waters are important for understanding the role of urea in the nitrogen cycle and its impact on ecosystems.

Table 1 Range of urea concentrations (μM urea-N) in surface water from coastal and estuarine areas reported in the literature

References	Location	Urea concentration (μM urea-N)
Remsen (1971)	Ogeechee River, Georgia	1.26–4.89
Kristiansen (1983)	Oslofjord, Norway	0.1–10.0
Price and Harrisson (1987)	Strait of Georgia, Canada	0.19–0.42
Lomas et al. (2002)	Chesapeake Bay, Maryland	<0.01–8.16
Glibert et al. (2004)	Florida Bay, Florida	0.36–1.7
Revilla et al. (2005)	Choptank River, Maryland	0.99–6.52
Glibert et al. (2006)	Coastal Bays, Maryland	<0.01–14.4
Cozzi et al. (2014)	Gulf of Trieste, Italy	0.67–2.30

At present, two main colorimetric techniques, namely, the indirect urease method or the direct diacetyl monoxime method, are used to measure dissolved urea concentrations in coastal waters. The enzymatic method of McCarthy (1970) is based on the indirect measurement of urea from the amount of ammonia formed by enzymatic hydrolysis. The enzymatic method is multifaceted and can yield underestimations of urea concentrations as a result of urease resistance (Price and Harrison 1987). In the direct method, a colored product forms when urea reacts with diacetyl monoxime in an acid solution. Newell et al. (1967) used this technique for the first time. Since then, researchers have tried to modify this technique to develop different automated (Chen et al. 2015; Cozzi 2004; DeManche et al. 1973; Price and Harrison 1987) and manual (Goeyens et al. 1998; Koroleff 1983; Mulvenna and Savidge 1992; Revilla et al. 2005) procedures for accurate urea measurements. The addition of two reagents successively is a commonly applied procedure for developing the color complex in the direct manual method. Nevertheless, for urea analysis in biological materials, a mixed regent can be used (Knipp and Vasák 2000; Rahmatullah and Boyde 1980). Additionally, a mixed reagent was used successfully to determine urea in rainwater and atmospheric aerosol samples (Cornell et al. 1998). Revilla et al. (2005) used a single mixed reagent instead of two separate reagents for analyzing dissolved urea by using a manual procedure at RT and made improvements to the precision of measurements (±0.02%). In their method, 3 days were required to complete the reaction. Therefore, this method is inconvenient for measurements of large numbers of samples and for studies conducted at field laboratories and onboard research vessels. The main purpose of this study is to develop a rapid and high-precision method for measuring dissolved urea in coastal waters.

Generally, two different temperature procedures are used for dissolved urea measurements; these include

a (1) room-temperature (RT) procedure and a (2) high-temperature (HT) procedure. In both procedures, various separately mixed chemical reagents (Goeyens et al. 1998) or a single mixed chemical reagent (Revilla et al. 2005) can be used. Additionally, both of these procedures can be performed through manual procedures (Goeyens et al. 1998; Mulvenna and Savidge 1992; Newell et al. 1967) or automatic procedures (Chen et al. 2015; Cozzi 2004; Price and Harrison 1987). In the case of the RT procedure, complete formation and stability of the red color complex requires at least 72 h, whereas in the case of the HT procedure, the complete color complex formation time and color stability depends on the chemical composition and temperature. At <80 °C, the time required for the reaction (i.e., complete color complex formation and color stability) is about 90–120 min, and at >80 °C, the time required is about 20–30 min (Chen et al. 2015; Cozzi 2004; Koroleff 1983; Mulvenna and Savidge 1992; Newell et al. 1967).

In this study, we assessed (1) the optimal temperature, color formation times, efficiency of using color-developing reagent (COLDER) after its preparation, illumination conditions, recovery rates, and lowest detection limit for both urea standards and natural samples by using different temperatures ranging from RT to 80 °C and (2) the precision of our optimized method in comparison to previously reported methods. The direct method with a single mixed reagent was used for both the RT and HT procedures, and both frozen and fresh samples were examined. Finally, recommendations for detecting urea levels in coastal waters are given.

2 Materials and methods

The procedure was based on the manual technique at RT given by Goeyens et al. (1998). This method was further modified by Revilla et al. (2005), who used a single mixed COLDER instead of separate reagents. In this work, we have introduced important modifications to these previous methods by optimizing the temperature; this allowed us to achieve a higher sensitivity and lower detection limit. Standards and reagents were prepared by using ultrapure water (Milli-Q) produced by a Millipore purification system. In addition, we ensured that no sources of contamination (e.g., from cleansing detergents) were present during the analysis.

2.1 Reagents and solutions

Diacetyl monoxime solution was prepared by dissolving 3.4 g of diacetyl monoxime (CH_3–CO–CNOH–CH_3; Wako Chemical Co., Japan) in 100 mL of Milli-Q water. Thiosemicarbazide solution was prepared by dissolving 0.19 g of thiosemicarbazide (NH_2–CS–NH–NH_2; Wako Chemical Co., Japan) in 20 mL of Milli-Q water. Reagent A was prepared by mixing 25 parts of the diacetyl monoxime solution with 1 part of the thiosemicarbazide solution. Reagent B was prepared by diluting 300 mL of concentrated sulfuric acid (H_2SO_4; Wako Chemical Co., Japan) to 535 mL with Milli-Q water together with 0.5 mL of an iron(III) chloride ($FeCl_3 \cdot 6H_2O$; Wako Chemial Co., Japan) solution (0.15 g of ferric chloride dissolved in 10 mL of Milli-Q water). The COLDER was prepared by mixing 1 part of reagent A with 3.2 parts of reagent B. The turbidity blank solution consisted of 1 part of Milli-Q water and 3.2 parts of reagent B. The diacetyl monoxime, thiosemicarbazide, and reagent B solutions were stable for at least 1 month when stored at 4 °C in the dark. Reagent A was prepared fresh prior to each analysis; the COLDER and blank solutions were used within 15 min. To prepare the standard stock solution (1200 mg urea-N), 1.2 g of urea (Wako Chemical Co., Japan) was dissolved in 1 L of Milli-Q water. This solution was stored at 4 °C in a high-density polyethylene (HDPE) bottle and used throughout the study; it can remain stable for more than 1 year. A secondary standard (200 μM urea-N) and working solutions (0, 0.5, 1, 2, 4, 8, and 10 μM urea-N) were prepared fresh in 100-mL volumetric glass flasks.

2.2 Apparatus

Water temperature was controlled by a temperature-controlled water bath (Sanyo SW-10, Japan) within ±0.1 °C. The absorbance of colored samples was measured at 520 nm by using a spectrophotometer with an optical path length of 1 cm (Shimadzu UV-mini 1240).

2.3 Procedure for sample analysis

For the analysis, five polypropylene tubes (15 mL, Fisher brand) were filled with 4 mL of sample. Among the five tubes, the turbidity blank solution was added to two of the tubes that served as blanks and the single mixed COLDER was added to the remaining three tubes at a volume of 1.2 mL. The five tubes were immediately capped and vortexed. In the case of the HT procedure, sample tubes were kept at 70 °C in the pre-heated temperature-controlled water bath under dark conditions for 1 h. After 1 h, samples were cooled by placing the tubes in cold tap water for 5 min, and then, the absorbance was measured immediately at 520 nm by using a spectrophotometer (optical path length of 1 cm). In the case of the RT procedure, tubes

were handled in a similar way but were kept in the dark for 72 h at RT to allow for the complete development of the color complex.

2.4 Statistical analysis

The molar absorption coefficient was calculated for both the calibration curves and standard solutions. For the urea standard solution, the absorption coefficient was defined as the absorbance divided by the optical path length and the concentration, which was calculated from the average slope of 10 calibration curves. For the 10 μM urea-N calibration curve, the molar absorption coefficient was calculated as follows:

$$\varepsilon = s/l, \tag{1}$$

where ε is the molar absorption coefficient, s is the slope of the calibration curve, and l is the optical path length of the spectrophotometer.

The concentration was calculated from the average slope of the 10 μM urea-N calibration curves obtained from 9 replicate measurements. The detection limit was calculated as the analyte concentration that gave a signal equal to the blank signal plus three standard deviations (SDs) of the blank [limit of detection (LOD) $= y_B + 3s_B$] according to Miller and Miller (2000). Precision was assessed by the SD as well as the coefficient of variation (CV) of a 2 μM urea-N standard; nine replicate measurements were used for this analysis. The slope ratio was calculated to determine the effect of temperature and illumination on the slope of the calibration curves according to Revilla et al. (2005):

$$\text{Slope ratio } (\%) = (m_1/m_0) \times 100, \tag{2}$$

where m_1 is the slope of the urea standard curve at HT or dark incubation conditions and m_0 is the slope of the urea standard curve at RT or light incubation conditions.

2.5 Natural samples

Sampling sites were located along the coast of Sesoko Island in Bise, Okinawa, Japan, and in the Fukido River estuary in the northern part of Ishigaki Island, Japan. All three sites belong to the subtropical climate zone. The Sesoko area is a coral reef lagoon, and the lagoon bed is covered by 50% coral rubble, 10% living corals, 30% sand, and 10% algae including micro-algae and turf algae (Nakano and Nakai 2008). The Bise area is a seagrass bed that is dominated by *Thalassia hemprichii*. The Fukido River estuary consists of tidal creeks and mangroves; the mangrove species cover an 18.7 ha^2 area along the Fukido River. The Fukido mangrove forest is mainly dominated by *Rhysophora stylosa* and *Bruguiera gymnorrhiza* (Yamaki et al. 2002). Samples were collected in acid-cleaned 100-mL HDPE bottles. As soon as possible (approximately 30–60 min), samples were transported to the laboratory under cool and dark conditions and were filtered immediately through pre-combusted (500 °C, 4 h) GF/F filters. The fresh, filtered water (20 mL) was used immediately for the urea analyses.

2.6 Accuracy assessment

Chemical interferences were assessed via recovery analysis of internal standards. Internal standards were run at 10, 20, and 50%; i.e., the 10% spiked samples contained 90% natural sample and 10% of each urea-N standard solution. In the same way, the 20 and 50% standards were added to the natural samples. The percentage of recovery for the internal standards was calculated as follows:

$$\text{For 10\% spiked, recovery } (\%) = \left[(y_2 - 0.9 \times y_1)/(0.1 \times y_0)\right] \times 100 \tag{3}$$

$$\text{For 20\% spiked, recovery } (\%) = \left[(y_2 - 0.8 \times y_1)/(0.2 \times y_0)\right] \times 100 \tag{4}$$

$$\text{For 50\% spiked, recovery } (\%) = \left[(y_2 - 0.5 \times y_1)/(0.5 \times y_0)\right] \times 100, \tag{5}$$

where y_2 represents the urea-N concentration (μM) of the spiked sample; y_1 represents the urea-N concentration (μM) of the natural sample; and y_0 represents the urea-N standard solution.

2.7 Procedural assessments

To optimize the conditions for the analytical procedure, we performed the following work: (1) assessed the effect of different temperatures on the development of the color complex, (2) determined the heating time durations needed for color complex development at RT and HT conditions, (3) compared calibration curves for urea measured with the RT and HT procedures, (4) investigated the efficiency of using a single mixed COLDER at HT, (5) assessed the effect of illumination on color complex formation, (6) assessed the stability of the colored complex at HT, (7) quantified the recovery of urea standards in natural samples, (8) calculated the precision of the whole procedure in regard to urea standard solutions and natural samples, (9) tested both fresh collected and frozen samples to determine appropriate storage methods, and (10) measured urea concentrations in fresh collected natural samples with both in the RT and HT procedures.

3 Results and discussion

3.1 Effect of temperature

Temperature is an important factor that affects the development of the color complex. In this study, the RT procedure required more time compared to the HT procedure for the complete development of the color complex. Comparative data were collected at different temperatures for standard solutions of 2, 4, and 10 μM urea-N in experiment 1 (Fig. 1). At ambient temperature (22 ± 2 °C), 72 h was required for complete color development, while at 50, 60, 70, and 80 °C, the color development times were only 1.5, 1.25, 1, and 0.5 h, respectively. The maximal absorbances were obtained at 70 and 80 °C, and the values for these temperatures were very similar. Though the 80 °C treatment required only 0.5 h for the complete development of the color complex, the 70 °C treatment yielded a better standard curve for the 10 μM urea-N with a high molar absorption coefficient (20,200 M^{-1} urea cm^{-1}) and low detection limit (0.03 μM urea-N; Table 2) compared to the 80 °C treatment.

In this study, 70 °C was selected as the optimum temperature for the HT procedure because of the associated low limit of detection and the high molar absorption coefficient with the 10 μM urea-N standard curve compared to the other temperatures. In addition, heating at temperatures >70 °C can accelerate the destruction of the red color (Newell et al. 1967).

The time duration needed for heating was determined to be 60 min in experiment 2 (Fig. 2). Heating at 70 °C for 60 min resulted the maximal absorbance. Over time, the absorbance first increased and peaked at 60 min, and then, it gradually decreased. Greater absorbances were obtained with higher concentrations (4–10 μM urea-N). In contrast, at intermediate concentrations (1–2 μM urea-N), the absorbance peaked at 60 min and then remained stable. In the case of the lowest concentrations (0–0.5 μM urea-N), where 0 μM represents the reagent blank, the absorbance increased over the full experiment, which was similar to the results achieved for the RT procedures of Revilla et al. (2005). We determined that 60 min was the optimum heating duration at 70 °C for the comprehensive development of color. Heating for longer periods of time could cause the absorbance of the reagent blank to increase. Simultaneously, the data suggest that the colored complex is not stable beyond 60 min in samples with high urea concentrations. In addition, these results also compared well with the prevailing urea analysis method introduced by Newell et al. (1967), in which longer times (90 min) are required at 70 °C for the complete reaction and production of the coloring

Table 2 Comparison of 10 μM urea-N calibration curves at different temperatures

Temperature (°C)	Molar absorption coefficient (M^{-1} urea cm^{-1})	Detection limit (μM urea-N)	SD	*n*
22	18,100	0.04	0.04	9
50	18,900	0.09	0.03	9
60	19,000	0.09	0.02	9
70	20,200	0.03	0.02	9
80	19,700	0.07	0.02	9

SD standard deviation, *n* number of replicates

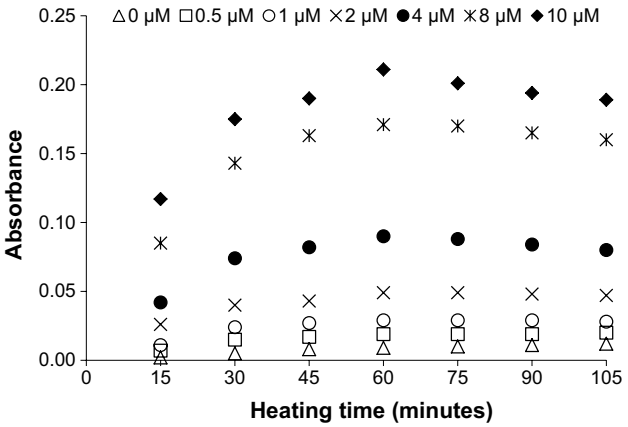

Fig. 2 Time-course for the development of color in standard solutions consisting of 0, 0.5, 1, 2, 4, 8, and 10 μM urea-N. Absorbance values are not corrected for blanks. *Error bars* (±SD, $n = 9$) are not displayed because they are smaller than the *symbols*

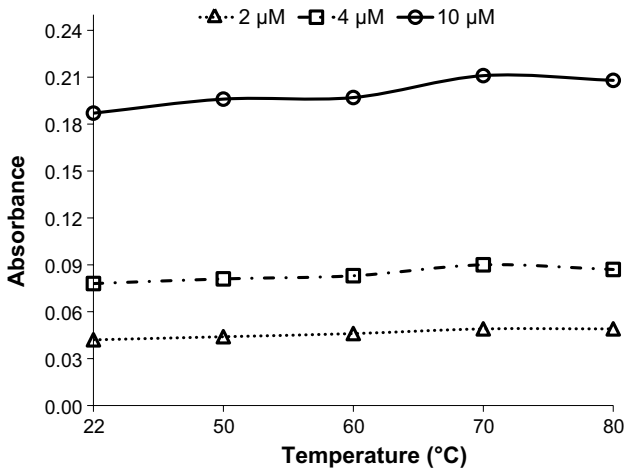

Fig. 1 Comparison of complete color development at different temperatures for standard solutions consisting of 2, 4, and 10 μM urea-N. Absorbance values are not corrected for blanks. *Error bars* for urea standards (±SD, $n = 9$) are not displayed because they are smaller than the symbols

complex. With the proposed method, the CV (1.2%) and molar absorption coefficient (20,200 M^{-1} urea cm^{-1}) were smaller and higher, respectively, compared to those of the prevailing method, where the CV = 4.3% and the molar absorption coefficient = 18,000 M^{-1} urea cm^{-1}, respectively. Two reagents are added separately in the prevailing method, whereas we used a single mixed COLDER and incubated under dark conditions thus decreasing the reaction time and enhancing the precision of this method.

The calibration curves for the RT and HT procedures were compared in experiment 3 (Fig. 3). The average slope of the calibration curve increased by 12% ($P < 0.01$) with the HT procedure compared to that for the RT procedure. The absorbance increase for the HT procedure was greater for lower concentrations of the standard solutions (0–2 μM urea-N) than higher concentrations (4–10 μM urea-N). Mean absorbances of the 1 μM standard solution amounted to 0.029 (SD = 0.002) for the HT method and 0.024 (SD = 0.001) for the RT method. On the other hand, mean absorbances of the 8 μM standard solution amounted to 0.171 (SD = 0.001) for the HT method and 0.150 (SD = 0.001) for the RT method. These absorbance values for the RT method were 83% (1 μM) and 88% (8 μM) of those obtained with the HT method.

3.2 Response of COLDER at a high temperature

The efficiency of the single mixed COLDER was tested at the HT of 70 °C in experiment 4 (Fig. 4). COLDER was added to the urea working solutions at 0, 15, 30, and 60 min after its preparation. When COLDER was added at 0 and 15 min after its preparation, similar absorbance values were obtained for each of the urea standard solutions according to the calibration curves. However, the slopes of the calibration curves decreased by 5% ($P < 0.05$) and 32% ($P < 0.01$) when COLDER was added after 30 min and 60 min, respectively. These results are similar to the results obtained for the RT procedure (Revilla et al. 2005), and the data indicate that it is best to use COLDER between 0 and 15 min after its preparation to ensure a high response corresponding to the complete development of the color complex.

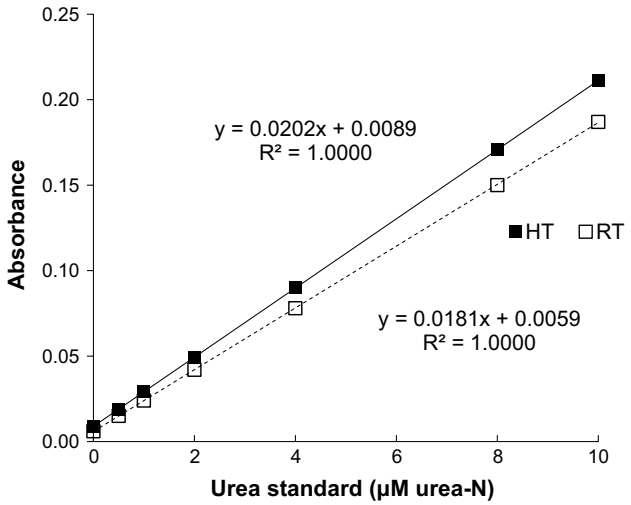

Fig. 3 Comparison of two calibration curves for urea obtained by the room temperature (RT) and high temperature (HT; 70 °C) procedures. Absorbance values are not corrected for blanks. Number of replicate measurements = 9

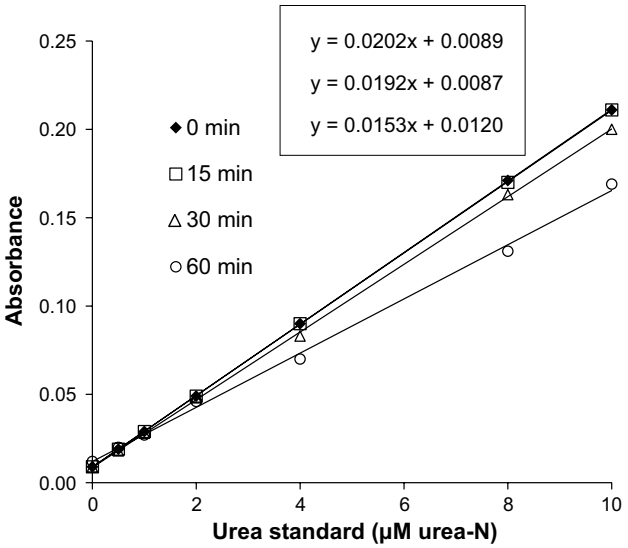

Fig. 4 Efficiency of COLDER at the high temperature (HT). Four calibration curves represent the addition of COLDER at 0, 15, 30, and 60 min after its preparation. Absorbance values are not corrected for blanks. *Error bars* of urea standards (±SD, $n = 9$) are not displayed because they are smaller than the *symbols*

3.3 Light effect on color complex formation

Illumination is an important factor that can influence the development and degradation of the color complex, and light typically has a negative effect on the development of color. The effect of light on color complex formation was assessed in experiment 5 (Table 3). The molar absorption coefficient of the calibration curve decreased (95%) under

Table 3 Illumination effect on complete color complex formation

Parameter	Illumination condition	
	Dark	Light
Molar absorption coefficient (M^{-1} urea cm^{-1})	20,200	19,300
Standard deviation	70.71	86.60
Detection limit (μM urea-N)	0.03	0.16
Number of replicate measurements	9	9

light conditions compared to dark conditions ($t_8 = 25$; $P < 0.01$). The urea detection limit was lowest (0.03 μM urea-N) for the dark conditions, while a higher limit of detection (0.16 μM urea-N) was observed for the light conditions. The constant ambient light intensity decreased the absorbance values during the complete development of the color complex. An earlier study found that the life time of the urea complex is halved when samples are held for more than 25 min at constant illumination conditions and a temperature of 27 °C (Goyens et al. 1998). Here, the stability of the absorbance was studied in a time-course experiment under dark conditions (experiment 6; Fig. 5).

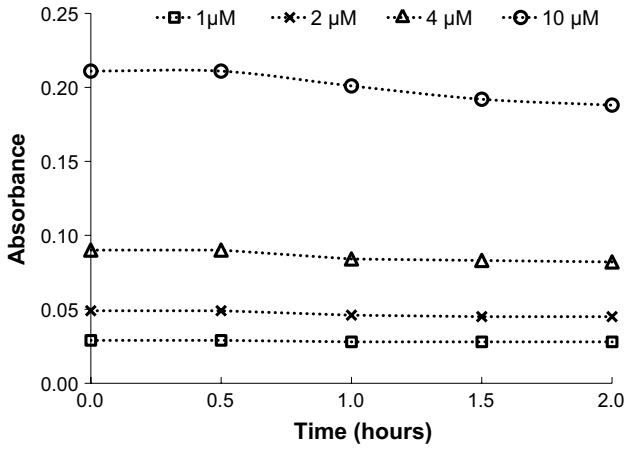

Fig. 5 Time-course of color stability in standard solutions consisting of 1, 2, 4, and 10 μM urea-N. Absorbance values are not corrected for blanks. Four lines show the measurements taken at 0, 0.5, 1, 1.5 and 2 h after the complete formation of color. *Error bars* of urea standards (±SD, $n = 9$) are not displayed because they are smaller than the symbols

Absorbance values remained stable until 30 min after the complete formation of the color complex. The absorbances values were similar between 0 to 30 min in dark conditions. In the case of the 10 μM urea-N standard solution, the absorbance decreased gradually with further increases in time. Rapid degradation occurred between 30 and 60 min (5%; $P < 0.01$), and then after half an hour, the degradation rate slowed to 4.5% ($P < 0.01$) and 4% ($P < 0.05$) at 1.5 and 2 h, respectively. Thus, measurements of absorbance within 30 min are required to ensure high absorbance values.

The HT procedure with the single mixed reagent method in the present study compares well with other techniques for urea analysis in terms of the detection limit (0.03 μM urea-N), precision (SD = 0.02 μM urea-N, CV = 1.2%), and sensitivity (molar absorption coefficient = 20,200 M^{-1} urea cm^{-1}) as shown in Table 4. The detection limit at HT when using the proposed protocol amounted to 0.03 μM urea-N, which was smaller than many of the detection limits published in previous reports including RT procedures (0.04 μM urea-N; Table 4). This result indicates that the HT procedure can detect low concentrations of dissolved urea. In addition, Chen et al. (2015) introduced an HT-based protocol with a 20 nmol L^{-1} detection limit for normal level urea analyses that uses a new reagent concentration, and this limit was nearly similar to our detection limit. The mean absorbance of a 2 μM urea standard solution amounted to 0.049 (SD = 0.001) for the HT method and 0.042 (SD = 0.001) for the RT method. This value implies that the absorbance at RT peaked at only 86% ($P < 0.01$) compared to the absorbance at HT.

In terms of the reproducibility, the coefficients of variation ($n = 9$) of the 2 μM standard amounted to 1.2 and

Table 4 Comparison results for urea analysis

References	Procedure	Molar absorption coefficient (M^{-1} urea cm^{-1})	Detection limit (μM urea-N)	Conc. tested	SD	CV (%)	*n*
Newell et al. (1967)	HT	18,000	–	33	–	4.3	10
DeManche et al. (1973)	AHT	7000	0.05	1	0.02	–	10
Aminot and Kerouel (1982)	AHT	18,000	0.02	1	0.01	–	2
Koroleff (1983)	HT	11,000	0.2	4	–	4.5	–
Price and Harrison (1987)	AHT	–	0.05	1	0.02	–	5
Mulvenna and Savidge (1992)	HT	16,000	0.14	2	0.02	0.3	10
Goeyens et al. (1998)	HT	21,000	0.28	4	–	2	2
Goeyens et al. (1998)	RT	19,000	0.20	4	–	1.6	10
Revilla et al. (2005)	RT	19,000	0.04	2	0.02	1.1	10
Present study	RT	18,100	0.04	2	0.04	1.8	9
Present study	HT	20,200	0.03	2	0.02	1.2	9

Adapted from Revilla et al. (2005). Precision is either expressed as the standard deviation (SD) or as the coefficient of variation (CV) for the concentration tested in a number of replicate measurements (*n*)

HT high temperature, *AHT* automated high temperature, *RT* room temperature

Table 5 Recovery of internal urea standards in natural samples

Urea standard (μM urea-N)	Coral reef			Seagrass			Mangrove		
	$10\%_{spk}$	$20\%_{spk}$	$50\%_{spk}$	$10\%_{spk}$	$20\%_{spk}$	$50\%_{spk}$	$10\%_{spk}$	$20\%_{spk}$	$50\%_{spk}$
	Recovery%								
0.5	96	94	95	96	96	95	95	95	96
1	94	96	95	99	95	97	97	97	98
2	95	95	96	96	97	99	95	95	97
4	97	95	98	98	96	97	96	96	98
8	97	97	98	98	97	99	99	99	97
10	98	98	99	99	98	99	97	97	98

Natural samples from coral reef, seagrass, and mangrove ecosystems were spiked ($_{spk}$) at 10, 20, and 50% with different urea standard solutions. Number of replicate measurements = 9

1.8% for the HT and RT procedures, respectively, and these data illustrate the high precision of the HT technique. The molar absorption coefficient was 20,200 M^{-1} urea cm^{-1} for the HT method and 18,100 M^{-1} urea cm^{-1} for the RT method, which were generally higher than those obtained in previous reports (Table 4). The data on the molar absorption coefficient are indicative of the high sensitivity of the HT procedure with high absorbance values.

3.4 Method validation with natural samples

Recovery analysis was conducted to measure the accuracy of the HT-based direct method with some natural samples that were collected from different types of ecosystems (coral reefs, seagrass beds, and mangrove forests) in experiment 7 (Table 5). The highest urea concentration (1.17 μM urea-N) was found in the mangrove ecosystem. Moderate and low urea concentrations were found at the coral reef site (0.68 μM urea-N) and the seagrass site (0.56 μM urea-N), respectively.

The natural samples were spiked with 0.5, 1, 2, 4, 8, and 10 μM urea-N internal standards at proportions of 10, 20, and 50% (i.e., the 10% spiked samples contained 90% natural sample and 10% of each urea-N standard solution and so forth). The recovery of standards ranged between 94 and 99%. The percentages tended to increase with higher concentrations of internal standards but the overall regression was not significant ($P > 0.05$); the regressions between the recovery percentages and standards were weak ($r^2 < 0.6$) for all samples. Results from a two-way analysis of variance (ANOVA) indicated that there was no significant difference ($F = 0.274$, $P > 0.05$) in the recovery rate depending on the study sites (coral reef lagoon, seagrass bed, and mangrove forest) and spiked percentages (10, 20, and 50%). High recoveries were obtained for all three spiked percentages in all three types of samples, thus indicating that this method may be applicable for ecological studies in waters of all major types of coastal ecosystems.

3.5 Urea measurement in natural samples

Dissolved urea concentrations were measured with the HT procedure by using both freshly collected samples and frozen samples in experiment 8 to study the effect of sample quality on the results. For this experiment, samples from the seagrass ecosystem were used. The analysis results for frozen samples showed 90% urea concentrations (0.50 μM urea-N) compared to levels detected in the freshly collected samples (0.55 μM urea-N; Fig. 6), and the difference was significant ($t_8 = 40.87$; $P < 0.01$). Thus, analysis of freshly collected samples with the HT procedure is vital for obtaining accurate levels of urea in natural samples. In the case of the frozen samples, the loss of urea may have been due to changes during melting and the effect of illumination. While these results reinforce the need to analyze samples as soon as possible after collection, if immediate analysis is not possible, the collected water samples can be

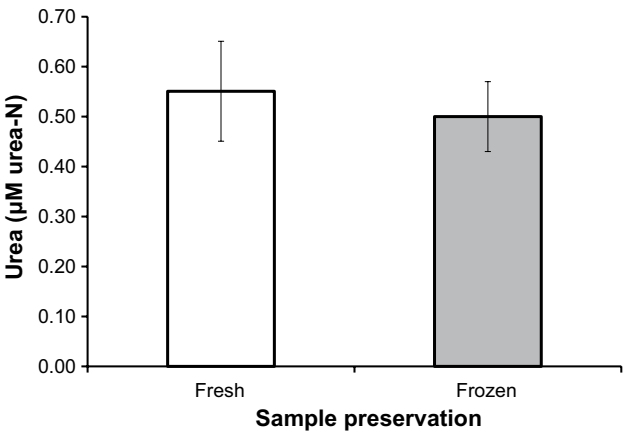

Fig. 6 Effects of sample preservation on urea concentrations. The *white bar* represents the immediate analysis results with freshly collected samples and the *gray bar* represents the late analysis results with frozen samples. Number of replicate measurements = 9

Table 6 Urea concentrations measured at RT and HT conditions in coastal and estuarine systems in Japan

System	Ecosystem	Location	No. of stations	Date	Urea-N (μM)	
					RT	HT
Coast	Coral reef	Sesoko, Okinawa, Japan (26°38′N, 127°51′E)	10	May 2015	0.59 ± 0.05–0.65 ± 0.07	0.65 ± 0.05–0.72 ± 0.08
Coast	Sea grass	Bise, Okinawa, Japan (26°42′N, 127°52′E)	9	May 2015	0.44 ± 0.06–0.52 ± 0.05	0.49 ± 0.07–0.58 ± 0.06
River estuary	Mangrove	Fukido, Ishigaki, Japan (24°34′N, 124°17′E)	12	Feb 2015	0.98 ± 0.06–2.00 ± 0.09	1.09 ± 0.05–2.28 ± 0.05

Number of replicate measurements = 3

HT high temperature, *RT* room temperature

preserved by freezing and analyzed within 1 month's time; this storage procedure should not result in too much loss of urea. Urea concentrations in the frozen samples from the seagrass ecosystem were measured in a time course study (10-d intervals) over a period of 1 month in experiment 9. Frozen samples maintained a less than 10% difference in urea values (0.50 μM urea-N) compared to the initial urea concentrations in freshly collected samples (0.55 μM urea-N) throughout the month. Results from a one-way ANOVA indicated that there was no significant difference in the urea concentrations of the preserved samples ($F = 3.17$, $P > 0.05$) depending on sample preserved times over the course of 30 days.

Urea concentrations in the freshly collected natural samples from the three different types of coastal ecosystems (coral reefs, seagrass beds, and mangrove forests) were also measured with both the RT and HT procedures in experiment 10. The urea results for the HT procedure were 10–12% higher in all three types of ecosystems than the results for the RT procedure (Table 6).

4 Conclusions and recommendations

This study demonstrates that the direct HT method with COLDER is effective for the manual determination of dissolved urea concentrations. Data on standards and natural samples from coastal ecosystems displayed an acceptable level of accuracy, reproducibility, and sensitivity, and the detection limit was low for low volumes of samples. Our proposed method proved to be more rapid and precise than existing dissolved urea analysis methods. Specifically, it is a very pragmatic and inexpensive method that can be completed in only a few hours; in contrast, the conventional RT procedure requires a 72-h-long waiting period before urea measurements can be taken. The use of a single mixed reagent (COLDER) in lieu of separate reagents is very convenient, but the stability of COLDER does decrease over time. Our data suggest that absorbance measurements should be taken within 60 min, and for this reason, it may not be possible to measure large numbers of samples in one batch. Furthermore, we recommend immediate analysis with freshly collected samples, although samples may be stored by freezing for about one month without excessive loss of urea concentrations. In conclusion, we hope that this new method for measuring urea concentrations will facilitate more data collection work that will reveal the importance of urea in the nitrogen cycle of coastal ecosystems and its potential role in triggering algal blooms.

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