

Urea analysis in coastal waters: comparison of enzymatic and direct methods

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Abstract

This study presents a comparison of two existing methods for the determination of urea concentration in seawater. These methods are referred to here as the enzymatic method, which is based on the use of the enzyme urease, and the direct method, which is based on the reaction of urea with diacetylmonoxime. A room temperature modification of the direct method was adapted for a single reagent and both the enzymatic and the direct method were tested in artificially prepared solutions and in natural samples from estuaries and shelf waters. We were particularly interested in the effects of salinity and humic acids on the accuracy of both methods. The effect of humic acids was negligible. In contrast, salinity ~34 caused a 15% to 40% underestimation in the urea concentrations measured by the enzymatic method and the degree of underestimation varied among enzyme batches. Urea concentrations corrected for the salt effect should, however, be considered estimations, as other factors also interfered with the enzymatic method in natural samples. The direct method as modified in this study presented a low detection limit (0.04 μM urea-N) and high precision (standard deviation: 0.02 μM urea-N; coefficient of variation: 1.1%) comparable to those of the enzymatic method. The direct method was more accurate and less salinity dependent than the enzymatic method. As urea levels could have been underestimated by the enzymatic method, our findings support previous conclusions regarding the important role of urea in the nitrogen cycle and its link with some harmful algal bloom phenomena.

Introduction

Urea is receiving increasing attention in phytoplankton physiology and ecology. In the last decades, the anthropogenic production of urea and its application in the environment have significantly augmented the input of this nutrient to coastal systems (Smil 2001). High urea levels have been observed prior to the development of harmful algal blooms in aquaculture ponds (Glibert and Terlizzi 1999) and coastal waters (Glibert et al. 2001, 2005). Preferential use of urea over inorganic nitrogen, high rates of urea uptake, and/or urease activity have been demonstrated for many phytoplankton species, some of which are associated with brown

tides (Lomas et al. 1996; Berg et al. 1997, 2002; Kana et al. 2004), red tides (Kudela and Cochlan 2000; Fan et al. 2003), paralytic shellfish poisoning (Dyhrman and Anderson 2003), or cyanobacterial blooms (Berman and Chava 1999; Berg et al. 2003; Glibert et al. 2004).

Accurate measurements of urea are needed to determine its role in the marine nitrogen cycle. There are currently two dominant methods for the analysis of urea in seawater. Both are colorimetric techniques. The enzymatic method of McCarthy (1970) is based on the use of jack bean urease to indirectly quantify urea from the amount of ammonia obtained after its hydrolysis by the enzyme. The use of this method has been supported in the handbooks of Strickland and Parsons (1972) and Parsons et al. (1984). The direct method is based on the formation of a colored product when urea reacts with diacetylmonoxime in acid solution. This technique was first used in seawater by Newell et al. (1967), who modified a clinical method for the analysis of urea in biological fluids. Since then, several modifications have been introduced to develop different automated (DeManche et al. 1973; Whitley et al. 1981; Aminot and Kerouel 1982; Price and Harrison 1987; Cozzi 2004) and manual procedures (Koroleff 1983; Mulvenna and Savidge 1992; Goeyens et al. 1998). The

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manual procedures involve the addition of two reagents sequentially. However, the use of a mixed reagent has been reported for urea and citrulline analysis in biological materials (Rahmatullah and Boyde 1980; Knipp and Vasák 2000), as well as for urea determination in rainwater and atmospheric aerosol (Cornell et al. 1998). The use of a single reagent can save time and also increase the precision of the analysis as only one step of dispensation is involved.

Although the direct method persists as the approach of choice for the analysis of urea in seawater (Francis et al. 2002), the enzymatic method has been extensively used, not only in the past, but also in recent studies (e. g., McCarthy 1972; Glibert et al. 1991; Cho and Azam 1995; Metzler et al. 1997; Bronk et al. 1998; Berg et al. 2001; Dyhrman and Anderson 2003; Bode et al. 2004; Lomas et al. 2004). In a comparative work on these techniques, Price and Harrison (1987) demonstrated that the enzymatic method underestimated urea concentrations in seawater compared to the direct method, and the effect was largely attributed to the inhibitory effect of seawater ions on the enzyme. They also found that high pH and, possibly, extracellular metabolites produced in unialgal cultures inhibited urease activity, and reported that the enzyme was not absolutely specific for urea. However, to date, there have been no studies comparing the enzymatic and the direct method in estuarine waters, where salinity can be lower and land-derived substances, such as humic acids, can be quantitatively important.

We thus conducted a comparison of the enzymatic and the direct method with focus on estuaries and other coastal waters. First, we assessed the direct method when adapted for the use of a single reagent. Then, the effects of humic acids and salinity on the accuracy of both methods were addressed in artificially prepared solutions and in natural samples. Next, we explored the implications of using the enzymatic method in ecological studies. Finally, recommendations for the analysis of urea in seawater are given.

Materials and procedures

Protocol for the enzymatic method—The method of McCarthy (1970) described in the manual of Parsons et al. (1984) was followed with minor modifications. All reagents, including the concentrated urease enzyme solution, were made according to the manual. The enzyme was purchased from Worthington Biochemical Corporation (jack bean urease, code URC). The dilute enzyme solution was prepared fresh by diluting 5 mL of the concentrated solution to 100 mL with de-ionized distilled water (DDW). Assuming the specifications of Worthington, the final concentration of the enzyme in the sample ranged 0.6 to 0.8 units mL⁻¹, where one unit produces 1 μmol of ammonia per min at 25°C and pH 7.6. Analysis was carried out in disposable polypropylene 17 × 100 mm tubes with snap caps (Fisher Brand). Five tubes, each one containing 5 mL aliquots of sample, were prepared. Three of them received 0.5 mL of the dilute enzyme solution and were incubated in a

water bath at 50°C for 20 min. Then, they were allowed to cool at room temperature for 30 min before adding the reagents for ammonia determination. The other two tubes were treated with urease and immediately with the reagents. Ammonia was analyzed by the method of Solórzano (1969). Each tube was sequentially treated with 0.2 mL of phenol solution, 0.2 mL of sodium nitroprusside solution, and 0.5 mL of oxidizing solution, mixing after each addition. All tubes were kept in the dark and their absorbance was read within 2.5 to 24 h at a wavelength of 640 nm, using a 5-cm cell in a Shimadzu 1601-UV spectrophotometer equipped with a Shimadzu ASC-5 auto sample changer. The absorbance of the unheated tubes was subtracted from the absorbance of the incubated tubes to correct for the background ammonia.

An external calibration was performed for each session of analysis. The standard stock solution (10 mM urea-N) was prepared by dissolving 0.3003 g of urea (Sigma, ACS reagent) in 1 L of DDW. This solution was stored in a high-density polyethylene (HDPE) bottle at 4°C and used throughout the study, being stable for more than 1 y. A secondary standard (100 μM urea-N) and working solutions (0, 0.5, 2, 5, and 10 μM urea-N) were prepared fresh in 100-mL volumetric flasks. Working solutions were analyzed as described above. Their absorbance was corrected for the background ammonia and then plotted against the concentration. The curve was fit by Model I linear regression.

Protocol for the direct method—The protocol was based on the room temperature manual technique of Goeyens et al. (1998). Three separate reagent solutions were made up according to Mulvenna and Savidge (1992): diacetylmonoxime solution (3.4 g of diacetylmonoxime in 100 mL of DDW), thiosemicarbazide solution (0.19 g of thiosemicarbazide in 20 mL of DDW) and reagent B, which contained 300 mL of concentrated sulfuric acid diluted to 535 mL with DDW together with 0.5 mL of a ferric chloride solution (0.15 g of ferric chloride in 10 mL of DDW). These reagents were stored in the dark at 4°C, and they were stable at least for 1 month. The reagent A (25 parts of diacetylmonoxime solution with 1 part of thiosemicarbazide solution) was made fresh prior to each analysis. The color developing reagent (COLDER) consisted of 1 part of reagent A and 3.2 parts of reagent B. The solution to determine the optical turbidity blank consisted of 1 part of DDW and 3.2 parts of reagent B. COLDER and the turbidity blank solution were used within 15 min. A sample volume of 4 mL was dispensed in each of five polypropylene tubes (Fisher Brand). Three tubes received COLDER (1.2 mL) and the other two tubes received the turbidity blank solution (1.2 mL). They were capped, stirred by vortex, and kept in the dark at room temperature (22 ± 2°C). After 72 h, the absorbance was measured at 520 nm in the same spectrophotometry system used for the enzymatic method. The absorbance of the samples treated with COLDER was corrected for their individual turbidity blanks.

External calibration in DDW was performed using the same urea stock solution and dilution procedure of the enzymatic

Table 1. The range in salinity and urea concentration measured by the enzymatic and the direct method in different coastal systems in the USA*

System	<i>n</i>	Date	Signed decimal degrees (°)		Salinity	Urea-N (μM)	
			Latitude	Longitude		Enzymatic	Direct
W. Florida shelf	16	May 03	24.50, 28.00	-81.00, -83.00	34.3 to 36.4	BDL† to 0.31	0.32 to 0.98
Florida Bay	7	Jul 03	24.90, 25.20	-80.50, -81.00	15.2 to 39.5	0.12 to 1.94	0.24 to 2.86
	7	Mar 04			15.8 to 37.3	0.23 to 0.92	0.78 to 1.98
	7	Aug 04			37.2 to 40.0	0.35 to 2.69	1.09 to 4.94
Chesapeake Bay‡	5	Apr 04	36.90, 37.30	-75.90, -76.10	11.5 to 27.4	0.07 to 0.68	0.27 to 1.10
Choptank River	9	Jul 03	38.60, 38.80	-75.90, -76.10	7.2 to 9.0	0.65 to 6.03	0.99 to 6.52
	5	Sep 03			0.2 to 12.8	0.54 to 0.93	0.84 to 1.24
	4	Dec 03			0.1 to 9.3	0.29 to 0.71	0.33 to 0.96
Corsica River	2	Oct 03	39.00, 39.10	-76.00, -76.10	6.2 to 7.3	BDL† to 0.58	0.45 to 1.15
	2	Nov 03			2.7 to 5.7	0.69 to 0.72	1.18 to 1.46

*The number of stations (*n*) is indicated with the sampling dates and the latitude and longitude limits.

†BDL, below detection limit.

‡Plume and lower segment of the estuary.

method protocol. The absorbance of the standards treated with COLDER was corrected for the average absorbance of two DDW tubes treated with the turbidity blank solution and then plotted against their respective concentrations. The curve was fit by Model I linear regression.

Figures of merit—The molar absorption coefficient, defined as the absorbance divided by the optical path length and the concentration, was calculated from the average slope of 10 calibration curves. The limit of detection was calculated according to Miller and Miller (2000) as the analyte concentration giving a signal equal to the blank signal plus three standard deviations of the blank. A reagent blank was prepared with DDW (*n* = 10), and it was analyzed together with a calibration curve for each method in a single experiment. Precision was evaluated by the standard deviation (SD) and the coefficient of variation (CV) of a 2 μM urea-N standard in 10 replicate measurements.

Experiments in artificially prepared solutions—Water rich in humic acids was prepared from a humic acid sodium salt, 39% carbon (Sigma-Aldrich). Seawater in the 3 to 35 salinity range was made up by dilution of low nutrient seawater (Sargasso seawater). Sargasso seawater was stored in 25-L HDPE containers for 1 y, and just before conducting the experiments, it was filtered through combusted (450°C, 2 h) GF/F filters. Standard solutions (0, 0.5, 2, 5, and 10 μM urea-N) were prepared in humic- and salt-containing water, as well as in DDW. A ratio was calculated to determine the effect of humic acids and salt on the slope of the calibration curves:

$$\text{Slope ratio (\%)} = (m_1/m_0) \times 100 \quad (1)$$

where m_1 is the slope of the urea standard curve in humic or salt containing water and m_0 is the slope of the urea standard curve in DDW.

The pH of the urea standard solutions in humic water, seawater, and DDW was measured with a Corning Ion Analyzer 150 calibrated with fresh buffer solutions.

Natural samples processing—Sampling sites were located in the coast of Florida and in the Chesapeake Bay and some of its tributaries (Choptank and Corsica rivers), USA (Table 1). Samples were collected in acid-cleaned buckets and Niskin-type bottles. The water was filtered through combusted GF/F filters and stored frozen in acid-cleaned HDPE bottles until its simultaneous analysis by the enzymatic and the direct method. Salinity was measured with an YSI Model 85 salinity sensor (precision: ± 0.1).

Internal standards were run in some samples to assess chemical interferences. Spiked samples contained 90% of water sample and 10% of a 10 μM urea-N standard solution. The percentage of recovery of the internal standard was calculated as:

$$\text{Recovery (\%)} = [(C_{\text{spiked sample}} - 0.9 \times C_{\text{sample}}) / (0.1 \times C_{\text{standard}})] \times 100 \quad (2)$$

where $C_{\text{spiked sample}}$ is the urea-N concentration (μM) of the spiked sample, C_{sample} is the urea-N concentration (μM) of the sample, and C_{standard} is 10 μM urea-N.

Assessment

Modifications of the direct method—In order to determine if the use of a single mixed reagent (COLDER) instead of two separate reagents produced comparable results, calibration curves in DDW were compared (Fig. 1). The curves did not present significant differences between slopes or y-intercepts ($P > 0.05$, *t* student test). The stability of COLDER was tested in another experiment. COLDER was added to the urea working solutions at 0, 15, 30, and 60 min after its preparation

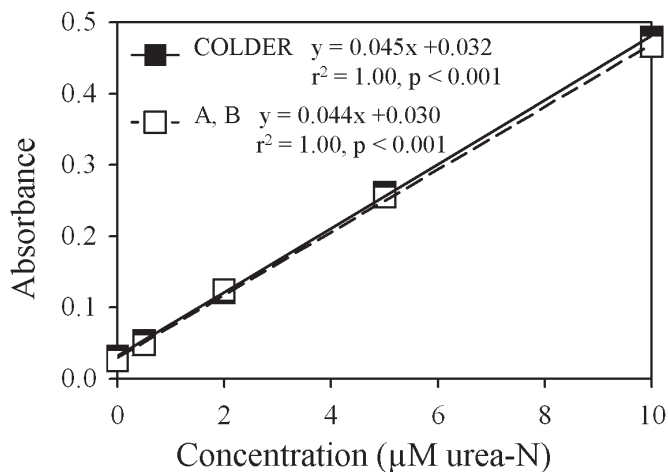


Fig. 1. Comparison of two urea calibration curves obtained by the direct method. Solid squares represent the addition of a single mixed reagent (COLDER) and open squares symbolize the addition of two reagents separately (reagents A and B).

(Fig. 2). The calibration curves presented the same slopes when using COLDER at 0 and 15 min. However, slopes decreased 5% at 30 min ($P < 0.05$) and 20% at 60 min ($P < 0.001$). These experiments indicate that similar results are obtained by the addition of COLDER as with the separate addition of the reagents A and B, provided the mixed reagent is used within 15 min after its preparation.

The color development with COLDER was studied in a time-course experiment and different trends were found depending on the urea concentration (Fig. 3). At the highest concentration (10 µM urea-N), the absorbance peaked after 3

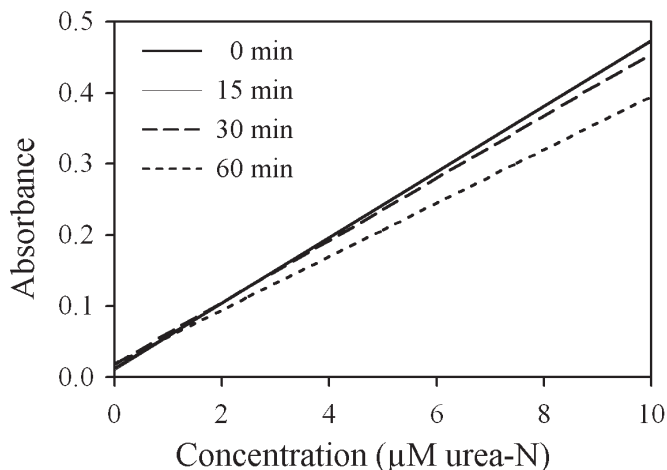


Fig. 2. Comparison of urea calibration curves obtained by the direct method. Four curves represent the addition of COLDER at 0, 15, 30, and 60 min, respectively, after its preparation.

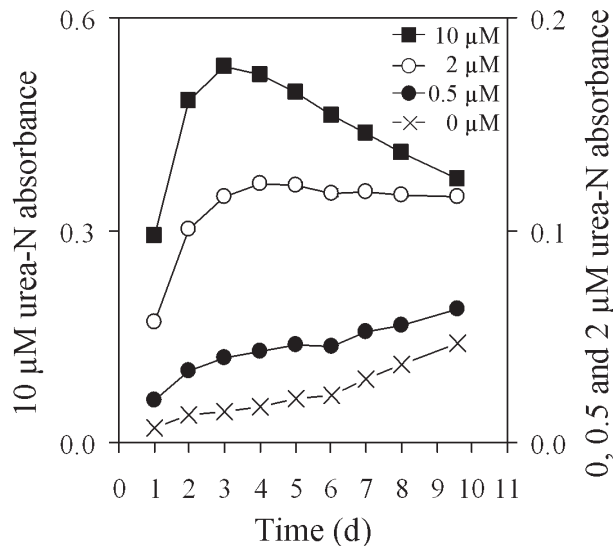


Fig. 3. Time-course for the development of color in standard solutions 0, 0.5, 2, and 10 µM urea-N treated with COLDER. Absorbance values are not corrected for blanks. Error bars (\pm SD, $n = 2$) not displayed because they are smaller than symbols.

d and then progressively decreased. At the intermediate concentration (2 µM urea-N), the absorbance reached its maximum after 4 d and then remained stable. At the lowest concentration (0.5 µM urea-N) and the reagent blank (0 µM urea-N), the absorbance increased over the 10 d of the experiment, which suggests an interfering reaction not related with urea. Absorbance corrected for the reagent blank peaked after 3 d at all concentrations and remained stable for 2 d only at the low and intermediate concentrations (data not shown). Similarly to Goeyens et al. (1998), we have demonstrated that 72 h is the optimum time to allow the complete formation of color at room temperature. Longer periods of incubation are discouraged because the absorbance of the reagent blank increases and the colored complex is not stable at high urea concentrations.

Our protocol involves a separate blank for each sample to correct the spurious absorbance derived from water turbidity. Apparent concentrations produced by turbidity blanks in estuarine samples were on the order of 0.10 µM urea-N. We did not observe optical effects when testing Sargasso seawater (salinity of 38.7). Optical blanks resulting from salt have been reported for automated direct methods (DeManche et al. 1973; Aminot and Kerouel 1982; Cozzi 2004). However, optical salt effects are ascribed to differences in the refractive index of seawater and DDW and are not obvious in manual techniques that employ flow cells with perpendicular ends (Stewart and Elliott 1996).

As shown in Table 2, the direct method in the present study compares well with other techniques for the analysis of urea in terms of detection limit (0.04 µM urea-N) and precision (SD = 0.02 µM urea-N, CV = 1.1%). Sensitivity (molar absorp-

Table 2. Figures of merit of various methods for urea analysis in seawater*

Reference	Method	Molar absorption coefficient (M ⁻¹ urea cm ⁻¹)	Detection limit (μM urea-N)	Conc. tested	SD	CV (%)	<i>n</i>
McCarthy (1970)	Enzymatic	31,000	—	1	0.01	—	10
Parsons et al. (1984)	Enzymatic	31,000	0.05	3	0.03	—	10
Price and Harrison (1987)	Enzymatic	32,000	—	1	0.02	—	5
Present study	Enzymatic	30,000	0.05	2	0.04	1.8	10
Newell et al. (1967)	HT Direct†	18,000	—	33	—	4.3	10
DeManche et al. (1973)	AHT Direct‡	7,000	0.05	1	0.02	—	10
Aminot and Kerouel (1982)	AHT Direct‡	18,000	0.02	1	0.01	—	2
Koroleff (1983)	HT Direct†	11,000	0.20	4	—	4.5	—
Price and Harrison (1987)	AHT Direct‡	—	0.05	1	0.02	—	5
Mulvenna and Savidge (1992)	HT Direct†	16,000	0.14	2	0.02	0.3	10
Goeyens et al. (1998)	HT Direct‡	21,000	0.28	4	—	2.0	2
Goeyens et al. (1998)	RT Direct§	19,000	0.20	4	—	1.6	10
Present study	RT Direct§	19,000	0.04	2	0.02	1.1	10

*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*).

†High Temperature Direct Method.

‡Automated High Temperature Direct Method.

§Room Temperature Direct Method.

tion coefficient = 19,000 M⁻¹ urea cm⁻¹) was typical of the direct techniques. Beer's law was obeyed from 0 to 15 μM urea-N, which is adequate for the range of concentrations typically found in these coastal waters (Table 1).

Accuracy of enzymatic and direct methods in artificial solutions—Laboratory experiments revealed that humic acids at concentration of 8 mg L⁻¹ would not interfere with either method (Fig. 4). The effect of humic acids on the enzymatic method was tested at concentrations as high as 24 mg L⁻¹ and similar results were found (data not shown). In contrast, salinity (34.4) caused an important underestimation when using the enzymatic method (33%) and a slight overestimation when using the direct method (6%). Synergetic effects were not observed when humic acids and salinity were combined.

The effect of salinity was further studied by seawater dilution experiments. Two enzyme batches were tested through a 3 to 35 salinity gradient (Fig. 5A). The slope ratio obtained by each of the urease batches was strongly related to salinity ($P < 0.01$). This experiment suggests that if an external calibration in DDW is employed, the enzymatic method could underestimate urea in waters of salinity > 10. In contrast, a slight overestimation would occur at salinity < 10. The interference of salt with the technique for ammonia determination was also studied using a similar experimental approach (Fig. 5A). Still, the salt effect on the ammonia analysis could not completely explain the observed effect on the enzymatic method. As for the direct method, the calibration curves performed in diluted seawater presented higher slopes than in DDW (3% to 10%). The slope ratio tended to increase with salinity but the regression was not significant (Fig. 5B).

On the basis of the above experiments, the humic content was shown not to affect the determination of urea in coastal waters as no chemical interferences were observed at a concentration of 8 mg L⁻¹ (265 μM C). Maximum humic concentrations in marsh creeks, estuaries, and nearshore areas are reported to be 25 to 170 μM C (Fox 1983; Eastman and Church 1984; Moran and Hodson 1994) and concentrations in the order of 10³ μM C are typical of blackwater rivers (Cai and Wang 1998). Nevertheless, individual blanks for each

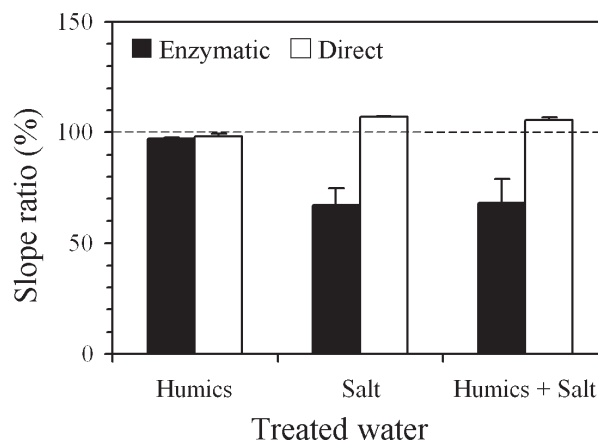


Fig. 4. The slope ratio obtained in laboratory experiments to evaluate the effect of high humic acid and/or salt concentration on the accuracy of the methods for urea determination. The dashed line indicates no effect. Treated water: humics (8 mg L⁻¹ humic acids in DDW); salt (34.3 salinity solution); humics + salt (8 mg L⁻¹ humic acids in 34.3 salinity solution). Error bars represent 1 SD (*n* = 2).

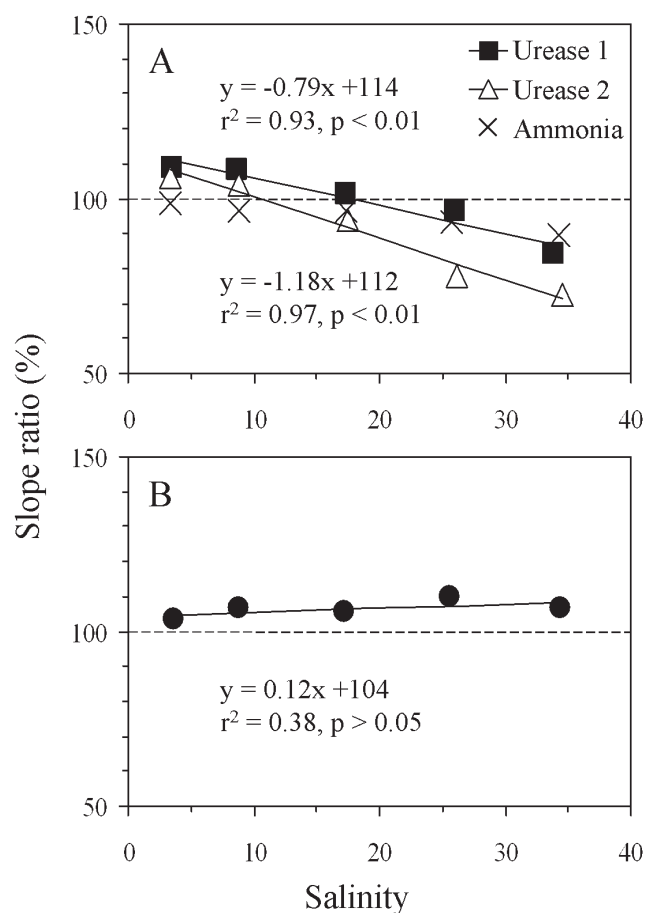


Fig. 5. The slope ratio as a function of salinity in laboratory experiments. (A) Enzymatic method for urea analysis (two enzyme batches tested) and ammonia determination technique. (B) Direct method for urea analysis. Equations were obtained by Model I linear regression and refer to the enzymatic method in the upper panel (Urease 1 and Urease 2) and to the direct method in the lower panel.

sample are indispensable in humic-rich environments to correct the background absorbance resulting from turbidity.

Our results differ from other works that show inhibitory effects of humic acids on urease activity (Vaughan and Ord 1991; Marzadori et al. 2000). Vaughan and Ord (1991) reported that 10 mg L⁻¹ humic acid solutions at pH 4.0 inhibited by 35% the activity of urease obtained from *Bacillus pasteurii*. They proposed that humic substances could act on the tertiary structure and/or the active sites of the enzyme. The effect was found to be pH dependent and similar solutions at pH 7.1 had either no effect, or a slight stimulating effect on the enzyme. Marzadori et al. (2000) reported inhibitory effects on jack bean urease in a 6 to 8 pH range. However, these authors indicated that the inhibition could have resulted from Cu²⁺ and Hg²⁺ ions adsorbed on the humic substances as these metals are considered strong urease inhibitors. We observed that urea hydrolysis comported similarly in humic solutions (pH 6 to 8) as in DDW (pH 4 to 5). Although we

cannot exclude the possibility of inhibitory effects mediated by humic acids at a lower pH, the analysis of urea in coastal waters would not be affected as their pH usually ranges from 7.5 to 8.5 (Price and Harrison 1987). In blackwater rivers, pH values about 4 are reported (Castillo et al. 2004). Therefore, in those systems, the enzymatic method could underestimate urea due to the high content in humic acids plus the low pH of the water.

The activity and kinetics of many enzymes vary with pH as a result of changes in the charges of the protein and/or the substrate (Nelson and Cox 2004). The pH optimum for the activity of jack bean urease is reported to be between 7 and 8 (Cesareo and Langton 1992). Price and Harrison (1987) indicated the importance of pH control for the performance of the enzymatic method when they observed the decrease in urea hydrolysis at pH > 8. In the present study, an effect due to pH on the enzymatic method is suggested. In the seawater dilution experiments (Fig. 5A), the slope of the urea calibration curves was higher in solutions for which salinity ranged 3 to 9 than in DDW. The stimulation of urea hydrolysis in the low salinity solutions could be related to the pH as it was in the optimum range for the enzyme in diluted seawater (pH 7 to 8), but not in DDW (pH 4 to 5).

The inhibition of urea hydrolysis at salinity > 10 is consistent with the work of Price and Harrison (1987). We observed a different degree of inhibition in two urease batches (Fig. 5A). At salinity ~34, another batch tested (urease 3) was even more inhibited (data not shown). Price and Harrison (1987) noted that the hydrolysis of urea in seawater was a function of the enzyme concentration. The activity of the enzyme batches purchased from Worthington ranged between 71.1 units mg⁻¹ (urease 1) and 50.8 units mg⁻¹ (urease 3), which coincided with the minimum (15%) and maximum (40%) inhibitory effect observed at salinity ~34. We did not attempt to establish a relationship between enzyme concentration and salt effect as only three batches were used. Moreover, the concentrated enzyme solutions corresponding to each batch differed in age by several months, which could also have influenced the strength of the enzyme. However, it is clear that a high variability existed in the response to salinity between different enzyme stock solutions.

Accuracy of enzymatic and direct methods in natural samples—Urea was analyzed by both methods in the 0.1 to 40.0 salinity range. Concentrations measured by the enzymatic method were lower and varied between 27% and 93% of those obtained by the direct method (Fig. 6). The salinity of the water was an important factor driving such variation ($r^2 = 0.47$, $P < 0.001$).

Experiments conducted with natural samples corroborated the salt effect observed in artificial solutions. With the enzymatic method, the recovery of standards ranged from 60% to 118% and tended to decrease with salinity (Fig. 7A). The regression was significant ($P < 0.01$) and its coefficient was among those obtained in laboratory experiments. However, salinity explained a much lower percentage of the vari-

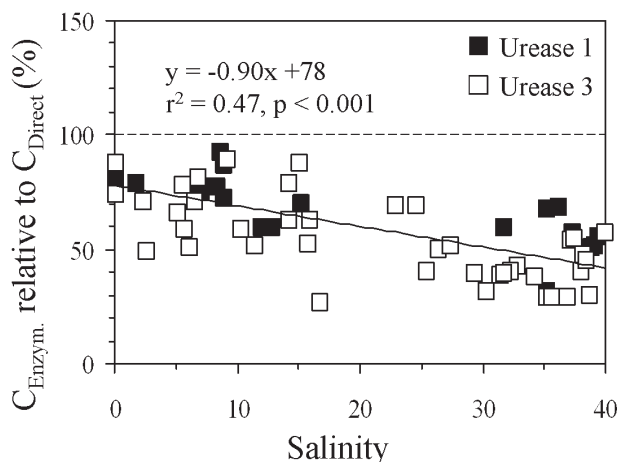


Fig. 6. Urea concentrations measured by the enzymatic method ($C_{Enzym.}$) as a percentage of the concentrations obtained by the direct method ($C_{Direct.}$). The relationship of this ratio with salinity was analyzed by Model I linear regression and includes data from two different enzyme batches.

ability in natural samples ($r^2 = 0.40$) than in artificial solutions ($r^2 > 0.90$), which suggests that other chemical interferences could affect the enzymatic method in natural waters. With the direct method, the recovery of standards varied between 103% and 119% and increased with salinity (Fig. 7B). Although significant, the regression between recovery and salinity was weak ($r^2 = 0.28$; $P < 0.05$).

Our experiments indicate that the direct method causes a slight overestimation somewhat related to salinity (Figs. 5B and 7B). Salt effects on the direct method were not reported in the works of Price and Harrison (1987) and Mulvenna and Savidge (1992) for automated and manual techniques, respectively. In contrast, other authors have identified interferences caused by salt. DeManche et al. (1973) observed a 5.2% enhancement of the reaction at 34 salinity and attributed it to the configuration of the autoanalyzer. Aminot and Kerouel (1982) determined a salt blank in low nutrient water from the deep ocean. The blank resulted from an optical blank (70%) plus a parasitic reaction that increased with temperature. More recently, Cozzi (2004) described an enhancement of the reaction with salinity that is minimized when the color reagent is prepared with a low content in diacetylmonoxime. This author explained the salt effect as a result of an increment in the ionic strength of the media that would stabilize the urea-chromogen, a molecule supposed to be positively charged (Butler et al. 1981).

Other factors could affect the direct method in coastal waters, as it gave higher urea concentrations than the enzymatic method also in low salinity samples (Fig. 6). Among several compounds tested, only citrulline is reported to react with diacetylmonoxime and this amino-acid has not been confirmed at high concentration in coastal waters (Aminot and Kerouel 1982; Price and Harrison 1987). As for humic acids, we

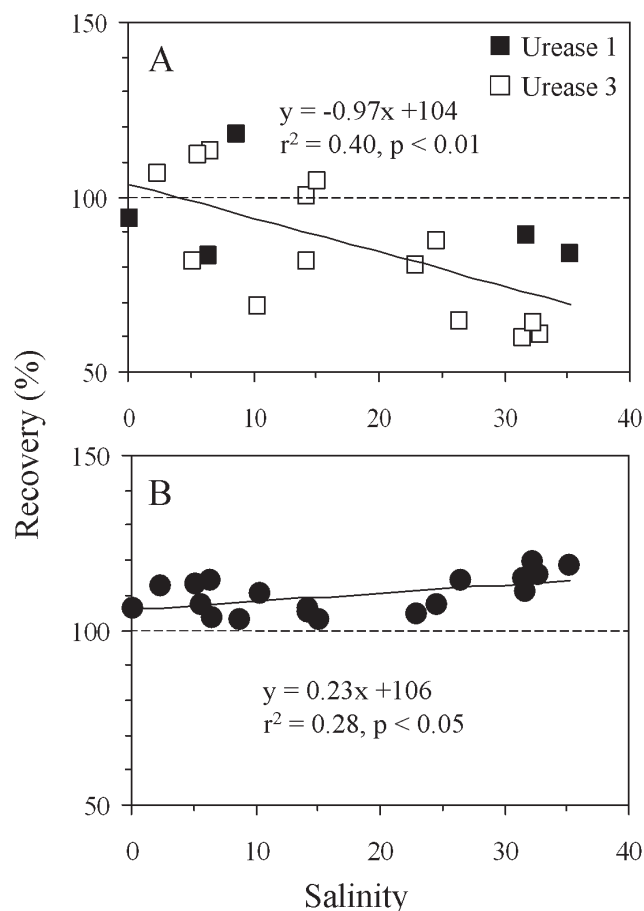


Fig. 7. The recovery of internal standards as a function of salinity in natural samples. (A) Enzymatic method for urea analysis (pooled data from two different enzyme batches). (B) Direct method for urea analysis. Equations were obtained by Model I linear regression analysis.

did not observe any chemical interference in artificial solutions, which is consistent with the high specificity of the diacetylmonoxime reaction (Butler et al. 1981). We hypothesize that in the coastal waters analyzed in the present study phosphate ions interfered. In this sense, Rahmatullah and Boyde (1980) investigated the composition of the chromogenic reagent and found that a mixture of sulfuric and phosphoric acid gave better color yield than either acid alone.

Implications of the methodology in ecological studies—In agreement with Price and Harrison (1987), this work has found the direct method to be more accurate than the enzymatic method. Therefore, it is important to know to what extent the enzymatic method underestimates urea in specific systems and if it affects the conclusions of ecological studies. In order to address that question, the recovery of standards was averaged for each system presented in Table 1. The enzymatic method caused ~40% underestimation in the urea concentrations on the Western Florida shelf, ~20% in Florida Bay, and ~10% in the plume and lower segment of the Chesapeake Bay. These systems represent neritic waters, coastal lagoons, and

meso-polyhaline estuaries, respectively. The average recovery was close to 100% at the Choptank and Corsica rivers, which are representative of oligo-mesohaline regions.

In coastal seas, total nitrogen availability and percentage of nitrogen available as urea would be higher than previously estimated by the enzymatic method. For example, the 30% to 40% contribution of urea to total nitrogen in inshore waters off Brazil by Metzler et al. (1997) should be considered a conservative value. Ambient nutrient ratios sometimes are used in ecological studies to infer nutrient limitation or preference for specific forms. Thus, conclusions could be biased if this source of regenerated nitrogen is not accurately measured.

In estuaries, with exception of hyperhaline and euhaline waters, underestimation of urea by the enzymatic method would have a weak impact. The surface concentration in the lower segment of the Chesapeake Bay was estimated in 0.46 μM urea-N (Lomas et al. 2002). Concentrations of urea in some of the upper reaches of Chesapeake Bay can, however, exceed 10 μM urea-N, but only in selected regions and at specific times of year following fertilization (Glibert et al. 2005). These concentrations would not be affected significantly. High contribution of urea to total nitrogen uptake has been reported in the lower segment (Glibert et al. 1995; Bronk et al. 1998) and plume of the Chesapeake Bay (Glibert et al. 1991). Conclusions regarding the role of urea in the nitrogen cycle would not be affected by the method employed in the above studies.

In euhaline and hyperhaline environments, urea would be available in higher concentration than previously estimated by the enzymatic method. Thus, in those systems, findings about the relationship of urea with harmful algal blooms, such as have been reported for *Alexandrium* sp. blooms in the Gulf of Maine (Dyhrman and Anderson 2003) and cyanobacterial blooms in Florida Bay (Glibert et al. 2004) are reinforced.

Comments and recommendations

Some investigators may prefer the enzymatic method in spite of its lower accuracy. The manual direct method requires laboratory conditions to be more controlled, first because the reagents are very toxic, and second because light and heating degrade the colored product. Also, if room temperature techniques are employed, urea concentrations are not known until 72 h later.

Improvements in the enzymatic method seem difficult to achieve. In oligotrophic marine systems the combination of high salinity and low urea concentrations can result in numbers below the detection limit. That was observed in 14 of 16 samples from the Western Florida shelf (Table 1). Therefore, the direct method is recommended in neritic and oceanic waters. In more eutrophic coastal systems, even if correction factors were calculated for the salinity effect, other chemical interferences could affect this method. Thus, internal standards should be run with each sample if the enzymatic method is employed.

Our modification of the room temperature direct method attained a satisfactory precision and detection limit in a low volume of sample. The use of a single mixed reagent would be particularly useful to adapt the manual technique to microvolumes of sample. However, COLDER presented a short stability over time. Therefore, if optimal laboratory conditions cannot be achieved, the method of Goeyens et al. (1998), which involves the addition of two separate reagents, is preferred.

In summary, laboratory conditions, time constraints, and the water system under study are factors to consider when the method to measure dissolved urea has to be decided. The manual direct method tends to measure higher urea concentrations than the enzymatic method. Therefore, comparisons of data sets should take into account the analysis procedure employed.

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