

## Measuring urease activity in aquatic environmental samples

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### Abstract

The current published method for measuring urease activity in phytoplankton involves measuring the hydrolysis of urea into ammonium. The method was previously optimized for studies of phytoplankton cultures, not for natural assemblages of phytoplankton. We tested several steps of the method using water samples from two distinct sites to optimize the urease assay method for field studies. We found interference of  $\text{NH}_4^+$  from extraction and assay reagents in the current published protocol, leading to inconsistencies in rates of urease activity. We recommend a reduction in the concentration or removal of two of these reagents. We also found a biomass effect and recommend collecting smaller sample volumes in field studies. These improvements lead to values that may be more reflective of rates of urease activity in situ, but unfortunately more variable due to the low volumes used.

Urea ( $\text{CO}(\text{NH}_2)_2$ ), a small neutral molecule, is a significant source of nitrogen for marine bacteria and phytoplankton in both freshwater and marine environments (Antia et al. 1991; Berman and Bronk 2003; Glibert et al. 2001, 2006). Many phytoplankton taxa use urea and many 'harmful' or 'toxic' species either have higher urea uptake rates than for  $\text{NO}_3^-$  or  $\text{NH}_4^+$  or are stimulated by elevated urea concentrations (Berg et al. 1997; Kudela and Cochlan 2000; Collos et al. 2004; Glibert et al. 2004; Mulholland et al. 2004). Urea was shown to contribute up to 90% of total nitrogen uptake during brown tide blooms of the pelagophyte *Aureococcus anophagefferens* in Shinnecock Bay, New York, and Chincoteague Bay, Maryland, despite higher concentrations of  $\text{NH}_4^+$  than urea (Berg et al. 1997; Mulholland et al. 2004). Elevated urea concentrations in aquaculture ponds were also found to be correlated with blooms of *Karlodinium veneficum* (as *Gyrodinium galatheanum*), *Gyrodinium nelsonii*, *Prorocentrum minimum*, and *Katodinium* sp. (Glibert and Terlizzi 1999). Other harmful species such as the red tide dinoflagellate *Karenia brevis* (Steidinger et al. 1988; Heil et al. 2007), *Lingulodinium polyedrum* (Kudela and

Cochlan 2000), *P. minimum* (Fan et al. 2003) and *Alexandrium catenella* and *A. fundyense* (Dyhrman and Anderson 2003; Collos et al. 2004) have been found to have the capacity to use urea either in culture or in the field.

Within a cell, urea is hydrolyzed by urease. Urease converts urea and water into  $\text{NH}_4^+$  and  $\text{CO}_2$  that are subsequently used by various biochemical pathways. The measurement of urease activity is essential for understanding whether urea, transported into the cell (Antia et al. 1991) or produced internally by catabolism of amino acids or purines (Antia et al. 1991), is assimilated into biomass by microbes. Estimations about the potential contribution of urea to total cellular nitrogen demand for phytoplankton and bacteria can be made using urease activity.

Urease activity can be measured by various techniques (Mobley and Hausinger 1989) but only two are commonly used in the aquatic sciences. The two methods measure different end products of urea catabolism:  $\text{CO}_2$  or  $\text{NH}_4^+$ . The first investigators measured urease activity after addition of  $^{14}\text{C}$ -urea (Leftley and Syrett 1973; Bekheet and Syrett 1977; Ge et al. 1990). Later investigators measured the liberation of  $\text{NH}_4^+$  by various  $\text{NH}_4^+$  analyses (Oliveira and Antia 1986; Jahns et al. 1995; Collier et al. 1999; Peers et al. 2000). The urease assay for culture studies was optimized by Peers et al. (2000) and further improved by Fan et al. (2003).

In recent years, urease activity has been measured in cultures of various species of phytoplankton using the method that measures production of  $\text{NH}_4^+$  from urea catabolism. While relatively few in number, these studies have shown that urease activity is present in the diatoms *Thalassiosira pseudonana* and *T. weissflogii* (Peers et al. 2000; Lomas 2004), dinoflagellates

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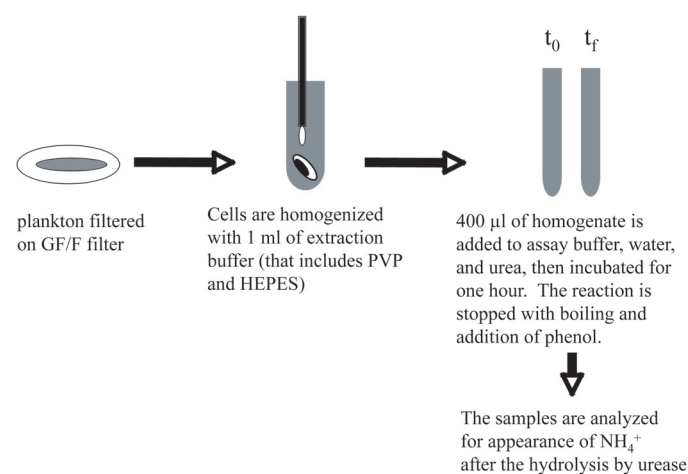
*P. minimum* (Fan et al. 2003), *Alexandrium* sp. (Dyhrman and Anderson 2003), the pelagophyte *A. anophagefferens* (Fan et al. 2003), as well as several other phytoplankton groups (Leftley and Syrett 1973; Antia et al. 1991). Urease activity varies from 0 to ~142 fg-N cell<sup>-1</sup> hr<sup>-1</sup> depending on growth conditions (Dyhrman and Anderson 2003; Fan et al. 2003; Lomas 2004).

To date, there has been relatively little research on urease activity in natural phytoplankton assemblages (e.g., Syrett and Leftley 1973; Dyhrman and Anderson 2003; Fan et al. 2003; Peers et al. 2003; Glibert et al. 2004; Lomas 2004; Heil et al. 2007). Preliminary field studies have hinted at variability in enzyme activity related to volume of sample filtered and other field manipulations. Urease activity in size fractionated samples with a lower chlorophyll *a* (Chl *a*) biomass composed of bacteria and small flagellates was often higher than in whole samples that included larger phytoplankton such as diatoms, suggesting interference from cell metabolites or that the method was not optimal. For example, in the upper Chesapeake Bay in October 2002, urease activity in the <1.6 μm size fraction (0.22 ± 0.38 μM-N h<sup>-1</sup>) was greater than in the whole sample (0.14 ± 0.12 μM-N h<sup>-1</sup>) (Solomon et al. unpubl. data). Hence, a thorough review of each step in the method was warranted.

The goal of this study was thus to refine and optimize the methods of Peers et al. (2000) and Fan et al. (2003) for field application. We investigated whether the high variability associated with manipulations of field samples was the result of steps in the method (NH<sub>4</sub><sup>+</sup> contamination from reagents, interference from buffers, boiling, and storage) or was the result of environmental heterogeneity.

## Materials and Procedures

**Overview of urease assay method**—The urease assay method of Peers et al. (2000) and Fan et al. (2003) first requires collection of phytoplankton and bacteria on GF/F filters (combusted at 450°C for 1 h; <5 mm Hg), which are then stored in liquid N<sub>2</sub> until analysis (Fig. 1). A storage time of a few days or less



**Fig. 1.** A flow chart showing the different treatment steps in the published methods (Peers et al. 2000; Fan et al. 2003).

was recommended in both methods. Urease is extracted from cells by homogenization with a tissue homogenizer in 1 mL of ice-cold extraction buffer (3.75 mL of 1 M potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) buffer adjusted to pH 7.9 with NaOH pellets, 2.5 mL 0.5 M HEPES (n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid) adjusted to pH 7.9 with NaOH pellets, 2.5 mL 3% PVP (polyvinyl pyrrolidone), 2.5 mL 1% Triton-X, 2.5 mL 50 mM EDTA (ethylenediamine-tetraacetic acid disodium salt), 11.25 mL deionized H<sub>2</sub>O for a total of 25 mL). The original extraction buffer had bovine serum albumin (BSA) (Peers et al. 2000) but its removal was recommended by Fan et al. (2003) because of high NH<sub>4</sub><sup>+</sup> contamination. After homogenization, samples are transferred to 1.5 mL centrifuge tubes and centrifuged for approximately 5 min.

The resulting supernatant is divided into two volumes of 0.4 mL that are transferred to two test tubes (t<sub>0</sub> and t<sub>r</sub>). The supernatant is combined with 700 μL of cold assay buffer (2.5 mL 0.5 M HEPES (pH 7.9), 3.75 mL 1 M potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) buffer (pH 7.9), 18.75 mL deionized H<sub>2</sub>O for a total of 25 mL) and 800 μL of deionized water. The t<sub>0</sub> test tubes are put in boiling water for 1 min, then 300 μL of 5 mM urea stock is added, followed immediately by 0.2 mL phenol (20 g phenol in 200 mL of 95% EtOH), the first reagent used in the indophenol method of NH<sub>4</sub><sup>+</sup> determination (Parsons et al. 1984). While the t<sub>0</sub> test tubes are in boiling water, 300 μL of 5 mM urea stock are added to the t<sub>r</sub> tubes, which are then incubated at environmental temperature for 30 min to 1 h. Peers et al. (2000) originally recommended halting the enzymatic reaction by adding 20 mL of HCl, followed by neutralization with 20 mL of 4N NaOH, but the indophenol method is sensitive to pH changes. Fan et al. (2003) suggested removing this step, but also found that boiling did not result in a complete inactivation of the enzyme and recommended immediately adding the phenol reagent to the samples to stop the reaction. After killing with phenol, 2.8 mL of deionized water are added to bring the total volume to 5.2 mL. Finally, the remaining reagents for the NH<sub>4</sub><sup>+</sup> assay (0.2 mL sodium nitroprusside prepared as 1 g of sodium nitroprusside in 200 mL deionized water; 0.5 mL oxidizing reagent prepared as 40 mL of 100 g sodium citrate and 5 g NaOH in 500 mL of deionized water and 10 mL of sodium hypochlorite; Parsons et al. 1984) are added and the test tubes are stored in the dark for a minimum of 2.5 h until analysis on a spectrophotometer at 640 nm.

**NH<sub>4</sub><sup>+</sup> contamination from reagents**—Since NH<sub>4</sub><sup>+</sup> is the end product of the urease assay, contamination of NH<sub>4</sub><sup>+</sup> by assay reagents is of concern. To address this concern, the contribution of NH<sub>4</sub><sup>+</sup> by each of the reagents in the extraction buffer was tested. One mL of each reagent (potassium phosphate buffer, HEPES, PVP, Triton-X, and EDTA) was diluted to 5 mL then analyzed for NH<sub>4</sub><sup>+</sup> concentration (Parsons et al. 1984). To examine further the contribution of PVP to NH<sub>4</sub><sup>+</sup> background levels, 3% PVP was diluted further to 0.3% and 0.03%. PVP was also dialyzed by placing a solution of 3% PVP in 6,000–8,000 MW membrane tubing in deionized water (replaced

several times) for 1 d. One mL of each PVP solution was diluted to 5 mL and analyzed for  $\text{NH}_4^+$  concentrations (Parsons et al. 1984).

To examine whether the contribution of contaminant  $\text{NH}_4^+$  could be reduced and the method would still yield the same results, different extraction buffers containing modified concentrations of PVP were tested. Originally, PVP was added to the method to act as an adsorbent for phenolic compounds that inhibit urease activity (Loomis and Battaile 1966; Peers et al. 2000). Furthermore, the addition of PVP reduced the variability in urease activity (Peers et al. 2000). The standard extraction buffer with 3% PVP was used as a positive control while the extraction buffer without PVP served as a negative control. The remaining three extraction buffers had 0.3% PVP, 0.03% PVP, and dialyzed PVP. Samples were collected from the dock of the Horn Point Laboratory on the Choptank River in July 2003 by filtering 50 mL onto combusted GF/F filters ( $n = 5$ ). Samples were then analyzed for urease activity using 400  $\mu\text{L}$  homogenate with the extraction buffers with varying concentrations of PVP.

**Buffer reagents**—The influence or interference of the extraction and assay buffers on detection of  $\text{NH}_4^+$  was examined in three experiments. The first experiment investigated the effect of each reagent (potassium phosphate buffer, HEPES buffer, 0.03% PVP, Triton X-100, EDTA) on the detection of  $\text{NH}_4^+$ . Two test tubes were used for each reagent: one had 1 mL of reagent diluted to 5 mL while the second had 1 mL of reagent, spiked with 5  $\mu\text{M}$ -N of  $\text{NH}_4^+$ , then diluted to 5 mL. The samples were then measured for  $\text{NH}_4^+$  concentrations (Parsons et al. 1984). The amount of  $\text{NH}_4^+$  detected was determined by subtracting the non-spiked sample from the spiked sample.

The combined effect of the reagents on the detection of  $\text{NH}_4^+$  was determined through a second experiment that tested a series of homogenate volumes (extraction buffer: 100, 200, 300, and 400  $\mu\text{L}$ ) with or without 700  $\mu\text{L}$  of assay buffer that were spiked with 5  $\mu\text{M}$ -N of  $\text{NH}_4^+$ , then diluted to 5 mL with deionized water. The samples were then measured for  $\text{NH}_4^+$  concentrations (Parsons et al. 1984). To further compare the combined effect of the reagents, four  $\text{NH}_4^+$  standard curves were measured in varying homogenate volumes (50, 100, 200, and 400  $\mu\text{L}$ ), but with the same volume of assay buffer (700  $\mu\text{L}$ ), and diluted to 5 mL. Two additional  $\text{NH}_4^+$  standard curves were measured in homogenate (100 and 400  $\mu\text{L}$ ) and assay buffers without HEPES. The seventh  $\text{NH}_4^+$  standard curve measured in deionized water served as a control.

The third experiment tested the specific effect of HEPES on urease activity measured in culture and field samples. Culture samples of the haptophyte *Isochrysis sp.* were obtained from the oyster hatchery at Horn Point Laboratory while field samples were collected from the dock of the Horn Point Laboratory on the Choptank River in March 2006. Samples were filtered (2 mL for culture samples, 75 mL for Choptank samples) onto combusted GF/F filters ( $n = 5$ ), and then analyzed for urease activity using homogenate (100 and 400  $\mu\text{L}$ ) and assay buffers with or without HEPES.

**Boiling**—The boiling step, thought to stop the enzymatic process, was investigated to determine if this step was necessary. In field conditions, manipulation of samples is facilitated if this step can be avoided or simplified. The current method requires putting test tubes in boiling water to stop the hydrolysis reaction by promoting the denaturation of urease, then adding phenol. Phenol (the first reagent for color development for ammonium) also can stop the hydrolysis reaction but is sensitive to the temperature of the samples (Stewart 1985).

To test the effect of boiling, water was collected from the dock of the Horn Point Laboratory in July 2003. River water (50 mL) was filtered onto combusted GF/F filters ( $n = 16$ ), and then analyzed for urease activity using different homogenate volumes with the only modification being in the boiling step. Samples ( $n = 4$  for each homogenate volume) were separated into a pair of  $t_0$  and  $t_f$  test tubes. The  $t_0$  test tubes of two samples were subjected to boiling while the test tubes of the other two samples were not.

**Storage**—Samples from a culture of *Isochrysis sp.* were used to test how long urease samples would remain stable in liquid  $\text{N}_2$  or in an  $-80^\circ\text{C}$  freezer after collection. In May 2004, aliquots of culture ( $n = 60$ ) of 25 mL were filtered onto combusted GF/F filters and kept in liquid  $\text{N}_2$  or in an  $-80^\circ\text{C}$  freezer over a period of three weeks. One set of filters ( $n = 3$ ) was analyzed for urease activity immediately using 0.03% PVP and 100 mL homogenate volume. Using the same protocol, the samples that were stored in liquid  $\text{N}_2$  were measured in triplicate every day for two weeks, then again at d 17 and 21. Samples that were stored in an  $-80^\circ\text{C}$  freezer were only measured in triplicate on a weekly basis (d 7, 14, and 21). Samples from liquid  $\text{N}_2$  storage that were analyzed on d 8–11 were removed from the data set as it was determined that those samples were at the top of the liquid  $\text{N}_2$  dewar and were not kept at the same cold temperatures due to liquid  $\text{N}_2$  evaporation.

**Environmental heterogeneity**—Water was collected from two sites: Choptank River, Maryland (a tributary of the Chesapeake Bay) and Duck Key, Florida (in Florida Bay) for the analysis of optimal filtration volumes and environmental heterogeneity. Experiments to test various filtration volumes and homogenate volumes were run using Choptank River water on two separate days in July 2003, while similar experiments manipulating filtration and homogenate volumes were run on Duck Key water the same month.

The range of filtration volumes tested was different between the two sites due to variable biomass levels, while the homogenate volumes were the same. Filtration volumes in the Choptank samples ranged from 25 mL to 100 mL and from 600 mL to 900 mL in the Florida Bay samples. For comparison between the two sites, filtration volumes were converted into chlorophyll content using the average chlorophyll concentrations over all filtration volumes in Choptank River and Duck Key ( $36.0 \pm 3.81 \mu\text{g Chl } a \text{ L}^{-1}$  and  $0.38 \pm 0.19 \mu\text{g Chl } a \text{ L}^{-1}$ , respectively). Homogenate volumes tested for both sites were the same at 50, 100, 200, and 400  $\mu\text{L}$ . The 400  $\mu\text{L}$  volume is the

**Table 1.** Concentration of  $\text{NH}_4^+$  ( $\pm$ SD) in each reagent in the extraction buffer and  $\text{NH}_4^+$  recovered from a 5  $\mu\text{M-N}$  spike

Reagent	% contribution			% recovery
	$\text{NH}_4^+$ concentration ( $\mu\text{M-N}$ )	$\text{NH}_4^+$ in 1 mL extraction buffer	$\text{NH}_4^+$ recovered ( $\mu\text{M-N}$ )	
HEPES	19.5 $\pm$ 0.18	2.55	-1.39 $\pm$ 1.92	-28
Phosphate Buffer (1M)	3.50 $\pm$ 0.07	0.69	3.10 $\pm$ 0.18	62
3% PVP	733 $\pm$ 22.4	95.8	6.55 $\pm$ 1.49	131*
Triton X-100	2.23 $\pm$ 0.05	0.06	4.68 $\pm$ 0.16	94
EDTA	6.63 $\pm$ 0.18	0.87	2.86 $\pm$ 1.56	57

\* for  $\text{NH}_4^+$  recovery, 0.03% PVP was used

original homogenate volume used in the methods of Peers et al. (2000) and Fan et al. (2003).

### Assessment

*NH<sub>4</sub><sup>+</sup> contamination from reagents*—The  $\text{NH}_4^+$  concentration in all reagents except 3% PVP was  $<20 \mu\text{M-N}$  (Table 1). While the  $\text{NH}_4^+$  concentrations of the reagents were high when measured independently, their contribution, with the exception of 3% PVP, to the final extraction buffer was lower ( $<2 \mu\text{M-N}$ ). PVP, with a  $\text{NH}_4^+$  concentration of  $733 \pm 22.4 \mu\text{M-N}$ , contributed 95.8% to the background  $\text{NH}_4^+$  in 1 mL of extraction buffer. Reduced concentrations of PVP led to lower  $\text{NH}_4^+$  concentrations (0.3% PVP =  $70.8 \pm 3.72 \mu\text{M-N}$ , 0.03% PVP =  $7.37 \pm 0.48 \mu\text{M-N}$  and dialyzed PVP =  $10.8 \pm 0.47 \mu\text{M-N}$ ).

Urease activity varied in extraction buffers with different concentrations of PVP, thus different  $\text{NH}_4^+$  background concentrations. Urease activity was significantly lower in the standard extraction buffer with higher  $\text{NH}_4^+$  concentrations than in the extraction buffer with 0.03% PVP (ANOVA, Tukey-Kramer HSD;  $P < 0.05$ ) (Table 2). Despite lower variance in urease activity in the extraction buffer with dialyzed PVP than with 0.03% PVP, the urease activities assayed with both buffer preparations ( $0.68 \pm 0.12 \mu\text{M-N h}^{-1}$  for dialyzed PVP;  $0.59 \pm 0.19 \mu\text{M-N h}^{-1}$  for 0.03% PVP) were not significantly different ( $P > 0.05$ ).

*Buffer reagents*—In theory,  $\text{NH}_4^+$  concentrations in the  $t_0$  and  $t_f$  samples should increase linearly with increasing homogenate volume if there is no interference with the urease enzyme or the indophenol-blue analytical method for

**Table 2.** Urease activity in extraction buffers containing different concentrations of PVP

Extraction buffer (EB)	Urease activity $\pm$ SD ( $\mu\text{M-N h}^{-1}$ )
Standard EB	0.23 $\pm$ 0.015
No PVP	0.29 $\pm$ 0.09
0.3% PVP	0.44 $\pm$ 0.11
0.03% PVP	0.59 $\pm$ 0.19
Dialyzed PVP (6-8,000 MW)	0.68 $\pm$ 0.12

detecting  $\text{NH}_4^+$ . An observed non-linear relationship in both  $t_0$  and  $t_f$  samples (Fig. 2) led to an investigation of whether reagents in this urease assay method were interfering with the indophenol-blue method for detecting  $\text{NH}_4^+$ .

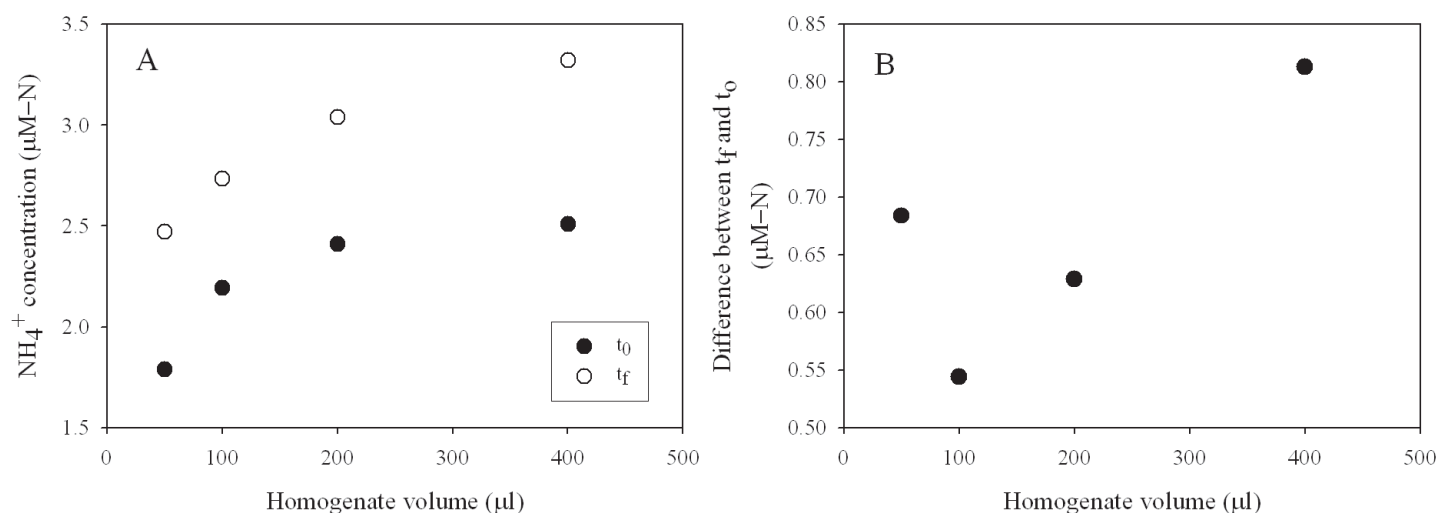
The reagents in the extraction and assay buffers had different effects on the ability to detect  $\text{NH}_4^+$  using the indophenol-blue method of Parsons et al. (1984). The HEPES buffer had a negative effect and none of the spiked  $\text{NH}_4^+$  was detected (Table 1). The other reagents, except PVP, had a less strong effect on the detection of the spiked  $\text{NH}_4^+$ . PVP was the only reagent that led to an amplification of the spiked  $\text{NH}_4^+$ . Overall, no reagent had a neutral effect on the detection of the spiked  $\text{NH}_4^+$ .

The combined effect of all the reagents in the buffer was investigated by testing a series of homogenate volumes with or without assay buffer. The amount of  $\text{NH}_4^+$  recovered from the spike increased when the homogenate volume was reduced from 400  $\mu\text{L}$  to 100  $\mu\text{L}$  in samples without assay buffer while the amount of  $\text{NH}_4^+$  recovered was unchanged with variable volumes of homogenate in samples with assay buffer (Fig. 3). The interference from the different reagents in the buffers was minimized at the lowest homogenate volume (100  $\mu\text{L}$ ).

The effect of the buffers was investigated further by comparing  $\text{NH}_4^+$  standard curves in deionized water and in different buffers. The  $\text{NH}_4^+$  standard curve measured in deionized water and without HEPES buffer was different than the other four standard curves with HEPES buffer (Fig. 4). The  $\text{NH}_4^+$  standard curve in deionized water resulted in a linear regression with a slope of 0.083 and y-intercept of 0.029. The regression of the  $\text{NH}_4^+$  standard curve in 50  $\mu\text{L}$  homogenate and 700  $\mu\text{L}$  assay buffer resulted in a lower slope of 0.050 and higher y-intercept of 0.145. As the homogenate volume increased, the slope decreased and the y-intercept increased.

HEPES had an influence on urease activity in both culture and field samples (Fig. 5). Rates of urease activity in HEPES buffer using the published method were almost two-fold lower with higher variability than in potassium phosphate buffer alone for *Isochrysis sp.* Rates of urease activity in samples from Choptank River were higher with lower variability using revised methods that either had a lower homogenate volume or no HEPES buffer than using the published methods. Urease activity in the Choptank River samples was not significantly different with or without HEPES when 100  $\mu\text{L}$  homogenate was used along with an appropriate ammonium standard curve.

A clear conclusion that can be made from the four experiments above is the interference of the HEPES buffer on the detection of  $\text{NH}_4^+$ . First, HEPES buffer had a negative detection of spiked  $\text{NH}_4^+$  while the potassium phosphate buffer did not (Table 1). Second, the HEPES concentration increased with increasing homogenate volume. The increase in homogenate volume led to a decrease in the detection of  $\text{NH}_4^+$  (Fig. 3), and smaller  $\text{NH}_4^+$  standard curve slopes (Fig. 4). Furthermore, urease activity measured in buffers without HEPES was higher than with HEPES (Fig. 5). These results agree with Peers et al. (2000) who suggested removing HEPES buffer, which was orig-



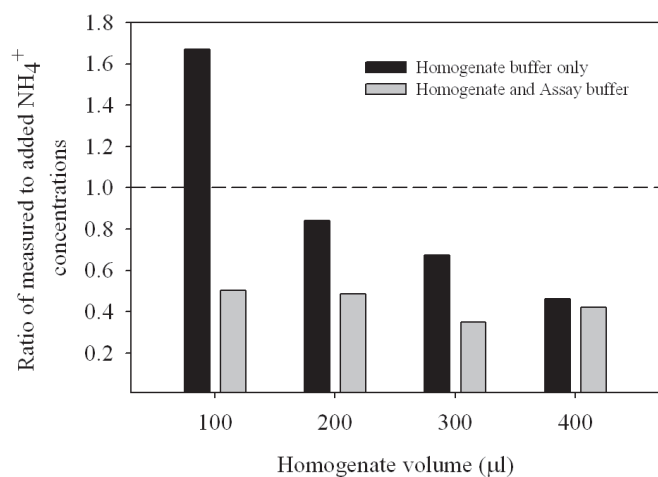
**Fig. 2.** (A) Concentration of  $\text{NH}_4^+$  in the assayed  $t_0$  and  $t_f$  samples and (B) difference in concentration of  $\text{NH}_4^+$  between assayed  $t_f$  and  $t_0$  and samples as a function of different homogenate volumes of a 25 mL filtered sample (using 0.03% PVP) from the Choptank River.

inally recommended by Mobley and Hausinger (1989), after observing lower activity in cultures of the diatom, *T. pseudonana*, in HEPES buffer than in potassium phosphate buffer. Therefore, the determination of urease activity (e.g., the detection of  $\text{NH}_4^+$ ) is best achieved by using lower homogenate volumes or the complete removal of HEPES buffer.

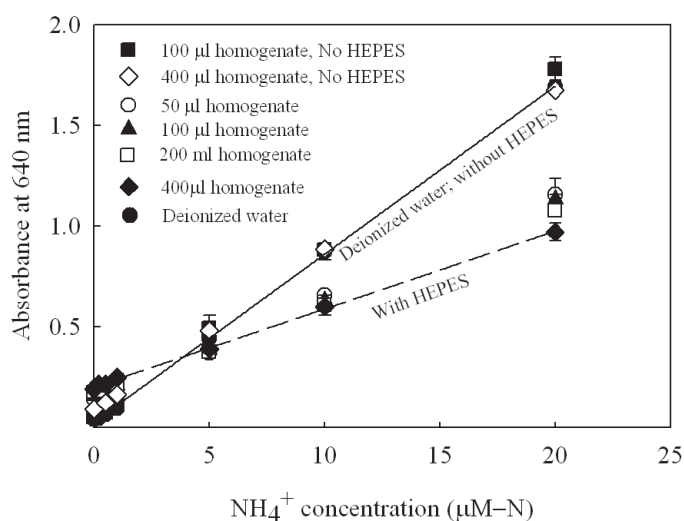
**Boiling**—Urease activity averaged over all homogenate volumes was higher in boiled samples than in non-boiled samples. Urease activity in the lower homogenate volumes of 50 and 100  $\mu\text{L}$  were significantly different between the boiled ( $0.66 \pm 0.15 \mu\text{M-N h}^{-1}$ ) and non-boiled samples ( $0.40 \pm 0.16 \mu\text{M-N h}^{-1}$ ; ANOVA, Tukey-Kramer HSD,  $P < 0.02$ ) while there was no significant difference in the higher homogenate volumes of 200

and 400  $\mu\text{L}$  ( $P > 0.37$ ; data not shown). The difference in  $\text{NH}_4^+$  concentration between the  $t_f$  and  $t_0$  boiled samples was on average higher than in the non-boiled samples ( $0.78 \pm 0.56 \mu\text{M-N h}^{-1}$  versus  $0.49 \pm 0.56 \mu\text{M-N h}^{-1}$ ).

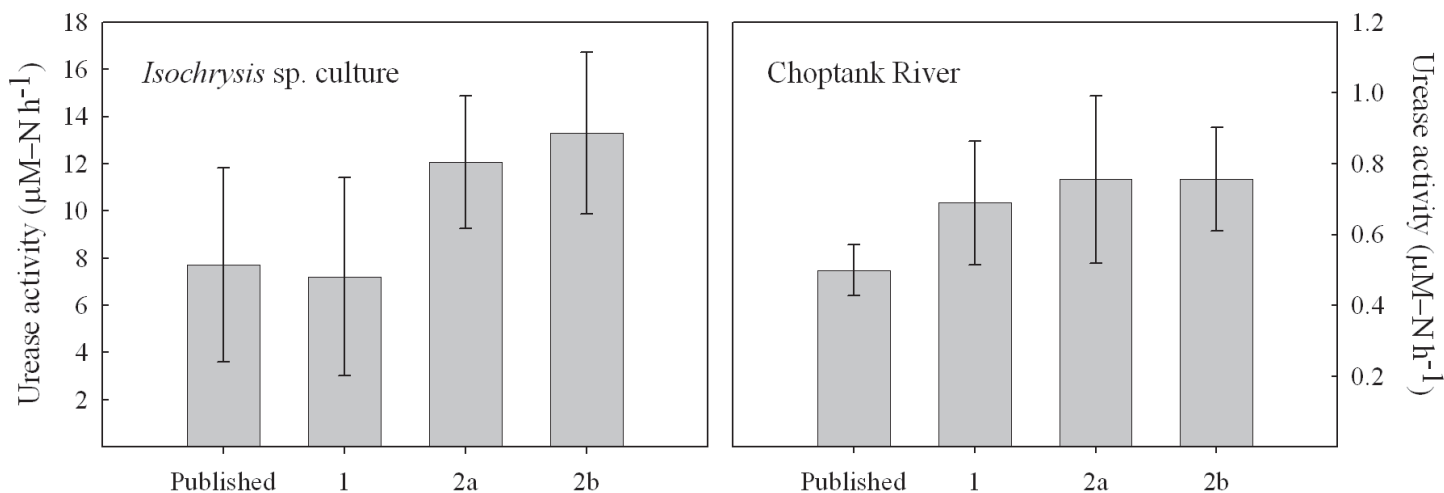
**Storage**—Urease activity of samples stored in liquid nitrogen remained at the same level (grand mean:  $5.83 \pm 0.71 \mu\text{M-N h}^{-1}$ ) for three weeks (Fig. 6). Urease activity was not significantly different (two-way ANOVA; Tukey-kramer HSD;  $P > 0.05$ ) between samples stored in both liquid  $\text{N}_2$  and in an  $-80^\circ\text{C}$  freezer the first, second, or third week (d 7, 14, and 21). The results suggest that urease samples can be preserved in either liquid  $\text{N}_2$  or an  $-80^\circ\text{C}$  freezer for at least up to three weeks.



**Fig. 3.** Ratio of measured to added  $\text{NH}_4^+$  concentrations in homogenate only and with assay buffer as a function of different homogenate volumes. The dotted line at 1.0 represents when the measured concentration of  $\text{NH}_4^+$  equaled the amount of  $\text{NH}_4^+$  added to the buffers.



**Fig. 4.**  $\text{NH}_4^+$  standard curves in deionized water and varying volumes of homogenate buffer and 700  $\mu\text{L}$  of assay buffer with and without HEPES buffer. Regression lines are shown for deionized water and 400  $\mu\text{L}$  homogenate volume with HEPES. Each point in the standard curves represents the average of the samples ( $n = 3$ ) with error bars ( $\pm\text{SD}$ ) removed for clarity.



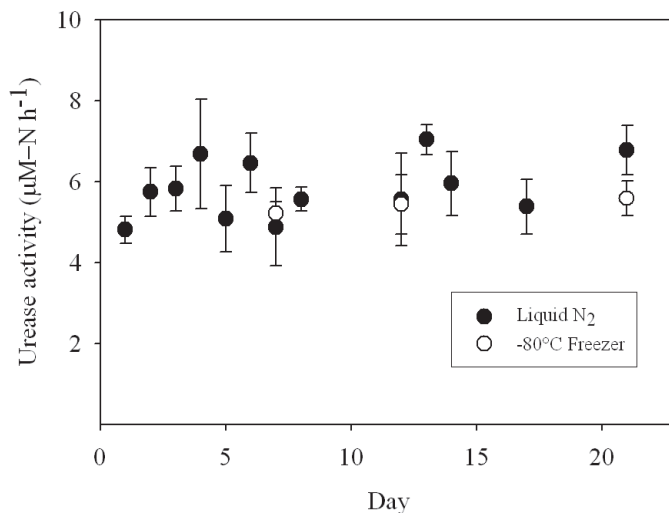
## Methods

**Fig. 5.** Comparison of urease activity values ( $\pm$ SD,  $n = 5$ ) obtained by using the published method (Fan et al. 2003) and revised methods with (1) 0.03% PVP, 100  $\mu$ L homogenate with HEPES buffer or 0.03 PVP, 100  $\mu$ L (2A) and 400  $\mu$ L (2B) homogenate without HEPES buffer in both *Isochrysis sp.* cultures and the Choptank River in March 2006.

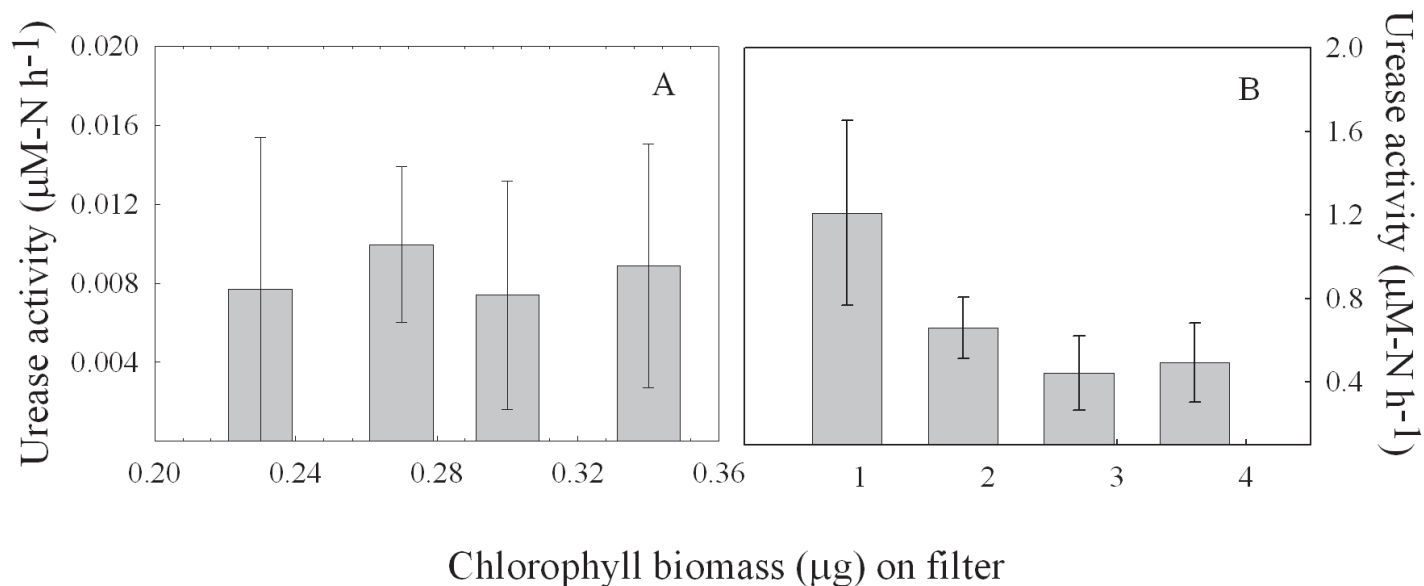
*Environmental heterogeneity*—Rates of urease activity were generally the highest at the lowest filtration and homogenate volumes, but the variability was much higher than at the highest filtration and homogenate volumes. Urease activity over a range of filtration volumes (averaged over all homogenate volumes) followed different trends in Duck Key, Florida than in Choptank River, Maryland. Samples from Duck Key, with a low chlorophyll biomass, did not have any statistical difference in urease activity among different filtration volumes (ANOVA, Tukey-Kramer HSD; Fig. 7A). Samples from Choptank River had higher chlorophyll biomass and decreasing average urease activity was observed with increasing biomass per sample filter (Fig. 7B). The only significant difference was between 25 mL (0.90  $\mu$ g Chl *a*) and 50 mL (1.80  $\mu$ g Chl *a*) filtration volumes (ANOVA, Tukey-HSD,  $P < 0.05$ ). The decrease in urease activity with increasing biomass may be due to interference from increasing concentrations of intracellular  $\text{NH}_4^+$  or other cell metabolites. The lowest filtration volume (25 mL; 0.90  $\mu$ g Chl *a*) with the highest urease activity was also adequate to run routine chlorophyll analyses.

Average urease activity decreased over increasing homogenate volumes at both sites (Fig. 8) which was consistent with earlier experiments. In Duck Key, urease activity decreased from 0.012 to 0.003  $\mu\text{M-N h}^{-1}$  as the homogenate volume increased from 50 to 400  $\mu\text{L}$ . Urease activities in the lowest homogenate volumes (50 and 100  $\mu\text{L}$ ) were not statistically different (ANOVA, Tukey-Kramer HSD,  $P = 0.80$ ). Urease activity in the homogenate volume of 100  $\mu\text{L}$ , which had a lower variance than 50  $\mu\text{L}$ , was significantly different than in the previously recommended homogenate volume of 400  $\mu\text{L}$

( $P < 0.05$  when the 50  $\mu\text{L}$  homogenate samples were removed from the data set). In the Choptank River, the decrease in activity was also four-fold as the homogenate volume increased. The urease activity between the 50 and 100  $\mu\text{L}$  volumes was significantly different ( $P < 0.05$ ) and urease activity in both volumes was significantly different than in the 400  $\mu\text{L}$  homogenate volume ( $P < 0.05$  and  $P = 0.06$ , respectively). Urease activity also followed the same trends when normalized for chlorophyll or carbon, as would be expected (data not shown).



**Fig. 6.** Average urease activity ( $\pm$ SD) in stored samples ( $n = 3$ ) at various time points over a 3 week period. Samples were stored in liquid  $\text{N}_2$  and in a  $-80^\circ\text{C}$  freezer.



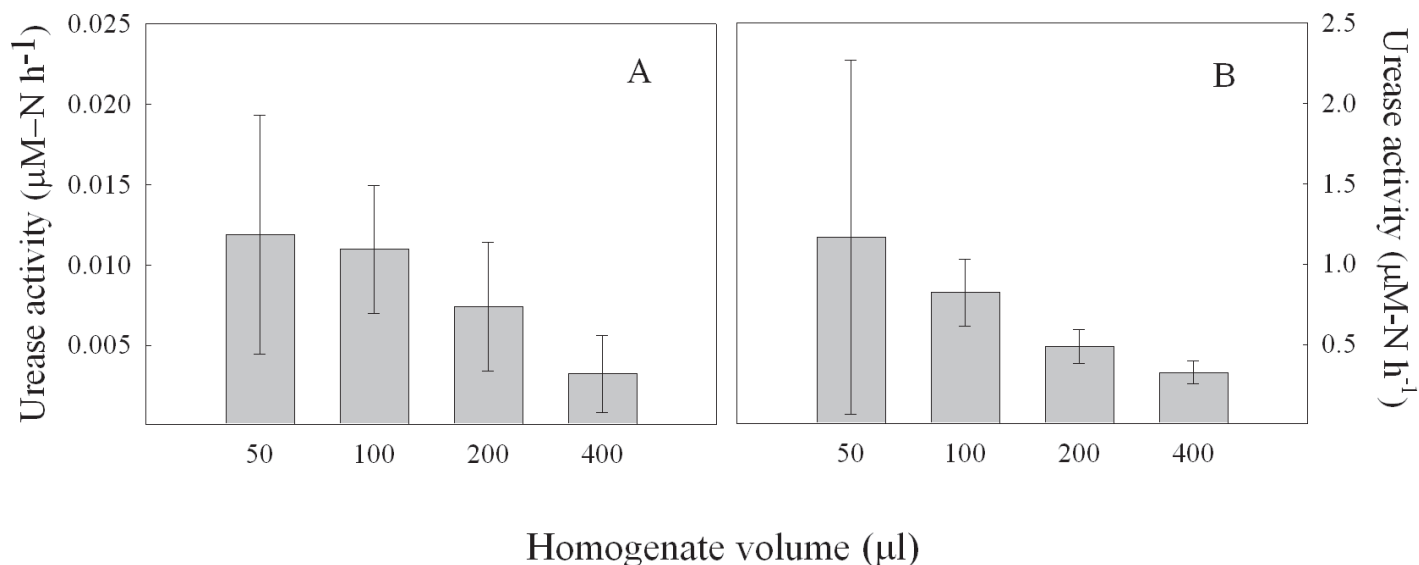
**Fig. 7.** Urease activity measured with different filtration volumes (normalized to chlorophyll biomass) averaged over all homogenate volumes in A) Duck Key in Florida Bay and B) Choptank River. Note difference in scale for urease activity.

The similarity in pattern of urease activity over increasing homogenate volumes between homogenate with cells (Fig. 8) and detection of  $\text{NH}_4^+$  in buffers without cells (Fig. 3) and  $\text{NH}_4^+$  standard curves (Fig. 4) suggests that the effects on the urease assay are not environmental but rather from the HEPES buffer on the indophenol-blue method for detecting  $\text{NH}_4^+$ .

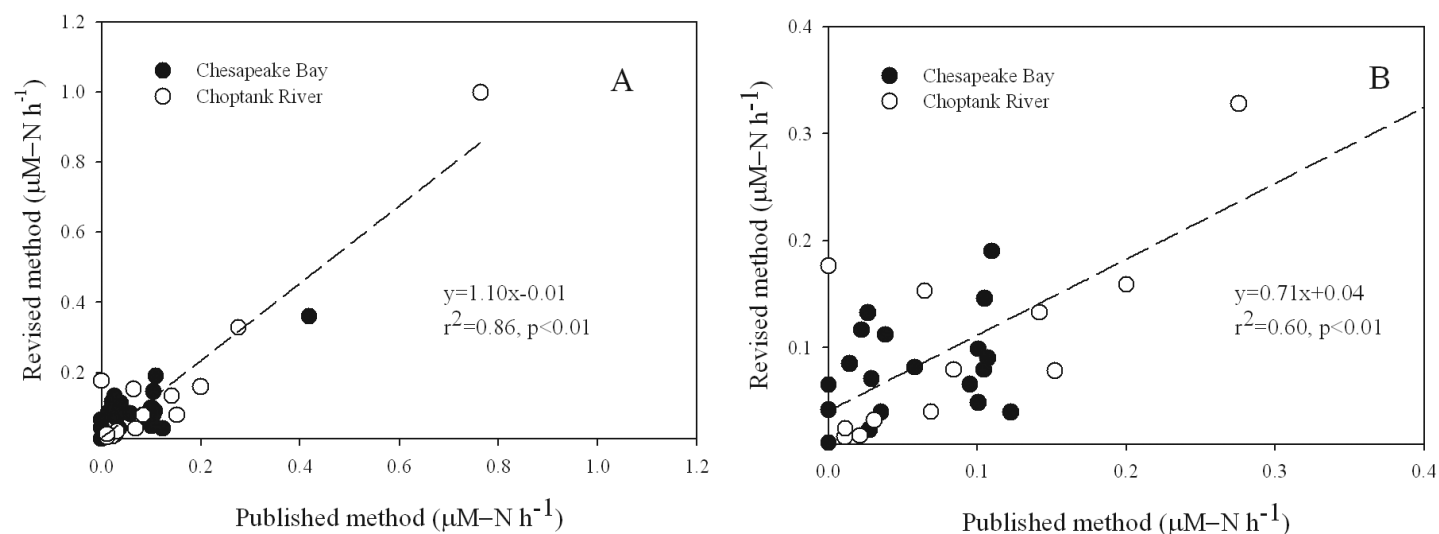
#### Suggested Modifications to the Method

Based on the data shown above, we suggest several modifications to Peers et al. (2000) and Fan et al. (2003) methods, the

only methods currently available for this analysis. First, the percentage of PVP used should be reduced from 3% to 0.03% to reduce background  $\text{NH}_4^+$ . The reduction of background  $\text{NH}_4^+$  is important in order to resolve lower levels of urease activity. Second, the  $\text{NH}_4^+$  standard curve should be measured in the same matrix as the assay, not in deionized water. Third, HEPES should not be used as a buffer, or 100 µL homogenate volumes should be used to minimize interference from the HEPES buffer. Fourth, the amount filtered onto GF/F filters should not exceed the minimum amount of seawater required for routine chlorophyll



**Fig. 8.** Urease activity measured with different homogenate volumes averaged over all filtrate volumes in A) Duck Key in Florida Bay and B) Choptank River. Note difference in scale for urease activity.



**Fig. 9.** (A) Comparison of urease activity values obtained by the published methods (Peers et al. 2000; Fan et al. 2003) and one of the revised methods (0.03% PVP, 100  $\mu\text{L}$  homogenate with HEPES buffer) on the same samples collected from Choptank River or Chesapeake Bay in April, July, August 2004. (B) Same data focusing on samples  $<0.4 \mu\text{M-N h}^{-1}$ .

analyses to minimize the biomass effect that may be a result of higher interference from cellular metabolites. With those modifications, results from natural samples should better reflect in situ field values and assay artifacts would be minimized.

Comparisons can be made between the published method (3% PVP, 400  $\mu\text{L}$  homogenate) to values obtained by one of the revised methods suggested here (0.03% PVP, 100  $\mu\text{L}$  homogenate; Fig. 9). Measured values by the previous method are similar to the ones in the revised method over a range of urease activity from undetectable to  $1.0 \mu\text{M-N h}^{-1}$  (Fig. 9A). However, a fundamental difference is the difference in interference with detection of  $\text{NH}_4^+$  and minimization of the biomass effect. Most urease activity is  $<0.2 \mu\text{M-N h}^{-1}$  with small differences between  $t_f$  and  $t_0$  and may be overestimated with the published method, thus it is important to measure  $\text{NH}_4^+$  concentrations accurately with low variability (Fig. 9B). Other methods that effectively measure  $\text{NH}_4^+$  at nanomolar concentrations (eg, Brzezinski 1987; Holmes et al. 1999) with different reagents should be tested in the near future. Until then, variability may be a reality, requiring more replicates to be performed.

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