

ABSTRACT

Title of Document:

**REGULATION OF ESTUARINE
PHYTOPLANKTON AND BACTERIAL
UREA UPTAKE AND UREASE ACTIVITY
BY ENVIRONMENTAL FACTORS**

Caroline Miller Solomon, Ph.D., 2006

Directed By:

Professor Patricia M. Glibert
Marine Estuarine Environmental Science

The regulation by environmental factors of two enzymes involved with urea utilization – urea transport and urease – in estuarine phytoplankton and bacteria was studied in cultures of five phytoplankton species, in Chesapeake Bay and Choptank River assemblages, and in bioassay and mesocosm experiments. In these experiments, temperature and nitrogen availability (NO_3^- , NH_4^+ , and urea) were found to regulate urea uptake and urease activity. However, regulation by these environmental factors was dependent on the composition of the plankton community.

Dinoflagellates were found to have the highest urease activity in culture among five phytoplankton species (*Prorocentrum minimum*, *Karlodinium veneficum*, *Heterocapsa triquetra*, *Storeatula major*, and *Isochrysis sp.*) in culture on a per cell and per cell volume basis with an optimized method to measure urease activity. Urease activity was also lower when the dinoflagellates were grown on NH_4^+ than when grown on NO_3^- or urea, suggesting repression by NH_4^+ .

Higher rates of urea uptake and urease activity in Chesapeake Bay and the Choptank River were often associated with the presence of dinoflagellates and cyanobacteria during the warmer months. Rates were also higher under N-limitation when these phytoplankton were present than under P-limitation when diatoms were present. Rates of urea uptake and urease activity in natural assemblages were repressed when NO_3^- and NH_4^+ concentrations exceeded 40 and 5 $\mu\text{g at N l}^{-1}$, respectively.

Rates of urea uptake and urease activity decreased in response to additions of NH_4^+ in bioassay and mesocosm experiments. In these experiments, dinoflagellates had the highest urea uptake and urease activity on a per cell basis while cyanobacteria had the highest urea uptake and urease activity on a per cell volume or per chlorophyll *a* basis. The difference in regulation of urea uptake and urease activity among the diatoms, dinoflagellates and cyanobacteria provide some biochemical explanations about how they utilize urea under contrasting environmental conditions.

REGULATION OF ESTUARINE PHYTOPLANKTON AND BACTERIAL UREA
UPTAKE AND UREASE ACTIVITY BY ENVIRONMENTAL FACTORS

By

Caroline M. Solomon

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2006

Advisory Committee:

Professor Patricia M. Glibert, Chair

Professor Diane K. Stoecker

Research Associate Professor Jeffrey C. Cornwell

Professor Bess B. Ward, Princeton

Assistant Professor Jackie L. Collier, SUNY-Stony Brook

Professor Daniel Terlizzi, Dean's Representative

© Copyright by
Caroline M. Solomon
2006

Dedication

I dedicate this dissertation to my loving and supportive family.

Acknowledgements

I must first thank Dr. James J. McCarthy of Harvard University who introduced me to two things: the field of biological oceanography and my doctoral advisor, Dr. Patricia M. Glibert. The first introduction sparked my interest and imagination. The second made it possible for me to develop as a scientist. I cannot thank Pat enough for the dedication, sensitivity and expertise that she brought to her work with me. She is, without doubt, the most supportive advisor a doctoral student could ever dream of having.

This dissertation would have not been possible without the unwavering support from my colleagues at the Biology Department at Gallaudet University, Horn Point Laboratory (HPL), my doctoral committee members and my family.

Dr. Ann Powell, Chair of the Gallaudet University Biology Department, showed endless flexibility in arranging my teaching schedule around my University of Maryland class, lab and field schedule for six years. Ann, along with my other departmental colleagues, Dr. Derek Braun, Dr. Jane Dillehay, and Dr. Kathy Arnos, were wonderful ‘cheerleaders’ along the way, rooting for me to finish my dissertation. I especially thank Kathy for allowing me to stay at her Cambridge home during my early years at HPL.

The faculty, staff, and students at Horn Point Laboratory were warm and welcoming during my time in Cambridge. They were patient and wonderfully supportive in obtaining communication access for me in classes and seminars. I appreciate their grace in understanding my need to obtain Power Point presentations ahead of time, letting me know when a seminar was scheduled and putting up with

periodic audio disconnections with the captioner. Anne Willey was instrumental in setting up the technology to make remote captioning possible. My heartfelt thanks goes to Dr. Michael Roman, the director of HPL, and to Pat for finding funding to cover interpreting and captioning expenses so that I could have a comprehensive graduate student experience.

I would like to thank my other committee members (Dr. Jackie Collier, Dr. Jeff Cornwell, Dr. Diane Stoecker, and Dr. Bess Ward) for challenging me through my comprehensive exam questions and pushing me to scrutinize and analyze my dissertation data closely. As a result, I have learned a great deal and have more questions now than when I began my dissertation. I also appreciate Dr. Daniel Terlizzi's willingness to serve as my Dean's Representative.

Analysis of samples and running experiments would have not been possible without the help of people in the Glibert Lab (Jeff Alexander, Cindy Shoemaker, Dr. Marta Revilla, and Ji Li) and Analytical Services (Lois Lane, Crystal Thomas and Laurie Van Heukelem) at HPL. I greatly appreciate the statistical advice that Dr. David Kimmel, also of HPL, gave me on PCA analyses. I thank Danielle Schmidt from Princeton University for providing her bacterial count data, and Dr. Jason Adolf at University of Maryland's Center of Marine Biotechnology for running CHEMTAX analyses. Dr. Micheal Lomas of the Bermuda Biological Station for Research (BBSR) gladly provided historical urea concentrations and urea uptake rates for the Chesapeake Bay. Dr. Byron Crump was instrumental in my education in molecular techniques. My undergraduate students from Gallaudet University, Nyas Dyckman and Heather McIntosh, helped out tremendously with the bioassay experiments.

My dissertation work would have not been possible without the funding support from the National Science Foundation (NSF) Biocomplexity Program, the American Association for University Women (AAUW) Fellowship, Gallaudet University Biology Department, Gallaudet University Small Research Grants, Gallaudet University Alumni Association Fellowship, Horn Point Laboratory Small Student Grants, Horn Point Laboratory Bridge Funds, NASA DC Space Consortium Fellowship.

Last but not least, I want to thank my entire family because they were my biggest source of support and inspiration. My father, an economics professor who understands the trials and tribulations of doing a dissertation, and my mother, my tireless editor, both provided support and encouragement. My husband, Barry, my biggest supporter, deserves the most thanks as he often had to deal with long absences while I did lab and field work. I now wish my brother, Jeff, the best of luck in finishing his own dissertation in Politics from Oxford University.

TABLE OF CONTENTS

Dedication	ii
Acknowledgements	iii
TABLE OF CONTENTS	vi
List of Tables	x
List of Figures.....	xi
Chapter 1: Introduction and Background.....	1
Introduction.....	2
Overview of cellular pathways of nitrogen utilization	3
Biochemical pathways of nitrogen utilization	4
The role of urea in estuarine ecosystems	6
Sources of urea.....	7
Biochemistry and regulation of urea uptake	9
Biochemistry and regulation of urease activity	12
Research Questions and Approaches	13
Primary Research Objectives	13
Chapter 2: A revised method to measure urease activity.....	15
Chapter 3: Regulation of urease by nitrogen sources in five phytoplankton species	15
Chapters 4 and 5: Seasonal and spatial patterns in urea uptake and urease activity in Chesapeake Bay and Choptank River.....	16
Chapters 6 and 7: Examination of regulation of urea uptake and urease activity in response to addition of nitrogen to biossays or mesocosms	17
Chapter 8: Summary and research conclusions	17
Literature cited	19
Table	28
Figures.....	29
Chapter 2: Measuring urease activity in environmental samples.....	34
Abstract	35
Introduction.....	35
Methods.....	38
Overview of urease assay method.....	38
NH ₄ ⁺ contamination from reagents	39
Buffers.....	40
Boiling.....	42
Storage	42
Environmental heterogeneity and effect of sample volume	43
Assessment.....	44
NH ₄ ⁺ contamination from reagents	44

Buffers.....	44
Boiling.....	47
Storage	47
Environmental heterogeneity and effect of sample volume	48
Suggested modifications to the method	49
Literature cited	51
Tables	54
Figures.....	56
Chapter 3: Urease activity in five phytoplankton species	64
Abstract	65
Introduction.....	65
Methods.....	68
Species studied.....	68
Culture conditions.....	69
Phytoplankton and bacteria biomass.....	69
Nutrient analyses.....	70
Urease activity	70
Data analysis	71
Results.....	73
Growth rates and biomass.....	73
Biochemical state of cells	74
Urease activity	74
Discussion.....	76
Urease activity and nitrogen demand.....	78
A brief review and conceptual model of urease regulation	80
Ecological implications.....	81
Conclusion	82
Literature cited.....	83
Tables	89
Figures.....	95
Chapter 4: Microbial urea uptake and urease activity in Chesapeake Bay	100
Abstract	101
Introduction.....	101
Methods.....	104
Field sampling.....	104
Hydrological data.....	104
Nutrients.....	104
Plankton composition and biomass.....	105
Urea uptake and urease activity	107
Statistical analysis	108
Results.....	109
Physical features	109
Dissolved nutrient distributions	110
Plankton composition and distribution	112

Urea uptake and urease activity rates.....	114
Relationships between physical factors, nitrogen availability, phytoplankton community composition and urea uptake and urease activity	115
Discussion.....	117
Seasonal changes in rates of urea uptake and urease activity.....	117
Regulation of urea uptake and urease activity by NO_3^- and NH_4^+ availability.....	118
Urea uptake and urease activity in different microbial assemblages	120
Gradient in urea uptake and urease activity rates	122
Chesapeake Bay: A historical perspective.....	123
Conclusion	125
Literature cited.....	126
Tables.....	132
Figures.....	136
Chapter 5: Urea utilization in Choptank River	147
Abstract.....	148
Introduction.....	148
Methods.....	150
Field sampling and hydrological data.....	150
Nutrients.....	151
Plankton composition and biomass.....	151
Urea uptake and urease activity	152
Statistical analyses	153
Results.....	154
Hydrological features.....	154
Dissolved nutrient distributions	154
Plankton distribution.....	155
Urea uptake and urease activity rates.....	156
Relationships between urea uptake, urease activity and environmental factors.....	157
Discussion.....	159
Differences between Upper and Lower Choptank.....	159
Influence of plankton composition on urea uptake and urease activity at two depths	160
Influence of NO_3^- and NH_4^+ availability on urea uptake and urease activity ...	161
Urea dynamics in the Choptank River	161
Possible impacts of urea-based agricultural fertilizers in the Choptank River.....	162
Conclusion	163
Literature cited.....	165
Tables.....	169
Figures.....	172
Chapter 6: Responses of urea uptake and urease activity to NH_4^+ additions in short-term bioassays	179
Abstract.....	180
Introduction.....	180
Methods.....	181

Plankton biomass measurements	182
Urea uptake and urease activity	183
Results	183
Discussion and Conclusion	184
Literature cited	186
Tables	189
Figures	191
Figures	191
Chapter 7: Urea uptake and urease activity under manipulated phosphorus and nitrogen conditions: a mesocosm study	193
Abstract	194
Introduction	194
Methods	196
Nutrient measurements	197
Plankton biomass measurements	197
Urea uptake and urease activity measurements	198
Genetic diversity	199
Statistical analyses	200
Results	201
Discussion	204
N- vs. P-limitation	206
Conclusion	207
Literature cited	208
Tables	212
Figures	213
Chapter 8: Summary and Research conclusions	219
Temperature	220
Nitrogen availability	221
Difference in regulation among phytoplankton taxonomic groups	222
Complexity in urea utilization	224
Comparison of field activity of enzymes involved in nitrogen assimilation	225
Future directions for understanding urea regulation in phytoplankton and bacteria	226
Literature cited	228
Table	231
Figures	233
Complete Literature Cited	237

List of Tables

1.1	Enzymes responsible for the conversion of various nitrogen substrates into NH_4^+	28
2.1	Concentration of NH_4^+ in each reagent in the extraction buffer and NH_4^+ recovered from a $5 \mu\text{g-at N l}^{-1}$ spike	54
2.2	Urease activity in extraction buffers containing different concentrations of PVP	55
3.1	Growth rates of the five phytoplankton species grown on NO_3^- , NH_4^+ , and urea	89
3.2	Intracellular NH_4^+ and urea concentrations, N content of cells and contribution of NH_4^+ and urea to total cellular N content of phytoplankton grown on different nitrogen sources	90
3.3	Average urease kinetic parameters, $V_{max-cell}$ ($\text{fg at N cell}^{-1} \text{ h}^{-1}$) and K_m ($\mu\text{g at N l}^{-1}$), for all five phytoplankton species	91
3.4	Comparison of urease activity kinetic parameters among different phytoplankton species in culture	92-95
4.1	Average streamflow, temperature and salinity at three Chesapeake Bay stations	132
4.2	Distribution of nitrogen and phosphate in the Chesapeake Bay during spring, summer and fall	133
4.3	Loadings for PC1, PC2, and PC3 for each Chesapeake Bay station	134
4.4	Regression coefficients and t-values for multiple regression models of urea uptake and urease activity at all three Chesapeake Bay Stations	135
5.1	Average streamflow, salinity and temperature in surface and bottom waters at both stations in the Choptank River	169
5.2	Loadings for PC1, PC2, and PC3 for each Choptank station	170
5.3	Regression coefficients and t-values for multiple regression models of urea uptake and urease activity at two Choptank River stations	171
6.1	Ambient concentrations of NO_3^- , NH_4^+ , urea and PO_4^{3-} for the bioassay experiments	189
6.2	Regression statistics for relationships between urea uptake and urease activity and NH_4^+ concentrations during three months in 2003	190
7.1	Sampling dates for nutrient additions, urea uptake, urease activity and genetic diversity in the mesocosm experiment	212
8.1	Comparison of field rates of enzymatic activities involved with nitrogen assimilation at various locations around the world	231-232

List of Figures

1.1	Division of molecular and biochemical regulation of nitrogen assimilation pathways in a phytoplankton cell	29
1.2	Various nitrogen assimilation pathways in a microbial cell	30
1.3	Intracellular pathways in a microbial cell involved with urea utilization and assimilation	31
1.4	Sources of urea and organisms that utilize urea in estuarine waters	32
1.5	Intracellular pathways in a microbial cell involved with urea utilization and assimilation	33
2.1	Concentration of NH_4^+ in the assayed t_0 and t_f samples as a function of different homogenate volumes of a 25 ml filtered sample (using 0.03% PVP) from the Choptank River	56
2.2	Concentration of NH_4^+ recovered in homogenate only and with assay buffer as a function of different homogenate volumes	57
2.3	NH_4^+ standard curves in deionized water and varying volumes of homogenate buffer and 700 μl of assay buffer with and without HEPES buffer	58
2.4	Comparison of urease activity values obtained by using the published method (Fan et al. 2003) and revised methods with 0.03% PVP, 100 μl homogenate with HEPES buffer or 0.03 PVP, 100 μl and 400 μl homogenate without HEPES buffer in both <i>Isochrysis sp.</i> cultures and the Choptank River in March 2006	59
2.5	Urease activity in stored samples over a three week period. Samples were stored in liquid N_2 and in a -80°C freezer	60
2.6	Urease activity measured with different filtration volumes (normalized to chlorophyll biomass) averaged over all homogenate volumes in Duck Key in Florida Bay and Choptank River	61
2.7	Urease activity measured with different homogenate volumes averaged over all filtrate volumes in Duck Key and Choptank River	62
2.8	Comparison of urease activity values obtained by measuring the published methods (Peers et al. 2000; Fan et al. 2003) and one of the revised methods with 0.03% PVP, 100 μl homogenate with HEPES buffer on the same samples collected from Choptank River or Chesapeake Bay in July 2003 and April, July, August 2004	63

3.1	Kinetic curves of urease activity per cell in five different phytoplankton species as function of urea concentration (on a per cell basis)	95
3.2	Kinetic curves of urease activity per cell in five different phytoplankton species as function of urea concentration (on a per cell volume basis)	96
3.3	Kinetic curves of urease activity per cell in five different phytoplankton species as function of urea concentration (on a per chlorophyll <i>a</i> basis)	97
3.4	Comparison of potential urease activity versus nitrogen demand calculated on an hourly basis for seven phytoplankton species	98
3.5	A comparison of biochemical regulation of enzymes involved with nitrogen acquisition (i.e. urease and nitrate reductase) in diatoms and dinoflagellates under two different environmental conditions	99
4.1	The five sampling sites in Chesapeake Bay and Sargasso Sea	136
4.2	Nutrient concentrations as function of month and year of sampling at three stations in the Chesapeake Bay during 2001-2004	137
4.3	Chl <i>a</i> biomass and bacterial abundance as function of month and year of sampling at three stations in the Chesapeake Bay during 2001-2004	138
4.4	Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage, as analyzed by CHEMTAX, as function of season and year of sampling during 2001-2004 at each Chesapeake Bay station	139
4.5	Normalized urea uptake and urease activity at three stations in Chesapeake Bay as function of month and year of sampling during 2001-2004	140
4.6	Contribution of the <1.6 μm fraction to total urea uptake or urease activity as function of month and year of sampling at the three stations in Chesapeake Bay during 2001-2004	141
4.7	Urea uptake rates and urease activity, normalized on a volumetric basis and chl <i>a</i> biomass basis, over a spatial gradient in Chesapeake Bay and the Sargasso Sea	142

4.8	The difference between urea uptake and urease activity rates from all stations in Chesapeake Bay, plume and the Sargasso Sea as a function of month and year of sampling during 2001-2004	143
4.9	Principal component analysis of temperature, salinity, nitrogen availability, and relative abundance of five phytoplankton taxonomic groups at three Chesapeake Bay stations from 2001-2004	144
4.10	Urea uptake and urease activity as function of phytoplankton:bacterial biomass in all Chesapeake Bay stations	145
4.11	Comparisons of surface urea concentrations and urea uptake rates during 2001-2004 with data collected prior to 1998 from Lomas et al. (2002)	146
5.1	Tonnage of urea fertilizer bought in the state of Maryland from 1989-2004	172
5.2	The two sampling stations in the Choptank River	173
5.3	Nitrogen concentrations (NO_3^- , NH_4^+ , and urea) and DIN:DIP ratios at the Upper Choptank station during 2001-2004 in both surface and bottom waters as a function of season and year	174
5.4	Nitrogen concentrations (NO_3^- , NH_4^+ , and urea) and DIN:DIP ratios at the Lower Choptank station during 2001-2004 in both surface and bottom waters as a function of season and year	175
5.5	Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage, as analyzed by CHEMTAX, during 2001-2004 at the Upper stations in both surface and bottom waters as a function of season and year	176
5.6	Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage, as analyzed by CHEMTAX, during 2001-2004 at the Upper stations in both surface and bottom waters as a function of season and year	177
5.7	Principal component analysis of temperature, salinity, nitrogen availability and relative abundance of six phytoplankton taxonomic groups at two Choptank River stations from 2001-2004	178
6.1	Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage, as analyzed by CHEMTAX, in the	191

bioassays during November 2002, March 2003, May 2003 and November 2003

6.2	Urea uptake and urease activity on a per chl <i>a</i> basis as function of NH_4^+ concentration	192
7.1	Nitrogen concentrations (NO_3^- , NH_4^+ , urea), DIN:DIP, and DON:DOP ratios in all N treatments as function of day of the experiment	213
7.2	Chl <i>a</i> biomass and phytoplankton composition in all mesocosms as function of day of experiment	214
7.3	Multidimensional scaling diagram of Dice distance matrix calculated from DGGE banding patterns of both prokaryotes and eukaryotes	215
7.4	Urea uptake and urease activity on both a volumetric and per μg chl <i>a</i> basis	216
7.5	Percentage of urea assimilated into the protein fraction on Days 5, 9, and 17 over one hour in each N treatment	217
7.6	A schematic synthesis of events during the mesocosm experiment	218
8.1	Urea uptake and urease activity as function of NO_3^- and NH_4^+ concentration	233
8.2	Comparison of urease activity between different phytoplankton species on a per cell or per cell volume basis	234
8.3	Comparison of urease activity in diatoms grown on different N substrates	235
8.4	Comparison of urease activity in dinoflagellates grown on different N substrates	236

Chapter 1: Introduction and Background

Introduction

Phytoplankton and bacteria control the amount of carbon transferred to higher trophic levels in marine ecosystems. Phytoplankton remove 30 to 50% of atmospheric CO₂ and transform it into particulate organic carbon (Siegenthaler and Sarmiento 1993). Bacteria utilize approximately one-half of the primary production (in terms of carbon) by phytoplankton that is later incorporated into the microbial loop (Azam 1998). The size, species, and growth rates of phytoplankton and bacteria determine how much carbon is exported or grazed by protozoa and zooplankton (Rivkin et al. 1996). These rates of phytoplankton and bacteria production directly depend on the supply of nutrients such as nitrogen (Kirchman 2000, Zehr and Ward 2002), phosphorus (Kirchman 1994, Lomas et al. 2004), and vitamins and trace metals (Martin et al. 1991, Reid and Butler 1991, Schulz et al. 2004). At times, phytoplankton and bacteria may experience co-limitation by two nutrients or one nutrient and a trace metal (Arrigo 2005, Shaked et al. 2006). Phytoplankton and bacteria convert dissolved forms of nutrients, minerals, and trace metals into cellular material, including proteins, carbohydrates, lipids, and nucleic acids (Falkowski and Raven 1997, del Giorgio and Cole 2000, Geider and LaRoche 2002), which are important for its own cellular growth, metabolism and production.

In most aquatic ecosystems, certain nutrients and trace metals are in limited supply, controlling rates of growth and production of phytoplankton and bacteria. Under nitrogen-limiting conditions that dominate during summer in areas near the mouth of temperate estuaries (Glibert et al. 1995, Fisher et al. 1999, Holmboe et al. 1999, Yin et al. 2001, Kemp et al. 2005), phytoplankton and bacteria compete for

nitrogenous substrates such as NO_3^- , NO_2^- , NH_4^+ , urea, and dissolved organic nitrogen (DON; Kirchman 2000). Which nitrogen substrate is utilized by phytoplankton or bacteria depends on energy costs of utilization, substrate concentrations, input of external energy (e.g. sunlight), as well as species-specific preferences (Falkowski and Raven 1997, Lomas and Glibert 1999, Capone 2000, Parker and Armbrust 2005). Phytoplankton generally prefer NH_4^+ to other nitrogen substrates because it is energetically favorable to utilize reduced forms of nitrogen (Dortch and Postel 1989, Capone 2000), although diatoms may prefer NO_3^- under some conditions (Collos et al. 1997, Lomas and Glibert 1999). Heterotrophic bacteria primarily use dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), and NH_4^+ before they use NO_3^- in order to reduce energetic costs of assimilation (Kirchman 2000). In estuarine ecosystems concentrations of NO_3^- and NH_4^+ are generally higher than those of other nitrogenous substrates, such as urea and other forms of dissolved organic nitrogen (e.g. urea, DFAA, DCAA; Antia et al. 1991). As a result, measuring NO_3^- and NH_4^+ uptake by phytoplankton and heterotrophic bacteria has received more attention than urea or DON uptake. This dissertation focuses on the utilization of urea, one of the less-studied nitrogenous substrates in estuarine ecosystems.

Overview of cellular pathways of nitrogen utilization

Nitrogen utilization pathways involve enzymes responsible for transport, conversion to NH_4^+ , and assimilation. These enzymes may be regulated on both biochemical and molecular levels (Fig. 1.1). Phytoplankton and bacteria that lack

genes for enzymes that are involved in the assimilation of inorganic or organic nitrogen cannot use these substrates. For instance, some strains of the cyanobacteria *Prochlorococcus* (MED4, MIT9313) that do not contain genes required for NO_3^- utilization such as NO_3^- permease (*napA*) and NO_3^- reductase (*narB*) cannot grow on NO_3^- (Moore et al. 2002). Another strain, SS120, lacks the urease gene (*ure*) (Dufrense et al. 2003).

The presence of genes required is a necessary but not a sufficient condition for a cell to utilize a particular form of nitrogen. In addition to the presence of the gene, the expression of the gene as mRNA is required. The mRNA is then translated into an enzyme responsible for a step in nitrogen utilization. The enzyme becomes active and performs its task in the nitrogen utilization pathway. Some phytoplankton contain the appropriate gene, yet lack the ability to utilize a nitrogen source. For example, a strain of *Synechococcus* (SH-94-5) contains both NO_2^- and NO_3^- reductase (*nir*, *nar*) genes, but they are not expressed (Miller and Castenholz 2001). Since regulation happens at each step in a process, it is vital to study regulation of both the activity and expression of those enzymes. However, only regulation of activity was studied in this dissertation.

Biochemical pathways of nitrogen utilization

Nitrogen utilization by phytoplankton and bacteria is *at least* a two-step enzymatic process regulated by light (Ohki et al. 1992, Falkowski and Raven 1997), temperature (Lomas and Glibert 1999a, Fan et al. 2003a, Parker and Armbrust 2005) and nitrogen concentrations (Wheeler et al. 1982, Glibert et al. 1991, Lomas and

Glibert 1999b, Lomas 2004) (Fig. 1.2). First, the nitrogen substrate must be transported across the cell membrane by either passive or active transport (Antia et al. 1991, Siewe et al. 1998, Beckers et al. 2004). After reaching the inside of the cell, the nitrogen substrate must be converted into NH_4^+ which is later incorporated into proteins by the enzymes, glutamine synthase (GS, *glnA*) and glutamine 2-oxoglutarate aminotransferase (GOGAT; also known as glutamate synthase; Wheeler 1983, Capone 2000). Nitrogen substrates are converted into NH_4^+ by a third (or a series of) enzyme(s). For example, the enzymes NO_3^- reductase (*narB*, *nasA*) and NO_2^- reductase (*nir*) convert NO_3^- to NO_2^- then NH_4^+ . (Table 1.1, Fig 1.2).

Utilization of urea in phytoplankton and bacteria is controlled by several biochemical pathways (Fig 1.3). Urea is transported into the cell by active or passive cell-membrane transport enzymes or produced internally as a by-product of the urea cycle and amino acid catabolism (Antia et al. 1991, Mobley and Hausinger 1989, Siewe et al. 1998, Beckers et al. 2004) or purine catabolism (McInich et al. 2003, Allen et al. 2005, Berg and Jørgensen 2006). Intracellular urea is then broken down into NH_4^+ and CO_2 by the enzyme urease (*ure*) (Antia et al. 1991, Mobley and Hausinger 1989, Zehr and Ward 2002) or by a series of enzymes in the UALase pathway (ATP: urea amidolyase; Antia et al. 1991, Hausinger 2004). The cell then uses the NH_4^+ produced by catabolism of urea for protein synthesis (Wheeler 1983, Capone 2000).

The role of urea in estuarine ecosystems

Urea concentrations in estuarine ecosystems are generally low ($< 1 \mu\text{g at N l}^{-1}$) (Lomas et al. 2002, Glibert et al. 2005), but have often been found to be higher in coastal waters, estuaries and tributaries (Glibert et al. 2005, 2006). Despite low urea concentrations, phytoplankton urea uptake rates can account for more than 50% of total nitrogen uptake, and can be greater than NH_4^+ and NO_3^- uptake rates (McCarthy 1972, Kaufman et al. 1983, Glibert et al. 1991, Bronk et al. 1998, Kudela and Cochlan 2000).

Heterotrophic bacteria, depending on environmental conditions, can be either consumers or producers of urea. However, rates of urea uptake or production are often variable and unpredictable (Jørgensen 2006). In a transect in the Gulf of Riga, bacteria produced 53% of the urea near the shore and progressively became consumers off-shore, utilizing 20% of the urea (Jørgensen et al. 1999). Bacteria can have low urea uptake rates ($< 3\%$) compared to total nitrogen uptake rates (Wheeler and Kirchman 1986, Middelburg and Nieuwenhuize 2000). Despite low urea uptake rates, heterotrophic bacteria can be responsible for a small ($< 10\%$) (Cho and Azam 1995, Cho et al. 1996) or large percentage (86%) of total urea uptake (Middelburg and Nieuwenhuize 2000). Benthic bacteria are important producers of urea (Pedersen et al. 1993, Cho et al. 1996, Therkildsen et al. 1997). Urease activity been studied to a lesser degree than urea uptake, but has been detected in both phytoplankton and bacteria (Antia et al. 1991, Mobley and Hausinger 1989, Nolden et al. 2000).

Recently, elevated urea concentrations ($>1 \mu\text{g at N l}^{-1}$) at several sites in the Chesapeake Bay (Glibert et al. 2001, 2004, 2005), Long Island Sound (Berg et al. 1997) and aquaculture ponds (Glibert and Terlizzi 1999) have been linked with increases in biomass of harmful algal species. Higher urea concentrations in estuarine waters may be a result of increasing use of urea-based fertilizers which now constitute more than 50% of the nitrogen fertilizers worldwide that become part of runoff during heavy rainfall (Soh 2001, Glibert et al. 2001, 2006). Urea is also a primary decomposition product of poultry manure also used as a fertilizer (Glibert et al. 2004, 2006). These two sources, along with input from sewage, probably contribute most of the urea to estuarine waters near the predominately agricultural regions of Chesapeake Bay and Choptank River which were studied in this dissertation. The link between increasing urea concentrations and frequency of harmful algal blooms is becoming a concern for scientists and environmental managers worldwide.

Sources of urea

Urea that is used by phytoplankton and bacteria can come from both extracellular and intracellular sources (Fig. 1.4). Urea is present in the surrounding water in concentrations of approximately $1 \mu\text{g-at N l}^{-1}$ due to natural sources of urea such as atmospheric deposition (Cornell et al. 1995, Peierls and Paerl 1997, Mace et al. 2003), or regeneration by heterotrophic bacteria and macro- and micro-zooplankton (Pedersen et al. 1993, Cho et al. 1996, Therkildsen et al. 1997, Miller and Glibert 1998, L'Helguen et al. 2005). Heterotrophic bacteria can be net

producers of urea in sediments (Pedersen et al. 1993, Cho et al. 1996, Therkildsen et al. 1997). Copepods such as *Acartia tonsa* excrete more urea during night-time hours (Miller and Glibert 1998) while microheterotrophs such as ciliates can excrete enough urea to meet phytoplankton requirements (L'Helguen et al. 2005). Benthic macrofauna are also producers of urea that diffuses upwards from sediments (Lomstein et al. 1989).

Urea is produced intracellularly in most organisms by either the urea cycle or by purine catabolism (Antia et al. 1991, Allen et al. 2005, Berg and Jørgensen 2006). The purpose of the urea cycle is to produce arginine and to excrete excess nitrogen produced by α -keto amino acid catabolism (e.g. glutamate, proline, arginine, and histidine; Garrett and Grisham 1995). Urea is produced when arginine is converted into ornithine by arginase (Fig. 1.3). The addition of amino acids or urea cycle intermediates to estuarine bioassays results in release of urea (Jørgensen et al. 1999, Berman et al. 1999). The discovery of the urea cycle genes in a phototroph, the diatom *Thalassosira pseudonana*, was a surprise because urea is not considered as a 'waste' product in those organisms (Armbrust et al. 2004). Likewise, cyanobacteria contain some of the urea cycle genes such as carbamoyl phosphate synthetase and arginase, but not a complete set of genes in their genomes (CyanoBase; www.kazusa.or.jp/cyano/). However, intermediates from the urea cycle were found to contribute to other aspects of diatom metabolism (Figure 1.5; Armbrust et al. 2004, Allen et al. 2005). The urea produced via the urea cycle either goes into a nitrogen reservoir or serves as osmolytes (Allen et al. 2005). Another intracellular source of urea is via the catabolism of purines into urea and glyoxylate by the enzyme

ureidoglycolate urea-lyase (McInich et al. 2003, Allen et al. 2005). The addition of the purines, guanine and hypoxanthine, to estuarine bioassays resulted in an increase of urea concentrations (Berman et al. 1999, Berg and Jørgensen 2006). Because phytoplankton and bacteria have several intracellular sources of urea, it is possible that they may exhibit low urea uptake rates and some urease activity.

Biochemistry and regulation of urea uptake

Urea, as a small neutral molecule, can be transported into the cell via passive diffusion, but can also be transported via active cell-membrane transporters (Antia et al. 1991). Passive transport occurs mostly at high urea concentrations that are mostly not seen in estuarine waters. For example, passive transport occurred at urea concentrations $> 70 \mu\text{M}$ while active transport occurred at lower urea concentrations in the green alga *Chlamydomonas reinhardtii* (Hodson et al. 1975). Likewise, in the bacterium *Corynebacterium glutamicum*, passive transport occurred at concentrations up to 50 mM (Siewe et al. 1998)

Active transport of urea is dependent on energy obtained from photophosphorylation (Rees and Syrett 1979, Siewe et al. 1998, Beckers et al. 2004), so rates tend to increase during the day and decrease during the night. A diel pattern was observed in Chesapeake Bay (Bronk et al. 1998), with the lowest rates during the night and the highest rates in mid-afternoon. Urea uptake also increased with increasing irradiance during a *Prorocentrum minimum* bloom in the Choptank River in spite of the fact that urea uptake in this species is not light-dependent on short time scales (30 min, Fan and Glibert 2005).

Significant urea uptake can take place during the dark in dinoflagellate-dominated assemblages (Kudela and Cochlan 2000, Fan and Glibert 2005) as well as in diatoms (*Melosira italica*; Cimberlis and Cáceres 1991). In the Chesapeake Bay plume, urea uptake also represented a larger proportion of total nitrogen utilization during the night than during the day during different seasons (Glibert et al. 1991). Production of energy (e.g. ATP) via oxidative phosphorylation is prevented by the addition of KCN, leading to inhibition of dark urea uptake (Cimberlis and Cáceres 1991). Because of significant uptake during dark periods in some species, it is not yet clear how urea uptake rates are regulated by light in different phytoplankton taxonomic groups.

Temperature has a positive influence on urea uptake rates. In contrast to NO_3^- , NH_4^+ and urea uptake rates have a positive relationship with temperature (5-30°C) in diatom-dominated assemblages (Lomas and Glibert 1999). Maximum urea uptake rates occur during the summer while minimum rates occur during the winter in various temperate estuaries (Kristiansen 1983, Glibert et al. 1991, Bronk et al. 1998, Lomas et al. 2002), but this may simply be due to seasonal changes in phytoplankton composition. Urea uptake increased as a function of temperature in the diatom, *Melosira italica*, with a Q_{10} coefficient of 1.94 (Cimberlis and Cáceres 1991). However, urea uptake remained constant over a narrow temperature range of 10 to 25°C for *Prorocentrum minimum* in the Chesapeake Bay and Neuse Estuary (Fan et al. 2003a). These findings suggest that the positive relationship between temperature and urea uptake may depend on species composition during various seasons.

Urea uptake rates may be influenced by nitrogen availability, especially NO_3^- and NH_4^+ , in both cultures and field incubations. Urea uptake rates of phytoplankton grown under nitrogen-starved or urea-replete conditions decrease after the addition of NH_4^+ and/or NO_3^- to cultures (Rees and Syrett 1979, Lund 1987, Lomas 2004). Most culture studies have focused on the response of diatoms when grown on these substrates or when the substrates are added to the culture. Urea uptake in the diatom, *Phaeodactylum tricornutum*, ceased after 24h in a NH_4^+ medium (Rees and Syrett 1979). Inhibition of urea uptake by NH_4^+ in various phytoplankton, including *P. tricornutum*, ranged from 8-34% (Molloy and Syrett 1988). In another experiment that investigated the effects of additions of different nitrogen substrates on ^{15}N -urea uptake in the diatom, *Skeletonema costatum*, Lund (1987) observed a decrease of 82-84% in urea uptake 3h after the addition of either NO_3^- and NH_4^+ or both. Lomas (2004) observed no changes in diel patterns of urea uptake in the cultures of the diatom, *Thalassiosira weissflogii*, after the additions of NO_3^- and NH_4^+ . However, urea uptake rates were lower in the culture that was grown on NO_3^- than NH_4^+ .

Urea uptake rates generally decrease in field incubations after the addition of NH_4^+ or when ambient NH_4^+ concentrations are high. In field incubations of Baltic seawater, urea uptake rates decreased after the addition of NH_4^+ but not after the addition of NO_3^- (Tamminen and Irmisch 1996). Furthermore, urea turnover rates did not immediately increase or decrease after nutrient additions. After 24h, however, there was a clear inhibition by the NH_4^+ addition. Urea uptake is inhibited or repressed by NH_4^+ concentrations higher than 1-2 $\mu\text{g at N l}^{-1}$ in Oslofjord, Norway (Kristansen 1983) and 40 $\mu\text{g at N l}^{-1}$ in the Neuse Estuary, NC (Twomey et al. 2005).

The results of these studies suggest that the suppression of urea uptake by NO_3^- and NH_4^+ may occur in both phytoplankton and bacteria.

Biochemistry and regulation of urease activity

Urease activity has been found in a diverse range of organisms including heterotrophic bacteria (Jahns 1992), cyanobacteria (Collier et al. 1999), diatoms (Peers et al. 2000, Lomas 2004), dinoflagellates (Dyhrman and Anderson 2003, Fan et al. 2003a), pelagophytes (Fan et al. 2003a), bangiophytes, chrysophytes, cryptophytes, euglenophytes, eustigmatophytes, phaeophytes, prasinophytes, and prymnesiophytes (see review by Antia et al. 1991).

Urease activity in phytoplankton and bacteria is carried out mostly by the cytoplasmic urease enzyme (urea amidohydrolase) that has one, two or three peptides of which sequences are highly conserved (Mobley et al. 1995, Collier et al. 1999). A minority of species of phytoplankton (e.g. Chlorophytes) and bacteria catabolize urea by UALase (Antia et al. 1991, Hausinger 2004) or by both urease and UALase (Hausinger 2004). Prokaryotic ureases have been found to be more closely related to each other than plant ureases (Todd and Hausinger 1987). In addition, ureases are nickel-requiring metalloenzymes (Mobley and Hausinger 1989, Collier et al. 1999). Often, urease activity is enhanced with the addition of Ni^{2+} (such as in the cultures of the diatom, *Cyclotella cryptica*; Oliveira and Antia 1991).

Urease activity, like urea uptake, appears to have a positive relationship with temperature, in part because it is a heat-stable enzyme (Mobley and Hausinger 1989). In the only study that investigated temperature effects on urease activity, urease activity generally increased in three phytoplankton species over a range of *in vitro*

temperatures in laboratory cultures (0-50°C). The study concluded that the optimal *in vitro* temperature for urease activity in the pelagophyte *Aureococcus anophagefferens* (~50°C) was higher than for the diatom *Thalassiosira weissflogii* and the dinoflagellate *P. minimum* (~20°C; Fan et al. 2003a).

Urease activity has been found to be present regardless of nitrogen source but differs among each nitrogen source (Antia et al. 1991, Collier et al. 1999, Peers et al. 2000, Dyhrman and Anderson 2003, Lomas 2004), suggesting that urease activity may be regulated. A clear example of regulation of urease activity was seen in the dinoflagellate *Alexandrium fundyense*, where urease activity was the highest in a urea grown culture, lower in NH_4^+ , and not detected in a NO_3^- grown culture (Dyhrman and Anderson 2003). Urease activity in *A. fundyense* was also derepressed by N-starvation (Dyhrman and Anderson 2003). Another dinoflagellate, *P. minimum*, had lower urease activity rates when grown on NH_4^+ than on NO_3^- and urea (Fan et al. 2003a). Yet, in the diatom *T. weissflogii*, different forms of regulation were seen. Urease activity was the same regardless of nitrogen source in one clone (Peers et al. 2000), but down-regulated in another clone when grown on NO_3^- (Fan et al. 2003a, Lomas 2004). The conflicting results suggest that urease activity is either regulated by nitrogen sources or constitutively expressed in different species of phytoplankton.

Research Questions and Approaches

Primary Research Objectives

The role of urea in estuarine ecosystems is beginning to be appreciated by the oceanography community (e.g. Glibert et al. 2006). Urea can be an important

source of nitrogen for phytoplankton or bacterial metabolism (Antia et al. 1991, Berman and Bronk 2003), especially under N-limiting conditions in coastal and estuarine waters (Bronk et al. 1998, Heil et al. *in press*). The main focus of this dissertation is to examine the biochemical regulation of urea uptake and urease activity by temperature and nitrogen substrates (e.g. NO_3^- , NH_4^+ and urea) in order to understand how urea is utilized by phytoplankton and bacteria in estuarine ecosystems. The regulation of urea uptake and urease activity was studied under laboratory (Chapter 3), field (Chapters 4, 5), and manipulated conditions (Chapters 6, 7).

Urea uptake and urease activity were hypothesized to be regulated by temperature and nitrogen substrates differently among phytoplankton and bacterial taxonomic groups, leading to seasonal and spatial pattern in rates in Chesapeake Bay and Choptank River. First, urea uptake and urease activity were predicted to increase with temperature among all phytoplankton taxonomic groups. This would result in higher rates during the summer. Second, nitrogen availability, especially NO_3^- and NH_4^+ , was expected to repress urea uptake and urease activity due to less demand for NH_4^+ by the phytoplankton cell via the urea uptake/urease pathway. Repression of urea uptake and urease activity by NO_3^- and NH_4^+ would lead to increasing rates from the Upper Chesapeake Bay to the Sargasso Sea as NO_3^- and NH_4^+ concentrations decreased. The degree and pattern of regulation by NO_3^- and NH_4^+ was also anticipated to differ between phytoplankton taxonomic groups – especially the diatoms and dinoflagellates.

Chapter 2: A revised method to measure urease activity

In order to assess urease activity, it was first necessary to examine and optimize the methodology (Peers et al. 2000, Fan et al. 2003a). This was prompted by observations that urease activity rates measured in the <1.6 μm fraction using the currently published methods were often higher than in whole samples, suggesting potential inference by reagents or cellular metabolites. In **Chapter 2**, different aspects of the urease activity method were scrutinized to find whether NH_4^+ contamination from reagents, interference from buffers on the indophenol assay for measuring NH_4^+ , or changes in the phytoplankton or bacterial biomass was responsible for the discrepancy. The simplification of two steps (boiling and longer storage times in liquid N_2) were also examined to provide ease in performing the method. In the end, substantial changes were made to the method. The comparison between the published and revised methods showed that previous measurements of urease activity (e.g. Fan et al. 2003a, Dyhrman and Anderson 2003, Lomas 2004) were most likely underestimated.

Chapter 3: Regulation of urease by nitrogen sources in five phytoplankton species

The regulation of urease activity by nitrogen sources in phytoplankton has only been examined in a few species that span few taxonomic groups. Prior to this research, levels of urease activity were measured only in the bacillariophytes (diatoms), dinophytes (dinoflagellates), pelagophytes, and cyanobacteria (Collier et al. 1999, Peers et al. 2000, Dyhrman and Anderson 2003, Fan et al. 2003a, Lomas

2004). The urease gene has been detected in many species of other taxonomic groups but the rates of urease activity under different conditions are unknown (Bruhn et al. 2002, Collier and Baker 2004). Experiments in **Chapter 3** investigated whether three dinoflagellates (*P. minimum*, *Karolodinium veneficum* and *Heterocapsa triquetra*), a cryptophyte (*Storeatula major*), and a haptophyte (*Isochrysis sp.*) had high levels of urease activity and whether the activity was regulated by nitrogen sources. *P. minimum* and *K. veneficum* were found to have higher urease activity rates than the other three species when measured on a per cell basis and also on a per cell volume basis. This chapter concludes with a conceptual synthesis of cellular regulation. It appears that dinoflagellate and cyanobacteria ureases are regulated by NH_4^+ while diatom urease is regulated by NO_3^- . Ureases of species in other taxonomic groups, such as the cryptophytes and the haptophytes, do not seem to be regulated by nitrogen sources.

Chapters 4 and 5: Seasonal and spatial patterns in urea uptake and urease activity in Chesapeake Bay and Choptank River

Most work done on urease activity prior to this dissertation was conducted in phytoplankton and bacterial cultures. Natural rates of urease activity in microbial assemblages were relatively unknown. One of the first field rates of urease activity came from the western Gulf of Maine where urease activity was found to be higher during an *Alexandrium sp.* bloom than during preceding periods (Dyhrman and Anderson 2003). **Chapters 4 and 5** advance the study of urease activity rates in natural assemblages. Urea uptake and urease activity rates were measured over a

seasonal and spatial gradient in Chesapeake Bay and Choptank River to investigate the regulation of urea utilization by temperature and nitrogen availability in different microbial assemblages. The highest urea uptake and urease activity rates in both bodies of water were associated with warmer temperatures, lower NO_3^- and higher urea availability and the presence of dinoflagellates, cyanobacteria and cryptophytes.

Chapters 6 and 7: Examination of regulation of urea uptake and urease activity in response to addition of nitrogen to bioassays or mesocosms

Urea uptake and urease activity rates measured in laboratory or the field, reported in Chapters 3-5, are from microbial assemblages adapted or acclimated to the cultural medium or environmental conditions in which they are found. In **Chapter 6**, different phytoplankton and bacterial communities were ‘challenged’ by NH_4^+ additions to see how quickly urea uptake or urease activity responded within a 3h time frame. Likewise in **Chapter 7**, different phytoplankton and bacterial communities were ‘challenged’ by NO_3^- , NH_4^+ and urea additions in a 3-wk mesocosm experiment. Urea uptake and urease activity (on a volumetric basis) in the mesocosms were the highest during a mixed bloom of diatoms and dinoflagellates. When cyanobacteria were present in the mesocosms towards the end of the experiment, the highest urea uptake and urease activity (on a per chl *a* basis) were observed.

Chapter 8: Summary and research conclusions

The last chapter (**Chapter 8**) is a synthesis of what has been learned about regulation of urea uptake and urease activity by temperature and nitrogen availability

with a focus on diatoms, dinoflagellates, and cyanobacteria. The difference in regulation between diatoms and dinoflagellate/cyanobacteria communities provides clues about patterns of urea utilization in natural environments. The pattern of urea utilization in these groups is considered in the context of the complexity of cellular pathways that may indirectly regulate urea uptake and urease activity. Natural rates of urease activity are put in context by comparisons with rates of other enzymes involved in nitrogen assimilation, such as nitrate reductase and glutamine synthase. Lastly, suggested future directions to better understand regulation of urea uptake and urease enzymes are discussed.

Literature cited

- Allen, A.E., Ward, B.B. & Bowler, C.P. 2005. Multi-lineage richness of functional genes in marine diatoms and transcriptional responses to nutrient stress. American Society of Limnology and Oceanography Summer Meeting. Santiago, Spain. June 2005.
- Antia, N.J., Harrison, P.J. & Oliveria, L. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia*. 1:1-89.
- Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S.G., Allen, A.E., Apt, K.E., Bechner, M., Brzezinski, M.A., Chaal, B.K., Chiovitti, A., Davis, A.K., Demarest, M.S., Detter, J.C., Glavina, T., Goodstein, D., Hadi, M.Z., Hellsten, U., Hildebrand, M., Jenkins, B.D., Jurka, J., Kapitonov, V.V., Kroger, N., Lau, W.W.Y., Lane, T.W., Larimer, F.W., Lippmeier, J.C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M.S., Palenik, B., Pazour, G.J., Richardson, P.M., Rynearson, T.A., Saito, M.A., Schwartz, D.C., Thamtrakoln, K., Valentin, K., Vardi, A., Wilkerson, F.P. & Rokhsar, D.S. 2004. The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. *Science*. 306: 79-86.
- Arrigo, K.R. 2005. Marine microorganisms and global nutrient cycles. *Nature*. 437: 349-355.
- Azam, F. 1998. Microbial control of oceanic carbon flux: The plot thickens. *Science*. 280: 694-696.
- Beckers, G., Bendt, A.K., Krämer, R. & Burkovski, A. 2004. Molecular identification of the urea uptake system and transcriptional analysis of urea transporter- and urease-encoding genes in *Corynebacterium glutamicum*. *J. Bacteriol.* 186: 7645-7652.
- Berg, G.M., Glibert, P.M., Lomas, M.W. & Burford, M.A. 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar. Biol.* 129: 377-387.
- Berg, G.M. & Jørgensen, N.O.G. 2006. Purine and pyrimidine metabolism by estuarine bacteria. *Aquat. Microb. Ecol.* 42: 215-226
- Berman, T., Béchemin, C. & Maestrini, S.Y. 1999. Release of ammonium and urea from dissolved organic nitrogen in aquatic ecosystems. *Aquat. Microb. Ecol.* 16: 295-302.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microbiol. Ecol.* 279-305.

- Bronk, D.A., Glibert, P.M., Malone, T.C., Banahan, S. & Sahlsten, E. 1998. Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux. *Aquat. Microb. Ecol.* 15: 177-189.
- Bruhn A., Buch-illing, H., LaRoche, J. & Richardson, K. 2002. Identification of two functional genes involved with phytoplankton metabolisms of dissolved organic nitrogen: the urease and the formamidase gene. American Society of Limnology and Oceanography Annual Meeting. Victoria, British Columbia, Canada. June 2002.
- Capone, D. 2000. The marine microbial nitrogen cycle. *In* Microbial Ecology of the Oceans [Ed. D. L. Kirchman]. Wiley-Liss. New York. pp. 455-493.
- Cho, B.C. & Azam, F. 1995. Urea decomposition by bacteria in the Southern California Bight and its implications for the mesopelagic nitrogen cycle. *Mar. Ecol. Prog. Ser.* 122: 21-26.
- Cho, B.C., Park, M.C., Shim, J.H. & Azam, F. 1996. Significance of bacteria in urea dynamics in coastal surface waters. *Mar. Ecol. Prog. Ser.* 142: 19-26.
- Cimberlis, A.C.P. & Caceres, O. 1991. Kinetics of urea uptake by *Melosira italica* (Ehr.) Kuetz at different luminosity conditions. *Hydrobiologia* 220: 211-216.
- Collier, J.L. & Baker, K.M. 2004. Urea-degrading microorganisms in Chesapeake Bay. American Society for Limnology and Oceanography Annual Meeting. Honolulu, Hawaii, USA. February 2004.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiology*. 145: 447-59.
- Collos, Y., Vaquer, A., Bibent, B., Slawyk, G., Garcia, N. & Souchu, P. 1997. Variability in nitrate uptake kinetics of phytoplankton communities in a Mediterranean coastal lagoon. *Estuar. Coastal Shelf Sci.* 44:369-375.
- Cornell, S. Rendell, A. & Jickells, T. 1995. Atmospheric inputs of dissolved organic nitrogen to the oceans. *Nature*. 376: 243-246.
- del Giorgio, P.A. & Cole, J.J. 2000. Bacterial energetics and growth efficiency. *In* Microbial Ecology of the Oceans. [Ed. D. L. Kirchman]. Wiley-Liss. New York. pp. 289-326.
- Dortch, Q. & Postel, J.R. 1989. Biochemical indicators of N utilization by phytoplankton during upwelling off the Washington Coast. *Limnol. Oceanogr.* 34: 758-773.

- Dufresne, A., Salanoubat, M., Partensky, F., Artiguenave, F., Axmann, I.M., Barbe, V., Duprat, S., Galperin, M.Y., Koonin, E.V., Le Gall, F., Makarova, K.S., Ostrowski, M., Oztas, S., Robert, C., Rogozin, I.B., Scanlan, D.J., de Marsac, N.T., Weissenbach, J., Wincker, P., Wolf, Y.I. & Hess, W.R. 2003. Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. *Proc Natl Acad Sci USA*. 100:9647-9649.
- Dyhrman, S.T., & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Falkowski, P.G. & Raven, J.A. 1997. Aquatic Photosynthesis. Blackwell Science.
- Fan, C. & Glibert, P.M. 2005. Effect of light on nitrogen and carbon uptake during a *Prorocentrum* minimum bloom. *Harmful Algae*. 4:629-641.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003b. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Fan, C., Glibert, P.M. & Burkholder, J.M. 2003a. Characterization of the affinity for nitrogen, uptake kinetics, and environmental relationships for *Prorocentrum minimum* in natural blooms and laboratory cultures. *Harmful Algae*: 2: 283-299.
- Fisher, T.R., Gustafson, A.B., Sellner, K., Lacouture, R., Haas, L.W., Wetzel, R.L., Magnien, R., Everitt, D., Michaels, B. & Karrh, R. 1999. Spatial and temporal variation of resource limitation in Chesapeake Bay. *Mar. Biol.* 133: 763-778.
- Garrett, R.H. & Grisham, C.M. 1995. *Biochemistry*. Harcourt Brace.
- Geider, R.J. & LaRoche, J. 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37: 1-17.
- Glibert, P.M., Alexander, J., Trice, T.M., Michael, B., Magnien, R.E. Lane, L., Oldach, D. & Bowers, H. 2004. Chronic urea loading: a correlate of *Pfiesteria* spp. in the Chesapeake and Coastal Bays of Maryland. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., and Vargo, G.A. (eds). Harmful Algae 2002, Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission and Intergovernmental Oceanographic Commission of UNESCO, USA. pp. 48-55.
- Glibert, P.M., Conley, D.J., Fisher, T.R., Harding, L.W. & Malone, T.M. 1995. Dynamics of the 1990 winter/spring bloom in Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 122: 27-43.

- Glibert, P.M., Garside, C., Fuhrman, J.A. & Roman, M.R. 1991. Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnol. Oceanogr.* 36: 895-909.
- Glibert, P.M., Harrison, J., Heil, C., & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry.* 77:441-463.
- Glibert, P.M., Magnien, R., Lomas, M.W., Alexander, J., Fan, C., Haramoto, E., Trice, M. & Kana, T.M. 2001. Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: Comparison of the 1997, 1998 and 1999 Events. *Estuaries.* 24: 875-883.
- Glibert, P.M. & Terlizzi, D.E. 1999. Cooccurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. *Appl. Environ. Microbiol.* 65: 5594-5596.
- Glibert, P.M., Trice, T.M., Michael, B. & Lane, L. 2005. Urea in the tributaries of the Chesapeake and Coastal Bays of Maryland. *Water, Air, and Soil Pollution.* 160: 229-243.
- Hausinger, R. P. 2004. Metabolic versatility of prokaryotes for urea decomposition. *J. Bacteriol.* 186: 2520-2.
- Heil, C.A., Revilla, M., Glibert, P.M. & Murasko, S. *In press.* Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Hodson, R.C., Williams, S.K. & Davidson, W.R. 1975. Metabolic control of urea catabolism in *Chlamydomonas reinhardtii* and *Chlorella pyrenoidsa*. *Journal of Bacteriology.* 121:1022-1035.
- Holmboe, N., Jensen, H.S. & Anderson, F.O. 1999. Nutrient addition bioassays as indicators of nutrient limitation of phytoplankton in an eutrophic estuary. *Mar. Ecol. Prog. Ser.* 186: 95-104.
- Jahns, T. 1992. Urea uptake by the marine bacterium *Deleya venusta* HG1. *J. Gen. Microbiol.* 138: 1815-1820.
- Jørgensen, N.O.G. 2006. Uptake of urea by estuarine bacteria. *Aquat. Microb. Ecol.* 42: 227-242.
- Jørgensen, N.O.G., Tranvik, L.J. & Berg, G.M. 1999. Occurrence and bacterial cycling of dissolved nitrogen in the Gulf of Riga, the Baltic Sea. *Mar. Ecol. Prog. Ser.* 191:1-18.

Kaufman, Z.G., Lively, J.S. & Carpenter, E.J. 1983. Uptake of nitrogenous nutrients by phytoplankton in a barrier-island estuary – Great South Bay, New York. *Est. Shelf. Coastal. Sci.* 17: 483-493.

Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde, E.D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M. & Stevenson, J.C. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. *Mar. Ecol. Prog. Ser.* 303:1-29.

Kirchman, D.L. 1994. The uptake of inorganic nutrients by heterotrophic bacteria. *Microb. Ecol.* 28:255-271.

Kirchman, D.L. 2000. Uptake and regeneration of inorganic nutrients by marine heterotrophic bacteria. *In* Microbial Ecology of the Oceans. [Ed. D. L. Kirchman]. Wiley-Liss. New York. pp. 261-288.

Kristiansen, S. 1983. Urea as a nitrogen source for the phytoplankton in the Oslofjord. *Mar. Biol.* 74:17-24.

Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.

L'Hulguen, S., Slawyk, G. & Le Corre, P. 2005. Seasonal patterns of urea regeneration by size-fractionated microheterotrophs in well-mixed temperate coastal waters. *J. Plank. Res.* 27: 263-270.

Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae): Interactions between nitrogen substrates. *Mar. Biol.* 144: 37-44.

Lomas, M.W. & Glibert, P.M. 1999a. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 556-572.

Lomas, M.W. & Glibert, P.M. 1999b. Interactions between NH_4^+ and NO_3^- uptake and assimilation: comparisons of diatoms and dinoflagellates at several growth temperatures. *Mar. Biol.* 133: 541-551.

Lomas, M.W., Swain, A., Shelton, R. & Ammerman, J.W. 2004. Taxonomic variability of phosphorus stress in Sargasso Sea phytoplankton. *Limnol. Oceanogr.* 49: 2303-2310.

- Lomas, M.W., Trice, T.M, Glibert, P.M., Bronk, D.A. & McCarthy, J.J. 2002. Temporal and spatial dynamics of urea uptake and regeneration rates and concentrations in Chesapeake Bay. *Estuaries*. 25: 469-482.
- Lomstein, B.A., Blackburn, T.H. & Henriksen, K. 1989. Aspects of nitrogen and carbon cycling in the northern Bering Shelf sediment. 1. The significance of urea turnover in the mineralization of NH_4^+ . *Mar. Ecol. Prog. Ser.* 57:237-247.
- Lund, B.A. 1987. Mutual interference of ammonium, nitrate, and urea on uptake of ^{15}N sources by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J. Exp Mar. Biol. Ecol.* 113:167-180.
- Mace, K.A., Kubilay, N. & Duce, R.A. 2003. Organic nitrogen in rain and aerosol in the eastern Mediterranean atmosphere: An association with atmospheric dust. *J. Geophysical Res. Atmos.* 108:4320-4331
- Martin, J.H., Gordon, R.M. & Fitzwater, S.E. 1991. The case for iron. *Limnol. Oceanogr.* 36: 1793-1802.
- McCarthy, J.J. 1972. The uptake of urea by natural population of marine phytoplankton. *Limnol. Oceanogr.* 17:738-748.
- McIninch, J.K., McIninch, J.D. & May, S.W. 2003. Catalysis, stereochemistry, and inhibition of ureidoglycolate lyase. *J. Biol. Chem.* 278: 50091-50100.
- Middelburg, J.J. & Nieuwenhuize, J. 2000. Nitrogen uptake by heterotrophic bacteria and phytoplankton in the nitrate-rich Thames estuary. *Mar. Ecol. Prog. Ser.* 203: 13-21.
- Milligan, A.J. & Harrison, P.J. 2000. Effects of non-steady-state iron limitation on nitrogen assimilatory enzymes in the marine diatom *Thalassosira weissflogii* (Bacillariophyceae). *J. Phycol.* 36: 78-86.
- Miller, C.A. & Glibert, P.M. 1998. Nitrogen excretion by the calanoid copepod *Acartia tonsa*: results of mesocosm experiments. *J. Plank. Res.* 20: 1767-1780.
- Miller, S.R. & Castenholz, R.W. 2001. Ecological physiology of *Synechococcus* sp. Strain SH-94-5, a naturally occurring cyanobacteria deficient in nitrate assimilation. *Appl. Environ. Microbiol.* 67: 3002-3009
- Mobley, H.L.T. & Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. *Microbiol. Rev.* 3: 85-108.
- Mobley, H.L.T., Island, M.D. & Hausinger, R.P. 1995. Molecular Biology of Microbial Ureases. *Microbiol. Rev.* 59: 451-480.

- Molloy, C.J. & Syrett, P.J. 1988. Interrelationships between uptake of urea and uptake of ammonium by microalgae. *J. Exp. Mar. Biol. Ecol.* 118: 85-95.
- Moore, L.R., Post, A.F., Rocap, G., & Chisholm, S.W. 2002. Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Limnol. Oceanogr.* 47: 989-996.
- Nolden, L., Beckers, G., Mockel, B., Pfefferle, W., Nampoothiri, K.M., Kramer, R. & Burkovski, A. 2000. Urease of *Corynebacterium glutamicum*: organization of corresponding genes and investigation of activity. *FEMS Microb. Let.* 189: 305-310.
- Ohki, K., Zehr, J.P. & Fujita, Y. 1992. Regulation of nitrogenase activity in relation to the light-dark regime in the filamentous non-heterocytosus cyanobacterium *Trichodesmium* sp. NIBB 1067. *J. Gen Microbiol.* 138:2679-2685.
- Oliveira, L. & Antia, N.J. 1986. Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J. Plank. Res.* 8: 235-242.
- Pantoja, S. & Lee, C. 1994. Cell-surface oxidation of amino acids in seawater. *Limnol. Oceanogr.* 39: 1718-1726.
- Parker, M.S. & Armbrust, E.V. 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 41:1142-1153.
- Pedersen, H., Lomstein, B.A. & Blackburn, T.H. 1993. Evidence for bacterial urea production in marine sediments. *FEMS Microb. Ecol.* 12:51-59.
- Peers, G.S., Milligan, A.J. & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.
- Peierls, B.L. & H.W. Paerl. 1997. Bioavailability of atmospheric organic nitrogen deposition to coastal phytoplankton. *Limnol. Oceanogr.* 42: 1819-1823.
- Rees, T.A.V. & Syrett P.J. 1979. The uptake of urea by the diatom *Phaeodactylum*. *New Phytol.* 82: 169-178.
- Reid, R.T. & Butler, A. 1991. Investigation of the mechanism of iron acquisition by the marine bacterium *Alteromonas luteoviolaceus* – characterization of siderophore production. *Limnol. Oceanogr.* 36:1783-1792.
- Rivkin, R.B., Legendre, L., Deibel, D., Tremlay, J-E., Klein, B., Crocker, K., Roy, S., Silverberg, N., Lovejoy, C., Mesple, F., Romero, N., Anderson, M.R., Matthews, P., Savenkoff, C., Vezina, A., Therriault, J-C., Wesson, J., Berube, C. & Ingram, R.G.

1996. Vertical flux of biogenic carbon in the ocean: Is there food web control? *Science*. 272: 1163-1166.
- Schulz, K.G., Zondervan, I., Gerringa, L.J.A., Timmermans, K.R., Veldhuis, M.J.W. & Riebesell, U. 2004. Effect of trace metal availability on coccolithophorid calcification. *Nature* 430: 673-676.
- Shaked, Y., Xu Y., Leblanc K., & Morel, F.M.M. 2006. Zinc availability and alkaline phosphatase activity in *Emiliana huxleyi*: Implications for Zn-P co-limitation in the ocean. *Limnol. Oceanogr.*, 51:299-309.
- Seigenthaler, U. & Sarmiento, J.L. 1993. Atmospheric carbon dioxide and the ocean. *Nature* 365: 119-125.
- Siewe, R.M., Weil, B., Burkovski, A., Eggeling, L., Krämer, R. & Jahns, T. 1998. Urea uptake and urease activity in *Corynebacterium glutamicum*. *Arch. Microbiol.* 169: 411-416.
- Soh, K.G. 2001. A review of the global fertilizer use by product. 7th AFA Annual Conference. Cairo.
- Tamminen, T. & Irmisch, A. 1996. Urea uptake kinetics of a midsummer planktonic community on the SW coast of Finland. *Mar. Ecol. Prog. Ser.* 130: 201-211.
- Therkildsen, M.S., Isaken, M.F. & Lomstein, B.A. 1997. Urea production by the marine bacteria *Delacya venusta* and *Pseudomonas stutzeri* grown in a minimal medium. *Aquat. Microb. Ecol.* 13: 213-217.
- Todd, M.J. & Hausinger, R.P. 1987. Purification and characterization of the nickel-containing multicomponent urease from *Klebsiella aerogenes*. *J. Biol. Chem.* 262: 5963-5967.
- Twomey, L.J., Piehler, M.F. & Paerl, H.W. 2005. Phytoplankton uptake of ammonium, nitrate, and urea in the Neuse River Estuary, NC, USA. *Hydrobiologia*. 533: 123-134.
- Wheeler, P. 1983. Phytoplankton nitrogen metabolism.. In Carpenter, E.J & Capone, D.G. [Eds] *Nitrogen in the Marine Environment*. Academic Press. New York, N.Y. pp. 309- 346.
- Wheeler, P.A., Glibert, P.M. & McCarthy, J.J. 1982. Ammonium uptake and incorporation by Chesapeake Bay phytoplankton: Short term uptake kinetics. *Limnol. Oceanogr.* 27:1113-1128.
- Wheeler, P.A. & Kirchman, D.L. 1986. Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnol. Oceanogr.* 31: 998-1009.

Yin, K., Qian, P., Wu, M.C.S., Chen, J.C., Huang, L., Song, X. & Jian, W. 2001. Shift from P to N limitation of phytoplankton growth across the Pearl River estuarine plume during summer. *Mar. Ecol. Prog. Ser.* 221:17-28.

Zehr, J.P. & Capone, D.G. 1996. Problems and promises of assaying the genetic potential for nitrogen fixation in the marine environment. *Microb. Ecol.* 32: 263-281.

Zehr, J.P. & Ward, B.B. 2002. Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Appl. Environ. Microbiol.* 68: 1015-1024.

Table

Table 1.1: Enzymes responsible for the conversion of various nitrogen substrates into ammonium

NO ₃	nitrite reductase (<i>nir</i>), nitrate reductase (<i>nar</i>)	Milligan and Harrison, 2000
Urea	urease (<i>ure</i>)	Peers et al. 2000
N ₂	nitrogenase (<i>nif</i>) (only in cyanobacteria)	Zehr and Capone, 1996
Amino acids	amino acid oxidation	Pantoja and Lee, 1994

Figures

Fig. 1.1 Division of molecular and biochemical regulation of nitrogen assimilation pathways in a phytoplankton cell.

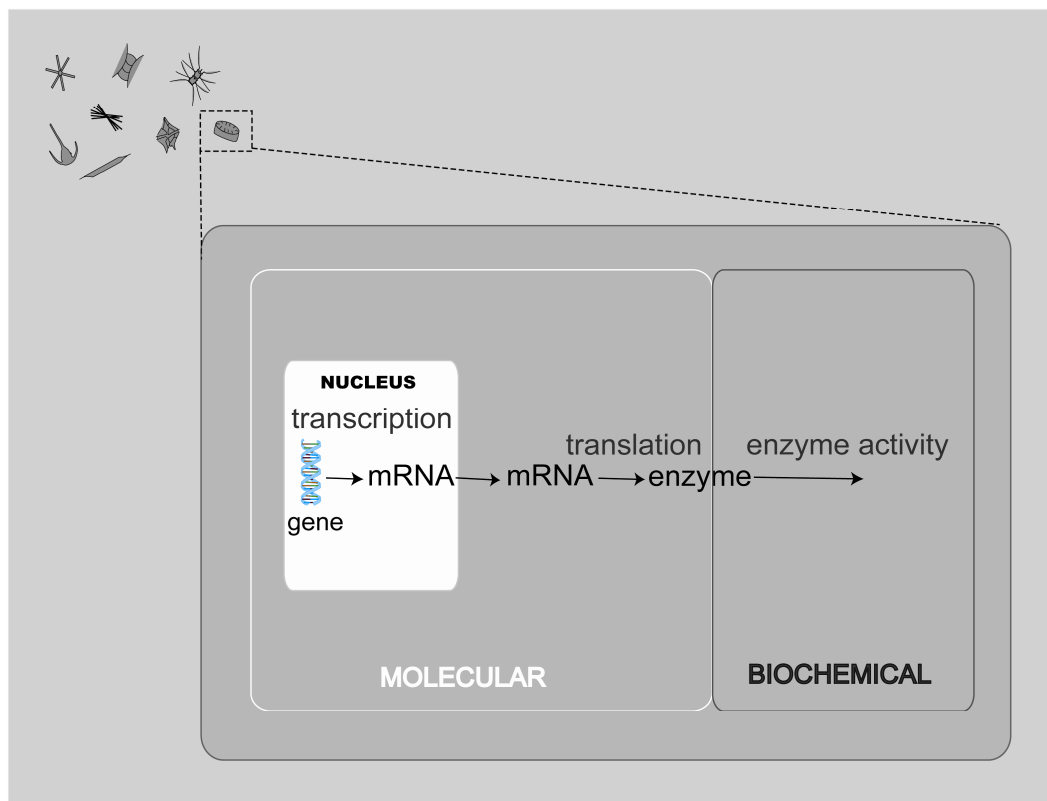


Fig. 1.2 Various nitrogen assimilation pathways in a microbial cell. Not all pathways will be present in different species of phytoplankton, cyanobacteria, and bacteria.

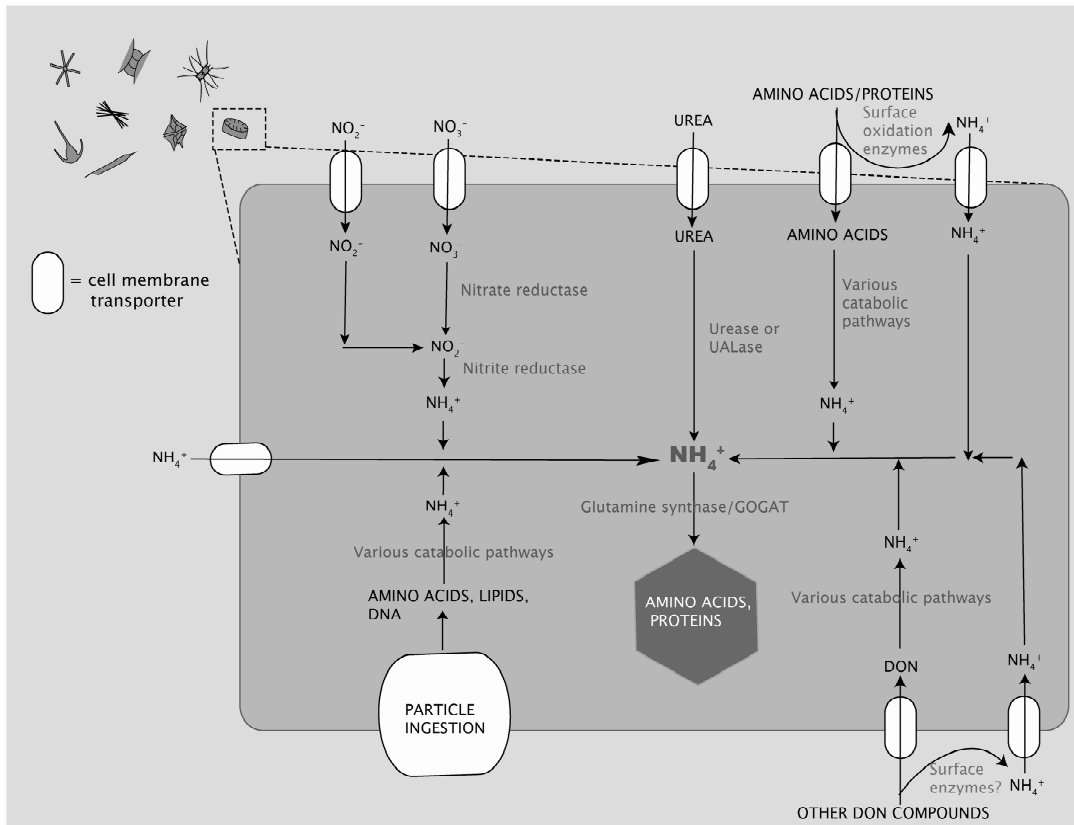


Fig. 1.3 Intracellular pathways in a microbial cell involved with urea utilization and assimilation.

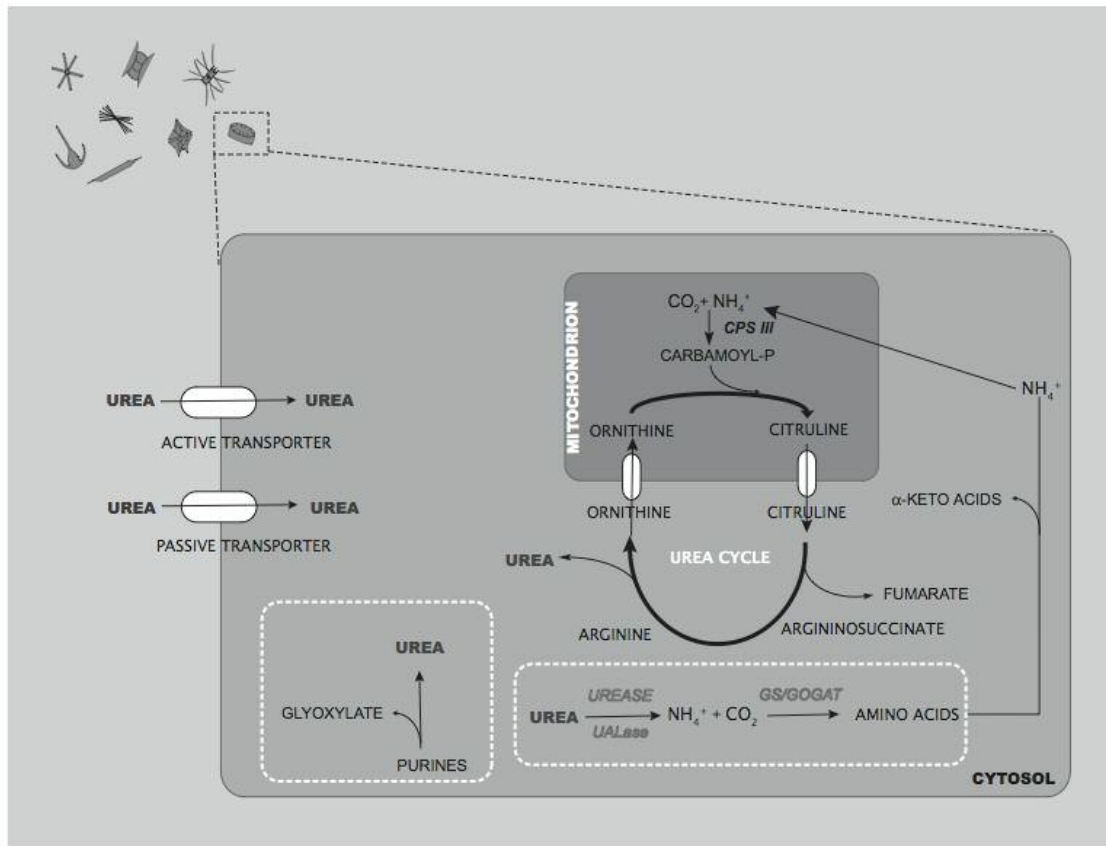


Fig. 1.4 Sources of urea and organisms that utilize urea in estuarine waters.

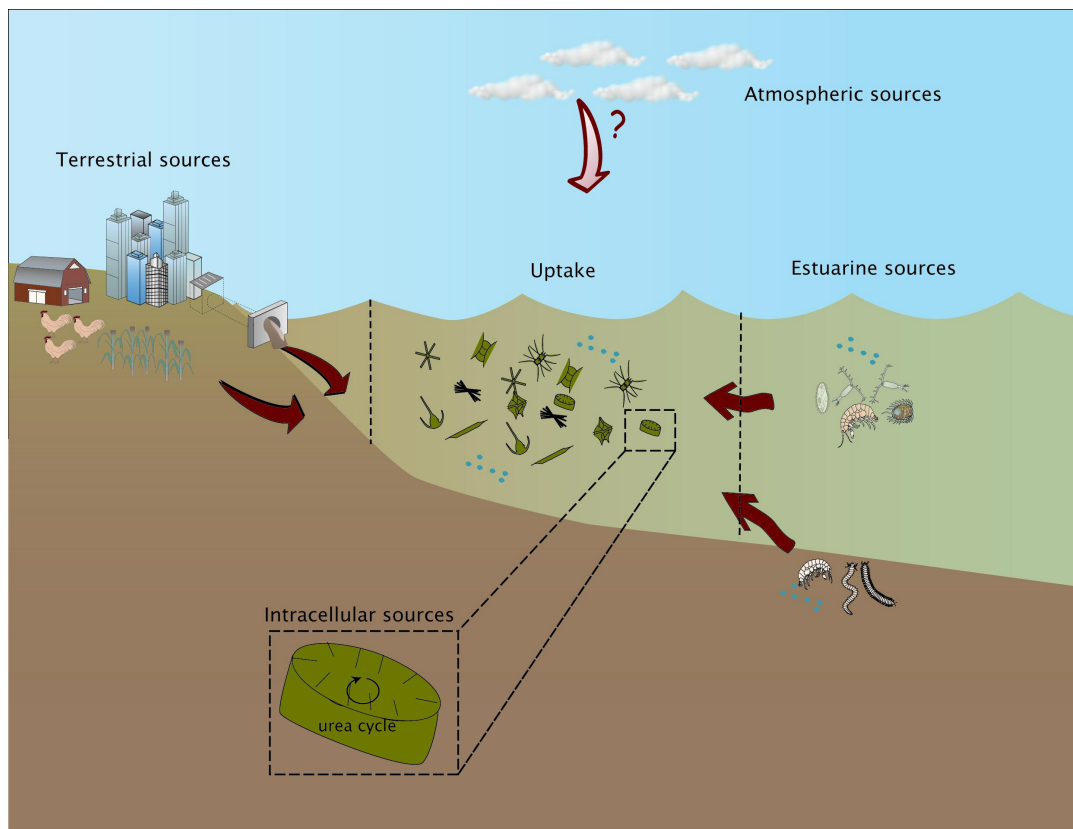
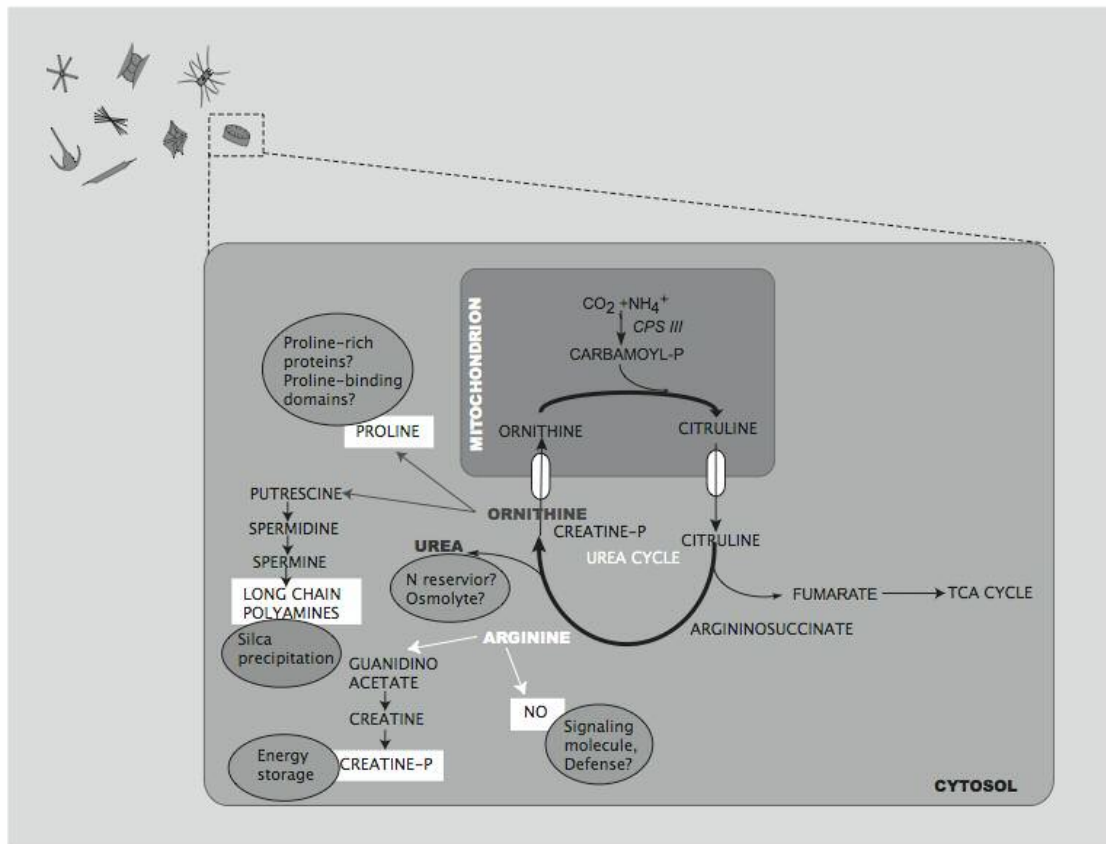


Fig. 1.5. Role of intermediates of the urea cycle in diatom metabolism (modified from Allen et al. 2005).



Chapter 2: Measuring urease activity in environmental samples

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 NH_4^+ from extraction and assay reagents in the current published protocol, leading to significant depression of urease activity. We recommend a reduction in the concentration or removal of two of these reagents. These improvements lead to values that are more accurate, but unfortunately more variable due to the low volumes used. We thus found a trade-off between increasing accuracy and increasing variability.

Introduction

Urea ($\text{CO}(\text{NH}_2)_2$), a small neutral molecule, is a significant source of nitrogen for 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 utilize urea and many 'harmful' or 'toxic' species either have higher urea uptake rates than for NO_3^- or NH_4^+ or are stimulated by elevated urea concentrations (Berg et al. 1997; Kudela and Cochlan 2000; Collos et al. 2004; Mulholland et al. 2004; Glibert 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 NH_4^+ than urea (Berg et al. 1997; Mulholland et al. 2004). Elevated urea concentrations in aquaculture ponds were also 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. *in press*), *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 NH_4^+ and CO_2 that are subsequently utilized by various biochemical pathways. The measurement of urease activity may help understand whether urea, transported into the cell (Antia et al. 1991) or produced internally by catabolism of amino acids or purines (Antia et al. 1991, Allen et al. 2005), is assimilated into biomass by microbes. Estimations about the potential contribution of urea to total cellular nitrogen demand for phytoplankton and bacteria may also 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: CO_2 or NH_4^+ . The first investigators measured urease activity by rates of release of $^{14}\text{CO}_2$ (Leftley and Syrett 1973; Bekheet and Syrett 1977; Ge et al. 1990). Later investigators measured the liberation of NH_4^+ by various 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 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 *P. minimum* (Fan et al. 2003) and *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-atom N cell⁻¹ hr⁻¹ 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 plankton assemblages (e.g. Syrett and Leftley 1973; Peers et al. 2003; Fan et al. 2003; Dyhrman and Anderson 2003; Lomas 2004; Glibert et al. 2004, Heil et al. *in press*). Preliminary field studies have hinted at variability in enzyme activity related to volume of sample filtered and other field manipulations. For example, urease activity in size fractionated samples with a lower chl *a* biomass was often higher than in whole samples, suggesting interference from cell metabolites or that the method was not optimal. 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_4^+ contamination from reagents, interference from buffers, boiling, and storage) or was the result of environmental heterogeneity.

Methods

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 hr; <5 mm Hg), which are then stored in liquid N_2 until analysis. A storage time of a few days or less 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 1M potassium phosphate buffer adjusted to pH 7.9 with NaOH pellets, 2.5 ml 0.5 M HEPES buffer (n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid, pH 7.9), 2.5 ml 3% PVP (polyvinyl pyrrolidone), 2.5 ml 1% Triton-X, 2.5 ml 50 mM EDTA (ethylenediamine-tetraacetic acid disodium salt, pH 7.9), 11.25 ml dionized H_2O 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_4^+ 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_0 and t_f). The supernatant is combined with 700 μl of cold assay buffer (2.5 ml 0.5 M HEPES, 3.75 ml 1 M potassium phosphate buffer, 18.75 ml dionized H_2O for a total of 25 ml) and 800 μl of deionized water. The t_0 test

tubes are put in boiling water for 1 min, then 300 μ l 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_4^+ determination (Parsons et al. 1984). While the t_0 test tubes are in boiling water, 300 μ l of 5 mM urea stock is added to the t_f 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 μ l of HCl, followed by neutralization with 20 μ l 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 NH_4^+ 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_4^+ assay (0.2 ml sodium nitroprusside prepared as 1g of sodium nitroprusside in 200 ml deionized water; 0.5 ml oxidizing reagent prepared as 40 ml of 100g sodium citrate and 5g 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 hours until analysis on a spectrophotometer at 640 nm.

NH_4^+ contamination from reagents

Since NH_4^+ is the end product of the urease assay, contamination with NH_4^+ by assay reagents is of concern. To address this concern, the contribution of NH_4^+ 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_4^+ concentration (Parsons et al. 1984). To examine further the contribution of PVP to NH_4^+ 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 (Spectra/Por®) in deionized water (replaced several times) for one day. One ml of each PVP solution was diluted to 5 ml and analyzed for NH_4^+ concentrations (Parsons et al. 1984).

To examine whether the contribution of contaminant 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.

Buffers

The influence or interference of the extraction and assay buffers on detection of 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 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 $5 \mu\text{g-at N l}^{-1}$ of NH_4^+ , then diluted to 5 ml. The samples were

then measured for NH_4^+ concentrations (Parsons et al. 1984). The amount of NH_4^+ recovered was determined by subtracting the non-spiked sample from the spiked sample.

The combined effect of the reagents on the detection of NH_4^+ was determined through a second experiment that tested a series of homogenate volumes (extraction buffer: 100, 200, 300 and 400 μl) with or without 700 μl of assay buffer that were spiked with 5 $\mu\text{g-at N l}^{-1}$ of NH_4^+ , then diluted to 5 ml with deionized water. The samples were then measured for NH_4^+ concentrations (Parsons et al. 1984). To further compare the combined effect of the reagents, four NH_4^+ standard curves were measured in varying homogenate volumes (50, 100, 200, and 400 μl), but with the same volume of assay buffer (700 μl), and diluted to 5 ml. Two additional NH_4^+ standard curves were measured in homogenate (100 and 400 μl) and assay buffers both with and without HEPES. The seventh 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 (2ml 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 μl) and assay buffers both 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 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 one t_0 and t_f test tubes. The t_0 and t_f 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 N₂ or a -80°C freezer after collection. In May 2004, aliquots of culture (n=60) of 0.025 l were filtered onto combusted GF/F filters and kept in liquid N₂ or a -80°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 µl homogenate volume. Using the same protocol, the samples that were stored in

liquid N₂ were measured in triplicate every day for two weeks, then again at days 17 and 21. Samples that were stored in a -80°C freezer were only measured in triplicate on weekly basis (days 7, 14, and 21). Samples from liquid N₂ storage that were analyzed on days 8-11 were removed from the data set after it was determined that those samples were at the top of the liquid N₂ dewar and were not kept at the same cold temperatures due to liquid N₂ evaporation.

Environmental heterogeneity and effect of sample volume

Water was collected from two sites: Choptank River, MD (a tributary of the Chesapeake Bay) and Duck Key, FL (in Florida Bay) for the analysis of optimal filtration volumes and environmental heterogeneity. Two separate experiments to test different 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 0.025 l to 0.1 l and from 0.6 l to 0.9 l in the Florida Bay samples. For comparison between the two sites, filtration volumes were converted into chlorophyll content using the average chlorophyll concentrations 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 μl . The 400 μl volume is the original homogenate volume used in the methods of Peers et al. (2000) and Fan et al. (2003).

Assessment

NH₄⁺ contamination from reagents

The NH₄⁺ concentration in all reagents except 3% PVP was < 20 µg-at N l⁻¹ (Table 2.1). While the NH₄⁺ concentrations of the reagents were high when measured independently, their contribution, with the exception of 3% PVP, to the final extraction buffer was significantly lower (<2 µg at N l⁻¹). PVP, with a NH₄⁺ concentration of 733 ± 22.4 µg-at N l⁻¹, contributed 95.8% to the background NH₄⁺ in 1 ml of extraction buffer. Reduced concentrations of PVP led to lower NH₄⁺ concentrations (0.3% PVP = 70.8 ± 3.72 µg-at N l⁻¹, 0.03% PVP = 7.37 ± 0.48 µg-at N l⁻¹ and dialyzed PVP = 10.8 ± 0.47 µg-at N l⁻¹).

Urease activity differed in extraction buffers with different NH₄⁺ background concentrations. Urease activity was lowest in the standard extraction buffer and was significantly different than urease activity in extraction buffers that had a lower concentration of PVP (0.03% PVP) or dialyzed PVP that had lower background NH₄⁺ (p<0.05; Table 2.2). Urease activity in the extraction buffer with dialyzed PVP had lower variance (\bar{x} = 0.68 ± 0.12 µg at N l⁻¹ h⁻¹) than in the extraction buffer with 0.03% PVP (\bar{x} = 0.59 ± 0.19 µg at N l⁻¹ h⁻¹), but the urease activities assayed with both buffer preparations were not significantly different (p=0.29).

Buffers

In theory, NH₄⁺ concentrations in the t₀ 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 detecting NH₄⁺. An observed non-

linear relationship in both t_0 and t_f samples (Fig. 2.1) led to an investigation of whether reagents in this urease assay method were interfering with the indophenol-blue method for detecting NH_4^+ .

The reagents in the extraction and assay buffers had different effects on the ability to detect NH_4^+ using the indophenol-blue method of Parsons et al. (1984). The HEPES buffer had a negative effect and none of the spiked NH_4^+ was recovered (Table 2.1). The other reagents, except PVP, did not have a 100% recovery of the spiked NH_4^+ . PVP was the only reagent that led to an amplification of the spiked NH_4^+ . Overall, no reagent had a neutral effect on the recovery of the spiked 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 NH_4^+ recovered from the spike increased when the homogenate volume was reduced from 400 μl to 100 μl in samples without assay buffer while the amount of NH_4^+ recovered did not change with volume of homogenate in samples with assay buffer (Fig. 2.2). The interference from the different reagents in the buffers was minimized at the lowest homogenate volume (100 μl).

The effect of the buffers was investigated further by comparing NH_4^+ standard curves in deionized water and in different buffers. The NH_4^+ standard curve measured in deionized water and without HEPES buffer was different than the other four standard curves with HEPES buffer (Fig. 2.3). The 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 NH_4^+ standard curve in 50 μl homogenate and 700 μl

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. 2.4). Rates of urease activity in HEPES buffer using the published method were almost two-fold lower and had higher variability than in potassium phosphate buffer alone for *Isochrysis sp.* Rates of urease activity in samples from the 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 was not significantly different with or without HEPES when 100 μl 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 with the detection of NH_4^+ . First, HEPES buffer had a negative effect on recovery of spiked NH_4^+ while the potassium phosphate buffer did not (Table 2.1). Second, the HEPES concentration increased with increasing homogenate volume, and an increase in homogenate volume led to a decrease in the detection of NH_4^+ (Fig. 2.2) and lower NH_4^+ standard curve slopes (Fig. 2.3). Furthermore, urease activity measured in buffers without HEPES was higher than with HEPES (Fig. 2.4). These results agree with Peers et al. (2000) who suggested removing HEPES buffer, which was originally 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 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 at the lower homogenate volumes of 50 and 100 μl was significantly different between the boiled (0.66 ± 0.15 $\mu\text{g at N l}^{-1} \text{ hr}^{-1}$) and non-boiled samples (0.40 ± 0.16 $\mu\text{g at N l}^{-1} \text{ hr}^{-1}$; $p < 0.02$) while there was no significant difference at the higher homogenate volumes of 200 and 400 μl ($p > 0.37$; data not shown). The difference in NH_4^+ concentration between the t_f and t_0 boiled samples was on average higher than in the non-boiled samples (0.78 ± 0.56 vs. 0.49 ± 0.56 $\mu\text{g at N l}^{-1}$).

Storage

Urease activity of samples stored in liquid nitrogen remained at the same level (grand mean: 5.83 ± 0.71 $\mu\text{g at N l}^{-1} \text{ hr}^{-1}$) for three weeks (Fig. 2.5). Urease activity was not significantly different (student's t-test; $p = 0.29$, $p = 0.46$) between samples stored in both liquid N_2 and an -80°C freezer the first and second week (day 7 and 14). Urease activity was borderline significantly different the third week (day 21; student's t-test: $p = 0.06$) between the two sets of samples. These results suggest that urease samples can be preserved in either liquid N_2 or an -80°C freezer for at least up to three weeks.

Environmental heterogeneity and effect of sample volume

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, FL than in Choptank River, MD. Samples from Duck Key, with a low chlorophyll biomass, did not have any statistical difference in urease activity among different filtration volumes (Fig. 2.6A). Samples from Choptank River had higher chlorophyll biomass and decreasing average urease activity was observed with increasing biomass per sample filter (Fig. 2.6B). There was no significant difference between filtration volumes with the exception of 0.025 l (0.90 $\mu\text{g chl } a$) and 0.05 l (1.80 $\mu\text{g chl } a$; $p < 0.005$). The decrease in urease activity with increasing biomass may be due to inference from increasing concentrations of intracellular NH_4^+ or other cell metabolites. The lowest filtration volume (0.025 l; 0.90 $\mu\text{g chl } a$) with the highest urease activity was also adequate to run routine chlorophyll analyses.

Average urease activity decreased with increasing homogenate volume at both sites (Fig. 2.7), which was consistent with earlier experiments. In Duck Key, urease activity decreased from 0.012 to 0.003 $\mu\text{g at N l}^{-1} \text{ h}^{-1}$ as the homogenate volume increased from 50 to 400 μl . Urease activities in the lowest homogenate volumes (50 and 100 μl) were not statistically different ($p = 0.80$). Urease activity in the homogenate volume of 100 μl , which had a lower variance than 50 μl , was significantly different than in the previously recommended homogenate volume of 400 μl ($p < 0.05$ when the 50 μl 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 μl volumes was significantly different ($p < 0.05$) and urease activity in both volumes was significantly different than in the 400 μl 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). The similarity in pattern of urease activity over increasing homogenate volumes between homogenate with cells (Fig. 2.7) and recovery of NH_4^+ in buffers without cells (Fig. 2.2) and NH_4^+ standard curves (Fig. 2.3) suggests that the effects on the urease assay are not environmental but rather from the HEPES buffer on the indophenol-blue method for detecting 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 NH_4^+ . The reduction of background NH_4^+ is important in order to resolve lower levels of urease activity. Second, the 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 analyses to minimize the biomass effect. Results from natural samples

should be closer to true field values and assay artifacts would be minimized with those modifications.

Conversions can be made from the published method (3% PVP, 400 μl homogenate) to values obtained by one of the revised methods suggested here (0.03% PVP, 100 μl homogenate; Fig. 2.8). Values measured by the previous method that were $>0.2 \mu\text{g at-N l}^{-1} \text{ h}^{-1}$ were underestimated while values $<0.2 \mu\text{g at-N l}^{-1} \text{ h}^{-1}$ were overestimated. Most urease activity is $<0.2 \mu\text{g at-N l}^{-1} \text{ h}^{-1}$ with small differences between t_f and t_0 , thus it is important to measure NH_4^+ concentrations accurately with low variability. Other methods that effectively measure NH_4^+ at nanomolar concentrations (e.g. 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.

Literature cited

- Allen, A.E., Ward, B.B. & Bowler, C.P. 2005. Multi-lineage richness of functional genes in marine diatoms and transcriptional responses to nutrient stress. American Society of Limnology and Oceanography. Santiago, Spain. June 2005.
- Antia, N.J., Harrison, P.J. & Oliveria, L.. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia*. 1: 1-89
- Bekheet, I.A. & Syrett, P.J. 1979. The uptake of urea by *Chlorella*. *New Phytol.* 82:179-186.
- Berg, G.M., Glibert, P.M., Lomas, M.W. & Burford, M.A. 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar. Biol.* 129: 377-387.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microb. Ecol.* 31:279-305.
- Brzezinski, M.A. 1987. Colorimetric determination of nanomolar concentrations of ammonium in seawater using solvent extraction. *Mar. Chem.* 20: 277-288.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiol.* 145:447-459.
- Collos, Y., Gagne, C., Laabir, M., Vaquer, A., Cecchi, P. & Souchu, P. 2004. Nitrogenous nutrition of *Alexandrium catenella* (Dinophyceae) cultures and in Thau Lagoon, Southern France. *J. Phycol.* 40:96-103.
- Dyhrman, S.T. & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Ge, X., Cain, K. & Hirschberg, R. 1990. Urea metabolism and urease regulation in the cyanobacterium *Anabaena varibilis*. *Can. J. Microbiol.* 36:218-222.
- Glibert, P.M., Harrison, J., Heil, C. & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry*. 77: 441-463.

- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Glibert, P.M., Magien, R., Lomas, M.W., Alexander, J., Fan, C., Haramoto, E., Trice M. & Kana, T.M. 2001. Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: Comparison of 1997, 1998, and 1999 events. *Estuaries.* 24: 875-883.
- Heil, C.A., Revilla, M., Glibert, P.M. & Murasko, S. In press. Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Glibert, P.M., & Terlizzi, D.E. 1999. Co-occurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. *Appl. Environ. Microbiol.* 65:5594-5596.
- Holmes, R.M., Aminot, A., Kerouel, R., Hooker, B.A. & Peterson, B.J. 1999. *Can. J. Fish. Aquat. Sci.* 56: 1801-1808.
- Jahns, T., Schafer, U. & Kaltwasser, H. 1995. Heat-stable ureases from two filamentous cyanobacteria. *Microbiol.* 141:737-741.
- Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.
- Leftley, J.W. & Syrett, P.J. 1973. Urease and ATP:Urea amidolyase activity in unicellular algae. *J. Gen. Microbiol.* 77:109-115.
- Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (*Bacillariophyceae*): Interactions between nitrogen substrates. *Mar. Biol.* 144: 37-44.
- Loomis, W.D. & Battaile, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5: 423-438.
- Mobley, H.L.T. & Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. *Microbiol. Rev.* 3: 85-108.
- Mulholland, M.R., Boneillo, G. & Minor, E.C. 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. *Harmful Algae* 3:361-376.

Oliveria, L. & Antia, N.J. 1986. Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J. Plank. Res.* 8: 235-242.

Parsons, T.R., Maita, Y. & Lalli, C.M. 1984. *A Manual of Chemical and Biological Methods for Seawater Analysis*. Pergamon, New York.

Peers, G.S., Milligan, A.J. & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.

Steidinger, K.A., Vargo, G.A., Tester, P.A. & Tomas, C.R. 1998. Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. In: Anderson D.M., Cembella A.D., Hallegraeff G.M. (eds) *Physiological ecology of harmful algal blooms*. Springer-Verlag, Heidelberg, p. 133-153.

Stewart, B.M. 1985. Effect of temperature on the formation of indophenol blue in a spectrometric method for the determination of ammonium. *Water Res.* 19:1443-1445.

Tables

Table 2.1 Concentration of NH_4^+ (\pm SD) in each reagent in the extraction buffer and NH_4^+ recovered from a 5 $\mu\text{g-at N l}^{-1}$ spike

Reagent	NH_4^+ concentration ($\mu\text{g at N l}^{-1}$)	% contribution to 1 ml extraction buffer	NH_4^+ recovered ($\mu\text{g at N l}^{-1}$)	% recovery
HEPES	19.5 \pm 0.18	2.55	-1.39 \pm 1.92	-28
Potassium phosphate buffer (1M)	3.50 \pm 0.07	0.68	3.10 \pm 0.18	62
3% PVP	222 \pm 6.73	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 0.08	0.87	2.86 \pm 1.56	57

*for NH_4^+ recovery, 0.03% PVP was used

Table 2.2 Urease activity in extraction buffers containing different concentrations of PVP

Extraction buffer (EB)	Urease activity ($\mu\text{g at N l}^{-1} \text{ h}^{-1}$)
Standard EB	0.23±0.15
No PVP	0.29±0.09
0.3% PVP	0.44±0.11
0.03% PVP	0.59±0.19
Dialyzed PVP (6-8,000 MW)	0.68±0.12

Figures

Fig 2.1 Concentration of NH_4^+ in the assayed t_0 and t_f samples as a function of different homogenate volumes of a 25 ml filtered sample (using 0.03% PVP) from the Choptank River.

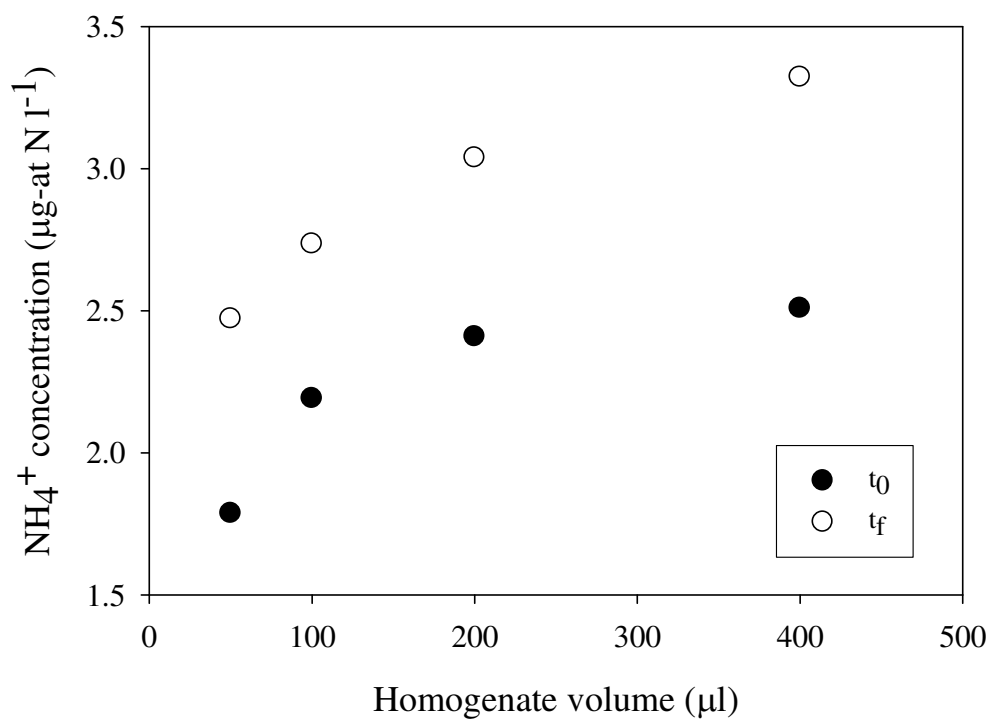


Fig. 2.2 Concentration of NH_4^+ recovered in homogenate only and with assay buffer as a function of different homogenate volumes. The dotted line represents the $5 \mu\text{g-at N l}^{-1} \text{NH}_4^+$ that should have been recovered.

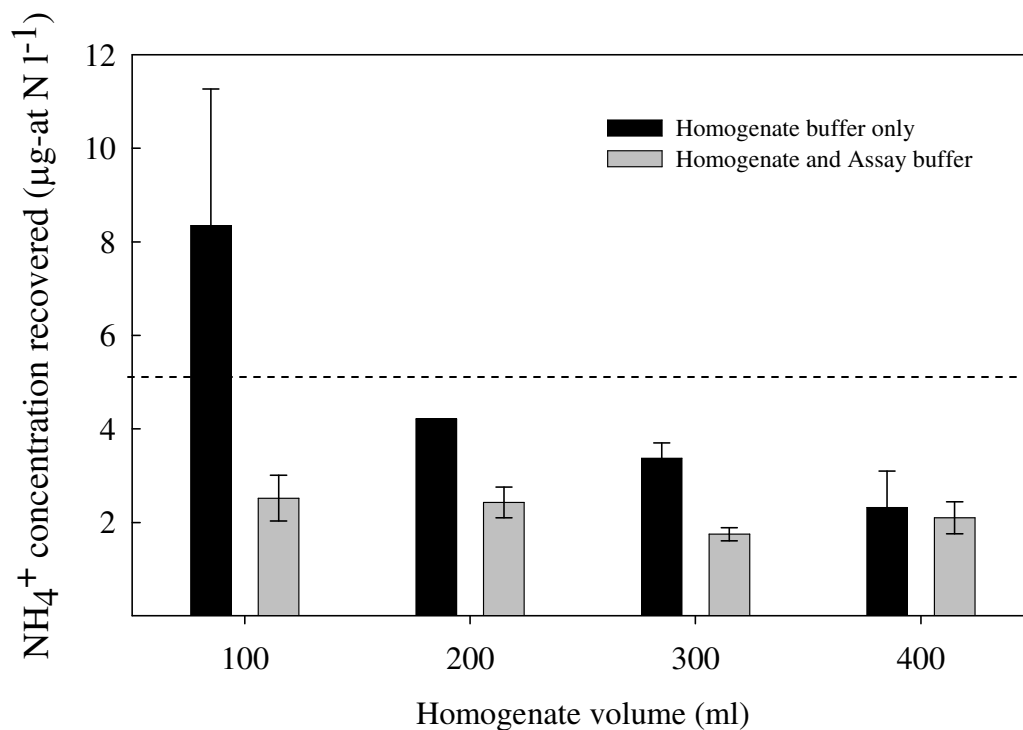


Fig. 2.3 NH_4^+ standard curves in deionized water and varying volumes of homogenate buffer and 700 μl of assay buffer with and without HEPES buffer. Regression lines are shown for deionized water and 400 μl homogenate volume with HEPES.

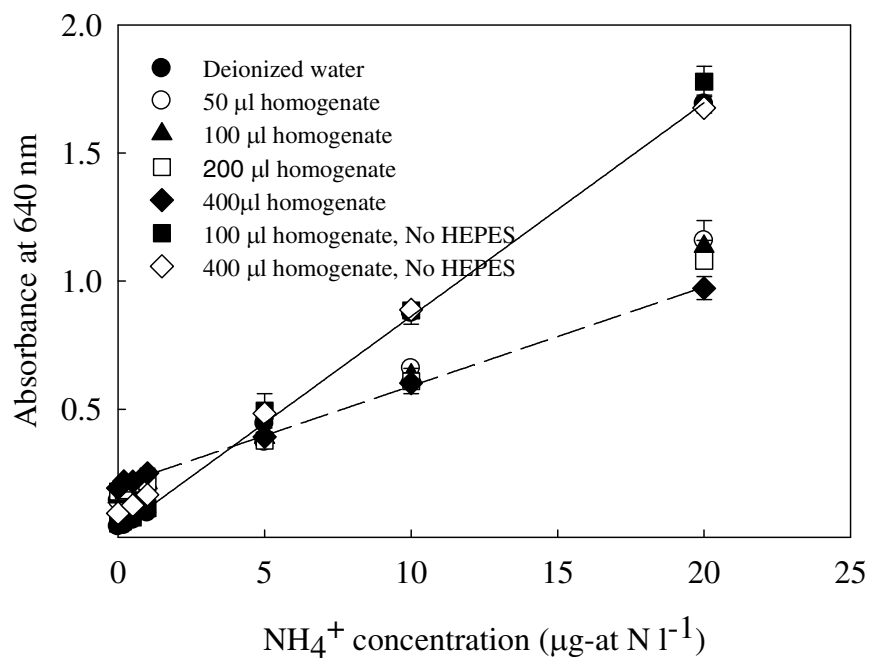


Fig. 2.4 Comparison of urease activity values obtained by using the published method (Fan et al. 2003) and revised methods with (1) 0.03% PVP, 100 μ l homogenate with HEPES buffer or 0.03 PVP, 100 μ l (2a) and 400 μ l (2b) homogenate without HEPES buffer in both *Isochrysis sp.* cultures and the Choptank River in March 2006.

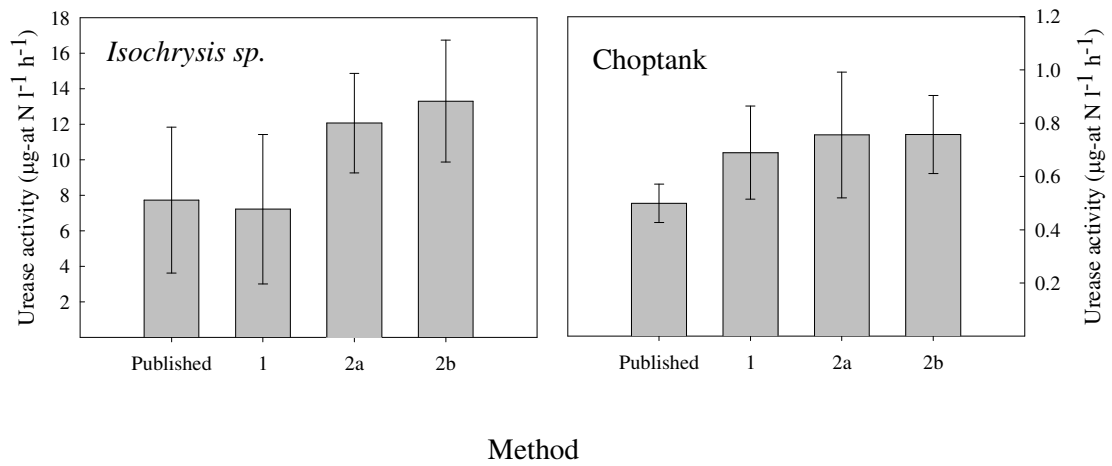


Fig. 2.5 Urease activity in stored samples over a three week period. Samples were stored in liquid N₂ and in a -80°C freezer.

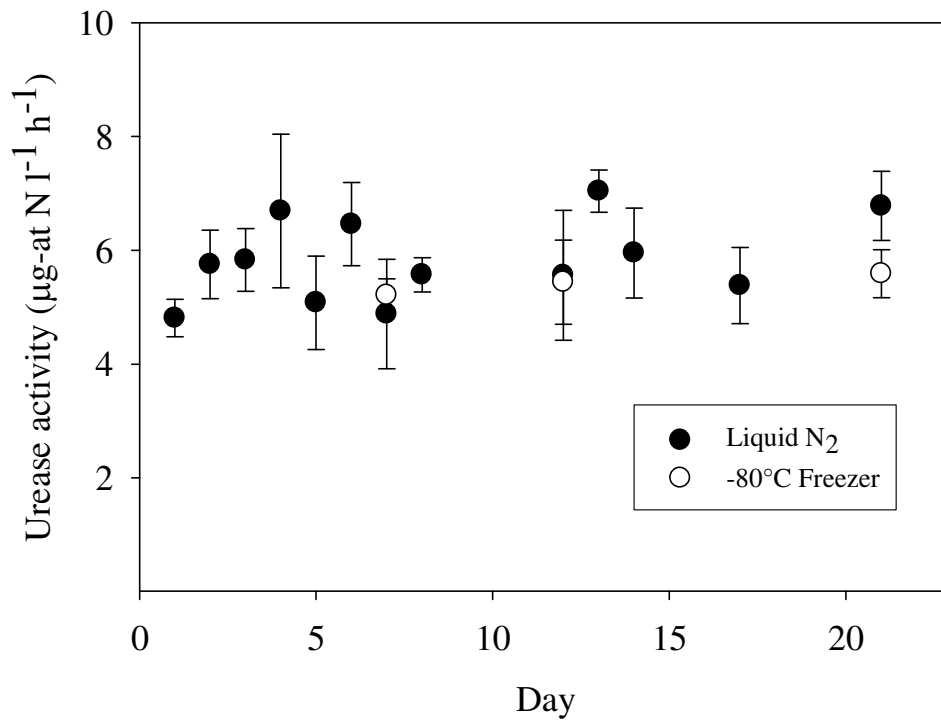


Fig. 2.6 Urease activity measured with different filtration volumes (normalized to chlorophyll biomass) averaged over all homogenate volumes in A) Duck Key in Florida Bay and B) the Choptank River. Note difference in scale for urease activity.

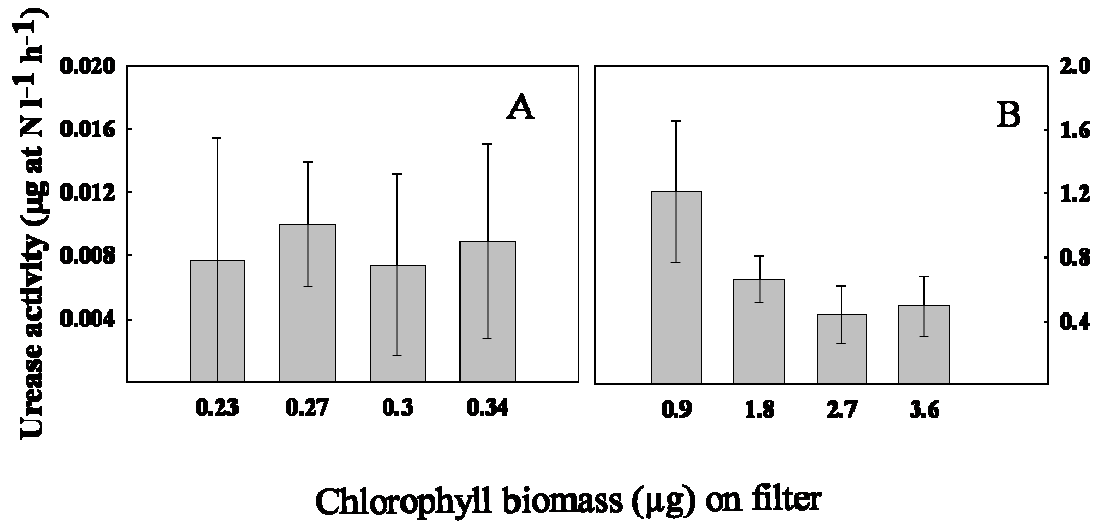


Fig. 2.7 Urease activity measured with different homogenate volumes averaged over all filtrate volumes in A) Duck Key in Florida Bay and B) the Choptank River. Note difference in scale for urease activity.

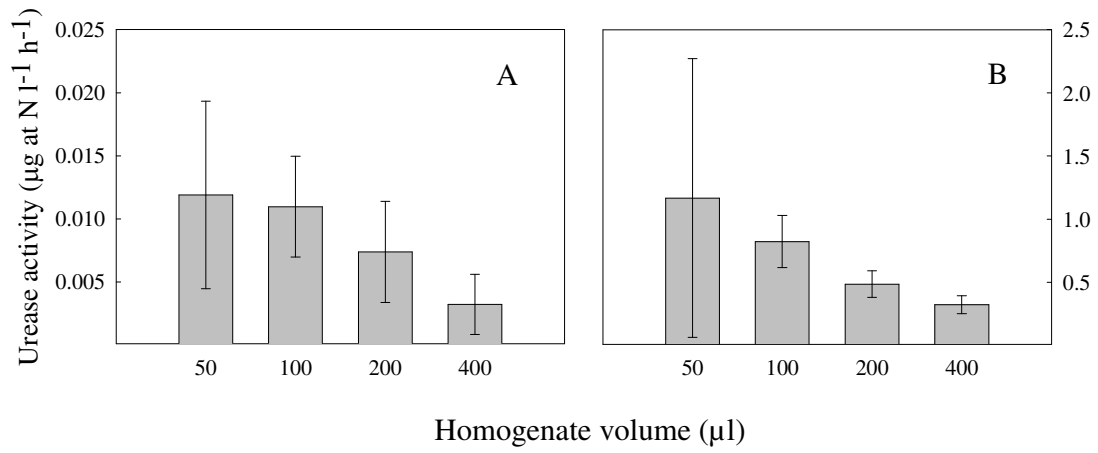
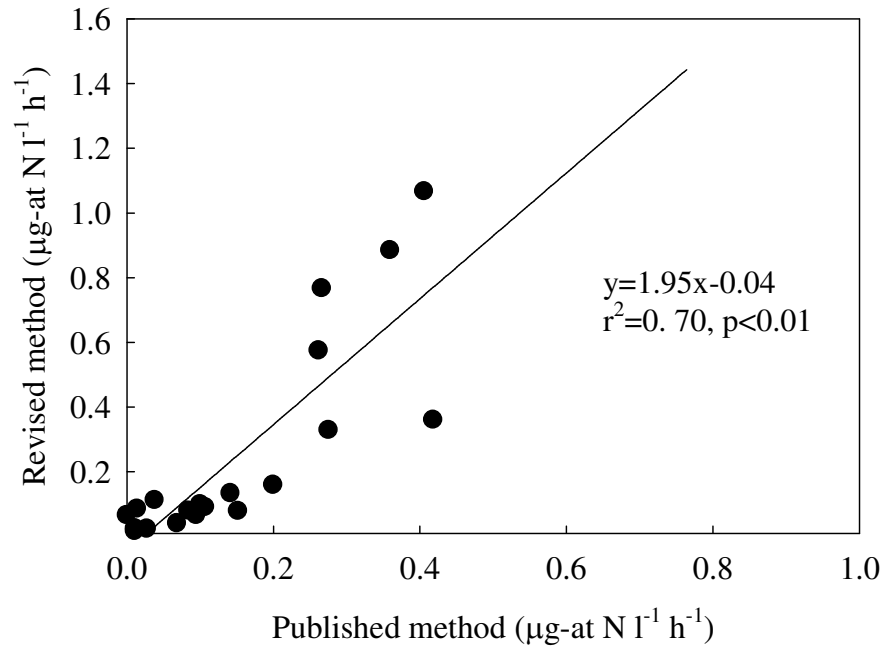


Fig. 2.8 Comparison of urease activity values obtained by measuring the published methods (Peers et al. 2000; Fan et al. 2003) and one of the revised methods with 0.03% PVP, 100 μ l homogenate with HEPES buffer on the same samples collected from the Choptank River or the Chesapeake Bay in July 2003 and April, July, August 2004.



Chapter 3: Urease activity in five phytoplankton species

Abstract

The regulation of urease, the enzyme responsible for the catabolism of urea to NH_4^+ and CO_2 , by different nitrogen compounds was investigated in laboratory cultures of five species of estuarine phytoplankton grown on NO_3^- , NH_4^+ and urea. Two of the species studied, dinoflagellates *Prorocentrum minimum* and *Karlodinium veneficum*, had higher urease activity rates on a per cell basis than the other species under investigation, the dinoflagellate *Heterocapsa triquetra*, the cryptophyte *Stoeatula major*, and the haptophyte *Isochrysis sp.* When urease activity was normalized on a per cell volume basis, *K. veneficum* had the highest rates while *S. major* had the lowest rates. Two dinoflagellates, *P. minimum* and *K. veneficum*, had lower urease activities when grown on NH_4^+ than when grown on NO_3^- or urea, suggesting that in some dinoflagellates, urease may be regulated by NH_4^+ . Results from this study and previous studies suggest that urease activity may be repressed by NO_3^- in diatoms and by NH_4^+ in dinoflagellates and cyanobacteria.

Introduction

Phytoplankton and bacteria can use a variety of nitrogenous substrates to meet their metabolic demand, including inorganic forms such as NO_3^- and NH_4^+ and organic nitrogen such as urea and amino acids. While urea in general contributes only a small percentage of total nitrogen uptake relative to NO_3^- and NH_4^+ , there are coastal regions and periods of the year when urea uptake can exceed 50% of total nitrogen uptake by phytoplankton (Glibert et al. 1991, Bronk et al. 1998, Kudela and Cochlan 2000, Tremblay et al. 2000, Twomey et al. 2005).

Some species of phytoplankton, including many harmful algal species, have been observed to utilize urea at higher rates than NO_3^- (Kudela and Cochlan 2000, Fan et al. 2003, Collos et al. 2004). In fact, many harmful dinoflagellates have been found to prefer urea both in culture and in the field. A large bloom of *Lingulodinium polyedrum*, a red tide dinoflagellate, off Newport Beach, California was found to have higher urea than NO_3^- and NH_4^+ uptake rates (Kudela and Cochlan 2000). A study of *Alexandrium catanella* nitrogen kinetics also found higher urea uptake rates compared to inorganic nitrogen uptake rates in culture (Collos et al. 2004). In addition, after elevated levels of urea were observed in aquaculture ponds, a consortium of harmful dinoflagellates, including *Karlodinium veneficum* (reported as *Gyrodinium galatheanum*), *Gymnodinium nelsonii*, *Prorocentrum minimum*, and *Katodinium* sp. increased in biomass (Glibert and Terlizzi 1999). Furthermore, the percent contribution of urea to total nitrogen uptake was found to be highly correlated with the percentage of dinoflagellates in the plankton community of Moreton Bay, Australia (Glibert et al. 2006).

Dinoflagellates are not the only taxa found to utilize urea at high rates. Blooms of the pelagophyte, *Aureococcus anophagefferens*, can be fueled by high urea concentrations in some environments (Berg et al. 1997, Mulholland et al. 2004, Kana et al. 2004). Blooms of the cyanobacterium, *Synechococcus elongatus*, in Florida Bay, have both higher rates of urea uptake and urease activity than surrounding areas (Glibert et al. 2004). Another cyanobacterium, *Trichodesmium* sp. (strain NIBB1067), had higher rates of urea uptake than nitrogen fixation when grown on urea (Mulholland et al. 1999). The estuarine species *Chloromorium toxicum*

(formerly the raphidophyte, *Chattonella cf. verriculosa*), grows better on urea or NH_4^+ than NO_3^- (Tomas 2005).

In order for a phytoplankton cell to utilize urea, it must first be transported into the cell via either passive or active transport. Many phytoplankton have active urea transport systems (Rees and Syrett 1979, Horrigan and McCarthy 1981, Antia et al. 1991). Urea is also produced intracellularly as the byproduct of the ornithine-urea cycle of arginine biosynthesis and catabolism (Antia et al. 1991) or catabolism of purines (Allen et al. 2005, Berg and Jørgensen 2006). Inside the cell, urea must be catabolized by urease or ATP: urea amidolyase (UALase) before urea-N enters the GS/GOGAT pathway as NH_4^+ (Antia et al. 1991). The urease catabolism pathway for converting urea into CO_2 and NH_4^+ is more common in phytoplankton than is UALase, which appears to be present only in some of the Chlorophyceae (Syrett and Leftley 1976, Bekheet and Syrett 1977, Anita et al. 1991).

In the past ten years, the study of urease activity has been limited to a few phytoplankton species including only the bacillariophytes (diatoms), dinophytes (dinoflagellates), pelagophytes and cyanobacteria (Collier et al. 1999, Peers et al. 2000, Dyrhman and Anderson 2003, Fan et al. 2003, Lomas 2004). The urease gene has been detected in many species of other taxonomic groups, but the rates of urease activity are unknown (Bruhn et al. 2002, Collier and Baker 2004). Due to the paucity of urease activity data, it is difficult to determine, along with urea uptake data, which taxonomic groups of phytoplankton are better competitors for urea and whether this ability is regulated by different nitrogen sources. There are indications that dinoflagellates have higher urease activity rates than diatoms and pelagophytes,

but this conclusion is based on one or very few species within each taxonomic group (Fan et al. 2003). Furthermore, experiments completed on the same few species have not revealed any clear patterns in the regulation of urease activity by nitrogenous substrates such as NO_3^- , NH_4^+ or urea. To broaden our understanding of urea utilization by phytoplankton, we investigated growth rates, internal N pools, and urease activity in laboratory cultures of five species from three taxonomic groups.

Methods

Species studied

Five phytoplankton species were investigated under controlled laboratory conditions. These included three harmful or toxic dinoflagellates (*Prorocentrum minimum*, *Karlodinium veneficum* (formerly *K. micrum*; Bergholtz et al. 2006), and *Heterocapsa triquetra*, one common cryptophyte, *Stoeatula major*, often a prey species for *K. veneficum* (Li et al. 2000, 2001), and the haptophyte, *Isochrysis sp.* Three species came from strains isolated from the Chesapeake Bay: *P. minimum* by M. Johnson (PM-1, Horn Point Laboratory, Cambridge, MD), *K. veneficum* (Leadbeater et Dodge) Larsen (strain GE) by A. Li and D. Stoecker (CCMP 1974), *S. major* Butcher ex Hill (strain g) by A. Lewitus (Baruch Marine Laboratory, Georgetown, SC). *H. triquetra* was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP 449) and was originally from the St. Lawrence estuary in Canada. *Isochrysis sp.* was isolated from near the island Providenciales in the Turks and Caicos Islands (Milford strain, C-ISO) and was

obtained from G. Wikfors (NOAA, National Marine Fisheries Service Laboratory, Milford, CT).

Culture conditions

The five species of phytoplankton were grown under identical nutrient and light conditions. Non-axenic cultures were grown in *f/2* media (Guillard and Ryther, 1962) with nitrogen (NO_3^- , NH_4^+ or urea) and phosphate substrates added at *f/20* concentrations (88 μM and 3.6 μM , respectively; N:P=24). All species were acclimated to the culture conditions described above for a period of several weeks to months before the experiments were conducted. Duplicate or triplicate cultures were grown in 2L glass bottles in a 20°C incubator room at 300 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on a 12h L:D cycle over the course of the experiment. Culture preparation and sampling were done under sterile conditions to maintain low bacterial biomass in the cultures.

Phytoplankton and bacteria biomass

During the two weeks of each experiment, 10-ml samples were taken each day for phytoplankton and bacterial counts and preserved in 4% glutaraldehyde. Depending on density of the culture, samples varying from 1-ml to 20-ml (and diluted with artificial seawater (salinity of 15) to a final volume of 20-ml) were also collected for determining cell counts and cell diameters using a Coulter Counter (Coulter Multisizer II). Bacterial biomass was calculated from bacterial counts made on a flow cytometer (Beckman Dickinson FACSCalibur) using the DNA stain SYTO 13

(del Giorgio et al. 1996) and an estimate of 19.5 fg-C bacterial cell⁻¹ for estuarine bacteria (Ducklow 2000).

Nutrient analyses

When each culture reached the mid- or late exponential phase, samples were collected for the analyses of particulate carbon (PC) and nitrogen (PN), and of internal cell nitrogen pools. For all analyses, phytoplankton were filtered (25-ml to 75-ml) onto a combusted (1 h at 450°C) GF/F filter. Samples for PC and PN were stored in a -20°C freezer, dried at 50°C for three days, and then analyzed on a Control Equipment CHN elemental analyzer. Internal cell nitrogen samples were placed into 5-ml of boiling water to break apart the cell walls, then immediately frozen (Raimbault and Mingazzini 1987). After thawing, samples were separated into two, 2-ml sub-samples and diluted to 5-ml for NH₄⁺ and 4-ml for urea. NH₄⁺ internal cell concentrations were measured using the indophenol method of Parsons *et al.* (1984), while urea internal cell concentrations were analyzed using the diacetylmonoxime method (Mulvenna and Savidge 1992, Revilla et al. 2005). Total cellular N was corrected by adding intracellular NH₄⁺ concentrations because of high volatilization rates of NH₄⁺ from filters during the drying process.

Urease activity

Replicate samples (n=15) for urease activity were collected at mid- to late exponential phase by filtering 25-ml to 75-ml of culture onto combusted GF/F filters

and then immediately freezing them in liquid N₂. The urease activity samples were transferred to a -80°C freezer for overnight storage. An exception to this procedure occurred for *K. veneficum* samples, which were immediately frozen in a -80°C freezer.

One day following sample collection, urease activity was assayed using a modified method based on Peers et al. (2000) and Fan et al. (2003) with reduced homogenate volumes and polyvinyl pyrrolidone (PVP) concentrations in order to reduce NH₄⁺ contamination and inference from buffers (Solomon et al. *submitted*). A range of urea additions were made in order to calculate Michealis-Menten kinetic parameters for urease activity. Final urea concentration additions of 250, 500 1360, and 3000 µg-at N•L⁻¹ were made to triplicate samples. One set of triplicate tubes had zero addition.

Data analysis

The kinetic parameters, K_m , and V_{max} are defined by the Michealis-Menten equation,

$$V = V_{max} \frac{S}{(K_m + S)}$$

where K_m is the half-saturation constant, V_{max} is maximum urease activity, and S is the concentration of the substrate, urea. In order to make comparisons among species and with other published studies, specific urease activity, V_{max} (µg-at N•L⁻¹•h⁻¹) was

normalized on a per cell ($V_{max-cell}$: fg-at N•cell⁻¹•h⁻¹), per cell volume basis ($V_{max-vol}$: fg-at N•μm⁻³•h⁻¹), and per chlorophyll *a* basis ($V_{max-chl}$; ng-at μg chl *a*⁻¹ h⁻¹).

Statistical testing was done to determine whether there were differences in growth rate and intracellular nitrogen concentrations between species grown on NO₃⁻, NH₄⁺, or urea. Significant differences among nitrogen sources were determined by one-way ANOVA tests and post-hoc comparisons (Tukey-HSD) using data from each individual species.

ANOVAs were also conducted to determine significant differences in K_m and V_{max} among species or nitrogen sources. Values of K_m and V_{max} for each culture were obtained using the best fit to the Michealis-Menten curve using SigmaPlot software (SYSTAT 2004). The calculated K_m and V_{max} values were checked to determine if the data had a normal distribution using the S-PLUS statistics program (Insightful Corporation 2002). Because the original data did not have a normal distribution, the data were transformed using the log₁₀(x+1) function. One-way ANOVAs with both K_m or V_{max} and nitrogen source as fixed effects for each species were run on the transformed data. Additional two-way ANOVAs were run to test for effect of species, nitrogen source, or any interaction between the two factors.

For graphical purposes, different calculations were performed to obtain K_m and V_{max} values. The mean overall urease activity at each assayed urea concentration was calculated. First, the replicates (n=3) of urease activity from each individual culture were averaged at each urea concentration. Next, sets of averages for each species (n=2 or 3 depending on species) grown on the same nitrogen source were combined to obtain an overall mean urease activity at each urea concentration.

Using the overall mean urease activity data, both K_m or V_{max} were calculated by using SigmaPlot software (SYSTAT 2004), using the best fit to the Michealis-Menten kinetic curve.

Results

Growth rates and biomass

Phytoplankton and bacterial growth rates in the cultures varied depending on the species and nitrogen growth source. Among the phytoplankton studied, the dinoflagellate *H. triquetra* had the lowest growth rates (0.21 ± 0.04 to 0.24 ± 0.01 d⁻¹) while the haptophyte *Isochrysis sp.* had the highest growth rates (0.72 ± 0.01 to 0.85 ± 0.01 d⁻¹; Table 3.1). Both *P. minimum* and *Isochrysis sp.* had higher growth rates on NO₃⁻ than the other nitrogen substrates, but these differences were only significant for *Isochrysis sp.* (ANOVA, Tukey-HSD, $p < 0.05$). All the other species had higher growth rates when grown on NH₄⁺, although only the cryptophyte *S. major* had a significantly higher growth rate on NH₄⁺ (0.71 ± 0.03 d⁻¹) than NO₃⁻ (0.65 ± 0.02 d⁻¹) ($p < 0.05$). For all the other species, differences in growth rate between nitrogen substrates were not significant (ANOVA, $p > 0.05$).

Bacteria net growth rates in the *K. veneficum* and *H. triquetra* cultures were less than 0.22 d⁻¹ and at times were not growing or were negative, likely due to being grazed by the dinoflagellates (data not shown). In the *P. minimum*, *S. major* and *Isochrysis sp.* cultures, bacteria net growth rates were comparable to or greater than the phytoplankton growth rates, varying from 0.25 to 0.91 d⁻¹. Regardless of growth

rates, bacterial carbon biomass contributed only 0.01 to 0.3% to the total carbon biomass in all the cultures.

Biochemical state of cells

Intracellular concentrations of urea (ranging from 0.16 to 3.46 mg-at N•L⁻¹) were 3- to 37-fold higher in cells grown on urea than on NO₃⁻ in *P. minimum*, *K. veneficum* and *H. triquetra* while there was no significant difference in *S. major* and *Isochrysis sp.* (Table 3.2, ANOVA, Tukey-HSD, p<0.05). Among species grown on urea, *K. veneficum* had the largest intracellular pool of urea. Urea composed 0.20% (*Isochrysis sp.*) to 42% (*K. veneficum*) of total cellular N (Table 3.2).

Intracellular concentrations of NH₄⁺ were higher than those of urea in all species regardless of growth N source, varying from 5.41 to 504 mg-at N•L⁻¹ (Table 3.2). Three species (*K. veneficum*, *H. triquetra*, and *Isochrysis sp.*) had higher NH₄⁺ concentrations when grown on NO₃⁻ than on other nitrogen sources. The other two species, *P. minimum* and *S. major*, had higher NH₄⁺ concentrations when grown on urea. *P. minimum* had significantly higher NH₄⁺ concentrations when grown on urea than on both NH₄⁺ and NO₃⁻ (ANOVA, Tukey-HSD, p<0.05). Intracellular NH₄⁺ contributed from 1.51 to 53.8% of total cellular N.

Urease activity

Patterns of maximal urease activity per cell ($V_{max-cell}$) among species followed similar trends when grown on NO₃⁻ or NH₄⁺ (Fig. 3.1). The dinoflagellates *P. minimum* and *K. veneficum* had the highest maximal urease activity (Fig. 3.1, Table

3.3). The next highest maximal urease activity rates were seen in the dinoflagellate species *H. triquetra*, followed by the cryptophyte *S. major*, and lastly the haptophyte *Isochrysis sp.* When grown on urea, a similar pattern existed with the exception of *S. major* having higher $V_{max-cell}$ than *H. triquetra*. The dinoflagellates, *P. minimum* and *K. veneficum*, had significantly higher $V_{max-cell}$ on average than the other three species (two-way ANOVA; $p < 0.05$). Among the three species with the lowest $V_{max-cell}$, *H. triquetra* had significantly higher maximal activity than *Isochrysis sp.* (two-way ANOVA; $p < 0.05$).

Significant differences in urease activity ($V_{max-cell}$) within a species when grown on different nitrogen sources were only seen in *K. veneficum*. The urease activity per cell ($V_{max-cell}$) of *K. veneficum* grown on NO_3^- and urea was significantly higher than in cells were grown on NH_4^+ (one-way ANOVA, Tukey-HSD; $p < 0.05$).

The patterns of maximal urease activity per cell volume ($V_{max-vol}$) among species differed from those of urease activity per cell ($V_{max-cell}$). *K. veneficum* had the highest $V_{max-vol}$, while *S. major* had the lowest $V_{max-vol}$ (Fig. 3.2). *K. veneficum* had significantly higher average $V_{max-vol}$ than the other four species regardless of nitrogen growth source (two-way ANOVA, Tukey-HSD; $p < 0.05$). *P. minimum*, *K. veneficum* and *Isochrysis sp.* grown on urea had significantly higher urease activity when grown on NH_4^+ (ANOVA; Tukey-HSD; $p < 0.05$).

Maximal urease activity per chlorophyll *a* ($V_{max-chl}$) was significantly higher in the three dinoflagellates than the other two species, *S. major* and *Isochrysis sp.* when grown on all three nitrogen sources (two-way ANOVA; Tukey-HSD; $p < 0.05$). *P. minimum*, *K. veneficum* and *S. major* had higher $V_{max-chl}$ when grown on urea than on

NO_3^- or NH_4^+ (Fig. 3.3) but significant differences in $V_{max-chl}$ were only observed in *K. veneficum* (ANOVA, Tukey-HSD; $p < 0.05$).

Overall differences in K_m between species or nitrogen growth source calculated using five urea concentrations were not significant (two-way ANOVA, $p > 0.05$). Cultures of *K. veneficum*, *S. major* and *Isochrysis sp.* had the lowest K_m when grown on NO_3^- , *P. minimum* when grown on NH_4^+ , and *H. triquetra* than when grown on urea. However, *K. veneficum* had a significantly higher K_m when grown on urea than NH_4^+ (one-way ANOVA, Tukey-HSD, $p < 0.05$).

Discussion

Urease may be expressed constitutively (e.g. urease activity remains the same regardless of cell physiology) or may be regulated by physiological factors (e.g. nitrogen or growth status). Both types of urease regulation have been observed in bacteria (Mobley and Hausinger 1989, Mobley et al. 1995) and may be true for phytoplankton. Regulation by nitrogen sources has been observed in various phytoplankton taxonomic groups for other enzymes involved with nitrogen acquisition. Nitrate reductase (NR) is induced by the presence of NO_3^- , and repressed by NH_4^+ in diatoms such as *Thalassiosira pseudonana* (Berges 1997, Parker and Armbrust 2005) and the chlorophyte *Dunaliella tertiolecta* (Song and Ward 2004), but does not appear to be repressed by NH_4^+ in dinoflagellates (Berges 1997). In the green alga, *Dunaliella primolecta*, *in vivo* glutamine synthetase (GS) activity, but not *in vitro* activity, has been shown to be inhibited by increasing NH_4^+ concentrations (Seguineau et al. 1987, 1989). Many bacterial and cyanobacterial

ureases are tightly regulated by the nitrogen regulatory system (e.g. nitrogen control gene A; *ntcA*) while some are expressed constitutively (Mobley and Hausinger 1989, Flores and Herrero 2005).

One way to determine whether urease is regulated or constitutively expressed in phytoplankton is to examine the kinetic parameter that describes the maximal urease activity rate (V_{max}) under different growth conditions. If a phytoplankton species has similar maximal urease activity rates (V_{max}) when grown on different nitrogen sources, urease may not be regulated by nitrogen source and therefore may be expressed constitutively. Two dinoflagellates, *P. minimum* and *K. veneficum*, in this study had higher urease activity rates in urea- and NO_3^- -grown cultures compared to NH_4^+ -grown cultures, suggesting regulation by NH_4^+ . Other dinoflagellate ureases also appear to be regulated. The dinoflagellate *Alexandrium fundyense* had the highest urease activity in an urea grown culture, lower in NH_4^+ and not detected in a NO_3^- grown culture (Dyhrman and Anderson 2003). Urease activity in *A. fundyense* was also induced by N-starvation (Dyhrman and Anderson 2003). The cryptophyte, *S. major*, and haptophyte, *Isochrysis sp.*, had similar urease activity when grown on all three nitrogen sources, suggesting that urease activity in these species is constitutive. However, different forms of regulation have been seen in the same species. In one clone of the diatom *Thalassosira weissflogii*, urease activity (V_{max}) was shown to be the same regardless of nitrogen source (Peers et al. 2000), but was down-regulated in another clone when grown on NO_3^- (Fan et al. 2003, Lomas 2004). Urease activity appears to be regulated in some but not all phytoplankton taxonomic groups.

Potential repression by NH_4^+ of urease activity occurs in some but not all dinoflagellates. Urease activity in *H. triquetra* when grown on urea was much lower than that of *P. minimum* and *K. veneficum* and had smaller pools of intracellular NH_4^+ . The lower growth rate of *H. triquetra* than *P. minimum* and *K. veneficum* may have reduced the metabolic demand for N, which in turn led to lower urease activity. These results suggest that regulation of urease within the dinoflagellates is complex, not simply dependent on nitrogen sources but also on other factors such as growth rates.

Urease activity and nitrogen demand

Potential urease contribution to cellular nitrogen demand varied among the five species examined in this study. The cellular nitrogen demand was calculated by multiplying the intracellular nitrogen concentrations (Q) and hourly growth rate (μ) for individual species grown on urea. Assuming that urease was operating at V_{max} measured *in vitro*, hourly urease activity accounted for more than enough nitrogen in the 3 dinoflagellates, sometimes by 4-fold (Fig. 3.4). In contrast, in both *S. major* and *Isochrysis sp.*, urease activity rates were not sufficient to meet the hourly nitrogen demand.

Whether the actual rate of urease activity *in vivo* reaches V_{max} depends on the intracellular urea concentration experienced by urease relative to its K_m . K_m is an intrinsic property of the enzyme and should not vary within a species grown on different nitrogen sources. Estimates of K_m made during this study (Table 3.3) were highly variable even within species, which likely reflects the sensitivity of calculated

K_m values to the number and distribution of urea concentrations assayed above and below K_m , and may also be influenced by the presence of other cell metabolites in the assays. Nevertheless, our values were consistent with previously reported values for phytoplankton urease K_m , which range from 120-460 μM (Table 3.4).

Intracellular urea concentrations of species studied here were comparable to concentrations found in other species, which range from non-detectable to 15 mg-at N l^{-1} in diatoms such as *Phaeodactylum tricornutum* and *Thalassiosira gravida* and the green alga, *Chlorella fusca* (Wheeler 1983). The diatom *Thalassiosira weissflogii* (0.42 ± 0.08 mg-at N l^{-1}) has been shown to have significantly lower urea intracellular concentrations than the dinoflagellate *P. minimum* (2.71 ± 0.21 mg-at N l^{-1}) and pelagophyte *A. anophagefferens* (4.65 ± 0.31 mg-at N l^{-1}) when grown on urea (Fan et al. 2003). When grown on urea, dinoflagellates in this study had higher intracellular urea concentrations than did the cryptophyte and haptophyte. The results from both past studies and this study suggest that dinoflagellates may have a more efficient mechanism for developing large intracellular urea pools, either through surface membrane transport proteins or via the urea cycle, and are able to retain urea within the cell. All species here had intracellular urea concentrations similar to or greater than estimated urease K_m . Therefore, as long as the intracellular urea pool is available to the enzyme, urease should operate between $\frac{V_{\max}}{2}$ and V_{\max} *in vivo*.

A brief review and conceptual model of urease regulation

A summary of kinetic data of urease activity from the literature is presented in Table 3.4. Urease activity often appears to be down-regulated in diatom cultures grown on NO_3^- . In contrast, urease activity in dinoflagellates, with the exception of *H. triquetra*, appears to be down-regulated in NH_4^+ grown cultures. The same is true in cyanobacteria where depressed urease activity is often seen when cultures are grown on NH_4^+ (Collier et al. 1999). In other phytoplankton taxonomic groups such as the cryptophytes and haptophytes, no evidence of regulation of urease activity has been observed.

Although the regulation of urease in diatoms and dinoflagellates is not fully understood, a conceptual model can be constructed based on general seasonal trends in temperate estuaries such as Chesapeake Bay (Fig. 3.5). When temperatures are seasonally low, spring diatom blooms are generally fueled by NO_3^- , (Lomas and Glibert 1999), leading to reduced urease activity because NO_3^- would be expected to suppress urease activity in diatoms (Table 3.4; Fig. 3.5) but not in the relatively few dinoflagellates present (Table 3.4; Fig. 3.5). Later during the summer, dinoflagellate blooms are frequently observed when NH_4^+ and urea, resulting from high regeneration rates, make up a large percentage of the nitrogen pool (Glibert et al. 2001), inhibiting nitrate uptake rates and NR activity in diatoms (Lomas and Glibert 1999, Lomas and Glibert 1999b, Lomas 2004) (Fig. 3.5). Supporting observations of low enzyme activities, low levels of mRNA of both NO_3^- transporters and NR have been observed in diatoms grown on NH_4^+ (Hildebrand and Dahlin 2000, Parker and Armbrust 2005). As in diatoms, NO_3^- uptake in dinoflagellates is suppressed by

NH_4^+ (Lomas and Glibert 1999b). However, it is not yet clear if NR activity or mRNA levels are regulated by NH_4^+ in dinoflagellates. The differences in repression of urease and NR, as well as enzymes involved in nitrogen uptake, between diatoms and dinoflagellates provide some biochemical explanations to why they bloom under contrasting environmental conditions.

Ecological implications

Urea concentrations in estuaries around the world have been generally low ($< 1 \mu\text{g-at N}\cdot\text{L}^{-1}$), but may be increasing due to a rise in urea fertilizer use in agriculture (Glibert et al. 2005, 2006). Harmful algal blooms have been observed after an increase in urea concentration in coastal waters, often after a fertilization event (Glibert et al. 2001, 2006). For example, blooms of *P. minimum* have been shown to be simulated by high urea concentrations in the Chesapeake Bay (Glibert et al. 2001). The harmful dinoflagellate, *K. veneficum*, as shown in this study, also has the ability to use urea and may also bloom under similar conditions as *P. minimum*. A *K. veneficum* bloom associated with fish kills in South Carolina retention ponds was associated with high levels of DON (Kempton et al. 2002). Based on what is known about *K. veneficum* physiology, urea has the potential for meeting its daily nitrogen demand and simulating blooms.

The potential regulation of urease activity by NH_4^+ in dinoflagellates that bloom during late spring and summer is important ecologically. Typically, NO_3^- concentrations are lower during those months in estuaries and NH_4^+ and urea make up a larger percentage of the available nitrogen pool than during the winter or early spring (Bronk et al. 1998, Glibert et al. 2005, Twomey et al. 2005). If NH_4^+ is not

present at concentrations that repress urease activity, then urea may be responsible for meeting most, if not all, of the nitrogen demand of the dinoflagellates *P. minimum* and *K. veneficum*. Ambient levels of NH_4^+ in the tributaries of Chesapeake Bay can reach $\sim 20 \mu\text{g-at N l}^{-1}$ (Glibert et al. 2005, Solomon et al. *submitted*), which may be enough to suppress urease activity during the late spring and summer months in estuaries and coastal areas. The possible regulation of urease by NH_4^+ in dinoflagellates, but not diatoms, is important to explore further for better understanding the differences in nitrogen metabolism and physiological ecology in these two phytoplankton taxonomic groups.

Conclusion

Urease activity is possibly regulated differently by nitrogen sources among and within phytoplankton taxonomic groups. Based on a review of the literature which only encompasses one to a few species in each taxonomic group, diatom urease may be repressed by NO_3^- while dinoflagellate and cyanobacterial ureases may be repressed by NH_4^+ . Urease in cryptophytes and haptophytes, at the present time, does not seem to be regulated by nitrogen source. The differential regulation of urease activity among various phytoplankton taxonomic groups may have implications for understanding differences in nitrogen metabolism and providing biochemical explanations to why diatoms and dinoflagellates bloom under different environmental conditions.

Literature cited

- Allen, A.E., Ward, B.B. & Bowler, C.P. 2005. Multi-lineage richness of functional genes in marine diatoms and transcriptional responses to nutrient stress. American Society of Limnology and Oceanography Annual Meeting. Santiago, Spain. June 2005.
- Antia, N.J., Harrison, P.J. & Oliveria, L. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia*. 1:1-89.
- Bekheet, I.A. & Syrett, P.J. 1977. Urea-degrading enzymes in algae. *Br. Phycol. J.* 12:137-43.
- Berg, G.M., Glibert, P.M., Lomas, M.W. & Burford, M.A. 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar. Biol.* 129: 377-387.
- Berg, G.M. & Jørgensen, N.O.G. 2006. Purine and pyrimidine metabolism by estuarine bacteria. *Aquat. Microb. Ecol.* 42: 215-226.
- Berges, J.A. 1997. Minireview: Algal nitrate reductases. *Eur. J. Phycol.* 32:3-8.
- Bergholtz, T., Daugbjerg, N., Moestrup, Ø. & Fernández-Tejedor, M. 2006. On the identity of *Karlodinium veneficum* and description of *Karlodinium armiger* sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. *J. Phycol.* 42: 170-193.
- Bronk, D.A., Glibert, P.M., Malone, T.C., Banahan, S. & Sahlsten, E. 1998. Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux. *Aquat. Microb. Ecol.* 15: 177-189.
- Bruhn A., Buch-illing, H., LaRoche, J. & Richardson, K. 2002. Identification of two functional genes involved with phytoplankton metabolisms of dissolved organic nitrogen: the urease and the formamidase gene. American Society of Limnology and Oceanography Annual Meeting. Victoria, British Columbia, Canada. June 2002.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiol.* 145: 447-459.
- Collier, J.L. & Baker, K.M. 2004. Urea-degrading microorganisms in Chesapeake Bay. American Society for Limnology and Oceanography Annual Meeting. Honolulu, Hawaii, USA. February 2004.

- Collos, Y., Gagne, C., Laabir, M., Vaquer, A., Cecchi, P., & Souchu, P. 2004. Nitrogenous nutrition of *Alexandrium catenella* (Dinophyceae) cultures and in Thau Lagoon, Southern France. *J. Phycol.* 40:96-103.
- del Giorgio, P.A., Bird, D.F., Prairie, Y.T., and Planas, D. 1996. Flow cytometric determination of bacterial abundance in lake plankton with the green nucleic acid stain SYTO 13. *Limnol. Oceanogr.* 41: 783-789.
- Ducklow, H. 2000. Bacterial production and biomass in the oceans. In Kirchman, D.L. [Ed.] *Microbial Ecology of the Oceans*. Wiley-Liss. New York., pp. 85-120.
- Dyhrman, S.T., & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Flores, E. & Herrero, A. 2005. Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochemical Society Transactions*. 33: 164-167.
- Ge, X., Cain, K. & Hirschberg, R. 1990. Urea metabolism and urease regulation in the cyanobacterium *Anabaena variabilis*. *Can. J. Microbiol.* 36: 218-222.
- Glibert, P.M., Garside, C., Fuhrman, J.A. & Roman, M.R. 1991. Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnol. Oceanogr.* 36: 895-909.
- Glibert, P.M., Harrison, J., Heil, C., & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry*. 77:441-463.
- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Glibert, P.M., Magnien, R., Lomas, M.W., Alexander, J., Fan, C., Haramoto, E., Trice, M. & Kana, T.M. 2001. Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: Comparison of the 1997, 1998 and 1999 Events. *Estuaries*. 24: 875-883.
- Glibert, P.M. & Terlizzi, D.E. 1999. Co-occurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. *Appl. Environ. Microbiol.* 65: 5594-5596.

- Glibert, P.M., Trice, T.M, Michael, B. & Lane, L. 2005. Urea in the tributaries of the Chesapeake and Coastal Bays of Maryland. *Water, Air, and Soil Pollution*. 160: 229-243.
- Guillard, R.R.L. & Ryther, J.H. 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Dentonula conferece* (Cleve) Gran. *Can. J. Microbiol.* 8:229-239.
- Hildebrand, M. & Dahlin, K. 2000. Nitrate transporter genes from the diatom *Cylindrotheca fusiformis* (Bacillariophyceae): mRNA levels controlled by nitrogen source and by the cell cycle. *J. Phycol.* 36: 702-713.
- Horrigan, S.G. & McCarthy, J.J. 1982. Phytoplankton uptake of ammonia and urea during growth on oxidized forms of nitrogen. *J. Plank. Res.* 4: 379-389.
- Kana, T.M., Lomas, M.W., MacIntyre, H.L, Cornwell, J.C.. & Gobler, C. J. 2004. Stimulation of the brown tide organism, *Aureococcus anophagefferens*, by selective nutrient additions to in situ mesocosms. *Harmful Algae* 3: 377-388.
- Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J. M. & Place, A.R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. *Harmful Algae* 1:233-241.
- Kramer, J.G & Morris, I. 1990. Growth regulation in irradiance limited marine *Synechococcus* sp. WH 7803. *Arch. Microbiol.* 154: 286-293.
- Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.
- Li, A., Stoecker, D.K. & Coats, D.W. 2000. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. *J. Plank. Res.* 22:2105-2124.
- Li, A., Stoecker, D.K. & Coats, D.W. 2001. Use of the 'food vacuole content' method to estimate grazing by the mixotrophic dinoflagellate *Gyrodinium galatheanum* on cryptophytes. *J. Plank. Res.* 23: 303-318.
- Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae): Interactions between nitrogen substrates. *Mar. Biol.* 144: 37-44.
- Lomas, M.W. & Glibert, P.M. 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 556-572.

- Lomas, M.W. & Glibert, P.M. 1999b. Interactions between NH_4^+ and NO_3^- uptake and assimilation: comparisons of diatoms and dinoflagellates at several growth temperatures. *Mar. Bio.* 133: 541-551.
- Lomas, M.W., Trice, T.M, Glibert, P.M., Bronk, D.A. & McCarthy, J.J. 2002. Temporal and spatial dynamics of urea uptake and regeneration rates and concentrations in Chesapeake Bay. *Estuaries.* 25: 469-482.
- Mobley, H.L.T. & Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. *Microbiol. Rev.* 3: 85-108.
- Mobley, H.L.T., Island, M.D. & Hausinger, R.P. 1995. Molecular Biology of Microbial Ureases. *Microbiol. Rev.* 59: 451-480.
- Mulholland, M.R., Ohki, K. & Capone, D.G. 1999. Nitrogen utilization and metabolism relative to patterns of N_2 fixation in cultures of *Trichodesmium* NIBB1067. *J. Phycol.* 35: 977-988.
- Mulholland, M.R., Boneillo, G. & Minor, E.C. 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. *Harmful Algae* 3:361-376.
- Mulvenna, P.F & Savidge, G. 1992. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. *Estuar. Coast. Shelf. Sci.* 34: 429-438.
- Oliveira, L. & Antia, N.J. 1986. Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J. Plank. Res.* 8: 235-242.
- Palinska, K.A., Jahns, T., Rippka, R. & Tandeau de Marsac, N. 2000. *Prochlorococcus marinus* strain PCC 9511, a picoplankton cyanobacterium, synthesizes the smallest urease. *Microbiology.* 146: 3099-3107.
- Parker, M.S. & Armbrust, E.V. 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 41:1142-1153.
- Parsons, T.R., Maita, Y. & Lalli, C.M. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon, New York.
- Peers, G.S., Milligan, A.J. & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.

- Rai, A.K. & Singh, S. 1987. Urease of blue-green algae (Cyanobacteria) *Anabaena doliolum* and *Anacystis nidulans*. *Curr. Microbiol.* 16: 113-117.
- Raimbault, P. & Mingazzini, P. 1987. Diurnal variations of intracellular nitrate storage by marine diatoms: Effects of nutritional state. *J. Exp. Mar. Biol. Ecol.* 112: 217-232.
- Rees, T.A.V. & Syrett P.J. 1979. The uptake of urea by the diatom *Phaeodactylum*. *New Phytol.* 82: 169-178.
- Rees, T.A.V. & Bekheet, I.A. 1982. The role of nickel in urea assimilation by algae. *Planta.* 156: 385-387.
- Revilla, M., Alexander, J. & Glibert, P.M. 2005. Analysis of urea in coastal waters: comparison of the enzymatic and the direct method. *Limnol. Oceanogr. Methods.* 3: 290-299.
- Seguineau, C., Batrel, Y. & Le Gal, Y. 1989. Glutamine synthase of *Dunaliella primolecta*. Partial characterization and possible adenylation control in relation to nitrogen nutrient levels. *Biochemical Systematics and Ecology.* 17: 503-508.
- Seguineau C., Batrel, Y., Martin-Jezequel, V. & La Gal, Y. 1987. Glutamine synthetase and the assimilation of inorganic nitrogen in *Dunaliella primolecta*. *Oceanis* 13: 515-520.
- Singh, S. 1990. Regulation of urease activity in the cyanobacterium, *Anabaena doliolum*. *FEMS Microbiol. Lett.* 67:79-84.
- Singh, S. 1993. Role of glutamine synthetase, glutamine and NH_4^+ regulation in urea metabolism in cyanobacterium, *Nostoc muscorum*. *Indian J. Exp. Biol.* 31: 256-259.
- Solomon, C.M, Alexander, J. & Glibert, P.M. Submitted. Measuring urease activity in environmental samples. *Limnol. Oceanogr. Methods.*
- Solomon, C.M. Collier, J.L., Alexander, J. & Glibert, P.M. Submitted. Microbial urea uptake and urease activity in Chesapeake Bay, USA. *Limnol. Oceanogr.*
- Song, B. & Ward, B.B. 2004. Molecular characterization of the assimilatory nitrate reductase gene and its expression in the marine green alga *Dunaliella tertiolecta* (Chlorophyceae). *J. Phycol.* 40:721-731.
- Syrett, P.J. & Leftley, J.W. 1976. Nitrate and urea assimilation by algae. In Sunderland, N. [Ed.] *Perspectives in Experimental Biology*. Pergamon Press, Oxford. pp. 221-234

Syrett, P.J. & Peplinksa, A.M. 1988. The effect of nickel and nitrogen deprivation on the metabolism of urea by the diatom *Phaeodactylum tricornutum*. *Br. Phycol. J.* 23: 387-390.

Tomas, C.R. 2005. Nitrogen preference of the fish-killing flagellate *Chattonella cf. verruculosa*. GEOHAB Open Science Meeting: HABs and Eutrophication. Baltimore, Maryland, USA. February 2005.

Tremblay, J-E, Legendre L., Klein, B. & Therriault, J-C. 2000. Size-differential uptake of nitrogen and carbon in a marginal sea (Gulf of St. Lawrence, Canada): significance of diel periodicity and urea uptake. *Deep-Sea Research II* 47:489-518.

Twomey, L.J., Piehler, M.F. & Pearl, H.W. 2005. Phytoplankton uptake of ammonium, nitrate, and urea in the Neuse River Estuary, NC, USA. *Hydrobiologia*. 533: 123-134.

Wheeler, P. 1983. Phytoplankton nitrogen metabolism.. In Carpenter, E.J & Capone, D.G. [Eds] *Nitrogen in the Marine Environment*. Academic Press. New York, N.Y. pp. 309-346.

Zubkov, M.V. & Tarran, G.A. 2005. Amino acid uptake of *Prochlorococcus* spp. in surface waters across the South Atlantic Subtropical Front. *Aquat. Microbiol. Ecol.* 40: 241-249.

Tables

Table 3.1 Growth rates ($d^{-1} \pm SD$) of the five phytoplankton species grown on NO_3^- , NH_4^+ , and urea

Phytoplankton species	Growth substrate - Nitrogen		
	NO_3^-	NH_4^+	Urea
Dinoflagellates			
<i>P. minimum</i> (n=3)	0.34±0.02	0.31±0.04	0.29±0.01
<i>K. veneficum</i> (n=3)	0.42±0.06	0.52±0.06	0.49±0.07
<i>H. triquetra</i> (n=2)	0.21±0.04	0.24±0.00	0.23±0.01
Cryptophyte			
<i>S. major</i> (n=3)	0.65±0.02	0.71±0.03	0.69±0.02
Haptophyte			
<i>Isochrysis sp.</i> (n=2)	0.85±0.01	0.72±0.01	0.78±0.00

Table 3.2 Intracellular NH_4^+ and urea concentrations, N content of cells and contribution of NH_4^+ and urea to total cellular N content (\pm SD) of phytoplankton grown on different nitrogen sources.

Phytoplankton species	Growth substrate	NH_4^+ (mg at N l ⁻¹)	% of total cellular N	Urea (mg at N l ⁻¹)	% of total cellular N	Total pg-at N/cell	C:N (molar)
Dinoflagellates							
<i>P. minimum</i> (n=3)	NO_3^-	4.39 \pm 2.44	15.3 \pm 5.79	0.80 \pm 1.15	2.29 \pm 2.84	2.02 \pm 0.38	9.90 \pm 1.23
	NH_4^+	41.8 \pm 18.4	25.2 \pm 8.86	0.39 \pm 0.60	0.98 \pm 1.20	1.76 \pm 0.24	9.65 \pm 0.24
	Urea	159 \pm 26.6	25.4 \pm 4.98	2.14 \pm 0.30	9.92 \pm 1.60	1.59 \pm 0.08	10.0 \pm 0.41
<i>K. veneficum</i> (n=3)	NO_3^-	504 \pm 148	45.0 \pm 9.62	0.26 \pm 0.40	2.54 \pm 2.21	1.49 \pm 0.22	5.01 \pm 1.18
	NH_4^+	477 \pm 71.2	53.8 \pm 4.99	0.29 \pm 0.39	2.76 \pm 2.32	1.19 \pm 0.13	7.90 \pm 4.47
	Urea	246 \pm 74.7	39.0 \pm 9.65	3.46 \pm 0.44	42.0 \pm 8.98	0.84 \pm 0.08	8.04 \pm 5.25
<i>H. triquetra</i> (n=2)	NO_3^-	86.3 \pm 52.0	30.9 \pm 13.9	0.08 \pm 0.16	0.79 \pm 1.12	0.35 \pm 0.06	8.89 \pm 0.94
	NH_4^+	79.5 \pm 6.66	23.1 \pm 4.90	0.56 \pm 0.75	3.55 \pm 1.92	0.46 \pm 0.13	7.89 \pm 0.40
	Urea	19.3 \pm 0.12	7.87 \pm 1.63	2.99 \pm 0.23	32.8 \pm 6.68	0.33 \pm 0.07	9.20 \pm 0.32
Cryptophyte							
<i>S. major</i> (n=3)	NO_3^-	5.41 \pm 1.10	2.33 \pm 0.47	2.03 \pm 1.45	2.47 \pm 1.83	0.85 \pm 0.09	6.45 \pm 0.39
	NH_4^+	5.48 \pm 0.57	3.44 \pm 0.29	2.95 \pm 1.91	3.61 \pm 1.71	0.58 \pm 0.06	8.16 \pm 0.25
	Urea	6.61 \pm 1.11	4.46 \pm 0.76	1.22 \pm 0.49	1.49 \pm 0.42	0.54 \pm 0.01	7.94 \pm 0.32
Haptophyte							
<i>Isochrysis sp.</i> (n=2)	NO_3^-	27.9 \pm 2.82	3.15 \pm 0.46	0.72 \pm 1.05	0.96 \pm 0.73	0.14 \pm 0.01	7.83 \pm 0.28
	NH_4^+	19.3 \pm 10.2	2.44 \pm 1.23	0.16 \pm 0.31	0.20 \pm 0.28	0.13 \pm 0.00	8.37 \pm 0.66
	Urea	10.5 \pm 1.28	1.51 \pm 0.25	0.53 \pm 0.56	0.72 \pm 0.62	0.12 \pm 0.01	8.09 \pm 0.50

Table 3.3 Average urease kinetic parameters (\pm SD), $V_{max-cell}$ (fg-at N cell⁻¹ h⁻¹) and K_m (μ g-at N l⁻¹) for all five phytoplankton species. Data represent mean (\pm SD) for all replicates. The correlation coefficient (r^2) represents the best fit to a non-linear model. All measurements were conducted during mid- to late exponential growth phase. *K. veneficum* (*) was the only species that had significantly higher $V_{max-cell}$ when grown on NO₃⁻ and urea than on NH₄⁺ as well as a higher K_m when grown on urea than NH₄⁺.

Phytoplankton species	Growth substrate - Nitrogen								
	NO ₃ ⁻			NH ₄ ⁺			Urea		
	$V_{max-cell}$	K_m	r^2	$V_{max-cell}$	K_m	r^2	$V_{max-cell}$	K_m	r^2
Dinoflagellates									
<i>P. minimum</i>	38.9 \pm 3.28	610 \pm 151	0.98	23.7 \pm 6.47	137 \pm 208	0.64	43.4 \pm 10.1	439 \pm 333	0.86
<i>K. veneficum</i> *	32.1 \pm 5.93	26.3 \pm 92.7	0.64	15.1 \pm 5.38	114 \pm 251	0.34	36.7 \pm 9.31	604 \pm 453	0.87
<i>H. triquetra</i>	9.14 \pm 2.82	924 \pm 722	0.85	12.1 \pm 3.23	1765 \pm 956	0.94	4.71 \pm 1.44	459 \pm 457	0.70
Cryptophyte									
<i>S. major</i>	8.46 \pm 2.26	266 \pm 284	0.49	9.23 \pm 3.90	553 \pm 696	0.66	9.22 \pm 1.77	476 \pm 291	0.90
Haptophyte									
<i>Isochrysis sp.</i>	2.24 \pm 0.42	608 \pm 337	0.92	1.15 \pm 0.19	867 \pm 381	0.95	2.03 \pm 0.44	1269 \pm 624	0.95

Table 3.4 Comparison of urease activity kinetic parameters among different phytoplankton species in culture. Units for V_{max} are fg-at N cell⁻¹ h⁻¹ (\pm SD) unless noted. Units for K_m are μ g-at N l⁻¹(\pm SD).

Growth nitrogen source Kinetic parameter	NO ₃ ⁻		NH ₄ ⁺		Urea	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Diatoms						
<i>Phaeodactylum tricornutum</i> (Syrett and Leftley 1976)						460
(Leftley and Syrett 1973)	2.22 nmol CO ₂ liberated/mg protein h ⁻¹					
(Rees and Bekheet 1982)	450 nmol C mg protein ⁻¹ h ⁻¹					
(Syrett and Peplinska, 1988)	5-9 nmol urea decomposed (10 ⁷ cells h) ⁻¹					
<i>Cyclotella cryptica</i> (Oliver and Anita 1986)	0.36 μ M urea hydrolyzed min ⁻¹ mg protein ⁻¹				0.47 μ M urea hydrolyzed min ⁻¹ mg protein ⁻¹	
<i>Thalassosira weissflogii</i> ¹ (Peers et al. 2000)	1667		1667		1667	
(Fan et al. 2003) ²	19.80 \pm 5.67		44.71 \pm 9.23		45.07 \pm 13.68	
(Lomas 2004)	17.9 \pm 5.9		41.7 \pm 8.3		46.26 \pm 3.6	120 \pm 53.8
					55.3 \pm 15.9	
<i>Thalassosira pseudonana</i> ³ (Peers et al. 2000)	167		41.7		312.5	
	718 nmol C mg protein ⁻¹ h ⁻¹				2.7 x 10 ³ nmol C mg protein ⁻¹ h ⁻¹	

Table 3.4 con't

	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Dinoflagellates						
<i>Prorocentrum minimum</i> (Fan et al. 2003) ²	61.75±10.74		48.47±22.02		64.96±12.35	
(Solomon et al., this study)	38.9±3.28	610±151	23.7±6.47	137±208	43.4±10.1	165±45.7 439±333
<i>Alexandrium fundyense</i> (Dyhrman and Anderson 2003)	not detectable 77.01 (low nitrate) 100.5 (low nitrate) 140.17 (low nitrate)		~10		~110	
<i>Karlodinium veneficum</i> (Solomon et al., this study)	32.1±5.93	26.3±92.7	15.1±5.38	114±251	36.7±9.31	604±453
<i>Heterocapsa triquetra</i> (Solomon et al., this study)	9.14±2.82	924±722	12.1±3.23	1765±956	4.71±1.44	459±457
Pelgaophytes						
<i>Aureococcus</i> <i>anophagefferens</i> (Fan et al. 2003) ²	6.54±2.07				6.03±1.53 4.22±0.22	144±39.4
Cryptophyte						
<i>Storeatula major</i> (Solomon et al., this study)	8.46±2.26	266±284	9.23±3.90	553±696	9.22±1.77	476±291
Haptophytes						
<i>Isochrysis sp.</i> (Solomon et al., this study)	2.24±0.42	608±337	1.15±0.19	867±381	2.03±0.44	1269±624

<i>Table 3.4 con't</i>	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Cyanobacteria (for more see Collier et al. 1999)						
<i>Anabaena doliolum</i> (Rai and Singh, 1987)	596.8 nmol urea hydrolyzed mg ⁻¹ protein h ⁻¹	120 μM ³	230.0 nmol urea hydrolyzed mg ⁻¹ protein h ⁻¹		630.0 nmol urea hydrolyzed mg ⁻¹ protein h ⁻¹	
<i>Anabaena variabilis</i> (Ge et al. 1990)			52.0±1.88 pmol CO ₂ released mg protein ⁻¹ h ⁻¹		34.2±0.92 pmol CO ₂ released mg protein ⁻¹ h ⁻¹	
<i>Anacystis nidulans</i> (Rai and Singh, 1987)	568.4 nmol urea hydrolyzed mg ⁻¹ protein h ⁻¹	250 μM, 1.66 mM ⁴ (biphasic)	214.0 nmol urea hydrolyzed mg ⁻¹ protein h ⁻¹		598.0 nmol urea hydrolyzed mg ⁻¹ protein h ⁻¹	
<i>Synechococcus</i> (WH7805) (Collier et al. 1999)	14, 22 nmol urea hydrolyzed min ⁻¹ mg protein ⁻¹ ⁵ 12, 20.2	232	3.2, 3.5 nmol urea hydrolyzed min ⁻¹ mg protein ⁻¹ ⁵ 2.94, 3.22		4.3, 5.7 nmol urea hydrolyzed min ⁻¹ mg protein ⁻¹ ⁵ 3.95, 5.23	
<i>Prochlorococcus marinus</i> (PCC 9511) (Palinska et al. 2000)					94.6 mol urea hydrolyzed min ⁻¹ mg protein ⁻¹ ⁶ 1.74 ng-N cell ⁻¹ h ⁻¹	0.23 mM
<i>Nostoc muscorum</i> (Singh 1993)			0.6 nmol urea hydrolyzed min ⁻¹ mg protein ⁻¹		9.8 nmol urea hydrolyzed min ⁻¹ mg protein ⁻¹	

¹Values from Peers et al. (2000) were taken from Day 1 of their experiment. ²Fan et al. (2003) used SE, not SD. ³Conversions from pg N cell⁻¹ d⁻¹ done by Peers et al. (2000). ⁴For cultures grown on combined nitrogen sources (NH₄Cl, Ca(NO₃)₃, urea) ⁵Conversions from nmol urea hydrolyzed min⁻¹ mg protein⁻¹ using 500 fg protein cell⁻¹ for WH7805 (Kramer and Morris, 1990) ⁶Conversions from mol urea hydrolyzed min⁻¹ mg protein⁻¹ using an average of 21.5 fg protein cell⁻¹ for *Prochlorococcus* sp. (Zubkov and Tarran, 2005).

Figures

Fig. 3.1 Kinetic curves of urease activity per cell in five different phytoplankton species as function of urea concentration. Each line represents data from cultures grown on a different nitrogen substrate. The kinetic parameters for each relationship are reported in Table 3.3.

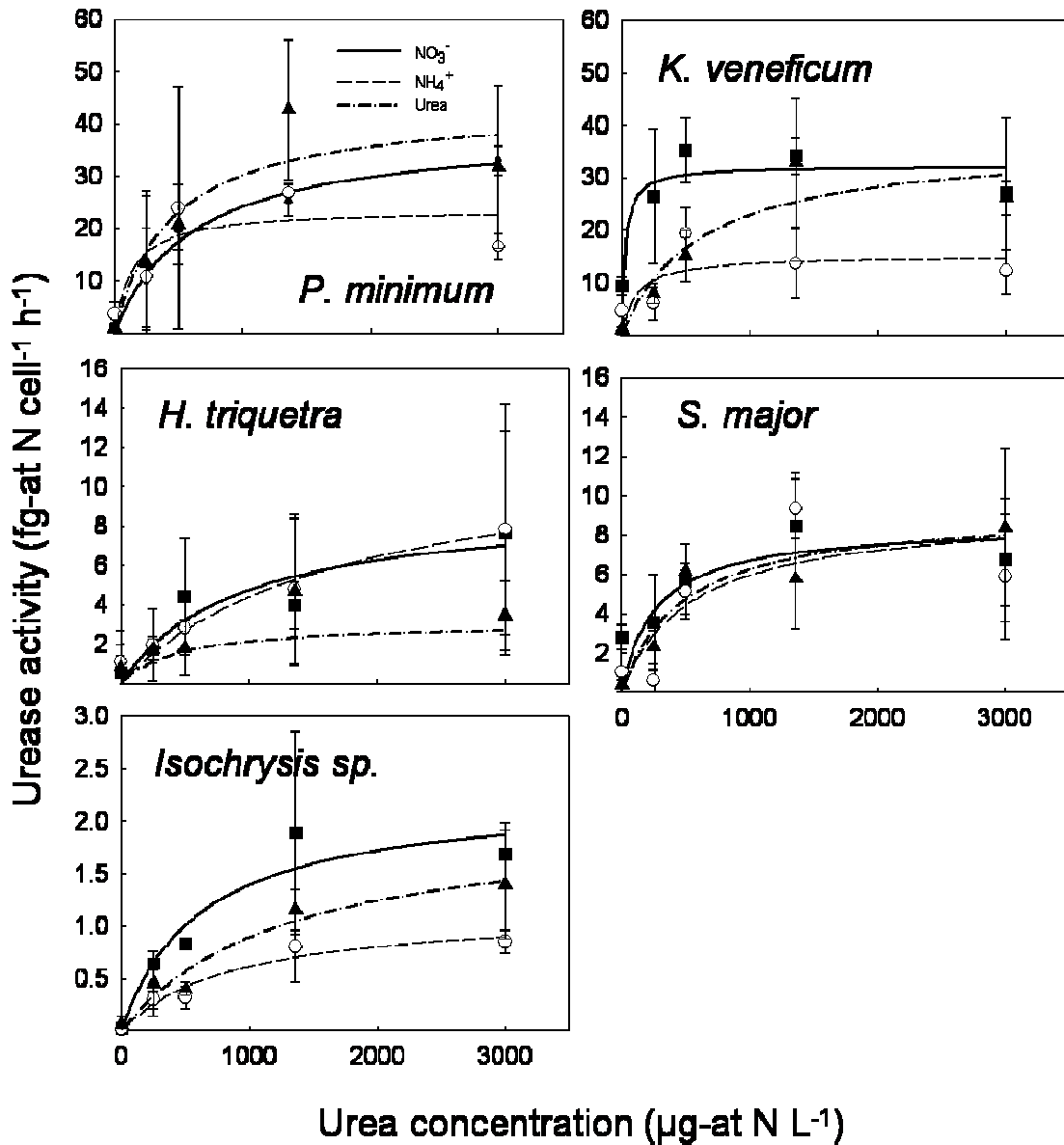


Fig. 3.2 Same as Fig. 3.1 except urease activity is given on a per cell volume basis. Note: the kinetic curve for *P. minimum* grown on NO_3^- was too low to be shown in the figure.

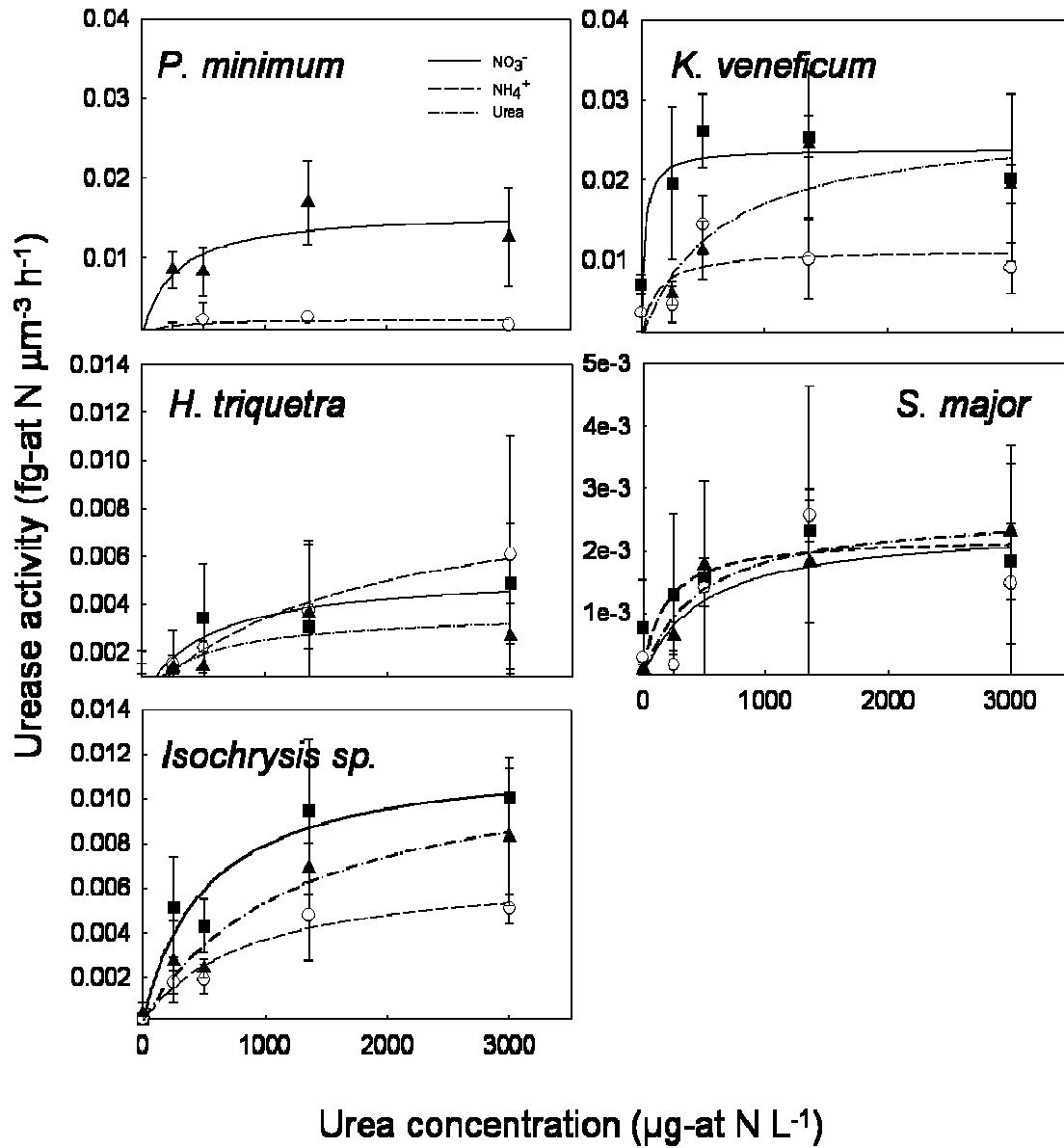


Fig. 3.3 Same as Fig. 3.1 except urease activity is given on a per chl *a* basis. Note: the kinetic curve for *K. veneficum* grown on NH_4^+ was too low to be shown in the figure.

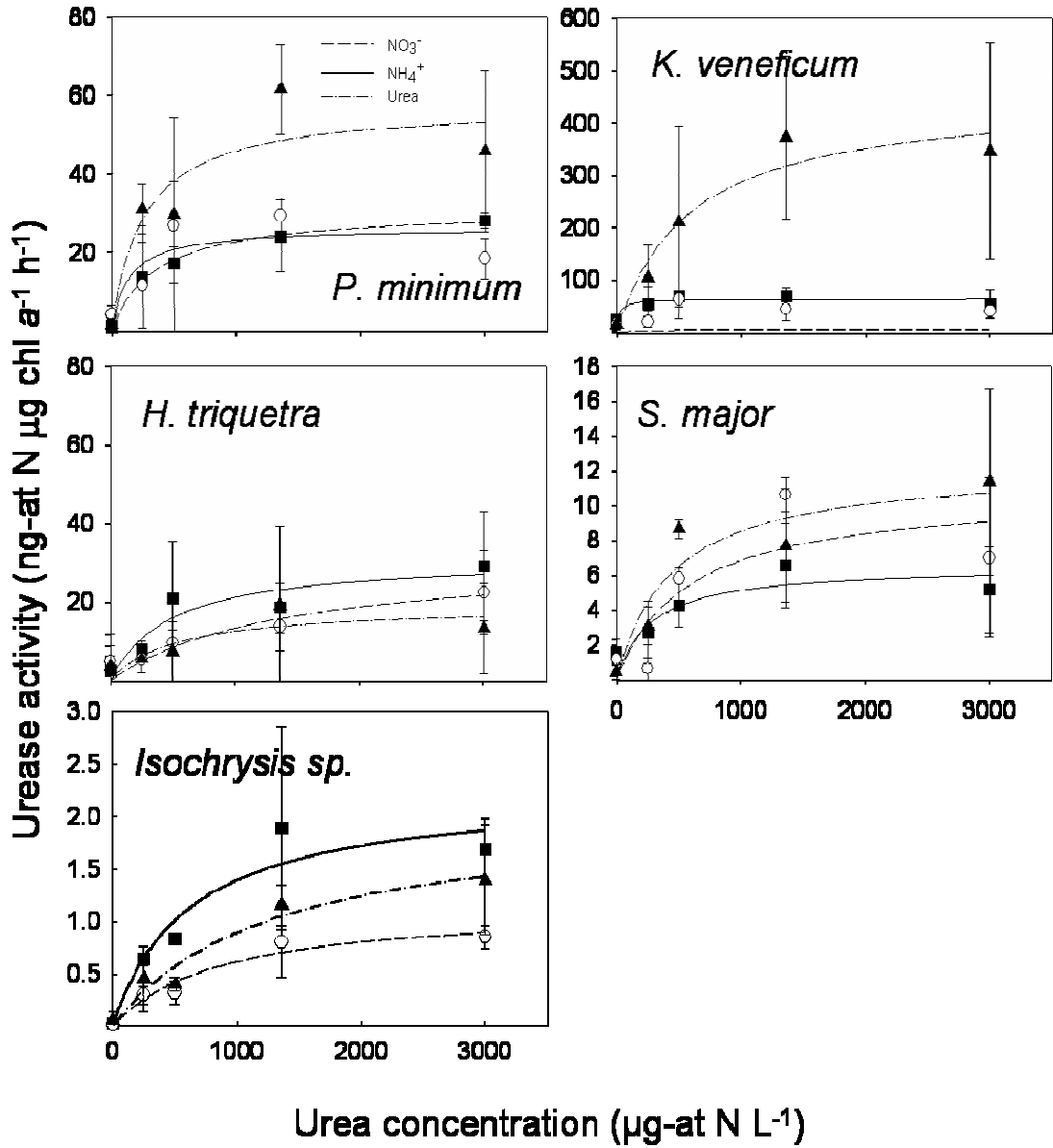


Fig. 3.4 Comparison of potential urease activity (dark bars) versus nitrogen demand (open bars) calculated on an hourly basis for the seven phytoplankton species on a log scale. Species denoted with an asterisk (*) are estimates from Fan et al. (2003).

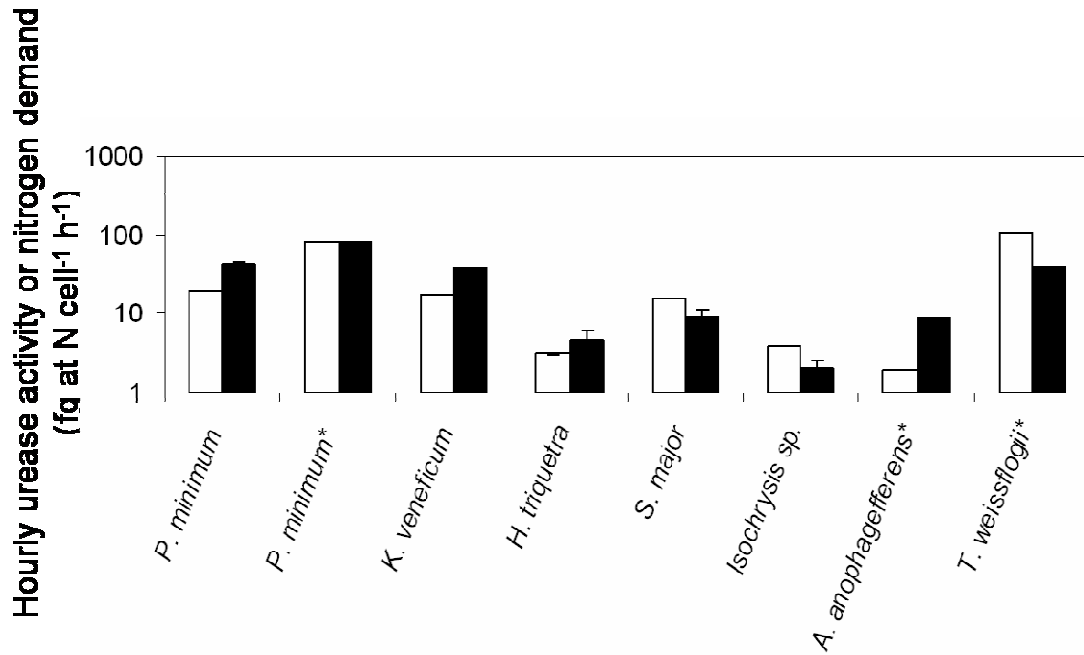
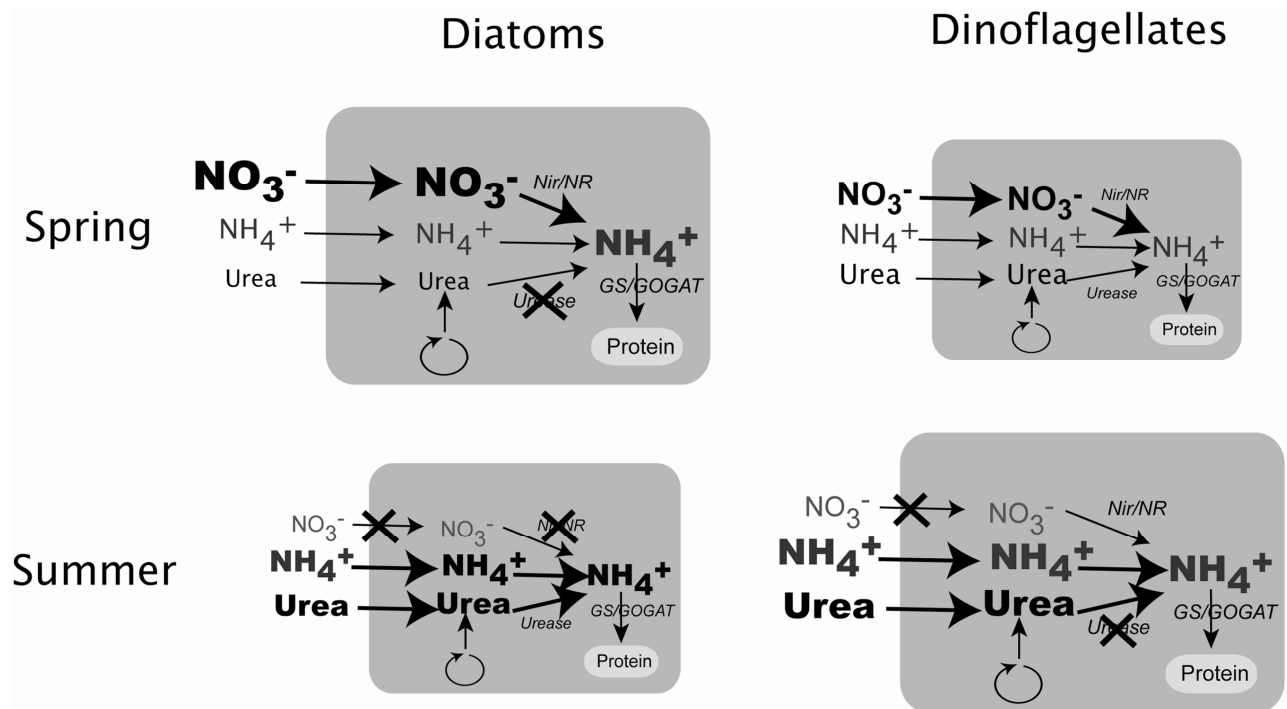


Fig. 3.5 A comparison of biochemical regulation of enzymes involved with nitrogen acquisition (i.e. urease and nitrate reductase) in diatoms and dinoflagellates under two different environmental conditions: high NO_3^- concentrations which occur in early spring and higher contribution of NH_4^+ /urea in late spring and summer in Chesapeake Bay. Enzymes responsible for the major pathways such as nitrite and nitrate reductase (Nir/NR), glutamate synthetase & glutamine 2-oxoglutarate aminotransferase (GS/GOGAT) and urease and those that are repressed by NO_3^- or NH_4^+ are shown with a X. The circular loop represents the urea cycle. The size of the font indicates relative concentrations of NO_3^- , NH_4^+ or urea.



**Chapter 4: Microbial urea uptake and urease activity in
Chesapeake Bay**

Abstract

Urea uptake and urease activity were studied over a nutrient gradient from Chesapeake Bay to the Sargasso Sea during two dry (2001, 2002) and two wet years (2003, 2004). The dry and wet years presented contrasting conditions in nitrogen concentrations and microbial communities, especially in the mesohaline Bay (Mid Bay), and possibly differences in regulation between urea uptake and urease activity. Urea uptake and urease activity changed seasonally, with the highest rates observed during the summer. Urea uptake was more tightly regulated by nitrogen availability than urease activity, sometimes leading to lower urea uptake than urease activity rates. The utilization of urea, as indicated by higher urea uptake and urease activity rates, increased from the Upper Bay to the Sargasso Sea, reflecting the importance of urea in N-limiting waters.

Introduction

Phytoplankton and bacteria utilize many nitrogenous substrates including NO_3^- , NH_4^+ , urea, and amino acids (Capone 2000; Kirchman 2000). Many enzymes are involved with the assimilation of these substrates. Urease, the enzyme that catabolizes urea to NH_4^+ and CO_2 , has been studied only in a few phytoplankton species (Collier et al. 1999; Fan et al. 2003; Dyhrman and Anderson 2003; Solomon and Glibert *submitted*). Utilization of most nitrogen sources, including urea, also requires the active uptake of the substrate into the cell (Capone 2000). The examination of both urea uptake and urease activity is necessary to understand how urea is utilized under different environmental conditions in estuaries.

The significance of urea utilization by phytoplankton and bacteria is beginning to be appreciated (Berman and Bronk 2003; Glibert et al. 2006). Urea uptake can account for > 50% of total nitrogen uptake in some coastal areas (Glibert et al. 1991, 1995; Kudela and Cochlan 2000) and can satisfy nitrogen metabolic requirements in some species in culture (Fan et al. 2003; Solomon and Glibert *submitted*). Higher urea uptake rates have been observed during blooms of both dinoflagellates and cyanobacteria than of other phytoplankton such as diatoms (Kudela and Cochlan 2000; Collos et al. 2004; Glibert et al. 2004, 2006).

In recent years, measurements of urease activity in culture and field studies have expanded our understanding of urea utilization. Urease activity seems to be inversely correlated with inorganic nitrogen and positively correlated with organic nitrogen. For example, urease activity rates were found to be higher when NO_3^- and NH_4^+ concentrations were low during a dinoflagellate bloom of *Alexandrium* sp., than prior to the bloom in the western Gulf of Maine (Dyhrman and Anderson 2003). Higher rates of urease activity were observed in a bloom of the cyanobacterium, *Synechococcus elongatus*, in Barnes Key in Florida Bay than in nearby areas that had higher dissolved inorganic nitrogen (DIN) concentrations (Glibert et al. 2004). On the West Florida Shelf, Heil et al. (*in press*) found the highest urease activity at the mouth of the Peace and Shark rivers where urea and dissolved organic nitrogen (DON) levels were higher than offshore sites. Urease activity, like urea uptake, also appears to be related to the taxonomic composition of phytoplankton. For example, urease activity rates in a dinoflagellate culture (*Prorocentrum minimum*) were observed to be higher on a per cell basis than in a diatom and a pelagophyte culture

(Fan et al. 2003). In a bloom of *P. minimum* in the Corsica River, Maryland, urease activity rates were four-fold higher than outside the bloom (Salerno 2005). On the West Florida Shelf, regions with high urease activity were dominated by dinoflagellates, including *Karenia brevis*, and cyanobacteria, as opposed to southern regions on the shelf with lower urease activity and higher percentage of diatoms (Heil et al. *in press*).

Chesapeake Bay offers a natural setting in which to examine the regulation of urea utilization by a variety of environmental factors. Freshwater flow into the Chesapeake Bay establishes a gradient in nitrogen availability (Fisher et al. 1992; Glibert et al. 1995; Bronk et al. 1998) and community structure (Adolf et al. 2006). The upper reaches of the estuary are dominated by oxidized forms of nitrogen (e.g. NO_3^- and NO_2^- ; Fisher et al. 1992; Kemp et al. 2005). Reduced forms such as NH_4^+ , urea, and DON become progressively more important from the upper to the lower parts of the estuary (Glibert et al. 1995; Bronk et al. 1998). If urea uptake and urease activity are regulated by NO_3^- and NH_4^+ , rates would be expected to increase from the upper to lower parts of Chesapeake Bay. If particular phytoplankton species are major users of urea, urea uptake and urease activity would be expected to vary with phytoplankton community composition. To test these hypotheses, we measured both urea uptake and urease activity and compared these rates to available taxonomic data over a period of four years (2001-2004) at three sites in the Chesapeake Bay, its plume, and the Sargasso Sea.

Methods

Field sampling

Samples were collected from three stations in Chesapeake Bay (Fig. 4.1) in the spring, summer, and fall from 2001-2004, with the exception that only one station (Mid Bay) was sampled in summer 2003. The first station was in the upper reaches of the Bay (Upper Bay), the second site was mid-Bay (Mid Bay), while the third site was near the mouth of the Bay (Lower Bay). During some cruises, additional sites were sampled in Chesapeake Bay plume (April 2002, October 2004) and the Sargasso Sea (April 2002, 2004).

Hydrological data

Water was collected from near-surface and near-bottom using Niskin bottles mounted on a 12 bottle General Oceanic 1015 rosette aboard the R/V Cape Henlopen. Only the surface data are presented here. Salinity and temperature data were taken with a Seabird 911 CTD. Monthly and daily streamflow data into Chesapeake Bay were obtained from the United States Geological Survey (www.usgs.gov/monthly/bay1.html).

Nutrients

At each station, water from the Niskin bottles was filtered through precombusted GF/F filters (450°C for 1 h) into acid-washed bottles for later determination of nutrients in the laboratory. Concentrations of NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} were determined with a Technicon Autoanalyzer II (Lane et al. 2000). Urea

concentrations were measured by the urease method described by Parsons et al. (1984) until April 2004. Samples taken after April 2004 were measured by the diacetylmonoxime method which was found to have a smaller salt interference than the urease method (Mulvenna and Savidge 1992; Revilla et al. 2005). Total dissolved nitrogen (TDN) concentrations were determined by persulfate oxidation (Bronk et al. 2000) while total dissolved phosphorus (TDP) was measured by the method of Solórzano and Sharp (1980). Concentrations of DON and dissolved organic phosphorus (DOP) were determined by subtracting inorganic nitrogen from TDN, and PO_4^{3-} from TDP. Concentrations of particulate carbon (PC) and particulate nitrogen (PN) were analyzed with an Exeter Analytical Incorporated CE-440 elemental analyzer.

Plankton composition and biomass

For chlorophyll *a* (chl *a*) and pigment analyses, water was also filtered through precombusted GF/F filters (450°C for 1 h), and the filters were immediately frozen onboard at -20°C. Once back in the laboratory, samples were stored in a -80°C freezer until analysis. Chl *a* samples were analyzed by extraction with acetone (Parsons et al. 1984), then measured on a 10-AU Turner Designs fluorometer. Pigment analyses were done according to Van Heukelem et al. (1994) and Van Heukelem and Thomas (2001) on a Hewlett Packard high-performance liquid chromatograph (HPLC; Model 110) system. Results from pigment analyses were analyzed using the CHEMTAX software program (Mackey et al. 1996) using a matrix calibrated for estuarine phytoplankton (Adolf et al. 2006) that gave the relative

abundance of seven taxonomic groups ($f_{chl-a^{taxa}}$): prasinophytes, dinoflagellates, cryptophytes, haptophytes, chlorophytes, cyanobacteria, and diatoms. Since CHEMTAX analysis is not completely robust (e.g. Lewitus et al. 2005), further analysis was conducted to ensure that certain groups of phytoplankton were detected such as dinoflagellates that lack peridinin. Dinoflagellates such as *Karlodinium veneficum* (formerly *K. micrum* and *Gyrodinium galatheaum*; Bergholtz et al. 2006) contain 19'-hexanoyloxy-fucoxanthin (Tengs et al. 2000; Bergholtz et al. 2006) which CHEMTAX uses as an indicator of haptophytes which leads to misidentification of this group of phytoplankton. To prevent misidentification, preserved samples (in Lugol's solution, 4% glutaraldehyde or 2% formalin) of a few selected time points and stations with high 19'-hexanoyloxy-fucoxanthin concentrations were analyzed by microscopy. Phytoplankton biomass estimates were based on conversions of chlorophyll to carbon biomass using an average estimate of C:Chl *a* of 75 (Hagy et al. 2005).

Samples for the enumeration of bacteria were collected from water at the same sites. Bacteria were preserved at the time of collection in 2% formalin and stored at 4°C until stained with DAPI (4',6-Diamidino-2-phenylindole) and counted on an epifluorescent microscope. Replicate counts during different months were conducted to ensure there was no cell loss over the storage period. Estimations of bacterial biomass were obtained by converting bacteria cell densities to carbon biomass using 19.5 fg C cell⁻¹ (Ducklow 2000).

Urea uptake and urease activity

Urea uptake and urease activity samples were collected for both whole and <1.6 μm fractions. The whole fraction consisted of unfiltered water while the <1.6 μm fraction was obtained by filtering water through precombusted GF/A filters (1.6 μm pore size; 450°C for 1 h). Urea uptake rates were determined on both size fractions using ^{15}N tracer techniques (Glibert and Capone 1993). Incubations were conducted in 1 L acid-washed polycarbonate bottles with 0.5 $\mu\text{g-at N L}^{-1}$ ^{15}N -urea (resulting in atom % enrichment of 13.1 to 100%) under 60% natural irradiance using neutral-density screening for 30 min. After the incubations, water was filtered onto one GF/F filter for the whole fraction and onto a double layer of GF/F filters for the <1.6 μm fraction to retain as much bacteria as possible. Once filtering was complete, samples were immediately frozen. Samples were dried at 50°C, packed into tin boats, and analyzed on a SerCon mass spectrometer.

Urease activity samples were collected from the same size fractions. Particulate matter was collected immediately onto filters in a similar fashion as for the urea uptake samples, and the filters were frozen in liquid N_2 until analysis. Samples were analyzed for urease activity within one week of sampling using the method of Peers et al. (2000) which was further improved by Fan et al. (2003). Beginning in August 2003, urease activity samples were analyzed using an optimized assay for field samples (Solomon et al. *submitted*). Conversions on data prior to August 2003 were made using an equation developed from samples collected from a range of sites and seasons analyzed by both methods (Solomon et al. *submitted*).

Urease activity was normalized on a per chl *a* biomass (ng at-N $\mu\text{g chl } a^{-1} \text{ h}^{-1}$) basis for temporal and spatial comparisons.

Statistical analysis

Data from all four years were analyzed to determine the extent to which environmental parameters explained variability in urea uptake and urease activity in Chesapeake Bay. The original urease activity and urea uptake data did not have a normal distribution, so the data were transformed using the square root function. Two-way ANOVAs were run to detect any significant relationships between seasons or stations in urea uptake and urease activity.

Physical factors (salinity and temperature), nitrogen availability (NO_3^- , NH_4^+ , urea), and phytoplankton community composition (diatoms, dinoflagellates, cryptophytes, cyanobacteria, and haptophytes) are strongly inter-correlated, so principal component analyses (PCA; S-PLUS, Insightful Corporation, 2002) were conducted to produce a new set of linear combined variables and reduce the number of variables to be used in a multiple regression model.

Two considerations were used in determining which variables should be combined to form a new set of linear variables. The first consideration was the relationship between urea uptake and urea concentration which is non-linear and follows Michealis-Menten enzyme kinetics. However, the relationship between the reaction rate and substrate concentration is close to linear below the half-saturation constant, K_s . Ambient urea concentrations from all sites were $<1.5 \mu\text{g-at N L}^{-1}$ which is close to K_s of $<1.0 \mu\text{g-at N L}^{-1}$ in many natural assemblages (*reviewed in Kudela*

and Cochlan 2000). Thus in most cases, the relationship between urea uptake and ambient urea concentrations is expected to be linear.

The second consideration was which values from the CHEMTAX analysis should be used to form the linear combined variables. Five major taxonomic groups (diatoms, dinoflagellates, cryptophytes, cyanobacteria and haptophytes) were chosen for the PCA analysis because they represented the largest percentage of the floral community in Chesapeake Bay (Adolf et al. 2006). Once the set of environmental variables to use was determined, the PCA analysis allowed for the visualization of how each combined variable changed over time at each Chesapeake Bay station. Urea uptake and urease activity were then modeled using multiple regressions with the new combined variables resulting from PCA analysis (PC1, PC2, PC3). The level of significance in the multiple regressions was set at $p < 0.1$.

Results

Physical features

All three stations in Chesapeake Bay exhibited an annual pattern in both temperature and salinity (Table 4.1). Of the sampled months, the coldest temperatures observed were recorded in April (7.3 to 11.6°C) and the warmest temperatures in July or August (25.5 to 27.7°C). Salinity increased from the mostly freshwater Upper Bay station (<9) to the Lower Bay station (>16.7). Salinity was lower in 2003 and 2004 than in 2001 and 2002 (Table 4.1). The average streamflow from the Susquehanna River in 2001 and 2002, considered to be dry years, was

$<1,700 \text{ m}^3 \text{ s}^{-1}$ which was below the range of normal streamflow ($1,900\text{-}2,600 \text{ m}^3 \text{ s}^{-1}$; USGS 2006). Average streamflow into Chesapeake Bay in 2003 and 2004, considered to be wet years, exceeded $3000 \text{ m}^3 \text{ s}^{-1}$ (USGS 2006) and reduced salinity levels by approximately 5 throughout Chesapeake Bay. The timing of the spring freshet differed each year, which had an influence on the nutrient concentrations at the time of sampling in April. In 2001, 2003, and 2004, the freshet occurred prior or during the spring sampling period. However, in 2002, the spring freshet occurred after the spring sampling (data not shown).

Dissolved nutrient distributions

Nitrogen concentrations followed a gradient from the Upper to Lower Bay. Average TDN during 2001-2002 was the highest in the Upper Bay ($98.7 \pm 28.7 \text{ } \mu\text{g-at N L}^{-1}$) and decreased southward to Lower Bay ($9.84 \pm 4.47 \text{ } \mu\text{g-at N L}^{-1}$; Table 4.2). NO_3^- concentrations were the highest in the Upper Bay and reached $>90 \text{ } \mu\text{g-at N l}^{-1}$ annually in April (Fig. 4.2), except in 2002 when the spring freshet, the main supply of NO_3^- , occurred in May (data not shown). NO_3^- levels dropped substantially from April to July/August at all stations, with the exception of the Upper Bay station in 2004. The sampling period in July 2004 was preceded by a peak in daily stream flow which kept NO_3^- levels high. NH_4^+ and urea concentrations were comparable at all stations (Fig. 4.2), but the percentage contribution to TDN increased southward. At the Upper Bay station, NH_4^+ and urea together on average contributed $<15\%$ of the TDN. The contribution of NH_4^+ and urea to TDN at the Mid and Lower Bay stations was 39 and 62%, respectively. Urea was a large percentage of TDN at the Lower

Bay station, with a maximum contribution of 75% occurring in October 2002. The ratio of DON (including urea) to TDN was the highest in Lower Bay.

Concentrations of PO_4^{3-} progressively decreased from Upper to Lower Bay (Table 4.2), while concentrations of DOP remained approximately constant, between 0.3-0.36 $\mu\text{g-at P L}^{-1}$ (data not shown). Average TDP concentrations were greater in the Upper Bay than in the other regions during each season (Table 4.2). The decrease in average TDP from Upper to Lower Bay was driven by the decrease in PO_4^{3-} (Table 4.2).

Seasonal patterns in the DIN:DIP ratio, which is indicative of inorganic N- or P-limitation in plankton (Redfield 1958; Falkowski 2000) were apparent (Table 4.2). Limitation by inorganic P was evident at all three sites in Chesapeake Bay during spring with DIN:DIP values 8-19 fold higher than the stoichiometric proportion of 16:1. The severity of P-limitation increased at Upper and Mid Bay stations from the dry years of 2001-2002 to the wet years of 2003-2004. During summer and fall, DIN:DIP ratios progressively decreased southward during the dry years, with Mid and Lower Bay showing N-limitation. During the wet years, average DIN:DIP values indicated possible N-limitation only at the Lower Bay station during the summer, and balanced nutrient availability during the fall. At the plume station, DIN:DIP ratios indicated a shift from N-limitation during the dry years to P-limitation during the wet years. N-limitation at the Sargasso Sea site was evident during both dry and wet years in both spring and the fall.

Plankton composition and distribution

Phytoplankton biomass (chl *a*) followed different trends at different stations (Fig. 4.3). During the dry years (2001-2002) at the Upper Bay station, chl *a* biomass peaked in the summer or fall. In contrast, during 2003 and 2004, the highest chl *a* biomass was found in spring. Chl *a* biomass at the Mid Bay station did not follow any consistent temporal patterns. Chl *a* biomass did not exceed $6.98 \mu\text{g chl } a \text{ L}^{-1}$ during 2001-2002 at the Lower Bay station, but was between 5.00 and $18.1 \mu\text{g chl } a \text{ L}^{-1}$ during 2003-2004.

The relative abundance of phytoplankton taxonomic groups differed between the three Chesapeake Bay stations (Fig. 4.4). Phytoplankton composition at the Upper Bay station was generally dominated by diatoms (e.g. *Navicula* sp., *Nitzschia* sp., *Skeletonema* sp. and *Cyclotella* sp.) and cryptophytes during the spring and fall. The phytoplankton composition in spring 2002 was unusual with a high haptophyte and chlorophyte signal. The summer phytoplankton community differed during the dry and wet years. Dinoflagellates composed 26-49% of the phytoplankton community during the summers of 2001 and 2002 while cryptophytes (50%), diatoms (30%) and chlorophytes (17%) were more abundant during 2004. The fall phytoplankton community in 2004 was different than preceding years with an unusually high percentage of chlorophytes (42%), followed by cryptophytes (37%) and cyanobacteria (12%).

Like Upper Bay, the phytoplankton community of the Mid Bay station shifted from dry to wet years. During the dry years, diatoms dominated in April (2001: 78%; 2002: 84%). The contribution of diatoms (e.g. *Cyclotella* sp., *Thalassosira* sp.,

Chaetoceros sp.) to the total phytoplankton community decreased during the wet years (2003: 69%; 2004: 52%), while the contribution of dinoflagellates and cryptophytes increased. The summer phytoplankton community saw a shift from dominance by cyanobacteria, dinoflagellates (such as *Ceratium* sp. and *Prorocentrum minimum*), and cryptophytes (2001: 55%; 2002: 63%) during the dry years to diatoms and cryptophytes (2003: 91%; 2004: 89%) during the wet years. In the small percentage of dinoflagellates present during the wet years, *Akashiwo sanguinea*, *P. minimum* and *Gonyaulax* sp. were observed. The fall phytoplankton community changed slightly from dry to wet years. Diatoms and cryptophytes consistently dominated the fall phytoplankton community, but their contribution increased from 74-79% during the dry years to 86-90% during the wet years.

The phytoplankton composition of the Lower Bay station changed from year to year, rather than from dry to wet years. Diatoms (e.g. *Skeletonema* sp., *Rhizolenia* sp., *Pleurosigma* sp. and centric diatoms <20 µm) and cryptophytes consistently dominated the spring community (76-99%). The summer community varied from one dominated by dinoflagellates and cyanobacteria (2001: 62%), to cyanobacteria (2002: 65%), and then cyanobacteria and cryptophytes (2004: 70%). In fall, diatoms and cyanobacteria dominated the phytoplankton community from 2001-2003 (73, 80, 84%), while diatoms and cryptophytes dominated in 2004 (79%).

The plume and Sargasso Sea stations had a different phytoplankton community composition than in the Bay (data not shown). The plume, which was only sampled in April 2002 and 2004, consisted of primarily diatoms (76 and 93% respectively). However, in 2002, there were also minor contributions from

haptophytes (7%) and prasinophytes (7%). The Saragasso Sea station had 30% diatoms, 23% cyanobacteria, 10% haptophytes, and 10% cryptophytes in April 2002. Two years later in October 2004, cyanobacteria and haptophytes collectively composed 89% of the phytoplankton community.

Bacteria abundance also exhibited a seasonal pattern (Fig. 4.3). In Upper and Mid Bay, bacteria abundance peaked either in summer or fall. The Lower Bay had a higher abundance of bacteria (reaching $> 8 \times 10^6$ cells mL⁻¹) which peaked during the summer months. Bacterial abundance was higher during 2001-2002 than 2003-2004 in both Upper Bay and Lower Bay (Fig. 4.3). In Mid Bay, bacterial abundance had similar seasonal patterns and levels throughout 2001-2004.

Urea uptake and urease activity rates

Both urea uptake and urease activity in surface waters exhibited significant seasonal patterns throughout the Bay (ANOVA, $p < 0.05$). Both urea uptake and urease activity in the whole fraction, when normalized to chl *a* biomass, generally had an annual peak in the summer months at all stations (Fig. 4.5). Urea uptake in the $< 1.6 \mu\text{m}$ fraction contributed on average $< 11\%$ of the total urea uptake in the Upper and Lower Bay (Fig. 4.6). In Mid Bay, average contribution by the $< 1.6 \mu\text{m}$ fraction to total urea uptake was 17% of total urea uptake rates, but did not exhibit any seasonal patterns. Urease activity from the $< 1.6 \mu\text{m}$ fraction contributed more of the total urease activity in April and October than during the summers of 2002-2004 at all stations. The percent contribution of the $< 1.6 \mu\text{m}$ fraction was the highest in Mid Bay, and where it often was responsible for total urease activity.

Urea uptake and urease activity had no spatial pattern on a volumetric basis (Fig. 4.7A, B) but both rates showed a significant increasing pattern from the freshwater to oceanic site when normalized for chl *a* biomass (Fig. 4.7C, D; ANOVA, $p < 0.05$).

Relationships between physical factors, nitrogen availability, phytoplankton community composition and urea uptake and urease activity

Generally, a positive linear relationship existed between urea uptake and urease activity in Chesapeake Bay. Urea uptake and urease activity were positively related bay wide ($n = 34$, $r^2 = 0.24$, $p < 0.05$), but the relationship was strongest in Upper Bay ($n=11$, $r^2 = 0.68$, $p < 0.05$) and Lower Bay ($n=11$, $r^2 = 0.59$, $p < 0.05$). However, urea uptake rates were, at times, not similar to urease activity rates (Fig. 4.8). During 2001-2002, urea uptake rates were often much higher (2.01-170 ng-at N $\mu\text{g chl } a^{-1} \text{ h}^{-1}$) than urease activity rates (0-88 ng-at N $\mu\text{g chl } a^{-1} \text{ h}^{-1}$), while during 2003-2004, uptake rates often were the same as urease activity or lower.

The PCA analysis created several combined principal components based on physical factors, nitrogen availability, and phytoplankton community composition. The proportion of variance in environmental variables explained from the first three principal components (PC1, PC2, PC3) varied from 0.78 to 0.82 at the three Bay stations (Table 4.3). At all stations, PC1 was primarily driven by temperature and salinity (Fig. 4.9). PC1 was also related to the relative abundance of several phytoplankton taxonomic groups which differed at each Bay Station. In the Upper Bay, warmer temperatures and more saline waters were associated with f_chl *a*^{dinoflagellates}. The same conditions were associated with f_chl *a*^{cyanobacteria} and f_chl

a^{haptophytes} but negatively with *f_chl a*^{diatoms} and *f_chl a*^{dinoflagellates} in the Mid Bay. The Lower Bay PC1 was positively related to all of the taxonomic groups except *f_chl a*^{diatoms}. PC2 was driven by different variables at each Bay station. In the Upper Bay, PC2 was positively related to *f_chl a*^{diatoms} and negatively related to *f_chl a*^{cyanobacteria}. The Mid Bay PC2 was primarily associated with salinity and *f_chl a*^{diatoms}, but negatively with *f_chl a*^{cryptophytes}. Nutrient availability, especially NH₄⁺, and urea, positively drove PC2 in the mostly nitrogen-limited Lower Bay. Salinity and *f_chl a*^{haptophytes} were also negatively associated with PC2 in the Lower Bay.

Urea uptake and urease activity were not related to any environmental variables in the Upper Bay (Table 4.4). However in the Mid and Lower Bay, urea uptake and urease activity were significantly related to one or more PC variables. Urea uptake was significantly correlated to PC1 in Mid Bay which represented warmer temperatures, higher salinity, and the presence of cyanobacteria and haptophytes, not diatoms and dinoflagellates, which occurred during the summer months (Fig. 4.9). Urea uptake was also significantly correlated to PC3, which was primarily driven by urea concentrations, in Lower Bay (Tables 4.3 & 4.4). Urease activity was significantly correlated to all three PC variables in Mid Bay ($n = 9$, $r^2 = 0.89$; Table 4.3). Urease activity was also strongly correlated with PC1 and PC2 in Lower Bay. Warmer temperatures were associated with the presence of cyanobacteria, dinoflagellates, and cryptophytes as well as NH₄⁺ and urea availability during the summer months (Fig. 4.9).

One factor not examined in the PCA analysis was the role of bacteria. Urea uptake and urease activity decreased with increasing ratio of phytoplankton:bacteria

biomass (Fig. 4.10). However, the relationship between urea uptake and urease activity rates and phytoplankton:bacteria biomass was weak ($n= 34$, $p >0.05$).

Discussion

A wide range of phytoplankton and bacteria can use urea to meet their nitrogen metabolic demand, especially in N-limiting waters (Anita et al. 1991; Kirchman 2000). The ability of those organisms to utilize urea depends on the regulation of urea uptake and urease activity in natural waters. The data presented here suggest that seasonal and spatial variations in urea uptake and urease activity were the result of regulation by environmental factors, such as temperature and nutrient availability, interacting with the species composition of the planktonic communities. These environmental factors often co-vary making it difficult to identify a single dominant factor. Principal component analysis (PCA) made it possible to identify groups of variables related to variations in urea uptake and urease activity. By combining environmental factors into one independent variable, PCA showed that higher urea uptake and urease activity were generally associated with warmer temperatures, lower NO_3^- but higher NH_4^+ and urea availability, and the presence of cyanobacteria, cryptophytes and haptophytes.

Seasonal changes in rates of urea uptake and urease activity

Both urea uptake and urease activity rates were generally higher during the summer in more saline waters in Chesapeake Bay. For instance, urea uptake and urease activity were positively correlated to warmer temperatures and higher salinity (PC1) in Mid Bay. Higher rates of urea uptake in the warmer months have also

previously been measured in temperate estuaries (Kristiansen 1983; Glibert et al. 1991; Bronk et al. 1998; Lomas et al. 2002). The importance of temperature is suggested by positive relationships between urea uptake and temperature observed in diatom-dominated assemblages (in contrast to negative relationships between NO_3^- uptake and temperature for similar diatom-dominated assemblages; Lomas and Glibert 1999). Urease activity was also found to be higher when growth temperatures were similar to temperatures observed during the summer months (20-30°C) in three phytoplankton species, *Aureococcus anophagefferens*, *Thalassiosira weissflogii* and *Prorocentrum minimum* (Fan et al. 2003).

Regulation of urea uptake and urease activity by NO_3^- and NH_4^+ availability

The results from Chesapeake Bay support a growing body of evidence that urea uptake is suppressed or inhibited by NH_4^+ . Urea uptake rates of phytoplankton grown under nitrogen-replete conditions decrease after the addition of NH_4^+ and/or NO_3^- to culture (Lund 1987; Lomas 2004) or field incubations (Tamminen and Irmisch 1996). Urea uptake rates are often low in the field when NH_4^+ concentrations are high. For an example, in a recent study in the Neuse River estuary, North Carolina, NH_4^+ concentrations exceeding $40 \mu\text{g-at l}^{-1}$ were associated with low urea uptake rates in the upper portion of the estuary (Twomey et al. 2005). Kristiansen (1983) found that urea uptake was inhibited by NH_4^+ concentrations higher than $1\text{-}2 \mu\text{g-at N L}^{-1}$ in Oslofjord, Norway. In the Mid Bay, during the dry years when NH_4^+ concentrations were below $2.5 \mu\text{g-at N L}^{-1}$, urea uptake rates were higher and exhibited a stronger seasonal pattern than during the following two wet years when NH_4^+ concentrations were often above $2.75 \mu\text{g-at N L}^{-1}$. Also, urea uptake was

consistently associated with PC variables that had negative loading values for NH_4^+ (PC1 in Mid Bay and PC3 in Lower Bay).

In contrast, little is known about regulation of urease activity by NO_3^- , NH_4^+ or urea. Diatoms grown on NO_3^- in culture have lower urease activity than those grown on NH_4^+ or urea (Solomon and Glibert *submitted*). Likewise, rates of urease activity were generally low during spring when NO_3^- concentrations were high and diatoms dominated the phytoplankton community in Chesapeake Bay. Urease activity rates were consistently the highest during the summer months, despite differences in the phytoplankton communities. Dinoflagellates and cyanobacteria, which dominated the summer community during the dry years, have been shown to have lower urease activity when grown on NH_4^+ in culture (Collier et al. 1999, Fan et al. 2003, Solomon and Glibert *submitted*). Low concentrations of NH_4^+ during the dry years may have allowed for higher urease activity in these species. Diatoms and cryptophytes, which were prevalent during the summer months in the wet years in both Upper and Mid Bay, do not have lower urease activity when grown on NH_4^+ in culture (Solomon and Glibert *submitted*). Thus, the higher summer NH_4^+ concentrations during the wet years may not repress urease activity in diatoms and cryptophytes. The difference in regulation of urease activity by NH_4^+ in diatoms and dinoflagellates may explain why at times urease activity was positively related to combined variables that included higher NH_4^+ availability. For example, urease activity was positively associated with PC3 in Mid Bay which had positive loadings for both diatoms and NH_4^+ . Both NO_3^- and NH_4^+ availability regulate urease activity

in Chesapeake Bay, but urease activity is also strongly dependent on the phytoplankton community composition.

Urea uptake and urease activity in different microbial assemblages

Dinoflagellates have been shown to have higher urease activity per cell than many other phytoplankton taxa in culture (Fan et al. 2003; Solomon and Glibert *submitted*). For this reason, urease activity would be expected to be positively related to combined variables that included the presence of dinoflagellates. However, urease activity was often negatively related to the presence of dinoflagellates. The discrepancy may be explained by how the CHEMTAX program identified dinoflagellates and haptophytes. Further investigation into specific dates (e.g. July 2002 in Mid Bay; PC1) when dinoflagellates were negatively related but haptophytes were positively related to urea uptake and urease activity, found high amounts of 19'-hexanoyloxy-fucoxanthin present during these times. *K. veneficum* is generally abundant in Mid Bay during the summer months (Li et al. 2000), lending support to the possibility that this species was present. Combined linear variables such as PC1 and PC3 in Mid Bay and PC3 in Lower Bay that had a negative relationship with dinoflagellates but a positive relationship with haptophytes may actually indicate a positive relationship with the presence of the dinoflagellate, *K. veneficum*. This possibility suggests that dinoflagellates, including *K. veneficum*, in Chesapeake Bay may be associated with high rates of urea uptake and urease activity.

Cyanobacteria have been shown to have high urease activity on a per cell volume basis in culture (Solomon et al. *submitted*). For instance, the

cyanobacterium, *Prochlorococcus marinus*, has the highest reported urease activity of any marine photosynthetic organism (Palinksa et al. 2000; Solomon and Glibert *submitted*). Supporting results from culture studies, urease activity was often positively related to the presence of cyanobacteria during the summer months in both Mid and Lower Bay.

The negative relationship between urease activity and phytoplankton:bacterial biomass and the large contribution to total urease activity from the <1.6 μm fraction in this study suggested that bacteria in Chesapeake Bay did utilize urea as a substrate. Similar observations were made in the Southern California Bight by Cho and Azam (1995) where high rates of urea decomposition were associated with the bacterial-size fraction especially at 100-200m. The contribution of the <1.6 μm fraction to total urease activity was higher during the spring and fall than during the summer months. Urease activity in the <1.6 μm fraction sometimes exceeded 100% which may be an artifact due to changes made in the urease method during the study. Yet, those times are probably when small flagellates, cyanobacteria and bacteria were actively utilizing urea. Nevertheless, the role of bacteria as consumers of urea should be considered in future studies on urea utilization.

Microbes in the <1.6 μm fraction that consisted of cyanobacteria, small flagellates and heterotrophic bacteria were sometimes responsible for most of the urease activity in Chesapeake Bay. In contrast, larger phytoplankton were mostly responsible for urea uptake, with some exceptions in Mid Bay. Luxury uptake of urea may be occurring in larger phytoplankton, resulting in large pools of intracellular urea. Dinoflagellates have been observed to have relatively large intracellular pools

of urea (Fan et al. 2003; Solomon and Glibert *submitted*). However, the possible mechanism of storage for urea, such as vacuoles for NO_3^- in diatoms (e.g Antia et al. 1963; Eppley and Catsworth 1968), is unknown. Urease activity may also be inhibited in larger phytoplankton from a lack of Ni^{2+} (Oliveira and Antia 1986) or by metabolites produced in the cell (Mobley and Hausinger 1989).

Gradient in urea uptake and urease activity rates

Chesapeake Bay, especially in the summer, has a nutrient gradient that is created by freshwater inputs during the spring (Fisher et al. 1988; Malone et al. 1988, Harding 1994; Kemp et al. 2005). The difference in freshwater discharge in Chesapeake Bay during the dry (2001-2002) and wet years (2003-2004) led to a change from N- to P-limitation in the Mid Bay, and a shift in phytoplankton community from dinoflagellate, cyanobacteria and cryptophytes during the dry years to diatoms and cryptophytes during the wet years. This observation is in agreement with Adolf et al. (2006) who observed during 1995-2000 that years with strong winter-spring freshets had a higher proportion of diatoms in the mesohaline region. The progressive shift from high to low total nitrogen concentrations and DIN:DIP ratios and change in nitrogen composition from mostly NO_3^- to organic nitrogen as a result of increasing stratification from Upper Bay to Lower Bay corresponded with increasing urea uptake and urease activity.

A pattern in urea uptake and urease activity rates over a nutrient gradient has also been observed on the West Florida Shelf (Heil et al. *in press*). Heil et al. (*in press*) classified the north-south gradient in nutrient availability into three zones.

The most northern zone, Zone I, was dominated by peridinin-containing dinoflagellates and the non-peridinin containing *Karenia brevis* and was primarily nitrogen-limited. The second zone, Zone II, contained mostly cyanobacteria and diatoms and had inorganic nitrogen and phosphorus pools close to Redfield proportions. The southern zone closest to the Everglades, Zone III, had the highest N:P ratios and supported mostly diatoms. Urea uptake and urease activity decreased southward with increasing inorganic nitrogen inputs and diatom-dominated phytoplankton assemblages. Like in Chesapeake Bay, urea uptake and urease activity increased with decreasing DIN:DIP and increasing presence of dinoflagellates and cyanobacteria.

Nutrient dynamics can differ over time in the same location. A study that compared *Alexandrium fundyense* blooms in the Gulf of Maine found different rates of urease activity during a period of two months (Dyhrman and Anderson 2003). An *A. fundyense* bloom that occurred in May was supported by lower urea and higher DIN concentrations (DIN:DIP = 4.76) than a bloom in June. Higher urease activity ($92.5 \pm 63.9 \text{ fmol cell}^{-1} \text{ h}^{-1}$) was reported in June with a lower DIN:DIP ratio of 0.77. Synthesizing results from field studies, urea uptake and urease activity are strongly associated with dinoflagellates and cyanobacteria under nutrient regimes with low DIN:DIP ratios.

Chesapeake Bay: A historical perspective

Urea concentrations and urea uptake rates from this study can be compared with historical data from Chesapeake Bay to examine whether changes in urea

dynamics have occurred in the recent decade. Urea concentrations were higher during the summer and fall of 2001-2004 than prior to 1998 (but not statistically significant; $p > 0.05$; Fig. 4.11A, Lomas et al. 2002). Increases in urea uptake rates during 2001-2004 were not sufficient to keep ambient urea concentrations at constant levels (Fig. 4.11B, Lomas et al. 2002). Urea concentrations may have increased as a result of higher urea regeneration by benthic macrofauna (Lomstein et al. 1989), micro- and macrozooplankton (Miller and Glibert 1998; L'Hulguen et al. 2005), and heterotrophic bacteria (Cho et al. 1996; Therkildsen et al. 1997) or from input from agricultural run-off (Glibert et al. 2005, 2006).

A comparison can be made with a study conducted during a dry year in mesohaline Chesapeake Bay (Mid Bay) by Bronk et al. (1998). In spring of 1988, urea uptake was ~33% of total nitrogen uptake with rates below $0.4 \mu\text{g-at N L}^{-1} \text{ h}^{-1}$, while in 2002 it was only 9% with a rate of $0.13 \mu\text{g-at N L}^{-1} \text{ h}^{-1}$. Later during the summer, urea uptake was ~33% of total nitrogen uptake with similar rates between $0.4\text{-}0.6 \mu\text{g-at N L}^{-1} \text{ h}^{-1}$ during both 1988 and 2002. Fall uptake rates were much lower ($\sim 0.02 \mu\text{g-at N L}^{-1} \text{ h}^{-1}$) and only contributed $< 20\%$ to total nitrogen uptake during both 1988 and 2002. The only difference between the two dry years was in the spring, which may have resulted from the timing of sampling. In 1988, sampling was done in May while in 2002 it was done in early April. The phytoplankton community can change dramatically during this time period, and it might have shifted from a predominantly diatom to a mixed diatom-dinoflagellate community that may utilize urea at higher rates. Nevertheless, it is remarkable that two dry years that are

more than a decade apart have similar urea uptake rates and urea contributions to total nitrogen uptake, despite higher urea concentrations in Chesapeake Bay.

Conclusion

Chesapeake Bay provided a natural laboratory for studying urea cycling along strong spatial and temporal gradients in temperature, nutrient availability, and plankton community composition. These factors often co-vary, making it difficult to identify a single dominant factor. Despite the complexity, urea uptake and urease activity appear to be regulated by temperature and nitrogen availability in different phytoplankton communities in Chesapeake Bay.

Literature cited

- Adolf, J.E., Harding, L.W., Yeager, C.L., Maloney, M.E. & Miller, W.D. 2006. Environmental forcing of phytoplankton floral composition, biomass and primary productivity in the Chesapeake Bay, USA. *Estuar. Coastal Shelf Sci.* 67: 108-122.
- Allen, A.E., Ward, B.B. & Bowler, C.P. 2005. Multi-lineage richness of functional genes in marine diatoms and transcriptional responses to nutrient stress. American Society of Limnology and Oceanography Summer Meeting. Santiago, Spain. June 2005.
- Antia, N.J., Harrison, P.J. & Oliveria, L. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia.* 1: 1-89.
- Antia, N.J., McCallister, C.D., Parsons, T.R., Stephens, K. & Strickland, J.D. 1963. Further measurements of primary production using a large-volume plastic sphere. *Limnol. Oceanogr.* 8: 166-183.
- Berges, J.A. 1997. Minireview: Algal nitrate reductases. *Eur. J. Phycol.* 32:3-8.
- Bergholtz, T., Daugbjerg, N., Moestrup, Ø. & Fernández-Tejedor, M. 2006. On the identity of *Karlodinium veneficum* and description of *Karlodinium armiger* sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. *J. Phycol.* 42: 170-193.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microb. Ecol.* 31: 279-305.
- Bronk, D.A., P.M. Glibert, T.C. Malone, S. Banahan and E. Sahlsten. 1998. Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux. *Aquat. Microb. Ecol.* 15: 177-189.
- Bronk, D.A., Lomas, M.W., Glibert, P.M., Schukert, K.J. & Sanderson, M.P. 2000. Total dissolved nitrogen analysis: comparisons between persulfate, UV, and high temperature oxidation methods. *Mar. Chem.* 69: 163-178.
- Cho, B.C. & Azam, F. 1995. Urea decomposition by bacteria in the Southern California Bight and its implications for the mesopelagic nitrogen cycle. *Mar. Ecol. Prog. Ser.* 122: 21-26.
- Cho, B.C., Park, M.C., Shim, J.H. & Azam, F. 1996. Significance of bacteria in urea dynamics in coastal surface waters. *Mar. Ecol. Prog. Ser.* 142: 19-26.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to

utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiol.* 145: 447-459.

Collos, Y., Gagne, C., Laabir, M., Vaquer, A., Cecchi, P. & Souchu, P. 2004. Nitrogenous nutrition of *Alexandrium catenella* (Dinophyceae) cultures and in Thau Lagoon, Southern France. *J. Phycol.* 40:96-103.

Ducklow, H. 2000. Bacterial production and biomass in the oceans. p. 85-120
In: D.L. Kirchman [ed.], *Microbial ecology of the oceans*. Wiley-Liss,

Dyhrman, S.T. & Anderson, D.A. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.

Eppley, R.W. & Coatsworth, J.L. 1968. Uptake of nitrate and nitrite by *Ditylum brightwellii* – kinetics and mechanisms. *J. Phycol.* 4: 151-156.

Falkowski, P.G. 2000. Rationalizing elemental ratios in unicellular algae. *J. Phycol.* 36:3-6.

Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.

Fisher, T.R., Harding, L.W., Stanley, D.W. & Ward, L.G. 1988. Phytoplankton, nutrients, and turbidity in Chesapeake, Delaware, and Hudson estuaries. *Estuar. Shelf Coastal Sci.* 27: 61-93.

Fisher, T.R., Peele, E.R., Ammerman, J.A. & Harding, L.W. 1992. Nitrogen limitation of phytoplankton in the Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 82:51-63.

Glibert, P.M. & Capone, D.G. 1993. Mineralization and assimilation in aquatic, sediment, and wetland systems, p. 243-272. *In* R. Knowles and T.H. Blackburn [eds], *Nitrogen isotope techniques*. Academic Press.

Glibert, P.M., Conley, D.J., Fisher, T.R., Harding, L.W. & Malone, T.C. 1995. Dynamics of the 1990 winter/spring bloom in Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 122:27-43.

Glibert, P.M., Garside, C., Fuhrman, J.A. & Roman, M.R. 1991. Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnol. Oceanogr.* 36: 895-909.

Glibert, P.M., Harrison, J., Heil, C. & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry.* 77: 441-463.

- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Glibert, P.M., Trice, T.M., Michael, B. & Lane, L. 2005. Urea in the tributaries of the Chesapeake and Coastal Bays of Maryland. *Water, Air, and Soil Pollution.* 160: 229-243.
- Hagy, J.D., Boynton, W.R. & Jasinski, D.A. 2005. Modelling phytoplankton deposition to Chesapeake Bay sediments during winter-spring: interannual variability in relation to river flow. *Estuar. Coastal Shelf Sci.* 62: 25-40.
- Harding, L.W. 1994. Long-term trends in the distribution of phytoplankton in Chesapeake Bay: roles of light, nutrients, and streamflow. *Mar. Ecol. Prog. Ser.* 104: 267-291.
- Heil, C.A., M. Revilla,, P.M. Glibert and S. Murasko. *In press.* Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde, E.D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M. & Stevenson, J.C. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. *Mar. Ecol. Prog. Ser.* 303:1-29.
- Kirchman, D.L. 2000. Uptake and regeneration of inorganic nutrients by marine heterotrophic bacteria. p. 261-288. *In*.D.L. Kirchman [ed.], *Microbial ecology of the oceans.* Wiley-Liss, New York.
- Kristiansen, S. 1983. Urea as a nitrogen source for the phytoplankton in the Oslofjord. *Mar. Biol.* 74:17-24.
- Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.
- Kudo, I., Yoshimura, T., Yanada, M. & Matsunaga, K. 2000. Exhaustion of nitrate terminates a phytoplankton bloom in Funka Bay, Japan: a change in SiO₄:NO₃ consumption rate during the bloom. *Mar. Ecol. Prog. Ser.* 193: 45-51.
- Lane, L., Rhoades, S., Thomas, C. & van Heukelem, L. 2000. Analytical Services Laboratory Standard Operating Procedures. Technical Report No. TS-264-00. Horn Point Laboratory, University of Maryland Center for Environmental Science.

- Lewitus, A.J., White, D.L., Tymowski, R.G., Geesey, M.E., Hymel, S.N. & Noble, P.A. 2005. Adapting the CHEMTAX method for assessing phytoplankton taxonomic composition in southeastern U.S. estuaries. *Estuaries*. 28: 160-172.
- L'Hulguen, S., Slawyk, G. & Le Corre, P. 2005. Seasonal patterns of urea regeneration by size-fractionated microheterotrophs in well-mixed temperate coastal waters. *J. Plank. Res.* 27: 263-270.
- Li, A., Stoecker, D.K. & Adolf, J.E. 1999. Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. *Aquat. Microb. Ecol.* 10:139-47.
- Li, A., Stoecker, D.K. & Coats, D.W. 2000. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. *J. Plankton Res.* 22:2105-2124.
- Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (*Bacillariophyceae*): Interactions between nitrogen substrates. *Mar. Biol.* 144: 37-44.
- Lomas, M.W. & Glibert, P.M. 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 556-572.
- Lomas, M.W., Trice, T.M., Glibert, P.M., Bronk, D.A. & McCarthy, J.J. 2002. Temporal and spatial dynamics of urea uptake and regeneration rates and concentrations in Chesapeake Bay. *Estuaries*. 25: 469-482.
- Lomstein, B.A., Blackburn, T.H. & Henriksen, K. 1989. Aspects of nitrogen and carbon cycling in the northern Bering Shelf sediment. 1. The significance of urea turnover in the mineralization of NH_4^+ . *Mar. Ecol. Prog. Ser.* 57:237-247.
- Lund, B.A. 1987. Mutual interference of ammonium, nitrate, and urea on uptake of ^{15}N sources by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar. Biol. Ecol.* 113:167-180.
- Mackey, M.D., Higgins, H.W., Mackey, D.J. & Wright, S.W. 1996. CHEMTAX - a program for estimating class abundances from chemical markers - application to HPLC measurements of phytoplankton. *Mar. Ecol. Prog. Ser.* 144: 265-283.
- Malone, T.C., Crocker, L.H., Pike, S.E. & Wendler, B.W. 1988. Influences of river flow on the dynamics of phytoplankton production in a partially stratified estuary. *Mar. Ecol. Prog. Ser.* 48: 235-249.
- Miller, C.A. & Glibert, P.M. 1998. Nitrogen excretion by the calanoid copepod *Acartia tonsa*: results of mesocosm experiments. *J. Plank. Res.* 20: 1767-1780.

- Mobley, H.L.T. & Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. *Microbiol. Rev.* 53: 85-108.
- Mulvenna, P.F. & Savidge, G. 1992. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. *Estuar. Coast. Shelf. Sci.* 34: 429-438.
- Oliveira, L. & Antia, N.J. 1986. Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J. Plank. Res.* 8: 235-242.
- Palinska, K.A., Jahns, T., Rippka, R. & Tandeau de Marsac, N. 2000. *Prochlorococcus marinus* strain PCC 9511, a picoplankton cyanobacterium, synthesizes the smallest urease. *Microbiology.* 146: 3099-3107.
- Parker, M.S. & Armbrust, E.V. 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 41:1142-1153.
- Parsons, R.T., Y. Maita and C.M. Lalli. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press, New York, N.Y.
- Peers, G.S., Milligan, A.J & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.
- Redfield, A.C. 1958. The biological control of chemical factors in the environment. *American Scientist* 46: 205-221.
- Revilla, M., Alexander, J. & Glibert, P.M. 2005. Analysis of urea in coastal waters: comparison of the enzymatic and the direct method. *Limnol. Oceanogr. Methods.* 3: 290-299.
- Salerno, M. 2005. Ectocellular enzyme activities in the mixotrophic dinoflagellate *Prorocentrum minimum* (Dinophyceae). Masters Thesis. University of Maryland.
- Solomon, C.M., J. Alexander and P.M. Glibert. *Submitted*. Measuring urease activity in environmental samples. *Limnol. Oceanogr. Methods.*
- Solomon, C.M and P.M.Glibert. *Submitted*. Urease activity in five phytoplankton species. *J. Phycol.*
- Soloranzo, L. & Sharp, J.H. 1980. Determination of the total dissolved phosphorus and particulate phosphorus in natural waters. *Limnol. Oceanogr.* 25: 754-752.

- Tamminen, T. & Irmisch, A. 1996. Urea uptake kinetics of a midsummer planktonic community on the SW coast of Finland. *Mar. Ecol. Prog. Ser.* 130: 201-211.
- Tengs, T., Dahlberg, O.J., Shalchian-Tabrizi, K., Klaveness, D., Rudi, K., Delwiche, C.F. & Jakobsen, K.S. 2000. Phylogenetic analyses indicate that the 19' hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol. Biol. Evol.* 17:718-729.
- Therkildsen, M.S., Isaken, M.F. & Lomstein, B.A. 1997. Urea production by the marine bacteria *Delacya venusta* and *Pseudomonas stutzeri* grown in a minimal medium. *Aquat. Microb. Ecol.* 13: 213-217.
- Twomey, L.J., Piehler, M.F. & Paerl, H.W. 2005. Phytoplankton uptake of ammonium, nitrate, and urea in the Neuse River Estuary, NC, USA. *Hydrobiologia.* 533: 123-134.
- van Heukelem, L., Lewitus, A., Kana, T.M. & Craft, N. 1994. Improved separation of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Mar. Ecol. Prog. Ser.* 114: 303-313.
- van Heukelem, L. & Thomas, C.S. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chromatogr A* 910:31-49.
- Zehr, J.P., Carpenter, E.J. & Villareal, T.A. 2000. New perspectives on nitrogen-fixing organisms in tropical and subtropical oceans. *Trends Microbiol.* 8:68-73.

Tables

Table 4.1 Average streamflow, temperature and salinity at Chesapeake Bay stations. Samples were not collected in summer 2003 in Upper Bay and Lower Bay so the range denoted by an asterisk (*) reflects spring and fall values.

	2001	2002	2003	2004
Average annual streamflow (m³ s⁻¹)	1416	1700	3964	3115
Temperature (°C)				
Spring	8.45±1.08	10.6±0.82	8.90±0.39	8.98±0.35
Summer	27.3±0.33	27.0±0.53	26.9	26.4±1.10
Fall	18.8±1.46	23.5±1.29	19.7±1.51	21.3±1.67
Salinity				
Upper Bay	2.76±1.98	4.53±4.64	0.13±0.00*	0.26±0.19
Middle Bay	13.2±2.80	15.2±3.37	9.27±2.75	7.89±2.00
Lower Bay	22.1±0.58	22.6±2.51	17.2±0.30*	16.9±0.26

Table 4.2 Distribution of nitrogen ($\mu\text{g at N l}^{-1}$) and phosphate ($\mu\text{g at P l}^{-1}$) in the Chesapeake Bay during three seasons. Values represent the mean of two years (with standard deviation). The Plume and Sargasso Sea data are from April 2002, April 2004, and October 2004.

Station	Season	2001-2002 (Dry years)					2003-2004 (Wet years)				
		TDN	TDP	DIN	DIP	DIN:DIP	TDN	TDP	DIN	DIP	DIN:DIP
Upper Bay	Spring	98.7 (28.7)	0.48 (0.21)	82.6 (21.6)	0.3 (0.04)	273 (34)	97.7 (28.7)	0.44 (0.24)	91.1 (10.7)	0.36 (0.18)	300 (183)
	Summer	54.9 (2.55)	1.0 (0.10)	21.1 (4.87)	0.37 (0.01)	56.7 (11.0)	107	1.13	97.9	0.95	103
	Fall	50.1 (3.61)	1.39 (0.07)	34.3 (10.0)	1.06 (0.03)	32.4 (0.07)	108.9 (24.3)	1.42 (0.72)	87.7 (24.2)	0.75 (0.08)	117 (20.3)
Mid Bay	Spring	38.8 (24.8)	0.07 (0.04)	26 (28.4)	0	8655* (9473)	67.4 (3.11)	0.29 (0.01)	52.9 (14.2)	0.21 (0.07)	255 (18.5)
	Summer	21.7 (3.25)	0.39 (0.07)	1.28 (0.27)	0.10 (0.04)	13.9 (2.35)	22.7 (4.74)	0.27 (0.19)	7.14 (5.43)	0.16 (0.01)	46.3 (38.0)
	Fall	20.75 (8.98)	0.84 (0.04)	3.15 (0.13)	0.34 (0.14)	10.2 (4.63)	48.7 (16.0)	1.41 (0.73)	29.1 (10.2)	0.53 (0.27)	57.4 (9.87)
Lower Bay	Spring	9.84 (4.47)	0.15 (0.01)	1.35 (1.00)	0	448.3* (332.3)	27.2 (2.12)	0.21 (0.04)	14.1 (1.28)	0.12 (0.01)	122 (3.61)
	Summer	18.4 (0.92)	0.59 (0.29)	2.43 (1.39)	0.18 (0.08)	17.3 (15.7)	19.9	0.47	1.2	0.11	10.9
	Fall	16.3 (3.32)	0.47 (0.12)	1.5 (2.04)	0.15 (0.02)	9.42 (12.7)	19.6 (5.87)	0.74 (0.28)	3.42 (1.48)	0.23 (0.01)	15.1 (7.35)
Plume	Spring	8.65	0.00	0.18	0.03	6.0	14.1	0.2	7.5	0.07	107
Sargasso Sea	Spring	9.79	0.27	0.27	0.05	5.4					
	Fall						12.8	0.19	1.24	0.13	9.54

Table 4.3 Loadings for PC1, PC2, and PC3 for each Chesapeake Bay station. Loadings <0.1 are not reported.

Variables	Upper Bay			Mid Bay			Lower Bay		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
<i>Physical</i>									
Temperature	0.400	-0.283	0.236	0.412	-0.195	0.269	0.410		
Salinity	0.560	0.147		0.314	0.484		0.135	-0.531	-0.191
<i>Chemical</i>									
NO ₃ ⁻	-0.492		0.170	-0.448		-0.134	-0.406	0.108	-0.201
NH ₄ ⁺	-0.117	-0.296	-0.573	-0.273	-0.180	0.105	0.200	0.423	-0.470
Urea		0.185	-0.487		-0.337	0.617	0.171	0.319	0.542
<i>Biological</i>									
Diatoms	-0.201	0.543		-0.319	0.343	0.442	-0.413		
Dinoflagellates	0.491	0.111	-0.129	-0.416	-0.313	-0.507	0.285		-0.585
Cryptophytes			0.163		-0.514		0.351	0.252	0.214
Cyanobacteria	-0.197	-0.396	-0.222	0.463		-0.188	0.403	-0.101	
Haptophytes		-0.274	0.492	0.334	-0.297	0.164	0.206	-0.582	0.100
<i>% cumulative variance explained</i>	0.35	0.61	0.78	0.41	0.64	0.78	0.46	0.68	0.82

Table 4.4 Regression coefficients and t-values for multiple regression models of urea uptake and urease activity at all three Chesapeake Bay Stations. The multiple correlation coefficient is denoted by r^2 .

Station		Urea uptake				Urease activity			
		Intercept	PC1	PC2	PC3	Intercept	PC1	PC2	PC3
Upper Bay	df	11	11	11	11	11	11	11	11
	coeff.	0.2525	0.0237	0.0080	-0.0196	0.2924	0.0357	-0.0002	0.0447
	(t-value)	(5.5896)	(0.9757)	(0.2879)	(-0.5707)	(3.7354)	(0.8477)	(-0.0042)	0.7509
	p-value	0.0008	0.3617	0.7817	0.5861	0.0073	0.4247	0.9967	0.4772
	r^2=	0.1627				0.1548			
Mid Bay	df	12	12	12	12	12	12	12	12
	coeff.	0.3019	0.0473	-0.0093	-0.0407	0.2917	0.0216	-0.0861	0.0628
	(t-value)	(6.1866)	(1.9540)	(-0.2908)	(-0.9843)	(15.0888)	(2.2541)	(-6.8075)	(3.8356)
	p-value	0.0003	0.0865	0.7786	0.3538	0.0000	0.0542	0.0010	0.0050
	r^2=	0.3785				0.8921			
Lower Bay	df	11	11	11	11	11	11		
	coeff.	0.5591	0.0521	0.0261	0.1171	0.3287	0.0406	0.0772	0.0335
	(t-value)	(7.7665)	(1.5585)	(0.5302)	(1.9449)	(8.8361)	(2.3486)	(3.0365)	(1.0765)
	p-value	0.0001	0.1631	0.6124	0.0929	0.0000	0.0512	0.0189	0.3174
	r^2=	0.4812				0.6942			

Figures

Fig. 4.1 The five sampling sites in the Chesapeake Bay and the Sargasso Sea.

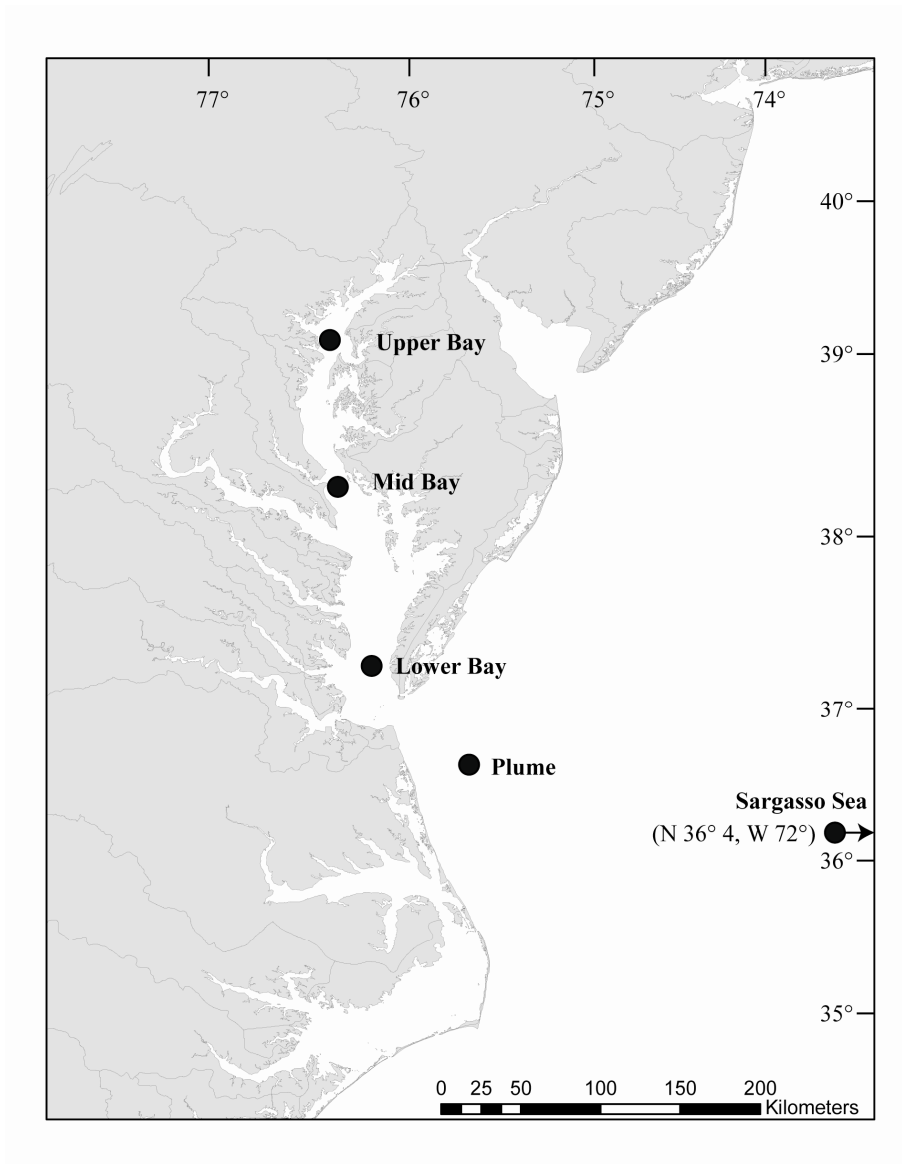


Fig. 4.2 Nutrient concentrations as function of month and year of sampling at three stations in the Chesapeake Bay during 2001-2004. Note the difference in scale for NO_3^- concentrations in Upper Bay as opposed to the Middle and Lower Bay stations.

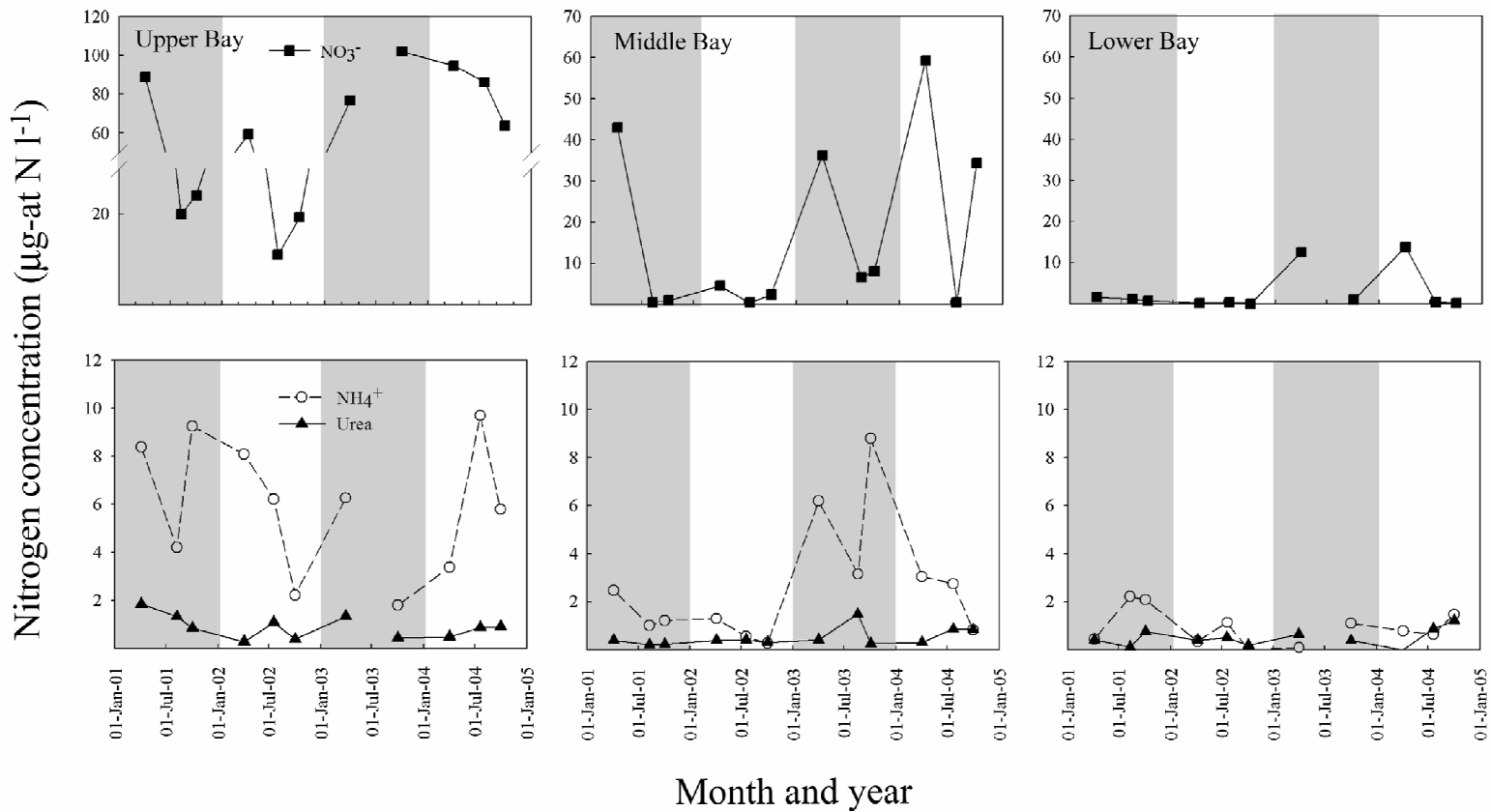


Fig. 4.3 Chl *a* biomass and bacterial abundance as function of month and year of sampling at three stations in the Chesapeake Bay during 2001-2004.

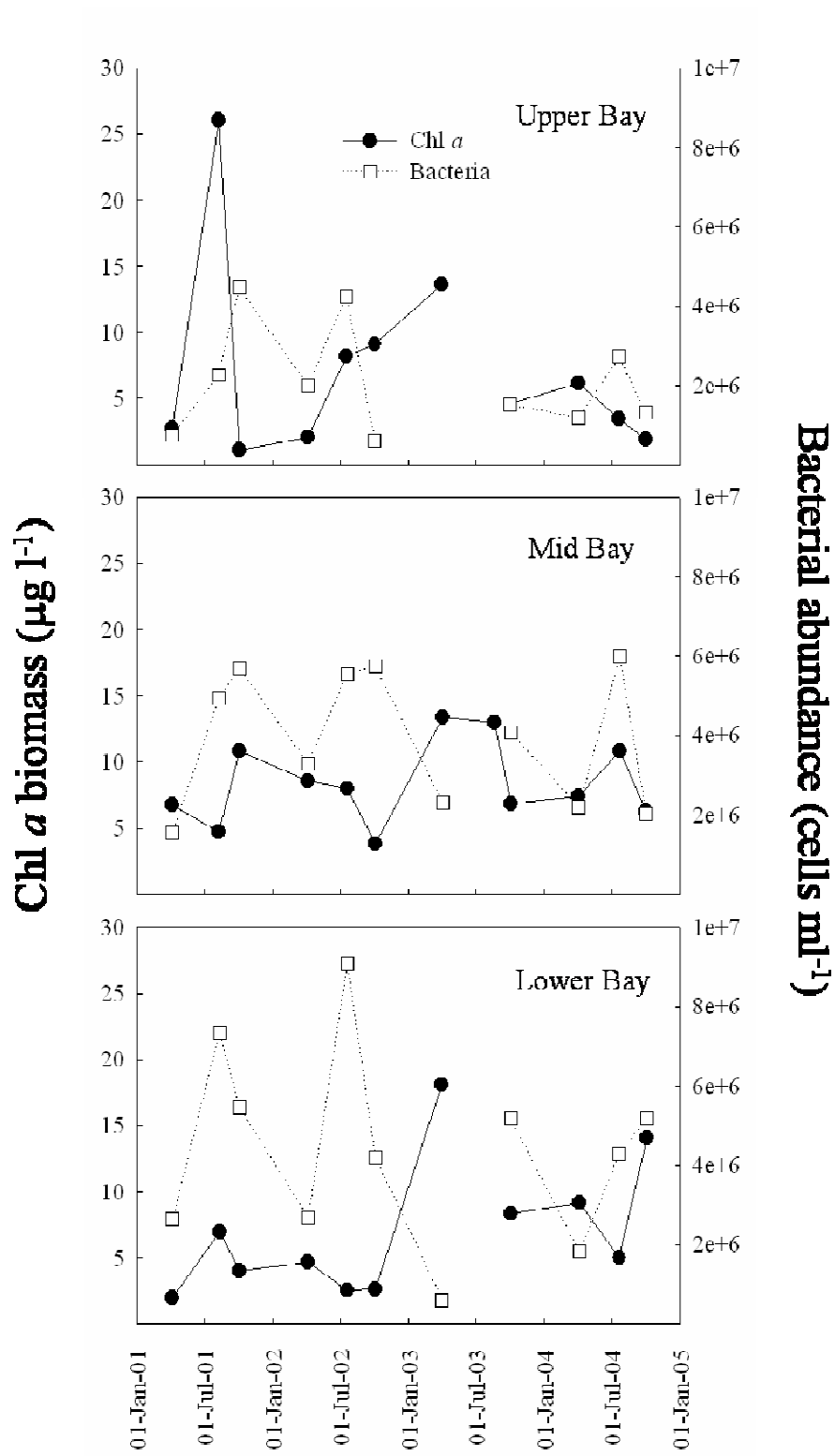


Fig. 4.4 Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage as analyzed by CHEMTAX as function of season and year of sampling during 2001-2004 at each Chesapeake Bay station.

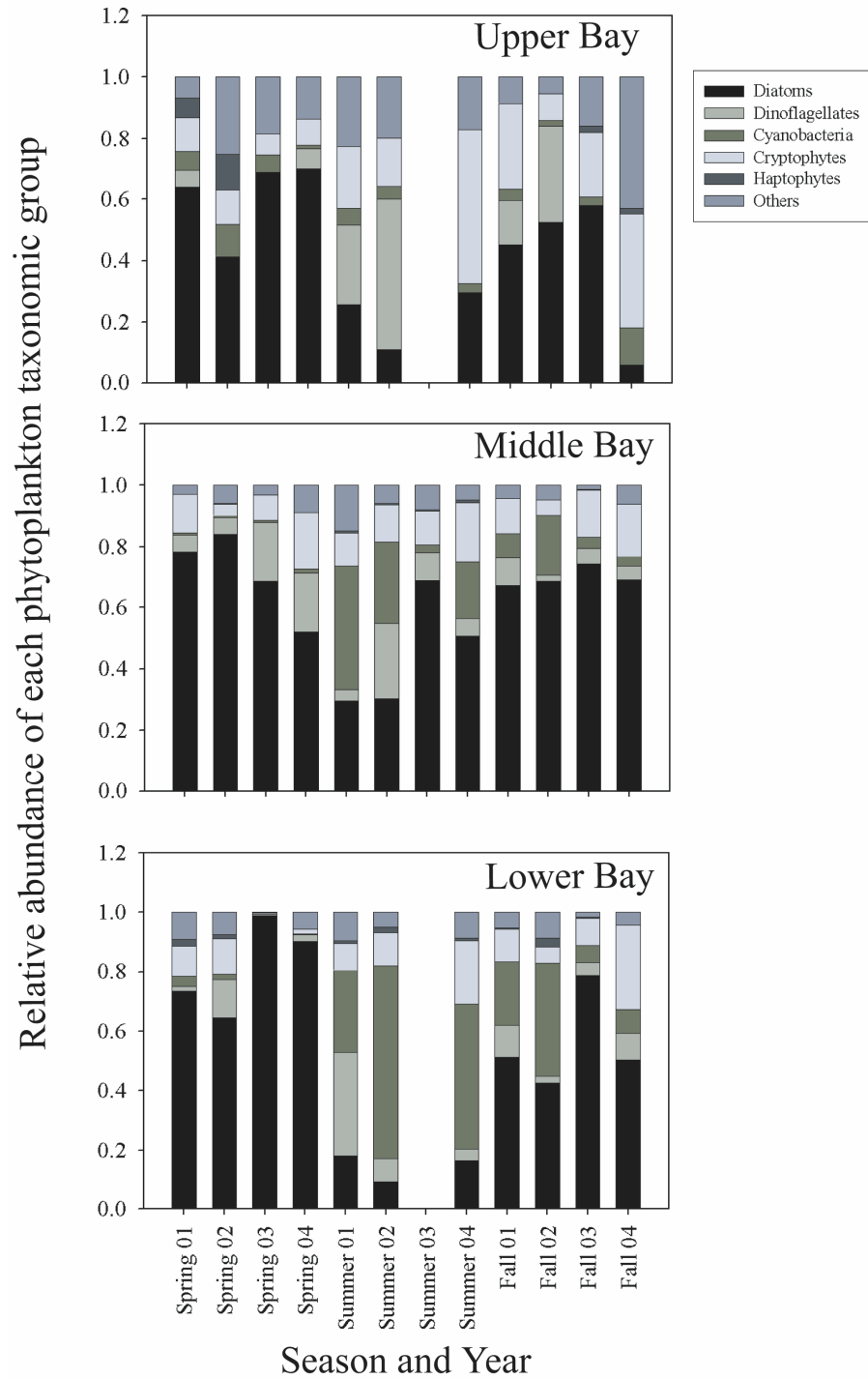


Fig. 4.5 Normalized urea uptake and urease activity at three stations in Chesapeake Bay as function of month and year of sampling during 2001-2004. The upper panels are urea uptake while the lower panels are urease activity.

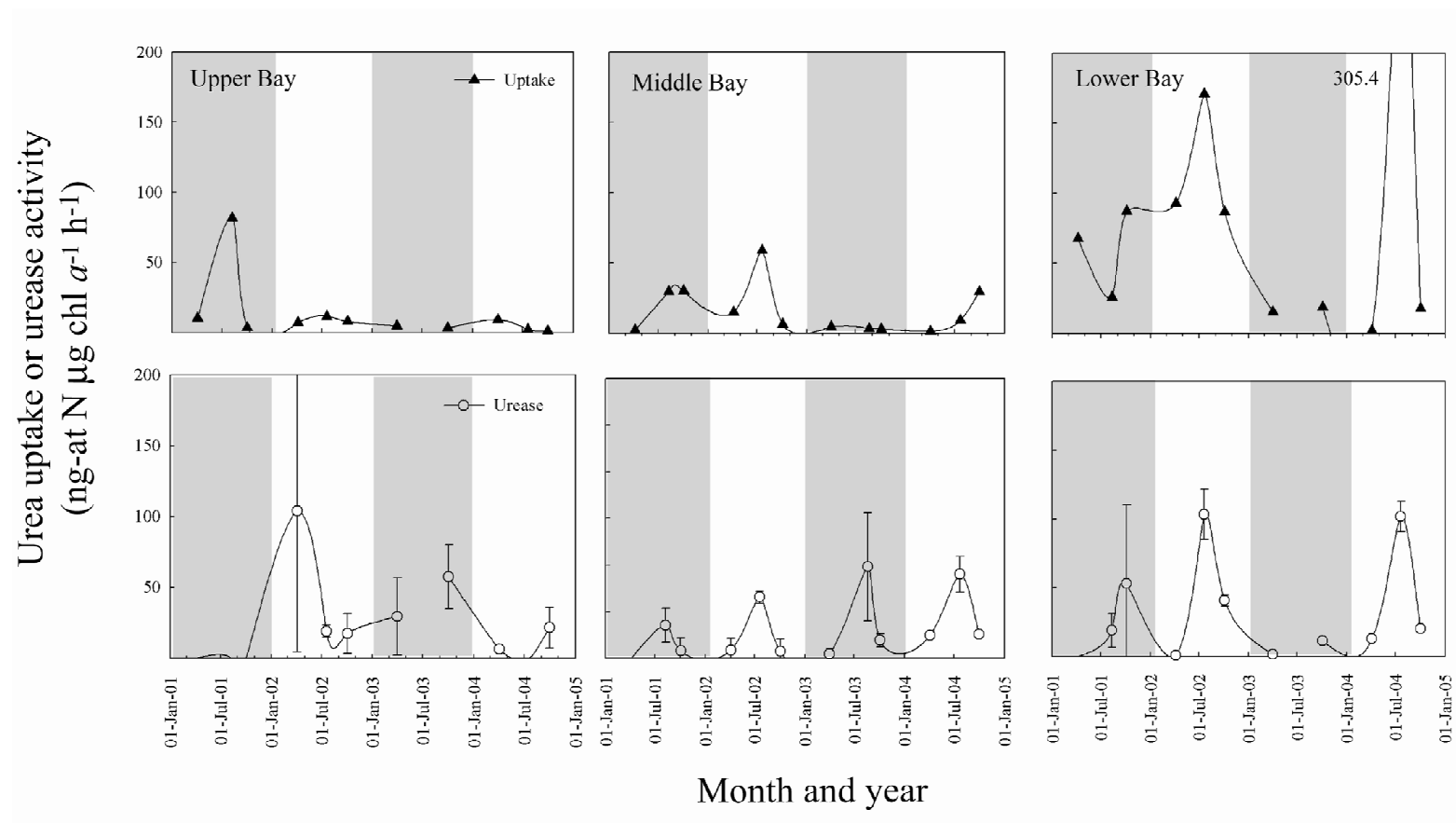


Fig. 4.6 Contribution of the <math><1.6 \mu\text{m}</math> fraction to total urea uptake or urease activity as function of month and year of sampling at the three stations in Chesapeake Bay during 2001-2004.

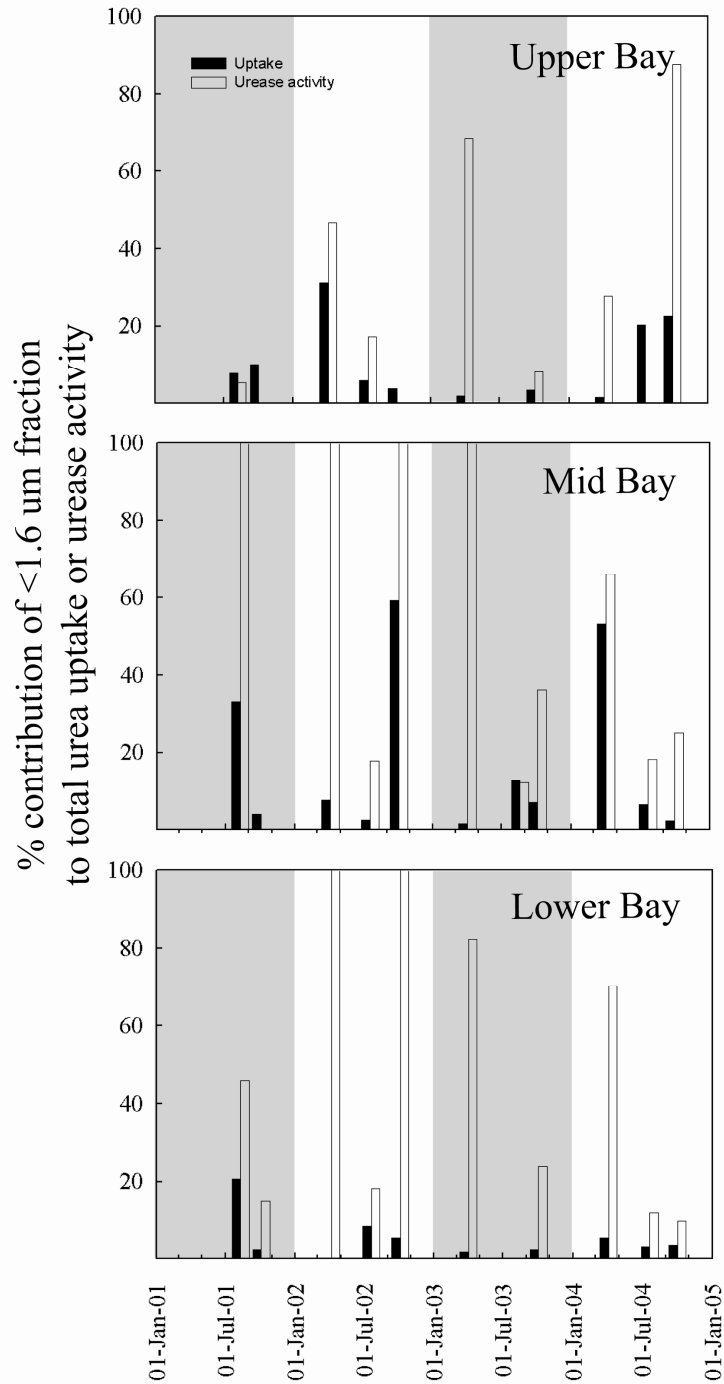


Fig. 4.7 Urea uptake rates and urease activity over a spatial gradient in Chesapeake Bay and the Sargasso Sea. A&B) normalized on a volumetric basis C&D) normalized on a chl *a* biomass basis.

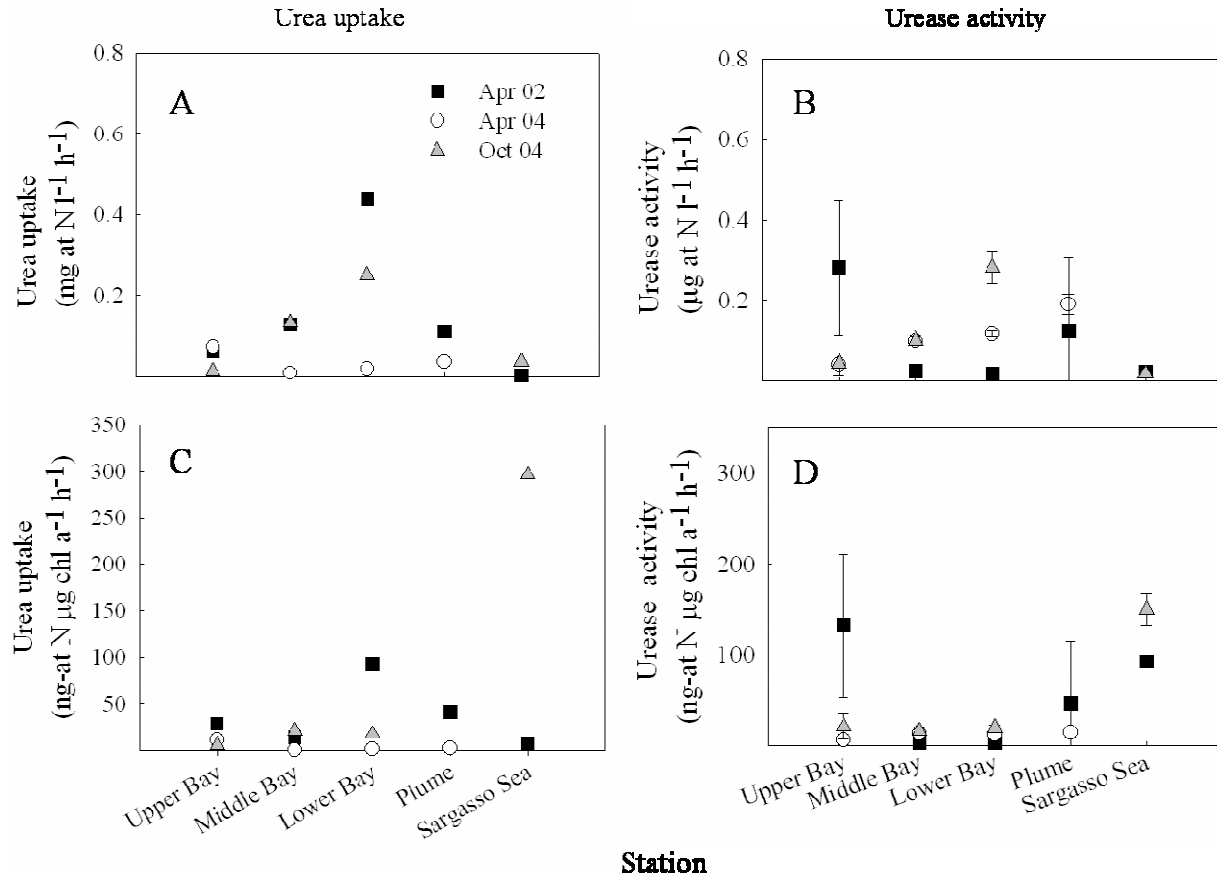


Fig. 4.8 The difference between urea uptake and urease activity rates from all stations in Chesapeake Bay, plume and the Sargasso Sea as a function of month and year of sampling during 2001-2004.

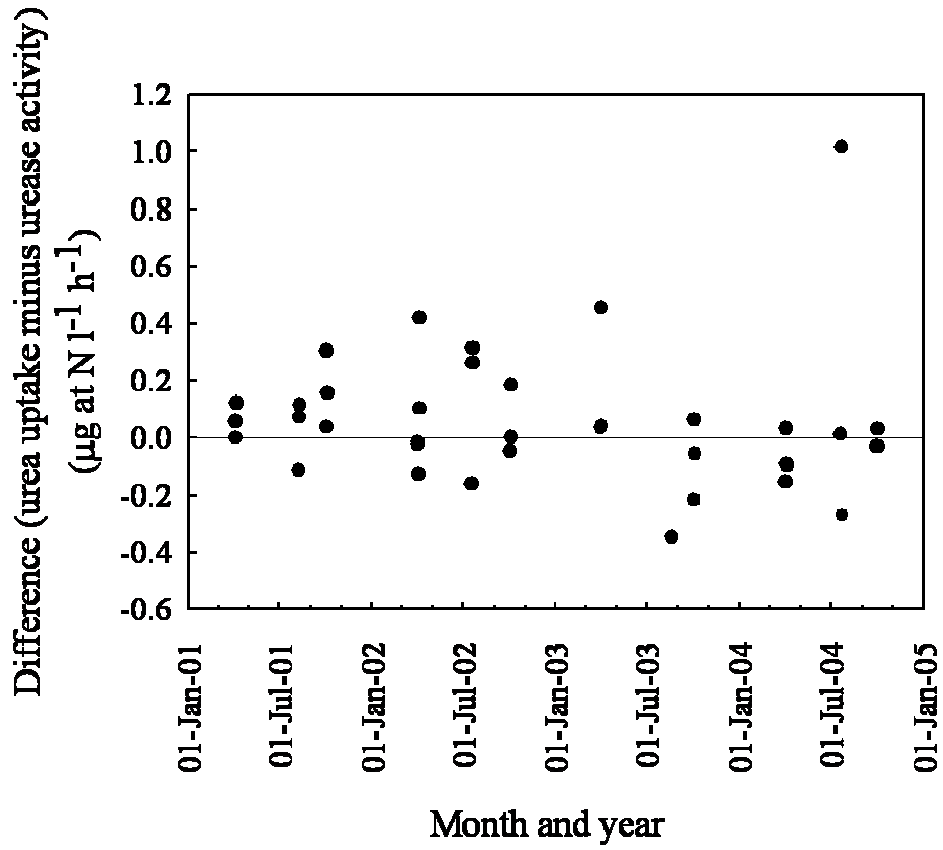


Fig. 4.9 Principal component analysis of temperature, salinity, nitrogen availability, and relative abundance of five phytoplankton taxonomic groups at three Chesapeake Bay stations from 2001-2004.

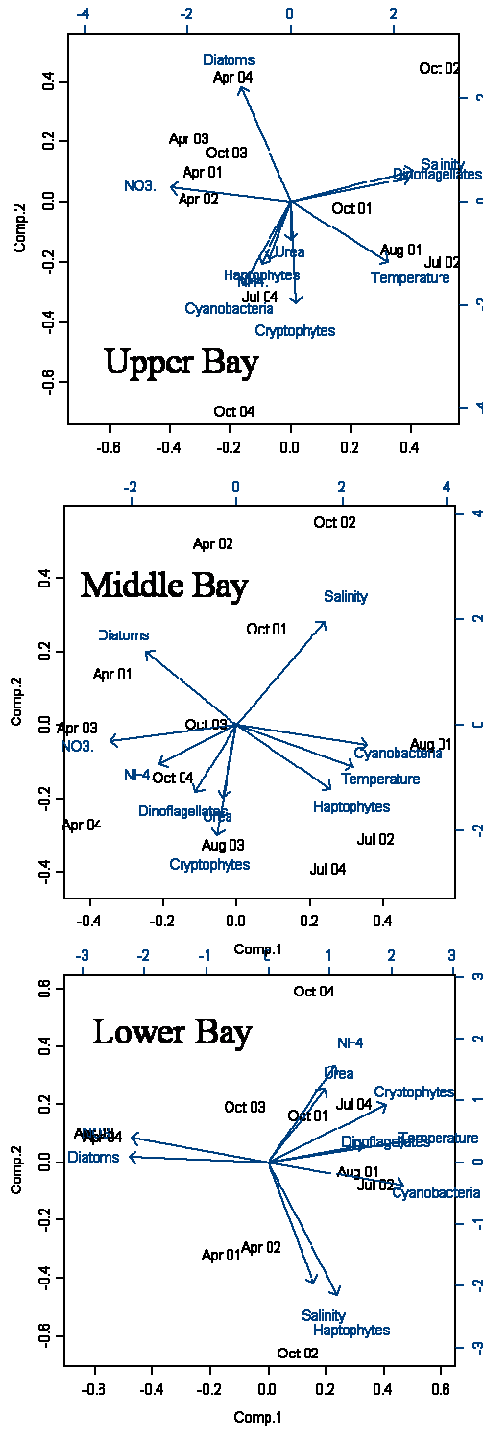


Fig. 4.10 A) Urea uptake and B) Urease activity as function of phytoplankton:bacterial biomass in all Chesapeake Bay stations.

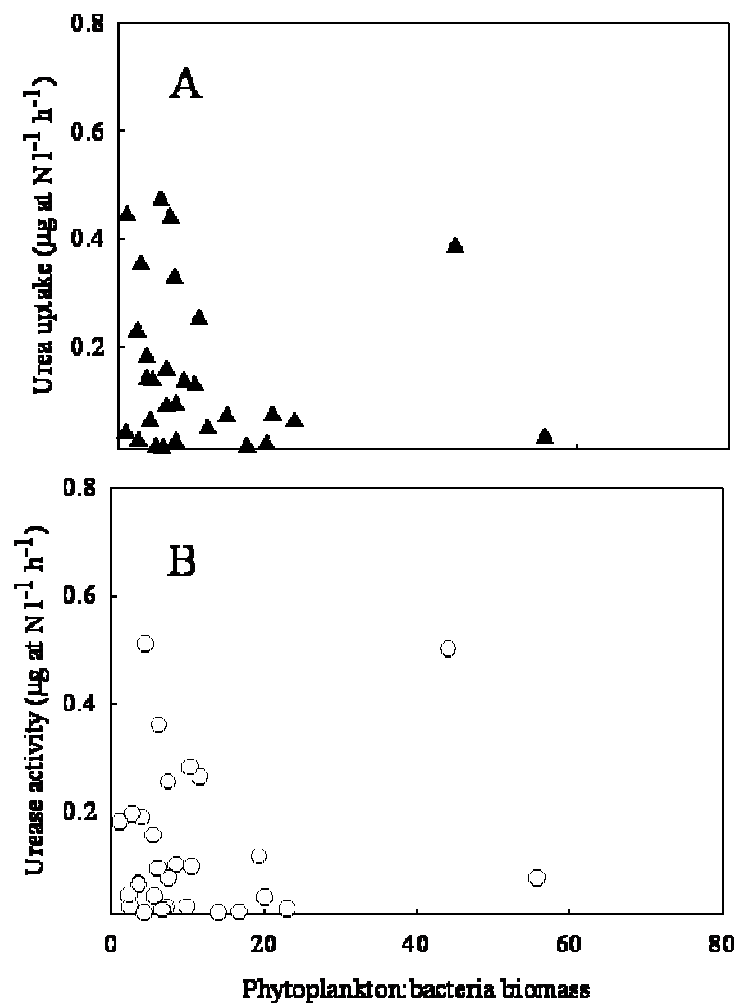
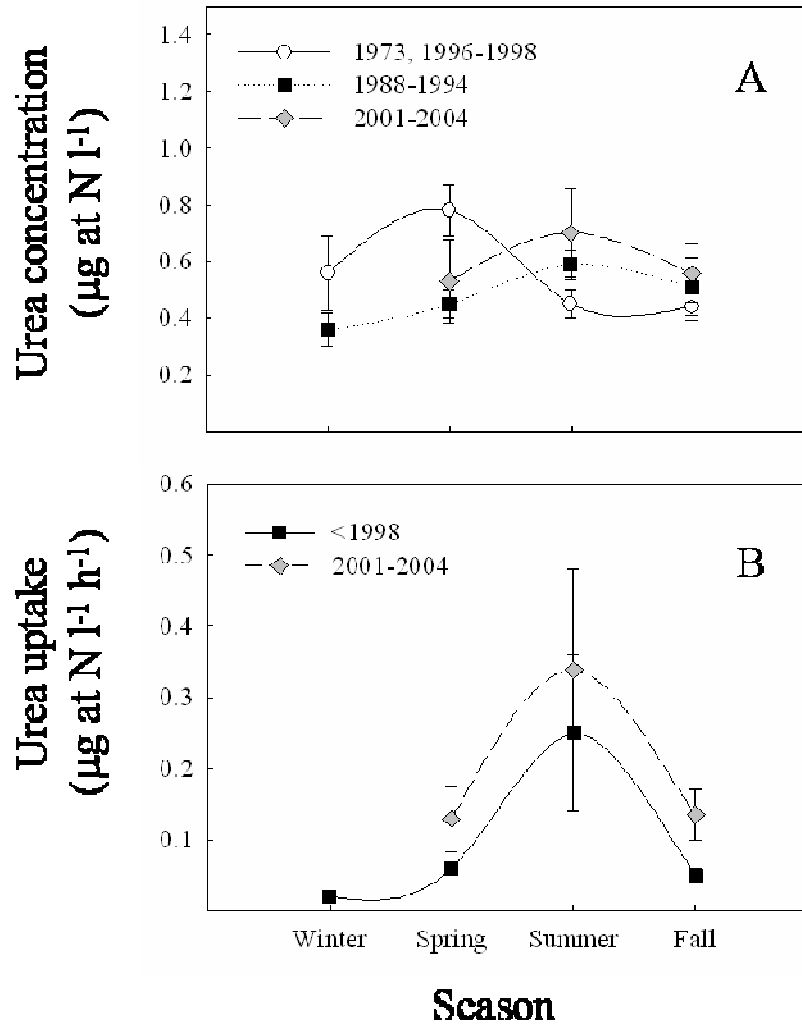


Fig. 4.11: Comparisons of A) surface urea concentrations and B) urea uptake rates during 2001-2004 with data collected prior to 1998 from Lomas et al. (2002). Urea concentration data prior to 1998 were recalculated, resulting in different means (\pm SE).



Chapter 5: Urea utilization in Choptank River

Abstract

Rates of urea uptake and urease activity were measured seasonally over a four-year period in the Choptank River, a tributary of the Chesapeake Bay, to understand how these processes are related to nutrient limitation, nitrogen availability, and plankton community composition. Urea uptake was lower in the diatom-dominated and predominately P-limited station in the upper tributary. At the N-limited station in the lower part of the Choptank River, dominated by dinoflagellates and cyanobacteria, during the summer and fall, urea was utilized at higher rates than in the upper Choptank. However, urea uptake was repressed if levels of NO_3^- and NH_4^+ were high. These findings have implications for the fate of urea-based fertilizers once they enter the Choptank River.

Introduction

The Choptank River, a tributary of the Chesapeake Bay, has been impacted by anthropogenic activity, especially by nitrogen inputs from agriculture (Staver et al. 1996, Fisher et al. 1998, Fisher et al. 2006). The predominately agricultural region of the Choptank watershed produces corn, soybeans, wheat, fruit and vegetables (Goel et al. 2005, Fisher et al. 2006). Total nitrogen concentrations in the river are strongly correlated with freshwater discharge through groundwater (Staver et al. 1996) and peak twice a year in late fall or winter and late spring (Fisher et al. 1998). Groundwater supplies mostly NO_3^- which has been increasing annually since 1980, possibly due to lags in leaching from agricultural lands enriched with N-based

fertilizers (Fisher et al. 1998). NH_4^+ , on average, represents a much smaller portion of the total nitrogen pool than NO_3^- (4%; Fisher et al. 1998, Fisher et al. 2006).

In the past decade, agriculturalists in Maryland have increased their use of urea-based fertilizers by more than two-fold (Fig. 5.1; Maryland Department of Agriculture, 2006). Urea fertilizers are preferred over NO_3^- -based fertilizers because of their longer storage times and retention in soils (*reviewed* in Glibert et al. 2006). The relationship between agricultural nutrient inputs and water quality has been a primary focus in the eutrophication of the Bay (e.g. Boesch et al. 2001, Kemp et al. 2005). The extent to which this shift in fertilizer use has impacted water quality is unknown. Glibert et al. (2001, 2005) found peaks in urea concentrations in spring and mid-summer that coincided with annual application of urea or manure to crops in other tributaries of the Chesapeake Bay over a period of five years, and such peaks coincided with blooms of the dinoflagellate, *Prorocentrum minimum*.

Although most of the agricultural urea is volatilized as NH_4^+ , consumed by crops, or converted to NH_4^+ , some does reach adjacent waterways via overland transport. Urea that enters the Choptank River via runoff from agricultural lands can be used by both phytoplankton and bacteria for cell metabolism. The contribution of urea from agricultural use may exceed urea produced *in situ* from zooplankton excretion (Miller and Glibert 1998, L'Hulguen et al. 2005), bacterial regeneration (Cho et al. 1996, Therkildsen et al. 1997), and release from sediments (Lomstein et al. 1989, Lund and Blackburn 1989, Therkildsen and Lomstein 1994). In order for external urea to be assimilated by a phytoplankton or bacterial cell, urea must first be transported into the cell via passive or active transport (Antia et al. 1991, Beckers et

al. 2004). The enzyme urease catabolizes urea into NH_4^+ and CO_2 . The NH_4^+ then enters the glutamate synthetase (GS)/glutamine 2-oxoglutarate aminotransferase (GOGAT) pathway.

Due to growing amounts of anthropogenic inputs of urea, rates of urea uptake and urease activity may be increasing in phytoplankton and bacteria to utilize this source of new nitrogen. The Choptank River has fluctuating nitrogen concentrations resulting from groundwater flow (Fisher et al. 2006) so rates of urea uptake and urease activity were hypothesized to vary seasonally between an upstream and downstream station as well as in surface and bottom waters.

Methods

Field sampling and hydrological data

Samples were collected from two sites in the Choptank River (Fig. 5.2) in April, July/August and October from 2001-2004. Additional sampling was done during February (2002, 2003), June (2002-2004), September (2002) and December (2001, 2003). The first site was downstream of the confluence of the Tuckahoe and Choptank Rivers (Upper Choptank) while the second site was near the mouth of the river where it enters the Chesapeake Bay (Lower Choptank). Water was collected from the near-surface and the near-bottom using a diaphragm pump, which minimizes damage to plankton. Water was stored in acid-washed Nalgene carboys for transport to the lab (<1 h) for processing. Salinity and temperature data was collected by both a YSI 85 probe and a Seabird CTD at both depths. Annual streamflow data into the

Choptank River at Greensboro, Maryland (Station 01491000) was obtained from the United States Geological Survey (www.usgs.gov).

Nutrients

In the laboratory, water from each site was filtered through precombusted GF/F filters (450°C for 1 h) into acid-washed bottles for nutrient analyses. Concentrations of NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} were determined with a Technicon Autoanalyzer II (Lane et al. 2000). Urea concentrations from April 2001-April 2004 were determined by the urease method described by Parsons et al. (1984) and after April 2004 by the diacetylmonoxime method (Mulvenna and Savidge 1992, Revilla et al. 2005). Total dissolved nitrogen (TDN) concentrations were determined by persulfate oxidation (Bronk et al. 2000) while total dissolved phosphate (TDP) was measured by the method of Solórzano and Sharp (1980). Concentrations of particulate carbon (PC) and particulate nitrogen (PN) were analyzed on particulate matter that was collected on precombusted GF/F filters (450°C for 1 h) with an Exeter Analytical Incorporated CE-440 elemental analyzer.

Plankton composition and biomass

Particulate matter was also collected on precombusted G/F filters (450°C for 1 h) for chlorophyll *a* (chl *a*) and pigment analyses, then immediately frozen in a -80°C freezer until analysis. Chlorophyll samples were analyzed by extraction with acetone (Parsons et al. 1984) and then measured on a 10-AU Turner Designs flourometer. Pigment analyses were done according to Van Heukelem et al. (1994) and Van

Heukelem and Thomas (2001) on a Hewlett Packard high-performance liquid chromatograph system (HPLC; Model 110). The HPLC data was analyzed using the CHEMTAX software program (Mackey et al. 1996, Adolf et al. 2006) in order to compare relative abundances (f_{chl} $a^{taxonomic\ group}$) of seven taxonomic groups (diatoms, dinoflagellates, cryptophytes, chlorophytes, haptophytes, prasinophytes and cyanobacteria). For a few selected time points and stations, the CHEMTAX results were compared to preserved samples of phytoplankton (in Lugol's, 4% glutaraldehyde or 2% formalin).

Urea uptake and urease activity

Urea uptake rates were determined using ^{15}N tracer techniques (Glibert and Capone 1993). Water samples of 500 mL were incubated in 1 L acid-washed polycarbonate bottles with $0.5\ \mu\text{g at N l}^{-1}\ ^{15}\text{N}$ -urea for 30 minutes. The atom % enrichment in samples varied from 10.2 to 100%. Surface water samples and bottom samples were incubated under 60% natural irradiance using neutral-density screening and in the dark, respectively. After the incubation period, particulate matter was collected on combusted GF/F filters. Once filtration was complete, the samples were frozen until they were dried at 50°C . Dried samples were packed into tin boats and analyzed on a SerCon Mass Spectrometer using urea as a standard.

Urease activity samples were also collected by collecting particulate matter on combusted GF/F filters which were frozen in liquid N_2 until analysis. Samples were analyzed for urease activity in the laboratory within one week of sampling using the method of Peers et al. (2000) which was further improved by Fan et al. (2003).

Beginning in August 2003, urease activity samples were analyzed using an optimized assay for field samples (Solomon et al. *submitted*). Urease activity data prior to August 2003 were corrected by a conversion between the currently published and revised methods using samples from a range of sites and seasons (Solomon et al. *submitted*).

Statistical analyses

Data from all four years were analyzed to see if physical factors (salinity and temperature), nitrogen availability (NO_3^- , NH_4^+ , urea), and phytoplankton community composition explained most of the variability in urea uptake and urease activity in the Choptank River. The urea uptake and urease activity data, which did not have normal distributions, were transformed using the square root function before analysis by statistical techniques.

The statistical analysis had two components. First, to detect any significant relationships between urea uptake or urease activity and season, station or depth, a three-way ANOVA was run using the entire data set. In addition, a two-way ANOVA was run to detect any seasonal or depth effects on urea uptake or urease activity at individual stations.

Second, to detect any significant relationships of urea uptake and urease activity with environmental variables that often co-vary, a principal component analyses (PCA; S-PLUS, Insightful Corporation, 2002) was conducted to produce a new set of linear combined variables and reduce the number of variables to be used in a multiple regression model. The PCA analysis also allowed for the visualization of

interaction among variables over time at each of the Choptank River stations. Rates of urea uptake and urease activity were then modeled using multiple regressions that incorporated the new combined variables resulting from PCA analysis (PC1, PC2, PC3). The level of significance in the multiple regressions was set at $p < 0.1$.

Results

Hydrological features

Streamflow, salinity, and temperature in the Choptank River changed annually during the four year period. During 2002, the annual streamflow into the Choptank River was lower than the long-term average (Table 5.1), with a historical daily low of $0.01 \text{ m}^3 \text{ s}^{-1}$ during August (data not shown). In contrast, during 2003-2004 streamflow into the Choptank River was two- to three-fold higher than the long-term average, and salinity decreased both on the surface and bottom of the Choptank River at both stations (Table 5.1). There was difference of 8-11 in salinity between the Choptank stations. Temperature at both Choptank stations followed a seasonal pattern with a winter low of 1.1°C in February 2003 to a summer high of 28.3°C in August 2003 (Table 5.1).

Dissolved nutrient distributions

In the Upper Choptank, concentrations of NO_3^- ranged from 41 to $>200 \text{ } \mu\text{g at N l}^{-1}$ during winter and spring in both surface and bottom waters, but were $<50 \text{ } \mu\text{g at N l}^{-1}$ (except fall 2003) during summer and fall. Concentrations of NH_4^+ were also higher during winter and spring in both surface and bottom waters ($>5 \text{ } \mu\text{g at N l}^{-1}$)

during summer and fall ($<5 \mu\text{g at N l}^{-1}$; Fig. 5.3). Urea concentrations lacked seasonality and were the highest in fall 2001 at $4.39 \mu\text{g at N l}^{-1}$. DIN:DIP ratios were generally higher than the stoichiometric proportion of 16:1 throughout all four years, indicating phosphorus limitation, especially in winter and spring (Fig. 5.3). DIN:DIP ratios at both the surface and bottom were similar during all seasons (Fig. 5.3).

Concentrations of nitrogen were lower at the Lower Choptank station than the Upper Choptank (Fig. 5.4). During winter and spring, NO_3^- concentrations in surface waters were $<7.5 \mu\text{g at N l}^{-1}$ in 2001-2002 and were between 40-54 $\mu\text{g at N l}^{-1}$ in 2003-2004. NH_4^+ concentrations remained below $<5 \mu\text{g at N l}^{-1}$ in the spring, winter and fall. Urea concentrations mostly were $<0.7 \mu\text{g at N l}^{-1}$ throughout the year (Fig. 5.4). The contribution of NH_4^+ and urea to the total nitrogen pool at the surface was $>80\%$ during summer and fall, except in 2003 (data not shown). DIN:DIP ratios at both the surface and bottom indicated P limitation ($>16:1$) during the winter and spring. During the summer and fall, DIN:DIP ratios suggested N limitation ($<16:1$) in both surface and bottom waters (Fig. 5.4).

Plankton distribution

Diatoms or chlorophytes and cryptophytes dominated the phytoplankton community in both surface and bottom waters in the Upper Choptank (Fig. 5.5). The relative abundance of diatoms (such as *Navicula* sp., *Skeletonema costatum*, *Cyclotella* sp. and other centric diatoms) at the surface was between 46-87% in the spring and 55-89% in the fall. The relative abundance of diatoms was lower and the relative abundance of chlorophytes (such as *Scenedesmus* sp. and *Pediastrum duplex*)

and cryptophytes was higher than rest of the sampling period during December 2001-June 2002 and June 2003-June 2004. The community in the bottom layer generally had more diatoms during the summer than the surface community, but followed the same trends during other times of the year (Fig. 5.5).

With the exception of spring 2004, diatoms were a large component of the spring community in the surface waters of the Lower Choptank (>74% with species such as *Cytotella* sp., *Ditylum brightwellii*, *Nitzschia* sp.) (Fig. 5.6). The spring 2004 community consisted of 31% diatoms, 31% chlorophytes, and 12% cryptophytes. The summer, fall and winter phytoplankton communities were mostly dominated by dinoflagellates (such as *Prorocentrum* sp., *Heterocapsa* sp., *Akashiwo sanguinea*) and cryptophytes, with contributions from cyanobacteria except in summer 2001 (Fig. 5.6). The summer of 2003 was unusual with 86% of the community consisting of the diatom, *Leptocylindrus minimus*. The bottom community was similar to the surface community but with more contributions by cyanobacteria and haptophytes during 2003-2004 (Fig. 5.6). The contribution of dinoflagellates at the surface (64%) was much higher than at the bottom (11%) in summer 2004.

Urea uptake and urease activity rates

Rates of urea uptake were higher during the summer and fall than during winter and spring at both Choptank stations (Figs. 5.5 and 5.6). Rates of urea uptake were higher in the surface than in the bottom waters during all seasons, but spring and summer 2002 was an exception to this trend. Rates of urease activity did not appear to follow any seasonal trend at either Choptank station. Maximum urease activity in

the surface waters in the Upper and Lower Choptank was 86.4 and 141 ng-at N $\mu\text{g chl } a^{-1} \text{ h}^{-1}$ (= 0.16 and 0.35 $\mu\text{g-at N l}^{-1} \text{ h}^{-1}$), respectively, which occurred in June 2004 (data not shown). Urease activity was higher in bottom waters with maximum urease activity of 126 ng-at N $\mu\text{g chl } a^{-1} \text{ h}^{-1}$ (= 0.60 $\mu\text{g-at N l}^{-1} \text{ h}^{-1}$) in winter 2002 in the Upper Choptank and 189 ng-at N $\mu\text{g chl } a^{-1} \text{ h}^{-1}$ (= 0.20 $\mu\text{g-at N l}^{-1} \text{ h}^{-1}$) in June 2004 in the Lower Choptank.

Relationships between urea uptake, urease activity and environmental factors

Urea uptake did not have any significant temporal (seasonal) or spatial (station and depth) patterns (ANOVA; $p > 0.19$) using results from both stations. On the other hand, urease activity did change significantly with seasons, but not between stations or depths (ANOVA; $p < 0.05$ for seasons and $p > 0.37$ for station and depth). When results from each station were analyzed individually, urea uptake was significantly different at the two depths in the Upper Choptank (ANOVA, $p = 0.07$) but not seasonally at either station (ANOVA, $p > 0.3$). Urease activity significantly changed seasonally but not between depths at both stations (ANOVA, $p < 0.05$ for seasons, $p > 0.13$ for depth). In addition, there was no significant relationship between urea uptake and urease activity in the Choptank or at individual stations ($r^2 < 0.01$).

The PCA analysis created several combined principal components based on physical factors, nitrogen availability, and phytoplankton community composition. The first three principal components (PC1, PC2, and PC3) explained 65% and 68% of the variance in the surface and bottom waters of the Upper Choptank, 63% and 74% in the Lower Choptank (Fig. 5.7; Table 5.2). PC1 for both surface and bottom waters

in the Upper Choptank was positively related to $f_chl\ a^{diatoms}$, but negatively to $f_chl\ a^{cryptophytes}$ and NO_3^- and NH_4^+ concentrations (Table 5.2). PC2 for surface waters and PC3 for bottom waters were associated with warm temperatures and higher urea concentrations, but negatively with $f_chl\ a^{dinoflagellates}$. Higher salinities and $f_chl\ a^{dinoflagellates}$ and $f_chl\ a^{cyanobacteria}$ represented PC2 in bottom waters and PC3 in surface waters. In surface waters, urea uptake was significantly positively correlated with PC1 while urease activity was similarly correlated with PC2 ($p < 0.06$; Table 5.3). Bottom water characteristics had no significant relationship with urea uptake or urease activity (Table 5.3).

Individual factors in the surface and the bottom waters grouped together differently in the Lower Choptank. PC1 in surface waters was associated with warmer temperatures, higher urea concentrations and $f_chl\ a^{dinoflagellates}$ and $f_chl\ a^{cyanobacteria}$ (Fig. 5.7B; Table 5.2). Like surface waters, PC1 in bottom waters was related to warmer temperatures and higher urea concentration but was negatively associated with higher salinity and $f_chl\ a^{dinoflagellates}$ (Fig. 5.7D; Table 5.2). PC2 at both depths was associated with higher NO_3^- and NH_4^+ concentrations, but with different phytoplankton communities. The surface community was associated with $f_chl\ a^{chlorophytes}$, $f_chl\ a^{cyanobacteria}$, $f_chl\ a^{dinoflagellates}$ and $f_chl\ a^{cryptophytes}$ and but not $f_chl\ a^{diatoms}$. The bottom community was associated with $f_chl\ a^{cryptophytes}$, $f_chl\ a^{haptophytes}$, and $f_chl\ a^{chlorophytes}$. PC3 at the surface was related with warmer temperatures and $f_chl\ a^{dinoflagellates}$, but not $f_chl\ a^{haptophytes}$ and $f_chl\ a^{cryptophytes}$. Bottom PC3 was associated with colder temperatures, higher NO_3^- and NH_4^+ concentrations, and lower $f_chl\ a^{cyanobacteria}$. Urea uptake in surface waters of the

Lower Choptank was significantly correlated to PC1 and PC2. However, urea uptake in the bottom waters and urease activity at both depths was not significantly related to any of the combined variables in the Lower Choptank (Table 5.3).

Discussion

Differences between Upper and Lower Choptank

The Upper Choptank had higher nitrogen concentrations than the Lower Choptank and remained mostly P-limited throughout the four year study. NO_3^- and NH_4^+ concentrations in the Upper Choptank were >40 and $5 \mu\text{g-at N l}^{-1}$, respectively, during the spring months. These concentrations may have repressed urea uptake rates in the dominant members of the phytoplankton community, the diatoms, during the spring months (Fig. 5.7A). Urea uptake rates, on average, were lower ($x = 11.8 \pm 19.0 \text{ ng-at N } \mu\text{g chl a}^{-1} \text{ h}^{-1}$) but not significantly different than those observed in the Lower Choptank. On the other hand, higher urease activity was associated with cyanobacteria and chlorophytes (Fig. 5.7A).

In contrast, the Lower Choptank station had lower nitrogen concentrations (TDN: $<83 \mu\text{g-at N l}^{-1}$ as opposed to the Upper Choptank where TDN reached $263 \mu\text{g-at N l}^{-1}$) and was often N-limited during the summer and fall months. Looking further into the PCA at individual dates (July 2002 and July 2004) when the Lower Choptank was N-limited, high urea uptake rates were associated with dinoflagellates and cyanobacteria (PC1; Fig. 5.7B). During times of P-limitation in the spring (April 2001), lower urea uptake, than other times, was associated with diatoms (PC2; Fig. 5.7B). Both rates of urea uptake and urease activity, on average, were higher in

surface waters of the Lower Choptank (24 ± 38 and 24.8 ± 5.66 ng-at N $\mu\text{g chl a}^{-1} \text{h}^{-1}$) than the Upper Choptank (11.8 ± 18.6 and 22.8 ± 4.87 ng-at N $\mu\text{g chl a}^{-1} \text{h}^{-1}$). In all, urea uptake and urease activity tended to be lower if diatoms were abundant and higher if dinoflagellates and cyanobacteria were abundant.

Influence of plankton composition on urea uptake and urease activity at two depths

Urea uptake and urease activity was higher when cyanobacteria or dinoflagellates were abundant in either surface or bottom waters in the Lower Choptank such as July 2002 (surface), August 2004 (both surface and bottom), October 2002 (both surface and bottom), and October 2004 (surface). Cyanobacteria in coastal regions such as Florida Bay (Glibert et al. 2004a), western Florida Shelf (Heil et al. *in press*), and the Chesapeake Bay (Solomon et al. *submitted*) have previously been associated with higher urea uptake and urease activity rates on a per chl *a* basis than found with other phytoplankton taxonomic groups in nearby waters. In contrast, dinoflagellates have higher urea uptake and urease activity on a volumetric basis than opposed to other phytoplankton taxonomic groups (Fan et al. 2003, Glibert et al. 2006, Solomon et al. 2006). For example, the association of higher rates of urea uptake with dinoflagellates and higher rates of urease activity with cyanobacteria was clearly seen in August 2004 in the Lower Choptank when urea uptake was higher at the surface while urease activity was higher at the bottom.

Influence of NO_3^- and NH_4^+ availability on urea uptake and urease activity

High levels of NO_3^- and NH_4^+ have previously been shown to repress urea uptake and urease activity rates both in culture and field studies (Solomon et al. 2006, Solomon and Glibert *submitted*). Repression of urea uptake by NO_3^- and NH_4^+ was evident in the Choptank both on spatial and temporal scales. On a spatial scale, urea uptake rates were lower in the Upper Choptank which has higher concentrations of NO_3^- and NH_4^+ than in the Lower Choptank. On a temporal scale, urea uptake rates were higher during the summer when NH_4^+ concentrations in the Lower Choptank were below $1.2 \mu\text{g-at N l}^{-1}$ in summers 2002 and 2004 than when NH_4^+ concentrations were $>1.2 \mu\text{g-at N l}^{-1}$ in summers 2001 and 2003. Urea uptake was also very high during April 2002 in the Lower Choptank when both NO_3^- and NH_4^+ concentrations were relatively low.

Urea dynamics in the Choptank River

Urea concentrations in the tributaries tend to be higher than in the mainstem of Chesapeake Bay (Glibert et al. 2001, 2004a,b, 2005). The highest urea concentration observed in the Choptank was $4.39 \mu\text{g-at N l}^{-1}$ while the maximum recorded observations in other tributaries range from 2.5 to $>24 \mu\text{g-at N l}^{-1}$ (Glibert et al. 2001, 2004b, 2005). However, the average concentration of urea in the Upper and Lower Choptank was 0.82 and $0.34 \mu\text{g-at N l}^{-1}$, respectively, which was within the range seen in the Chesapeake Bay during the same period ($0.50\text{-}0.89 \mu\text{g-at N l}^{-1}$; Solomon et al. *submitted*). As in other tributaries, urea concentrations were highly variable with no seasonal trend and not related to seasonally averaged flow (Glibert et

al. 2005). Higher transient urea concentrations may be associated with precipitation events (e.g. Glibert et al. 2001), but such events were not observed during this study.

Possible impacts of urea-based agricultural fertilizers in the Choptank River

Application of urea fertilizers on farmland will have a different impact at the two stations in the Choptank River. At the primarily P-limited Upper Choptank station, a response to a possible increase in urea concentrations will unlikely occur due to repression of urea utilization by high NO_3^- and NH_4^+ concentrations. During years of low flow (2001-2002), NH_4^+ concentrations during the spring in the Upper Choptank were often $>15 \mu\text{g-at N l}^{-1}$, while during years of high flow (2003-2004), NO_3^- concentrations were often $>200 \mu\text{g-at N l}^{-1}$. These are concentrations that have previously been observed to repress urea uptake and urease activity (Solomon et al. 2006, Solomon et al. *submitted*). Thus, even during a sporadic precipitation event in the spring, most likely urea will not be utilized at high rates in this region of the river.

Anthropogenic urea, however, may have a bigger impact at the Lower Choptank station during the summer or fall when the plankton is N-limited. The ability of the dinoflagellate and cyanobacteria community to respond to increases in urea concentrations may depend on NH_4^+ availability. Dinoflagellates and cyanobacteria have lower urease activity when grown on NH_4^+ than other nitrogen substrates (Solomon et al. 2006, Solomon and Glibert *submitted*), so if NH_4^+ concentrations are low enough not to repress urease activity, then urea will be readily utilized by these phytoplankton. Dinoflagellates in the region have previously responded to inputs of urea. High urea concentrations in the Chicamacomico River,

another tributary of the Chesapeake Bay, coincided with major periods of urea fertilization (Glibert et al. 2005). Likewise, *Prorocentrum minimum* blooms were observed in tributaries after periods of high ambient concentrations of urea soon after a rainfall event (Glibert et al. 2001). Many harmful algal species have been observed to have the ability to efficiently utilize urea (Glibert et al. 2006, Solomon and Glibert *submitted*), and may potentially bloom after a precipitation event washes in agricultural urea if other conditions permit. The ability of phytoplankton and bacteria to utilize urea from both anthropogenic and natural sources in the Lower Choptank River thus appears to depend on nitrogen availability, not only of urea but other forms of nitrogen as well.

Conclusion

The contrasting conditions of the Upper and Lower Choptank stations, despite only being separated by 28 km, in terms of nutrient limitation, nitrogen availability and plankton community composition allowed for comparisons to understand what conditions may regulate urea uptake and urease activity. Urea uptake and urease activity were lower in the primarily P-limited Upper Choptank than the often N-limited Lower Choptank. Under P-limiting conditions, diatoms were dominant members of the community and were associated with urea uptake, albeit at lower rates, while most of the urease activity was associated with cyanobacteria, cryptophytes, and chlorophytes. Under N-limiting conditions, higher urea uptake and urease activity were associated with dinoflagellates, cyanobacteria, and haptophytes. In these plankton communities, high levels of NO_3^- and NH_4^+ repressed urea uptake and urease activity. The difference in enzymatic rates between

surface and bottom waters was also explained by differences in the presence of diatoms, dinoflagellates, and cyanobacteria. Urea uptake and urease activity are influenced by the state of nutrient limitation and plankton community composition which in turn has implications on whether urea from agricultural runoff will impact phytoplankton communities at the two stations in the Choptank River.

Literature cited

- Adolf, J.E., Harding, L.W., Yeager, C.L., Maloney, M.E & Miller, W.D. 2006. Environmental forcing of phytoplankton floral composition, biomass and primary productivity in the Chesapeake Bay, USA. *Estuar. Shelf Coastal Sci.* 67: 108-122.
- Beckers, G., Bendt, A.K., Krämer, R. & Burkovski, A. 2004. Molecular identification of the urea uptake system and transcriptional analysis of urea transporter- and urease-encoding genes in *Corynebacterium glutamicum*. *J. Bacteriol.* 186: 7645-7652.
- Boesch, D.F., Brinsfield, R.B. & Magnien, R.E. 2001. Chesapeake Bay eutrophication: scientific understanding, ecosystem restoration, and challenges for agriculture. *J. Environ. Qual.* 30: 303-320
- Bronk, D.A., Glibert, P.M., Malone, T.C., Banahan, S. & Sahlsten, E. 1998. Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux. *Aquat. Microb. Ecol.* 15: 177-89.
- Bronk, D.A., Lomas, M.W., Glibert, P.M., Schukert, K.J. & Sanderson, M.P. 2000. Total dissolved nitrogen analysis: comparisons between persulfate, UV, and high temperature oxidation methods. *Mar. Chem.* 69: 163-178.
- Cho, B.C., Park, M.C., Shim, J.H. & Azam, F. 1996. Significance of bacteria in urea dynamics in coastal surface waters. *Mar. Ecol. Prog. Ser.* 142: 19-26.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Fisher, T.R., Hagy, J.D., Boynton, W.R. & Williams, M.R. 2006. Cultural eutrophication in the Choptank and Patuxent estuaries of Chesapeake Bay. *Limnol. Oceanogr.* 51: 435-447.
- Fisher, T.R., Lee, K., Berndt, H, Benitez, J.A & Norton, M.M. 1998. Hydrology and chemistry of the Choptank River Basin. *Water, Air, and Soil Pollution* 105: 387-397.
- Glibert, P.M., Alexander, J., Trice, T.M., Michael, B., Magnien, R.E. Lane, L., Oldach, D. & Bowers, H. 2002. Chronic urea loading: a correlate of *Pfiesteria* spp. in the Chesapeake and Coastal Bays of Maryland. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., and Vargo, G.A. (eds). Harmful Algae 2002, Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission and Intergovernmental Oceanographic Commission of UNESCO, USA. pp. 48-55.

- Glibert, P.M. & Capone, D.G. 1993. Mineralization and assimilation in aquatic, sediment, and wetland systems. *In* Knowles, R., Blackburn, T.H. (eds). Nitrogen isotope techniques. Academic Press, New York, pp. 243-272
- Glibert, P.M., Garside, C., Fuhrman, J.A. & Roman, M.R. 1991. Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnol. Oceanogr.* 36: 895-909.
- Glibert, P.M., Harrison, J., Heil, C., & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry.* 77: 441-463.
- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Glibert, P.M., Magnien, R., Lomas, M.W., Alexander, J., Fan, C., Haramoto, E., Trice, M. & Kana, T.M. 2001. Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: Comparison of the 1997, 1998 and 1999 Events. *Estuaries.* 24: 875-883.
- Glibert, P.M., Trice, T.M, Michael, B. & Lane, L. 2005. Urea in the tributaries of the Chesapeake and Coastal Bays of Maryland. *Water, Air, and Soil Pollution.* 160: 229-243.
- Goel, A., McConnell, L.L. & Torrents, A. 2005. Wet deposition of current use pesticides at a rural location on the Delmarva peninsula: impact of rainfall patterns and agricultural activity. *J. Agric. Food Chem.* 53: 7915-7924.
- Heil, C.A., Revilla, M., Glibert, P.M. & Murasko, S. *In press*. Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde, E.D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M. & Stevenson, J.C. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. *Mar. Ecol. Prog. Ser.* 303:1-29.
- Lane, L., Rhoades, S., Thomas, C. & Van Heukelem, L. 2000. Analytical Services Laboratory Standard Operating Procedures. Technical Report No. TS-264-00. Horn Point Laboratory, University of Maryland Center for Environmental Science.

L'Hulguen, S., Slawyk, G. & Le Corre, P. 2005. Seasonal patterns of urea regeneration by size-fractionated microheterotrophs in well-mixed temperate coastal waters. *J. Plank. Res.* 27: 263-270.

Lomstein, B.A., Blackburn, T.H. & Henriksen, K. 1989. Aspects of nitrogen and carbon cycling in the northern Bering Shelf sediment. 1. The significance of urea turnover in the mineralization of NH_4^+ . *Mar. Ecol. Prog. Ser.* 57:237-247.

Lund, B.A. & Blackburn, T.H. 1989. Urea turnover in a coastal marine sediment measured by ^{14}C -urea short-term incubation. *J. Microbiol. Methods* 9:297-308.

Mackey, M.D., Higgins, H.W., Mackey, D.J. & Wright, S.W. 1996. CHEMTAX - a program for estimating class abundances from chemical markers – application to HPLC measurements of phytoplankton. *Mar. Ecol. Prog. Ser.* 144: 265-283.

Maryland Department of Agriculture. 1990-2004. Annual Fertilizer Tonnage Report.

Miller, C.A. & Glibert, P.M. 1998. Nitrogen excretion by the calanoid copepod *Acartia tonsa*: results of mesocosm experiments. *J. Plank. Res.* 20: 1767-1780.

Mulvenna, P.F & Savidge, G. 1992. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. *Estuar. Coastal Shelf Sci.* 34: 429-438.

Parsons, R.T., Maita, Y. & Lalli, C.M. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press, New York, N.Y.

Peers, G.S., Milligan, A.J. & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.

Revilla, M., Alexander, J. & Glibert, P.M. 2005. Analysis of urea in coastal waters: comparison of the enzymatic and the direct method. *Limnol. Oceanogr. Methods* 3: 290-299.

Solomon, C.M., Alexander, J.A., Collier, J.L. & Glibert, P.M. *Submitted*. Microbial urea uptake and urease activity in Chesapeake Bay, USA. *Limnol. Oceanogr.*

Solomon, C.M., Alexander, J. & Glibert, P.M. *Submitted*. Measuring urease activity in environmental samples. *Limnol. Oceanogr. Methods*

Solomon, C.M & Glibert, P.M. *Submitted*. Urease activity in five phytoplankton species. *J. Phycol.*

Solomon, C.M., Glibert, P.M. & Collier, J.L. 2006. Potential regulation of urea utilization by nitrogen sources: a synthesis from laboratory and field experiments.

American Society of Limnology and Oceanography Summer Meeting. Victoria, B.C. June 2006.

Soloranzo, L. & Sharp, J.H. 1980. Determination of the total dissolved phosphorus and particulate phosphorus in natural waters. *Limnol. Oceanogr.* 25: 754-752.

Staver, L.W., Staver, K.W. & Stevenson, J.C. 1996. Nutrient inputs to the Choptank River estuary: implications for watershed management. *Estuaries* 19:342-358.

Therkildsen, M.S., Isaken, M.F. & Lomstein, B.A. 1997. Urea production by the marine bacteria *Delacya venusta* and *Pseudomonas stutzeri* grown in a minimal medium. *Aquat. Microb. Ecol.* 13: 213-217.

Therkildsen, M.S. & Lomstein, B.A. 1994. Seasonal variation in sediment urea turnover in a shallow estuary. *Mar. Ecol. Prog. Ser.* 109: 77-82.

van Heukelem, L., Lewitus, A., Kana, T.M. & Craft, N. 1994. Improved separation of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Mar. Ecol. Prog. Ser.* 114: 303-313.

van Heukelem, L. & Thomas, C.S. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chrom. A.* 910:31-49.

Wheeler, P.A., Glibert, P.M. & McCarthy, J.J. 1982. Ammonium uptake and incorporation by Chesapeake Bay phytoplankton: Short term uptake kinetics. *Limnol. Oceanogr.* 27:1113-1128.

Tables

Table 5.1 Average (\pm SE) streamflow, temperature (at both stations over both depths) and salinity of both depths in the Choptank River. *Winter temperature values for 2001 are from December, 2002-2003 are from February, and 2004 from December 2003.

	2001	2002	2003	2004
Average annual streamflow at Greensboro, MD ($\text{m}^3 \text{s}^{-1}$) (long-term average 1949-2005; $3.86 \text{ m}^3 \text{ s}^{-1}$)	4.33	2.89	9.12	5.28
Temperature ($^{\circ}\text{C}$)				
Winter	12.8	5.43 \pm 0.31*	1.20 \pm 0.31*	12.7 \pm 3.11*
Spring	15.3 \pm 0.91	12.7 \pm 0.70	11.6 \pm 0.62	9.96 \pm 0.49
Summer	27.0 \pm 0.45	26.7 \pm 0.29	26.3 \pm 0.97	26.3 \pm 0.35
Fall	18.1 \pm 0.21	23.7 \pm 0.32	20.8 \pm 0.07	23.0 \pm 0.08
Salinity				
Upper Choptank	0.58 \pm 0.20	3.68 \pm 0.27	0.29 \pm 0.12	0.98 \pm 0.19
Lower Choptank	12.0 \pm 0.78	15.1 \pm 0.60	9.43 \pm 0.70	10.33 \pm 0.19

Table 5.2 Loadings for PC1, PC2, and PC3 for each Choptank station

Variables	Upper Choptank Surface			Upper Choptank Bottom			Lower Choptank Surface			Lower Choptank Bottom		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
<i>Physical</i>												
Temperature		0.608	0.177	0.135	0.176	0.671	0.304	0.110	0.302	0.373		-0.467
Salinity	-0.134		0.371		0.643	0.103	0.139	-0.265	-0.196	-0.486		-0.139
<i>Chemical</i>												
NO ₃ ⁻	-0.329		-0.372	-0.388			-0.348	0.351	0.184	0.174	0.218	0.563
NH ₄ ⁺	-0.272		-0.476	-0.402			-0.415	0.311		0.453	0.159	0.204
Urea		0.407	-0.414	-0.207		0.400	0.353	-0.191	0.149	0.374	-0.157	
<i>Biological</i>												
Diatoms	0.520			0.438	-0.201		-0.347	-0.433		0.148	-0.520	
Dinoflagellates	-0.201	-0.557	0.133	-0.114	0.422	-0.566	0.460	0.175	0.231	-0.304		
Cryptophytes	-0.426			-0.421	0.220			0.222	-0.701	-0.108	0.511	-0.214
Cyanobacteria	-0.286	0.121	0.416		0.433		0.311	0.356	-0.235	0.124	0.271	-0.546
Haptophytes	-0.208		-0.295	-0.298	-0.206		0.177	0.149	-0.403	0.237	0.364	0.111
Chlorophytes	-0.423	0.348		-0.393	-0.200	0.187	-0.103	0.494	0.216	-0.225	0.395	0.196
<i>Cum. prop of variance</i>	0.286	0.472	0.648	0.402	0.561	0.684	0.273	0.493	0.632	0.305	0.551	0.743

Table 5.3 Regression coefficients and t-values for multiple regression models of urea uptake and urease activity at two Choptank River stations. The multiple correlation coefficient is denoted by r^2 .

Station		Urea uptake				Urease activity			
		Intercept	PC1	PC2	PC3	Intercept	PC1	PC2	PC3
Upper Choptank Surface	df	13	13	13	13	12	12	12	12
	coeff.	0.3633	0.0731	0.0451	0.0415	0.3866	0.0624	0.1162	0.0954
	(t-value)	(6.9681)	(2.3332)	(1.2427)	(0.9739)	(5.3432)	(0.13672)	(2.0535)	(1.7043)
	p-value	0.0000	0.0363	0.2359	0.3479	0.0002	0.1966	0.0625	0.1141
	$r^2=$	0.4312				0.4299			
Upper Choptank Bottom	df	12	12	12	12	12	12	12	12
	coeff.	0.2132	0.0238	0.0251	0.0273	0.5202	0.0121	0.0380	-0.0285
	(t-value)	(7.3483)	(1.6650)	(1.0118)	(0.8317)	(6.4065)	(0.2955)	(0.6348)	(-0.4335)
	p-value	0.0000	0.1218	0.3316	0.4218	0.0000	0.7727	0.5375	0.6723
	$r^2=$	0.3233				0.0542			
Lower Choptank Surface	df	12	12	12	12	12	12	12	12
	coeff.	0.4112	0.1067	-0.0864	0.0369	0.4286	0.0699	0.0311	0.0804
	(t-value)	(5.8988)	(2.6526)	(-1.9274)	(0.6537)	(5.8236)	(1.6468)	(0.6565)	(1.3483)
	p-value	0.0001	0.0211	0.0779	0.5256	0.0001	0.1255	0.5239	0.2024
	$r^2=$	0.4823				0.2925			
Lower Choptank Bottom	df	10	10	10	10	9	9	9	9
	coeff.	0.3041	-0.0608	-0.1008	0.0546	0.3515	0.0522	-0.0219	0.0663
	(t-value)	(2.8430)	(-1.1212)	(-1.5187)	(0.7614)	(5.6370)	(1.5317)	(-0.5903)	(1.6700)
	p-value	0.0175	0.2884	0.7614	0.4640	0.0003	0.1600	0.5695	0.1293
	$r^2=$	0.2901				0.3768			

Figures

Fig. 5. 1 Tonnage of urea fertilizer bought in the state of Maryland from 1989-2004.

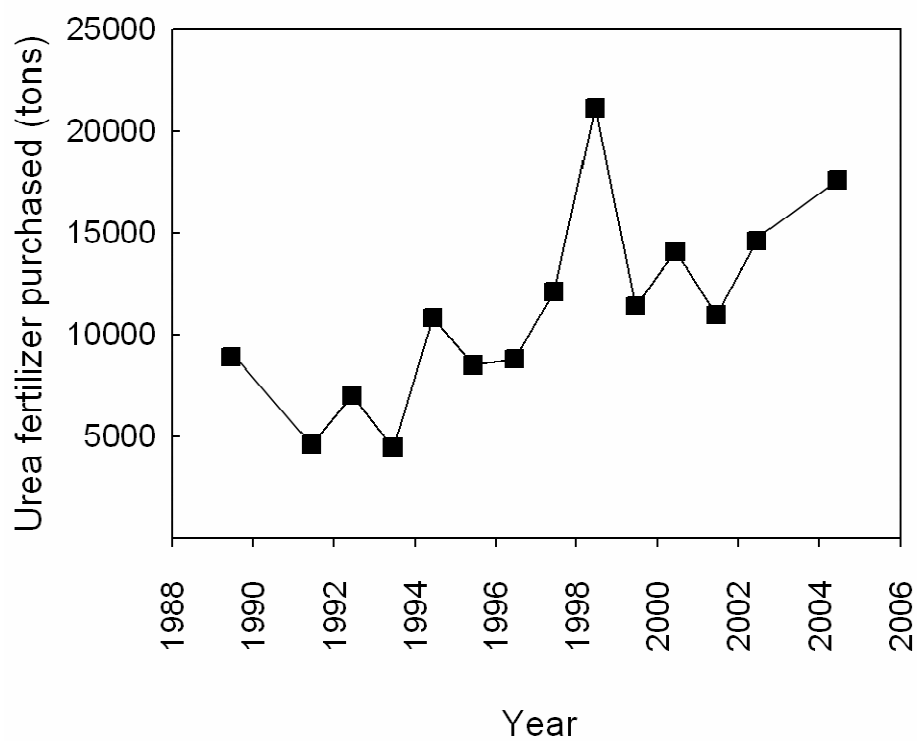


Fig. 5.2 The two sampling stations in the Choptank River

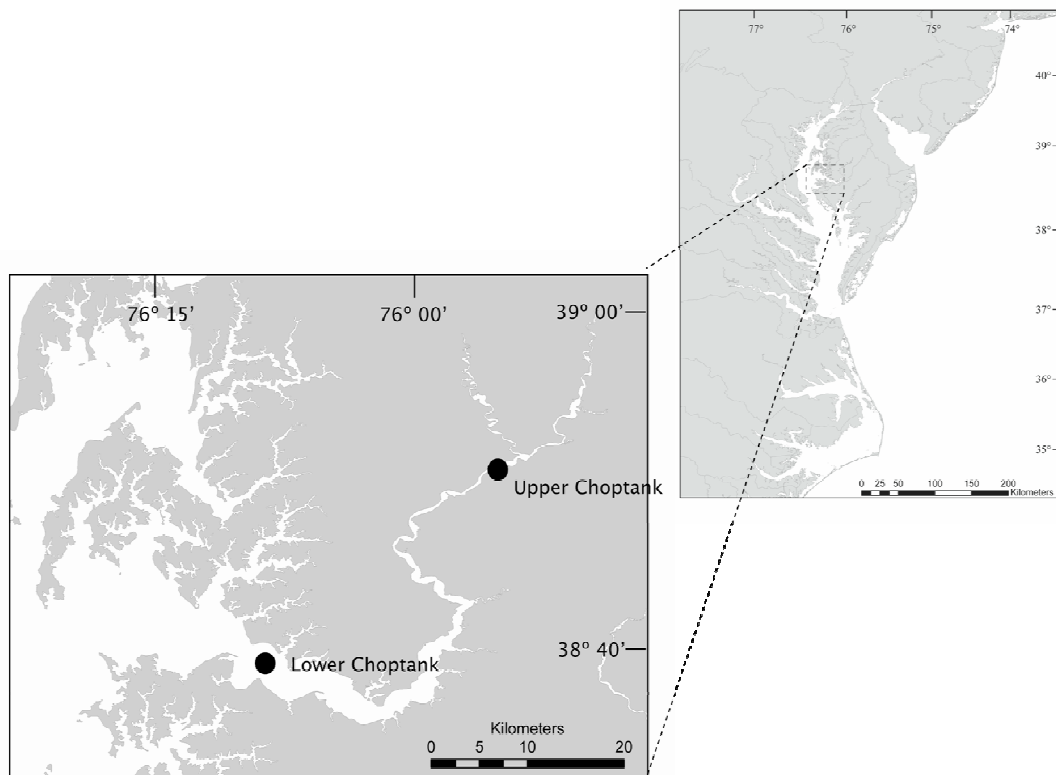


Fig. 5.3 Nitrogen concentrations (NO_3^- , NH_4^+ , and urea) and DIN:DIP ratios at the **Upper Choptank** station during 2001-2004 in both surface (left panel) and bottom (right panel) waters as function of season and year.

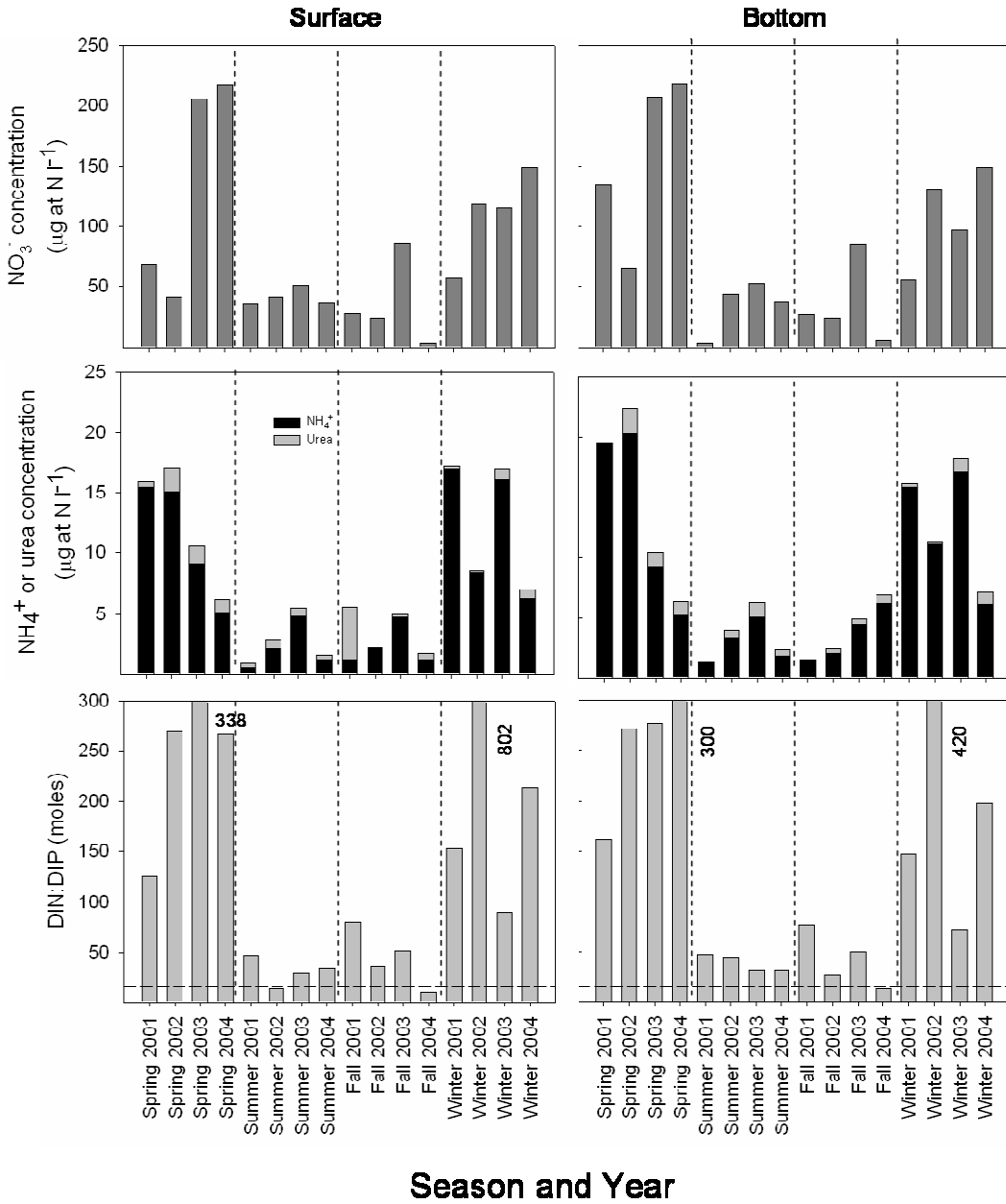


Fig. 5.4 Nitrogen concentrations (NO_3^- , NH_4^+ , and urea) and DIN:DIP ratios at the **Lower Choptank** station during 2001-2004 in both surface (left panel) and bottom (right panel) waters as function of season and year.

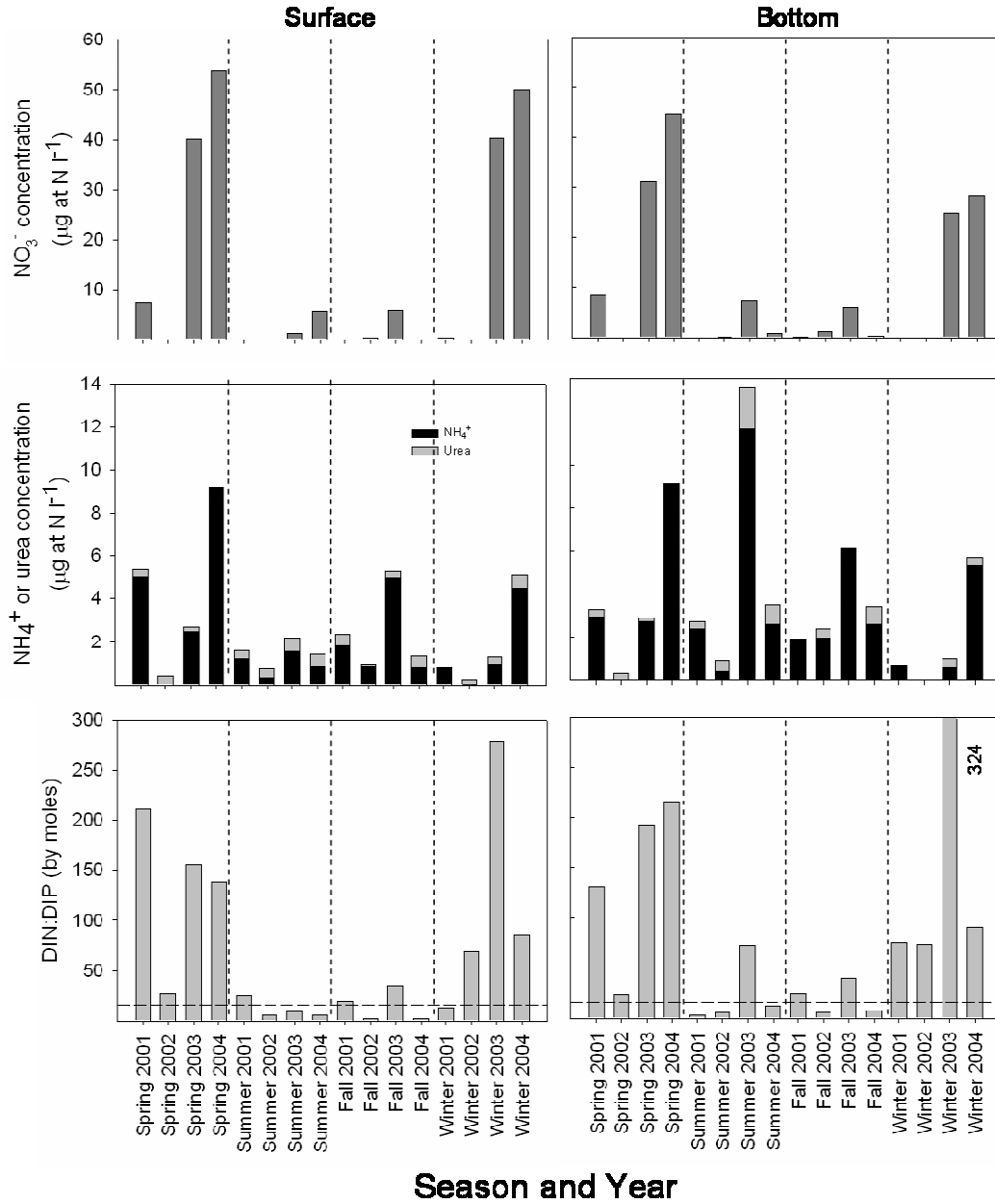


Fig. 5.5 Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage, as analyzed by CHEMTAX during 2001-2004, urea uptake and urease activity (on a per chl a basis) at the **Upper Choptank** in both surface (left panel) and bottom (right panel) waters as a function of season and year.

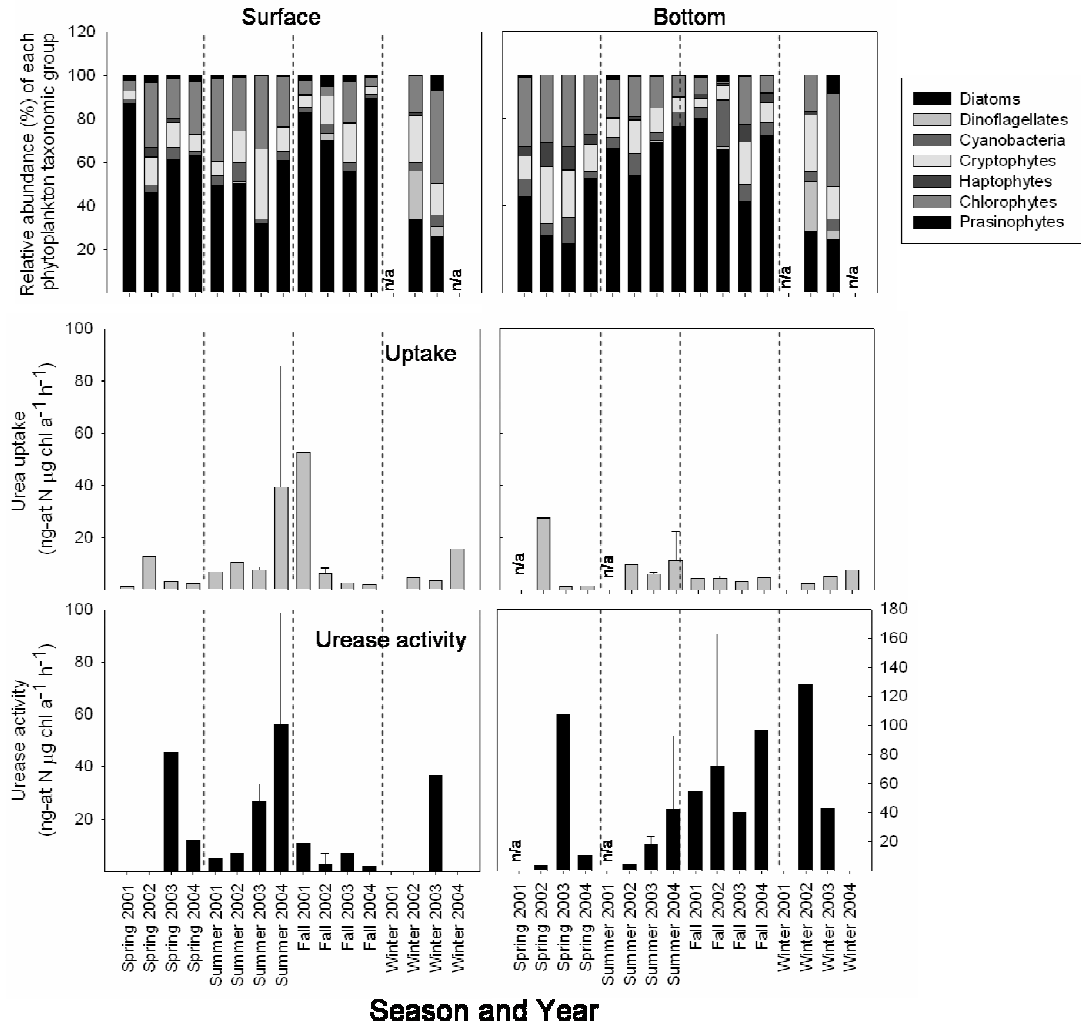


Fig. 5.6 Relative contribution of each phytoplankton taxon to the total phytoplankton assemblage, as analyzed by CHEMTAX during 2001-2004, urea uptake and urease activity (on a per chl a basis) at the **Lower Choptank** in both surface (left panel) and bottom (right panel) waters as a function of season and year.

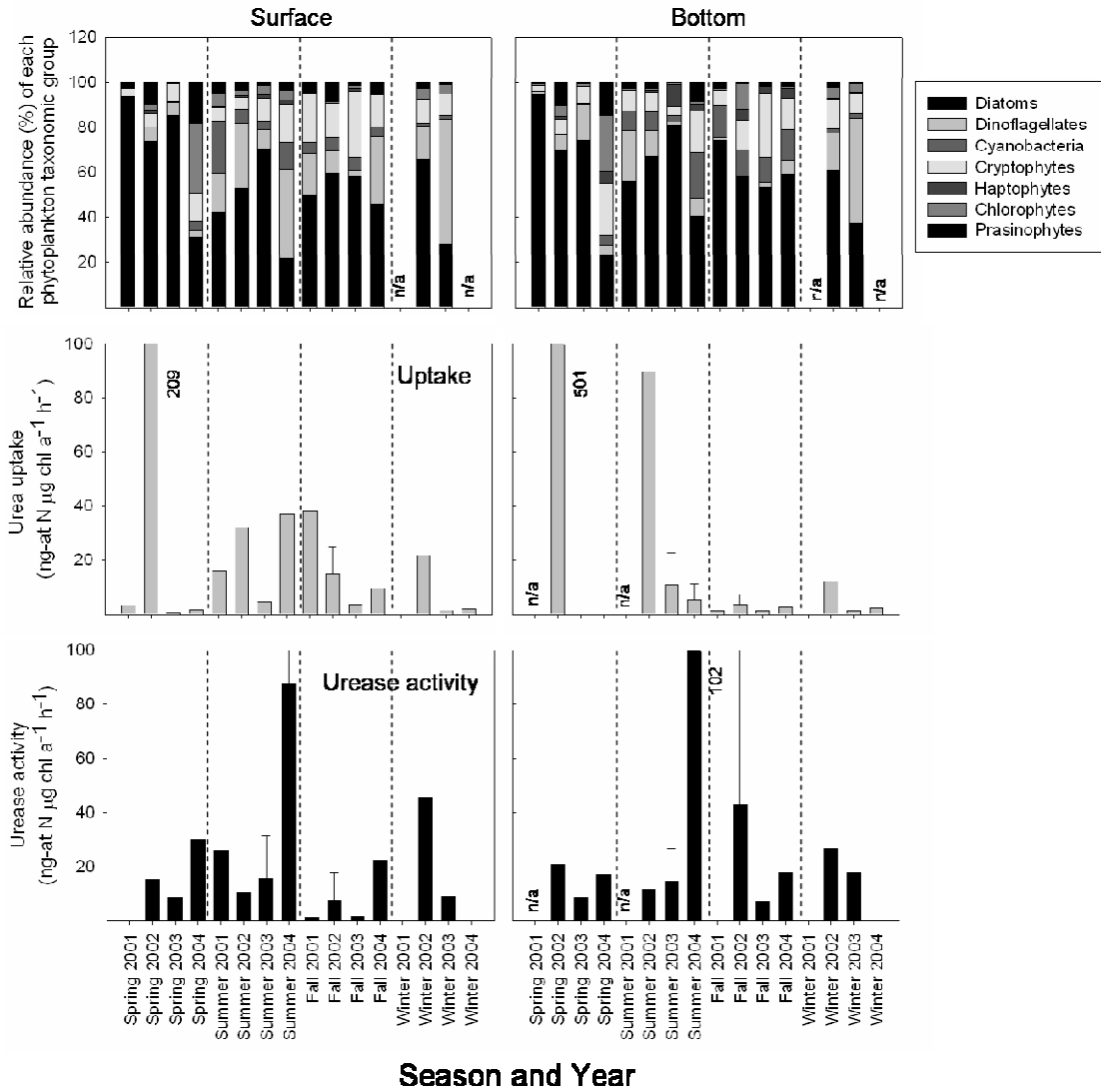
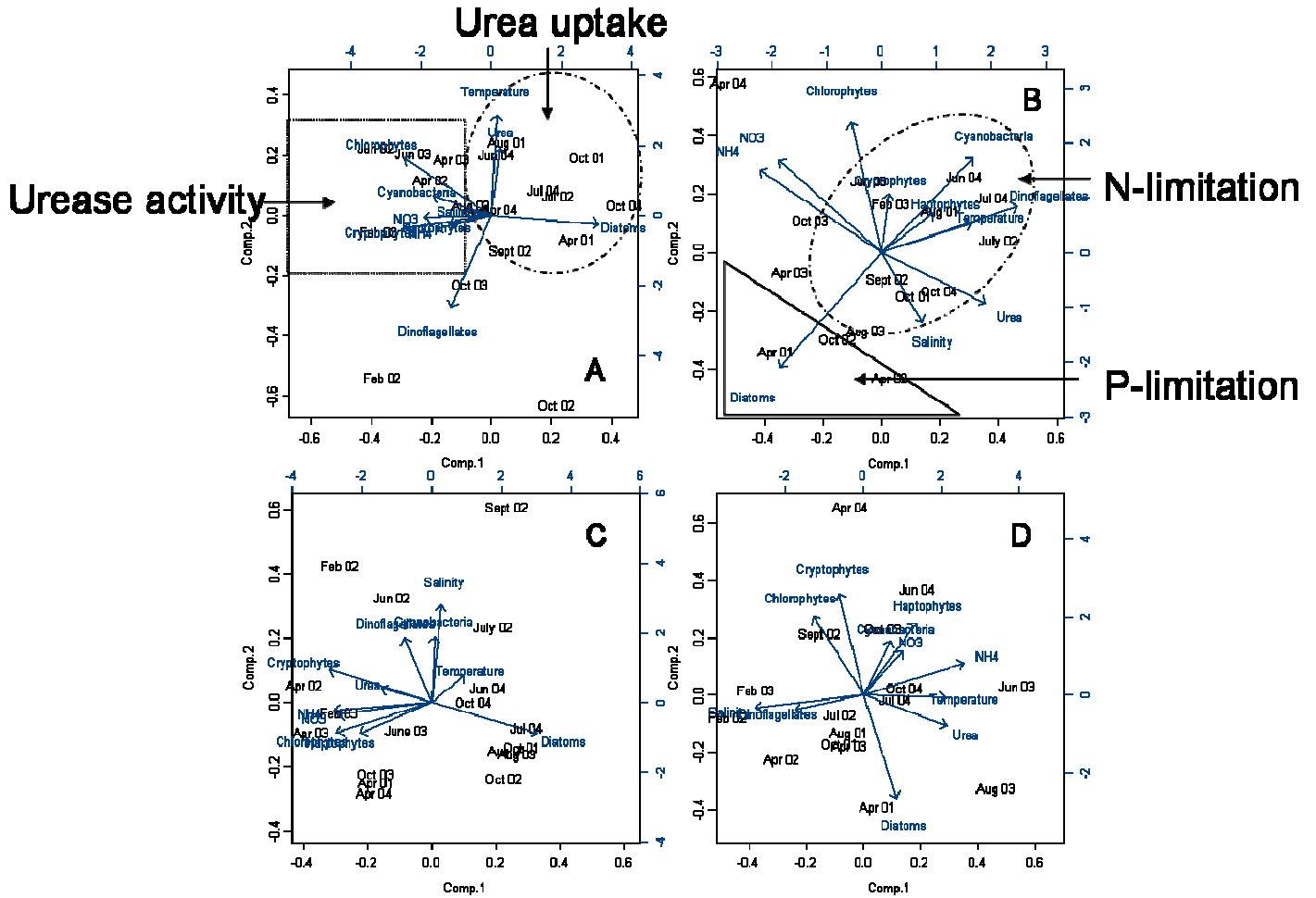


Fig. 5.7 Principal component analysis of temperature, salinity, nitrogen availability and relative abundance of six phytoplankton taxonomic groups at two Choptank River stations from 2001-2004. A) Surface Upper Choptank B) Surface Lower Choptank C) Bottom Upper Choptank D) Bottom Lower Choptank.



Chapter 6: Responses of urea uptake and urease activity to NH_4^+ additions in short-term bioassays

Abstract

Short-term (3h) responses of urea uptake and urease activity to *in vivo* changes in NH_4^+ availability were examined in bioassay experiments conducted on water from the Choptank River, Maryland, four times during the course of one year. Responses by the two processes were dependent on the phytoplankton community composition in the bioassays. Rates of urea uptake were more responsive to changes in NH_4^+ availability in a mixed diatom-cryptophyte than in a dinoflagellate-dominated assemblage, while rates of urease activity were generally not responsive to changes in NH_4^+ availability. The different degree of responses of urea uptake to *in vivo* changes in NH_4^+ availability among different phytoplankton assemblages suggests that influence of NH_4^+ is not similar among various phytoplankton taxonomic groups.

Introduction

Urea, an organic nitrogen substrate, can be readily used by both phytoplankton and bacteria in coastal, estuarine and oceanic regions (Antia et al. 1991, Kirchman 2000, Kudela and Cochlan 2000, Berman and Bronk 2003, Glibert et al. 2006). The ability of different phytoplankton and bacteria species to use urea depends on how two enzymes involved with urea assimilation, urea transport and urease, are regulated by environmental factors such as NH_4^+ availability (Solomon et al. *submitted*).

Enzyme activity is determined by the concentration of its substrate and potential competing or interfering substrates. For instance, urea uptake in estuaries is generally reduced under high ambient NH_4^+ concentrations (Twomey et al. 2005, Solomon et al.

submitted). Expression of urease activity, which directly responds to intracellular nitrogen levels, is not as greatly influenced by ambient NH_4^+ concentrations as are rates of urea uptake (Solomon et al. 2006). The influence of NH_4^+ availability also may be taxon-specific. Diatoms appear to have lower urease activity when grown on NO_3^- than NH_4^+ and urea, while dinoflagellates and cyanobacteria generally have lower urease activity when grown on NH_4^+ than on NO_3^- and urea (Solomon and Glibert *submitted*). Urea uptake and urease activity in both phytoplankton and bacteria may also be induced under N-starvation (Nolden et al. 2000, Dyhrman and Anderson 2003).

These observed rates may be the result of environmental conditions or the plankton community composition and those influences are difficult to separate. One possible way to tease out the differences is by conducting bioassay experiments and exposing different phytoplankton and bacterial assemblages to similar changes in NH_4^+ availability on shorter time scales. From these experiments, a better understanding of whether urea uptake or urease activity is more strongly dependent on environmental conditions, such as NH_4^+ availability, or plankton community composition can emerge.

Methods

Near-surface water was collected from Choptank River, MD (a tributary of Chesapeake Bay) on four occasions (November 2002, March 2003, May 2003 and November 2003). Samples of 10 L were then subjected to changes *in vivo* NH_4^+ concentrations for 3 h. NH_4^+ concentrations were amended by adding NH_4Cl , resulting in final concentrations of approximately 0.5, 1, 5, or 20 $\mu\text{g at-N l}^{-1}$. When initial NH_4^+ concentrations were $>0.5 \mu\text{g at-N l}^{-1}$ no amendments were made for the lowest NH_4^+

concentration treatment. Nutrient samples were taken at the beginning and the end of the bioassay experiments, which were 3 h in duration, by filtering water through precombusted GF/F filters (450°C for 1 h) into acid-washed bottles for later determination in the laboratory. Concentrations of NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} in the filtrates were then determined with a Technicon Autoanalyzer II (Lane et al. 2000) while urea concentrations were determined by the diacetylmonoxime method (Revilla et al. 2005).

Plankton biomass measurements

Samples were also collected before and after the 3h incubation from each bioassay for particulate carbon (PC) and particulate nitrogen (PN), chl *a* and pigment analyses. Particulate matter was collected on precombusted GF/F filters (450°C for 1 h) for all analyses, and then stored in a -20°C or -80°C freezer. Concentrations of PC and PN were analyzed on the filters that had been dried at 50°C with an Exeter Analytical Incorporated CE-440 elemental analyzer. Chlorophyll samples were analyzed by extraction with acetone (Parsons et al. 1984), and then measured on a 10-AU Turner Designs fluorometer. Pigment analyses from the four sampling dates were done according to van Heukelem et al. (1994) and van Heukelem & Thomas (2001) on a Hewlett Packard high-performance liquid chromatograph (HPLC Model 110) system.

Results from pigment analyses were analyzed using the CHEMTAX software program (Mackey et al. 1996) using a matrix calibrated for estuarine phytoplankton (Adolf et al. 2006) that gave the relative abundance of seven phytoplankton taxonomic groups (prasinophytes, dinoflagellates, cryptophytes, haptophytes, chlorophytes, cyanobacteria and diatoms).

Urea uptake and urease activity

For urea uptake rates, water samples (500 ml) were incubated with $0.5 \mu\text{g at-N l}^{-1}$ ^{15}N -urea in acid-washed polycarbonate bottles for 30 min at ambient temperature after the 3h incubation period was complete. Following a 30 min uptake incubation, particulate matter was collected on precombusted GF/F filters (450°C for 1 h), which were then frozen at -20°C, and subsequently dried at 50°C. Dried filters were packed into tin boats then analyzed on a SerCon mass spectrometer using urea as a standard.

Urease activity samples were collected immediately at the end of the 3h bioassay incubation period by collecting particulate matter on GF/F filters which were immediately frozen in liquid nitrogen. Samples collected after March 2003 were analyzed using the method of Solomon et al. (*submitted*) that revised the currently published methods of Peers et al. (2000) and Fan et al. (2003). Prior to March 2003, the methods of Peers et al. (2000) were used and were subsequently corrected by a conversion between the revised and previous methods (Solomon et al. *submitted*).

Statistical analyses of urea uptake and urease activity over a range of NH_4^+ concentrations were conducted for each sampling period (SigmaPlot; SYSTAT, 2004).

Results

General characteristics of the bioassays

The bioassay experiments were conducted during different seasons over the course of one year, which resulted in different NO_3^- concentrations and phytoplankton communities at the beginning of each experiment (Table 6.1). The highest concentration of NO_3^- ($74 \mu\text{g at N l}^{-1}$) occurred during March while the lowest was in November ($1.4 \mu\text{g at N l}^{-1}$).

The concentrations of the other nutrients, NH_4^+ , urea, and PO_4^{3-} , did not vary as much between sampling periods.

The phytoplankton community in November 2002 was dominated by diatoms and cryptophytes (Fig. 6.1). By March 2003, the abundance of diatoms decreased while the abundance of dinoflagellates increased compared to the previous November. The May 2003 phytoplankton community composition was similar to March but with more cryptophytes, haptophytes and diatoms. The November 2003 phytoplankton community was dominated by cryptophytes with minor contributions from dinoflagellates and diatoms.

Influence of NH_4^+ availability

Urea uptake and urease activity both decreased as NH_4^+ concentrations increased in March and November 2003 (Table 6.2). Results were different in May 2003 because urea uptake decreased while urease activity increased over the same range of NH_4^+ concentrations. None of the trends were significant due to few data points ($n=3$, Table 6.2). When all the data were combined ($n=9$) then the significance of the negative relationship between urea uptake and NH_4^+ concentrations improved (Table 6.2). Urea uptake decreased on average by 38% while urease activity slightly increased on average (9%) with increasing NH_4^+ concentrations (Fig. 6.2).

Discussion and Conclusion

Urea uptake responded more to changes in ambient NH_4^+ availability than urease activity. The lesser response by urease was not surprising because urease responds directly to intracellular rather than ambient NH_4^+ concentrations. Urea uptake always

decreased with increasing NH_4^+ concentrations, suggesting repression of urea uptake by this substrate. During all three sampling periods, diatoms were present and repression of urea uptake by NH_4^+ in diatoms has previously been observed (Lund 1987, Molloy and Syrett 1988, Lomas 2004). However, the clearest decrease in urea uptake was observed when cryptophytes were present in addition to diatoms in November 2003. Surprisingly, urea uptake rates did not decrease as much when dinoflagellates were dominant. Lower rates of urea uptake have been observed in mixed field assemblages when ambient NH_4^+ concentrations are higher relative to other areas or time of the year (Kristansen 1983, Twomey et al. 2005, Burford 2005) suggesting that repression of urea uptake by NH_4^+ occurs but at different degrees across many phytoplankton taxonomic groups.

Responses of urea uptake and urease activity to environmental factors such as NH_4^+ availability can be difficult to separate from the composition of the estuarine community. The degree of changes in urea uptake and urease activity in bioassays with different phytoplankton communities that were treated to similar changes in NH_4^+ availability were ultimately dependent on phytoplankton taxonomic differences. Taxonomic-specific responses in urea uptake and urease activity to changes in NH_4^+ availability helps elucidate how urea is utilized in estuarine and marine ecosystems.

Literature cited

- Adolf, J.E., Harding, L.W., Yeager, C.L., Maloney, M.E & Miller, W.D. 2006. Environmental forcing of phytoplankton floral composition, biomass and primary productivity in the Chesapeake Bay, USA. *Estuar. Shelf Coastal Sci.* 67: 108-122.
- Antia, N.J., Harrison, P.J., & Oliveria, L. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia.* 1:1-89.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microb. Ecol.* 31: 279-305.
- Burford, M.A. 2005. Relative uptake of urea and ammonium by dinoflagellates or cyanobacteria in shrimp mesocosms. *Hydrobiologia.* 549: 297-303.
- Dyhrman, S.T., & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Glibert, P.M., Harrison, J., Heil, C. & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry.* 77: 441-463.
- Kirchman, D.L. 2000. Uptake and regeneration of inorganic nutrients by marine heterotrophic bacteria. [Ed. D.L. Kirchman] *In* Microbial Ecology of the Oceans. Wiley Liss, New York. pp. 261-288.
- Kristiansen, S. 1983. Urea as a nitrogen source for the phytoplankton in the Oslofjord. *Mar. Biol.* 74:17-24.
- Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.
- Lane, L., Rhoades, S., Thomas, C., van Heukelem, L. 2000. Analytical Services Laboratory Standard Operating Procedures. Technical Report No. TS-264-00. Horn Point Laboratory, University of Maryland Center for Environmental Science.
- Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae): interactions among nitrogen substrates. *Mar. Biol.* 144: 37-44.

- Lund, B.A. 1987. Mutual interference of ammonium, nitrate, and urea on uptake of ^{15}N sources by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar. Biol. Ecol.* 113:167-180.
- Mackey, M.D., Higgins, H.W., Mackey, D.J. & Wright, S.W. 1996. CHEMTAX - a program for estimating class abundances from chemical markers – application to HPLC measurements of phytoplankton. *Mar. Ecol. Prog. Ser.* 144: 265-283.
- Molloy, C.J. & Syrett, P.J. 1988. Interrelationships between uptake of urea and uptake of ammonium by microalgae. *J. Exp. Mar. Biol. Ecol.* 118:85-95.
- Nolden, L., Beckers, G., Möckel, B., Pfefferle, W., Nampoothiri, K.M., Krämer, R. & Burkovski, A. 2000. Urease of *Corynebacterium glutamicum*: organization of corresponding genes and investigation of activity. *FEMS Microbiology Letters.* 189: 305-310.
- Parsons, T.R., Maita, Y. & Lalli, C.M. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon, New York.
- Peers, G.S., Milligan, A.J. & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.
- Revilla, M., Alexander, J. & Glibert, P.M. 2005. Analysis of urea in coastal waters: comparison of the enzymatic and the direct method. *Limnol. Oceanogr. Methods.* 3: 290-299.
- Solomon, C.M, Alexander, J. & Glibert, P.M. *Submitted.* Measuring urease activity in environmental samples. *Limnol. Oceanogr. Methods.*
- Solomon, C.M. Collier, J.L., Alexander, J. & Glibert, P.M. *Submitted.* Microbial urea uptake and urease activity in Chesapeake Bay, USA. *Limnol. Oceanogr.*
- Solomon, C.M & Glibert, P.M. *Submitted.* Urease activity in five phytoplankton species. *J. Phycol.*
- Solomon, C.M., Glibert, P.M. & Collier, J.L. 2006. Potential regulation of urea utilization by nitrogen sources: a synthesis from laboratory and field experiments. American Society of Limnology and Oceanography Summer Meeting. Victoria, B.C. June 2006.
- Twomey, L.J., Piehler, M.F. & Paerl, H.W. 2005. Phytoplankton uptake of ammonium, nitrate, and urea in the Neuse River Estuary, NC, USA. *Hydrobiologia.* 533: 123-134.
- van Heukelem, L., Lewitus, A., Kana, T.M., Craft, N. 1994. Improved separation of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Mar. Ecol. Prog. Ser.* 114: 303-313.

van Heukelem, L. & Thomas, C.S. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chromatogr A*. 910:31-49.

Tables

Table 6.1 Ambient concentrations of NO_3^- , NH_4^+ , urea, and PO_4^{3-} for the bioassay experiments.

Month and year	NO_3^- ($\mu\text{g at N l}^{-1}$)	NH_4^+ ($\mu\text{g at N l}^{-1}$)	Urea ($\mu\text{g at N l}^{-1}$)	PO_4^{3-} ($\mu\text{g at P l}^{-1}$)
November 2002	1.38	3.14	0.38	0.39
March 2003	74.3	1.90	0.47	0.24
May 2003	23.5	2.22	0.48	0.10
November 2003	9.02*	1.77	0	0.04

* $[\text{NO}_3^- + \text{NO}_2^-]$

Table 6.2 Regression statistics for relationships between urea uptake and urease activity and NH_4^+ concentrations during three months in 2003. Note: All sampling dates have at least n=3 (control + treatments).

Month	Uptake				Urease			
	slope	y-intercept	r ²	p	slope	y-intercept	r ²	p
March 2003	-0.12	2.24	0.91	0.19	-0.08	1.88	0.38	0.58
May 2003	-0.08	1.88	0.38	0.58	0.07	5.40	0.03	0.90
November 2003	-0.61	13.2	0.95	0.15	-0.05	1.48	0.94	0.16
Overall	-0.38	7.09	0.28	0.14	0.09	2.85	0.03	0.66

Figures

Fig. 6.1 Average relative contribution of each phytoplankton taxon to the total phytoplankton assemblage as analyzed by CHEMTAX in the bioassays during November 2002, March 2003, May 2003, and November 2003.

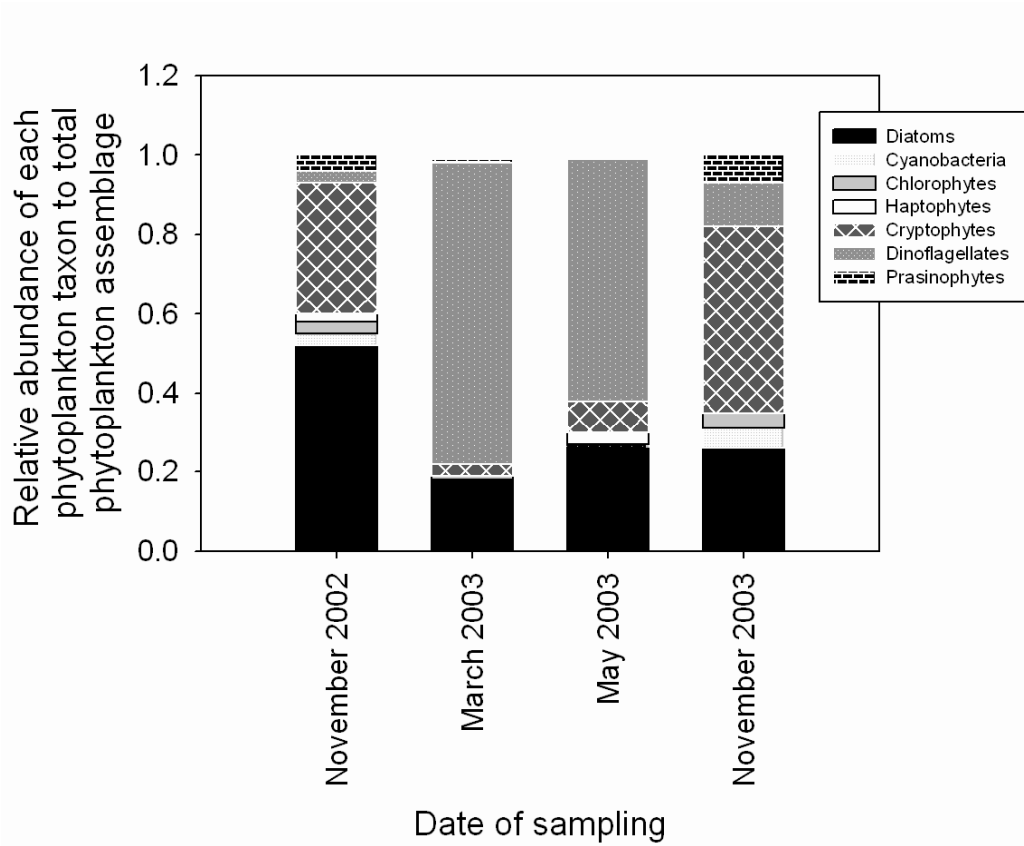
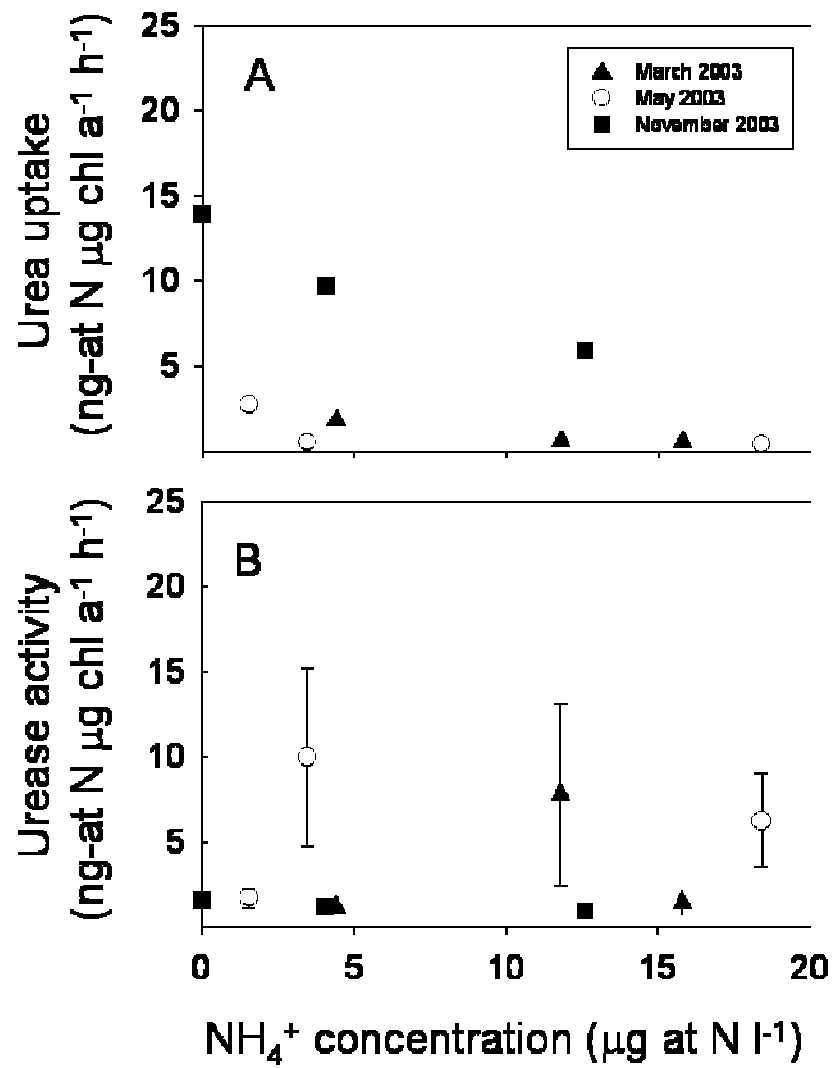


Fig. 6.2 A) Urea uptake and B) urease activity on a per chl *a* basis as function of NH_4^+ concentrations.



Chapter 7: Urea uptake and urease activity under manipulated phosphorus and nitrogen conditions: a mesocosm study

Abstract

The response of urea uptake and urease activity to manipulation of phosphorus and nitrogen sources was studied in a three week mesocosm experiment. Mesocosms first received PO_4^{3-} then N additions (NO_3^- , NH_4^+ , or urea) several days later. Neither the plankton community composition nor rates of enzymatic activity differed among N treatments, but there were significant changes during the course of the experiment. The mesocosms were initially P-limited, then after the addition of PO_4^{3-} became N-limited. During the period of N-limitation, a mixed bloom of diatoms and dinoflagellates occurred. Urea uptake and urease activity, normalized on a volumetric basis, was the highest during this period. Five days after the addition of N, the mesocosms became P-limited again. Cyanobacteria appeared and urea uptake and urease activity, normalized on a per chl *a* basis, began to increase. This study supports previous work suggesting that dinoflagellates and cyanobacteria are important consumers of urea under both N- and P-limitation.

Introduction

Urea transporters and urease, enzymes involved in urea assimilation, in different phytoplankton and bacteria species may be regulated differently by nitrogen substrates. Most of our current understanding of regulation of urea uptake and urease activity by nitrogen substrates is based on cultures grown on NO_3^- , NH_4^+ or urea (Fan et al. 2003, Dyrman and Anderson 2003, Solomon and Glibert *submitted*) or field studies (Dyrman and Anderson 2003, Glibert et al. 2004, Heil et al. *in press*, Solomon et al. *submitted*). Further understanding of urea utilization may be obtained from ‘challenging’ the

phytoplankton and bacteria community *in vivo* with rapid changes in nutrient concentrations.

Previous work has shown that urea uptake may be repressed or inhibited by NO_3^- and/or NH_4^+ additions in phytoplankton cultures or field incubations (Lund 1987, Tamminen and Irmisch 1996). In the diatom, *Skeletonema costatum*, Lund (1987) observed an 82-84% decrease in urea uptake 3 h after the addition of either NO_3^- or NH_4^+ or both. Tamminen and Irmisch (1996) added NH_4^+ , NO_3^- , and glucose to field incubations of Baltic seawater during mid-summer. Urea turnover rates did not respond immediately to the nutrient additions, but after 24 h there was a clear inhibition of urea turnover by the NH_4^+ additions. However, addition of PO_4^{3-} removed any inhibitory effect of NH_4^+ . There have been few other studies in which the effects of added PO_4^{3-} on urea uptake or urease activities have been examined.

Only one study reported changes in *both* urea uptake and urease activity after ‘challenging’ a culture grown on one nitrogen substrate by adding a different substrate. In cultures of the diatom, *Thalassiosira weissflogii*, diel patterns in urea uptake were not immediately influenced by the additions of NO_3^- and NH_4^+ within hours, but urea uptake rates were lower when grown on NO_3^- than NH_4^+ and urea (Lomas 2004). A larger decrease in urease activity took place within 30 min after addition of NH_4^+ than NO_3^- to the *T. weissflogii* cultures. These studies suggest that urea uptake and urease activity may be down-regulated by ambient NO_3^- and NH_4^+ , but many questions remain.

The goal of this study was to examine how urea uptake and urease activity respond to additions of NO_3^- , NH_4^+ , urea and/or PO_4^{3-} in a long term mesocosm experiment (~3 weeks). It is unclear whether urea uptake and urease activity are

regulated similarly by NO_3^- , NH_4^+ or urea availability or nitrogen status in phytoplankton and bacteria. The mesocosm experiment was designed to expose phytoplankton and bacteria to different conditions to allow observation for changes in urea uptake and urease activity to better understand how these two enzymes are regulated by nitrogen or phosphorus status and sources.

Methods

During May and June 2004 for 19 days, eight mesocosms (1 m³ volume) (labeled C2-C9) were established in the Multiscale Experimental Ecosystem Research Center (MEERC) facility at University of Maryland Center for Environmental Science, Horn Point Laboratory (Berg et al. 1999, Petersen et al. 2003). Mesocosms were filled with water from Choptank River, MD which was filtered through a 2 µm mesh filter before entering the mesocosms, but there was some evidence of introduction of >2 µm cells because copepods were later observed in the mesocosms. Light above the mesocosms was provided by fluorescent bulbs at between 365-382 µmol photons m⁻² s⁻¹ at the water surface on a 12:12 light:dark cycle. The mesocosms were well mixed with paddles that rotated in cycles of 4h on/2h off and the walls scrubbed frequently. Temperature was maintained between 24 and 26°C while salinity remained at 8.2 during the course of the three week experiment.

Concentrations of N and P in the mesocosms were manipulated during the three weeks of the experiment. On Day 3, NaH_2PO_4 was added to all eight mesocosms, resulting in a final concentration of 2 µg at-P l⁻¹. On Day 8, N additions were made to six mesocosms, leaving two as controls. Of the six N-enriched mesocosms, each form of

N (NaNO_3 , NH_4Cl , or urea; final concentration $32 \mu\text{g at-N l}^{-1}$) was added to two mesocosms. No further nutrient additions were made during the rest of the experiment (Table 7.1).

Nutrient measurements

On a daily basis, 10 L samples were removed from each mesocosm 1 h after the lights came on and were subsequently used in various chemical or experimental assays. Some of the sample was filtered through combusted GF/F filters (450°C for 1 h) into acid-washed bottles for later determination of nutrients in the laboratory. The particulate matter collected on the filters was dried for later determination of particulate carbon (PC) and particulate nitrogen (PN) on an Exeter Analytical Incorporated CE-440 elemental analyzer. Concentrations of NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} in the filtrates were determined with a Technicon Autoanalyzer II (Lane et al. 2000) while concentrations of urea were determined by the diacetylmonoxime method (Revilla et al. 2005). Total dissolved nitrogen (TDN) concentrations were determined by persulfate oxidation (Bronk et al. 2000) and total dissolved phosphorus (TDP) was measured by the method of Solórzano and Sharp (1980). Dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) concentrations were determined by subtracting inorganic nitrogen from TDN, and PO_4^{3-} from TDP.

Plankton biomass measurements

Particulate matter was also collected on combusted GF/F filters for both chl *a* and pigment analyses, and the filters were stored in a -80°C freezer until analyzed.

Chlorophyll samples were extracted with acetone (Parsons et al. 1984), and then measured on a 10-AU Turner Designs fluorometer. Pigment analyses on Days 5, 9 and 17 (Table 7.1) were done according to Van Heukelem et al. (1994) and Van Heukelem & Thomas (2001) on a Hewlett Packard high-performance liquid chromatograph (HPLC; Model 110) system. Relative contributions of each of seven phytoplankton taxonomic groups (prasinophytes, dinoflagellates, cryptophytes, haptophytes, chlorophytes, cyanobacteria, and diatoms) to the total phytoplankton assemblage were determined by CHEMTAX analysis using HPLC results (Mackey et al. 1996, Adolf et al. 2006). Phytoplankton samples were preserved in both Lugol's solution and 4% glutaraldehyde to compare with results obtained from CHEMTAX analyses.

Urea uptake and urease activity measurements

Rates of urea uptake were determined using ^{15}N tracer techniques on Days 5, 9, and 17 (Glibert and Capone, 1993). Concurrently, the rate of urea assimilated into the protein fraction was measured using trichloroacetic acid (TCA) (Wheeler et al. 1982, Glibert and McCarthy 1984, Lomas and Glibert 1999). Sample volumes of 500 ml were incubated with $0.5 \mu\text{g at-N l}^{-1} \text{ }^{15}\text{N}$ -urea in acid-washed polycarbonate bottles for 30 min at ambient temperature by floating the bottles in a mesocosm filled separately for this purpose. At intervals during the incubation period (5, 10, 15, 30 min and 1 h), two samples were collected. The first sample, which represented the ^{15}N pool in the whole cell, was immediately filtered then frozen. Uptake rates were calculated using this sample. The second sample was filtered until about 5-10 ml remained in the filtering tower. Approximately 25 ml of cold 5% TCA was added to the tower and incubated

between 30 sec to 1 min, and then the remaining volume was filtered. Once filtration was complete, the filter was then frozen. This sample represented the protein pool and was used to calculate assimilation rates. After three weeks in the freezer, all filters were dried at 50°C, packed into tin boats, and then analyzed on a SerCon mass spectrometer using urea as a standard.

Activity of the enzyme urease was also measured on 9 days (Days 1, 3, 4, 6, 8, 9, 10, 13, 16 and 19) during the experiment using the method of Solomon et al. (*submitted*) (Table 7.1).

Genetic diversity

In order to assess genetic diversity during the course of the experiment, 2L of water from each of the eight mesocosms was incubated with 0.67 $\mu\text{g at-N l}^{-1}$ or 8 $\mu\text{g at-N l}^{-1}$ ^{15}N -urea on Days 1, 8, and 16 (Table 7.1). After the three day incubation period was complete, samples were then filtered into 0.2 μm Sterivex filters using a peristaltic pump then flash-frozen in liquid nitrogen, and transferred to a -80°C freezer. DNA was extracted from the Sterivex filters using the protocol of Tillett and Neilan (2000).

PCR amplification was carried out to generate products for DGGE (denaturing gradient gel electrophoresis). PCR amplification of prokaryotic DNA used 341fGC (GC clamped) and 519r primers, while for eukaryotic DNA used 980fGC (GC clamped) and 1200r (Gast et al. 2004). The amplification with a BioRad iCycler began at 65°C for 2 cycles (45s at 95°C, 45s at 65°C, 45s at 72°C) then followed with a decrease in annealing temperature by 2°C every two cycles until 55°C. Twenty five cycles were then carried

out at 55°C annealing temperature (45s at 95°C, 45s at 65°C, 45s at 72°C) (Gast et al. 2004).

DGGE gels were run with a denaturing gradient of 35-60% urea for prokaryotic diversity and 25-50% urea for eukaryotic diversity at 65°C at 75 V for 24 h using a DGGE gel apparatus (CBS Scientific). Band patterns were analyzed using GelCompar II (Applied Maths).

Statistical analyses

Changes in floral composition

Statistical analyses were conducted on CHEMTAX data to determine whether if any significant shifts occurred in phytoplankton composition after nutrient additions in all the mesocosms during the experiment. A multivariate ANOVA (MANOVA) was first run to determine whether there were any overall significant changes in relative contribution of each phytoplankton taxonomic group among nutrient treatments or during the course of the three week experiment. Next, individual ANOVAs were conducted to see how the relative contributions of each phytoplankton taxonomic group varied with fixed factors such as nitrogen treatment and day of experiment.

Urea uptake and urease activity

Rates of urea uptake and urease activity were analyzed with a two-way ANOVA using N treatment and day as main factors. The original urease data was normal but skewed, so the data was transformed using the log (x+1) function. Multiple comparisons between N treatments and days were done using pre-planned contrasts (Tukey-HSD).

DGGE bands

Similarity in DGGE patterns between mesocosms was evaluated using a presence/absence data set produced by GelCompar II. Bands that had a pixel density of at least 5% of the densest band in the sample were scored as present in the sample. A pairwise distance matrix (Dice; $S_d = 2a/(b+c)$ where a is the number of shared bands, b and c are the number of bands in each sample) was calculated using the presence/absence data by the multidimensional scaling (MDS) module of the SPSS software package (SPSS, Inc.). The MDS analysis resulted in a graphical representation in which the degree of similarity among mesocosms could be visualized. Mesocosms that contained many of the same bands were plotted in close proximity to each other.

Results

Nutrient concentrations in all the mesocosms varied somewhat at the beginning of the experiment due to the length of the time required to fill the mesocosms and the variable standing time before the experiment was begun ($\text{NO}_3^- = 33\text{-}39 \mu\text{g-at N l}^{-1}$; $\text{NH}_4^+ = 4.95\text{-}7.03 \mu\text{g-at N l}^{-1}$; urea = 1.24-9.88 $\mu\text{g-at N l}^{-1}$, $\text{PO}_4^{3-} = 0.52\text{-}1.89 \mu\text{g-at P l}^{-1}$) (Fig. 7.1). NH_4^+ and urea were rapidly drawn down to $<1 \mu\text{g-at N l}^{-1}$ when PO_4^{3-} was added on Day 3. By Day 4, PO_4^{3-} concentrations in the mesocosms were drawn down to between 0.13 to 1.22 $\mu\text{g at P l}^{-1}$, approximately the initial conditions (data not shown). On Day 8, concentrations of nitrogen in the NO_3^- , NH_4^+ and urea treatments increased to 23-27 $\mu\text{g at N l}^{-1}$ after addition of those substrates. Following the N addition in the NO_3^- treatment, levels of NH_4^+ increased almost immediately while in the NH_4^+ and urea treatments, NO_3^- concentrations increased beginning on Day 11 (Fig. 7.1).

DIN:DIP and DON:DOP ratios varied significantly over the course of the experiment (Fig. 7.1). Before the addition of PO_4^{3-} on Day 4, the average DIN:DIP ratios in the mesocosms were >200 , suggesting strong inorganic P-limitation. Once PO_4^{3-} was added, DIN:DIP ratios were reduced to <65 . On Day 8 after N was added, DIN:DIP in the NO_3^- , NH_4^+ and urea mesocosms once again indicated P-limitation (>250). The control mesocosms maintained DIN:DIP ratios at <20 . Towards the end of the experiment (Day 13), DIN:DIP ratios increased further and reached >700 in the mesocosms that received urea additions on Day 19. DON:DOP ratios often followed the same trends as DIN:DIP ratios but were frequently lower (Fig. 7.1). DON:DOP ratios were <100 except during the third period in control and urea mesocosms, and the first period in NO_3^- treatments. DON:DOP was also high during the fourth period in the urea mesocosms.

The planktonic community composition and biomass changed during the course of the experiment, but did not differ among N treatments (MANOVA; $p < 0.05$ for day). Of the seven phytoplankton groups, there were significant changes in relative contributions over the course of the experiment by diatoms, dinoflagellates, and cyanobacteria (ANOVA, $p < 0.05$). There were almost no prasinophytes or haptophytes in the mesocosms, while the relative contribution of cryptophytes and chlorophytes did not significantly change as the experiment progressed. Chl *a* biomass began to increase on Day 3 and consisted of mostly diatoms (Fig. 7.2). By Day 6, chl *a* peaked with an average biomass of $101 \pm 5.60 \mu\text{g chl } a \text{ l}^{-1}$ and was composed of a bloom of diatoms and some dinoflagellates (mostly *Prorocentrum minimum*). By Day 17, chl *a* biomass declined to $<2 \mu\text{g chl } a \text{ l}^{-1}$ and was dominated by diatoms and cyanobacteria.

The sequential changes in the planktonic community and similarity among mesocosms were confirmed by MDS analyses of DGGE bands of prokaryotes and eukaryotes (Fig. 7.3). While the plankton community throughout the experiment may have included some of the same species, the overall genetic composition of the community on each day (Days 1, 8 and 16) was distinct with very little overlap. The similarity of genetic prokaryotic diversity between replicate N treatments was the greatest on Day 16 and the least on Day 1, while for genetic eukaryotic diversity the opposite was true (Fig. 7.3).

Rates of urea uptake and urease activity on a volumetric basis ($\mu\text{g at N l}^{-1} \text{ h}^{-1}$) changed similarly across all N treatments, including the control (Fig. 7.4). Rates of urea uptake were $<0.4 \mu\text{g at N l}^{-1} \text{ h}^{-1}$ for all mesocosms on the days tested. In contrast, rates of urease activity increased to $>1.0 \mu\text{g at N l}^{-1} \text{ h}^{-1}$ on Day 6, and were significantly higher on Days 3, 4, 6 and 8 than during the other days of the experiment (ANOVA, Tukey-HSD, $p<0.05$).

When normalized on a per chl *a* basis ($\text{ng at N } \mu\text{g chl } a^{-1} \text{ h}^{-1}$), a different pattern emerged (Fig. 7.4). Urea uptake was not significantly different between Day 5 (prior to N addition) and 9 (after N addition) but was significantly higher on Day 17 (ANOVA, Tukey-HSD, $p<0.05$). Urease activity was significantly lower on Day 6 (prior to N addition) than Day 9 (after N addition) but did not differ among N treatments over the course of the experiment (ANOVA, $p<0.01$ for day, and $p=0.96$ for mesocosms). Towards the end of the experiment, urease activity was significantly higher than prior to N addition, especially after Day 16 (ANOVA, Tukey-HSD, $p<0.05$).

The rate of urea assimilation into protein was faster on Days 5 and 9 than Day 17 (Fig. 7.5). On both Days 5 and 9, uptake rates, on average, were between 0.18-0.23 μg at N l^{-1} with 60-80% of urea incorporated into protein within the first 15 min. In contrast, average uptake rates were lower on Day 17 (0.13 μg at N l^{-1}) with <50% of urea incorporated into protein within the first 15 min. On each day, urea uptake and assimilation rates were similar among all treatments.

Discussion

The mesocosm experiment can be divided into four stages based on plankton succession and type of nutrient limitation (I-IV; Fig. 7.6). Stage I (Days 1-2) was the initial P-limited condition prior to any nutrient manipulation. The eukaryotic plankton community consisted of mostly diatoms and was very genetically similar among the mesocosms. Stage II (Days 3-7) began after PO_4^{3-} was added to all the mesocosms, resulting in N-limitation. During this period, a bloom of diatoms and some dinoflagellates occurred. Stage III (Days 8-14) occurred during the end of the bloom when N was added to the mesocosms. P-limitation was again observed in the plankton community that was more genetically diverse among the N treatments than during Stage I (eukaryotes) or Stage IV (prokaryotes). Stage IV (>day 15) was a period of severe P-limitation with DIN:DIP and DON:DOP ratios >400 and ~100, respectively. Phytoplankton biomass was low (<2 $\mu\text{g chl } a \text{ l}^{-1}$) and was dominated by both diatoms and cyanobacteria. During this stage, prokaryotic genetic diversity was the lower while the eukaryotic genetic diversity was greater than during the rest of the experiment.

The highest urea uptake and urease activity on a volumetric basis were observed during Stage II (N-limitation) while highest rates on a per chl *a* basis were observed during Stage IV (severe P-limitation). During Stage II, urea uptake and urease activity (on a volumetric basis) increased concurrently with chl *a* biomass when the plankton experienced N-limitation. The prokaryotic and eukaryotic community was similar among mesocosms during this period and consisted of some dinoflagellates which in the past have been shown to have high rates of urea uptake and urease activity on a per cell basis (Glibert et al. 2006, Solomon et al. 2006). Dinoflagellates such as *Prorocentrum minimum* and *Karlodinium veneficum* have higher urea uptake and urease activity on a per cell basis than diatoms, pelagophytes, cryptophytes and haptophytes (Fan et al. 2003, Glibert 2006, Solomon et al. 2006, Solomon and Glibert *submitted*). In this community with some dinoflagellates, urea was rapidly incorporated into protein 15 min after addition of N.

Later during the experiment during Stage IV, urea uptake and urease activity (on a per chl *a* basis) began to increase under severe P-limitation with mostly diatoms and cyanobacteria present. The genetic diversity of prokaryotes during this time was less similar than during other stages, suggesting that same assemblage of bacteria or cyanobacteria may have been present in all the mesocosms. High rates of urea uptake have previously been associated with cyanobacteria in other areas such as Lake Kinneret, Florida Bay, and the western Florida Shelf, as well as urease activity (Berman 1997, Berman and Bronk 2003, Glibert et al. 2004, Heil et al. *in press*, Solomon et al. 2006). Despite P-limitation, the total concentration of N was also low. Under these circumstances, urea was assimilated more slowly into protein.

N- vs. P-limitation

The mesocosm experiment originally set out to investigate whether urea uptake and urease activity was regulated by nitrogen sources. Instead, we found that the mesocosms had similar planktonic communities that responded to different stages of N- and P-limitation over time. Urea uptake and urease activity rates, normalized on a volumetric basis, were the highest during N-limitation. Both rates of urea uptake and urease activity have previously been observed to increase in both phytoplankton and bacteria under N-limitation. Urea uptake in some bacteria is mediated by ATP binding cassette (ABC)-type transporters that are transcribed under N-starvation (Beckers et al. 2004) and transcription can be inhibited by the addition of NH_4^+ , glutamine or glutamate (Siewe et al. 1998). Like urea uptake, urease activity in bacteria and cyanobacteria increase under nitrogen starvation and lower rates are observed when grown on NH_4^+ than urea (Ge et al. 1990, Collier et al. 1999, Nolden et al. 2000).

In contrast, not much is understood about how urea uptake or urease activity responds to P-limitation. Urease is not an energy-dependent enzyme, so does not require ATP or other energy sources. However, urea uptake in bacteria is an ABC-type uptake system that is linked with proton motive forces (Siewe et al. 1998, Beckers et al. 2004). Urea uptake at low concentrations depends on the membrane potential which can be disrupted by the addition of CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) in both bacteria (Siewe et al. 1998, Beckers et al. 2004) and diatoms (Rees and Syrett 1979). The membrane potential is produced by oxidative phosphorylation which requires PO_4^{3-} , thus the addition of P (as PO_4^{3-}) may help facilitate urea uptake. Due to the need for

PO_4^{3-} , urea uptake may be repressed under P-limitation. Supporting this reasoning, urea uptake rates (on a volumetric basis) were low during stages of P-limitation (stages I, III, IV). Also, urea was assimilated into protein more slowly during this period.

Conclusion

Despite additions of different nitrogen substrates, the mesocosms did not differ in plankton community composition nor urea uptake or urease activity rates. However, there was a succession of phytoplankton and nitrogen status throughout the mesocosm experiment which allowed for comparison of urea uptake and urease activity among different photosynthetic taxonomic groups under N- and P-limitation. Urea uptake and urease activity, normalized on a volumetric basis, was the highest when dinoflagellates increased in the mesocosms during N-limitation, during which chl *a* biomass was the highest. Later during the experiment when mesocosms were P-limited, urea uptake and urease activity when normalized on a per chl *a* basis was the highest when cyanobacteria had an increased presence in the mesocosms. This study supports previous work (Solomon and Glibert *submitted*, Solomon et al. 2006) that urea is utilized at higher rates in select groups of phytoplankton and bacteria.

Literature cited

- Adolf, J.E., Harding, L.W., Yeager, C.L., Maloney, M.E & Miller, W.D. 2006. Environmental forcing of phytoplankton floral composition, biomass and primary productivity in the Chesapeake Bay, USA. *Estuar. Shelf Coastal Sci.* 67: 108-122.
- Beckers, G., Bendt, A.K., Krämer, R. & Burkovski, A. 2004. Molecular identification of the urea uptake system and transcriptional analysis of urea-uptake- and urease-encoding genes in *Corynebacterium glutamicum*. *J. Bacteriol.* 186: 7645-7652.
- Berman, T. 1997. Dissolved organic nitrogen utilization by an *Aphanizomenon* bloom in Lake Kinneret. *J. Plank. Res.* 19: 577-586.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microb. Ecol.* 31: 279-305.
- Berg, G.M., Glibert, P.M. & Chen, C. 1999. Dimensional effects of enclosures on ecological processes in pelagic systems. *Limnol. Oceanogr.* 44: 1331-1340.
- Bronk, D.A., Lomas, M.W., Glibert, P.M., Schukert, K.J. & Sanderson, M.P. 2000. Total dissolved nitrogen analysis: comparisons between persulfate, UV, and high temperature oxidation methods. *Mar. Chem.* 69: 163-178.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiol.* 145: 447-459.
- Dyhrman, S.T., & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Gast, R. J., Dennett, M.R. & Caron, D.A. 2004. Characterization of protistan assemblages in the Ross Sea, Antarctica, by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70: 2028-2037.
- Ge, X., Cain, K. & Hirschberg, R. 1990. Urea metabolism and urease regulation in the cyanobacterium *Anabaena variabilis*. *Can. J. Microbiol.* 36: 218-222.
- Glibert, P.M. & Capone, D.G. 1993. Mineralization and assimilation in aquatic, sediment, and wetland systems, p. 243-272. In R. Knowles and T.H. Blackburn [eds], Nitrogen isotope techniques. Academic Press.

- Glibert, P.M., Conley, D.J., Fisher, T.R., Harding, L.W. & Malone, T.C. 1995. Dynamics of the 1990 winter/spring bloom in Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 122:27-43.
- Glibert, P.M., Harrison, J., Heil, C.A. & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry*. 77: 441-463.
- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Glibert, P.M. & McCarthy, J.J. 1984. Uptake and assimilation of ammonium and nitrate by phytoplankton: indices of nutritional status for natural assemblages. *J. Plank. Res.* 6: 677-697.
- Heil, C.A., M. Revilla, P.M. Glibert & S. Murasko. *In press*. Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Lane, L., Rhoades, S., Thomas, C., & van Heukelem, L. 2000. Analytical Services Laboratory Standard Operating Procedures. Technical Report No. TS-264-00. Horn Point Laboratory, University of Maryland Center for Environmental Science.
- Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (*Bacillariophyceae*): Interactions between nitrogen substrates. *Mar. Biol.* 144: 37-44.
- Lomas, M.W. & Glibert, P.M. 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 556-572.
- Lund, B.A. 1987. Mutual interference of ammonium, nitrate, and urea on uptake of ¹⁵N sources by the marine diatom, *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar. Biol. Ecol.* 113: 167-180.
- Mackey, M.D., Higgins, H.W., Mackey, D.J. & Wright, S.W. 1996. CHEMTAX - a program for estimating class abundances from chemical markers – application to HPLC measurements of phytoplankton. *Mar. Ecol. Prog. Ser.* 144: 265-283.
- Nolden, L., Beckers, G., Möckel, B., Pfefferle, W., Nampoothiri, K.M., Krämer, R. & Burkovski, A. 2000. Urease of *Corynebacterium glutamicum*: organization of corresponding genes and investigation of activity. *FEMS Microbiology Letters*. 189: 305-310.

- Parsons, T.R., Maita, Y. & Lalli, C.M. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon, New York.
- Petersen, J.E., Kemp, W.M., Bartleson, R., Boynton, W.R., Chen, .CC., Cornwell, J.C., Gardner, R.H., Hinkle, D.C., Houde, E.D., Malone, T.C., Mowitt, W.P., Murray, L., Sanford, L.P., Stevenson, J.C., Sundberg, K.L., Suttles, S.E. 2003. Multiscale experiments in coastal ecology: Improving realism and advancing theory. *Bioscience*. 53: 1181-1197.
- Rees, T.A. & Syrett, P.J. 1979. The uptake of urea by the diatom, *Phaeodactylum*. *New Phytol.* 82: 169-178.
- Revilla, M., Alexander, J. & Glibert, P.M. 2005. Analysis of urea in coastal waters: comparison of the enzymatic and the direct method. *Limnol. Oceanogr. Methods*. 3: 290-299.
- Siewe, R.M., Weil, B., Burkovski, A., Eggeling, L., Krämer, R. & Jahns, T. 1998. Urea uptake and urease activity in *Corynebacterium glutamicum*. *Arch. Microbiol.* 169: 411-416.
- Solomon, C.M. Alexander, J., Collier, J.L. & Glibert, P.M. *Submitted*. Microbial urea uptake and urease activity in Chesapeake Bay, USA. *Limnol. Oceanogr.*
- Solomon, C.M, Alexander, J. & Glibert, P.M. *Submitted*. Measuring urease activity in environmental samples. *Limnol. Oceanogr. Methods*.
- Solomon, C.M & Glibert, P.M. *Submitted*. Urease activity in five phytoplankton species. *J. Phycol.*
- Solomon, C.M., Glibert, P.M. & Collier, J.L. 2006. Potential regulation of urea utilization by nitrogen sources: a synthesis from laboratory and field experiments. American Society of Limnology and Oceanography Summer Meeting. Victoria, B.C.
- Soloranzo, L. & Sharp, J.H. 1980. Determination of the total dissolved phosphorus and particulate phosphorus in natural waters. *Limnol. Oceanogr.* 25: 754-752.
- Tamminen, T. & Irmisch, A. 1996. Urea uptake kinetics of a midsummer planktonic community on the SW coast of Finland. *Mar. Ecol. Prog. Ser.* 130: 201-211.
- Tillett, D. & Neilan, B.A. 2000. Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. *J. Phycol.* 36: 251-258.
- van Heukelem, L., Lewitus, A., Kana, T.M. & Craft, N. 1994. Improved separation of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Mar. Ecol. Prog. Ser.* 114: 303-313.

van Heukelem, L. & Thomas, C.S. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chromatogr A* 910:31-49.

Wheeler, P.A., Glibert, P.M & McCarthy, J.J. 1982. Ammonium uptake and incorporation by Chesapeake Bay phytoplankton: short term uptake kinetics. *Limnol. Oceanogr.* 27: 1113-1128.

Tables

Table 7.1: Sampling dates for nutrient additions, urea uptake, urease activity, and genetic diversity in the mesocosm experiment.

Date	Day	Nutrient additions to mesocosms	HPLC	Urea uptake (TCA)	Urease activity	Genetic diversity
24 May	1				X	Incubation 1
25 May	2					
26 May	3	PO ₄ ³⁻			X	
27 May	4				X	Collected 1
28 May	5		X	X		
29 May	6				X	
30 May	7					
31 May	8	NO ₃ ⁻ , NH ₄ ⁺ , Urea			X	Incubation 2
1 June	9		X	X	X	
2 June	10				X	
3 June	11					Collected 2
4 June	12					
5 June	13				X	
6 June	14					
7 June	15					
8 June	16				X	Incubation 3
9 June	17		X	X		
10 June	18					
11 June	19				X	Collected 3

Figures

Fig. 7.1 *Left panels:* Nitrogen concentrations (NO_3^- , NH_4^+ and urea) in all N treatments as a function of day of the experiment. Data points represent the average of two mesocosms for each N treatment. *Right panels:* Average DIN:DIP and DON:DOP ratios over four periods. The vertical lines represent when PO_4^{3-} (dashed line), or N was added (dotted line).

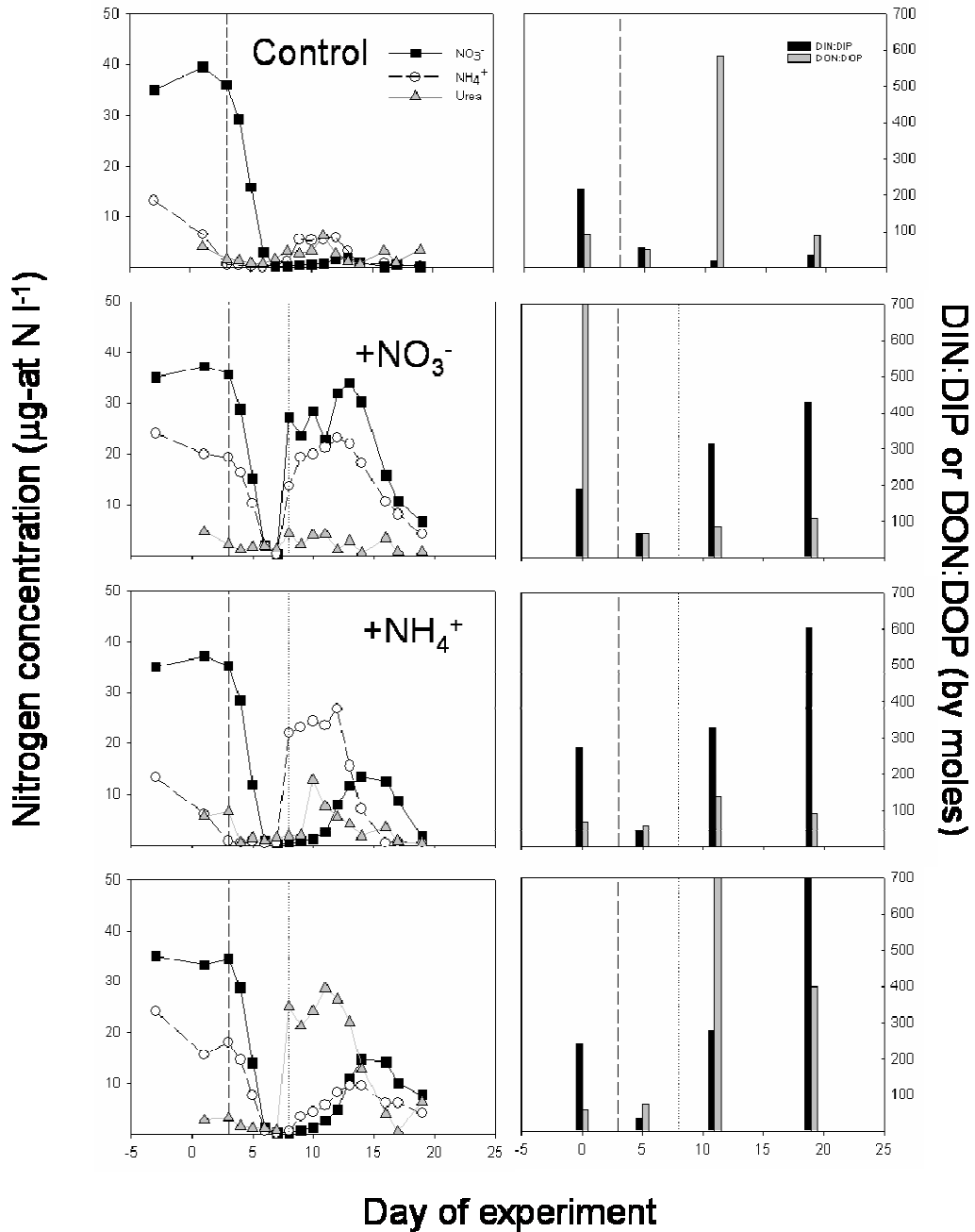


Fig. 7.2 A) Chl *a* biomass in all eight mesocosms as a function of day. The two mesocosms for each treatment are represented by filled and empty symbols. The line is the mean of all mesocosms. B) Mean relative contribution of each phytoplankton taxon to the total phytoplankton assemblage in all eight mesocosms as a function of sampling date (Days 5, 9, 17) in the mesocosms. The vertical lines are the same as in Fig. 7.1

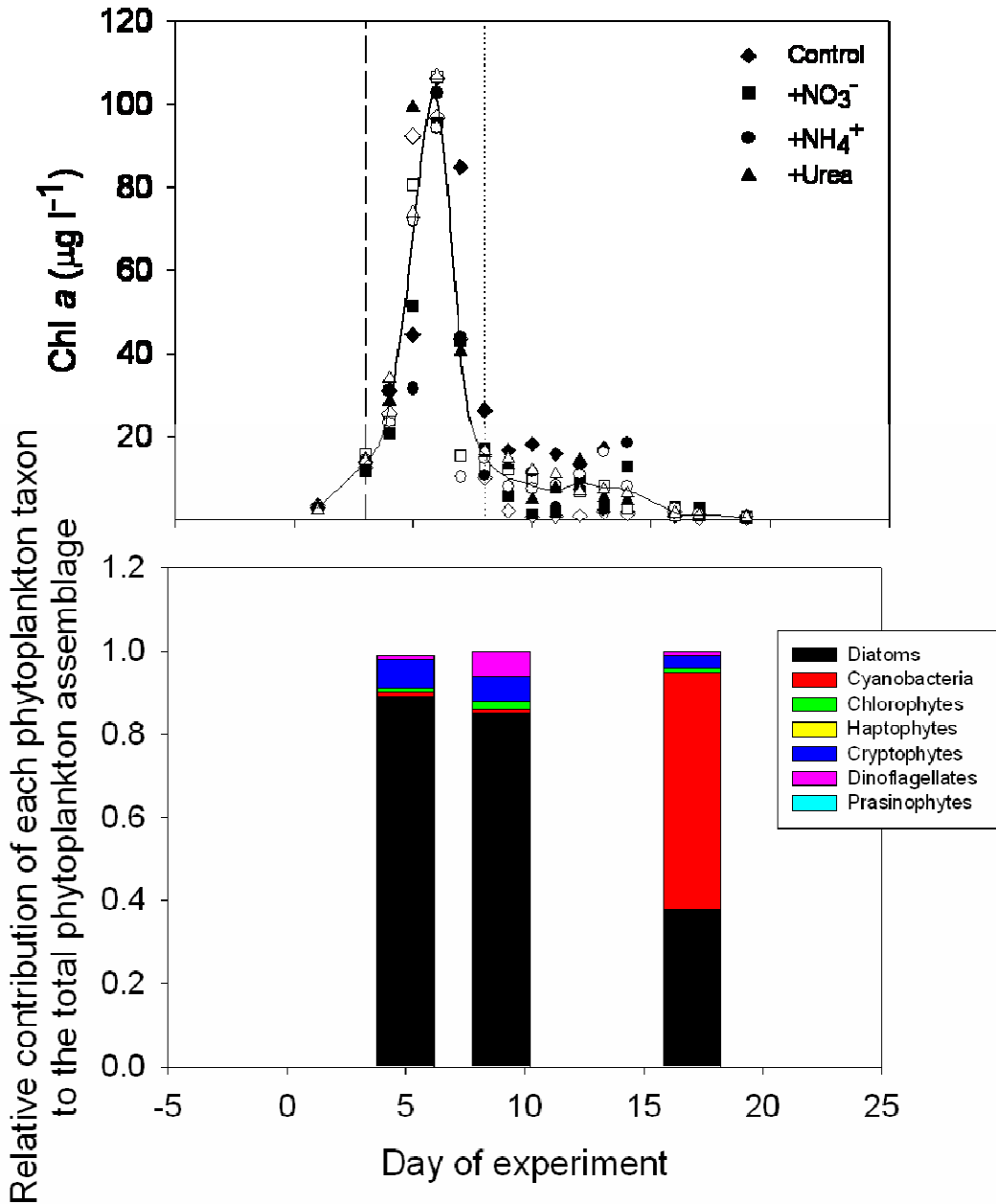


Fig. 7.3 Multidimensional scaling diagram with stress value of the Dice distance matrix calculated from DGGE banding patterns of both prokaryotic and eukaryotic samples collected from each mesocosm on Days 1, 8 and 16.

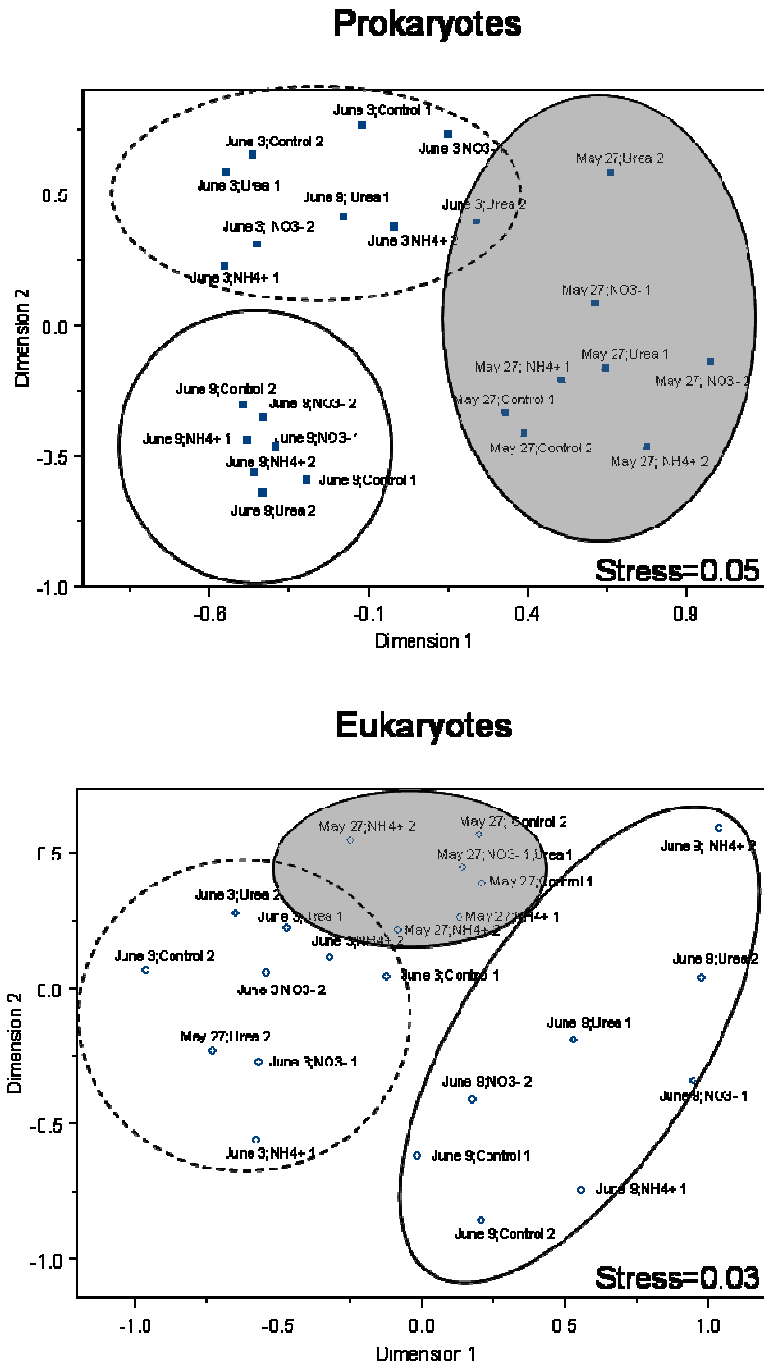


Fig. 7.4 *Left panel:* Urea uptake and urease activity on a volumetric basis in all N treatments as a function of day of experiment. Data points represent the average of two mesocosms for each N treatment. Lines were drawn by eye for easier visualization of trends. *Right panel:* Urea uptake and urease activity on a per μg chl *a* basis in all N treatments as a function of day of experiment. The vertical lines are the same as in Fig. 7.1.

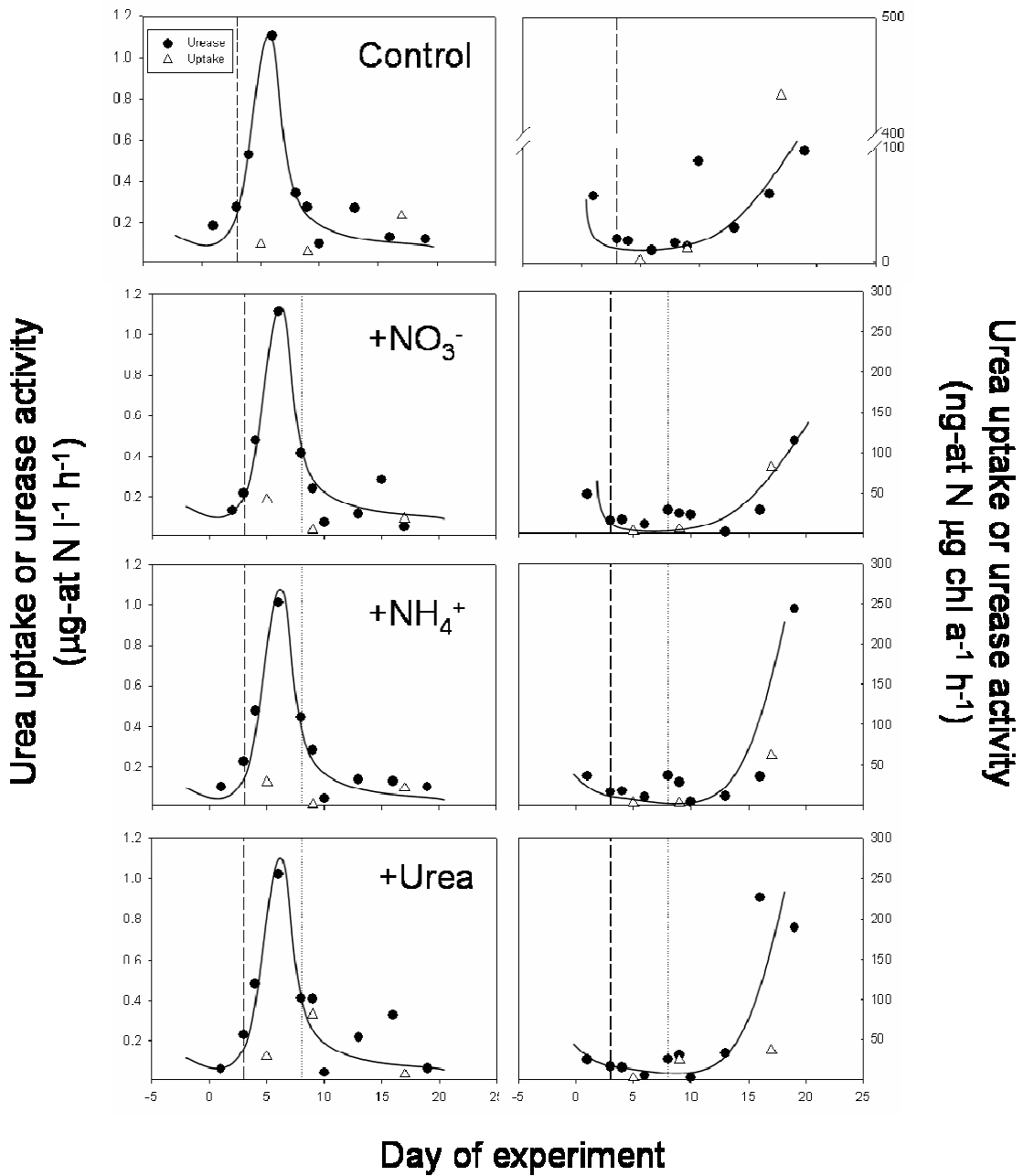


Fig. 7.5 Percentage of urea assimilated into the protein fraction on days 5, 9 and 17 over one hour in each N treatment. Each point represents the mean (\pm SE) of the two mesocosms for each treatment.

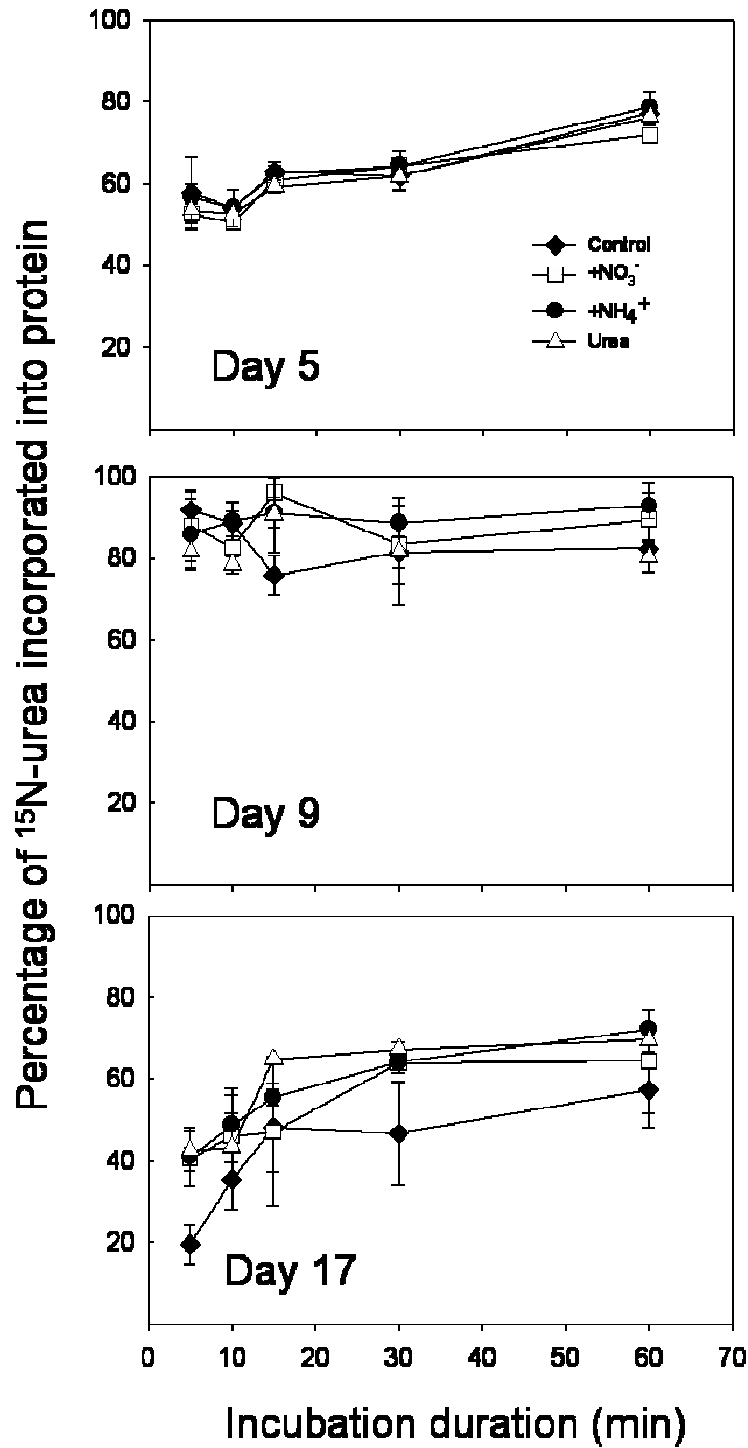
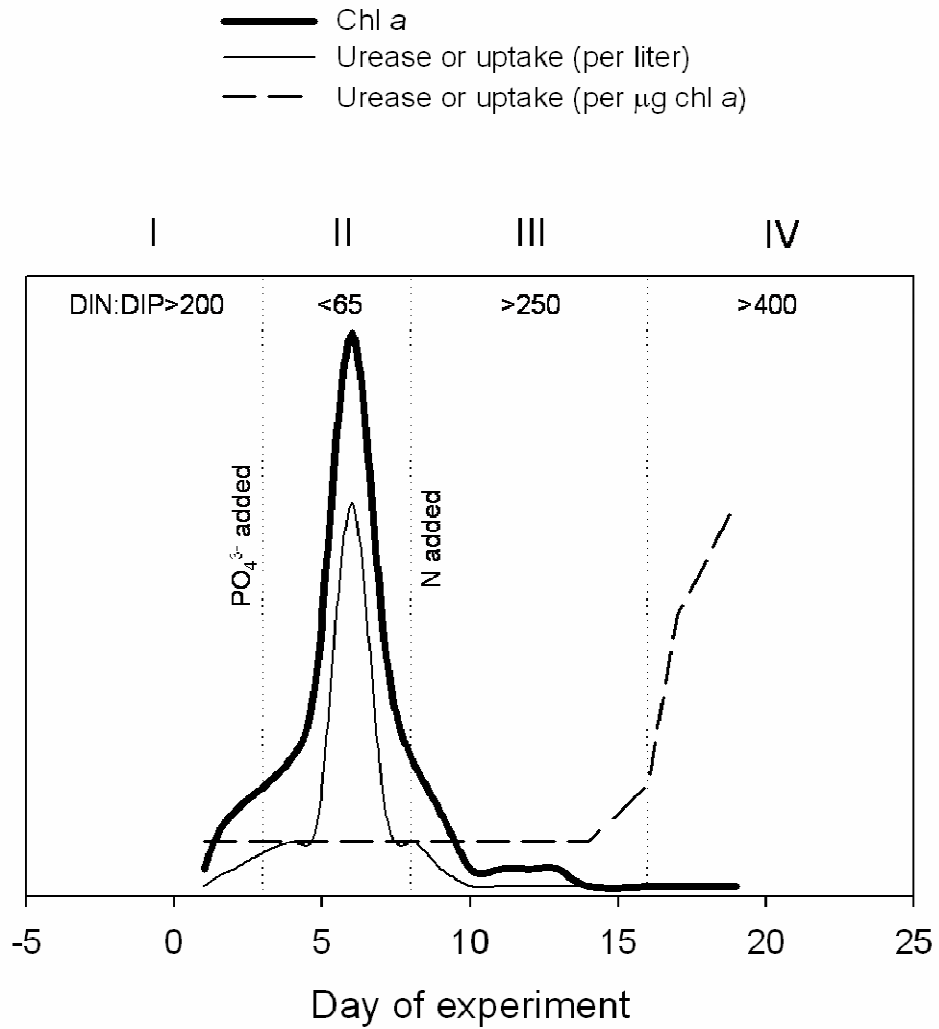


Fig. 7.6 A schematic synthesis of events during the mesocosm experiment. The mesocosms experiment is divided into four stages according to when nutrients were added and DIN:DIP ratios



Chapter 8: Summary and Research Conclusions

The research conducted for this dissertation set out to understand how urea uptake and urease activity are regulated in estuarine ecosystems. The framework of this dissertation was guided by work done on another enzyme involved with N assimilation, NO_3^- reductase. The regulation of NO_3^- reductase by temperature (Lomas and Glibert 1999a, Gao et al. 2000) and nitrogen availability (Lomas and Glibert 1999a, Parker and Armbrust 2005) is different among the diatoms and dinoflagellates (Lomas and Glibert 1999b, Lomas and Glibert 2000). NO_3^- reductase activity is able to exceed cellular N demand in diatoms but not in dinoflagellates (Lomas and Glibert 2000). In contrast, urease activity can meet the N demand of the dinoflagellate, *Prorocentrum minimum* (Fan et al. 2003). This led to the question of whether urea (as well as NH_4^+) could be meeting the cellular N demand in other dinoflagellates and other phytoplankton and bacterial taxonomic groups and whether urea utilization was regulated by the same factors - temperature and nutrient availability.

Temperature

Consistent with previous studies, urea uptake and urease activity generally increased with temperature. Rates were the highest during the summer months throughout Chesapeake Bay and Choptank River unless repressed by NH_4^+ availability. Dinoflagellates were generally abundant during the summer months and the higher rates of urea uptake and urease activity suggests that they are able to use urea under warmer conditions. This may be explained by the work of Fan et al. (2003) who found urease activity in the diatom, *Thalassosira weissflogii*, increased from 0 to 20°C before decreasing at higher temperature while urease activity in the dinoflagellate, *P. minimum*, did not vary much from 20-50°C. The difference in physiological responses between

diatoms and dinoflagellates to temperature may help explain the ability of dinoflagellates to utilize urea during the warmer months (>20°C) in Chesapeake Bay and Choptank River.

Nitrogen availability

Urea uptake and urease activity are regulated by ambient nitrogen availability, but the degree and pattern of repression differs among phytoplankton taxonomic groups. Urea uptake enzymes are located in the phytoplankton or bacterial cell membrane and are directly exposed to ambient NH_4^+ and NO_3^- and are more tightly regulated by these N sources than urease. Urea uptake rates were the highest when the ambient NO_3^- and NH_4^+ concentrations were <40 and <5 $\mu\text{g-at N l}^{-1}$, respectively, in Chesapeake Bay and Choptank River (Fig. 8.1). Supporting these observations, urea uptake rates decreased with increasing NO_3^- and NH_4^+ concentrations in bioassay and mesocosm experiments. Repression of urea uptake by NO_3^- and NH_4^+ may occur because the metabolic cost of assimilating NH_4^+ directly for protein synthesis is less energetically expensive than converting urea or NO_3^- into NH_4^+ (Falkowski and Raven 1997). The repression of urea uptake by NO_3^- and NH_4^+ would allow for more NH_4^+ to be transported into the cell to be directly assimilated into protein.

Urease activity is not as tightly regulated by ambient nitrogen availability as urea uptake (Fig. 8.1) maybe because urease activity responds to intracellular N concentrations or N status (e.g. N starvation). Regardless, urease activity generally was lower when ambient NO_3^- and NH_4^+ concentrations were high under both field and manipulated conditions. When less NO_3^- and NH_4^+ are transported into the cell, urease activity may increase in response to limitation of inorganic N or an increase in

intracellular urea. The size of the intracellular urea pool can change with variations in rates of urea uptake or production of urea from amino acid and purine catabolism. Urease in phytoplankton also may respond to intracellular NO_3^- , NH_4^+ or urea concentrations which vary as a result of growing on different media or the activities of other cellular pathways. Lower urease activity is sometimes found in NH_4^+ grown cultures, especially of the dinoflagellates *P. minimum* and *K. veneficum*, than in NO_3^- and urea grown cultures. In conclusion, urease activity may be indirectly regulated by ambient NO_3^- and NH_4^+ , but how urease activity is regulated by intracellular N concentrations or N status requires further study.

Difference in regulation among phytoplankton taxonomic groups

It is well established in the literature that a wide range of phytoplankton and bacteria species can use urea to meet their N metabolic demand (Anita et al. 1991, Kirchman 2000, Berman and Bronk 2003, Glibert et al. 2006). This dissertation went a step further and investigated how rates of urea uptake and urease activity differed among several specific taxonomic groups. Fan et al. (2003) provided clues that dinoflagellates may have higher urease activities than diatoms or pelagophytes on a per cell basis. However, *P. minimum*, the dinoflagellate studied by Fan et al. (2003) is summer-time estuarine species but many other dinoflagellates exist in estuaries throughout the year and their ability to utilize urea may change over time and space. The dinoflagellates are a physiologically diverse group, so it is difficult to generalize about the ability of dinoflagellates to use urea based on one species. High urea uptake and urease activity could also possibly be attributed to other phytoplankton taxonomic groups such as

cyanobacteria, cryptophytes, chlorophytes, and haptophytes that are present in the Chesapeake Bay.

It was possible to tease out which phytoplankton taxonomic group was responsible for the high urea uptake or urease activity rates during different seasons throughout the Chesapeake Bay and the Choptank River by conducting principal component analysis (PCA) which combined environmental factors including the relative abundance of the five to seven major taxonomic groups. Dinoflagellates and cyanobacteria consistently were related to periods of high urea uptake and urease activity, not only in Chesapeake Bay and Choptank River but in culture, bioassay and mesocosm experiments. The dinoflagellates, *Prorocentrum minimum* and *Karlodinium veneficum*, had the highest urease activity in culture on both per cell, per cell volume, and per chl *a* basis. Finally, a synthesis of published rates of urease activity confirmed that dinoflagellates and cyanobacteria had the highest rates on either per cell or per cell volume basis (Fig 8.2).

The greater ability of dinoflagellates and cyanobacteria to utilize urea during the summer than diatoms lies in the possible difference in regulation of urea uptake and urease activity by N sources. Urea uptake and urease activity are lower in diatoms grown on NO_3^- (Fig 8.3) and in dinoflagellates grown on NH_4^+ than on urea (Fig. 8.4). Conceptually, spring-time conditions with high NO_3^- concentrations would repress urease activity in diatoms but not the relatively few dinoflagellates present (Fig. 8.4). Later during the summer, high NH_4^+ concentrations would repress NO_3^- uptake in diatoms, dinoflagellates, and cyanobacteria allowing for higher NH_4^+ and urea utilization. However, if NH_4^+ concentrations are high enough, urea uptake can be repressed in diatoms and dinoflagellates, and subsequently urease activity in dinoflagellates and

cyanobacteria. The optimal conditions for high urea uptake and urease activity in dinoflagellates and cyanobacteria are under N-limitation.

Large phytoplankton were mostly responsible for urea uptake observed in Chesapeake Bay and Choptank River. In contrast, some of the urease activity observed was due to smaller phytoplankton and bacteria. Luxury uptake of urea may be occurring in larger phytoplankton, resulting in large pools of intracellular urea. Dinoflagellates such as *P. minimum* and *K. veneficum* had large intracellular pools of urea, but the possible storage mechanisms are unknown. Urease activity in larger phytoplankton may be inhibited from the lack of Ni^{2+} (needed for the metalcenter of urease; Oliveira and Antia 1991) or by metabolites produced in the cell. Supporting such a possibility, urease activity decreased with increasing biomass, hence more cell metabolites, in tests of the urease activity method.

Complexity in urea utilization

Urea uptake and urease activity may be influenced by many factors including ambient and intracellular N concentrations. The study of the regulation of these processes by intracellular NH_4^+ and urea is especially complicated by many cellular production and consumption pathways. For example, NH_4^+ represses both urea uptake and urease activity but these two enzymes may be more directly regulated by intracellular concentrations of 2-oxoglutarate than NH_4^+ (Muro-Pastor et al. 2005, Flores and Herrero 2005). The pool of 2-oxoglutarate may be influenced by pathways that produce NH_4^+ from inorganic and organic compounds that are present or transported into the cell. Urease activity is higher in phytoplankton that are grown on urea with larger intracellular pools of urea. The size of the intracellular urea pool depends on the supply of urea via

transport and the catabolism of amino acids and purines (Antia et al. 1991, Allen et al. 2005) and the demand by urease.

The discovery of urea cycle genes in the diatom, *T. pseudonana*, brought some insights in how the intermediates for the urea cycle, which remains to be found in other phytoplankton, may be important for many aspects of diatom metabolism (Armbrust et al. 2004, Allen et al. 2005). Urease activity may respond to an increase in urea from enhanced urea cycle activity to provide the needed intermediates for silica precipitation, signaling molecules, osmolytes, or energy storage (Allen et al. 2005). Changes in urease activity are not regulated only by fluctuations in ambient and intracellular urea concentrations but also by variations in metabolic needs of the phytoplankton or bacterial cell.

Comparison of field activity of enzymes involved in nitrogen assimilation

Before work began on this dissertation, urease activity had not been measured in field samples. Subsequent work by Dyhrman and Anderson (2003), Glibert et al. (2004), and Heil et al. (*in press*) has shown that urease activity can supply NH_4^+ to phytoplankton and bacteria (Table 8.1). The highest bulk urease activity reported was during a bloom of the dinoflagellate *P. minimum* (Salerno 2005) while the highest urease specific activity reported (on a per chl *a* basis) was during a bloom of the cyanobacterium *Synechococcus elongatus* (Glibert et al. 2004). The average urease activity reported for the Chesapeake Bay was lower than during bloom conditions reported elsewhere. NO_3^- reductase activity, generally, was higher than urease activity under either non-bloom or bloom conditions (Table 8.1). Further studies measuring both NO_3^- reductase and urease

activity simultaneously will be needed in addition to ^{15}N tracer studies to fully understand the relative contribution of each pathway to microbial N assimilation. Regardless, urease activity can supply sufficient NH_4^+ to phytoplankton and bacteria in estuaries such as the Chesapeake Bay.

Future directions for understanding urea regulation in phytoplankton and bacteria

Recent studies on regulation of transcription of several genes that code for enzymes involved with N metabolism are beginning to explain observations in the field. Most of this work has focused on the utilization of NO_3^- and NH_4^+ in diatoms, in attempt to understand why NO_3^- is utilized during spring blooms rather than the normally preferred NH_4^+ , which is less energetically expensive to assimilate. Lomas and Glibert (1999) first provided a hypothesis to explain this phenomenon: under cool temperatures, the NO_3^- reductase (NR) pathway accepts excess electrons from light stress that cannot go through the temperature-limited RUBSICO pathway. The excess NO_3^- reduction would result in release of DON and NH_4^+ generated as a byproduct of NR and subsequent reactions. Parker and Armbrust (2005) went a step further and found that the transcript abundance (mRNA) for NR increased under high light and cool temperatures in the diatom, *Thalassiosira pseudonana* when grown on NO_3^- . Levels of mRNA of other enzymes involved with NO_3^- assimilation, such as NO_3^- transporters (NAT) and glutamine synthetase II (GSII), which is specific for NH_4^+ produced by NR also increase when grown on NO_3^- (Hildebrand and Dahlin 2000, Parker and Armbrust 2005). However, diatoms grown on NH_4^+ had lower levels of NR, NAT and GSII NR mRNA. On the other hand, the NH_4^+ transporters (AMT1 and AMT2) in the diatom,

Cylindrotheca fusiformis, are not as tightly regulated and exhibit different expression patterns (Allen 2005, Hillebrand 2005). The highest mRNA levels were found in the diatom under N starvation and lower levels were found in NO_3^- and NH_4^+ grown cultures. AMT mRNA was quickly degraded, suggesting a high turnover of AMT transcripts (Hildebrand 2005). Since NH_4^+ is the preferred and energetically cheaper nitrogen source (Falkowski and Raven 1997) there is probably no need to prevent NH_4^+ assimilation, except under N starvation.

Similar work on how transcription of urea transporters (*urt*) and urease (*ure*) genes is regulated by different nitrogen sources need to be done to understand the interactions between enzymes involved with N assimilation. A beginning study on urea transport genes found that additions of 5 mM of NH_4^+ , glutamine and glutamate prevented the synthesis of the urea uptake system in the bacterium, *Corynebacterium glutamicum* (Siewe et al. 1998), while N starvation promoted synthesis (Beckers et al. 2004). In contrast, down-regulation of the urea uptake system occurred in the haptophyte, *Emiliana huxleyi*, when grown in NO_3^- poor medium (5 μM ; A. Bruhn, personal communication). It would be expected from this dissertation that transcript abundance of urea transport and urease enzymes would be regulated by global nitrogen regulators (such as NctA, AmtR), and to some degree nitrogen availability (NO_3^- , NH_4^+ , glutamine, and amino acids). However, the degree and pattern of regulation of transcript abundance of urea transporters and urease would be expected to differ among the diatoms, dinoflagellates and the cyanobacteria.

Literature cited

- Allen, A.E., Ward, B.B. & Bowler, C.P. 2005. Multi-lineage richness of functional genes in marine diatoms and transcriptional responses to nutrient stress. American Society of Limnology and Oceanography Annual Meeting. Santiago, Spain. June 2005.
- Antia, N.J., Harrison, P.J. & Oliveria, L. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia*. 1:1-89.
- Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S.G., Allen, A.E., Apt, K.E., Bechner, M., Brzezinski, M.A., Chaal, B.K., Chiovitti, A., Davis, A.K., Demarest, M.S., Detter, J.C., Glavina, T., Goodstein, D., Hadi, M.Z., Hellsten, U., Hildebrand, M., Jenkins, B.D., Jurka, J., Kapitonov, V.V., Kroger, N., Lau, W.W.Y., Lane, T.W., Larimer, F.W., Lippmeier, J.C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M.S., Palenik, B., Pazour, G.J., Richardson, P.M., Rynearson, T.A., Saito, M.A., Schwartz, D.C., Thamtrakoln, K., Valentin, K., Vardi, A., Wilkerson, F.P., Rokhsar, D.S. 2004. The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. *Science*. 306: 79-86.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microbiol. Ecol.* 31: 279-305.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiology*. 145: 447-459.
- Dortch, Q. & Maske, H. 1982. Dark uptake of nitrate and nitrate reductase activity of a red-tide population off Peru. *Mar. Ecol. Prog. Ser.* 9: 299-303.
- Dyhrman, S.T., & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Falkowski, P.G. & Raven, J.A. 1997. Aquatic Photosynthesis. Blackwell Science.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Flores, E. & Herrero, A. 2005. Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochemical Society Transactions*. 33: 164-167.
- Gao, Y., Smith, G.J. & Alberte, R.S. 2000. Temperature dependence of nitrate reductase activity in marine phytoplankton: biochemical analysis and ecological implications. *J. Phycol.* 36: 304-313.

- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Heil, C.A., Revilla, M., Glibert, P.M. & Murasko, S. *In press*. Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Iriarte, J.L., Quiñones, R.A. & Gonzalez, R.R. 2005. Relationship between biomass and enzymatic activity of a bloom-forming dinoflagellate (Dinophyceae) in southern Chile (41°S): a field approach. *J. Plank. Res.* 27: 159-166.
- Kirchman, D.L. 2000. Uptake and regeneration of inorganic nutrients by marine heterotrophic bacteria. *In Microbial Ecology of the Oceans.* pp. 261-288. Wiley Liss.
- Kramer, J.G & Morris, I. 1990. Growth regulation in irradiance limited marine *Synechococcus* sp. WH 7803. *Arch. Microbiol.* 154: 286-293.
- Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.
- Kudo, I., Yoshimura, T., Yanada, M. & Matsunaga, K. 2000. Exhaustion of nitrate terminates a phytoplankton bloom in Funka Bay, Japan: change in SiO₄:NO₃ consumption rate during the bloom. *Mar. Ecol. Prog. Ser.* 193: 45-51.
- Lomas, M.W. & Glibert, P.M. 1999a. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 556-572.
- Lomas, M.W. & Glibert, P.M. 1999b. Interactions between NH₄⁺ and NO₃⁻ uptake and assimilation: comparisons of diatoms and dinoflagellates at several growth temperatures. *Mar. Biol.* 133: 541-551.
- Lomas, M.W. & Glibert, P.M. 2000. Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. *J. Phycol.* 36: 903-913.
- Menden-Duer, S. & Lessard, E.J. 2000. Carbon to volume relationships for dinoflagellates, diatoms and other protist plankton. *Limnol. Oceanogr.* 45:569-579.
- Muro-Pastor, M.I., Reyes, J.C. & Florencio, F.J. 2005. Ammonium assimilation in cyanobacteria. *Photosynthesis Research* 83: 135-150.

- Oliveira, L. & Antia, N.J. 1986. Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J. Plank. Res.* 8: 235-242.
- Palinska, K.A., Jahns, T., Rippka, R. & Tandeau de Marsac, N. 2000. *Prochlorococcus marinus* strain PCC 9511, a picoplankton cyanobacterium, synthesizes the smallest urease. *Microbiology.* 146: 3099-3107.
- Parker, M.S. & Armbrust, E.V. 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 41:1142-1153.
- Salerno, M. 2005. Ectocellular enzyme activities in the mixotrophic dinoflagellate *Prorocentrum minimum* (Dinophyceae). Masters of Science Thesis. University of Maryland, College Park.
- Siewe, R.M., Weil, B., Burkovski, A., Eggeling, L., Krämer, R. & Jahns, T. 1998. Urea uptake and urease activity in *Corynebacterium glutamicum*. *Arch. Microbiol.* 169: 411-416.
- Takayanagi, K., Wong, G.T.F. & Filardo, M.J. 1989. Nitrate reductase activity and the speciation of selenium at the mouth of the Chesapeake Bay. *J. Ocean. Soc. Japan.* 45:129-133.
- Wheeler, P. 1983. Phytoplankton nitrogen metabolism.. In Carpenter, E.J & Capone, D.G. [Eds] *Nitrogen in the Marine Environment*. Academic Press. New York, N.Y. pp. 309- 346.
- Zubkov, M.V. & Tarran, G.A. 2005. Amino acid uptake of *Prochlorococcus* spp. in surface waters across the South Atlantic Subtropical Front. *Aquat. Microbiol. Ecol.* 40: 241-249.

Table

Table 8.1. Comparisons of field rates of enzymatic activities involved with nitrogen assimilation at various locations around the world. Units for bulk activity are $\mu\text{g-at N l}^{-1} \text{h}^{-1}$ while for specific activity are $\text{ng-at N } \mu\text{g chl } a^{-1} \text{h}^{-1}$ unless noted. Conversions for rates denoted with an asterisk (*) were not possible because no chl *a* data were available.

Location (Reference)	Nitrate reductase activity		GS activity		Urease activity	
	Bulk	Specific	Bulk	Specific	Bulk	Specific
Chesapeake Bay mouth (Takayanagi et al. 1989)	0.71-1.62	0-0.78				
Chesapeake Bay (Solomon et al. <i>submitted</i> ; Chapter 4)					0.12±0.06	21.3±4.99
Choptank River (Solomon et al. <i>submitted</i> ; Chapter 5)					0.21±0.02	20.0±4.84
<i>Alexandrium</i> sp. (dinoflagellate) bloom in Gulf of Maine (Dyhrman and Anderson 2003)					0.02-0.03	141.7-115.7 $\text{fmol cell}^{-1} \text{h}^{-1}$
<i>Synechococcus elongatus</i> (cyanobacteria) bloom in Florida Bay (Glibert et al. 2004)					0.4	50
West Florida Shelf (Heil et al. <i>in press</i>)					0.02-0.15	6-190
<i>Prorocentrum minimum</i> bloom in Corsica River, MD (Salerno 2005).					2.8	10.8

<i>Table 8.1 con't</i> Location (Reference)	Nitrate reductase activity		GS activity		Urease activity	
	Bulk	Specific	Bulk	Specific	Bulk	Specific
<i>Gymnodinium cf. chlorophorum</i> (dinoflagellate) bloom in Puerto Montt Bay, Chile (Iriarte et al. 2005)	0.006	0	1600	<1		
<i>Chaetoceros socialis</i> (diatom) bloom in Funaka Bay, Japan (Kudo et al. 2000)	0.78	52				
<i>Gymnodinium splendens</i> (dinoflagellate) bloom off the coast of Peru (Dortch & Maske 1982)	0.03	*				
East China Sea (Hung et al. 2000)	0-15,000	0-18.75				
East China Sea (Wong and Hung 2001)	0-8; coastal zone 0.2-0.6; Kuroshiro current	*				

Figures

Fig. 8.1 Urea uptake or urease activity as a function of NO_3^- concentration and NH_4^+ concentration using all field data.

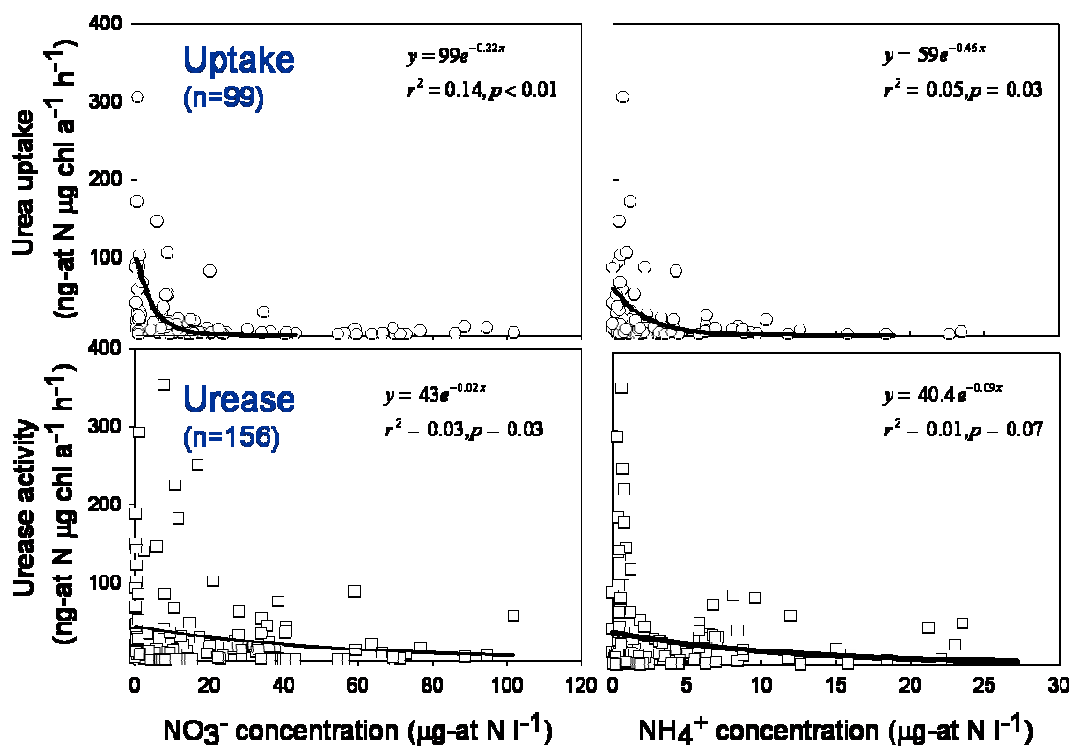


Fig. 8.2 Comparison of urease activity rates between different phytoplankton species on per cell or per cell volume basis. Data for the cyanobacteria *Prochlorococcus marinus* were obtained from Palinska et al. (2000) and *Synechococcus* WH7805 from Collier et al. (1999). Rates were converted from μM urea hydrolyzed min^{-1} protein $^{-1}$ to $\text{fg-at N cell}^{-1} \text{h}^{-1}$ using $21.5 \text{ fg protein cell}^{-1}$ for *P. marinus* (Zubkov & Tarran 2005) and $500 \text{ fg protein cell}^{-1}$ for WH7805 (Kramer and Morris 1990). *P. marinus* on a per cell volume basis (*) was divided by 10 to allow for visualization of other species. Data source of other species are described in Figs. 7.3 and 7.4. Cell volumes were calculated assuming a sphere or cylinder and diameters obtained from readings on the Coulter Counter or from CCMP.

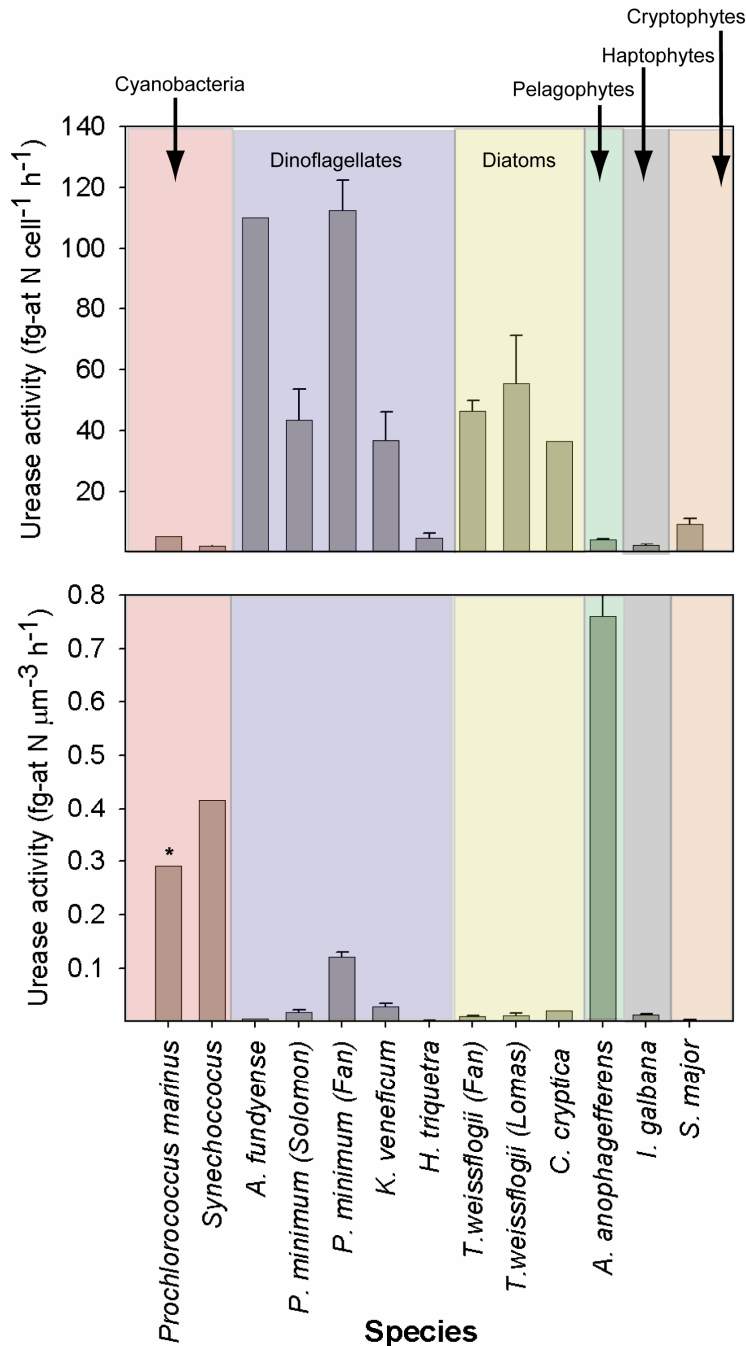


Fig. 8.3 Comparison of rates of urease activity in diatoms grown on different N substrates. Data for *Thalassosira weissflogii* were obtained from Fan et al. (2003) and Lomas (2004). Data for *Cyclotella cryptica* were obtained from Oliveira and Antia (1986) and rates were converted from μM urea hydrolyzed min^{-1} protein $^{-1}$ to $\text{fg-at N cell}^{-1} \text{h}^{-1}$ using the regression from Menden-Duer and Lessard (2000) to get N cell^{-1} and the fact that 70-90% of cellular N is protein (Wheeler et al. 1983).

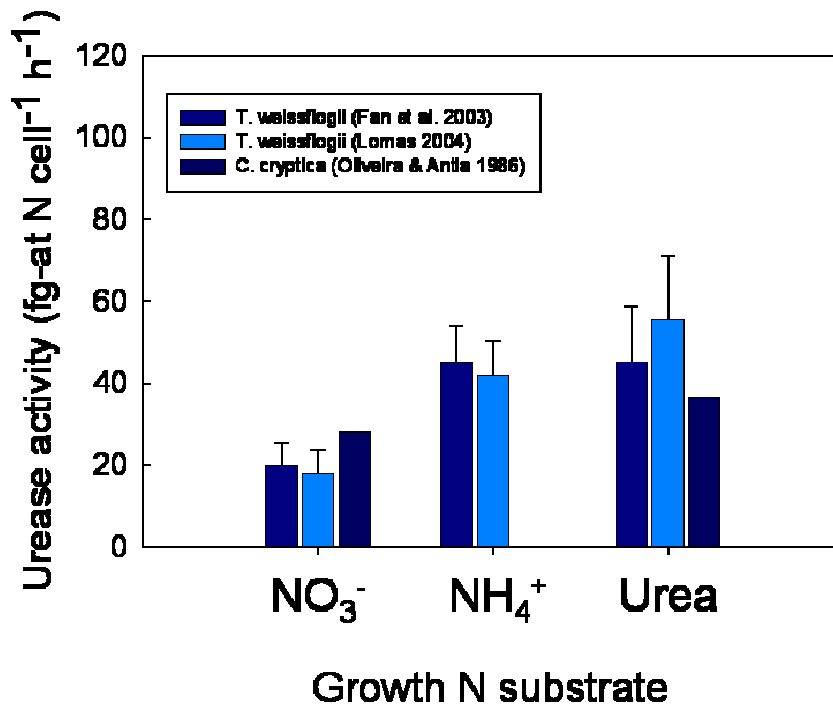
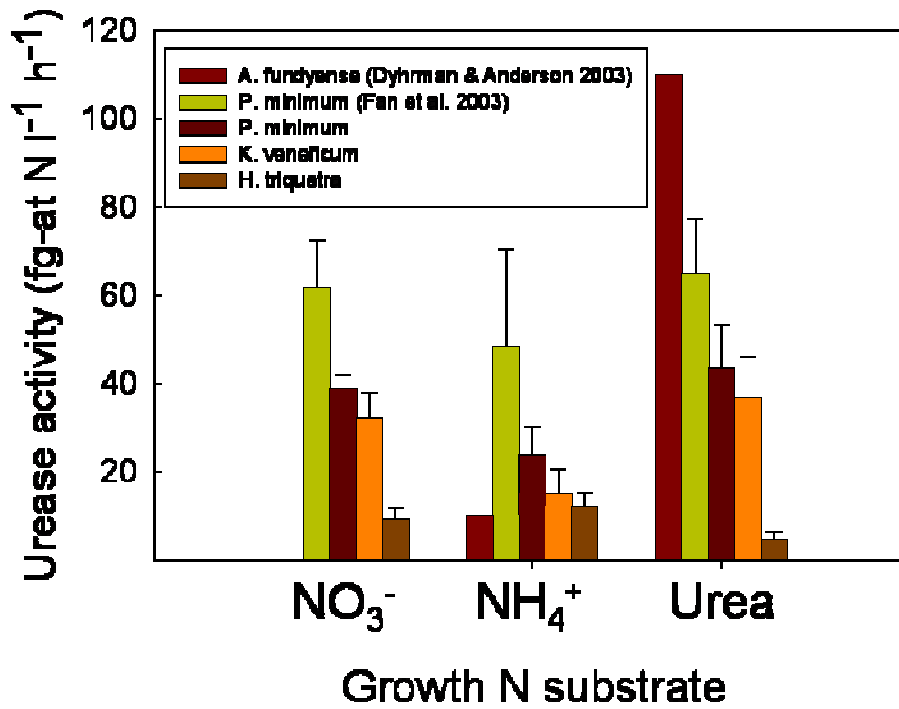


Fig. 8.4 Comparison of rates of urease activity in dinoflagellates grown on different N substrates. Data for *Alexandrium fundyense* were obtained from Dyhrman & Anderson (2003), for *Prorocentrum minimum* from Fan et al. (2003), and for the remaining species from this dissertation.



Complete Literature Cited

- Adolf, J.E., Harding, L.W., Yeager, C.L., Maloney, M.E & Miller, W.D. 2006. Environmental forcing of phytoplankton floral composition, biomass and primary productivity in the Chesapeake Bay, USA. *Estuar. Shelf Coastal Sci.* 67: 108-122.
- Allen, A.E., Ward, B.B. & Bowler, C.P. 2005. Multi-lineage richness of functional genes in marine diatoms and transcriptional responses to nutrient stress. American Society of Limnology and Oceanography Summer Meeting. Santiago, Spain. June 2005.
- Antia, N.J., Harrison, P.J. & Oliveria, L. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology, and ecology. *Phycologia* 1:1-89.
- Antia, N.J. McCallister, C.D., Parsons, T.R., Stephens, K. & Strickland, J.D. 1963. Further measurements of primary production using a large-volume plastic sphere. *Limnol. Oceanogr.* 8: 166-183.
- Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S.G., Allen, A.E., Apt, K.E., Bechner, M., Brzezinski, M.A., Chaal, B.K., Chiovitti, A., Davis, A.K., Demarest, M.S., Detter, J.C., Glavina, T., Goodstein, D., Hadi, M.Z., Hellsten, U., Hildebrand, M., Jenkins, B.D., Jurka, J., Kapitonov, V.V., Kroger, N., Lau, W.W.Y., Lane, T.W., Larimer, F.W., Lippmeier, J.C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M.S., Palenik, B., Pazour, G.J., Richardson, P.M., Rynearson, T.A., Saito, M.A., Schwartz, D.C., Thamtrakoln, K., Valentin, K., Vardi, A., Wilkerson, F.P. & Rokhsar, D.S. 2004. The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. *Science* 306: 79-86.
- Arrigo, K.R. 2005. Marine microorganisms and global nutrient cycles. *Nature* 437: 349-355.
- Azam, F. 1998. Microbial control of oceanic carbon flux: The plot thickens. *Science* 280: 694-696.
- Beckers, G., Bendt, A.K., Krämer, R. & Burkovski, A. 2004. Molecular identification of the urea uptake system and transcriptional analysis of urea transporter- and urease-encoding genes in *Corynebacterium glutamicum*. *J. Bacteriol.* 186: 7645-7652.
- Bekheet, I.A. & Syrett, P.J. 1977. Urea-degrading enzymes in algae. *Br. Phycol. J.* 12:137-143.
- Bekheet, I.A., & Syrett, P.J. 1979. The uptake of urea by *Chlorella*. *New Phytol.* 82:179-186.
- Berg, G.M., Glibert, P.M. & Chen, C. 1999. Dimensional effects of enclosures on ecological processes in pelagic systems. *Limnol. Oceanogr.* 44: 1331-1340.

- Berg, G.M., Glibert, P.M., Lomas, M.W. & Burford, M.A. 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar. Biol.* 129: 377-387.
- Berg, G.M. & Jørgensen, N.O.G. 2006. Purine and pyrimidine metabolism by estuarine bacteria. *Aquat. Microb. Ecol.* 42: 215-226
- Berges, J.A. 1997. Minireview: Algal nitrate reductases. *Eur. J. Phycol.* 32:3-8.
- Bergholtz, T., Daugbjerg, N., Moestrup, Ø. & Fernández-Tejedor, M. 2006. On the identity of *Karlodinium veneficum* and description of *Karlodinium armiger* sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. *J. Phycol.* 42: 170-193.
- Berman, T. 1997. Dissolved organic nitrogen utilization by an *Aphanizomenon* bloom in Lake Kinneret. *J. Plank. Res.* 19: 577-586.
- Berman, T., Béchemin C. & Maestrini, S.Y. 1999. Release of ammonium and urea from dissolved organic nitrogen in aquatic ecosystems. *Aquat. Microb. Ecol.* 16: 295-302.
- Berman, T. & Bronk, D.A. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat. Microbiol. Ecol.* 279-305.
- Berman, T. & Chava, S. 1999. Algal growth on organic compounds as nitrogen sources. *J. Plank. Res.* 1423-1437.
- Boesch, D.F., Brinsfield, R.B. & Magnien, R.E. 2001. Chesapeake Bay eutrophication: scientific understanding, ecosystem restoration, and challenges for agriculture. *J. Environ. Qual.* 30: 303-320
- Brzezinski, M.A. 1987. Colorimetric determination of nanomolar concentrations of ammonium in seawater using solvent extraction. *Mar. Chem.* 20: 277-288.
- Bronk, D.A., Glibert, P.M., Malone, T.C., Banahan, S. & Sahlsten, E. 1998. Inorganic and organic nitrogen cycling in Chesapeake Bay: autotrophic versus heterotrophic processes and relationships to carbon flux. *Aquat. Microb. Ecol.* 15: 177-189.
- Bronk, D.A., Lomas, M.W., Glibert, P.M., Schukert, K.J. & Sanderson, M.P. 2000. Total dissolved nitrogen analysis: comparisons between persulfate, UV, and high temperature oxidation methods. *Mar. Chem.* 69: 163-178.
- Bruhn A., Buch-illing, H., LaRoche, J. & Richardson, K. 2002. Identification of two functional genes involved with phytoplankton metabolisms of dissolved organic nitrogen: the urease and the formamidase gene. American Society of Limnology and Oceanography Annual Meeting. Victoria, British Columbia, Canada. June 2002.

- Burford, M.A. 2005. Relative uptake of urea and ammonium by dinoflagellates or cyanobacteria in shrimp mesocosms. *Hydrobiologia* 549: 297-303.
- Capone, D. 2000. The marine microbial nitrogen cycle. *In* Microbial Ecology of the Oceans [Ed. D. L. Kirchman]. Wiley-Liss. New York. pp. 455-493.
- Cho, B.C. & Azam, F. 1995. Urea decomposition by bacteria in the Southern California Bight and its implications for the mesopelagic nitrogen cycle. *Mar. Ecol. Prog. Ser.* 122: 21-126.
- Cho, B.C., Park, M.C., Shim, J.H. & Azam, F. 1996. Significance of bacteria in urea dynamics in coastal surface waters. *Mar. Ecol. Prog. Ser.* 142: 19-26.
- Cimberlis, A.C.P. & Caceres, O. 1991. Kinetics of urea uptake by *Melosira italica* (Ehr.) Kuetz at different luminosity conditions. *Hydrobiologia* 220: 211-216.
- Collier, J.L. & Baker, K.M. 2004. Urea-degrading microorganisms in Chesapeake Bay. American Society for Limnology and Oceanography Annual Meeting. Honolulu, Hawaii, USA. February 2004.
- Collier, J.L., Brahamsha, B. & Palenik, B. 1999. The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiology*. 145: 447-459.
- Collos, Y., Gagne, C., Laabir, M., Vaquer, A., Cecchi, P. & Souchu, P. 2004. Nitrogenous nutrition of *Alexandrium catenella* (Dinophyceae) cultures and in Thau Lagoon, Southern France. *J. Phycol.* 40:96-103.
- Collos, Y., Vaquer, A., Bibent, B., Slawyk, G., Garcia, N. & Souchu, P. 1997. Variability in nitrate uptake kinetics of phytoplankton communities in a Mediterranean coastal lagoon. *Estuarine Coastal Shelf Sci.* 44:369-375.
- Cornell, S. Rendell, A. & T. Jickells. 1995. Atmospheric inputs of dissolved organic nitrogen to the oceans. *Nature*. 376: 243-246.
- del Giorgio, P.A. & Cole, J.J. 2000. Bacterial energetics and growth efficiency. *In* Microbial Ecology of the Oceans. [Ed. D. L. Kirchman]. Wiley-Liss. New York. pp. 289-326.
- Dortch, Q. & Maske, H. 1982. Dark uptake of nitrate and nitrate reductase activity of a red-tide population off Peru. *Mar. Ecol. Prog. Ser.* 9: 299-303.
- Dortch, Q. & Postel, J.R. 1989. Biochemical indicators of N utilization by phytoplankton during upwelling off the Washington Coast. *Limnol. Oceanogr.* 34: 758-773.

- Ducklow, H. 2000. Bacterial production and biomass in the oceans. *In* Kirchman, D.L. [Ed.] *Microbial Ecology of the Oceans*. Wiley-Liss. New York., pp. 85-120.
- Dufresne, A., Salanoubat, M., Partensky, F., Artiguenave, F., Axmann, I.M., Barbe, V., Duprat, S., Galperin, M.Y., Koonin, E.V., Le Gall, F., Makarova, K.S., Ostrowski, M., Oztas, S., Robert, C., Rogozin, I.B., Scanlan, D.J., de Marsac, N.T., Weissenbach, J., Wincker, P., Wolf, Y.I. & Hess, W.R. 2003. Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. *Proc Natl Acad Sci USA*. 100 :9647-9649.
- Dyhrman, S.T., & Anderson, D.M. 2003. Urease activity in cultures and field populations of the toxic dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48: 647-655.
- Eppley, R.W. & Coatsworth, J.L. 1968. Uptake of nitrate and nitrite by *Ditylum brightwellii* – kinetics and mechanisms. *J. Phycol.* 4: 151-156.
- Falkowski, P.G. 2000. Rationalizing elemental ratios in unicellular algae. *J. Phycol.* 36:3-6.
- Falkowski, P.G., Raven, J.A. 1997. Aquatic Photosynthesis. Blackwell Science.
- Fan, C. & Glibert, P.M. 2005. Effect of light on nitrogen and carbon uptake during a *Prorocentrum minimum* bloom. *Harmful Algae*. 4:629-641.
- Fan, C., Glibert, P.M., Alexander, J. & Lomas, M.W. 2003. Characterization of urease activity in three marine phytoplankton species, *Aureococcus anophagefferens*, *Prorocentrum minimum*, and *Thalassiosira weissflogii*. *Mar. Biol.* 142:949-958.
- Fan, C., Glibert, P.M. & Burkholder, J.M. 2003. Characterization of the affinity for nitrogen, uptake kinetics, and environmental relationships for *Prorocentrum minimum* in natural blooms and laboratory cultures. *Harmful Algae*: 2: 283-299.
- Fisher, T.R., Gustafson, A.B., Sellner, K., Lacouture, R., Haas, L.W., Wetzel, R.L., Magnien, R., Everitt, D., Michaels, B. & Karrh, R. 1999. Spatial and temporal variation of resource limitation in Chesapeake Bay. *Mar. Biol.* 133: 763-778.
- Fisher, T.R., Hagy, J.D., Boynton, W.R. & Williams, M.R. 2006. Cultural eutrophication in the Choptank and Patuxent estuaries of Chesapeake Bay. *Limnol. Oceanogr.* 51: 435–447.
- Fisher, T.R., Harding, L.W., Stanley, D.W. & Ward, L.G. 1988. Phytoplankton, nutrients, and turbidity in Chesapeake, Delaware, and Hudson estuaries. *Estuar. Shelf Coastal Sci.* 27: 61-93.

- Fisher, T.R., Peele, E.R., Ammerman, J.A. & Harding, L.W. 1992. Nitrogen limitation of phytoplankton in the Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 82:51-63.
- Fisher, T.R., Lee, K., Berndt, H., Benitez, J.A. & Norton, M.M. 1998. Hydrology and chemistry of the Choptank River Basin. *Water, Air, and Soil Pollution* 105: 387-397.
- Flores, E. & Herrero, A. 2005. Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochemical Society Transactions.* 33: 164-167.
- Gao, Y., Smith, G.J. & Alberte, R.S. 2000. Temperature dependence of nitrate reductase activity in marine phytoplankton: biochemical analysis and ecological implications. *J. Phycol.* 36: 304-313.
- Garrett, R.H., Grisham, C.M. 1995. *Biochemistry.* Harcourt Brace.
- Gast, R. J., Dennett, M.R. & Caron, D.A. 2004. Characterization of protistan assemblages in the Ross Sea, Antarctica, by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70: 2028-2037.
- Ge, X., Cain, K. & Hirschberg, R. 1990. Urea metabolism and urease regulation in the cyanobacterium *Anabaena variabilis*. *Can. J. Microbiol.* 36: 218-222.
- Geider, R.J. & LaRoche, J. 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37: 1-17.
- Glibert, P.M., Alexander, J., Trice, T.M., Michael, B., Magnien, R.E., Lane, L., Oldach, D. & Bowers, H. 2002. Chronic urea loading: a correlate of *Pfiesteria* spp. in the Chesapeake and Coastal Bays of Maryland. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., and Vargo, G.A. (eds). *Harmful Algae 2002, Proceedings of the Xth International Conference on Harmful Algae.* Florida Fish and Wildlife Conservation Commission and Intergovernmental Oceanographic Commission of UNESCO, USA. pp. 48-55.
- Glibert, P.M. & Capone, D.G. 1993. Mineralization and assimilation in aquatic, sediment, and wetland systems. In Knowles, R., Blackburn, T.H. (eds). *Nitrogen isotope techniques.* Academic Press, New York, pp. 243-272
- Glibert, P.M., Conley, D.J., Fisher, T.R., Harding, L.W. & Malone, T.M. 1995. Dynamics of the 1990 winter/spring bloom in Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 122: 27-43.
- Glibert, P.M., Garside, C., Fuhrman, J.A. & Roman, M.R. 1991. Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnol. Oceanogr.* 36: 895-909.

- Glibert, P.M., Harrison, J., Heil, C., & Seitzinger, S. 2006. Escalating worldwide use of urea – a global change contributing to coastal eutrophication. *Biogeochemistry*. 77:441-463.
- Glibert, P.M., Heil, C.A., Hollander, D., Revilla, M., Hoare, A., Alexander, J. & Murasko, S. 2004. Evidence for dissolved organic nitrogen and phosphorus uptake during a cyanobacterial bloom in Florida Bay. *Mar. Ecol. Prog. Ser.* 280: 73-83.
- Glibert, P.M., Magnien, R., Lomas, M.W., Alexander, J., Fan, C., Haramoto, E., Trice, M. & Kana, T.M. 2001. Harmful algal blooms in the Chesapeake and Coastal Bays of Maryland, USA: Comparison of the 1997, 1998 and 1999 Events. *Estuaries*. 24: 875-883.
- Glibert, P.M. & McCarthy, J.J. 1984. Uptake and assimilation of ammonium and nitrate by phytoplankton: indices of nutritional status for natural assemblages. *J. Plank. Res.* 6: 677-697.
- Glibert, P.M & Terlizzi, D.E. 1999. Cooccurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. *Appl. Environ. Microbiol.* 65: 5594-5596.
- Glibert, P.M., Trice, T.M, Michael, B. & Lane, L. 2005. Urea in the tributaries of the Chesapeake and Coastal Bays of Maryland. *Water, Air, and Soil Pollution*. 160: 229-243.
- Goel, A., McConnell, L.L. & Torrents, A. 2005. Wet deposition of current use pesticides at a rural location on the Delmarva peninsula: impact of rainfall patterns and agricultural activity. *J. Agric.Food Chem.* 53: 7915-7924.
- Guillard, R.R.L. & Ryther, J.H. 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Dentonula conferece* (Cleve) Gran. *Can. J. Microbiol.* 8:229-39.
- Hagy, J.D., Boynton, W.R. & Jasinski, D.A. 2005. Modelling phytoplankton deposition to Chesapeake Bay sediments during winter-spring: interannual variability in relation to river flow. *Estuar. Coastal Shelf Sci.* 62: 25-40.
- Harding, L.W. 1994. Long-term trends in the distribution of phytoplankton in Chesapeake Bay: roles of light, nutrients, and streamflow. *Mar. Ecol. Prog. Ser.* 104: 267-291.
- Hausinger, R. P. 2004. Metabolic versatility of prokaryotes for urea decomposition. *J. Bacteriol.* 186: 2520-2522.

- Heil, C.A., Revilla, M., Glibert, P.M. & Murasko, S. *In press*. Nutrient quality drives differential phytoplankton community composition on the West Florida Shelf. *Limnol. Oceanogr.*
- Hildebrand, M. & Dahlin, K. 2000. Nitrate transporter genes from the diatom *Cylindrotheca fusiformis* (Bacillariophyceae): mRNA levels controlled by nitrogen source and by the cell cycle. *J. Phycol.* 36: 702-713.
- Hodson, R.C., Williams, S.K. & Davidson, W.R. 1975. Metabolic control of urea catabolism in *Chlamydomonas reinhardtii* and *Chlorella pyrenoidsa*. *Journal of Bacteriology* 121:1022-1035.
- Holmboe, N., Jensen, H.S. & Anderson, F.O. 1999. Nutrient addition bioassays as indicators of nutrient limitation of phytoplankton in an eutrophic estuary. *Mar. Ecol. Prog. Ser.* 186: 95-104.
- Holmes, R.M., Aminot, A., Kerouel, R., Hooker, B.A. & Peterson, B.J. 1999. *Can. J. Fish. Aquat. Sci.* 56: 1801-1808.
- Horrigan, S.G. & McCarthy, J.J. 1982. Phytoplankton uptake of ammonia and urea during growth on oxidized forms of nitrogen. *J. Plank. Res.* 4: 379-89.
- Iriarte, J.L., Quiñones, R.A. & Gonzalez, R.R. 2005. Relationship between biomass and enzymatic activity of a bloom-forming dinoflagellate (Dinophyceae) in southern Chile (41°S): a field approach. *J. Plank. Res.* 27: 159-166.
- Jahns, T. 1992. Urea uptake by the marine bacterium *Deleya venusta* HG1. *J. Gen. Microbiol.* 138: 1815-1820.
- Jahns, T., Schafer, U. & Kaltwasser, H. 1995. Heat-stable ureases from two filamentous cyanobacteria. *Microbiology* 141:737-741.
- Jørgensen, N.O.G. 2006. Uptake of urea by estuarine bacteria. *Aquat. Microb. Ecol.* 42: 227-242.
- Jørgensen, N.O.G., Tranvik, L.J. & Berg, G.M. 1999. Occurrence and bacterial cycling of dissolved nitrogen in the Gulf of Riga, the Baltic Sea. *Mar. Ecol. Prog. Ser.* 191:1-18.
- Kana, T.M., Lomas, M.W., MacIntyre, H.L., Cornwell, J.C. & Gobler, C. J. 2004. Stimulation of the brown tide organism, *Aureococcus anophagefferens*, by selective nutrient additions to in situ mesocosms. *Harmful Algae* 3: 377-388.
- Kaufman, Z.G., Lively, J.S. & Carpenter, E.J. 1983. Uptake of nitrogenous nutrients by phytoplankton in a barrier-island estuary – Great South Bay, New York. *Est. Shelf. Coastal. Sci.* 17: 483-493.

- Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde, E.D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M. & Stevenson, J.C. 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. *Mar. Ecol. Prog. Ser.* 303:1-29.
- Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J. M. & Place, A.R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. *Harmful Algae* 1:233-241.
- Kirchman, D.L. 1994. The uptake of inorganic nutrients by heterotrophic bacteria. *Microb. Ecol.* 28:255-271.
- Kirchman, D.L. 2000. Uptake and regeneration of inorganic nutrients by marine heterotrophic bacteria. In *Microbial Ecology of the Oceans*. [Ed. D. L. Kirchman]. Wiley-Liss. New York. pp. 261-288.
- Kramer, J.G & Morris, I. 1990. Growth regulation in irradiance limited marine *Synechococcus* sp. WH 7803. *Arch. Microbiol.* 154: 286-293.
- Kristiansen, S. 1983. Urea as a nitrogen source for the phytoplankton in the Oslofjord. *Mar. Biol.* 74:17-24.
- Kudela, R.M. & Cochlan, W.P. 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21:31-47.
- Kudo, I., Yoshimura, T., Yanada, M. & Matsunaga, K. 2000. Exhaustion of nitrate terminates a phytoplankton bloom in Funka Bay, Japan: change in SiO₄:NO₃ consumption rate during the bloom. *Mar. Ecol. Prog. Ser.* 193: 45-51.
- Lane, L., Rhoades, S., Thomas, C. & Van Heukelem, L. 2000. Analytical Services Laboratory Standard Operating Procedures. Technical Report No. TS-264-00. Horn Point Laboratory, University of Maryland Center for Environmental Science.
- Leftley, J.W., & Syrett, P.J. 1973. Urease and ATP:Urea amidolyase activity in unicellular algae. *J. Gen. Microbiol.* 77:109-115.
- Lewitus, A.J., White, D.L., Tymowski, R.G., Geesey, M.E., Hymel, S.N. & Noble, P.A. 2005. Adapting the CHEMTAX method for assessing phytoplankton taxonomic composition in southeastern U.S. estuaries. *Estuaries.* 28: 160-172.
- L'Hulguen, S., Slawyk, G. & Le Corre, P. 2005. Seasonal patterns of urea regeneration by size-fractionated microheterotrophs in well-mixed temperate coastal waters. *J. Plank. Res.* 27: 263-270.

- Li, A., Stoecker, D.K. & Adolf, J.E. 1999. Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. *Aquat. Microb. Ecol.* 10:139-147.
- Li, A., Stoecker, D.K. & Coats, D.W. 2000. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. *J. Plank. Res.* 22:2105-2124.
- Li, A., Stoecker, D.K. & Coats, D.W. 2001. Use of the 'food vacuole content' method to estimate grazing by the mixotrophic dinoflagellate *Gyrodinium galatheanum* on cryptophytes. *J. Plank. Res.* 23: 303-318.
- Lomas, M.W. 2004. Nitrate reductase and urease enzyme activity in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae): Interactions between nitrogen substrates. *Mar. Biol.* 144: 37-44.
- Lomas, M.W. & Glibert, P.M. 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 556-72.
- Lomas, M.W. & Glibert, P.M. 1999. Interactions between NH_4^+ and NO_3^- uptake and assimilation: comparisons of diatoms and dinoflagellates at several growth temperatures. *Marine Biology*. 133: 541-551.
- Lomas, M.W. & Glibert, P.M. 2000. Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. *J. Phycol.* 36: 903-913.
- Lomas, M.W., Swain, A., Shelton, R. & Ammerman, J.W. 2004. Taxonomic variability of phosphorus stress in Sargasso Sea phytoplankton. *Limnol. Oceanogr.* 49: 2303-2310.
- Lomas, M.W., Trice, T.M, Glibert, P.M., Bronk, D.A. & McCarthy, J.J. 2002. Temporal and spatial dynamics of urea uptake and regeneration rates and concentrations in Chesapeake Bay. *Estuaries* 25: 469-482.
- Loomis, W.D. & Battaile, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5: 423-438.
- Lomstein, B.A., Blackburn, T.H. & Henriksen, K. 1989. Aspects of nitrogen and carbon cycling in the northern Bering Shelf sediment. 1. The significance of urea turnover in the mineralization of NH_4^+ . *Mar. Ecol. Prog. Ser.* 57:237-247.
- Lund, B.A. 1987. Mutual interference of ammonium, nitrate, and urea on uptake of ^{15}N sources by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J. Exp Mar. Biol. Ecol.* 113:167-180.

- Lund, B.A. & Blackburn, T.H. 1989. Urea turnover in a coastal marine sediment measured by ^{14}C -urea short-term incubation. *J. Microbiol. Methods* 9:297-308.
- Mace, K.A., Kubilay, N. & Duce, R.A. 2003. Organic nitrogen in rain and aerosol in the eastern Mediterranean atmosphere: An association with atmospheric dust. *J. Geophysical Res. Atmos.* 108:4320-4331
- Mackey, M.D., Higgins, H.W., Mackey, D.J. & Wright, S.W. 1996. CHEMTAX - a program for estimating class abundances from chemical markers – application to HPLC measurements of phytoplankton. *Mar. Ecol. Prog. Ser.* 144: 265-283.
- Malone, T.C., Crocker, L.H. Pike, S.E. & Wendler, B.W. 1988. Influences of river flow on the dynamics of phytoplankton production in a partially stratified estuary. *Mar. Ecol. Prog. Ser.* 48: 235-249.
- Martin, J.H., Gordon, R.M. & Fitzwater, S.E. 1991. The case for iron. *Limnol. Oceanogr.* 36: 1793-1802.
- Maryland Department of Agriculture. 1990-2004. Annual Fertilizer Tonnage Report.
- McCarthy, J.J. 1972. The uptake of urea by natural population of marine phytoplankton. *Limnol. Oceanogr.* 17:738-748.
- McIninch, J.K., McIninch, J.D. & May, S.W. 2003. Catalysis, stereochemistry, and inhibition of ureidoglycolate lyase. *J. Biol. Chem.* 278: 50091-50100.
- Menden-Duer, S. & Lessard, E.J. 2000. Carbon to volume relationships for dinoflagellates, diatoms and other protist plankton. *Limnol. Oceanogr.* 45:569-579.
- Middelburg, J.J. & Nieuwenhuize, J. 2000. Nitrogen uptake by heterotrophic bacteria and phytoplankton in the nitrate-rich Thames estuary. *Mar. Ecol. Prog. Ser.* 203: 13-21.
- Milligan, A.J. & Harrison, P.J. 2000. Effects of non-steady-state iron limitation on nitrogen assimilatory enzymes in the marine diatom *Thalassosira weissflogii* (Bacillariophyceae). *J. Phycol.* 36: 78-86.
- Miller, C.A. & Glibert, P.M. 1998. Nitrogen excretion by the calanoid copepod *Acartia tonsa*: results of mesocosm experiments. *J. Plank. Res.* 20: 1767-1780.
- Miller, S.R. & Castenholz, R.W. 2001. Ecological physiology of *Synechococcus* sp. Strain SH-94-5, a naturally occurring cyanobacteria deficient in nitrate assimilation. *Appl. Environ. Microbiol.* 67: 3002-3009
- Mobley, H.L.T. & Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. *Microbiol. Rev.* 3: 85-108.

- Mobley, H.L.T., Island, M.D. & Hausinger, R.P. 1995. Molecular Biology of Microbial Ureasases. *Microbiol. Rev.* 59: 451-480.
- Molloy, C.J. & Syrett, P.J. 1988. Interrelationships between uptake of urea and uptake of ammonium by microalgae. *J. Exp. Mar. Biol. Ecol.* 118: 85-95.
- Moore, L.R., Post, A.F., Rocap, G., & Chisholm, S.W. 2002. Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Limnol. Oceanogr.* 47: 989-996.
- Mulholland, M.R., Boneill, G. & Minor, E.C. 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. *Harmful Algae* 3:361-376.
- Mulholland, M.R., Ohki, K. & Capone, D.G. 1999. Nitrogen utilization and metabolism relative to patterns of N₂ fixation in cultures of *Trichodesmium* NIBB1067. *J. Phycol.* 35: 977-988.
- Mulvenna, P.F & Savidge, G. 1992. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. *Estuar. Coastal Shelf Sci.* 34: 429-438.
- Muro-Pastor, M.I., Reyes, J.C. & Florencio, F.J. 2005. Ammonium assimilation in cyanobacteria. *Photosynthesis Research* 83: 135-150.
- Nolden, L., Beckers, G., Mockel, B., Pfefferle, W., Nampoothiri, K.M., Kramer, R. & Burkovski, A. 2000. Urease of *Corynebacterium glutamicum*: organization of corresponding genes and investigation of activity. *FEMS Microb. Let.* 189: 305-310.
- Ohki, K., Zehr, J.P. & Fujita, Y. 1992. Regulation of nitrogenase activity in relation to the light-dark regime in the filamentous non-heterocytosus cyanobacterium *Trichodesmium* sp. NIBB 1067. *J. Gen Microbiol.* 138:2679-2685.
- Oliveira, L. & Antia, N.J. 1986. Some observations on the urea-degrading enzyme of the diatom *Cyclotella cryptica* and the role of nickel in its production. *J. Plank. Res.* 8: 235-342.
- Palinska, K.A., Jahns, T., Rippka, R., Tandeau de Marsac, N. 2000. *Prochlorococcus marinus* strain PCC 9511, a picoplankton cyanobacterium, synthesizes the smallest urease. *Microbiology* 146: 3099-3107.
- Pantoja, S. & Lee, C. 1994. Cell-surface oxidation of amino acids in seawater. *Limnol. Oceanogr.* 39: 1718-1726.

- Parker, M.S. & Armbrust, E.V. 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 41:1142-1153.
- Parsons, R.T., Maita, Y. & Lalli, C.M. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press, New York, N.Y.
- Pedersen, H., Lomstein, B.A. & Blackburn, T.H. 1993. Evidence for bacterial urea production in marine sediments. *FEMS Microbiology Ecology.* 12:51-59.
- Peers, G.S., Milligan, A.J. & Harrison, P.J. 2000. Assay optimization and regulation of urease activity in two marine diatoms. *J. Phycol.* 36: 523-528.
- Petersen, J.E., Kemp, W.M., Bartleson, R., Boynton, W.R., Chen, .CC., Cornwell, J.C., Gardner, R.H., Hinkle, D.C., Houde, E.D., Malone, T.C., Mowitt, W.P., Murray, L., Sanford, L.P., Stevenson, J.C., Sundberg, K.L., Suttles, S.E. 2003. Multiscale experiments in coastal ecology: Improving realism and advancing theory. *Bioscience.* 53: 1181-1197.
- Peierls, B.L. & H.W. Paerl. 1997. Bioavailability of atmospheric organic nitrogen deposition to coastal phytoplankton. *Limnol. Oceanogr.* 42: 1819-1823.
- Rai, A.K. & Singh, S. 1987. Urease of blue-green algae (Cyanobacteria) *Anabaena doliolum* and *Anacystis nidulans*. *Curr. Microbiol.* 16: 113-117.
- Raimbault, P. & Mingazzini, P. 1987. Diurnal variations of intracellular nitrate storage by marine diatoms: Effects of nutritional state. *J. Exp. Mar. Biol. Ecol.* 112: 217-232.
- Redfield, A.C. 1958. The biological control of chemical factors in the environment. *American Scientist* 46: 205-221.
- Rees, T.A.V. & Bekheet, I.A. 1982. The role of nickel in urea assimilation by algae. *Planta* 156: 385-387.
- Rees, T.A.V. & Syrett P.J. 1979. The uptake of urea by the diatom *Phaeodactylum*. *New Phytol.* 82: 169-178.
- Reid, R.T. & Butler, A. 1991. Investigation of the mechanism of iron acquisition by the marine bacterium *Alteromonas luteoviolaceus* – characterization of siderophore production. *Limnol. Oceanogr.* 36:1783-1792.
- Revilla, M., Alexander, J. & Glibert, P.M. 2005. Analysis of urea in coastal waters: comparison of the enzymatic and the direct method. *Limnol. Oceanogr. Methods* 3: 290-299.

Rivkin, R.B., Legendre, L., Deibel, D., Tremlay, J-E., Klein, B., Crocker, K., Roy, S., Silverberg, N., Lovejoy, C., Mesple, F., Romero, N., Anderson, M.R., Matthews, P., Savenkoff, C., Vezina, A., Therriault, J-C., Wesson, J., Berube, C. & Ingram, R.G. 1996. Vertical flux of biogenic carbon in the ocean: Is there food web control? *Science*. 272: 1163-1166.

Salerno, M. 2005. Ectocellular enzyme activities in the mixotrophic dinoflagellate *Prorocentrum minimum* (Dinophyceae). Masters of Science Thesis. University of Maryland, College Park.

Schulz, K.G., Zondervan, I., Gerringa, L.J.A., Timmermans, K.R., Veldhuis, M.J.W. & Riebesell, U. 2004. Effect of trace metal availability on coccolithophorid calcification. *Nature* 430: 673-676.

Seguineau, C., Batrel, Y. & Le Gal, Y. 1989. Glutamine synthase of *Dunaliella primolecta*. Partial characterization and possible adenylation control in relation to nitrogen nutrient levels. *Biochemical Systematics and Ecology* 17: 503-508.

Seguineau C., Batrel, Y., Martin-Jezequel, V. & La Gal, Y. 1987. Glutamine synthetase and the assimilation of inorganic nitrogen in *Dunaliella primolecta*. *Oceanis* 13: 515-20.

Shaked, Y., Xu Y., Leblanc K., & Morel, F.M.M. 2006. Zinc availability and alkaline phosphatase activity in *Emiliana huxleyi*: Implications for Zn-P co-limitation in the ocean. *Limnol. Oceanogr.*, 51:299-309.

Seigenthaler, U., Sarmiento, J.L. 1993. Atmospheric carbon dioxide and the ocean. *Nature*. 365: 119-125.

Siewe, R.M., Weil, B., Burkovski, A., Eggeling, L., Krämer, R. & Jahns, T. 1998. Urea uptake and urease activity in *Corynebacterium glutamicum*. *Arch. Microbiol.* 169: 411-416.

Singh, S. 1990. Regulation of urease activity in the cyanobacterium, *Anabaena doliolum*. *FEMS Microbiol. Lett.* 67:79-84.

Singh, S. 1993. Role of glutamine synthetase, glutamine and NH_4^+ regulation in urea metabolism in cyanobacterium, *Nostoc muscorum*. *Indian J. Exp. Biol.* 31: 256-259.

Soh, K.G. 2001. A review of the global fertilizer use by product. 7th AFA Annual Conference. Cairo.

Solomon, C.M., Glibert, P.M. & Collier, J.L. 2006. Potential regulation of urea utilization by nitrogen sources: a synthesis from laboratory and field experiments. American Society of Limnology and Oceanography Summer Meeting. Victoria, B.C. June 2006.

- Soloranzo, L. & Sharp, J.H. 1980. Determination of the total dissolved phosphorus and particulate phosphorus in natural waters. *Limnol. Oceanogr.* 25: 754-752.
- Song, B. & Ward, B.B. 2004. Molecular characterization of the assimilatory nitrate reductase gene and its expression in the marine green alga *Dunaliella tertiolecta* (Chlorophyceae). *J. Phycol.* 40:721-731.
- Staver, L.W., Staver, K.W. & Stevenson, J.C. 1996. Nutrient inputs to the Choptank River estuary: implications for watershed management. *Estuaries* 19:342-358.
- Steidinger, K.A., Vargo, G.A., Tester, P.A. & Tomas, C.R. 1998. Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. In: Anderson D.M., Cembella A.D., Hallegraeff G.M. (eds) *Physiological ecology of harmful algal blooms*. Springer-Verlag, Heidelberg, p. 133-153.
- Stewart, B.M. 1985. Effect of temperature on the formation of indophenol blue in a spectrometric method for the determination of ammonium. *Water Res.* 19:1443-1445.
- Syrett, P.J. & Leftley, J.W. 1976. Nitrate and urea assimilation by algae. In Sunderland, N. [Ed.] *Perspectives in Experimental Biology*. Pergamon Press, Oxford. pp. 221-234
- Syrett, P.J. & Peplinksa, A.M. 1988. The effect of nickel and nitrogen deprivation on the metabolism of urea by the diatom *Phaeodactylum tricorutum*. *Br. Phycol. J.* 23: 387-390.
- Takayanagi, K., Wong, G.T.F. & Filardo, M.J. 1989. Nitrate reductase activity and the speciation of selenium at the mouth of the Chesapeake Bay. *J. Ocean. Soc. Japan.* 45:129-133.
- Tamminen, T. & Irmisch, A. 1996. Urea uptake kinetics of a midsummer planktonic community on the SW coast of Finland. *Mar. Ecol. Prog. Ser.* 130: 201-211.
- Tengs, T., Dahlberg, O.J., Shalchian-Tabrizi, K., Klaveness, D., Rudi, K., Delwiche, C.F. & Jakobsen, K.S. 2000. Phylogenetic analyses indicate that the 19' hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol. Biol. Evol.* 17:718-729.
- Therkildsen, M.S., Isaken, M.F. & Lomstein, B.A. 1997. Urea production by the marine bacteria *Delacya venusta* and *Pseudomonas stutzeri* grown in a minimal medium. *Aquat. Microb. Ecol.* 13: 213-217.
- Therkildsen, M.S. & Lomstein, B.A. 1994. Seasonal variation in sediment urea turnover in a shallow estuary. *Mar. Ecol. Prog. Ser.* 109: 77-82.
- Tillett, D. & Neilan, B.A. 2000. Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. *J. Phycol.* 36: 251-258.

- Todd, M.J. & Hausinger, R.P. 1987. Purification and characterization of the nickel-containing multicomponent urease from *Klebsiella aerogenes*. *J. Biol. Chem.* 262: 5963-5967.
- Tomas, C.R. 2005. Nitrogen preference of the fish-killing flagellate *Chattonella cf. verruculosa*. GEOHAB Open Science Meeting: HABs and Eutrophication. Baltimore, Maryland, USA. February 2005.
- Tremblay, J-E, Legendre L., Klein, B. & Therriault, J-C. 2000. Size-differential uptake of nitrogen and carbon in a marginal sea (Gulf of St. Lawrence, Canada): significance of diel periodicity and urea uptake. *Deep-Sea Research II* 47:489-518.
- Twomey, L.J., Piehler, M.F. & Paerl, H.W. 2005. Phytoplankton uptake of ammonium, nitrate, and urea in the Neuse River Estuary, NC, USA. *Hydrobiologia*. 533: 123-134.
- van Heukelem, L., Lewitus, A., Kana, T.M. & Craft, N. 1994. Improved separation of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Mar. Ecol. Prog. Ser.* 114: 303-313.
- van Heukelem, L. & Thomas, C.S. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chrom. A*. 910:31-49.
- Wheeler, P. 1983. Phytoplankton nitrogen metabolism.. In Carpenter, E.J & Capone, D.G. [Eds] *Nitrogen in the Marine Environment*. Academic Press. New York, N.Y. pp. 309-346.
- Wheeler, P.A., Glibert, P.M. & McCarthy, J.J. 1982. Ammonium uptake and incorporation by Chesapeake Bay phytoplankton: Short term uptake kinetics. *Limnol. Oceanogr.* 27:1113-1128.
- Wheeler, P.A. & Kirchman, D.L. 1986. Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnol. Oceanogr.* 31: 998-1009.
- Yin, K., Qian, P., Wu, M.C.S., Chen, J.C., Huang, L., Song, X., Jian, W. 2001. Shift from P to N limitation of phytoplankton growth across the Pearl River estuarine plume during summer. *Mar. Ecol. Prog. Ser.* 221:17-28.
- Zehr, J.P. & Capone, D.G. 1996. Problems and promises of assaying the genetic potential for nitrogen fixation in the marine environment. *Microb. Ecol.* 32: 263-281.
- Zehr, J.P., Carpenter, E.J. & Villareal, T.A. 2000. New perspectives on nitrogen-fixing organisms in tropical and subtropical oceans. *Trends Microbiol.* 8:68-73.

Zehr, J.P. & Ward, B.B. 2002. Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Appl. Environ. Microbiol.* 68: 1015-1024.

Zubkov, M.V. & Tarran, G.A. 2005. Amino acid uptake of *Prochlorococcus* spp. in surface waters across the South Atlantic Subtropical Front. *Aquat. Microbiol. Ecol.* 40: 241-249.