CHAPTER 32

ENZYMES AND N CYCLING

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1. Principles and Practice

1.1. Importance of enzyme-mediated reactions

Enzyme-mediated reactions are responsible for most of the transformations of nitrogen (N) that occur in the marine environment, thus measurements of enzymes have figured prominently in oceanographic work for some time, especially in terms of N assimilation (see Falkowski, 1983).

Many different measurements of enzymes are undertaken to acquire different types of information. For example, the presence of genes for an enzyme has been used to infer whether organisms are capable of performing particular functions, the expression of those genes or the appearance of the enzyme protein is used to indicate if and under what conditions the gene is functioning, while assay of the activity of the enzyme has been used to infer rates of particular processes (see Section 2). In fact, virtually all measurements classified as “molecular” and most rate measurements of “uptake” are, in fact, measurements of enzymes. Because strictly molecular methods (i.e., the capacity to perform a reaction; see Chapter 30 by Zehr and Jenkins, this volume) and N uptake (i.e., the net result of enzymatically mediated processes; see Chapter 6 by Mulholland and Lomas, this volume) are discussed elsewhere in this book; in this chapter, we will focus primarily on measurements of enzyme activities.
Conceptually, measurements of enzyme activity are easier to interpret than measurements of the presence or absence of particular enzymes, genes, or transcripts or rates of transcription and translation, and are more likely to be related to physiological rates. Measurements of enzyme activity have often been proposed as quantitative indices/estimators of biological rate reactions. Moreover, enzyme activity can often be measured quickly and using conventional ocean-going equipment, so that these measurements are more easily incorporated into oceanographic studies. That said, assays of enzyme activity are subject to different biases than molecular methods, and the interpretation of enzyme activity relies on specific knowledge of reaction pathways and their regulation (see Section 1.3.5). Interpretation of enzyme activity is often complicated when pathways are branched, cyclic, or reversible; when different enzymes produce similar products or compete for similar substrates; or when different pools of the same enzyme are regulated differently.

Paradoxically, as we have gained more information about enzymes from the molecular perspective, the interpretation of enzyme activity measurements and the classification of enzymes have actually become more complicated. First, there are different classification schemes for gene or protein sequences (derived from molecular biology) and biochemical function (derived from classic enzymology). For example, enzyme genes and proteins are classified in terms of homologous structures and sequences. In contrast, in biochemical classification schemes, enzyme nomenclature is based on the reaction that is mediated (the International Union of Biochemistry and Molecular Biology (IUMB) uses Enzyme Commission (EC) numbers for this purpose; Table 32.1). Thus, two evolutionary divergent enzymes that catalyze the same reaction would fall into the same EC category, while their functional proteins might be quite distant based on genetic analysis. In addition, enzymes that catalyze processes such as membrane transport, where there is no actual chemical change, cannot easily be accommodated in the biochemical classification scheme. For example, because they mediate no chemical transformation “permases” or “translocases” are generally not included in enzymological classifications, yet are frequently adopted in the molecular biology literature. In this discussion, we will generally classify enzymes according to activity (i.e., EC numbers) but we will also consider pathways that mediate no chemical transformation.

In 1983, Falkowski observed, “there is little information on the N enzymology of marine organisms” and also noted that there was “scanty” information about transporters (Falkowski, 1983). At that time, most of the information available for enzymes was based on activity assays with relatively few attempts to purify enzymes and characterize their structural and functional diversity, and very few attempts to use molecular or other techniques to probe the full range of enzymes mediating
<table>
<thead>
<tr>
<th>Enzyme name</th>
<th>EC numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Uptake, reduction and assimilation</td>
<td></td>
</tr>
<tr>
<td>Membrane transporters</td>
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</tr>
<tr>
<td>Polar-amino-acid-transporting ATPase</td>
<td>3.6.3.21</td>
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<td>Nonpolar-amino-acid-transporting ATPase</td>
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<td>Oligopeptide-transporting ATPase</td>
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<td>Nitrate-transporting ATPase</td>
<td>3.6.3.26</td>
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<td>Polyamine-transporting ATPase</td>
<td>3.6.3.31</td>
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<tr>
<td>Quaternary-amine-transporting ATPase</td>
<td>3.6.3.32</td>
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<tr>
<td>Taurine-transporting ATPase</td>
<td>3.6.3.36</td>
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<tr>
<td>Guanine-transporting ATPase</td>
<td>3.6.3.37</td>
</tr>
<tr>
<td>Peptide-transporting ATPase</td>
<td>3.6.3.43</td>
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<tr>
<td>Nitrate reductase (assimilatory)</td>
<td>1.7.1.1(NADH-specific)</td>
</tr>
<tr>
<td></td>
<td>1.7.1.2(NADH(P)H-bispecific)</td>
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<tr>
<td></td>
<td>1.7.1.3(NAD(P)H-specific)</td>
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<tr>
<td></td>
<td>1.7.7.2(Ferredoxin-specific)</td>
</tr>
<tr>
<td>Nitrite reductase (assimilatory)</td>
<td>1.7.7.1(Ferredoxin),</td>
</tr>
<tr>
<td></td>
<td>1.7.1.4(NAD(P)H-bispecific)</td>
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<td>Glutamate dehydrogenase</td>
<td>1.4.1.2 (NADH)</td>
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<td></td>
<td>1.4.1.3(NAD(P)H)</td>
</tr>
<tr>
<td></td>
<td>1.4.1.4(NADPH)</td>
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<td>Glutamate synthase</td>
<td>1.4.1.13(NADPH)</td>
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<td>1.4.1.14(NADH)</td>
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<td></td>
<td>1.4.7.1(Ferredoxin)</td>
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<tr>
<td>Glutamine synthetase</td>
<td>6.3.1.2</td>
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<tr>
<td>Nitrogenase</td>
<td>1.18.6.1 (Ferredoxin)</td>
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<tr>
<td></td>
<td>1.19.6.1(Flavodoxin)</td>
</tr>
<tr>
<td>Urease</td>
<td>3.5.1.5</td>
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<td>Urea carboxylase</td>
<td>6.3.4.6</td>
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<td>Allophanate hydrolase</td>
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(Continued)
Table 32.1  Enzyme activities relevant to marine nitrogen cycling (continued)

<table>
<thead>
<tr>
<th>Enzyme name</th>
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</tr>
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<tbody>
<tr>
<td>(b) N compounds as electron sources or sinks</td>
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<tr>
<td>Nitrate reductase (dissimilatory)</td>
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<td>Nitrite reductase (dissimilatory)</td>
<td>1.9.6.1 (Cytochrome)</td>
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<tr>
<td>Nitric oxide reductase</td>
<td>1.7.2.1 (NO-forming)</td>
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<tr>
<td>Nitrous oxide reductase</td>
<td>1.7.99.7</td>
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<tr>
<td>Nitrite reductase</td>
<td>1.7.99.6</td>
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<tr>
<td>Hydroxylamine oxidase</td>
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</tr>
<tr>
<td>Nitrite oxioreductase</td>
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</tr>
<tr>
<td>Hydroxylamine reductase</td>
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<td>Hydroxylamine oxioreductase</td>
<td>1.7.99.8</td>
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<tr>
<td>(c) Synthetic pathways for secondary compounds</td>
<td></td>
</tr>
<tr>
<td>(d) Turnover and maintenance of internal N</td>
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<tr>
<td>Proteases</td>
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<tr>
<td>Aminopeptidases</td>
<td>3.4.11.x</td>
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<td>Dipptidases</td>
<td>3.4.13.x</td>
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<td>Du/tripeptidyl-peptidases</td>
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<td>Peptidyl-dipeptidases</td>
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<td>3.4.16.x</td>
</tr>
<tr>
<td>Metallocarboxypeptidases</td>
<td>3.4.17.x</td>
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<tr>
<td>Cysteine carboxypeptidases</td>
<td>3.4.18.x</td>
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<tr>
<td>Omega peptidases</td>
<td>3.4.19.x</td>
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<td>Serine endopeptidases</td>
<td>3.4.21.x</td>
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<tr>
<td>Cysteine endopeptidases</td>
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<tr>
<td>Aspartic endopeptidases</td>
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<td>Metalloendopeptidases</td>
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<td>Threonine endopeptidases</td>
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<td>Other endopeptidases</td>
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<tr>
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<td>D-/-L-Amino acid oxidases</td>
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<td>D-Amino-acid oxidase</td>
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<td>Amine oxidase (flavin-containing)</td>
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<td>Amine oxidase (copper-containing)</td>
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<td>D-Glutamate oxidase</td>
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<td>Putrescine oxidase</td>
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<td>L-Glutamate oxidase</td>
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<td>D-Glutamate(D-aspartate) oxidase</td>
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<td>L-Aspartate oxidase</td>
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<td>Glycine oxidase</td>
<td>1.4.3.19</td>
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<td>Transaminases</td>
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<td>(e) Other enzymes relevant to N cycling</td>
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<td>Rubisco</td>
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<td>Isocitrate dehydrogenase</td>
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<td></td>
<td>1.1.1.42 (NADP)</td>
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<td>Succinate dehydrogenase</td>
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<td>Citrate synthase</td>
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</tr>
<tr>
<td>Carbon–nitrogen lyases</td>
<td>4.3.x.x</td>
</tr>
<tr>
<td>Forming carbon–nitrogen bonds</td>
<td>6.3.x.x</td>
</tr>
<tr>
<td>Acting on carbon–nitrogen bonds, other than peptide bonds</td>
<td>3.5.x.x</td>
</tr>
<tr>
<td>Chitinase</td>
<td>3.2.1.14</td>
</tr>
</tbody>
</table>
reactions affecting the marine N cycle. Much of the information regarding N assimilation was based on analogies with processes in higher plants and freshwater green algae. Remarkably, much of this information has proven to be correct. Since 1983, the technical aspects of enzyme measurements have changed radically. For perspective, the first monoclonal antibodies were produced less than a decade before (Köhler and Milstein, 1975), practical DNA sequencing methods were in their infancy (Maxam and Gilbert, 1977; Sanger et al., 1977), mainstays such as the polymerase chain reaction had yet to be put forward (Saiki et al., 1988), and automated DNA sequencers were still the stuff of science fiction (Smith et al., 1986). A remarkable suite of molecular measurements are now considered routine (see Chapter 30 by Jenkins and Zehr, this volume). While the principles of enzyme activity measurements remain largely the same, technical innovations such as improved spectrophotometers (including diode array detectors), compact scanning fluorometers, more sophisticated optics for fluorescence microscopes, and multiwell plate readers (e.g., Butler et al., 1988), as well as the development of highly fluorescent enzyme substrates (e.g., Molecular Probes, Bachem) have now revolutionized the practice. We contend that in order to understand how genetic capacity is expressed in the environment, it is critical to couple molecular measurements with rate estimates of activity in the environment.

In this chapter, our goal is to review the practical aspects of enzyme activity measurements and their interpretation and to illustrate how such measurements have advanced our knowledge of marine N cycling. While our main focus will be the assimilatory pathways found in marine photoautotrophs, we will also consider degradation pathways and secondary metabolism, and will point out other groups of marine organisms where enzyme activity has proven useful in examining aspects of the N cycle.

1.2. Overview of enzymes involved in marine nitrogen cycling

There are an enormous number of enzymes that are relevant to the marine N cycle and we still know little about many of them. Indeed, since all enzymes are proteins, all enzymes are intrinsically part of the N cycle. We can broadly divide enzymes into: (1) those involved in inorganic N acquisition (including uptake, reduction, and assimilation, and acquisition of organic N), (2) those involved in using N compounds as electron sources or sinks (i.e., dissimilatory enzymes), (3) those involved in incorporating N into macromolecules or synthesizing secondary compounds that contain N, (4) those responsible for turnover and maintenance of internal N, and (5) those involved in pathways strongly coupled to N metabolism. Table 32.1 provides a summary of relevant enzymes and enzyme classes, including prokaryotic and eukaryotic autotrophs and heterotrophs.

1.2.1. Nitrogen acquisition

Mulholland and Lomas (this volume, Chapter 7) have reviewed the general uptake and assimilation pathways for photoautotrophs (summarized in Fig. 7.2). Transporter proteins are not enzymes in the strictest sense because they do not catalyze particular chemical transformations, but facilitate entry of specific compounds into
A distinct Transporter Classification (TC) system exists that is analogous to the EC system (http://www.tcdb.org), although there remain areas of overlap (e.g., ATPases are in TC 2.2.2.1, but also fall under EC 3.6.3.x; Table 32.1). Although it is possible to make measurements of nutrient transport and infer “activities” of transporters using techniques that measure membrane potential such as patch-clamping (Boyd and Gradmann, 1999a,b; Taylor and Brownlee, 2003), it is not possible to unambiguously measure such activities using traditional enzyme assays. Immunochemical and gene sequencing methods have been most useful for isolating transporters (Hildebrand and Dahlin, 2000; Lara et al., 1993; Tischner et al., 1989; Chapter 30 by Jenkins and Zehr’s review, this volume).

Reduction of N to NH₄⁺ may be viewed as a committing step on the way to assimilation. Enzymes mediating these reactions are attractive targets for study because they generally represent linear pathways relating enzyme activity to metabolic flux of particular compounds: nitrogenase (N₂), nitrate reductase (NO₃⁻), and nitrite reductase (NO₂⁻) (Table 32.1). Assimilation of N generally refers to the synthesis of small organic molecules, such as amino acids, from inorganic N and some cellular C compound, and enzymes responsible for this process include glutamine synthetase (GS), glutamate synthase, and glutamate dehydrogenase (Table 32.1).

There are also a number of enzymes that degrade organic N compounds, inside or outside the cell, thereby making N available for assimilation or uptake, respectively. Examples we will consider include urease, amino acid oxidases, and extracellular peptidases (Mulholland et al., 2002; Mulholland and Lee, submitted for publication, Chapter 7 by Mulholland and Lomas, Fig. 2, this volume; Table 32.1). In addition, for heterotrophic organisms, digestive enzymes, especially proteases, are important for internal N cycling, recouping cellular N, and excretion.

1.2.2. Nitrogen compounds as electron sources or sinks

In addition to N assimilation, N compounds in nature can also be used as electron sources or sinks, especially by prokaryotes (but not exclusively, see Zvyagil’skaya et al., 1996). The enzymes that mediate these dissimilatory reactions have been studied extensively in the context of inputs and losses of fixed N in the marine N budget. Many of these measurements have been based on enzyme activities (e.g., dissimilatory nitrate reductase). Since dissimilatory reactions have been reviewed elsewhere in this volume (Chapter 5 by Ward and Chapter 6 by Devol, this volume), these will not be discussed further and we refer the reader to these chapters.

1.2.3. Incorporation of nitrogen into macromolecules and secondary compounds

Incorporation of N is typically viewed as flux of inorganic N into macromolecules such as proteins and nucleic acids. Pathways of protein and nucleic acid synthesis are complex, and highly regulated so that single enzyme activities are unlikely to provide useful information regarding their production and turnover. In addition to what we think of as “conventional” metabolites, marine organisms contain a bewildering array of N-containing secondary compounds, many of which are responsible for toxic effects, cell–cell communication, and other processes we still know little
In particular, enzymes of EC classes 4.3 (C–N lyases), 6.3 (forming C–N bonds), and 3.5 (acting on C–N bonds, other than peptide bonds) are likely to be important in cells, as are transaminases and carboxylases (Table 32.1). Few of these enzymes have been measured in marine organisms, especially with respect to N cycling. However, there is increasing recognition of the importance of these enzymes, for understanding toxic secondary metabolites such as domoic acid (Pan et al., 1998; Shimizu, 1996), and there have been recent advances in appreciating the importance of such enzymes including the role of N-sulfotransferase in saxitoxin production (Sako et al., 2001).

1.2.4. Turnover and maintenance of internal nitrogen

Intracellular N turnover is poorly understood in marine species despite the fact that N turnover is often used to estimate productivity. In addition, N turnover is often estimated based on C turnover and Redfield stoichiometry but these may not be in balance in natural systems (Mulholland et al., 2006). Since the major pool of cellular N in organisms is in protein, understanding protein turnover is likely to shed light on cellular N turnover in general. Based on our understanding of eubacteria, plants, and animals (e.g., Bond and Butler, 1987; Gottesman and Maurizi, 1992; Vierstra, 1993), it seems likely that the major catabolic pathways for proteins are conserved amongst all of these groups (Fig. 32.1).

Protein turnover is essential for acclimation to changes in the external environment (e.g., irradiance or N supply), release of stored N during N-limited periods, removal of damaged or mis-synthesized N-containing compounds, reorganization of cell morphology to generate resting stages, production of specialized structures such as

**Figure 32.1** Pathways of protein turnover and maintenance in a hypothetical unicellular eukaryotic photoautotroph (after Collos and Berges, 2002). Ub represents ubiquitin and polyubiquitin. Question marks indicate current uncertainties about proteases present in different cell compartments.
heterocysts, cell cycle functions, cellular defense mechanisms, and the creation and removal of intra- and intercellular signaling compounds. Within cells, degradation of protein is accomplished by the activity of hydrolytic enzymes and is regulated by cellular compartmentalization, selectively activating or inactivating particular groups of proteases, or other energy dependent pathways (see Vierstra, 1993). In some cases, the degradation products are then removed from cells or cellular compartments through specific pathways, e.g., urea cycle enzymes, or glutamate dehydrogenase in the case of heterotrophs (e.g., Mayzaud, 1987).

Proteases are traditionally divided into four classes, based on the key amino acids found in the active site of the enzyme: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases (Bond and Butler, 1987), although more recently a number of enzymes have been characterized that defy such a classification systems because they exhibit mixed characteristics and have multiple activities (Liu et al., 2003). Protease activities are also classified by the nature of the cleavage they catalyze: endoproteases (i.e., cleaves within a peptide chain; also called proteinases) versus exoproteases (cleave at the ends of peptide chains) such as aminopeptidases or carboxypeptidases. Proteases are sensitive to pH and as such, their location in cellular compartments may vary (Berges and Falkowski, 1998). In eukaryotic cells large (600–900 kDa), multisubunit proteases with multiple activities (proteasomes) are present. Many details of proteasomes remain unclear (Liu et al., 2003), but this protease complex is involved in the ubiquitin-dependent proteolytic pathway. Ubiquitin (Ub) is a highly-conserved 76-amino-acid peptide that is covalently coupled to proteins targeted for degradation (Kerscher et al., 2006). Proteins labeled with multiple Ub molecules (polyubiquitinated proteins) are rapidly degraded by proteasomes. It has also become clear that there are several other polypeptides found in cells that share similarities with Ub and function in protein modification, e.g., SUMO, ISG15, Nedd8, and Atg8 (Kerscher et al., 2006). For example, SUMO is a 100-amino-acid peptide with 20% homology to Ub. While some of these polypeptide-tagging systems may have proteolytic functions (e.g., SUMO, Colby et al., 2006), others may function to regulate transcription, signal transduction, and the cell cycle (Kerscher et al., 2006).

There is evidence that the majority of protein turnover within higher plant cells may occur via the Ub/proteasome pathway (Rock et al., 1994; Vierstra, 2003); the few data available from marine organisms suggest that the pathway is very well conserved (e.g., Moriyasu and Malek, 2004; Tonon et al., 2003; Berges, unpublished annotation from Thalassiosira pseudonana genome sequencing project). Within prokaroytes and organelles of eukaryotic cells that have evolutionary roots in the prokaryotic domain, Ub and proteasomes are not found, but ATP-dependent proteases such as the Clp, Lon, and Fts proteases are thought to have similar functions (Clarke et al., 2005; Sakamoto, 2006).

Beyond proteases, there is virtually nothing known about other enzymes mediating N turnover, e.g., the nucleases that degrade RNA and DNA (cf., Falkowski and La Roche, 1991), or about the degradation of complex molecules like toxins and phytochelatins. Some details are available for chlorophyllases from marine diatoms and they may be compartmentally regulated, because degradation proceeds very rapidly when cells are homogenized (Owens and Falkowski, 1982).
1.2.5. Other enzymes connected with nitrogen cycling

A number of other enzymes deserve consideration because they are involved in pathways that are closely linked to N metabolism, such as Rubisco and other carboxylases that are critical to the coupling of C and N metabolism (Huppe and Turpin, 1994), or electron transport system (ETS) enzymes such as isocitrate dehydrogenase that are involved in respiratory metabolism (Roy and Packard, 2001). Others are relevant to N losses (e.g., esterases that are used as indices of cell lysis; Agusti et al., 1998 or proteases associated with active cell death processes, e.g., Berman-Frank et al., 2004) (Table 32.1).

1.3. Measuring enzyme activity

What we generally want to know is the rate at which enzymes are catalyzing reactions in nature. True in situ assays of enzymes are not yet practical outside of the laboratory, although technological developments may make these sorts of observations possible in the future. In practice, enzyme-specific assays are developed based on the chemical reaction catalyzed (Table 32.2). Because all EC enzymes consume substrates to generate products, the chemical reaction and its energetics are examined to determine logical, and chemically measurable, endpoints for evaluating enzyme activity. Commonly endpoints are selected based on: disappearance of a substrate, production of an end-product, consumption of cellular energy, production of byproducts, or oxidation of a reducing equivalent (Table 32.2).

Unfortunately, enzyme activity measurements are considerably more complex than simply quantifying enzyme proteins or the production of metabolites. Achieving efficient extraction of proteins of interest can be challenging and the cellular environment or environments in which the enzyme operates may be difficult to simulate in vitro. In this section we consider general approaches to activity assays (e.g., strategies and types of assays), practical considerations (e.g., precautions and controls), kinetic measurements, and interpretation (e.g., deciding whether an enzyme activity can serve as an index of a metabolic rate).

1.3.1. Approaches to enzyme activity measurements

Assaying enzyme activity is often a trade off between the desire to measure activity in living cells under natural environmental conditions (a strict in vivo assay) and extracting and purifying the enzyme and making measurements under precisely controlled conditions (a strict in vitro assay). While maintaining natural, and thus relevant, conditions is often important in understanding rate reactions in situ, it is often difficult to measure the reaction in a way that is satisfactory. Therefore, in practice, all assays fall somewhere in between the two extremes: even the gentlest in vivo assay involves some perturbation to the natural environment, and even the most rigorous in vitro assay involves assumptions (see Kornberg, 1990).

In general, the best candidates for in vivo assays are extracellular or cell surface associated enzymes because there are a variety of fluorescent or fluorogenic substrates that can be added to intact cells in natural seawater whose disappearance or products can be measured (e.g., leucine aminopeptidases (LAPs), cell surface amino
### Table 32.2  Overview of N assimilatory enzymes, their chemical reactions, and assay endpoints

<table>
<thead>
<tr>
<th>N Assimilation</th>
<th>Reaction catalyzed</th>
<th>Energy</th>
<th>Reductant</th>
<th>Cofactors</th>
<th>Inhibitors</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reductase</td>
<td>( \text{NO}_3^- + 2e^- + 2H^+ \rightarrow \text{NO}_2^- + H_2O )</td>
<td>ATP</td>
<td></td>
<td>NAD(P)H; NADH; NADPH;</td>
<td>( \text{NH}_4^+ ), ( \text{CN}^- ), hydroxylamine, vanadate ( \text{CN}^- )</td>
<td>( \text{NO}_2^- ) production, NADH oxidation</td>
</tr>
<tr>
<td>Nitrite reductase</td>
<td>( \text{NO}_2^- + 6e^- + 8H^+ \rightarrow \text{NH}_4^+ + 2H_2O )</td>
<td></td>
<td>Ferredoxin; NAD(P)H</td>
<td></td>
<td></td>
<td>Disappearance of ( \text{NO}_2^- ), ( \text{NH}_4^+ ) production</td>
</tr>
<tr>
<td>Nitrogenase</td>
<td>( \text{N}_2 + 8\text{H}_2 \rightarrow 2\text{NH}_4^+ )</td>
<td>ATP</td>
<td>Ferredoxin or flavodoxins</td>
<td>Mg(^{2+} )</td>
<td></td>
<td>Acetylene reduction, (^{15}\text{N}_2 ) uptake, ( \text{NH}_4^+ ) production</td>
</tr>
<tr>
<td>Urease</td>
<td>( \text{CO}[\text{NH}_2]_2 + 2\text{H}_2\text{O} \rightarrow 2\text{NH}_4^+ + \text{CO}_2 )</td>
<td></td>
<td>Ferredoxin or flavodoxins</td>
<td></td>
<td>Biotin; Avidin</td>
<td>(^{14}\text{CO}_2 ) production</td>
</tr>
<tr>
<td>Urea carboxylase/allophanate</td>
<td>( \text{CO}[\text{NH}_2]_2 + 3\text{H}_2\text{O} + \text{HCO}_3^- \rightarrow 2\text{CO}_2 + 2\text{NH}_4^+ )</td>
<td>ATP</td>
<td>Ferredoxin or flavodoxins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolase</td>
<td>( \text{NH}_4^+ + \alpha\text{-ketoglutarate} \rightarrow \text{glutamate} )</td>
<td></td>
<td>NAD(P)H; NADH; NADPH</td>
<td>Methionine sulfloxamine azaserine, albizzine</td>
<td></td>
<td>NAD(P)H oxidation or NAD(P) (^+ ) production, Hydroxylamine, ( P ) formation, ATP hydrolysis and NADH oxidation, glutamine production</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>( \text{Glu} + \text{NH}_4^+ \leftrightarrow \text{Gln} )</td>
<td>ATP</td>
<td></td>
<td>Mg(^{2+} ); Mn(^{2+} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
### Table 32.2  Overview of N assimilatory enzymes, their chemical reactions, and assay endpoints *(continued)*

<table>
<thead>
<tr>
<th>N Assimilation</th>
<th>Reaction catalyzed</th>
<th>Energy</th>
<th>Reductant</th>
<th>Cofactors</th>
<th>Inhibitors</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate synthase</td>
<td>Gln + α-kg → 2Glu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14C glu production; NADH oxidation</td>
</tr>
<tr>
<td>Extracellular</td>
<td>Various</td>
<td></td>
<td>NADH;</td>
<td>NADPH; ferredoxin</td>
<td></td>
<td>Fluorogenic and fluorescent substrates;</td>
</tr>
<tr>
<td>proteases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>substrate disappearance; product formation</td>
</tr>
<tr>
<td>Amino acid</td>
<td>Amino acid + H₂O + O₂ → NH₄⁺ + keto or other acid + H₂O₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H₂O₂ production; fluorescent substrates;</td>
</tr>
<tr>
<td>oxidases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>substrate disappearance; product formation</td>
</tr>
</tbody>
</table>

Capone et al., 978-0-12-372522-6
Intracellular enzymes are usually measured in chemically defined homogenates in vitro (e.g., nitrate reductase, Berges et al., 1995). Recently, novel noninvasive assays have been developed using magnetic resonance techniques (e.g., Callies et al., 1992; Mesnard and Ratcliffe, 2005), and some of these may be applicable to natural plankton assemblages (see Feuillade et al., 1995). There are also a number of hybrid assays, where intracellular enzymes are measured by permeabilizing cell membranes without extraction (sometimes referred to as “in situ” assays, see Corzo and Niell, 1991). In some respects, hybrid assays represent the worst of both worlds: permeabilization (typically with alcohols) alters the local environment of the enzyme so in vivo conditions are not realized, yet the substrate concentrations are not controlled as in in vitro assays (see Berges, 1997). The availability of membrane-permeable substrates and the ability to monitor changes in them on a single-cell basis using flow cytometry has also led to the development of new enzymological methods, and though these have yet to be applied to enzymes of the N cycle, there is enormous potential for a growing number of reactions for which fluorescent substrates are available (Collier, 2000; Jellett et al., 1996; Jochem, 1999).

The vast majority of in vitro enzyme assays measure activity in a reaction vessel to which reductant, substrates, and cofactors are supplied in excess (so-called \( V_{\text{max}} \) assays, see Section 1.3.3). Under these conditions, one assumes that enzyme activity is not limited by any chemical factor so the enzyme-mediated reaction proceeds at its optimal rate. Consequently, \( V_{\text{max}} \) assays are sometimes referred to as measuring “potential” activity, even though this term can be deceptive (see Section 1.3.5). In vivo assays, on the other hand are thought to better estimate “actual” physiological rates in situ because reductant, substrates, and cofactors are not augmented. However, it is conceivable that energy supply or substrates may limit activity, at least over time in a closed reaction vessel, and so care must be taken to select appropriate time scales over which to measure activity. As a further complication, there may be more than one enzyme catalyzing the same reaction in an organism or extract, or multiple organisms with different forms of the same enzyme in a given samples. In this case, kinetic parameters at subsaturated substrate concentrations may well be impossible to interpret.

1.3.2. Assays

Several considerations are common for both in vivo and in vitro assays. For example, many enzymes can be assayed using artificial rather than physiological substrates. This may simplify detection and quantification of substrate or product pools, but rates measured are not necessarily equivalent to those achieved using natural substrates. Enzyme assays are usually either stop-time (i.e., a substrate is added, the
reaction is allowed to proceed, and is then stopped at a precise time; comparing initial and final time points, depletion of substrate or increase in product can be calculated), or continuous (i.e., a variable such as absorbance or fluorescence is continuously monitored over time, and changes in these properties can be related to changes in substrate or product) (see Rossomando, 1990). Absorbance measurements have traditionally been used, based on specific peaks (e.g., NADH absorbs strongly at 340 nm), but fluorescence measurements are more precise (since two wavelengths are specified, e.g., fluorescein isothiocyanate (FITC)-labeled substrates have absorption peaks near 480 nm and fluorescence peaks close to 530 nm), and generally offer detection limits over an order of magnitude lower. Care must be taken in selecting fluorescent compounds because many substrates developed for medical use have red fluorescence (e.g., Texas Red), and are almost useless in organisms that have chlorophylls; green fluorescing substrates (e.g., FITC) are generally more adaptable.

1.3.2.1. Storage and extraction Because enzymes are highly labile, care must be taken during sample collection, storage, and handling to preserve enzyme activity. Appropriate holding times for preserved samples, holding temperature, and selection of preservatives or preservation methods all need to be considered on an enzyme-specific basis. For example, algal nitrate reductases are extremely labile and require immediate storage at liquid N₂ temperatures (e.g., −196°C) to maintain activity (see Berges and Harrison, 1995a, Young et al., 2005), while many algal proteases retain activity at room temperatures for many hours (e.g., Berges and Falkowski, 1998). Enzymes must be protected during sample handling and homogenization to avoid pH fluctuations, oxidation of cellular material, and other compounds such as phenolics or exogenous heavy metals (Gegenheimer, 1990; Volkin and Klibanov, 1989). In addition, selection of extraction buffers is important because, in addition to controlling the pH, activities of different enzymes are enhanced or depressed by different buffers (Berges and Harrison, 1995a; Stoll and Blanchard, 1990). Finally, intracellular proteases can degrade enzymes, either specifically or nonspecifically. A number of protease inhibitors can be used to prevent this (unless of course proteolytic enzymes are the target of assays) (Gegenheimer, 1990; Volkin and Klibanov, 1989); however, in phytoplankton and macroalgae, there is evidence that commonly-used protease inhibitors are not effective (see Berges and Falkowski, 1996; Perez-Llorens et al., 2003).

1.3.2.2. Assay conditions To measure realistic enzyme activities, pH, temperature, and ionic strength should be kept as close to physiological conditions as possible, but in many cases we may not know the conditions that are relevant to a particular enzyme within a cell or cellular compartment. One strategy is to assume that the conditions that produce maximum activity are those for which the enzyme is adapted. However, while changing pH and temperature may produce higher rates of activity in a test tube, the resulting measurements often become further distanced from reality. For example, raising the assay temperature generally increases reaction rates up to a point. However, high temperatures can also denature enzymes and result in lower enzyme activity (e.g., Berges et al., 2001). Rather than “maximizing”
activity, a better process might be “optimization,” wherein temperature, pH, and ionic strength are kept close to what are believed to be physiological conditions, and substrate and cofactor concentrations are adjusted so as to be nonlimiting for a given assay duration (see also Newsholme and Crabtree, 1986).

In the case of *in vivo* assays, there are tradeoffs in selecting the concentrations of substrates (including fluorescent or fluorogenic compounds being used as tracers) to add to incubations. Concentrations of substrates are ideally adjusted to levels found in the environment. However, sometimes these concentrations are not known or ambient concentrations are so low that substrates are rapidly depleted in bottle incubations, precluding an accurate rate measurement. It is therefore paramount that enzyme assays are conducted on appropriate time scales (and these may vary temporally, spatially and depending on the enzyme and organism) so that substrates are not significantly depleted in incubation bottles. Conversely, if substrate additions are too high relative to ambient environmental concentrations, enzyme activity can be stimulated over natural conditions (e.g., see Chapter 7 by Mulholland and Lomas, this volume). A common strategy is to add substrates at saturating levels. This allows for stable measurements, and can be an indication of maximal rates of activity under the environmental conditions. These so-called “potential” rates should be used cautiously because rate processes in organisms often depend on nutrient and environmental “prehistory,” and because other elements and factors also in short supply in nature may become limiting over time in incubation bottles.

For intracellular enzymes, the picture is complicated because we do not have a clear idea of physiological substrate concentrations and turnover times inside cells; indeed, compartmentalization, metabolic channeling, and tight coupling of reactions make it difficult to estimate intracellular concentrations even under ideal conditions (Albe *et al*., 1990; Srere, 1987). For these reasons, intracellular enzyme assays are typically run at saturating substrate concentrations (i.e., \( V_{\text{max}} \) assays); the reaction rate should then depend only on the quantity of active enzyme (see Section 1.3.3).

### 1.3.2.3. Quality-control measures

There are a number of basic quality-control steps that need to be taken to ensure that rate measurements are not biased by assay artifacts. First, it must be demonstrated that the rate reaction is linear over time for the period of the assay. If this is not the case, then either the enzyme is not stable, the substrates are being depleted over the course of the assay, or there is some sort of inhibition of the reaction over time, perhaps by a reaction product. Second, the activity should vary directly with the amount of enzyme (or homogenate) added. If this is not true, it suggests a problem with the assay such as degradation of enzyme or exhaustion of substrates. Third, for a \( V_{\text{max}} \) assay, all reaction substrates must be saturating. Typically, this is evaluated by running parallel reactions varying the concentration of each substrate independently; however, even this is not simple because reactions can also be inhibited by high substrate concentrations.

### 1.3.2.4. Scaling activity

Once activity measurements have been made, they must be scaled to something ecologically or physiologically relevant. *In vitro* enzyme activity is typically scaled to cell protein because it is straightforward to make protein...
determinations in the same homogenate that is used for assays, but this may not always be appropriate (see Berges and Harrison, 1995b). In many studies of N cycling, it is relevant to simply express enzyme activity as the amount of nitrogenous product generated or substrate consumed per unit time per unit biomass. Alternative scaling variables include cell number, N, C, or chlorophyll biomass; the choice of scaling factors depends largely on the process being measured and the context in which the measurement was made. Enzyme activity is typically expressed as units (U) (or, less commonly, International Units, IU), where one unit is the amount of enzyme needed to catalyze the conversion of one micromole substrate to product in one minute. Since minutes and micromoles are not SI units, some investigators prefer to use moles per second, although this results in small values. The katal (kat), a derived SI unit representing the catalytic activity that will raise the rate of reaction by one mole per second in a specified assay system is technically correct, but is not often used (see Dybkaer, 2000).

1.3.3. Enzyme kinetic measurements

Beyond the instantaneous rate measurements described above, determining how enzyme activity varies with substrate concentration can provide useful information about enzyme capacity and the rates that are likely to be observed under different environmental conditions. These kinetic measurements probably work best in *in vivo* assays of exo- and ectoenzymes where substrate concentrations can be measured directly from the external environment and there are no metabolic intermediary pools or reactions to complicate the picture. Kinetic measurements also have limitations as there can be bottle effects, diffusion boundary layers around organisms can be important, and it is often difficult to make measurements at the "low" substrate end-members in all but the most oligotrophic environments. Further, kinetic parameters are physiological variables themselves, dependent on the preconditioning of cells and so can vary widely even in the same organism, across environments (see Chapter 7 by Mulholland and Lomas, this volume).

For *in vitro* assays, it is extremely difficult to determine the relevant intracellular concentrations of substrates due to compartmentalization of particular reactions, competing reactions that produce and consume common substrates, and metabolic channeling (Albe *et al.*, 1990; Srere, 1987). Consequently, *in vitro* kinetic measurements may not be very useful for assessing *in situ* or *in vivo* reaction rates. However, provided assays conditions are carefully controlled, kinetic parameters may be very useful for characterizing enzyme isoforms and enzymes from different metabolic pools, different organisms, or from organisms collected from different environments.

In most cases where enzyme kinetics have been measured in marine settings, simple Michaelis–Menten models have been used to characterize enzyme activity. This is sensible, because such rectangular hyperbolic models suit many biological processes (e.g., Berges *et al.*, 1994). Aquatic biogeochemical models use a "classical" kinetics formulation to describe N uptake (e.g., Fasham *et al.*, 1990; Moore *et al.*, 2002). Similarly, physiological models use enzyme kinetic principles to formulate mathematical equations describing enzymatically mediated N assimilation (Packard *et al.*, 2004). However, it is still unclear whether such kinetic descriptions are ecologically meaningful for the reasons described earlier. In addition, many enzymes
display non–Michaelis–Menten kinetics due to regulatory pathways, and multiple enzymes or enzyme isoforms accomplishing the same reaction but with different substrate affinities. For example, allosteric enzymes have multiple subunits, regulatory sites that bind modulators other than the substrate, and/or multiple binding sites for substrate. Michaelis–Menten kinetics assume that there is no interaction among binding sites when in fact, binding of a modulator can cause activation/inhibition of enzyme activity, while binding of a substrate on another subunit may increase or decrease binding at another subunit (cooperativity) (Cornish-Bowden, 2004; Ottaway, 1988). Such mechanisms make it difficult to interpret enzyme activity, especially in the context of the commonly used kinetic models (see also Section 1.3.5).

**1.3.4. Enzyme inhibitors**

Enzyme inhibitors represent powerful tools for isolating specific enzyme activities in crude homogenates (e.g., proteases; Berges and Falkowski, 1996), verifying assays, and determining whether alternative metabolic pathways exist. They can also provide a means to differentiate the roles of different taxa that perform identical reactions. For example, cyanobacterial assimilatory nitrate reductases are quite distinct from eukaryotic forms and so the relative contributions of the two groups to total nitrate assimilation might be distinguished using a carefully selected inhibitor. Very few specific inhibitors are known (e.g., vanadate for nitrate reductase, methionine sulfoximine [MSX] for GS, and azaserine for glutamate synthase), and so their use has been limited.

To be used effectively, the mechanisms through which inhibitors exert their effects need to be understood. For example, competitive inhibitors compete with physiological substrates for binding sites. Their effect depends on the relative concentrations of substrates and the inhibitor and the degree of inhibition depends on the number of active sites occupied by the inhibitor versus the metabolic substrate. In contrast, noncompetitive inhibitors bind to parts of the enzyme other than the substrate binding site, so the degree of inhibition depends only on the inhibitor and not the substrate concentration. This type of inhibition is typically irreversible and reduces the amount of total enzyme available to catalyze a particular reaction. Uncompetitive inhibition occurs when the inhibitor binds to the enzyme–substrate complex and prevents the reaction from being catalyzed.

There are also inhibitors that affect enzyme synthesis. Inhibitors of transcription (e.g., dibromothymoquinone [DBMIB]) and inhibitors of translation (e.g., cycloheximide [CHX]) are available but these are not specific to particular enzymes. In addition, because protein synthesis takes place within the chloroplast and mitochondrion in eukaryotes, prokaryotic protein synthesis inhibitors (e.g., chloramphenicol [CAP]) may be necessary to distinguish prokaryotic versus eukaryotic activity (e.g., Segovia and Berges, 2005).

**1.3.5. Interpreting enzyme measurements**

There are many factors that can affect enzymes in vivo that are not simulated in in vitro assays. Consequently, in vitro enzyme activities may or may not reflect actual in situ rates. In nature enzymes may not be substrate-saturated as they are in in vitro assays;
N is often limiting in the marine environment. Further, many enzymes are sensitive to cellular substrate concentrations rather than extracellular concentrations and it is difficult to measure the relevant intracellular metabolite pools. In vitro assays may affect the conformation of enzymes and the degree to which they are modified. For example, allosteric effects (see Kinetics section above) may be modified under in vitro conditions. Many enzymes undergo posttranslational regulation wherein enzyme activity is affected by binding of activator/inactivator proteins and covalent modification of the enzyme (e.g., adenylylation, phosphorylation or carbamylation) (Ottaway, 1988). When there is posttranslational modification of enzymes, enzyme activity measured in assays may be unrelated to in vivo activity (see Section 2.2.1) and there are few ways to determine the extent of enzyme modification in nature.

To understand why it is often difficult to interpret in vitro enzymes assays, it is useful to consider the principles of metabolic control theory, derived from engineering control theory (Raven, 1981). Consider a series of enzyme catalyzed reactions, where \( a, b, c, \) and \( d \) represent substrates and or products of reactions catalyzed by enzymes represented by \( A, B, \) and \( C \):

\[
a \xrightarrow{A} b \xrightarrow{B} c \xrightarrow{C} d
\]

Control of the whole series of reactions generally rests at one point (the rate-limiting step), which is often the first step (Newsholme and Crabtree, 1986), catalyzed in this case by enzyme \( A \). Further, it has been argued that it is advantageous that control of a metabolic pathway not shift between steps as conditions change, otherwise regulation would be unreasonably complex, i.e., in this example, the step catalyzed by \( A \) should continue to be rate-limiting. For this to be true, the concentration of \( b \) and substrates downstream must always be below saturation for the enzymes catalyzing their transformations, such that they do not accumulate and enzymes \( B \) and \( C \) do not become rate limiting. It follows that enzyme \( A \) is always functioning at close to its capacity, an thus \( V_{\text{max}} \) assays for \( A \) would give information about the rate of flux through the whole pathway, but that \( V_{\text{max}} \) assays for enzymes \( B \) and \( C \) would not. Another way cells could maintain a single rate-limiting step in a metabolic pathway is for downstream enzymes (e.g., \( B \) and \( C \)) to have \( K_m \) values that are close to the steady state concentrations of \( b \) and \( c \), respectively, when the pathway is functioning at its maximum rate (see Heinrich and Hoffmann, 1991). In this way, even small changes in \( b \) or \( c \) will result in relatively large changes in the rates of enzymes \( B \) and \( C \) (respectively) and thus prevent these enzymes from becoming rate limiting. Although this example is theoretical, there are considerable empirical data supporting such relationships (see Albe et al., 1990; Pettersson, 1991). Moreover, if conditions change so that fluxes through a pathway increase or decrease, parallel adjustments in concentration occur in all the enzymes along the particular metabolic pathway (see discussion in Fell, 2005), opening the possibility that even nonrate-limiting enzymes (e.g., \( B \) or \( C \)) could provide information about prevailing metabolic fluxes (see Newsholme and Crabtree, 1986).

In addition to determining where metabolic pathways are regulated it is also essential to understand how particular enzymes are regulated in order to interpret...
enzyme activity assays. In the case where an enzyme or enzyme pathway is controlled by an enzyme whose concentration or activity is regulated at the level of synthesis and degradation, an in vitro enzyme activity is likely to be a good reflection of in situ rate. It is much more difficult to interpret enzyme activity for enzymes that are regulated allosterically or posttranslationally because simple $V_{\text{max}}$ measurements may not reflect enzyme availability or potential.

Analysis of metabolic networks may help us properly interpret enzyme activities from enzyme abundance, and ultimately extrapolate to the whole organism using models. Application of such computational modeling to enzyme systems is still limited but promising. For example, Su et al. (2006) were able to develop computational models of N assimilation in *Synechococcus* sp. WH 8102 that closely corresponded to results from microarray analyses of gene expression.

Ultimately, if enzyme activity measurements are to be useful we need to be able to validate them in cultures under controlled laboratory conditions, and then in controlled field settings where there is basic understanding of the important processes, resident organisms, and their metabolic pathways. In natural samples, it is often difficult to specifically target organisms of interest using bulk collection methods. As a result, it is not always clear to whom enzyme activities can be attributed (e.g., cooccurring organisms or endosymbionts). Unfortunately, the field-oriented focus of aquatic sciences, lack of appropriate cultured organisms, and the time-pressures of most project funding cycles mean that such validations are seldom done.

## 2. CASE STUDIES OF SPECIFIC ENZYMES

### 2.1. Inorganic nitrogen acquisition

Inorganic N is taken up by microbes and marine plants in a variety of forms and uptake is facilitated through a variety of transporters (see Chapter 7 by Mulholland and Lomas, this volume). Inorganic N compounds must first be reduced to ammonium prior to their assimilation into organic compounds and these reduction steps are enzymatically mediated and are described here.

#### 2.1.1. Nitrogenase

**2.1.1.1. Overview** Nitrogenase, the enzyme complex catalyzing N$_2$ fixation, is found in phylogenetically diverse prokaryotes (archaea and bacteria, including cyanobacteria), ranging from aerobic heterotrophs, aerobic and anaerobic phototrophs to strict anaerobes (see Chapter 4 by Carpenter and Capone, this volume). The enzyme is extremely sensitive to O$_2$ and N$_2$ fixation is energetically expensive, “costing” a cell up to 16 molecules of ATP per molecule N$_2$ reduced (Postgate, 1998). Nitrogenase enzymes are numerous but biochemically similar and so the whole family of enzymes is referred to as “nitrogenase” even though the name does not conform to systematic enzyme nomenclature (Postgate, 1998). The enzyme complex nitrogenase consists of two proteins, a molybdenum iron protein (Protein 1) that binds N$_2$ (dinitrogenase reductase) and a smaller iron protein (Protein 2) that acts...
as an electron donor to Protein 1 (nitrogenase reductase), although alternative nitrogenases that contain vanadium or only iron in the larger subunit have been identified. As well as catalyzing the reduction of N₂ to NH₃, nitrogenase can reduce other small triply bonded molecules, including acetylene, azide, and cyanide, and nitrogenase reduces hydrogen ions to gaseous hydrogen, even when N₂ is present. Many assays of nitrogenase activity take advantage of these competing reactions. Although N₂ consumption or H₂ production theoretically can be used to estimate N₂ fixation rates, N₂ concentrations in aquatic systems are high (and generally saturated in seawater) and simultaneous production and consumption of H₂ both intracellularly and extracellularly through a variety of processes confounds interpretation of this measurement.

2.1.1.2. Assay techniques Because robust in vitro assays have not been developed, nitrogenase activity has been estimated based on the use of isotopic tracers and substrate analogs. Nitrogen fixation rates have been estimated based on ¹⁵N₂ uptake using tracer techniques (e.g., see Chapter 31 by Lipschultz, this volume). This method is a sensitive measure of net ¹⁵N₂ uptake into particulate organic nitrogen (PON) (Montoya et al., 1996; Mulholland et al., 2004a,b, 2006), however, because diazotrophs can release fixed N₂ prior to its assimilation into biomass (e.g., Capone et al., 1994; Glibert and Bronk, 1994; Mulholland et al., 2004a,b, 2006), ¹⁵N₂ uptake can underestimate total or gross N₂ fixation (Gallon et al., 2002; Mulholland et al., 2004a,b, 2006). Other assays of nitrogenase activity rely on reduction of other triply bound compounds that serve as N₂ analogs.

Most often, assays of nitrogenase take advantage of the enzymes’ ability to reduce acetylene (C₂H₂) (Capone, 1993). The acetylene reduction method is convenient, very sensitive and relatively simple. However, acetylene reduction assays should not be excessively long because nitrogenase preferentially reduces C₂H₂ as opposed to its natural substrate N₂ and thus “starves” cells for N. Because C₂H₂ reduction measures just the reduction step, it is a measure of gross N₂ fixation, while movement of ¹⁵N₂ from the dissolved to the particulate pool measures net N assimilation (see Gallon et al., 2002; Mulholland and Bernhardt, 2005; Mulholland et al., 2004a,b). In addition, the stoichiometry of these two reactions is different and a conversion factor is needed to estimate N₂ fixation rates; theoretically a ratio of 3:1 mol C₂H₂:N₂ are reduced (Montoya et al., 1996; Postgate, 1998). However, because nitrogenase reduces H⁺ along with N₂, ratios >3:1 are often assumed and observed; for example, if 1 mole of H₂ is also produced for each mole of N₂ reduced, the ratio of C₂H₂:N₂ fixation increases to 4:1. Despite this potential bias, for the marine diazotroph Trichodesmium, little net H₂ production has been observed under environmental conditions (Scranton, 1984; Scranton et al., 1987) suggesting either: (1) that hydrogen production from nitrogenase is low in nature (and so a 3:1 conversion factor is justified), or (2) that cells efficiently recoup H₂ produced during N₂ fixation, arguing for higher ratios and the need for a thorough evaluation of hydrogen cycling within cells.

Estimates of acetylene reduction are usually calibrated against ¹⁵N₂ to determine the appropriate ratio extrapolating N₂ fixation from C₂H₂ reduction. However, release of recently reduced N₂ and the difficulty in chemically recovering all possible
dissolved pools into which products of N\textsubscript{2} fixation might be released, may make intercalibration between the two methods impossible (Mulholland, 2007). In numerous paired comparisons, ratios of C\textsubscript{2}H\textsubscript{2} reduced to N\textsubscript{2} taken up varied by at least an order of magnitude (see Mulholland, 2007; Mulholland 	extit{et al.}, 2006). Even though it may be impossible to intercalibrate the two methods, the difference between N\textsubscript{2} reduction (gross N\textsubscript{2} fixation) and net N\textsubscript{2} assimilation may provide an excellent index of the release of recently fixed N\textsubscript{2} that has proven promising in culture systems (Mulholland, 2007; Mulholland and Bernhardt, 2005; Mulholland 	extit{et al.}, 2004a,b, 2006).

More recently, the capacity for N\textsubscript{2} fixation has been evaluated by amplifying \textit{nif} genes (which encode nitrogenase enzymes), from natural samples using appropriate primers (see Chapter 30 by Jenkins and Zehr, this volume). DNA macro- and microarrays have been used to explore the diversity of N\textsubscript{2} fixing organisms (Jenkins \textit{et al.}, 2004) and quantitative PCR approaches have been used to determine the abundance of particular groups of diazotrophs (Church \textit{et al.}, 2005). These molecular methods have high sensitivity but they cannot determine if there is actually nitrogenase activity. Recent studies have coupled gene expression assays with uptake experiments to demonstrate active N\textsubscript{2} fixation in open ocean unicellular diazotroph populations (Montoya \textit{et al.}, 2004).

### 2.1.1.3. Enzyme regulation and interpretation of activity assays

For 	extit{Trichodesmium}, probably the best studied marine N\textsubscript{2} fixer, nitrogenase is regulated both at the level of transcription and activity and so gene presence and even the presence of gene products, are not quantitative measures of nitrogenase activity (Zehr and Capone, 1996; Zehr \textit{et al.}, 1993; and see Chapter 30 by Jenkins and Zehr and Chapter 7 by Mulholland and Lomas, this volume). Nitrogenase, like many enzymes involved in N reduction and metabolism, appears to be regulated through negative feedback from intracellular NH\textsubscript{4}\textsuperscript{+}, other downstream metabolites (amino acids), and cellular N:C status (Flores and Herrero, 1994; Guerrero and Lara, 1987; Mulholland \textit{et al.}, 1999). N\textsubscript{2} fixation also appears to be sensitive to or inhibited by other forms of N in the growth medium (Flores and Herrero, 1994; Guerrero and Lara, 1987; Smith and Gallon, 1993). Though it was previously thought that virtually any NH\textsubscript{4}\textsuperscript{+} or NO\textsubscript{3}\textsuperscript{-} in the growth medium inhibited the synthesis and activity of nitrogenase, recent studies suggest that environmentally-relevant concentrations (e.g., 1 or 2 \textmu M) of these compounds do not inhibit N\textsubscript{2} fixation in the oligotrophic or coastal ocean (Mulholland \textit{et al.} 1999, 2001). In addition to N, nitrogenase activity is dependent on the provision of ATP (and so is tied in with the light cycle in photoautotrophs) and Mg\textsuperscript{2+} and the absence of O\textsubscript{2}. As for other N assimilation, pathways, further metabolism of NH\textsubscript{4}\textsuperscript{+} produced through N\textsubscript{2} fixation can also be limited by the provision of carbon skeletons.

In addition to regulation at the level of transcription, at least some nitrogenases can be regulated by covalent modification (inactivation) of the Fe protein (Capone \textit{et al.}, 1990; Gallon, 1992; Zehr \textit{et al.}, 1993). However, regulation of nitrogenase appears to be complex since nitrogenase is also subject to circadian rhythms and may be related to heterocyst formation in heterocystous species (Chen \textit{et al.}, 1996, 1998; Herrero \textit{et al.}, 2001).
2.1.1.4. Information gained from assays and future potential

Nitrogenase activity assays remain the most important means to estimate rates of N₂ fixation and acetylene reduction assay is a good example of an in vivo assay that does not require much manipulation of natural samples. Thus, there should be a good relationship between what the assay measures and the actual rate of N₂ fixation in situ. Finding means to convert measures of acetylene reduction to N₂ reduction remains a critical issue, and H₂ production is an important part of the problem. Physiological studies of N₂ fixation and H₂ production catalyzed by nitrogenase in Trichodesmium are hampered by the lack of in vitro assays.

2.1.2. Nitrate reductase

2.1.2.1. Overview

Because N is thought to limit primary productivity in much of the world ocean, and nitrate thought to be the primary form of new N available in most systems (Dugdale and Goering, 1967), nitrate reductase (NR) activity was one of first enzyme measurements to be broadly applied in an oceanographic context (Eppley, 1978; Eppley et al., 1969). Features of the enzyme that made it suitable for assay included high specific activity, relatively easy extraction, and a straightforward, sensitive assay.

In eukaryotes, NR is a complex enzyme containing molybdenum, iron–heme, and flavin adenine dinucleotide that uses NADH, NADPH, or both as a reductant (the basis for division of the enzyme into subclasses: EC 1.7.1.1, 1.7.1.2, or 1.7.1.3). The eukaryotic enzyme is best characterized from higher plants (Campbell, 1999; Fischer et al., 2005), but generalizations hold well in the green algae (Solomonson and Barber, 1990; Song and Ward, 2004; Wang et al., 2003) and diatoms (NR’s in T. pseudonana and P. tricornutum are 50–60% identical to higher plant and green algal gene sequences; Allen et al., 2005) where the genes and proteins have been examined. In most cases, the enzyme appears to be dimeric, with subunits of about 100 kDa in size, however, Iwamoto and Shirawa (2003) have suggested that NR from Emiliania huxleyi is different, possibly a hexamer with 85 kDa subunits, and there are reports that NR from dinoflagellates may differ as well (Ramalho et al., 1995). Assimilatory nitrate reductases are quite distinct enzymes in prokaryotes where they are ferredoxin-dependent and contain iron–sulfur clusters (Flores et al., 2005).

2.1.2.2. Assay techniques

One of the major problems in assaying NR activity has been the stability of the enzyme. Consequently, it has been difficult to measure any NR activity in some field samples (e.g., Hochman et al., 1986), and measured NR activity has often been insufficient to account for observed rates of nitrate incorporation (e.g., Blasco et al., 1984). Attempts to overcome this problem have included: assaying the parts of the enzyme catalyzed reaction that are less affected such as NADH oxidizing (diaphorase) activity, assaying NO₃⁻ reducing activity in the presence of artificial reducing compounds such as methyl viologen, or developing “in vivo” assays (Corzo and Niell, 1991; Hochman et al., 1986). Problems extracting enzyme may be related to the high proteolytic activities observed in phytoplankton, which appear resistant to classical protease inhibitors (see Berges and Falkowski, 1998). Some success in countering this problem has been achieved by adding
“carrier” proteins like BSA to provide abundant alternative targets for proteases, thereby protecting NR (Berges and Harrison, 1995a). NR activity is also very sensitive to storage conditions. In many cases, there are significant losses of activity when samples are stored frozen at $-80^\circ$C, and preservation at liquid N$_2$ temperature is essential (Berges and Harrison, 1995a; Vergara et al., 1998; Young et al., 2005).

Typically, NR assays measure production of nitrite, using the sulphanilamide/$N$-(1-naphyl) ethylenediamine dihydrochloride reaction after a timed incubation period, and correcting for any nitrite present initially in extracts. Continuous spectrophotometric assays that measure NADH oxidation over time are feasible when cell biomass is high (e.g., in cultures), but corrections must be applied because enzymes other than NR present in cell homogenates can also oxidize NADH (Berges and Harrison, 1995a). Complicating things further, high levels of NADH and nitrate inhibit NR activities in some species and so assays need to be optimized on a species-specific basis (Berges and Harrison, 1995a,b). It is equally important to optimize assays for all types of NR because some NR enzymes require flavin adenine nucleotide (FAD) as a cofactor and this is also variable among species (Berges et al., 1995a; Everest et al., 1984).

2.1.2.3. Enzyme regulation and interpretation of activity assays A diverse range of regulatory mechanisms have been described for NR in higher plants, green algae, and cyanobacteria, and these include induction by nitrate, repression by NH$_4^+$ or other products of N metabolism, and modulation by light (Campbell, 1999; Herrero et al., 1984; Solomonson and Barber, 1990; Song and Ward, 2004). In addition to feedback inhibition of NR, NH$_4^+$ can interfere with NO$_3^-$ transport, thereby inhibiting NO$_3^-$ uptake and reduction. The effect of NH$_4^+$ on NR is rapid in diatoms, indicating a relatively fast turnover of the enzyme (Berges et al., 1995; Vergara et al., 1998). In contrast, in some intertidal macroalgae, additions of ammonium have little effect on NR activity (Young et al., 2007b).

In eukaryotic algae (at least in diatoms), the primary mode of regulation of NR appears to be at the level of synthesis and degradation (Berges, 1997; Vergara et al., 1998). Consequently, the interpretation of NR activity measurements is relatively straightforward, and NR activity and NO$_3^-$ incorporation rates are correlated (Berges and Harrison, 1995a,b; Berges et al., 1995). While posttranslational modification of NR by phosphorylation in response to changes in irradiance is well-described in higher plants (Huber et al., 1992), it has not been demonstrated for algae, and in diatoms, the NR protein appears to lack the key phosphorylation site found in higher plants (Allen et al., 2005) making it unlikely that this is a viable regulatory pathway.

Diel variability in NR activity, and NO$_3^-$ uptake, has been observed in a variety of N-replete marine phytoplankton communities (e.g., Packard et al., 1971). Patterns vary among species (e.g., Lopes et al., 1997; Ramalho et al., 1995). In diatoms, NR activity typically has a peak near the middle of the light period, but there is also an increase in activity just prior to the beginning of the light period (Berges et al., 1995). This periodicity does not appear to be under the control of a circadian oscillator (cf., Chow et al., 2004; Vergara et al., 1998), but is more likely to be
linked to changes in internal carbon pools. The diel variability of NR activity is also affected by N status and N-deplete or starved cells will take up NO$_3^-$ even during the dark (e.g., Cochlan et al., 1991). Interestingly, while macroalgae in the lower intertidal zone show diel variations in NR activity, this is not observed in Fucus species in the upper intertidal (Young et al. 2007b).

NR activity is also sensitive to temperature, at least in diatom species. (Gao et al. 2000) demonstrated that NR activity in diatoms such as Skeletonema costatum tended to lose activity above 16°C, while other eukaryotic enzymes were stable at over 30°C. Lomas and Glibert (1999) combined laboratory and field data to argue that this temperature sensitivity effectively limited diatoms to particular environments. Berges et al. (2002) speculated that features of the N-terminus of the NR protein might be responsible for temperature sensitivity, and these appear to be borne out in data from recent sequencing projects (Allen et al., 2005).

In cyanobacteria, transcription of NR seems to be triggered by a high intracellular C:N ratio, as for nitrogenase (see Section 2.1.1.3). Further, regulations of NR and nitrogenase appear to share common features in a variety of organisms (e.g., Flores and Herrero, 1994). We are just beginning to understand many of the complex regulatory systems that control total N acquisition by aquatic microbes.

2.1.2.4. Information gained from assays and future potential
NR activity measurements have not been all that useful in predicting NO$_3^-$ incorporation rates or new production as once hoped (see Blasco et al., 1984), though these earlier assays may have underestimated true NR activities. However, NR assays have proven useful in examining the effects of iron limitation on nitrate metabolism (e.g., Boyd et al., 1998; Timmermans et al., 1994; see also NiR section below). In addition, NR activity measurements have been used extensively to examine patterns of nitrate incorporation in macroalgae (e.g., Corzo and Niell, 1991; Davison and Stewart, 1984; Davison et al., 1984; Hurd et al., 1995; Young et al., 2007a,b) and to assess the contribution of epiphytes to N cycling in seagrass meadows (Young et al., 2005).

Differences among NR enzymes might be exploited to provide taxon-specific information. For example, since cyanobacterial and eukaryotic enzymes differ markedly, there may be potential for using different assays to distinguish eukaryotic and prokaryotic NO$_3^-$ uptake in natural communities (see Berges, 1997). At the protein level, NR from diatom species is remarkably variable in the N-terminal region, so that targeting NR genes could provide community information at a functional level (Allen et al., 2005).

Interestingly, despite the fact that nitrate is one of the largest pools of inorganic N in many marine systems, not all species of phytoplankton can use NO$_3^-$ . For example, some “brown tide” organisms are unable to grow on nitrate (DeYoe and Suttle, 1994), and at least some marine Prochlorococcus apparently lack nitrate assimilation gene clusters (Rocap et al., 2003). NR activity assays could confirm such findings.

Finally, in addition to playing a key role in nitrate acquisition, NR may play a role in cell signaling. Desikan et al. (2002) found that NR mediates nitric oxide (NO) synthesis in Arabidopsis guard cells. NO signaling has also been established in Chlamydomonas (Sakihama et al., 2002) and quite recently in diatoms (Vardi et al., 2006).
2.1.3. Nitrite reductase

2.1.3.1. Overview

Despite early interest in nitrite reductase (NiR), relatively fewer measurements of the enzyme have been made, compared to NR (e.g., Eppley and Rogers, 1970; Eppley et al., 1971). Traditionally, it has been considered that algae and cyanobacteria have only the ferredoxin-dependent form of the enzyme (Campbell and Kinghorn, 1990; Flores et al., 2005; Luque et al., 1993), but annotation of the T. pseudonana genome indicates the presence of both a nuclear-encoded ferredoxin-dependent enzyme, targeted to the chloroplast (homologous to vascular plant/cyanobacterial forms), and a second NiR with homology to a NAD(P)H-dependent form found only in heterotrophic bacteria and some fungi (Allen, unpublished data).

2.1.3.2. Assay techniques

NiR is typically measured by following the disappearance of NO$_2^-$ in cell homogenates, when provided with either reduced ferredoxin or alternative electron acceptors such as methyl viologen (Vega et al., 1980). The assay is somewhat more complicated than that for NR. If methyl viologen is used, then sodium dithionite is typically used to reduce it and to maintain reducing conditions throughout the assays, though strictly anaerobic conditions may not be necessary (Milligan and Harrison, 2000; Vega et al., 1980). It seems from the genome annotation data that diatoms, like yeast, may also have NAD(P)H-dependent NiR, but this has not been reported on in the literature.

2.1.3.3. Enzyme regulation and interpretation of activity assays

Available evidence suggests that there is a tight coregulation between NR and NiR in both eukaryotes and prokaryotes. Indeed, in cyanobacteria, N assimilation genes show gene clusters of NiR-permease gene–NaR (nitrate reductase genes) with an operon structure (Flores et al., 2005). In prokaryotes, the two enzymes are very closely linked (Faure et al., 1991), and both probably respond to the presence of NO$_3^-$ (e.g., Galvan et al., 1992; Wray, 1993). Such a regulatory system would presumably prevent the build-up of pools of nitrite that could prove toxic to cells. In many cases, NiR activity is greater than NR activity, which provides additional support that NR is the rate limiting enzyme of the pair (see above). Carbon limitation may also affect NiR, as in the case of NR (Suzuki et al., 1993, 1995).

2.1.3.4. Information gained from assays and future potential

NiR assays have much more seldom been applied in the field, perhaps because of the more complicated assay, but also because it is not the rate limiting step of the NiR–NR pair. However, there may be special cases where NiR does become limiting for NO$_3^-$ acquisition. (Kessler and Czygan1968) provided evidence that because NiR in green algae contains more iron than NR, iron limitation more strongly affects NiR. This also appears to be the case for at least one species of diatom; (Milligan and Harrison, 2000) demonstrated that under iron limitation, NiR was more strongly affected than NR, and speculated that control of nitrate acquisition might shift from NR to NiR under these circumstances.
2.2. Ammonium assimilation pathways

Ammonium is the common denominator in inorganic N assimilation and most forms of N taken up by cells are first reduced to NH$_4^+$ prior to their assimilation into cellular material. Ammonium assimilation also metabolically links the C and N cycles because C skeletons are required for amino acid synthesis. Consequently, the enzymes involved in N assimilation are extensively regulated in response to a variety of intracellular cues (N and C) and environmental variables. The regulatory mechanisms for NH$_4^+$ assimilation in microorganisms generally build upon detecting the presence or absence of NH$_4^+$ in the environment or in the cell or feedback from other downstream metabolites. The regulatory networks of well-studied microorganisms, including bacteria and cyanobacteria, include a transcriptional factor, the global N regulator, NtcA, that is involved in regulating transcription of co-regulated genes, and the signal transduction protein PII, which broadcasts and amplifies cellular N status within cells; together, these coordinate N (and to some extent C) metabolism in cells (García-Fernández et al., 2004; Herrero et al., 2001; Tandeau de Marsac et al., 2001). Recently, (Su et al. 2006) demonstrated that models of the N assimilation network agreed well with gene expression data from microarray studies for a cyanobacterial system, offering hope that even quite complex metabolic systems may prove tractable to this type of modeling analysis.

The assimilation of ammonium in most marine phytoplankton and bacteria is via a cyclic pathway involving the enzyme pair GS and glutamate synthase (glutamine oxoglutarate aminotransferase [GOGAT]). GS first assimilates ammonium into the amide group of glutamine (gln) and then, GOGAT transfers the amide group to a molecule of $\alpha$-ketoglutarate ($\alpha$-kg) to form two molecules of glutamate (glu), one of which is recycled to GS to synthesize another glutamine (gln). While the reaction is fully reversible, in cells, it is thought that GS/GOGAT works primarily in the forward or assimilatory direction. However, it does so at the expense of cellular energy since the GS reaction requires one ATP for each molecule of gln produced. More is known about GS because that is generally considered the rate-limiting enzyme in the pair (see above).

Glutamate dehydrogenase offers an alternative pathway by which ammonium can be incorporated through the reductive amination of $\alpha$-kg. Because of its higher $K_m$ for ammonium (millimolar range for GDH versus micromolar range for GS; Ahmed et al., 1977; Bressler and Ahmed, 1984), GDH is thought to play a minor role in N assimilation in marine microbes (see Muro-Pastor et al., 2005). However, GDH may offer a competitive advantage for cells when energy supplies are limited because it does not require ATP (see below). GDH activity is also common in marine animals, where it appears to operate primarily in the reverse direction resulting in the excretion of NH$_4^+$ (see Chapter 8 by Steinberg and Bronk, this volume). GS, GOGAT, and GDH are all fully reversible; many assays of their activity take advantage of this and as well as the hydrolysis of an ATP, the oxidation of reduced forms of specific coenzymes such as NADH or NADPH, or the consumption of substrates or production of products, or their analogs, over time.
In addition to their central role in amino acid biosynthesis, as primary products of N assimilation, glutamine (glu) and glutamate (glu) are thought to play an important role in regulating cellular N metabolism and determining cellular N status (Flynn and Gallon, 1990; Flynn et al., 1989, 1994; Mulholland and Capone, 1999; Mulholland et al., 1999). In enteric bacteria, the global N regulatory system (Ntr) is sensitive to intracellular C:N ratios (e.g., α-kg/gln) (Guerrero and Lara, 1987; Magasanik, 1993; Muro-Pastor et al., 2001; Reitzer and Magasanik, 1987). Global N control has also been observed in cyanobacteria but the cellular cues resulting in the expression of the transcriptional activator, NtcA, are unknown (Flores and Herrero, 1994; Lindell et al., 1998).

2.2.1. Glutamine synthetase

2.2.1.1. Overview

Glutamine synthetase (GS) (EC 6.3.1.2) catalyzes the condensation of glutamate and ammonium to form glutamine at the expense of ATP. There are three families of GS enzymes that have been identified. GS I, encoded by the glnA gene, is the typical eubacterial GS found in bacteria and archaea (Robertson et al., 1999, 2001), but also occurs in cyanobacteria (Herrero et al., 2001), and possibly some vascular plants (Mathis et al., 2000). It has 12 equally sized subunits. Type II GS, encoded by glnII, is found in eukaryotes and a few bacteria (Robertson et al., 2001). It has eight equally sized subunits. Type III GS, encoded by glnT and glnN, was thought to occur only in limited numbers of bacteria and in nondiazotrophic cyanobacteria (Herrero et al., 2001), but now appears to be more broadly distributed among the algae, e.g., diatoms and haptophytes (Robertson et al., 2001). In cyanobacteria, type III GS (glnN) consists of six identical subunits that are much larger than those of GSI or GSII (Merrick and Edwards, 1995).

The three classes of GSs are structurally related but their sequence similarities vary. Isoenzymes of GS are expressed in different cellular compartments, in microalgae, the cytoplasm and chloroplasts. The evolutionary origin of isoenzymes is the subject of research as are the relationships between different forms of GS (e.g., Robertson et al., 1999, 2001). Recent sequencing projects have provided data that indicate even greater complexities. For example, all of the genomes sequenced for Prochlorococcus have glnA and the amino acid sequence, molecular size and kinetic parameters are similar to those of other cyanobacteria (García-Fernández et al., 2004). Unexpectedly, the T. pseudonana genome contained all three forms of GS including the prokaryotic GSI type. The function of the different isoenzymes is also unclear. In higher plants, GS1 (cytosolic form) is thought to catalyze an N-recovery pathway and is typically used as a stress marker (e.g., Pageau et al., 2006).

2.2.1.2. Assay techniques

GS is an excellent example of a reversible enzyme that is highly regulated and modified at multiple levels. GS is assayed in both the forward and reverse directions. In the forward direction, GS assays the biosynthesis of glutamine (glu) in the presence of the divalent cation Mg^2+ (biosynthetic activity). Because Mg^2+ appears to inhibit the activity of modified enzyme in the forward reaction, biosynthetic assay is thought to represent in vivo potential for glu synthesis via GS (Lee et al., 1988). In the reverse direction, GS assays measure total potential GS activity of both active and inactive enzyme in the presence of Mn^2+ as the divalent cation (transferase activity) (Lee et al., 1988; Stadtman et al., 1979). In both of these
assays, hydroxylamine can be substituted for NH$_4^+$ and provides a stable product, γ-glutamylhydroxamate, which is easily measured and does not undergo further metabolism in vitro (Mulholland and Capone, 1999). The ratio between the forward and reverse direction assays may also provide an index of enzyme modification and the proportion of the total enzyme that is biosynthetically active in cells (Lee et al., 1988; Mulholland et al., 1999).

A variety of endpoints have been used to estimate GS activity in in vitro assays run in both the forward and reverse directions. Colorimetric assays for glutamyl hydroxamate and inorganic phosphate production and the release of NH$_4^+$ associated with the hydrolysis of gln (Bressler and Ahmed, 1984; Clayton and Ahmed, 1987; Falkowski and Rivkin, 1976; Shapiro and Stadtman, 1970; Stadtman et al., 1979) are commonly employed, as are radioactive assays employing $^{14}$C-glutamate (Pahuja and Reid, 1982; Prusiner and Milner, 1970; Rhee et al., 1976; Thomas et al., 1977). The GS/GOGAT pathway was first identified as the primary pathway of N metabolism in cyanobacteria using radioactive $^{15}$NH$_4^+$ (Meeks et al., 1978); however, this method is impractical for routine assays given the short half-life of $^{15}$N.

Although this seems to paint a bright picture for GS assays, there are different forms of GS doing different things in different places within cells. In vitro assays using cell homogenates mix these enzyme pools and confounds results measuring total activity in either direction. Currently, it is impossible to distinguish activity among isoforms in bulk enzyme assays. For example, in the diatom, S. costatum, GSII appears to play a role in assimilating NH$_4^+$ derived specifically from NO$_3^{-}$/CO$_2$ reduction (Takabayashi et al., 2005).

Inhibitors have been used not only to identify pathways of N metabolism but also to distinguish between uptake and assimilation of inorganic N. For example, MSX is an irreversible inhibitor of GS and has been used to explore the role of N assimilation products (e.g., gln) and intracellular NH$_4^+$ on the feedback inhibition of processes such as nitrogenase activity and NO$_3^{-}$ uptake (Arp and Zumft, 1983). In addition, the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) has been used to distinguish between uptake and assimilation of NH$_4^+$ in marine algae (Rees et al., 1998).

2.2.1.3. Enzyme regulation and interpretation of activity assays  GS is a highly regulated enzyme and its regulation may preclude the ability to use simple activity assays to derive estimates of N assimilation in marine microbes. Different families of GS are regulated and modified differently. As with many enzymes, GS has characteristics that make interpretation of assay results difficult (e.g., sensitivities to feedback inhibition and divalent cation specificity), regulation occurs at both the level of synthesis and activity, and activity varies with growth conditions. In addition, subunits of GS are differentially and reversibly modified; by adenylylation in bacteria (Silman et al., 1995), covalent modification by ADP-ribosylation (Merrick and Edwards, 1995), phosphorylation (Miflin and Habash, 2002), and by interaction of two inhibitory polypeptides in cyanobacteria (García-Dominguez et al., 1999). The degree of inhibition appears to be governed by the number of modified subunits.
GS is regulated in response to cumulative feedback inhibition by multiple end products of glutamine metabolism (Stadtman, 2001) and the different families of GS are sensitive to different cellular cues (Shatters et al., 1993). Nitrogen regulation of GS occurs under all growth conditions. Under N-replete conditions, nitrogen assimilatory capacity is controlled to coordinate nitrogen and carbon assimilation (balance). Under nitrogen-depleted conditions the cell’s assimilatory capacity is modified. Severe starvation can result in expression of alternative N acquisition pathways (Ninfa and Atkinson, 2000).

Modification of GS is mediated through regulatory proteins (e.g., P II) that are themselves highly regulated and reversibly modified (Arcondégui et al., 2001; Lee et al., 2000; Magasanik, 2000). P II proteins serve as signal processing proteins that integrate cellular carbon and nitrogen status and signal the control of N assimilation via GS in enteric bacteria, archaea, and some cyanobacteria (Arcondégui et al., 2001). The complexities of these regulatory pathways are beyond the scope of this review but regulation appears to occur in response to environmental factors such as dissolved inorganic nitrogen concentrations and feedback from intracellular metabolites. A glnB gene encoding for P II protein has also been identified in marine cyanobacteria including, Trichodesmium erythraeum, Prochlorococcus, Synechococcus (García-Fernández et al., 2004) as well as Synechocystis sp. and Synechococcus sp. (Forchhammer and Tandeau de Marsac, 1995; García-Domínguez and Florencio, 1997; Wyman, 1999).

As for GS, activation of the P II protein is regulated by nitrogen availability and is dependent on photosynthetic electron transport (García-Domínguez and Florencio, 1997; Reyes and Florencio, 1995). P II proteins have been reported so far only for red algae (Reith and Munholland, 1993), and marine cyanobacteria (García-Fernández et al., 2004). However, it has been suggested that the P II protein in Prochlorococcus marinus, like that of Synechocystis may serve more as a sensor for C acquisition than for N control (Palinska et al., 2002). In addition to P II signal transducer proteins, GlnK and GlnK-like proteins may also contribute to N regulation and signal sensing in bacteria and archaea. These proteins are structurally similar but functionally distinct (Ninfa and Atkinson, 2000).

Transcription of both the GS gene (glnA) and the P II gene (glnB) are regulated by the transcription activator NtcA in enteric bacteria (Magasanik, 2000). However, there appear to be simpler regulatory pathways in some cyanobacteria such as Prochlorococcus (Gómez-Baena et al., 2001). We still know little about the regulatory pathways affecting GS across algal taxa or among different GS pools. GS is not the only enzyme that is under N control in cyanobacteria (e.g., nitrogenase and NR above). Other enzymes involved in N uptake and assimilation are also regulated in response to N through common regulatory pathways. Because GS is needed to assimilate all N, GS activity must be maintained for cellular N acquisition. However, it appears that not only are there different GS pools but that they may be regulated differently and are transcribed from different promoters during growth on different forms of N (e.g., N2; Tumer et al., 1983). For example, nonheterocystous N2 fixing bacteria and cyanobacteria have been shown to have elevated GS activity when fixing N2 (Meeks et al., 1987; Rai et al., 1992; Smith and Gallon, 1993). For Trichodesmium spp., glnA transcript abundance was elevated both during the
pre-dawn period corresponding with the peak \textit{nifH} transcript abundance and another peak late in the afternoon that was not correlated with nitrogenase-associated gene products (e.g., constitutive; Kramer \textit{et al.}, 1996; Mulholland \textit{et al.}, 1999; Wyman \textit{et al.}, 1996). Consistently, the ratio of GS transferase/biosynthetic activity, or the proportion of the enzyme that was biosynthetically active, changed in response to the daily pattern of \textit{N}_2 fixation in \textit{Trichodesmium} IMS101, consistent with the idea of two separate pools of GS regulated differently, at the level of enzyme synthesis (Mulholland \textit{et al.}, 1999).

2.2.1.4. Information gained from assays and future potential

GS activity is critical to our understanding of N assimilation in marine organisms but, is clearly a very complicated enzyme both from the standpoint of activity and regulation. Understanding GS activity and its regulation might offer insights into the marine N cycle but, in practice, it may be beyond our abilities to measure it effectively and to gain useful information from the interpretation of bulk enzyme assays at this time.

2.2.2. Glutamate synthase

2.2.2.1. Overview

Glutamate synthase (GOGAT; formerly glutamine: 2-oxoglutarate amidotransferase) is a complex iron–sulfur flavoprotein that catalyzes the synthesis of two glu molecules from a molecule each of gln and \(\alpha\)-kg. The eubacterial version of the enzyme is composed of two subunits. The small subunit is a flavin adenine dinucleotide-dependent NADPH oxidoreductase which shows sequence similarity to several other protein domains and enzyme subunits. Indeed, the small subunit of eubacterial glutamate synthase has been proposed to be a prototype of protein domains or enzyme subunits used in many different contexts to transfer electrons from NAD(P)H to an acceptor protein or protein domain. In the case of glutamate synthase, the small subunit provides electrons to the large subunit, which binds L-gln and 2-oxoglutarate and forms L-glu.

There appear to be three classes of GOGAT based on the source of the electron donor used for the conversion of glutamine and \(\alpha\)-kg to glutamate: NADPH-GOGAT (EC 1.4.1.13) found primarily in bacteria and archaea, both NADH-GOGAT (EC 1.4.1.14) and Fd-GOGAT (EC 1.4.7.1) are found in eukaryotes (higher plants and algae), cyanobacteria, and animal cells. Different forms of the enzyme have distinct genes (Suzuki and Knaff, 2005). However, distribution of various forms of the enzyme appear to be complex. All three types of GOGAT were found in the \textit{T. pseudonana} genome; \textit{glsF}, the cyanobacterial ferredoxin dependent version, and two NAD(P)H dependent forms, \textit{gltB} and \textit{glt D}. It had been previously speculated that these two genes were typically fused to form \textit{glnN} in eukaryotes; however, this is clearly not the case in \textit{T. pseudonana} where these genes are separate (Armbrust \textit{et al.}, 2004; Berges, unpublished data).

2.2.2.2. Assay techniques

Assays of GOGAT rely on measuring fluorometrically or spectrophotometrically the oxidation of reductant (Ahmed \textit{et al.}, 1977; Avila \textit{et al.}, 1987; Clayton and Ahmed, 1986; Inokuchi \textit{et al.}, 1999), or measuring products of enzyme activity (e.g., \textsuperscript{14}C-glu production; Cullimore and Sims, 1981; Prusiner and Milner, 1970). As for other enzyme assays, these assays are sensitive to
pH, temperature and substrate concentrations. Azaserine, a gln analog, inhibits GOGAT and has been used to probe GOGAT activity in nature.

### 2.2.2.3. Enzyme regulation and interpretation of activity assays

GOGAT activity does not appear to be as heavily regulated as GS activity, or perhaps it is just less studied (Florencio et al., 1987; Muro-Pastor et al., 2005). However, GOGAT does appear to be regulated by light and metabolite sensing systems in higher plants (Suzuki and Knaff, 2005). Cyanobacterial systems are well studied compared to other marine phytoplankton, but no regulation of GOGAT expression by N source has been observed for this group (Herrero et al., 2001). Interestingly, in heterocystous cyanobacteria, GS is present but GOGAT is absent from the heterocysts suggesting that gln or some other amino acid has to be transported from the heterocysts to vegetative cells to be further metabolized (Muro-Pastor et al., 2005).

### 2.2.2.4. Information gained from assays and future potential

GOGAT may be an excellent example of an enzyme that plays a secondary role in a cyclic enzyme pathway. However, if this is the case, it is unclear why it is so highly regulated in some cases. Additional functions for this enzyme in N metabolism may yet be identified.

### 2.2.3. Glutamate dehydrogenase

#### 2.2.3.1. Overview

Glutamate dehydrogenase (EC 1.4.1.3) is an alternative pathway for NH$_4^+$ assimilation and glu synthesis in some species of algae, such as green microalgae and macroalgae, but it does not appear to be the dominant pathway of N assimilation in most marine phytoplankton. GDH is thought to function primarily in a catabolic role and likely only plays a role in ammonium assimilation when intracellular ammonium concentrations are very high (Melo-Oliveira et al., 1996; Moorhead and Smith, 2003).

As for GS and GOGAT, there appear to be several families of GDH with many isoenzymes in each family. NADP-GDHs have been found in the chloroplasts of green algae as well as in the mitochondria and cytosol of other species including macroalgae (Inokuchi et al., 1999). Organelle-specific GDH isozymes (chloroplastic, mitochondrial, and cytosolic) and two distinct GDH genes were isolated in the green macroalgae Bryopsis maxima (Inokuchi et al., 1999). NADP-dependent GDH activities were found in all three cellular compartments while NAD-dependent GDH was observed only in the chloroplasts.

Genes encoding GDH are not present in most cyanobacteria; however, they are present in one Prochlorococcus genome and in T. erythraeum (García-Fernández et al., 2004; Muro-Pastor et al., 2005). Interestingly, Trichodesmium appear to release NH$_4^+$ during active N$_2$ fixation (Mulholland et al., 2004a,b) and destroy and resynthesize much of their nitrogenase daily (Zehr et al., 1993), suggesting a catabolic role. Both NADH- and NADPH-specific forms of GDH are present in the diatom. T. pseudonana (Armbrust et al., 2004).

In heterotrophs, GDH is involved in the production of NH$_4^+$ during catabolism of protein (Park et al., 1986). Indeed, zooplankton often account for the majority of
GDH activity in marine systems (Bidigare et al., 1982; Park et al., 1986). Assays of the enzyme in the reverse direction are virtually identical to those discussed below.

### 2.2.3.2. Assay techniques
GDH utilizes both nicotinamide nucleotide cofactors; NAD$^+$ in the direction of N liberation (catabolic) and NADP$^+$ for N incorporation (assimilatory). In the forward reaction, GDH catalyzes the synthesis of amino acids from free ammonium and α-ketoglutarate. The reverse reaction links amino acid metabolism with TCA cycle activity. In the reverse reaction, GDH provides an oxidizable carbon source used for the production of energy as well as a reduced electron carrier, NADH, and production of NH$_4^+$.

As for other enzymes, spectrophotometric methods have been developed for measuring oxoglutarate and aminotransferase activities by assaying substrates and products of the GDH catalyzed reaction (Ahmad and Hellebust, 1989).

### 2.2.3.3. Enzyme regulation and interpretation of activity assays
GDH is ubiquitous in both higher and lower organisms where it serves in both biosynthetic and catabolic capacities. Little is known about its regulation in marine microbes because it is not thought to be biosynthetically active in most marine environments and because of low N concentrations. Although it is thought that GDH functions primarily in the reverse direction based on kinetic arguments, activity measurements are sparse as are measurements of intracellular NH$_4^+$ concentrations. It has recently been argued that in higher plants, GDH may have a role in stress response (Dubois et al., 2003), but this has not been examined in marine algae.

### 2.2.3.4. Information gained from assays and future potential
This enzyme is easily assayed in vitro but results from assays have rarely shown relationships with N assimilation, perhaps because this is not its major function. It might be time to revisit GDH assays and consider relationships with nonassimilatory pathways.

### 2.3. Organic nitrogen acquisition
Although inorganic N has traditionally been thought to be the primary N source for marine autotrophs, it is now apparent that this is not always true (see Chapter 7 by Mulholland and Lomas, this volume) and that organic N can be a major N source in many marine environments. With that in mind, enzymes involved in the hydrolysis or mobilization of organic N are examined.

#### 2.3.1. Urease

##### 2.3.1.1. Overview
Urease is found in diverse groups of marine bacteria, plants, and animals. In most organisms, urease serves to break down urea to mitigate its toxic effects in the cellular environment. However, urea is the most abundant identifiable form of dissolved organic nitrogen (DON) in most aquatic ecosystems and most organisms can use urea as an N source by taking it up into the cytoplasm where it can be hydrolyzed into two ammonia molecules which can then be assimilated. Intracellular hydrolysis of urea and its ultimate assimilation occurs through one of two enzymatic pathways: urease (urea amidohydrolase, EC 3.5.1.5) or ATP:urea
amidolyase (UALase). The latter has been demonstrated to exist exclusively in the chlorophyte algae (Leftley and Syrett, 1973). UALase complex catalyzes the production of two ammonia and two bicarbonate ions from urea and bicarbonate via a carboxylating reaction and then the produced allophanate, water, and hydroxyl group by hydrolysis (Bekheet and Syrett, 1977).

Urease is widespread in phytoplankton, including diatoms (Lomas, 2004; Milligan and Harrison, 2000; Oliveira and Antia, 1986; Peers et al., 2000), chrysophytes (Leftley and Syrett, 1973), dinoflagellates (Dyhrman and Anderson, 2003; Fan et al., 2003), a pelagophyte (Fan et al., 2003), and cyanobacteria (Herrero et al., 2001); and in bacteria, (Oliveira and Antia, 1986) and other organisms (see Antia et al., 1991; Leftley and Syrett, 1973). Urease activity involves the nickel-dependent hydrolysis of urea into two NH\textsubscript{4}\textsuperscript{+} molecules and CO\textsubscript{2}. Urease is constitutive in many cases for algae but not for bacteria. Urease is generally cytoplasmic (Mobley and Hausinger, 1989).

Urease was the first enzyme whose functional structure was elucidated (see Zimmer, 2000). All together seven polypeptides are needed for functional urease. UreC is the major functional subunit of urease and is large (Collier et al., 1999) relative to the ureA and ureB subunits; all are highly conserved. In addition, four accessory genes, ureD, ureE, ureF, ureG, are needed for enzyme assembly.

### 2.3.1.2. Assay techniques

As for other N compounds, uptake is often estimated using isotopic tracers and urease activity is inferred from incorporation of labeled atoms into biomass. In addition to molecular methods of purifying and characterizing ureases in organisms, in vitro colorimetric methods for assaying urease activity in marine microbes have been developed (Peers et al., 2000). Hydrolysis of urea produces two NH\textsubscript{4}\textsuperscript{+} and a CO\textsubscript{2} molecule, both of which have been used as endpoints to estimate urease activity in marine organisms. \textsuperscript{14}CO\textsubscript{2} has been measured as an endpoint of urea hydrolysis using \textsuperscript{14}C-labeled urea (Bekheet and Syrett, 1977; Ge et al., 1989; Leftley and Syrett, 1973). Assays have also measured NH\textsubscript{4}\textsuperscript{+} production from urease to estimate activity (Collier et al., 1999; Fan et al., 2003; Jahns et al., 1995; Oliveira and Antia, 1986; Peers et al., 2000). Unfortunately, easily measurable products of urease, NH\textsubscript{4}\textsuperscript{+} and CO\textsubscript{2}, are subsequently released or consumed in both downstream and competing biochemical pathways.

### 2.3.1.3. Enzyme regulation and interpretation of activity assays

There appear to be species-specific differences in the degree of constitutive expression versus upregulation of urease activity depending upon nutrient prehistory. For example, urease activity has been shown to be upregulated in \textit{T. pseudonana} grown on NO\textsubscript{3}\textsuperscript{−} and urea (Peers et al., 2000), downregulated in \textit{T. weissflogii} grown on NO\textsubscript{3}\textsuperscript{−} (Lomas, 2004), and upregulated in \textit{Synechococcus WH7805} growing on NO\textsubscript{3}\textsuperscript{−} (Collier et al., 1999). The pelagophyte, \textit{Aureococcus anophagefferens}, and dinoflagellate, \textit{Prorocentrum minimum}, exhibited high urease activities regardless of the N source (Fan et al., 2003a) while the dinoflagellate \textit{Alexandrium fundyense} displayed inducible urease activity (Dyhrman and Anderson, 2003). Constitutive and ammonium-repressible ureases are present in cyanobacteria. However, expression of high affinity urea
uptake activity is subject to N control and has an ABC-type high-affinity transporter (Herrero et al., 2001; Valladares et al., 2002). As for nitrogenase and NR, urease expression in marine Synechococcus (WH7805, WH8102) is part of the NtcA operon (e.g., therefore, N regulated).

### 2.3.1.4. Information gained from assays and future potential

Urea uptake may be much more important than previously thought in the open ocean (see Chapter 7 by Mulholland and Lomas, this volume). Not only do autotrophs use urea, but bacteria also have ureases, suggesting that they can readily use urea, although the available data suggest that autotrophic phytoplankton have consistently lower \( K_m \) values for urea than do bacteria (Fan et al., 2003; Jahns, 1992; Mobley and Hausinger, 1989). Another surprise arising from a genomic analysis of *T. pseudonana* is that this diatom has a fully-functional urea cycle. Such a cycle is typical of animals, where \( \text{NH}_4^+ \) generated during catabolism is converted into urea for excretion. It is unclear whether diatoms actually excrete “waste” urea since they also possess an apparently constitutively active urease that can hydrolyze urea to \( \text{NH}_4 \) and \( \text{CO}_2 \) and three urea transporters (Armbrust et al., 2004). The function of the urea cycle in this diatom is still a mystery, but bacteria are known to produce urea and in general, we know little about the production or sources of urea in the marine environment, despite the observation that it can fuel much of the productivity at times (see Chapter 7 by Mulholland and Lomas, this volume). In toad fish, urea synthesis appears to act as part of a N salvage mechanism (Mommsen and Walsh, 1989), and in teleostean fish and elasmobranches, urea serves as an osmolyte. If in fact diatoms produce urea internally in order to turnover amino acids and recycle N, reports of constitutive urease activity indeed make sense.

### 2.3.2. Extracellular enzymes acting on organic nitrogen

#### 2.3.2.1. Overview

In general, less is known about cell surface than intracellular enzymes, particularly for phytoplankton. One of the reasons is that although dissolved organic matter (DOM) is the largest pool of combined N in the ocean, its availability to organisms is unknown because DOM is a complex mixture of compounds, most of which are uncharacterized (see Hansell and Carlson, 2002). Because of this complexity, a variety of different substrate-specific extracellular enzymes are necessary to remineralize DOM in nature (Hoppe, 1991) so that it is available to microorganisms. Most of the DOM is ultimately derived from primary production and proteins typically represent at least 75% of phytoplankton cell N (Dortch et al., 1984; Nguyen and Harvey, 1994) and 80% of bacterial cell N (Kirchman, 2000). Dissolved combined amino acids (DCAA) typically represent between 5 and 20% of the dissolved organic nitrogen (DON) pool (Bronk, 2002; Keil and Kirchman, 1991; Sharp, 1983), and 3–4% of the dissolved organic carbon (DOC) pool in seawater (Benner, 2002). The high molecular weight (HMW) DOM is thought to be the more bioactive fraction of the bulk DOM pool and it is estimated that 75–85% of the nitrogen in HMW DOM is in amide structures (Benner, 2002). While much of this is resistant to chemical hydrolysis in the laboratory, organisms are clearly able to hydrolyze or use some of this N because the C:N ratio of particulate organic material (POM) and DOM decreases significantly as material is degraded.
Extracellular degradation of HMW DOM into usable components is necessary for microorganisms to take advantage of organically-bound N because most microbes and phytoplankton can take up only inorganic or small organic compounds (Antia et al., 1991; Nikaido and Vaara, 1985) and most HMW DOM is just too big. Therefore, extracellular hydrolysis of proteins and peptides is required before this N can be used for growth. Particularly important are extracellular enzymes that degrade large polymeric biomolecules to small, labile compounds. In addition, some organisms may not be able to use organic compounds at all and require remineralization of DON prior to its uptake. Extracellular amino acid oxidases can facilitate production of dissolved inorganic nitrogen (DIN) from amino acids (Mulholland et al., 1998; Palenik and Morel, 1990a,b; Palenik et al., 1989) and there is close coupling between amino acid oxidation and uptake of NH\textsubscript{4}\textsuperscript{+} produced from these reactions (Mulholland et al., 2003).

Extracellular enzymes include exoenzymes that occur free in the water or adsorbed to surfaces other than those of their producers whereas ectoenzymes are bound to the cell surface or are found in the periplasmic space (Chrost, 1991). Extracellular enzymes responsible for degrading proteins, peptides, and amino acids have been widely attributed to heterotrophic bacteria (Rosso and Azam, 1987; Sinsabaugh et al., 1997; Hoppe, 1983, 1991; Hoppe et al., 2002), although phytoplankton have been found to possess cell surface enzymes that can oxidize amino acids (Mulholland et al., 1998; Palenik and Morel, 1990a,b; Palenik et al., 1989; Pantoja and Lee, 1994) and hydrolyze peptides (Mulholland et al., 2002, 2003; Stoecker and Gustafson, 2003). LAP activity has also been observed in cyanobacteria (Martinez and Azam, 1993; Stoecker et al., 2005). Other types of plankton may also be responsible for dissolved protease activities. For example, heterotrophic flagellates are a source of protease activity (Karner et al., 1994; Sala and Güde, 1999), and mesozooplankton grazers may also be important (Bochdansky et al., 1995).

2.3.2. Assay techniques Fluorogenic compounds and fluorescent derivatives have been commonly used to evaluate extracellular enzyme activity. Fluorogenic substrates are comprised of an artificial fluorescent molecule bound to a more natural molecule (e.g., amino acids) with a specific linkage (e.g., peptide binding). Fluorescence is observed after enzymatic cleavage of the bond. Assays employing fluorogenic compounds are very sensitive. Extrapolating enzyme activity from assays employing fluorogenic compounds can be complicated, however, by competing or inhibitory compounds naturally occurring in the environment. Two commonly used fluorogenic compounds are 4-methylumbelliferyl (MUF) and 7-amino-4-methylcoumarin (AMC). Both L-leucyl-B-naphthylamide and L-leucine-4-methylcoumarinyl-7-amide (Leu-MCA) have been used to assay aminopeptidases in aquatic systems and MUF-N-acetyl-glucosaminide has been used to investigate chitinase activity (Berg et al., 2002). These compounds are too large to cross cell membranes.

There are many types of proteases, but an important division is endo- vs. exopeptidases. Endopeptidases (= proteinases) that cleave within polypeptide chains are likely important in the early stages of protein degradation, while exopeptidases that act on the termini of polypeptide chains (i.e., amino- or carboxypeptidases) are
probably more important during the intermediate and late stages of protein degradation (Barrett, 1986). A number of endo- and exopeptidases with different substrate specificities have been described, some of which are active at alkaline pH (Barrett, 1986). Only a few types have been reported in seawater, probably because of the limited number of artificial substrates available for use in investigating extracellular enzyme activity in aquatic ecosystems. Other extracellular enzymes have been examined in aquatic systems using fluorogenic substrates in natural assemblages or cultures (Berg et al., 2002; Hoppe, 1983; LeCleir and Hollibaugh, 2006; Martinez et al., 1996; Sherr and Sherr, 1999; Sinsabaugh et al., 1997).

There are fewer measurements of endopeptidase activity in aquatic samples (see below). In contrast, there have been numerous measurements of LAP, an exopeptidase, in freshwater and marine environments using artificial fluorogenic substrates, such as Leu-AMC (Martinez et al., 1996; Rosso and Azam, 1987; Sinsabaugh et al., 1997; Stoecker and Gustafson, 2003; Stoecker et al., 2005). LAP hydrolyzes a broad spectrum of substrates with a free amino group, but has a preference for N-terminal leucine and related amino acids (Mahler and Cordes, 1966). Measuring LAP may underestimate peptide utilization not only because dipeptides are directly incorporated (see below), but also because endopeptidases are not the only enzymes that hydrolyze protein-like material.

Alternative methods for measuring rates of amino acid oxidation and peptide hydrolysis in seawater samples are the application of fluorescently labeled amino acids and peptides, Lucifer Yellow Anydride-lysine (LYA-lysine) and Lucifer Yellow Anydride-tetraalanine (LYA-ala4), respectively (Mulholland et al., 1998, 2002, 2003; Pantoja and Lee, 1999; Pantoja et al., 1993, 1994, 1997 Fig. 32.2). These methods have the advantage of using real amino acids and peptides with the availability of several peptide bonds to cleave. Uptake of $^{15}$NH$_4^+$ from oxidation of $^{15}$N-LYA-lysine has been demonstrated (Mulholland et al., 2003). In addition, the primary products of LYA-ala4 hydrolysis appear to be dipeptides (Mulholland et al., 2002, 2003, unpublished data) suggesting that it may measure endopeptidase activity and that endopeptidase activity exceeds exopeptidase activity in the environment. Direct uptake of dipeptides has also been observed suggesting that uptake competes with exopeptidase activity and that LAP assays may underestimate the hydrolysis and utilization of peptides by marine microbes (Mulholland and Lee, submitted for publication).

Assays of extracellular enzyme activity must take care not to disrupt cells and thereby bias results by including intracellular enzyme activity in rate estimates. Berges and Falkowski (1996, 1998) found cell-associated LAP with peak activity between pH 7.5 and 8.5 (the pH range of seawater) in homogenates of marine phytoplankton, however, in this study no attempt was made to distinguish intracellular from ectocellular enzymes.

No molecular methods for cell surface enzymes are yet available, but one study by (Palenik and Koke 1995) found an N-regulated cell surface (membrane or wall) protein, nrp1, present in N-limited cells and during growth on urea. This method located enzyme on the cell surface using a biotinylation process. Derivatizing the cell-surface with a biotin-containing reagent causes the biotin to be transferred to cell-surface proteins which then allows the labeled proteins to be detected on
western blots because of the high affinity of the protein avidin to the biotin moiety (Palenik and Koke, 1995). While they were unable to identify the function of nrp1, they were able to rule out a cell surface amino oxidase, but it is possible that nrp1 is another type of degradative enzyme or transporter for organic N.

Other endpoints that have been used to estimate extracellular enzyme activity include monitoring the concentrations of substrates and products of oxidation or hydrolysis reactions (e.g., \( \text{H}_2\text{O}_2 \), \( \alpha \)-keto acids, amino acids). These methods are limited however, because there are multiple sources and sinks of these compounds in the environment.

### 2.3.2.3. Enzyme regulation and interpretation of activity assays

Little to no information is available on the regulation of these enzymes although extracellular peptide hydrolysis and amino acid oxidation appear to be sensitive to the nutrient environment (Mulholland et al., 2004a,b; Mulholland and Lee, submitted for publication).
2.3.2.4. Information gained from assays and future potential  

The contribution of eukaryotic phytoplankton to protease activity in the water has only recently been reconsidered (Sala et al., 2001). Most of the evidence for exo- or endopeptidase activity in phytoplankton is from research with cultures (Stoecker and Gustafson, 2003).

Langheinrich (1995) investigated plasma membrane-associated leucine and alanine aminopeptidase activities in *Chlamydomonas reinhardtii* as well as in several other freshwater microalgae. *C. reinhardtii*, can grow on histidine, thanks to presence of histidinase and urocanase (Hellio et al., 2004). Berg et al. (2002) found that axenic cultures of the “brown tide” pelagophyte, *A. anophagefferens* had high rates of extracellular hydrolytic enzyme activity and could out-compete several groups of bacteria for organic compounds. Further, these cultures used the hydrolysis products to meet their N demands for growth. Mulholland et al. (2002) found high rates of peptide hydrolysis both in nature and in nonaxenic cultures of *A. anophagefferens* and measured rates of peptide hydrolysis varied depending on whether cultures were conditioned on NO$_3^-$, NH$_4^+$, or urea as an N source suggesting that enzyme activity is at least partially controlled by nutrients (Mulholland et al., 2004a,b; Mulholland and Lee, submitted for publication). Surveying cultures across a wide range of taxa suggests that the ability to hydrolyze peptides may be common (Fig. 3 from Mulholland and Lee, submitted for publication). Other extracellular enzymes may also be common in the marine environment but far less is known about them.

2.3.3. Digestive proteases

2.3.3.1. Overview  

Heterotrophs represent a major sink for primary production, and thus a critical part of the marine N cycle. In the pelagic realm, there have been attempts to estimate zooplankton grazing (both micro- and macro-zooplankton) using two major enzymatic approaches: activities of digestive enzymes (especially proteases in the case of N) (e.g., Gonzalez et al., 1993) and the activity of GDH, the
key step in the pathway of excretion of \( \text{NH}_4^+ \) following catabolism of protein (e.g., Bidigare et al., 1982; Mayzaud, 1987; Park et al., 1986). Attention has also been given to the digestive enzymes of benthic heterotrophs, though this has been related more to assessing digestive acclimation and food quality than to quantifying rates. Digestive enzymes have been examined in connection with food supply and feeding rates (e.g., Mayer et al., 1997), and to help determine what materials might be degraded by different species in different environments (Roberts et al., 2001).

### 2.3.3.2. Assay techniques

Traditional assays for digestive proteases were based on spectrophotometric measurements of substrate hydrolysis, but in some cases these substrates lacked the sensitivity necessary for field work and there may also be problems with specificity. In the past 10 years, due largely to the demands of the emerging proteomics field, a wide range of fluorometric substrates have become available, especially amino methyl-coumarin conjugates (AMC or MCA), which have been discussed above in the section on extracellular enzymes (see also Sarath et al., 1989). A wide range of highly-specific substrates are available (e.g., Suc–Ala–Ala–Phe–AMC for chymotrypsin [http://www.bachem.com/]), as well as corresponding inhibitors.

### 2.3.3.3. Enzyme regulation and interpretation of activity assays

Because digestive enzymes are released into the guts of multicellular organisms, or contained within structures like food vacuoles of unicellular ones, they are not under the same degree of regulation as are many other enzymes that have been discussed. Control is exerted during synthesis by having inactive forms (proenzymes or zymogens), which are activated just prior to release. As a result, the major difficulty in interpreting digestive enzyme activities is that they may respond to feeding conditions with variable and unpredictable time lags (e.g., Hassett and Landry, 1990; Mayzaud et al., 1992; Roche-Mayzaud et al., 1991). There are also complications in comparing activities among species with differing gut morphology (see Han et al., 2002). In terms of assays, generic substrates have often been used so that the precise identities of the proteases remain unknown; it is likely that intracellular and gut enzymes have often been measured together. For benthic macroorganisms, fitting digestive enzyme activities into N budgets has been challenging because the long recovery times and sharp changes in environment following capture of organisms are not ideal for preserving activities.

Despite the recognition that GDH may be more important in its catabolic role, GDH activities have been difficult to correlate with nitrogen excretion because of the complications of size-scaling; GDH activities scale differently with body size than do N-excretion rates (see Berges et al., 1993). Assays are also potentially complicated by the presence of assimilatory GDH in other organisms in samples.

### 2.3.3.4. Information gained from assays and future potential

Digestive enzyme activities have proven most useful in indicating the potential for an organism to metabolize a given substrate, and also for inferring that an organism has had the substrate in its diet. In some cases, variation in enzyme activity has proven useful in establishing the presences of diel feeding rhythms (e.g., Head et al., 1984).
Quantitative interpretations of digestive enzyme activities have proven challenging, but newer, more specific assays (involving highly fluorescent substrates) and improvements in image analyses may offer potential solutions, and these measurements are worth revisiting. Digestive enzyme assays have seldom been used for microzooplankton (but see Gonzalez et al., 1993), but they may be more interpretable in these species because the enzyme is intracellular and thus may be under tighter control than those found in the guts of metazoans. Very few molecular characterizations of digestive enzymes have been conducted in any group of zooplankton.

### 2.4. Other enzymes connected with nitrogen cycling

There are a variety of other enzymes that cannot be excluded in our discussion of N assimilation.

#### 2.4.1. Enzymes associated with Carbon metabolism: Carbon-fixation and respiratory pathways

**2.4.1.1. Overview** While enzymes associated with C metabolism are not directly involved in marine N cycling, they are significant for two reasons. First, N is inextricably linked with C metabolism; without C skeletons, N cannot be assimilated by autotrophs (Huppe and Turpin, 1994; Young and Beardall, 2003), and virtually all N degradation also results in respiration of C (Collos et al., 1992). Second, there is some evidence that algal species, like higher plants, may use the enzymes (especially Rubisco) as forms of N storage. This is clearest in the green algae where the pyrenoid of the chloroplast can contain the greater part of the Rubisco pool in crystalline form. Although it is not yet known how widespread such a strategy might be (see Ekman et al., 1989 for an example in red macroalgae), starvation does result in degradation of Rubisco in several species (Falkowski et al., 1989; Garcia-Ferris and Moreno, 1994). Carboxylase activities have been measured in marine systems for some time (e.g., Fontugne et al., 1991; MacIntyre et al., 1996) and more recently, species-specific measurements of gene expression of Rubisco in natural communities have become feasible (e.g., Pichard et al., 1997; Wyman, 1999).

Because of the links between rates of N acquisition and C metabolism (e.g., Grobbelaar et al., 1991), there have been attempts relate the two using the activities of respiratory enzymes and metabolic modeling (see Packard et al., 2004). Metabolic rate has been estimated using specific enzymes (succinate dehydrogenase, isocitrate dehydrogenase, cytochrome c oxidase, citrate synthase), but also by using a multi-enzyme assay known as ETS activity (see Packard, 1985), or bulk reduction of tetrazolium substrates (Bamstedt, 2000).

**2.4.1.2. Assay techniques** For Rubisco and other carboxylases, while some spectrophotometric methods exists (e.g., Gerard and Driscoll, 1996), the sensitivity necessary for measurements in the field makes radiochemical methods based on $^{14}$C most useful (Ashton et al., 1990; Keys and Parry, 1990). Most respiratory enzyme assays are spectrophotometric in nature, based on use of NADH or tetrazolium salts (e.g., Packard, 1985; Thuesen and Childress, 1993).
2.4.1.3. Enzyme regulation and interpretation of activity assays

Carboxylases and respiratory enzymes are heavily regulated in vivo, making interpretation of activity assays challenging. In the case of Rubisco, for example, there is a second enzyme system involved in posttranslational activation (Rubisco activase, Macintyre et al., 1997; Pierce et al., 1982). For respiratory enzymes, as well, control of fluxes through pathways may not rest with a single enzyme and may involve posttranslational regulation, so individual enzyme activities may be of limited use in predicting overall fluxes (Arnold and Kadenbach, 1997). As is the case for GDH (see above), there are issues involving metabolic size scaling of respiratory enzymes, as well (see Berges et al., 1993). Moreover, enzymes involved in carbon metabolism are linked to those involved in nitrogen metabolism on a number of levels. The regulatory networks of well-studied microorganisms including bacteria and cyanobacteria include a transcriptional factor, the global nitrogen regulator, NtcA, and signal transduction proteins (e.g., P, glnK see above) which coordinate the metabolism of N and C in the cell (García-Fernández et al., 2004; Herrero et al., 2001; Tandeau de Marsac et al., 2001).

2.4.1.4. Information gained from assays and future potential

Because of the high degree of regulation, C cycle enzyme activities may prove difficult to interpret by themselves. The potential for combining such measurements with activities of N cycle enzymes has been discussed (see Packard et al., 2004) but not yet realized.

2.4.2. Esterases

2.4.2.1. Overview

The presence of esterase activity in seawater has been used as evidence for lysis of cells, which represents a potentially important pathway from PON to DON in marine environments (e.g., Brussaard et al., 1995; van Boekel et al., 1992). Esterases represent a large group of enzymes (EC 3.1.x.x) that catalyze the hydrolytic cleavage of an ester bond. These enzymes have very broad specificity, and a variety of other enzymes including proteinases have “esterolytic” action. Esterase methods are based on the assumptions that these enzymes are solely intracellular, and that they do not retain activity for very long outside the cell. Additional assumptions permit estimates of lysis rate to be calculated (e.g., Agusti et al., 1998). Criticisms of the lysis rate methods have led to a number of modifications (see Agusti and Duarte, 2002; Riegman et al., 2002), and the accuracy of the quantitative method is in some doubt, however, the basic measurements have been correlated with independent measurements of cell losses in at least some cases (e.g., Brussaard et al., 1995). There is evidence to suggest that phytoplankton are the main source of dissolved esterase activity, and that grazing does not result in significant release of esterases.

2.4.2.2. Assay techniques

Esterases are measured using the substrate fluorescence diacetate (FDA). FDA cleavage leads to an increase in fluorescence, which can be measured with high sensitivity in a fluorometer. A number of controls for spontaneous degradation of substrate are necessary (Agusti and Duarte, 2002; Agusti et al., 1998; Riegman et al., 2002; van Boekel et al., 1992).
2.4.2.3. Enzyme regulation and interpretation of activity assays A major uncertainty in using esterases to estimate cell lysis is the very broad nature of the assay. It is quite clear that FDA can be hydrolyzed by a number of different enzymes, including some of the extracellular enzymes described above (e.g., Schnurer and Rosswall, 1982); obviously this is not compatible with the assumption that they are solely intracellular. Within cells, esterases are typically regulated by compartmentalization rather than other mechanisms, and so regulatory features are not likely to complicate interpretations of activity assays.

2.4.2.4. Information gained from assays and future potential The application of esterases assays have provided the first direct evidence that cell lysis (as distinguished from loss terms such as grazing and sedimentation) may be an important factor in marine systems (see Kirchman, 1999). While the meaning of cell lysis, its incidence and ecological importance remain unclear (see Franklin et al., 2006), and there are concerns about the assay, we currently lack objective, independent methods with which to measure lysis (cf., Agusti and Duarte, 2002). In future, it may be possible to identify particular esterases that are truly found only within cells and choose substrates that are more specific for them.

2.4.3. Cell death-associated proteases

2.4.3.1. Overview The esterase assays described above provide evidence for cell lysis, but they do not give information about the causes of cell lysis. One type of cell lysis that has received attention recently involves mortality driven by internal factors (e.g., nutrient or energy stress), often referred to as "cell death." This process is poorly characterized, but it applies to both eukaryotic (Berges and Falkowski, 1998; Bidle and Bender, 2008; Segovia et al., 2003; Vardi et al., 1999, 2007) and prokaryotic cells (Berman-Frank et al., 2004). There is considerable speculation about the origins and function of cell death pathways. Hypotheses about origins from transfer of genes following viral infections have been put forward, and suggestions of roles in preventing mass viral lysis, allowing survival of subpopulations of cells under stress, and facilitating formation of resting stages have been advanced (e.g., Bidle and Falkowski, 2004; Franklin et al., 2006; Segovia et al., 2003; Vardi et al., 1999). Recently, activation of cell death-associated proteases have been associated with viral lysis in the coccolithophore, Emiliania huxleyi (Bidle et al. 2007).

Cell mortality of this type appears to be related to the process of apoptosis described in multicellular systems. Apoptosis is mediated by a group of cysteine endopeptidases (EC 3.4.22.x), termed caspases, that cleave after aspartate residues. Thus, caspase activity (or what is better termed "caspase-like activity," see Section 2.4.3.2) may prove to be a good marker of this form of cell mortality.

2.4.3.2. Assay techniques Because cleavage after aspartate residues is uncommon among proteases, caspase substrates are highly specific. In animal systems, over a dozen caspases have been described, and specific substrates have been developed. Relatively little work has been done exploring caspase activities in other organisms, but gene sequence comparisons reveal that algae and bacteria appear to have variant forms of the animal caspases, termed "metacaspases"; metacaspases are also the form found in higher plants (Aravind et al., 2001; Bidle et al. 2007; Uren et al., 2000). Although sequence
homologies between caspases and metacaspases are striking (especially in the active site regions), information about metacaspase proteins and the specificities of these enzymes are largely lacking. For this reason, the conservative term “caspase-like” has often been used to describe enzymes in algae and cyanobacteria that cleave caspase substrates (but note that use of “metacaspase” is becoming more common, e.g. Biddle and Bender, 2008).

As for the proteases described above, a number of spectrophotometric and fluorescent methods are available, but typically AMC conjugates are used (Stennicke and Salvesen, 2000). Aldehyde inhibitors can be used in order to verify specificity of the assays (Bedner et al., 2000), and labelled inhibitor can also be used for direct cell staining (Bidle and Bender, 2008).

2.4.3.3. Enzyme regulation and interpretation of activity assays
Caspases are best characterized in animal species where a complex system of enzymes, proenzymes, and activation cascades exist (see Aravind et al., 2001). There is evidence suggesting that some components of these systems exist in algae (e.g., Segovia et al., 2003), but the nature of the regulation remains unknown. Some level of caspase activity is likely to be constitutive (Berman-Frank et al., 2004; Segovia et al., 2003), and it is also possible that algae and cyanobacteria contain non-caspase proteases that are able to cleave caspase substrates. Work in culture has correlated higher caspase-like activities with cell lysis events (e.g., Berman-Frank et al., 2004; Segovia et al., 2003), but relatively little can be deduced from activities measured using different substrates that are specific for mammalian caspases (see Segovia et al., 2003).

2.4.3.4. Information gained from assays and future potential
So far, few field measurements have been made of caspase-like activities (Berman-Frank et al., 2004; Vardi et al., 1999), but the assay methods appear to be sensitive enough to allow use in natural communities. As work using cultures proceeds, and our understanding of cell death processes improves, assays of capase-like activity may offer an important means to distinguish different forms of cell mortality. Aside from bulk in vitro assays, the availability of cell-permeable substrates, coupled with flow cytometry will provide improved resolution and specificity (e.g. Biddle and Bender, 2008).

Interestingly, there is evidence that cell death processes in phytoplankton may be driven by allelopathy (e.g., Casotti et al., 2005), and that similar processes may also occur in zooplankton (e.g., Romano et al., 2003). So far, caspase activities have not been detected in these situations, but the potential usefulness of the assays should be assessed.

3. Summary
For many enzymes, the period surrounding publication of the first edition of this volume was a peak in the application of activity assays to marine environments. Problems with interpretation of such measurements and inadequate characterization of the enzymes in marine organisms led to a period of decline in their use. In the years that have followed, we have made considerable progress in understanding...
enzymes, the genes that encode them, and their regulatory pathways. Studies of gene expression in marine environments are now feasible and have provided new understanding. Now, as many areas of biological research move from genomics to proteomics and “metabolomics,” it seems timely to revisit enzyme activity measurements in marine systems using our new knowledge and technical advances in substrates and equipment. There are still very few integrated studies that examine relationships between in situ uptake rates, enzyme activities, and transcription and translation, yet such work is critical to understanding regulation of marine N cycling. It is also humbling that many of the problems identified in 1983 remain exactly the same as today. We are still not much closer to reconciling the species-specific measurements in cultures with the sort of bulk assays that can be made in the field. We now know that in many marine systems, bacteria compete for the same resources as phytoplankton (Chapter 7 by Mulholland and Lomas, this volume), but we still cannot effectively determine which organisms are responsible for enzyme activities in natural samples.

ACKNOWLEDGMENTS

This research was supported by NSF grants OCE 0095923 and OCE 0136367 and a US ECOHAB Program grant to M. R. M. and grants from the Natural Environment Research Council (UK), British Council and European Union to J. A. B. The ECOHAB Program is sponsored by the National Oceanographic and Atmospheric Administration, Environmental Protection Agency, National Science Foundation, National Aeronautics and Space Administration, and Office of Naval Research. This is contribution number 285 from the US ECOHAB Program.

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