

## Expression of glutamine synthetase and glutamate dehydrogenase by marine bacterioplankton: Assay optimizations and efficacy for assessing nitrogen to carbon metabolic balance in situ

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### Abstract

Expression of glutamate metabolism enzymes, glutamate dehydrogenase (GDH), and glutamine synthetase (GS) are proposed to yield information on the nitrogen (N) to carbon (C) metabolic balance within bacterioplankton communities. Whole-cell assay conditions were optimized, and reactions were linear with time and biomass. Enzyme activities were assayed in seawater cultures from four ecosystems of contrasting trophic state amended with different regimes of glucose, amino acids, and ammonium (NH<sub>4</sub><sup>+</sup>) to validate expression patterns of GDH and GS in various combinations of N and C limitation or excess. In three of four experiments, glucose amendment enhanced GS expression by 2-fold but repressed GDH by 10% to 40% relative to the control. In contrast, addition of amino acids or NH<sub>4</sub><sup>+</sup> resulted in 20% to 90% repression of GS and enhanced GDH expression by 20% to 900%. The GDH:GS activity ratio ( $\times 10^{-3}$ ) ranged from 6 to 22 in glucose added treatments and 63 to 264 in NH<sub>4</sub><sup>+</sup>-amended treatments and appears to be a more sensitive index of bacterial N bioavailability relative to C supply than either enzyme alone. Cluster analysis was used to identify the condition of ambient bacterioplankton by matching enzyme expression with the validation results. This enzymatic approach overcomes some of the biases and limitations of other methods for assessing N metabolism in marine bacteria while providing unique information to further understand constraints of bacterioplankton respiration, production, and N flux.

Supply and quality of nitrogen (N) substrates for heterotrophic marine bacterioplankton can control biomass and production, N uptake or regeneration, and overall oceanic carbon (C) cycling and phytoplankton community dynamics (Kirchman 2000). Stoichiometric models constrain dissolved inorganic nitrogen (DIN) uptake and ammonium (NH<sub>4</sub><sup>+</sup>) regeneration by bacterial growth efficiency (BGE) and C:N ratio of organic substrates and bacterial biomass (Billen 1984; Goldman et al. 1987). A model based on bioenergetics has included substrate molecular complexity and oxidation state

(Vallino et al. 1996). Despite the sound theoretical emphasis on the coupling N and C metabolism and how it influences bacterial N fluxes, application of the models to predicting status of C and N metabolism in natural marine bacterioplankton communities is limited by difficulties in accurately measuring key model parameters (Kirchman 2000).

Our knowledge of N metabolism in heterotrophic marine bacterioplankton assemblages largely comes from experimental studies of seawater cultures (e.g., Keil and Kirchman 1991; Tupas and Koike 1991; Jørgensen et al. 1994), and ambient measurements of uptake and regeneration rates using <sup>15</sup>N or <sup>13</sup>N tracers (e.g., Wheeler and Kirchman 1986; Fuhrman et al. 1988; Hoch and Kirchman 1995). These approaches have been useful in determining the preferential use of nitrogenous substrates and the conditions limiting bacterioplankton growth. Seawater culture incubations often decouple fluxes of DOM and nutrients to bacterioplankton (Nagata 2000) and typically last longer than a day, leading to substantial changes in prokaryote community composition (González and Moran 1999; Massana et al. 2001; Carlson et al. 2002), viral proliferation and host lysis (Øvreås et al. 2003; Williams and Paul 2004),

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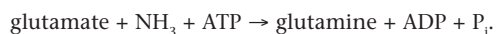
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and protist growth (Hoch et al. 1994). These limitations may bias interpretation of in situ biogeochemical processes if not recognized. Alternatively, ambient rate measurements of N uptake and  $\text{NH}_4^+$  regeneration via short-term incubations with tracer compounds provide opportunities to observe and test conditions that influence N metabolism with little disturbance to other plankton processes. For example, studies of  $\text{NH}_4^+$  uptake and its inhibition by amino acids, particularly glutamine, suggests N starved bacterioplankton possess a high affinity  $\text{NH}_4^+$  transport system and  $\text{NH}_4^+$  assimilation enzymes similar to enteric bacteria (Fuhrman et al. 1988; Kirchman et al. 1989). However, these rate measurements are technically more challenging and costly than seawater cultures, particularly if multiple compounds are studied simultaneously.

Physiological approaches to understanding bacterioplankton N metabolism in situ have largely focused on N-substrate use and defining N replete versus N deplete conditions. Aminopeptidase activity has greatly contributed to the understanding of protein degradation and use by bacterioplankton (Someville and Billen 1982; Smith et al. 1992). Recently, enhanced leucine-aminopeptidase activity (LAPA) in total plankton communities was proposed as an indicator of N-depleted plankton conditions (Sala et al. 2001). Whether LAPA increases specifically in N-depleted bacterioplankton remains uncertain given that other studies have identified the inverse relationship (Christian and Karl 1998; Jørgensen et al. 1999). The C:N ratio of bacterial biomass has also been proposed to increase with more N deplete metabolic conditions (Fagerbakke et al. 1996). This parameter reflects a coupling of N and C metabolism, but the range in biomass C:N ratio is small for natural bacterioplankton (3.8 to 9.9) and may not vary significantly (Fukuda et al. 1998). Another direct approach to exploring bacterioplankton N and C metabolism is to analyze concentrations and isotope dilution of intracellular amino acids (Simon 1991; Simon and Rosenstock 1992), especially glutamate, which couples N and C metabolism via  $\text{NH}_4^+$  assimilation. However, an assumption that high intracellular glutamate concentration indicates  $\text{NH}_4^+$  as primary N source under N deplete conditions has not been verified. Glutamate can also be produced by  $\text{NH}_4^+$  assimilation under N replete conditions and catabolism of numerous other nitrogenous metabolites (Hudson and Daniel 1993). However, the relative activities of enzymes involved in glutamate biosynthesis and catabolism could be a useful indicator of N bioavailability in heterotrophic marine bacterioplankton.

Prokaryotes have two major pathways for intracellular  $\text{NH}_4^+$  assimilation that involve glutamate (Merrick and Edwards 1995). All prokaryotes studied have a high affinity pathway ( $K_m \leq 0.1$  mM intracellular  $\text{NH}_4^+$ ) whereby ammonia is captured by glutamate to form glutamine via the glutamine synthetase (GS) reaction:



The GS reaction can couple with the glutamate synthase (GOGAT) reaction, which catalyzes transamination between

glutamine and  $\alpha$ -ketoglutarate to form two glutamates:



The glutamate dehydrogenase (GDH) reaction is an alternate lower affinity ( $K_m \geq 1.0$  mM intracellular  $\text{NH}_3$ ) pathway for many prokaryotes, whose reaction is similar to the net GS/GOGAT reaction except ATP is not required for ammonia assimilation:

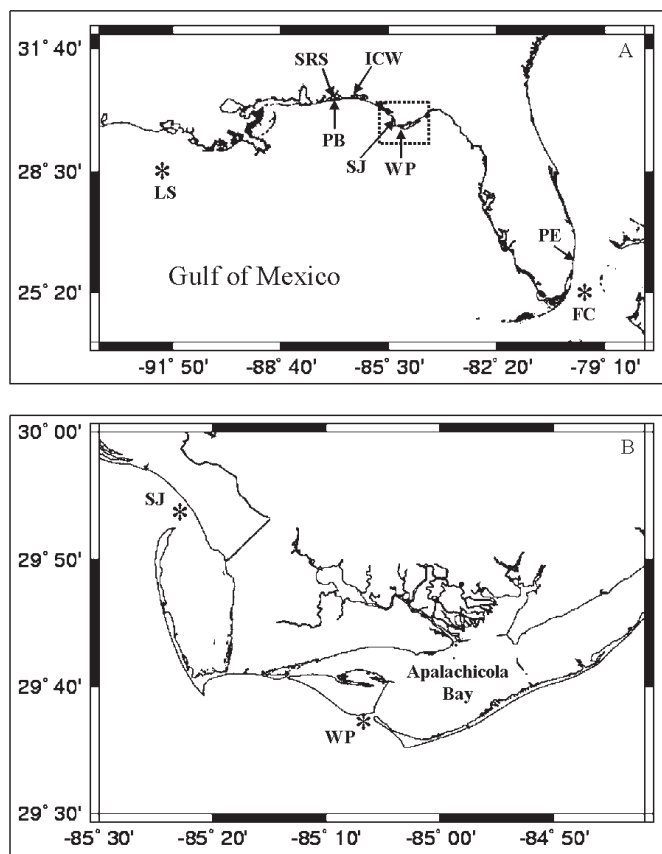


The reverse reaction permits oxidative deamination of glutamate in addition to the reductive amination of  $\alpha$ -ketoglutarate to glutamate. Generally, the anabolic reaction requires the NADPH as coenzyme and the catabolic reaction uses NADH (Hudson and Daniel 1993). There are multiple isozymes for the  $\text{NH}_4^+$  assimilation enzymes.

Four GS isozymes have been identified (Pesole et al. 1995; Eisenburg et al. 2000). Some bacteria have multiple copies of their GS gene and even more than one GS isozyme. The transcriptional regulatory mechanisms are also diverse among GS genes and even within the *glnA* gene family of enterics (Reitzer 2003), cyanobacteria (Herrero et al. 2001), and bacilli (Fisher 1999; Hu et al. 1999). Post-translational regulation of GS activity is by allosteric inhibition, and the GSI- $\beta$  isozyme is also subject to covalent modification (adenylation or ADP-ribosylation). Conditions controlling gene expression and enzyme activity are consistent for GS isozymes. Many prokaryotes have a constitutive level of GS expression, which can be greatly enhanced when N bioavailability is low relative to the organic C substrate supply and vice versa (Merrick and Edwards 1995).

There are four GDH isozymes that vary in subunit number and size as well as coenzyme specificity (Hudson and Daniel 1993; Anderson and Roger 2003). Like GS types, there are different regulatory motifs of gene expression for GDH isozymes, but in contrast to GS, expression of GDH isozymes is increased when N substrates are in excess of organic C substrates. These conditions include high (mM)  $\text{NH}_4^+$  or N-rich amino acids like arginine, ornithine, or histidine (Smith et al. 1975; Hudson and Daniel 1993; Baggio and Morrison 1996). Excess organic C and energy substrates like glucose will repress GDH, particularly catabolic GDH types (e.g., Bonete et al. 1996; Belitsky et al. 2004). Overall, the conditions that lead to GDH expression are associated with GS repression and vice versa, as has been demonstrated for putative marine pseudomonads and marine vibrios (Brown et al. 1972; Hoch et al. 1992). Thus, measurements of the GS and GDH specific activities in natural bacterioplankton may provide community level information on the average balance of N to C metabolism.

Activities of GS and GDH have been measured in a few studies of N metabolism in natural phytoplankton communities (Ahmed et al. 1977; Dorch et al. 1985; Clayton and Ahmed 1987; Mulholland et al. 2001) and one experimental study of heterotrophic bacterioplankton (Jørgensen et al. 1999). In the



**Fig. 1.** Map of the northern Gulf of Mexico and eastern Florida, USA, coasts (A), with the tidal cycle sampling area indicated by a dashed box (expanded in panel B). Asterisks or arrows specify sites of surface seawater collection at Santa Rosa Sound (SRS), Pensacola Beach (PB), the Northwest Florida Intra-coastal Waterway (ICW), Port Everglades (PE), over the Louisiana shelf break (LS), and in the Florida Current (FC). Map of the Apalachicola Peninsula (B), with the St Joseph's Bay (SJ) and west pass of Apalachicola Bay (WP) sampling sites indicated by asterisks. Latitude and longitude are indicated along map borders.

latter study, bacterioplankton in seawater cultures from a more N replete estuary had about 10-fold greater GDH and repressed GS compared with cultures from an N deplete open-ocean site. Although, there was agreement between these enzymatic results and other parameters of N bioavailability and metabolism in these incubation experiments, no controlled experiments were performed to test assumptions on conditions controlling enzyme activities nor were optimal enzyme assay conditions presented.

This paper presents optimized enzyme assay conditions, calibration of the assays with amended seawater experiments, assessment of any phytoplankton bias in assays of bacterioplankton (<0.8  $\mu\text{m}$  size fractionated seawater), and a case study of bacterioplankton GS and GDH activities in situ. We conclude that specific activity measurements of GDH and GS, or the biomass-independent GDH:GS ratio, are simple, inexpensive, rapid, and informative means of assessing N to

C metabolic balance in ambient bacterioplankton communities that will contribute to understanding microbial biogeochemical processes.

### Materials and procedures

**Ammonia assimilation enzyme assays**—GS activity was assayed as the  $\gamma$ -glutamyltransferase (GT) activity by colorimetric detection of  $\gamma$ -glutamyl hydroxymate ( $\gamma$ -GH) (Stadtman and Shapiro 1970). Unlike GS biosynthetic activity, GT activity is insensitive to adenylation state of subunits for GSI- $\beta$  types when reacted in the presence of  $\text{Mn}^{2+}$  and properly optimized for reaction pH (e.g., Bender et al. 1977). All other known GS isozymes also have GT activity (Eisenburg et al. 2000). Therefore, at substrate saturating conditions, we assume that GT activity is proportional to GS protein content (expression) for all possible isozymes. GDH isozymes were assayed as the biosynthetic activity, i.e., reductive amination activity, by the fluorometric detection of oxidized nicotinamide adenine dinucleotide (phosphate),  $\text{NAD(P)}^+$  (Ahmed et al. 1977). Activity dependent on NADPH is referred to as anabolic GDH ( $\text{GDH}_A$ ) and that dependent on NADH is referred to as catabolic GDH ( $\text{GDH}_C$ ). As with GS, at substrate saturating assay conditions, we assume that the optimized GDH activities reflect GDH expression. All initial experiments to optimize GS and GDH activity used seawater collected in 10% HCl washed carboys from beyond the surf zone in the Gulf of Mexico (GOM) at Pensacola Beach, Florida, at 30.328°N, 87.152°W and/or from Santa Rosa Sound, Florida, at 30.339°N, 87.156°W (Fig. 1). The bacterioplankton communities were isolated from total plankton by size fractionation.

GS and GDH assays were run in triplicate along with their appropriate “blank” assay, which was used to correct results for glutaminase and other  $\text{NAD(P)H}$ -oxidase activities, respectively. A modified GT reaction reagent for GS assays was prepared from stock solutions (Table 1) and included all substrates except for glutamine, which was added upon initiation of each assay. Preparation of a modified GDH reaction reagent excluded reduced  $\text{NAD(P)H}$  (Table 2), which was added separately to initiate each assay. The blank reagent for the GS assay omitted arsenate and ADP (Table 1) and that for GDH assays omitted  $\alpha$ -ketoglutarate (Table 2). Further step-by-step details for assaying GS and GDH activity in parallel are given below.

The bacterioplankton size fraction (<0.8  $\mu\text{m}$  or <0.6  $\mu\text{m}$  where indicated) was used for all enzymes assayed, unless stated otherwise. A large volume (10 to 20 L) of this size fraction was rapidly collected by low positive-pressure pumping (<5 psi) of ambient seawater through serial 10  $\mu\text{m}$  and 0.8  $\mu\text{m}$  pleated polypropylene cartridge filters (Nucleopore) using a pneumatic double diaphragm pump (model LNT-1/2 in.; Lutz Pumps). Bacterioplankton in 0.2 to 2 L of the <0.8  $\mu\text{m}$  size fraction (ca. 0.5 to 1.0  $\times 10^9$  cells) were collected on 25 mm diameter 0.22  $\mu\text{m}$  Millipore GS membranes with gentle vacuum (<5 psi) using an eight-place filtration manifold with 250 mL towers and 25 mm diameter base. Four replicate filters were

**Table 1.** Stock solutions and proportions used to prepare GT reaction reagent and GT blank reagent for GS assays

Stock solution	Final reaction concentration	Stock solution volume (mL)	
		GT reaction reagent	GT blank reagent
Deionized water	—	76.2	16.7
1.0 M imidazole (pH 7.1)	100 mM	10.0	3.0
0.1 M manganese chloride	300 $\mu$ M	0.30	0.09
0.8 M hydroxylamine	20 mM	2.50	0.75
0.1 M potassium arsenate (pH 7.1)	20 mM	20.0	—
0.1 M adenosine diphosphate*	400 $\mu$ M	0.40	—
1.28 M 2-mercaptoethanol	500 $\mu$ M	0.039	0.012

Add stock solution in descending order to avoid precipitation. The final volumes are enough to assay 30 samples each with triplicate reactions and a single blank

\*Dissolve in 1.0 M imidazole buffer, pH 7.1.

required for each enzyme assayed (triplicate reactions and one blank). An additional set of four filters were used for protein analysis. To remove residual  $Mg^{+2}$  cations content that can reduce GT activity, filters were rinsed with 3 mL sterile NaCl solution of salinity equal to the sample. Membrane filters with bacteria were transferred to 2 mL conical bottom tubes with o-ring screw caps that contained 0.5 g of 0.1 mm diameter sterile zirconium beads and 0.89 mL of the appropriate ice-cold reaction (or blank) reagent. Filters were inserted so that the bacteria side faced inward. Tubes for each assay were bead beaten simultaneously in an eight-place Mini-Bead Beater (Biospecs Products, Bartlesville) set on "high" for 1 min, followed by chilling on ice. Cell membranes not sheared by bead beating were made permeable by adding 10  $\mu$ L 10 mg/mL cetyltrimmonium bromide (CTAB) solution (0.01% wt/vol final concentration) and incubated at 25°C for 5 min (Bender et al. 1977).

GS assays were initiated by adding 100  $\mu$ L 0.2 M glutamine (made fresh daily) to each CTAB treated assay tube. Assays were incubated at 30°C for 30 to 60 min and terminated by addition of 0.5 mL "GT stop-mix" (22 g  $FeCl_2 \cdot H_2O$ , 8.0 g trichloroacetic acid, 8.2 mL concentrated HCl to 200 mL with sterile deionized  $H_2O$ ). Terminated GS assays were centrifuged

at 10,000 rpm for 10 min, 1.0 mL supernatant was transferred to 1.5 mL acrylic cuvettes with 1 cm path length, and the concentration of  $\gamma$ -GH was determined colorimetrically as absorption at 540 nm. Standards of  $\gamma$ -GH were prepared in GT reaction reagent and then treated with GT stop-mix as for the GS assay. Specific-activity of GS was defined as GT activity units (1  $\mu$ mol  $\gamma$ -GH produced per hour) per milligram protein.

GDH assays were initiated by adding 100  $\mu$ L 2 mM NAD(P)H to CTAB-treated assay tubes. NAD(P)H was made fresh daily in 0.1 M carbonate-bicarbonate buffer (pH 10.6). This alkaline solution was boiled 3 min to destroy oxidized forms (NAD(P)<sup>+</sup>) then cooled on ice prior to use in assays (Ahmed et al. 1977). Assays were incubated at 30°C for 30 to 60 min and terminated with 0.5 mL 1.2 N HCl. The acidified GDH assays were frozen and stored at -70°C until fluorometric analysis of NAD(P)<sup>+</sup>. Thawed GDH reaction tubes were centrifuged at 10,000 rpm for 10 min and 0.75 mL supernatant was added to a fresh tube with 0.5 mL 0.3%  $H_2O_2$  in 12.5 N NaOH and reacted at 60°C for 10 min. The reaction was diluted 1:7 with deionized water before reading fluorescence with a Turner Designs fluorometer at 360 nm excitation (Turner 7-60) and 460 nm emission (Turner 2A and 10% T) wavelengths. Standards of NAD(P)H were prepared in GDH

**Table 2.** Stock solutions and proportions used to prepare reaction reagent and blank reagent for GDH assays

Stock solution	Final reaction concentration	Stock solution volume (mL)	
		GDH reaction reagent	GDH blank reagent
Deionized water	—	73	22.2
1.0 M Tris-HCl (pH 7.7)	100 mM	10	3
1.0 M $\alpha$ -ketoglutaric acid*	10 mM	1	—
0.5 M ammonium sulfate	25 mM	5	1.5
0.05 M EDTA (pH 7.7)	50 $\mu$ M	0.1	0.1
1.28 M 2-mercaptoethanol	500 $\mu$ M	0.039	0.012

The final volumes are enough to assay 30 samples each with triplicate reactions and a single blank.

\*Dissolve in 1.0 M NaOH when using the free acid.

reaction reagent and treated as the GDH assays. Specific-activity of GDH<sub>A</sub> and GDH<sub>C</sub> was defined as GDH activity units (1  $\mu\text{mol NAD(P)H}$  oxidized per hour) per milligram protein assayed. Total GDH activity (GDH<sub>T</sub>) refers to the sum of GDH<sub>A</sub> and GDH<sub>C</sub> activities, and ratios of GDH to GS activities (GDH<sub>T</sub>:GS) reported here were multiplied by 1000 to generate whole numbers.

These standard procedures for GS and GDH assays were established after assessing (1) cell disruption and whole cell treatments to maximize activity, (2) substrate saturation concentrations, (3) reaction linearity over incubation time, (4) reaction linearity with biomass amount per assay, (5) incubation temperature affect on activity, and (6) optimum pH.

**Bacterial biomass and activity**—Bacterial biomass was determined as bacteria abundance or as protein content in the bacterial size fraction. Bacterial abundance was determined on Lugol's acid-fixed samples (Pomeroy 1984) using DAPI staining and epifluorescent microscopy (Porter and Feig 1980). Bacterial abundance was converted to C and N content using 20 fg C cell<sup>-1</sup> and 5 fg N cell<sup>-1</sup> (Lee and Fuhrman 1987). Protein content of bacterioplankton was determined by the bicinchoninic acid (BCA) assay (Smith et al. 1985) using assay reagents supplied in Peirce kit #23235 (Rockford) as previously described (Jeffrey et al. 1996). Briefly, bacteria size particles (<0.8  $\mu\text{m}$ ) were collected on Millipore GV-type (Durapore) membranes (0.22  $\mu\text{m}$  pore size). Protein on filters was extracted in 1 mL 1% sodium dodecyl sulfate by heating for 10 min at 100°C. Samples were centrifuged at 10,000 rpm for 10 min at room temperature, and the supernatant was assayed according to Pierce product literature. Protein standards were prepared with bovine serum albumin diluted in 1% sodium dodecyl sulfate.

Bacterial activity was measured by the incorporation of [<sup>3</sup>H-methyl] thymidine (TdR) and [<sup>14</sup>C]leucine (Leu) into total macromolecules using a dual label approach (Chin-Leo and Kirchman 1988). Final concentration was 10 nM TdR and 20 nM Leu. Specific-activity of the external leucine pool was corrected for ambient concentration of leucine measured by high pressure liquid chromatography (HPLC). Growth rates were estimated by multiplying TdR incorporation rates by  $1.8 \times 10^{18}$  cells mole<sup>-1</sup> TdR (Fuhrman and Azam 1982) and dividing by bacterial abundance.

**Phytoplankton biomass and activity**—Chlorophyll *a* (Chl *a*) was measured fluorometrically and potential photosynthesis rate ( $P_{\text{max}}$ ) was determined from the net uptake of [<sup>14</sup>C]HCO<sub>3</sub><sup>-</sup> (light minus dark incubations) (Parsons et al. 1984). Triplicate light (90% surface irradiance) and dark incubations for  $P_{\text{max}}$  determination were performed using 60 mL borosilicate BOD bottles spiked with 15  $\mu\text{Ci}$  of [<sup>14</sup>C]HCO<sub>3</sub><sup>-</sup>. Biomass for both Chl *a* and  $P_{\text{max}}$  incubations was collected on 0.45  $\mu\text{m}$  mixed-ester cellulose filters (Millipore HA-type). Filters from  $P_{\text{max}}$  incubations were treated for 6 h in a HCl vapor-saturated environment to remove any residual [<sup>14</sup>C]HCO<sub>3</sub><sup>-</sup>. A C:Chl *a* ratio of 50 (Ahlgren 1983) was assumed in calculating phytoplankton biomass from Chl *a* content.

**Seawater chemistry**—Salinity, temperature, and depth were determined with a Neil-Brown III CTD system, which was connected to the hydrocast sample rosette and operated by SeaSoft version 4.0 software (Sea-Bird Electronics). Nutrient concentrations (NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, and soluble reactive phosphate [SRP]) were assayed according to Parsons et al. (1985). Dissolved free amino acids (DFAA) were fluorometrically detected as o-phthalaldehyde derivatives separated by reverse-phase HPLC (Mopper and Lindroth 1982). Dissolved organic carbon (DOC) was measured in ambient seawater samples by high-temperature catalytic oxidation using a Shumadzu TOC-5000, and values were corrected for both instrument and water blanks (Benner and Strom 1993). Total dissolved nitrogen (TDN) concentrations were determined using high-temperature catalytic oxidation with an ANTEK-720 chemiluminescent detector (López-Veneroni and Cifuentes 1994). Dissolved organic nitrogen (DON) was calculated by subtracting the sum of inorganic N species (NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, and NO<sub>2</sub><sup>-</sup>) from the TDN value. Concentration of DOC and DON at the end of amendment experiments were calculated as the initial concentration plus any amendment minus bacterial demand (cell abundance increase converted to N or C units), which for C was adjusted for a BGE of 30%.

**Bacterioplankton amendment experiments**—Four different experiments were performed to assess bacterioplankton growth and N metabolism response to different amendments of glucose, NH<sub>4</sub><sup>+</sup>, phosphate, amino acids, or selected combinations of these compounds. Different surface (0.5 m to 4 m depth) seawater collection sites were used for these experiments to encompass a range of physicochemical conditions and trophic status of the GOM and Florida coastal waters (Fig. 1). The <0.8  $\mu\text{m}$  size fraction was used for all experimental incubations except those performed with Florida current seawater, where the <0.6  $\mu\text{m}$  size fraction was used to better exclude picophytoplankton, like *Prochlorococcus* spp. and *Synechococcus* spp., which were presumed to be a larger fraction of the total phytoplankton community in these more oligotrophic waters. Size fraction seawater was pooled before redistribution among incubation carboys to assure the same initial bacterioplankton community composition. All carboys were washed with 10% HCl and rinsed with sterile deionized water until the pH of rinse water was greater than 6.0. Prior to filling, carboys were further rinsed with the pooled size-fractionated seawater. Amendments to incubation carboys were staggered by an hour interval to account for sample processing time between treatments. All experiment treatments were incubated in darkness at ambient surface water temperature.

Experiments on Florida current (FC; 25.41°N, 80.09°W) seawater and dock water from Port Everglades, Florida (PE; 26.22°N, 80.34°W) were sampled every 6 h for about 24 h. Seawater collection and experimentation was performed aboard the OSV *Anderson*. Time-course sampling required about 40 L size-fractionated seawater per treatment, which were incubated in 50 L Nalgene carboys with spigots for easy sampling.

The PE time-course experiment (PE-exp) treatments included amendments to the following approximate concentrations: 10  $\mu\text{M}$  glucose; 5  $\mu\text{M}$  total amino acids as an equal molar mixture of the 20 genetically encoded amino acids; 9  $\mu\text{M}$   $\text{NH}_4^+$  plus 2  $\mu\text{M}$   $\text{PO}_4^{3-}$ ; and an unamended control. For the more oligotrophic FC time-course experiment (FC-exp), amendments were reduced by 5-fold as follows: 2  $\mu\text{M}$  glucose; 1  $\mu\text{M}$  total amino acids; 2  $\mu\text{M}$   $\text{NH}_4^+$  plus 0.4  $\mu\text{M}$   $\text{PO}_4^{3-}$ ; and an unamended control. All enzyme and bacterial analyses and chemical analyses except DOC and TDN were performed at each time point sampled for PE and FC experiments.

The two other amendment experiments were only sampled at the beginning and end of the incubation. The smaller volume of size-fractionated seawater required for each treatment was incubated in 10 L Cubitainers with spigots. Seawater was collected from the OSV *Anderson* over the Louisiana Shelf (LS; 28.34°N, 92.15°W), west of the Achafalaya River and Mississippi River plumes in the GOM. The LS experiment (LS-exp) incubations were amended as follows: 40  $\mu\text{M}$  glucose; 4  $\mu\text{M}$   $\text{NH}_4^+$  plus 2  $\mu\text{M}$   $\text{PO}_4^{3-}$ ; 40  $\mu\text{M}$  glucose plus 4  $\mu\text{M}$   $\text{NH}_4^+$  plus 2  $\mu\text{M}$   $\text{PO}_4^{3-}$ ; and an unamended control. For the second of these "endpoint" experiments, seawater was collected from the R/V *Bellows* in the Intracoastal Waterway of northwest Florida (ICW; 30.40°N, 86.77°W). The ICW experiment (IWC-exp) incubations were amended as follows: 10  $\mu\text{M}$  glucose; 7  $\mu\text{M}$   $\text{NH}_4^+$ ; 5  $\mu\text{M}$  total amino acids; 5  $\mu\text{M}$  glutamine, and an unamended control. The incubation endpoint was at 24 and 18 h for LS-exp and IWC-exp, respectively. Biological and chemical analyses for these experiments were the same as the two time-course experiments, except DFAA was not analyzed in the LS-exp.

**Field sampling of ambient conditions**—All enzymatic, biological, and chemical parameters were performed on the unfiltered seawater (referred to as "total plankton") collected for each amendment experiment and during ebb tide flow sampled from the R/V *Bellows* at fixed sites at the mouth of Saint Joseph's Bay, Florida (SJ; 29.98°N, 85.37°W) and the West Pass (WP; 29.63°N, 85.09°W) of Apalachicola Bay, Florida (Fig. 1). Enzyme activities, bacterial abundance, protein content, concentration of Chl *a*, and  $\text{P}_{\text{max}}$  were also measured in the bacterial size fraction at these sites and compared to respective total plankton values to assess the influence of phytoplankton on the bacterial size fraction enzyme activities.

**Statistical analyses**—Factor analysis by principle components, stepwise multiple regressions, and hierarchical cluster analysis were performed with SPSS for Windows, release 11.5.0 (LEAD Technologies, Inc.), and Excel 2003 (Microsoft) was used for all other statistics.

## Assessment

**Enzyme assay optimization**—GS and GDH assays were performed at initial pH values of 7.0 and 7.5, respectively. GDH was assayed with equimolar amounts of NADH and NADPH to approximate  $\text{GDH}_T$  activity. For each enzyme, bacterioplankton (< 0.8  $\mu\text{m}$  size fraction) from Santa Rosa Sound (Fig. 1) was

**Table 3.** Activity of GS and  $\text{GDH}_T$  for different whole cell assay treatments relative to activity of the optimized assay conditions

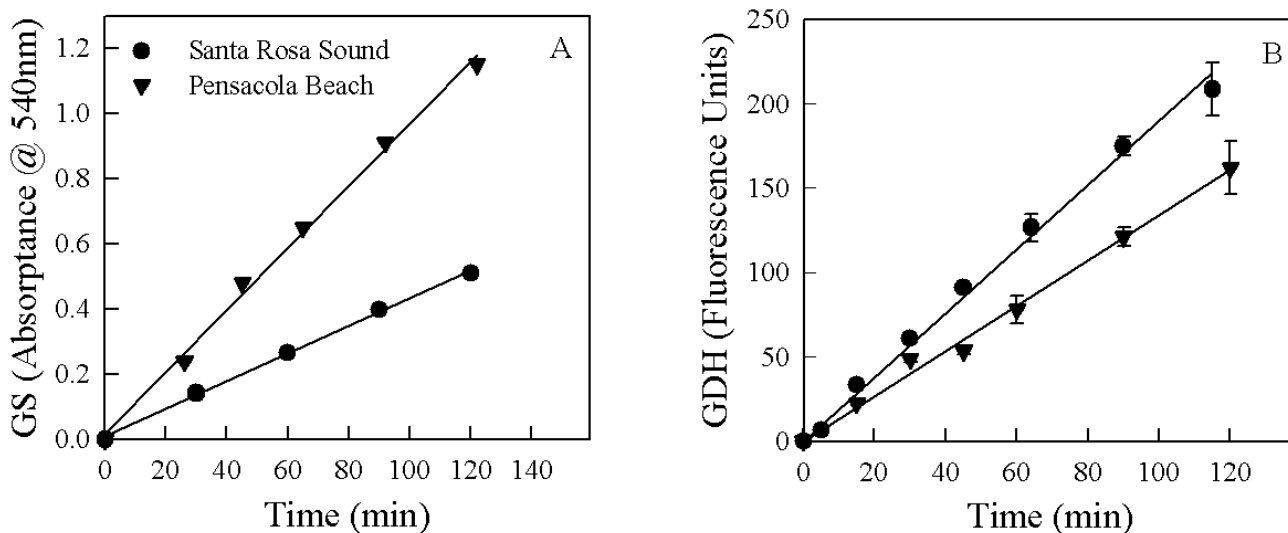
Condition(s)	Relative enzyme activity*	
	GS	$\text{GDH}_T$
No treatment	0.07 $\pm$ 0.006	0.08 $\pm$ 0.010
0.5 $\times$ bead beating alone (0.5 min)	0.32 $\pm$ 0.019	0.21 $\pm$ 0.032
1 $\times$ bead beating alone (1 min)	0.73 $\pm$ 0.053	0.50 $\pm$ 0.043
2 $\times$ bead beating alone (2 min)	0.43 $\pm$ 0.058	0.20 $\pm$ 0.016
1 $\times$ CTAB alone (5 min)	0.75 $\pm$ 0.056	0.73 $\pm$ 0.052
2 $\times$ CTAB alone (10 min)	0.78 $\pm$ 0.043	0.72 $\pm$ 0.033
Optimized (1 $\times$ bead beating & 1 $\times$ CTAB)	1.00 $\pm$ 0.033	1.00 $\pm$ 0.054
Optimized with 0.5 $\times$ substrate	0.88 $\pm$ 0.032	0.86 $\pm$ 0.063
Optimized with 2 $\times$ substrate	1.03 $\pm$ 0.036†	1.08 $\pm$ 0.051†

Standard deviations for propagated errors are given for triplicate assays. \*Relative enzyme activity equals treatment activity divided by the optimized assay activity.

†No significant difference (df = 2,  $P > 0.05$ ) from the optimized assay activity.

harvested on membrane filters and then subjected to (1) cell shearing via bead beating, (2) chemical permeabilization of cell membranes with CTAB, or (3) a combination of both. The optimal cell treatment was 1 min bead beating followed by 5 min CTAB conditioning, and all treatments were subject to 30 min incubation at 30°C (Table 3). Cells without any treatment had less than 10% of optimal activity for either enzyme. Epifluorescence microscopy with acridine orange staining confirmed that bead beating dispersed cells from the filter surface and appeared to shear apart about 25% of cells. Longer bead beating sheared more cells, but compromised activity by warming the preparations. Optimal assay substrate concentration (Tables 1-3) was at saturating substrate concentrations for both enzymes.

GS and GDH activity assays were tested for reaction linearity with time and optimized with respect to biomass assayed. Bacterioplankton communities used in these experiments were from offshore of Pensacola Beach and overlying seagrass beds in Santa Rosa Sound (Fig. 1). The latter was presumed to be more N replete due to seagrass habitat and riverine influences (Jørgensen et al. 1999). Both GS and GDH activity was linear with incubation time (adjusted  $R^2 > 0.994$ ) to at least 2 h (Fig. 2) for both communities. Different amounts of bacterial biomass on the filter did not affect assay results for either enzyme or source habitat (Fig. 3). The slope of the linear regression lines for activity versus biomass represents enzyme specific activity, and fitting with prior assumptions on N bioavailability at the two seawater collections sites (Jørgensen et al. 1999) the Santa Rosa Sound samples has 2.8-fold greater GDH specific activity and 83% less GS specific activity than the more N deplete GOM. Standard errors associated with these slope estimates were less than 3.2% for both enzymes. In contrast, the average coefficient of variation, using the propagated errors of activity and bacterial counts for each data point in Fig. 3, was

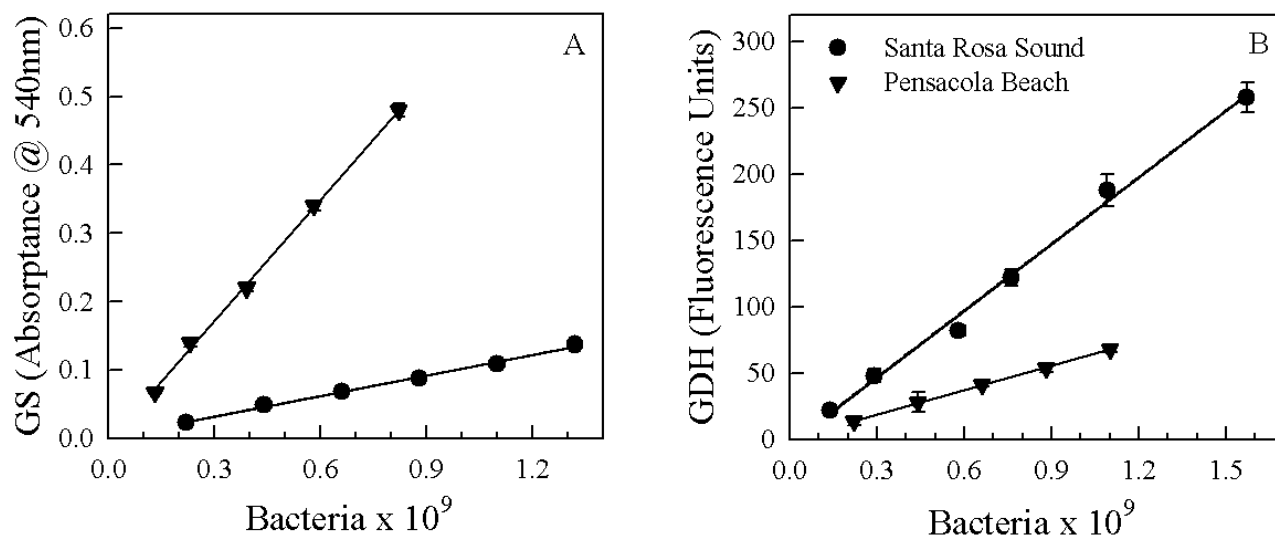


**Fig. 2.** GS (A) and GDH<sub>T</sub> (B) product formation versus assay incubation time for bacterioplankton from two locations of different trophic status. Error bars represent  $\pm$  SD.

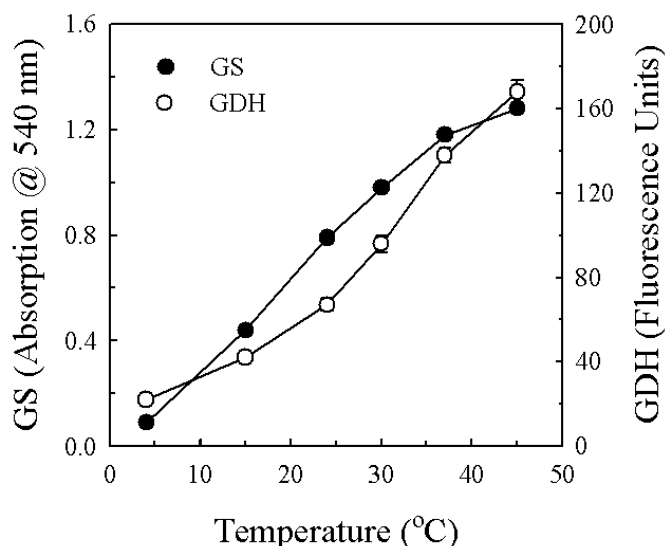
8.1% ( $n = 11$ ; SD = 1.1%) for GS and 10.9% ( $n = 11$ ; SD = 3.7%) for GDH<sub>T</sub>. Therefore, precision of specific activity estimates maybe improved by performing many assays of differing biomass content; however, little accuracy is relinquished by performing fewer replicate assays of the same biomass content within the range  $2.0 \times 10^8$  to  $1.6 \times 10^9$  cells.

No temperature optimum was found for either enzyme. Both GS and GDH activity increased within an environmentally relevant incubation temperature range (5°C to 45°C; Fig. 4). To enhance sensitivity, assays were incubated at 30°C, which was 7°C above the mean temperature of surface seawater in this study, 23°C ( $n = 16$ , SD = 2°C).

Last, both GS and GDH assays were optimized for reaction pH. GS assay was specifically tested for an isoactivity pH of adenylylated and deadenylylated GSI- $\beta$  forms (Shapiro and Stadtman 1970; Bender et al. 1977). Cells were incubated with excess glucose and phosphate (50  $\mu$ M glucose plus 5  $\mu$ M phosphate) for 24 h to express all GS types and to minimize GSI- $\beta$  subunit adenylylation. Half of this incubation volume was adjusted to 1 mM NH<sub>4</sub><sup>+</sup> and incubated 2 h to maximize subunit adenylylation of any GSI- $\beta$ . Assays were performed on both treatments over a pH range of 6 to 8.5. Optimum pH for the GS assay was 7.1 for NH<sub>4</sub><sup>+</sup> spiked cells and 7.2 for the glucose only treatment (Fig. 5), and the intercept of the two



**Fig. 3.** GS (A) and GDH<sub>T</sub> (B) activity after 1 h incubation at 30°C versus bacterial cell abundance per assay for two locations of different trophic status. Error bars represent  $\pm$  SD.



**Fig. 4.** GS and GDH<sub>T</sub> activity after 1 h incubation at different temperatures for bacterioplankton from Santa Rosa Sound, Florida. Bacterioplankton used in GS assays were pre-cultured for 24 h with excess glucose and phosphate, and that used for GDH assays were pre-cultured for 28 h with excess ammonium. Error bars represent  $\pm$  SD.

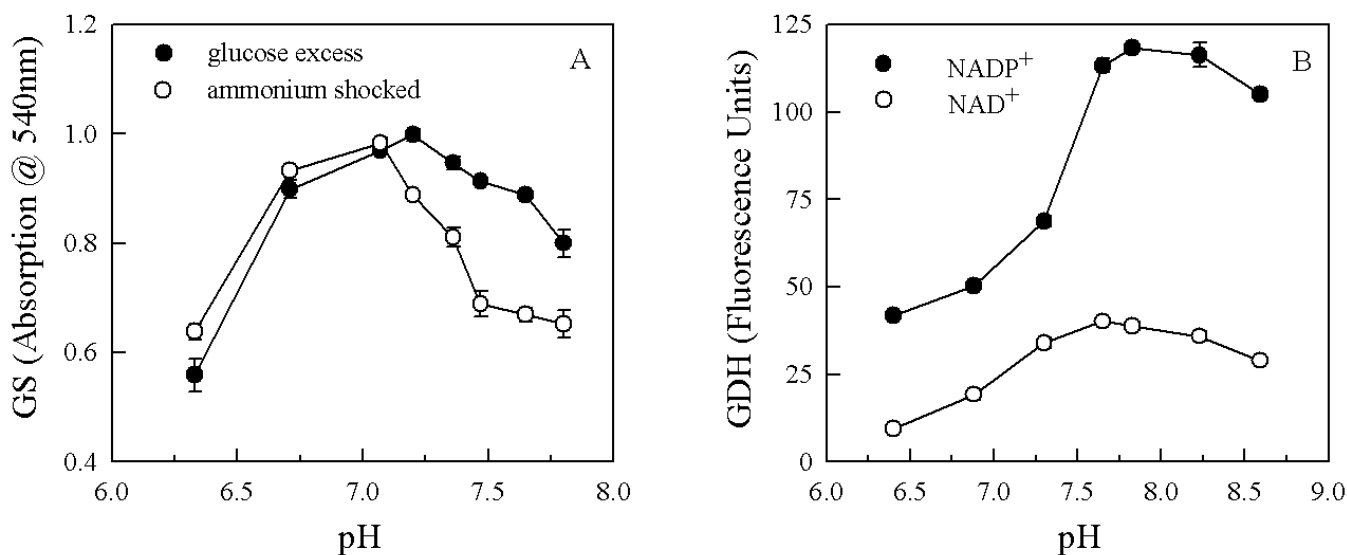
curves is the isoactivity pH, 7.15. Bacterioplankton used in determining the pH optimum of GDH assays were incubated with excess  $\text{NH}_4^+$  (1 mM  $\text{NH}_4^+$ ) for 28 h. Activity of GDH<sub>A</sub> was 4 to 10 times greater than GDH<sub>C</sub> activity; however, pH optima were both about 7.7 (Fig. 5). Based on these results, all amendment experiment and ambient samples were assayed at pH 7.15 and 7.7 for GS and GDH, respectively.

**Amendment experiments**—Use of GS and GDH enzyme activities as an indicator of N-metabolism was tested by manipu-

lating the C:N ratio of organic and inorganic growth substrates in seawater cultures of bacterioplankton. GS specific activity was expected to be greatest for glucose-amended treatments (increased C:N ratio of growth substrates) and least for amino acid- or excess  $\text{NH}_4^+$ -amended treatments (decreased C:N ratio of growth substrates). Inversely, we hypothesized that GDH-specific activities would increase with N-rich amendments and decrease or remain unchanged for glucose amendments. More specifically, the ratio of anabolic GDH to catabolic GDH activities ( $\text{GDH}_A:\text{GDH}_C$ ) was expected to increase with excess N and decrease with excess glucose. Overall, the ratio of GDH<sub>T</sub> to GS activities ( $\text{GDH}_T:\text{GS}$ ) was expected to be a more sensitive index of bacterioplankton community N to C metabolic balance than either enzyme reaction alone.

Due to practical constraints of size fraction volume, there was no replication of treatments within our experiments to assess reproducibility. However, Jørgensen et al. (1999) ran triplicate incubations on two experiments similar to our controls, for which there were average coefficients of variation of 8%, 10%, and 15% for GS- and GDH-specific activities and the GDH<sub>T</sub>:GS ratio, respectively. Instead, we ran four separate amendment experiments to test our hypotheses, and each used seawater with different ambient physicochemical and biological conditions. The enzyme response among experiments was expected to be partly influenced by ambient conditions at collection sites and not by amendments alone.

**Ambient seawater conditions for amendment experiments.** Seawater was collected from mesotrophic coastal sites at the shipyard docks of PE, in the ICW and from more oligotrophic offshore sites within FC adjacent to Miami, Florida, and in the GOM at a site over the LS, west of direct influence of either the Achafalaya River or Mississippi River plumes (Fig. 1). The more



**Fig. 5.** Enzyme activity versus assay pH for GS (A) NADH-specific GDH<sub>C</sub> and NADPH-specific GDH<sub>A</sub> (B). Bacterioplankton were treated as in Fig. 4 for GS and GDH assays. GS assays were also performed on bacterioplankton pre-cultured in excess glucose and then exposed to excess ammonium for 2 h (ammonium shocked). Error bars represent  $\pm$  SD.

**Table 4.** Physicochemical and biological parameters at seawater collection sites for amendment experiments and at tidal cycle stations\*

Site (time)	Temp (°C)	Salinity (ppt)	Chl $\alpha$ ( $\mu\text{g/L}$ )	$P_{\text{max}}$ ( $\mu\text{g C/L/h}$ )	Bact ( $10^9/\text{L}$ )	Leu (pM/h)	Tdr (pM/h)	Protein ( $\mu\text{g/L}$ )	DIN ( $\mu\text{M N}$ )	SRP ( $\mu\text{M P}$ )	DIN:SRP	DON ( $\mu\text{M N}$ )	DOC ( $\mu\text{M C}$ )	DOC:DON
PE	24.37	36.13	1.70	3.19	1.23	443	9.6	174	4.40	0.145	30.3	10.56	192	18.2
FC	23.01	36.34	0.20	1.92	0.60	96	1.7	40	0.36	0.018	20.2	2.30	112	48.5
LS	26.96	35.02	0.16	0.45	1.08	27	0.37	70	0.32	0.010	32.0	5.90	104	17.6
ICW	20.55	10.41	0.70	1.75	3.91	428	15.1	269	1.08	0.053	20.3	10.55	297	28.2
SJ 8:30 low	20.86	28.68	1.15	8.27	2.39	755	23.6	279	0.41	0.036	11.6	8.24	177	21.5
SJ 10:30	20.96	29.65	2.38	—	1.90	646	21.0	—	0.35	0.036	9.7	10.43	163	15.6
SJ 12:30	21.18	29.46	2.32	5.46	1.93	599	25.8	256	0.43	0.032	13.5	8.84	176	19.9
SJ 14:30 high	21.85	29.82	0.52	—	1.48	816	24.7	—	0.66	0.025	26.3	9.47	246	26.0
SJ 16:30	21.21	30.84	3.11	11.11	1.26	820	31.5	509	0.35	0.018	19.8	9.52	182	19.1
SJ 18:30	21.63	31.09	4.28	—	1.01	1340	43.4	406	0.32	0.039	8.2	8.05	202	25.1
SJ 20:30 low	21.71	30.75	2.09	9.63	1.31	587	28.8	287	0.34	0.039	8.6	9.15	189	20.7
WP 14:00 high	24.35	18.29	7.72	11.88	1.39	766	17.8	907	0.41	0.039	10.5	10.48	180	17.2
WP 15:30	24.61	16.63	2.15	6.02	1.37	1390	36.7	519	6.40	0.057	112.5	11.03	208	18.9
WP 17:30	24.42	13.56	6.58	—	1.97	3130	83.4	—	9.77	0.082	118.9	17.05	272	16.0
WP 19:30	24.87	15.99	3.95	4.11	2.10	6290	136.0	579	16.96	0.071	237.9	3.98	251	63.1
WP 21:30 low	24.09	17.59	3.75	5.36	2.34	1680	40.6	906	6.12	0.060	101.2	10.56	243	23.0

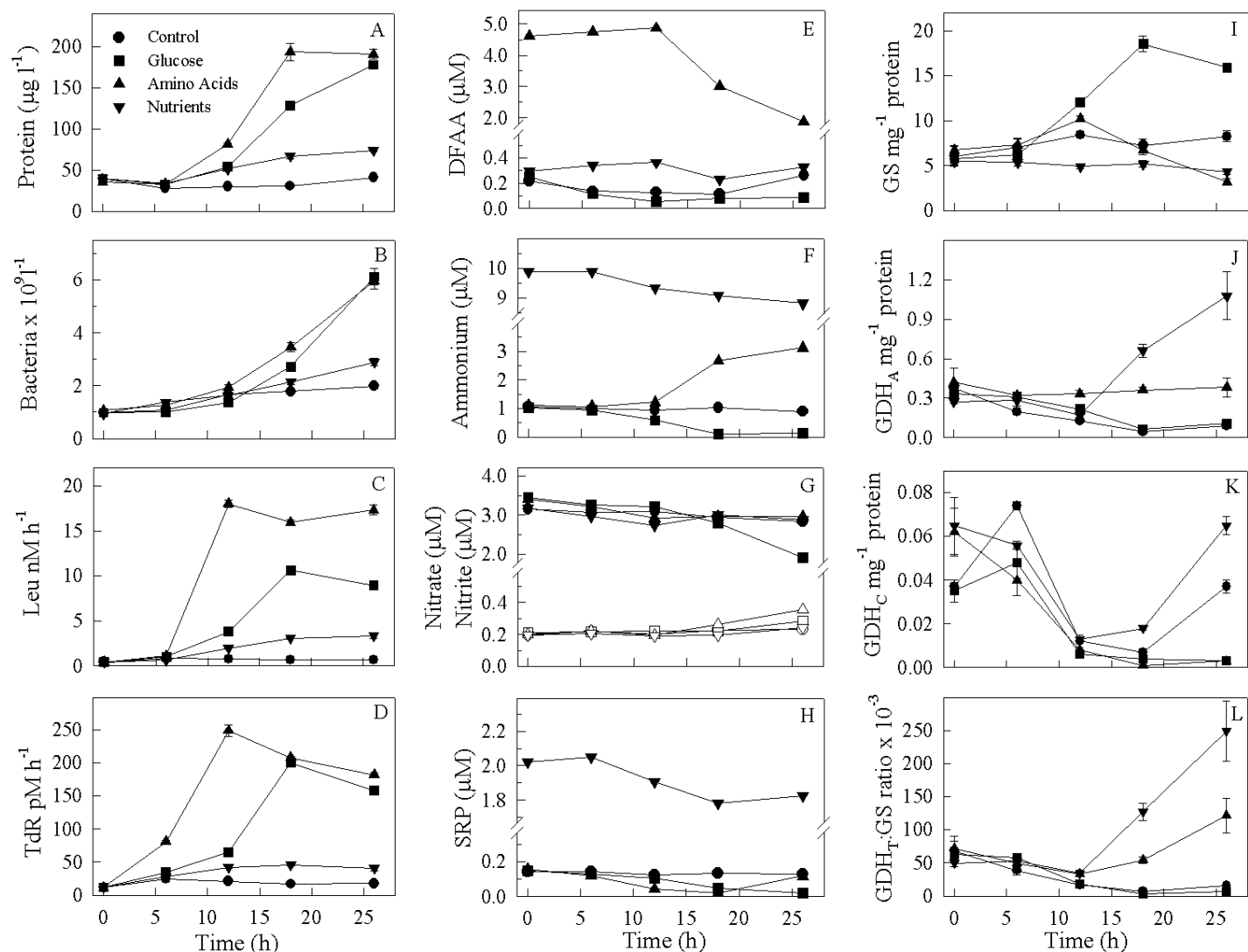
\*Approximate times of low and high tides at St. Joseph's Bay (SJ) and west pass of Apalachicola Bay (WP) are noted next to the respective sample collection time. Mean values, standard deviation (SD), and percent coefficient of variation (%CV) given at the bottom.

mesotrophic PE and IWC sites generally had 2-fold to 20-fold greater values for algal and bacterial biomass and production parameters, inorganic nutrient concentrations, and DOM concentrations than at the FC and LS sites (Table 4). Inorganic nutrients and phytoplankton parameters were greater at PE than ICW, whereas DOC concentration and bacterial parameters were greater at ICW (Table 4), which suggests PE was an autotrophically dominated marine system in contrast with the more heterotrophic brackish waters of the ICW. Oligotrophic sites were less distinct. The FC site had greater biological production and DOC:DON ratio and lower DIN:SRP ratio, i.e., it was more C and phosphate replete and N deplete than the LS site.

Time-course amendment experiments. In the PE experiment (PE-exp), there was little deviation from the control in amendment treatments for any parameter until after a 6- to 12-h lag period (Fig. 6). Overall, the ranking of treatments for growth stimulation was amino acids > glucose > nutrients > control (Fig. 6A-D). In the amino acid treatment, 3  $\mu\text{M}$  DFAA was consumed (Fig. 6E) and about 2  $\mu\text{M}$  of this DFAA-N was released as  $\text{NH}_4^+$  (Fig. 6F). Net uptake of DFAA,  $\text{NH}_4^+$ , and nitrate occurred in the glucose treatment. In the nutrient treatment, net uptake of  $\text{NH}_4^+$  was about 1  $\mu\text{M}$  and that for SRP was 0.2  $\mu\text{M P}$  (Fig. 6H), and this 5:1 ratio of net inorganic N to P uptake is less than N:P ratios for bacterial biomass (6 to 60; Kirchman 2000), which suggests luxury consumption of phosphate by cells that were P-stressed partly due to a high ambient DIN:SRP ratio of 30 at the PE site (Table 4). Given these results alone, bacteria at PE seemed to be more organic substrate (C and energy) limited than nutrient limited; although there was some stimulation by P in the nutrient amendment.

The highest GS specific activities were measured in the glucose treatment at 18 h incubation (Fig. 6I). The slight decrease in GS activity per cell in the glucose treatment at 26 h may be related to depletion of the glucose. There was a 20% increase in GS-specific activity in the amino acid treatment relative to the control at 12 h, but this transient increase was coincident with a maximum in cell-specific incorporation rates of TdR and Leu (not shown), which suggests a peak in growth rate. Once amino acids were consumed and  $\text{NH}_4^+$  regenerated in the amino acid treatment the GS-specific activity was reduced to levels of the nutrient-amended treatment by 26 h, which was about 50% that in the control. The specific activity of GS was lowest and constant in the nutrient amended treatment throughout the experiment. In contrast to GS, the specific activity of  $\text{GDH}_A$  increased 4-fold by 26 h in the nutrient treatment, remained constant in the amino acid treatment, and decreased to about 25% of the initial activity in both the glucose treatment and control (Fig. 6J). After declines in specific activity of  $\text{GDH}_C$  during the first 12 h in all treatments, by 26 h the levels increased by 6-fold and 4-fold in the nutrient treatment and control, respectively (Fig. 6K), suggesting increased C-limitation. Specific activity levels of  $\text{GDH}_C$  were about 10% those for  $\text{GDH}_A$ , meaning the former had little influence on  $\text{GDH}_T$ :GS ratio values (Fig. 6L). The  $\text{GDH}_T$ :GS ratio reduced by half in the control and glucose treatments but increased by 3 and 5 times in the amino acid and nutrient treatments, respectively, so that the ranking of treatments was nutrients > amino acids >> control > glucose.

During the FC-exp bacterial growth was about 5 times lower than in the PE-exp (Fig. 7A-D) and the response to treatments was not until 12 to 18 h. The ranking of treatments for

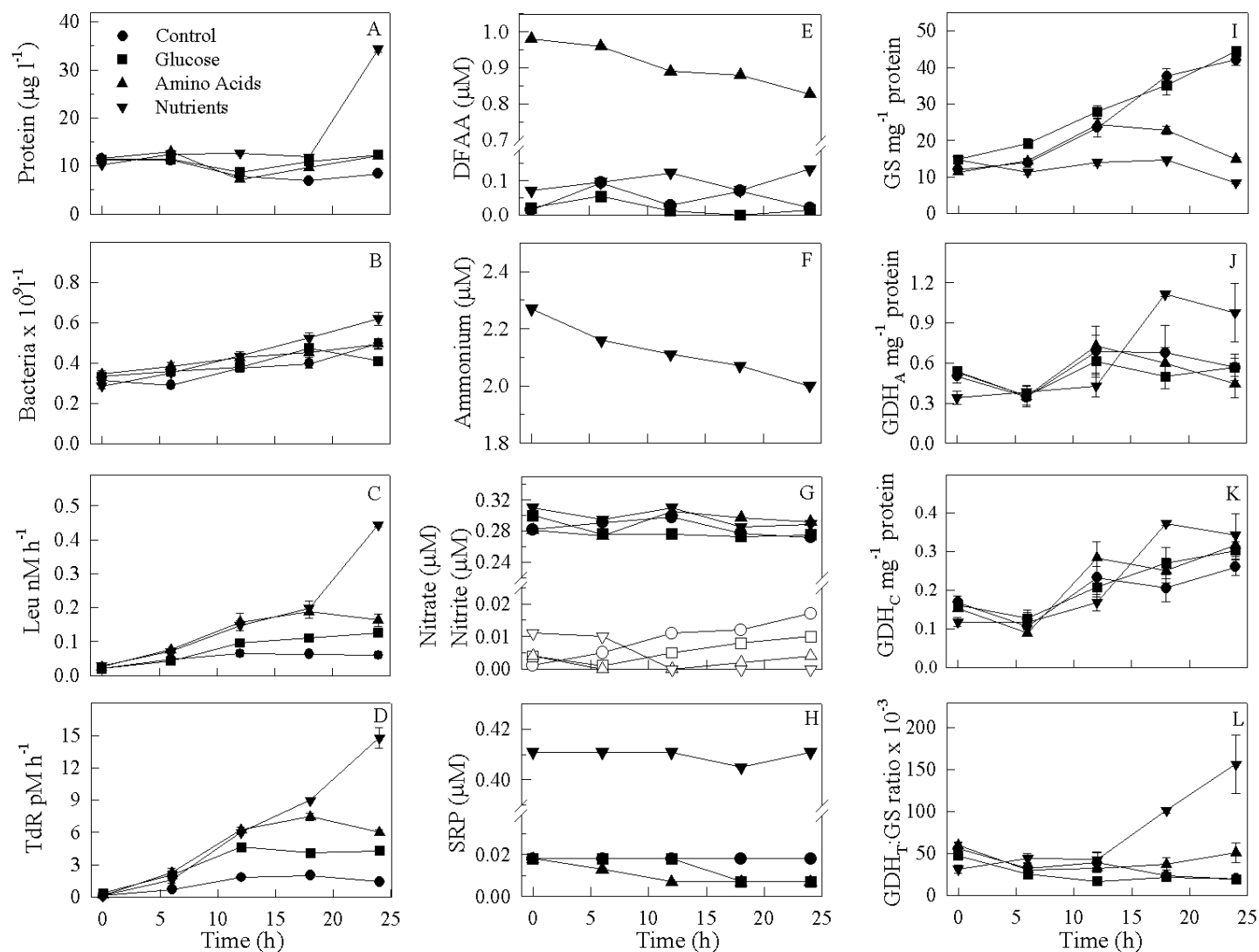


**Fig. 6.** Time-course amendment experiment performed with water collected from Port Everglades, Florida. Parameters include measurements of bacterial biomass and activity (A-D), DFAA and nutrient concentrations (E-H), and enzyme-specific activities and ratio of GDH<sub>T</sub> to GS activity (I-L). Error bars represent  $\pm$  SD.

growth stimulation was nutrients > amino acids > glucose > control, which was most obvious in Leu and TdR incorporation rates (Fig. 7C-D). DFAA concentration declined by only 0.13  $\mu\text{M}$  over 24 h in the amino acid treatment and remained < 0.1  $\mu\text{M}$  in other treatments and control (Fig. 7E). Ammonium concentrations steadily decreased in the nutrient treatment, totaling a net loss of about 0.3  $\mu\text{M}$   $\text{NH}_4^+$  by 24 h; in all other treatments concentrations remained < 0.1  $\mu\text{M}$   $\text{NH}_4^+$  (Fig. 7F). No net regeneration of  $\text{NH}_4^+$  was observed in the amino acid treatment. Nitrate concentrations remained at about 0.3  $\mu\text{M}$  throughout all experimental treatments (Fig. 7G). Concentrations of SRP did not decrease more than about 0.01  $\mu\text{M}$  P in any treatment (Fig. 7H). In the nutrient treatment the ratio of net uptake of inorganic N to P was about 60, which is high compared to the N:P needs of bacteria and suggests a N-

deficient community. These results and ambient seawater chemistry (Table 4) suggest these bacterioplankton had both an ample P and organic C supply and were more limited by N-supply, specifically as  $\text{NH}_4^+$ .

The initial GS specific activity in the FC-exp was twice that for PE (Fig. 7I). The addition of glucose increased GS specific activity by three times during 24 h in the FC-exp. However, the control response for GS was nearly identical to the glucose treatment. As seen in the PE-exp, GS specific activity in the amino acid treatment increased concomitant with growth stimulation (Leu and TdR incorporation; Fig. 7C-D) in the first 12 h of incubation and then decreased by 50%. Specific activity of GS in the nutrient treatment remained constant throughout the experiment. The only significant change in the activity of either GDH (Figs. 7J-K) was for the nutrient



**Fig. 7.** Time-course amendment experiment performed with water collected from the Florida current. Parameters include measurements of bacterial biomass and activity (A-D), DFAA and nutrient concentrations (E-H), and enzyme-specific activities and ratio of  $\text{GDH}_T$  to GS activity (I-L). Error bars represent  $\pm$  SD.

treatment, where the specific activity of  $\text{GDH}_A$  was about 40% greater than the other treatments after 24 h. Ranking  $\text{GDH}_T:\text{GS}$  ratios at 24 h in the FC-exp treatments was nutrients  $\gg$  amino acids  $>$  glucose  $\approx$  control (Fig. 7L). Overall, an 18 to 24 h incubation of amended cultures appears to be long enough to induce or repress expression of GS and GDH in marine bacterioplankton communities consistent with theoretical expectations.

Single endpoint amendment experiments. Experiments at LS (LS-exp) and ICW (ICW-exp) were only sampled initially and after 24 and 18 h, respectively (Table 5). Like the FC-exp, the LS-exp bacterioplankton were from more oligotrophic seawater than PE or ICW. Nutrient treatment stimulated the TdR incorporation rate to over three times the glucose treatment, which was about 60% greater than the control (Table 5). There was no amino acid treatment in the LS-exp; instead a fourth

treatment included glucose plus nutrients, which stimulated TdR incorporation rate to ten times the nutrient alone treatment and 58 times the control value. Net uptake of  $\text{NH}_4^+$  and nitrate plus nitrite totaled about 0.2  $\mu\text{M}$  DIN in the control and glucose treatments, 0.8  $\mu\text{M}$  DIN in the nutrient treatment, and 4.3  $\mu\text{M}$  DIN in the glucose plus nutrient treatment. Phosphate may have partly limited growth given the DIN:SRP ratio at LS was 32 (Table 4) and net uptake ratio for inorganic N to P in the nutrient and nutrient plus glucose treatments was 11 and 8, respectively. The LS bacterioplankton appeared to be primarily limited by nutrient supply and then by organic C.

The specific activity of GS in LS-exp was high (38 GS units  $\text{mg}^{-1}$  protein) at the start of the experiment, suggesting greater N-limitation than in other experiments. However, GS decreased by 50% after 24 h in the control. Presumably, the ambient labile DOC was consumed during the incubation, decreasing C supply

**Table 5.** Nutrient and organic matter concentrations, bacterial biomass and activity, and enzyme specific activities ( $\pm$  SD) for the start and endpoints of incubation experiments using seawater from over the Louisiana Shelf (LS) and the northwest Florida intracoastal waterway (ICW)

Experiment treatment	NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup>		DIN ( $\mu$ M N)	SRP ( $\mu$ M P)	DON* ( $\mu$ M N)	DOC† ( $\mu$ M C)	Protein ( $\mu$ g/L)	Bact (10 <sup>9</sup> /L)	Tdr (pM/h)	GDH <sub>C</sub> (U/mg protein)	GDH <sub>A</sub> (U/mg protein)	GS (U/mg protein)
	NH <sub>4</sub> <sup>+</sup> ( $\mu$ M N)	NO <sub>3</sub> <sup>-</sup> ( $\mu$ M N)										
LS T0	0.1	0.22	0.54	0.01	5.9	104	17.1	0.91	0.31	0.38 $\pm$ 0.08	0.48 $\pm$ 0.08	38.5 $\pm$ 3.10
LS T24 Control	0.07	0.13	0.33	0.03	5.9	104	19.5	0.85	2.24	0.23 $\pm$ 0.00	0.35 $\pm$ 0.02	18.5 $\pm$ 0.60
LS T24 + 40Gluc	0.06	0.13	0.32	0.01	5.9	343	21.2	0.94	3.56	0.08 $\pm$ 0.00	0.28 $\pm$ 0.00	40.2 $\pm$ 0.35
LS T24 + 4N + 2P	3.41	0.17	3.75	1.94	6	103	23.2	0.85	12.37	0.34 $\pm$ 0.02	1.35 $\pm$ 0.01	14.8 $\pm$ 0.50
LS T24 + 40Gluc + 4N + 2P	0.01	0.12	0.25	1.47	8.4	327	144.7	3.73	131.56	0.33 $\pm$ 0.02	0.63 $\pm$ 0.00	42.5 $\pm$ 2.66
ICW T0	0.42	0.66	1.08	0.053	10.6	297	62.6	2.87	1.51	0.06 $\pm$ 0.01	0.27 $\pm$ 0.00	6.9 $\pm$ 0.17
ICW T18 Control	0.34	0.65	1	0.053	10.5	296	72.9	3.15	2.11	0.07 $\pm$ 0.01	0.28 $\pm$ 0.02	7.8 $\pm$ 0.23
ICW T18 + 10Gluc	0.34	0.57	0.91	0.053	10.6	356	72.6	3.24	1.86	0.05 $\pm$ 0.00	0.25 $\pm$ 0.00	14.5 $\pm$ 0.61
ICW T18 + 5AA	0.96	0.57	1.53	0.057	15.3	313	76.1	3.42	2.88	0.28 $\pm$ 0.00	0.38 $\pm$ 0.01	5.5 $\pm$ 0.06
ICW T18 + 5gln	0.44	0.6	1.04	0.057	20.5	321	72.3	3.18	2.1	0.08 $\pm$ 0.02	0.17 $\pm$ 0.00	3 $\pm$ 0.09
ICW T18 + 7N	7.14	0.59	7.73	0.053	10.4	295	70.1	3.48	2.65	0.05 $\pm$ 0.02	0.36 $\pm$ 0.00	6.4 $\pm$ 0.12

\*DON values are estimated from ambient measurement, amendment N, and biomass N change; see *Materials and Procedures*.

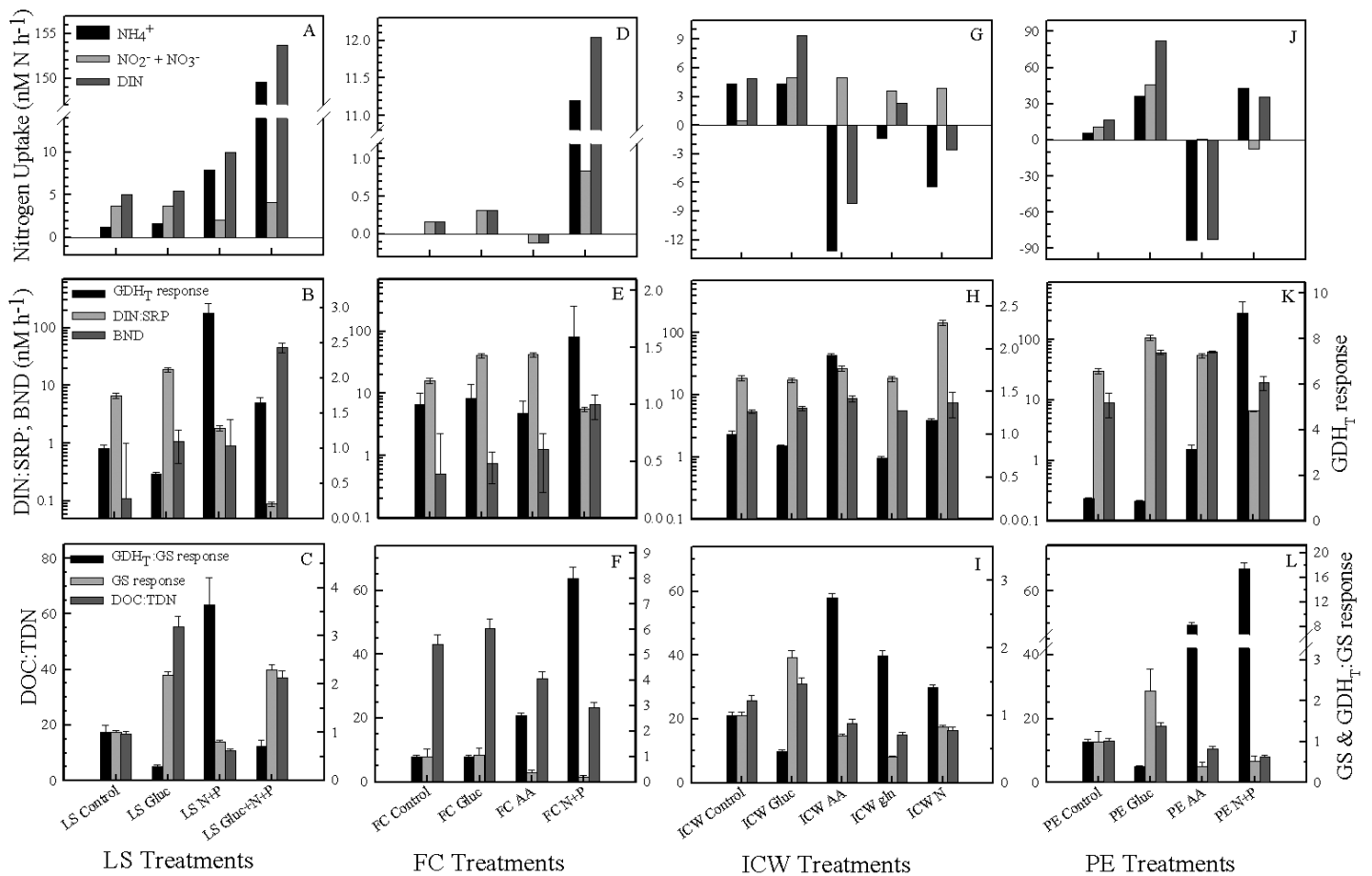
†DOC values are estimated from ambient measurement, amendment C, biomass C, and 30% growth efficiency; see *Materials and Procedures*.

relative to N, resulting in a down-shift in GS expression. In contrast, GS specific activity after 24 h in the glucose treatment did not change due to the ample addition of highly labile DOC relative to the ambient nutrient supply; GDH<sub>C</sub> was repressed in this treatment. The GDH<sub>T</sub>:GS ratio nearly doubled in the control and decreased by over half in the glucose treatment. After 24 h, the nutrient treatment (added in a N:P ratio of 2:1) reduced GS-specific activity to less than the control, tripled the specific activity of GDH<sub>A</sub>, and increased the GDH<sub>T</sub>:GS ratio from 22 to 114, which is consistent with an excess supply of N relative to labile C. When provided nutrients with excess labile C (40  $\mu$ M glucose) growth rates and specific activities of each enzyme increased (Table 5), yet the balance of C and N metabolism seemed unchanged from the initial control population with a GDH<sub>T</sub>:GS ratio of 22. Enhanced constitutive expression would be expected due to the increased growth rate, similar to what was observed to lesser degree for GS in amino acid treatments of PE-exp and FC-exp.

Responses of growth to amendments of glucose, amino acids, glutamine, or NH<sub>4</sub><sup>+</sup> in the ICW-exp were at least an order of magnitude less than in other experiments and revealed few clear conclusions on what limited bacterioplankton growth at ICW (Table 5). The only pronounced increase in growth parameters above the control was in the amino acid treatment. Despite this unclear growth limitation, GS-specific activity doubled with glucose amendment, remained nearly constant in amino acids and NH<sub>4</sub><sup>+</sup> treatments, and decreased by half in the glutamine treatment (Table 5). The latter likely reflects the role of glutamine as a signal ligand for repressing the N control regulon and as an allosteric inhibitor of GS (Merrick and Edwards 1995; Arcondéguy et al. 2001). Four-fold increase in GDH<sub>C</sub> specific activity in the amino acid treatment suggests catabolism of glutamate and glutamate family

amino acids to support energy or C demands. The GDH<sub>A</sub> specific activity doubled in the amino acid and NH<sub>4</sub><sup>+</sup> treatments but was reduced by 37% in the glutamine treatment, possibly due to repression by glutamate accumulated during deamination or transamination of glutamine (Brown et al. 1974). Treatments ranked by GDH<sub>T</sub>:GS ratio with decreasing N bioavailability such that amino acids > glutamine > NH<sub>4</sub><sup>+</sup> > control > glucose. These enzyme data alone suggest ICW bacterioplankton to be dual limited by N and labile organic C.

Comprehensive analysis of amendment experiments. To summarize all four amendment experiment results, incubation endpoint values for net DIN uptake, BND, DIN:SRP, and DOC:TDN were compared to the response of GDH<sub>T</sub>, GS, and the GDH<sub>T</sub>:GS ratio in each treatment relative to the respective experimental control (Fig. 8). Of the 17 experimental incubations (all experimental treatments and controls), NH<sub>4</sub><sup>+</sup> regeneration was only seen in the more eutrophic seawater cultures (ICW-exp and PE-exp) amended with amino acid or just NH<sub>4</sub><sup>+</sup> (Fig. 8A, 8D, 8G, and 8J). No net change in NH<sub>4</sub><sup>+</sup> was observed in the amino acid addition of the FC-exp. All other treatments and controls had some degree of net NH<sub>4</sub><sup>+</sup> uptake, which was greatest in the NH<sub>4</sub><sup>+</sup> plus phosphate plus glucose treatment of LS-exp. Ammonium treatments that received excess phosphate (LS-exp, FC-exp, and PE-exp) consistently had greater net NH<sub>4</sub><sup>+</sup> uptake than the respective glucose treatment and higher GDH<sub>T</sub>:GS ratios. Some of the NH<sub>4</sub><sup>+</sup> taken up in NH<sub>4</sub><sup>+</sup> treatments with excess phosphate may also have been converted to low molecular weight DON, such as urea (Jørgensen et al. 1999), and then released. Uptake of NH<sub>4</sub><sup>+</sup> at high concentrations has been previously observed in seawater cultures (Tupas and Koike 1991) and in ambient estuarine waters of the upper Delaware Bay (Hoch and Kirchman 1995). The degree that nitrification contributes to this net uptake of NH<sub>4</sub><sup>+</sup> is



**Fig. 8.** Compilation of all amendment experiment endpoint values for the following: net uptake of DIN, ammonium, and nitrate plus nitrite (A, D, G, and J); response (relative to the control value) of total GDH specific activity ( $GDH_T$ ), average bacterial nitrogen demand (BND) based on change in bacterial abundance and protein content (error bars reflect the range), and the DIN:SRP ratio (B, E, H, K); and the response of GS specific activity, the response in the  $GDH_T$ :GS activity ratio, and the DOC:TDN ratio (C, F, I, and L). Error bars represent  $\pm$  SD.

unclear; although, nitrite concentration did increase in some PE-exp and FC-exp treatments (Fig. 6G and Fig. 7G). Net uptake of nitrate also occurred in most experiments, even when  $NH_4^+$  was being regenerated in the ICW-exp amino acid and  $NH_4^+$  treatments. This observation contradicts observations of bacterial preference for  $NH_4^+$  over nitrate as a growth substrate (Kirchman 2000), yet nitrate respiration as an alternative explanation of net nitrate uptake (loss) is speculative at best. Evidently, uptake of  $NH_4^+$  is not exclusive to N deplete conditions. When concentration is high enough, marine bacteria use the more energy efficient  $GDH_A$  pathway for  $NH_4^+$  assimilation. Response of GS and  $GDH$  expression may provide a clearer picture of the N metabolic condition of the heterotrophic bacterioplankton without having to account for the complexities of DIN reactions.

The response of  $GDH_T$  specific activity did not covary with bacterial N demand (BND) or net DIN uptake (Fig. 8B, 8E, 8H, and 8K). The largest relative responses in  $GDH_T$  specific activity were for amino acids and nutrient treatments, which also

caused a decrease in DIN:SRP ratio. Most of this response was due to induction of  $GDH_A$  expression by elevated  $NH_4^+$  concentration, particularly when excess phosphate was present. Note that nutrient effects on  $GDH_T$  in FC-exp and PE-exp were greater than amino acid effects, but the magnitude of these effects was less for the ICW-exp where no phosphate was added in the  $NH_4^+$  treatment. The only  $GDH_T$  response to glucose addition was for the LS-exp where the specific activity dropped to about half that of the control, most of which was due to repression of  $GDH_C$ .

In three of four experiments, there was about a 2-fold increase in GS specific activity in response to glucose treatment relative to the control (Fig. 8C, 8F, 8I, and 8L). There was no significant response ( $P < 0.05$ ) to glucose addition in the FC-exp where the ambient DOC:DON ratio was about 48 and the amount of glucose added was  $< 20\%$  that in other experiments. Apparently, FC bacterioplankton had ample organic C supply so that the  $2 \mu M$  glucose addition did not drive further N limitation and GS expression. Conversely, GS specific activi-

**Table 6.** Rotated components matrix for first four principal components (PC) extracted from all non-enzymatic parameters measured at the experimental endpoint of all treatments and experiments ( $n = 17$ )\*

Factor	Principal components			
	PC1 (25.80%)	PC2 (25.10%)	PC3 (20.80%)	PC4 (17.10%)
ln NH <sub>4</sub> <sup>+</sup>	<b>0.84</b>	0.13	0.41	-0.01
ln NO <sub>2</sub> <sup>-</sup>	0.37	0.67	0.34	0.12
ln NO <sub>3</sub> <sup>-</sup>	0.55	0.55	0.15	0.38
ln DIN	0.85	0.33	0.06	0.07
ln SRP	0.48	0.24	0.01	<b>-0.81</b>
ln DIN:SRP	0.16	0	0.04	<b>0.96</b>
ln DON	0.45	0.28	<b>0.8</b>	-0.05
ln DOC	-0.14	0.24	<b>0.93</b>	0.03
ln DOC:DON	<b>-0.82</b>	-0.16	-0.2	0.11
ln DOC:TDN	<b>-0.94</b>	-0.22	-0.02	0.16
ln BND	0.18	<b>0.83</b>	0.41	-0.11
ln protein	0.28	0.68	0.61	-0.14
ln bacteria	0.32	0.58	0.73	-0.02
ln TdR incorporation	0.12	<b>0.91</b>	-0.2	-0.3
ln Leu incorporation	0.24	<b>0.9</b>	0.04	-0.29
ln Salinity	-0.16	0.35	<b>-0.84</b>	-0.21
ln DOC:SRP	-0.49	-0.17	0.22	<b>0.8</b>
Net DIN uptake	-0.47	0.31	0.18	-0.65

\*Percent data set variation explained by each PC are given in parentheses. Boldface values indicate dominant factors of each PC.

ity decreased in response to treatments with NH<sub>4</sub><sup>+</sup>, glutamine, or amino acids. Generally, the GS and GDH<sub>T</sub>:GS ratio responses covaried with the DOC:TDN ratio, such that greater DOC:TDN ratios were coincident with higher GS and lower GDH<sub>T</sub>:GS ratio responses (Fig. 8C, F, I, and L).

To quantitatively explore the environmental and physiological factors contributing to variation in enzyme parameters among experiments, the chemical and growth parameters from all experiments and treatments ( $n = 17$ ) were subjected to principal component (PC) analysis (Table 6). All parameter values from experiment endpoints were first natural log transformed, except for net DIN uptake due to positive and negative values. The analysis extracted four principal components with Eigenvalues > 1.0, which were rotated using the Varimax method with Kaiser normalization. Only 11% of variation in the data set remained unexplained by these four PCs. Concentration of NH<sub>4</sub><sup>+</sup> and DIN and the ratios of DOC:DON and DOC:TDN dominated PC1, and PC2 was dominated by bacterial production rates and average bacterial N demand (BND). PC1 and PC2 each explained about 25% of the data set variation (Table 6). PC3 explained 20.8% data set variation and was dominated by salinity, DOC, and DON. PC4 was dominated by SRP parameters and explained 17% variation. Using the four PC regression equations, dimensionless values were calculated for each experimental treatment and control endpoint, and

tested for correlations with enzyme results. The dominant variables represented in each significantly correlated PC were used in a stepwise analysis to construct multivariate regression models for enzyme parameters.

GS specific activity negatively correlated with PC1 and PC3 (adjusted  $R^2 = 0.696$ ), and a positive regression model with salinity and DOC:TDN ratio explained 74% of GS variation:

$$\ln \text{GS} = 0.647 \times (\ln \text{Salinity} + 1.202) \times (\ln \text{DOC:TDN} - 3.26).$$

Both GDH<sub>C</sub> and GDH<sub>A</sub> specific activities negatively correlated with PC4, which suggests high phosphate concentration relative to DOC and DIN partly controls expression of these enzymes. GDH<sub>C</sub> specific activity also negatively correlated with PC2 (growth parameters). The resulting multivariate model included bacteria, salinity and DIN:SRP ratio and explained 88% variation:

$$\ln \text{GDH}_C = -1.24 \times (\ln \text{Bacteria} - 0.452) \times (\ln \text{DIN:SRP} - 1.047) \times (\ln \text{Salinity} + 2.939).$$

GDH<sub>A</sub> specific activity negatively correlated with PC3 in addition to PC4, and its multivariate regression model included DON and DOC:SRP ratio to explain 62% of variation:

$$\ln \text{GDH}_A = -0.247 \times (\ln \text{DOC:SRP} - 0.578) \times (\ln \text{DON} + 2.128).$$

The GDH<sub>A</sub>:GDH<sub>C</sub> ratio correlated with PC1 and PC2. This means that anabolic activity will be greater as DIN concentration and growth increases and DOC:TDN ratio decreases, which is consistent with faster growing bacterioplankton limited by energy or C relative to N and P (e.g., PE-exp). Conversely, slower growing bacterioplankton as in LS and FC, had greater GDH<sub>C</sub> specific activity. The multivariate regression model that best explained variation (adjusted  $R^2 = 0.70$ ) in the GDH<sub>A</sub>:GDH<sub>C</sub> ratio included positive correlations with nitrate concentration and leucine incorporation rate:

$$\ln \text{GDH}_A:\text{GDH}_C = 0.554 \times \ln \text{NO}_3 + 0.259 \times \ln \text{Leu} + 0.223$$

Last, GDH<sub>T</sub>:GS ratio correlated with PC1 (adjusted  $R^2 = 0.40$ ). Regression analysis revealed that the DOC:TDN ratio alone explained about 35% of variation in the GDH<sub>T</sub>:GS ratio:

$$\ln \text{GDH}_T:\text{GS} = -1.111 \times \ln \text{DOC:TDN} + 7.137.$$

Expression of GS and both GDH<sub>A</sub> and GDH<sub>C</sub> were all partially explained by either DIN, DON, DOC, or some combination of these variables and in accordance to expectation based on pure culture studies. It is important to note that much of the variation in DOC and DON between experimental treatments was due to large amendments (60% to 300% of ambient DOC) of glucose and amino acids. One might expect more variation in enzyme expression and GDH<sub>T</sub>:GS ratio to be explained if only concentrations of labile fractions of DOC and DON pools were included in the regression analyses, because variation in bulk DOC:DON ratios under ambient conditions is likely due to the larger more refractory component than the labile substrates. Regardless, the GDH<sub>T</sub>:GS ratio was correlated

**Table 7.** Percent of biomass or activity parameter value for the total plankton (TP) recovered in the bacterial size fraction (SF)

Site (time)	% TP in SF				% SF biomass as phytoplankton*	% TP as bacterial protein	% TP activity in SF			Specific activity ratio SF:TP		
	Chl <i>a</i>	P <sub>max</sub>	Bacteria	Protein			GDH <sub>C</sub>	GDH <sub>A</sub>	GS	GDH <sub>C</sub>	GDH <sub>A</sub>	GS
PE	0.16	5.65	81.4	22.6	1.42	38	5.6	49.2	74.1	0.46	5.83	3.27
FC*	2.6	1.91	52.6	30.9	3.43	43	11.2	12.7	23.5	0.36	0.61	0.76
LS	8.81	0.02	84.4	20	3.33	23.7	32.5	67.4	49.7	1.62	3.3	2.47
ICW	0.68	ND	73.4	23.2	0.36	27.8	7.2	35	28.7	0.31	1.5	1.24
SJ 8:30 low	0.17	ND	64.4	12.3	0.27	19	80.7	74.4	27.7	6.59	6.07	2.26
SJ 10:30	0.96	—	93.7	—	2.71	—	—	—	—	—	—	—
SJ 12:30	0.23	ND	72	12.7	0.83	17.6	7.4	75	37.1	0.59	5.91	2.93
SJ 14:30 high	0.51	—	58.8	—	0.66	—	—	—	—	—	—	—
SJ 16:30	1.85	0.08	46	5.8	17.76	12.7	4.7	84.1	30.3	0.81	14.43	5.19
SJ 18:30	2.54	—	96	6.8	19.59	7.1	—	—	—	—	—	—
SJ 20:30 low	3.86	0.08	90.8	15.9	12.86	17.5	92.8	96.8	47	13.69	14.28	6.94
WP 14:00 high	0.16	0.03	60.7	4.1	3.08	6.7	2.1	21.4	14.1	0.52	5.23	3.46
WP 15:30	0.4	0.25	94.2	8.4	1.43	8.9	4.5	35.7	34.5	0.53	4.27	4.12
WP 17:30	0.67	—	71.1	—	6.41	—	—	—	—	—	—	—
WP 19:30	0.29	0.06	64.8	6.8	1.8	10.5	2.6	33.7	19.2	0.38	4.94	2.81
WP 21:30 low	0.38	0.1	67.5	5.6	1.92	8.3	5.9	34.1	21.9	1.05	6.1	3.92
Mean	1.52	0.68	73.2	13.5	4.87	18.52	21.44	51.6	34	2.24	6.04	3.28
± SD	2.24	1.65	15.5	8.5	6.21	11.76	31.66	27	16.5	4	4.27	1.67
%CV	147	242	21	63	128	63	148	52	48	179	701	51

Approximate times of low and high tides and other table details are as indicated in Table 4.

\*The bacterioplankton size fraction was < 0.6 μm filtered seawater for the Florida Strait site.

with the DOC:TDN ratio, which supports its use as a biomass independent index of bacterioplankton N to C metabolic balance for bacterioplankton in situ, where bulk DOC:DON ratios may not reflect the substrates supplied to bacteria.

**Ambient sample assessment**—Application GS and GDH specific activities to assessment of N to C metabolic balance of bacterioplankton communities in situ requires confirmation of at least two additional assumptions: (1) the ambient size fraction assayed is dominated by heterotrophic marine bacteria; and (2) minimal enzymatic activity is due to picophytoplankton contamination of the size fraction. To address these assumptions, we measured biomass, productivity, and enzyme activity parameters on both unfiltered seawater (total plankton; TP) and the bacterial size fraction (SF) at sites of seawater collection for amendment experiments and during ebb tide flow at the mouth of Saint Joseph Bay (SJ) and the West Pass (WP) of Apalachicola Bay (Fig. 1).

Bacterioplankton size fraction efficacy. Bacterial abundance and protein content were used to assess the efficiency of separating bacterioplankton from the total plankton. On average 73.2% of total bacterial abundance and 13.5% of total protein content was recovered in the size fraction (Table 7). Bacterial abundance explained 85% of protein content in the size fraction significant relationship ( $n = 13$ ,  $P < 0.05$ ). Overall, protein content used to calculate enzyme-specific activity values was predominantly bacterial in the size fraction and mostly phytoplankton or other sources in the total plankton.

Concentration of Chl *a* and P<sub>max</sub> in the total plankton and size fraction were measured to assess the abundance and activity of picophytoplankton contaminants in bacterial size fractions (Table 7). The average percent of total plankton Chl *a* in the bacterial size fraction was 1.5%, with higher values at the more oligotrophic FC and LS sites and during low tide at the SJ site. Average percent total P<sub>max</sub> in the size fraction was 0.7% and only the PE and FC sites had values greater than 1%. Although a small amount of total phytoplankton biomass was recovered in the size fraction, its contribution to size fraction biomass was less than 5% on average and considered negligible (Table 7). Furthermore, phytoplankton biomass in the size fraction did not explain any variation in size fraction protein content. In contrast, total plankton Chl *a* correlated ( $n = 13$ ,  $P < 0.05$ ) with total plankton protein (not shown) and explained 72% of its variation. The percent bacterial protein contribution to the total plankton protein was estimated as 18.5% on average (Table 7).

Comparison of enzyme activities in the bacterial size fraction and total plankton of ambient samples reveals further evidence that the size fraction activity is due to heterotrophic bacteria and not residual phytoplankton contamination. On average, the percent total plankton activity in the size fraction for GDH<sub>C</sub>, GDH<sub>A</sub>, and GS was 21%, 52%, and 34%, respectively (Table 7). When these values are corrected for the average bacterial protein content in the total plankton (divide by 82%) and phytoplankton biomass in the size fraction (multiply by

**Table 8.** Specific activity ( $\pm$  SD) of GS, GDH<sub>C</sub>, and GDH<sub>A</sub> for the bacterial size fraction (SF) and enzyme ratios for both the bacterial size fraction and total plankton (TP)

Site (time)	SF – GDH <sub>C</sub> (U/mg protein)	SF – GDH <sub>A</sub> (U/mg protein)	SF – GS (U/mg protein)	SF GDH <sub>A</sub> : GDH <sub>C</sub> ratio	SF GDH <sub>A</sub> : GS ratio	SF GDH <sub>T</sub> : GS ratio	TP GDH <sub>A</sub> : GDH <sub>C</sub> ratio	TP GDH <sub>A</sub> : GS ratio	TP GDH <sub>T</sub> : GS ratio
PE	0.037 $\pm$ 0.001	0.379 $\pm$ 0.040	6.08 $\pm$ 1.04	10.24	62.3	68.4	0.81	34.7	77.6
FC*	0.169 $\pm$ 0.016	0.5 $\pm$ 0.050	12.03 $\pm$ 0.90	2.97	41.6	55.6	1.42	41.6	70.9
LS	0.38 $\pm$ 0.080	0.481 $\pm$ 0.083	38.5 $\pm$ 3.08	1.27	12.5	22	0.61	9.2	24.2
ICW	0.064 $\pm$ 0.011	0.275 $\pm$ 0.003	6.93 $\pm$ 0.17	4.3	39.6	48.9	0.89	36.6	77.9
SJ 8:30 low	0.066 $\pm$ 0.004	0.325 $\pm$ 0.054	7.78 $\pm$ 0.39	4.94	56.1	66.6	4.94	11.6	13.9
SJ 10:30	0.266 $\pm$ 0.012	0.376 $\pm$ 0.004	7.91 $\pm$ 0.24	1.41	47.6	81.3	—	—	—
SJ 12:30	0.072 $\pm$ 0.021	0.499 $\pm$ 0.005	9.46 $\pm$ 0.01	6.97	52.7	60.3	0.69	26.1	63.9
SJ 14:30 high	0.046 $\pm$ 0.009	0.285 $\pm$ 0.006	6.75 $\pm$ 0.23	6.2	42.3	49.1	—	—	—
SJ 16:30	0.024 $\pm$ 0.005	0.356 $\pm$ 0.019	9.7 $\pm$ 0.90	14.8	36.7	39.1	0.83	13.2	29
SJ 18:30	0.267 $\pm$ 0.071	0.548 $\pm$ 0.052	11.77 $\pm$ 1.04	2.05	46.6	69.3	—	—	—
SJ 20:30 low	0.057 $\pm$ 0.010	0.344 $\pm$ 0.004	8.83 $\pm$ 0.01	6.08	38.9	45.3	1.97	22.6	34.1
WP 14:00 high	0.056 $\pm$ 0.028	0.462 $\pm$ 0.004	5.71 $\pm$ 0.21	8.21	80.8	90.6	0.82	53.5	118.9
WP 15:30	0.083 $\pm$ 0.001	0.354 $\pm$ 0.010	8.44 $\pm$ 0.52	4.29	42	51.8	0.54	40.6	116.5
WP 17:30	0.166 $\pm$ 0.004	0.257 $\pm$ 0.002	8.29 $\pm$ 0.58	1.55	31	50.9	—	—	—
WP 19:30	0.033 $\pm$ 0.017	0.584 $\pm$ 0.001	9.63 $\pm$ 0.01	17.48	60.7	64.1	1.33	34.6	60.5
WP 21:30 low	0.129 $\pm$ 0.004	0.61 $\pm$ 0.047	8.04 $\pm$ 0.25	4.72	75.9	92	0.81	48.8	108.9
Mean	0.119	0.412	10.3	6.09	48	59.7	1.3	31.1	66.4
$\pm$ SD	0.104	0.111	7.7	4.69	16.8	18.5	1.22	14.6	36.1
%CV	87	27	75	77	35	31	93	47	54

Approximate times of low and high tides and other table details are as indicated in Table 4.

\*The bacterioplankton size fraction was  $< 0.6 \mu\text{m}$  filtered seawater for the Florida Strait sample.

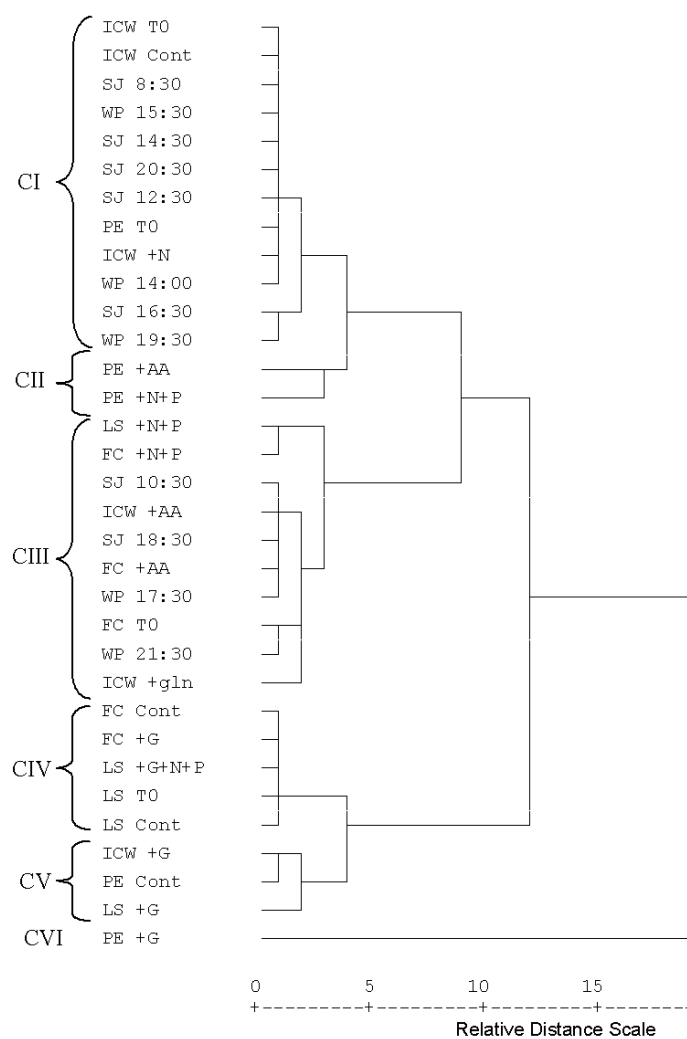
5%), then only 1.3%, 3.2%, and 2.1% of the total non-bacterial plankton activity for GDH<sub>C</sub>, GDH<sub>A</sub>, and GS appears to be recovered in the size fraction, respectively. Dividing these percentages by the average ratio of size fraction to total plankton (SF:TP) specific activities for each enzyme (Table 7) yields estimates of percent size fraction activity due to phytoplankton contamination, as follows: 0.6% for GDH<sub>C</sub>, 0.5% for GDH<sub>A</sub>, and 0.6% for GS. A more conservative estimate of the average contribution of phytoplankton to size fraction enzyme activities is made by simply multiplying the average percent phytoplankton biomass in the size fraction (5%) by the SF:TP specific activity ratios, which yields 2.2% for GDH<sub>C</sub>, 0.8% for GDH<sub>A</sub>, and 1.5% for GS. These values are less than the average coefficient of variation for enzyme-specific activity estimates.

Variation in ambient N metabolism. In addition to the four sites used to collect seawater for amendment experiments, two sites of dynamic tidal exchange on the northwest Florida coast (St. Joseph Bay and the West Past of Apalachicola Bay) were sampled to assess the range in N enzyme-specific activities and variation of N metabolism status of bacterioplankton in situ (Table 8). The range of enzyme specific activities in the size fraction was 5.71 to 38.5 Units mg<sup>-1</sup> protein for GS, 0.024 to 0.38 Units mg<sup>-1</sup> protein for GDH<sub>C</sub>, and 0.26 to 0.61 Units mg<sup>-1</sup> protein for GDH<sub>A</sub>. There was no correlation ( $n = 16$ ,  $P < 0.05$ ) between GS and either GDH<sub>C</sub> or GDH<sub>A</sub>. Lack of correlation between specific activities of GDH<sub>C</sub> and GDH<sub>A</sub> suggests that these enzymes are regulated dif-

ferently among bacterioplankton populations. The average ratio of anabolic to catabolic GDH isozyme activities (GDH<sub>A</sub>:GDH<sub>C</sub>) was 6.1 in the size fraction, but near unity in the total plankton (GDH<sub>A</sub>:GDH<sub>C</sub> = 1.3). This difference emphasizes the importance of the GDH<sub>C</sub> reaction in the total plankton, and that could be due to phytoplankton, heterotrophic protists, or particle attached bacteria excluded from the size fraction.

Enzyme specific activities measured during the tidal exchange at either SJ or WP sites did not follow any trend, nor did the enzyme specific activities measured on either the size fraction or total plankton of WP and SJ ambient samples correlate with any of the chemical or biological parameters, and regression models derived from amendment experiment results were poor predictors of ambient enzyme values ( $< 20$  variation, data not shown). This was not unexpected given the dynamic shifts in tidal fronts that establish at both sites as illustrated by the variation in physicochemical and biological parameters at each site (Table 4).

To draw inferences on ambient conditions controlling N to C metabolic balance of bacterioplankton in situ, ambient samples (unknowns) were grouped with known experimental treatment results (Fig. 9) by performing a cluster analysis of bacterioplankton enzyme parameters (specific activities, GDH<sub>A</sub>:GDH<sub>C</sub>, and GDH<sub>T</sub>:GS values). Six clusters were defined by furthest neighbor analysis of squared Euclidean distances for all natural log transformed enzyme parameters (Fig. 9).



**Fig. 9.** Hierarchical cluster analysis of all ambient samples and all experimental incubation endpoints combined, based on the furthest neighbor method using squared Euclidean distances for all enzymatic parameter. Six clusters discussed in the text are indicated by brackets.

Clusters I, III, and IV included at least one ambient sample from a tidal cycle station or an amendment experiment collection sites ( $T_0$ ). Cluster I (C-I) had five WP, three SJ, and the ICW ambient samples clustered with PE and ICW controls and ICW  $\text{NH}_4^+$  treatment. Based on the association with experimental results, C-I ambient samples were experiencing some degree of organic C limitation on N metabolism, but not to the extreme extent seen for PE-exp amino acid or nutrient treatments of Cluster II (C-II) nor to the degree of other experimental amino acid and nutrient treatments in Cluster III (C-III). The remaining two SJ and two WP samples and the FC ambient sample were in C-III. Average specific activity of  $\text{GDH}_c$  for C-III samples was about 4-fold greater than in C-I (Table 9). Comparable elevated expression of  $\text{GDH}_c$  was only seen in Cluster IV (C-IV) samples, which included the ambient sample from the LS site and oligotrophic experiment controls and

FC-exp glucose treatment. Overall, the C-IV ambient samples were N limited as indicated by having the highest mean GS expression of all clusters, which may partly be a result of lower DIN ( $< 1 \mu\text{M}$ ) and DON ( $< 6 \mu\text{M}$ ) concentrations in C-IV (Table 9). Clusters V and VI were N-limited to a greater degree than C-IV, yet included only experimental treatments. Measurements of these enzyme parameters in other ambient GOM and Florida Strait waters reveal bacterioplankton communities in a state of excessive N-limitation similar to CVI (Hoch unpubl. data). Clearly cluster analysis provides a means of comparing ambient measurements of enzyme parameters with responses in controlled assessments of bacterioplankton resource limitations.

**Discussion**—Activity assays for GDH and GS isozymes provide an approach to assessing the bioavailability of N relative to labile organic C supply in bacterioplankton communities in situ. Response in bacterioplankton GDH and GS expression to amendments of labile organic C and N are generally as predicted from numerous studies of diverse prokaryotic taxa (Merrick and Edwards 1995; Hudson and Daniel 1993). These experimental results can be used to infer the N to C metabolic balance of ambient bacterioplankton from GDH and GS activity in the  $< 0.8 \mu\text{m}$  (or  $< 0.6 \mu\text{m}$ ) size fraction, which has minimal photoautotrophic bias. Assays of in situ GS and GDH specific activities reflect the cumulative bioavailability of N and C substrates without problems associated with the long incubations of nutrient limitation experiments or requiring measurements of uptake rate and concentration of multiple tracer compounds. In fact, the  $\text{GDH}_T:\text{GS}$  ratio may be a more practical and accurate index of the balance of N to C metabolism than the specific activity of either enzyme alone.

Use of the  $\text{GDH}_T:\text{GS}$  ratio eliminates the need for an additional measurement of biomass, which adds to variability and costs. We chose to use protein content in our specific activity calculations to be consistent with pure-culture studies but recognize that, in ambient seawater, some particulate protein entering the size fraction could be non-bacterial (Long and Azam 1996). Alternatively, bacterial abundance and DNA content could be used, because they strongly correlate with protein in bacterioplankton of the GOM (Jeffrey et al. 1996).

Calculating the  $\text{GDH}_T:\text{GS}$  ratio appears to account for growth rate or growth state influences on their specific activities. Bacterial growth rate or imbalances in growth were seen to influence specific activity, even for levels of constitutive expression. For example, the GS-specific activity in our time-course amendment experiments was seen to increase transiently during shift-up in growth for some amino acid and glucose treatments, but there was no change in  $\text{GDH}_T:\text{GS}$  ratio. Also, specific activity of GS and GDH in the extremely fast growing LS-exp treatment with glucose,  $\text{NH}_4^+$  plus phosphate (Table 5) was greater than in the LS-exp control; however, the  $\text{GDH}:\text{GS}$  ratios were similar. Hence the ratio of activities is independent of biomass and growth dynamics.

The range of the  $\text{GDH}_T:\text{GS}$  ratio (5 to 260, or 50-fold) is also greater than that of the specific activity for either enzyme (12-

**Table 9.** Mean ( $\pm$  SD) of enzymatic and chemical parameters for each clusters defined in Fig. 9

Parameter	CI ( $n = 12$ )	CII ( $n = 2$ )	CIII ( $n = 10$ )	CIV ( $n = 5$ )	CV ( $n = 3$ )	CVI ( $n = 1$ )
GS	7.8 ( $\pm$ 1.4)	3.8 ( $\pm$ 0.8)	9.4 ( $\pm$ 3.8)	37.2 ( $\pm$ 10.7)	21 ( $\pm$ 17.0)	18.5
GDH <sub>C</sub>	0.054 ( $\pm$ 0.017)	0.039 ( $\pm$ 0.037)	0.235 ( $\pm$ 0.095)	0.3 ( $\pm$ 0.058)	0.057 ( $\pm$ 0.022)	0.003
GDH <sub>A</sub>	0.375 ( $\pm$ 0.095)	0.73 ( $\pm$ 0.493)	0.558 ( $\pm$ 0.355)	0.518 ( $\pm$ 0.107)	0.206 ( $\pm$ 0.104)	0.107
GDH <sub>A</sub> :GDH <sub>C</sub>	7.9 ( $\pm$ 4.3)	22.9 ( $\pm$ 9.0)	2.4 ( $\pm$ 1.2)	1.7 ( $\pm$ 0.36)	3.5 ( $\pm$ 1.1)	35.7
GDH <sub>T</sub> :GS	57.6 ( $\pm$ 14.2)	194.1 ( $\pm$ 98.8)	87.6 ( $\pm$ 34.7)	23 ( $\pm$ 4.9)	15 ( $\pm$ 5.8)	5.9
TdR pM h <sup>-1</sup>	29.5 ( $\pm$ 35.3)	111.6 ( $\pm$ 99.6)	22.8 ( $\pm$ 26.1)	28 ( $\pm$ 57.9)	7.8 ( $\pm$ 8.8)	158
SRP	0.05 ( $\pm$ 0.03)	2.97 ( $\pm$ 4.03)	0.27 ( $\pm$ 0.60)	0.31 ( $\pm$ 0.65)	0.06 ( $\pm$ 0.06)	0.02
DIN	3.35 ( $\pm$ 5.01)	9.2 ( $\pm$ 3.89)	2.54 ( $\pm$ 3.16)	0.24 ( $\pm$ 0.08)	1.69 ( $\pm$ 2.01)	2.34
DIN:SRP	54.6 ( $\pm$ 72.5)	28.3 ( $\pm$ 37.2)	34.8 ( $\pm$ 41.5)	16.3 ( $\pm$ 15.4)	22.1 ( $\pm$ 7.1)	108.3
DON	9.4 ( $\pm$ 1.9)	11.5 ( $\pm$ 0.6)	9.6 ( $\pm$ 6.4)	5.1 ( $\pm$ 2.8)	9.2 ( $\pm$ 2.7)	11.2
DOC:TDN	18.6 ( $\pm$ 4.9)	9.3 ( $\pm$ 1.7)	20.6 ( $\pm$ 10.1)	31.7 ( $\pm$ 14.0)	33.1 ( $\pm$ 21.5)	17.5
DOC:SRP	5430 ( $\pm$ 2470)	837 ( $\pm$ 1140)	5120 ( $\pm$ 4530)	6500 ( $\pm$ 6230)	14200 ( $\pm$ 17600)	11000

fold for GDH<sub>T</sub>; 22-fold for GS). This larger range is because the specific activities of GS and GDH are typically of opposing magnitudes, i.e., GS is high and GDH low under N-deplete conditions, and vice versa. Furthermore, GDH and GS expression may remain unchanged after some degree of increasing N or C limitation, respectively, but expression of the opposing enzyme may continue to increase (Brown et al. 1972; Gräzer-Lampert et al. 1986; Hoch et al. 1992). Therefore, the GDH<sub>T</sub>:GS ratio better reflects the continuum of severe N-deplete to excessively N-replete conditions than either enzyme alone. This continuum is reflected in the clustering of amendment experiment treatments (Fig. 9 and Table 9), where the most N-replete (C-limited) conditions of Clusters II and III (amino acid and NH<sub>4</sub><sup>+</sup> plus PO<sub>4</sub><sup>3-</sup> treatments) yield GDH<sub>T</sub>:GS ratio values > 100, and the most N-deplete conditions of Clusters IV and V (glucose treatments) yield values < 20. Values of GDH<sub>T</sub>:GS ratio for ambient bacterioplankton along the northwest Florida coast were 40 to 90 (Table 8), which are within the range of the more definitive N-deplete versus N-replete values. Lack of correlation between ambient enzyme values and chemical parameters emphasizes how concentrations do not necessarily reflect the supply (flux) and lability of substrates.

Measurements of GDH<sub>T</sub>:GS ratio should prove useful to understanding how N replete versus N deplete conditions in situ influences BGE, because their expression is regulated in response to the intracellular balance of N and C signal molecules (NH<sub>4</sub><sup>+</sup>, glutamine, and  $\alpha$ -ketoglutarate) whose levels effectively reflect the metabolic integration of all substrates for heterotrophic bacterioplankton respiration and growth. Furthermore the dominant pathway for NH<sub>4</sub><sup>+</sup> assimilation has a direct influence on BGE, in that GS requires expenditure of ATP and GDH does not. For example, GS pathway dependence during N-limited growth of *E. coli* increased biosynthetic energy costs by 10% to 20% over that for N replete growth using the GDH<sub>A</sub> pathway (Helling 1994), i.e., under conditions of excess labile organic C expression of GS dominates and at low organic C conditions (energy-limited) GDH domi-

nates. The only study that has measured GDH and GS activity of bacterioplankton involved seawater cultures from two pelagic ecosystems of contrasting trophic conditions and N supply (Jørgensen et al. 1999). Although incubations lasted days, regeneration of N in the more eutrophic SRS incubations was associated with more efficient growth (BGE = 47% to 57%) and GDH<sub>T</sub>:GS ratios > 200. Whereas, oligotrophic GOM incubations had lower BGE (17% to 37%), GDH<sub>T</sub>:GS ratios were < 50, and more DIN was taken up. Our controlled studies of GS and GDH expression and seawater culture experiment results of Jørgensen et al. (1999) collectively demonstrate that GDH<sub>T</sub>:GS ratio measurements will advance our understanding N supply effects on BGE.

*Comments and recommendations*—Establishing this suite of enzyme assays by others requires little specialized equipment or instrumentation outside what would already exist in most aquatic ecology laboratories. Overall, the method is very amenable to complete shipboard operation. A single practiced individual can perform all three enzyme assays (GS, GDH<sub>A</sub>, and GDH<sub>C</sub>) within 1 to 2 h, which includes an assay incubation time of 15 to 75 min. Time is saved by omitting filtrations for protein analysis, which is not a concern if only the GDH:GS ratio is of interest. Furthermore, it is feasible to triple the sample processing efficiency if assay incubation time is used to start processing the next sample and an assistant is working in tandem.

Enzyme assays reported here were optimized using northwest Florida coastal waters of the GOM, and most amendment experiments and ambient field measurements were performed in this general biogeographic region under similar climatic conditions. In adopting these measurements to other ocean regions or freshwater systems, we recommend repeating experiments to determine the optimal (isoactivity) pH, temperature, and substrate saturating conditions. A cursory review of pH optima of both enzymes from numerous mesophilic prokaryotes (not shown) suggests that the values vary by about  $\pm$  0.3 pH units, and substrate concentrations in our

assays were similar to saturating conditions for numerous studies of prokaryotes. There are few studies of temperature effects for these enzymes as most prokaryotes studied have been mesophilic and assays performed at about 30°C, which would be inappropriate for psychrotrophic and psychropilic aquatic prokaryotes at higher latitudes, as demonstrated for GDH of *Psychrobacter* sp. TAD1 (Di Fraia et al. 2000).

In cases where changes to assay conditions are required or a new ecosystem is studied, we recommend performing amendment experiments for the purpose of interpreting ambient field results. The time and cost of running amendment experiments can be minimized by performing single endpoint analyses, but caution should be taken not to underestimate the lag time for induction or repression of enzyme expression. Conversely, until more is known on the influence of community composition on GS and GDH activity, amendment experiment duration should be kept as short as possible. Deciding on amendment experiment incubation duration is best approached with some prior knowledge of growth rate response, so that incubations can be stopped within a few generation times. Replication of treatments within amendment experiments may be impractical due to large volumes of size-fractionated seawater; instead replicating the amendment experiment should be considered or assume about 15% error in treatments. The number of amendment experiments performed should be proportionate to the diversity of habitats in the ecosystem studied, i.e., more in an estuary than open ocean.

Community composition influences on the relationship between enzyme parameter values and degree of N bioavailability is untested. We recommend incorporating rapid molecular techniques based on rRNA gene profiling (e.g., TRFLP, DGGE, or ARISA) or taxon-specific probing (e.g., FISH) to explore community dynamics during amendment experiments. These techniques and microscopy assessment of cell size and particle attachment should be used to address the underlying assumption that bacteria in the size fractions are representative of the whole bacterioplankton community, as there may be large (> 0.8 µm) and particle attached bacteria of a different physiological state being removed from the size fraction.

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