

## Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation

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### Abstract

In a combination of batch and continuous culture experiments involving natural assemblages of marine bacteria we examined the impact of varying both the carbon and nitrogen substrates and the substrate C:N ratio ( $C:N_S$ ) on the bacterial C:N ratio ( $C:N_B$ ), the gross growth efficiency (GGE), and the ability of marine bacteria to cometabolize amino acids and  $NH_4^+$ . We varied the  $C:N_S$  ratio from 5:1 (carbon limitation) to 30:1 (nitrogen limitation) with varying combinations of glucose, glutamate, and  $NH_4^+$  (and in some cases with an amino acid mixture and with other simple carbon compounds). We found that GGE was unaffected by growth rate, the  $C:N_S$  ratio, or different combinations of carbon and nitrogen substrates, averaging ~50%. However, the 95% confidence interval was as high as  $\pm 20\%$ , suggesting that experimental error may be a common problem in determining GGE in laboratory studies and a far more serious problem in making such estimates in field studies. The  $C:N_B$  ratio was invariant at ~4.5:1 (by atoms) under all conditions when carbon was limiting growth, but increased to ~7:1–9:1 when reaching the stationary phase during batch growth and up to 13:1 at very low growth rates in continuous culture. These results indicate that the  $C:N_B$  ratio has some value as a diagnostic tool for determining the physiological state of bacterial populations, although there are some concerns about its general applicability. Finally, there was absolutely no evidence that  $NH_4^+$  uptake was inhibited in the presence of amino acids. The key to  $NH_4^+$  uptake is the presence of a readily assimilated carbon source to balance nitrogen uptake stoichiometrically. These findings have some bearing on the recent suggestions that gradients exist in both GGE and nitrogen preference of marine bacteria from productive inshore regions to the oligotrophic open ocean.

Bacteria clearly play a key role in the cycling of energy and materials within the marine food chain and, as such, they are the subject of intensive research in contemporary microbial ecology (Ducklow and Carlson 1992; Azam 1998). Yet, despite these efforts, persistent difficulties in identifying the substrates available for growth of natural bacterial populations and in making accurate measurements of key parameters such as the cellular carbon and nitrogen contents and the gross growth efficiency (GGE) of these microbes has greatly hampered a comprehensive and quantitative understanding of their actual importance (del Giorgio and Cole 1998).

To address these questions in a quantitative and controlled fashion, we have been carrying out a series of laboratory studies with natural bacterial populations (Goldman et al. 1987; Goldman and Dennett 1991). Previously, we showed that marine bacteria were capable of utilizing amino acids and  $NH_4^+$  simultaneously as nitrogen sources as long as a readily assimilated carbon source was available to balance  $NH_4^+$  uptake (Goldman and Dennett 1991). In effect, two separate systems for nitrogen metabolism seemed to be operating concurrently. Regeneration of  $NH_4^+$  occurred only through amino acid catabolism and only when the C:N ratio of the organic substrate ( $C:N_S$ ) was lower than the corre-

sponding ratio in bacterial biomass ( $C:N_B$ ), corrected for respiratory and other losses. In contrast, uptake of  $NH_4^+$  in the absence of amino acids occurred only in a stoichiometric balance with uptake of a carbon source and without any regeneration. The relative amounts of nitrogen taken up (amino acids and  $NH_4^+$ ) and regenerated (amino acids) were defined in a quantitative fashion by three parameters, the  $C:N_S$  ratio of the total available carbon and nitrogen sources, the  $C:N_B$  ratio and the GGE based on carbon utilization. These results were in contrast to those of Kirchman et al. (1989) who argued that  $NH_4^+$  uptake could be inhibited by the presence of amino acids. As we pointed out earlier (Goldman and Dennett 1991), their observation of apparent inhibition of  $NH_4^+$  uptake may have been due simply to the exhaustion of a carbon source needed to balance  $NH_4^+$  uptake. Results from later studies of Kirchman and colleagues (Kirchman 1990; Kirchman and Rich 1997; Kirchman et al. 1990) seemed to support this possibility.

We also showed that when both amino acids and  $NH_4^+$  were cometabolized some fraction of the  $NH_4^+$  produced through amino acid catabolism was recycled (possibly internally) and incorporated into bacterial biomass. However, net  $NH_4^+$  regeneration occurred only after complete utilization of the carbon source, which in this case was glucose (Goldman and Dennett 1991). Tupas and Koike (1990, 1991) similarly showed concurrent uptake of the two forms of nitrogen and stressed the point that bacteria are capable of acting simultaneously as sinks and sources for  $NH_4^+$ . However, it is important to stress that, irrespective of the degree of  $NH_4^+$

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Table 1. Summary of batch culture conditions and results.

Substrate characteristics					
Source*	C:N <sub>s</sub> (by atoms)	$\mu$ (h <sup>-1</sup> )	N recovered (%)	GGE (%)	C:N <sub>B</sub> † (by atoms)
Glu + NH <sub>4</sub> <sup>+</sup> + Gluc	10:1	0.50	99	59	4.7:1
Glu + NH <sub>4</sub> <sup>+</sup> + Gluc	30:1	0.49	96	50	8.4:1
NH <sub>4</sub> <sup>+</sup> + Gluc	30:1	0.56	90	70	8.9:1
Glu + NH <sub>4</sub> <sup>+</sup> + Gly	5:1	0.38	101	53‡	4.9:1
Glu + NH <sub>4</sub> <sup>+</sup> + Cit	5:1	0.33	106	38‡	5.8:1
Glu + NH <sub>4</sub> <sup>+</sup> + Ace	5:1	0.26	105	38‡	4.8:1
Glu + NH <sub>4</sub> <sup>+</sup> + Gly	30:1	0.39	99	—	7.7:1
Glu + NH <sub>4</sub> <sup>+</sup> + Cit	30:1	0.32	107	—	7.1:1
Glu + NH <sub>4</sub> <sup>+</sup> + Ace	30:1	0.28	103	—	7.8:1
AA Mix + NH <sub>4</sub> <sup>+</sup> + Gluc	30:1	0.52	106	34	7.6:1
NH <sub>4</sub> <sup>+</sup> + Gluc	30:1	0.46	98	50	7.4:1

\* Glu, glutamate; Gluc, glucose; Gly, glycerol; Cit, citrate; Ace, acetate; AA Mix, amino acid mix.

† C:N ratio at end of experiment.

‡ Estimated GGE based on complete utilization of carbon substrate by end of exponential growth.

recycling, the net amount of NH<sub>4</sub><sup>+</sup> regenerated is described quantitatively by the three parameters identified above (Caron and Goldman 1990; Goldman and Dennett 1991). Moreover, NH<sub>4</sub><sup>+</sup> regeneration during exponential growth or to meet maintenance requirements can occur only if an organic nitrogen compound is present (Goldman and Dennett 1991). These catabolic processes need to be distinguished from nutrient regeneration processes that may result from cell autolysis.

Our earlier studies (Goldman and Dennett 1991) were limited to batch culture experiments in which the C:N<sub>s</sub> ratio was kept at ≤10:1 to establish conditions of carbon limitation and in which glucose was the sole organic carbon compound added along with different amino acids. In those experiments the C:N<sub>B</sub> ratio was relatively constant at ~4.5:1–5:1 and GGE was in the range of ~40–60%. In the current experiments we expand the C:N<sub>s</sub> ratio to 30:1 to include conditions of extreme nitrogen limitation, examine the effect of several organic carbon compounds on growth and nitrogen metabolism, and utilize both batch and continuous culture techniques. Our aim is to explore in more detail a broader range of conditions bacteria might experience in the marine environment. In particular, we want to demonstrate more completely that, while cometabolism of amino acids and NH<sub>4</sub><sup>+</sup> is a common response of marine bacteria for exploiting all available nitrogen sources, there is a strict stoichiometry involving organic carbon in this process. Additionally, we want to determine if the C:N<sub>B</sub> ratio of bacteria is rigid or changes with a switch from carbon to nitrogen limitation and if the GGE is influenced by the C:N<sub>s</sub> ratio over a broader range of C:N<sub>s</sub> ratios than previously examined.

## Materials and methods

Our experimental protocols for the batch experiments were identical to those used previously (Goldman and Dennett 1991). We grew natural assemblages of bacteria in 1,000

ml of medium contained in 2.5-liter glass Fernbach flasks. The vessels were stirred with magnetic bars and kept in the dark at 24°C. The growth medium was an artificial seawater recipe (Goldman and McCarthy 1978) supplemented with a fixed concentration of 500 μg-atoms L<sup>-1</sup> N made from equal amounts of NH<sub>4</sub><sup>+</sup> and organic nitrogen (derived from glutamate or an amino acid mixture) and varying amounts of different organic carbon sources to yield C:N<sub>s</sub> ratios from 5:1 to 30:1 (by atoms). The carbon sources were added singularly either as glucose, acetate, citrate, or glycerol. We chose these carbon substrates to span a range of oxidation states from the most oxidized (citrate) to the most reduced (glycerol) in order to test the effect of carbon source on bacterial GGE. Three sets of batch experiments were conducted, one involving NH<sub>4</sub><sup>+</sup> plus glutamate along with either acetate, citrate, or glycerol (C:N<sub>s</sub> ratios of 5:1 and 30:1), another involving NH<sub>4</sub><sup>+</sup> plus either glutamate or an amino acid mixture along with glucose (C:N<sub>s</sub> ratios of 10:1 and 30:1 for glutamate and 30:1 for the amino acid mixture), and the third with only NH<sub>4</sub><sup>+</sup> and glucose (C:N<sub>s</sub> ratio of 30:1). This latter experiment was repeated. The amino acid mixture was a commercial mixture (Pierce 20089) of 17 amino acids (C:N = 3.6:1). Sufficient PO<sub>4</sub><sup>3-</sup> (150 μg-atoms L<sup>-1</sup> P) was added to ensure that either carbon or nitrogen was limiting growth. The treatments are summarized in Table 1.

The continuous culture experiments were performed under conditions similar to those in the batch studies. We maintained 1,100 ml of media in 2-liter glass reaction vessels at 24°C. Both aeration and stirring with magnetic bars were used to keep the cultures well mixed. The basic medium, except for NaHCO<sub>3</sub> and the nitrogen and organic carbon sources, was autoclaved in glass carboys. The other nutrients were autoclaved separately, cooled, and added individually to the sterile medium. The NaHCO<sub>3</sub> solution was sealed in a glass ampoule before autoclaving to avoid any loss of CO<sub>2</sub>. The culture vessels and feed lines were autoclaved before continuous flow commenced. Laboratory air for aeration was

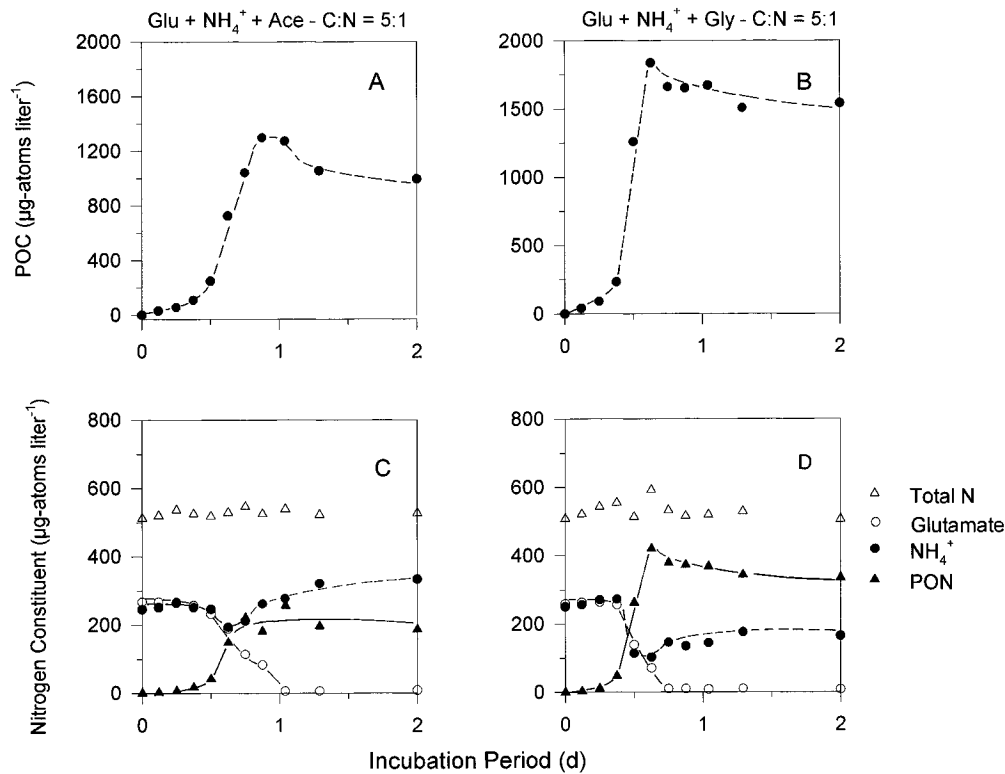


Fig. 1. Time course of growth and nitrogen transformations by natural assemblages of marine bacteria grown in batch culture on combinations of glutamate,  $\text{NH}_4^+$ , and either (A,C) acetate or (B,D) glycerol for a substrate C:N ratio ( $\text{C:N}_s$ ) of 5:1. Curves in all figures were drawn by visual inspection to represent trends only.

passed through activated carbon and autoclaved cotton wool columns. Medium was pumped into the culture at varying flow rates with a peristaltic pump to maintain dilution rates in the range of  $\sim 0.02\text{--}0.60\text{ h}^{-1}$ . We conducted two sets of experiments, one under carbon limitation ( $\text{C:N}_s = 5:1$ ) and other under nitrogen limitation ( $\text{C:N}_s = 30:1$ ). In all cases we added nitrogen at a fixed concentration of  $500\ \mu\text{g-atoms L}^{-1}$ , either with  $\text{NH}_4^+$  or glutamate, and varied the  $\text{C:N}_s$  ratio by adding the required amount of glucose. Because glutamate has a C:N ratio of 5:1, it was not necessary to add glucose when glutamate was used under carbon limiting conditions. The continuous culture treatments are summarized in Table 2. We conducted all experiments at the Woods Hole Oceanographic Institution.

Inocula for both the batch and continuous culture exper-

iments were natural assemblages of marine bacteria obtained by filtering seawater from Vineyard Sound, Massachusetts through a sterile  $0.6\text{-}\mu\text{m}$  membrane filter (Nuclepore). We made time-series measurements of a suite of chemical constituents in the batch culture experiments at intervals of 3–4 h during the first day of incubation and then at longer intervals through 2 d. In the continuous culture studies, we took measurements when the cultures reached steady state at each dilution rate (steady state was assumed when absorbance measured at 600 nm did not vary for 2–3 d). Among the chemical constituents we measured were particulate carbon (POC), particulate nitrogen (PON), residual and medium  $\text{NH}_4^+$ , dissolved free amino acids [DFAA as glutamate (Glu) or the amino acid mixture], and glucose (Gluc). We analyzed POC and PON on 25–100 ml samples retained on precom-

Table 2. Summary of continuous culture conditions and results.

Substrate characteristics			$\mu$ (range) ( $\text{h}^{-1}$ )	N recovered* (%)	GGE* (%)	$\text{C:N}_b$ (by atoms)
Source	$\text{C:N}_s$ (by atoms)	$n$				
$\text{NH}_4^+$ + Gluc	5:1	15	0.02–0.58	$98.7 \pm 4.84$	$52.8 \pm 5.67$	$4.4:1 \pm 0.20^*$
Glu	5:1	9	0.02–0.58	$97.2 \pm 5.32$	$42.9 \pm 8.88$	$4.5:1 \pm 0.19^*$
$\text{NH}_4^+$ + Gluc	30:1	7	0.02–0.60	$100.1 \pm 5.81$	$57.6 \pm 9.18$	$5.7:1\text{--}11.4:1^\dagger$
Glu + Gluc	30:1	7	0.02–0.60	$95.0 \pm 8.34$	$51.3 \pm 6.90$	$4.9:1\text{--}13.0:1^\dagger$

\* Average  $\pm 2$  SD.

† Range from lowest value at max dilution rate to highest value at min dilution rate.

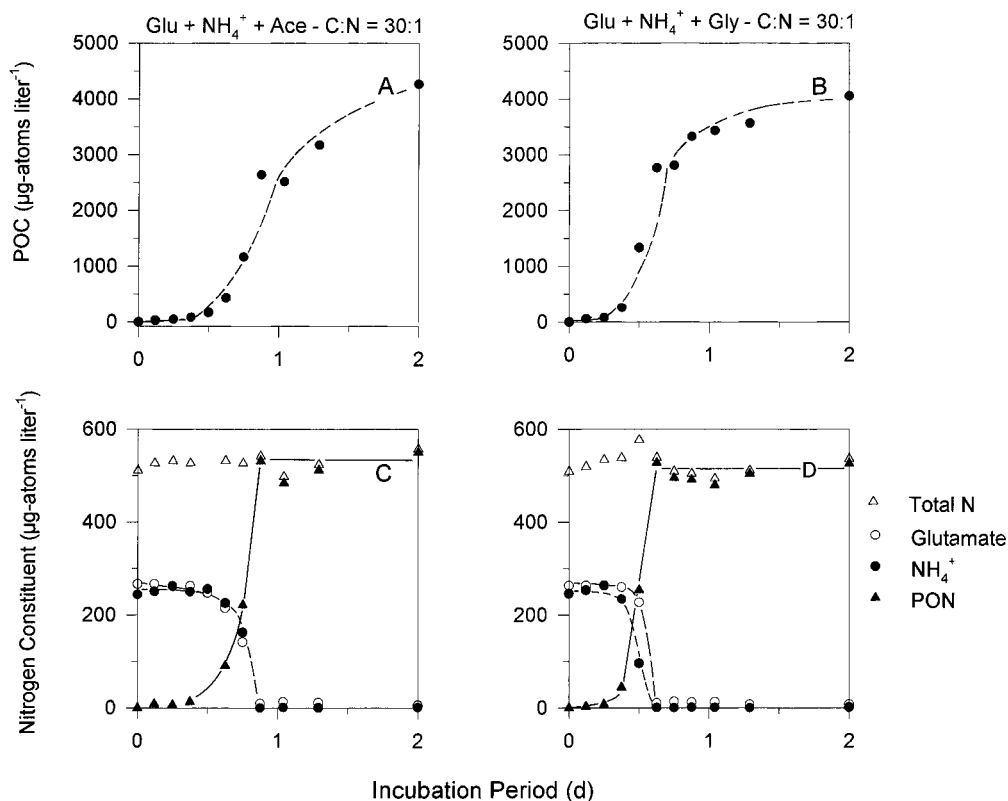


Fig. 2. As Fig. 1 except  $\text{C:N}_s = 30:1$ .

busted glass-fiber filters (Whatman GF/F) with a Perkin Elmer 240C elemental analyzer. We used the filtrate to analyze for  $\text{NH}_4^+$  by the modified hypochlorite procedure (McCarthy and Kamykowski 1972), glucose by the phenol sulfuric acid method (Kochert 1978), and DFAA by the fluorescamine technique (North 1975). Details of these methods are given elsewhere (Goldman and Dennett 1991). We did not analyze for glycerol, citrate, or acetate.

We calculated specific growth rates ( $\mu$ ) in the batch studies by regression analyses of the slope of the linear (exponential) portion of the curves of  $\ln$  POC versus time and GGE as  $100 \times \Delta\text{POC}/\text{total substrate C consumed}$  during this time interval. We estimated GGE in the experiments involving glycerol, citrate, and acetate at  $\text{C:N}_s = 5:1$  by assuming that all the carbon substrate was utilized by the end of exponential growth. Considering that we would expect some residual carbon when  $\text{C:N}_s$  was 30:1 for these same carbon substrates, it was not possible to estimate GGE in these experiments. For the continuous culture experiments  $\mu$  was equal to the steady state dilution rate and GGE was  $100 \times \text{POC at steady state}/\text{total substrate C consumed}$ .

## Results

**Batch culture experiments**—Recovery of substrate nitrogen in the batch experiments as the sum of PON, DFAA, and  $\text{NH}_4^+$  was virtually complete, varying from 90% to 107% (Table 1). Specific growth rates were slightly higher when glucose was the carbon substrate (0.46–0.56  $\text{d}^{-1}$ ) than

when glycerol, citrate, or acetate were used (0.26–0.39  $\text{d}^{-1}$ ) (Table 1). There appeared to be no systematic effect of the  $\text{C:N}_s$  ratio on  $\mu$  within the range 5:1–30:1.

Because we did not analyze for glycerol, citrate, or acetate, it was impossible to determine the GGE in experiments involving these substrates. However, we estimated GGE in the portion of these experiments involving carbon limitation ( $\text{C:N}_s = 5:1$ ) by assuming that all of the carbon substrate was consumed by the end of exponential growth. The estimated GGE varied between 38% and 53% in these experiments and when calculated for experiments involving glucose as the carbon source it was within the range 34–70% (Table 1). The lowest value occurred when the nitrogen source was a combination of the amino acid mixture and  $\text{NH}_4^+$  and the highest value was found in one of the replicate experiments involving only  $\text{NH}_4^+$ . In both experiments the  $\text{C:N}_s$  ratio was 30:1. In the other experiments GGE varied between 50% and 59%. The  $\text{C:N}_B$  ratio at the end of the 2 d incubation period varied from 4.7:1–5.8:1 when carbon was limiting ( $\text{C:N}_s$  ratio = 5:1–10:1) to 7.1:1–8.9:1 under nitrogen limitation ( $\text{C:N}_s$  ratio = 30:1) (Table 1). The carbon source appeared to have no impact on this result.

The patterns of carbon and nitrogen uptake primarily were a function of the  $\text{C:N}_s$  ratio and not of the carbon source (Figs. 1–4) (figures for citrate uptake and for one of the replicate experiments involving uptake of  $\text{NH}_4^+$  and glucose were excluded for clarity, as trends were similar for all carbon sources). In experiments involving glutamate and  $\text{NH}_4^+$  or the amino acid mixture we observed concurrent uptake

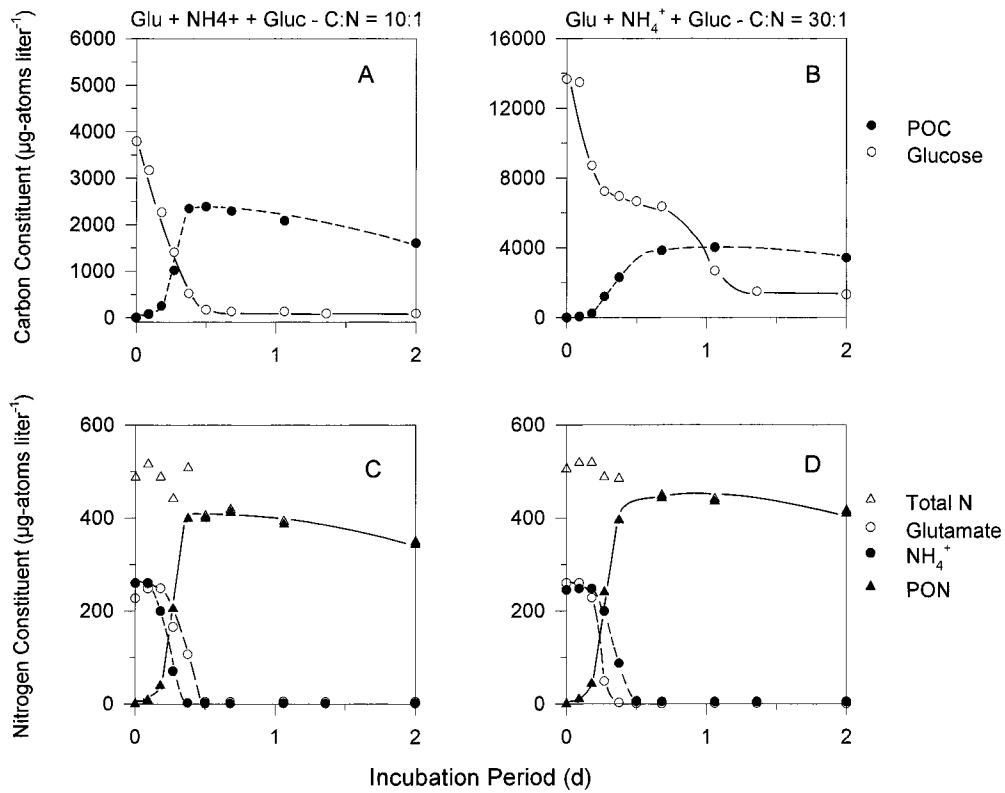


Fig. 3. As Fig. 1 except glucose, glutamate, and  $\text{NH}_4^+$  are the carbon and nitrogen substrates and  $\text{C:N}_s = (\text{A,C}) 10:1$  or  $(\text{B,D}) 30:1$ .

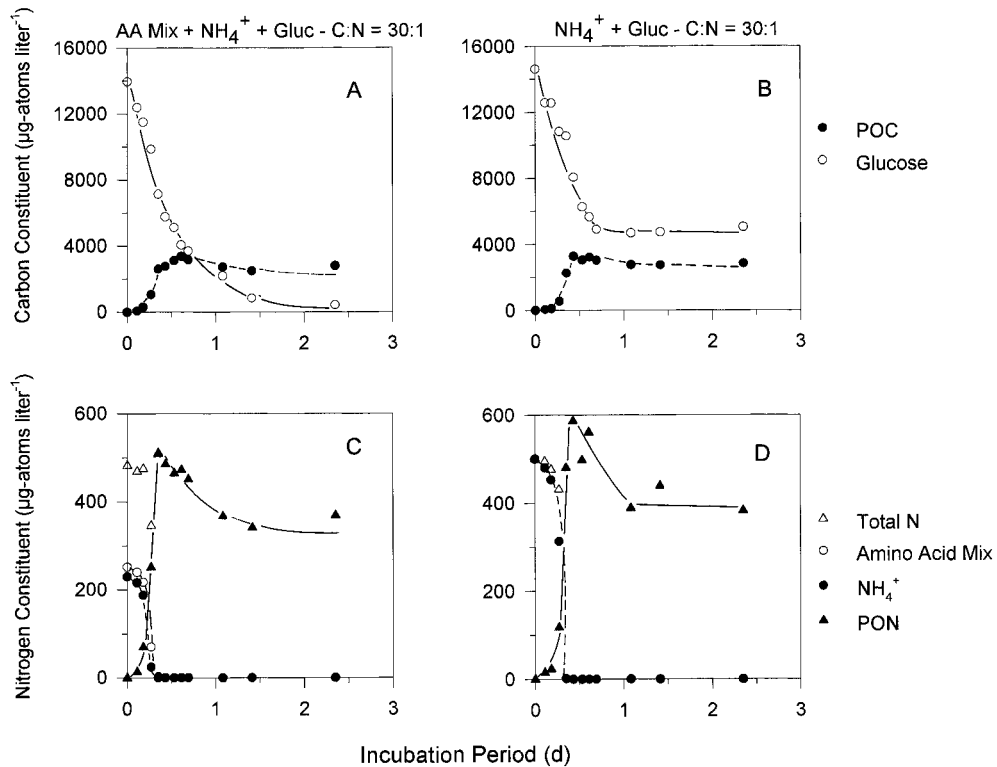


Fig. 4. As Fig. 3 except (A,C) amino acid mixture, glucose and  $\text{NH}_4^+$  or (B,D) glucose and  $\text{NH}_4^+$  are the carbon and nitrogen substrates and  $\text{C:N}_s = 30:1$ .

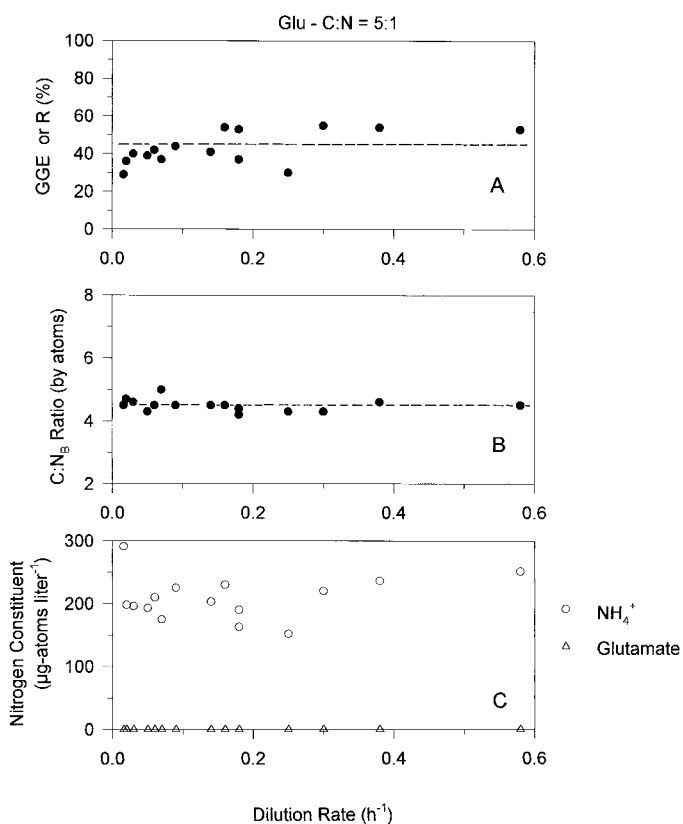


Fig. 5. Effect of dilution rate on (A) GGE, (B) C:N<sub>B</sub> ratio, and (C) residual chemical constituents in continuous culture studies involving growth of natural assemblages of marine bacteria on glutamate (C:N<sub>S</sub> = 5:1).

of both nitrogen sources regardless of the C:N<sub>S</sub> ratio and the carbon source. When the C:N<sub>S</sub> ratio was 5:1 there were initial decreases in the concentrations of both nitrogen sources, followed by increases in NH<sub>4</sub><sup>+</sup> which started between the middle to the end of exponential growth. In contrast, uptake of glutamate continued until it was completely exhausted by the end of exponential growth (Figs. 1C,D). When the C:N<sub>S</sub> ratio was 10:1 (Fig. 3C) or 30:1 (Figs. 2C,D, 3D, 4C) concurrent utilization of glutamate and NH<sub>4</sub><sup>+</sup> occurred until both substrates were exhausted by the end of exponential growth. Finally, when NH<sub>4</sub><sup>+</sup> was the sole nitrogen source and the C:N<sub>S</sub> ratio was 30:1 uptake was continuous and complete by the end of exponential growth (Fig. 4D).

Glucose uptake was virtually complete by the end of exponential growth when the C:N<sub>S</sub> ratio was 10:1 (Fig. 3A). Under nitrogen limiting conditions (C:N<sub>S</sub> = 30:1) and when the nitrogen source was NH<sub>4</sub><sup>+</sup> alone (Fig. 4B) or NH<sub>4</sub><sup>+</sup> plus glutamate (Fig. 3B) substantial amounts of glucose remained well into the stationary phase when the experiments were terminated. However, when the nitrogen source was the amino acid mixture (Fig. 4A) any glucose remaining at the end of exponential growth was consumed during the stationary phase.

*Continuous culture experiments*—Under carbon limiting conditions (C:N<sub>S</sub> = 5:1) there were large residuals of NH<sub>4</sub><sup>+</sup>

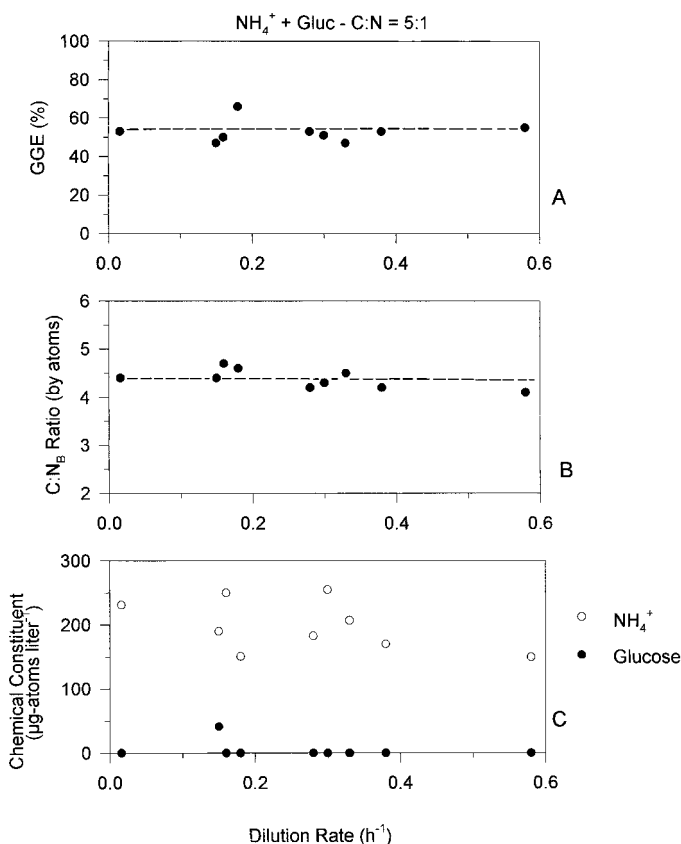


Fig. 6. As Fig. 5 except glucose and NH<sub>4</sub><sup>+</sup> (C:N<sub>S</sub> = 5:1) were the carbon and nitrogen substrates.

at all dilution rates both when glutamate was the sole carbon and nitrogen source (regenerated NH<sub>4</sub><sup>+</sup>, Fig. 5C) and when glucose and NH<sub>4</sub><sup>+</sup> were the substrates (unused NH<sub>4</sub><sup>+</sup>, Fig. 6C). Hence, recovered nitrogen was the sum of PON and NH<sub>4</sub><sup>+</sup>. In contrast, when nitrogen was limiting (C:N<sub>S</sub> = 30:1) all substrate nitrogen was utilized regardless of the source, there were large residuals of unused glucose (Figs. 7C, 8C) and recovered nitrogen was solely as PON. In all experiments recovery of substrate nitrogen was virtually complete, varying from 95.0% to 100.1% (Table 2).

Regardless of the carbon and nitrogen substrates and the C:N<sub>S</sub> ratios, there was little variation in GGE over the entire range of dilution rates. Under carbon limiting conditions GGE varied from 42.9 ± 8.88% (2 SD) when glutamate was the sole carbon and nitrogen source (Table 2; Fig. 5A) to 52.8 ± 5.67% (2 SD) when NH<sub>4</sub><sup>+</sup> plus glucose were the substrates (Table 2; Fig. 6A). Similarly, under nitrogen limiting conditions GGE varied from 51.3 ± 6.90% (2 SD) when glutamate plus glucose were the substrates (Table 2; Fig. 7A) to 57.6 ± 9.18% (2 SD) when they were replaced by NH<sub>4</sub><sup>+</sup> plus glucose (Table 2; Fig. 8A).

The C:N<sub>B</sub> ratio, in contrast to GGE, was markedly affected by the type of nutrient limitation. When carbon was limiting the C:N<sub>B</sub> ratio remained relatively invariant at 4.4:1–4.5:1 over the range of dilution rates (0.02–0.60 h<sup>-1</sup>) regardless of the form of available nitrogen (Table 2; Figs. 5B, 6B). However, the C:N<sub>B</sub> ratio was relatively constant (~5:

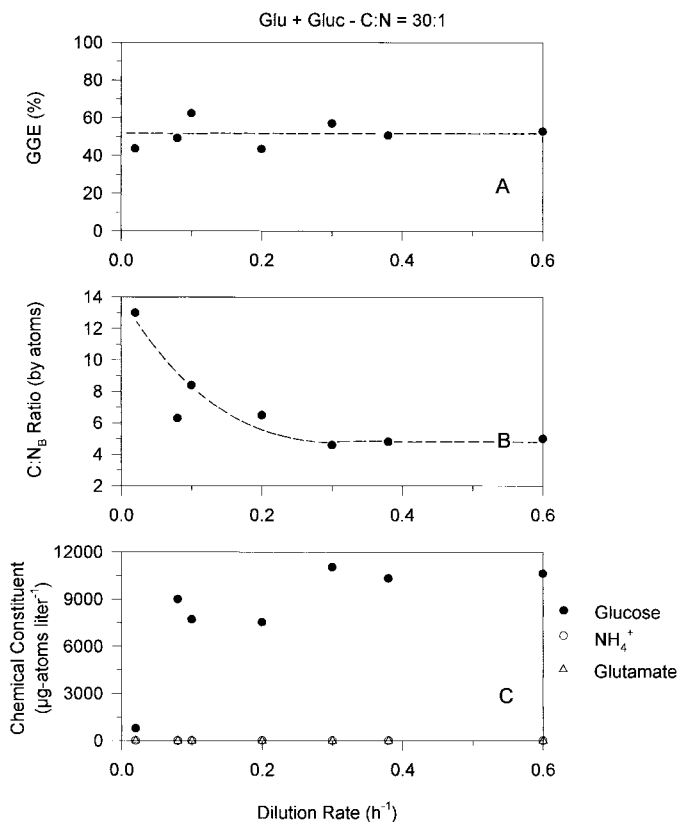


Fig. 7. As Fig. 5 except glucose and glutamate ( $C:N_s = 30:1$ ) were the carbon and nitrogen substrates

1) from the highest dilution rates down to about  $0.2\text{--}0.3\text{ h}^{-1}$  when nitrogen was limiting, but then increased dramatically to  $11:1\text{--}13:1$  at the lowest dilution rates ( $0.02\text{ h}^{-1}$ ) (Table 2; Fig. 7B, 8B). The response was the same whether glutamate (Fig. 7B) or  $\text{NH}_4^+$  (Fig. 8B) was the nitrogen source.

## Discussion

As we have emphasized in previous studies, the simple equation:

$$E_t : C_t = 1/C : N_s - \text{GGE}/(C : N_B \times 100)$$

where the ratio  $E_t : C_t$  is the amount of nitrogen (as  $\text{NH}_4^+$ ) regenerated relative to total carbon uptake and the GGE is expressed in % (Goldman et al. 1987; Caron and Goldman 1990; Goldman and Dennett 1991), provides a straightforward and quantitative description of the conditions under which  $\text{NH}_4^+$  regeneration occurs. The qualifying condition for the equation to be applicable is that the nitrogen source must come from an organic compound (Goldman and Dennett 1991).

**Variable  $C:N_B$  ratios**—The three variables describing the above equation have all been the subject of much research, but still there is ambiguity as to their magnitude and nature. Of the three variables, the one which is best understood is the  $C:N_B$  ratio. From numerous previous studies including our own there is abundant evidence that the  $C:N_B$  ratio is

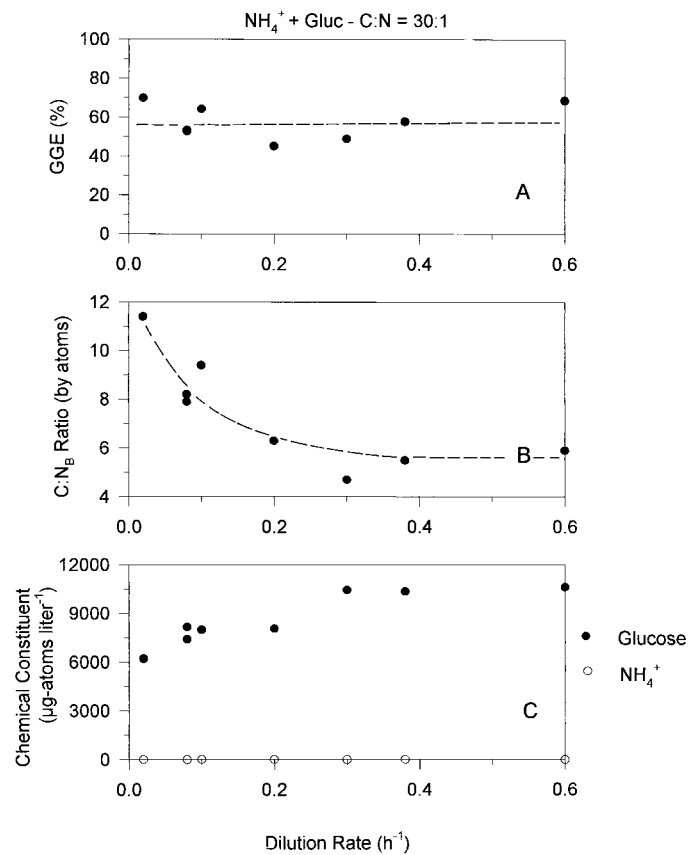


Fig. 8. As Fig. 5 except glucose and  $\text{NH}_4^+$  ( $C:N_s = 30:1$ ) were the carbon and nitrogen substrates.

relatively invariant at  $\sim 4.5:1\text{--}5:1$ , but only when the  $C:N_s$  ratio is less than  $\sim 10:1$ , which is a rough cutoff for carbon limiting conditions (Egli 1991). Our current results, both from the batch (Table 1) and continuous culture studies (Table 2) confirm this general observation.

However, as seen in both our batch (Table 1) and continuous culture results (Table 2), the ratio is not a constant, but does vary considerably when nitrogen is the limiting nutrient, and that this variability seems to be dependent to some degree on growth conditions. We suspect that the difference between the maximum  $C:N_B$  ratios of  $\sim 7:1\text{--}9:1$  (Table 1) that occurred after the stationary growth phase was reached in the batch studies versus the considerably higher maximum  $C:N_B$  ratios observed at the lowest dilution rates tested ( $0.02\text{ h}^{-1}$ ) with the continuous cultures ( $\sim 11:1\text{--}13:1$ ) reflects a fundamental difference between batch and continuous cultures. For example, when referring to batch cultures as being carbon or nitrogen limited based on the substrate  $C:N_s$  ratio, limitation of yield rather than growth rate is inferred, except perhaps for very short periods near the end of exponential growth when nitrogen levels are low enough to influence growth rate. In contrast, carbon or nitrogen limitation of growth rate (the intensity of which increases with decreasing dilution rate) is a major feature of continuous cultures at steady state. It's not entirely clear why maximum  $C:N_B$  ratios in nitrogen-limited batch cultures should be lower than in nitrogen-limited continuous cultures at low dilution rates,

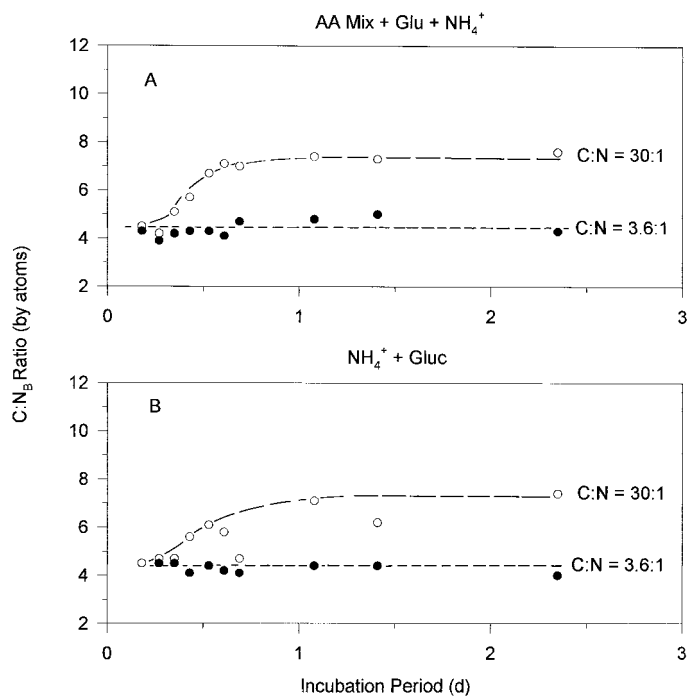


Fig. 9. Comparison of time course changes in  $C:N_B$  ratio for experiments where either (A) an amino acid mixture, glucose and  $NH_4^+$  or (B) glucose and  $NH_4^+$  were the carbon and nitrogen substrates: Data for  $C:N_S = 30:1$  from current experiments described in Fig. 4; data for  $C:N_S = 3.6:1$  from Goldman and Dennett (1991).

but it may be related to the transient nature of true nitrogen limitation of growth rate in the batch culture and the resulting impact on the physiological state of the culture as nitrogen limitation becomes increasingly severe. This point is clearly seen in Fig. 9, where the time course of changes in the  $C:N_B$  ratio for nitrogen limited batch cultures ( $C:N_S = 30:1$ ) grown either with an amino acid mixture and  $NH_4^+$  (Fig. 9A) or  $NH_4^+$  alone (Fig. 9B) as the nitrogen source is compared with the invariant  $C:N_B$  ratio over the entire growth rate range for growth with similar nitrogen sources, but under carbon limitation ( $C:N_S = 3.6:1$  from Goldman and Dennett 1991). In all cases the  $C:N_B$  ratio was around 4.5:1 during exponential growth and only increased in the nitrogen limited cultures for a short period during the transition phase before leveling off at  $\sim 7.5:1$  in the stationary phase. Perhaps differences in maintenance requirements between the two types of culture systems contribute to these different metabolic patterns. Also, we can not discount the possibility that different bacterial populations were selected for in the two systems and even during different growth phases in each of the two systems (Harder and Kuenen 1977). However, if species selection occurred then we would have expected the maximum response for a given growth phase, not unlike what would be expected under natural conditions.

For the purposes of our study the important point is that the elemental chemical composition of bacteria is quite plastic and reflects, to a large degree, the form of nutrient limitation, similar to what is found in phytoplankton (Goldman

et al. 1979). In fact, large variations in the N:P and C:P ratios of bacteria have been found in recent studies when phosphorus was limiting growth (Tezuka 1990; Chrzanowski and Kyl 1996; Cotner et al. 1997). Thus it is evident that like its phytoplankton counterpart, the C:N:P ratio of bacteria may be useful as a diagnostic tool to estimate the form of nutrient limitation occurring in natural waters. However, given that we observed an increase in the  $C:N_B$  ratio  $>4.5:1$  only below a dilution rate of  $\sim 0.3\text{ h}^{-1}$ , which is about 50% of the maximum growth rates obtained in the current and previous studies (Goldman and Dennett 1991), it is conceivable that even when nitrogen was the apparent limiting nutrient, the expression of this limitation only occurred over a restricted (low) growth rate regime. Hence, there may be zones of growth rate for a given substrate C:N ratio where there is a transition from carbon to nitrogen limitation (Thingstad 1987; Egli 1991). The diagnostic value of the  $C:N_B$  ratio under such conditions would be limited and it would be possible to distinguish only grossly between carbon (energy) and extreme nitrogen limitation of bacteria. Additionally, it is important to note that since both carbon (energy) limitation of growth and nonnutrient limitation (maximal growth rate) lead to a minimum  $C:N_B$  ratio of 4.5–5:1, it is impossible to distinguish between these two growth modes with just elemental chemical composition data. Accurate in situ measurements of  $C:N_B$  ratios of marine bacteria are notoriously difficult to make, but from recent field studies (Fagerbakke et al. 1996; Cotner et al. 1997; Fukuda et al. 1998), it appears that they typically are in the range  $\sim 4.5\text{--}7:1$  (by atoms) for natural populations. While these values are slightly higher than the minimum values possible under carbon limitation, they provide some support, albeit indirect, for the notion that extreme nitrogen limitation is not a common occurrence in the ocean. (cf. Jorgensen et al. 1999).

**Bacterial GGE**—In the current and previous studies (Goldman et al. 1987; Goldman and Dennett 1991) only data for exponentially growing cultures were used to calculate GGE. From this composite data pool it appears that, with exception of some GGE values  $>70\%$  from our first study (Goldman et al. 1987) obtained at very low  $C:N_S$  ratios ( $\leq 3:1$ ) with single nitrogen sources (either  $NH_4^+$ , arginine, or glycine) and either single (amino acid or glucose) or double carbon sources (amino acid + glucose), all of the GGE data fell within the broad range of 30–70% over a wide range of  $C:N_S$  ratios (3:1–30:1) (Fig. 10). These results generally confirm our previous conclusion (Goldman and Dennett 1991) that there is no relationship between GGE and the  $C:N_S$  ratio when multiple nitrogen sources are being cometabolized. It appears that the very high GGE values we observed earlier (Goldman et al. 1987), and which we originally thought were indicative of an effect of the  $C:N_S$  ratio on GGE, seem to be restricted to when single nitrogen sources are being used, and only at very low  $C:N_S$  ratios ( $\leq 3:1$ ). As we stated earlier (Goldman and Dennett 1991), the energy requirements (possibly due to increased enzymatic activity) required for metabolizing multiple nitrogen sources offsets any gains in reduced carbon catabolism that might occur under severe carbon limitation. Similarly, we observed no discernible effect of carbon source on GGE, at least with

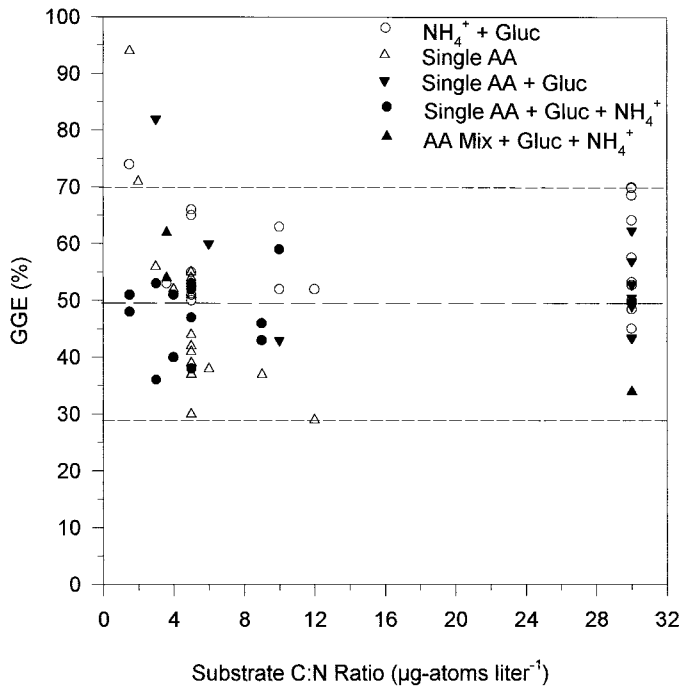


Fig. 10. Summary of GGE data as a function of substrate C:N<sub>s</sub> ratio from current and previous experiments (Goldman and Dennett 1991; Goldman et al. 1987) with natural assemblages of marine bacteria grown with different carbon and nitrogen substrates in batch and continuous cultures. Average GGE from Table 3 is broken line and represents all data encompassed by dashed lines.

the several carbon sources we tested (Table 1; Fig. 10). If the cultures grown at a C:N<sub>s</sub> ratio of 5:1 were energy rather than carbon limited, then, as shown by Linton and Stephenson (1978), GGE should have increased along a gradient of carbon oxidation states from the most oxidized (citrate), followed by acetate, then glucose, to the most reduced (glycerol). Once again, since multiple carbon and nitrogen substrates (glutamate and NH<sub>4</sub><sup>+</sup>) were used along with the single carbon compounds (Table 1), the complexity of energy allocation and expenditure for multiple substrate metabolism probably masked any effect of the different carbon sources on GGE. Unfortunately, our inability to estimate GGE for citrate, acetate, and glycerol use when the C:N<sub>s</sub> ratio was 30:1 made it impossible to observe if a similar effect occurred under nitrogen limiting conditions.

In this context, the invariance of GGE with changing dilution rate for each of the different substrate combinations in the continuous culture experiments (Table 2) is of some interest; to the best of our knowledge, this result has not been observed before. Intuitively, decreases in GGE with decreasing dilution rate are expected. Such variations in GGE most likely are due to a switch in macromolecular biosynthesis from protein to polysaccharide rich cells, as dilution rate decreases and nitrogen limitation becomes more severe. The higher C:N<sub>B</sub> ratios at the lower dilution rates we observed is suggestive of such a switch in biosynthesis. But it is perplexing that we saw no corresponding reduction in GGE. To examine this question in more detail we pooled the composite data set from the two previous studies and the

Table 3. Determination of average GGE for different combinations of substrate sources from composite data from current and previous studies, as described in Fig. 10 legend.

Substrate source	<i>n</i>	GGE (%)*
All	72	49.8 ± 18.54
NH <sub>4</sub> <sup>+</sup> + Gluc	24	55.9 ± 15.39
All - (NH <sub>4</sub> <sup>+</sup> + Gluc)	48	45.1 ± 23.03

\* Average ± 2 SD.

current one, which includes 72 separate measurements of GGE from experiments encompassing a wide range of substrates and growth conditions. The composite GGE averaged 49.8 ± 18.54% (±2 SD) (Table 3), which is the value often cited as representative of bacterial growth (Fenchel and Blackburn 1979), but the 95% confidence interval in GGE was ±20%. We strongly suspect that this rather large deviation in GGE around 50%, which not coincidentally has been found in other experimental studies (Søndergaard and Theil-Neilson 1997) and in literature surveys (Cole and Pace 1994), does not have a physiological basis, but rather is due simply to experimental error. For example, in one case, after obtaining a relatively high GGE of 70% (involving NH<sub>4</sub><sup>+</sup> + glucose as substrates and C:N<sub>s</sub> = 30:1), we repeated the experiment and found GGE to be 50% (Table 1). However, without extensive replication, it is difficult to resolve this question satisfactorily. Similarly, in the continuous culture studies in which the most accurate measurements of GGE should have been possible, the 95% confidence interval for GGE was ±5–10% (Table 2) and the data varied irregularly with varying dilution rate. There is some indication that GGE values from the composite data were higher when NH<sub>4</sub><sup>+</sup> and glucose were the nitrogen and carbon substrates (GGE ≈ 56%) than when multiple carbon and nitrogen sources were used (GGE ≈ 45%), regardless of the C:N<sub>s</sub> ratio (Table 3); but once again, the differences are not statistically robust, and we suspect experimental error as the cause of the variability. Søndergaard and Theil-Neilson (1997) discussed in detail many of the problems in making accurate measurements of GGE and came to a similar conclusion that experimental error was often the cause of the wide variation in this parameter within and among experiments. Hence, perhaps the only meaningful interpretation of these results (ours included) is that when easily assimilated nitrogen and carbon sources are available for bacterial growth (whether singularly or in combination) the average GGE will be approximately 50%, irrespective of growth conditions (Table 3).

We conclude that a GGE value of 50% may be typical for situations where easily assimilated carbon and nitrogen substrates (e.g., glucose, amino acids, NH<sub>4</sub><sup>+</sup>) are utilized by bacteria. Then does this value represent a “maximum” GGE for natural bacterial populations and should lower GGE values be expected along a gradient of increased substrate complexity? In recent studies del Giorgio and colleagues (del Giorgio et al. 1997; del Giorgio and Cole 1998) dealt with this issue by comparing historical data on bacterial respiration and production rates from numerous marine and fresh-

water studies. From their analyses, they concluded that bacterial growth efficiencies do indeed increase along a gradient of bacterial productivity (and correspondingly in proportion to primary productivity) from values as low as a few percent in extremely unproductive open ocean waters up to about 50% in highly productive estuaries and coastal waters. They suggested that one possibility for the gradient in GGE is that cell maintenance requirements increase as substrate availability decreases. Under such conditions energy allocations would be increased for active nutrient transport and for extracellular enzyme production necessary for breaking down complex polymeric materials, compounds which are known to be major components of total dissolved organic carbon in oceanic waters (Benner et al. 1992). From their analyses they concluded that net heterotrophy exceeds net primary productivity in oligotrophic regions, in effect, resulting in an organic carbon deficit. Recent estimates of very low values of GGE (<10%) for oligotrophic marine waters (Kirchman et al. 1991; Hansell et al. 1995; Carlson and Ducklow 1996; Cherrier et al. 1996; Carlson et al. 1999) are consistent with this conclusion. They are in marked contrast to the more common GGE values in the range 20–80% (and typically averaging ~50%) that have been measured for a broad class of marine waters (Jahnke and Craven 1995). Such a conclusion, which has profound implications for the way that carbon is cycled in the ocean, has not gone unchallenged. Geider (1997), for example, has argued that no accounting was made in the del Giorgio study for the contribution of phototrophic picoplankton to total respiration, thereby leading to substantial overestimates of bacterial respiration rates, and concomitantly to low values of GGE. Additionally, Williams (1998) analyzed depth-integrated estimates of production and respiration from different open ocean locales and could find no evidence of respiration exceeding production in the top 100 m. A similar conclusion is readily apparent from the long-term time series analysis of net oxygen production in the water column at Sta. S off Bermuda. At this site the seasonal accumulation of oxygen in the euphotic zone begins in late winter and extends well into the summer months before being eroded through mixing and gas exchange in the fall-early winter periods (see Fig. 5 in Jenkins and Goldman 1985). Such a result could only have occurred if primary production exceeded respiration.

The controversy over the magnitude of respiration in the open ocean highlights the problem of making accurate measurements of GGE. In principle, direct measurements of bacterial POC production and organic carbon utilization should provide the most accurate measurements of GGE. But, as discussed previously, serious problems are common with this technique even under exacting laboratory conditions. The problem is exacerbated in field studies and often indirect and more inaccurate estimates of GGE based on surrogate methods for bacterial POC production and substrate utilization are made (Pomeroy et al. 1994; Søndergaard and Theil-Nielson 1997; del Giorgio and Cole 1998). Hence, there still is great uncertainty regarding the accuracy of field measurements of bacterial GGE, particularly in open ocean studies where small and not easily measurable changes in bacterial productivity and respiration are occurring. Additionally, as pointed out by Søndergaard and Theil-Nielson (1997) and

ourselves (Goldman and Dennett 1991), the magnitude of GGE and nutrient regenerative processes are greatly influenced by those data from the growth cycle which are included in the calculations. Inclusion of lag and stationary phase data generally leads to reductions in GGE values, and hence, should be avoided (Søndergaard and Theil-Nielson 1997). Thus, while it is intuitively appealing to suggest that bacteria have higher cell maintenance requirements and, concomitantly, lower GGE values in low nutrient waters, further confirmation of this concept is needed before it becomes more widely accepted.

*The C:N<sub>s</sub> ratio and multiple substrate sources*—Our current results confirm our previous conclusions (Goldman and Dennett 1991) and those of Tupas and Koike (1990; 1991) that cometabolism of amino acids and NH<sub>4</sub><sup>+</sup> is a common occurrence in marine bacteria and that there is absolutely no evidence that NH<sub>4</sub><sup>+</sup> uptake is inhibited in the presence of amino acids, as has been suggested previously (Kirchman et al. 1989; Kirchman 1994; Jørgensen et al. 1999). The key factor regulating the uptake of NH<sub>4</sub><sup>+</sup> is the availability of a carbon source to provide a stoichiometric balance between C and N uptake, as regulated by the C:N<sub>B</sub> ratio and GGE of the bacterial population. This balance between C and N is clearly seen in the results of the continuous culture studies. In this case under carbon limiting conditions all glutamate (Fig. 5C) or glucose (Fig. 6C) was utilized and excess or regenerated NH<sub>4</sub><sup>+</sup> remained at all dilution rates and under nitrogen limiting conditions all glutamate (Fig. 7C) or NH<sub>4</sub><sup>+</sup> (Fig. 8C) was utilized and excess glucose remained. The lack of preference between these different nitrogen sources has direct relevance to the recent suggestion that there is a gradient of decreasing bacterial dependence on amino acids from estuarine to oligotrophic waters and just the opposite for NH<sub>4</sub><sup>+</sup> utilization (Kirchman 1994). If true, bacterial production in marine waters would have to be dependent, not only on the relative availability of the two nitrogen sources, but on the availability of utilizable carbon sources to balance NH<sub>4</sub><sup>+</sup> uptake.

The great difficulty in determining the C:N<sub>s</sub> ratio of available substrates for bacterial growth in marine waters makes it virtually impossible to test the above hypothesis definitively. For example, hidden within the maze of a large pool of dissolved biopolymeric compounds such as acyl oligosaccharides (Aluwihare et al. 1997) and amide containing compounds (McCarthy et al. 1997), found throughout the world's oceans and believed to be of phytoplankton origin, are the much smaller quantities of likely carbon and nitrogen sources for bacterial growth such as simple sugars, free amino acids and inorganic N sources (NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>). Given that the bulk of the biopolymeric materials are believed to resist enzymatic degradation (McCarthy et al. 1997) and have a composite C:N<sub>s</sub> ratio of about 15:1 in surface waters (Benner et al. 1992; McCarthy et al. 1997) and also that the C:N<sub>B</sub> ratio of marine bacteria typically is ~4.5:1–7:1, it is clear that only some fraction of the total pools of dissolved organic carbon and nitrogen are being utilized by bacteria and that most likely carbon (Kirchman 1990; Kirchman and Wheeler 1998) or possibly even phosphorus (Cotner et al. 1997) is in the shortest supply. Determination of this pool

of carbon and nitrogen compounds that support bacterial production in the ocean together with better methods for determining both the C:N<sub>B</sub> ratio and GGE of bacteria in different environments are major challenges in contemporary marine microbial ecology. Moreover, they are vitally necessary before definitive answers will be found to current hypotheses regarding differences in important physiological characteristics of marine bacteria along gradients from productive inshore regions to nutrient deplete open ocean waters.

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