Growth of marine bacteria and ammonium regeneration from substrates in different C:N ratios

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Abstract
Natural assemblages of marine bacteria were chosen in a batch culture experiments. The impact of varying nitrogen substrate concentrations and the substrate C:N ratios (C:Ns) on the bacterial C:N ratio (C:Nb), the bacterial growth efficiency (BGE) and ammonium regeneration was mainly examined. The C:Ns ratios varied from 5:1 (carbon limitation) to 40:1 (nitrogen limitation) with varying combinations of glucose and NO3
. The C:Nb ratio had positive relationship with the C:Ns ratio (r=0.93, n=8), whose value was 3.77 when the C:Ns ratio was 5:1 but increased to 6.47 when the C:Ns ratio was 40:1. These results indicate that the C:Nb ratio is a potential diagnostic tool for determining the bacterial growth in natural waters controlled by either, carbon or nitrogen. BGE decreased with the declining nitrate concentration and negatively related to C:Ns (r=−0.51, n=8). The average value of BGE was 0.20. This value was a little lower than other reports, which could be induced by the nitrogen source used in our experiments. Finally, regeneration time of ammonium delayed with the increasing C:Ns ratio, which indicates that there were different metabolism mechanisms when bacterial growth was limited by carbon source and nitrogen source.

Key words: marine bacteria, C:N, bacteria growth efficiency, ammonium

1 Introduction
Heterotrophic bacteria decompose dissolved organic carbon (DOC) released by phytoplankton and serve as food for protozoa in the microbial loop. More than 50% of total net primary production was grazed by marine bacteria in the euphotic zone (Michael and Phillip., 1999; Del Giorgie and Cole., 1997; Jennifer et al., 1996; Fuhrman and Azam., 1989). Bacterial DOC uptake significantly influences energy flows and material flux of marine ecosystem, and global CO2 cycles.

Only marine bacteria are uniquely involved in both taking up and releasing inorganic nutrients in aquatic ecosystems (Wheeler and Kirchman., 1986; Laws et al., 1985). But this does not mean that they can do both at the same time. Three vital variables, which are the C:N ratio of the bacterial biomass(C:NB), the C:N ratio of the available substrate(C:Ns) and the bacterial growth efficiency (BGE), control when and where heterotrophic bacteria taking up or excreting dissolved inorganic nitrogen (DIN) (Goldman et al., 1987). Usually, C:NB ratios vary little, and the average value is a little lower than the Redfield C:N ratios. Since it is still unknown about the utilization of DOC fractions to bacteria, C:Ns always changes temporally and spatially. The value of BGE depends on the quality and availability of substrates, indicating the adaptation degree of bacteria to different substrates.

Bacteria normally prefer ammonium and amino acid more than nitrate, but Kirchman and Wheeler (1998) found that bacteria consumed relatively more nitrate than ammonium. Few reports were published about the bacterial uptake on nitrate in the oceans (Goldman and Dennett., 2000; Kirchman, and Wheeler, 1998; Kirchman, 1994; Kirchman et al., 1991; Jorgensen et al., 1993; Goldman and Dennett., 1991; Goldman et al., 1987; Wheeler and Kirchman., 1986). Heterotrophic bacteria could release ammonium. However, it could not oxidize ammonium to nitrate. So in this paper, nitrate is used as nitrogen source to explore the ammonium regeneration.

2 Material and method

Natural assemblages of bacteria were obtained by filtering seawater from the Jiaozhou Bay, Qingdao,
through a precombusted (450°C, 3 h) Whatman GF/F glass-fiber filter. The growth medium was artificial seawater (NaCl, 23.96 g/dm³; MgSO₄, 5.06 g/dm³; MgCl₂, 3.05 g/dm³; KCl, 0.60 g/dm³; CaCl₂, 0.30 g/dm³; NaHCO₃, 0.20 g/dm³) supplied with different combinations of carbon and nitrogen sources (Table 1), which C:N₅ ratios were changed from 5:1 to 30:1 (by atoms). Glucose was added to all experiments as carbon source at 200 μmol/dm³, when nitrate was added from 40 μmol/dm³ to 5 μmol/dm³ as nitrogen source. PO₄³⁻ was added to all cultures with different concentrations to ensure that carbon or nitrogen was limiting final yield. The culture was incubated in dark at 20°C for about one month. Duplicate samples for each treatment were incubated.

Water samples were collected when the incubation began and on the 1st, 2nd, 3rd, 5th, 9th, 14th, and 31th day after the experiment started. Glass syringes, after autoclaving, were used in the sampling process. To determine the total number of bacteria, 10 ml sample was fixed with formalin (final concentration of 5%) and stored at 4°C in dark. 25 ml sample was filtered through a 0.2 μm millipore polycarbonate filter. The filtrate was frozen for later determination of nutrient concentration. 10 ml unfiltered water sample was collected and frozen for determination of total organic carbon (TOC) and total nitrogen (TN). 10 ml sample was filtered through a Whatman 0.2 μm filter. The filtrate was frozen for the determination of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN).

To determine the total number of bacteria, sample was stained with DAPI (4′6-diamino-2-phenylindole; final concentration of 5 µg/cm³; sigma) and observed by epifluorescence UV microscope (Leica microscope) by using the method of Porter and Feig (1980).

Inorganic nutrient (NH₄⁺, NO₂⁻, NO₃⁻) was analyzed on a Technicon autoanalyzer. Nitrate was reduced to nitrite using Cd-Cu method with a standard deviation of 0.2 µg/dm³; diazotization method was used to analyze nitrite concentration with a standard deviation of 0.022 µmol/dm³; ammonium concentration was determined by the indophenol-blue method with a standard deviation of 0.05 µmol/dm³ (Grasshoff. et al, 1999). DIN was the sum of NH₄⁺, NO₂⁻, and NO₃⁻.

Table 1. Culture condition and parameters of bacteria growth

<table>
<thead>
<tr>
<th>Substrate characteristics</th>
<th>Concentration/µmol-dm⁻³</th>
<th>C:N₅ (by atoms)</th>
<th>C:N₁₀ (by atoms)</th>
<th>BGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>NO₂⁻</td>
<td>PO₄³⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>40</td>
<td>8</td>
<td>5</td>
<td>3.77</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>4</td>
<td>10</td>
<td>3.14</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td>2</td>
<td>20</td>
<td>5.01</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>1</td>
<td>40</td>
<td>6.47</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>4.60</td>
<td>0.20</td>
</tr>
</tbody>
</table>

TOC, DOC, TN and TDN were measured with a Shimadzu TOC-V analyzer (appending nitrogen analysis). Detection limit was 4 µg/dm³, and the coefficient of variation was less than 1.5%. To calculate particulate organic carbon (POC) concentration, DOC concentration was subtracted from the TOC concentration, and also TDN from TN for particulate nitrogen (PN). The concentration of DON was the concentration of TDN minus the DIN concentration.

Bacterial growth efficiency (BGE) was calculated from the ratio of the decline of dissolved organic carbon (DOC) in substrate and the increase of particulate organic carbon (POC) in bacterial biomass during the exponential growth stage. C:N₅ was calculated from the ratio of POC and PN when maximum biomass was attained. Ammonium regeneration rate was calculated from the increase rate of the ammonium concentration with time.

3 Results

3.1 Bacterial abundance

Bacterial abundance changed little (3×10^4 cells/dm³) on the first day. Exponential growth started from the second day (Fig.1). For the different C:N₅ ratios, the growth of bacteria behaved differently during the exponential growth and stationary phases. On the same glucose concentration, the bacterial abundance tended to increase with the nitrate concentration increased. A maximum of 1.19×10^7 cells/dm³ appeared on the 14th day when the C:N₅ ratio was 5:1.

3.2 Transformation of different carbon forms

Figure 2 shows the change of POC production and DOC consumption. POC increased quickly in the
Fig. 1. Time-course of bacterial cell growth.

Fig. 2. Time-course of POC production and DOC consumption. a. C: N=5:1, b. C:N=10:1, c. C:N=20:1 and d. C:N=40:1.

exponential growth stage but was followed by a slowly decrease. Coincident with the increase of POC, DOC sharply declined. The main part of DOC was glucose at the beginning of the experiment, the concentration was about 200 μmol/dm³, but it decreased to about 8 μmol/dm³ when the experiment was terminated in all the cultures. The remained DOC was inert organic compound that could not support bacteria growth. We can see that DOC has already been down to 8 μmol/dm³ at the end of exponential growth and has little change in the stationary phase in Figs. 2a and b, when it was slowly down to 8 μmol/dm³ in the stationary phase in Figs 2c and d.

3.3 Transformation of different nitrogen forms

The pattern of nitrogen distribution was a function of the C:N$_{S}$ ratio (Fig. 3). Uptake of NO$_3^-$, the main nitrogen source, occurred only in the exponential growth. By the end of the experiment, NO$_3^-$ still remained when the C:N$_{S}$ ratio was 5:1 (Fig. 3a) or 10:1 (Fig. 3b), but NO$_3^-$ was completely exhausted when the C:N$_{S}$ ratio was 20:1 (Fig. 3c) or 40:1 (Fig. 3d), which indicated that nitrogen was limiting growth. The NO$_3^-$ amount consumed during the culture was about 18 μmol/dm³ in

Fig. 3. Time-course of different forms of nitrogen. a.C: N=5:1, b.C: N=10:1, c.C: N=20:1, and d.C: N=40:1.
Figs. 3a and b. Similar to POC, PN was also increased quickly in the exponential growth stage and followed by a slowly decrease. When the C:N ratio was 40:1, about 91% of nitrogen in the medium was in the form of PN, the highest value was 98% in the third day; when the C:N ratio was 5:1, the average value was only 26% (Table 2). DON had no big change during all cultures. The low concentration of $\text{NH}_4^+$ initially contained in the substrate was completely exhausted at the second day but began to excrete concomitant with the losses of PN. The excretion started from the third day in Figs. 3a and b, but the excretion appeared later in Figs. 3c and d.

3.4 C:N$_B$ and BGE

C:N$_B$ ratios ranged from 3.77–6.47, average value was 4.60, presenting a positive relationship with C:N$_S$ ($r=0.93$, $n=8$) (Table 1). The estimated BGE varied between 0.18 and 0.21, negatively related to C:N$_S$ ($r=-0.51$, $n=8$) (Table 1).

4 Discussion

4.1 C:N$_B$ and C:N$_S$

C:N$_B$ ratios varied little in natural marine ecology. Because of their relative high nucleic acid and protein content, bacteria normally attain low C:N ratio (5:1), lower than the Redfield C:N ratio. The average C:N$_B$ ratio was calculated to 4.6, which agreed with some of other researches (Goldman and Dennett., 2000; Tezuka, 1990).

The variation of C:N$_S$ was small. As only minor fraction of the total DOM can be used by bacteria (Münster and Chro’st., 1990), the bacterial secondary production is related more to the composition and availability of DOM than to the total amount of DOM. The composition of DOM is always changing, so we could not simply use the DOC:DOM to be equal to C:N$_S$. In our experiments, the C:N$_B$ was less than 4 when the C:N$_S$ ratio was no more than 10:1 (carbon-limited), while the C:N$_B$ was as high as 6.47 when the C:N$_S$ ratio was 40:1 (nitrogen-limited). A positive correlation between C:N$_S$ and C:N$_B$ was attained ($r=0.93$, $n=8$). It has been found that the C:N$_B$ ratio of mixed bacterial populations, grown in nitrogen-limited batch and continuous cultures, can be 13:1 to 17:1 (Tezuka, 1990). It can be seen that the C:N$_B$ ratio is a potential diagnostic tool for determining which one, carbon or nitrogen, control the bacterial growth in natural waters. Many evidences show that organic carbon limitation is common in many types of marine waters (Kirchman et al., 1990; Nagata, 1986). However, that the bacteria cannot be separated from other detritus effectively limits its use.

<table>
<thead>
<tr>
<th>C:N$_S$ (by atoms)</th>
<th>PN/TN$^{11}$ (%)</th>
<th>POC/TOC$^{11}$ (%)</th>
<th>$\text{NH}_4^+$ regeneration rate/μmol·dm$^{-3}$·d$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>26</td>
<td>76</td>
<td>0.23</td>
</tr>
<tr>
<td>10</td>
<td>74</td>
<td>79</td>
<td>0.16</td>
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<td>20</td>
<td>82</td>
<td>66</td>
<td>0.12</td>
</tr>
<tr>
<td>40</td>
<td>91</td>
<td>44</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Notes: 1) Average value of the latter exponential growth and the stationary phases.

4.2 BGE

BGE is a fundamental attribute of microbial metabolism (Del Giorgio et al., 2000). It largely determines the ecological and biogeochemical roles of bacteria in microbial food webs and in aquatic systems (Del Giorgio et al., 1997; Sherr and Sherr., 1996), and is significantly important for global carbon cycles and nutrient regeneration. Bacterial growth efficiencies were found to range from less than 0.10 to greater than 0.60 in the report of Cole et al. (1989). But Goldman and Dennett. (2000) found that all their BGE data fell within the board range of 0.30–0.70. The BGE for all marine data averaged at 0.20 (mean 0.23±0.11), calculated by Del Giorgio and Cole. (2000). The BGE data in our experiments are shown in Table 1, and a little lower than the data of Goldman and Dennett. (2000) calculated. This could be induced by the different nitrogen sources: ammonium and amino acid were used in their culture.

BGE is determined by the concentrations and relative availability of nutrient and organic carbon in the culture environment, and low BGE generally reflect increased maintenance energetic costs, by which cell must face in extremely dilute aquatic systems (Del Giorgio and Cole, 2000). Some published works show that BGE depended on the category of nitrogen source (Jorgensen et al., 1999; Kroer et al., 1993; Goldman...
and Dennett 1991; Benner et al., 1988). When nitrate was used as substrate, bacteria need the help of nitrate and nitrite reductase to take up nitrate. The use of nitrate requires five NADHs whereas one to ammonium (Vallino et al., 1996). So in our culture, most of the DOC assimilated by bacteria was used for energy production, while little was used for increasing cell material.

Some reports support that the BGE should be negatively related to the C:N_S when nitrogen was limited (Goldman et al., 1987; Billen, 1984), but some believed the relationship between the BGE and the C:N_S was weakened (Goldman and Dennett, 2000). In our experiments, BGE declined with the decrease of the nitrate concentration (Table 1), but it had a negative relationship with C:N_S (r = -0.51, n = 8). It shows, as well as the DOM, the growth and secondary production of bacteria in aquatic systems may be determined by the species of nitrogen (Xiao and Wang, 2000; Ning, 1997; Jost, 1992). Linley and Newell (1984) believed that bacteria need consume more carbon to get enough nitrogen when the C:N_S ratio was relatively high.

4.3 Ammonium regeneration

Excess nitrate remained in Figs. 3a and b, which implies that nitrogen was surplus and carbon was limited. The release of ammonium started in the latter exponential growth when carbon was exhausted. In Figs 3c and d, nitrate was quickly used up during the exponential growth, but the concentration of DOC was relatively high (glucose remained) at the same time, which means that nitrogen was the limiting factor when the C:N_S ratio was kept at not being less than 20. But the ammonium regeneration (Figs. 3c and d) did not start until glucose was slowly exhausted during the stationary phase in Figs 2c and d. Some reports found that the uptakes of ammonium and amino acids (either singularly or mixed) continued until glucose was used up, net regeneration of ammonium could start only after glucose was completely utilized, that may be the result of two separate metabolic processes (uptake of amino acids and uptake of ammonium and glucose) working simultaneously (Goldman and Dennett, 1991; Goldman et al., 1987; Martinussen and Thingstand, 1987).

A clear distinction must be made between the biochemical processes and those regulating regenerations. Biochemical processes controlled the regeneration of nutrient during the exponential growth phase of bacteria. But endogenous functions dominated bacterial metabolism or cell death occurring in regulating regeneration during the stationary phase (Goldman and Dennett., 1991; Goldman et al., 1987). During the stationary phase, when carbon source was exhausted, bacteria had to consume organic carbon surpluses in cell to maintain the requirements (Figs. 2a and b), which led to the decline of POC, and ammonium was released to keep the C:N_B ratio invariant. When the carbon remained, bacteria could continue to use carbon to maintain cell viability, and this will not change the C:N_B ratio (Figs. 2c and d). So no ammonium was released until glucose was exhausted. Goldman and Dennett. (1991) found when amino acids were used as carbon source and remained during the stationary phase, bacteria only assimilated their organic carbon, and ammonium of the amino acids was released.

The release rate of ammonium also changed (Table 2), which declined with the rise of the C:N_S ratio.

5 Conclusions

Our research on the growth of bacteria on different nitrate concentrations shows that the C:N_B, BGE and ammonium regeneration starting time vary with the C:N_S. It suggests that the nitrogen, as well as the DOM, also influenced the marine bacterial secondary production, which offered us a significantly implication for the research on the energy flows and the material flux in the microbial loop. Now, more investigations are being done in the purpose to explore a broad range of conditions that bacteria might experience in marine environment.

References