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Bacterial Regeneration of Ammonium and Phosphate as Affected by the Carbon : Nitrogen : Phosphorus Ratio of Organic Substrates

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Abstract. The effect of carbon : nitrogen : phosphorus (C:N:P) ratio of organic substrates on the regeneration of ammonium and phosphate was investigated by growing natural assemblages of freshwater bacteria in mineral media supplemented with the simple organic C, N, and P sources (glucose, asparagine, and sodium glycerophosphate, respectively) to give 25 different substrate C:N:P ratios. Both ammonium and phosphate were regenerated when C:N and N:P atomic ratios of organic substrates were $\leq$10:1 and $\leq$16:1, respectively. Only ammonium was regenerated when C:N and N:P ratios were $\leq$10:1 and $\geq$10–20:1, respectively. On the other hand, neither ammonium nor phosphate was regenerated when C:N and N:P ratios were $\geq$15:1 and $\geq$5:1, respectively. In no case was phosphate alone regenerated. As bacteria were able to alter widely the C:N:P ratio of their biomass, the growth yield of bacteria appeared primarily dependent on the substrate carbon concentration, irrespective of a wide variation in the substrate C:N:P ratio.

Introduction

Regeneration of inorganic nitrogen and phosphorus by microbial decomposition of organic matter is an important aspect of nutrient cycling in both terrestrial and aquatic ecosystems. Numerous investigations have considered how inorganic nitrogen and/or phosphorus are regenerated by microbial decomposition of naturally occurring organic matter. When barley straw with a high C:N ratio (30:1, by atoms) is decomposed in the soil, there is no net nitrogen regeneration, at least in the early stage of decomposition [1]. Upon decaying, proteinous material such as fish meat releases abundant ammonium. Thus, the C:N ratio of organic substrates is an important parameter determining the degree of nitrogen regeneration. Recently, Goldman et al. [12] studied the relationship between substrate C:N ratio and nitrogen regeneration by marine bacteria and found that the higher the C:N ratio of organic substrates, the smaller the proportion of nitrogen regenerated, the remaining nitrogen being incorporated into bacterial biomass. They also found no net release of inorganic nitrogen at a substrate C:N ratio higher than 10:1.

However, there has been almost no systematic study on the way in which

C:N:P ratio of organic matter affects inorganic nitrogen and phosphorus regeneration by microbial decomposition. In Lake Biwa, which is warm monomictic, phosphorus-limited, mesotrophic and the largest (surface area, 674 km^2; maximum depth, 104 m) lake in Japan, an abundant accumulation of nitrate in the hypolimnion can be found during the summer stagnation period, in contrast with almost no such accumulation of dissolved inorganic phosphorus [18, 19]. This suggests that dead plankton sinking through the hypolimnion and undergoing microbial decomposition release inorganic nitrogen but not phosphorus. To test this possibility, I collected seston (composed mainly of phytoplankton) from the euphotic zone of Lake Biwa in summer, and the seston was then decomposed under aerobic conditions in the laboratory for three months. As expected, there was an abundant release of inorganic nitrogen, in contrast with almost no release of inorganic phosphorus [20]. This lack of phosphorus release was probably due to the high N:P or C:P ratio of the seston in the euphotic zone of Lake Biwa during the summer stagnation period (the mean C:N and N:P atomic ratios of the seston in the euphotic zone from May to October were 10:1 and 36:1, respectively [19]). To test this possibility, I cultivated the three algal assemblages with different C:N:P ratios, which were then decomposed under aerobic conditions in the dark. As expected, the two algal assemblages with the C:N:P ratios of 817:122:1 and 270:38:1 released no inorganic phosphorus during a three months incubation period, whereas the algal assemblage with a C:N:P ratio of 144:12:1 released an abundant amount of inorganic phosphorus [21]. In this study, however, I could not clarify whether the C:P or N:P ratio of phytoplankton was more important in determining the degree of phosphorus regeneration, since the algal assemblage with a high N:P ratio also had a high C:P ratio.

In contrast to C:P or N:P ratios, it is not as easy to prepare artificially algal assemblages with different C:N ratios, since most algae utilize CO_2 or bicarbonate as their carbon source. Moreover, regeneration of nitrogen and phosphorus by microbial decomposition of dead plankton in natural environments is affected not only by the chemical composition of plankton themselves but also by the food web, including bacteria, protozoa, zooplankton, etc. Hence, I tested the effect of substrate C:N:P ratio on the regeneration of inorganic nitrogen and phosphorus by combining the simple model substrates (glucose, asparagine, and glycerophosphate, respectively) as organic C, N, and P sources and by using freshwater bacteria as decomposers. These organic substrates were selected for their susceptibility to bacterial utilization and for the low C:N ratio (2:1) for asparagine and low C:P ratio (3:1) for glycerophosphate.

Materials and Methods

Composition of Culture Media and Growth Conditions

The basal mineral medium used for the cultivation of natural freshwater bacteria contained the following ingredients in mg per liter of distilled water: KCl, 37; $MgSO_4 \cdot 7H_2O$, 130; $CaCl_2 \cdot 2H_2O$, 14; Na·Fe-EDTA, 7 (pH, 7.3). To this basal medium, glucose, asparagine, and sodium glycerophosphate were added at concentrations to give 25 different substrate C:N:P ratios as shown in

Table 1. C:N:P and C:N ratios of organic substrates

Culture no.	C:N:P (atomic ratio)	C:N	Culture no.	C:N:P	C:N	Culture no.	C:N:P	C:N
1	7:2:1	3.5	10	112:16:1	7	19	100:5:1	20
2	16:4:1	4	11	120:40:1	3	20	150:5:1	30
3	28:4:1	7	12	180:60:1	3	21	240:16:1	15
4	35:16:1	2.2	13	300:30:1	10	22	480:16:1	30
5	40:8:1	5	14[b]	400:40:1	10	23	450:30:1	15
6	50:5:1	10	15	448:64:1	7	24	800:40:1	20
7[a]	50:7:1	7	16	500:50:1	10	25	1,200:40:1	30
8	60:20:1	3	17	900:60:1	15			
9	100:10:1	10	18	75:5:1	15			

[a] Phosphorus was added at a concentration of 20 μM. Other cultures were all added at a concentration of 2 μM

[b] Protozoa contaminated

Table 1. In all cultures but one (no. 7), phosphorus concentrations of media were kept constant at 2 μM. Since for cultures with low C:N:P ratios bacterial growth yields were too low to determine the bacterial carbon and nitrogen, culture no. 7 (substrate C:N:P = 50:7:1) contained phosphorus at a concentration of 20 μM. Two hundred ml of each medium was poured into an L-shaped glass tube (500 ml capacity). Bacterial assemblages used as inocula were prepared as follows: Inshore water of Lake Biwa was filtered through a sterilized glass-fiber filter (Whatman, GF/C), and 2 ml of the filtrate was inoculated into each tube. The tubes were incubated under Monod-type shaking at 25°C in the dark for 3 to 4 days, during which most of the cultures reached stationary phase.

After cultivation, absorbances (410 nm, 5 cm cell) of all cultures were measured to compare relative bacterial growth yields. At the same time, all cultures were examined with a microscope to check for protozoan contamination. Only one culture (no. 14) was heavily contaminated with ciliates. In almost all cultures, predominant bacteria were motile rods.

Chemical Analyses

A discrete volume of each culture was filtered through an ignited glass-fiber filter (Whatman, GF/F). The filtrate was used for determinations of ammonium [6], dissolved inorganic phosphorus (DIP) [16], and total dissolved phosphorus (TDP) [15]. Total phosphorus (TP) of each culture was determined by the same method as that of TDP but using nonfiltered culture. Particulate phosphorus (PP) and dissolved organic phosphorus (DOP) were calculated as the differences between TP and TDP and between TDP and DIP, respectively. For eight cultures, which gave high bacterial growth yields, particulate carbon and nitrogen were determined with a CHN recorder (Yanaco, MT-3), using the filters retaining bacteria. Detection limits of the methods adopted for this study were about 0.5 μM for ammonium and 0.1 μM for phosphorus. For several cultures which released ammonium, the formation of nitrate or nitrite due to nitrification was tested, but such forms of nitrogen were not detected in all cases.

Results

Regeneration of Ammonium and Phosphate

Regeneration of ammonium and phosphate in relation to C:N and N:P ratios of organic substrates is shown in Fig. 1. It is evident that the regeneration

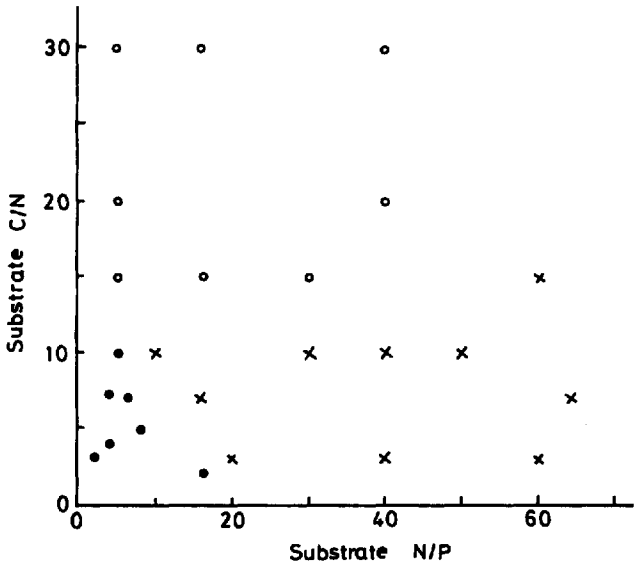

Fig. 1. Relationship between bacterial regeneration of ammonium (N) and phosphate (P) and the C:N and N:P atomic ratios of organic substrates. ●: N and P were regenerated; ×: only N was regenerated; O: neither N nor P was regenerated.

pattern of ammonium and phosphate can be classified into three groups according to the C:N:P ratio of organic substrates. In the first group (culture no. 1 to 7 in Table 1), both ammonium and phosphate were regenerated, although percentage recovery of ammonium and phosphate from organic substrates was different with different substrate C:N:P ratios (see below). This pattern was observed when C:N and N:P ratios of substrates were $\leq$10:1 and $\leq$16:1, respectively. In other words, this pattern was observed for the organic substrates whose nitrogen and phosphorus contents were high. In the second group (culture no. 8 to 17 in Table 1) only ammonium was regenerated. This pattern was observed when C:N and N:P ratios of substrates were $\leq$10:1 and $\geq$10–20:1, respectively. In this group, however, the minimum N:P ratio bringing about ammonium regeneration shifted from 10:1 toward 20:1 with decrease of C:N ratio. In this group, except for cultures of no. 8, 11, and 12, phosphorus added as organic phosphorus was fully incorporated into bacterial biomass. For cultures of no. 8, 11, and 12 whose substrate C:N ratios were as low as 3, only a small amount of ammonium (equivalent to 4 to 5% of substrate nitrogen) was regenerated, and most (>95%) of total phosphorus was detected in the DOP fraction after cultivation. It is not known whether this DOP fraction was composed of the substrate glycerophosphate or other metabolic products. However, it seems likely that bacteria utilized only a small amount of organic substrates, since growth yields of these three cultures were very low. In the third group (culture no. 18 to 25 in Table 1), neither ammonium nor phosphate was regenerated. This pattern was observed when C:N ratio of substrates was $\geq$15:1, irrespective of a wide variation of the N:P ratios (5:1 to 40:1). One exception was culture no. 17, whose substrate C:N and N:P ratios were 15:1 and 60:1, respectively. In this case, a small amount of ammonium was regenerated. Except for culture no. 18, all phosphorus added as organic phosphorus was incorporated into bacterial biomass. For culture no. 18, about 30% of phosphorus

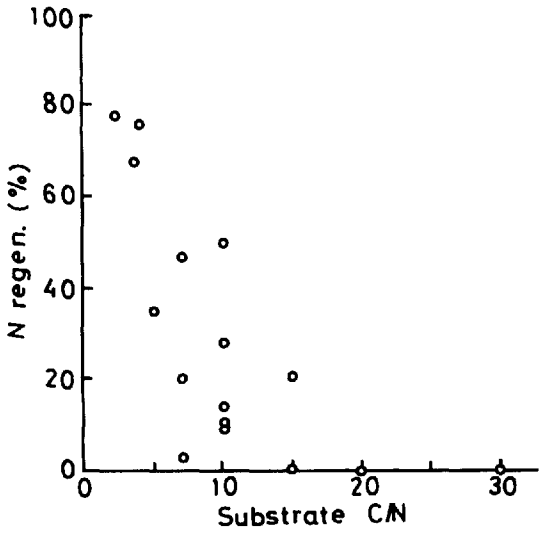

Fig. 2. Relationship between percentage of ammonium regeneration and the C:N atomic ratio of organic substrates.

remained as DOP after cultivation. Within the range of substrate C:N:P ratios tested, there was no case in which phosphate alone was regenerated.

Percentage Recovery of Ammonium and Phosphate

The percentage of organic nitrogen recovered as ammonium in relation to substrate C:N ratio is shown in Fig. 2, where the data on three cultures (no. 8, 11, and 12) are omitted for the reason already mentioned above. The relative amount of nitrogen regenerated increased with decrease in substrate C:N ratio. The degree of ammonium regeneration was also related to the N:P ratio of substrates. For example, for cultures to which the substrates were added at a C:N ratio of 10:1, the highest recovery (50%) of ammonium was observed for the substrate N:P ratio of 50:1, while the lowest one (9%) was observed for the substrate N:P ratio of 5:1. Of all cultures, the highest recovery (78%) of ammonium was observed for the lowest substrate C:N ratio of 2.2:1 (culture no. 4).

The percentage recovery of phosphate in relation to the substrate C:P ratio is shown in Fig. 3. In contrast to ammonium regeneration, the degree of phosphate regeneration was highly dependent on only the substrate C:P ratio. Below the C:P ratio of 40:1, percentage recovery of phosphate was high (about 60%) and rather constant, but it decreased abruptly with increase in C:P ratio and was zero above a C:P ratio of 60:1.

Growth Efficiency of Bacteria in Terms of Carbon, Nitrogen, and Phosphorus

As already stated, for many cultures with low substrate C:N:P ratios, bacterial carbon and nitrogen could not be determined because of the low bacterial

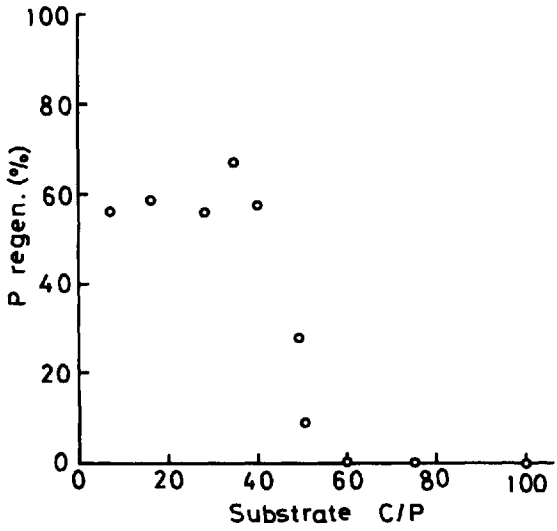

Fig. 3. Relationship between percentage of phosphate regeneration and the C:P atomic ratio of organic substrates. The values at the substrate C:P ratios higher than 100:1 are not plotted, since no phosphate was regenerated for these ratios.

Table 2. Percentage of substrate carbon, nitrogen, and phosphorus converted to bacterial biomass

Culture no.	C:N:P	Carbon (%)	Nitrogen (%)	Phosphorus (%)
7	50:7:1	46	73	91
14	400:40:1	30	40	100
16	500:50:1	26	32	100
17	900:60:1	35	44	100
22	480:16:1	32	78	100
23	450:30:1	42	74	100
24	800:40:1	50	69	100
25	1,200:40:1	41	72	100

growth yields. For eight cultures which gave high growth yields, bacterial carbon and nitrogen were determined, and growth efficiencies were calculated (Table 2). The values of growth efficiency in terms of carbon and nitrogen may not be highly accurate, because actual concentrations of organic carbon and nitrogen in the media just before incubation were not determined in this study. Growth efficiency in terms of carbon ranged between 26 and 50%, but no clear relationship was found between growth efficiency and substrate C:N:P ratio. This was also true for growth efficiency in terms of nitrogen, with relatively higher efficiency (up to 78%) than carbon. Particulate (bacterial) phosphorus was determined for all cultures, and the percentage of phosphorus incorporated into the bacterial biomass as a function of substrate C:P ratio is shown in Fig. 4, where the data for the three cultures (no. 8, 11, and 12) with exceptionally low phosphorus recovery (see above) are omitted. It is clear that phosphorus uptake into biomass increases with increase of the substrate C:P ratio and attains 100% at C:P ratio of about 100:1. As already stated, the lower the substrate C:P ratio, the higher the percentage of phosphate released.

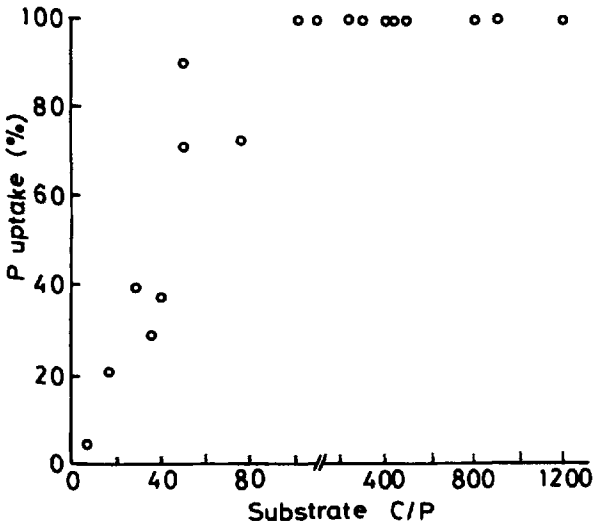

Fig. 4. Relationship between percentage of substrate phosphorus incorporated into bacterial biomass and the C:P atomic ratio of organic substrates.

Table 3. Relationship between substrate C:N:P ratio and the C:N:P ratio of bacterial biomass

Culture no.	Substrate C:N:P	Bacteria C:N:P
7	50:7:1	31:7:1
14	400:40:1	176:24:1
16	500:50:1	165:20:1
17	900:60:1	515:42:1
22	480:16:1	178:15:1
23	450:30:1	230:27:1
24	800:40:1	434:30:1
25	1,200:40:1	464:27:1

C:N:P Ratio of Bacterial Biomass

The C:N:P ratio of bacterial biomass was determined for eight cultures, which gave high growth yields (Table 3). It is evident that the C:N:P ratio of bacterial biomass changes widely depending on the substrate C:N:P ratio. The lowest bacterial C:N:P ratio (31:7:1) was observed for the substrate C:N:P ratio of 50:7:1, while the highest one (515:42:1) was observed for that of 900:60:1. The C:N and C:P ratios of bacterial biomass as functions of the substrate C:N and C:P ratios are shown in Figs. 5 and 6, respectively. The lowest and highest bacterial C:N ratios were 4.5:1 and 17.2:1, respectively, and bacterial C:N ratio increased with increase of substrate C:N ratio, showing a tendency to saturate at a substrate C:N ratio >30:1. It is unknown whether bacterial C:N ratio can decrease below 4.5:1, since the lowest substrate C:N ratio tested was 7:1. Similarly, bacterial C:P ratio increased with increase of substrate C:P ratio. The lowest and highest bacterial C:P ratios observed were 31:1 and 515:1, respec-

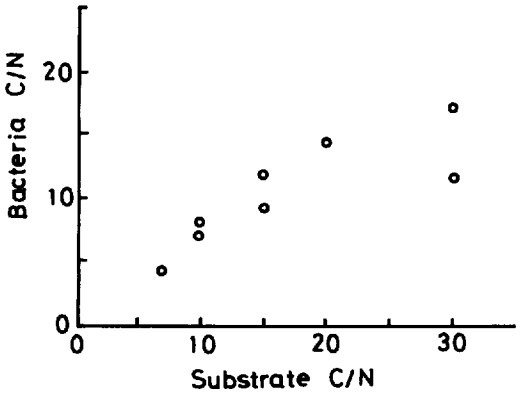

Fig. 5. Relationship between the C:N atomic ratio of bacterial biomass and that of organic substrates.

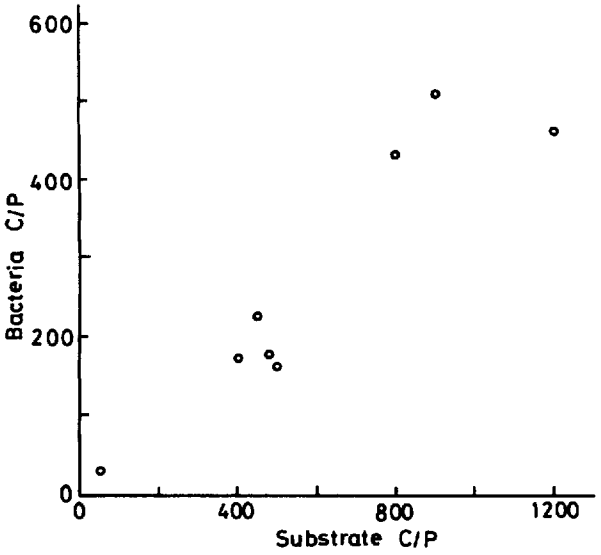

Fig. 6. Relationship between the C:P atomic ratio of bacterial biomass and that of organic substrates.

tively. Thus, the range of variation in bacterial C:P ratio was much wider than that of bacterial C:N ratio. As in the case of C:N ratio, however, it is unknown whether bacterial C:P ratio can increase or decrease outside the range of the bacterial C:P ratios observed.

Bacterial Growth Yield in Relation to Substrate C:N:P Ratio

Figure 7 shows relative growth yields of bacteria measured as absorbances of cultures and plotted against substrate carbon concentration. It can be seen that the growth yield of bacteria is fairly dependent on the concentration of substrate carbon, irrespective of a wide variation of substrate C:N:P ratio. This is easily understood from the fact that bacteria have the capability to alter widely the C:N:P ratio of their biomass, depending on the substrate C:N:P ratio.

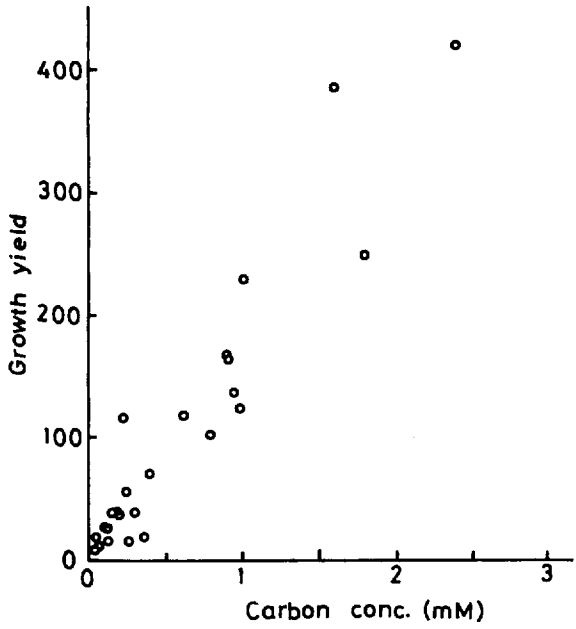

Fig. 7. Relationship between relative bacterial growth yields (absorbance at 410 nm × 1,000) and substrate carbon concentration.

Discussion

The results of this study have demonstrated clearly that the C:N:P ratio of organic substrates is an important parameter determining the degree of ammonium and phosphate regeneration by bacterial decomposition. They shed light on the reasons why the seston in the euphotic zone of Lake Biwa during the summer stagnation period does not release inorganic phosphorus during aerobic decomposition, while there is an abundant release of inorganic nitrogen [20]. This provides an explanation for why inorganic nitrogen (nitrate) but not phosphorus accumulates abundantly in the hypolimnion [18, 19]. The fact that phosphorus regeneration from decomposing algae depends on the C:P ratio of the algae has also been reported recently by Uehlinger [23].

The percentage of substrate nitrogen released as ammonium was primarily determined by the C:N ratio of organic substrates, but it was also affected by the N:P ratio of organic substrates (Fig. 2). When the C:N ratio of organic substrates was higher than 15:1, there was no release of ammonium, irrespective of a wide variation of the substrate N:P ratio (5:1 to 40:1). Recently, Goldman et al. [12] studied the effect of substrate C:N ratio on the ammonium regeneration by marine bacteria using several amino acids and glucose. They found that the percentage regeneration of ammonium increased approximately linearly with decrease of substrate C:N ratio. In their experiment, however, substrate N:P ratio was kept constant at 10:1. Hence, their conclusion must be modified, when considering the effect of substrate N:P ratio.

In contrast with ammonium regeneration, phosphate regeneration seems to be determined mainly by C:P ratio alone (Fig. 3). There was no phosphate regeneration at low substrate C:P ratios, such as 60:1. This is probably due to

the capability of bacteria to store excess phosphorus within the cells. Moreover, according to the results of the present study, organic substrates with the so-called Redfield ratio (C:N:P = 106:16:1), which has often been considered as the representative C:N:P ratio of well-nourished phytoplankton, may not release phosphate during aerobic decomposition. In this study, however, a simple system composed of only bacteria and simple organic substrates was used, and all chemical components were analyzed in the early stage of stationary phase. Even if bacteria at this phase do not release ammonium or phosphate, they may release these nutrients during much longer times of incubation, i.e., at death phase. In this respect, the results of the present study must be evaluated with some caution.

Our knowledge on the C:N:P ratio of bacterial biomass is quite fragmentary. There has been almost no systematic study on how bacterial C:N:P ratio changes with changing environmental conditions, especially nutritional conditions. It is generally agreed that bacterial C:N:P ratio must be lower than that of larger organisms. For example, the values of 52:9:1 [11] and 130:9:1 [3] were reported. Using a pure culture of *Pseudomonas putida* and mixed bacterial populations, Bratbak [7] found that the C:N:P ratio of bacterial biomass changed between 8:2:1 and 500:90:1, depending on the substrate C:N:P ratio. My data support this finding. On the other hand, Goldman et al. [12] studied the effect of substrate C:N:P ratio on the elemental composition of marine bacteria and concluded that the C:N:P ratio of bacteria was rather constant (45:9:1) within a range of substrate C:N ratios from 1.5 to 10:1. As already mentioned above however, substrate N:P ratio was kept constant at 10:1 in their experiment. Therefore, such a narrow range of the C:P ratio (15:1 to 100:1) tested by them might bring about a constant C:N:P ratio of bacterial biomass.

When we consider nutrient regeneration in natural environments, the results of this study must be evaluated with caution for the following reasons: Naturally occurring organic matter is usually a complicated mixture of low and high molecular weight substances, and if organic matter contains a high percentage of inert or poorly decomposable carbon compounds like lignin, such organic matter may behave like a substrate with low C:N:P ratio, even if the total C:N:P ratio of the organic matter is high. In a previous study [22], for example, I collected the two cyanobacteria *Microcystis* and *Anabaena* from their natural eutrophic environments and found the C:N:P ratio of 191:29:1 for the former and 150:18:1 for the latter. According to the results of the present study, it would be predicted that the two cyanobacteria do not release phosphate during aerobic decomposition because their C:P ratios were high. Contrary to this expectation, they released large amounts of both ammonium and phosphate. This fact suggests that the results of this study cannot be applied directly to the natural environment.

Second, nutrient regeneration in nature is not accomplished by bacteria alone. In natural environments where bacteria are produced, food chains beginning with bacteria would be more or less functioning. Even if bacteria do not regenerate ammonium and/or phosphate, primary or secondary consumers of bacteria may regenerate these nutrients. In particular, the importance of bacterivorous protozoa in nutrient regeneration has recently attracted the attention of many investigators [2, 4, 5, 8–11, 13, 14, 17]. In the present study, culture

no. 14 (substrate C:N:P = 400:40:1) was heavily contaminated with ciliates. Nevertheless, the pattern of nutrient regeneration was similar to other protozoa-free cultures with similar substrate C:N:P ratios. This suggests that the regeneration of ammonium and phosphate due to protozoan feeding on bacteria may also be dependent on the C:N:P ratio of the prey. This subject, however, awaits further systematic study.

In spite of the above situation, the results of the present study will give a basic knowledge of nutrient regeneration occurring in nature. The C:N:P ratio of organisms must be different in different taxonomic groups and under different nutritional conditions. For example, the two macrophytes, *Elodea nuttallii* and *Phragmites communis*, have C:N:P ratios of 132:13:1 and 459:30:1, respectively (Y. Sakurai and Y. Watanabe, pers. comm.). From these values, it is expected that upon decaying the former will release larger amounts of inorganic nitrogen and/or phosphorus than the latter. The knowledge obtained by this study will be also useful for the research fields of applied nature, e.g., how to remove nitrogen and phosphorus efficiently in microbial sewage treatment.

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