Microflora and Mineralization Activity in the Bottom Sediment of Lake Biwa

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Microflora and mineralization activity in the bottom sediment taken from two distinct sites in Lake Biwa were investigated. From the colony formation process, the following three common features for soil bacteria were also confirmed in the sediment bacteria: 1) the number of colonies increased not continuously but intermittently with incubation time; 2) the number of colonies formed on diluted nutrient agar medium was considerably higher than that on nutrient agar; 3) the total number of bacteria counted by epifluorescence microscopy was 100 to 10000 times higher than the number of colonies formed on both media. The following features were also found for sediment bacteria: 1) the deeper the layer of the sediment, the smaller the number of colonies; 2) the southern basin sediments yielded more colonies than northern basin sediments; 3) the number of bacteria that can form colonies increased from spring to summer in the sediment sample contrary to the smaller count of total bacteria in the summer sample. From the bacterial activity of glucose mineralization the following points were revealed: 1) the upper layer of the sediments are richer in aerobes than facultative anaerobes; and 2) under oxygen-deficient conditions even the upper layer of the sediments becomes rich in facultative anaerobes.

As revealed in this study, the combination of observing the colony-forming process on agar medium and the measuring the glucose-mineralizing activity of the bacteria in the sediments seems to be useful for understanding the complex microflora in the sediments and changes relating to various factors, such as, nutrient and oxygen concentration, and temperature.

Key words: sediment, microflora, colony formation curve, mineralization activity, Lake Biwa

It is generally accepted that bacteria are the primary decomposers and mineralizers of organic matter in aquatic ecosystems. It has also been revealed that usually there are more bacteria in lake sediment than in lake water⁵). Thus, the bacteria in lake sediment seem to play an important role on the flow and conversion of organic matter. However, little is known about bacterial flora and their activity in sediment with

only a few exceptions¹³), due to the lack of a systematic method of investigating them.

The time necessary for bacterial cells to form visible colonies on a nutrient agar plate can be a criterion for classifying isolates based on the growth rate of the bacteria⁹): A longer time is necessary for a slow-growing cell to develop into a colony of visible size, while a shorter time is expected for a fast-growing cell to do so.

This study characterized the bacterial flora in the sediment of Lake Biwa (the largest lake in Japan and

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Fig. 1. Location of the sampling stations in Lake Biwa.

an important water reservoir for the 14 million people living in the surrounding area) according to the growth rate using the method described above. Furthermore, the mineralization activity of the bacteria in the sediment was also investigated using a convenient method, the double vial method.

We found that the bacterial flora and mineralization activity varied depending on the site and the depth of the lake sediment, i.e., the distance from the boundary between the lake water and sediment. A seasonal change in these characteristics is also discussed.

Materials and Methods

Sampling stations

Sediment samples were taken with a K-K core sampler using a transparent acrylic tube measuring 5 cm in

internal diameter from the following two characteristic sites (Fig. 1), i.e., station 1 (St. 1) in the northern basin and station 2 (St. 2) in the southern basin. Station 1 (depth ca. 87 m) was located near the deepest point of Lake Biwa and St. 2 (depth 3 m) was near a small artificial island in the southern basin. Samples were taken on June 11 and September 10 in 1996 from St. 1 and on June 24 and Sept. 5 in 1996 from St. 2. Each sediment sample was divided into four layers with a sterilized spatula, i.e., 0–1 cm, 2–3 cm, 5–6 cm and 9–10 cm layers from the boundary surface where the sediment contact the bottom lake water. Each of the divided samples was placed into a sterilized plastic bottle and brought back to our laboratory within several hours and kept in a refrigerator (4°C). The experiments described below were carried out within two days to minimize possible change in the microflora.

Culture media and colony count

The sediment samples were serially diluted in sterilized water to 10^4 – 10^5 folds. The samples were cultured on a nutrient broth (NB) agar plate medium (containing 10 g of polypepton (Japan Pharmaceuticals), 10 g of nutrient broth (Wako Pure Chemicals, Japan), 5 g of NaCl, and 15 g of agar in 1000 ml of ion exchanged water; pH was adjusted to 7.2) and DNB agar (1.5%) plate medium; in which nutrients were diluted 100-folds compared to those plated on the NB medium except agar. The plates (5 for each sample) for the northern and southern basin samples were incubated at 10°C and 20°C, respectively. Colonies appearing on the plates were counted everyday for about 30 days. On the backside of the Petri dish, colonies were marked using a pen with a different color for each day in order to distinguish them.

Total number of bacteria

The total number of bacteria in the sediment was counted by an epifluorescence microscope (OLYMPUS BX50 BX-FLA) as follows. Samples were diluted in distilled water to 10^4 – 10^5 folds and were fixed with glutaraldehyde (final concentration: 1%). Then, the cells were stained with DAPI (4', 6-diamidino-2-phenylindole) solution (final concentration: 1 mg/l)

and were filtrated on a membrane filter (0.20 μm , MILLIPORE). Particles which showed a bright blue fluorescence were counted as microbes.

Mineralization activity of microorganisms in the sediment

The double-vial radiorespirometry method²⁰⁾ was used to measure the mineralization activity of microorganisms in the sediment. In this method two vials were used, a standard glass scintillation vial (WHEATON, Disposable Scintillation Vials) and a smaller vial (NICHIDEN RIKKA screw vial S-1) which can be placed inside a standard vial along with a filter paper (40 $\times$ 80 mm, ADVANTEC Toyo No. 2). The paper was soaked beforehand with a few ml of an alkaline scintillation mixture (containing 2 ml of 6 N NaOH and 8 ml of methanol in 100 ml of Scintisol 500 (DOJIN)) and dried under a vacuum. This paper was placed into the standard scintillation vial to line its inside; the paper can adsorb ^{14}C mineralized from the ^{14}C -labelled glucose by microbes in the sediment. A wet sediment sample (1 g) was placed into an inner vial, then the following were added: sterilized water (1 ml), 200 μl of 1% glucose solution and 5 μl of radio-active glucose solution (9.25 kBq of D- ^{14}C (U)-Glucose] (1 nmol), American Radiolabeled Chemicals Inc., in each vial). By following this procedure, two sets were prepared for each sediment sample. To one set 20 μl of 1 M KCN solution and to another set 20 μl of sterilized water was added, respectively. By comparing these two sets, it was possible to estimate whether mineralization of glucose was due to respiration or fermentation.

At the end of the experiment, 300 μl of 1 M HCl aq. solution was added to some representative samples. However, there was no further CO_2 evolution observed. Thus, the time course of CO_2 production obtained by the double-vial method seem to correctly reflect the mineralization activity of bacteria in the sediments.

To confirm the experimental conditions, we applied the double-vial method to two kinds of bacteria, *Pseudomonas syringae* NIAES 1309 and *Escherichia coli* IAM 12119. The cells of *P. syringae* were cultured in King's B medium (containing 20 g of pol-

ypepton (Japan Pharmaceuticals), 1.5 g of K_2HPO_4 , 1.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 g of glycerol in 1000 ml of ion exchanged water; pH was adjusted to 7.2)¹⁹⁾. The cells of *E. coli* were cultured in the NB medium. The cells at the stationary phase were centrifuged at 8000 g for 10 min at 4°C. The cells were washed twice with 0.1 M NaCl aqueous solution, and resuspended in the solution; the cell concentration was adjusted to 10^8 cells/ml. To 1 g of sterilized lake sediment 1 ml of the cell suspension was added, and the CO_2 production was followed as described above.

Results and Discussion

Number of colonies

Figure 2 and 3 show the change in the number of bacterial colonies along with the incubation time on the NB and DNB medium, respectively. It is apparent that the number of colonies of the sediment taken from St. 2 was greater compared with St. 1 at each depth of sediments on both media. This may be due to differences in environmental factors, e.g., water temperature and nutrient concentration, between these two sites.

The total number of colonies appearing on the DNB plate (Fig. 3) were greater than that on the NB plate (Fig. 2) in each layer of the sediment. The number of colonies of 0–1 cm appearing on the DNB plate were about 5 times greater than that on the NB plate both in St. 1 and St. 2. At other layers the number of colonies on the DNB plate were about 2 to 4 times greater than that on the NB plate. This tendency, more colonies being formed on DNB medium than on NB medium, was also observed for bacteria in the grassland soil and paddy field soil^{4,7,9)}.

Colony-forming time

The number of colonies increased not constantly, but intermittently during incubation especially on the DNB plate (Figs. 2, 3). Most colonies (ca. 70% of total colonies) on the NB plate appeared during the first 10 days after inoculation as shown in Fig. 2. After 10 days, the number of colonies increased slightly on the NB plate. Contrary to this, a different colony formation pattern was observed on the DNB plate, i.e., many

Fig. 2. Increase in the number of bacterial colonies on NB agar medium as a function of incubation time for the samples taken from St. 1 (a) and St. 2 (b). The samples were taken on June 11 (a) and on June 24 (b) in 1996 and plated on the NB medium. (○): 0~1 cm, (●): 2~3 cm layer, (□): 5~6 cm layer, and (■): 9~10 cm layer. Average of five plates was plotted as the colony number.

Fig. 3. Increase in the number of bacterial colonies on DNB agar medium as a function of incubation time for the samples taken from St. 1 (a) and St. 2 (b). The samples were taken on June 11 (a) and on June 24 (b) in 1996 and plated on the DNB medium. (○): 0~1 cm, (●): 2~3 cm layer, (□): 5~6 cm layer, and (■): 9~10 cm layer. Average of five plates was plotted as the colony number.

more colonies appeared at a later stage of inoculation (after 15 days) compared to that which appeared during the first 15 days of incubation (Fig. 3). This tendency was more apparent in the sample taken from St. 1. Suwa *et al.*¹⁸⁾ and Kasahara *et al.*⁹⁾ reported that

soil bacteria appearing on DNB medium could be divided into several groups based on the colony forming time, i.e., the growth rate. Supposing that this can be applied to the Lake Biwa sediment samples, the ratio of bacteria having a high growth rate seems to be

greater in the sample from St. 2 compared with that of those from St. 1.

The relationship between the depth of sediment and the number of colonies

The largest number of colonies was observed at the surface layer (0–1 cm) of sediment, whereas the number of colonies decreased as the sediment depth increased, regardless of the sampling site and season. In this experiment obligate anaerobes were not able to form colonies since the experiments were carried out under an aerobic condition. Thus, only aerobes and facultative anaerobes seem to have been able to form colonies under the experimental condition. In generally, the dissolved oxygen concentration seems to be very low at the deeper layer of the lake sediment²⁾. Therefore, the results shown in Fig. 2 and Fig. 3 may be due to the decrease in the number of aerobes at the deeper sediment sample.

Seasonal change of colony formation curve

Figure 4 shows the colony formation curve of samples taken in spring and summer both for St. 1 and St. 2, and the difference between these two samples. The results, obtained by subtracting spring data from summer data, indicate that the number of colonies was greater in the summer sample than in the spring sample, especially for the shallow layer of sediment (0–1 cm) compared to the deeper layer. In addition to this, the difference between the spring and summer sample was mainly due to the change in the number of colonies appeared during shorter incubation time. This seems to indicate that from spring to summer the number of fast-growing bacteria increased more than the slow-growing ones. The water temperature reached as high as ca. 30°C at St. 2 (southern basin) in the summer. This high temperature may enhance bacterial growth, especially in the shallow layer of the sediment, resulting in greater colony numbers compared with that in the spring sample as shown in Fig. 4 (c). Contrary to this, the water temperature at St. 1 (northern basin) remains constant through the year (ca. 7°C). The smaller variation in colony number between the two seasons in the sample from St. 1 compared with that of St. 2 may

be due to the stable temperature of the water.

The total number of bacteria in sediment

The total number of bacteria in the sediment samples was counted using an epifluorescent microscope after staining with DAPI (Fig. 5). At each sediment layer, the total number of bacteria was about 100 to 10000 times greater than the number of colonies in both the spring and summer samples. This tendency, for the number of colonies in sediment to be below the total number of bacteria, was also observed in other regions.^{3,8,11)} Thus, the bacteria in the Lake Biwa sediment hardly seemed to form colonies even on DNB medium.

It is obvious that the total number of bacteria is greater in the southern basin (St. 2) sample compared with the northern basin (St. 1) sample as well as the number of colonies. This may reflect the difference in the trophic level between the two basins; the trophic level of the northern basin has changed from oligotrophic (total nitrogen (TN) 0.02–0.2 mg/l, total phosphorus (TP) 0.002–0.02 mg/l) to mesotrophic (TN 0.1–0.7 mg/l, TP 0.01–0.03 mg/l) and that of the southern basin from mesotrophic to eutrophic^{14,15)}. Inorganic nutrients are usually produced by the decomposition of organic substances. Thus, changes in the trophic level described above seem to reflect the change in the concentrations of organic substances. So, the greater total number of bacteria at the 0–1 cm layer of the sediment compared to that of those at deeper layers may also be due to the availability of organic substances for bacterial growth.

The total number of bacteria in the summer sample decreased to as low as 1/10 to 1/20 of that in the spring sample at both St. 1 and St. 2 (Fig. 5). In the summer, thermal stratification occurs at the northern basin. This inhibits the oxygen supply to the bottom water and renders it anaerobic¹²⁾. Thus, the decrease in the overall number may be caused by a decrease in the number of strict aerobes in the sediment. Although the thermal stratification does not occur at the southern basin, the high water temperature in the summer season (temperature of the bottom water at the southern basin was ca. 20°C in June and ca. 30°C in September) seems to enhance the respiratory activ-

Fig. 4. Comparison of colony formation curves between two seasons, spring and summer in 1996. (a) The number of colonies appeared on the DNB medium at a certain incubation time for the sample taken in spring (St. 1: June 11, St. 2: June 24), (b) the sample taken in summer (St. 1: Sept. 10, St. 2: Sept. 5), and (c) the difference between the summer and spring sample; the number of colonies appeared on the medium at a certain incubation time for the spring samples was subtracted from that of the samples taken in summer. (○): 0~1 cm, (●): 2~3 cm layer, (□): 5~6 cm layer, and (■): 9~10 cm layer.

ity of the bacterial cells resulting in the formation of an anaerobic condition. This may also cause a decrease in the number of aerobes, hence a decrease in the total number of sediment bacteria.

It is noteworthy that the direction of change from spring to summer is different between the total number of bacteria and the number of colonies formed on nutrient agar medium: From spring to summer, the overall number of bacteria decreased (Fig. 5), while the colony number increased (Fig. 4). The sediment samples were mixed with nutrient agar medium and the colony number was counted. During incubation, oxygen diffusion through the agar may have been hindered. As discussed above, the bacteria in the summer season experienced an anaerobic condition in the sediment, so they may more easily form colonies on oxygen-deficient agar medium compared with bacteria obtained in spring. Here we must remember that strict anaerobes could not form colonies, because the sediment samples were mixed with agar media under an aerobic experimental condition.

Mineralization activity of bacteria in the sediment

Firstly, we applied the double-vial method to two kinds of bacteria, *Pseudomonas syringae* (a strict

Fig. 5. Total numbers of bacteria in the lake sediment. The numbers of bacteria stained with DAPI (final concentration 1 $\mu\text{g}/\text{ml}$) were counted with an epifluorescent microscope. (a) St. 1 and (b) St. 2. $\square$: Spring sample in 1996 (St. 1: June 11, St. 2: June 24). $\blacksquare$: Summer sample in 1996 (St. 1: Sept. 10, St. 2: Sept. 5).

aerobe) and *Escherichia coli* (a facultative anaerobe), to confirm the experimental conditions. The time course of CO_2 production differed greatly between these two organisms (Fig. 6). The activity of CO_2 production by *P. syringae* was smaller during the first

Fig. 6. $^{14}\text{CO}_2$ production by microorganisms metabolizing ^{14}C -labelled glucose added to Lake Biwa sediment. The production of $^{14}\text{CO}_2$ was measured for 93 h with a liquid scintillation counter using the double-vial radiorespirometry method. 1 ml of cell suspension (10^8 cells/ml) of (a) *P. syringae* NIAES 1309 and (b) *E. coli* IAM 12119 were added to 1 g of sterilized lake sediment. ($\circ$): without addition of KCN. ($\bullet$): with addition of KCN (final concentration 10 mM).

Fig. 7. Mineralization activity of microbes metabolizing glucose to CO_2 . The activity of CO_2 production during the incubation time from 0–38 h in Fig. 6 was adopted to avoid the disturbance caused by the metabolite accumulation, change of medium conditions etc. during the incubation (see text). (□): without addition of KCN. (■): with addition of KCN (final concentration 10 mM).

half of the incubation period compared with the second half. While the activity of *E. coli* was greater during the first half than the second half. During prolonged incubation, metabolites may accumulate or medium conditions may be altered. These unknown factors seemed to cause a change in activity with increased incubation time. Thus, we regarded activity during the first half as intact activity.

When KCN was added the response of *P. syringae* and *E. coli* was totally different (Fig. 6). A strict aerobe *P. syringae* could hardly produce CO_2 , while a facultative anaerobe *E. coli* produced CO_2 , although at a small amount, by fermentation even while its respiratory pathway was blocked by KCN. To avoid disturbance by unknown factors during the longer incubation as described above and to evaluate the effect of KCN before its loss by decomposition and/or evaporation¹⁾, only data from the first half of the incubation was used. Thus, as shown in Fig. 7, activities during the incubation time from 0 to 38 h were compared. It is clearly shown that the activity of a strict aerobe (*P. syringae*) was almost perfectly depressed by KCN addition. Whereas a facultative anaerobe (*E. coli*) showed some activity, although depressed, even after the addition of KCN. These results indicate that from the difference between

CO_2 production with and without addition of KCN we can distinguish the kind of bacteria responsible for CO_2 production, i.e., whether the bacteria are aerobes or facultative anaerobes. Thus, this method of comparison seems to enable us to evaluate the microflora in the sediment, i.e., which organisms are dominant, aerobes or facultative anaerobes.

Based on the above preliminary experiment, we examined the mineralization activity of the Lake Biwa sediment (Fig. 8). In this experiment the incubation time was considerably longer than that usually adopted⁶⁾. Thus, the bacteria in the sediments might utilize the glucose added externally and the nitrogen-source contained originally in the sediments, resulting in the increase in the number of bacteria. However, almost linear relationships were observed between CO_2 production and incubation time for the sediment samples, during the shorter period of incubation, 0–38 hr (data not shown) as was observed for *P. syringae* and *E. coli* (Fig. 6). Thus, it seems that the effect of the bacterial growth, if any, is slight.

The activity without the addition of KCN decreased along with the depth of the sediment. At the bottom of the lake, oxygen is supplied from the lake water to sediment and is consumed during the diffusion from the surface into the sediment of the deeper layer. Thus, the respiratory activity seems to decrease along with the depth of the sediment. This may indicate that respiratory activity is dependent on the sediment depth as shown in Fig. 8.

When KCN was added, the activity of CO_2 production was greatly depressed at the upper (0–1 cm) layer of the sediment except for the sample from St. 1 in the summer season, whereas the effect of KCN was smaller in the deeper layer of the sediment. These results can be explained as follows. The upper layer can easily obtain oxygen from lake water^{10,17)}. Thus, at this layer, the CO_2 production will be mainly via the respiratory process and the respiratory activity will be decreased greatly by adding KCN. However, at deeper layers of sediment, oxygen diffusion is limited. It seems that facultative anaerobes adapt to this condition better than strict aerobes. The smaller effect of KCN on CO_2 production at the deeper

Fig. 8. Mineralization activity of the microorganisms in the sediments of Lake Biwa. The samples were taken in spring (A: June 11, B: June 24) and summer (B: Sept. 10, D: Sept. 5). □: without addition of KCN. ■: without the addition of KCN (final concentration 10 mM).

layer seems to reflect the change in microflora, from aerobic rich to facultative anaerobic rich, with increases in the sediment depth.

In the northern basin of Lake Biwa, a thermal stratification, which prevents water exchange between the upper part and the bottom part of the basin, occurs from summer to late autumn due to higher water temperature at the upper part of water. Thus, in the summer season, oxygen is limited (DO in the bottom water of the northern basin is ca. 10 mg/l in the spring season and in the summer season DO is ca. 4.5 mg/l¹⁶) even at the upper layer of the sediment. This may force a change in microflora from aerobic rich to facultative anaerobic rich. The slight effect of KCN addition on the 0–1 cm layer of the St.1 sediment taken in the summer season (Fig. 8c) may be

due to the change of microflora. In the southern basin, thermal stratification does not occur in summer. Thus, the microflora will change little from spring to summer as can be deduced from Figs. 8B and D.

We investigated microflora in the bottom sediment of Lake Biwa and found a greater number of bacteria that can form colonies on agar media, 1) in the upper rather than the deeper layer of sediments, 2) in southern basin rather than northern basin sediments, and 3) in samples taken in summer rather than those taken in spring. Measurement of bacterial activity by glucose-mineralization clarified that 1) the upper layer of sediment is richer in aerobes than facultative anaerobes and 2) that under oxygen-deficient conditions even the upper layer of the sediment becomes rich in facultative anaerobes.

Through this study microflora in the sediments and their changes relating to various factors, such as, nutrient and oxygen concentration, and temperature was partly understood. However, from this study no information concerning strict anaerobes was available because of the experimental aerobic condition present. Strict anaerobes are now being investigated for a more precise understanding of the microflora in the sediments.

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