CHAPTER 2

EXPERIMENTAL PROCEDURES
2-1. Photometrical characterization of bacteria by Gram stain index (GSI) and its application to natural bacterial populations in the aquatic environment
Introduction

Gram's method is the most important and fundamental orthodox method for bacterial identification. It classifies bacteria into two groups, Gram negative and Gram positive. The mechanism of Gram staining is based on the fundamental structural and chemical attributes of bacterial cell walls. The cell walls of Gram positive bacteria have a high percentage of peptidoglycan, while those of Gram negative bacteria have only a thin peptidoglycan layer (Beveridge, 1981; 1990; Davies et al., 1983). In Gram's method, an insoluble dye-iodine complex is formed inside bacterial cells and is extracted by alcohol from Gram negative but not from Gram positive bacteria (Davies et al., 1983; Murray et al., 1994; Rodina, 1972). There are taxonomically gram variable species, but some cells of Gram negative or Gram positive species may show gram variable characteristics due to environmental stress, such as unsuitable nutrients, temperature, pH, or electrolytes (Beveridge, 1983).

Functional differences between Gram positive and Gram negative cell walls have been studied with special emphasis on nutrient uptake from the ambient environment. Gram negative bacteria have a periplasmic space between the lipopolysaccharide layer and the plasma membrane. In this space, binding proteins initially attach to nutrients and take them to a membrane carrier. Gram positive bacteria lack the periplasmic
space and are believed to have no binding proteins (Harold, 1972). Therefore, nutrient uptake from the environment is easier for Gram negative bacteria than for Gram positive bacteria. Because of this difference, the population density of Gram negative bacteria in more oligotrophic environments could be higher than that of Gram positive bacteria (Seki et al., 1984).

Gram staining is commonly used only to reflect cell wall structure. If Gram staining characterizes not only simple taxonomical dichotomy, but also multiple biological functions, it may also be used to correlate bacterial cell wall structure with related physiological responses to the environment. In particular, Gram staining could supply ecological information on natural bacterial populations that are difficult to culture by the present technology.

Membrane filter methods are widely used for microscopy in aquatic microbiology because of the low population densities of bacteria in many aquatic environments (Bowden, 1977; Hbbie et al., 1977; Rodina, 1972). However, these methods sometimes have problems associated with microscopic observations, causing unclear images of bacterial cells on Nuclepore filters when used with the conventional mounting medium (immersion oil nd (refractive index) =1.514). Hence, a suitable
mounting agent must be applied for the precise image analyses of Gram stained bacteria on Nuclepore filters.

In this study, the author has established a distinct method to characterize photometrical Gram stain images, it involves the Gram stain index (GSI) for specifying natural bacterial populations in various aquatic environments. For this purpose, the author has standardized the GSI of typical Gram negative and Gram positive bacteria by using *Escherichia coli* and *Bacillus subtilis*, respectively, and compared these GSI values to those of natural bacterial populations of several freshwater environments. The investigated natural waters were Hyoutaro-ike pond, Matsumi-ike bog, and Lake Kasumigaura, which are oligotrophic, mesotrophic, and eutrophic water bodies, respectively, as previously investigated (Hara *et al.*, 1983; Higashi *et al.*, 1997; Naganuma and Seki, 1985; Seki and Takahashi, 1983; Shiraishi *et al.*, 1985; Tsuchida *et al.*, 1984).
Materials and Methods

Samples

As standards for Gram negative and Gram positive bacteria, *E. coli* JCM 1649 and *B. subtilis* JCM 1465 (Nakamura et al., 1995) were used, respectively. The standard strains were supplied by Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN, Saitama). Both strains were cultured for 20 h at 37°C in nutrient broth with 0.5% NaCl (5.0 g of peptone 3.0 g of meat extract and 5.0 g of NaCl in 1.0 liter of distilled water) with the pH adjusted to 7.0, and used at the exponential growth phase to evaluate Gram stainability.

A predominant strain of the bacterial population in a strongly acidic hot spring was used as a representative of the most difficult Gram staining cases. This sample was grown at 60°C in TB medium, consisting of (per liter of tap water) 10 g of Na₂S₂O₃·5H₂O, 0.25 g of K₂HPO₄, 0.5 g of NH₄Cl, and 0.25 g of MgSO₄·7H₂O, adjusted to pH 5.0 (Seki and Naganuma, 1989).

The author selected natural bacterial populations from an oligotrophic pond, Hyoutaro-ike, a mesotrophic bog (Matsumi-ike) and a eutrophic lake (Kasumigaura) and adjacent water bodies of the lake as representative of various trophic systems for analytical case studies. The bacterial cells in the water samples were fixed by adding
37% buffered formalin (sample/formalin ratio = 10:1) to each water sample immediately after collection.

**Gram staining**

Bacterial cells on polycarbonate Nuclepore filters (no. 110606; pore size, 0.2 μm; diameter, 25 mm) were stained by the basic Gram method (contemporary modification) (Rodina, 1972). The bacterial cells, which were vacuumed on the filters by a filtration apparatus (Millipore Co., MA) were stained for 1 min with a few drops of carbolic gentian violet (Wako solution A; Wako Pure Chemical Industries Ltd., Osaka) and then rinsed with prefiltred (Nuclepore filter; pore size, 0.2 μm) distilled water for 30 s under vacuum. The filters were then immersed for 1 min in several drops of Lugol solution (Wako solution B) and again rinsed with distilled water under vacuum. After removal of all the liquid, 95% ethanol was poured on the cells for 30 s to decolorize them. The bacteria were then counterstained for 1 min with Ziehl- diluted fuchsin (Wako solution C), and rinsed with distilled water. After being dried under vacuum, the filters were removed from the filtration apparatus. The bacteria on the filters were mounted on a glass slide with immersion oil, covered with a glass coverslip (thickness: ca. 0.15 mm), and sealed with nail enamel to avoid drying.
Evaluation of Gram staining

The mounted specimens were observed microscopically and analyzed photometrically under an optical microscope with Fluor 100/1.30 oil Ph4DL 160/0.17 objective lens (Optiphot-2; Nikon, Tokyo), and the images were recorded (total magnification, \( \times 2,500 \)) with a 3-CCD charge-coupled device video camera (Sony, Tokyo). Each output from the video camera, red, green, and blue, was separately transferred to an image analyzer (Luzex III U; Nireco Co. Ltd., Tokyo) to digitize the intensity of each of the three primary colors on each pixel at 8-bit resolution (256 levels). Although every color of each bacterial cell can be described by digitized intensities of red, green, and blue, the colors of Gram stained cells distributed in the spectrum area of only red and blue (Fig. 2-2-1). Hence, the intensities of only red and blue were used to determine the characteristics of bacterial Gram staining (GSI) by the following equation:

\[
GSI = [(B - R) / (B + R)]
\]

where \( R \) and \( B \) are the intensities of red and blue, respectively. The GSI is zero when the intensities of red and blue are equal and positive if the blue intensity is higher than the red intensity. Theoretically, GSI values should range from -1 without blue
light to +1 without red light. By dividing the difference of light intensities by the sum of light intensities, the fluctuations in the light source at each observation are canceled.

Devising a satisfactory mounting medium

The bacterial cells from a hot spring in the Tateyama Jigokudani Valley (Sasa et al., 1996) were hardly visible when Gram stained on a Nuclepore filter and embedded in a standard immersion oil (nd = 1.514; Olympus, Tokyo), although they could be seen clearly when stained on a glass slide directly by the traditional method.

To solve this problem, improvements to the mounting agent were studied. The immersion oil and 1-bromonaphthalene (nd = 1.657; Wako Pure Chemical Industries Ltd.) were mixed in various ratios to achieve various refractive indices to try to minimize the light scattering by membrane filters in the mounting agent. To determine these ratios within the possible range for actual applications, the refractive indices of the mixtures were determined with a refractive index meter (1-T; Atago, Tokyo) and membrane transmittances were determined with a spectrophotometer (UV-550; Jasco, Tokyo). The effects of these mounting agents in actual microscopic observations were determined with double-stained standard bacterial samples (see below).
Double staining with the Gram stain and acridine orange

Cultured bacterial cells (E. coli, B. subtilis; and a bacterial strain isolated from the Tateyama Jigokudani hot spring) at the exponential growth phase in liquid media (nutrient broth for E. coli and B. subtilis and TB medium for the Jigokudani strain) were fixed by adding 37% buffered formalin (sample / formalin ratio = 10:1). These were stained on the Nuclepore filters with both the Gram staining reagents and an acridine orange solution (acridine orange at 1:10,000 in 6.6 mM phosphate buffer [pH6.6]). The Gram stain was made by the basic Gram method of staining (contemporary modification)(Rodina, 1972). After regular Gram staining with carbolie gentian violet and Lugol solution and decolorization, acridine orange solution was used for the epifluorescence microscopy (Seki et al., 1984) to ensure that all bacterial cells present were made visible by the Gram stain. Then, the bacterial sample on the filter was counterstained with Ziehl-diluted fuchsine to complete the Gram staining. After staining, each filter was cut into eight pieces. Each piece was mounted with a different mounting agent, which had various refractive indices. The number of bacterial cells in each field was counted under an epifluorescence microscope (EFD2; Nikon, Tokyo) with a B-2A filter and Hg 100 w bulb, using fluorescence for the acridine orange and
transmitted light for the Gram stain. The fraction of bacterial cells observable by the Gram staining compared to that observable by fluorescence staining was determined.

Confirmation of water sample type

The author performed chemical analysis to characterize the extent of eutrophication of the various water bodies sampled. Water samples were filtered, immediately upon collection, through precombusted (450°C for more than 2 h) Whatman GF/C fiberglass filters (Parsons et al., 1984) and stored at -20°C until used for analysis. The filtrates of these water samples were analyzed for inorganic nutrients (ammonium, nitrite, nitrate, and phosphate) with an autoanalyzer (TARAKO, 8000; Technico, Tokyo) that operates based on the standard method (Greenberg et al., 1992) and for dissolved organic carbon with a TOC Analyzer (TOC-5000; Shimadzu, Kyoto).
Results and Discussion

GSI characteristics of *Escherichia coli* and *Bacillus subtilis*

The GSI spectra of *E. coli* and *B. subtilis* were distinctly different (Figs. 2-1-2a and b). The GSI spectrum of *E. coli* cells, a typical Gram negative bacterium, ranged from -0.095 to 0 (average and 95% confidence limit for 300 bacterial cells; \(-0.047 \pm 0.017\)) with a sharp maximal peak at -0.06 and a broad shoulder at higher GSI values. On the other hand, the GSI spectrum of *B. subtilis* cells, a typical Gram positive bacterium, was in the broader range of -0.070 to 0.175 (average and 95% confidence limit for 300 bacterial cells; 0.082 \(\pm\) 0.043) with a low maximal peak at 0.070. Clearly, Gram negative and Gram positive bacteria showed distinctly different GSI profiles. Hence, the GSI of these two typical bacteria can be used as the standards for typical Gram negative and Gram positive bacteria for comparison with those of other bacteria, as indicated by the average and range of the GSI peaks.

Mounting medium

To obtain reasonably clear images of Gram stained bacteria on Nuclepore filters, the appropriate mounting medium was selected. The optical transmittance of Nuclepore filters at a wavelength of 600 nm depended on the refractive index of the
immersion medium. The highest transmittance (96%) was obtained at a refractive
index of 1.600 by mixing the immersion oil (nd = 1.514) and 1-bromonaphthalene in a
ratio of 1:3.6. By contrast, the transmittance of Nuclepore filters mounted in the
commercial immersion oil (nd = 1.514) was 56% (Fig. 2-1-3a). In the medium with a
refractive index of 1.600, the Gram stained bacteria were clearly observed on the
Nuclepore filter. the author mounted E. coli and B. subtilis on Nuclepore filters
embedded in mounting agents having different refractive indices (1.500, 1.514, and
1.600) (Fig. 2-1-4). The background of the bacteria embedded in the mounting agent
with a refractive index of 1.600 was flat, and the brightness was uniform, so that the
bacterial cells could be clearly seen. In contrast, when I used a mounting agent with
refractive index of 1.514, the filter pores were observed and the brightness was not
uniform; the image appeared as if the whole membrane undulated, making the bacterial
cells extremely difficult to observe. Observation of Gram negative cells was more
difficult. The number of bacterial cells observable in the field when the filter was
mounted with the agent of nd =1.514 was only about half that when mounted with the
agent with nd of 1.600.
Effect of new mounting medium

Comparison of bacterial number in the same microscopic field under epifluorescence and light microscopy, even using a bacterial strain isolated from the Tateyama Jigokudani hot spring, showed a much clearer image with the mounting agent whose nd is 1.600. In samples embedded in the mounting agent with nd of 1.600, all the bacterial cells counted under the epifluorescence microscope were recognized under the light microscope. In contrast, only one-third of the bacterial cells counted with the nd = 1.514 mounting agent under epifluorescence were observable under the light microscope (Fig. 2-1-3b). Thus, observation of Gram stained bacteria with an appropriate mounting agent is very important for the differentiation of Gram negative from Gram positive cells by the GSI method.

The presence of organic solvents in the mounting agent can affect the Gram stain spectra. 1-bromonaphthalene was used to adjust the commercial immersion oil to the most appropriate refractive index. The GSI values of *E. coli* and *B. subtilis* cells mounted in the best medium (nd = 1.600) and stored at room temperature were examined every 10 days for 1 month. No decolorization of *E. coli* was observed during this observation period. Although there was no significant change in the GSI profile of *B. subtilis* until day 20 after mounting, a small peak at 0.080 had decreased on
day 30 to the height level of Gram negative cells. Therefore, I recommend that bacterial specimens be examined by microscopy within 3 weeks of fixation.

**GSI of natural bacterial populations**

The GSI spectra of natural bacterial populations in Hyoutaro-ike pond, Matsumi-ike bog, and Lake Kasumigaura showed different profiles over the ranges of -0.095 to -0.025, -0.080 to 0.000, and -0.090 to 0.025, respectively. However, the highest peaks in each of these profiles appeared at the same GSI value of -0.055, (Figs. 2-1-2c to e). The averages and 95% confidence limit of GSI in those populations were -0.056 ± 0.011 at Hyoutaro-ike pond, -0.054 ± 0.009 at Matsumi-ike bog, and -0.051 ± 0.021 at Lake Kasumigaura. Therefore, by comparing these GSI spectra with those of *E. coli* and *B. subtilis*, it was clear that all these freshwater environments were similar in having almost exclusively Gram negative bacterial species. The fraction of bacterial cells having more Gram positive GSI characteristics tended to increase in the more eutrophic waters. Statistical differences of the frequency distribution of GSI values in differently eutrophicated waters were significant based on the F test: \( F = 1.48 \) (\( F_{0.01}[302,315] = 1.34 \)) between Hyoutaro-ike pond (oligotrophic) and Matsumi-ike bog (mesotrophic), \( F = 3.51 \) (\( F_{0.01}[297,302] = 1.35 \)) between Hyoutaro-ike pond and Lake
Kasumigaura (eutrophic), and \( F = 5.17 \) (\( F_{0.01(297,315)} = 1.35 \)) between Matsumi-ike bog and Lake Kasumigaura, respectively.

Laboratory experiments with \( E. \ coli \) and \( B. \ subtilis \) in their different growth phases showed slightly variable GSI profiles; i.e., the GSI spectrum of each species shifted to more negative sides (by 0.14 and 0.11 in average in \( E. \ coli \) and \( B. \ subtilis \), respectively) when the growth phase advances from the exponential phase to the stationary phase (Fig. 2-1-5). They could be good examples for application to the natural bacterial community, as follows:

Figure 2-1-6 shows an analytical example of a GSI spectrum of the natural bacterial community from Lake Kasumigaura. The water in the lake is influenced greatly by waters from a lotus field and an irrigation creek. Three GSI spectra of the bacterial populations from the water and the mud of the lotus field and from the water of the creek that connects the lotus field and Lake Kasumigaura, were weighed with factors of 0.20, 0.45, and 0.30, respectively. In this case, the GSI spectrum of the bacterial populations from the lotus field water and the creek were shifted -0.01 in GSI (to negative) and +0.01 in GSI (to positive) to improve the reproduction of three peaks of the GSI spectrum observed at the different physiological conditions of \( E. \ coli \). The difference between the actual GSI spectrum of the lake community and the synthesized
spectrum of three bacterial populations from inflowing waters to the lake is shown in
Fig. 2-1-7. The peaks of the residue GSI spectrum resemble those of \textit{B. subtilis}, which
is a dominant bacterial population in the sediment of Lake Kasumigaura.

From these case studies of representative freshwater environments, I conclude that
GSI histograms and their analysis of bacterial cells in natural populations may indicate
the possible correlation between bacterial populations and their living milieu.
Fig. 2-1-1. Schematic diagram of the equipment used for GSI determination, with a standard example analyzed by the equipment.
Fig. 2-1-2. GSI of bacterial strains and populations. GSI standards of the Gram-negative bacterial strains (a) and the Gram-positive bacterial strains (b). Typical GSI standards of the natural bacterial populations from the oligotrophic water (c), mesotrophic water (d), and eutrophic water (e).
Fig. 2-1-3. Optical transmittance of Nuclepore filters (pore size, 0.2 μm) mounted in immersion media with different refractive indices (a). Detectability of bacterial cells from the Tateyama Jigokudani hot spring with different mounting agent refractive indices (b).
Fig. 2-1-4. Gram stained images of *E. coli* and *B. subtilis* with mounting agent refractive indices of 1.514 (a), 1.550 (b), and 1.600 (c).
Fig. 2-1-5. GSI spectra at different growth phases of *E. coli* (a) and *B. subtilis* (b). 
---: exponential growth phase, ----: stationary growth phase
Fig. 2-1-6. Gram stained images of a natural bacterial population from Lake Kasumigaura with mounting agent refractive indices of 1.514 (a) and 1.600 (c).
Fig. 2-1-7. GSI of natural bacterial populations in water samples from Lake Kasumigaura and its adjacent environments (top four panels), with an analytical example of the component of GSI spectrum of the lake bacterial population (bottom panel).
2-2. Simulated *in situ* experiment
i) Instrumentation: *in situ* gradostat
Introduction

The aquatic habitat is an open system where natural water masses exist as a patchwork and where living organisms and non-biotic materials are exchanged continuously. The dynamics inside a natural water mass have to be studied by taking into account the exchanges between neighbouring water masses (Lovitt and Wimpenny, 1981). These parameters may be more important in such places as upwelling zones or aquaculture areas around fishnets, where eutrophication occurs frequently because of continuous inflow of nutrients and the consequent formation of nutrient gradients. Bacteria dynamics in nutrient gradients have frequently been observed in situ (e.g. Naganuma and Seki, 1993; Rheinheimer, 1991; Seip and Reynolds, 1995; Seki et al., 1975; Yamaguchi and Seki, 1977). But there have been no experimental studies in situ to clarify the dynamic mechanisms because the appropriate apparatus has been lacking.

In the laboratory, on the other hand, continuous culture has been used to extend the understanding of growth and physiology as a contribution to ecological studies (Aiba et al., 1973; Antia et al., 1991; deNoyelles and O'Brien, 1974; Fritzche et al., 1991). One example is the gradostat (Lovitt and Wimpenny, 1981) which was designed to simulate more natural conditions in the laboratory by the application of multi-chambers chemostat principles. The system provided experimentally a desired
heterogeneity of nutrients and microbial populations. If a gradostat could be devised
to establish a nutrient gradient in situ, it would allow experimental study of
spatial-temporal changes in the natural community.

Based on the principles of the laboratory gradostat laid out by Lovitt and
Wimpenny (1981), an in situ gradostat was devised, as part of a semi-open mesocosm,
to satisfy the following conditions: (1) an experimental semi-enclosed system in the
natural environment; (2) to maintain equivalence with the external water except for the
experimental factors; and (3) to form a manipulable gradient of experimental factors
across the mesocosm.
Device of *in situ* gradostat apparatus

The *in situ* gradostat consisted of three components; a culture chamber, a medium reservoir and a pump to transfer the medium into the culture chamber (Fig. 2-2-i-1).

The culture chamber consisted of three concentric cylinders with volumes, 1st to 3rd, of 24.1 liter; 32.4 liter; 20.8 liter, inner to outer, respectively. The cylindrical walls of each chamber were perforated by holes to allow a desired water exchange between neighbouring compartments. Both the diameter of each hole and the number of holes per unit area were equal over all walls; the rate of water exchange was regulated by putting silicon rubber plugs into the holes. A lid with a seal to each wall tightly covered these chambers to restrict water exchange only through holes in the walls. Each chamber had stirring paddles to establish and maintain homogeneity within each compartment (Fig. 2-2-i-1). The stirring paddles were driven by a single shaft connected to a geared DC motor. The central (1st) chamber had an input of sterile nutrient from the medium reservoir. The nutrient passed to the outer chambers through the perforated chamber walls, so that a desired nutrient gradient could be established by adjusting the number of holes (Fig. 2-2-i-2). Four buoys were connected to the device to keep it afloat. A transfusion pump (TERUMO STC-608, Tokyo) supplied nutrients to the central compartment at a predetermined rate. The power for the stirring paddles
and the pump was supplied from a 12 V DC lead-acid battery on the pond side. Batteries were exchanged recharged in every two days.

The fate of the experimental nutrient in each coaxial chamber was either turbulent diffusion through chamber walls or consumption by the resident organisms. The rate of diffusion was adjusted by the hole density in the chamber wall. The fluxes between each chamber are shown schematically in Figure 2-2-i-2. The concentration, $C_i$, in the $i$-th chamber, of volume $V_i$, was kept homogeneous by mixing with stirring paddles. The quantity of solute in the $i$-th chamber, $V_iC_i$, increased by the solute influx from the $(i-1)$-th (inner) and the $(i+1)$-th (outer) chambers and decreased by the efflux to the $(i-1)$-th and the $(i+1)$-th chambers. The quantity of solute $V_iC_i$ in the $i$-th chamber can be expressed by the following differential equation as:

$$V_i \frac{dC_i}{dt} = v_{i-1,i}C_{i-1} + v_{i+1,i}C_{i+1} - v_{i,i-1}C_i - v_{i,i+1}C_i$$

... Equation (1)

where $v_{i,i+1}$ is the flux from the $i$-th chamber to the $(i+1)$-th chamber, for example. The flux $v_{i,i+1}$ is an instrument parameter, controlled both by the input from the nutrient reservoir and the mixing by stirring paddles. Backward fluxes (from outer to inner chamber) are due to diffusion mixing only, whereas the pumped nutrient supply
maintains a net outward flow so that forward fluxes have a pump-component and a mixing component.
Examination and improvement of the apparatus at an oligotrophic pond

Parameters characterizing the *in situ* gradostat were determined experimentally using a fluorescent solute. Uranin L, being biologically inert, approximately the same molecular weight as inorganic nutrient molecules and detectable by spectrometry with great sensitivity, was a good indicator of the gradient inside the *in situ* gradostat. To measure both fluxes with and without pumping, all holes of both side-walls of the 2nd chamber were covered with plastic sheets prior to filling with 4 mM uranin. After mixing the water in all chambers for 10 min, the vinyl sheets were removed and filtered pond water from the medium reservoir was introduced into the central chamber at a constant rate (480 ml h⁻¹). After 300 min, the pumping was stopped to determine the dilution effect of mixing. Ten ml of solution was sampled from each chamber at 0, 10, 20, 30, 60, 90, 120, 180, 240 and 300 min after the pumping was started. Samples were also taken at 0, 10, 20, 30, 60, 90, 120 and 180 min after the pumping was stopped. The uranin concentration was determined using a fluorescence spectrophotometer (HITACHI F-4010, Tokyo). This showed that uranin in the 2nd chamber spread into both the 1st and 3rd chambers during the period of 90 min from the start of the experiment, and then the uranin concentration in all chambers decreased exponentially (Fig. 2-2-i-3). The dilution rates were different with pumping on and off. During the
period of decrease, the concentration in the $i$-th chamber, $C_i$, can be expressed by an equation as

$$C_i = A_i \exp (-D_i t)$$  \hspace{1cm} ... Equation (2)

where the constants, $A_i$ and $D_i$, are different depending on whether pumping is on and off.

By analyzing the experimental data based on relationships between these equations, the estimated values in this study using the *in situ* gradostat are summarized in Table 2-2-i-1.

Nitrate (15 mg NO$_3$-N l$^{-1}$) and uranin (4 μM) were simultaneously supplied at 480 ml h$^{-1}$ to the system to compare their dynamics in each chamber (Fig. 2-2-i-4).
Results and Discussion

Physical characteristics of the in situ gradostat

When 4 µM uranin was supplied to the system, its concentrations in all chambers increased and became almost constant within 2 hours, but the real steady-state was attained in 12 hours. Parameter values shown in Table 2-2-i-1 were applied to equation 1 and predicted the changes of solute concentrations with high significance \( r^2=0.98, n=59; \) Fig. 2-2-i-4). A concentration gradient of as much as 9.5-fold could be achieved across the system. The nitrate dynamics were almost the same as those of uranin \( r^2=0.97, n=59; \) Fig. 2-2-i-4). The relationship between flux \( v \) and the hole density (the open area in the chamber walls) were expressed as: 

\[
\begin{align*}
\nu_{1,2} &= 2.92 \times 10^{-1} \times s; \\
\nu_{2,1} &= 2.17 \times 10^{-1} \times s; \\
\nu_{2,3} &= 6.45 \times 10^{-1} \times s; \\
\nu_{3,2} &= 3.75 \times 10^{-1} \times s; \\
\nu_{3,4} &= 4.00 (s = 11.1 \%); \\
\nu_{4,3} &= 1.89, (s = 11.1 \%), \\
\end{align*}
\]

where \( s \) is open area ratio. From these relationships, the operational conditions for the in situ gradostat could be chosen.

In this particular semi-open mesocosm, little difference existed between the physico-chemical and biological factors inside and outside each chamber except for the experimental parameters. As shown in Table 2-2-i-1, there was exchange between the gradostat and its surrounding environment which was maintained inside the chambers as natural as possible. The exchange of living organisms and non-biotic materials as well
as solute has to be an important parameter to simulate the dynamics of natural phytoplankton community in such an environment as upwelling area or aquaculture site around a fish net. The exchange enables one to elucidate the influence of the experimental parameter on a natural community.

In conclusion, the in situ gradostat allows various natural conditions to be simulated for the study of the dynamics of natural microbial communities in various environmental gradients. The in situ gradostat is appropriate to the simulation of highly dynamic environmental gradients, common in such places as estuarine and up-welling areas. Experimental results obtained by this gradostat will give necessary information to bridge a gap between the in situ observation and laboratory results using conventional equipment.
Table 2-2-i-1. Estimated value of dilution rate \( (D) \) and flux \( (\nu) \) at each wall. Concentration fractions at steady state for each chamber \( (j) \) are given in Figure 3. Volumes \( (V) \) are given in text.

<table>
<thead>
<tr>
<th>Wall</th>
<th>( D_{on} ) (min(^{-1}))</th>
<th>( D_{off} ) (min(^{-1}))</th>
<th>( v_{mix} ) (l min(^{-1}))</th>
<th>( v_{pump} ) (l min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>( 1.44 \times 10^{-2} )</td>
<td>( 6.14 \times 10^{-3} )</td>
<td>0.25</td>
<td>0.20</td>
</tr>
<tr>
<td>II</td>
<td>( 1.55 \times 10^{-2} )</td>
<td>( 7.11 \times 10^{-3} )</td>
<td>0.72</td>
<td>0.78</td>
</tr>
<tr>
<td>III</td>
<td>( 1.56 \times 10^{-2} )</td>
<td>( 7.35 \times 10^{-3} )</td>
<td>7.66</td>
<td>8.57</td>
</tr>
</tbody>
</table>
Fig. 2-2-i-1. The *in situ* gradostat with a reservoir, a pump, three culture chambers, three sampling tubes, four buoys, a battery and stirring paddles. The central chamber has an input of sterile nutrients from the medium reservoir. The nutrients move to the outer chambers through the holes on chamber walls establishing a nutrient gradient. Water samples were collected through the sampling tubes. Four buoys were used for floating the *in situ* gradostat. Electrical power was supplied from lead-acid batteries to the stirring paddles and the pump.
Fig. 2-2-i-2. Schematic solution fluxes in the *in situ* gradostat. Fluxes of solute through the holes in the chamber walls determine the solute concentrations in each chamber.
Fig. 2-2-i-3. Uranin dilution in the in situ gradostat. (a) Uranin amount and (b) the fraction of uranin in each 1st, 2nd and 3rd chamber (symbols ○, □, △). (a) Uranin that had been preloaded into the 2nd chamber propagated to adjacent chambers. The dilution rate was dependent on supply from the reservoir and attained steady-state equilibrium among chambers within 120 min. The dilution rates are summarized in Table 1. (b) Uranin concentration expressed as exponential time course as \( f_1 = 0.64 - 0.62 \times \exp(-0.013t) \) \((r^2 = 0.99)\) in the 1st chamber (○), \( f_2 = 0.34 + 0.58 \times \exp(-0.018t) \) \((r^2 = 0.98)\) in the 2nd chamber (□) and \( f_3 = 0.20 + 0.10 \times \exp(-0.009t) \) \((r^2 = 0.81)\) in the 3rd chamber (△).
Fig. 2-2-i-4. Concentration change of solutes in each chamber. Solid lines are calculated from equations 1. Symbols ○, □, and △ indicate the solution concentration in the 1st, 2nd and 3rd chambers. The open and closed symbols indicate the uranin and nitrate concentration, respectively. The regression of experimental data onto the theoretical equations was highly significant ($r^2 = 0.96$ and $r^2 = 0.93$ for uranin and NO$_3$, respectively).
ii) Simulated *in situ* experiment of eutrophication in an oligotrophic pond
Introduction

The terms "eutrophic", "mesotrophic" and oligotrophic" were first introduced into science by Weber (1907) to describe the general nutrient conditions determined by the chemical nature of soil water in German bogs. Anthropogenic organic pollutants that cause eutrophication originate primarily from domestic sewage, run-off from agricultural fertilizer and waste materials from animal feed lots (e.g., Hutchinson, 1970; Seki et al., 1975; Southwick, 1976; Nixon, 1995). These organic nutrients stimulate the growth of heterotrophic microorganisms at any aquatic environment (e.g., Seki and Nakano, 1981; Tranvik, 1988; Miyamoto and Seki, 1992; Naganuma and Seki, 1993; Utsumi et al., 1994).

It has been suggested (Saida et al., 1998) that Gram stain index (GSI) can be used as a potential indicator for assessment of trophic levels in aquatic environments because Gram stain characteristic of natural aquatic bacterial populations indicates a particular profile of predominant bacteria inhabiting in a certain trophic level. Gram stain has been used for a century as the first step in identification of bacteria, which can be classified into two groups, gram negative and gram positive, based on bacterial cell wall composition (e.g. Beveridge, 1981; 1990; Davies et al., 1983; Brock et al., 1994). Some cells of gram negative or gram positive species have shown gram variable
characteristics due to environmental stress, such as unsuitable nutrients, temperature, pH, or electrolytes (Beveridge and Davies, 1983). Variability of Gram stain is caused not only by changing of stainability of individual cells but also by changes in bacterial species composition in natural aquatic environments. Unfortunately, however, there is no research on the nutrient effect onto Gram stain characteristics of bacterial populations in any eutrophicated aquatic environment. Most of the predominant bacterial species in aquatic environments are unculturable by our present microbiological technology, so that in situ methods of biomass and activity assessment are necessary. One of the best resolutions on this matter is to determine the GSI characteristics of natural bacterial populations in various eutrophicated environments formed experimentally.
Materials and Methods

Study site

An *in situ* study of natural bacterial communities was performed in an experimental organic nutrient gradient inside the *in situ* gradostat (Higashi *et al.*, 1998) deployed in the oligotrophic Hyoutaro-ike Pond (lat 36°03' N, long 140°27' E; surface area $1.4 \times 10^3$ m²; mean depth 80 cm; maximum depth 100 cm) once each season from 1997 summer to 1998. Rainfall is only the source of water supply to the pond, and the pond water has been kept at the oligotrophic type throughout the year (Higashi *et al.*, 1998).

Samples

Sampling during these seasonal deployments was made at appropriate times in the following three periods: i.e., at 0 h during period I at the initiation of experiment; at 24 h at spring and summer, at 72 h at autumn, and at 120 h at winter during period II when a few species of dominant bacteria had adapted to the experimental conditions to proliferate actively and exclusively; and at 144 h (final day of the experiment) during period III when only few species of predominant bacteria had acclimatized to be able to continue their proliferation at the experimental condition. In order to establish an
organic nutrient gradient inside the gradostat as natural as possible, the pond water as
the basal medium for the gradostat was sampled from Hyoutaro-ike pond a day before
the initiation of each experiment. The pond water for the basal medium was filtered
through glass-fiber filters (Whatman GF/D and GF/C), thereafter peptone was added to
complete the nutrient medium (1 g peptone l⁻¹) before sterilization by autoclaving.
The nutrient medium in a reservoir flask was pumped into the central chamber of the
gradostat at 480 ml h⁻¹. By adjusting the number of holes in the chamber walls of the
gradostat, the peptone concentrations of water in the 1st, 2nd, and 3rd chambers were
adjusted to a highly eutrophic level (25 mgC l⁻¹), a moderately eutrophic level (10 mgC
l⁻¹) and a mesotrophic level (4 mgC l⁻¹), respectively. Water samples (800 ml) for
chemical and microbiological analyses were collected from every chamber at 20 cm
depth into 50 ml sterile syringes. For chemical analyses, water samples were filtered
immediately through Whatman GF/C glass-fiber filters, and kept at -20 °C until
analyses. These filtrates were used to determine the concentrations of dissolved
organic carbon (DOC).

Analyses

The concentration of dissolved oxygen (DO) was determined by the Winkler
method (Strickland and Parsons, 1972). The DOC measurement was made with the high temperature combustion technique (Greenberg et al., 1992) on a Shimadzu TOC-5000, equipped with an ASI-5000A autosampler.

Water samples for the microbiological analyses were fixed by adding 2.5 ml of buffered formalin to each 10 ml of sample water immediately after sampling, and filtered onto polycarbonate Nuclepore filters (pore size, 0.2 μm; diameter, 25mm). Bacterial cells on half of each filter were counted under an epifluorescent microscope after staining with an acridine orange solution (acridine orange at 1:10,000 in 6.6 mM phosphate buffer of pH 6.6).

Bacterial cells on the other half of each filter were stained by the basic Gram method (Rodina, 1972), and were analysed photometrically using an optical microscope with a Fluor 100/1/30 oil Ph4DL 160/0.17 objective lens (Optiphot-2; Nikon, Tokyo). The image of bacterial cells was recorded (total magnification, ×2,500) by a 3-CCD charge-couple device video camera (XC-009; Sony, Tokyo). Each output from the video camera, three primary colours (red, green, and blue), were separately transferred to an image analyzer (Luzex III U; Nireco, Tokyo) and light intensity was digitized on each pixel at 8-bit resolution (256 levels). The intensities of only red and blue were used to determine the characteristics of bacterial Gram staining (GSI) (Saida et al., 2001).
1998). GSI histograms of 300 bacterial cells in each sample were compared based on their modes and ranges.

The nutrient effect should affect on the GSI mode of a bacterial population through the mechanism of a Michaelis-Menten Kinetics (Monod, 1949). The least squares method was applied to obtain the best-fit equation, \( r = a \times N / (0.5 a + N) + b \), where \( r \) is mode of the bacterial GSI histogram; \( N \) is the organic nutrient concentration (DOC) of ambient water; \( a \) and \( b \) are constants. All the statistical regression analyses were performed by t test. The modes in lag phase of bacterial growth were excluded from the correlation analyses, because the bacterial population in this phase has not yet been adapted to the experimental conditions.

The population growth rate of the bacterial community in each chamber of the gradostat was calculated based on the chemostat theory (Higashi and Seki, 1998).
Results and Discussion

Physico-chemical and biological conditions of Hyoutaro-ike Pond

The water temperature (mean \( \pm \) SD, \( n=25 \)) during the investigation was \( 18.1 \pm 5.3 \) at spring, \( 28.7 \pm 4.1 \) at summer, \( 12.1 \pm 5.7 \) at autumn and \( 6.3 \pm 4.8 \) °C at winter, respectively (Table 2-2-ii-1). The pH of water inside every chamber of the gradostat at every season was around 7 and not significantly different from that of the pond water.

Although the DO concentration in the pond water fluctuated only within the DO concentration range from 3 to 7 mg O\(_2\) l\(^{-1}\) throughout each experimental period, those in the chambers decreased (the minimum down to 0.1 PDL) because of the active respiration during proliferation of bacterial populations (Fig. 2-2-ii-1). The final DO decrease during the experiments in spring and summer could have affected greatly the bacterial metabolism and species composition, because the concentration attained at Pasteur point to affect bacterial metabolism from aerobic respiration to fermentation (0.43 mg O\(_2\) l\(^{-1}\)) (Pamatmat, 1971; ZoBell, 1940; Seki and Iwami, 1984).

The DOC concentration (Table 2-2-ii-1) shows that the peptone solution added into the \textit{in situ} gradostat contributed to eutrophication up to eutrophic levels of waters in the 1st and 2nd chambers, a mesotrophic level in the 3rd chamber (the definitions of eutrophic and mesotrophic levels; see Wetzel \textit{et al.}, 1972; Ochiai and Honda, 1980; Seki
and Nakano, 1981; Søndergaard and Schierup, 1982). The pond water outside the
gradostat was oligotrophic (the definitions of oligotrophic level; see Kaplan and Bott,
1982; Søndergaard, 1984). This artificial perturbation facilitated the dynamic study of
the natural bacterial population under various eutrophicated conditions.

Bacterial growth and GSI characteristics

Bacterial growth rate and species successions were dependent primarily on water
temperature and DOC concentration (Table 2-2-ii-2, Figs. 2-2-ii-1 and 2-2-ii-2). The
successions of the same natural bacterial population showed different peculiarities in
GSI histograms due to the experimental exposures to a eutrophic environment (1st
chamber) and an oligotrophic environment (the pond water outside the gradostat); i.e.,
the peak positions and peaks of GSI profiles in the eutrophic environment fluctuated
with fewer species composition (narrower width) of gram negative bacteria showing
GSI spectrum of more gram positive characteristics (higher GSI peak positions) with
progression of the experiment period (Fig. 2-2-ii-2). These GSI profiles should have
reflected the species succession of a natural bacterial community in the oligotrophic
environment adapting and acclimatizing to the experimental perturbation of
eutrophication (e.g., Utsumi et al., 1994).
Such a species succession can be illustrated with the example observed in the bacterial community in the 1st chamber during summer (Fig. 2-2-ii-3). During period I, most bacterial cells were 1 μm long with their stainability of bright red. The bacterial community then consisted of bacterial rods with different sizes including more dividing cells during period II (24 h), when more bacterial cells were stained blue. Species composition of the bacterial populations should have been further simplified to become one or two predominant species at period III as a result of the experimental eutrophication. This phenomenon has been confirmed by enrichment culture of natural bacterial communities from all kinds of environment since Koch established Koch's Postulates for the pure culture of bacterium (Koch, 1884). Thus, these three periods could be defined as follows:

Period I: natural conditions.

Period II: species succession of dominant bacterial species adapted to the Perturbation.

Period III: natured period of species succession comprised of predominant bacteria acclimatized to the perturbation.

Those profiles during each period at each season suggest general transition of the GSI spectrum of a natural bacterial community at different stages of adaptation and
acclimatization to the eutrophication phenomenon (Fig. 2-2-ii-4). The same profiles of GSI spectrum were determined for the bacterial communities from samples in the 1st and 3rd chambers and outside the gradostat during period I at all seasons. However, during period II, the GSI profiles in the eutrophic (1st chamber) and mesotrophic (3rd chamber) environments widened and shifted to the right (gram positive side), showing a possible increasing number of dominant species (Fig. 2-2-ii-4). The GSI spectrum of a bacterial community with its wider histogram and more multiple peaks could be composed of various GSI spectra of greater number of dominant bacterial species. During period III, the histogram profiles converged with the histogram summits at −0.06 and −0.05 at spring and summer, respectively, showing that the species succession were mature with very few predominant species of facultatively psychrophilic bacteria acclimatized to those experimental eutrophicated environments. On the other hand, during period III, the profiles at autumn and winter widened with the histogram peaks at −0.04, showing possible climax stages of succession process with very few predominant species of obligately psychrophilic bacteria.

These peculiar GSI profiles with the given eutrophicated condition at each season reflected the stage of species succession of natural bacterial communities (Figs. 2-2-ii-2 and 2-2-ii-4, Table 2-2-ii-2). The GSI profiles of bacterial communities at the initial
stage of *in situ* experiment (0 h) with all any nutrient conditions showed almost the same spectrum (same summit and width) at any season (Fig. 2-2-ii-4a, d, g and j), thereafter they showed different profiles with the progress of species succession at differently eutrophicated conditions (Fig. 2-2-ii-4). At spring season, the GSI profiles (Fig. 2-2-ii-4b) were typical of dominant bacteria (12th generation at 24 h since the experiment was commenced) adapted to the new eutrophicated condition, whereas those in Fig. 2-2-ii-4c were typical predominant bacteria (63rd generation at 144 h since the experiment was commenced) typically acclimatized. At summer season, the GSI profiles in Fig. 2-2-ii-4e were typical of dominant bacteria (15th generation at 24 h since the experiment was commenced) adapted to the new eutrophicated condition and Fig. 2-2-ii-4f were typical predominant bacteria (76th generation at 144 h since the experiment was commenced) acclimatized to the given condition. At autumn season, the GSI profiles (Fig. 2-2-ii-4h) were typical of dominant bacteria (11th generation at 72 h since the experiment was commenced) adapted to the new eutrophicated condition, whereas those in Fig. 2-2-ii-4i were typical predominant bacteria (35th generation at 144 h since the experiment was commenced) typically acclimatized. At winter season, the GSI profiles (Fig. 2-2-ii-4k) were typical of dominant bacteria (10th generation at 120 h since the experiment was commenced) adapted to the new eutrophicated
condition, whereas those in Fig. 2-2-ii-41 were typical predominant bacteria (19th generation at 144 h since the experiment was commenced) typically acclimatized.

The population growth rates of bacterial communities in the pond were experimentally shown to be higher at higher DOC concentrations (Table 2-2-ii-2). DOC concentration and water temperature are the primarily limiting environmental factors of population growth rate of natural bacterial communities in ponds at the same site as this experimental investigation (Seki and Nakano, 1981; Shiraishi et al., 1985; Miyamoto and Seki, 1992; Naganuma and Seki, 1993; Utsumi et al., 1994). As the temperature effect in this study can be ignored, because of possible predominant occurrence of facultatively psychrophilic bacteria during spring and summer and obligately psychrophilic bacteria during autumn and winter in the pond, the heterotrophic uptake of soluble organic compounds in this natural water should be analyzed primarily based on Michaelis-Menten kinetics. (Seki, 1992).

Nutrient uptake kinetics can be described using Michaelis-Menten uptake kinetics as

\[ V = \frac{V_m S}{(K_m + S)} \]

where \( V \) is the rate of substrate uptake, \( V_m \) is the maximum rate of substrate uptake, \( K_m \) is the substrate concentration at which \( v = V_m/2 \) and \( S \) is the substrate concentration.
(Parsons and Strickland, 1962). The constant \( K_m \) reflects the microbial capability to utilize a substrate. Certain bacterial species, such as those found in marine and freshwater bacterioplankton, have very low \( K_m \) values (Seki, 1992; 1993).

Based on the assumption that the species predominance of a natural bacterial community is controlled by growth rate at a given nutrient concentration (Seki, 1982; 1992; 1994), the effect of DOC concentration in the ambient water at each season was analyzed with reference to the value at the peak \( r \) (GSI) of GSI histograms (Fig. 2-2-ii-5). The relationship between \( r \) (GSI) and DOC concentration \( (N) \) could be expressed as highly significant regressions:

- **Spring:** \( r \) (GSI) = \( 0.27N / (0.14+N) - 0.329 \) (p<0.01)
- **Summer:** \( r \) (GSI) = \( 0.42N / (0.21+N) - 0.46 \) (p<0.01)
- **Autumn:** \( r \) (GSI) = \( 0.49N / (0.25+N) - 0.53 \) (p<0.01)
- **Winter:** \( r \) (GSI) = \( 0.32N / (0.16+N) - 0.36 \) (p<0.01)

This analytical result endorses mathematically the former finding that the GSI spectrum of a natural bacterial population can be correlated with the trophic level of the aquatic environment (Saida et al., 1998). The present result suggests also that the GSI of natural bacterial populations is a good indicator of organic pollution in the aquatic
environment.
Table 2-2-ii-1

Physico-chemical conditions (mean ± SD, n = 25) in each chamber and outside the gradostat during the experiments.

<table>
<thead>
<tr>
<th></th>
<th>1st chamber</th>
<th>2nd chamber</th>
<th>3rd chamber</th>
<th>outside</th>
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<td><strong>Water temperature</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>6.8 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>6.7 ± 0.1</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>Summer</td>
<td>7.2 ± 0.3</td>
<td>7.3 ± 0.2</td>
<td>7.3 ± 0.2</td>
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<tr>
<td>Autumn</td>
<td>7.1 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>7.3 ± 0.3</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>Winter</td>
<td>7.1 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>7.2 ± 0.1</td>
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<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Spring</td>
<td>46.4 ± 22.1</td>
<td>18.3 ± 7.9</td>
<td>2.9 ± 0.8</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Summer</td>
<td>30.1 ± 8.2</td>
<td>11.2 ± 4.2</td>
<td>4.9 ± 1.1</td>
<td>3.8 ± 1.1</td>
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<tr>
<td>Autumn</td>
<td>18.5 ± 7.6</td>
<td>8.9 ± 1.7</td>
<td>4.7 ± 0.5</td>
<td>3.4 ± 0.4</td>
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<tr>
<td>Winter</td>
<td>25.2 ± 7.7</td>
<td>9.8 ± 2.6</td>
<td>2.7 ± 0.4</td>
<td>1.9 ± 0.2</td>
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</table>

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Table 2-2-ii-2. Growth rate (mean ± SD; h⁻¹) of bacterioplankton in each gradostat chamber and its outside.

<table>
<thead>
<tr>
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<th>1st chamber</th>
<th>2nd chamber</th>
<th>3rd chamber</th>
<th>Outside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td>0.36 ± 0.08</td>
<td>0.27 ± 0.06</td>
<td>0.043 ± 0.02</td>
<td>0.017 ± 0.01</td>
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<tr>
<td>Summer</td>
<td>0.42 ± 0.10</td>
<td>0.33 ± 0.03</td>
<td>0.077 ± 0.03</td>
<td>0.047 ± 0.02</td>
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<tr>
<td>Autumn</td>
<td>0.18 ± 0.02</td>
<td>0.17 ± 0.09</td>
<td>0.031 ± 0.04</td>
<td>0.018 ± 0.02</td>
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<tr>
<td>Winter</td>
<td>0.19 ± 0.04</td>
<td>0.051 ± 0.01</td>
<td>0.029 ± 0.01</td>
<td>0.011 ± 0.01</td>
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</tbody>
</table>
Fig. 2-2-ii-1. Bacterial population growth and DO concentration of ambient water at each season. ○, 1st chamber; □, 2nd chamber; △, 3rd chamber; ▼, outside
Fig. 2-2-ii-2. Gram stain index (GSI) spectra at different growth phases of a natural oligotrophic bacterial community with experimental eutrophication in 1st chamber of the gradostat, as compared with those in the oligotrophic pond with environment (outside). ●, 1st chamber; ○, outside; ■, 1st chamber; □, outside.
Fig. 2-2-ii-3. Photomicrographs of bacterial cells in the 1st chamber at 0 h in period I (top), at 24 h in period II (middle), and at 144 h in period III during the summer experiment.
Fig. 2-2-ii-4. Characteristics of Gram stain index (GSI) spectra at the beginning of the experiment (upper), the timing of bacterial adaptation (middle), and acclimatization (bottom) to the experimental eutrophication (eutrophic in 1st chamber; mesotrophic in 3rd chamber; oligotrophic in the pond outside of the in situ gradostat) at every season. ———, 1st chamber; ————, 3rd chamber; ————, outside.
Fig. 2-2-ii-5. Effect of dissolved organic carbon (DOC) concentration in the ambient water on Gram stain index (GSI) of bacterial population.