Abundance, Production and Viability of Bacterioplankton in the Seto Inland Sea, Japan

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The abundance, specific growth rate, estimated production rate and viability of bacterioplankton were determined in surface waters of the Seto Inland Sea, Japan. Bacterioplankton were counted by epifluorescence microscopy, stained with either acridine orange (AO) or two-color dye mixture to differentiate live/dead cells based on membrane integrity. The AO-based abundance showed area-to-area variation corresponding to eutrophication levels of the areas. The abundance varied from $4.0 \times 10^8$ to $2.2 \times 10^9$ cells l$^{-1}$, and the overall average was $9.0 \times 10^8$ cells l$^{-1}$. Specific growth rate varied from 0.037 to 0.174 h$^{-1}$, with an overall average of 0.125 h$^{-1}$. Bacterial production rate, estimated by multiplying the abundance and growth rate, was within the range of 24 to 172 $\mu$g C l$^{-1}$ day$^{-1}$, with an overall average of 67 $\mu$g C l$^{-1}$ day$^{-1}$, which may correspond to or exceed phytoplankton primary production. A correlation of chlorophyll-a concentration with bacterioplankton abundance was shown, but not to bacterioplankton growth rate. The viability of bacterioplankton based on membrane integrity varied slightly from 61.8 to 75.1%, with an average of 69.7%. This viability estimate is in good agreement with those measured in coastal and inland waters by other protocols. The technique employed can serve as an alternative for estimating bacterial viability in natural waters. It is concluded that a large fraction of bacterioplankton in the Seto Inland Sea is viable and involved in bacterial production which may exceed the primary production of the area.

Keywords: Bacterioplankton, marine bacteria, abundance, production, viability, Seto Inland Sea.

1. Introduction

Marine pelagic ecosystems are sustained primarily by two types of food chains, viz. a grazing food chain and a microbial food chain (e.g., Valiela, 1995). The microbial food chain serves to recover organic drop-outs from grazing food chains, such as phytoplankton exudates and detritus (Pomeroy and Wiebe, 1993). Pelagic microbial food chains have their basis in abundantly occurring bacterioplankton (Sherr and Sherr, 1987). Bacterioplankton have considerable heterotrophic versatility (Egli, 1995) as well as a large component of pelagic secondary production (Cole et al., 1988), and thus affect the whole pelagic food web structure. The abundance and production of bacterioplankton are in good agreement with the phytoplankton abundance and production (e.g., Bird and Kalff, 1984; Cole et al., 1988; Cho and Azam, 1990) and eutrophication (e.g., Naganuma and Seki, 1993).

The Seto Inland Sea, about $2.2 \times 10^4$ km$^2$ in area, is a highly productive area that yields an annual fish catch of $> 8 \times 10^5$ tones (e.g., Okaichi et al., 1996). On the other hand, the Seto Inland Sea is heavily affected by industry, agriculture and mariculture (Yanagi, 1988), with major cities located in the close vicinity (Fig. 1). Some areas in the sea are highly eutrophicated and suffer from occasional red tides (e.g., Kamiyama, 1994, 1995; Nakamura et al., 1995). However, environmental and ecological studies have been rather limited to selected areas, and there have been only a few transverse studies to date (Okaichi et al., 1996; Uye et al., 1996); little has been reported on bacterial dynamics. This communication reports the transverse distribution of abundance and production of bacterioplankton in the Seto Inland Sea, with reference to phytoplankton abundance and production. We also report the transverse distribution of bacterial viability based on cell membrane integrity (Naganuma, 1996).

2. Materials and Methods

2.1 Sample collection

Surface water was collected during the Toyoshiomaru cruises in March, June and September 1995, and September 1996, in the areas of the Seto Inland Sea, Japan (Fig. 1, Table 1). These cruises collectively allowed transverse sampling, though a single cruise was limited to selected areas (Table
Fig. 1. Site locations of sample collection in the Seto Inland Sea, Japan.

Table 1. Sites and months of sample collection, and corresponding area names in the Seto Inland Sea, Japan.

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<tr>
<td>22</td>
<td>Harima-nada</td>
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<td>—</td>
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<td>—</td>
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<tr>
<td>23, 24</td>
<td>Osaka Bay</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>25, 26</td>
<td>Kii Channel</td>
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</table>

1) Chlorophyll-a concentration was not measured.
2) Range of surface water temperature observed during each cruise.
3) Samples were not collected.
4) Samples were collected.
5) Site 27 is not included in the Seto Inland Sea.

1) Surface water temperature was measured with a Sea Bird CTD; the temperature ranges observed during the cruises are shown in Table 1. Chlorophyll a (chl-a) concentration was measured using a Sea-Tech fluorometer and expressed as the average over the water column.

2.2 Bacterial cell count
Samples were fixed with 0.05 vol. formalin and refrigerated on board. Bacterioplankton in a known volume of a sample were collected on a Nuclepore filter (pore size, 0.2 µm) pre-blackened with Sudan Black, and fluoro-stained with 0.01% acridine orange (AO) in 10 mM phosphate buffer (pH8.0) (Hobbie et al., 1977; Zimmermann et al., 1978). The total number of AO-stained bacterial cells (NAO, cells l⁻¹) were counted with an epifluorescence microscope (Olympus BH-2).
2.3 Bacterial growth and production

Growth of bacterioplankton in the surface waters was determined by a dilution-incubation technique. Sample water was first passed through a 5 μm filter to remove bacterivores such as heterotrophic nanoflagellates. The 5 μm filtrate was then diluted 10 times in the same water; but 0.2 μm filtered. Bacteria existed in the 0.2 μm-filtrate, at a density as low as less than 10^3 cells l^-1, and the diluting water did not affect the estimate. Specific growth rates (μ, h^-1) of the bacterioplankton were calculated from the increase of bacterial numbers during incubation over 12 h at room temperature. The difference between water and room temperatures was within 3.5°C. Linearity of the regression line for time vs. logarithm of bacterial cell number was always significant, with the regression coefficient being higher than 0.9 for the sample numbers of at least 3.

Production of bacterioplankton (μg C l^-1 day^-1) was calculated by multiplying N_AO and μ (Moriarty, 1990). The cell-to-carbon conversion factor was assumed to be 2.42 × 10^-14 g C per cell, based on: an approximate average cell volume of 0.1 μm³ determined by microscopy; a specific gravity of 1.1; and a carbon content of 22% of the wet weight (Bratbak and Dundas, 1984; Simon and Azam, 1989). The approximate cell volume was determined with epifluorescence micrographs. Most of the cells were rod-shaped (as rotating ellipsoids) of 0.4–0.5 μm × 0.8–1 μm, and the volume range was 0.067–0.131 μm³, with an approximate average of 0.1 μm³. This was very close to the previous estimate for bacteria in a limited area of the Seto Inland Sea (average 0.09 to 0.1 μm³; Imai, 1984, 1987).

2.4 Differential staining based on bacterial membrane integrity

The viability of bacterioplankton in the surface waters was estimated during the cruise in March 1995. Bacterial cells were stained with mixed green and red dyes (Live/Dead BacLight Viability Kit; Molecular Probes Inc., Eugene, Oregon, U.S.A.). The dye mixture differentiates live (=green fluorescent) and dead (=red fluorescent) cells based on the membrane integrity (Molecular Probes Inc., 1993; Haugland, 1996). Bacterial cells in the sample water were green- or red-stained by incubation with the dye mixture for 15 min at room temperature, and were collected on pre-blackened Nuclepore filters (0.2 μm) for epifluorescence microscopy. The numbers of the green and red cells were counted respectively (N_G and N_R) by epifluorescence microscopy (Naganuma, 1996). The sum of the green and red cells (N_GR) was compared with the N_AO counts. The ratios of the green and red cells to N_GR were also determined.

3. Results and Discussion

3.1 Abundance of bacterioplankton

The abundance of the bacterioplankton in the Seto Inland Sea (Fig. 2) varied within the range from 4.0 × 10^8 (site 16, Aki-nada) to 2.2 × 10^9 cells l^-1 (site 13, Hiroshima Bay), with an overall average of (9.0 ± 3.9) × 10^8 cells l^-1 (n = 39). The highest abundance, at site 13, was within a range of temporal fluctuation of 1.1–4.9 × 10^9 cells l^-1, reported from a nearby site (Iwamoto et al., 1994). Our overall range of 0.4–2.2 × 10^9 cells l^-1 was in good agreement with the range of 0.7–1.5 × 10^9 cells l^-1 (annual average) for the whole Seto Inland Sea (Okaichi et al., 1996; determined by K. Tada, Kagawa University, Japan). These ranges correspond to the low to middle level in the overall review (but relatively middle for marine waters) of Bird and Kalff (1984). These ranges are also known to meso- to eutrophic coastal waters such as Shimoda Bay (Naganuma and Seki, 1993) and Kiel Bight (Rheinheimer, 1991).

The bacterioplankton abundance showed site-to-site variation, corresponding to the degree of eutrophication. The variation was limited to a relatively narrow range of magnitude 10^3 at most, but it was well correlated with the variation of chl-a concentration as a measure of eutrophication. A high abundance of > 1.5 × 10^9 cells l^-1 was found in Hiroshima Bay (site 13) and Osaka Bay (sites 23 and 24), where chl-a concentration was higher than 8 μg l^-1. The two areas are ranked as most eutrophicated points in the Seto Inland Sea (e.g., Okaichi et al., 1996; Uye et al., 1996). On the other hand, low abundance (< 10^8 cells l^-1) was commonly observed in the areas where chl-a concentration was less than 4 μg l^-1. The correlation between bacterial abundance and chl-a concentration is discussed in Subsection 3.4.

3.2 Bacterial specific growth rate

The specific growth rate (and the doubling time) of bacterioplankton was within the range of 0.037 h^-1 (18.7 h; June 1995, site 1) to 0.174 h^-1 (4.0 h; September 1995, site 23), with an overall average of 0.125 ± 0.033 h^-1 (5.5 h; n = 27), as shown in Fig. 2. This range was rather low for a eutrophic water, compared with 0.14–0.22 h^-1 in Shimoda Bay (Naganuma and Seki, 1993). The slowest growth was found at the site 1 in June, the westernmost site in the Suo-nada area. Organic input to the surface water by vertical mixing is probably depressed by strong stratification of the area (a stratification index, log H/U^3, of 3.5–4; Yanagi and Okada, 1993). Also, inflow of less polluted water from the Japan Sea might reduce the bacterial growth rate, although the bacterial abundance at site 1 did not deviate from the relationship with chl-a concentration (Fig. 3). On the other hand, the fastest growth was found at site 23, the innermost site of the highly productive Osaka Bay. However, the bacterial growth rate was not always correlated with chl-a concentration, showing a good contrast with the correlation between bacterio- and phytoplankton abundances. This is probably because the bacterial growth is easily controlled by environmental conditions such as
temperature and substrate availability. Kirchman et al. (1989) reported that the correlation between bacterio- and phyto-plankton was disrupted by the changes in temperature and salinity as well as by the input of allochthonous organic matter. Accordingly, the bacterial growth rate was thought to be controlled more by allochthonous organic carbon than phytoplankton production in the Seto Inland Sea.

The growth measurement was done with the 5 μm filtered water, from which bacterivores such as heterotrophic nanoflagellates (HNF) were to be removed. About 25% of

Fig. 2. Transverse distribution of chlorophyll-a concentration, bacterioplankton abundance, specific growth rates and estimated production rates in the Seto Inland Sea, Japan.
The estimated bacterial production was equivalent to, or seems typical of a coastal water, compared with 0.4–150 g C m$^{-2}$ yr$^{-1}$ in the Seto Inland Sea (Tada et al., in preparation, cited in Uye et al., 1996). This imbalance contradicts the generally held view that bacterial secondary production is 20–30% of primary production (Cole et al., 1988). The imbalance was also pointed out from the study of micro- and net-zooplankton production in the Seto Inland Sea, suggesting that primary production only was insufficient to support the zooplankton production (Uye et al., 1996).

The imbalance between primary and bacterial productions may be explained by the input of terrigenous dissolved organic matter (DOM). Estuaries and coastal waters are subsidized with terrigenous DOM (Hopkinson, 1985; Moran et al., 1991; Findlay et al., 1992), and terrigenous DOM is subjected to microbial utilization (Benner et al., 1995; Opsahl and Benner, 1997). The Inland Sea may receive large quantity of naturally occurring terrigenous DOM through river discharge of total $>3 \times 10^7$ m$^3$yr$^{-1}$, in addition to the organic loads from industry, agriculture and aquaculture (Yanagi, 1988). Thus, bacterial productivity based on allochthonous organic input can be larger than primary production.

### 3.3 Estimated bacterial production

Bacterial production rate (Fig. 2), estimate from the abundance by growth rate amounted from 24 µg C 1$^{-1}$day$^{-1}$ (June 1995, site 1) to 172 µg C 1$^{-1}$day$^{-1}$ (September 1996, site 23) with the overall average of 67 ± 33 µg C 1$^{-1}$day$^{-1}$ ($n = 27$). The lowest and highest productions were found at the sites 1 and 23, respectively, affected by the slowest and fastest growth rates as stated above.

The bacterial production of 24–172 µg C 1$^{-1}$day$^{-1}$ seems typical of a coastal water, compared with 0.4–150 µg C 1$^{-1}$day$^{-1}$ for various pelagic systems (Cole et al., 1988). The estimated bacterial production was equivalent to, or exceeded, the photosynthetic production in the Seto Inland Sea of Japan. Assuming the bacterial production is evenly distributed throughout the water column of 32.6 m average depth, average bacterioplankton productivity is simply calculated to be 2200 mg C m$^{-2}$day$^{-1}$, or 800 g C m$^{-2}$yr$^{-1}$. The estimated yearly bacterial production should be somewhat lower, because the estimate was based only on the July and September data. Nevertheless, a large imbalance still remains between the bacterial production and the primary production of 285 g C m$^{-2}$yr$^{-1}$ in the Seto Inland Sea (Tada et al., 1995; Findlay et al., 1996). This imbalance contradicts the generally held view that bacterial secondary production is 20–30% of primary production (Cole et al., 1988). The imbalance was also pointed out from the study of micro- and net-zooplankton production in the Seto Inland Sea, suggesting that primary production only was insufficient to support the zooplankton production (Uye et al., 1996).

### 3.4 Correlation of bacterio- and phytoplankton abundances

There was a clear correlation between phytoplankton (chl-a) and bacterioplankton abundances in the Seto Inland Sea (Fig. 3), although primary and bacterial productions showed a remarkable imbalance as discussed above. Chl-a concentration (Fig. 2) varied from 0.09 µg l$^{-1}$ (June 1995, site 6) to 18.94 µg l$^{-1}$ (June 1995, site 13), with an overall average of 4.53 ± 4.80 µg l$^{-1}$ ($n = 27$). Chl-a concentration [CA, µg l$^{-1}$] was well correlated with bacterioplankton abundance [BA, × $10^8$ l$^{-1}$] and bacterioplankton production rates in the Seto Inland Sea (Table 2). A theoretical correlation, using the Lineweaver-Berk equation, was not significant; bacterial growth was not significantly correlated, either. The CA-BA linear correlation was statistically significant ($p < 0.01$; Fig. 3), being expressed as:

<table>
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<th>Chlorophyll-a vs.</th>
<th>Mode of correlation</th>
<th>$r^*$</th>
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</thead>
<tbody>
<tr>
<td>Bact. Abundance</td>
<td>Linear</td>
<td>0.78**</td>
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<tr>
<td>Bact. Growth</td>
<td>Linear</td>
<td>0.28</td>
</tr>
<tr>
<td>Bact. Growth</td>
<td>Lineweaver-Berk</td>
<td>0.01</td>
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<tr>
<td>Bact. Production</td>
<td>Linear</td>
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<tr>
<td>Bact. Production</td>
<td>Lineweaver-Berk</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*$Correlation coefficient.

**Significant at the $p < 0.01$ level.

![Fig. 3. Correlation between the average water-column chlorophyll-a concentration and the bacterioplankton abundance in the surface water of the Seto Inland Sea, sampled in June 1995 (filled triangle), September 1995 (filled circle) and September 1996 (open circle). Bacteria data collected in other studies (×; from Okaichi et al. (1996) and Uye et al. (1996)) were overlaid but not included in the correlation analysis.](image-url)
\[ [BA] = 0.69[CA] + 6.49 \quad (r = 0.778, \ n = 27). \]

Combined with nine data from previous studies (Okaichi et al., 1996; Uye et al., 1996), the correlation is still statistically significant as:

\[ [BA] = 0.63[CA] + 7.04 \quad (r = 0.704, \ n = 36). \]

This type of correlation is well known for a wide variety of waters over large temporal and spatial scales (e.g., Bird and Kalff, 1984; Cole et al., 1988; Cho and Azam, 1990). The Seto Inland Sea is regarded as included in such waters, with the red tide (>20 $\mu$g chl-a l $^{-1}$) being an exception.

The classical view of the BA-CA correlation is that the bacterial abundance depends on the photosynthetic exudation (e.g., Pomeroy and Wiebe, 1993). However, it was recently suggested that coastal primary production may be supported by microbial regeneration of nutrients from terrigenous DOM (Opsahl and Benner, 1997). If so, it would be hard to tell the cause from the result of the BA-CA correlation.

It seems likely that the BA and CA interact as a positive feedback and are thus interdependent. This may explains why the BA and CA were well correlated against the large imbalance where bacterial production exceeds primary production. A recent report suggested that the BA and CA are correlated against the imbalance where bacterial respiration exceeds primary production in oligotrophic waters (del Giorgio et al., 1997). Our result suggests that the BA-CA correlation is still maintained in coastal waters that are heavily subsidized with allochthonous organic matter.

3.5 Differential staining vs. acridine orange staining

The sum of the green and red cells ($N_{GR}$) was positively correlated with the AO-based total bacterial count ($N_{AO}$) with a statistical significance of $p < 0.01$ (Fig. 4). The slope of the regression line was 0.972, and was close enough to 1:1 within the range of evaluation. The 1:1 relationship for $N_{AO}:N_{GR}$ has been previously reported for western areas of Hiroshima Bay and Iyo-nada (Naganuma, 1996), and this study presents the 1:1 relationship for eastern areas of the Seto Inland Sea. Thus, the 1:1 relationship can be assumed throughout the Seto Inland Sea, although applicability to other seas and fresh waters is not confirmed. If the 1:1 relationship can be assumed for any waters, the use of BacLight dyes facilitates simultaneous estimation of total bacterial numbers and the numbers presumably live cells therein.

The occurrence of “empty” bacterial cells may affect the 1:1 relationship for $N_{AO}:N_{GR}$, since the empty cells will be counted as false $N_{AO}$, but will be uncounted as $N_{GR}$. Recent estimation of “empty” cells in the free-living marine bacterial populations was as high as 24% in the Adriatic Sea (Heissenberger et al., 1996). Assuming that “empty” cells were absent in the Adriatic Sea, the proportion of “intact” cells was 45% on average, which was not far from the average “viability” of 69.7% (see below) and 59.7% (Naganuma, 1996) in the Seto Inland Sea. Simultaneous determination of “empty” cell proportion and $N_{GR}$ data will provide more meaningful information to assess the role of bacterioplankton in secondary production and carbon cycling.

3.6 Bacterial viability by differential staining

The average proportion of green (presumably live) cells was 69.7 ± 4.1%, with some geographical fluctuation from 61.8% (site 16) to 75.1% (site 24), as shown in Fig. 5. There was no significant correlation between the green cell proportion and the $N_{AO}$ or $N_{GR}$ counts. Similarly, Naganuma (1996) reported that the green cell proportion, which was 59.7 ± 5.2% on average, was little related to the level of eutrophication. The green cell proportion in this study is 10% higher than those collected during another cruise in the Seto Inland Sea (Naganuma, 1996). The difference can possibly be attributed to the difference in the sampling sites. The samples for this study were collected mainly in the eastern part of the Seto Inland Sea, while the samples for Naganuma (1996) were collected mainly in Iyo-nada, located in the western part of the Seto Inland Sea. However, the environmental factors that may explain the 10% difference in the green cell proportion remains to be explained.

Assuming that green cells are regarded as being “live” based on membrane integrity, the green cell proportion can be used to estimate the viability of the natural bacterioplankton assemblage. The green cell proportion in the previous and present studies (49.3–75.1%) were generally higher than the viability estimated by metabolic activities such as: sensitivity to nalidixic acid (maximum 39.8% in Tokyo Bay; Kogure et al., 1980); autoradiography (maxi-
Fig. 5. Distribution of bacterioplankton viability (green cell %) based on membrane integrity. Average viability of 69.7% is shown by the horizontally drawn line.

maximum > 85% in Chesapeake Bay; Tabor and Neihof, 1984; and respiratory activity (maximum 25% eutrophic lakes; Dutton et al., 1986). These metabolism-based assays have limitation due to the diverse preference for substrates and resistance to antibiotics. On the other hand, the membrane-based protocol has an advantage over the metabolic specificity, and this may be a reason for the higher viability estimates in our study. A recent viability assay based on esterase activity was as high as 75% (polluted river water; Porter et al., 1995). Our viability estimate of 49.3–75.1% is in good agreement with the data stated above, and thus can serve as an alternative to the previous protocols for estimating bacterial viability in natural waters.

Our viability estimate based on membrane integrity was among the highest of those reported by other methods based on metabolic activities. This raises a question about the definition of microbial death. A situation of membrane-active but metabolism-dead is conceivable and possibly occurs in the natural environment. Further study is needed to correlate and combine viabilities estimated by different methods and to give a background to the live/dead definition.

In conclusion, we summarized that bacterioplankton in the Seto Inland Sea are thought to make a large contribution to the biological production of the area, as most of them were viable and involved in the bacterial production, which may exceed the primary production.

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References


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