水研センター研報，第19号，53－68，平成19年
Bull. Fish. Res. Agen. No. 19, 53-68, 2007

Microbial activity and community structure in a net pen aquaculture area

Tomoko SAKAMI [*1], Katsuyuki ABO [*1] and Kazufumi TAKAYANAGI [*2]

Abstract Excess organic matter production from net-pen aquaculture farms operated in open water causes serious seawater pollution. To develop sustainable aquaculture systems we need to consider the mechanisms of organic matter cycling. Microbial communities have very important roles in cycling organic matter in seawater. To elucidate how microbial communities are affected by aquaculture activities, we examined the bacterial activity and community structure in a red sea bream aquaculture area and a neighboring, non-aquaculture, reference area in Gokasho Bay, Japan.

The bacterial activity parameters examined - abundance, production rate, and extracellular hydrolytic enzyme activity- were always higher in the aquaculture area than in the reference area, and these differences were most pronounced in surface waters in summer. The annual mean bacterial abundance and production rate in the aquaculture area were 4.7 $\times 10^9$ cells L^{-1} and 82 mg C m^{-3} day^{-1}: about 1.4 and 3.5 times, respectively, those in the reference area. The annual bacterial production per unit area was estimated as 608 g C m^{-2} y^{-1} in the aquaculture area. The difference of bacterial production between the two examined areas was 444 g (37 mol) C m^{-2} y^{-1}, which was equivalent to the organic matter loads from fish farming. This fact suggests that bacterial community in seawater could utilize organic matters as much as fish farms released into the surrounding water in this aquaculture area.

The annual mean extracellular leucine aminopeptidase activity (which represents bacterial protein degrading activity) in seawater in the aquaculture area was about twice that in the reference area. On the other hand, the activity of β-glucosidase (which represents polyhydrocarbon degrading activity) in the aquaculture area was about five times that in the reference area, indicating that, overall, β-glucosidase activity was promoted more than leucine aminopeptidase activity. These microbial activity parameters were positively correlated with the organic matter concentrations in the water, suggesting that input of organic matter from the fish farms to the surrounding waters promoted microbial activity.

We examined bacterial community structure in seawater by using a PCR-denaturant gradient gel electrophoresis (DGGE) method based on 16S-rRNA gene fragment fingerprinting. Since the level of leucine aminopeptidase activity was closely correlated with the particulate organic matter concentration, the bacterial community was separated into two categories, particle-associated (>1 μm) and free-living (<1 μm). In the free-living bacterial community, the number of DGGE bands (which corresponded to bacterial species) ranged from 2 to 12, and the DGGE profiles were similar in the aquaculture area and in the reference area. However, some bands identified as representing alpha and gamma proteobacteria were observed only in the aquaculture area from spring to autumn. In the particle-associated bacterial community, the number of DGGE bands was less than in the free-living one, and most of the bacteria were identified as cyanobacteria. However, in summer, when the particle-associated bacterial community had high hydrolytic enzyme activity, bands identified as representing

2006年6月26日受理（Received. June 26, 2006）
[*1] National Research Institute of Aquaculture, Nansei, Mie, 516-0193, Japan
[*2] Seikai National Fisheries Research Institute, 1551-8, Tairacho, Nagasaki, 851-2213, Japan

the *Cytophaga-Flavobacterium-Bacteroides* group, most isolates of which have the ability to degrade biomacromolecules, were detected in the aquaculture area, together with those of alpha and gamma proteobacteria. These results suggest that the variation in bacterial activity was related to bacterial community structure and that aquaculture activity affects the bacterial community in seawater, both quantitatively and qualitatively.

Key words: Bacterial production, hydrolytic enzyme, particle-associated bacteria, DGGE

Net pen culture is the most common form of fish aquaculture in Japan. Because it is usually conducted in small inlets where water circulation is rather small and a large amount of organic matter is input into the net pen as fish food, it can lead to the formation of harmful algal blooms and oxygen-deficient water masses, which are detrimental to cultured fish (Ministry of Agriculture, Forestry and Fisheries of Japan, 2000). To resolve these problems we first need to elucidate the cycling of the organic matter that is added to the aquaculture area. In a fish cage aquaculture, it is estimated that approximately 80% and 75% of input fish food carbon and nitrogen are released into the environment (Hall *et al.,* 1990, 1992). It is consumed by bacteria directly or after reconstructed to organic matters by phytoplankton. Bacterial production contributes to biological production at higher trophic levels in aquatic ecosystems via bacterivorous nanozooplankton (Carrick *et al*., 1991; Sanders *et al*., 1992; Jurgens *et al*., 1996; Fukami *et al*., 1999; Wieltschnig *et al*., 2000). This process is now viewed as an important pathway for the energy and material flow as well as the large-organisms-dominated classic food chain. There have been many studies of the balance of organic matter cycling in aquaculture areas (Tanaka, 1977; Hall *et al*., 1990, 1992; Foy and Rosell, 1991; Holby and Hall, 1991; Johnsen *et al.*, 1993). However, there have been few studies of microbial activity and its contribution to organic matter cycling in these areas (Moriarty, 1997; Patel *et al*., 2000; Sakami *et al.*, 2003), though the microbial community plays an important role in degradation and remineralization of organic matters.

Most dissolved organic matter in seawater is polymeric and is degraded mainly by bacterial ectoenzymes. Extracellular hydrolysis is thought to be the limiting step of organic matter degradation in aquatic ecosystems, and therefore ectoenzymatic activity is a good indicator of organic matter degradation by bacterial communities (Chrost, 1990; Chrost *et al*., 1993). We can expect the quality and quantity of organic matter loaded into an aquaculture area to be different from that in oligotrophic areas where the major organic matter source is phytoplanktonic primary production. The main ingredient of fish food is fishmeal and effluents from fish farms contain large amounts of polymeric nitrogen compounds (Foy and Rosell, 1991; McCaig *et al*., 1999). Mesocosm studies have reported that protein enrichment triggers a dynamic response in ectoenzymatic activity and population dynamics from the microbial community, whereas enrichment with starch has no effect (Chrost, 1993; Pinhassi *et al*., 1999). We can also expect the qualitative differences in organic matter between the two areas to lead to differences in bacterial organic matter utilization property indicated by ectoenzymatic activity (Sinsabaugh *et al*., 1997).

We also need to elucidate structures of microbial communities to understand their qualitative variation. However, there are few studies about whole community structure of bacteria in aquaculture areas because most of bacteria in seawater cannot culture in ordinal bacterial culturing methods (Giovannoni and Rappe, 2000). Because bacteria in seawater are the first organisms that act on organic matters loaded from fish farms, their variations of community structure may become a good indicator to assess effects of aquaculture activities on surrounding environment.

The object of this study is to elucidate how microbial communities are affected by aquaculture. We examined bacterial activity parameters such as abundance, production rate, and extracellular hydrolytic enzyme activity in a red seabream

aquaculture area. We found that these parameters were always greater in the aquaculture area than those in the reference area, and positively correlated with the concentrations of organic matter in seawater. We also examined bacterial community structure in seawater and could detect some distinctive bacteria in the aquaculture area in warm water season. These facts indicates that aquaculture activity affects the bacterial community in seawater, both quantitatively and qualitatively.

Materials and methods

Study area Gokasho Bay is located in the Sea of Kumano, which is contiguous with the Pacific Ocean (Fig. 1). The bay's mean depth is 18 m, and its area is 22 km^2. The main current runs into the eastern part of the bay. Intensive fish farms are operated in a small inlet in the western part of the bay, the Hasama-ura inlet. The area where net-pens are set covers about 2×1 km. The major product of this area is red sea bream. In 1999 the annual fish production was 1578 t and the total area of net-pens was 23,000 m^2 (Tokai Regional Agricultural Administration Office, 1999). In 1996 the fish feed added to the Hasama-ura aquaculture area was equivalent to a total of about 2225 t of carbon or 328 t of nitrogen (Yokoyama, 2002). This Hasama-ura aquaculture area often suffers hypoxic water conditions and harmful algal blooms in summer (Toda *et al*., 1994; Abo and Toda, 1995).

Bacterial abundance, production and ectoenzymatic activity

1. Sampling Water samples were collected from the aquaculture area of Hasama-ura inlet (Stn A, water depth about 18 m) and from the central part of the bay as a reference area (Stn R, water depth about 23 m) monthly from June 1999 to September 2000. Water samples were obtained at depths of 0.5 m, 10 m and 1 m above bottom for bacterial production and ectoenzymatic activity measurements. In addition to them, water samples were also obtained at depths of 2.5 m, 5 m (Stn R, Stn A) and 15 m (Stn R) for measurement of chemical parameters. Because water samples for bacterial production measurement had to be treated quickly after collection, they were collected separately (generally

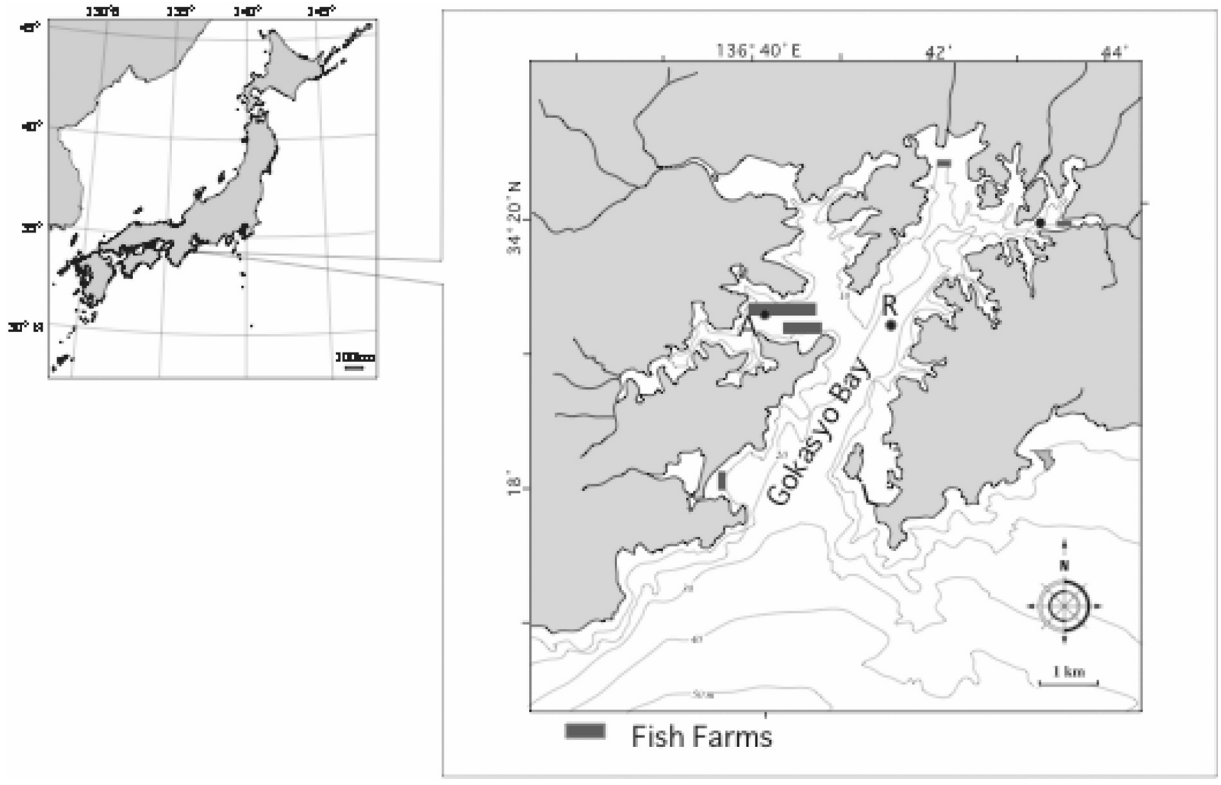

Fig. 1. Map of the Gokasho Bay study area, showing sampling stations.

the next day) from those collected for assessment of ectoenzymatic activity and measurement of chemical parameters. We checked bacterial abundance, chlorophyll-*a* concentrations and dissolved organic carbon concentrations in both water samples. Water temperature, salinity and chlorophyll-*a* concentrations were measured *in situ* with a CTD device (Alex, Kobe, Japan). Chlorophyll-*a* concentrations were calibrated on each sampling occasion by testing 3 or 4 water samples. The chlorophyll-*a* concentrations were measured by the N, N-dimethyl formamide extraction and fluorescence method (Suzuki and Ishimaru, 1990).

The water samples were carried to a land-based laboratory under cool and dark conditions immediately after collection and treated as described below.

2. Bacterial abundance Samples (30 mL) for assessment of bacterial abundance were preserved with filtered formaldehyde (2% final concentration) and stored at 4 ℃. All samples were analyzed within 1 month of collection. Bacterial abundance was determined by epifluorescence microscopy in DAPI-stained samples collected on 0.2-μm black Nuclepore filters (Porter and Feig, 1980). Duplicate filters were prepared for each sample, and more than 400 cells were counted per filter.

3. Bacterial production Bacterial production was estimated from [^{3}H]-thymidine and [^{14}C]-leucine incorporation rates (Chin-Leo and Kirchman, 1988). Duplicate 5-mL water samples were incubated in the dark at the *in situ* surface water temperature for 30 min with 10 nM [^{3}H]-thymidine and 50 nM [^{14}C]-leucine. Duplicate controls killed with formaldehyde (4% final concentration) were run in tandem with every live incubation. Samples were ice-cooled and precipitated by adding 0.5 mL of ice-cold 50% trichloroacetic acid (TCA) for more than 5 min, then passed through a 0.2-μm membrane filter and washed 3 times with 1mL of 5% ice-cold TCA. Filters were dissolved in 10mL of a scintillation cocktail (Clear-sol Ⅱ, Nacalai tesque, Kyoto, Japan), and radioactivity was read in a liquid scintillation counter (LSC-6101, Aloka, Tokyo Japan). The average difference between the two replicates was 11%.

4. Ectoenzymatic activity To estimate bacterial extracellular hydrolytic activity, we measued the potential activity of two enzymes: β-D-glucosidase and leucine aminopeptidase, which are responsible for the hydrolysis of predominating organic constituents in the dissolved organic carbon pool, i.e., β-linked polysaccharides and proteins (Chrost, 1993).

Aminopeptidase and β-glucosidase activities were measured by using fluorophore-labeled analog substrates, namely L-leucine 7-amino-4-methyl-couma rylaminide (leucine-MCA) and 4-methylumbelliferyl β-D-glucoside (MUF-β-glucoside) respectively (Hoppe, 1983). The substrates were added to duplicate water samples and 1 heat-killed (boiled for 20 min) blank at 4 different concentrations, ranging from 1 to 20 μM for leucine-MCA and 0.04 to 4 μM for MUF-β-glucoside. We chose these substrate concentrations after Rath *et al.* (1993) and from the results of preliminary experiments. The samples were incubated for 1 to 3 h at the *in situ* surface water temperature. Fluorescence was measured with a spectrophotometer (RF-5300PC, Shimadzu, Kyoto, Japan) at 365 nm excitation and 445 nm emission. The average difference between the two replicates was less than 10%. The increase in fluorescence was linear with time for incubation times used. Calibration was performed with standard solutions of 7-amino-4-methylcoumarin and 4-methylumbelliferone. The potential maximum substrate degradation rate (V_{max}) was estimated by the Michaelis-Menten kinetic model (Dowd and Riggs, 1965; Unanue *et al.*, 1999).

5. Chemical analysis Seawater samples for dissolved organic carbon (DOC) analysis were passed through pre-combusted (450℃, 4 h) Whatman GF/F glass-fiber filters. The filtrate was collected directly in muffled glass tubes sealed with Teflon-lined caps, and stored frozen until analysis. DOC concentrations were determined by high-temperature catalytic oxidation using a Shimadzu TOC-5000 analyzer.

Seawater samples for dissolved organic nitrogen (DON) analysis were passed through Whatman GF/F glass-fiber filters and stored frozen until analysis. Dissolved nitrogen was determined as total dissolved nitrogen (TDN), including both DON and dissolved inorganic nitrogen (DIN), by catalytic oxidation followed by chemiluminescent detection

of nitric oxide in a Mitsubishi Kasei Corporation TN-05 analyzer. Ammonium, nitrate, and nitrite concentrations were determined by standard colorimetric techniques (Strickland and Parsons, 1977). DON was calculated by subtracting the sum of nitrate plus nitrite and ammonia from TDN.

For particulate organic carbon and nitrogen (POC and PON) determination, seawater samples were passed through pre-combusted (450 ℃, 4 h) Whatman GF/F glass-fiber filters. The filters were dried at 80 ℃ and kept in a desiccator until analysis. POC and PON concentrations were determined by using a CHN analyzer (EA1110; Thermoquest, Milano, Italy).

Particle associated and free-living bacterial communities and their community structure

Since the level of leucine aminopeptidase activity was closely correlated with the particulate organic matter concentration, we separated the bacterial community into two categories, particle-associated (>1 μm) and free-living (<1 μm), and examined their abundance, hydrolytic enzyme activities and community structures respectively.

Water samples were collected at a depth of 0.5 m at Stn A and Stn R monthly from March 2000 to March 2001. Collected water was filtrated through a nucleopore filter of 1 μm pore size with natural gravity. The filtrated fluid was again filtrated through a nucleopore filter of 0.2 μm pore size. Bacterial abundance and extracellular hydrolytic enzyme activity was measured as described above both in the intact seawater sample and the filtrated fluid through 1 μm pore size filter. The abundance and enzymatic activity in the filtrated fluid were regarded as those of free-living bacterial community and the differences between intact seawater and filtrated fluid were regarded as those of particle associated community.

Particles trapped on the filter of 1 μm pore size was regarded as a particle associated community and that on the filter of 0.2 μm pore size was regarded as a free-living community. Each of the filters were put into a NET buffer (400 mM NaCl -20 mM EDTA-50 mM Tris; pH 9.0) and freeze immediately at −80℃ until further analysis. DNA was extracted by phenol-chloroform-isoamyl-alchole mixture and purified by ethanol precipitation. Bacterial 16S-rRNA genes were amplified by PCR using a primer set reported by Muyzer et al. (1993). The amplified gene fragments were analyzed by a denaturant gradient gel electrophoresis method (Muyzer *et al.,* 1993). Denaturant concentrations in the gel ranged from 30 to 60%. Electrophoresis was performed at 110V for 16hours in TAE buffer using the D-Code system (BioRad). The gel was stained by ethydiumbromide and its image was recorded using a Polaroid camera. Some bands were excised from the DGGE gel for sequence analysis. DNA was extracted in distilled water and reamplified. Sequence was determined after cloning using the TA Cloning Kit (Qiagen).

Results

Bacterial abundance, production and ectoenzymatic activity

Water temperature and salinity at both stations showed typical seasonal variations, and the profiles at the 2 stations were almost identical. The temperature ranged from 14.2 to 30.0 ℃ and salinity from 25.37 to 34.78 PSU. These parameters

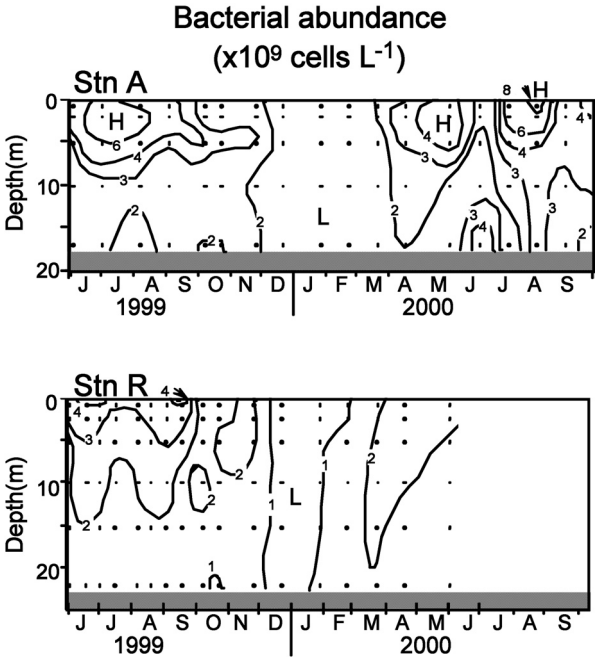

Fig. 2. Isopleth diagrams of seasonal and spatial distribution bacterial abundance in aquaculture area (Stn A) and reference area (Stn R).

indicated that the water column was stratified at around 10 m depth in summer (June to September) and well mixed in winter (October to May).

Bacterial abundance exhibited similar profiles at both stations (Fig. 2). It was high in the surface waters in summer (with the exception of June 2000) and vertically uniform in winter. Bacterial abundance at Stn A was significantly higher than that at Stn R ($p < 0.0001$, *t-test*). It ranged from 0.99 to 8.4 (2.4 in annual average) $\times 10^9$ cells L^{-1} at Stn A and from 0.51 to 4.5 (1.8 in annual average) $\times 10^9$ cells L^{-1} at Stn R.

In general, at both stations bacterial production was high in the surface waters in summer, except in August 2000, and vertically uniform in winter (Fig. 3). The thymidine incorporation rates ranged from undetectable (nd) to 144 (23 in annual average) pmol $L^{-1} h^{-1}$ at Stn R and from nd to 436 (85 in annual average) pmol $L^{-1} h^{-1}$ at Stn A. The leucine incorporation rate ranged from 0.04 to 2 (0.64 in annual average) nmol $L^{-1} h^{-1}$ at Stn R and from 0.18 to 9.2 (1.9 in annual average) nmol $L^{-1} h^{-1}$ at Stn A.

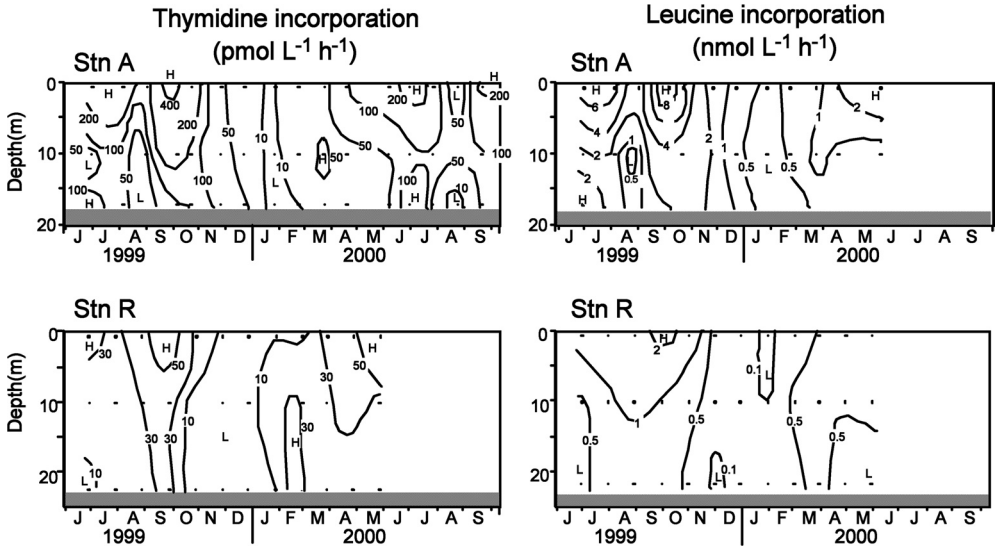

Fig. 3. Isopleth diagrams of seasonal and spatial distribution of thymidine and leucine incorporation rate in aquaculture area (Stn A) and reference area (Stn R).

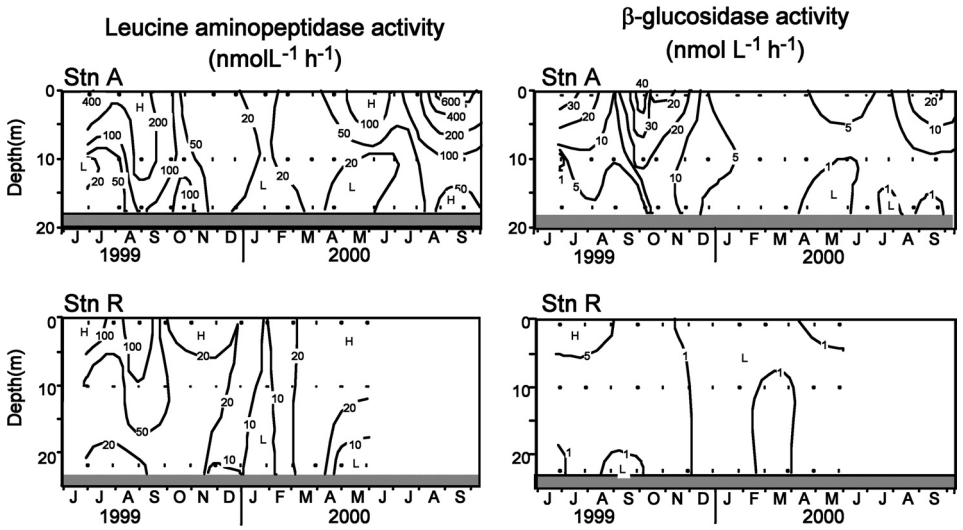

Fig. 4. Isopleth diagrams of seasonal and spatial distribution of leucine aminopeptidase and β-glucosidase activity in aquaculture area (Stn A) and reference area (Stn R).

The bacterial production at Stn A was significantly higher than that at Stn R in both of the parameters ($p < 0.0001$ in thymidine incorporation rate and $p=0.0003$ in leucine incorporation rate, *t-test*).

The profiles of potential ectoenzymatic activity were similar to those of bacterial abundance and bacterial production (Fig. 4). The activities were markedly higher in the surface water in summer, and were vertically uniform in winter at both stations. However, high leucine aminopeptidase activity was observed in the bottom waters in October, just after destratification, at Stn A. Leucine aminopeptidase activity ranged from 1.7 to 165 (32 in annual average) nmol $L^{-1} h^{-1}$ at Stn R and from 13 to 604 (74 in annual average) nmol $L^{-1} h^{-1}$ at Stn A, whereas β-glucosidase activity ranged from nd to 9.5 (1.8 in annual average) nmol $L^{-1} h^{-1}$ at Stn R and from 0.4 to 37 (8.9 in annual average) nmol $L^{-1} h^{-1}$ at Stn A. Both of the leucine aminopeptidase and β-glucosidase activities at Stn A were significantly higher than those at Stn R ($p = 0.0004$ in leucine aminopeptidase and $p<0.0001$ in β-glucosidase, *t-test*).

Organic matter concentration at both stations was shown in Table 1. The concentrations of DOC and DON were also high in the surface waters in summer and vertically uniform in winter at both stations. DOC concentrations were in the range from 51 to 116 (76 in annual average) μM at Stn R and 50 to 161 (85 in annual average) μM at Stn A. DON concentrations were in the range of 4.7 to 10 (7.8 in annual average) μM at Stn R and 3.9 to 23 (8.6 in annual average) μM at Stn A. Both of the DOC and DON concentrations were significantly higher at Stn A than at Stn R ($p=0.0002$ in DOC and $p= 0.0245$ in DON, *t-test*).

The profiles of the POC, PON, and chlorophyll-*a* concentrations were different between the two stations. Particulate organic matter concentrations were high in the surface and middle layers in May, June, and October at Stn R, and high in the surface layer in the summer at Stn A. POC concentrations

Table 1. Ranges of values (annual average from June 1999 to May 2000) of estimated parameters related to microbial activity and organic matter concentrations in aquaculture area (Stn A) and reference area (Stn R) of Gokasho Bay. (nd-not detected)

Estimated parameter	Stn A	Stn R
Bacterial abundance ($\times 10^9$ cells L^{-1})	0.99–8.4 (2.4)	0.51–4.5 (1.8)
Thymidine incorporation (pmol $L^{-1} h^{-1}$)	nd–436 (85)	nd–144 (23)
Leucine incorporation (nmol $L^{-1} h^{-1}$)	0.18–9.2 (1.9)	0.04–2 (0.64)
Leucine aminopeptidase activity (nmol $L^{-1} h^{-1}$)	13–604 (74)	1.7–165 (32)
β-Glucosidase activity (nmol $L^{-1} h^{-1}$)	0.4–37 (8.9)	nd–9.5 (1.8)
Dissolved organic carbon (μM)	50–161 (85)	51–116 (76)
Dissolved organic nitrogen (μM)	3.9–23 (8.6)	4.7–10 (7.8)
Particulate organic carbon (μM)	7.5-117 (8.0)	3.3-101 (7.8)
Particulate organic nitrogen (μM)	0.86-18 (1.1)	0.7-14 (1.0)
Chlorophyll-*a* (μg L^{-1})	0.2–45 (4.7)	0.1–26 (3.7)

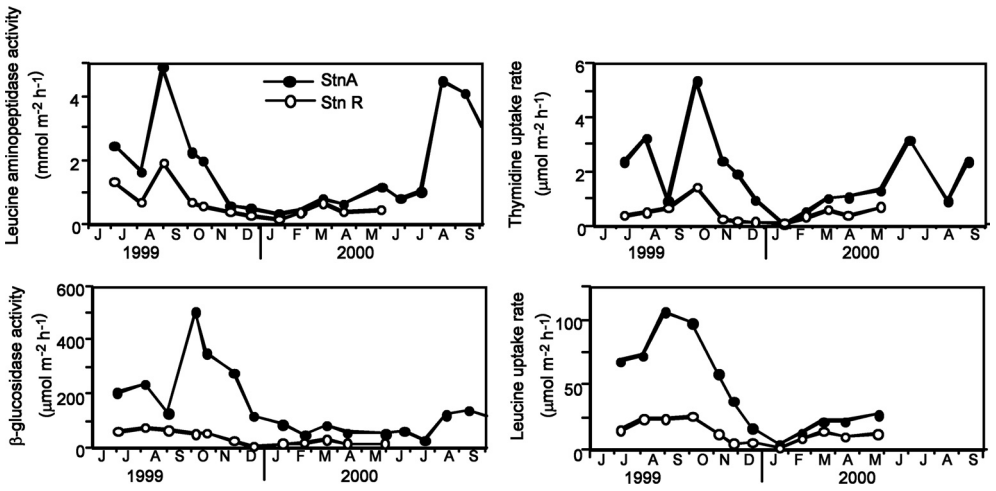

Fig. 5. Seasonal variations in bacterial production and ectoenzymatic activity per unit area in aquaculture area (Stn A; solid symbols) and reference area (Stn R; open symbols).

Table 2. Correlation coefficients between microbial activity parameters and environmental parameters in aquaculture area (Stn A) and reference area (Stn R) of Gokasho Bay. ($P< 0.05$ are shown)

Stn A	DOC	DON	POC	PON	Chl-*a*	Temp	Sal
Bacterial abundance	0.56	0.56	0.77	0.73	0.57	0.61	-0.48
Bacterial production (TdR)	0.44		0.55	0.53	0.40	0.46	-0.77
Leucine aminopeptidase	0.49		0.87	0.86	0.83	0.57	-0.58
β-glucosidase	0.37		0.63	0.62	0.59		-0.54

Stn R	DOC	DON	POC	PON	Chl-*a*	Temp	Sal
Bacterial abundance	0.59	0.42				0.44	
Bacterial production (TdR)	0.36	0.40	0.46	0.50	0.46	0.37	
Leucine aminopeptidase	0.39		0.66	0.67	0.64	0.54	-0.55
β-glucosidase	0.44		0.73	0.70	0.69	0.54	-0.53

TdR: thymidine incorporation rate; DOC / DON: dissolved organic carbon / nitrogen concentration; Chl-*a*: chlorophyll-*a* concentration; POC / PON: particulate organic carbon / nitrogen concentration: Temp: water temperature; Sal: salinity.

were in the range of 3.3 to 101 (7.8 in annual average) μM at Stn R and 7.5 to 117 (8.0 in annual average) μM at Stn A. PON concentrations were in the range of 0.70 to 14 (1.0 in annual average) μM at Stn R and 0.86 to 18 (1.1 in annual average) μM at Stn A. Chlorophyll-α concentrations were in the range of 0.1 to 26 (3.7 in annual average) μg L^{-1} at Stn R and 0.20 to 45 (4.7 in annual average) μg L^{-1} at Stn A. Differences in POC, PON, and chlorophyll-α concentrations between the stations were not significant ($p>0.05$, *t-test*).

The annual fluctuations of bacterial production and ectoenzymatic activity per unit area (water column integrated values) are shown in Figure 5. All of these microbial parameters showed greater values at Stn A than at Stn R, especially in late summer/early autumn (August and September). Total annual bacterial production was about three times greater at Stn A than at Stn R in terms of both thymidine and leucine incorporation. Total annual leucine aminopeptidase activity was about twice, and that of β-glucosidase was about five times greater at Stn A than at Stn R.

Statistical analysis indicated that the microbial activity parameters were positively well correlated with organic matter concentrations in the water at both of the stations, with exception of DON at Stn A (Table 2). Comparing the relationship between the two stations, the correlation between POC and bacterial abundance was significantly stronger at Stn A than at Stn R. Bacterial production (TdR) were significantly negatively correlated with salinity (Sal) at Stn A but not at Stn R. Leucine aminopeptidase activity showed a strong positive correlation with particulate organic matters (POC, PON, and Chl α), which was significantly greater than that with DOC at Stn A.

Abundance, hydrolytic enzyme activity and community structure of particle associated and free-living bacterial communities

Bacterial abundance of particle associated community was high in warm water season (Fig. 6). Percentage of particle associated bacterial number to the total bacterial abundance was 20% in average, indicating that free-living community was a major part in view of the abundance. In β-glucosidase activity, percentage of particle associated bacterial community was fluctuated widely but 30% in average, indicating that 70% of the enzyme activity was in the free-living community. On the other hand in leucine aminopeptidase, the particle-associated community was responsible to about 60% of the total activity, and the ratio was greater in summer with the exception of August. This fact agrees with the former observation that leucine aminopeptidase activity was strongly correlated with POM

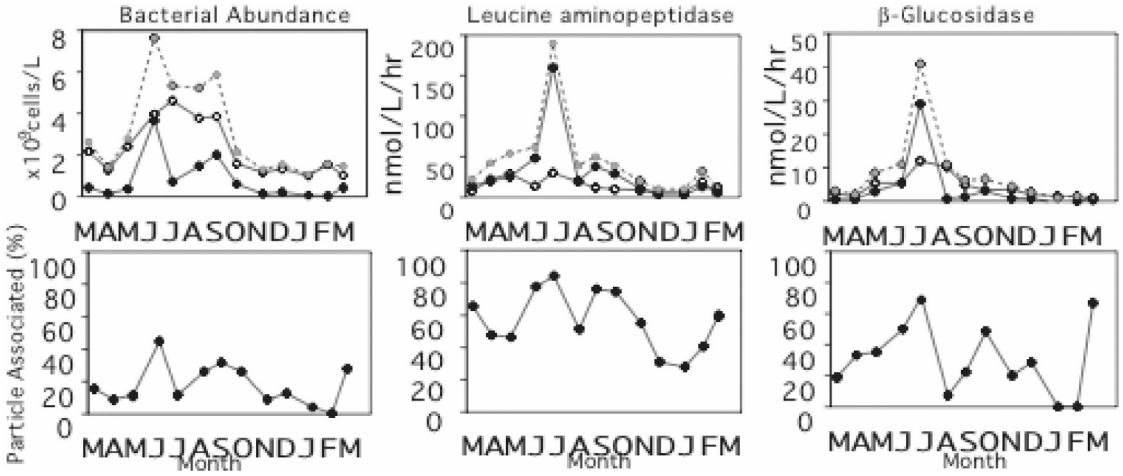

Fig. 6. Bacterial abundance and hydrolytic enzyme activities in total (gray circle with dotted line), particle associated (solid circle), and free-living (open circle) bacterial communities in surface water in aquaculture area (Stn A), and percentage of the values of particle associated community to the total.

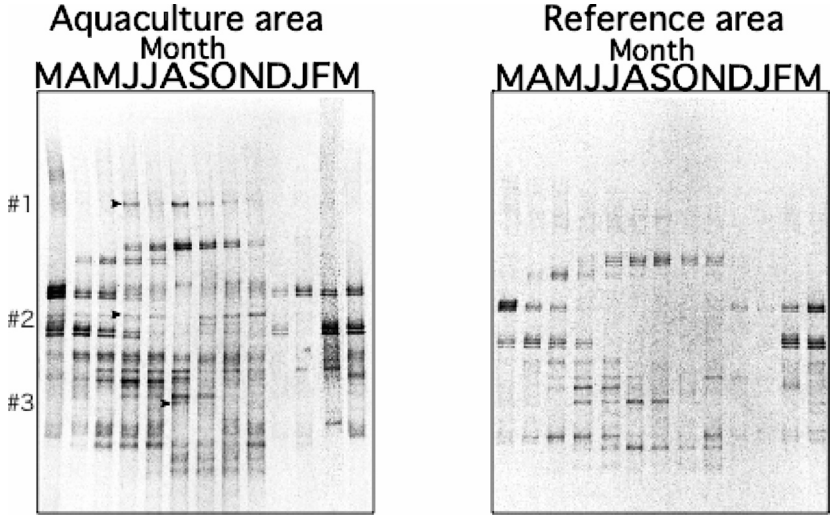

Fig. 7. DGGE profiles of the free-living (<1 μm) bacterial community composition over a year in surface water at aquaculture area and reference area.

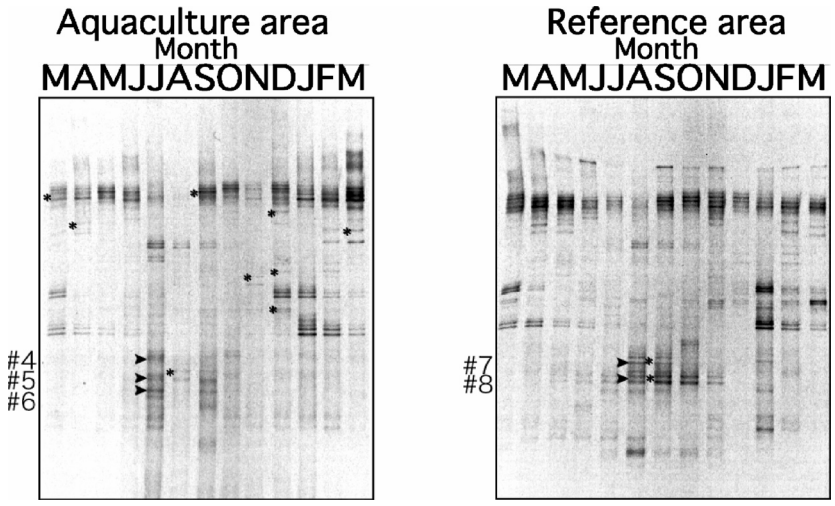

Fig. 8. DGGE profiles of the particle associated (>1 μm) bacterial community composition over a year in surface water at aquaculture area and reference area. Bands marked by star symbols were identified as representing the cyanobacteria.

concentration in the seawater.

In free-living community, the community structure shows clear seasonal variation (Fig. 7). The number of DGGE bands ranged from 2 to 12. The DGGE profile changed obviously between November and December. The DGGE profile in the aquaculture area was similar to that in the reference area. We have confirmed that most of the bands were identical position at both stations in the same month when the samples were loaded on one gel. However, some bands marked by star symbols as #1～#3 in the figure were observed only in the aquaculture area from March to November. From sequence analysis, the band of #1 was identified as representing alpha subclass of the proteobacteria whose closest identified relatives was *Roseovarius* sp.. The band of #2 was as representing gamma subclass of the proteobacteria whose closest identified relatives

was *Pseudomonas* sp., and the band of #3 was as representing *Cytophaga-Flavobacterium-Bacteroide*s group whose closest identified relatives was *Cytophaga* sp.

In the particle-associated bacterial community, the number of DGGE bands was less than in the free-living community (Fig. 8). Most of the bands were identified as representing cyanobacteria. In July and September when the particle-associated bacterial community had high hydrolytic enzyme activity, the DGGE profiles were different between aquaculture area and reference area. The bands observed specifically in the aquaculture area in July were identified as representing the *Cytophaga-Flavobacterium-Bacteroides* group (#4), and alpha (#5) and gamma (#6) subclass of the proteobacteria. On the other hand, the bands observed in the reference area (#7, 8) were identified as representing cyanobacteria, indicating that bacterial community composition was different from that in the aquaculture area.

Discussion

Bacterial production in the aquaculture area in Gokasho Bay In this study, we examined annual fluctuation of bacterial production in the aquaculture area and found that the bacterial production was correlated with organic matter concentrations in the water (Table 2) and that it was strongly stimulated in late summer/early autumn (Fig. 5). Yokoyama (2002) has shown seasonal fluctuation of organic carbon and nitrogen load in the form of fish feed into fish cages in this study area. It exhibited a similar pattern with those of bacterial production measured in this study. This fact may suggest that organic matter from fish farms supported the high bacterial production in the aquaculture area. We show the annual mean of organic carbon components in seawater measured in this study in Fig. 9. Bacterial cellular carbon and production was estimated using the ordinal conversion factors; 2×10^{18} cells mol^{-1} thymidine incorporated, and 20 fg C $cell^{-1}$ (Ducklow and Carlson, 1992). It shows that organic matters deposited in the aquaculture area by 9 μM as dissolved organic carbon and by 1 μM as bacterial cellular carbon. Bacterial production rate stimulated due to aquaculture activity (the difference between both stations) was estimated to 5 μmol C L^{-1} day^{-1}. This indicates that potential turn over time of the deposited organic matters in seawater was about 2 days in this aquaculture area.

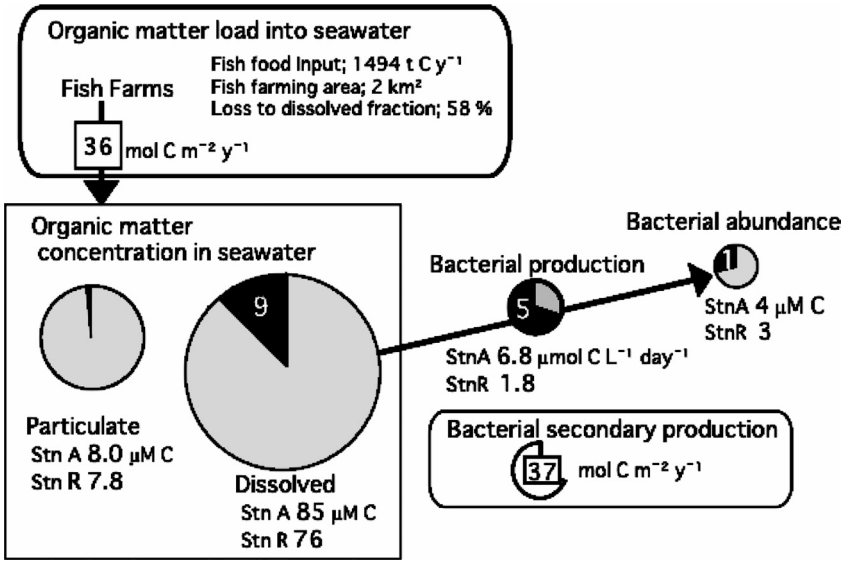

Fig. 9. Mean values of organic matter components in seawater at the aquaculture area (whole circle area) and at the reference area (shaded part), and organic matter input from fish farms.

The Bacterial production rate stimulated due to aquaculture activity was also calculated to be 37 mol C $m^{-2} y^{-1}$ as the sum of depth - integrated values of each month for a year. It was also estimated to be 6.3 mol N $m^{-2} y^{-1}$ in the aquacultrue area, using the conversion factors as C to N ratio of 5.9 (Fukuda *et al.,* 1998). On the other hand, the total amount of fish feed added to this aquaculture area in 1998 was estimated as 1494 t of carbon or 238 t of nitrogen (Yokoyama H, pers. comm. 2001). If the loss to the environment in the dissolved fraction of fish feed is assumed to be 58% for carbon (Hall *et al.,* 1990) or 48% for nitrogen (Hall *et al.,* 1992), then 867 t of carbon and 124 t of nitrogen would have been released into the surrounding water. Considering that the actual fish farming area is 2 km^2, it is calculated that 36 mol C or 4.4 mol N $m^{-2} y^{-1}$ would have been released into the surrounding water. Although these estimations depend heavily on conversion factors translating thymidine incorporation rate into bacterial carbon production (Fukuda *et al.,* 1998) and the extents of the area affected by aquaculture and feed loss, it seems that bacterial secondary production was equivalent to the amount of the organic carbon and nitrogen entering into the water from fish farming.

When the bacterial growth efficiency in the aquacultrue area was assumed to be 0.43 from del Giorgio and Cole's empirical model (del Giorgio and Cole, 1998), the bacterial carbon demand exceeded the carbon input from fish farms. This fact suggests that primary production by phytoplankton might have been also accelerated by the high load of nitrogen and phosphorous from fish farms in this study area. Sakami *et al.* (2003) has shown from a short-term observation conducted at the same study area in summer that the relative bacterial production to the chlorophyll *a* concentration was high only in the deep water in the aquaculture area, but it was invariable in surface to middle water compared with reference area. It seems that most of organic matters released from fish farms utilized by bacteria not directly but after mineralized and reconstructed by phytoplankton. On the other hand, we has shown in this annual study that the chlorophyll *a* concentration in the aquaculture area did not differ significantly from that in the reference area although the bacterial production was much higher in the aquaculture area. Functions of phytoplankton community must be cleared to understand whole organic matter flow in the aquaculture area.

Ectoenzymatic activities in the aquaculture area It should be evident that we measured the V_{max}, which is the estimated maximum enzymatic potential at saturated substrate concentration, not the direct *in situ* activity. Nevertheless, our values for both of the ectoenzymatic activities were within the range of reported activities in eutrophic estuaries (Karner *et al.*, 1992; Rath *et al.,* 1993; Talbot *et al.,* 1997; Nausch *et al,* 1998; Patel *et al.,* 2000). It should also be noted that higher values of leucine aminopeptidase activity, ranging from about 1000 to 4000 nmol $L^{-1} h^{-1}$ have been reported with organic matter enrichment in some mesocosm studies (Chrost and Rai 1993; Pinhassi *et al.,* 1999; Riemann *et al.,* 2000) and in a yellowtail aquaculture area (Patel *et al.,* 2000). The somewhat lower peaks of leucine aminopeptidase activity at the study site (604 nmol $L^{-1} h^{-1}$) may indicate a relatively low organic matter burden in this area.

Seasonal fluctuation of the ratio of β-glucosidase to leucine aminopeptidase activity was shown in Fig. 10. In general, the ratio was always high in the aquaculture area (Stn A) than that in the reference area (Stn R). Especially the ratio was high in the surface water at September, October, and the surface and middle layer water at November when fish food input was high (Yokoyama, 2002). These

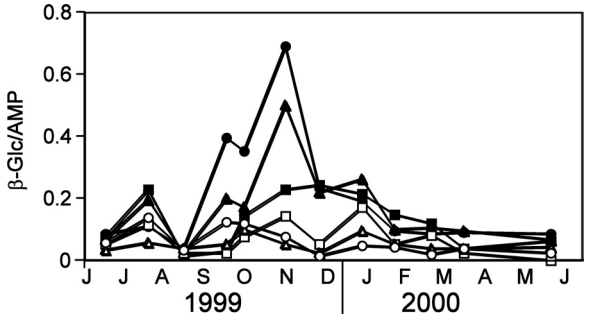

Fig. 10. Seasonal variations in ratio of β-glucosidase (β-Glc) to leucine aminopeptidase (AMP) activity at surface (circle symbols), middle (triangle symbols) and bottom (square symbols) water in aquaculture area (Stn A; solid symbols) and reference area (Stn R; open symbols).

results may indicate that aquaculture activity have stronger stimulatory effect on β-glucosidase activity than on leucine aminopeptidase activity in the aquaculture area. Previous studies have also shown that glucosidase activity is accelerated more prominently than aminopeptidase activity in some eutrophic environments (Chrost and Rai 1993; Rath *et al.,* 1993; Sinsabaugh *et al.,* 1997). On the other hand, some studies also indicated that the synthesis of glucosidase and aminopeptidase in aquatic bacteria is repressed in the presence of low-molecular weight substrates, including inorganic nitrogen, directly available for bacterial metabolism in concentrations that do not limit their growth (Chrost, 1990). Ammonia is the major excretory product of cultured fish and it might repress the aquatic microbial aminopeptidase activity in the aquaculture area. Moreover, if the β-glucosidase activitiy was related to bacterial carbon requirements, the observation that bacterial community in the aquaculture area had relatively higher β-glucosidase activity than leucine aminopeptidase activity might suggest that the bacterial community was under more carbon limit condition than that in the reference area. We measured β-glucosidase activity to assess bacterial extracellular hydrolytic activity because β-linked polysaccharides are a predominating organic constituent of DOC pool in an aquatic environment (Chrost and Rai, 1993). On the other hand, a main carbohydrate ingredient in fish food is flour, which is α-linked polysaccharides rich, and β-glucosidase activity does not concern its degradation directly. α-Glucosidase activity might promote more in the aquacultrue area than in the case of β-glucosidase.

Particle associated and free-living bacterial community

Leucine aminopeptidase activity was strongly correlated with particulate organic matter or chlorophyll-α concentrations at the aquaculture area (Table 2). The strong relationship between aminopeptidase activity and POM concentration has been reported in several previous studies (Middelboe *et al.*, 1995; Talbot *et al.*, 1997; Nausch *et al.*, 1998). It has also been shown that particle associated bacterial communities have higher per-cell activity and predominantly degrade the polymeric nitrogen substances on the particle surface where concentration of organic nitrogenous substrate is high (Karner *et al.,* 1992; Hoppe *et al.,* 1988; Smith *et al.,* 1992; Grossart and Simon, 1998; Unanue *et al.,* 1998). We also showed that particle-associated community was responsible for only 20% of abundance but 60% of LAP activity (Fig.6). In summer when their activity was very high, we could observe some specific bacteria and their closest identified relatives were *Pseudomonas* sp., *Cytophaga* sp., and *Bactroides* sp. (Fig. 8). Generally, in the *Cytophaga-Flavobacterium Bacteroides* group, most isolates have the ability to degrade biomacromolecules, such as protein, nucleic acid, and polysaccharide. Its member is expected to have a high organic matter degrading ability. Moreover, beta-subclass proteobacteria group includes a majority of culturable marine bacteria, suggesting that this group may adapt to eutrophic condition (Giovannoni and Rappe, 2000). The bacteria observed particularly in the aquaculture area probably have high organic matter degrading abilities, which explainin the high hydrolytic enzyme activities observed in the aquaculture area.

On the other hand, free-living bacterial community in the aquaculture area was responsible for 80% of abundance and 70% of β-Glc activity in average (Fig. 6). In this community, some specific species (DGGE bands) were also observed in warm water, namely high activity season (Fig. 7). The closest identified relatives of them were *Pelagibacter* (alpha subclass of proteobacteria), *Pseudomonas* (gamma subclass of proteobacteria), and *Bactroides*(*Cytophaga Flavobacterium-Bacteroides* group). They also might have high organic matter degrading ability, especially in β-linked polysaccharides.

In conclusion, we examined microbial community in an aquaculture area and have shown that aquaculture activity affects the microbial community in seawater, both quantitatively and qualitatively. The examined microbial activities were promoted markedly in the aquaculture area and the stimulated bacterial secondary production was estimated to be equivalent to the organic matter loads from fish farming. In the organic matter degrading activity, poly-hydrocarbon degrading activity was promoted more than protein degrading activity. From community structure study, some specific bacterial

species were observed at high activity season both in the particle associated and free-living bacterial community. In further study, we need to elucidate dynamics and functions of these particular bacteria to promote a better understanding of effects of aquaculture to the surrounding environment.

References

Abo, K. and Toda, S., 1995: Effects of water movement on the fluctuation of oxygen concentration in the lower layer of Gokasho Bay on the east coast of Honshu Island, Japan, in "*The twenty-fourth U.S.-Japan aquaculture panel symposium*", (ed. by Keller, B.J., Park, P. K., McVey, J.P., Takayanagi, K., and Hosoya, K.), Corpus Christi, Texas, Texas A and M University Sea Grant College Program, 1995; 85-89.

Carrick, H.J., Fahnenstiel, G.L., Stoermer, E.F., and Wetzel, R.G., 1991: The importance of zooplankton-protozoan trophic coupling in Lake Michigan. *Limnol. Oceanogr.*, **36**, 1335-1345.

Chin-Leo, G. and Kirchman, D.L., 1988: Estimating bacterial production in marine waters from the simultaneous incorporation of thymidine and leucine. *Appl. Environ. Microbiol.*, **54**, 1934-1939.

Chrost, R.J., 1990: Microbial ectoenzymes in aquatic environments. in "*Aquatic Microbial Ecology: Biochemical and Molecular Approaches*" (ed. by Overbeck, J., and Chrost, R.J.), Springer Verlag, New York, pp. 47-78.

Chrost, R.J. and Rai, H., 1993: Ectoenzyme activity and bacterial secondary production in nutrient-impoverished and nutrient-enriched freshwater mesocosms. *Microb. Ecol.*, **25**, 131-150.

del Giorgio, P.A. and Cole, L.L., 1998: Bacterial growth efficiency in natural aquatic systems. Ann. *Rev. Ecol. Syst.*, **29**, 503-541.

Dowd, J.E. and Riggs, D.S., 1965: A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. *J. Biol. Chem.*, **240**, 863-869.

Ducklow, H.W. and Carlson, C.A., 1992: Oceanic bacterial production. in "*Advances in Microbial Ecology*" (ed. by Marshall, K.C.), Vol.12, Plenum Press, New York, 113-181.

Foy, R.H. and Rosell, R., 1991: Loadings of nitrogen and phosphorus from a Northern Ireland fish farm. *Aquaculture*, **96**, 17-30.

Fukami, K., Watanabe, A., Fujita, S., Yamaola, K. and Nishijima, T., 1999: Predation on naked protozoan microzooplankton by fish larvae. *Mar. Ecol. Prog. Ser.*, **185**, 285-291.

Fukuda, R., Ogawa, H., Nagata, T., and Koike, I., 1998: Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. *Appl. Environ. Microbiol.*, **64**, 3352-3358.

Giovannoni, S. and Rappe, M., 2000: Evolution, diversity, and molecular ecology of marine prokaryotes. in *Microbial ecology of the ocean* (ed. by Kirchman, D. L.), Wiley-Liss, New York, pp. 47-84.

Grossart, H.P., and Simon, S., 1998: Bacterial colonization and microbial decomposition of limnetic organic aggregates (lake snow). *Aquat. Microb. Ecol.*, **15**, 127-140.

Hall, P.O.J., Anderson, L.G., Holby, O., Kollberg, S., and Samuelsson, M.O., 1990: Chemical fluxes and mass balances in a marine fish cage farm. I. Carbon. *Mar. Ecol. Prog. Ser.*, **70**, 61-73.

Hall, P.O.J., Holby, O., Kollberg, S., and Samuelsson, M.O., 1992: Chemical fluxes and mass balances in a marine fish cage farm. IV. Nitrogen. *Mar. Ecol. Prog. Ser.*, **89**, 81-91.

Holby, O. and Hall, P.O.J., 1991: Chemical fluxes and mass balances in a marine fish cage farm. Ⅱ. Phosphorus. *Mar. Ecol. Prog. Ser.*, **70**, 263-272.

Hoppe, H.G., 1983: Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. *Mar. Ecol. Prog. Ser.*, **11**, 299-308.

Hoppe, H.G., Kim, S.J., and Gocke, K., 1988: Microbial decomposition in aquatic environments-combined process of extracellular enzyme activity and substrate uptake. *Appl. Environ. Microbiol.*, **54**, 784-790.

Johnsen, R.I., Grahl-Nielsen, O., and Lunestad, B.T., 1993: Environmental distribution of organic waste from a marine fish farm. *Aquaculture*, **118**, 229-244.

Jurgens, K., Wickham, S.A., Rothhaupt, K.O.,and

Santer, 1996: B. Feeding rate of macro- and microzooplankton on heterotrophic nanoflagellates. *Limnol. Oceanogr.*, **41**, 1833-1839.

Karner, M., Fuks, D., and Herndl, G.J., 1992: Bacterial activity along a trophic gradient. *Microb. Ecol.*, **24**, 243-257.

McCaig, A.E., Phillips, C.J., Stephen, J.R., Kowalchuk, G.A., Harvey, S.M., Herbert, R.A., Embley, T.M., and Prosser, J.I., 1999: Nitrogen cycling and community structure of proteobacterial β-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. *Appl. Environ. Microbiol.*, **65**, 213-220.

Middelboe, M., Sondergaard, M., Letarte, Y., and Borch, N.H., 1995: Attached and free-living bacteria: production and polymer hydrolysis during a diatom bloom. *Microb. Ecol.*, **29**, 231-248.

Ministry of Agriculture, Forestry and Fisheries of Japan, 2000: Fiscal 1999 *Annual Report on Fisheries* (*White Paper*), MAFF, Tokyo, 267 pp.

Moriarty, D.W., 1997: The role of microorganisms in aquaculture ponds. *Aquaculture*, **151**, 333-349.

Muyzer, G., De Wall, E. C., and Uitterlinden, A. G., 1993: Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S r RNA. *Appl. Environ. Microbil.*, **59**, 695-700.

Nausch, M., Pollehne, F., and Kerstan, E., 1998: Extracellular enzyme activities in relation to hydrodynamics in the Pomeranian Bight (Southern Baltic Sea). *Microb. Ecol.*, **36**, 251-258.

Patel, A.B., Fukami, K., and Nishijima, T., 2000: Regulation of seasonal variability of aminopeptidase activities in surface and bottom waters of Uranouchi Inlet, Japan. *Aquat. Microb. Ecol.*, **21**, 139-149.

Pinhassi, J., Azam, F., Hemphala, J., Long, R.A., Martinez, J., Zweifel, U.L., and Hagstrom, A., 1999: Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. *Aquat. Microb. Ecol.*, **17**, 13-26.

Porter, K.G. and Feig, Y.S., 1980: The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.*, **25**, 943-948.

Rath, J., Schiller, C., and Herndl, G.J., 1993: Ectoenzymatic activity and bacterial dynamics along a trophic gradient in the Caribbean Sea. *Mar. Ecol. Prog. Ser.*, **102**, 89-96.

Riemann, L., Steward, G.F., and Azam, F., 2000: Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl. Environ. Microbiol.*, **66**, 578-587.

Sakami, T., Abo, K., and Takayanagi, K., 2003: Effects of water mass exchange on bacterial communities in an aquaculture area during summer. *Estuar. Coast. Shelf. Sci.*, **56**, 111-118.

Sanders, R.W., Caron, D.A., and Berninger, U.G. 1992: Relationships between bacteria and heterotrophic nanoplankton in marine and fresh waters: an inter-ecosystem comparison. *Mar. Ecol. Prog. Ser.*, **86**, 1-14.

Sinsabaugh, R.L., Findlay, S., Franchini, P., and Fischer, D., 1997: Enzymatic analysis of riverine bacterioplankton production. *Limnol. Oceanogr.*, **42**, 29-38.

Smith, D.C., Simon, M., Alldredge, A.L., and Azam, F., 1992: Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. *Nature*, **359**, 139-142.

Strickland, J.D.H. and Parsons, T.T., 1977: *A practical handbook of seawater analysis*. Fisheries Research Board of Canada, Ottawa.

Suzuki, R. and Ishimaru, T., 1990: An improved method for the determination of phytoplankton chlorophyll using N,N-dimethylformamide. *J. Oceanogr. Soc. Jpn.*, **46**, 190-194.

Talbot, V., Giuliano, L., Bruni, V., and Bianchi, M., 1997: Bacterial abundance, production and ectoproteolytic activity in the Strait of Magellan. *Mar. Ecol. Prog. Ser.*, **154**, 293-302.

Tanaka, Y., 1977: Decomposition process of pollutants, in "*Fish Culture in Coastal Areas and Self-Pollution* (*Senkai Yousyoku to Jika Osen*" (ed. by the Japanese Society of Scientific Fisheries). Kouseisha-Kouseikaku, Tokyo, pp. 42-51.

Toda, S., Abo, K., Honjyo, T., Yamaguchi, M., and MatsuyamaY., 1994: Effect of water exchange on the growth of red-tide dinoflagellate *Gymnodinium nagasakiense* in an inlet of Gokasho Bay, *Japan. Bull. Natl. Res. Inst.*

Aquaculture Suppl. **1**, 21-26.

Tokai Regional Agricultural Administration Office., 2000: Statistical Yearbook by Fishing District, Mie(1999)., *Association of Agriculture and Forestry Statistics*, Tsu, Japan. 2000.

Unanue, M., Azua, I., Arrieta, J.M., Labirua-Iturburu, A., Egea, L., and Iriberri, J., 1998: Bacterial colonization and ectoenzymatic activity in phytoplankton-derived model particles: cleavage of peptides and uptake of amino acids. *Microb. Ecol.*, **35**, 136-146.

Unanue, M., Ayo, B., Agis, M., Slezak, D., Herndl, G.J., and Iriberri, J., 1999: Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. *Microb. Ecol.*, **37**, 36-48.

Wieltschnig, C., Kirchner, A.K.T., Steitz, A., and Velimirov, B., 2001: Weak coupling between heterotrophic nanoflagellates and bacteria in a eutrophic freshwater environment. *Microb. Ecol.*, **42**, 159-167.

Yokoyama, H. 2002: Impact of fish and pearl farming on the benthic environments in Gokasyo Bay: Evaluation from seasonal fluctuation of the macrobanthos. *Fish. Sci.*, **68**, 258-268.