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Salinity effect on the growth rate of bacterioplankton in the Teshio River estuary during winter*

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Abstract: The Teshio River estuary in northern Japan has a "salt wedge", creating three layers—surface (river water), bottom (seawater) and intermediate (river-sea interface). Growth rates of bacterioplankton from these layers were determined under variable salinity conditions (0-50‰) during winter including freezing period. Sample bacterioplankton showed varying degree of halophilism (growth in salt conditions), ranging from nonhalophilic (surface) to slight halophilic (bottom) with the interface (intermediate) layer neither distinctly nonhalophilic nor halophilic characteristics. Bacterioplankton were also shown to be salt-tolerant at or near their optimal growth temperatures. These results suggest that bacterioplankton contribute more to estuarine productivity in winter when primary phytoplankton production declines.

1. Introduction

Since primary productivity is low in subarctic estuaries, heterotrophic bacteria greatly influence their productivity. The bacterial support of estuarine food webs through detritus food chains has been shown by: 1) high bacterial activities in water column (SEKI *et al.*, 1969) and sediments (SIZEMORE *et al.*, 1973), 2) nutrient value of detritus bacteria (DARNELL, 1961; PAERL, 1974), and 3) interaction between bacteria and bacteriovors in waters and sediments (ZOBELL, 1946; CHUA and BRINKHURST, 1973).

Estuarine bacteria have been categorized as halophiles (requiring salt) of marine origin, nonhalophiles of freshwater origin, and indigenous bacteria acclimatized to the brackish environment (e.g., SEKI *et al.*, 1969; COLWELL *et al.*, 1981). Their response to salinity, however, has been studied primarily with isolates (e.g., STANLEY and MORITA, 1968; SINGLETON *et al.*, 1982a; 1982b). To better understand bacteria halophilism and its ecological importance in estuaries, growth studies of natural bacterioplankton are preferable. Such bacteria are free-living and probably more numerous than attached epibacteria, but show relatively less metabolic activities (review in PEDROS-ALIO and BROCK, 1983; else, BELL and ALBRIGHT, 1982; PALUMBO *et al.*, 1984; ALBRIGHT *et al.*, 1986; CLARKE and JOINT, 1986). Furthermore, bacterioplankton can be more easily studied since standing stock and *in situ* growth rates can be estimated by direct microscopy, giving an approximation of their importance to estuarine productivity.

Estuarine environments in the subarctic zone are physically stable during winter because of both low water discharge from terrestrial snow and the dampening effect of surface ice on river water movement. This stability makes winter the best season to study the relationship between bacterial halophilism and the salt environment of the estuary.

In northern Japan, the Teshio River estuary shows these conditions, hence was suitable for studying salinity effects on the growth of bacterioplankton collected from layers of varying salinity.

2. Materials and methods

The Teshio River of Hokkaido is the fourth longest river in Japan (256 km) and has the tenth largest area of watershed (5,590 km^2). Its estuary is a "positive estuary", i.e., evaporation is less than the volume of entering freshwater (MCLUSKY, 1981). The seawater intrusion is more than 15 km along the river bottom, thus it is a "salt wedge type" (SEKI and EBARA, 1980). At its lowest course, the river flows parallel to the coast line but is forced to be nearly perpendicular to the Sea of Japan by a

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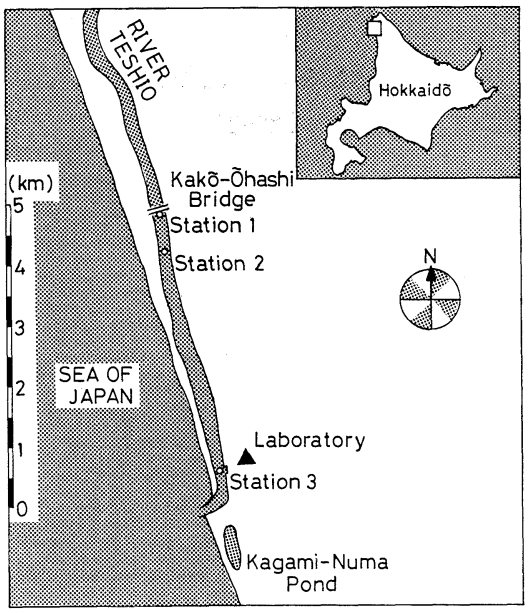

Fig. 1. Station locations in the Teshio River estuary, Hokkaido, Japan.

concrete breakwater at the estuary mouth (Fig. 1). The maximum depth of the study area was 4 to 5 m (within 5 km above the estuary mouth). This area is frozen from December to March or April every winter, with the ice cover developing first upstream. Samples were taken during four winter periods as follows: late non-freezing (October 1984), just pre-freezing (December 1985), early freezing (January 1985), late freezing (March 1984). Sampling sites (and times) were as follows: Station 1—beneath the Kakoh-Ohashi Bridge (October, January, and March), Station 2—near the edge of ice cover (December), Station 3—off the Teshio Harbour (October and December). Station 2 was selected as an alternative of Station 1 in December when thin ice cover made sampling at Station 1 dangerous. In January and March, Stations 2 and 3 were not sampled because of unavailability of boat. All samples were taken in the morning, with those from the boat when and where water was ice free (October and December) and those on the ice cover when the ice thickness was 30–50 cm at Station 1 (January and March). Vertical profiles of both water temperature and salinity were measured with a Tohodentan EST-3. Water samples were collected with sterilized Hyroht bottles (1,000 ml) from the surface to the bottom. The intermediate sampling depth was determined at each investigation according to the vertical salinity profile. In the estuary, surface water was low in salinity (below 0.2 ‰), due to the influence of river water. The bottom layer, however, received seawater from the Sea of Japan, thus its salinity was comparable to that outside the river. Samples from the intermediate layer were brackish and varied in salinity.

Immediately after sampling, pH of each water sample was measured with a Toa-Denpa DMIA, and dissolved oxygen (DO) was determined by the Winkler method. Each water sample was filtered through glass fiber filters (Whatman GF/C), thereafter both filtrate and filters were frozen for later chemical analyses.

Inorganic nitrogen (total of NO_3-N, NO_2-N and NH_4-N) and inorganic phosphorus (PO_4-P) were determined according to GOLTERMAN (1969) and STRICKLAND and PARSONS (1972). Dissolved organic carbon (DOC) in each filtrate was measured with a Beckman TOC Analyzer 915B. Particulate organic carbon (POC) on the filter was measured with a Yanagimoto CHN Analyzer MT-2. Chlorophyll *a* was determined by the methods of STRICKLAND and PARSONS (1972), using the formula of JEFFREY and HUMPHREY (1975).

To estimate standing stock and growth rate (increase in cell number), bacterioplankton were counted by the acridine orange direct count (AODC, HOBBIE *et al.*, 1977; ZIMMERMANN *et al.*, 1978; PETTIPHER, 1983) as follows: a known volume of sample water was filtered through a Nuclepore membrane filter (pore size =0.2 μm) prestained with Sudan black B solution (5 mg dissolved in absolute ethanol, which was then diluted to 50 % with distilled water). Bacterial cells on the filter were stained with several drops of acridine orange solution (10 mg dissolved in 6.6 mM phosphate buffer, pH 6.7) and thereafter were counted under a Nikon epifluorescent microscope (NAGANUMA and SEKI, 1985).

Specific *in situ* growth rates of bacterioplankton were determined with chemostats (NAGANUMA and SEKI, 1985). These rates were determined within the initial 12 hours at a dilution rate of

0.16 hr^{-1} in dark at *in situ* temperature.

Bacterial growth rates under various experimental salt conditions were determined for the following samples: October—all layers from Station 3 only, December—surface sample from Station 2 but intermediate and bottom samples from Station 3, January and March—all samples from Station 1 only. A 2 ml water sample from each depth was added to a series of test tubes, each with 20 ml of sterilized medium (sodium chloride dissolved in tap water). For growth rate determination, quintuple inoculates of each of ten salinity media (0, 5, 10, 15, 20, 25, 30, 35, 40 and 50 ‰) were incubated for a maximum of 12 hours. Inoculates were incubated at 15 and 25°C for samples from October, but at 0°C for those from December, January and March. The incubations were stopped with 1 ml of 37% formalin at known intervals in the time-course.

3. Results

Environmental Parameters

Distinct vertical stratification was observed in temperature and salinity profiles in the Teshio River estuary for all samples taken, except at Station 1 in October when river flushing water increased with heavy rain (50-80 mm) just before sampling (Fig. 2). At that time, all environmental parameters were similar to those of the surface layer (SL) at Station 3. The surface salinity of all samples was below 0.2‰. Due to intruding seawater, the bottom layer (BL) salinity ranged from 19.6‰ (January) to 35.1‰ (October). The brackish intermediate layer (IL) had salinities between those of the surface and bottom layers.

The pH was nearly neutral (6.8-7.4) at the surface, but at the bottom it was similar to seawater (8.0-8.3), except at Station 1 in March when it was 7.2. Intermediate layer pH values were between those of the surface and bottom layers.

The dissolved oxygen (DO) was high throughout the water column in all samples (Fig. 2), ranging from 10.4 to 13.1 $mgO_2 \cdot l^{-1}$ in the surface layer, 9.3 to 11.2 $mgO_2 \cdot l^{-1}$ in the intermediate layer, 7.9 to 11.7 $mgO_2 \cdot l^{-1}$ in the bottom

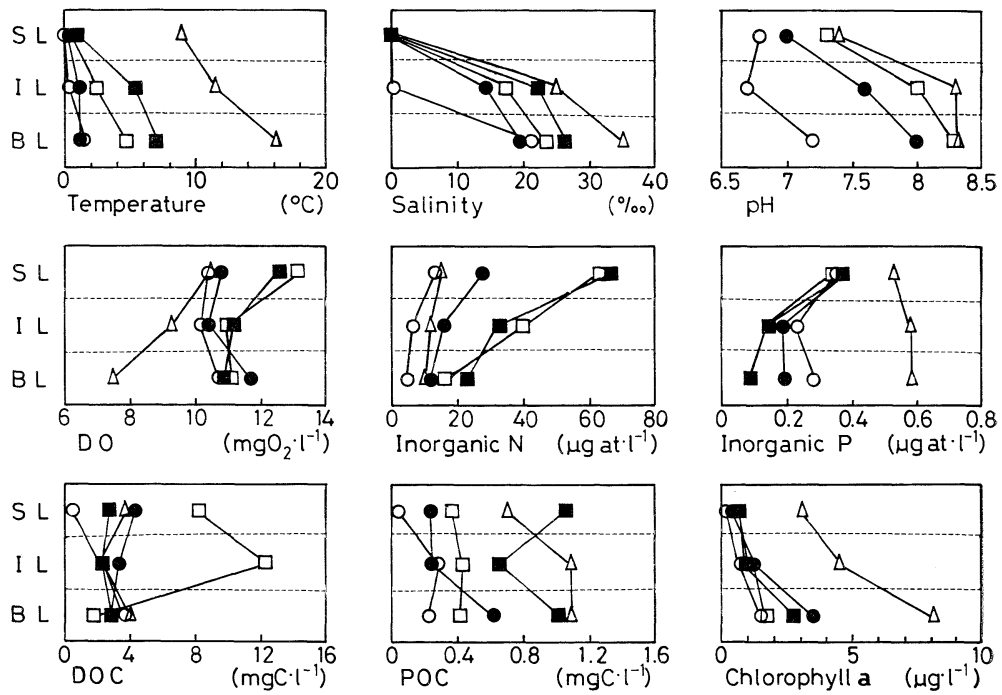

Fig. 2. Environmental measurements in the Teshio River estuary: triangle—October at Station 3, open and filled square—December at Stations 2 and 3, filled circle—January at Station 1, open circle—March at Station 1.

layer. Even the lowest DO (7.9 $mgO_2 \cdot l^{-1}$, October, Station 3, bottom) was 93 % of saturation.

The surface concentration of inorganic nitrogen (inorganic-N) was higher than those in both intermediate and bottom layers (Fig. 2). This difference was most distinct in December when concentrations of surface, intermediate and bottom layers were 63.3–67.4, 32.8–39.7 and 15.8–23.1 $\mu g\text{-at.} \cdot l^{-1}$, respectively. Throughout the study, nitrate-N made up the largest component of total inorganic-N, except in March when ammonium-N increased to more than 70% of the total. The highest nitrate-N fraction, above 90 % of the total, was observed in October.

Overall inorganic phosphorus (inorganic-P) level in the surface layer was only slightly higher than in other layers (Fig. 2), However, the inorganic-P level varied the most in the bottom layer, ranging from 0.09 to 0.58 $\mu g\text{-at.} \cdot l^{-1}$, and the least in the surface layer, 0.34 to 0.53 $\mu g\text{-at.} \cdot l^{-1}$ (Fig. 2). In winter, the inorganic-P level was higher in river water than in seawater. It was highest in October when river flushing was the greatest, but distributed evenly throughout the water column.

Dissolved organic carbon (DOC) levels were nearly the same (0.04–4.39 $mgC \cdot l^{-1}$), except those taken at Station 2 in December (Fig. 2). During that time, early winter, river flushing decreased sharply and the $mgC \cdot l^{-1}$ level rose to 8.14 $mg \cdot l^{-1}$ in the surface (river water) and 12.29 $mgC \cdot l^{-1}$ in the intermediate (brackish water).

Particulate organic carbon (POC) increased with depth in the estuary throughout the study, except at Station 3 in December when it was the highest in the surface and the lowest in the intermediate (Fig. 2). This increase was the most distinct at Station 3 in October and Station 1 in January when POC difference between river and sea water was 0.4 $mgC \cdot l^{-1}$. An increase with depth was even more obvious in the vertical profile for chlorophyll *a* content (Fig. 2).

Bacterioplankton Standing Stock Density and In Situ *Growth Rate*

Standing stock densities varied most with time of sampling, but there was some depth variation (Fig. 3). Sample densities ranged from 1.3×10^8 cells $\cdot l^{-1}$ at Station 1 in March (bottom) to 9.8×10^8 cells $\cdot l^{-1}$ at Station 3 in October (surface).

In situ growth rates, however, varied most by depth. These rates were usually the highest and showed the greatest range at the surface, 0.035 to 0.211 hr^{-1} (generation time of 19.8–3.3 hours). The overall highest surface rate was 0.211 hr^{-1} at Station 2 in December. The bottom layers usually showed the lowest growth, with a range from 0.002 to 0.87 hr^{-1} (generation time of 346–8.0 hours). The averall lowest rate was 0.002 hr^{-1} at Station 1 in January. The

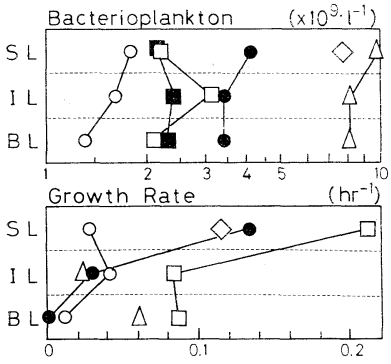

Fig. 3. Standing stock density and growth rate of bacterioplankton in the Teshio River estuary. Symbols are as in Fig. 2, with an additional symbol of diamond—October at Station 1.

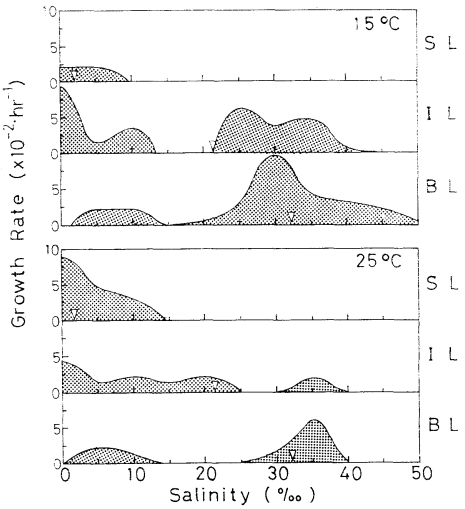

Fig. 4. Bacterioplankton growth in variable salinity in the surface, intermediate and bottom layers in the Teshio River estuary in October. Incubated at 15 and 25°C. Wedges indicate *in situ* salinities.

intermediate layer growth rate varied the least, 0.024-0.084 hr^{-1} (generation time of 28.9-8.2 hours).

Bacterioplankton Growth in Variable Salinity Conditions

October (Fig. 4)—At 15°C, surface layer growth occurred only below 10‰, with an unclear peak at 0‰. The intermediate layer growth pattern showed double bimodality with a gap (no growth) between 15 and 20‰, and bimodal curves above and below the gap. The latter showed the greatest growth around 0 and 10‰, while the former peaked at about 25 and and 35‰. Bottom layer growth was bimodal but the gap was minimal. The peaks were at 5-10‰ and 30‰, with the latter showing a much higher rate of growth.

At 25°C, surface layer growth occurred up to nearly 15‰, but was much higher at or near 0‰. Intermediate layer growth showed a gap between 25 and 30‰, with three peaks below (0, 10 and 20‰) and one above (35‰). Bottom layer growth was bimodal with peaks at 5 and 35‰ and a gap between 15 and 25‰. The latter peak was at a much higher salinity than at 15°C-incubation.

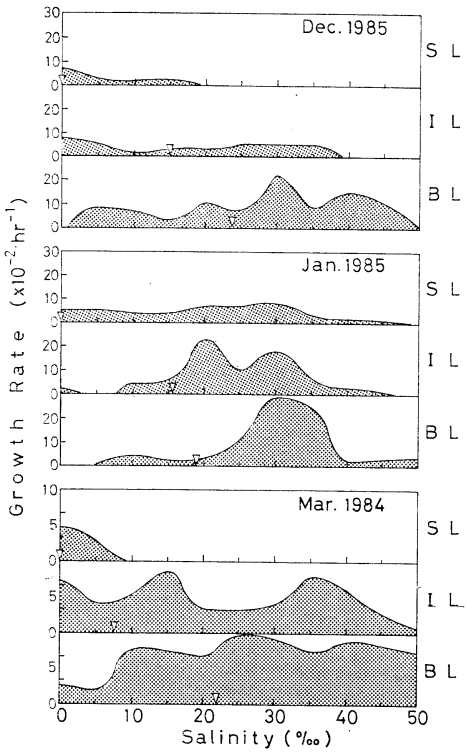

Fig. 5. Bacterioplankton growth in variable salinity in the surface, intermediate and bottom layers in the Teshio River estuary in December, January and March. Incubated at 0°C. Wedges indicate *in situ* salinities.

December (Fig. 5)—Surface layer growth occurred below 20‰, with a minimal peak around 0‰. Intermediate layer growth was not distinctly bimodal but did show slight maxima at about 0 and 30‰. Bottom layer growth was continuous from near 0 to 50‰, with peaks at 5, 20, 30 and 40‰. The most growth was at 30‰.

January (Fig. 5)—Surface layer growth occurred over the entire salinity range of 0 to 50‰, with two slight peaks at about 0 and 30‰. The intermediate layer pattern showed a small growth peak around 0‰, a short gap, and continuous growth between 15 and 45‰ with peaks at about 20 and 30‰. The bottom layer growth had two peaks, a small one at about 10‰ and much larger one at about 30‰. There was minimal growth at 20‰, although that was the concentration where the intermediate layer bacteria showed their geatest growth.

March (Fig. 5)—Surface layer growth occurred only below 10‰, with a peak at about 0‰. The intermeniate layer growth showed a continuous pattern from 0 to 50‰ but with distinct peaks at 0, 15 and 35‰, the greatest of which was at 15‰. The bottom layer showed the same range but growth from 0 to 5‰ was minimal and there were peaks at about 10, 25 and 40‰.

4. Discussion

Environment

The Teshio River, typical of those flowing through the peaty plains in Hokkaido, has a well developped bottom salt wedge in the estuary (SEKI and EBARA, 1980). During our winter study, levels of both organic and inorganic nutrients were in the mesotrophic range (nutrient-middle, The Oceanographical Society of Japan, 1975). These conditions may have been constant for many years, as evidenced by the continual commercial harvest of *Corbicula ja-*

ponica, a bivalve which inhabits the sandy bottom of nonpolluted estuaries.

The inorganic-N level (Fig. 2), especially nitrate-N, correlated negatively with salinity, forming an "estuarine sink" (SHARP *et al.*, 1982; BARNES, 1984). This "sink" was most distinct in December when the surface inorganic-N level was the highest. In October, there was a predominance of nitrate-N (above 90 % of inorganic-N), attributable to record rain fall (50–80 mm) just before sampling, as observed elsewhere (ZEDLER and ONUF, 1984). This surface-rich and bottom-poor inorganic-N distribution suggests an important role of the Teshio River as a nitrogen source for the coastal waters as well as a source of particulate organic matter (SEKI and EBARA, 1980).

The inorganic-P level was highest at the surface, except in October when the intermediate and bottom levels were higher (Fig. 2). At that time, phosphorus, bound in sediment, may have diffused into the water as soluble phosphate, perhaps due to redox potential change caused by the autumn temperature decrease and the low DO content ("estuarine source", SHARP *et al.*, 1982). The Teshio River is also a source of phosphorus for the coastal waters.

The dissolved organic carbon (DOC) level was below 4 mgC l^{-1}, except at Station 2 in December when surface and intermediate layers reached 8.14 and 12.29 mgC$\cdot l^{-1}$, respectively (Fig. 2). These local highs may have been due to both seasonal humus increase in the Sarobetsu River discharge, a tributary to the Teshio (SEKI and EBARA, 1980), and plasmolytic release from freshwater halophobic phytoplankton at the freshwater-seawater interphase ("osmotic hypothesis", MORRIS *et al.*, 1978). The plasmolytic release, however, was minor since it was only 90 μgC$\cdot l^{-1}$ at the surface, based on the relationship between carbon and chlorophyll *a* concentration (C:Chl.*a*=60:1, VOLLENWEIDER, 1974; soluble cell contents=50‰). The December Station 2 highs may also have resulted from: 1) increased nutrient quality and quantity, 2) greater light availability at the edge of the ice, 3) increased residence time of water, and 4) population interactions (CHRISTIAN *et al.*, 1984).

The particulate organic carbon (POC) increased with depth (Fig. 2), but this increase was not necessarily caused by either the bottom liquid mud flow (BARNES, 1984) or sedimentation, since the difference in the POC level between surface and bottom layers was, at most, 0.4 mgC$\cdot l^{-1}$.

The chlorophyll *a* concentration was the highest in the bottom layer (Fig. 2), as also noted by SEKI and EBARA (1980), probably from concentrations of marine diatoms such as *Chaetoceros* or allied species. The highest chlorophyll *a* levels (μg$\cdot l^{-1}$) for each layer reached in October, a time when both temperature and inorganic-P were high—surface 3.1, intermediate 4.5 and bottom 8.1. Based on the 8.1 bottom chlorophyll *a* level, diatom biomass was estimated to a maximum of 0.24 mgC$\cdot l^{-1}$ (diatom-C:Chl. *a*=31:1, RIEMANN *et al.*, 1982), comprising 22 % of the POC there.

Bacterioplankton Standing Stock Density and In Situ *Growth Rate*

Our bacterioplankton densities, made by the acridine orange direct count (AODC) method, were similar to those using phase contrast microscopy for the same river in October 1979 (SEKI and EBARA, 1980). Minor differences between the two methods can be expected in rivers with low turbidity, like the Teshio. Bacterioplankton standing stock varied by sampling times rather than by layers (Fig. 3) and was the highest in October and lowest in March. This temporal variation suggests that winter decrease of bacterioplankton standing stock is due to a high bacteriovory : bacterial propagation ratio.

The *in situ* growth rate (GR, hr^{-1}) was highly correlated with DOC concentration (C, mgC$\cdot l^{-1}$), despite the small sample size (n=4), as follows:

surface layer, $GR = 0.023 \times C + 0.024$ ($r = 0.998$)

intermediate layer, $GR = 0.995 \times C + 0.017$ ($r = 0.954$)

bottom layer, $GR = -0.043 \times C + 0.159$ ($r = -0.894$)

where r is the correlation coefficient. The high correlation coefficient suggests a close relationship between bacterial growth and DOC of terrestrial origin discharged into this subarctic estuary in winter. *In situ* growth rate was not correlated with *in situ* temperature when analyzed with the Arrhenius equation, suggesting thermal selection or acclimatization of bacterioplankton.

Table 1. Estimation of bacterioplankton production in the Teshio River estuary ($\mu gC \cdot l^{-1} \cdot day^{-1}$).

	October	December	January	March
Surface	8.5	4.5	5.2	0.6
Intermediate	1.9	2.5	0.9	0.6
Bottom	4.7	1.8	0.1	0.1

Bacterioplankton production can be estimated from standing stock and *in situ* growth rate (assuming cell volume=0.1 μm^3, specific gravity =1, water content=80 %, and carbon content in dry weight=20 %), and is summarized in Table 1. Although this bacterioplankton production is low (e.g., MURRAY and HODSON, 1985), it undoubtedly is an important component of overall estuarine productivity during frozen conditions when the primary phytoplankton production declines.

Bacterioplankton Growth in Variable Salinity Conditions

Surface layer growth of bacteria from all sample times occurred only in media with a salinity below 10–20‰, except in January when the upper limit was 50‰, perhaps because of river-sea water mixing prior to sampling (Figs. 4 and 5). Growth below 10–20‰ is characteristic for non-halophilic bacteria (LARSEN, 1962), if one accounts for some variation in nutritional conditions (JONES, 1964). Other than in January, surface layer growth showed the widest salinity range in December when the surface DOC levels peaked (8.14 $mgC \cdot l^{-1}$)—maybe organic matter can extend saline tolerance ranges of surface layer bacteria. The organic nutrient, for example, trypon has been shown to make an estuarine bacterium more salt-tolerant (SINGLETON *et al.*, 1982a). In the presence of optimal quantity and quality of organic matter estuarine bacteria may be more resistant to salinity change than expected.

Bottom layer growth showed multiple peaks, with gaps (no growth) between peaks in October, but little or no growth around 0‰ salinity (Figs. 4 and 5). These multiple peaks probably reflect various levels of halophilism within the category of "slight halophiles" (best growth at 20–50‰, LARSEN, 1962) in this estuary.

Intermediate layer growth also showed multiple peaks, probably reflecting both river bacteria, which grow best at about 0‰ salinity, and marine bacteria, approximately 35‰. Peaks which were not observed in both surface and bottom layers, however, did occur in October and January. Hence the intermediate layer was not simply a river-sea water mixture but included a unique habitat for bacteria found only in estuaries. That indigenous estuarine bacteria, neither strictly nonhalophilic nor marine, do exist. COLWELL *et al.* (1981) found an estuarine-indigenous bacterium, confirmed as such by its physiological features (SINGLETON *et al.*, 1982a; 1982b). Accordingly it is no wonder that similar or other indigenous bacteria may occur in various estuaries such as at the Teshio River mouth, despite differences in climate, topography, water quality, etc.

Temperature affected halophilism (growth in salt environment) in two ways (Fig. 4). First, for surface bacteria, the higher the temperature (25°C), the higher the growth peak, whereas for intermediate and bottom bacteria, the lower the temperature (15°C), the higher the growth peaks. Second, for surface and lower intermediate (below a gap) bacteria, the higher the temperature (25°C), the wider the range of salt tolerance, while for bottom and upper intermediate (above a gap) bacteria, the ranges were narrower. Reciprocal experiments gave reverse results. These effects of temperature-salinity interactions were similar to those associated with growth of a marine bacterium (STANLEY and MORITA, 1986; MORITA, 1975). Temperature-salinity-growth relationships can be seen in an idealized diagram that shows both halophilic-psychrophilic marine bacteria and nonhalophilic-psychrotrophic river bacteria (psychrophilic=able to grow at 0°C; psychrotrophic=able to grow at both 0°C and 25–30°C; BROCK *et al.*, 1984).

Estuarine bacterioplankton, such those in the Teshio River estuary, may be more active and contribute more to estuarine productivity than expected. This is because: 1) bacterioplankton salt-tolerance increases in the presence of organic matter, 2) "slight halophiles" (i.e., grow best at 20–50‰ salinity), an important component of the estuarine bacterioplankton community, are more responsible for various salinity, 3) indige-

nous estuarine bacteria may be more important than previously recognized, and 4) bacterioplankton are more resistant to salinity shift at optimal temperatures.

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天塩川河口域冬季におけるバクテリオプランクトン群集の成長速度におよぼす塩分の影響

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要旨：北海道北部に位置する天塩川河口域には，表層（河水）・底層（海水）・中層（両者の混合水）の3層からなる顕著な「塩水くさび」現象が観察される。これら3層に生息しているバクテリオプランクトン群集の好塩性特性を，河口域結氷期を含む冬季に研究した。バクテリオプランクトン群集の好塩性特性は，非好塩性（表層）から低度好塩性までであり，特に中層においては明らかにそのどちらでもない特徴もみられた。これらは，成長最適温度付近で耐塩性を増加させている。河口域の生産におけるバクテリオプランクトンの寄与は，特に植物プランクトンの一次生産が低下する冬季において大きくなりうることが，細菌の好塩性特性の上から明確に示された。