An Examination on the Bacterial Number and Flora in the Water of Uranouchi Inlet, Kochi Prefecture

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Abstract : The number of aerobic heterotrophic bacteria in the surface water of Uranouchi Inlet, Kochi Prefecture, was examined by using two kinds of counting media which were different in the nutrient strength. Viable counts on the 1/10-diluted medium were on the average 2.2 times larger than those on the original medium containing 0.5 % peptone and 0.01 % yeast extract. On closer examination, the ratios of chromogenic bacteria to total viable bacteria appeared on the 1/10-diluted medium were higher than those on the original medium. The proportion of the bacteria attached to suspended solids to total viable bacteria was higher in the mouth and the innermost area than in the middle area of the inlet, but the proportion was not correlated with the concentration of suspended solids. The bacterial flora in the seawater was dominated by *Acinetobacter-Moraxella* and/or *Pseudomonas*, and the latter genus having the strong ability to hydrolyze some organic substances was abounding in the middle of the inlet, near the area influenced by aquaculture.

Introduction

Uranouchi Inlet is about 12 km long ranging east and west, and lies in the central coast of Tosa Bay open to the Pacific Ocean. Since the open seawater is led to the inner area through its narrow mouth, the inlet is said to behave like a salt lake or something[1]. This means that there seems to be a gradient of environmental factors ranging from the open flushed area to the innermost stagnant area.

In the present survey, by making use of a variety of the seawater mentioned above, the effect of the dilution of counting medium on viable counts and generic composition of aerobic heterotrophic bacteria was examined.

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Materials and Methods

Sampling stations : Study area and sampling stations are illustrated in Fig. 1. The field survey was carried out on October 7, 1982. The oceanographic description in detail has been previously reported[2].

Sampling of seawater : Seawater samples for the bacteriological examination were collected from the surface layer with sterile glass bottles. Those for the determination of the amount of suspended solids were collected from the same layer with polyethylene bottles. Samples were ice-cooled on board and brought back to the laboratory. The time shared between sampling and inoculation never exceeded 3 hrs.

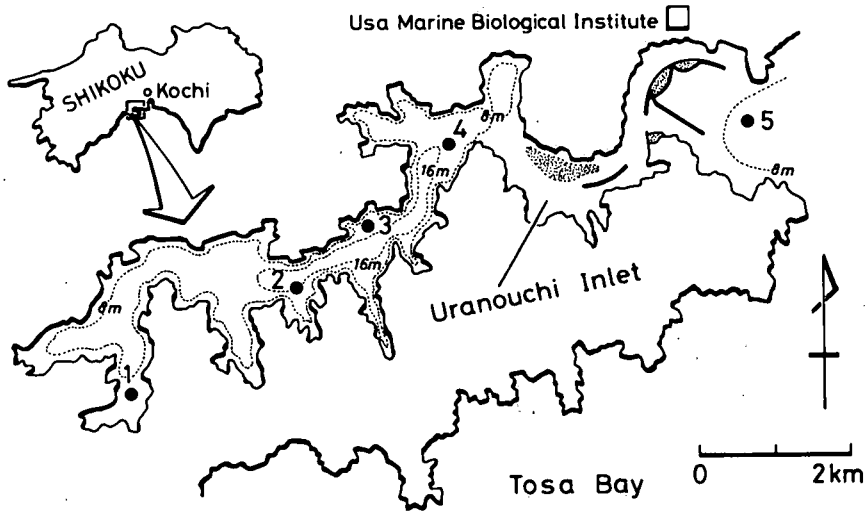

Fig. 1. Location of sampling stations in Uranouchi Inlet.

Treatment of samples: (1) Distinction of attached and unattached bacteria: A 10 ml portion of the asceptically collected seawater was screened through a 1 μm Nuclepore filter to separate the bacteria adhering to suspended solids from those floating freely. The apparatuses for screening were sterilized with a cross-fire type UV-sterilizer (Millipore, USA) before use. Nuclepore filters are said to use as screens for particulate matter in seawater[3], and the filtrate of the 1 μm filter was referred to as the suspension of discrete cells of bacteria[4]. (2) Estimation of attached bacteria: The number of attached bacteria was determined by subtracting viable count in the filtrate of a 1 μm filter from total viable count in the unscreened sample. (3) Determination of suspended solids: An adequate amount of seawater was filtered through a 0.45 μm Millipore filter, and the residue on it was weighted after having dried at 80 ℃ for 1 hr.

Enumeration of bacteria: The number of aerobic heterotrophic bacteria was determined by the spread plate technique. To disperse the attached bacteria in the unscreened seawater sample, the treatment with a Wahring blender (at 16000 rpm for 5 min) was employed in advance of enumeration. A 0.1 ml portion of each sample (unscreened seawater and 1 μm filtrate) diluted with sterile seawater was spread on the agar plates prepared with the following two kinds of media: Namely, a medium A contained 5 g Bacto-peptone (Difco), 1 g Bacto-yeast extract (Difco), 12 g Bacto-purified agar (Difco) in 1000 ml of seawater purified with activated charcoal, and the final pH value was adjusted to 7.6; while a medium B was diluted with the purified seawater to a 1/10-strength of organic nutrient in the medium A. Viable counts were obtained after incubation at 25 ℃ for 30 days. Then the colored bacterial colonies which appeared on the counting plates was recorded as chromogenic bacteria.

Isolation and identification of bacteria: About 40 colonies were picked out randomly

from the counting plates of seawater and transferred to other plates prepared with the marine agar 2216E medium. Then the bacterial isolates were roughly grouped according to the scheme of Cowan and Steel[5].

Hydrolyses of organic substances : The ability to hydrolyze the following substrates was examined: 1.0 % casein, 0.1 % chitin, 0.5 % tributyrin and 0.5 % starch with the marine agar 2216E as the basal medium. The result was recorded after having incubated at 30 ℃ for 7 days and expressed semi-quantitatively on the basis of the width of hydrolyzed zone around the bacterial colony on the plate[6].

Results

Effect of dilution of medium on bacterial counts : Fig. 2 shows the number of aerobic

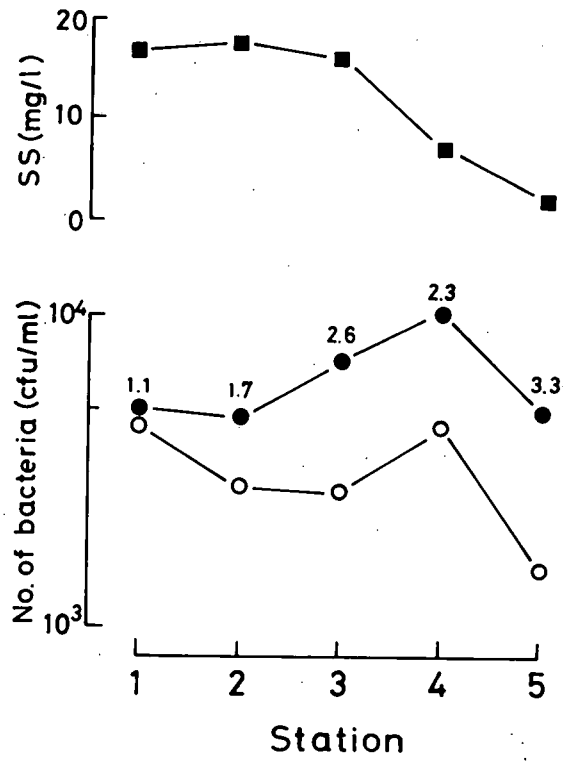

Fig. 2. Distribution of suspended solids and aerobic heterotrophic bacteria in the surface water of Uranouchi Inlet on October 7, 1982. ■ : Suspended solids (SS), ○ : Medium A, ● : Medium B. Figures indicate the ratio of viable counts (medium B/medium A).

heterotrophic bacteria determined by using two kinds of media, which were different in the concentration of organic nutrient, and the distribution of suspended solids, ranging from the mouth to the innermost area of Uranouchi Inlet. Suspended solids in the surface

water were distributed more abundantly in the inner area than in the outer area of the inlet. The amount of suspended solids varied from 2.0 to 18.4 mg/l. Other environmental properties are listed in Table 1. Viable counts of the bacteria on the medium B were

Table 1. *Some environmental properties in the surface water of Uranouchi Inlet (on October 7, 1982)*

Station No.	1	2	3	4	5
Water temp.	22.8	23.0	22.4	22.8	22.0
pH*	8.0	8.2	8.2	8.1	8.3
E_{220}**	0.204	0.232	0.226	0.203	0.184

* pH value was determined by a pH meter.
** Ultraviolet absorption of the 0.45 μm filtrate at 220 nm was determined by using a 1 cm quartz cell.

more or less larger than those on the medium A, that is, the difference ranged from 1.1 to 3.3 (on the average 2.2) times larger, and it tended to enlarge in the outer area of the inlet.

The difference in the ratio of chromogenic bacteria occurred on two kinds of media is presented in Table 2. The percentage of chromogenic bacteria to total viable bacteria

Table 2. *Percentage of the occurrence of chromogenic bacteria in the surface water of Uranouchi Inlet, which were counted with media of different nutrient strength*

Station No.	1	2	3	4	5
Medium A*	12.8 ***	11.3	12.1	13.3	19.1
Medium B**	39.4	31.2	45.0	46.2	36.8
B/A	3.1	2.8	3.7	3.5	1.9

* Medium A contains 0.5 % peptone and 0.01 % yeast extract.
** Medium B contains a 1/10-strength of the medium A.
*** The figures indicate the percentage of the number of colored colonies to total colonies appeared on counting plates.

on the medium A was on the average 13.7 %, while the percentage of those on the medium B was on the average 39.7 %.

The percentage of heterotrophic bacteria attached to suspended solids to total viable bacteria is shown in Fig. 3. It varied from 3.6 to 42.0 % on the medium A, and from 1.0 to 40.0 % on the medium B, respectively. Overall, the ratios of attached bacteria estimated on the undiluted medium were slightly higher than those on the diluted medium in all the stations surveyed.

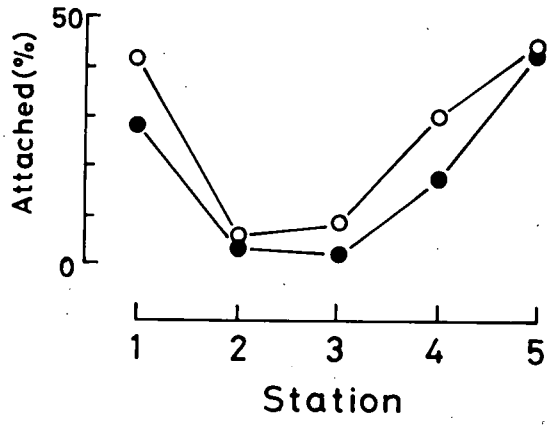

Fig. 3. Proportion (attached bacteria/total bacteria) of the bacteria attached to suspended particles larger than 1 μm in the surface water of Uranouchi Inlet. ○: Medium A, ●: Medium B.

Bacterial flora in the seawater: The percentage of generic composition of the bacterial isolates from the seawater of Uranouchi Inlet is shown in Fig. 4. Most of the bacterial

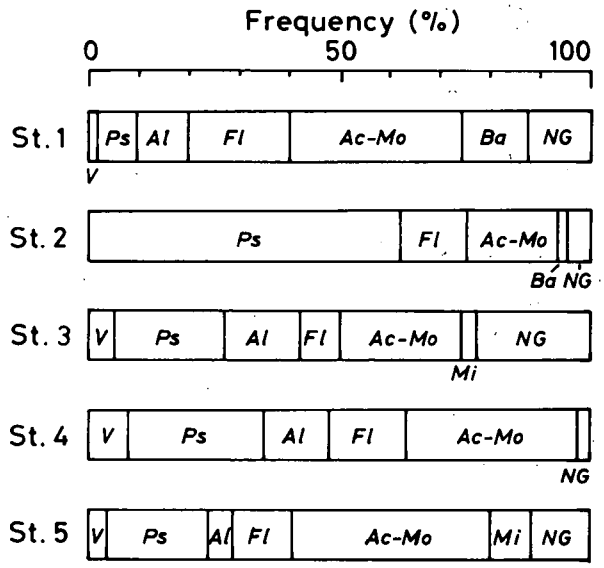

Fig. 4. Generic composition of the bacteria isolated by using medium A from the seawater of Uranouchi Inlet.
V: *Vibrio*, *Ps*: *Pseudomonas*, *Al*: *Alcaligenes*,
Fl: *Flavobacterium*, *Ac-Mo*: *Acinetobacter-Moraxella*,
Ba: *Bacillus*, *Mi*: *Micrococcus*, *NG*: No Growth.

Table 3. *Generic composition of bacterial isolates from the surface water of St. 4, Uranouchi Inlet, which were isolated with media of different nutrient strength*

Genera	Medium A *	Medium B **
Vibrio	7.5	15.0
Pseudomonas	27.5	10.0
Alcaligenes	12.5	12.5
Flavobacterium	15.0	22.5
Acinetobacter ***	35.0	20.0
No Growth	2.5	20.0

*, ** see the notes in Table 2.
*** includes *Moraxella*.

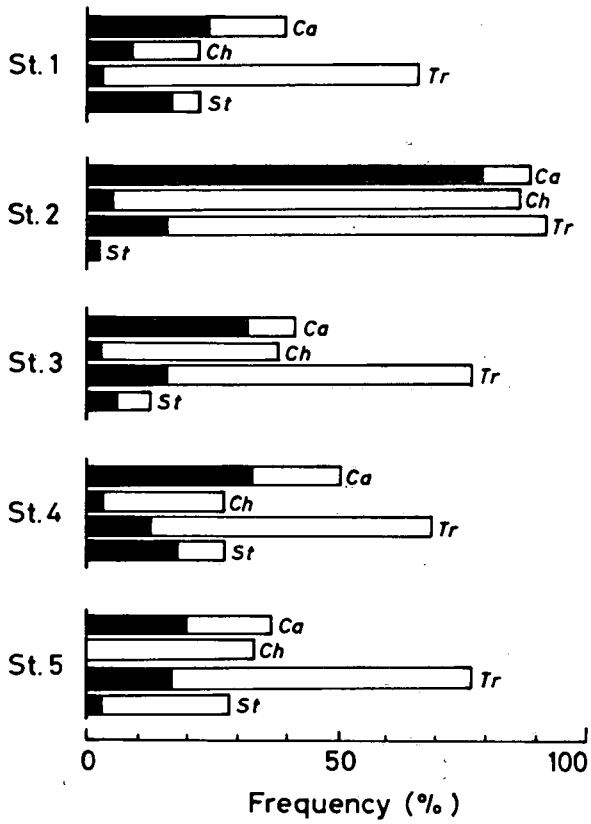

Fig. 5. Percentage of occurrence of the bacteria having the ability to hydrolyze several organic substrates.
Ca: Casein, *Ch*: Chitin, *Tr*: Tributyrin, *St*: Starch ;
□: Hydrolyzed, ■: Strongly hydrolyzed.

isolates from all the stations were Gram-negative bacteria. *Acinetobacter-Moraxella* and/or *Pseudomonas* predominated in the seawater, particularly about 60 % of the isolates from St. 2 belonged to *Pseudomonas*. *Vibrio* was not so common in every station, that is, it accounted for up to 8.0 % of the bacterial isolates. Gram-positive bacteria were scarce. *Micrococcus* was detected in the mouth of the inlet, whereas *Bacillus* was found in the inner stagnant area of the inlet.

The difference of generic composition in using the two media mentioned above is shown in Table 3. By using the diluted medium, the relative abundance of *Vibrio* and *Flavobacterium* became higher, whereas that of *Pseudomonas* and *Acinetobacter-Moraxella* became lower. In addition, 20 % of the bacteria isolated from the medium B could not grow after the first isolation.

The percentage of the bacterial isolates having the ability to hydrolyze several organic substrates is presented in Fig. 5. The bacterial isolates from St. 2 were noticed to have the greatest ability to hydrolyze casein, chitin and tributyrin. However, there was no difference in the degree of the bacterial abilities among other four stations.

Discussion

It has been known that the concentration of organic nutrient, as well as the ingredients, in counting media should affect the bacterial counts. As summarized by Hattori[7], viable counts of heterotrophic bacteria in the various kinds of natural samples were obtained 1.2 to 6.7 times larger by using the 1/10 to 1/100-diluted medium than the original medium. The same tendency was recongnized in the present survey, that is, the bacterial counts on the 1/10-diluted medium were 2.2 times greater than the undiluted original medium, which was almost alike to the marine agar 2216E and lacking for ferric phosphate. And higher counts of bacteria were obtained in the outer area than in the inner area of Uranouchi Inlet, by using the 1/10-diluted medium. Therefore, the diluted medium was effective in increasing the bacterial counts of the seawater which contained less dissolved organic matter like the outer area of the inlet.

The dilution of counting medium was effective not only in increasing the bacterial counts but also in the ratios of chromogenic bacteria to total bacteria. According to Simidu[8], the relative abundance of chromogenic bacteria in the seawater of the Pacific Ocean off New Guinea tended to become higher according as the concentration of counting medium was diluted, though it was not always a general rule. This suggests that the nutrient strength of counting media should be noted in determining the bacterial flora as well as the bacterial number.

With respect to the habitat status of heterotrophic bacteria in seawater, a large proportion of the attached bacteria was found either in the innermost or in the outermost area, but not in the central area of Uranouchi Inlet. However, this abundance of the attached bacteria is not likely to be due to the same reason, because there was a great difference between the two stations in the amount of suspended solids. On the one hand, bacterial aggregates or flocculates may be formed in the innermost stagnant waters, in which filament-

type detritus are often said to be visible to the unaided eye. On the other hand, the bacteria attached to suspended solids are thought to have somewhat of an advantage, which is based on the effect of solid surfaces[9], in the seawater with lack of enough nutrient to grow in the free-living state.

The bacterial flora in the surface water of Uranouchi Inlet was dominated by *Acinetobacter-Moraxella* and/or *Pseudomonas*. *Acinetobacter* was previously found to predominate in the highly polluted and eutrophic waters like Tokyo Bay[10]. The predominance of *Pseudomonas* was thought to be influenced by the water mass contaminated with the leavings and feces of fish in the aquaculture farms of yellow-tail. In addition, most of the bacterial isolates from the site near the farms (St. 2) had the strong ability able to decompose the organic substrates tested. The appearance of *Bacillus* in the inner area of the inlet seemed to be transferred from the surrounding land.

The result of the present study suggests that further examinations may be needed in that what concentration of counting media should be employed for the determination of the number and the flora of bacteria inhabited in natural waters.

References

1) Ohno, M., Imoto, S. and Yatsuzuka, K., Oceanographic observation on the Uranouchi Inlet, Tosa Bay, 1968-1969. *Repts. Usa Mar. Biol. Sta., Kochi Univ.*, 17, 1-41 (1970).
2) Yatsuzuka, K. and Imamura, T., On the marine conditions of Uranouchi Inlet. *Repts. Usa Mar. Biol. Sta., Kochi Univ.*, 12, 1-22 (1965).
3) Sheldon, R.W., Size separation of marine sestone by membrane and glass-fiber filters. *Limnol. Oceanogr.*, 17, 494-498 (1972).
4) Sieburth, J.McN., Smetacek, V. and Lenz, J., Pelagic ecosystem structure: Heterotrophic compartments of the plankton and their relationship to plankton size fractions. *Limnol. Oceanogr.*, 23, 1256-1263 (1978).
5) Cowan, S.T., in "Cowan and Steel's Manual for the identification of Medical Bacteria, 2nd ed." (translated by Sakazaki, R.) 62-164, Kindai Shuppan, Tokyo (1974).
6) Konda, T. and Tezuka, Y., Physiological properties of aerobic heterotrophic bacteria isolated from the water and sediment of Lake Motosu-ko. *Bull. Japan. Soc. Sci. Fish.*, 46, 781 (1980).
7) Hattori, T., in "Biseibutsu Seitai Nyumon" (Introduction to Microbial Ecology), 14-18, Tokyo Daigaku Shuppan-kai, Tokyo (1978).
8) Simidu, U., in "Umi no Biseibutsu-tachi" (Microbes in the Sea), 29-30, Otsuki Shoten, Tokyo (1982).
9) ZoBell, C.E., The effect of solid surfaces upon bacterial activity. *J. Bacteriol.*, 46, 39-56 (1943).
10) Simidu, U., Kaneko, E. and Taga, N., Microbiological studies of Tokyo Bay. *Microb. Ecol.*, 3, 173-191 (1977).

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