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Silica Gel Medium for Enumeration of Petroleumlytic Microorganisms in the Marine Environment

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A silica gel medium was developed for the enumeration of petroleumlytic microorganisms in the marine environment. The medium was satisfactorily used for the investigation of the vertical distribution of bacteria in seawater from the surface to 1,000 m depth of western north Pacific central water as well as the neritic region of Japan.

Petroleum pollution has been chronic in seas and oceans (9, 10). Although many bacteria and allied microorganisms are known to decompose and utilize this pollutant, there have been only a few reports on the precise distribution of these microorganisms in marine environments (9). One of the reasons might be the absence of a desirable method for the determination of their population densities in seawater.

Liquid media have usually been used for the viable count of petroleumlytic bacteria (1, 10, 11), but the use of plate media is desirable with the use of filters for the concentration of microorganisms in seawater where population densities are low. Agar is usually used as the solidifying agent for plate media. However, as agar-decomposing bacteria are often more numerous than petroleumlytic microorganisms in seawater (H. Seki, H. Abe, and H. Numanoi, Proc. Annu. Meet. Oceanogr. Soc. Japan, no. 236, 1973), agar plates may be liquefied during incubation and colony counting becomes impossible. The use of biochemically inert solidifiers such as silica is necessary. Moreover, for the concentration of microorganisms, inert filters, such as glass filters, may be used.

In this study, a silica gel medium (3) was developed for the enumeration of petroleumlytic microorganisms and used in the microbiological investigation aboard the research vessel Hakuho-maru of the University of Tokyo during the Leg KH-73-1 expedition in 1973 (A. Hattori, in press).

MATERIALS AND METHODS

Preparation of silica gel medium: (i) silica solution. Sodium silicate (30 g) (Kokusan Chemical Works Ltd, Tokyo) was dissolved in 500 ml of distilled water at 50 C in a water bath. After the distilled water in the ion-exchange column (3) was displaced, the silica solution was passed through the resin at a rate of 5 ml per min. The pH of the silicic acid solution collected was approximately 2.0.

(ii) Petroleum emulsion. The mixture of 10 ml of light oil and 90 ml of distilled water with 0.05 g of Tween 80 was emulsified by an ultrasonic oscillator for about 10 min.

(iii) Inorganic nutrients. Na_2HPO_4 (1.5 g) and KNO_3 (0.5 g) were dissolved into 40 ml of distilled water. Then the solution was made to the final volume of 50 ml with distilled water.

Aged seawater was concentrated from 1,000 ml to 350 ml by a rotary evaporator. The ammonium salts in the concentrated seawater were adjusted to approximately 0.5 mg of N per ml after autoclaving.

(iv) Autoclaving. Each solution was autoclaved separately at 120 C for about 30 min.

(v) Mixture. After autoclaving, the petroleum emulsion and inorganic nutrient solutions were mixed, and the pH of the mixture was adjusted with autoclaved 1 N NaOH or HCl to make a final pH of 7.8 for the silica gel medium.

After each solution was cooled in a water bath to 5 C, 10 ml of the mixture of petroleum emulsion and inorganic nutrients was poured into a petri dish, followed by the addition of 10 ml of silica solution. The solutions were mixed thoroughly by swirling each dish. The medium started to gel about 60 min after mixing but the medium should be stored at 20 C for at least a week before use.

Enumeration of microorganisms in seawater. Seawater samples were collected with sterilized J-Z type bacteriological samplers (ORIT samplers). All the microbiological treatments were done within several hours of sampling on board the research vessel.

Total bacteria and heterotrophic bacteria were enumerated by a direct microscope count method and a plate count method, respectively (5). The microorganisms in 100-ml samples were collected onto autoclaved Gelman glass fiber filters type A (diameter, 4.7

cm; pore size, 0.3 μm) by filtration. The inoculated filter was placed on the silica gel medium and incubated at 20 C for 30 days before counting colonies.

RESULTS

The results with this method for the enumeration of petroleumlytic microorganisms showed good agreement with those obtained by using a liquid medium (1) at the coastal region (Table 1).

Petroleumlytic bacteria could be enumerated by the method employed here in the pelagic region of the Pacific Ocean, as well as in the coastal region of Japan. Vertical distribution of petroleumlytic bacteria in the microbial flora of seawater at a station (22° 00.2' N, 125° 51.9' E) is shown in Fig. 1. Petroleumlytic bacteria formed colonies from only 44 samples among the 104 samples from the pelagic region, and usually less than 10 colonies of petroleumlytic bacteria were enumerated for each 100-ml seawater sample from the surface to 1,000 m depth at stations in the western north Pacific central water (7), whereas more than 10 colonies of the bacteria were enumerated for each 100-ml seawater sample in Tokyo Bay (Table 1). This means that less than 0.001% of the total bacteria or less than 0.1% of the heterotrophic bacteria are petroleumlytic at the pelagic region.

DISCUSSION

When a glass filter was placed on the silica gel medium before the medium was completely gelled, water leaked out from the medium onto the filter, probably because the petroleum emulsion is inhibitory to the gelation of silica

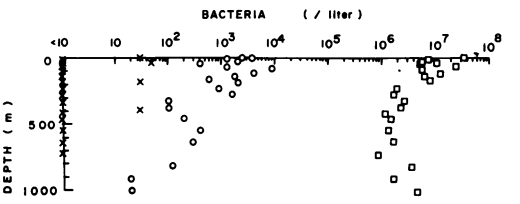

FIG. 1. *Vertical distribution of petroleumlytic bacteria, heterotrophic bacteria, and total bacteria in seawater at a station in the northwestern Pacific central water on 2 February 1973. Symbols: □, total bacteria; ○, heterotrophic bacteria; ×, petroleumlytic bacteria.*

solution. This caused spreading and made it impossible for each petroleumlytic microorganism to form an individual colony. Such leaking water is usually not expected in a silica gel medium without oil emulsion (3, 4, 6). The medium must be completely gelled before use.

The pH or temperature has been shown to relate to the gelation of silica solution media (3). Before and after autoclaving, the pH of the prepared silica solution was approximately 2.0. As the optimal pH for marine microorganisms has been shown to be between 7.6 and 7.8 (8), and as the silica solution media started to gel quickly (within 60 min) at 20 C and pH 7.8 (3), the mixture of solutions prepared for the medium must be carried out at pH 7.8.

When storage of plates with the mixture of solutions was at about 5 C, no water leaked out unless a glass filter was placed on the medium. On the other hand, when the filter was placed on the medium after different periods of storage, less water leaked from the medium after

TABLE 1. *Petroleumlytic microorganisms (bacteria/100 ml) in seawater of Tokyo Bay as enumerated by a liquid medium (1) and the silica gel medium, January 1973*[a]

Sample	At a station in the gyre in the inner part of Tokyo Bay (35° 31.6' N, 139° 53.9' E)			At a station in the gyre in the middle part of Tokyo Bay (35° 18.1' N, 139° 44.2' E)			At a station in the entrance part of Tokyo Bay (35° 08.5' N, 139° 44.2' E)		
	Silica gel medium	Liquid medium	Count index[b]	Silica gel medium	Liquid medium	Count index[b]	Silica gel medium	Liquid medium	Count index[b]
1	46	43	107	38	46	83	1	1.8	56
2	48	46	104	33	31	106	7	3.6	194
3	69	76	91	51	49	104	4	5.4	74
4	53	58	91	40	43	93	8	3.6	222
5	40	33	121	35	33	106	1	0	
6	53	28	189	38	46	83	2	5.6	36
7	49	58	84	64	84	76	8	3.7	216
8	45	31	145	33	29	114	4	7.4	54
9	70	84	83	30	25	120	3	1.8	167
10	54	49	110	53	64	83	6	7.2	83

[a] Seki, Abe, and Numanoi, Proc. Annu. Meet. Oceanogr. Soc. Japan, no. 236, 1973.

[b] Count index: (bacterial number by silica gel medium/bacterial number by liquid medium) × 100 (%). Average of count index: 113, 97, 122, respectively.

longer storage, probably due to the progress of the gelation before placing the filter. Finally, no water leaked onto the filter from the medium if the storage was more than 7 days before placing the filter (Fig. 2).

Organic nutrients available in the medium for inoculated microorganisms are petroleum (10 g/liter of medium), Tween 80 (0.05 g/liter of medium), and particulate organic matter collected with microorganisms on the filter. The medium without petroleum was shown to sustain no visible colonial growth of microorganisms for more than 100 samples collected at both the neritic and pelagic regions. As the concentration of particulate organic matter in seawater is 0.1 mg/liter on the average (2), the collected organic matter is calculated to be approximately 0.01 mg in about 1 ml of seawater absorbed onto the filter when 100 ml of water sample was examined. This corresponds to concentrations of 0.003 g of carbohydrate and 0.005 g of amino acids per liter of water in the filter. As these nutrients in natural water and Tween 80 can only be used as trace elements for the multiplication of microorganisms which form macrocolonies on the plate, only petroleumlytic microorganisms can form macrocolonies.

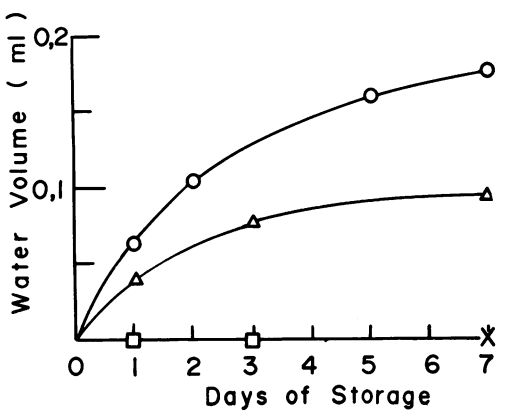

FIG. 2. *Water volume leakage from the silica gel medium when the glass filter was placed on the plate after plate preparation of:* O, *2 days;* Δ, *3 days;* ×, *7 days; and* □, *17 days.*

Although it was shown that petroleumlytic microorganisms in the sea could be satisfactorily enumerated by this method, more than 100 ml of seawater samples must be filtered to estimate precisely their numerical distribution from the surface to 1,000 m at the pelagic region of the oceans.

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