海域における有機物の無機化について

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Mineralization of Organic Substances in Sea Water*1

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A simplified method for the determination of rate of mineralization of organic matter is devised by using a diffusion technique instead of an aeration method. The apparatus is a 50 ml amber glass Erlenmyer flask with an inner chamber. In the inner chamber, Hyamine solution in methanol and an accordion-folded piece of filter paper are placed, and in the outer chamber, microorganisms collected on a Millipore HA filter by filtering sea water, the filtrate and uniformly [^{14}C] labelled substrate are added. After sealing tightly with a serum rubber cap, the reaction is initiated in a motor-drive incubator. The reaction is stopped by adding acid through the rubber cap with a syringe. Further incubation is continued to accomplish the absorption of CO_2 into the inner chamber. The radioactivity of [^{14}C] CO_2 derived from the mineralization is measured by a liquid scintillation spectrometer. The error of this method was under 10% caluculating from the 25 pairs of experiments.

The mineralization rate was estimated by applying this method to field surveys in Beppu Bay.

Marine pollution is a serious problem at coastal waters in a closed area such as the Seto Inland Sea today. The contents of organic materials are influenced by the eutrofication and inflow from lands. The blooming of phytoplankton in coastal areas results in the accumulation of dead marine organisms in the bottom. Therefore, the amounts of organic matter become higher at the eutrophicated area where the red tides often occurred. The marked decrease of dissolved oxygen is also observed in these regions, especially in summer as the result of high heterotrophic activity, and causes for damage of edible organisms in these areas. Therefore, it is important to know the function of the microorganisms in the heterotrophic biodegradation of organic materials and to estimate the mineralization rate in the sea.

Up to now the mineralization rate of sea water has been evaluated by measuring 1) the consumption of dissolved oxygen (BOD method), 2) the decrease of organic materials (*e.g.* DOC) in sea water[1], and 3) the increase of carbon dioxide derived from mineralization of organic materials.[2–4] The first and second methods require a long incubation time, and may possibly change number and population of microorganisms during the incubation period. Accordingly, the mineralization rate by those methods seems to be overestimated. The last one is rather inconvenient for workers who deal with many samples, because considerably large scale of apparatus is required. Here, the authors present a simplified method, using small Erlenmyer flask with inner chamber.

Experimentals

Mineralization of Organic Substances

The apparatus used is shown in Fig. 1. It is a 50 m*l* amber glass Erlenmyer flask, in which the inner chamber (internal diameter, 10 mm) is equipped. Microorganisms retained on ultrafine

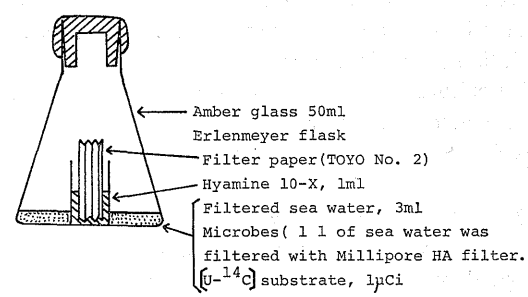

Fig. 1. Apparatus used in this study.

Millipore HA filter by filtering 1 liter of sea water, 1 μCi of [U-^{14}C] labelled substrate, and 3 m*l* of filtered sea water were put in the outer chamber, 1 m*l* of 1 M methanol solution of hydroxide Hyamine 10-X (p. (diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide) and an ac-

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cordion-folded piece of filter paper (3×4 cm) were introduced into the inner chamber. The Hyamine 10-X was used as a reagent for absorbing carbon dioxide evolved, and the filter paper was used to expand the surface area.

The incubation was carried out after sealing with a double rubber cap in a shaking incubator (TOYO Monoshin II) for 1 h. At the end of this incubation time, the reaction was stopped and the medium was acidified with 1 m*l* of 6 N HCl. The acid was introduced to the outer chamber through the rubber cap by a syringe with a needle. The flask was incubated again on a shaker for 1 h after acidifying to liberate carbon dioxide derived from mineralization and to absorb it into Hyamine 10-X.

After the incubation, the Hyamine solution and the filter paper were put into a glass vial and the inner chamber was rinsed with scintillation solution (5.005 g PPO (2, 5-diphenyloxazole) and 0.495 g POPOP (1, 4-bis-2-(4-methyl-5-phenyloxazole)-benzene) per 1 liter of toluene) and the rinsed toluene solution was added to the glass vial. The radioactivities based on absorbed [^{14}C] CO_2 were measured by a liquid scintillation spectrometer.

Bacterial Counts

One m*l* of sea water samples was added to 9 m*l* of sterile sea water and stirred vigorously. The mixture was serially diluted by decimal intervals in sterile sea water and the samples of 1 m*l* of each dilution were inoculated to the plate medium. The culture medium was similar to ZoBell 2216E. Inoculated media were incubated at 25°C for two weeks. The colonies appeared were counted.

The Outline of Beppu Bay

The Beppu Bay where the surveys have been carried out is located at the western part of the Seto Inland Sea. It has comparatively narrow area of 475 km^2, 20 km wide at the entrance and 36 km deep. Although oceanic water inflows in this region through the Bungo Channel between Kyushu and Shikoku islands, the inner part of this bay is apt to be polluted. Because two constant currents of which the one turning to right is located at the inner part of this region and the other turning to left is located at the outer part of this region are observed, the inner part is closed.

Since the load of pollutants gradually increase (the load of COD, nitrogen and phosphorus in 1971 are 4, 2 and 2 times that in 1955 respectively[5]), the inner part of this region is polluted or eutrophicated now.

Results and Discussion

Preliminary Experiments

1. The relationship between [^{14}C] CO_2 production and incubation time:

As shown in Fig. 2, the linear relationship was observed within the incubation period of 2 h. The radioactivity after 2 h incubation is about twice of 1 h incubation. Judging from the linearity of the curve, only 2–3% of substrate is decomposed during 2 h incubation.

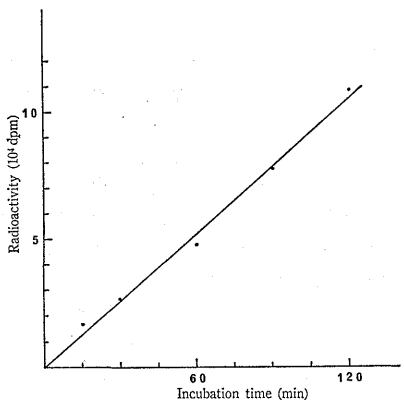

Fig. 2. Relationship between [^{14}C] CO_2 production and incubation time.

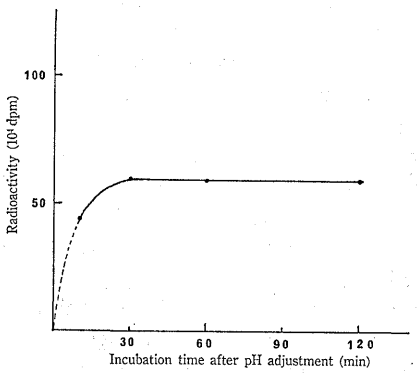

Fig. 3. Relationship between [^{14}C] CO_2 production and second incubation time.

The relationship between [^{14}C] CO_2 production and the incubation time for trapping of carbon dioxide is shown in Fig. 3. The radioactivity was almost saturated after 30 min. It is obvious that

[^{14}C] CO_2 evolved is absorbed entirely to Hyamine solution during 1 h incubation after acidifying.

2. The effect of incubation temperature on the mineralization rate:

The effect of incubation temperature on the relative mineralization rate is shown in Fig. 4. The maximum value is expressed as 1.00 in this

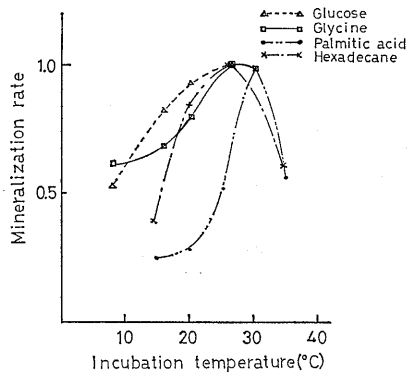

Fig. 4. The relationship between mineralization rate and incubation temperature.

figure. The maximum mineralization rates for the four kinds of substrates ranged between 25°C and 30°C. It is described that the optimum temperatures of marine microorganisms lie between 25°C and 30°C[6].

3. The relationship between mineralization rate and the amount of substrate:

As shown in Fig. 5, it is obvious that the higher the concentration of substrate was used, the higher the mineralization rate was obtained. This figure showed the hyperbolic curve and resembled to the curve obtained from the enzyme-substrate relation. It is obvious from Fig. 5 that the mineralization rate is dependent on the amount of substrate added.

The temperature and the concentration of substrate in the sea water are different from each other. Therefore, when mineralization rate is estimated at the field, the mineralzation rate determined at a fixed temperature must be corrected according to the field temperature. Moreover, since the microorganisms are collected on the ultrafine Millipore filter from 1 liter of sea water in this method, the substrate must be also adjusted to the amount dissolved in 1 liter of sea water. When the substrate is insufficiently added, the mineralization rate may be underestimated.

According to OKAICHI *et al.*[7], the carbohydrate content in sea water ranged from 0.80 and 0.20 mg glucose/*l* in eastern part of Hiuchi Nada. As it is considered that the average value of carbohydrate content in sea water is 0.3 mg glucose/*l*, 300 μg of glucose is introduced as a carrier on the survey at Beppu Bay.

4. Kind of ultrafine filter:

As the microorganisms are collected on an ultrafine Millipore filter by filtering sea water, it is important what type of Millipore filter is to be used. As far as three type of filter (Millipore GS, PH and HA) were tested, higher radioactivity was

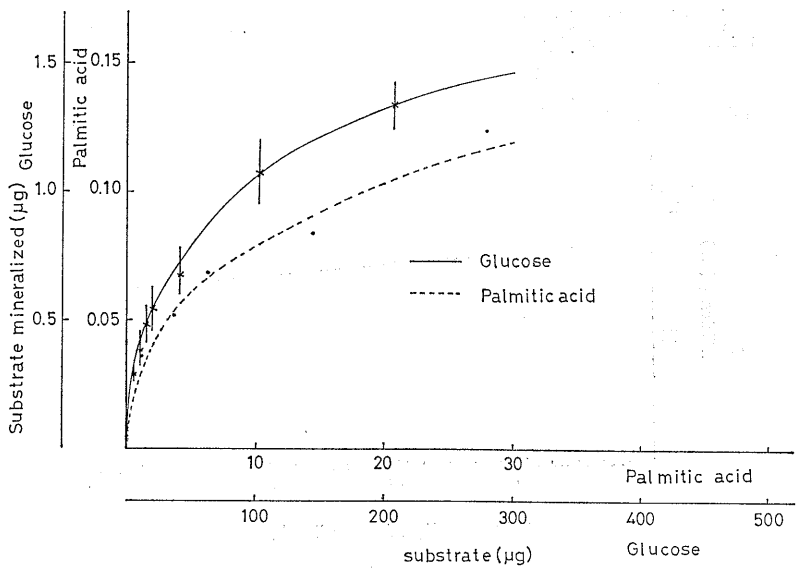

Fig. 5. The relationship between mineralization rate and an amount of substrate.

obtained in Millipore HA filter as shown in Table 1. It is supposed that the microorganisms on Millipore filter would be partly damaged by reduced pressure inversely proportional to the pore size of Millipore filter. Furthermore, since the filtration of sea water with Millipore HA filter is easier than that with Millipore GS or PH, and microorganisms are effectively collected on Millipore HA filter,[8] it is recommended to use Millipore HA filter for the filtration of sea water.

Table 1. The differences in mineralization rate between millipore filters.

Kind of millipore filter	Pore size (μ)	Radioactivity* (dpm)
Millipore GS	0.22	1.4×10^4
Millipore PH	0.30	2.3×10^4
Millipore HA	0.45	2.9×10^4

* [U−^{14}C] palmitic acid was used as the substrate.

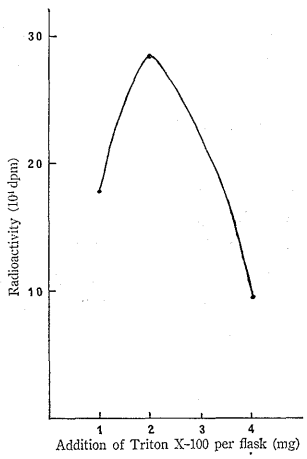

Fig. 6. Effect of amount of Triton X-100 on mineralization rate of palmitic acid.

5. Amount of Triton X-100 and Hyamine 10-X:

When the mineralization of lipids was estimated, Triton X-100 (octyl phenoxy polyethoxyethanol) was used to emulsify the substrate. Excess amounts of Triton X-100 injured the microorganisms, however, if the amount of Triton X-100 is small, the substrate is not well decomposed by microorganisms. Therefore, the suitable amount of Triton X-100 must be determined. The relationship between [^{14}C] CO_2 production and the amount of Triton X-100 is shown in Fig. 6. When 2 mg of Triton X-100 per flask was used, the maximum radioactivity was obtained.

Since methanol solution of Hyamine 10-X is a kind of quenture in the case of radioactivity estimation by a liquid scintillation spectrometer, the efficiency based on the amount of Hyamine 10-X was examined. The curve is shown in Fig. 7. The efficiencies declined gradually from 88% to 72% in the range below 2.0 m*l* of 1 M methanol solution of Hyamine 10-X. As 72% efficiency is not too low to estimate the radioactivity by a liquid scintillation spectrometer, it is better to use 1 m*l* as absorber of carbon dioxide and additional 1 m*l* for rinsing the inner chamber.

6. Kind of substrate:

The mineralization rates of four kinds of substrate were compared. As shown in Table 2, it is obvious that glucose and glycine are decomposed easily, while palmitic acid and n-hexadecane are more stable than the former two. It is considered that glucose and glycine are decomposed by almost all sorts of microorganisms, on the contrary, the decomposition of palmitic acid and n-hexadecane is concerned with special microorganisms. As it is reported by KESTER and FOSTER[9]

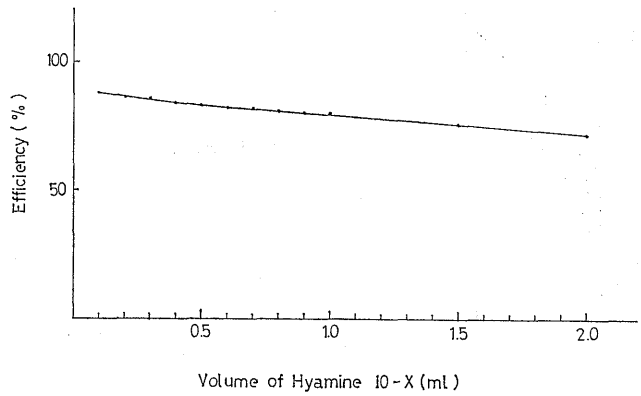

Fig. 7. The relationship between counting efficiency and volume of Hydroxide of Hyamine 10-X in liquid scintillation solution.

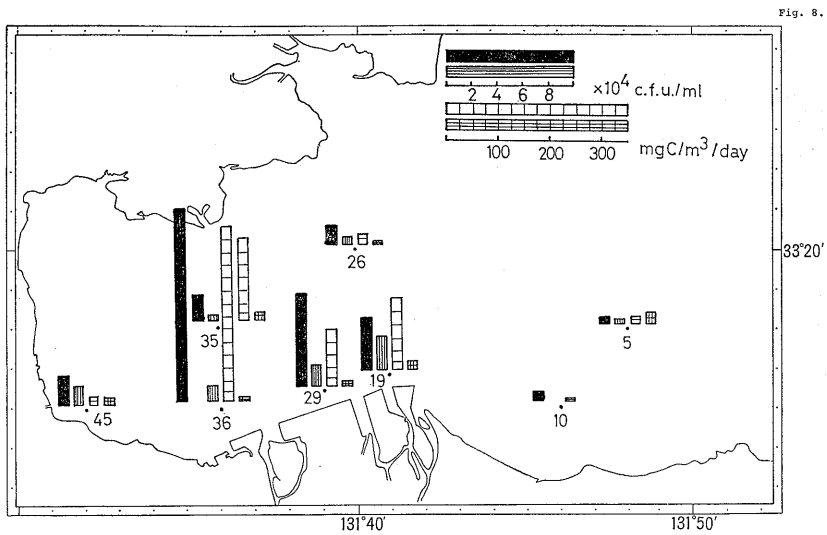

Fig. 8. Mineralization rate of glucose and bacterial counts in Beppu Bay.

Table 2. The differences in mineralization rate between substrates used

Kind of substrate	Radioactivity (dpm)
[1—^{14}C] *n*-Hexadecane	2.4×10^4
[U—^{14}C] Palmitic acid	29.3×10^4
[U—^{14}C] Glucose	150.3×10^4
[U—^{14}C] Glycine	151.5×10^4

that n-alkane is converted to fatty acid first, and then it is decomposed, it is reasonable that n-hexadecane is inferior to palmitic acid in the mineralization rate.

Survey at Beppu Bay

The mineralization rate of glucose and number of heterotrophs are shown in Fig. 8. Both values were larger in June than in October, and also were larger at the inner part than at the outer part of the bay. From the observation on vertical distribution, it was shown that the mineralization rate was larger at surface than at middle or bottom layer (Table 3). This result indicates that the mineralization rate may depend on the number of heterotrophs.

Although the mineralization rate of glucose in Beppu Bay falls between 4.06 mgC/m^3/day and 432.37 mgC/m^3/day, these values were similar ro that at Hiuchi Nada communicated personally by HATA. However, it is remarkable that the values obtained at the coastal or inner part of Beppu Bay (St. 19,35,36) were very large in June. It is considered that the environmental condition at the coastal or inner part of this region is worse in June. The more polluted, the more heterotrophs exist.

The mineralization acitivity per cell (μgC/day) was caluculated from the results of mineralization rate and the number of heterotrophs (Table 3). It is interesting that the mineralization activity per cell is low at surface layer of coastal and inner part of this region where the mineralization rate and the number of heterotrophs are large. OKUTANI *et al.*[10] reported similar result in Hiuchi Nada that the mineralization activity was smaller at the coastal area than at the off area. It is reported by ISHIDA and KADOTA[11] that bacterial community at coastal and polluted areas is different from that offshore area. They also reported that the ratio in number of bacteria grown on the nutrient-rich medium to those on the nutrient-poor medium increased in pararell to the concentration of dissolved organic substances. According to these results, some bacteria in sea water collected at offshore area of Beppu Bay do not grow on the nutrient-rich medium (ZoBell 2216E) used in this study. It is supposed that bacteria not grown on the nutrient-rich medium are also concerned with the actual mineralization of organic substances in the sea water.

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Table 3. The heterotrophs number (c. f. u.*/m*l*), mineralization rate (mgC/m³/day) and mineralization activity per cell (μgC/day) at several stations in Beppu Bay

Stn. No.	Depth (m)	Mineralization rate	Heterotrophs number	Mineralization activity	Mineralization rate	Heterotrophs number	Mineralization activity
		June, 1977.			Oct., 1977.		
	0	14.06	4.7×10^{3}	3.0×10^{-6}	20.36	3.8×10^{3}	5.4×10^{-6}
5	20	6.53	1.0×10^{2}	65.3×10^{-6}	53.65	3.0×10^{2}	178.8×10^{-6}
	49	4.52	2.0×10^{2}	22.6×10^{-6}	6.93	5.0×10^{2}	13.9×10^{-6}
	0	5.28	7.4×10^{3}	0.7×10^{-6}	—	—	—
10	20	5.84	2.0×10^{2}	29.2×10^{-6}	—	—	—
	33	4.17	2.0×10^{2}	20.9×10^{-6}	—	—	—
	0	139.74	4.0×10^{4}	3.5×10^{-6}	15.56	2.5×10^{4}	0.6×10^{-6}
19	20	13.06	1.0×10^{3}	13.1×10^{-6}	—	—	—
	24	—	—	—	4.66	3.0×10^{2}	15.5×10^{-6}
	35	10.94	1.0×10^{3}	10.9×10^{-6}	—	—	—
	0	20.79	1.4×10^{4}	1.5×10^{-6}	7.09	5.8×10^{3}	1.2×10^{-6}
26	20	6.10	6.0×10^{2}	10.2×10^{-6}	—	—	—
	25	—	—	—	4.98	3.0×10^{2}	16.6×10^{-6}
	28	6.27	6.0×10^{2}	10.5×10^{-6}	—	—	—
	0	109.85	7.2×10^{4}	1.5×10^{-6}	9.09	8.4×10^{3}	1.1×10^{-6}
29	20	21.84	1.0×10^{3}	21.8×10^{-6}	6.40	1.3×10^{3}	4.9×10^{-6}
	30	—	—	—	4.36	7.0×10^{2}	6.2×10^{-6}
	35	10.06	3.0×10^{3}	3.4×10^{-6}	—	—	—
	0	162.21	2.0×10^{4}	8.1×10^{-6}	15.70	3.9×10^{3}	4.0×10^{-6}
35	20	10.96	1.0×10^{2}	109.6×10^{-6}	12.33	9.0×10^{2}	13.7×10^{-6}
	49	9.01	1.0×10^{2}	90.1×10^{-6}	4.35	6.0×10^{2}	7.3×10^{-6}
	0	342.37	1.5×10^{5}	2.3×10^{-6}	10.21	1.1×10^{4}	0.9×10^{-6}
36	20	43.19	1.0×10^{3}	43.2×10^{-6}	7.90	3.2×10^{3}	2.5×10^{-6}
	50	22.95	2.0×10^{3}	11.5×10^{-6}	5.20	1.7×10^{3}	3.1×10^{-6}
	0	14.93	2.3×10^{4}	0.6×10^{-6}	11.85	1.4×10^{4}	0.8×10^{-6}
45	20	7.36	4.0×10^{2}	18.5×10^{-6}	7.97	5.0×10^{2}	15.9×10^{-6}
	50	—	—	—	4.06	6.0×10^{2}	6.8×10^{-6}
	66	9.43	2.0×10^{2}	47.2×10^{-6}	—	—	—

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