On the relation between methane production and sulfate reduction in bottom muds containing sea water sulfate

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The processes of methane production in bottom muds containing sea water sulfate were investigated in relation to both sulfate reduction and organic acid fermentation.

The methane production in bottom muds was dependent on the degree of sulfate reduction, and did not occur until about 80% of sea water sulfate was removed by sulfate reduction. The activity of methane production in bottom muds was not inhibited even under the sulfide-S concentration of 160mg per 100g wet mud, and was directly dependent on the amount of organic acids.

When the bottom mud added with phyto-plankton was incubated at 20°C for 20 days, sulfate decreased with consumption of lactic acid, and acetic and propionic acids were formed as the successive metabolites of lactic acid. Of the three organic acids added individually to bottom muds, lactic acid was utilized as hydrogen donor for the sulfate reduction, and acetic and propionic acids were produced with the passage of sulfate reduction. Acetic and propionic acids were not utilized for the sulfate reduction, but they were converted into methane, when the degree of sulfate reduction attained more than 80%.

INTRODUCTION

The phenomenon of methane fermentation in bottom muds is considered to occupy an important position in the carbon cycle of the hydrosphere.

On the mineralization process of organic matter in lake sediments, KOYAMA and NIKAIDO (1971) and KOYAMA *et al.* (1973) have reported that methane fermentation takes place mainly according to two processes which consist of the formation of organic acids from proteinous substances and the production of methane from organic acids. NIKAIDO (1972) has pointed out that the seasonal patterns of organic acid formation and methane fermentation in brackish lake sediments are affected by the seasonal variation of mud temperature and of characteristics of organic matter. However, in the bottom muds of coastal and estuarine regions and brackish lake which contain large amounts of organic matter are often found methane fermentation coupled with sulfate reduction. TEZUKA *et al.* (1963) have pointed out that sulfate reduction in polluted river water under anaerobic condition occurs along with organic acid fermentation. MARTENS and BERNER (1974, 1977) have reported that methane production does not

occur in anoxic marine sediment in the presence of sea water sulfate.

From these points of view, it can be considered that methane production in bottom muds containing sea water sulfate is related to both sulfate reduction and organic acid fermentation.

The present studies were carried out to make clear the relation of methane production to organic acid formation and to sulfate reduction in bottom muds of two bays and a brackish lake.

MATERIALS AND METHODS

Collection of mud samples Twelve sampling stations were selected in Lake Kojima, 5 stations in Oura Bay and 2 stations in Iyo-mishima Bay. These stations indicated different chlorine contents for interstitial waters of bottom muds. Lake Kojima (max. depth: 9.0m; area: 11.0km²) is a man-made brackish and eutrophic lake located in Okayama Prefecture. It was constructed in 1956 by damming up estuaries to keep water for irrigation and industrial uses. Oura Bay (mean depth: 10.0m) is located about 30km northwest of Matsuyama City, Ehime Prefecture, on the northeastern coast of Naka-

jima Island in the Seto Inland Sea, and receives very little drainage from the land. Iyo-mishima Bay (mean depth: 15.0m) is located near Iyo-mishima City, Ehime Prefecture. It faces the Hiuchi-nada in the Seto Inland Sea, and receives 40,000 tons of drainage per day from pulp factories.

Surface mud samples of about 20cm in thickness were collected with a 30cm Ekman dredge at each station during the summer in 1972. Each dredge sample was divided into two parts, one part was taken into a wide-mouthed plastic bottle of 250ml in capacity and was kept in a freezer for later analyses of organic acids. The other part of the dredge sample was used for the determination of some chemical properties such as chloride, sulfate, sulfide and methane contents.

Incubation of mud samples Dredge samples from each station were put in a polyethylen beaker, homogenized immediately, and were taken in a number of soil injectors (KOYAMA, 1953) of 50ml in capacity, and the mouth of injector was tightly rubber-stoppered leaving no air space. A number of soil injectors thus prepared were incubated in darkness at 20°C for 30 days. At appropriate intervals some injectors were taken out for analyses of methane and sulfate contents.

Incubation of mud samples added with phytoplankton A mud sample was collected with an Ekman dredge from the deepest part (9.0m) of Lake Kojima in April, 1972. The dredge sample was divided into two parts, one part was put in a polyethylen beaker and homogenized immediately, and to the other part was added phyto-plankton which was collected with a plankton net (XX 13) from surface water of Lake Kojima and homogenized immediately. The plankton was composed mainly of blue-green algae such as *Osillatoria limnetica*, *O. tenuis* and *Microcystis flos-aque*, and the amount of added plankton was 200mg per 100g wet mud as organic carbon. Homogenized samples contain an appropriate amount of sea water sulfate. A number of injectors with or without plankton added were incubated at 20°C for 20 days. At appropriate intervals, some injectors were taken out for analyses of methane, organic acids and sulfate contents.

In addition to the experiment mentioned above, the other experiments on the relation of methane production to sulfate reduction and

to added organic acids such as lactic, propionic and acetic acids were also carried out as follows; the mud sample was put in a polyethylen bottle of 3.0l in capacity, and the polyethylen bottle was kept at 20°C for 30 days. During this time a greater part of easily attackable organic matter in the mud sample was decomposed by microbes. To the mud sample thus incubated was added some of sea water, it was mixed well and then taken in a number of soil injectors. To each of the soil injectors were added 5 or 10ml of 0.2mol sodium lactate solution, 5ml of 0.1mol sodium acetate solution, and 0.5ml of 0.1mol sodium propionate solution, respectively. All of these soil injectors were incubated at 20°C for 20 days. At appropriate intervals, some injectors were taken out for analyses of methane, organic acids and sulfate contents.

Analytical method For the determination of chloride and sulfate contents, the mud samples were put in centrifuge tubes of 50ml in capacity, and were tightly stoppered leaving no air space, and then were centrifuged. Supernatant solutions were used for determining chloride and sulfate contents. Sulfate was determined by turbidimetric analysis for samples of low concentration and by gravimetric analysis as BaSO₄ for samples of high concentration. Chloride was determined by MOHR's method. Sulfide was determined by the methylen blue method of NIKAIDO (1970), which is similar to the method of CLINE (1969). Methane was determined by KOYAMA's method (KOYAMA, 1953) and gas chromatography using molecular sieve 13 X columns. Organic acids were first extracted from the samples at pH 2. It was adjusted by adding diluted H₂SO₄. The extracted organic acids were determined by using the silicagel chromatography of KOYAMA and THOMPSON (1964). Degree of sulfate reduction in bottom muds was calculated as follows:

$$(\text{Calc. SO}_4 - \text{Obs. SO}_4) / \text{Calc. SO}_4 \times 100$$

The calculative sulfate (Calc. SO₄) was estimated from the chloride content of the interstitial water in bottom muds as 2,649mg/kg of sulfate as against chloride concentration of 18,980mg/kg in sea water.

RESULTS AND DISCUSSION

Presence of methane and its related properties in bottom muds The chloride content of bot-

Table 1. Methane, sulfide-sulfur, total organic acid and degree of sulfate reduction in bottom muds collected from Lake Kojima, Oura Bay and Iyo-mishima Bay

Locations	Samples	Methane (ml/100g)	Sulfide-S (mg/100g)	Total organic acid (μ e/100g)	Degree of sulfate reduction (%)
Lake Kojima	1	0	26.8	25.4	43.8
	2	0	34.6	10.2	63.1
	3	0	32.4	20.1	70.0
	4	0.6	54.4	230.2	81.4
	5	1.0	87.5	181.2	90.8
	6	1.3	76.5	652.0	90.9
	7	1.8	65.4	426.5	86.3
	8	1.9	78.9	351.2	82.8
	9	2.1	76.2	847.6	95.5
	10	3.1	147.6	762.7	89.1
	11	3.3	165.2	983.2	84.3
	12	3.7	131.5	1,038.1	96.8
Oura Bay	1	0	12.1	7.5	12.6
	2	0	13.1	12.9	15.8
	3	0	17.4	15.8	22.1
	4	0	25.5	22.3	29.0
	5	0	30.4	28.9	30.0
Iyo-mishima Bay	1	2.5	54.8	1,346.2	91.0
	2	2.6	126.5	389.0	91.9

tom muds ranged from 1.76 to 18.22g/l at each station. As seen in Table 1, methane was not detected until the degree of sulfate reduction exceeded about 80%. The methane content was large for bottom muds containing greater amounts of sulfide or total organic acid. This fact suggests that methane fermentation in bottom muds was closely related to both sulfate reduction and organic acid fermentation. The organic acids found at each station were composed of butyric, propionic, acetic, formic, lactic and unidentified organic acids, and their composition differed somewhat from each other. Among these organic acids, acetic acid was predominant at all stations.

Methane content of bottom mud reveals a balance between the methane produced in the bottom mud and the methane released into bottom water from the bottom mud. Therefore, the activity of methane production was also examined on the bases of incubation experiments. Methane production during the incubation period of 30 days exhibited a high value for mud samples of high degree of sulfate reduction. Methane did not occur until the sulfate reduction became about 80% (Fig. 1). This fact is similar to the results of MARTENS and BERNER (1974, 1977). A higher activity of methane production was also recognized in mud samples with greater concentrations of sulfide (Fig. 2) or total organic acid (Fig. 3). The results

Fig. 1. Relation between the degree of sulfate reduction and the amount of methane produced in the bottom muds collected from Lake Kojima, Oura Bay and Iyo-mishima Bay during the incubation period.

Fig. 2. Relation between the sulfide content at the initial stage of incubation and the amount of methane produced for the bottom muds collected from Lake Kojima, Oura Bay and Iyo-mishima Bay during the incubation period.

Fig. 3. Relation between the total organic acid content at the initial stage of incubation and the amount of methane produced for the bottom muds collected from Lake Kojima, Oura Bay and Iyo-mishima Bay during the incubation period.

of incubation experiments are similar to the relation between methane content and its related properties in the field observation. The results show also that the methane production is not inhibited in the presence of 160mg of sulfide-sulfur per 100g of wet mud. LAWRENCE *et al.* (1966) have reported that the gas production (CH_4 and CO_2) in the operation of laboratory digesters is affected in the presence of a large amount of sulfide-sulfur and is also more largely affected in the presence of soluble hydrogen sulfide than insoluble iron sulfide. According to NIKAIDO (1970, 1971), sulfides in bottom muds of Lake Kojima are present mainly as ferrous sulfide. The effect of sulfide on the methane fermentation is considered to be dependent on the form of sulfide sulfur.

From all of these findings mentioned above, it is apparent that the methane production in bottom muds is dependent on the degree of sulfate reduction, and does not occur until about 80% of sea water sulfate is removed as sulfide by sulfate reducers, and that the activity of methane production is not inhibited even in the presence of 160mg of ferrous sulfide per 100g of wet mud, and is directly dependent on the amounts of organic acids.

Relation of methane production to formation of organic acids and to sulfate reduction deduced from incubation experiments

To get more detailed information concerning the phenomena mentioned above, various components such as plankton and lactic, propionic and acetic acids were added to bottom mud, and the mud samples were incubated at 20°C for 20 days. In the bottom mud without phyto-plankton, the degree of sulfate reduction increased from 60 to 83% towards the end of incubation period

(Fig. 4). Of the organic acids, lactic acid decreased remarkably after 10 days of incubation, while acetic and propionic acids increased till 15 days of incubation. When the degree of sulfate reduction reached 83% at the end of incubation, a small amount of methane was produced with a decrease of acetic and propionic acids. In the bottom mud with phyto-plankton, sulfate decreased more rapidly than in the bottom mud without phyto-plankton, and the degree of sulfate reduction increased from 60 to 97% towards the end of incubation. Of the organic acids, lactic acid increased towards 5 days of incubation and then decreased for some time, and again increased till the end of incubation. The final increase of lactic acid might have been caused by the completion of sulfate reduction. Methane production occurred after 10 days of incubation, but acetic and propionic acids did not decrease throughout the incubation period. It is considered that the amounts of acetic and propionic acids show a balance between the production of organic acids from organic matter and that of methane from the organic acids, and that the accumulation of acetic and propionic acids was caused by a higher activity of organic acid formation from the plankton added than methane production from the organic acids formed. Similar results have been reported also by NIKAIDO (1972).

Fig. 4. Changes of sulfate, organic acids and methane in the bottom mud from Lake Kojima with or without phyto-plankton added during the incubation period. Of calc. SO_4 , observed SO_4 is represented by white. Organic acids comprise lactic acid (black), acetic acid (oblique lines), propionic acid (reticulate) and other organic acids (white).

According to HANDA and MIZUNO (1969), monosaccharides which are extracted by water from *Microcystis* sp. are composed mainly of glucose, and this glucose is easily decomposed by microbes in lake water or in bottom muds. On the other hand, TEZUKA *et al.* (1964) have pointed out that lactic acid is formed by anaerobic decomposition of glucose inoculated with microflora from polluted river-water. From these facts, it is considered that the formation of lactic acid in the incubation experiment was caused by the decomposition of carbohydrates such as glucose which is contained in phytoplankton. The results of experiments suggest also that lactic acid is closely related to both sulfate reduction and formation of acetic and propionic acids in bottom muds.

In the bottom mud added with 5 ml of 0.2 mol lactic acid, sulfate was consumed slightly throughout the incubation period (Fig. 5). Lactic acid decreased rapidly after 10 days of incubation, and acetic and propionic acids increased on incubation. Methane production, however, did not occur throughout the incubation. In the bottom mud added with 10 ml of 0.2 mol lactic acid, the change of lactic acid during the incubation was similar to the above results. Methane production, however, occurred after 15 days of incubation, and acetic and propionic acids decreased with methane production. This fact suggests that lactic acid was utilized as hydrogen donor for sulfate reduction, and that acetic and propionic acids were formed as the successive metabolites of lactic acid. TEZUKA (1965) has proved the following reaction from the physiological studies on a strain of sulfate reducing bacterium, *Desulfovibrio desulfuricans*, isolated from polluted river-water.

Fig. 5. Changes of sulfate, organic acids and methane in the bottom mud from Lake Kojima added with lactic acid during the incubation period.

Of calc. SO_4 , observed SO_4 is represented by white. Organic acids comprise lactic acid (black), acetic acid (oblique lines), propionic acid (reticulate) and other organic acids (white).

On the propionic acid formation through lactic acid fermentation, TEZUKA *et al.* (1963) have pointed out that acetic and propionic acids are formed from lactic acid in polluted river-water. This process is generally understood as the following equation.

The relation between consumption of lactic acid

Table 2. Changes of metabolites and their mole ratios to the sulfate consumed in the bottom mud from Lake Kojima added with lactic acid and incubated at 20°C

Initial concentration of lactic acid	Incubation time (days)	Sulfate consumed (μM)	Lactic acid consumed (μM)	Acetic acid produced (μM)
1 mM	10	315	850 (2.7)	736 (2.3)
	15	361	903 (2.5)	806 (2.2)
2 mM	5	142	354 (2.5)	335 (2.4)
	10	710	1908 (2.7)	1631 (2.3)

Numerical values in parentheses show the mole ratios of metabolites to the sulfate consumed.

and changes of metabolites was examined on the mud samples in which methane production did not occur throughout the incubation period. Table 2 shows the changes of metabolites and their mole ratios to the sulfate reduced in the bottom mud from Lake Kojima added with lactic acid and incubated at 20°C. The mole ratios of metabolites to the sulfate consumed ranged from 2.5 to 2.7 in the lactic acid consumed and from 2.2 to 2.4 in the acetic acid produced. These mole ratios were slightly higher in comparison with the calculative ones which were estimated from equation (1). This fact suggests that lactic acid was utilized mainly for sulfate reduction as hydrogen donor and acetic acid was formed through the successive metabolism of sulfate reduction with lactic acid, and that a part of lactic acid was consumed in other metabolisms than sulfate reduction, and a part of acetic and propionic acids was formed in those metabolisms.

Table 3 shows the changes of metabolites and their mole ratios to propionic acid formed in the bottom mud from Lake Kojima added with lactic acid and incubated at 20°C. In this table, the amounts of lactic acid consumed and acetic acid produced were calculated as the difference between the amounts of metabolites shown in Table 2 and those calculated from sulfate consumed according to equation (1). The mole ratios of metabolites to lactic acid consumed ranged from 0.8 to 1.3 in the propionic acid produced and from 0.4 to 0.7 in the acetic acid produced. These mole ratios are slightly higher in comparison with the calculative ones which

are estimated from equation (2). This fact suggests that acetic and propionic acids were produced mainly from lactic acid, but some of these acids were also produced from other organic matter besides lactic acid. It may be considered that lactic acid accelerated the propagation of bacteria which decompose organic matters in bottom muds. From these findings, it is apparent that lactic acid is utilized as hydrogen donor for sulfate reduction, and that acetic and propionic acids are formed through the successive metabolism of lactic acid.

As to organic matters related to sulfate reduction, RAMM and BELLA (1974) have reported that sulfide production is dependent on the concentration of soluble organic carbon in marine sediments, and BERNER (1972) has reported that carbohydrates and amino acids are utilized for sulfate reduction as hydrogen donors. These organic matters also seem to be related to sulfate reduction in bottom muds in the absence of lactic acid.

Furthermore, HATA *et al.* (1965) have pointed out that some refractory substances such as cellulose may play important roles in the production of sulfides by micro-organisms in coastal region polluted with pulp wastes. It can be considered that there is a difference between bottom muds of Lake Kojima and those of Iyo-mishima Bay as to organic matters related to sulfate reduction. The results suggest also an interesting problem that there is a difference between fresh water lake and brackish water lake as to the process of organic acid formation in bottom muds. KOYAMA and NIKAIDO (1971)

Table 3. Changes of metabolites and their mole ratios to the propionic acid formed in the bottom mud from Lake Kojima added with lactic acid and incubated at 20°C

Initial concentration of lactic acid	Incubation time (days)	Lactic acid* consumed (μ M)	Propionic acid produced (μ M)	Acetic acid** produced (μ M)
1 mM	10	220	176 (0.8)	106 (0.5)
	15	181	231 (1.3)	84 (0.5)
2 mM	5	70	62 (0.9)	51 (0.7)
	10	488	556 (1.1)	211 (0.4)

* Lactic acid consumed = Lactic acid consumed given in Table 1 - Theor. lactic acid consumed which is calculated from sulfate consumed.

** Acetic acid produced = Acetic acid produced given in Table 1 - Theor. acetic acid produced which is calculated from sulfate consumed.

Numerical values in parentheses show the mole ratios of metabolites to the lactic acid consumed.

and KOYAMA *et al.* (1973) have reported that organic acids in bottom muds were formed mainly by de-amination of amino acids in fresh water lake such as Lake Suwa. From the results obtained in these experiments, it may be considered that organic acids such as acetic acid and propionic acid are formed through the successive metabolism of lactic acid in relation with sulfate reduction, but the process of organic acid formation varies depending on the degree of sulfate reduction.

To make clear the role of acetic and propionic acids concerning sulfate reduction and methane production, acetic acid or propionic acid was added to the bottom mud of 60 or 90% reduction of sulfate, and the mud samples were incubated at 20°C for 20 days.

Methane was produced in the bottom mud of 90% sulfate reduction, but it did not occur in the bottom mud of 60% sulfate reduction (Fig. 6). The amount of methane observed (methane produced during 20 days of incubation) as against calculative methane (methane is calculated from the amount of acetic acid or propionic acid added) was 98% for acetic acid, and 110% for propionic acid. This fact suggests that acetic and propionic acids were not utilized for sulfate reduction as hydrogen

Fig. 6. Relation between sulfate reduction and methane production in the bottom mud from Lake Kojima added with acetic acid or propionic acid.

Of calc. SO_4 , observed SO_4 is represented by white. Calc. CH_4 was estimated according to the following reactions from the amount of added acetic acid or propionic acid:

Fig. 7. Scheme of the main process of methane production and organic acid formation in relation to sulfate reduction in the bottom muds of Lake Kojima.

donors, but they were utilized for methane production. KOYAMA and NIKAI (1971) and KOYAMA *et al.* (1973) have pointed out that methane in bottom muds of fresh water lake is produced mainly from organic acids such as acetic, propionic and butyric acids. Although the process of organic acid formation in fresh water lakes differs from that in brackish water lakes, the process of methane production is the same.

Figure 7 shows the main process of methane production and organic acid formation in relation to sulfate reduction in muds from brackish lakes such as Lake Kojima which contain large amounts of easily decomposable planktonic carbohydrates. Methane is fermented in brackish lake muds mainly according to the following three processes: 1) Formation of lactic acid from carbohydrates, 2) formation of acetic and propionic acids in relation with sulfate reduction, and 3) methane production from acetic and propionic acids, when the degree of sulfate reduction is more than 80%.

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