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# Methane Production in Shallow-Water, Tropical Marine Sediments[1]

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The in situ production of methane was monitored in several types of tropical benthic communities. A bed of *Thalassia testudinum* located in Caesar Creek (Florida Keys) exhibited the highest methanogenic activity (initial rates = 1.81 to 1.86 μmol $CH_4/m^2$ per h) as compared with another seagrass (*Syringodium* sp., 0.15 to 0.33 μmol/$m^2$ per h) and two coral reef environments (Hydro-Lab, 0.016 to 0.10 μmol/$m^2$ per h; Curaçao, 0.14 to 0.47 μmol/$m^2$ per h). The results suggest that a wide variety of benthic metabolic processes (e.g., photosynthetic oxygen production) influences methane production rates.

Prior to the development of sensitive techniques for extraction and determination of low-molecular-weight hydrocarbons from seawater (17, 18), little was known about methane in the oceanic water column and marine sediments (1). The high concentration of sulfate ions in seawater (2.7 g/liter) led to the belief that the metabolic activities of sulfate-reducing bacteria dominated the anaerobic regions of the seas (e.g., the Cariaco Trench, marine sediments). Sulfate ions and methane have been reported in the same shallow-water sediments (10), and recently Martens and Berner (14) reported that a demarcation exists between regions of interstitial sulfate (upper sediment layers) and dissolved methane (lower layers). The latter workers concluded, based on 90-day laboratory incubations of sediments, that methane production occurs only when sulfate ions are depleted. Bryant (Abstr. 158th Meet. Am. Chem. Soc., p. 18, 1969) has cultured *Methanobacterium* MoH with *Desulfovibrio desulfuricans* and *D. vulgaris* and concluded that methane production from an organic carbon source (lactate) proceeded, only in the absence of sulfate, via the sulfate-reducer's oxidation of the compound and transfer of hydrogen to the methanogen. Because of these facts, as well as other reports of high levels of methane in shallow marine sediments (15) and in the water column of shallow regions of the seas (4, 12), it appears that methanogenic bacteria are active in shallow benthic areas. Measurements of methane concentrations are analogous to "standing crop" determinations of primary productivity and, therefore, say little concerning its actual rate of production. An estimation of the production rates of methane or the in situ activity of methanogenic bacteria is of importance because of methyl mercury contamination of aquatic food chains (9, 20), production of atmospheric carbon monoxide from methane (8), and the fact that a knowledge of the ecological parameters that influence methanogenic bacteria in natural environments may prove useful in the design of fuel-producing systems.

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## MATERIALS AND METHODS

The in situ production of methane was measured in cylindrical Plexiglas incubation chambers (volume, 4 liters; diameter, 19.5 cm) placed with their open ends inserted 5 cm into the sediment. The chambers were fitted with a serum stopper for sampling and additions (surface area, 0.04% of chamber) and with a pressure relief valve to retard the exchange of water in the chamber with interstitial water during sampling. Dark chambers, covered with silver tape, were used to measure the rates of methane production in the absence of benthic photosynthetic activity. "Light" chambers were merely subjected to local variations in diurnal illumination. The experiments required the use of SCUBA equipment. Samples were withdrawn with plastic syringes (Becton-Dickinson Inc.; volume, 50 ml) and, after being inserted into rubber bungs, were stored at −20 C until assayed. Storage times lasted up to 4 weeks with negligible loss of methane. Samples were injected into a 55-ml stripping chamber, and methane was removed and trapped by the procedure of Swinnerton and Linnenbom (17). A flame ionization gas chromatograph (Varian series 1700) fitted with a Poropak R (100- to 200-mesh) column (10 feet long, 1/8-inch bore [ca. 3.4 m by 3.2 mm]) was used to detect methane. The column was operated at 120 C with $N_2$ as the carrier and purge gas (flow rate, 25 ml/min).

Standards were prepared by bubbling finely dispersed methane through distilled water. After 45 min, the water was assumed to be saturated and the temperature was measured. A small fraction (0.1 ml) was removed and pipetted into 3,900 ml of distilled water previously purged with $N_2$. After several seconds of gentle stirring, portions were withdrawn into Glaspak syringes (Becton-Dickinson; volume, 2.5 ml; 0.5-ml increments) and inserted into rubber bungs. Standards were injected into the stripping chamber which contained previously purged seawater. The amount of methane in the standards was calculated from its solubility (13) and the dilution factor. The gas chromatograph response was found to be linear, with a lower limit of detection around $1.0 \times 10^{-5}$ ml/liter or 0.5 nmol/liter. A maximum possible error of 13% was associated with any sample as determined by the largest variation among replicate standards. With the assumption that methane production remained constant between sampling times, the rates of methane production were calculated from changes in chamber concentrations.

Dissolved oxygen concentrations in the chambers were determined by withdrawing 55-ml samples in a syringe and "pickling" the sample with Winkler reagents (16). Titration with thiosulfate was performed immediately upon return to the research vessel.

Particle size distributions of the upper 8 cm of the sediments were obtained from dry weights of sediments washed through sieves (W, S. Tyler Co., mesh sizes, 63 $\mu$m to 2 mm). The mean sediment particle sizes were calculated from plots of size fractions versus cumulative weight percentages. Sediments were recovered and brought back to the ship for the Eh measurements within 0.5 h after collection.

An Analytical Measurements Redox 700 meter equipped with a combination platinum electrode was employed to obtain depth profiles of Eh. The electrode was inserted into holes on the sides of plastic suction cores. The Eh was checked against a standard $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution (21).

To demonstrate that the observed production of methane was of microbial origin, 60-ml sediment portions collected from the upper 10 cm of a local *Thalassia testudinum* bed were placed in Erlenmeyer flasks (total volume, 550 ml) that contained 300 ml of seawater previously boiled and purged with $N_2$. The flasks were sealed with no. 8 rubber stoppers fitted with serum stoppers for gas sample withdrawal. The flasks were purged for 15 min with the desired gas phase, resealed, and incubated at 30 C, in the dark, with constant shaking. Nitrogen, $H_2$-$CO_2$ (70:30), and air were used as atmospheres. $CHCl_3$ (0.05%, vol/vol), which is a specific inhibitor of methanogenic bacteria (2), and autoclaving were used as controls. The methane concentration of the gas phase was monitored during the course of the experiment by injecting 0.25-ml samples into a Varian 1700 gas chromatograph.

## RESULTS AND DISCUSSION

Characteristics of the areas investigated are shown in Table 1. The fine sediments of Caesar Creek were the most reducing, exhibiting an Eh of −150 mV at a depth of 1 cm. These sediments also gave the highest methanogenic activity (Table 2). The Hydro-Lab and Bimini sites had deeper, ill-defined redox potential discontinuity layers, probably caused by a greater degree of flushing of the upper sediment layers by surrounding waters (7). The fine coral reef sediments of Curacao were surprisingly reducing.

Dissolved-methane levels within the dark chambers increased markedly over the course of the incubation periods as compared with those in the light chambers (Fig. 1). Oxygen

TABLE 1. *Description of sediment areas investigated*[a]

| Areas | Dates | Bottom type[b] | Eh[c] (mV) | Water depth[d] (feet) | Final concn of enrichment substrate |
|---|---|---|---|---|---|
| Caesar Creek (Florida Keys) | 5–8 Oct. 1973 | *Thalassia testudinum*; MSPS = 200 | −150 | 5 (1.52) | 1 mM sodium formate + 1 mM sodium acetate |
| Hydro-Lab (Grand Bahama Island) | 10–15 Dec. 1973 | Coral reef; MSPS = 515 | +365 | 45 (13.7) | 5 mM sodium formate |
| Bimini (Bahama Island) | 7–9 Feb. 1974 | *Syringodium* sp.; MSPS = 225 | +260 | 35 (10.7) | |
| Curaçao (Dutch Antilles) | 12–15 Apr. 1974 | Coral reef; MSPS = 265 | −130 | 60 (18.3) | 7 mM sodium ascorbate |

[a] Enrichment substrates were prepared in concentrated form (pH 7.5) and injected into chambers at the beginning of the experiment (volume injected, 5 ml).
[b] MSPS, Mean sediment particle size (micrometers).
[c] Depth, 1 cm.
[d] Numbers in parentheses indicate depth in meters.

TABLE 2. *Estimates of the annual production of methane by in situ incubations*[a]

| Area | Conditions[b] | Initial rates[c] | Final rates[c] | No. of chambers | Incubation period (h) |
|---|---|---|---|---|---|
| Caesar Creek | Lt/endogenous | 2.54–2.61 | 1.52–2.86 | 2 | Initial, 0.0–22.0 |
| | Lt + substrate | 0.74–1.86 | 3.78–15.80 | 2 | Final, 69.0–92.0 |
| | Dk/endogenous | 0.64–4.37 | 1.21[d]–29.80 | 2 | |
| | Dk + substrate | 1.78–2.84 | 29.00–45.10 | 2 | |
| Hydro-Lab | Lt/endogenous | 0.02–0.15 | 0.10–0.32 | 2 | Initial, 0.0–19.0 |
| | Lt + substrate | 0.07–0.11 | 0.13–1.93 | 2 | Final, 92.0–115.0 |
| | Dk/endogenous | 0.11–0.42 | 1.20[d]–7.40 | 2 | |
| | Dk + substrate | 0.01–0.03 | 2.00–2.30 | 2 | |
| Bimini | Lt/endogenous | 0.21–0.47 | (−)0.19–0.06 | 3 | Initial, 0.0–24.5 |
| | Dk/endogenous | 0.31–0.40 | 0.31–0.76 | 2 | Final, 24.5–47.0 |
| Curaçao | Lt/endogenous | 0.08–0.66 | (−)0.03–0.48 | 3 | Initial, 0.0–19.5 |
| | Lt + substrate | 0.03–0.04 | 0.19–0.39 | 2 | Final, 44.0–68.0 |
| | Dk/endogenous | 0.13–0.21 | 4.17–24.35 | 3 | |
| | Dk + substrate | (−)0.02–0.01 | 0.17–1.15 | 2 | |

[a] Ranges of the final calculated values are given for each sediment area investigated. The values obtained by Koyama (11) are as follows: paddy soils, 804 kg/ha per year; upland forests, 4.2 kg/ha per year; forest soils, 9.09 g/ha per year.

[b] Lt, Light; Dk, dark.

[c] Kilograms per hectare per year.

[d] Disturbed (open) chamber. Chambers found open were not included in interpretation of data.

tensions within the dark chambers dropped steadily over the course of the incubations, and anaerobiosis was achieved after 68 and 93 h in the Curacao and Hydro-Lab experiments, respectively. Dissolved oxygen was not monitored during the first experiment at Caesar Creek, but it is assumed that the dark chambers were anaerobic by 60 h. The Bimini incubation was terminated early due to poor weather, but by 47 h the mean oxygen tension within the dark chambers had decreased to 0.8 ml/liter as compared with 3.75 ml/liter for the light chambers. The light chambers in all the experiments maintained an aerobic environment (e.g., Hydro-Lab: 2.8 to 4.4 ml/liter) that was lower in dissolved oxygen than the surrounding water (e.g., Hydro-Lab: 5.5 to 5.8 ml/liter), but higher than the dark chambers. The only exception to this occurred at the final determination at Curacao, when two out of the three light/endogenous chambers had higher internal values of dissolved oxygen than did the surrounding waters (7.8 versus 4.7 ml/liter).

There appears to be an inverse relationship between dissolved oxygen and methane levels for the dark chambers as compared with those for the light chambers (Fig. 2). The higher levels of dissolved methane achieved in the dark chambers are, therefore, due to the absence of photosynthetic oxygen production. Methane-producing bacteria are the strictest anaerobes known (19), and thus their activity is repressed by actively photosynthesizing benthic flora occupying the upper portion of the sediment column. The variations encountered in chamber-dissolved methane apparently reflect an uneven distribution of benthic microbial populations and activity (3).

Light chambers exhibited low, fluctuating rates of methane production (Fig. 3), whereas the dark chambers that fluctuated did so only in the early stages of the incubation period, after which rates increased markedly. The fluctuations (i.e., decreases) in production rates may be due to the activity of methane-oxidizing bacteria. Bacterial oxidation of methane would result in a net decrease in methane production rates, but only during the early stages of dark-chamber incubations, before the onset of anaerobiosis. By contrast, methane-oxidizing activity would persist within the light chambers due to the presence of oxygen, thereby giving rise to continuing rate fluctuations.

Substrate additions (acetate and formate) did not appear to significantly increase methane levels over endogenous levels (Caesar Creek and Hydro-Lab); therefore, either these substances are not limiting to growth of methanogens or a situation of competition exists which decreases the availability of these compounds

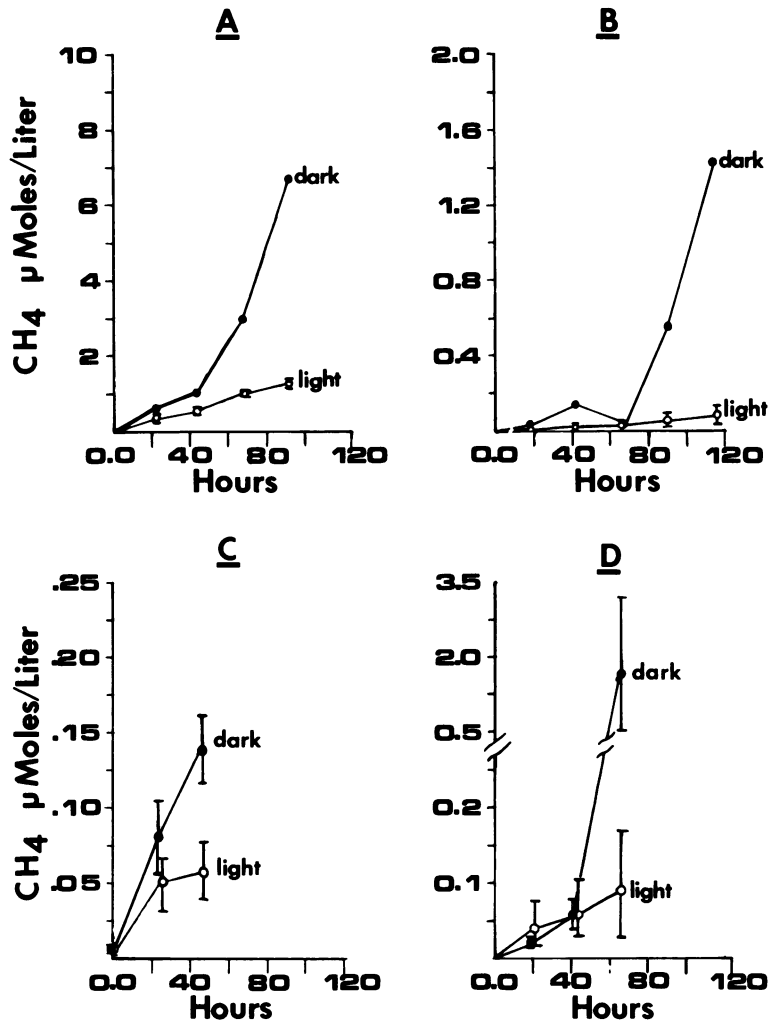


FIG. 1. *Dissolved-methane concentrations over the course of the incubation periods for the light/endogenous and dark/endogenous chambers. Mean values are plotted, and bars indicate the range of values observed for two or three replicate chambers. Caesar Creek and Hydro-Lab dark-chamber levels represent only single determinations since the duplicates were found to be disturbed during the incubation. (A) Caesar Creek; (B) Hydro-Lab; (C) Bimini; (D) Curaçao. Symbols: ○, light chambers; ●, dark chambers. Limit of $CH_4$ detection: 0.5 ± 0.065 µmol/liter. Values of dissolved $CH_4$ were determined on 50-ml samples and extrapolated to 1-liter volumes; conversion of milliliters per liter to micromoles per liter is achieved by multiplying by 44.6.*

to methanogenic bacteria. Sodium ascorbate was added to the Curacao enrichment chambers in an attempt to promote methanogenesis by removing oxygen, providing a substrate for benthic anaerobes and also decreasing methane-oxidizing activity. Ascorbate, however, initially inhibited methanogenesis, although production rates increased towards the end of the incubation (Fig. 3). Samples of the sediment surface under each chamber were examined with a microscope at the end of the experiment. The light/endogenous chambers harbored numerous benthic diatoms and dinoflagellates, whereas microbes were generally lacking under dark/endogenous chambers. Both light and dark ascorbate-containing chambers were overgrown with bacteria. Apparently the reducing conditions and the large bacterial population resulting from growth upon ascorbate created unsuitable conditions in these chambers for microalgae and, being motile, they escaped (an algal ring formed at the outside base of the ascorbate chambers). The initial inhibitory effect of ascorbate upon methane production ap-

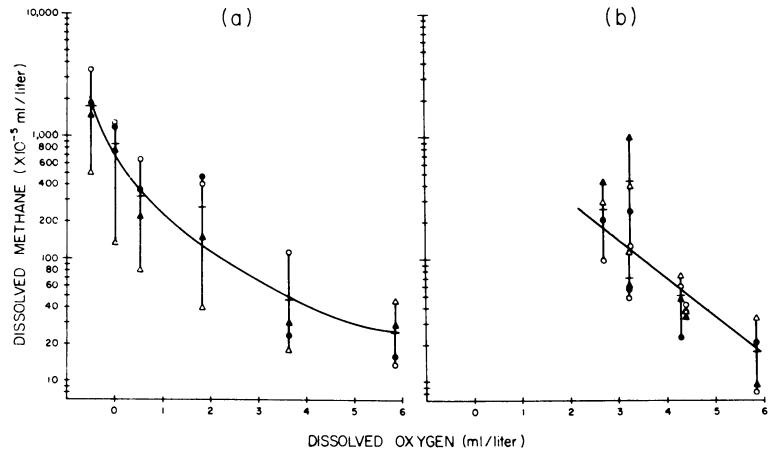


FIG. 2. *Relationship between dissolved oxygen and methane in the dark (a) and light (b) chambers of the Hydro-Lab experiment. Oxygen concentrations were determined in an additional two chambers (one light, one dark) and do not represent the values in individual chambers. In subsequent incubations (Bimini and Curaçao), dissolved-oxygen measurements were performed on all chambers, and results were similar to those of Hydro-Lab. Closed symbols, Chambers enriched with sodium formate; slash marks, mean values of dissolved methane.*

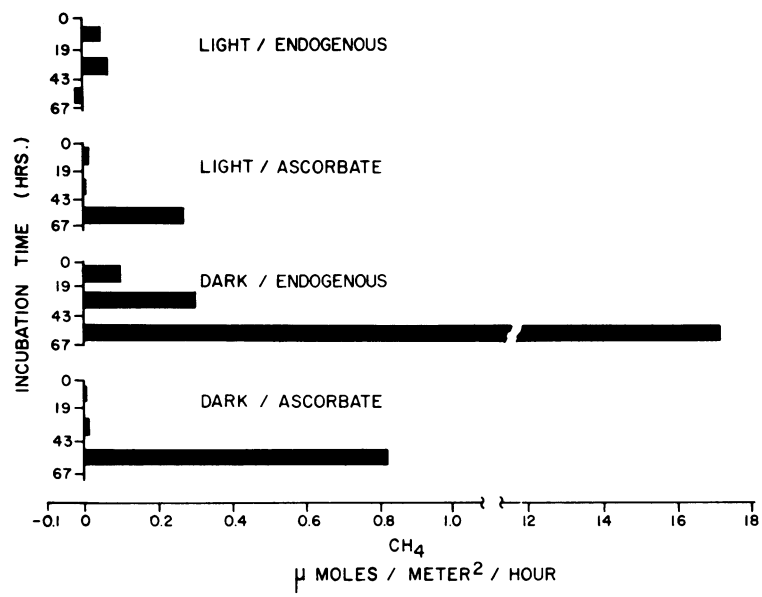


FIG. 3. *Methane production rates during the Curaçao experiment for some representative chambers. Chambers occasionally exhibited negative rates (light/regular).*

pears to be due to a lag phase of growth for anaerobes capable of metabolizing ascorbate. The addition of large quantities of ascorbate (1.25 g/liter) may have inhibited the degradation of endogenous substates and thereby temporarily interrupted substrate flow to the methanogens. Methanogenic substrates presumably became available after 68 h of incubation.

Yearly rate estimates of methane production for tropical sediments are shown in Table 2 and are compared with results obtained by Koyama (11), who based his estimates on 30-day laboratory incubations (dark) of rice paddy soils. Length of the in situ incubation period as well as conditions of the incubation (illumination, additions, etc.) strongly influenced production

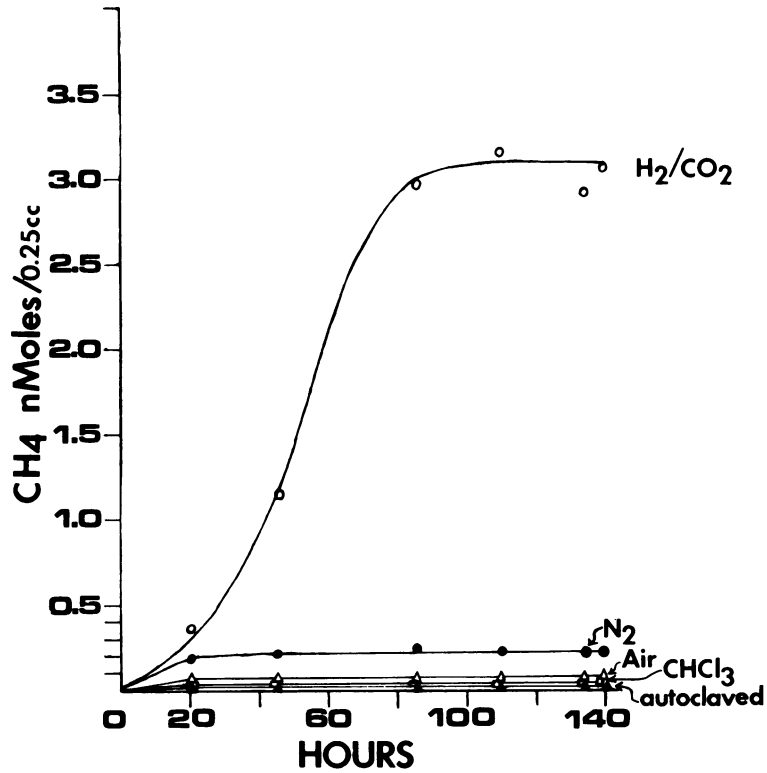


FIG. 4. *Results of laboratory sediment incubations. Symbols:* ○, $H_2$-$CO_2$ *(70:30);* ●, $N_2$; △, *air;* ▲, *autoclaved under* $N_2$ *atmosphere;* □, $CHCl_3$ *inhibited under* $N_2$ *atmosphere. Similar inhibitory effects occurred under an* $H_2$-$CO_2$ *atmosphere.*

rates. Apparently then, conditions within the chambers changed as time progressed, and thus estimates that are closest to "reality" or reflect normal environmental rates are those obtained for initial rates within the light/endogenous chambers. Rate measurements occurring at the end of the incubation period for the dark chambers greatly overestimate actual rates and, therefore, represent the capacity for methanogenesis under more optimal ecological conditions. The estimates of Koyama, therefore, may reflect methane produced under optimized conditions and thus may be an overestimate of natural rates since activities that would tend to depress observed methane production (i.e., methane oxidation, photosynthetic oxygen production, sulfate reduction) were minimized or eliminated. These activities are probably of great significance in sediments influenced by sunlight, such as shallow, tropical seas.

The observed production was microbial in origin (Fig. 4). Near-total inhibition was achieved by autoclaving, use of low levels of chloroform, and use of air. An $H_2$-$CO_2$ atmosphere stimulated methane production, and hydrogen is a recognized substrate of all methanogenic bacteria (5).

The fact that methane was produced in these in situ chambers over relatively short periods of time implies that sulfate suppression was rapidly overcome. This could be explained by the following: (i) a high rate of sulfate reduction is associated with these sediments, which rapidly depletes the chamber dissolved sulfate; (ii) the existence of sulfate-depleted zones of methane production (either macro- or microscopic) beneath layers of sulfate reduction (6, 14); (iii) enzymatic mechanisms (e.g., a low $K_m$) which enable methanogens to compete with sulfate-reducing bacteria (and other anaerobes) for available substrates ($H_2$ and acetate) at low sulfate concentrations.

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