

## Influence of Light Intensity on Methanotrophic Bacterial Activity in Petit Saut Reservoir, French Guiana

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**One year after impoundment in January 1994, methanotrophic bacteria in Petit Saut Reservoir (French Guiana) were active at the oxic-anoxic interface. This activity was revealed by the sudden extinction of diffusive methane emission (600 metric tons of CH<sub>4</sub> · day<sup>-1</sup> for the whole lake surface area, i.e., 360 km<sup>2</sup>). Lifting of inhibition was suspected. After reviewing the potential inhibitors of this physiological guild (O<sub>2</sub>, NH<sub>4</sub><sup>+</sup>, sulfides) and considering the similarities with nitrifiers, we suggest that sunlight influenced the methanotrophic bacteria. On the basis of phospholipid analysis, only a type II methanotrophic community was identified in the lake. Both growth and methanotrophic activity of an enriched culture, obtained in the laboratory, were largely inhibited by illumination over 150 microeinsteins · m<sup>-2</sup> · s<sup>-1</sup>. These results were confirmed on a pure culture of *Methylosinus trichosporium* OB3B. In situ conditions showed that water transparency was quite stable in 1994 and 1995 and that the oxycline moved steadily deeper until January 1995. Considering the mean illumination profile during this period, we showed that removal of methanotrophic growth inhibition could only occur below a 2-m depth. The oxycline reached this level in October 1994, allowing methanotrophic bacteria to develop and to consume the entire methane emission 4 months later.**

Within the three past decades, methanotrophic bacterial guilds have been studied in the laboratory for industrial applications, and the major factors controlling their activity have been examined in detail in pure and mixed cultures (8, 20, 26, 32). These experiments contributed to the understanding of how methanotrophs operate in natural environments, such as termite mounds, lake water, sediments, and soils (7, 28, 35, 39). Most of the data set obtained in tropical or equatorial lakes concerned only measurements of methane emissions or concentrations (21, 22, 36, 38). Only a few works reported experimental determination of bacterial methane oxidation in such environments (19, 31). So we chose to develop such an approach for a new equatorial reservoir.

Petit Saut Dam was built on the Sinnamary River, by Electricité de France Company, in order to sustain the economic development of French Guiana. More than 360 km<sup>2</sup> of equatorial rain forest was flooded. The anaerobic degradation of submerged organic matter rapidly induced strong emission of methane into the atmosphere (12). Because of the huge emission of reduced elements and the very little mixing by the wind, the oxygenated layer of the lake remained thin during the two first years.

The aim of this study was to determine the role played by methanotrophic bacteria in regulating methane emission and the factors controlling their growth in this equatorial medium.

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### MATERIALS AND METHODS

**In situ measurements.** Since the dam has been closed, water analyses have been regularly performed at a floating station (Barge Petit Saut [BPS]) located 300 m upstream of the dam (35-m maximum depth). Sampling was performed and physical-chemical profiles were determined by means of a peristaltic pump fitted with silicone rubber tubing (7.9-mm inside diameter; Masterflex Ltd.). A septum located just before the pump head allowed the water to be sampled without gas stripping. The water was pumped at 800 ml · min<sup>-1</sup>, and the line was flushed for 3 min before any samples were taken.

Methane emission fluxes were measured at the surface of the lake in a floating stainless steel chamber (50 by 50 by 18 cm). The concentration of methane gas was determined in the chamber by gas chromatography analyses at time zero and 15, 30, and 60 min later. Before the samples were taken, the atmosphere in the chamber was mixed by connecting the chamber to the peristaltic pump in a closed circuit. The linear regression determined from the experimental points allowed the methane flux to be calculated. During each trial, fluxes were measured at four different sites (two were in the flooded forest, one was the BPS site, and one was above the former river bed) distributed over the whole lake, in order to evaluate an average total methane emission, expressed as moles of CH<sub>4</sub> per square meter per day. The method used did, however, lead to an underestimation of the methane flux since the chamber reduced wind effects on surface roughness and transfer velocity.

Methane concentrations were determined by the headspace method. A 20-ml water sample was injected into a 57-ml glass flask which had been sealed with a Teflon septum and previously emptied with a vacuum pump. The methane concentration in the water was calculated after analyzing the headspace with a Hewlett Packard HP 5890 A gas chromatograph fitted with a flame ionization detector and a Poraplot Q semicapillary column. Details concerning the analysis conditions are described elsewhere (11).

The concentration of dissolved O<sub>2</sub> was determined in situ with an OXY 196 Wissenschaftlich Technische Werkstätten (Weilheim, Germany)-specific probe. Ammonium ion was analyzed immediately after sampling by spectrophotometry with the Nessler reagent. Total sulfides were determined by the colorimetric method of Cline (5) in samples preserved with zinc acetate.

Illumination in the water column was measured with a LI-COR 1000 luxmeter (wavelengths integrated from 400 to 700 nm).

Transparency was assessed with a 33-cm-diameter white disk (Secchi disk). The average depth at which the disk disappeared from view was noted after three readings.

The actual methane consumption rate was measured to document the methanotrophic activity in the water column. For each depth, four 26-ml glass flasks, closed with a Teflon septum, were filled with lake water. Twenty-milliliter vol-

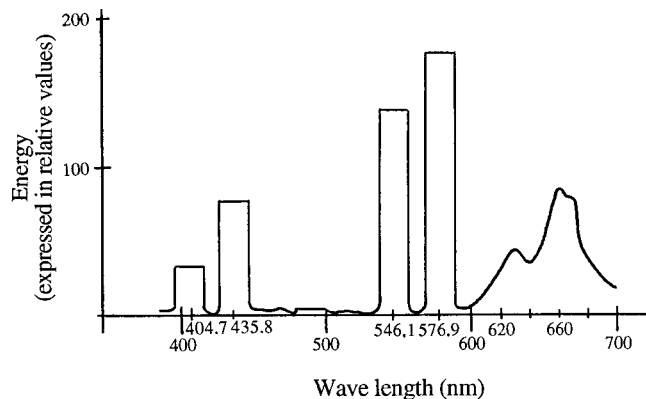


FIG. 1. Emission spectrum of the Phytoclaude lamp used to demonstrate the inhibitory effect of light on methanotrophic bacterial growth and activity.

umes of the water contained in two flasks were rapidly sampled to measure the initial methane concentration as described above. The two other glass flasks were incubated under in situ conditions for 1 h and the final methane concentration was measured. The difference between the two average measurements gives an estimation of the methane consumption rate in milligrams of  $\text{CH}_4$  per liter per hour. This determination was repeated three times at the BPS station in December 1995. The values were very similar over the whole lake. No abiotic methane disappearance was observed in samples preserved with thimerosal (BDH Chemicals Ltd., Poole, England), a chemical compound blocking bacterial enzyme activity in the same way mercuric chloride does.

**Laboratory experiments.** Water samples were taken at the BPS site from around the oxycline in September 1995 for enrichment of their methanotrophic bacterial population. Ten-milliliter aliquots of water complemented with 10% glycerol were frozen at  $-18^\circ\text{C}$  until required for enrichment. The samples were thawed and centrifuged three times to eliminate the glycerol carbon source. The resulting pellet was resuspended in 20 ml of nitrate mineral salt medium (41), buffered at pH 6.8, and incubated in a 250-ml glass Erlenmeyer flask at  $30^\circ\text{C}$  in darkness, with magnetic stirring (130 rpm). The headspace of the flask was filled with a methane-air atmosphere (50/50, vol/vol) complemented with 4 ml of  $\text{CO}_2$ , to enhance the start of bacterial growth (17). The atmosphere was replaced every day and the bacteria were subcultured to obtain a large active bacterial biomass. A freeze-dried pure culture of *Methylosinus trichosporium* OB3B (purchased at the National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland) was also resuscitated by the same method in order to perform control experiments.

Bacterial growth was monitored by spectrophotometry (Anthelie; Secomam, Domont, France) in a 1-cm cell (optical density [OD] measured at 540 nm).

Replicates of the bacterial enrichment were placed in a  $30^\circ\text{C}$  regulated incubator with different illumination intensities (from 10 to 1,100 microeinsteins  $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). The white light was delivered for 12 h a day (the day length in French Guiana) with two 400-W lamps (Phytoclaude; Claude, Paris, France). The emission spectrum is given in Fig. 1. The incident light was integrated from 400 to 700 nm by a LI-COR 189 luxmeter.

The total methane concentration was monitored during the incubations by analyzing the flask's headspace with a gas chromatograph fitted as described in the previous paragraph.

Phospholipid fatty acid (PLFA) analysis was performed on the enriched culture and on water taken directly from the lake, which had been freeze-dried after filtration on a Nuclepore polycarbonate membrane (0.22- $\mu\text{m}$  pores, 47-mm diameter). Briefly, total lipids were extracted by a modified Bligh and Dyer extraction procedure (40). The extracted lipids were fractionated by silica column chromatography with dichloromethane, acetone, and methanol as eluants. The methanol fraction (polar lipids) was dried under a stream of nitrogen and submitted to acid methanolysis. Nonadecanoic acid was added before methanolysis as an internal standard. The resulting fatty acid methyl esters were resuspended in 50  $\mu\text{l}$  of dichloromethane and analyzed by gas chromatography. The double bond positions in the monounsaturated fatty acids were determined by dimethyl disulfide derivatization (25). The double bond positions in the polyunsaturated fatty acids were determined after 4,4-dimethylloxazoline derivatization (10). Detailed procedures are reported elsewhere (16).

## RESULTS AND DISCUSSION

**In situ measurements.** Since the beginning of reservoir impounding, the anaerobic degradation of submerged organic matter caused the production of large quantities of reduced

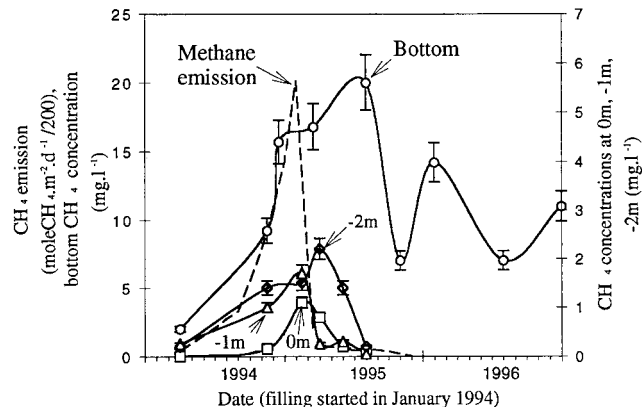


FIG. 2. Variation of total methane emission and methane concentrations in the water column (at depths of 0, 1, and 2 m and at the bottom), since the beginning of filling (day zero), in January 1994, d, day.

compounds. The bottom methane concentration increased until the filling was finished, i.e., in July 1995 (Fig. 2). After that time the bottom methane concentration evolved in a cyclic manner, with a maximum during the dry season. This phenomenon could be due to a variation in methane production or dilution by the huge amounts of rain in the wet season.

Average diffusive methane emission reached a maximum of  $0.103 \text{ mol of } \text{CH}_4 \cdot \text{m}^{-2} \cdot \text{d}^{-1}$  (600 metric tons of  $\text{CH}_4 \cdot \text{day}^{-1}$  for the whole lake surface area) in February 1995 (Fig. 2) and then decreased suddenly, falling to a level close to zero. This phenomenon could be attributed not to a drop in production, since the bottom concentration was still increasing, but rather to the activation of methanotrophic populations present in the upper layers of the water column. The biological methane-oxidizing activity was directly demonstrated by measuring the actual methane consumption rate at the BPS site (Fig. 3). This

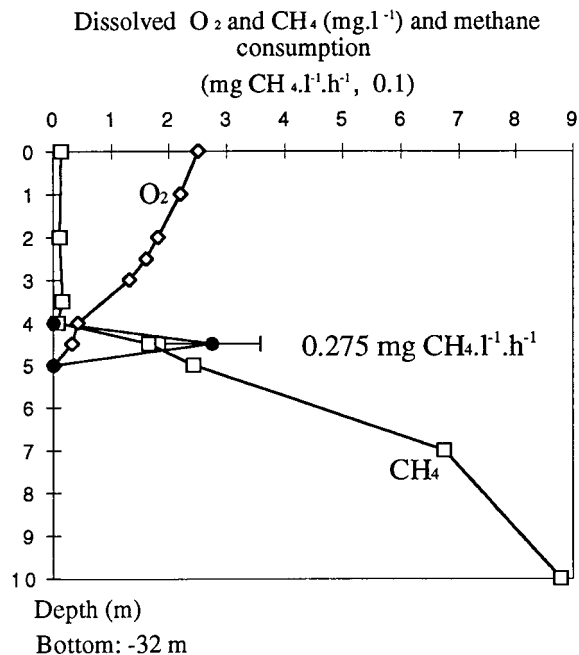


FIG. 3. Dissolved oxygen and methane profiles and methane consumption measured at the BPS site in December 1995. Values are in tenths.

TABLE 1. Measurements of ammonium and sulfide at the BPS site on 28 May 1995<sup>a</sup>

| Depth (m) | Concn of:   |   |
|-----------|---|---|
|           | Ammonium<br>( $\mu\text{mol} \cdot \text{liter}^{-1}$ ) | Sulfide ( $\mu\text{g} \cdot \text{liter}^{-1}$ ) |
| 0         | 37.07   | ND  |
| 2         | ND  | 10.68   |
| 5         | 40.37   | 139.2   |
| 7         | ND  | 171.2   |
| 10        | 139.46  | 175.36  |
| 15        | 203.32  | 178.24  |
| 20        | 235.25  | 180.8   |
| 25        | ND  | 185.28  |
| 32        | 476.37  | 188.48  |

<sup>a</sup> The oxycline was located at a depth of 3 m. ND, not determined.

vertical profile was obtained in December 1995 and showed that the methanotrophic activity was localized at the oxycline level, where methane and oxygen were both available in low but sufficient quantities. Sharp stratifications of this bacterial activity have already been shown to occur in aquatic environments (19, 30) and in sediments (6). The methanotrophic activity was assumed to act in a 1-m-thick stratum with a maximum activity of  $0.275 \text{ mg of } \text{CH}_4 \cdot \text{liter}^{-1} \cdot \text{h}^{-1}$  in the middle of the stratum (4.5 m from the surface) and over a surface area of about  $250 \text{ km}^2$ , at the oxycline (34). Considering that the methanotrophic activity was equally distributed across the lake, we obtained a total methane consumption of 825 metric tons of  $\text{CH}_4 \cdot \text{day}^{-1}$ , which could explain the sudden drop to zero of the methane emission recorded in February 1995.

**Hypothesis of methanotrophic bacterial inhibition.** This sharp decrease in methane emission occurred more than 1 year after the beginning of filling, suggesting a long lag phase or inhibition by environmental factors. During preliminary measures realized in 1994, Gosse and Grégoire (13) demonstrated downstream of the dam a strong oxygen depletion due to methane oxidation. This phenomenon occurred only when bottom water masses (anaerobic with methane) and upper water masses (aerobic with methanotrophic bacteria) were evacuated simultaneously. When bottom water was discharged alone into the Sinnamary River, oxygen consumption was reduced. So, we hypothesized that methanotrophic bacteria were present and inhibited in the upper water layers of the lake during the first year of filling.

A few inhibitory factors have been well documented in the literature. In Petit Saut Lake, a marked gradient in oxygen concentrations has been observed a few centimeters or meters below the lake surface since the beginning of the filling phase (Fig. 3). Oxygen concentrations were sufficiently low to enable methanotroph development. So, the hypothesis of inhibition by excessive concentrations of dissolved oxygen was improbable (29).

The ammonium concentrations observed around the oxycline, at the BPS site (Table 1), were very low compared with the reported inhibitory concentrations, which range from 1,000 to  $10000 \mu\text{mol of } \text{NH}_4^+ \cdot \text{liter}^{-1}$  (1, 4). So,  $\text{NH}_4^+$  could only have had only a trivial inhibitory effect in Petit Saut Lake in 1994.

Great amounts of sulfides were produced, and a maximum of  $188 \mu\text{g}$  of sulfides  $\text{S} \cdot \text{liter}^{-1}$  was measured at the bottom of the water column in May 1995 (Table 1). However, the sulfides could not attain the oxycline level in sufficiently high quantities, because they were totally consumed by phototrophic sulfur-oxidizing bacteria, located in the anaerobic zone, 1 or 2 m

TABLE 2. PLFA profiles determined on methanotrophic cultures and on a water sample from around the BPS oxycline in December 1996.

| Fatty acid                                  | % of composition <sup>a</sup> of: |             |
|---|-----------------------------------|-------------|
|   | Culture                           | Oxycline    |
| i14:0                                       | 0.33                              | 0.73        |
| 14:1  | 0.1                               |             |
| 14:0  | 0.49                              | 8.85        |
| i15:0                                       | 0.89                              | 2.26        |
| a15:0                                       | 0.21                              | 1.84        |
| 15:0  | 0.24                              | 2.05        |
| i16:0                                       | 0.25                              | 0.94        |
| 16:1 $\omega$ 9                             | 0.64                              | 0.25        |
| 16:1 $\omega$ 7                             | 0.69                              | 10.5        |
| 16:0  | 2.93                              | 30.7        |
| 10Me16:0                                    | 0.06                              |             |
| i17:0                                       | 0.22                              | 0.62        |
| a17:0                                       | 0.19                              | 1.39        |
| 17:1  | 0.09                              | 0.78        |
| 17:0  | 0.16                              | 0.96        |
| 18:2(8,14)                                  | 7.06                              | 0.52        |
| 18:2(7,14)                                  | 1.65                              | 0.31        |
| 18:1 $\omega$ 9                             | 1.78                              | 3.78        |
| <b>18:1<math>\omega</math>8<sup>b</sup></b> | <b>57.6</b>                       | <b>1.63</b> |
| 18:1 $\omega$ 7                             | 19.63                             | 4.16        |
| 18:0  | 3.08                              | 26.8        |
| 19:1  | 0.08                              |             |
| 20:0  | 0.13                              | 0.7         |

<sup>a</sup> For culture and the oxycline, the concentrations of PFLA were  $61,436 \times 10^{-10}$  and  $357 \times 10^{-10} \text{ mol} \cdot \text{liter}^{-1}$ , respectively.

<sup>b</sup> Specific to type II bacteria.

below the oxycline (9). So, the inhibitory effect of sulfur-containing compounds on methanotroph growth (1) can be also excluded.

**Laboratory experiments.** Methanotrophic bacteria are genetically related to nitrifying bacteria (18). This second physiological guild has an ammonia monooxygenase (AMO) which is inactivated by light (15, 33), specifically in the near-UV region (300 to 375 nm) and in the blue region of the spectrum (400 to 475 nm). AMO and methane monooxygenase (MMO) are closely related by their substrate specificities, the structures of their active sites, and their sensitivities to inhibitors (1). As a consequence, we suspected light as a possible environmental factor able to influence methanotrophic guilds in the upper layers of the water column. So, the effect of light on methanotrophic growth was tested in the laboratory on enriched samples.

Methane-oxidizing bacteria contain unusual PLFAs in their membranes (2, 14, 23, 24, 37). On the basis of the PLFA analyses and the presence of 18:1 $\omega$ 8 acid, a biomarker for methanotrophs, only type II was present in our enrichments (Table 2). Moreover, it seemed to be widespread in the whole lake and in the Sinnamary River.

Our mixed methane-oxidizing bacterial culture was capable of stable and predictable growth in batch culture. It exhibited a fatty acid profile with 18:1 $\omega$ 8 and 18:1 $\omega$ 7 acids accounting for 57.6 and 19.63% of the total PLFAs, respectively (Table 2). This profile is very close to those of *Methylosinus* or *Methylocystis* strains previously described (3), with 18:1 $\omega$ 8 acid (from 52.9 to 73.6%) and 18:1 $\omega$ 7 acid (from 14.8 to 37.7%) as major fatty acids. However, we can assume that the exact composition of the bacterial community remained unknown and that our enrichment conditions probably gave not a pure culture but a

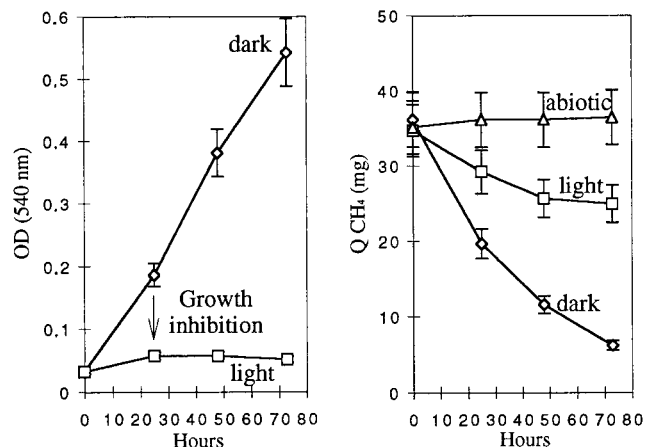


FIG. 4. Bacterial growth determined by OD at 540 nm and by total methane measurements in the incubated flasks. Replicates were incubated in darkness or under illumination at about  $168 \text{ microeinsteins} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 12 h a day. Abiotic experiments were run without an inoculum. Percent growth inhibition was calculated after 1 day of incubation. Q, quantity.

mixed culture composed essentially of methanotrophic bacteria.

During our enrichment procedure and for every assay submitted to illumination, the exponential growth phase stopped at the end of the first day of culture. So, we calculated for that time the percent inhibition versus the bacterial growth measured in the reference culture, after subtracting the OD value measured at the initial time. Figure 4 shows the results of an experiment run with an illumination of  $168 \text{ microeinsteins} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . The change in the methane quantity (in water-air) in the different culture flasks clearly showed that the growth inhibition was related to a strong decrease of actual methane consumption. We did not observe any abiotic methane oxidation with noninoculated flasks submitted to illumination. The experiments were performed with two separately enriched cultures showing almost identical PLFA compositions. Considering all the data obtained for both enrichments, the percent inhibition increased rapidly to reach high values (over 90% inhibition, taking into account the standard error) from an illumination about  $150 \text{ microeinsteins} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . This inhibition profile was confirmed with experiments using a pure *M. trichosporium* OB3B culture that exhibited about the same sensitivity to light (Fig. 5).

**Relation between laboratory experiments and environmental conditions.** So, under our experimental conditions, light inhibited the growth and the activity of the type II methanotrophic bacteria sampled in Petit Saut Lake. In order to relate this observation to environmental data, we monitored the oxycline level (where methanotrophic activity is located) and the Secchi disk depth since the beginning of reservoir filling. The average Secchi disk depth was about 1.5 m during the first 2 years of filling, revealing quite steady illumination of the water column during this period (Fig. 6). Small variations corresponding to periods of low sunshine during the rainy season were observed. On the other hand, the oxycline level sank deeper until January 1995. In consequence, the illumination that would have been measured at the oxycline decreased regularly. If we consider an average illumination profile in the water column obtained at the end of 1994 (Table 3), and the levels of inhibition versus illumination previously measured, we can see that the percent inhibition significantly dropped only for depths below 2 m (Table 3).

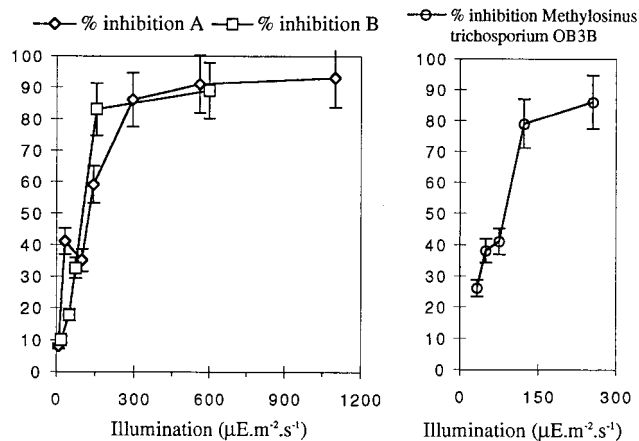


FIG. 5. Percent growth inhibition versus illumination delivered during bacterial growth. Experiments were run on two identical enriched cultures (A and B) obtained from water sampled at the oxycline in September 1995 and on a pure culture of *M. trichosporium* OB3B.  $\mu\text{E}$ , microeinsteins.

The average depth of the oxycline fell below 2 m in October 1994 (Fig. 6). Considering our laboratory results, the inhibition of methanotrophic guild activity could have been largely lifted during this period. The extinction of methane emissions occurred only 4 months later, a duration in agreement with the well-known low growth rate of methanotrophs in natural environments. Since this time, we have not detected any diffusive methane emission from the lake surface into the atmosphere. Currently, methane production at the bottom is still high, but most of the methane is evacuated through the hydroelectric plant (12) or rises as bubbles in the shallow flooded forest.

In conclusion, this work allowed us to formulate a plausible explanation for the sudden decrease in diffusive methane emissions from Petit Saut Lake in French Guiana. Environmental observations were closely paralleled by laboratory experiments which showed that light intensity has a serious inhibitory effect on the growth of the methanotrophic guild present in the water column around the oxycline and also on pure methanotroph cultures. So, the light intensity in the upper water column is thought to have seriously inhibited the methanotrophic activity during the year 1994. However, other unknown inhibitory factors might have been active during this first year of filling. Our experiments allowed us to conclude that the lifting of sunlight

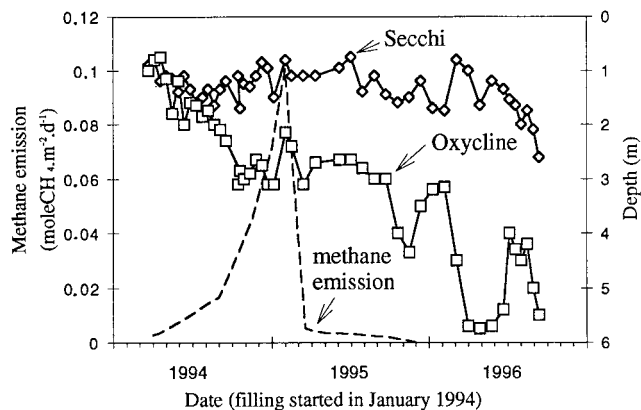


FIG. 6. Variation of the total methane emission, the Secchi disk depth, and the oxycline depth in Petit Saut reservoir since the beginning of the filling in January 1994. d, day.



TABLE 3. Illumination and growth inhibition at BPS site

| Depth (m) | Illumination<br>(microeinsteins · m <sup>-2</sup> · s <sup>-1</sup> ) | % Inhibition<br>(±10%) |
|-----------|---|------------------------|
| 0         | 760   | 92                     |
| 1         | 260   | 85                     |
| 1.5       | 200   | 84                     |
| 2         | 130   | 40                     |
| 3         | 74  | 32                     |
| 4         | 21  | 11                     |
| 6         | 2   | 0                      |

inhibition on this physiological guild occurred about 4 months before the methanotrophs had consumed large amounts of methane. Unfortunately, this study does not explain the actual mechanism by which light inhibits MMO. However, there is a great similarity of structure with the AMO of nitrifying bacteria, which is also photosensitive. Shears and Wood (33) speculated on the existence of a particular configuration of the active site of AMO having two copper atoms which become photosensitive after binding to oxygen (O<sub>2</sub>). Taking into account our experimental illumination conditions, one hypothesis is that the soluble MMO is inhibited by the UV or blue region of the spectrum, as Guerrero and Jones showed for nitrifying bacteria (15). We suggest that immunochemical and structural analyses after isolation of soluble MMO from methanotroph cultures may contribute, in further works, to explaining the inactivation by high illumination.

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