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Acetylene Reduction Assays for Nitrogen Fixation in Freshwaters: a Note of Caution

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Lake water samples were observed to transform [^{14}C]ethylene into water-soluble compounds that were undetectable by conventional acetylene reduction assay procedures. Methane oxidizing bacteria, which are known to be common in freshwaters, appeared to be responsible for this activity. As much as 28% of added ethylene has been observed to be transformed and this figure is probably an underestimate. It is suggested that acetylene reduction assays may not be accurately applied to samples containing methane oxidizing bacteria.

Organisms that fix molecular nitrogen also reduce acetylene to ethylene (7). As concentrations of both gases are easily determined by simple gas chromatography, this indirect assessment of nitrogen fixation has gained wide acceptance and has been applied to very diverse environments.

Current procedures depend upon at least three major assumptions: (i) that there exists a constant relationship between the amount of ethylene produced and the amount of nitrogen fixed, an assumption that is still being debated (5, 8); (ii) that ethylene is essentially insoluble in water, an assumption which is invalid but which we have found can be circumvented using suitable techniques; (iii) that ethylene produced during the assay is neither biologically nor abiologically altered during the incubation period.

In this report, it is shown that the third assumption is invalid in some lakes. It is suggested that our observations imply possible errors in the application acetylene reduction assays to certain aquatic and terrestrial environments.

Recently, precise techniques for the in situ assay of rates of methane oxidation were developed and applied to several Canadian Shield lakes in the Experimental Lakes Area of Northwestern Ontario (9, 10). Microorganisms that oxidized methane were found to be quite active in a variety of lakes (9, 10). In view of these findings, it was thought important to determine if they had the ability to utilize ethylene since reproducible recovery of ethylene produced in the acetylene reduction assay was absolutely essential to concurrent studies of nitrogen fixation in these lakes.

MATERIALS AND METHODS

Methane oxidation techniques (9, 10), substituting [^{14}C]ethylene (Amersham-Searle, uniformly labeled, 22 mCi/mmol) for [^{14}C]methane, were applied to three quite different lakes. Lake 227 is extremely eutrophic due to experimental additions of nitrogen and phosphorus (12), lake 383 is naturally mesotrophic, and lake 120 is naturally meromictic.

Profiles of [^{14}C]ethylene uptake as a function of depth. Water samples (125 ml) were precisely collected from a range of depths in a manner which was found to maintain in situ dissolved gas concentrations (9, 10). Small amounts of labeled ethylene (0.1 μmol/liter) were dissolved in the samples which were then incubated in the dark at in situ temperatures. After incubation for 4 h all samples were adjusted to pH 11 to stop biological activity. From these alkaline samples 25 ml of water were withdrawn by syringe for gas chromatographic analysis of dissolved methane and ethylene concentrations (10). Additional 10-ml portions of these alkaline samples were then sparged with air for 5 min, a period of time previously determined to remove all [^{14}C]ethylene without detectable loss of $^{14}CO_2$. A 4-ml portion of each sparged sample was then assayed for radioactivity using liquid scintillation procedures suitable for water samples (11). The results were assumed to represent ^{14}C originating from ethylene, which was present as cell material, soluble organic compounds, and CO_2.

Similarly, 10-ml portions of each original 125-ml alkaline sample were acidified to pH 2.5 with H_2SO_4 and then sparged to remove [^{14}C]ethylene and $^{14}CO_2$. The residual liquid was assayed for radioactivity as before. These results were assumed to represent ^{14}C in cell material and soluble organic compounds.

Substrate competition. To determine if substrate competition was occurring between methane and ethylene, approximately 0.4 μmol or [^{14}C]ethylene per liter was added to five 125-ml samples (lake 227, 4.12-m depth, 20 June 1974) and a range of methane concentrations (0.04 to 8.05 μmol/liter) was introduced into the five samples. In a second experiment,

0.42 μmol of [^{14}C]methane per liter was added to five 125-ml samples (lake 227, 4.12-m depth, 20 June 1974) and a range of ethylene concentrations (0 to 12.15 μmol/liter) was introduced. All samples were incubated for 4 h at in situ temperatures (~6 C) in the dark. Analytical methods were the same as those described above.

Temperature response. The rate of [^{14}C]ethylene metabolism was determined as a function of temperature. Five 125-ml samples (lake 227, 4.25-m depth, 11 June 1974) were each incubated with 0.5 μmol of ethylene per liter at different temperatures (6.5, 13, 20, 30, 40 C) for 4 h in the dark. Analytical methods were as mentioned above; in situ temperature was 6.5 C.

RESULTS

Profiles of [^{14}C]ethylene uptake as a function of depth. Every attempt of obtain profiles of ethylene transformation rates as a function of depth was successful. Acidic and alkaline sparged samples yielded similar rates of ethylene transformation and therefore only rates determined from the acidic samples are considered here. Figure 1 is typical of profiles done during summer stratification in that a peak of activity was found in association with sharp transitions in the concentrations of methane and oxygen and in association with high methane oxidation rates. In lake 227 this peak occurred at about 4.5 m, in lake 383 it was found at 7 m, and at 15 m in lake 120. Conservative measurements of the amount of ethylene transformed in the zones of peak activity ranged from 5 to 17% of that added to the sample.

A profile of ethylene transformation was also obtained during fall circulation of lake 227. In Fig. 2 it can be seen that ethylene is transformed throughout the mixed water column at this time. The methane oxidation profile has also been included to further illustrate the similar distribution of the two processes. On this occasion, the ethylene transformed represented an average of at least 28% of that added to the 1-, 3-, and 5-m samples.

Substrate competition. Ethylene was found to markedly inhibit methane oxidation when the ethylene concentration was equal to or greater than the methane concentration (Fig. 3). In contrast, increasing methane concentrations, up to approximately 5:1 (CH_4:C_2H_4) molar ratio, enhanced ethylene transformation rather than inhibiting it.

Temperature response. The effect of temperature upon the transformation rate of ethylene was quite pronounced (Fig. 4). Maximal rates of activity were exhibited in the 24 to 30 C range and decreased sharply at higher temperatures but only gradually between 24 and 5 C. These temperature response curves are very similar to such curves, obtained in our laboratory, for methane oxidation rates (9).

It was also observed (Fig. 4) that ethylene transformation rates based upon acid sparged

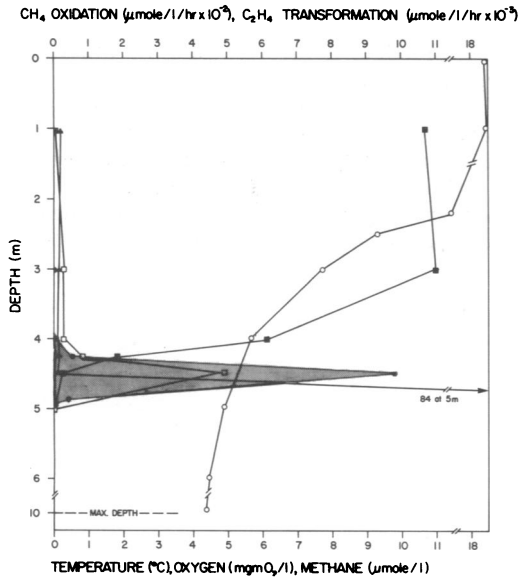

FIG. 1. *Typical summer distribution (6 June 1974) of oxygen (■), temperature (○), and methane (▲) with depth in lake 227, together with rates of ethylene transformation (●) and methane oxidation (□).*

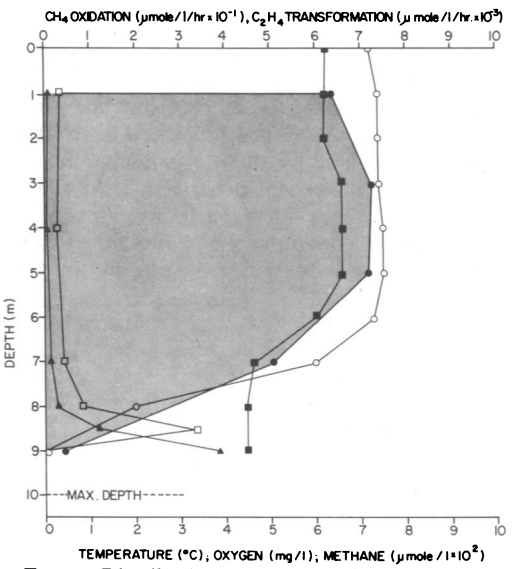

FIG. 2. *Distribution of oxygen (■) temperature (○), and methane (▲) with depth in lake 227 during fall circulation (8 October 1974), together with rates of ethylene transformation (●) and methane oxidation (□).*

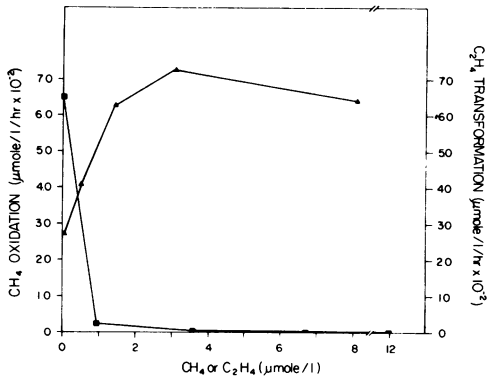

FIG. 3. *The oxidation rate of .42 μmol of methane per liter in the presence of various concentrations of ethylene* (■) *and the transformation of ~0.4 μmol of ethylene per liter in the presence of various concentrations of methane* (▲).

samples were consistently higher than alkaline sparged samples, especially at optimum temperatures. The significance of this will be discussed later.

DISCUSSION

The similar depth distribution of methane oxidizing and ethylene transforming activities, the similar temperature response curves, and the substrate competition experiments lead us to conclude that methane oxidation and ethylene transformation are mediated by the same organisms.

It also appears that whenever and wherever the methane oxidizing bacteria are active the results of acetylene reduction assays for nitrogen fixation may be in error due to the transformation of ethylene by these organisms. This is supported by the recent observation (3) that a methane-oxidizing, nitrogen-fixing culture of microorganisms degraded ethylene to the extent that the acetylene reduction assay could not be effectively employed.

In the course of the ethylene uptake studies, it was found that when acidic or alkaline samples were sparged and then filtered through 0.22-μm membrane filters (Millipore Corp.), very little radioactivity was retained on the filters. It was therefore concluded that metabolized ethylene was not incorporated into cellular material. Activity was detected in the filtrate, whether acidic or alkaline, and it was concluded that at least some of the metabolites must be water-soluble, nonvolatile compounds. In studying the temperature response, it was noted that acidic-sparged samples contained consistently higher activity than alkaline-sparged samples, especially at optimum temperatures (Fig. 4). Such findings indicate that CO_2 was probably not a major end product of ethylene metabolism. Depth profiles of ethylene transformation did not appear to exhibit these higher activities in the acidic samples. This was probably due to the low incubation temperatures used, as the absolute differences in activities between acidic and alkaline samples were much reduced and more difficult to detect.

Further work revealed that samples sparged at neutral pH contained higher activities than either acidic or alkaline samples. It appears that, depending upon pH, different quantities of the labeled metabolites were lost. It follows that there is no reason for believing that samples at any given pH value represent the total amount of ethylene transformed. In particular, it is concluded that such estimates of rates of ethylene transformation are probably underestimates.

Although the metabolism of ethylene is not well understood (6), it is possible to propose a mechanism for the ethylene transformation observed. It is known that hydrocarbon oxidizing micoorganisms can use ethylene, in some cases, as a nongrowth or cooxidation substrate (4), (Fig. 3). If one can extrapolate from the manner in which such organisms dihydroxylate olefinic double bonds (4) the expected product of ethylene transformation would be ethylene glycol. This product would be consistent with our observation of volatility at room temperature and active aeration. However, it is doubted that a single product is involved and attempts are being made to resolve the problem.

The biological instability of ethylene does not appear to be limited solely to the aquatic environment. In soils the distribution of methane oxidizing bacteria is so unbiquitous that they cannot be used in biological prospecting for natural gas fields (2). In addition, ethylene

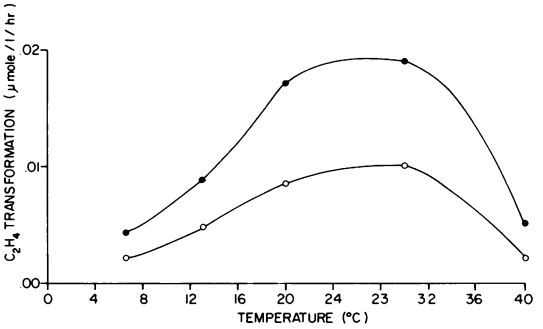

FIG. 4. *The apparent transformation rate of ethylene as a function of temperature, determined from acidic sparged samples* (●) *and alkaline sparged samples* (○).

uptake has been previously observed in soils and rice histospheres; it was suggested to be biologically mediated but no mechanisms have been postulated (1, 13). If methane oxidizing bacteria were responsible for the uptake of ethylene, there is reason to suspect the accuracy of acetylene reduction assays performed on soils.

Greater confidence could be placed in the acetylene reduction technique, when applied to soil or water, if ethylene stability were routinely demonstrated by radiochemical techniques. Should ethylene transformation be found to occur, it would appear that only $^{15}N_2$ techniques could be used accurately under such circumstances.

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