Flowthrough Reactor Flasks for Study of Microbial Metabolism in Sediments†

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Flowthrough reactor flasks are described that allow continuous low-level nutrient input to mixed anoxic sediments without dilution of the sediment. The flasks were tested by simulating sulfate inputs into sediments collected from a freshwater eutrophic lake. After an initial 2-day adaptation within the reactor system, rates of methane production and sulfate consumption were constant for the duration of a 12-day incubation. A sulfate input rate of 0.15 mmol liter of sediment⁻¹ day⁻¹ resulted in an equivalent rate of sulfate removal, which was unaffected by inputs of acetate (1.0 mmol liter of sediment⁻¹ day⁻¹). The rate of methane production in control reactors, 0.18 mmol liter of sediment⁻¹ day⁻¹, was doubled by the addition of acetate, whereas sulfate consumption was only stimulated by additions of high concentrations of sulfate plus acetate (1.5 and 1.0 mmol liter of sediment⁻¹ day⁻¹, respectively). The reactor system appears to be effective in maintaining the balance between sulfate reduction and methane production in freshwater sediments and is potentially useful for study of the response of sediment populations to varying inputs of naturally occurring substrates, selected inhibitors, or xenobiotic compounds.

The response of a naturally occurring microbial community as it is subjected to environmental fluctuations can yield valuable information regarding the activities and makeup of that community. As such, samples from various microbial ecosystems frequently have been subjected to artificial manipulations (e.g., see references 1–3, 6, 13, 14, 18, 22). For example, laboratory experiments may be designed to simulate seasonal changes in physicochemical parameters, to introduce exotic factors, such as selective inhibitors or xenobiotic compounds, or to alter the concentration of naturally occurring substrates. One obvious advantage of laboratory manipulations, as compared with field measurements of natural fluctuations, is the ability to conduct controlled experiments. However, interpretation of the results often is predicated on the ability to maintain the structure of the microbial community. Depending upon the type of sample and the set of circumstances, this situation may not be easily accomplished. This is particularly true for microbial communities that have adapted to and in many cases are limited by a flux of nutrients into or out of the habitat. Without maintenance of the flux of nutrients, the interrelationships of the microbial populations that compose the community will almost certainly change and thereby confound the interpretation of the results of the experiment.

Freshwater sediments are habitats in which the microbial community has adapted to nutrient fluxes across the sediment-water interface. However, these fluxes are difficult to maintain in laboratory experiments by conventional methods. A good example is testing the effect of low-level inputs of natural substrates upon specific microbial populations within anaerobic sediments. Batch additions will not simulate the natural steady-state flux of nutrients into the sediment; hence, they are inappropriate for experiments of this type. This report describes the use of sediment-containing flowthrough flasks designed to maintain a nutrient flux into sediments. The criteria used in designing the experimental apparatus were as follows. The system must maintain anaerobiosis, mix the sediment to maintain a homogeneous microbial population, continually add the substrate to maintain the desired input, and yet prevent sediment dilution. The flasks were tested by simulating low-level inputs of sulfate to freshwater sediments. The system was able to maintain the relationship exhibited between the rates of sulfate reduction and methane production within the freshwater habitat and therefore could be used to examine the effect of added acetate upon these activities.

MATERIALS AND METHODS

Profound surface sediment from Wintergreen Lake, a shallow (cₘ = 6.5 m) hypereutrophic lake located in southwestern Michigan (11), was sampled with an Eckman dredge. Jars were completely filled with sediment, sealed, and stored at 10°C; experiments were initiated on the day of collection. Sediment was homogenized in the jars with a paint shaker, transferred to an anaerobic glove box, and pooled. Sediment subsamples (700 ml each) were transferred to preassembled flowthrough flasks (called reactors for brevity: Fig. 1). The reactors then were stoppered with a butyl rubber stopper, sealed with pressure-sensitive tape, and removed from the glove box. They were then placed in a 10°C water bath, and each stirring assembly was connected to a continuous-drive 50-rpm motor. Each reactor was also connected in series to a check valve (1/3 lb/in²) and a water displacement gas trap by 3/16-in.-outside-diameter Tygon tubing connected to a syringe needle (1 in. = 2.54 cm); the needle was inserted through the reactor stopper. The gas trap contained a solution of 20% NaCl-0.5% citric acid to minimize CO₂ solubility (9). Headspace gas was flushed for 10 min with O₂-free N₂ through a syringe needle and then exhausted from a vent in the gas trap stopper.

The reactors were designed to maintain a continuous flow of an input solution to the sediments contained within and to remove sediment interstitial water from the reactor at the same rate. Input solutions were added by connecting each
reactor to a reservoir flask with sterile 0.03-in.-internal-diameter Tygon microbore tubing. Sediment interstitial water was pumped from the bottom of each reactor after having passed through a combination of three consecutive filters (Fig. 1). Input and output flow rates were maintained at 2 ml/h with a proportioning pump (Technicon Instruments Corp.). Effluent was collected in traps maintained in an ice bath, removed daily, and frozen until analysis. The residence time of the effluent was less than 1 h in the effluent lines. Analysis of sediment interstitial water, obtained directly from the reactor flasks by centrifuging the sediment at the end of a set of experiments, indicated that the concentrations of acetate, propionate, and sulfate in the effluent were the same as those in the reactor sediment. Hence, microbial metabolism does not appear to significantly affect the effluent as it passes through the filters and into the traps.

The reactor input solution contained the following (per liter): 10 ml of a trace element solution (15); Na$_2$SO$_4$, 0.031, or 3.1 g; NaHCO$_3$, 0.5 g; KH$_2$PO$_4$, 0.2 g; and sodium acetate·3H$_2$O, 1.98 g when included. A solution containing the first two items was adjusted to pH 7.2 in the reservoir flasks, autoclaved, and flushed with O$_2$-free N$_2$ while cooling. Filter-sterilized stock solutions of the latter three items (pH 7.2) were added when the reservoir flasks were cool. The flasks were stoppered and sealed to preclude the entrance of O$_2$ and then were connected to the reactors. A constant pressure of N$_2$, 3 lb/in$^2$, was maintained in each reservoir headspace.

The concentration of methane in the headspace of each reactor was subsampled daily with a syringe. Methane was analyzed by gas chromatography as previously described (12). Effluent sulfate concentrations were determined by high-pressure liquid chromatography (8); alkalinity was monitored with a Hach Chemical Corporation test kit. Volatile fatty acids were assayed with a Varian 3700 gas chromatograph equipped with a flame ionization detector. Analysis was made with a glass column (1.8 m by 2 mm) packed with 15% SP 1220 plus 1% H$_3$PO$_4$ on a solid support of Chromosorb W AW (100/120 mesh) at 110°C.

**RESULTS AND DISCUSSION**

The suitability of the reactors for sediment incubations was determined by examining methane production and sulfate reduction throughout the course of a 12-day incubation. Methane production, which is the predominant terminal electron-accepting process in Wintergreen Lake profundal sediments (7, 17), was unaffected by continuous low-level inputs of sulfate (Fig. 2). Two distinct rates of methane production were observed in the control reactor, an initial rate of 0.58 mmol of CH$_4$ produced liter of sediment$^{-1}$ day$^{-1}$ for 2 days followed by a second, lower rate of 0.18 mmol of CH$_4$ produced liter of sediment$^{-1}$ day$^{-1}$ for the duration of the experiment. The first value is very similar to rates of methane production observed in Wintergreen Lake profundal surface sediments (7, 12, 18). Corresponding drops in the rate of sulfate consumption (Fig. 3) and in the rate of $\delta^{35}$SO$_4^{2-}$ reduction (data not shown) were also evident in these same reactors. However, the rates of both methane production and sulfate consumption were constant after day 2. A threefold decrease in the rate of methane production was also reported by Strayer and Tiedje (19) for continuously agitated Wintergreen Lake sediments when the sediments were incubated in culture tubes. The mechanism responsible for the decrease in activity is unknown, but it may have been physical mixing; inhibition of methanogenesis by stirring has been reported by others (4, 20). Thus,
the mechanism does not appear to be unique to the reactor system. The decrease was not due to a depletion of essential nutrients, since the interstitial water retention time is 12 to 13 days, and the rates do not continually decrease, as might be expected from a dilution of an essential nutrient. The important factor is that, once the sediment had adapted to the reactor conditions (2 to 3 days), metabolic activities in control reactors appeared to be constant for the duration of the experiment. Thus, changes in activity during this time period (days 2 to 11) relative to controls can be inferred to be responses to the given substrate added.

The reactor system was capable of maintaining low-level inputs of sulfate without diluting the sediment. Sediment porosity was 0.94 both at \( T_0 \) and after an 11-day incubation, whereas the effluent pH at day 11 ranged from 6.95 to 7.15 for five different reactors. When the rate of sulfate addition was 150 \( \mu \text{mol} \) liter of sediment \(^{-1} \) day \(^{-1} \), the rate of sulfate consumption quickly approached the same value, effectively removing the added sulfate (Fig. 3). As would be expected, in the absence of added sulfate the sediment sulfate concentration was depleted and the apparent rate of sulfate consumption was low. (In this case, the rates are based on a decrease in the effluent sulfate concentration.) Thus, the reactor design provided the capability of controlling the rate of sulfate reduction during the course of the incubation. The addition of acetate had no effect upon the rate of sulfate removal when the sulfate input was 0.15 mmol liter of sediment \(^{-1} \) day \(^{-1} \). Since acetate serves as an electron donor for sulfate reduction in Wintergreen Lake sediments (17), this finding suggests that, for this rate of sulfate input, sulfate reduction was sulfate limited in the reactor system. The same situation exists in situ in Wintergreen Lake, where sulfate reduction is sulfate limited (16), and accounts for less than 15\% of the acetate mineralization (17). High inputs of sulfate (1.5 mmol liter of sediment \(^{-1} \) day \(^{-1} \)) and acetate (1.0 mmol liter of sediment \(^{-1} \) day \(^{-1} \)) resulted in a large increase in the rate of sulfate consumption (Fig. 3), most likely due to growth by sulfate-reducing bacteria when sulfate concentrations were no longer limiting.

Acetate inputs to the sediments contained within the reactor stimulated the rate of methane production over that of control reactors (Fig. 2), but the effect was not evident until after 24 h of continuous addition. A similar result was reported by Strayer and Tiedje (19) for batch additions of acetate to Wintergreen Lake sediment. They found that the initial (0 to 24 h) rate of methanogenesis was independent of the acetate concentration added, which led them to conclude that methane production from acetate was occurring in these sediments at a rate very near the \( V_{\text{max}} \) of the given population of methanogens. Only after 24 h of their 48-h experiment did the acetate amendments affect the rate of methane production. The reactor experiments, then, were able to duplicate the results of the short-term batch experiment and, because of the design of the reactor system, add additional information by overcoming some of the limitations of the batch experiments. First, since the acetate input can be maintained at a constant and continuous rate, the time course can be extended for much longer, and the effect of other amendments, such as changes in sulfate concentration, can be examined as well. Second, the concentration of the added substrates in the sediment interstitial water can be monitored easily without disrupting the incubation. The results demonstrate that the rate of methane production in reactors receiving an acetate input was constant for the day 2 to day 11 interval (Fig. 2) and that the addition of 0.15 mmol of sulfate liter of sediment \(^{-1} \) day \(^{-1} \) had no effect upon methane production. In addition, the effluent acetate concentration increased over time for those reactors receiving acetate (Fig. 4). The effluent acetate concentration of reactors not receiving an acetate input did not exceed 32 \( \mu \text{M} \). This indicates that pathways responsible for acetate metabolism appear to have been saturated with respect to acetate at this level of acetate addition for the entire 11-day incubation. This saturation might be expected since both methanogenic and sulfate reducers that utilize acetate are relatively slow growing (5, 10, 21) and would be slow to adapt to the added acetate. The rate of increase of acetate in the effluent was approximately 33 \( \mu \text{mol} \) liter of sediment \(^{-1} \) day \(^{-1} \) for reactors receiving acetate plus sulfate as compared with 17 \( \mu \text{mol} \) liter of sediment \(^{-1} \) day \(^{-1} \) for a reactor receiving only acetate. Since acetate is produced internally within the reactor as well as consumed, interpretation of this difference is complicated by potential differences in the rates of both acetate consumption and production. Hence, the results in Fig. 4 are presented relative to the effluent acetate concentration rather than to the amount of input acetate consumed.

In summary, both methane production and sulfate reduction could be studied simultaneously with this reactor system by controlling the rate of sulfate input. This input is accomplished without diluting the sediment or the microbial populations contained within. At the same time, the effect upon each activity of adding a naturally occurring substrate (acetate) could be examined, a task which would be difficult with batch experiments at low sulfate concentrations. Although continuous-flow and semi-continuous-flow sludge digestors have long been used to study anaerobic metabolism in sludge, few studies have investigated anaerobic
activities in freshwater sediments with this technique. The reactor system described here appears to be well suited for such a task, as it allows manipulation of the sediment to either maintain or alter the microbial community structure. Those sediments that have relatively high porosity and sufficient fluidity to allow mixing will probably work best in this system. Given sediments of this nature, this system can be useful for studying, for example, the fate of naturally occurring organic compounds or xenobiotic compounds within sediments. Although such results cannot be considered indicative of in situ activity, the response of sediment ecosystems to experimental manipulations serves as the basis for a better understanding of the activities of natural populations of bacteria.

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LITERATURE CITED