Associations of methanotrophs with the roots and rhizomes of aquatic vegetation

G. M. King

University of Maine

Follow this and additional works at: https://digitalcommons.lsu.edu/biosci_pubs

Recommended Citation


This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Digital Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Digital Commons. For more information, please contact ir@lsu.edu.
Associations of Methanotrophs with the Roots and Rhizomes of Aquatic Vegetation†

GARY M. KING*
Darling Marine Center, University of Maine, Walpole, Maine 04573

Received 25 April 1994/ Accepted 8 July 1994

Results of an in vitro assay revealed that root-associated methane consumption was a common attribute of diverse emergent wetland macrophytes from a variety of habitats. Maximum potential uptake rates ($V_{\text{max}}$) varied between about 1 and 10 μmol g (dry weight)$^{-1}$ h$^{-1}$, with no obvious correlation between rate and gross morphological characteristics of the plants. The $V_{\text{max}}$ corresponded to about $2 \times 10^6$ to $2 \times 10^7$ methanotrophs g (dry weight)$^{-1}$, assuming that root-associated methanotrophs have cell-specific activities comparable to those of known isolates. $V_{\text{max}}$ varied seasonally for an aquatic grass, Calamagrostis canadensis, and for the cattail, Typha latifolia, with highest rates in late summer. $V_{\text{max}}$ was well correlated with ambient temperature for C. canadensis but weakly correlated for T. latifolia. The seasonal changes in $V_{\text{max}}$ as well as differences from apparent half-saturation constants for methane uptake ($K_{\text{app}}$; generally 3 to 6 μM), indicated that oxygen availability might be more important than methane as a rate determinant. In addition, roots incubated under anoxic conditions showed little or no postanoxia aerobic methane consumption, indicating that root-associated methanotrophic populations might not tolerate variable oxygen availability. Hybridization of oligodeoxynucleotide probes specific for group I or group II methylotrophs also varied seasonally. The group II-specific probe consistently hybridized to a greater extent than the group I probe, and the relative amount of group II probe hybridization to C. canadensis root extracts was positively correlated with $V_{\text{max}}$.

Methane transport through aboveground plant tissues often exceeds diffusive and ebullitive fluxes across the sediment-water (or air) interface in vegetated wetlands (e.g., see references 11, 12, 16, 37, and 43). In rice paddies and natural wetlands, up to 90% of the total methane emission occurs via plants (e.g., see references 7, 8, 27, 28, 36, and 38), with the relative extent of emission varying as a function of shoot development. At present, the significance of plant emissions appears independent of physiognomy, even though absolute emission rates vary substantially among wetland systems. For example, plant emissions dominate methane flux in a Canadian fen (primarily Carex spp.; low total methane flux [42]), the Florida Everglades (primarily Cladium jamaicense; intermediate total flux [43]), and rice paddies (high total flux [8, 36, 38]). The uniformly large contribution of plants to total wetland emissions suggests that belowground plant surface area and gas phase transport within plant tissues are more important determinants of methane dynamics than is diffusional transport across the sediment-water interface. The net result is that wetland plants provide not only the reduced carbon fuel that drives methanogenesis but also the exhaust system that vent it to the atmosphere.

Although the mechanisms and dynamics of methane emissions from aboveground plant tissues have been well documented, the controls of methane flux into belowground plant tissues are poorly known. In addition to physical processes (e.g., diffusion), biological controls likely determine the characteristics of methane transport into roots and rhizomes. In particular, root-associated, bacterial methanotrophy probably functions as a filter that removes much of the methane influx. DeBont et al. (13) have observed enhanced populations of methane-oxidizing bacteria in the rice rhizosphere, and Holtzapfel-Pschorn et al. (20, 21) have reported that methane oxidation (presumably in the rhizosphere) consumed up to 80% of total methane production in vegetated rice paddy soils. Stimulation of methane efflux from rice plants incubated with nitrogen headspaces or acetylene, a potent inhibitor of methane oxidation (1), has provided additional evidence for rhizospheric methane consumption.

The rhizospheres (or roots) of other aquatic plants also oxidize methane. Epp and Chanton (14) have reported methane consumption by roots of Pontederia cordata and Sagittaria lancifolia. Increases in methane efflux from the leaves of "weed" species incubated in the presence of acetylene or nitrogen provide other evidence (21) for oxidation. King et al. (26) have observed rapid methane oxidation by live, sediment-free roots of several plant species from the Florida Everglades. These observations suggest that root tissues and their methanotrophic associations are an important locus controlling methane flux to the atmosphere.

In this study, basic characteristics of root-associated methanotrophy have been assessed by using an in vitro approach with multiple plant species from a number of different systems. Activities have been measured on a seasonal basis and correlated with temporal variations in the hybridization of oligodeoxynucleotide probes specific for sequences from the 16S rRNA of group I and group II methylotrophic bacteria. Results of these analyses indicate that associations of methanotrophs with aquatic plants are very common, that activities are probably limited by oxygen availability, and that group II methylotrophs dominate the associations.

MATERIALS AND METHODS

Field sites. Plants for methane uptake and production assays were collected primarily from two wetlands, Vihelmsborg So, near Aarhus, Denmark (described previously by King [22, 23]), contained a diversity of herbaceous emergent macrophytes in a silty sand of the riparian zone of a stream feeding a small lake.
The degree of submergence of this zone fluctuated with stream level. A second site located near Newcastle, Maine, was formed as a result of beaver dams on a small stream. Water levels fluctuated seasonally, with lowest levels from approximately July through October. The canopy at this site was dominated by the grass *Calamagrostis canadensis*, but sedges such as *Scirpus* sp. and the cattail, *Typha latifolia*, were also abundant. Plants from Vilhelmsborg Sø were collected from May to August, 1990; plants from the Newcastle site were collected periodically during 1991 and 1992. Live plants at the Newcastle site were marked with surveyor's tape to facilitate identification and collection during winter months. At this site, both *C. canadensis* and *T. latifolia* senesced during late September to early October; stems and shoots that remained above the water surface were frozen in place from mid-December to early March. In spite of the ice and snow pack, a continuous connection between sediments, roots, and the atmosphere was evident from the high methane concentrations (up to 11,100 ppm) in the lacunar spaces of *T. latifolia* stems during February.

**Rates of methane production and maximum potential consumption.** Intact plants were returned to the laboratory within a few hours of collection. The roots and rhizomes were typically covered with sediments or peats from the site during transport and prior to further processing. After excision, the roots and rhizomes of each species were washed separately and extensively with tap water to remove all loosely adhering sediment or peat. A comparison of tap and filtered (0.45-μm pore-size filter), deionized water revealed no effect of the type of wash water used on rates of methane production or maximum potential consumption. In addition, routine controls revealed no detectable methanotrophic or methanogenic activity in the rinse water. The roots and rhizomes of each species were sorted to remove nonliving organic matter, dead plant tissues, or other foreign material and then blotted to remove excess water. In most cases, washed roots and rhizomes from multiple individuals of a given species were pooled before use in RNA or activity assays. Material for RNA extractions and oligonucleotide probing was placed in sealable freezer bags and stored at −80°C. Triplicate subsamples (about 1 g [fresh weight]) of the remaining roots or rhizomes of individual species were then placed in 40-ml culture tubes (Belco, Inc.) containing 1 ml of deionized water for activity assays. In some instances, the roots of specific individuals of a given taxon were separated and compared in triplicate with the roots of other individuals of the same taxon.

For maximum potential methane consumption assays, culture tubes containing a headspace of ambient air were sealed with green neoprene stoppers. Ultrahigh-purity methane was added to a final concentration of 1 to 3%; uptake at ambient field temperature, ambient laboratory temperature, or both was assayed by gas chromatography with 0.3-ml headspace samples as described previously (26). For some assays, methane was added at initial concentrations of 100 ppm. For these samples, methane uptake was monitored until concentrations were <10 to 20 ppm (usually within 24 to 48 h); the tubes were then opened briefly, and uptake during a second incubation was monitored after addition of 1- to 3% methane. Deposition of methane when initial concentrations were 100 ppm typically behaved as a first-order process, and uptake was expressed as a rate constant (k, with units of t −1 gram [dry weight] −1 [gdw −1]); depletion when methane concentrations were 1 to 3% was zero order or linear, and consumption was expressed as a maximum potential uptake rate (micromoles of methane gdw −1 hour −1). For one set of *Sparganium erectum* and *Menhida aquatica* roots, methane was added at initial concentrations of 1 to 3%; methane depletion was monitored at intervals until final concentrations were <0.01%. Maximum potential consumption rates (Vapp) and apparent half-saturation constants (Kapp) were determined from progress curve analyses, based on the nonlinear parameter estimation algorithm of the software package Kaldiedagraph (Synergy Software). Root and rhizome dry weights were determined by incubating the tubes at 105°C for 48 h.

Rates of methane production were assayed by incubating roots and rhizomes in culture tubes containing a headspace of nitrogen. The root material for these assays was processed as described above; however, no attempt was made to minimize or avoid exposure to oxygen during washing. Anoxic headspaces were established by flushing the culture tubes with nitrogen for at least 30 min; the tubes were sealed with green neoprene stoppers. Methane accumulation was measured at intervals during incubations up to 120 h.

**RNA extractions and oligonucleotide probing.** The washed, sediment-free roots and rhizomes from pooled samples of each taxon for a given collection date were ground with a mortar and pestle at −80°C. This was accomplished by precooling the mortar and pestle in an ultracold freezer before use. One- to 2-g portions of the resulting flour were transferred to 15-ml polystyrene tubes containing 5 ml of 4 M guanidine isothiocyanate (GTC; from a Promega RNAgent kit [Promega, Inc.]). After being vortexed for 30 s, the suspension was subjected three times to a freeze-thaw cycle based on a period of about 30 min at −80°C followed by a 5-min incubation at 65°C. After the final heating cycle, the suspension was centrifuged at 1,000 × g for 10 min and the supernatant was decanted. Macromolecules in the supernatant were precipitated with an equal volume of isopropanol at −20°C for at least 1 h. The precipitate was collected by centrifugation at 10,000 × g for 10 min, washed with 70% ice-cold ethanol, air dried, and then redissolved in a minimal volume of 4 M GTC. Five-microtiter volumes of the GTC solutions were blotted onto nylon membranes (Zeta-probe; Bio-Rad, Inc.) with a slot blotter and by following the manufacturer’s (Bio-Rad, Inc.) instructions. The membranes were dried at 37°C for 30 to 60 min after blotting. Prior to hybridization, membranes were incubated for at least 1 h at 65°C in a solution (HS) containing 7% sodium dodecyl sulfate (SDS), 0.5 M Na2HPO4, and 1 mM EDTA. Nonspecific oligonucleotide binding with this solution was comparable to that with a more complex solution containing few seaward’s solution and denatured herring sperm DNA (e.g., see reference 40). The prehybridization solution was decanted and replaced with a minimum volume of HS to which was added a 32P-end-labeled oligonucleotide probe at a final activity of >106 dpm cm−2 of membrane. The probe was freshly labeled with T4 kinase according to the manufacturer’s instructions (Promega, Inc.). Two oligodeoxynucleotide probes were used for duplicate membranes: 10-γ, 5′GGTCCGAA GATCCCCGGCTT3′, for group I methylotrophs and 9-α, 5′CCTGAGTTTCTCGAAC3′, for group II methylotrophs (see references 40 and 41 for details on probe specificity). The membranes were hybridized for 9 h at 43°C and then washed according to the following scheme. Washes 1 and 2 were at 42°C with a solution containing (final concentrations) 5% SDS, 3× SSC (20× SSC is 175.3 g of NaCl liter−1 and 88.2 g of sodium citrate liter−1, pH 7.0), 1 mM EDTA, and 34 mM Na2HPO4, pH 7.2; wash 3 was at a temperature of 58 or 50°C for the respective probe with a solution containing 1% SDS and 1× SSC. A higher final wash temperature than that recommended by Tisen et al. (40) was required for the 10-γ probe to ensure appropriate specificity. The washed membranes were wrapped with saran; autoradiograms were pro-
produced by incubating the membranes at \(-80^\circ\text{C}\) for 24 to 48 h with Kodak X-Omat-AR X-ray film and an intensifying screen. After the autoradiograms had been developed, the membranes were sectioned and the bound radioactivity corresponding to each sample slot was determined by liquid scintillation counting. Crude nucleic acid extracts derived from the cell pellets of *Methylobacter albus* BG8 (group I; formerly *Methylomonas albus* BG6 [3]) were used as a positive control for the 10-\(\gamma\) probe and a negative control for 9-\(\alpha\); extracts of *Methylisus trichosporium* OB3b (group II) served as a positive control for 9-\(\alpha\) and a negative control for 10-\(\gamma\); a commercial preparation (Sigma Chemical Co.) of 16S rRNA from *Escherichia coli* was used as a negative control for both probes. Group I and II methanotrophs have been previously defined as coherent clades on the basis of a variety of physiological and biochemical traits; for example, group II but not group I methanotrophs fix nitrogen, possess an incomplete tricarboxylic acid cycle, and assimilate methane via serine (see references 3 and 24 and references therein).

**RESULTS**

**Methane consumption.** The washed, excised roots and rhizomes of all of the macrophytes sampled at Vilhelmsborg Sø consumed methane actively and without a lag (Fig. 1). Substantial differences in \(V_{\text{max}}\) were observed among the taxa (Table 1), but these differences were not associated with any apparent taxonomic characteristics (e.g., aboveground morphology). Variability within pooled root samples was generally relatively low, with a few exceptions. Variation for replicate samples from individual plants was more substantial for three taxa (*Sparganium erectum*, *Rorippa amphibia*, and *M. aquatica* [Table 2]), but uptake estimates based on the means from three individuals for each of these species were comparable in magnitude and variability to estimates from a separate assay based on pooled samples from multiple individuals (Table 1). Methane consumption also occurred without a lag for roots and rhizomes of plants collected near Newcastle, Maine (Fig. 2; Tables 1 and 2). Levels of variability for these pooled samples were comparable to those for samples from Vilhelmsborg Sø. \(V_{\text{max}}\) for *T. latifolia* from Denmark were also comparable to rates for the same species collected in Maine at a similar time.

**TABLE 1. Methane consumption by live, sediment-free roots and rhizomes**

<table>
<thead>
<tr>
<th>Species, habitat</th>
<th>Type</th>
<th>Rate (\pm) SE</th>
<th>Locale</th>
<th>Date*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veronica beccabunga</td>
<td>H</td>
<td>6.4 (\pm) 1.9</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Mentha aquatica L.</td>
<td>H</td>
<td>1.7-3.9</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Lycopus europaeus L.</td>
<td>H</td>
<td>28.0 ± 0.5</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Ranunculus sceleratus L.</td>
<td>H</td>
<td>1.7 ± 0.3</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Rorippa amphibia (L.) Bess</td>
<td>H</td>
<td>1.0-1.6</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Alisma plantago aquatica L.</td>
<td>WP</td>
<td>4.5 ± 1.2</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Typha latifolia L.</td>
<td>C</td>
<td>5.4 ± 1.1</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Sparganium erectum</td>
<td>B</td>
<td>4.5 ± 0.2</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Glyceria maxima</td>
<td>G/S</td>
<td>5.1 ± 1.6</td>
<td>DK</td>
<td>Ju-JI</td>
</tr>
<tr>
<td>Carex spp.</td>
<td>G/S</td>
<td>0.1-4</td>
<td>ME</td>
<td>YR</td>
</tr>
<tr>
<td>Calamagrostis canadensis</td>
<td>G/S</td>
<td>0.1-5.3</td>
<td>ME</td>
<td>YR</td>
</tr>
<tr>
<td>Typha latifolia</td>
<td>B</td>
<td>0.1-3.5</td>
<td>ME</td>
<td>YR</td>
</tr>
<tr>
<td>Cladium jamaicense, peat</td>
<td>G/S</td>
<td>0.7 ± 0.4</td>
<td>FL</td>
<td>Oc</td>
</tr>
<tr>
<td>Cladium jamaicense, marl</td>
<td>G/S</td>
<td>ND</td>
<td>FL</td>
<td>Oc</td>
</tr>
<tr>
<td>Eleocharis interstincta</td>
<td>G/S</td>
<td>ND</td>
<td>FL</td>
<td>Oc</td>
</tr>
<tr>
<td>Sagittaria lancifolia, peat</td>
<td>WP</td>
<td>0.5 ± 0.04</td>
<td>FL</td>
<td>Oc</td>
</tr>
<tr>
<td>Sagittaria lancifolia, marl</td>
<td>WP</td>
<td>ND</td>
<td>FL</td>
<td>Oc</td>
</tr>
<tr>
<td>Crinum americanum</td>
<td>Am</td>
<td>0.3-0.1</td>
<td>FL</td>
<td>Oc</td>
</tr>
</tbody>
</table>

* \(V_{\text{max}}\) are micromoles gdw\(^{-1}\) hour\(^{-1}\); location codes are as follows: DK, Vilhelmsborg Sø, Denmark; ME, Newcastle, Maine; FL, Florida Everglades (see reference 26). Broad taxonomic groupings are as follows: H, herbs; G/S, grasses and sedges; C, cattails; B, bur reeds; WP, water plantain; Am, amylris.
* Ju-Ju, June; Ju, July; Oc, October; YR, periodically through a seasonal cycle.
* ND, not determined.

\(V_{\text{max}}\) (Table 1) were typically estimated from the linear depletion of methane at saturating concentrations. However, for two species, *S. erectum* and *M. aquatica*, \(V_{\text{max}}\) were also calculated from nonlinear parameter estimation based on progress curves (Fig. 1). These rates compared favorably with estimates based on a linear regression of uptake at the beginning of the progress curve incubations (Table 2) and with initial rates of depletion by separate samples at saturating methane concentrations (Table 1). In addition, progress curve analyses provided estimates of \(K_{\text{app}}\); these values were similar to estimates calculated by assuming that \(K_{\text{app}} = V_{\text{max}}\). The first-order rate constant, \(k\), was determined from regression analysis of the region of the progress curves where methane depletion was exponential. Rate constants determined from incubations of *C. canadensis* with 100 ppm of methane, and the corresponding \(V_{\text{max}}\) values determined from incubations with 1- to 3% methane, were also used to estimate \(K_{\text{app}}\) (Table 2). All of the \(K_{\text{app}}\) values ranged between 3 and 6 \(\mu\text{M}\), irrespective of the plant species examined or calculation procedure.

\(V_{\text{max}}\) varied seasonally over a 30-fold range (Fig. 3A) for roots and rhizomes of *T. latifolia* and *C. canadensis* incubated at ambient field temperatures. Through winter, spring, and early summer, changes in \(V_{\text{max}}\) basically paralleled changes in field temperatures, although anomalously low \(V_{\text{max}}\) were observed for *T. latifolia* in April 1992. In addition, \(V_{\text{max}}\) for both *T. latifolia* and *C. canadensis* remained high while temperatures were relatively low in September 1992. \(V_{\text{max}}\) for *C. canadensis* were typically greater than \(V_{\text{max}}\) for *T. latifolia*, but the differences were statistically significant (\(t\) test; \(P \leq 0.05\)) only during the late spring-summer months. Seasonality in methane consumption was also evident for incubations of *C. canadensis* at ambient laboratory temperatures (22 to 25°C; Fig. 3B). In particular, \(V_{\text{max}}\) for methene consumption increased through spring and summer, as did rates for samples incubated at ambient field temperatures. \(V_{\text{max}}\) declined

**FIG. 1. Methane consumption by washed, sediment-free excised roots and rhizomes of *M. aquatica* from Vilhelmsborg Sø; the symbols ○, □, and ■ represent time course data from 1-g (fresh weight) samples from three individual plants.**
Table 2. Summary of kinetic data for washed, sediment-free roots and rhizomes

<table>
<thead>
<tr>
<th>Species and sample type (n)</th>
<th>( V_{\text{max}} \pm 1 \ SE )</th>
<th>( K_{\text{app}} \pm 1 \ SE )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. amphibia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (3)</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>B (3)</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>C (3)</td>
<td>1.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Mean, A, B, C</td>
<td>1.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Pooled (3)</td>
<td>1.3 ± 0.2</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td><strong>S. erctum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (3)</td>
<td>3.4 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>B (3)</td>
<td>2.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>C (3)</td>
<td>2.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Mean, A, B, C</td>
<td>2.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Pooled (3)</td>
<td>3.6 ± 0.4</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>NPE (6)</td>
<td>1.7 ± 0.3</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td><strong>M. aquatica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (3)</td>
<td>1.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>B (3)</td>
<td>1.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>C (3)</td>
<td>2.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Mean, A, B, C</td>
<td>1.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Pooled (3)</td>
<td>2.0 ± 0.3</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>NPE (3)</td>
<td>3.9 ± 2.2</td>
<td>4.2 ± 2.7</td>
</tr>
<tr>
<td><strong>C. canadensis (pooled)</strong></td>
<td>1.6 ± 0.4</td>
<td>5.8 ± 0.5</td>
</tr>
</tbody>
</table>

* All rates are in micromoles g dw \(^{-1} \) h \(^{-1} \) and \( K_{\text{app}} \) is in micromolar. NPE, nonlinear parameter estimation for kinetic data. For *R. amphibia*, *S. erctum*, and *M. aquatica*, samples either are from individual plants (A, B, or C) or are replicates from the pooled roots of multiple plants; all other data are based on replicates from pooled material only. Numbers of replicates are given in parentheses.

sharply during late fall and remained low but measurable through the winter.

On the basis of an analysis of the natural logarithm of methane uptake data, the activity of *C. canadensis* was strongly correlated with ambient field temperature during an annual cycle, with the exception of a point in late fall (Fig. 4). A much weaker correlation was observed for *T. latifolia* (unpublished data). The apparent activation energy for methane uptake by *C. canadensis* derived from an analysis of the data according to the Arrhenius relationship was 97.3 kJ °K \(^{-1} \) mol \(^{-1} \). Because of the weak correlation between temperature and activity, activation energies were not calculated for *T. latifolia*.

**Oligonucleotide hybridization with RNA extracts.** Seasonality was also evident in the binding of radiolabeled oligonucleotide signature probes to total root and rhizome nucleic acid extracts. Liquid scintillation assays provided a relative indication of the extent of hybridization by the probes, since the probes were used at equivalent specific activities and comparable stringencies. Extracts from both *C. canadensis* and *T. latifolia* hybridized with both the group I and group II methylotroph probes (Fig. 5), with the strongest signals in spring and summer. The group I and II probes hybridized with the appropriate positive controls, but not with the negative controls. For both plant extracts and all sampling dates, the group II probe hybridized to a greater extent than did the group I probes.
probe (Fig. 5). The extent of group II probe hybridization with *C. canadensis* extracts was well correlated with potential methane consumption rates measured for parallel root and rhizome samples, with the exception of the September sampling date (Fig. 6). A weaker correlation was observed for data from roots and rhizomes of *T. latifolia* (Fig. 6).

**Methane production.** Methane production occurred with little or no lag for roots and rhizomes of *C. canadensis* and *T. latifolia* incubated with an anoxic headspace (Fig. 7). Rates of methane production were typically higher for *C. canadensis*, even though this species was characterized by roots and rhizomes of much smaller diameter than those of *T. latifolia*. Rates of methane production were much lower than maximum rates of methane consumption for samples assayed in parallel. Shifts of roots and rhizomes from anoxic to oxic conditions appeared to substantially or totally inhibit methane production but did not stimulate active methane consumption. In contrast, parallel samples of roots and rhizomes incubated under oxic conditions readily consumed methane (Fig. 7).

**DISCUSSION**

The roots and rhizomes of many aquatic plants actively consume methane (Table 1). In fact, the absence of root-associated methanotrophic activity appears exceptional. King et al. (26) reported negligible activities for plants collected from Everglades marls, but otherwise, the roots and rhizomes of grasses, sedges and rushes, plantains, and the reeds from a variety of sites consume methane at substantial rates (Table 1). Since this activity occurs either on the rhizoplane or in the root tissues themselves, aquatic plants are not simply conduits for methane transport that bypass oxidation zones at the sediment surface or in the water column (e.g., see reference 39). In addition to serving as a gas transport system, the belowground tissues of aquatic plants function as a dynamic, oxygenated biofilter that facilitates methane consumption. The dynamics of this system are a function of spatial, temporal, and taxonomic variations in root and rhizome growth and gas transport. $V_{\text{max}}$ values provide an initial, surrogate estimate of the methanotrophic densities associated with roots and rhizomes. If per-cell $V_{\text{max}}$ values are comparable for roots and known methanotrophic isolates, one can estimate the population numbers necessary to account for the observed uptake. With methane oxidation rates of 20 mmol gdw$^{-1}$ cell biomass$^{-1}$ h$^{-1}$ (1) and a conversion factor of 4 $\times$ 10$^{12}$ cells gdw$^{-1}$, the range of observed plant consumption rates requires populations of about 2 $\times$ 10$^3$ to 2 $\times$ 10$^9$ methanotrophs gdw$^{-1}$ root$^{-1}$. These densities compare with CFU gdw$^{-1}$ estimates for sediments and soils of about 10$^2$ to 10$^6$ (18) and 10$^6$ to 10$^7$ (2), respectively. By using the assumptions given above and reported $V_{\text{max}}$ values, population estimates are about 0.9 $\times$ 10$^9$ to 1.8 $\times$ 10$^{10}$ cells gdw$^{-1}$ for a very active wetland sediment (22) and about 0.5 $\times$ 10$^9$ cells gdw$^{-1}$ for a deep lake sediment (29). Thus, the rhizoplane or interior of roots and rhizomes appears to offer a relatively favorable habitat for methanotrophic growth.

Assuming that the $V_{\text{max}}$ are proportional to methanotrophic biomass, the seasonality of uptake measured at a constant

![Graph](https://journals.asm.org/journal/aem/11�October%202021%20by%2026201053001%2010010%2C57%2C51%2Cker%3A%2C1%3A%2C1%3A%2C2%3A%2C2%3A%2C7%3A)
temperature (22 to 25°C) (Fig. 3B) provided evidence that changes in methanotrophic populations contributed to the dynamics of subsurface methane consumption (Fig. 3A). In general, methane uptake was greatest in the late spring and summer when the plant hosts were most active metabolically. This suggested that changes in methanotrophic populations were coupled to plant processes, with root oxygenation likely of greatest significance. However, the extent of seasonal change was not equally apparent in the two taxa examined. Changes in activities associated with T. latifolia roots and rhizomes were much less pronounced than in C. canadensis. The differences between taxa in seasonal responses (Fig. 3) and the more general differences in uptake rates among taxa (Table 1) indicated that species-specific characteristics, e.g., aerenchyma architecture, root morphology, and root physiology, among others, constrain the extent and activities of methanotrophic associations.

The differential association of methanotrophs with aquatic plants and differences in responses to seasonal changes in temperature and plant physiology have important implications for predicting the behavior of wetland systems in the context of climate change. Current climate change models predict wetland gas exchange as a parameter that changes mostly as a function of wetland area or hydrology (e.g., see references 33 and 34). Wetland area and hydrology are certainly key determinants, but future emissions may not be accurately predictable without considering the impact of changes in wetland species composition and variabilities in plant-methanotroph interactions.

Seasonality was also evident in the hybridization signals observed with oligonucleotide probes for 16S rRNA (Fig. 6 and 7). Probe binding to extracts from the roots and rhizomes of C. canadensis correlated well with maximum potential methane uptake rates, if data from late September were excluded from the analysis (Fig. 6). In late September, methane uptake rates for C. canadensis were high, even though ambient temperatures had declined sharply, the plants had begun to senesce, and the level of probe hybridization was relatively low. This pattern may have been the result of a smaller but more active methanotrophic population. Lower rates of root respiration accompanying senescence and decreased temperatures might temporarily have reduced competition between methanotrophs and plant tissues for oxygen, thus enhancing uptake.

The weak correlation between probe hybridization and uptake rates for T. latifolia suggests that several factors may confound interpretation of the results. Perhaps the most significant limitation is the fact that the 9-α and 10-γ probes are specific for methylotrophs, but not methanotrophs (40). As a consequence, an unknown fraction of the observed signal may arise from bacteria that utilize nonmethane C₃ substrates (e.g., methanol). Methylotrophs (e.g., the pink-pigmented facultative methylotrophs) have been previously documented as among the most common components of the phyllosphere (e.g., see reference 9) and likely occur in the rhizosphere as well. The relative abundances of methylotrophs versus methanotrophs may vary both temporally and spatially for a given plant and vary among plant taxa. Other confounding factors include the variability in ribosome (i.e., oligonucleotide target) content as a function of physiological state and variations among the methanotrophic and methylotrophic taxa in ribosome content for a given physiological state.

In spite of these caveats, the probe data indicate that bacteria from the group II methanotroph-methylotroph cluster (17) dominate root and rhizome associations throughout the year for both C. canadensis and T. latifolia. A similar pattern has been observed for limited analyses of RNA extracts from the methane-consuming roots of a Carex sp. obtained from a different site (25a), and for extracts of the floating, stemless Lemna minor (17). Naphthalene oxidation by C. canadensis plant roots (25a) is consistent with domination by group II methanotrophs, since group I methanotrophs typically cannot facilitate this process (5).

Although it is not possible by using existing data to assign definitively the methanotrophic activity of aquatic plants to either group I or group II taxa, it appears that the phyllosphere generally favors or promotes associations of group II species. Group II methanotrophs also appear to dominate in methane-enriched soils (4, 30). In contrast, group I methanotrophs...
appear to dominate the metalimnia of two lakes (17) and the symbionts of mytilid mussels (e.g., see reference 6). It is tempting to speculate that the diazotrophic character of group II methanotrophs provides a selective advantage in systems where nitrogen is potentially limiting (e.g., methane-enriched soils and plant roots), while greater growth yields provide an advantage for group I methanotrophs in systems where nitrogen is relatively abundant or where methane is limiting (e.g., the water column and animal tissues). This pattern conforms to the outcome of competition studies using the group I (M. albus BG8) and the group II (M. trichosporium OB3b) methanotrophs in continuous-flow reactors (15). A more detailed analysis of root-associated methanotroph phylogeny and the determinants of methanotroph distribution should provide important insights into the controls and dynamics of root-associated activities in situ. Recently developed signature oligonucleotide probes specific for various phylogenetic and physiological subgroups of methanotrophs, as well as probes specific for methyloths, will allow much greater resolution of these issues (4).

Kinetic data (Table 1) provide insights into other controls of root-associated methane uptake. In spite of differences in root and rhizome morphology, the K_s values for all plants used in this study were about 3 to 6 μM. These values are comparable to results from sediments and some cultures (see review in reference 24) but are generally lower than dissolved methane concentrations in the root zones of typical wetlands (e.g., see references 10, 35, and 44). Pore water methane concentrations on the order of 10 to 100 μM are often observed, with values of <10 μM uncommon. Although bulk methane values are likely higher than those in the rhizosphere or root tissues, aerenchyma methane concentrations can be used to establish lower limits for root and rhizome methane concentrations. After conversion to equivalent dissolved concentrations, typical methane partial pressures in the stem aerenchyma of a number of different plants approximate the range of K_s values reported here. These observations suggest that methane concentrations per se are not the primary limiting factor for uptake rates. Other parameters, e.g., oxygen concentrations, may be much more critical. Since the supply of oxygen for root methanotrophs must come from the aboveground plant tissues, and since methanotrophs must compete for oxygen with a diversity of other aerobes, including the root cells, limitation by oxygen is likely.

A somewhat different aspect of oxygen limitation is illustrated by results from oxic and anoxic incubations of roots and rhizomes (Fig. 7). The accumulation of methane with little or no lag during anoxic incubations suggests that microzones suitable for a fermentative-methanogenic metabolism exist on (or more likely in) living roots. The significance of these microzones and their associated methanogenesis is undoubtedly underestimated in this study, since root and rhizome tissues were processed under oxic conditions prior to the anoxic incubations. The intimate association of fermentors and methanogens with living roots is important for at least three reasons: (i) methane produced by these associations is more immediately available for plant transport than methane produced in bulk sediments or peats, (ii) fermentors and methanogens directly associated with plant tissues may have greater access to more readily degradable organic substrates, and (iii) methane produced in these associations may have significantly different isotopic signatures than has bulk methane. If root-associated methanogenesis is quantitatively significant in situ, the isotopic signatures of bulk sediment methane may not represent an accurate reference for estimating the extent of physical and biological isotopic fractionation that occurs during methane transport to the atmosphere (e.g., see reference 18).

The results of incubations with both C. canadensis and T. latifolia also indicated that methanogenesis is strongly sensitive to oxygen, as expected, and that methanotrophic activity does not appear to recover from relatively brief exposures to anoxia (Fig. 7). The sensitivity of root-associated methanotrophy to anoxia differs distinctly from the sensitivities observed for peats, sediments, and cultures (22, 25, 26, 31, 32). In each of the latter two cases, it is evident that methanotrophs possess a notable ability to survive anoxia, and to rapidly consume methane after reintroduction of oxygen. The anoxia intolerance of root-associated methanotrophy observed in this study may arise from toxic effects of fermentation end products resulting from both plant cell and microbial activity. Whether a similar intolerance of anoxia occurs in situ is unclear. However, it is certain that the roots of at least some aquatic plants are variably oxygenated because of diurnal and seasonal shifts in plant metabolism. As a consequence, root-associated methanotrophy may be restricted to tissues that are continuously oxygenated. Any such limitation could prove an important determinant of the relative significance of root-associated methanotrophy for the magnitude of wetland methane fluxes.

In conclusion, the rhizoplane, and likely the interior, of the roots and rhizomes of aquatic vegetation promotes the growth of methanotrophs, thereby substantially expanding the volume of the methanotrophic zone below the sediment surface. The activity of root-associated methane-oxidizing bacteria varies seasonally and among plant species, may be dominated by group II species, appears relatively sensitive to oxygen, and may be limited to a greater extent by oxygen than by methane. Because aquatic plants are a major conduit for methane flux to the atmosphere, root-associated methanotrophy is an important component of global methane dynamics. The characteristics and controls of root-associated methanotrophy are therefore key elements that must be incorporated into models of the response of wetland methane flux to global and regional climate change.

ACKNOWLEDGMENTS

This work was supported by NASA grants NAGW-1428 and NAGW 3746 and by NSF grant DEB-9107315.

I thank A. P. Adamsen for assistance with plant identification and assays in Denmark and G. Brusseau and R. S. Hanson for advice on RNA processing and the use of 16S rRNA probes for methylotrophic bacteria.

REFERENCES


6. Cavanaugh, C. M. 1993. Methanotroph-invertebrate symbioses in...


