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Aram Calhoun
University of Maine

Gary M. King
University of Maine

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Regulation of Root-Associated Methanotrophy by Oxygen Availability in the Rhizosphere of Two Aquatic Macrophytes†

ARAM CALHOUN AND GARY M. KING*

Darling Marine Center, University of Maine, Walpole, Maine 04573

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The relative importance of oxygen for root-associated methanotrophy was examined by using sediment-free, intact freshwater marsh plants (*Pontederia cordata* and *Sparganium eurycarpum*) incubated in split chambers. The root medium contained approximately 100 μM methane. Methane oxidation was calculated from the difference between methane loss from chambers in the presence and absence of 1 mM 1-allyl-2-thiourea, a methanotrophic inhibitor. When the root medium was oxic, methane oxidation accounted for 88 and 63% of the total methane depletion for *S. eurycarpum* and *P. cordata*, respectively; the remainder represented diffusional loss to the atmosphere via roots, stems, and leaves. Under suboxic conditions, methane oxidation was not detectable for *S. eurycarpum* but accounted for 68% of total methane depletion for *P. cordata*. The introduction of a biological oxygen sink, *Pseudomonas aeruginosa*, resulted in complete loss of methane oxidation in *S. eurycarpum* chambers under oxic conditions, while methane consumption continued (51.6% of total methane depletion) in *P. cordata* chambers. The differences between plant species were consistent with their relative ability to oxygenate their rhizospheres: during a suboxic incubation, dissolved oxygen decreased by 19% in *S. eurycarpum* chambers but increased by 232% for *P. cordata*. An in situ comparison also revealed greater methanotrophic activity for *P. cordata* than *S. eurycarpum*.

Natural and agricultural wetlands contribute an estimated 40 to 50% of the total methane emitted to the atmosphere annually (8, 40). Given the key role of methane in atmospheric chemistry (8, 16), understanding of the controls of methane flux is essential for predicting methane dynamics in the context of global climate change.

The magnitude of methane emission from wetlands to the atmosphere reflects the balance between methanogenesis and methanotrophy. Some of the key parameters affecting this balance include temperature, plant distribution and productivity, sediment type and organic content, and hydrologic dynamics (35, 42). The role of each of these has been elucidated largely in the context of methanogenesis. Methane fluxes from subarctic to tropical ecosystems have been quantified, and various controls of production have been analyzed (1, 7, 19, 26, 49). Mechanisms of gas transport (plant mediated as well as diffusive flux and ebullition through water and sediment) have also been studied in detail (6, 8, 12–14, 21, 25, 39).

More recently, the focus of attention has shifted from methane production to the dynamics of methane oxidation. Methane oxidation occurs at two loci in wetlands: (i) oxic sediment or peat surfaces and (ii) the rhizosphere and roots of aquatic plants. A number of studies have shown that methane oxidation is important in rice paddies (15, 20, 23, 25, 48), with rates accounting for up to 95% of the potential methane flux. Similarly high relative activities have been recorded for sediments and peats of natural freshwater wetlands, including temperate and boreal systems (11, 18, 27, 41, 50) as well as subtropical and tropical wetlands (17, 20, 32, 42).

The potential importance of root-associated methanotrophy has been established for several aquatic species (22, 23, 28, 32, 40, 44). However, plant-associated methanotrophy is less well understood than methanotrophy in sediments. Estimates of the relative extent of rhizospheric methane oxidation vary widely from 10 to 90% of methane production (14, 22, 24, 25, 29, 32, 46), with relative activity in situ somewhat lower than in vitro activity (15, 17, 27, 44).

Gilbert and Frenzel (23) have examined the effect of rice plants on oxygen distribution, pore water methane concentrations, and the distribution and number of methanotrophic bacteria associated with the rhizoplane (live plant root surface), the rhizosphere, and bulk sediment. Their results suggest that rice plants support methanotrophy by increasing oxygen availability in the root environment. Gilbert and Frenzel (23) have also concluded that methane limits methanotrophy associated with rice plants. In a series of greenhouse experiments, Schipper and Reddy (44) measured the extent of methane production and oxidation in the rhizosphere of *Sagittaria lancifolia*. They found that over 50% of the potential methane flux was oxidized. In contrast to Gilbert and Frenzel (23), Schipper and Reddy suggest that rhizosphere methanotrophy is dependent on oxygen transport through the plants. King (28, 29) has also postulated a major role for oxygen limitation on the basis of indirect observations.

In order to distinguish between rhizospheric and rhizoplane methanotrophy and to specifically examine controls of the latter, we have used intact, sediment-free plants with a root chamber that allowed manipulation and monitoring of methane uptake. The availability of and competition for oxygen as controls of methane oxidation were examined by using two common freshwater marsh species, *Pontederia cordata* (pickerelweed) and *Sparganium eurycarpum* (bur-reed). The possible role of ammonium as a control and the relative abilities of the two plant species to oxygenate the root chamber medium were also investigated. Results indicate that oxygen availability is paramount in importance for root-associated methanotrophy and that root oxygenation may vary markedly among wetland macrophytes, with proportional changes in methanotrophy.

* Corresponding author. Phone: (207) 563-3146, ext. 207. E-mail: GKing@Maine.Maine.Edu.

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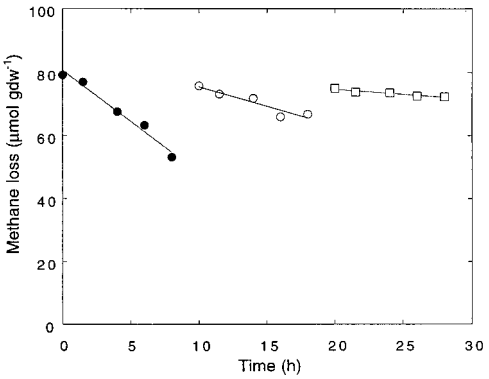

FIG. 1. Example of methane consumption by *P. cordata*. Methane loss rates (micromoles per gram [dry weight] of root) during oxic (●), suboxic (○), and 1 mM ATU (□) incubations are shown. Methanotrophic activity was calculated as the difference between total methane loss rates and total methane loss rates with ATU.

MATERIALS AND METHODS

Plant chambers. Plants for chamber experiments were collected from two freshwater marshes in Bristol and Orono, Maine. Plants were freshly harvested for each experiment; root damage was minimal, since the substrate from which they were extracted was a sapric muck. Sediment was removed from the plant roots through a series of successive rinses in plastic tubs filled with nonchlorinated well water at 20°C. The sediment-free roots of intact *P. cordata* and *S. eurycarpum* were incubated in cylindrical acrylic chambers (10-cm diameter, 21-cm height) consisting of a flanged cylindrical bottom (1.5-liters) and a top fitted with a central port and well through which leaves or stems extended (see Fig. 7). Roots and rhizomes were contained entirely in the chamber bottom. The plant was secured in the top with porous foam to support it firmly without crushing the stems or leaves. The top and bottom were submerged in nonchlorinated, air-saturated ($O_2 > 200$ μM) well water to eliminate bubbles. A well (3.5 cm high) holding 45 ml of water surrounded the plant stem and facilitated transpiration. Transpiration rates were estimated as the difference between water loss from plant chambers and control chambers. Loss from controls was approximately 10% of loss from plant chambers. The wells were filled with water and topped off with 5 mm of mineral oil to reduce gas diffusion (3, 10). The outer rim between the chamber top and body was sealed with silicone cement for each chamber experiment.

Methane concentrations in the chambers were adjusted by simultaneously adding methane-saturated water and removing an equal volume from the chamber, resulting in a final concentration of about 100 μM. One milliliter of root medium was removed by needle and syringe through sample ports at intervals to measure methane loss (with a Shimadzu 14A gas chromatograph and a flame ionization detector); samples were replaced simultaneously with an equal volume of water. The mean initial oxygen concentrations were >250 μM for oxic incubations.

For assays with suboxic rooting media, chambers were assembled as described above. Subsequently, the chambers were flushed with about 4 volumes of deoxygenated well water. A volume of methane-saturated, deoxygenated water was added to the chambers as needed to yield a final methane concentration of about 100 μM. The initial oxygen concentrations were <40 and ≤25 μM for *P. cordata* and *S. eurycarpum* chambers, respectively. Totally anoxic conditions could not be established due to oxygen leakage from plant roots.

Initial experiments showed that the chambers were gastight. Controls consisted of solid glass rods in lieu of live plants. Changes in the controls were negligible for oxygen and methane over the experimental period. The chambers were incubated without stirring at a constant temperature (20°C) in a water bath during any given assay. Leaves and stems were illuminated with a metal halide light source at about 400 microeinsteins m^{-2} s^{-1}. Methane uptake for a given plant was measured by sequential assays with one of the following sets of incu-

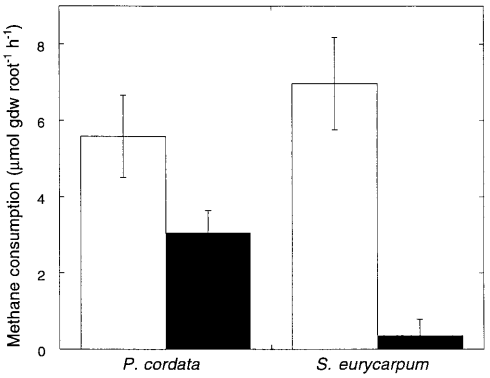

FIG. 2. Rates of methane consumption by sediment-free, intact roots of *P. cordata* (n = 18) and *S. eurycarpum* (n = 10) in whole-plant chamber experiments under oxic (open bars) and suboxic (closed bars) conditions. Error bars indicate ±1 standard error. gdw, gram (dry weight).

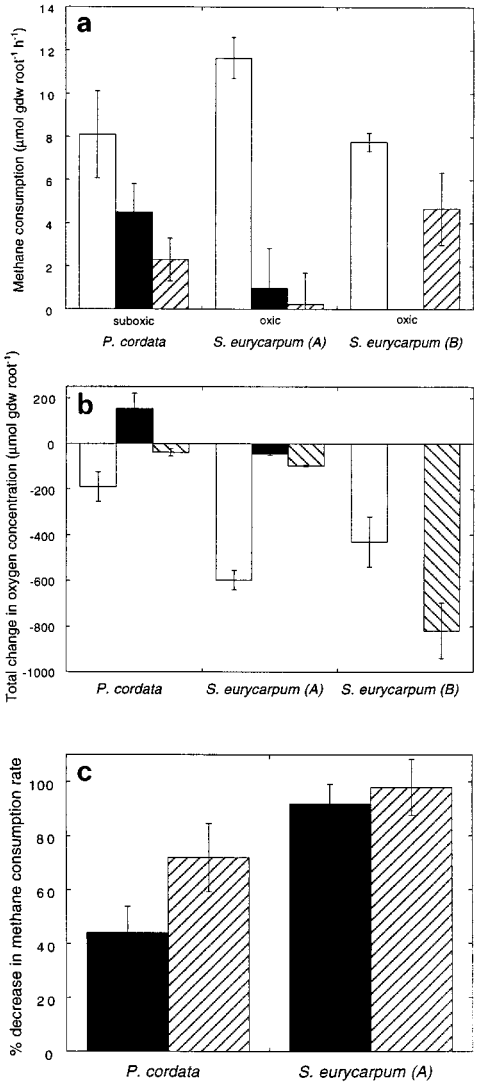

FIG. 3. (a) Rates of methane consumption by sediment-free, intact roots of *P. cordata* (n = 3) and *S. eurycarpum* (n = 6) in whole-plant chamber experiments with the following treatments: oxic (open bars), suboxic (closed bars), and suboxic plus *P. aeruginosa* (cross-hatched bars). *P. aeruginosa* was added to oxic chambers for *S. eurycarpum* assays A and B at concentrations of 10^9 and 10^8 cells ml^{-1}, respectively. Error bars indicate ±1 standard error. (b) Oxygen change during an 8-h experimental period with the following treatments: oxic (open bars), suboxic (closed bars), and suboxic plus *P. aeruginosa* (cross-hatched bars). Root dry weights were 0.4 and 0.3 g for *P. cordata* and *S. eurycarpum*, respectively. Error bars indicate ±1 standard error. (c) Percent decrease in methane consumption rates from oxic to suboxic conditions (closed bars) and oxic to suboxic plus *P. aeruginosa* conditions (cross-hatched bars) by sediment-free, intact roots of *P. cordata* (n = 3) and *S. eurycarpum* (A) (n = 3) in whole-plant chamber experiments. Error bars indicate ±1 standard error. gdw, gram (dry weight).

TABLE 1. Rates of methane consumption by intact *P. cordata* and *S. eurycarpum* roots[a]

Treatment	Consumption rate (μmol g [dry wt] of root^{-1} h^{-1}) ± SE		
	P. cordata	*S. eurycarpum*	
		Assay A	Assay B
Oxic only	7.3 ± 0.3	9.0 ± 4.0	5.5 ± 1.3
Oxic + NH_4Cl	7.3 ± 0.6		5.2 ± 1.3
Suboxic only		3.0 ± 1.0	
Suboxic + NH_4Cl		3.1 ± 1.3	

[a] Roots were incubated with 1 mM NH_4Cl (n = 3).

bation regimens: (i) oxic conditions and then oxic conditions plus 1-allyl-2 thiourea (ATU) or (ii) oxic conditions, suboxic conditions, and then suboxic conditions plus ATU. Each incubation involved an 8-h time course with 100 μM methane initially. ATU was added as 13 ml of a 0.1 M stock solution to yield a final concentration of 1 mM. At this concentration, ATU completely inhibited microbial methane oxidation. Rates of methane oxidation were calculated as oxidation = oxic or suboxic uptake − ATU uptake (see Fig. 1). Losses in the presence of ATU were attributed to diffusive transport through roots and rhizomes to stems and leaves.

In some cases, ammonium chloride was added to a final concentration of 1 mM coincident with the addition of methane under both oxic and suboxic conditions. Ammonium chloride also was added to a set of chambers 10 h prior to methane addition. Preincubation with ammonium was used to maximize the potential response.

Nine chamber assays involved the addition of *Pseudomonas aeruginosa* as an alternative to chemical reductants (e.g., sulfide and thioglycolate) that might affect methanotrophy or plant metabolism. Cultures of *P. aeruginosa* were grown in 150 ml of Luria-Bertani medium (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g) and incubated overnight at 33°C. Cells were harvested during exponential growth by centrifugation for 10 min at 4°C and 10,000 × *g*. The pellets were washed and resuspended in 150 ml of Higgins phosphate buffer (10 mM; pH 7) and recentrifuged. The resulting pellets were resuspended in 5 ml of a succinate salts medium (sodium succinate, 4 g; KNO_3, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; pH 7) and then injected into the chambers at final concentrations ranging from 2.3×10^8 to 1.6×10^9 cells ml^{-1}. *P. aeruginosa* was added to chambers following a suboxic time course for *P. cordata* and to oxic chambers for *S. eurycarpum*. In the assays with consecutive suboxic incubations, the chambers were made oxic and reamended with methane overnight to maintain the methanotrophic population. Changes in oxygen status were measured by withdrawing 10 ml of chamber medium initially and again after completion of the experiment 8 h later. Oxygen was monitored by using a Winkler titration (2).

Changes in methane concentration were measured by withdrawing 1 ml of medium from the chambers at fixed intervals over 8 h. All samples were withdrawn following 1 min of stirring via a magnetic stir bar in the chambers. The samples were transferred to gastight 8-ml blood collection tubes and vigorously shaken for 2 min to equilibrate dissolved methane with the tube headspace. For each time point, two headspace samples of 0.2 cm^3 were collected with 1-ml disposable syringes and needles for methane analysis with a Shimadzu 14A gas chromatograph and a flame ionization detector operated at 150°C. Methane was separated with a Porpak Q column in series with a wide-bore capillary column (DB-1; 30 m by 0.53 mm [outside diameter]; J&W Scientific, Inc.). Uptake rates were estimated from regression analysis of the methane time courses, all of which were zero order.

Typically, each assay used three experimental chambers and one control chamber. Comparisons of oxic and suboxic rates and oxygen budgets were based on pooled data from 18 and 10 plant chambers for *P. cordata* and *S. eurycarpum*, respectively. Results from ammonium chloride additions were based on six chambers for each plant species. Bacterial amendments were made to three and six *P. cordata* and *S. eurycarpum* root chambers, respectively.

Dry weights were determined for all experimental plants by oven drying at 105°C for 12 h. Emergent plant biomass (stems and leaves) and submerged biomass (roots and rhizome) were weighed separately.

Root oxygenation. A dye technique to visually monitor oxygen leakage patterns from roots was adapted from the methods of Armstrong et al. (4) and Smits et al. (47). The plant chambers previously described were filled with a 0.3% molten agar solution containing 25 mg of methylene blue liter^{-1}. The solution was bubbled with nitrogen overnight in a water bath (55°C). Subsequently, sodium dithionite (0.2 g liter^{-1}) was added as a reductant; the agar solution was cooled to 35°C and carefully poured into chambers containing intact plants. A layer of molten paraffin was solidified at the surface, and the remaining space was filled with water to serve as a diffusion barrier. The chambers were photographed at regular intervals to monitor patterns of oxygen leakage from the roots.

The effect of the rhizoplane microbiota on oxygen leakage from the roots of both plant species was estimated by agitating the roots of whole plants in 0.2% sodium dodecyl sulfate (SDS) for 5 min to remove surface films and bacteria. The roots were subsequently soaked overnight in a solution containing the indicated amounts (in micrograms per milliliter) of the following antibiotics: ampicillin, 50; nalidixic acid, 100; streptomycin, 100; and amphotericin B, 5. The plants were then assayed with the dye method as described above. The efficacy of the SDS and antibiotic treatments was determined by staining treated and control sediment-free excised roots with Baclight Live/Dead fluorescent stain (Molecular Probes, Inc.). Fine roots on agar-coated slides were examined with a Zeiss Axioscope and epifluorescent illumination using 40× achrostigmat and 100× plan-neofluar phase-contrast objectives. The effects of SDS and antibiotic treatments on methane consumption rates were tested on sediment-free excised roots by using the same methods for SDS and antibiotic rinses used for whole plant roots incubated in the methylene blue chambers. For *P. cordata*, methane consumption rates for untreated sediment-free excised roots and roots treated with antibiotics were compared; comparisons were also made for untreated roots and roots washed in 0.01, 0.05, and 0.1% SDS.

The effect of root respiration on patterns of oxygen leakage was assayed after roots of intact plants were soaked in 10 mM sodium azide for 3 h and in 3% formaldehyde for 2 h. The plants were then incubated in chambers with methylene blue agar as described above. The efficacy of inhibition of root cell respiration was tested on excised roots receiving the same treatment as whole plant roots. Oxygen consumption of treated roots was measured by incubating excised roots in 10-cm^3 airtight glass syringes filled with oxygen-saturated water and measuring oxygen consumption over 8 h. Oxygen was assayed as described above. Oxygen consumption rates were compared with those of untreated excised roots.

Statistical analysis. Statistical analyses of differences among chamber treatments were determined through analysis of variance; post hoc pairwise differences in means were determined with Tukey's test (SYSTAT, Inc., 1994).

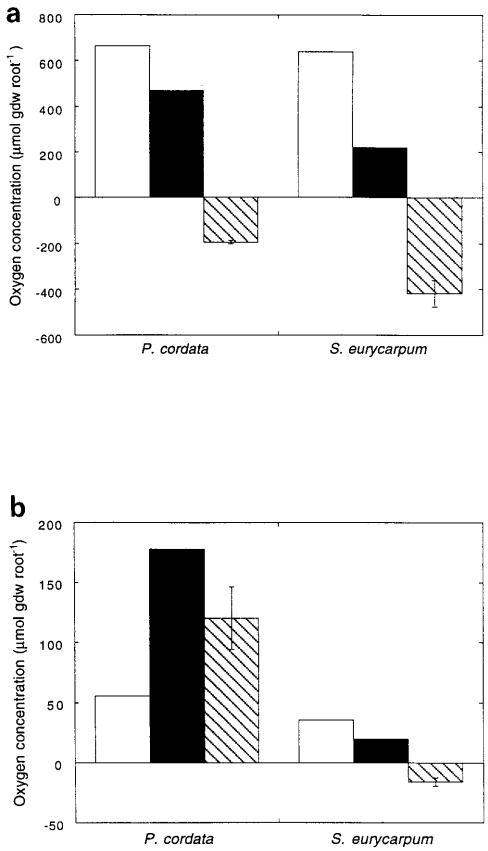

FIG. 4. (a) Changes in oxygen concentration under oxic conditions in whole-plant *P. cordata* (n = 18) and *S. eurycarpum* (n = 10) chambers over an 8-h experimental period. Open bars, initial oxygen concentrations; closed bars, final oxygen concentration; cross-hatched bars, change from initial concentrations. Error bars indicate ±1 standard error. (b) Changes in total oxygen concentration under suboxic conditions in whole-plant *P. cordata* (n = 18) and *S. eurycarpum* (n = 10) chambers over an 8-h experimental period. Open bars, initial oxygen concentrations; closed bars, final oxygen concentrations; cross-hatched bars, change from initial concentrations. Error bars indicate ±1 standard error.

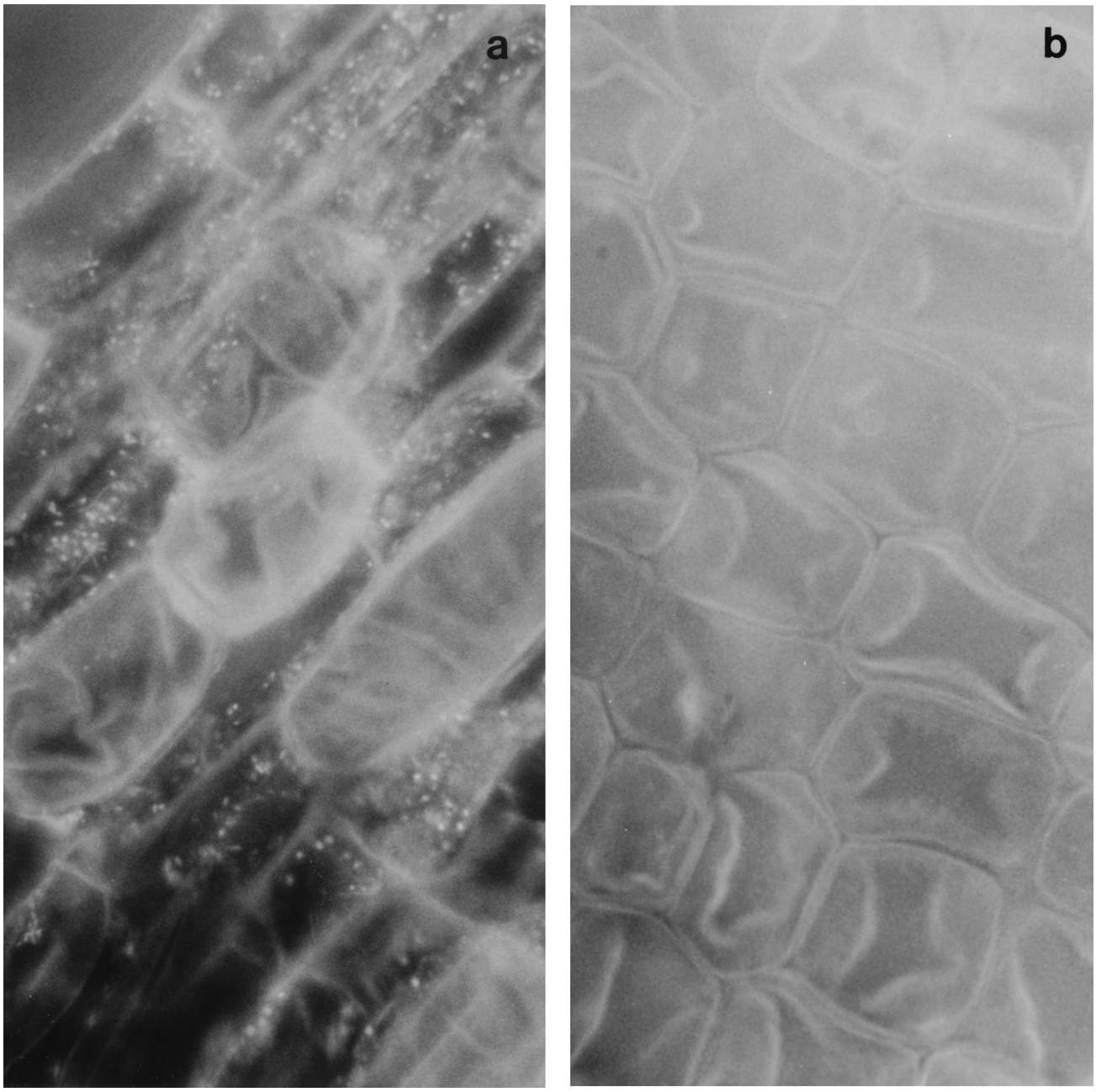

FIG. 5. Excised roots stained with Baclight Live/Dead fluorescent stain (Molecular Probes, Inc.) viewed at ×1,000. (a) *P. cordata* control; (b) *P. cordata* following SDS and antibiotic treatment.

RESULTS

Methane consumption under oxic and suboxic conditions. Controls over a 2-day experimental period showed no difference in methane oxidation rates for either species under oxic and suboxic conditions. Similarly, there were no significant differences in suboxic methane oxidation rates during two sequential assay periods with *P. cordata* chambers. Methane loss from the chambers was linear with time (i.e., zero order; Fig. 1). Under oxic conditions, 87.6 and 62.6% of the total methane loss was due to methanotrophic activity for *P. cordata* and *S. eurycarpum*, respectively; the remainder was attributed to diffusive flux through the stems and leaves.

For *P. cordata*, the mean methane consumption rate (± 1 standard error) under oxic conditions was 5.6 ± 1.1 μmol g (dry weight) of root^{-1} h^{-1}. Methane consumption rates decreased by 45.3% under suboxic conditions (Fig. 2). Methane consumption rates during the oxic and suboxic incubations were significantly different ($P = 0.05$). In *S. eurycarpum* chambers, the mean methane consumption rate (± 1 standard error) under oxic conditions was 7.0 ± 1.1 μmol g (dry weight) of root^{-1} h^{-1}. Under suboxic conditions, rates decreased by 94.8% (Fig. 2); the differences between oxic and suboxic rates were highly significant ($P < 0.001$).

Effect of *P. aeruginosa* on methane uptake. Additions of *P. aeruginosa* to chambers with either *S. eurycarpum* or *P. cor-*

data inhibited methane consumption. In *P. cordata* chambers, mean methane consumption rates (± 1 standard error) under oxic and suboxic conditions and under suboxic conditions with addition of *P. aeruginosa* were 8.1 ± 2.0, 4.5 ± 1.3, and 2.3 ± 1.0 μmol g (dry weight) of root^{-1} h^{-1}, respectively (Fig. 3a), with the addition of *P. aeruginosa* resulting in a decrease of methane consumption rates by up to 72%. The oxic and suboxic uptake rates were not significantly different ($P = 0.22$), but the oxic and suboxic plus *P. aeruginosa* rates were ($P = 0.04$).

Under suboxic conditions, the root medium in *P. cordata* chambers gained 152.9 ± 67.5 μmol of O_2 g (dry weight) of root^{-1} h^{-1} (mean ± 1 standard error; 365.8% increase). In contrast, average net oxygen losses of 190.1 ± 65.0 (26.3% decrease) and 38.2 ± 16.7 (77% decrease) μmol of oxygen g (dry weight) of root^{-1} were measured for the oxic and suboxic plus *P. aeruginosa* incubations, respectively (Fig. 3b).

Methane consumption by *S. eurycarpum* was completely inhibited during suboxic and oxic plus *P. aeruginosa* incubations (Fig. 3a), with rates for the oxic treatments significantly different from all others ($P < 0.005$)). However, no significant differences were found between rates for suboxic and oxic plus *P. aeruginosa* incubations ($P = 0.94$; Fig. 3a) or between rates for suboxic and ATU incubations ($P = 0.81$). In addition, net oxygen consumption occurred during each of the incubations (597.4 ± 42.9, 43.6 ± 6.2, and 96.6 ± 2.9 μmol g [dry weight] of root^{-1} h^{-1} for oxic, suboxic, and oxic plus *P. aeruginosa* incubations, respectively [Fig. 3b]). Methane consumption rates decreased by 92 and 98% from oxic to suboxic and oxic to oxic plus *P. aeruginosa* incubations, respectively (Fig. 3c).

In a second set of *S. eurycarpum* chamber experiments (designated B in Fig. 3), the mean methane consumption rates (± 1 standard error) were 7.8 ± 0.4 μmol g (dry weight) of root^{-1} h^{-1} for oxic conditions and 4.7 ± 1.7 μmol g (dry weight) of root^{-1} h^{-1} for oxic plus *P. aeruginosa* conditions. The latter differed significantly ($P = 0.026$) from the former but not from rates with ATU (5.2 ± 1.25 μmol g (dry weight) of root^{-1} h^{-1}; $P = 0.31$). Both incubations resulted in net oxygen consumption (429.2 ± 109 and 818.4 ± 121.8 μmol g [dry weight] of root^{-1} for oxic and oxic plus *P. aeruginosa* incubations, respectively); however, the bacterium-amended chamber decreased in total oxygen concentration by 47%, compared to 16.7% for the unamended oxic chambers (Fig. 3b).

Effect of ammonium chloride. Ammonium chloride additions did not change methane consumption rates for *P. cordata* or *S. eurycarpum* (Table 1) under oxic conditions. Results for suboxic incubations with 1 mM ammonium chloride for *P. cordata* were not significantly different ($P = 0.22$) from those of oxic assays, nor was there a significant difference ($P = 0.94$) between suboxic and suboxic plus ammonium treatments. Preincubation of *P. cordata* roots in 1 mM ammonium chloride with no methane enrichment 12 h prior to monitoring of uptake rates had no effect on methane consumption rates.

Oxygen dynamics in root chambers. Data from all the chamber assays involving oxic and suboxic time courses ($n = 18$ for *P. cordata*; $n = 10$ for *S. eurycarpum*) were pooled to evaluate patterns of oxygen uptake and leakage. Dramatic differences between the two species were observed. Under oxic conditions, total oxygen concentrations (micromoles per gram [dry weight] of root) from the beginning of one incubation to completion 8 h later decreased by 29.3 and 65.5% for *P. cordata* and *S. eurycarpum*, respectively (Fig. 4a). Under suboxic conditions, the total oxygen concentration in chambers containing *P. cordata* increased by 219.5%, while a decrease of 44.0% was observed for *S. eurycarpum* (Fig. 4b).

Patterns of root oxygenation. Following SDS and antibiotic treatments, microscopic analysis showed that root surfaces were largely bacterium-free relative to control roots that were densely colonized (Fig. 5). Rates of methane consumption by sediment-free excised roots of *P. cordata* treated with the antibiotic mixture were depressed by 75.8%.

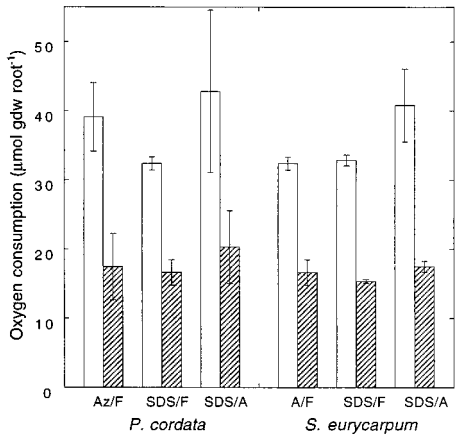

FIG. 6. Oxygen consumption by sediment-free excised roots of *P. cordata* and *S. eurycarpum* with the following treatments: Az/F = 10 mM sodium azide plus 3% formaldehyde; SDS/F = 0.1% SDS plus 3% formaldehyde; and SDS/A = 0.1% SDS plus antibiotic. Open and cross-hatched bars represent controls and treatments, respectively. Error bars indicate ±1 standard error.

Treatments with 10 mM sodium azide plus 3% formaldehyde, SDS plus 3% formaldehyde, and SDS plus antibiotic lowered oxygen consumption rates by excised, sediment-free fine roots of both species incubated in air-saturated water in 10-cm^3 gastight glass syringes (Fig. 6). Autoclaved roots consumed negligible amounts of oxygen.

Qualitative analyses based on methylene blue oxidation in reduced, semisolid agar confirmed the pattern established in the chamber experiments (Fig. 7). Methylene blue was consistently oxidized to a greater extent and more rapidly by *P. cordata* ($n = 12$) than by *S. eurycarpum* ($n = 12$). Within 4 h, more than 50% of the chamber was blue for *P. cordata*, whereas after 48 h, no more than 20% of the chamber was oxidized by *S. eurycarpum.*

DISCUSSION

Previous studies have established the potential importance of rhizospheric methane oxidation (15, 17, 20, 23, 25, 28, 29, 44) by documenting ranges of relative activity, primarily using in vitro flux measurements. More limited analyses have focused on activity in situ (17, 29). Methodological development and the relative importance of activity have been emphasized thus far, with somewhat less attention given to distinctions between rhizospheric and rhizoplane activity and controls of methane oxidation. The rhizoplane is particularly interesting, since it may represent an optimal habitat with respect to the availability of both oxygen and methane.

We have evaluated controls of rhizoplane methanotrophy using intact, sediment-free roots of plants in split chambers that allowed us to manipulate initial methane, oxygen, and ammonium concentrations. We measured methanotrophic activity using approximately 100 μM methane concentrations. Such concentrations saturate root-associated methane oxidation, which has a K_s of <10 μM (28). Since in situ methane concentrations during summer exceed >100 μM at the Bristol marsh site (ranging from 400 to 1,130 μM over the upper 20 cm of peat), the concentrations used in the in vitro assays are reasonable lower limits. A range of oxygen concentrations

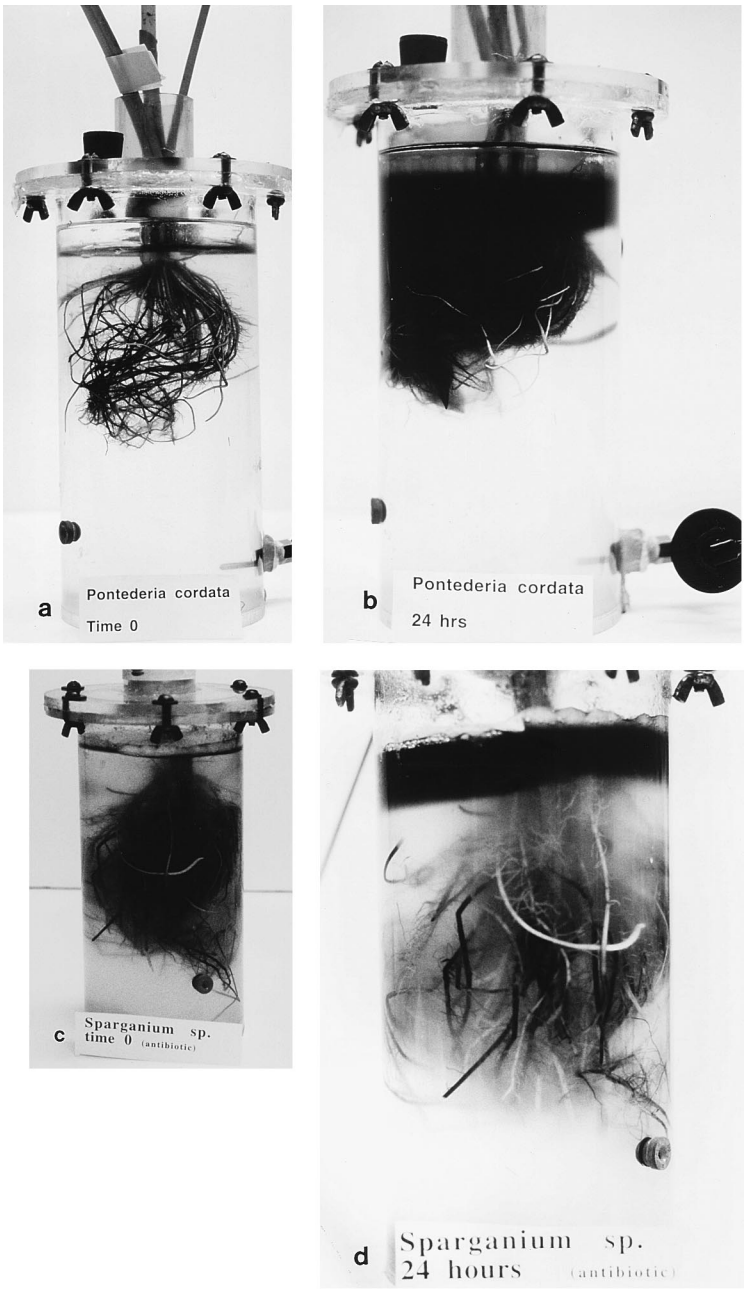

FIG. 7. Pattern of root oxidation of methylene blue in whole-plant chambers with intact sediment-free roots of *P. cordata* at 0 h (a), *P. cordata* at 24 h (b), *S. eurycarpum* at 0 h (c), and *S. eurycarpum* at 24 h (d).

from <10 to >250 μM was used in our analyses. Since oxygen penetrates the peat surface <7 mm at the Bristol marsh site (29), bulk peat throughout the root zone is undoubtedly anoxic. Although oxygen concentrations at the rhizoplane in situ are unknown, an equilibrium with an aerenchyma containing 21% gas phase oxygen provides a suitable upper limit for comparison.

We did not vary ammonium concentrations, choosing instead a value of 1 mM to facilitate an analysis of the potential for ammonium to control activity. Although ammonium inhibits methanotrophy when methane concentrations are low (<1 μM), minimal or negligible inhibition occurs at higher concentrations (31, 37, 38, 45). We observed no inhibition when 1 mM ammonium was added to chambers containing 100 μM methane (equivalent to an equilibrium with a headspace methane concentration of about 6%). These results suggest that ammonium regulation of root methanotrophy in situ is unlikely. However, ammonium might prove more important in wetlands during spring, when concentrations are relatively high, or in wetlands characterized by relatively low root zone methane concentrations. A limited role for ammonium as a root methanotrophy control is consistent with the lack of ammonium-induced inhibition of methane consumption reported for freshwater sediments (5, 9, 30, 45).

Rates of methane consumption were comparable to rates measured throughout the summer for freshly collected excised

TABLE 2. In situ methane flux and methane oxidation rates

Species	Mean rate ± SE[a] (mg of CH_4 m^{-2} day^{-1})			% Methane oxidation (mean ± SE)
	Control[b]	Post-acetylene treatment	Methane oxidation	
P. cordata	40.7 ± 15.1	80.5 ± 32.1	39.8 ± 17.1	48.3 ± 1.6
S. eurycarpum	51.4 ± 18.3	67.8 ± 21.7	16.4 ± 4.5	26.2 ± 6.8

[a] $n = 3$.
[b] Pre-acetylene treatment as described in the text.

roots incubated in vitro with a 1% methane headspace (data not shown). In addition, methanotrophy accounted for 38 to 86% of the total methane loss from *P. cordata* chambers under anoxic (suboxic plus *P. aeruginosa*) conditions. These values compare favorably with those reported by Epp and Chanton (17) for *P. cordata* and *S. lancifolia* (23 to 90%) and with those reported by Schipper and Reddy (44) for *S. lancifolia* (65 to 79%) in greenhouse experiments. The relative activity of *P. cordata* in chambers also agreed well with results from a field analysis (Table 2). Collectively, these results suggest that the chamber approach provided a useful tool for analysis of methanotrophic controls.

However, the chamber approach has some limitations. Methane consumption was largely inhibited in *S. eurycarpum* chambers under suboxic and anoxic conditions. In contrast, *S. eurycarpum* supports active methanotrophy in situ, although at considerably lower levels than those measured for *P. cordata* (Table 2). It is thus likely that estimates for total potential activity associated with the rhizoplane in the chamber analyses are lower limits. In addition, plants in chambers lack a true rhizosphere, which may further constrain the oxidation estimates.

Nevertheless, the results of the oxic, suboxic, and suboxic plus *P. aeruginosa* incubations suggest that oxygen availability determines oxidation rates to a great degree. The chamber results reveal dramatic differences in oxygen transport between *P. cordata* and *S. eurycarpum*. The dissolved oxygen initially present plus any root oxygen loss was sufficient to support oxidation of 87.6 and 62.6% of the total methane consumed (under oxic conditions) by *P. cordata* and *S. eurycarpum*, respectively. However, under suboxic conditions, *P. cordata* not only supplied 33 μmol of oxygen g (dry weight) of $root^{-1}$ for the observed methanotrophic activity, but also increased the chamber oxygen content by threefold, from a mean (± standard error) of 32.9 ± 2.2 to 104.6 ± 15.9 μM. In contrast, oxygen was reduced by an average of 8.2 ± 2.8 μmol g (dry weight) of $root^{-1}$ and methanotrophic activity was negligible in suboxic chambers with *S. eurycarpum*. Thus, *S. eurycarpum* roots appear either to leak less or to respire more oxygen than *P. cordata* roots. Assays with *P. aeruginosa* supported this pattern. Significant methane consumption by *P. cordata* occurred after addition of *P. aeruginosa* in spite of nearly anoxic conditions in the chambers. In contrast, additions of *P. aeruginosa* to *S. eurycarpum* chambers resulted in total oxygen depletion and complete inhibition of methane oxidation.

The methylene blue oxidation experiments provided qualitative evidence that despite a common root and stem architecture, most notably aerenchymous tissue (12, 34, 43, 47), oxygen availability for rhizoplane bacteria can vary substantially among plant taxa. *P. cordata* roots oxidized reduced methylene blue agar almost completely, in many cases within 8 h; *S. eurycarpum* roots supported little oxidation even after 48 h (Fig. 7). Moorehead and Reddy (36) also found distinct variations in the extent of oxygen transport from aerial plant tissue into the root zone. As in this study, they found that *P. cordata* had the highest oxygen transport capacity of the four emergent plants they evaluated for wastewater treatment. In our chamber studies, differences in rhizosphere oxygenation were reflected in rates of root-associated methanotrophy in the chambers. Relative rates of methane consumption for *P. cordata* and *S. eurycarpum* (Fig. 2 and 3) were also consistent with differences in oxygen transport. These differences were reflected in situ, with *P. cordata* and *S. eurycarpum* oxidizing (48.3 ± 1.6) and (26.2 ± 6.8)% of potential methane efflux, respectively (Table 2).

Differences in rhizosphere oxygenation do not appear to result from differences in oxygen consumption by root bacteria. Removal of microbial films with SDS and subsequent soaking in antibiotics did not visibly increase oxygen loss from *S. eurycarpum* or *P. cordata* roots. Likewise, inhibition of root respiration by formaldehyde and sodium azide slightly enhanced oxygen loss from *S. eurycarpum* but did not result in losses comparable to those of untreated *P. cordata* roots.

Interestingly, neither SDS nor antibiotic treatments completely inhibited root-associated methanotrophy, in spite of the fact that the former treatment removed virtually all of the rhizoplane microbiota (Fig. 5). This strongly suggests that methanotrophs not only colonize the root exterior, but also grow within the root itself. Preliminary analyses based on in situ hybridization of fluorescent oligonucleotides suggested a similar phenomenon for roots of another aquatic macrophyte, *Calamogrostis canadensis* (33). Colonization of the root interior may represent a common response of methanotrophs and other rhizospheric bacteria to oxygen limitation. It may also result in niche separation, increase the diversity of root methanotrophs, and explain the presence of both group I and II methanotrophs on roots (e.g., see reference 28).

In summary, two common freshwater marsh species varied dramatically in their ability to support methane oxidation. Methanotrophy accounted for more than half of the total methane loss from intact, sediment-free plants in chambers when excess exogenous oxygen was available. However, when exogenous oxygen was limiting, methanotrophy decreased markedly. Differences in the relative activities of *P. cordata* and *S. eurycarpum* under these conditions were related to differences in oxygen transport capacity. These results, along with data from field studies (e.g., see references 28 and 29), suggest that oxygen availability is a major control of root-associated methanotrophy and that oxygen availability differs markedly among plant taxa. Thus, future predictions of methane efflux from wetlands will have to take into consideration the impact of changes in wetland species composition on methane oxidation in addition to changes in parameters such as temperature and hydrologic regimens.

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