WINTER VERTEBRATE BROWSING OF BIRCH: EFFECTS ON THE USE OF LEAF LITTER LEACHATES BY STREAM MICROORGANISMS

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A

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ABSTRACT

Winter browsing of birch leads to chemical changes in leaves of the following growing season, potentially generating differences in the quality of leachates derived from leaf litter and in leachate use by stream microorganisms. The effects of moose browsing were tested on leachates from leaves collected from browsed and unbrowsed trees and inoculated with microbial communities. Respiration and bacterial abundance were used to assess qualitative differences in leachates. Microbes cultured in leachates derived from leaves of browsed trees had significantly higher rates of oxygen uptake. There were no significant differences in bacterial abundance between treatments. The basis for the qualitative difference in leachates is likely due to an 89 % greater concentration of amino acids in leachates derived from leaves of previously browsed trees. This study provides evidence that winter herbivory of birch can influence the use of leaf leachates by stream microbes, demonstrating coupling between riparian zones and stream ecosystems.

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INTRODUCTION*

Dissolved organic carbon in streams

Dissolved organic carbon (DOC) is the most abundant form of organic carbon found in natural waters (Fisher and Likens 1973, Hobbie and Likens 1973, McDowell and Fisher 1976, Lush and Hynes 1978, Thurman 1985, Meyer *et al.* 1987, Volk *et al.* 1997). Hobbie and Likens (1973) calculated that 79 to 88 % of the organic carbon exported in two small northeastern watersheds was in the dissolved form, while 70 % of the annual exported organic carbon in a second order northeastern stream was dissolved (Fisher and Likens 1973). Generally, DOC transport in streams ranges from 60 to 90 % of the total organic carbon (Dahm 1981, Hope 1994), and DOC transport in rivers worldwide, on average, exceeds particulate organic carbon (POC) transport (Thurman 1985) by a 2:1 ratio (Hope 1994, Allan 1995). Factors such as stream type and discharge dictate the relative amounts of DOC and POC in transport.

DOC in streams derives from organisms and organic detritus. Some is transported from the surrounding landscape, while the remainder generated *in situ* (e.g., exudates from algae; Fisher and Likens 1973, Thurman 1985). DOC reaches the stream by overland, interflow, and groundwater flow. DOC concentrations in rivers and streams vary with latitude, climate, position along the river continuum, season, vegetation composition in the river's basin, and watershed primary production. Concentrations can

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be as low as 1 $mg \cdot L^{-1}$ in small arctic streams (although concentrations have been reported as high as 13 $mg \cdot L^{-1}$; see Oswood *et al.* 1996) where primary production is low and decomposition is slow, to as high as 60 $mg \cdot L^{-1}$ in rivers draining swamps and wetlands, where primary production is high and decomposition is slow (Thurman 1985). Values between 1 and 4 $mg \cdot L^{-1}$ are common, with concentrations rarely exceeding 10 $mg \cdot L^{-1}$; high concentrations are often associated with larger rivers and disturbances (Allan 1985).

Leaf litter, and the DOC leachates derived from it, make important contributions to the energy budgets of streams by providing large amounts of organic matter (McDowell and Fisher 1976, Dahm 1981). Leaf litter enters a stream by direct fall and by lateral movement (e.g., wind blown into the stream). The relative amounts of leaf litter entering the stream by these routes is influenced by wind patterns, aspect of the surrounding landscape, bank slope, and other site specific factors (Fisher and Likens 1973, McDowell and Fisher 1976, Benfield 1997). Location and the vegetation composition of the surrounding landscape also influence the amount of litter entering a stream.

Leaf litter inputs can be pulsed, such as in deciduous boreal forests where leaf fall occurs in autumn, or may be relatively continuous year round, such as in tropical areas (Stout 1980). Leaf litter inputs have been recorded for many of streams worldwide. Litterfall into Monument Creek, a small boreal forest stream in interior Alaska was 62 g·AFDM (ash-free dried matter)$\cdot m^{-2}\, y^{-1}$, while litterfall into two small boreal forest streams located at the Caribou-Poker Creeks Research Watershed was 37 g·AFDM$\cdot m^{-2}\, y^{-1}$ (Cowan

and Oswood 1983). The total litter input into Roaring Brook, a small, forested, second order stream in Massachusetts was 345 g AFDM $m^{-2} y^{-1}$ (McDowell and Fisher 1976), while litter inputs for three first order streams in the Coweeta Hydrologic Laboratory in North Carolina were approximately 500 g AFDM $m^{-2} y^{-1}$ (Wallace *et al.* 1995). The amount of leaf litter inputs recorded for 33 streams worldwide averaged 600 $g \cdot m^{-2} y^{-1}$, with amounts ranging from 3 $g \cdot m^{-2} y^{-1}$ for a mixed deciduous forest in Canada to 4363 $g \cdot m^{-2} y^{-1}$ for a mixed deciduous forest in Georgia (Benfield 1997).

Upon entering the stream, leaf litter immediately begins a leaching process in which 5-30 % of the leaf's mass can be lost as soluble compounds within 1-2 days (Cummins 1974, Webster and Benfield 1986). Considering the quantity of leaf litter entering the stream and the amount of the soluble fraction leached from the leaf, the flux of DOC from leaf leachates is substantial (Lock and Hynes 1976).

DOC removal from the water column

DOC is retained on benthic surfaces by both abiotic and biotic processes (Lock and Hynes 1976, Dahm 1981, McDowell 1985). Abiotic processes include physical and chemical mechanisms such as adsorption, flocculation, and aggregation of DOC (Allan 1995). Biotically, DOC is removed from the water by microorganisms associated with the stream sediments. Current evidence indicates that in lotic systems, benthic bacteria are more abundant than suspended bacteria (Edwards *et al.* 1990) and play a larger role in DOC uptake. Lock and Hynes (1976) reported very little uptake of DOC derived from

maple leaves in stream water alone. However, DOC concentrations were reduced to 15 % of initial concentrations when in water overlying stream sediments. Dahm (1981) calculated that 97 % of DOC derived from red alder leaves was removed within 48 hours from recirculating chambers filled with stream sediments: approximately 20 % by adsorption and 77 % by microbial utilization. Lock and Hynes (1976) found that initial concentrations of DOC derived from maple leaves were reduced by 85 % within 9 hours after exposure to stream sediments, and estimated that 40 - 100 % of the DOC removal was due to microbial uptake. Leachates produced from sugar maples and white cedar leaves were removed from a small Ontario stream at rates ranging from 0.5 to 1.1 $g \cdot C \cdot m^{-2} h^{-1}$ (Lush and Hynes 1978), while the rate of microbial uptake of DOC derived from red alder leaves was calculated to be 14 mg $C \cdot m^{-2} h^{-1}$ (Dahm 1981). These studies show the quick removal of DOC from the water column by microorganisms associated with the sediment. In addition, DOC concentrations rarely increase in a river with input and leaching of autumnal leaf litter, indicating the rapid uptake within a short distance from the source (Allan 1995).

DOC flux through microbial pathways

Bacterial assimilation of DOC from the overlying water column leads to the fixation of organic carbon into bacterial biomass. This conversion provides the basis for the transfer of organic carbon through microorganisms to higher consumers in streams. This parallels the role microbes play in the breakdown of particulate organic matter (e.g.,

leaf litter). Microbial (bacterial and fungal) colonization conditions leaf litter allowing detritivore consumption. The question remains, however, whether microbial colonization provides nutrition directly to the detritivore (in the form of microbial biomass), or if colonization modifies the leaf litter allowing the detritivore access to nutrients within the litter (Allan 1995). It has been suggested that detritivores gain most of their energy from microbial biomass rather than leaf litter per se (Cummins 1974). Invertebrate consumers undoubtedly gain energy from both the detritus and the microbial consumers. It is important to know how much carbon is gained from microbial biomass relative to detrital matter to understand the amount of detrital carbon reaching higher consumers (Hall and Meyer 1998).

Bacteria can provide the trophic base in heterotrophic systems, such as forested headwater streams, where they can be more productive than primary producers (Hall and Meyer 1998). There is, however, some question about the importance of the flux of energy through the microbial food web (Pomeroy and Weibe 1988). Aspects of this problem include the efficiency in which bacteria convert DOC to microbial biomass, and the quantification of the number of trophic transfers from microbes to metazoans (Allan 1995). The unresolved questions are how much energy reaches higher consumers through the microbial food web, and how much is lost to respiration. A key to understanding the flux of carbon through the microbial loop is determining what components of DOC are being metabolized, and the efficiency with which microbes convert these components to microbial carbon. This information is important in

estimating the microbially-mediated turnover of DOC and the amount of carbon reaching higher trophic levels (Coffin *et al.* 1993).

DOC found in rivers originates from many sources that change both temporally and spatially. The efficiencies with which microogrganisms convert DOC to biomass varies with source. Efficiencies range from 53% for DOC originating from alligatorweed to 10 % for DOC originating from leaf leachate (reviewed by Pomeroy and Weibe 1988). Efficiencies likewise can vary within a single source of DOC as DOC concentrations change (Ho and Payne 1979), suggesting variation in efficiencies with changing conditions.

Measuring bacterial growth on specific sources of DOC is useful as certain reaches of a stream receive large amounts of DOC from specific sources (e.g., headwater streams receiving seasonal pulses of leaf litter, and subsequent leachates, from the riparian vegetation surrounding them). However, DOC originating from a single source is composed of a number of chemical constituents. Much of the DOC found in the lower reaches of a river is imported from upstream, originating from a number of sources, thus having a complex chemical composition. Not all DOC is labile or biodegradable, and the chemical composition of the DOC likely determines its nutritional quality (Amon and Benner 1996, Volk *et al.* 1997). Bacteria selectively remove components of DOC from water, likely assimilating substrates that can be used for energy and growth. Understanding what components of DOC are selectively removed by bacteria and how these components are used in bacterial growth is important in understanding bacterially-

mediated carbon turnover and the flux of this energy to higher trophic levels in lotic systems.

Chemical composition of DOC

The chemical composition of DOC found in natural waters can be classified into six major groups: humic substances, hydrophilic acids, carbohydrates, carboxylic acids, amino acids, and hydrocarbons. Excellent classifications and detailed descriptions of organic compounds in natural waters can be found in Keskitalo and Eloranta (1999) and Thurman (1985). Humic substances, which can be divided into humic and fulvic acids, are polymeric molecules from which constituent compounds are difficult to isolate and purify. Humic substances are operationally defined as fractions of DOC that are removed from water by XAD resins or weak-base ion exchange. Humic substances are terrestrially derived from plants and soils, and enter streams via groundwater and overground flow. Typically, concentrations of humic substances range from 0.5 to 4 $mg \cdot L^{-1}$ in freshwater systems, and account for 30 to 50 % of the DOC in natural waters, with contributions as high as 50 to 90 % in blackwater systems (Keskitalo and Eloranta 1999). Generally, humic substances are thought to be refractory and of little use biologically (Allan, 1995), although they may play an important role in freshwater ecosystem stability, providing a large but slowly metabolized pool of DOC (Wetzel 1995). Hydrophilic acids make up about 30 % of the DOC in natural waters and are operationally defined as organic acids that are not retained by XAD resins at pH 2.

Likewise, hydrophilic acids are difficult to isolate and purify, and little is known about them (Thurman 1985). Of the remaining groups, carbohydrates, amino acids, and phenolics have received much attention, as they are easily identified, isolated, and have been shown to be of the greatest biological importance to bacteria.

Carbohydrates are the most abundant class of compounds produced. They originate terrestrially from plant and organic matter. The oxidation of carbohydrates is the central energy-yielding pathway in most non-photosynthetic cells. Carbohydrates are also important in providing structure within a cell, and serve as the structural and protective elements in cell walls. Concentrations of carbohydrates in river water range from 100 to 2000 $\mu g \cdot L^{-1}$, with the average concentration being 500 $\mu g \cdot L^{-1}$ (Thurman 1985). Carbohydrates can account for 5 to 10 % of the total dissolved organic carbon in rivers (Thurman 1985).

Amino acids are essential for the growth and function of bacteria, as they provide the building blocks for proteins, which are used for structure and transport, for catalyzing cellular reactions, and for numerous other functions within cells. Dissolved amino acids can be broken down into two groups: free amino acids or combined amino acids (combined into peptide and protein form). Combined amino acids are 4 to 5 times more abundant than free amino acids (Thurman 1985). Terrestrial plants and soil organic matter make important contributions to the total dissolved amino acids in rivers. Amino acids range in concentration from 100 to 500 $\mu g \cdot L^{-1}$, and account for 2 to 3 % of the DOC in rivers (Thurman 1985). The dominant amino acids found in rivers worldwide include

glutamate, glycine, methylhistidine, serine, and aspartate (reviewed by Thurman 1985). Volk *et al.* (1997) in White Clay Creek in Pennsylvania, found that glycine and aspartate were the dominant amino acids accounting for as much as 40 % of the total amino acid pool. Other amino acids found in relatively high concentrations were alanine, glutamate, serine, and threonine.

Condensed tannins, a group of phenolic compounds that originate from plant material, comprise less than 2 % of the DOC in water, likely due to their low solubilities (Thurman 1985). A characteristic that sets tannins apart from other phenolic compounds is their high reactivity with proteins, which interferes with the normal functioning of proteins (such as enzymes) in biological systems (Fields and Lettinga 1992). Tannins may have antibacterial and antifungal properties (reviewed by Stout 1989). From an evolutionary standpoint, tannins bound to plant pectins and celluloses (Stout 1989) protect the plant from a wide range of phytopathological microorganisms and their extracellular enzymes (Field and Lettinga 1992). Increased tannin production is associated with induced responses by plants in reaction to herbivory (Karban and Baldwin 1997). It is widely accepted that plants high in tannin content are less palatable (Bryant *et al.* 1991), and leaf litter high in tannin content has slower decomposition rates in streams (Stout 1989).

Selective removal of DOC by microorganisms

The biodegradable components of DOC are selectively removed by microorganisms and used to support microbial respiration and growth. Assimilable components include carbohydrates (Kaplan and Bott 1983), combined humic bound amino acids (Volk *et al.* 1997), dissolved free amino acids (Crawford *et al.* 1974, Keil and Kirchman 1991), and low molecular weight fractions (Kaplan and Bott 1983, Meyers *et al.* 1987, Amon and Benner 1996). Volk *et al.* (1997) examined the biodegradable fraction of DOC collected from headwaters of a small southeastern stream in Pennsylvania using plug-flow biofilm reactors. They determined that 25 % of the total DOC was biodegradable, and the biodegradable fraction was composed of 75 % humic substances, 30 % carbohydrates (primarily humic bound polysaccharides), and 4 % amino acids (present in combined form and humic bound, with glycine and aspartate the dominant amino acids). Meyer *et al.* (1987) measured bacterial growth on DOC of different nominal molecular weights (nMW) collected from a southeastern blackwater stream. Bacterial growth and DOC assimilation were greatest in the low nMW enrichment (< 1,000), were lowest in the intermediate nMW enrichment (1,000 - 10,000), and intermediate on high nMW (> 10,000) enrichments. These results indicate that lower nMW compounds are more available to bacteria as substrates than higher nMW compounds. The greater growth of bacteria on the higher nMW fraction was likely due to lower nMW compounds being complexed with more refractory high nMW compounds.

Bacteria presumably were able to use exoenzymes to cleave low nMW compounds from high nMW compounds.

Kaplan and Bott (1983) examined microbial utilization of DOC in microcosms using stream water and stream-bed sediments collected from a third order stream in Pennsylvania. They likewise found selective uptake of DOC components from the DOC pool. They found that low MW compounds (carbohydrates, and amino acids) were preferentially used. They reasoned that bacterial energetics may be responsible for the preferential selection of low MW fractions. High MW molecules are difficult to transport across cell membranes, thus bacteria would require exoenzymes to cleave off smaller molecules in order to move them into the cell. Also, smaller molecules are likely more easily recognized by bacterial permease systems.

Amon and Benner (1996) examined bacterial utilization of high and low MW dissolved organic carbon collected from a wide spectrum of marine and freshwater systems. They measured oxygen consumption, bacterial abundance, and bacterial production, and found that bacterial growth efficiencies were higher for bacteria cultured in low MW DOC than high MW DOC. However, bacterial growth rates and respiration were greatest in high MW DOC incubations, and DOC assimilation rates were greater in the high MW than in the low MW incubations. Amon and Benner's (1996) results contrast with other studies; their results showed bacterial growth and respiration were greater in bacteria cultured on high MW DOC than low MW DOC. They reasoned that high MW components may be more recent in origin, thus more labile than low MW

compounds. Low MW compounds may be older and more recalcitrant, and are the remains of high MW compounds after extensive biotic processing - essentially what remains after the higher quality components have been cleaved.

DOC quality

The quality of DOC leachates from leaf litter is likely determined by the chemistry of the leaves from which it is derived. Litter high in nitrogen content is more palatable and processed faster than litter high in cellulose, lignin, and tannins. The intrinsic properties of the leaves, and the nutritional status of the leaf, are factors that likely dictate the quality of the leachates derived from leaves. Irons *et al.* (1988) examined the influence of tree species (alder, birch, willow, and poplar) and nutrient status (trees fertilized with nitrogen and phosphorus) on feeding preferences of leaf litter to a stream shredder. Larvae preferred leaf detritus originating from trees grown with nitrogen and phosphorus fertilization, and preferred alder over the other tree species Their results showed that leaf consumption was positively associated with nitrogen content and negatively associated with condensed tannin content of leaf litter.

The source of DOC determines its chemical composition, which in turn determines its nutritional quality to stream microorganisms. Sources of DOC can change spatially with changing riparian vegetation (McArthur and Marzolf 1986, Koetsier *et al.* 1997), and with selective biological uptake during transport (Kaplan *et al.* 1980) along the stream continuum. Seasonal variations in DOC occur with changing plant

metabolism (green leaves vs. senescent leaves; Koetsier *et al.* 1997), and with seasonal primary production (Kaplan and Bott 1982). Bacteria can react to changing sources of DOC by undergoing changes in physiology and with shifts in bacterial communities (Kaplan and Bott 1983). Bacterial communities may be acclimated to specific sources of DOC generated by the surrounding landscape, and unable to readily use foreign sources of DOC. McArthur and Marzolf (1986) found DOC leachates originating from grasses disappeared rapidly from *in situ* chambers at upstream sites in grasslands, and at downstream sites in the forested area. Leachates originating from litter in the forested area were readily taken up by bacteria in the forested area, but not as readily by bacterial assemblages inhabiting the grassland reaches. Bacterial assemblages in the forested region were able to use DOC originating from the upstream grassland reaches because not all of the DOC generated in the grassland reaches is used immediately. Thus, some of this DOC is exported downstream, where bacterial assemblages in the forested reaches would have been exposed to it prior to the experiment. Similar results were obtained by culturing bacteria sampled from a southeastern blackwater stream on leachates from common riparian species along the river continuum (Koetsier *et al.* 1997). Bacteria from the lower site were able to utilize leachates from all sources, while bacteria from the upper sites could not. In addition, (Koetsier *et al.* 1997) found bacteria were not able to grow on green leaf leachate, but were able to grow on senescent leaf leachate.

In contrast, Kaplan and Bott (1985) found that bacteria, when introduced to foreign DOC sources, were able to acclimate to the new source within a matter of days.

However, the time needed to acclimate often exceeded the DOC residence time in that stretch of stream. Acclimation to differing sources of DOC could result in a shift in species composition, or a physiological adaptation by the bacteria (McArthur and Marzolf 1986). Induction, the production of new enzymes in response to changing substrate, is common in bacteria. The ability of bacteria to utilize various sources of DOC would be an evolutionary advantage, especially when coping with pulses of varying sources of DOC in streams (Kaplan and Bott 1983), as well as dealing with abrupt changes in riparian conditions resulting from terrestrial disturbances.

Terrestrial disturbances and DOC

Terrestrial disturbances can influence DOC inputs by causing changes in riparian ecosystems, translating into differential use by stream microorganisms. Fire, for example, can cause abrupt changes in the structure, size, and taxonomic composition of the riparian vegetation (Romme 1982), causing abrupt changes in the source of DOC to streams adjacent to these areas. Both fire and logging in riparian zones alter the amount of woody debris entering a stream. Woody debris, such as logs, impede the movement of organic debris (e.g., leaf litter) down stream, creating debris dams which can become water tight. Pools form on the upstream side of debris dams where sediments are deposited. A study in which organic dams were removed from a 175 m stretch of a second order stream at the Hubbard Brook Experimental Forest found that the removal of the organic debris dams increased DOC export by 18 % (Bilby and Likens 1980). Debris

dams retain organic matter within the system, allowing it to be processed on site rather than being transported downstream. In general, effects of wildfire on streams include alterations in water runoff, nutrient cycling, leaf litter input, as well as decreases in woody debris entering the stream, and increases in suspended sediments (Minshall *et al.* 1989). Other disturbances, such as mammalian herbivory, can have more subtle effects than those observed with fire and logging.

Mammalian browsing of woody plants can affect their food value (Bryant *et al.* 1991). Studies dealing with plant-herbivore interactions often focus on browsing during the summer, in which herbivory results in defoliation of the plant. The effects of defoliation are generally an increase in toxic or repellant secondary compounds and a decrease in nitrogen content, thus decreasing the palatability and nutritional quality of the plant (Bryant *et al.* 1991, Karban and Baldwin 1997).

Browsing does not always result in a decrease in food value (Danell *et al.* 1985, Danell and Huss-Danell 1985, Bryant *et al.* 1991). For example, winter browsing by moose can increase the nutritional quality of birch leaves grown on trees during the following growing season. The removal of terminal buds on birch by moose during the winter releases apical dominance of the terminal bud, and reduces the number of buds (Bergstrom and Danell 1987). During senescence, birch translocate nutrients from the leaves to the roots and stems where they are stored until spring, when they are then translocated back to new developing leaves in the spring. Thus, the stored nutrients are allocated to the remaining leaves (Danell *et al.* 1985, Bryant *et al.* 1991, Oswood *et al.*

1992). Birch browsed the previous winter produce larger leaves with higher chlorophyll contents, have higher nitrogen contents, and decreased tannin contents compared to leaves from trees with no previous browsing (Bryant *et al.* 1991). Danell and Huss-Danell (1985) observed an increase in leaf size, photosynthesis rates, and nitrogen content, and decreased tannin content in *Betula pendula* and *B. pubescens* Ehrh. exposed to natural and simulated moose browsing. Similar results were observed by Danell *et al.* (1985) and Bryant *et al.* (1991) for *Betula resinifera* Britt. (Dugle 1966) = *B. papyrifera* var. *humuis* (Reg.) Fern & Raup (Hulten 1968).

Changes in leaf chemistry induced by herbivory can influence the nutritional quality of plant material to consumers. Summer defoliation causes chemical changes in leaves that deter future herbivory. In contrast, winter herbivory can increase the nutritional value of leaves grown in the following growing season. Leaf eating and sucking insects preferred leaves from previously winter browsed *B. pendula* and *B. pubescens* trees (Danell and Huss-Danell 1985) over leaves from unbrowsed trees, the preference attributed to higher nitrogen content. Irons *et al.* (1991) compared the in-stream breakdown rates of leaf litter derived from previously browsed birch, and litter from trees with no previous browsing. Leaves from browsed trees had faster breakdown rates than leaves from unbrowsed trees. The difference in breakdown rates was likely due to a higher nitrogen content and the faster leaching rate of tannin from the browsed litter.

Long-term browsing by moose can facilitate landscape level changes in riparian areas. Pastor *et al.* (1988) used exclosures to examine the long term ecosystem effects of

moose browsing at Isle of Royale in Lake Superior. Moose browsing facilitated changes in species composition, from deciduous to coniferous. Changes in species composition altered the quantity and quality of leaf litter production, which translated into differences in soil properties, further affecting soil microbial communities. The chemistry of organic matter is an important component in its decomposition. Compared to litter from deciduous plants (e.g., birch), litter derived from conifer needles has lower nitrogen and phosphorus concentrations, while having higher concentrations of compounds such as lignin, tannin, and resins, which are toxic and recalcitrant (Kielland *et al.* 1997). Changes in species composition facilitated by long term browsing changes the quality of leaf litter to soil and streams, generating differential use by stream organisms, which can cascade up the food chain.

Objectives

Winter browsing changes the chemical and morphological characteristics of birch leaves in the following growing season, and hence, in leaf litter. These changes can alter the size, structure, and solubility of the components of dissolved organic matter leached from leaves, in turn affecting the uptake of dissolved organic matter by stream microbes. In forested headwater streams, where primary production is greatly reduced, leaf litter leachates provide an important trophic input to benthic bacteria, which in turn provide the trophic base for these systems. Changes in bacterial growth in response to changes in substrate may affect the amounts of energy reaching higher consumers.

The objective of this study was to determine if chemical changes in leaf litter caused by winter herbivory translate into differential use of leaf leachates by stream microbes. The effects of moose browsing were tested by producing leachate from leaves collected from birch trees showing signs of previous winter browsing and from trees exhibiting no previous browsing. A microbial inoculum was then added to each leachate and microbial respiration and bacterial growth were used to assess qualitative differences in leachates. Previous examinations of the biodegradable components of DOC have similarly used microbial oxygen consumption (Kaplan and Bott 1983, 1985, Coffin *et al.* 1993) and changes in bacterial abundance using microscopic direct counts (Kaplan and Bott 1983, 1985, Meyer *et al.* 1987, Coffin *et al.* 1993, Amon and Benner 1996) as measures of microbial activity. Analyses of condensed tannins, carbohydrates, and amino acids were performed to provide clues to the basis of any differences in the abilities of leachates to support microbial growth and respiration.

METHODS

Leaf collection

Senescent leaf samples (leaves that were yellow and still attached to the tree) were collected in early September from Alaska birch, *Betula resinifera* Britt. (Dugle 1966) = *B. papyrifera* var. *humuis* (Reg.) Fern & Raup (Hulten 1968), a species commonly found in the riparian areas of Interior Alaska. Birch trees were located at the Bonanza Creek Long Term Ecological Research Site near Fairbanks, Alaska. *Unbrowsed* refers to leaves

that were collected from trees within an 8 yr old, 20 x 20 x 3 m exclosure (designed to prevent vertebrate herbivory) that showed no signs of previous browsing. *Browsed* refers to leaves that were collected from trees outside the exclosure (within a 50 m radius of the exclosure) having branches that had regrown from recent moose (*Alces alces*) browsing. In addition, only leaves that exhibited no signs of additional herbivory (such as damage from insect feeding) were collected. Leaves were sampled from trees approximately 3 cm in diameter, and leaves were removed at approximately breast height. Leaves were sampled from at least five browsed trees and five unbrowsed trees. Birch was chosen as previous studies showed it to be particularly responsive to winter browsing (Danell *et al.* 1985, Bryant *et al.* 1991, Irons *et al.* 1991). After collection, leaves were air dried at room temperature (approx. 21-23 °C) for several days, then stored at room temperature in plastic bags until use.

Leachate preparation

In three separate experiments (Table 1), browsed and unbrowsed leachates were prepared by combining dried leaves with water and allowing the leaves to leach for 48 h while being aerated. After leaching, large particulate matter was removed by filtration through fine Nytex. The preparations were then re-filtered through glass-fiber filters (nominal pore size 0.45 μm, Gelman Sciences, Ann Arbor, Michigan). In experiment 1 (Table 1), 100 mL samples for chemical analyses were removed from each leachate preparation and stored at 5 °C until analyses were made (within 48 h of leaching).

Table 1. Experimental Protocol

	Leachate preparation and experimental set-up						analyses performed		
	treatment	dry wt leaf (g)	water source[a] leaching/dilution	water amt (L) leaching	inoculum[b]	amino acid amendment	chemical[c] analyses	direct counts	MPN
experiment 1	browsed	20	A	6	A	no	yes	yes	no
	unbrowsed	25	A	6	A	no	yes	yes	no
experiment 2	browsed	5	B	2	B	no	no	no	yes
	unbrowsed	5	B	2	B	no	no	no	yes
experiment 3	browsed	10	B	3	C	no	no	yes	no
	unbrowsed	10	B	3	C	no	no	yes	no
	suppl. brow.	10	B	3	C	yes	no	no	no
	suppl unbrow.	10	B	3	C	yes	no	no	no

[a] A)-water collected in December from Chena River, Alaska
B)-1:1 mixture of spring water collected in Fox, Alaska and reverse osmosis water

[b] A)-course particulate organic matter collected in September from pond near Fairbanks, Alaska
B)-course particulate and benthic fine particulate organic matter collected from Smith Lake near Fairbanks, Alaska
C)-course particulate and benthic fine particulate organic matter collected from O'Conner Creek near Fairbanks, Alaska

[c] analyses for carbohydrates, condensed tannins, and amino acids

Leachate preparations were analyzed for dissolved organic carbon (DOC) concentration and then diluted with filtered (glass fiber filters - nominal pore size 0.45 μm, Gelman Sciences, Ann Arbor, Michigan) water to a calculated concentration of 10 mg/L DOC (near the upper range reported for two small, subarctic streams near Fairbanks; MacLean *et al.* 1999). After dilution, all preparations were aerated until oxygen levels were approximately 90 % saturation at 21 °C. In experiment 3, leachate preparations were prepared as described above and enriched with 13.78 μM glutamate, 22.72 μM alanine, 17.22 μM valine, and 15.44 μM leucine.

Microbial activity

Microbial activity was determined by adding 10 mL of inoculum (Table 1) to 290 ml of the diluted leachate preparation in 300 mL BOD (biological oxygen demand) bottles. As a control, 10 mL of inoculum was added to 290 mL of filtered water. Twenty-four bottles were prepared (8 time series measurements x 3 replicates) for each treatment and control. After inoculation, BOD bottles were incubated at room temperature (approx. 21-23 °C) with minimal exposure to light for the duration of the experiment. At each sampling period, dissolved oxygen measurements were performed on each replicate using a YSI 5905 BOD probe and YSI Model 57 oxygen meter (Yellow Springs Instrument Company, White Springs, Ohio). In experiments where bacterial direct counts and most probable number (MPN) estimates were performed (Table 1), a 20 mL sample was removed from each replicate at each oxygen measurement period. In

experiment 1, the sample was fixed with 2 mL of 45 % formalin and used for bacterial direct count estimations; in experiment 2, the sample was used immediately for MPN estimation.

Microbial abundance

Total bacterial numbers were estimated by direct count microscopy. Formalin-fixed samples were shaken thoroughly, sonicated, and then 1 mL subsamples were removed and stained by adding 0.01 mL of DAPI (4'-6-diamidino-2-phenylindole dihydrochloride; concentration 50 μg/mL) stain for a final stain concentration of 0.5 μg/mL (Porter and Feig 1980). The mixture was allowed to react for 1 h, and then filtered through a black polycarbonate membrane filter (0.2 μm, Poretics Corporation, Livermore, California). The filter was then placed on a microscope slide and viewed. Counts were determined from ten randomly chosen fields per slide (Kirchman *et al.* 1982). In subsamples where bacterial numbers were high (>150 bacteria per field), subsamples were serially diluted with filtered water (American Society for Testing Standards Type 1 quality) until countable numbers were obtained. Slides were viewed under oil immersion at 1000X power under 390 nm wavelength light using an Olympus BHS-RFL light fluorescence attachment and an Olympus BHS microscope (Olympus Optical Co. LTD., Japan). A miniaturized most-probable-number (MPN) method (Braddock and Catterall 1999) was used to enumerate viable heterotrophic microorganisms using R2A (Difco Laboratories, Detroit, MI) as a growth medium.

Chemical analyses

Condensed tannin content was determined using a butanol-HCl reaction for proanthocyanidin (Martin and Martin 1982); two sequential extractions of 10 min each were made in 20 mL aqueous methanol (50 % V/V) at 95 °C. Extracts were analyzed spectrophotometrically at 550 nm (A_{550}) using condensed tannin purified from Alaska paper birch leaves as the standard.

To determine amino acid concentrations, samples were hydrolyzed using 6 N HCl at 110 °C for 24 h, and then freeze-dried to remove acid vapor. Ninhydrin derivatization and high pressure liquid chromatography with an amino acid analyzer (Beckman 6300, Beckman Instruments Inc., Palo Alto, California) were then used to determine concentrations.

Soluble carbohydrates were determined colorimetrically by the manual phenol/ sulfuric reaction method of McCown (1979).

Samples for dissolved organic carbon measurement were filtered through 0.45 μm membrane filters (Whatman Filtration, England), and concentrations were determined by the combustion/non-dispersive infrared gas analysis method using a Shimadzu TOC 5000-A total organic carbon analyzer (Shimadzu Corp., Japan).

Data analyses

Data were analyzed using the Statistica (5.1, 1997) statistical package (Statsoft, Tulsa, OK). Differences in means for experiments 1 and 2 were tested using 2 way

ANOVA with time and treatment (browsed vs. unbrowsed) as the main effects. In experiment 3, differences in means were tested using 3 way ANOVA with time, treatment (browsed vs. unbrowsed), and amendment (with and without amino acid enrichment) as the main effects.

RESULTS

Microbial activity

In experiment 1, oxygen uptake of microorganisms cultured in leachate from browsed leaves was significantly greater than for microorganisms cultured in the unbrowsed leachate (Fig.1). In a two way ANOVA, the treatment effect was highly significant ($p < 0.001$), as were the time effect and the time x treatment interaction ($p < 0.001$). Respiration rates for both treatments exhibited similar trends during the course of the experiment. Similar results were observed in experiment 2 (Fig. 2). Oxygen uptake by microbes cultured in leachates amended with amino acids (experiment 3) was three times greater than by microbes cultured in non-amended leachates (Fig. 3). In addition, respiration by microbes cultured in the amended unbrowsed leachate was significantly greater than by microorganisms cultured in the amended browsed leachates (three way ANOVA; time, treatment, amendment as the main effects, $p < 0.001$). Although there was a significant treatment effect, the amendment effect was also highly significant ($p < 0.001$), as were time, time x amendment interaction, time x treatment x amendment interaction ($p < 0.001$), and time x treatment interaction ($p < 0.002$).

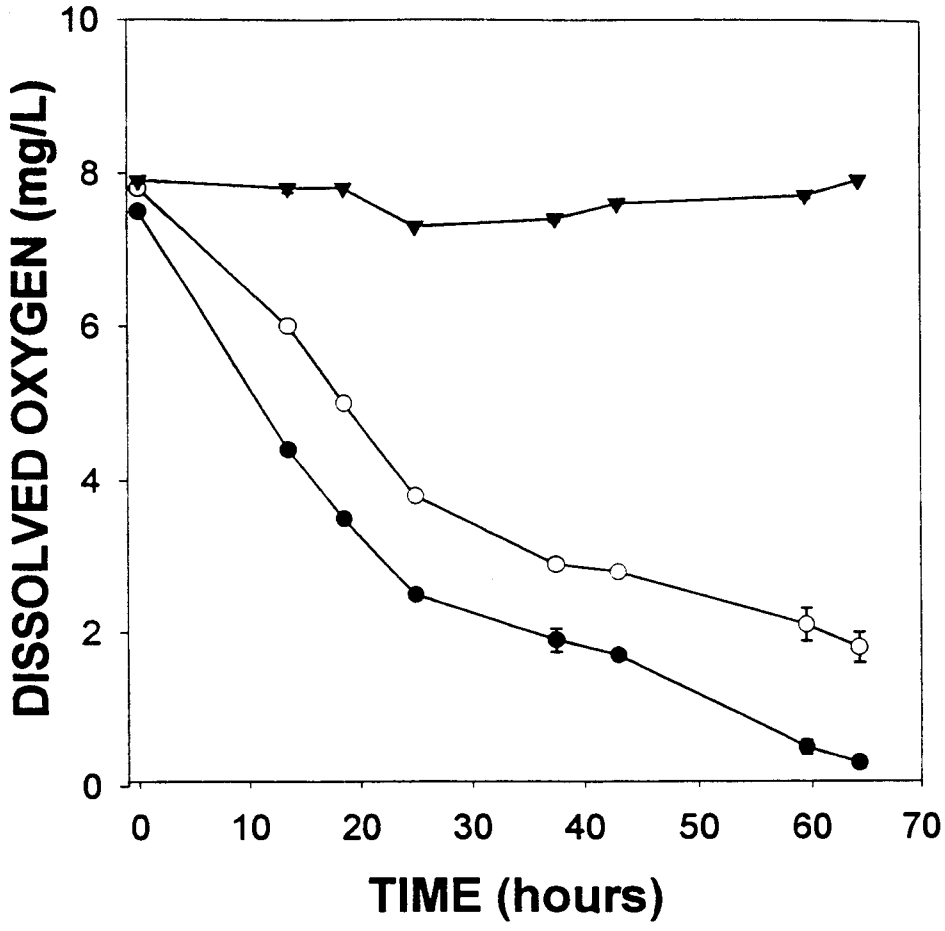

Figure 1. Oxygen uptake of microbes over time for experiment 1. Closed circles represent browsed treatment (leachates derived from leaves of trees browsed by moose), open circles unbrowsed treatment (leachates derived from leaves of trees not browsed by moose), and triangles represent the controls. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

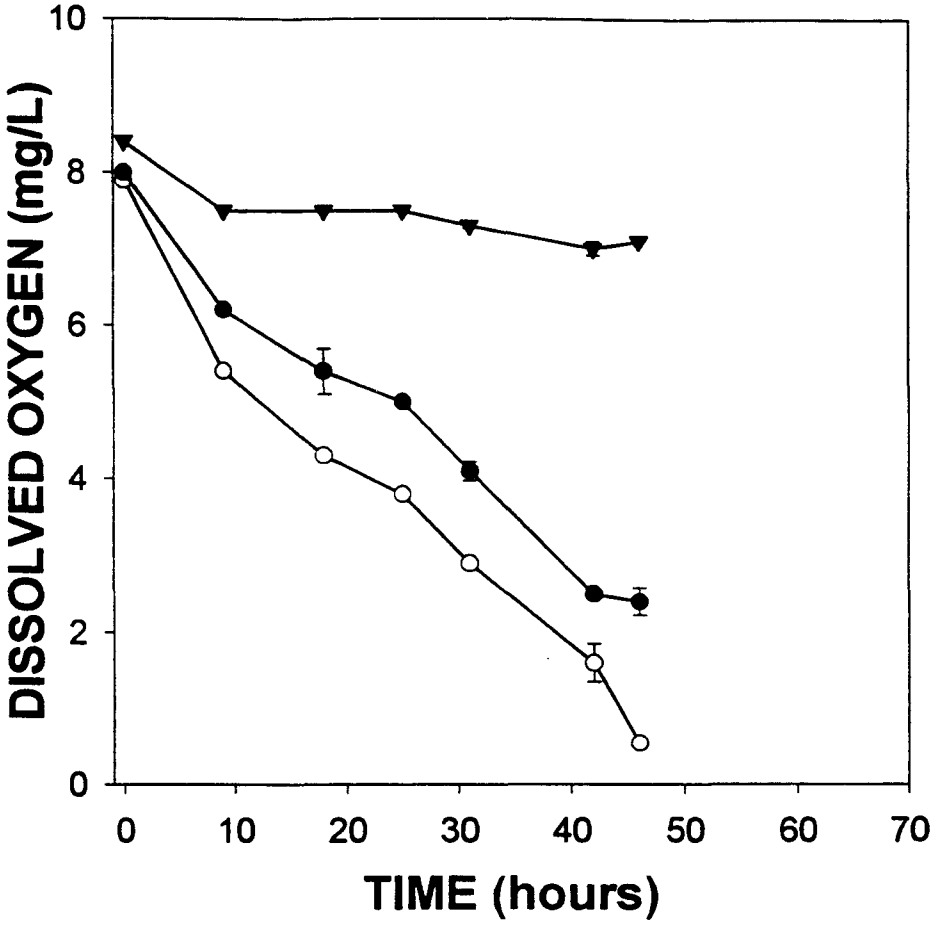

Figure 2. Oxygen uptake of microbes over time for experiment 2. Closed circles represent browsed treatment (leachates derived from leaves of trees browsed by moose), open circles unbrowsed treatment (leachates derived from leaves of trees not browsed by moose) and triangles represent the controls. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

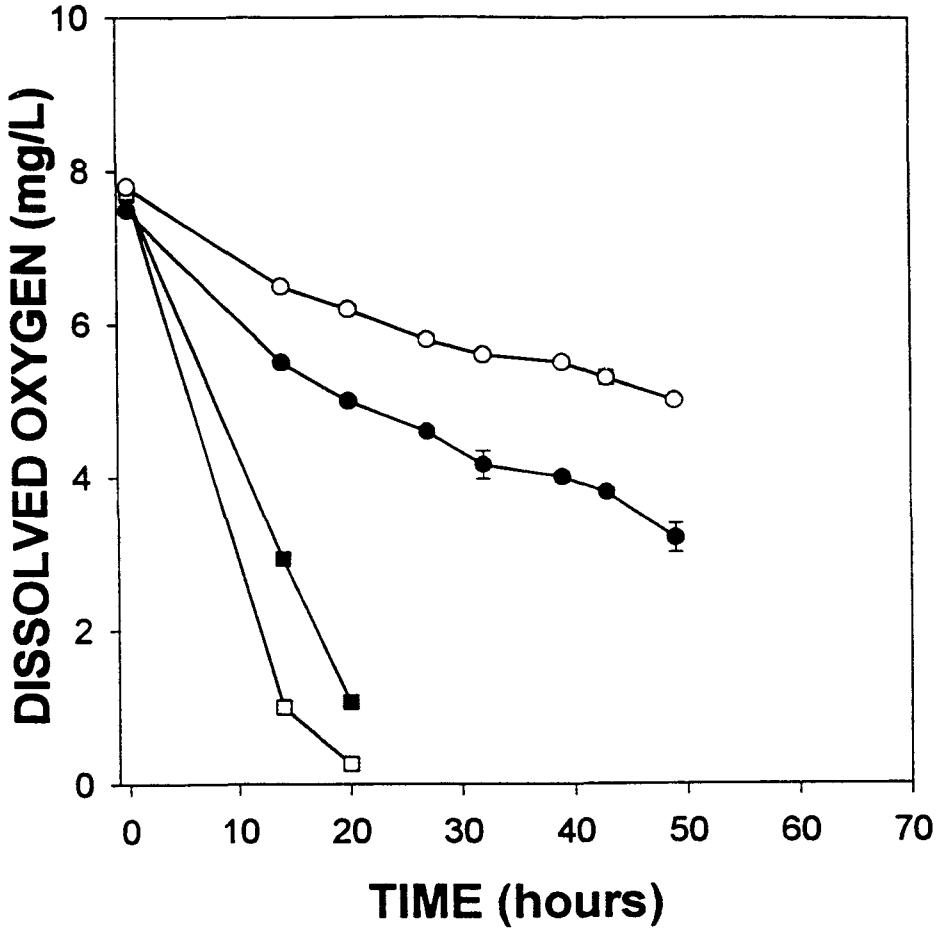

Figure 3. Oxygen uptake of microbes over time for experiment 3. Closed circles represent browsed treatment (leachates derived from leaves of trees browsed by moose), open circles unbrowsed treatment (leachates derived from leaves of trees not browsed by moose), closed squares represent supplemented (with exogenous amino acids) browsed treatment, open squares supplemented unbrowsed treatment, and triangles represent the controls. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

Microbial abundance

There was no significant treatment effect for bacterial abundance in experiment 1 (two way ANOVA), although both time; $p < 0.001$, and the time x treatment interaction; $p = 0.0222$, were significant; (Fig. 4). Bacterial populations in both treatments grew steadily for the first 25 h, at which point cell numbers leveled off and then decreased. Bacterial populations in the control increased slightly over the course of the experiment. Similar results were seen in a replicate experiment (Fig. 5). There was also no significant difference in viable microbial populations in browsed and unbrowsed leachates (Fig. 6). Microbial populations in the leaf leachates increased slightly in the first 10 h of the incubation and after 25 h decreased steadily to about 10^5 cells/mL. In the control, microbial populations increased in the first 10 h then declined. Microbial biomass in these experiments was predominately from the inoculum but in leachate treatments was supplemented by microorganisms in the leachate solutions (see population in controls compared to the leachate solutions at time 0, Figs. 4 and 6).

Chemical analyses

There were no measurable condensed tannins (< 80 μg/mL; the detection limit of the analysis) in leachates from leaf litter of either browsed or unbrowsed trees, nor was there a significant difference in carbohydrate concentrations between the leachates (0.039 and 0.038 % of the leachates, respectively). There was an 89 % greater concentration of

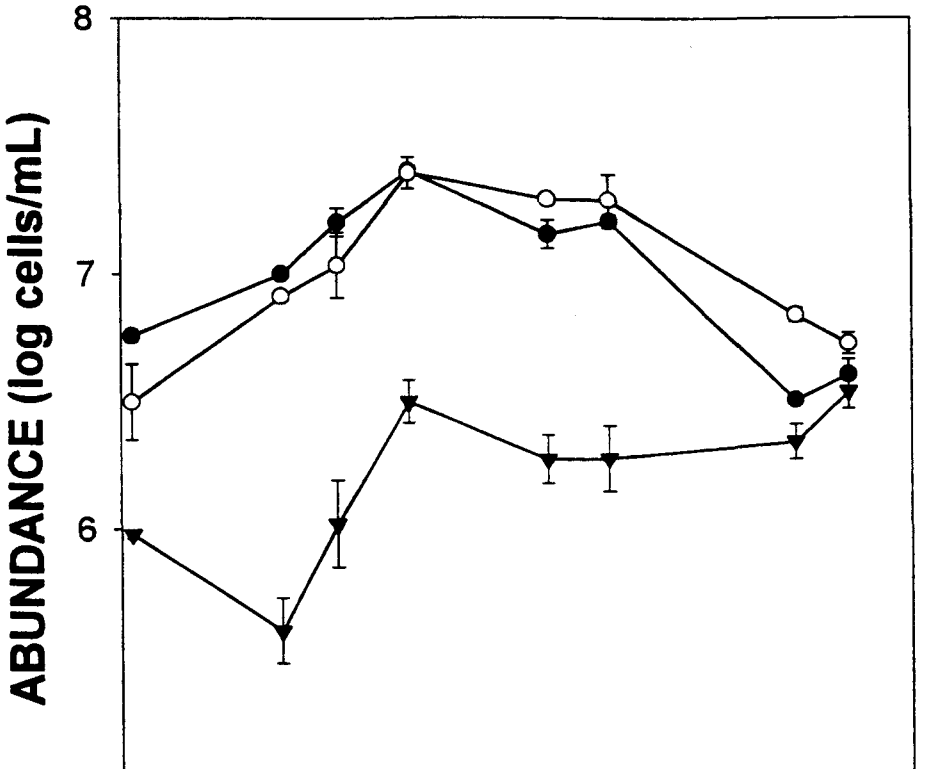

Figure 4. Bacterial abundance over time estimated by microscopic direct counts for experiment 1. Closed circles represent browsed treatment, open circles represent unbrowsed treatment, and triangles represent the control. Values shown are means ± 1 SE based on n = 3 BOD bottles.

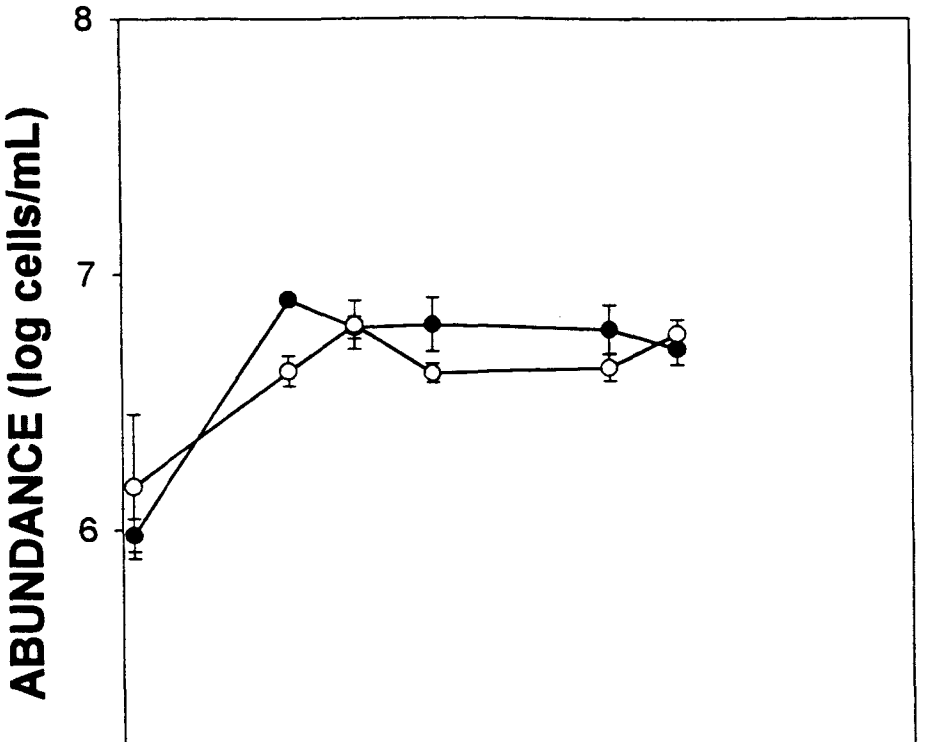

Figure 5. Bacterial abundance over time estimated by microscopic direct counts for bacteria cultured in unsupplemented leachates in experiment 3. Closed circles represent browsed treatment, open circles represent unbrowsed treatment. Values shown are means ± 1 SE based on n = 3 BOD bottles.

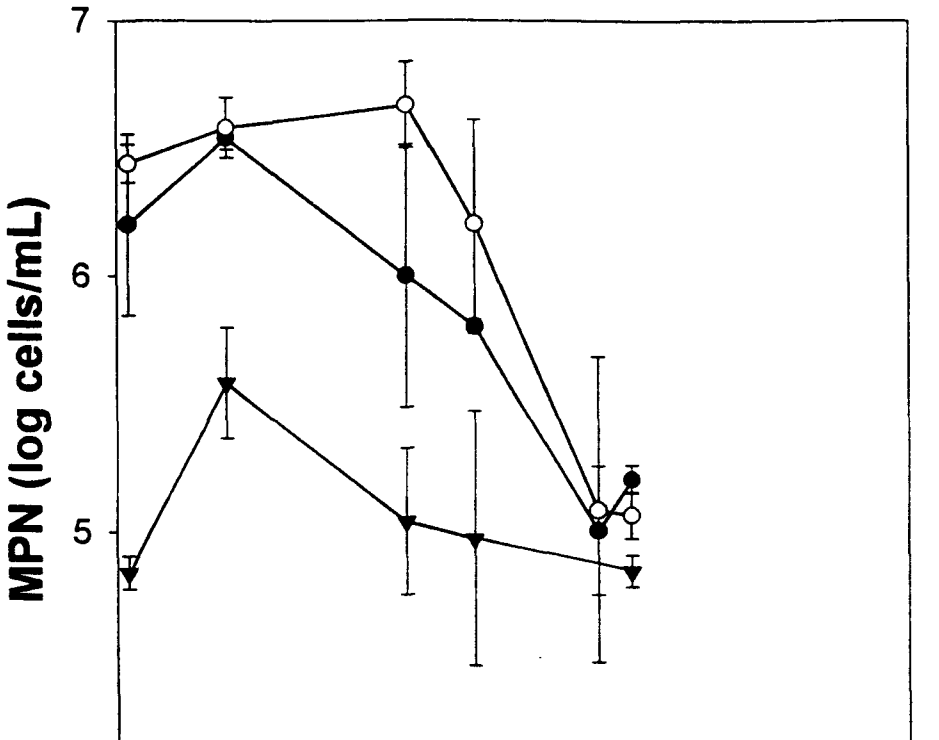

Figure 6. Bacterial abundance over time estimated by the MPN technique in experiment 2. Closed circles represent browsed treatment, open circles represent unbrowsed treatment, and triangles represent the control. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

amino acids present in leachates derived from the leaves of browsed trees than in leachates derived from leaves of trees not previously browsed (Fig. 7). Glutamate, glycine, aspartate, valine, and alanine were the most abundant amino acids accounting for over 40 % of the total amino acid pool in both leachates.

DISCUSSION

Winter browsing of birch is associated with chemical changes in the leaves in the following growing season and consequent effects on consumers and decomposer communities. Winter browsing by moose increased in-stream decomposition of *B. resinifera* leaf litter the following autumn (Irons *et al.* 1991). Leaf eating and sucking insects prefer previously browsed *B. pendula* and *B. pubescens* leaves (Danell and Huss-Danell 1985). Both investigations found that leaves originating from previously browsed trees had higher nitrogen contents compared to leaves from unbrowsed trees. Our results likewise suggest cascading effects of vertebrate browsing of riparian trees.

In experiments 1 and 2, microbes cultured in leachates originating from leaves of previously browsed birch had greater rates of oxygen consumption (Fig. 1 and 2). This could be caused, in part, by higher concentrations of amino acids (Fig. 7) in leachates derived from leaves of browsed trees. Specific molecular sizes and types of molecules are selectively removed from DOC by microorganisms in stream sediments (Kaplan and Bott 1983, 1985) and in non-benthic bacteria (Meyer *et al.* 1987; Amon and Benner 1996), and amino acids are a constituent of biodegradable dissolved organic carbon

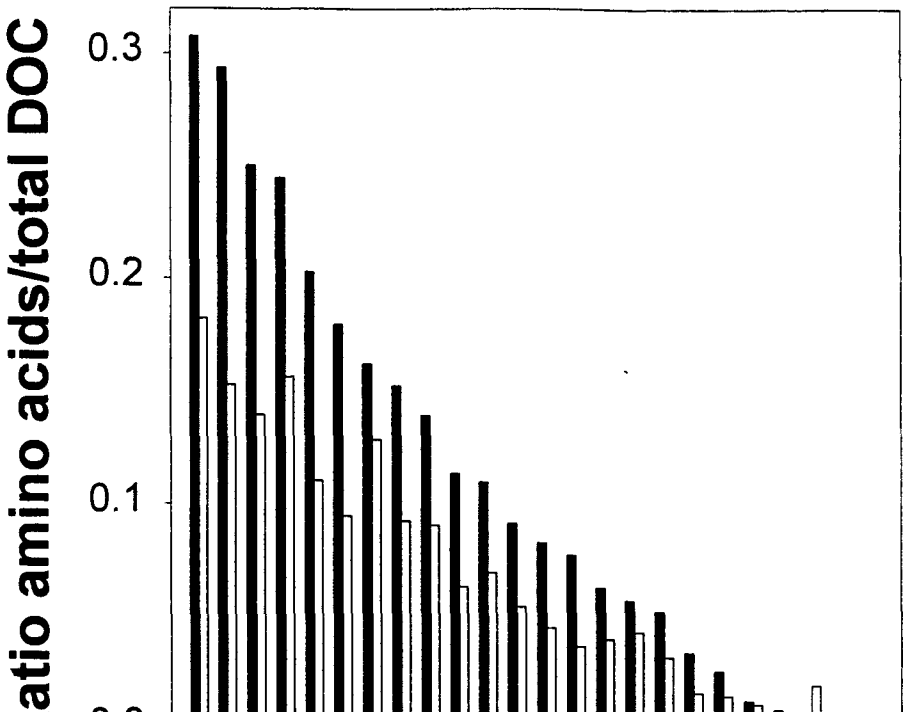

Figure 7. Individual amino acid concentrations of leachates expressed as the ratio of amino acids to total DOC. Black bars represent browsed treatment and open bars represent unbrowsed treatment. Amino acid abbreviations: glutamate, GLU; alanine, ALA; aspartic acid, ASP; glycine, GLY; valine, VAL; leucine, LEU; serine, SER; threonine, THR; lysine, LYS; isoleucine, ILE; proline, PRO; arginine, ARG; phenylalanine, PHE; tyrosine, TYR; hisidine, HIS; 1-methylhisidine, MHS; methionine, MET; ornithine, ORN; ethanoalanine, ETH; citrulline, CIT; phosphoethanolamine, PET; α-aminoadipic acid, AAD; γ-aminobutyric acid, GAB; phosphoserine, PSR; α-amino-n-butyric acid, ABU.

(Kaplan and Bott 1983, 1985, Volk *et al.* 1997). The threefold increase in oxygen uptake exhibited by the microbes cultured in the amended leachates compared to microbes cultured on unamended leachates (Fig. 3) demonstrates the nutritional value of amino acids. Our supplement of 8000 μg/L of four selected amino acids (chosen because they were present in the highest concentrations in the unamended leachates) is a concentration more than 15 times that reported in streams (concentrations range from 100 to 500 μg/L; Thurman 1985). Although the much greater rate of oxygen uptake for microbes cultured in the augmented leachates is likely a consequence of our substantial enrichment of amino acids, amino acid availability seems clearly implicated as a measure of DOC quality and as a limiting factor to stream microbes.

In experiment 1, and for the microbes cultured in the non-augmented leachates in experiment 3, bacterial growth (Fig. 4 and 5) corresponds with the highest rates of oxygen uptake in both treatments (Fig. 1 and 3). These results suggest that bacteria are assimilating labile components (primary substrate) of the leachates quickly, and that these components support bacterial growth. Bacterial abundance in experiment 1 decreased to initial numbers (Fig. 4), which is likely attributable to a steady decrease in higher quality substrate capable of supporting growth, or a decrease in dissolved oxygen as the experiment progressed, or both. In addition, there may have been protozoan/micrometazoan grazing during the course of the experiments as we did not take measures to remove grazers from the inoculum. A similar growth pattern was observed in experiment 3 (Fig. 5). Microbial oxygen uptake in experiment 2 was similar to

experiment 1 and for microbes cultured in unamended leachates in experiment 3. However, assessment of microbial abundance via a MPN technique (which estimates viable cell numbers) (Fig. 6) rather than the direct count method (which includes all bacterial cells) showed no evidence of microbial growth. Microbial abundance decreased an order of magnitude below the initial concentrations. These differences between viable and total cells in temporal patterns of microbial abundance suggest considerable turnover of microbial populations, with some populations reproducing rapidly in response to abundant DOC resources in the early phase but then becoming non-viable as easily assimilated resources are depleted. As a note, controls exhibited modest microbial growth (Figs. 4 and 6), likely supported by DOC introduced by the inoculum. However, oxygen uptake by microbes in the control treatments was very small compared to treatments with leachates (Fig. 1 and 2). In addition, differences in initial bacterial abundance between the treatments and the controls clearly show a bacterial contribution from the leachates.

Our results suggests that the qualitative difference between leachates from leaves of browsed and unbrowsed trees were manifested as differences in microbial metabolism rather than differences in microbial abundance. The lack of differences in bacterial growth between treatments is interesting in that high quality organic nitrogen should be limiting to bacteria (organic nitrogen is used in making structural proteins, enzymes, and nucleic acids), thus increased organic nitrogen availability would be predicted to facilitate increases in bacterial growth. Our analysis of amino acids measured the concentration of

individual amino acids after the complete hydrolysis of proteins and peptides. Changes in leaf chemistry associated with winter browsing may include changes in the size, structure, and solubility of leaf proteins and peptides, which can affect their utilization by stream microorganisms.

Our results suggest cascading effects of vertebrate browsing of riparian trees. Long term browsing by vertebrates can facilitate landscape-level changes in forest ecosystems, such as on Isle Royale, where long term moose herbivory altered plant species composition, quantity and quality of leaf litter, soil chemistry, and microbial biomass (Pastor *et al.* 1988). Lotic trophic systems are tightly coupled to the riparian zones that surround them, and terrestrial disturbances by keystone herbivores can change the chemical characteristics of leaf litter. In turn, changes in the chemical characteristics of leaf litter generate changes in the components of dissolved organic matter leached from leaves and subsequent effects on uptake of dissolved organic matter by stream microbes.

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List of Appendicies

Appendix 1: Raw data from experiments 1-3.

Appendix 1 contains the raw microbial respiration and bacterial abundance data from experiments 1-2, and raw microbial respiration data from experiment 3 (Table 1) included in the manuscript. Measurement periods which data were not collected are denoted as 'na' in the raw data tables.

Table A1.1: Microbial Respiration Data for Experiment 1

	Dissolved oxygen (mg/L)		
	treatment		
time (hours)	browsed	unbrowsed	control
0	7.5	7.8	7.8
	7.5	7.8	7.9
	7.4	7.8	7.9
13	4.5	6.1	7.9
	4.4	6.0	7.8
	4.4	6.0	7.7
18	3.5	5.1	7.8
	3.5	5.0	7.8
	3.4	5.0	7.8
25	2.4	3.7	7.3
	2.4	3.9	7.3
	2.6	3.8	7.3
37	1.8	2.9	7.4
	2.2	2.9	7.3
	1.7	2.8	7.4
43	1.6	2.7	7.6
	1.7	2.8	7.6
	1.7	2.8	7.6
59	0.6	1.8	7.7
	0.5	2.5	7.8
	0.3	1.9	7.7
64	0.4	1.6	7.9
	0.4	2.2	7.9
	0.2	1.6	7.9

Table A1.2: Microbial Abundance Data for Experiment 1

	Abundance (LOG cells/mL)		
	treatment		
time (hours)	browsed	unbrowsed	control
0	na	6.2	6.0
	6.8	6.6	na
	6.7	6.7	6.0
13	7.0	6.9	5.9
	7.0	6.9	5.5
	7.0	6.9	5.9
18	7.1	7.1	6.4
	7.2	6.8	5.8
	7.3	7.2	5.9
25	7.4	na	6.6
	7.5	7.3	6.6
	7.5	7.5	6.3
37	7.1	7.3	6.1
	7.3	7.3	6.3
	7.1	7.3	6.4
43	7.3	7.1	6.2
	7.3	7.5	6.5
	7.2	7.3	6.1
59	na	na	6.2
	6.5	6.8	6.4
	6.5	6.9	6.4
64	6.6	6.6	6.6
	6.8	6.8	6.6
	6.6	6.8	6.4

Table A1.3: Microbial Respiration Data for Experiment 2

	Dissolved oxygen (mg/L)		
	treatment		
time (hours)	browsed	unbrowsed	control
0	7.9	8.0	8.4
	7.9	8.0	8.4
	7.9	8.0	8.4
9	5.4	6.2	7.5
	5.4	6.1	7.5
	5.3	6.2	7.5
18	4.2	5.8	7.5
	4.4	5.5	7.5
	4.4	4.8	7.5
25	3.8	5.1	7.5
	3.8	5.0	7.5
	3.7	5.1	7.5
31	3.0	4.3	7.2
	3.0	3.9	7.3
	3.0	4.0	7.3
42	1.1	2.6	6.9
	1.9	2.6	7.2
	1.8	2.4	7.0
46	0.5	2.1	7.1
	0.5	2.7	na
	0.6	2.4	7.1

Table A1.4: Microbial Abundance Data for Experiment 2

	MPN estimation (LOG cells/mL)	
	treatment	
time (hours)	browsed	unbrowsed
0	na	6.4
	5.9	6.6
	6.5	6.3
9	6.5	6.6
	6.5	6.3
	6.6	6.7
18	na	na
	na	na
	na	na
25	na	6.9
	5.3	6.3
	6.6	6.7
31	5.9	7.0
	5.8	5.8
	5.8	5.8
42	4.5	4.9
	5.0	5.1
	5.4	5.1
45	5.2	5.1
	5.1	4.9
	5.2	na

Table A1.5: Microbial Respiration Data for Experiment 3

	Dissolved oxygen (mg/L)			
	treatment			
time (hours)	browsed	unbrowsed	ammended browsed	ammended unbrowsed
0	7.5	7.7	7.7	7.7
	7.5	7.8	7.7	7.7
	7.5	7.9	7.7	7.7
14	5.5	6.5	2.9	1.0
	5.5	6.5	3.0	1.0
	5.5	6.5	2.9	1.0
20	5.0	6.3	1.1	0.10
	5.1	6.3	1.1	0.40
	4.9	6.3	1.0	0.30
27	4.6	5.9	na	na
	4.6	5.9	na	na
	4.6	5.7	na	na
32	4.4	5.7	na	na
	4.3	5.7	na	na
	3.8	5.6	na	na
39	3.9	5.6	na	na
	4.1	5.6	na	na
	4.1	5.4	na	na
43	3.8	5.3	na	na
	3.8	5.4	na	na
	3.8	5.1	na	na
49	2.8	5.1	na	na
	3.5	5.0	na	na
	3.3	5.1	na	na

Table A1.6: Data from Amino Acid Analysis of Leachates.

	Concentration (uM)	
amino acid	browsed	unbrowsed
glutamate	24.3	19.2
alanine	23.2	16.1
aspartate	19.7	14.7
glycine	19.3	16.5
valine	16.0	11.6
leucine	14.1	9.95
serine	12.8	13.5
threonine	12.0	9.73
lysine	11.0	9.54
isoleucine	8.96	6.68
proline	8.65	7.30
arginine	7.21	5.71
phenylalanine	6.52	4.75
tyrosine	6.10	3.87
hisidine	4.96	4.20
1-methylhisidine	4.50	4.53
methionine	4.10	3.34
ornithine	3.32	3.68
ethanoalanine	2.65	1.68
citrulline	2.01	1.52
phosphoethanolamine	0.964	1.13
α-aminoadipic acid	0.660	0.000
γ-aminobutyric acid	0.580	2.07
phosphoserine	0.478	0.673
α-amino-n-butyric acid	0.146	0.089

Appendix 2: Supplementary Data.

Appendix 2 contains data and discussion from experiments not included in the manuscript. These data were omitted due to constraints imposed by the guidelines of the journal to which the manuscript was submitted. The data included in this appendix do not offer any additional information that would alter the interpretations or conclusions drawn from this study. Rather, the data contained in this appendix supplement that reported in the manuscript. Measurement periods which data were not collected are denoted as 'na' in the data tables.

In experiment 3 (see Table 1), respiration was compared between microbes cultured in non-amended leachates and those cultured in leachates amended with an amino acid supplement. The results showed that respiration rates of microbes cultured in the amended leachates were more than 3 times greater than the rates of microbes cultured in non-amended leachates (Fig. 3). As a result, these microbes exhausted their available dissolved oxygen within 20 hours after inoculation. In addition, microbes cultured in the amended unbrowsed leachates had a significantly greater rate of respiration than microbes cultured in amended browsed leachates. This in contrast with experiments 1 and 2, and with microbes cultured in non-amended leachates in experiment 3, in which microbes cultured in browsed leachates had significantly greater rates of respiration (Figs. 1-3).

This supplementary experiment (experiment 4) was designed to examine the respiration of microorganisms cultured in amino acid amended leachates during a short term incubation (12 hours), and to see if, as in experiment 3 (Fig. 3), the greater

respiration rates of microbes cultured in amended unbrowsed leachates would be repeated. Leachates were prepared with the amino acid amendment following the methods described in the manuscript. Methods for measuring microbial respiration and estimation of bacterial abundance by microscopic direct counts likewise follow those described in the manuscript.

The high rates of microbial respiration for both treatments were consistent with results of microbes cultured in amended leachates in experiment 3 (Appendix 3A). However, microbes cultured in the amended browsed leachates had a significantly higher rate of respiration, 2 way ANOVA, time, treatment as the main effects ($p < 0.001$), and time x treatment interaction ($p < 0.001$), than microbes cultured in amended unbrowsed leachates, which is in contrast to the results found with microbes cultured in amended leachates in experiment 3 (Fig. 3). In a similar experiment, microbes cultured in amended unbrowsed leachates had higher rates of respiration compared to microbes cultured in amended browsed leachates (Experiment 5; Appendix 3B). The observed 'switching' of higher respiration rates between the browsed and unbrowsed treatments among the amended leachate experiments is likely the result of the browsed and unbrowsed treatment effect simply being an artifact. The amino acid supplements provide microorganisms with large supply high quality substrate. Any observed treatment effect is likely due to metabolic variation within microbial populations, opposed to differences in microbial metabolism due to a difference in leachate quality.

Total organic carbon (TOC) uptake for the respiration experiment of microorganisms cultured in non-amended leachates was determined in experiment 3. At each respiration measurement, a 20 mL sample removed and acidified with HCl (pH < 2), then refrigerated at 10° C until analysis was performed. TOC concentrations were then determined using the combustion/non-dispersive infrared gas analysis method using a Shimadzu TOC 5000-A total organic carbon analyzer (Shimadzu Corp., Japan).

Microbes cultured in the browsed leachate had a significantly higher rate of TOC uptake than microbes cultured in the unbrowsed leachate (Appendix 3C), (two way ANOVA, both time and treatment effects were significant ; $p < 0.001$). TOC concentrations in both treatments decrease steadily until 25 h, at which point, there is a decrease in the rate of TOC assimilation for both treatments.

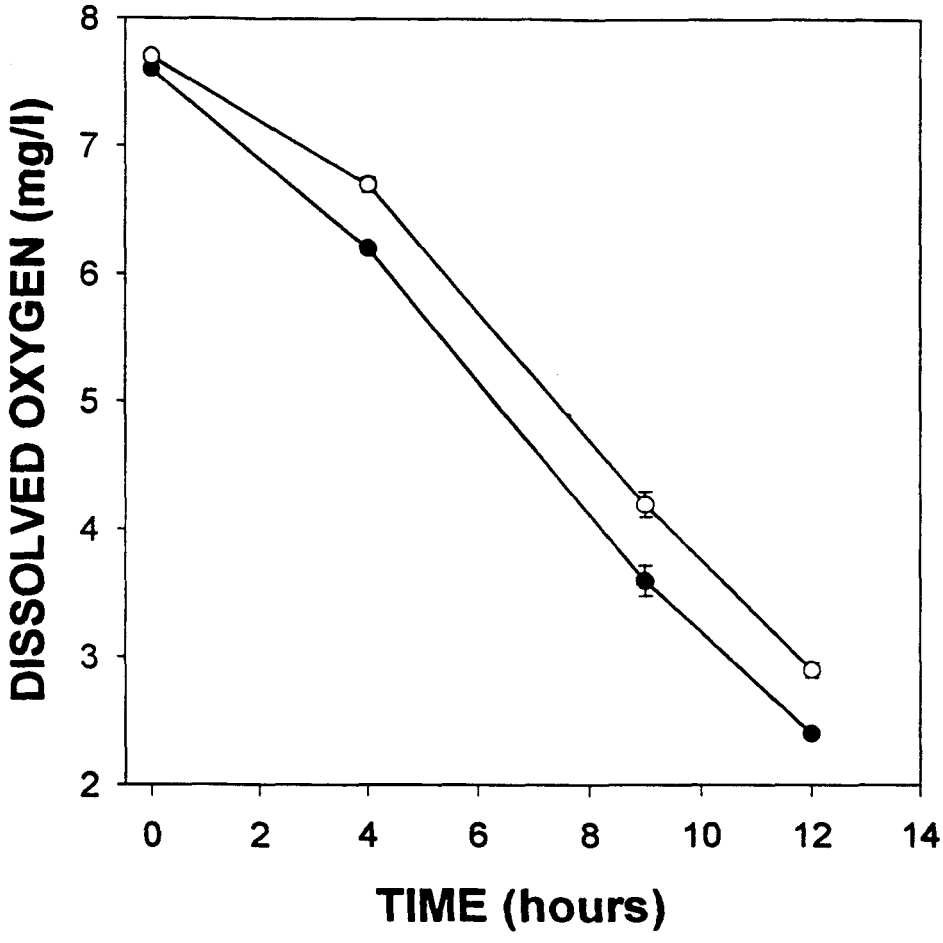

Figure A2.1. Oxygen uptake of microbes over time for experiment 4. Closed circles represent browsed treatment, open circles unbrowsed treatment. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

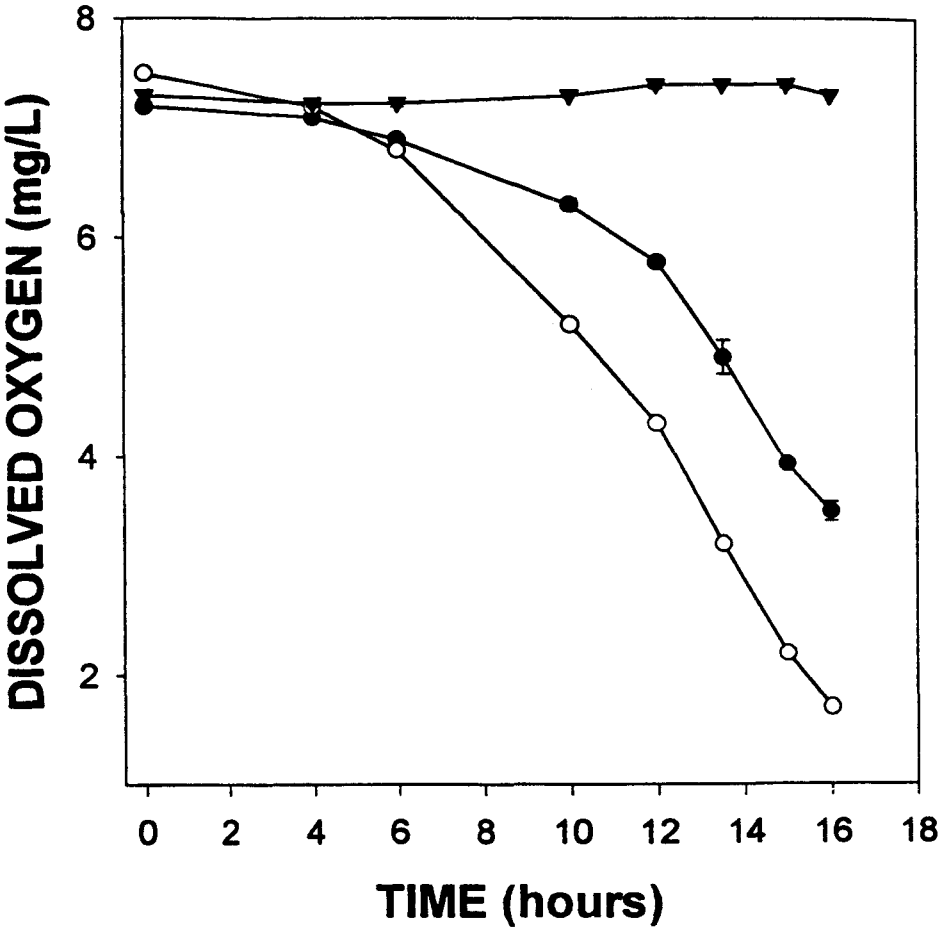

Figure A2.2. Oxygen uptake of microbes over time for experiment 5. Closed circles represent browsed treatment, open circles unbrowsed treatment, and triangles represent the controls. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

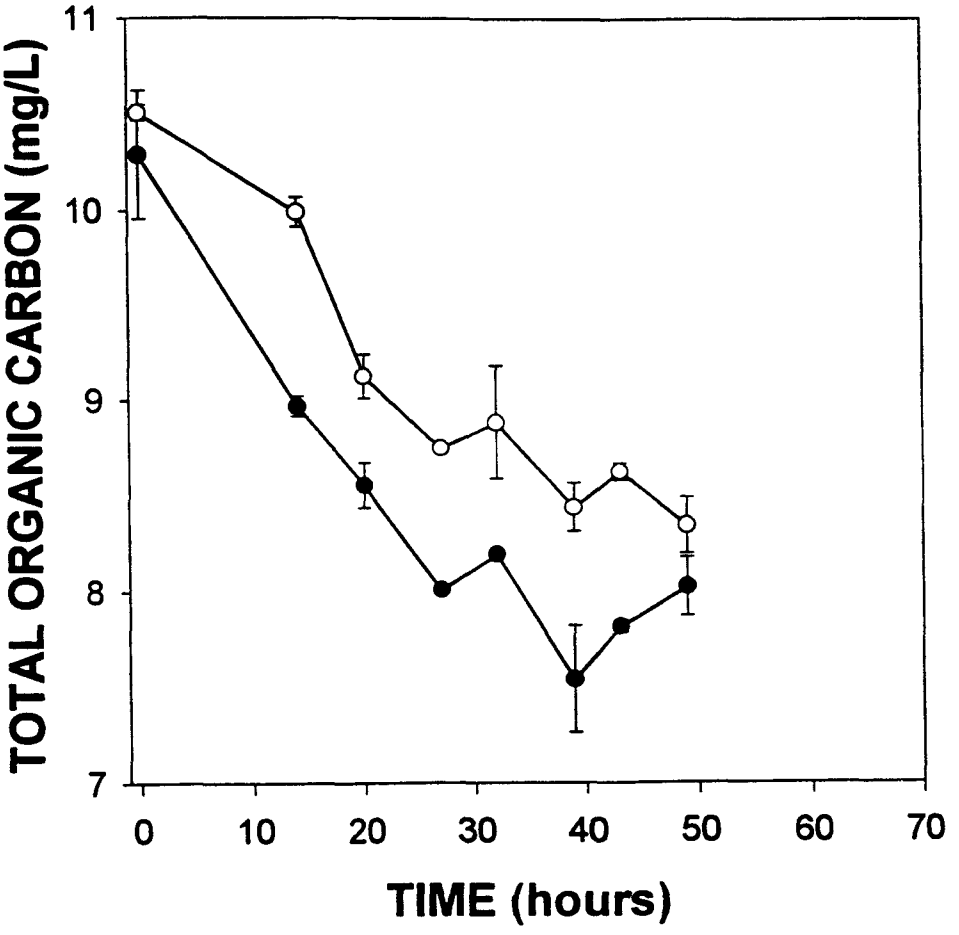

Figure A2.3. Total organic uptake for microbes over time. Closed circles represent browsed treatment, open circles unbrowsed treatment. Values shown are the means ± 1 SE based on n = 3 BOD bottles.

Table A2.1: Microbial Respiration Data for Experiment 4

	Dissolved oxygen (mg/L)	
	treatment	
time (hours)	amended browsed	amended unbrowsed
0	7.5	7.7
	7.6	7.7
	7.6	7.7
4	6.2	6.7
	6.2	6.8
	6.1	6.7
8	3.4	4.2
	3.5	4.3
	3.8	4.1
12	2.4	2.8
	2.4	2.9
	2.3	2.9

Table A2.2: Microbial Respiration Data for Experiment 5

	Dissolved oxygen (mg/L)		
	treatment		
time (hours)	browsed	unbrowsed	control
0	7.3	7.5	7.3
	7.2	7.4	7.2
	7.2	7.5	7.3
4	7.1	7.1	7.2
	7.0	7.2	7.3
	7.1	7.2	7.2
6	6.9	6.8	7.2
	6.9	6.8	7.3
	6.9	6.8	7.2
10	6.3	5.2	7.3
	6.4	5.2	7.3
	6.2	5.3	7.3
12	5.7	4.3	7.3
	5.8	4.3	7.4
	5.8	4.4	7.4
13	4.6	3.2	7.4
	5.0	3.2	7.4
	5.1	3.3	7.4
15	4.0	2.3	7.4
	3.9	2.2	7.3
	3.9	2.2	7.4
16	3.3	1.7	7.3
	3.6	1.7	7.3
	3.5	1.7	7.3

Table A2.3: Total Organic Carbon Uptake Data for Experiment 3

	Total organic carbon (mg/L)	
	treatment	
time (hours)	browsed	unbrowsed
0	10.9	10.2
	9.87	10.3
	10.1	11.0
14	9.04	9.17
	na	9.40
	8.90	9.63
20	8.35	8.92
	8.70	9.27
	8.61	9.18
27	7.89	8.83
	7.89	8.81
	8.23	8.61
32	8.23	8.65
	8.15	9.54
	8.20	8.45
39	7.33	8.17
	8.17	8.55
	7.14	8.60
43	7.69	8.32
	7.78	8.18
	7.98	9.34
49	7.83	7.99
	8.23	8.43
	8.00	8.61