# CONTRIBUTION OF ALLOCHTHONOUS CARBON TO THE MICROBIAL COMMUNITY IN AN ACIDIC GEOTHERMAL LAKE

_____

A Thesis

Presented

to the Faculty of

California State University, Chico

_____

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biological Sciences

_____

by

Areeje Almasary

Spring 2013

# CONTRIBUTION OF ALLOCHTHONOUS CARBON TO THE MICROBIAL COMMUNITY IN AN ACIDIC GEOTHERMAL LAKE

A Thesis

by

Areeje Almasary

Spring 2013

APPROVED BY THE DEAN OF GRADUATE STUDIES
AND VICE PROVOST FOR RESEARCH:

_____
Eun K. Park, Ph.D.

APPROVED BY THE GRADUATE ADVISORY COMMITTEE:

_____
Gordon V. Wolfe, Ph.D., Chair

_____
Larry F. Hanne, Ph.D.

_____
Andrea White, Ph.D.

_____
Kristopher A. Blee, Ph.D.

_____
David L. Brown, Ph.D.

ACKNOWLEDGMENTS

A special thanks to my advisor Dr. Gordon Wolfe for giving me a chance to have my masters degree and work in his lab, and for the continued advice and help which was instrumental to this study, especially with the carbon budget calculations and isotopic experiments. I also thank my committee members, Dr. Andrea White, Dr. Larry Hanne, Dr. Kristopher Blee, and Dr. David Brown for support and the time dedicated for review of my thesis. I also thank the Saudi Arabian Cultural Mission, and the National Science Foundation grant MCB-0702069 for funding this project.

I am also grateful to my husband Majed Alayuni whose support and help was vital to go through the ups and downs of my path toward completing my degree. I also like to express appreciation to my family, my mother Huda Alzahrani, my father Dr. Nasser Almasary, my sisters Abeer, Shatha and Reem, and my brother Khalid for their continued support through this journey.

I want to extend my thanks to Dr. Patricia Siering and her student Connor Fitzhugh (Humboldt State Univ.) for sharing unpublished data. I also thank Erika Keshishian who helped me during my first months in CSU Chico, and William Reeder for help, advice and sharing unpublished data. I thank Bryan Ervin for helping maintain some of the samples and sharing unpublished data as well. I also thank the 2010 BIOL 402 class for collecting leaf litter for the *in situ* decomposition experiment. Last but not least, I would like to thank Lindsey Wallace and Sarah Hoddick for their support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

ABSTRACT

CONTRIBUTION OF ALLOCHTHONOUS CARBON TO THE MICROBIAL COMMUNITY IN AN ACIDIC GEOTHERMAL LAKE

by

Areeje Almasary

Master of Science in Biological Sciences

California State University, Chico

Spring 2013

Microorganisms can live in environments where macroscopic organisms cannot survive, such as in the extremely acidic geothermal Boiling Spring Lake (BSL) in Lassen Volcanic National Park, CA. BSL is an oligotrophic lake with thriving microbial populations whose carbon (C) sources are still poorly understood. In this study, I examined the possibility that allochthonous leaf litter (mostly pine needles) observed falling in the lake can fuel microbial production in BSL. Coniferous needles are known to be particularly difficult to colonize by microbes and have low decay rates, but no studies have examined the combined effects of low pH and high temperature on pine needle leaching and decomposition. In both lab- and field-based experiments, I found coniferous leaves had high decay rates during leaching and decomposition in BSL

compared to those reported in the literature for other neutral pH and mesophilic lakes and streams. Thus, the acid and high temperatures appear to help condition the leaves and make their C more biologically available. I also found little difference in decay between fresh and dry leaves, but when needles were cut, I observed faster leaching rates and slower decomposition rates, suggesting the effects of mechanical weathering are important, possibly by degrading the waxy cuticle barrier. I also observed that leaves decayed more slowly in 0.2 μm-filtered BSL water, suggesting both native and introduced microbes contribute to leaf breakdown. Based upon bacterial growth experiments and recovery of an ascomycete fungus, both prokaryotes and eukaryotes seem to contribute to leaf decay. The addition of a nitrogen (N) source (yeast extract) enhanced prokaryote growth more than adding a simple carbon such as glucose, suggesting BSL is both C and nitrogen limited. I also used radioisotopes to assess primary and secondary production rates, and observed autotrophic growth was low, and dominated by chemoautotrophy, while heterotrophic uptake rates varied depending on the organic substrate used, and were highest for acetate. These results suggest that allochthonous carbon is important in fueling microbial production in the lake. I constructed a preliminary C budget for the lake by measuring leaf deposition and estimating other dissolved organic carbon (DOC) sources from BLS's hydrologic budget.

# CHAPTER I

# INTRODUCTION

## Boiling Springs Lake: An Extreme Aquatic Ecosystem

Boiling Springs Lake (BSL) in Lassen Volcanic National Park (LVNP), California is a 1.8 ha pool of geothermal water lying along a geologic fault that developed from a flooded fumarole (Figure 1). The lake temperature is about 52 $^{o}$C and it is extremely acidic (pH 1.7-2.2) (Siering et al. 2006, Wilson et al. 2008). The lake's temperature varies seasonally and spatially, and is thought to be fueled primarily through a 'hot zone' at the south end of the lake, where gases and bubbling water can reach temperatures of 90 $^{o}$C or more. The lake shore has an abundance of other volcanic geological features such as mud pots, steam vents, and springs, while seasonal side-pools surrounding the lake vary in temperature and pH. BSL is situated in high-elevation (1806 m) (Siering et al. 2006) coniferous forest typical of the western Cascade mountains, and represents an 'island' type biogeographical community (Reeder 2011). BSL is a microbial observatory funded by the National Science Foundation (NSF), with the goal of understanding the microbial assemblages and their interactions in the lake, including prokaryotes, eukaryotes, and their viruses. BSL is thriving with microbial populations (Siering et al. 2006, Wilson et al. 2008, Reeder 2011, Diemer & Stedman 2012, Siering et al. 2013). The assemblage in BSL consists of prokaryotes (chemolithotrophs and

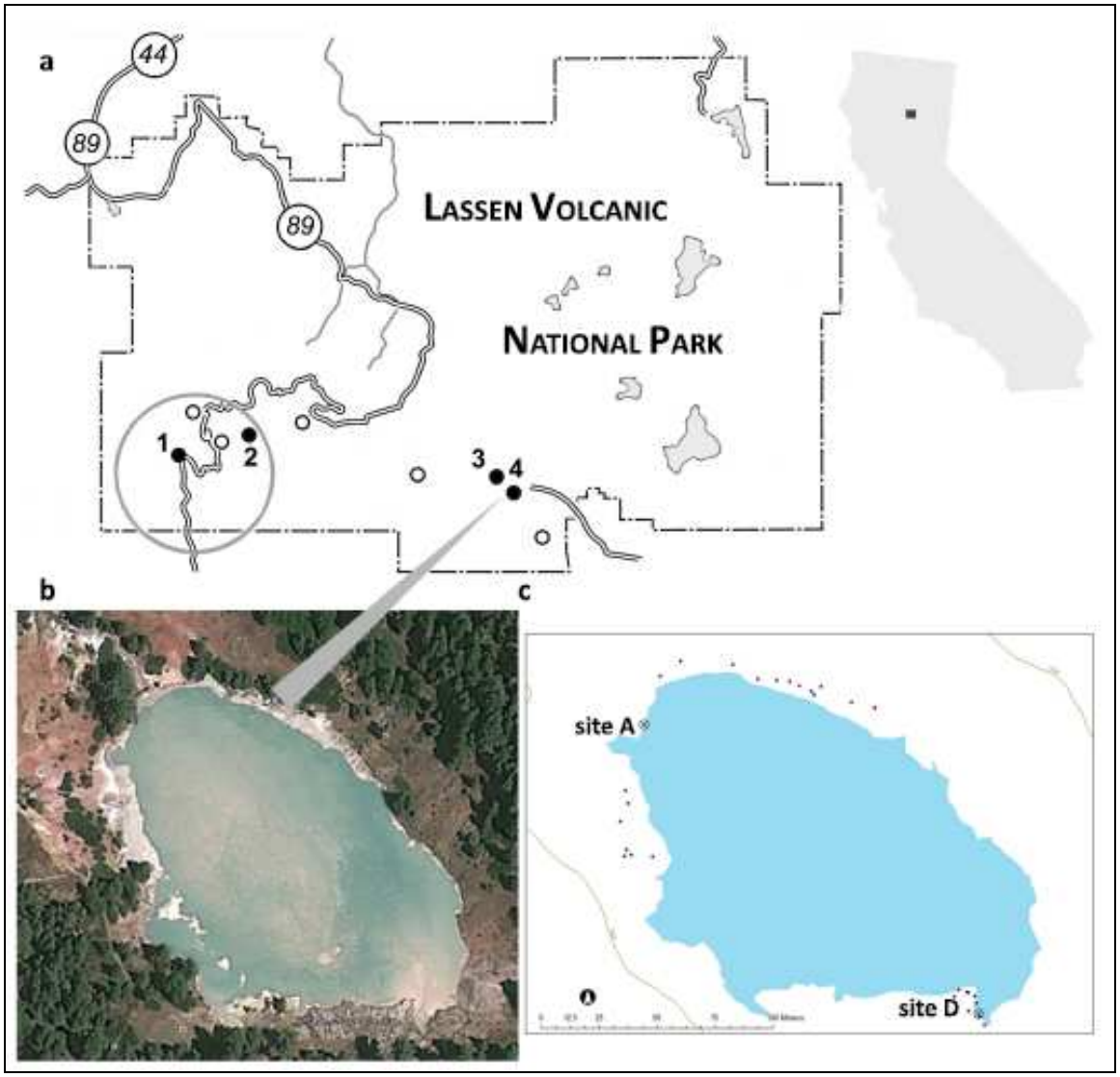


Fig. 1. Map of study site. (a) Lassen Volcanic National Park and its approximate location in Northern California. Black circles are geothermal sites (1, Sulfur Works; 2, Bumpass Hell; 3, Devil's Kitchen; 4, Boiling Springs Lake). Large gray circle denotes former Brokeoff Mountain caldera. (b) Satellite photo of the lake from Google maps. (c) BSL 30-cm resolution GPS map. Water sampling sites A and D are shown, along with major near-shore geothermal features (black diamond)

heterotrophs), and microeukaryotes (photoautotrophs, predominantly Cyanidiales;osmotrophs, predominantly ascomycete fungi (Wilson et al. 2008); and phagotrophic protists, predominantly heterolobosean ameobae (Reeder 2011) (Table 1).

Not all the microbes living in BSL are active during the high temperature season; some of the isolated organisms were not able to grow in temperatures

Table 1. Microbial guilds in BSL

| guild | prokaryotes | Eukaryotes |
|---|---|---|
| Primary producers | Iron oxidizers, S oxidizers (*Acidimicrobium ferrooxidans)* (Wilson et al. 2008) | Cyanidiales (main lake); diatoms *Pinnularia*) |
| Osmotrophs | G+ (*Sulfobacillus, Clostridium*) (Wilson et al. 2008) | Ascomycete fungi, possibly Cyanidiales (Bryan Ervin, Gordon Wolfe, unpublished) |
| Phagotrophs | | Amoeboflagellate *Tetramitus* sp., kinetoplastids (Reeder 2011) |

around 55 $^{o}$C (Wilson et al. 2008), and it was suggested that the structure of the microbial community changes with seasons (Siering et al. 2013). Microorganisms are the only life form found in BSL, higher plants and metazoans are absent from the lake water.

## Carbon Sources Available for BSL Microbes

All organisms need carbon to grow. In BSL chemo- and photo- autotrophs use inorganic carbon ($CO_2$) to build their organic matter, and heterotophs use organic carbon (dissolved or particulate organic carbon, DOC or POC respectively) which can be simple sugars, amino acids, or complex polymers. As in other aqueous environments organic carbon (OC) is either autochthonous or allochthonous. Autochthonous carbon is introduced to the water from within the system from primary producers, and allochthonous carbon is introduced from outside the ecosystem like from terrestrial plants surrounding the lake (Figure 2).

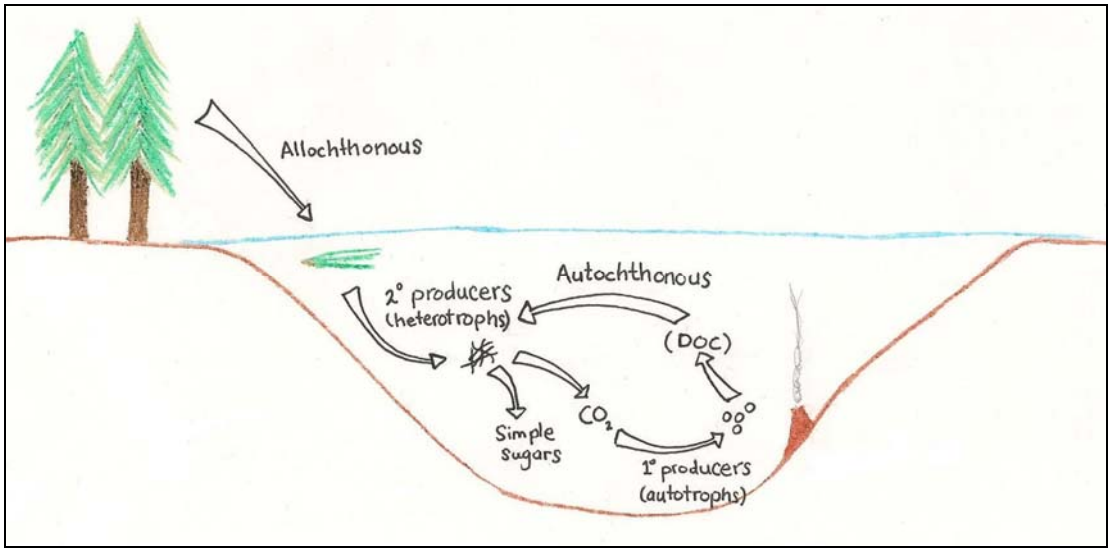


Fig. 2. The carbon cycle in a lake showing two carbon sources: autochthonous and allochthonous

Autochthonous carbon comes from microbial primary production. Even though there are several hydrothermal vents in the lake that are strong sources of $CO_2$ (Janik & McLaren 2010), the high acidity prevents $H_2CO_3$ from converting to $HCO_3^-$, and the high temperature reduces $CO_2$ solubility, limiting the availability of inorganic carbon (IC) in the lake. In fact, it was found that the concentration of total inorganic carbon in an extremely acidic lake to be undetectable, but it was higher in the layer close to sediment (Nixdorf & Kapfer 1998). Suspended sediments limit photosynthesis further by preventing light penetration (Michaelides & Kendrick 1978). The only eukaryotic life forms found in the lake are microscopic, animals and plants cannot live in the harsh conditions of BSL, limiting the availability of organic carbon for BSL microbes creating an oligotrophic, carbon-poor, environment which is characteristic of acidic lakes (Grahn et al. 1974).

On the other hand, allochthonous carbon comes from chemicals that are released from decaying leaves that fall into the lake. BSL is surrounded by three dominant plant species; Ponderosa pine, *Pinus pondorosa*; incense cedar, *Calocedrus decurrens;* and pineleaf manzanita*, Arctostaphylos nevadensis.* These plants are situated such that their leaves can fall into the lake (Figure 3), or deposited onto the ground and can be washed into the lake by seasonal rains. Some sections of the lake shore have no trees, but in other areas trees are healthy despite the high temperature (Figure 3).

Fig. 3. View of BSL showing surrounding coniferous vegetation, usually 2-10 m from the lake's perimeter, but in some cases overhanging the water. Picture taken 8/03/2010

## Carbon Cycling in Acidic Aquatic Systems

In extremely acidic waters, such as the acidic mining lakes in Germany, where pH is 2.3-3, primary production was limited and heterotrophy exceeded primary

production, suggesting that primary production is not the main C source for the heterotrophic bacteria in the lakes (Nixdorf et al. 2003, Kamjunke et al. 2005). In such lakes heterotrophy is more prevalent than autotrophy, this is especially true in oligotrophic lakes (Forsström et al. 2013). Limited availability of autochthonous carbon for bacteria in acidic lakes suggests the importance of allochthonous carbon in which leaf litter can have a significant role (Rau 1978). Because light availability further limits primary production in BSL, allochthonous C is more important than autochthonous C as in similar environments (Webster & Benfield 1986). Leaf litter was shown to be the main carbon source for microbes in fresh water streams where the microbial population is predominantly heterotrophic (Chamier 1987).

## Leaf Decay Processes

Leaf decay goes through many stages: leaching, fungal and bacterial colonization and mineralization of organic matter (this stage is termed decomposition in this thesis), and shredding by micro invertebrates (Webster & Benfield 1986, Schlief 2004, Löhr et al. 2006). When leaves fall into a water body, most of soluble compounds including organic and inorganic material are leached in the first few days (Mathews & Kowalczewski 1969, Petersen & Cummins 1974, Webster & Benfield 1986). Rau (1978) studied hemlock and fir needles, and observed the leaching period to be 10 days, when needles lost from 20-30% of their initial dry weight. Water temperature, water turbulence, and the leaf species are factors controlling how much the leaves lose in the leaching period (Webster & Benfield 1986).

Microbial activity is important for leaf decay in aqueous environments; in fact, it is more important than physical factors such as water current and fragmentation. Rau (1978) studied fir needle decay in Finley lake in Washington state and compared his results with other studies done in streams in the Pacific northwest. Rau's results were similar to the other study which indicates that physical fragmentation by water current in streams does not have a significant effect on leaf decomposition. It is thought that both bacteria and fungi contribute to leaf degradation, but from work done by Chamier (1987) it was observed, specifically in low pH streams, that bacterial populations on oak leaves rise after the leaf is partially degraded by fungi. Furthermore, in other studies it was stated that aquatic fungi are the major leaf decomposers in the early stages of leaf litter breakdown (Webster & Benfield 1986, Niyogi et al. 2001), especially aquatic hyphomycetes. In another study, the bacterium *Pectobacteirum carotovorum* did not contribute to the decay of beech leaf litter as much as the fungus *Aspergillus nidulans* (Schneider et al. 2010). However, microbial activity can be affected by many factors, which include acidity (Niyogi et al. 2001) and presence of waxy cuticles on leaves that act as barriers and delay fungal colonization of decaying leaves (Kelly et al. 1984, Canhoto & Graça 1999). Microbial activity is affected by pH, in a study done by Mulholland et al. (1987), it was observed that thymidine uptake and microbial respiration was highest in streams with near-neutral pH (6.4) and lowest in streams with more acidic pH (4.5). In another study done on leaf maceration of *Nymphaea alba* in acidic waters (Kok et al. 1992), it was noted that all fungal species being studies (*Colletotrichum nymphaeae, Epicoccum nigrum, Botrytis cinerea, Trichoderam viride, Ulocladium oudemansii, Arthrinium phaeospermum, Pythium undulatum, Fusarium avenaceum*, and

*Coprium* sp.) grew more slowly at pH 4 than at higher pH, and a reduction in the ability of the isolated fungi to degrade some substrates was observed. Fungal diversity in the population can be affected by low pH and dissolved zinc, as observed by Niyogi et al. (2002), and fungal conidia spores were not observed to be produced in a stream of pH <3. However, another study (Rousk et al. 2010) found that pH changes over 4-8.3 affect bacterial communities more severely than fungi, and that fungi have a wider range of optimal pH for growth.

Shredding macroinvertebrates play a role in leaf breakdown, but many invertebrate species are sensitive to water acidity (Dangles & Guerold 1998). It was observed that streams affected by acid mine drainage have few or no shredders (Niyogi et al. 2001), which was associated with low breakdown rate of leaves. Microbes are thought to be more important than shredding macroinvertebrates in such environments. Macro-invertebrates are absent from BSL, which is both too acidic and too hot.

## Techniques Used to Measure Leaf Decomposition

All leaf decay experiments follow a standard method of incubating the leaves in the environment in question (aquatic or terrestrial), and measuring the loss of organic matter (as measured by dry weight) over time, which is then modeled by a first-order (exponential) decay equation. However, there are numerous variations in experimental details. Some researchers pre-treat the leaves before the experiment by leaching (Mulholland et al. 1987) or drying (Hodkinson 1975, Rau 1978, Carpenter et al. 1983, Niyogi et al. 2001). However, such pretreatments alter the leaf chemistry and can affect the decay rate (Boulton & Boon 1991, France et al. 1997). Several methods are also used

to immerse leaves in water. The leaf pack technique exposes the leaves or needles to the water and insects without restriction. Problems associated with this technique, such as fragmentation from the water current, cause leaf weight loss not attributed to leaching or decomposition (Witkamp & Olson 1963, Rau 1978). Rau noted that no fragmentation occurred in needles that were put into the lake inside mesh bags of fine mesh size (0.073 mm) or a bigger mesh size (0.8 mm). Another problem is weight gain caused by attachment of protozoa, hydras, etc. (Rau 1978). Another method used to immerse leaves is the litter bag technique, used by most investigators (Francisco et al. 1973, Rau 1978, Carpenter et al. 1983, Chamier 1987, Mulholland et al. 1987, Canhoto & Graça 1999, Niyogi et al. 2001, Siefert & Mutz 2001, Schlief 2004). Usually mesh bags made of nylon are used in such experiments. Mesh sizes can regulate shredder accessibility to the leaves. Hodkinson (1975) and Rau (1978) found more macroinvertebrates present in coarse mesh bags, but this did not affect leaf decay rates (Hodkinson 1975), and Rau did not observe difference in needles weight loss between the two different mesh sizes.

## The Effect of Leaf Characteristics on Leaf Decomposition

The chemical and physical characteristics of leaves are important factors that control leaf decomposition rates (Chamier 1987). Lignified leaves and leaves with tannins or resins, such as pine needles, resist microbial degradation. Another important factor is leaf initial nitrogen content; the more initial nitrogen concentration in the leaf (such as alder leaves), the faster the leaf decays (Webster & Benfield 1986). Waxy cuticles (Kelly et al. 1984) and presence of leaf oils, tannic acids, and polyphenols inhibits fungal growth; however, fungal sensitivity to polyphenols depends on the species

(Webster & Benfield 1986, Canhoto & Graça 1999). Polyphenols complex with fungal enzymes rendering them useless and slow down breakdown, or they interact with plant proteins and form decay resisting complexes (Webster & Benfield 1986). Phenolic substances were found greatest in fresh leaves, and lower in dry fallen leaves (Albariño & Balseiro 2002), but water chemistry may affect phenols, as one study of leaf breakdown in acidic waters did not detect phenolic compounds (Siefert & Mutz 2001).

## The Effect of pH on Leaf Decomposition Rate

It was evident from many studies that the rate of leaf decomposition is lower in acidic lakes (McKinley & Vestal 1982, Hoeniger 1985) or streams (Siefert & Mutz 2001, Dangles & Chauvet 2003) than in neutral pH waters. Siefert and Mutz (2001) compared leaf decay of *Betula pendula* in an acidic stream (pH 2.8, temperature 0-24 $^{o}$C) with its decay in a neutral pH stream. In the acidic stream, the leaf litter bags used to submerge leaves into the stream did not include macroinvertebrates that belong to the feeding group (shredders), so the leaves were not fragmented. They stated that leaves in the acidic water had a very low decay rate ($k=0.0014$ to $37\ d^{-1}$). The cause of this decrease in decay rate was explained by three reasons: (i) low temperature and acidity; (ii) absence of shredders; (iii) precipitation of ferric iron oxides. McKinley and Vestal (1982) reported that leaves of *Carex aquatilis* degraded at a slower rate in pH 3 than in other pH values tested (4-6) and that there was no associated trend in microbial biomass, as measured by ATP concentration. Chamier (1987), using water from streams of varying pH (4.9-6.8), concluded that at low pH, water chemistry can affect microbial enzyme systems.

Most of the studies examining leaf decay in acidic waters have studied sites affected by anthropogenic acid mine drainage (Carpenter et al. 1983, Niyogi et al. 2001). These frequently have high concentrations of dissolved metals, (Carpenter et al. 1983, Mulholland et al. 1987, Niyogi et al. 2001), which can significantly lower decay rates. Metals are almost absent from BSL (Siering et al. 2013), which is also a naturally-occurring environment where microbes have likely adapted over longer time periods than acid mine drainage sites.

## Study Objectives

BSL is a highly oligotrophic system with <1 ppm DOC because of the rapid loss of dissolved IC (DIC) as $CO_2$ due to rapid degassing, and limited light penetration from suspended sediments (Siering et al. 2013). These conditions suggest that as an 'island' in a coniferous montane forest, leaf litter observed in the lake might provide significant C for microbial growth. As mentioned above, coniferous leaves are known to be difficult to be colonized by microbes, but BSL's combination of high temperature and extremely low pH might help microbes to access this C source. In this study I assessed the possibility that the microbial community in BSL can utilize leaf material, especially pine needles, as a carbon source in the harsh conditions in BSL. My objectives were to examine allochthonous inputs (leaf litter) to the lake as a C source for BSL microbes, including:

1. Determining leaching and decomposition rates for different plant types;
2. Determining the relative contributions of BSL microbes to breakdown of leaf litter;

3. Estimating total infall of terrestrial vegetation to the lake;

4. Examine the ability of the BSL microbial community to utilize inorganic and organic C sources;

5. Construct a preliminary C budget for BSL.

I hypothesized that leaf material would be rapidly degraded in BSL's warm, acidic conditions, fueling microbial metabolism dominated by heterotrophic activity. I further hypothesized that both prokaryote and eukaryotes would contribute to leaf breakdown in BSL's waters.

# CHAPTER II

# MATERIALS AND METHODS

## Estimation of Leaching and Decomposition of Leaf Material

Incubations were done with 2 g leaves or leaf litter in 50 mL BSL water in tubes. Depending on the experiment, BSL water was either unfiltered, or 0.2 µm membrane-filtered. I incubated the tubes at a constant temperature of 48-55 $^{o}$C for different times; at each time point 2 replicates were dried to constant dry mass (DM). Varying temperatures (50-105 $^{o}$C) and periods (12 hours-5 days) have been used to dry leaf material in different studies (Francisco et al. 1973, Rau 1976, Rau 1978, McKinley & Vestal 1982, Carpenter et al. 1983, Chamier 1987, Mulholland et al. 1987, France et al. 1997, Schultheis et al. 1997, López et al. 2001, Siefert & Mutz 2001, Niyogi et al. 2002, Dangles & Chauvet 2003, Dangles et al. 2004). From previous work (Erika Keshishian, personal communication), drying leaves at 70 $^{o}$C for 24 hr was used to obtain a constant weight.

At the beginning of the experiment, 2 g of each leaf litter was oven dried to account for initial DM. The DM was subtracted from the initial DM of the leaves to calculate the mass loss and remaining DM percentages. The decay rate (k) was calculated as a first-order negative exponential function, used in most leaf decay studies (Hodkinson

1975, Carpenter et al. 1983, Webster & Benfield 1986, Chamier 1987, Niyogi et al. 2001, Siefert & Mutz 2001, Niyogi et al. 2002):

$$M_t = M_o e^{-kt}$$

where $M_t$ is the remaining DM, $M_o$ is the initial DM, t is incubation time. The decay constant k was obtained from the slope of the line in linear regression of ln $M_t$ vs. time as was described by Carpenter et al. (1983).

Effect of Leaf Type and Age on Breakdown Rates

Fresh leaves from each species were collected from plants near BSL, including ponderosa pine (*Pinus ponderosa*) incense cedar (*Calocedrus decurrens)* and pineleaf manzanita *(Arctostaphylos nevadensis)* in July 2010. Dried fallen incense cedar needles were also collected to examine the effect of leaf age on decay rate. Leaves were transported to the lab and processed the same day. I incubated the leaf material in filtered BSL water, to see how the chemistry of BSL affects leaching and decay of leaf litter, at temperature 55 $^{o}$C for 0, 1, 2, 3, 11, 28, 51, and 77 days.

Effect of Leaf Quality on Decay and Contribution of BSL Microbes to Leaf Decay

In this experiment, pine needles were used because they are the most abundant leaf species observed to fall in and around the lake (Figure 4a). I left the needles intact or cut them with surface sterilized scissors into ~4 cm pieces. The needles were incubated in filtered or unfiltered BSL water at 48 $^{o}$C for 0, 7, 21, 42, and 70 days.

## Characterization of the Metabolism of the Microbial Community in BSL

BSL water was collected from site A (Figure 2) in January 2011, returned to the lab, and stored at 47 $^{o}$C overnight. For primary production, samples were incubated in 20 mL scintillation vials with $NaH^{14}CO_3$ (Moravek Inc.) under simulated full-light conditions, or in foil-wrapped vials to simulate dark conditions. Vials were filled to avoid outgassing of $CO_2$ under acidic conditions. Killed controls were pre-treated with 0.5% glutaraldehyde. Samples were incubated for 1-2.3 hrs and then filtered onto 0.2 µm-pore size polycarbonate filters (Poretics), rinsed, and counted in ScintiVerse cocktail (Thermo-Fisher). Heterotrophic potential was measured by incorporation of $^{14}$C[U]-acetate or glucose (Moravek, Inc.). 'Bacterial' production (likely all prokaryotes) was measured by $^{3}$H-thymidine incorporation (Gyure et al. 1987). Following incubation, samples were fixed with glutaraldehyde (0.5% v/v final) and ice-cold trichloroacetic acid (TCA; 5% v/v final). After 30 min. incubation, these were filtered as with other samples, and filters were rinsed with ice-cold TCA and counted as for $^{14}$C. All additions were used at 0.1-1 µCi per sample, and label did not exceed 8 ppm additions. Typical counting efficiencies were 62% ($^{3}$H) and 95% ($^{14}$C) as determined by the internal standards method. Turnover times of primary production was calculated as the amount of assimilated substance*concentration of DOC in BSL.

## Characterizing BSL Prokaryote

### Growth Response to Nutrients, $O_2$, and T

Erlenmeyer flasks (250 mL) were filled with 100 mL of BSL water. Yeast extract (YE, 0.02% final concentration) was used as a nitrogen and carbon source. A

control was prepared without YE addition; two replicates were prepared for both the control and the amended samples. The flasks were shaken at 100 rpm at 37 $^{o}$C. For anaerobic treatments, 15 mL Falcon tubes were filled completely with BSL water and 0.02% yeast extract was added to one tube, and tubes were incubated at 37 $^{o}$C without shaking. Subsamples were taken from each treatment after 0, 1, 2, 3, 4, 5, and 7 days. The samples were then stained with acridine orange for cell counts as described below. Another experiment was performed with BSL water amended with glucose to see if amendment with a simple carbon would give more growth. 40 mL of BSL water with or without the addition of 0.5% (final concentration) glucose was incubated at 50 $^{o}$C. Subsamples were taken from both treatments and stained with acridine orange for cell counts after 0, 1, 2, 4, and 5 days of incubation.

To measure growth response to temperature, 50 mL Falcon tubes were filled with BSL water with 0.02% (final concentration) YE, and were incubated at 21, 37, or 50 $^{o}$C. Two replicate were prepared for each treatment. Subsamples were taken from each treatment after 0, 1, 2, and 6 days of incubation and stained with acridine orange for cell counting.

Acridine Orange Cell Counts

Samples were fixed with 10% v/v formalin (37% formaldehyde) and incubated at room temperature for at least 1 hr. Then a dilution was prepared depending on the density of the culture: a subsample from the fixed samples was transferred to a new 2 mL tube and was neutralized with Tris buffer (pH 7). Acridine orange (0.1% in water) was added to 15% v/v. The sample was incubated with the dye for 15 min., and then the volume was adjusted to 2 mL by adding Tris-EDTA (TE) buffer to neutralize the

sample. The dyed sample was then filtered using 0.2 μm polycarbonate filter (Millepore) and viewed on a fluorescence microscope (Olympus) under blue excitation. Two replicates for each treatment were prepared, and at least 200 cells/slide were counted for most samples.

## Isolation of Fungus from Decomposition Experiments

I observed in the experiment comparing leaching and decomposition of different leaf species that fungi grew in the tube that had pine needles after 28 days of incubation (see Results). The water with the fungal mass was poured into a sterile tube and left at the same temperature (55 $^{o}$C). A sample of the fungal growth was plated onto a mycological agar (Difco) and incubated at 37 $^{o}$C. A cube of mycological agar was cut from a new mycological agar plate and placed on the surface of the new media. A loop was then used to transfer fungal spores from the mycological agar plate that has the fungal growth and was smeared onto the sides of the square of media. A clean slide cover was gently placed on top of the square and the plate was sealed with film and incubated at 37$^{o}$C (right side up) till fungal growth was observed. A drop of lactophenol blue was put onto a clean slide. Then the cover was removed from the plated and placed on top of the slide. The slide was then viewed under the microscope.

## *In Situ* Leaf Decomposition

Fresh leaves (pine needles, manzanita, and cedar) were collected Sept. 5, 2010 and 10 g fresh weight (FW) was placed in nylon mesh bags (0.5 mm mesh size; three replicates each). The bags were immersed in the lake and 3 bags of each species were

retrieved after 14, 28, and 45 days. Bags were returned to the lab for processing. Leaves were washed to remove sediment and dried at 70 $^{o}$C for 24 hr to obtain the dry weight. First-order decay rates were calculated as for lab samples (described above).

## Quantification of Leaf Litter Fall

Litter traps (0.6 x 0.6 m) were constructed from commercial window screen material (~1 mm$^2$ mesh; Figure 4a). The traps were distributed around the northern side of BSL at different distances from the lake in September 2011, at the locations indicated in Figure 4b, and were tethered to the ground using wire. Captured leaf material was collected every 2 weeks for 2 months. Leaves were taken to the lab and processed for DM as previously described.

## Estimating BSL's Hydrologic and C Budgets

I used precipitation data collected by LVNP from the Manzanita Lake Fire Station in the park (http://12.45.109.6/data.aspx). LVNP receives roughly 60-80 cm precipitation yr$^{-1}$, most as snow during October-April. Seasonal stream inflow was set to be similar to outflow, estimated from videos. Evaporation was calculated using the Ryan-Harleman equation (Rosenberry et al. 2007), which takes into consideration the elevated temperature of BSL. Soil DOC runoff and snow melt were estimated using a watershed basin 4X BSL's area (7.2 x 10$^4$ m$^2$), assuming all of 0.6 m precipitation runoff reaches the lake. Groundwater and geothermal upwelling were set so that net annual water flux was zero.

For water DOC ranges, I used estimates from the literature shown in table 2. Rainwater DOC levels are very low, especially in pristine environments such as LVNP,

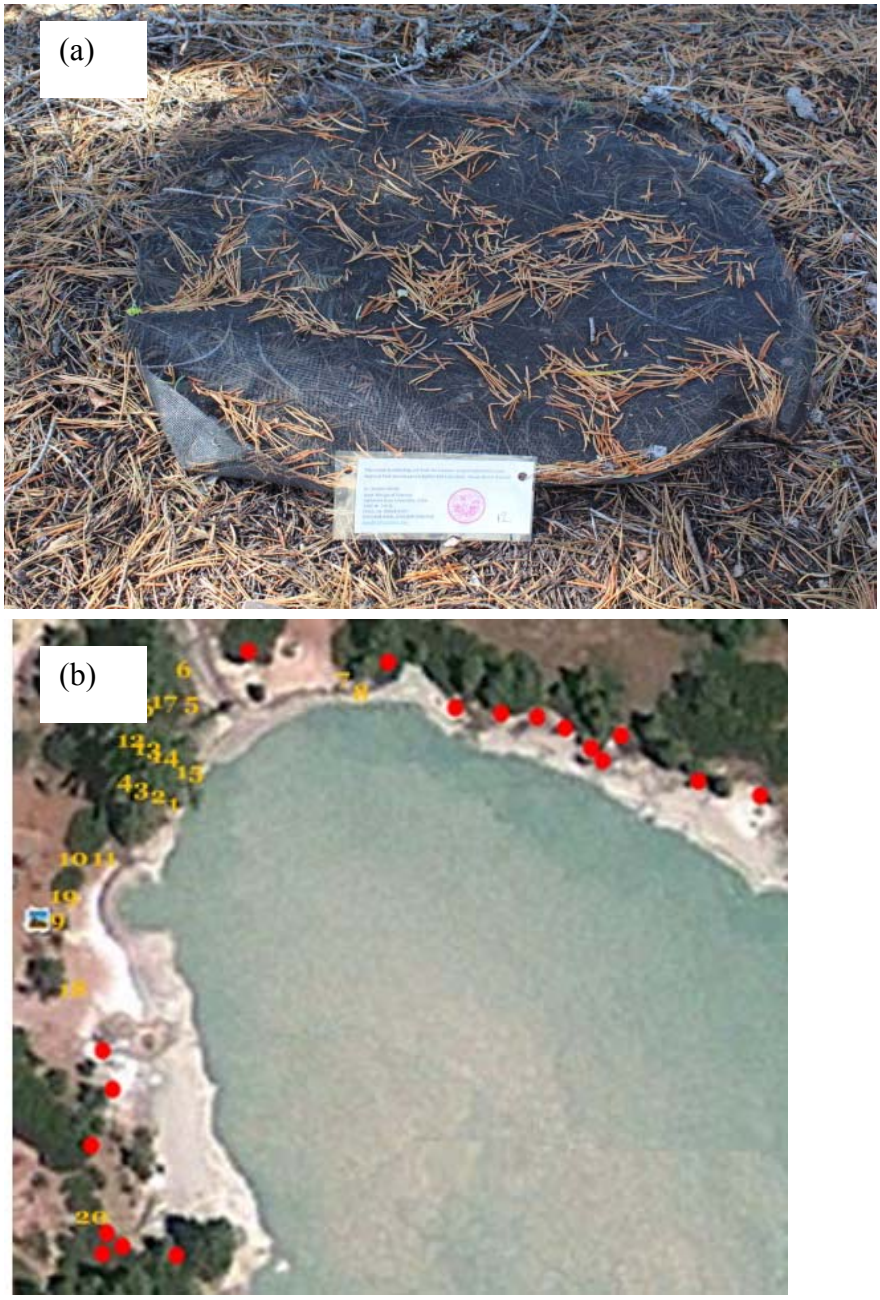


Fig. 4. (a) example of leaf litter trap. (b) Locations of the traps. Red dots indicate thermal features

roughly 20-160 µM. The concentration of DOC measured in a stream in Humboldt county, northern California was ~1.15 mg $L^{-1}$ (Triska et al. 1989). In another stream in the Kananaskis Valley of Alberta it was ~2.2 mg $L^{-1}$ (Wallis et al. 1981). The

concentration of DOC in groundwater (containing mainly humic and fulvic acids, tannins, and lignins) in the same valley was ~5.9 mg $L^{-1,}$ while soil runoff DOC was ~21.2 mg $L^{-1}$ (Wallis et al. 1981) (Table 2).

Table 2. DOC concentration ranges for different water sources

| **DOC source** | **DOC range (mg $L^{-1}$)** | **Reference** |
|---|---|---|
| rainwater | 0.02-0.16 | (Willey et al. 2000) |
| stream inflow | 1.15-2.2 | (Wallis et al. 1981, Triska et al. 1989) |
| soil runoff | 21.2 | (Wallis et al. 1981) |
| Groundwater or geothermal upwelling | 5.9 | (Wallis et al. 1981) |

# CHAPTER III

# RESULTS

## *In Vitro* Leaf Decay Studies

### Leaching and Decomposition of Different Plant Species

The first *in vitro* decay study was done using 0.2 μm-filtered BSL water to compare different plant species, and fresh green leaves were used for all but one treatment. All leaves exhibited a rapid loss in DM in the first week of incubation, followed by slow decrease in mass over 2-8 weeks (Figure 5). The first stage of rapid mass loss is due to leaching of soluble compounds (Webster & Benfield 1986), which caused the release of yellow-brown material (Figure 6), while the second period is due to microbial conditioning and decomposition. Pine leached notably less colored material than cedar or manazanita, and this was reflected by different leaching kinetics. Manzanita lost mass most rapidly between days 1 and 2, while cedar between days 2 and 3 (Figure 7), so leaching periods for these taxa were judged to be 3 days. In contrast, pine leached more slowly, and the leaching period was judged to be 11 days (Figure 7). These leaching rates were proportional to the DM:FM ratios calculated for the different taxa (manzanita: 55-60%; cedar: 45-55%; pine: 40-50%) in summer-fall 2010; these values were similar to others collected in 2009 (Erika Keshishian, personal communication). Decomposition rates, in contrast, showed opposite trends: highest for pine, and lower for manzanita and

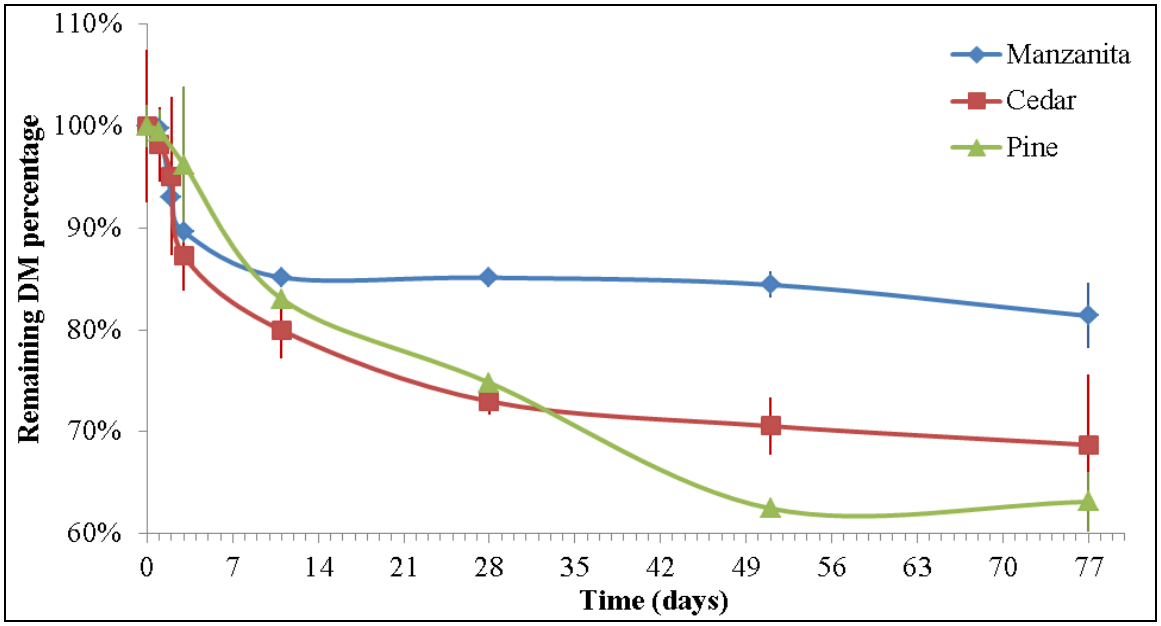


Fig. 5. Decomposition kinetics of different leaf species (green foliage) found near BSL. Data are averages of duplicates, and error bars represent +/- one standard deviation

cedar, so that by week 11, all taxa had lost 20-40% of DM. Both dry and fresh cedar leaves had similar leaching and decomposition kinetics (Figure 8). First-order decay rates for leaching and microbial decomposition are shown in Table 3.

Effect of Leaf Quality on Decay, and Contribution of BSL Microbes to Leaf Decay

In order to determine how leaf leaching and decomposition depend on leaf quality, and the contributions of microbes in lake water to the decay of leaf material, I compared decomposition of pine needles that were intact vs. cut, in both unfiltered and 0.2-µm filtered BSL water. Pine was chosen because most of the leaf litter entering the lake was observed to be pine needles (see below). Two-gram samples were incubated in 20 mL BSL water for 10 weeks at 48 $^{o}$C. Cut pine needles leached mass dramatically faster than needles left intact (Figure 9), while decomposition rates were higher for intact

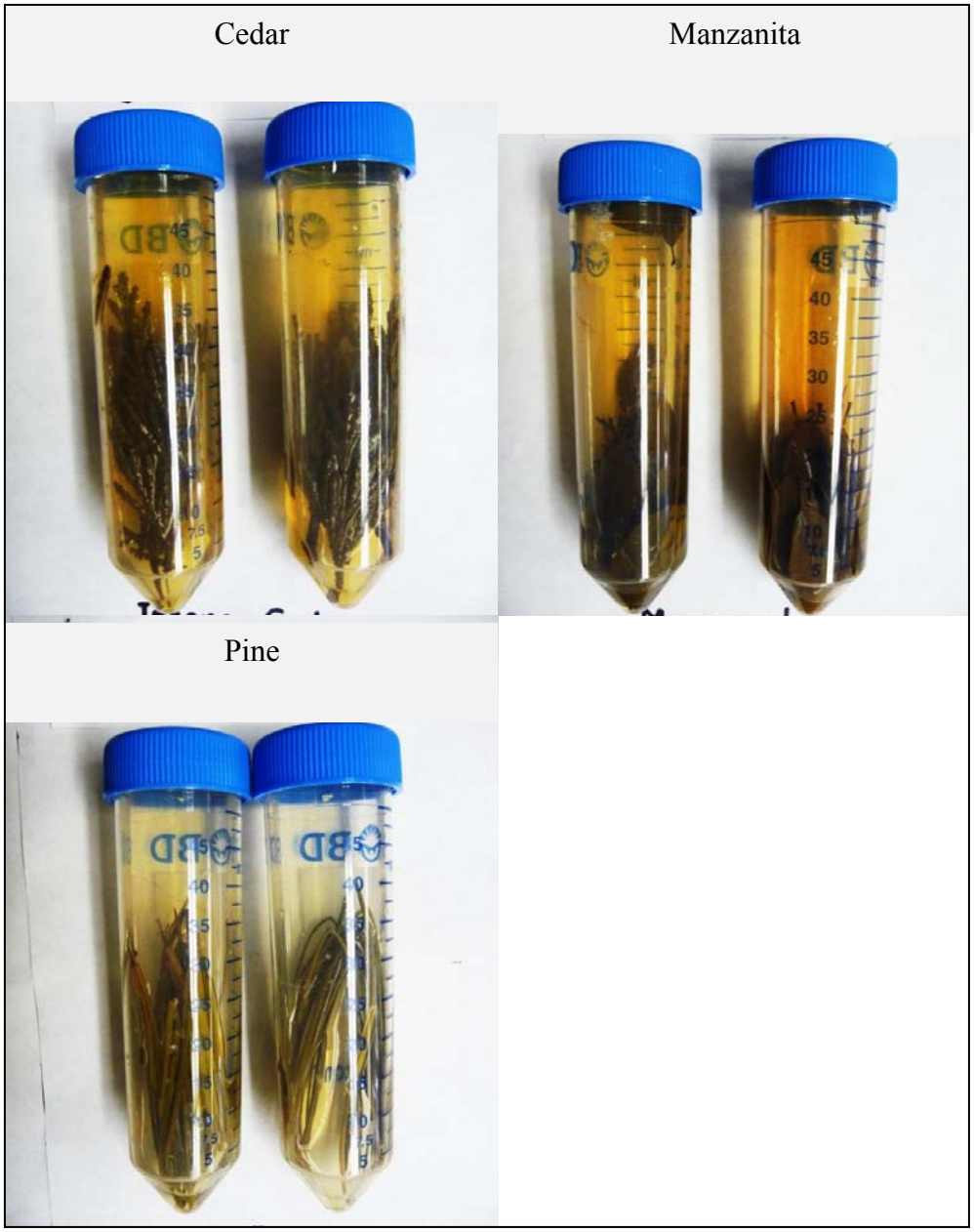


Fig. 6. The color of the material leached from the different leaf species after the leaves were incubated for 3 days in filtered BSL water at 55$^{o}$C

needles. For both cut and intact needles, total loss of mass was significantly larger for unfiltered BSL water compared to 0.2-µm filtered BSL water (Figure 9). These results

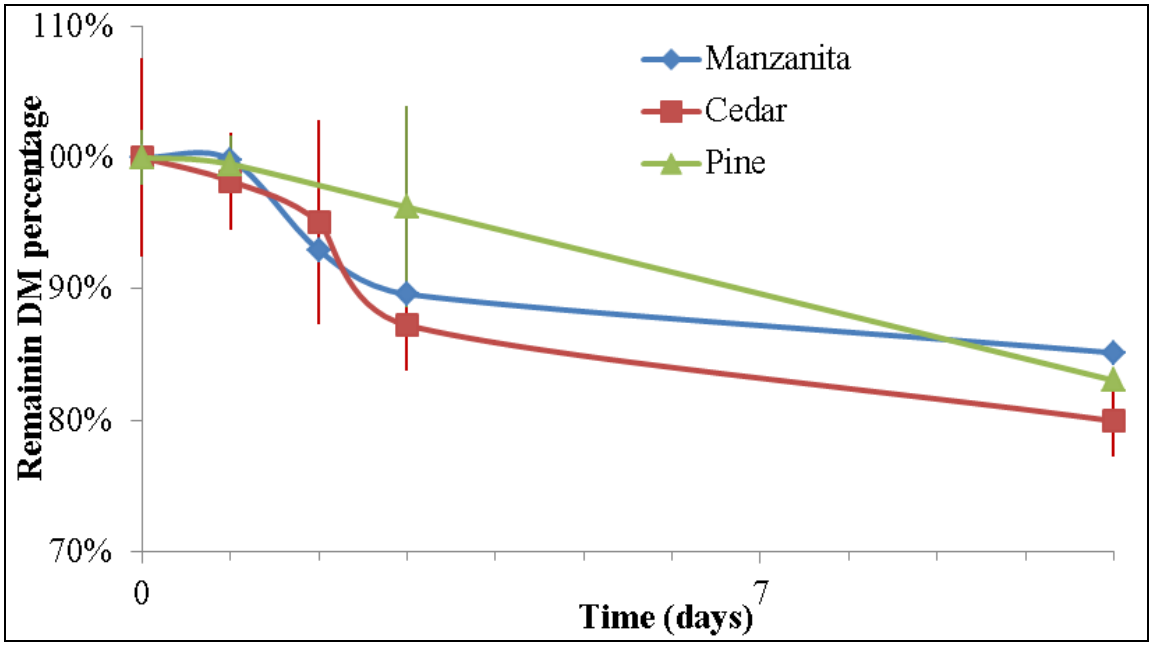


Fig. 7. Leaching kinetics for the different leaf species (pine, manzanita, and cedar). The second data point for pine was removed because it had an abnormal increase which might be caused by experimental error. Data are averages of duplicates, and error bars represent +/- one standard deviation

suggest that leaf breakage allows soluble solutes to be leached more quickly, and that microbes in BSL contribute to decomposition.

Isolation of Fungus from Decomposition Experiments

Fungal growth was observed during the leaching and decomposition experiment in one tube that had pine needles after 28 days of incubation (Figure 10a). After the fungal mass and the leachate were poured into a new sterile tube the fungal mass continued to grow (Figure 10b). This fungal mass produced a powdery substance (spores) 65 days after the fungal mass was observed (Figure 10c), which was viewed under the microscope. The fungus had an appearance similar to *Penicillium* sp. (Figure 11).

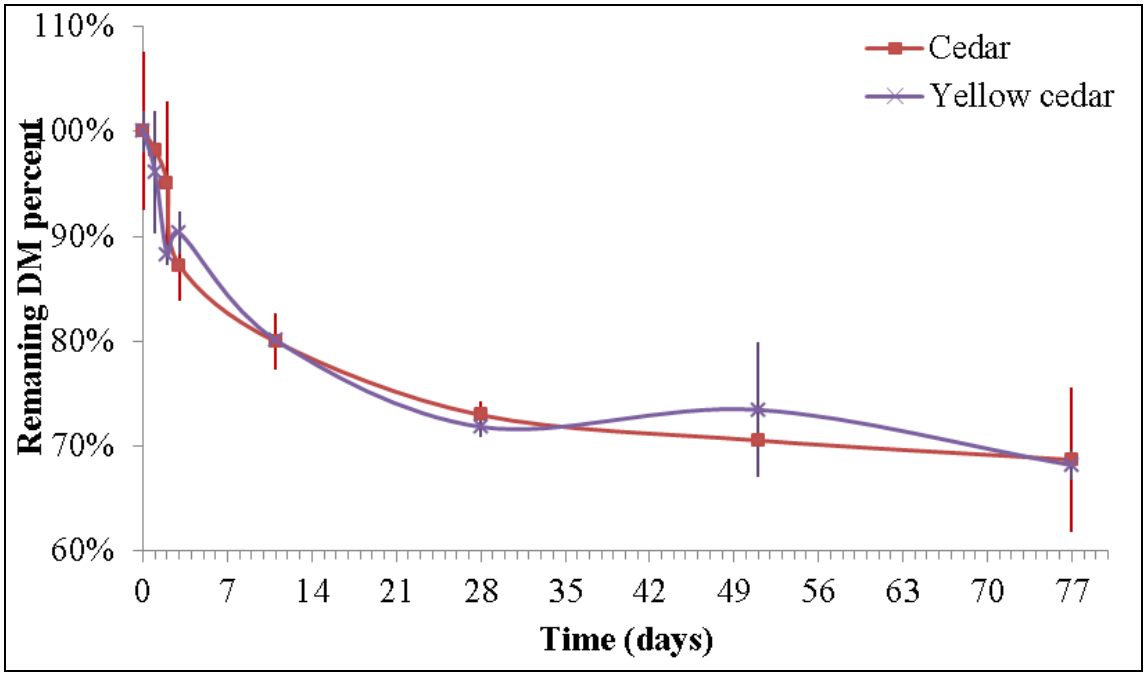


Fig. 8. Decomposition kinetics of dry (yellow) and fresh (green) cedar leaves. Data are averages of duplicates, and error bars represent +/- one standard deviation.

Table 3. Decay rates of the different leaf species found around BSL during leaching and microbial decomposition

| | | ---------- decay rate k ($d^{-1}$) ---------- | |
|---|---|---|---|
| **Plant type** | **Leaching period (days)** | **During Leaching** | **During microbial decomposition** |
| Manzanita | 3 | 0.0400 | 0.0011 |
| Ponderosa pine | 11 | 0.0174 | 0.0044 |
| Incense cedar | 3 | 0.0442 | 0.0025 |
| Incense cedar* | 3 | 0.0390 | 0.0031 |

*dried fallen material

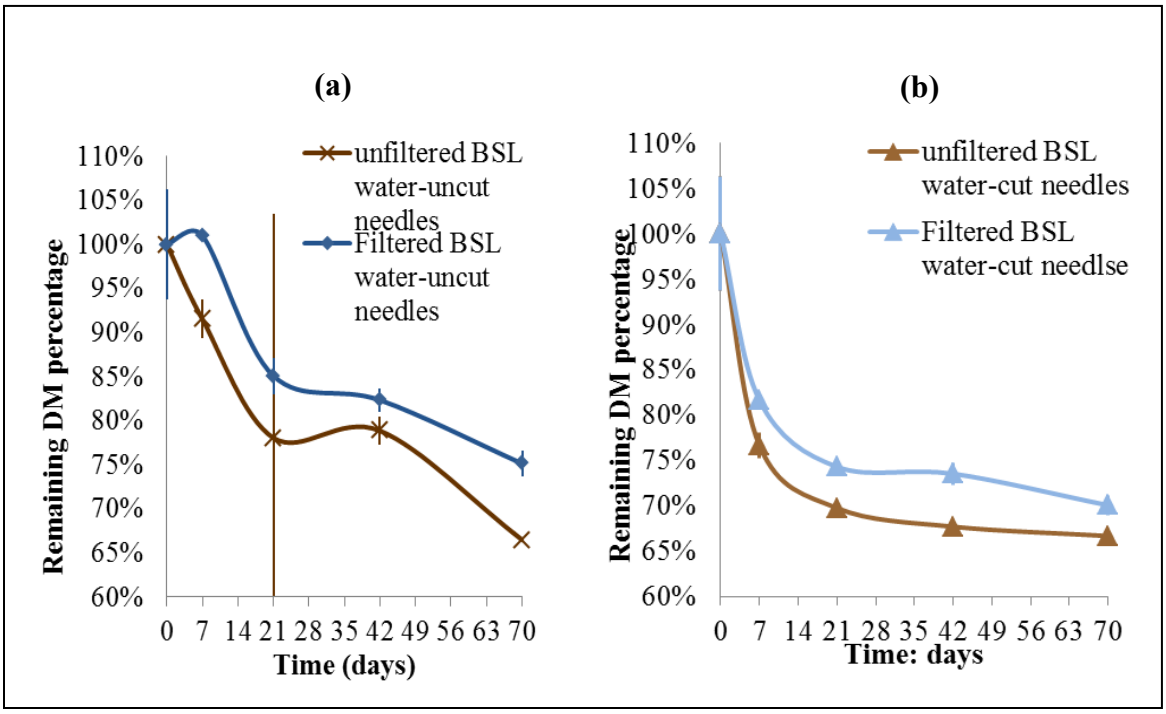


Fig. 9. Effects of leaf condition and source of microbes on pine needle mass loss. (a) uncut pine needles; (b) cut pine. Data are averages of duplicates, and error bars represent +/- one standard deviation. Large standard deviation on day 21 (a) was caused by experimental error.

## *In Situ* Leaf Decomposition

*In situ* leaf decomposition was examined in fall 2010, with 10 g fresh leaf material suspended at 1-3 m depth over 6 weeks, when BSL water temperatures were 48-50 $^{o}$C. Litter bags retrieved from the lake after 45 days were covered with algae (Cyanidiales) (Figure 12a). Algae were also observed on ropes left in the lake which shows us that when these algae have a surface to attach to, which keeps them near the water surface where light is available, they can grow. This observation demonstrates that primary production is limited by light in BSL. Although variation was considerably greater than in the lab and detailed leaching trends were not measured, the patterns of

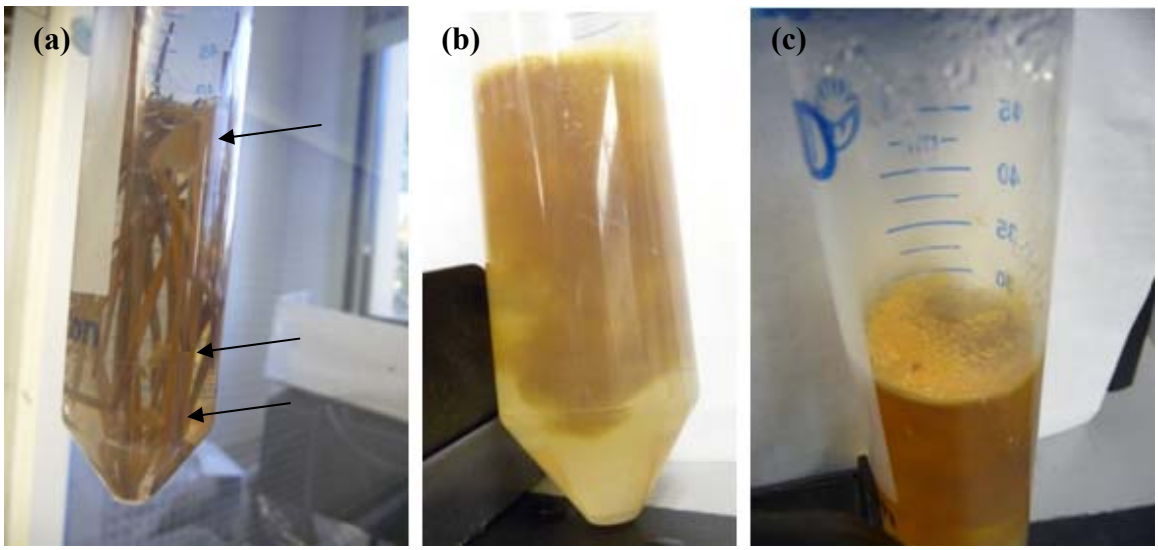


Fig. 10. Fungus observed in the tube that had pine needles decomposing in filtered BSL water. (a) Fungal mass was observed after 28 days incubation in filtered BSL water at temperature in the upper 40 $^{o}$C. (b) The leachate was poured into a sterile tube and incubated at the same temperature and the fungal mass continued to increase. (c) After 65 days of incubation spores were produced at the surface of the liquid

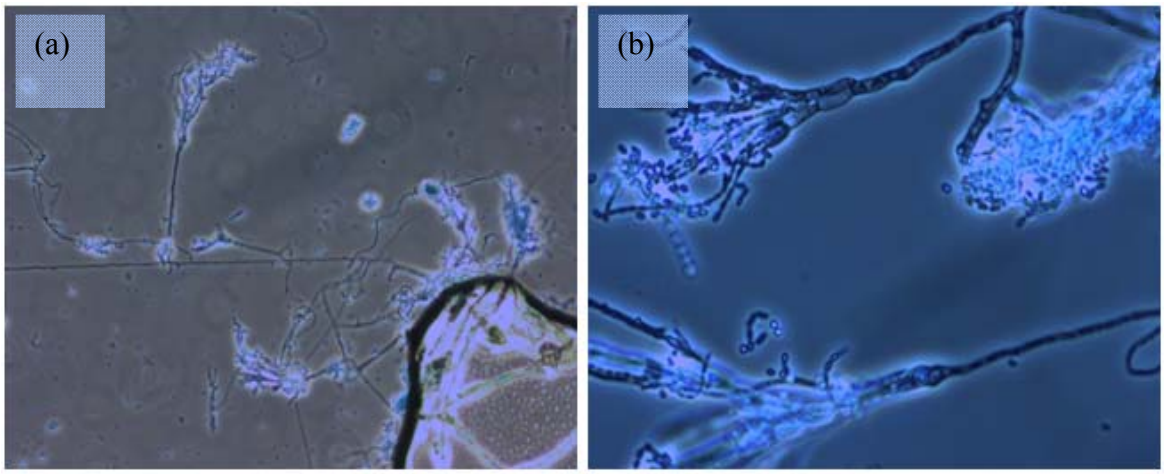


Fig. 11. Micrograph of the fungus isolated from decaying pine needles. A was captured at 100X magnification showing conidiophores. B was captured at 400X showing the conidiospores

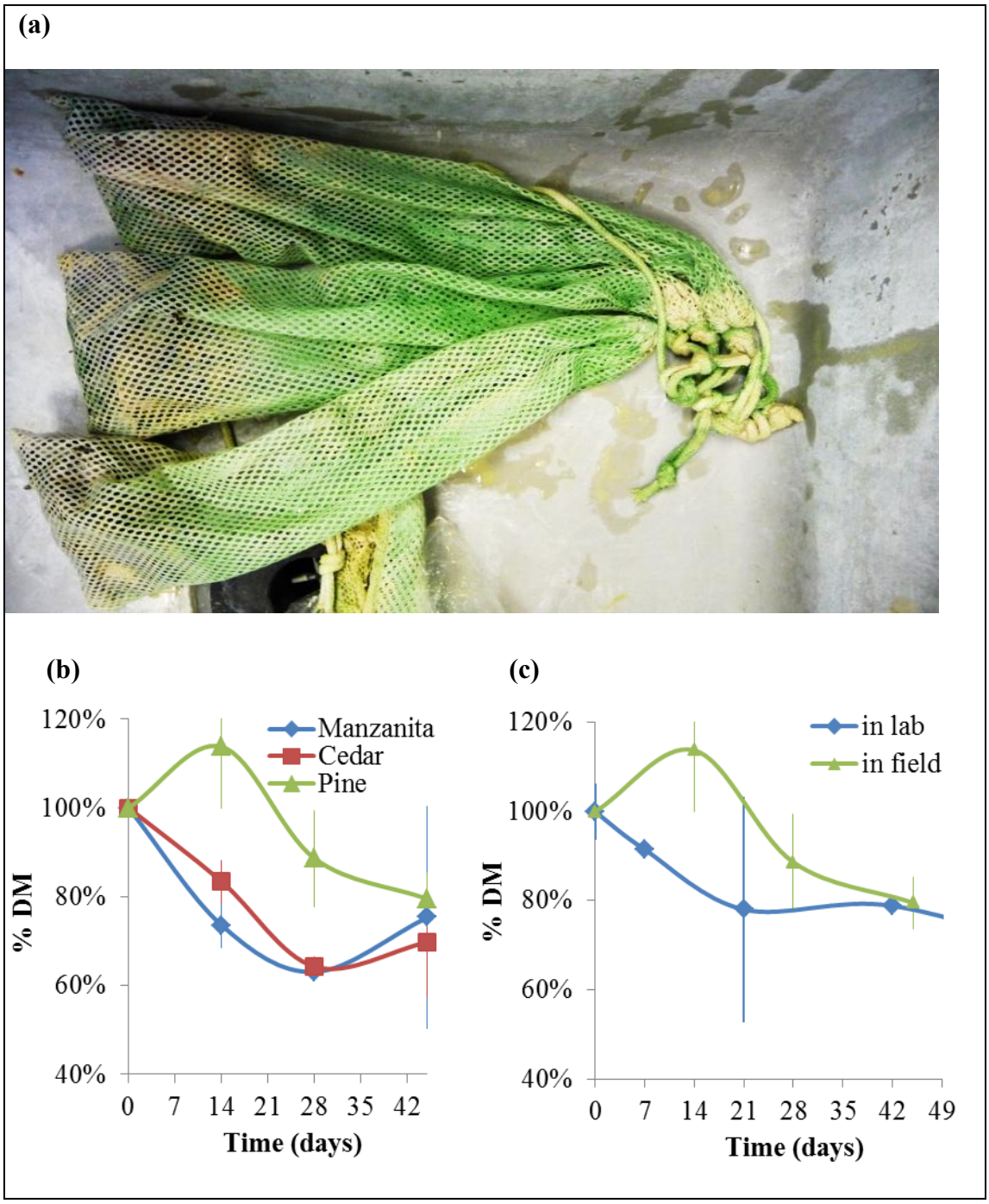


Fig. 12. *In situ* leaf decomposition experiment, fall 2010. (a) Mesh bags retrieved after 45 days were covered with algae (Cyanidiales). (b) Loss of DM for three taxa. (c) Comparison of pine decomposition *in situ* vs. *in vitro*. Data are averages of duplicates, and error bars represent +/- one standard deviation

leaching and decomposition appeared to be similar to those found *in vitro*, with manzanita and cedar leaching more quickly than pine, and all taxa losing about 20% of initial DM over 6 weeks (Figures 12b, c). This suggests that *in vitro* rates, although possibly affected by artifacts such as putting leaf material in small volumes of lake water, were a reasonable prediction of *in situ* leaching and decomposition (Figure 12c).

## Quantification of Plant Material Inputs to BSL

Terrestrial leaf litter traps were deployed in late summer-fall 2011, the time of year when the majority of tree species typically shed old leaves. Traps collected 0-50 g DM, with dry pine needles comprising the majority of leaf litter species observed (Figure 4a). Leaf litter fall was highly episodic, probably relying on wind conditions, but the amounts of litter collected over time followed one of three trends (Figure 13). Some traps recorded the largest fall in mid-September, while others peaked in early or late October. When the amounts of material collected from each trap over time was averaged and graphed vs. distance from the lake, there was a clear drop-off of litter fall in traps closer than 8 m to the lake (Figure 14), reflecting the scarcity of trees immediately next to the lake shore. Although I did not place any litter trips on the lake itself, this suggests that most leaf material reaches the lake by first being deposited on the ground, and then being washed into the lake with episodic winter rains and snow.

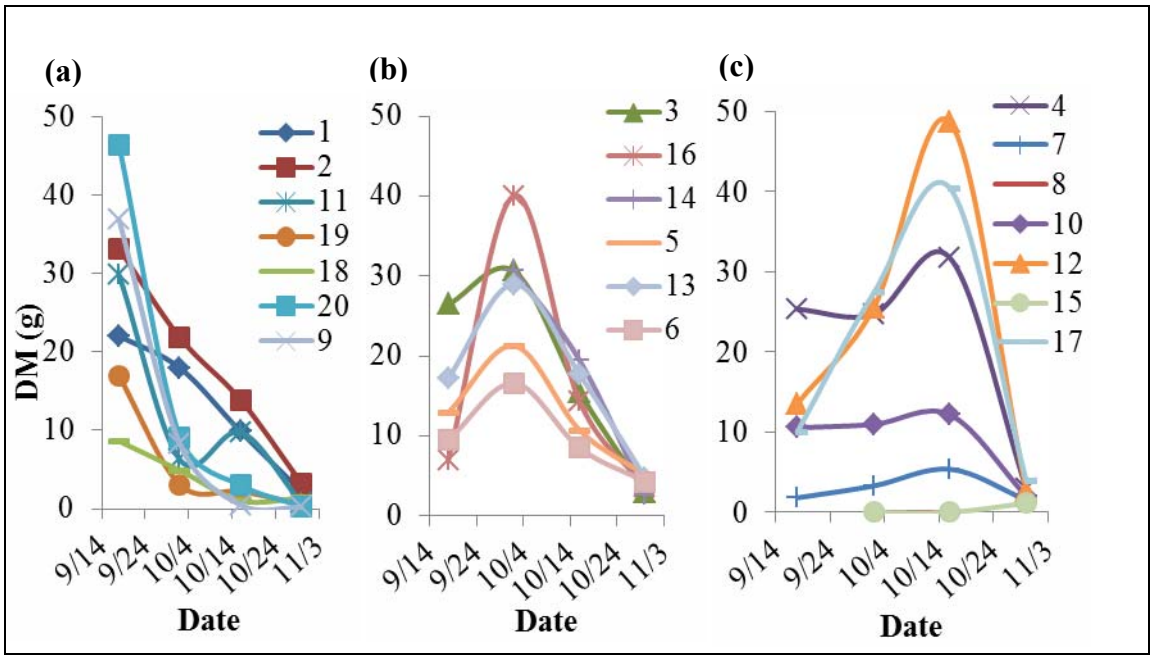


Fig. 13. Pine needles (DM) collected in the traps over time follow different patterns. The numbers (1-20) represent the trap IDs, whose locations are indicated in Figure 3

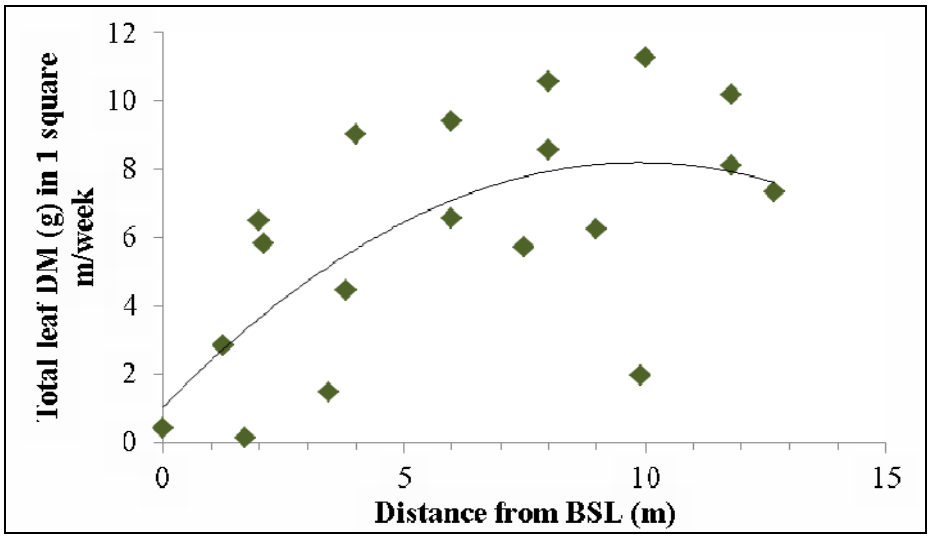


Fig. 14. The collected leaf litter amount increase as the distance from BSL increases. The line is a polynomial trend

# Characterization of the Metabolism of the Prokaryote Community in BSL

## Uptake of Inorganic Vs. Organic C

In January 2011, Dr. Wolfe was able to retrieve water samples from BSL during a period of good weather. Although temperature data were not available, data logger temperatures from other years showed that winter lake water temperatures were typically 46-48 $^{o}$C in January (Siering et al. 2013). The water was stored overnight at 47 $^{o}$C, and the next day I conducted C uptake experiments to estimate autotrophic production from $^{14}$C-bicarbonate (which becomes $^{14}CO_2$ in the acid BSL water) or bacterial production (BP) using the $^{3}$H-thymidine method (Gyure et al. 1987). I also investigated uptake of several simple heterotrophic C sources, U-$^{14}$C-acetate or glucose. All incubations were conducted both in the light and dark, as well as with killed controls, over 140 min (2.3 hr), at 47 $^{o}$C.

Primary production, as measured by $^{14}CO_2$ uptake, was very low, barely above background (Figure 15a), with <0.1% of label taken up in 2.3 hr. $^{14}CO_2$ uptake in light was notably less than in the dark, suggesting chemoautotrophy was more active than photoautotrophy. In contrast, heterotrophic uptake of all substrates showed no difference between light and dark treatments. However, uptake rates differed dramatically among substrates. Uptake of $^{14}$C-acetate was strong, with 12% of the label incorporated in 2.3 hr (Figure 15b), noting the difference in the y-axis scale between the figures, while uptake of glucose was much lower, with only ~0.16% of label assimilated. Uptake of $^{3}$H-thymidine, supposedly specific for bacterial secondary production, was also low, with 0.5% taken up in 2.3 hr.

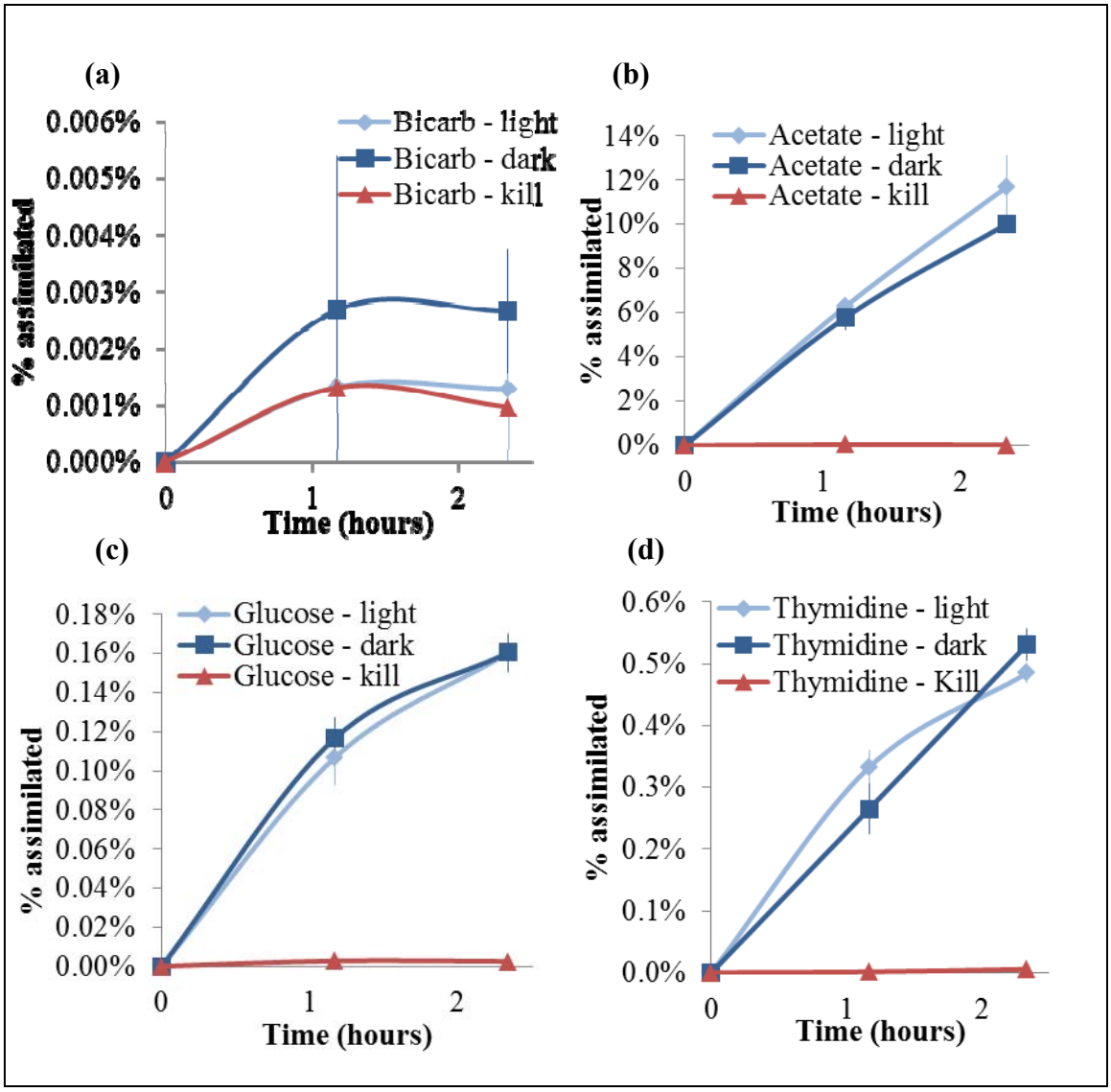


Fig. 15. The majority of the microbial community in BSL is heterotrophic. Comparison of incorporation of inorganic (a) or organic (b, c, and d) carbon into microbial cells in BSL water. Data presented are averages of duplicates, and error bars represent +/- one standard deviation

## Characterizing Growth Conditions for the Prokaryote Community in BSL

In order to estimate *in situ* growth rates of BSL prokaryotes, as well as limiting nutrients, I conducted growth tests with the acridine orange method modified

from that used by Francisco et al. (1973). I compared treatments with no C amendment and 0.02% YE, in both aerobic (shaking) and anaerobic conditions at 37 $^{o}$C. Cell counts clearly increased in samples incubated with YE over 2-4 days by nearly 2 orders of magnitude (Figure 16), while there was no significant effect of aeration. In contrast, adding 0.5% glucose to BSL water incubated without shaking at 50 $^{o}$C did not significantly increase microbial growth above controls as the samples that were amended with YE (not shown). To check if the lack of glucose response was due to the higher temperature, the YE experiment was performed again at 21, 37, and 50 $^{o}$C. This showed clearly that growth on YE is faster at 50 $^{o}$C than at 37 $^{o}$C, and little growth was observed at room temperature (Figure 17). This suggests that the BSL microbial community is adapted to growth at *in situ* temperatures of 48-52 $^{o}$C, and responds to N sources like YE rather than glucose.

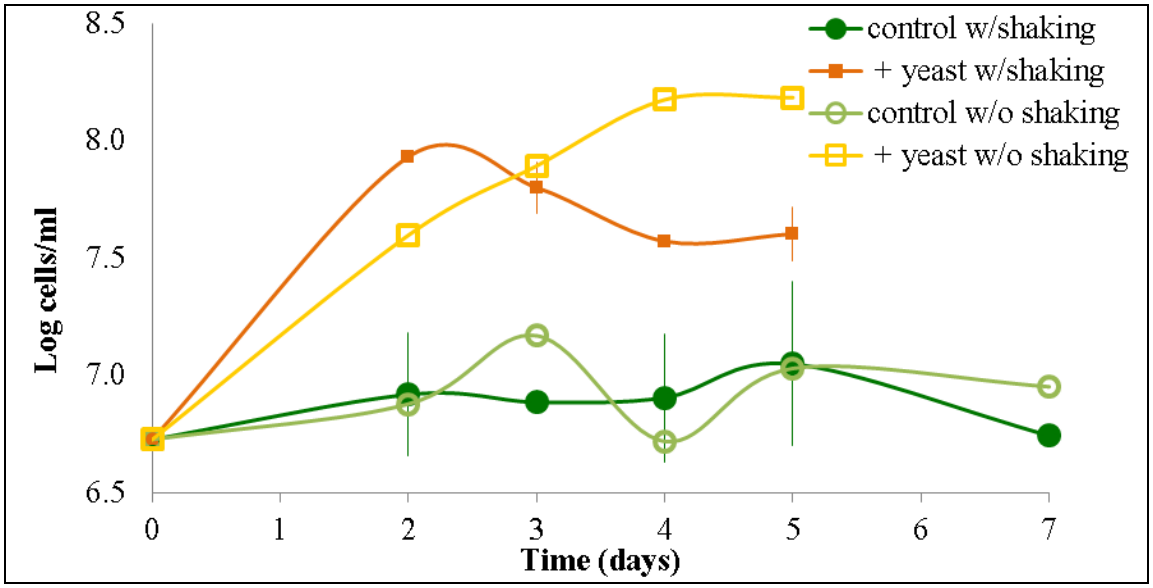


Fig. 16. A nitrogen source is more important for BSL microbes' growth than oxygen. BSL water was either amended with yeast extract (final concentration 0.02%) or left without amendment and incubated with or without shaking. Data are averages of duplicates and error bars represent +/- one standard deviation, except for samples that were incubated without shaking, which were performed without replicates

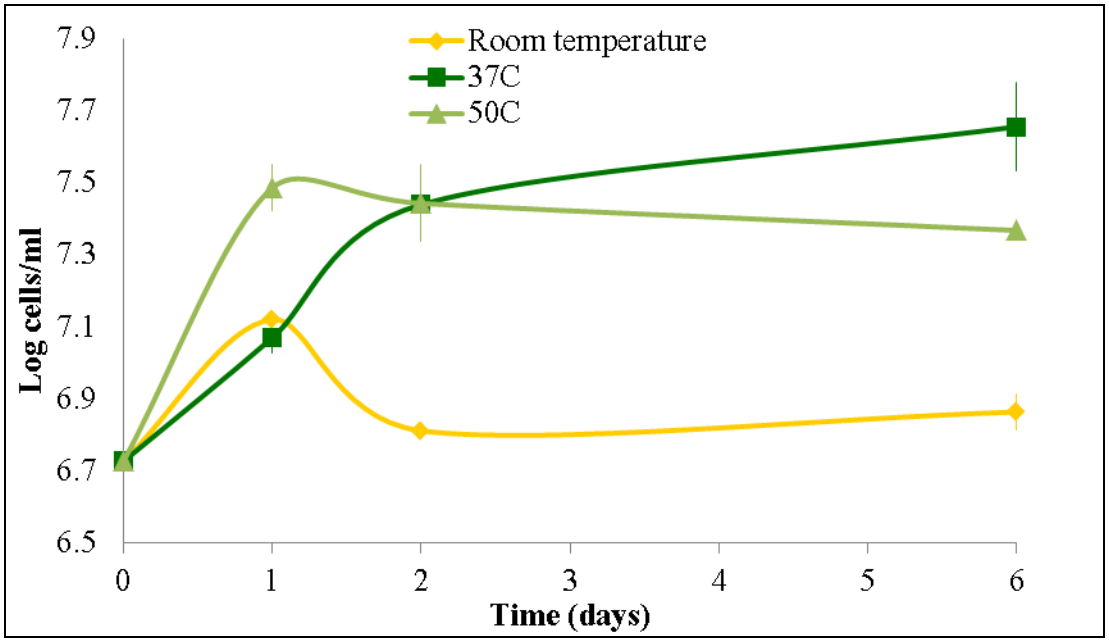


Fig. 17. The majority of the microbial community in BSL grows optimally at temperature above room temperature (37°C and 50°C). 50 mL of BSL water was amended with 0.02% yeast extract and incubated without aeration. Data are averages of duplicates and error bars represent +/- one standard deviation

# CHAPTER IV

# DISCUSSION

## Major Findings

For my thesis, I set out to determine if leaf material observed in BSL can be utilized by the microbes in BSL. Here I present my major findings and compare my results with those from other studies.

### Leaching and Decomposition Rates Are Fast Compared to Freshwater Coniferous Systems

Leaf degradation occurs in two major phases: initial chemical leaching of soluble solutes, followed by microbial colonization and decomposition (Sigee 2004). Leaching is a strong function of leaf type; for example, Petersen and Cummins (1974) reported first-order leaching rates of 0.11-0.18 $d^{-1}$ for *Betula, Alnus*, and *Quercus*, broadleaf deciduous species, at 20-25 $^{o}$C, much higher than I observed for the coniferous species that surround BSL (Table 3). A few leaching studies were conducted using coniferous leaves and when compared to results from this study, pine and cedar had higher leaching decay rates (Figure 18). Leaching rates also increase with temperature (Petersen and Cummins 1974), and at 50 $^{o}$C leaching rates of deciduous leaves would be even higher. Although I have not found studies of leaching from conifer needles, their thick waxy coating should make them leach more slowly, which fits my observation that cut pine needles leached much more quickly than uncut. However, by 11 days, all BSL

taxa had lost about 20% of DM (Figure 6). This is surprisingly high compared to other studies. For example, France et al. (1997) compared 'early mass loss' (14 d) for a number of leaf types in oligotrophic Canadian Shield lakes, where the water is neutral and the temperature is 14°C. This period includes leaching and initial microbial colonization. They observed that pine taxa lost 5-12% of DM, while cedar lost about 15% (Figure 18). The observation of 20% DM loss in BLS water suggests that higher temperature accelerated leaching rates, and allowed microbial decomposition to occur more quickly.

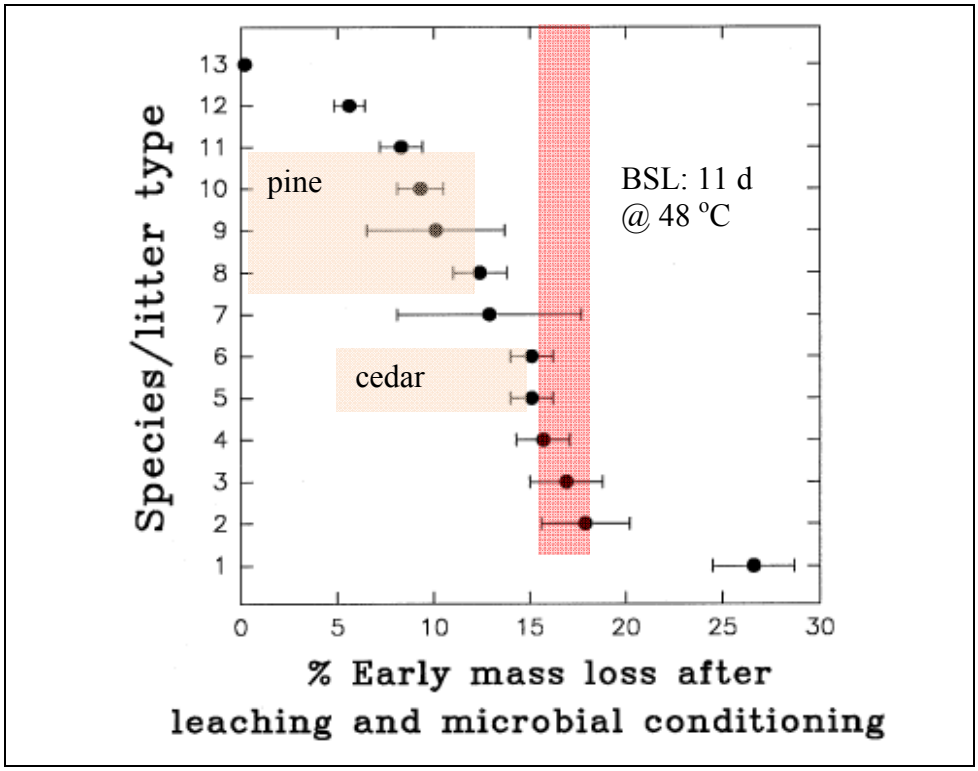


Fig. 18. % DM loss over 11-14 days for BSL compared to other systems.

Source: Modified from France R, Culbert H, Freeborough C, Peters R (1997) Leaching and early mass loss of boreal leaves and wood in oligotrophic water. Hydrobiologia 345:209-214

I found that decomposition rates over 2-11 weeks were lower than leaching rates, but inversely related: pine had the most rapid rate, followed by cedar and manzanita. This may reflect the more slow release of soluble material from pine, leaving more utilizable C in the needles for microbial consumption. This hypothesis is supported by my observations that cut pine needles leached more quickly than intact, and decayed significantly more slowly (Figure 9). Another explanation might be that all three leaves leached inhibitory substances such as polyphenolics, abundant in most coniferous taxa. For example, pine needles are known to have high content of polyphenols (Webster & Benfield 1986, Sigee 2004). These are usually thought to be inhibitory to fungi (Webster & Benfield 1986, Canhoto & Graça 1999, Sigee 2004), affecting enzyme activities, or interacting with the leaf's proteins to create decay-resistant compounds (Webster & Benfield 1986). Although I did not measure polyphenols or characterize the leachates chemically, I observed more yellow-brown coloration of manzanita and cedar leachate compared to pine, which might constitute high polyphenolics. However, it is possible that low pH negates inhibitory effects of polyphenolics. Although the inhibitory activity of polyphenols is active at pH 4-4.5 (Bärlocher & Oertli 1978), Siefert & Mutz (2001) found that phenolics were undetectable in waters of pH $\leq$3 compared to neutral. My observation that fungi grow on pine needle leachates at pH ~2.5 supports the idea that inhibitors were not important.

Coniferous leaves are difficult for microbes to degrade because of the waxy cuticle that covers the surface (Kelly et al. 1984), and usually have very low decay rates in neutral pH environments, especially without invertebrate shredders to attack the material and degrade the cuticle mechanically. BSL is too warm and acidic to support

metazoan shredders, making attack of waxy conifer needles by microbes even harder, and again, I observed faster decomposition rates on cut pine needles than on intact. Even in freshwater systems with shredders, conifers and ericaceous taxa typically are among the lowest decomposition rates among all plant taxa (Webster & Benfield 1986), and were characterized as 'slow' when compared to categories generated by Petersen and Cummins (1974) (Figure 19). My observed BSL decay rates for cedar (0.0021 $d^{-1}$) and pine (0.0056 $d^{-1}$) were also higher than those reported by Harmon et al. (1990) for *Thuja plicata, Pinus montocola*, or *Pseudotsuga menzeisii* (0.0008-0.0011 $d^{-1}$) in Olympic National Park, WA. These all suggest that BSL decomposition rates are relatively fast compared to freshwater systems, especially in the absence of invertebrate shredders.

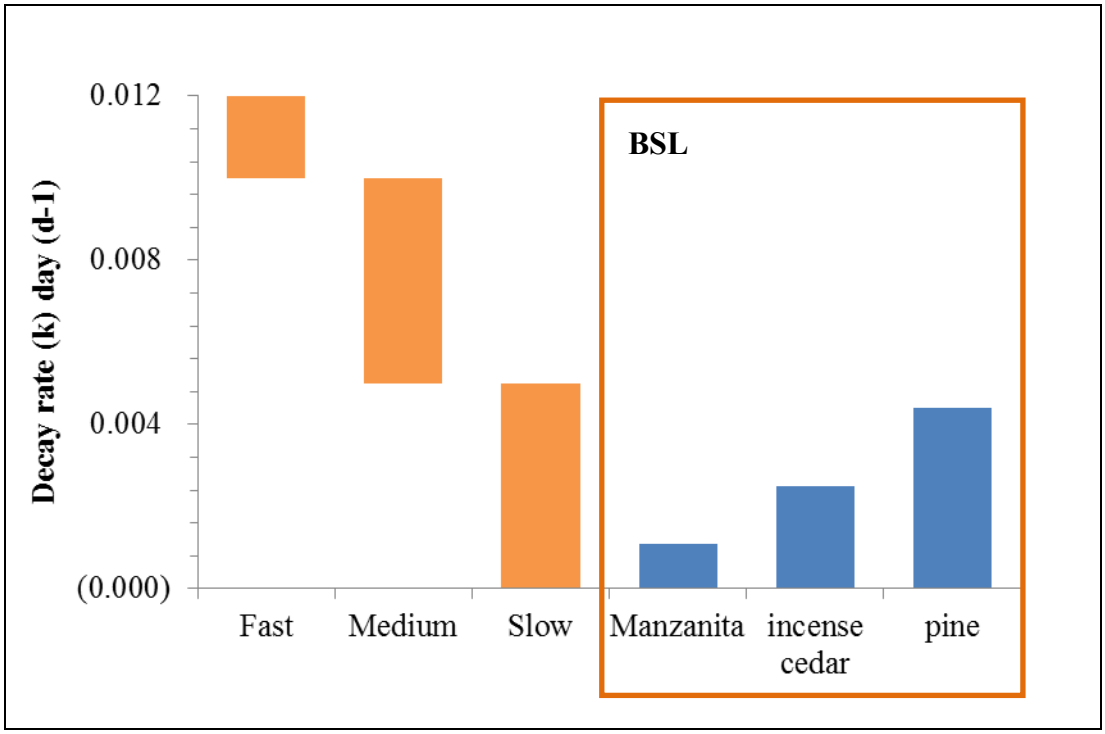


Fig. 19. Comparison of first-order decay rates for BSL manzanita, cedar and pine compared to categories established by Petersen & Cummins (1974) for freshwater systems

Although my lab-based decomposition studies used much more concentrated leaf material than is dispersed in the lake, several factors suggest my rates are reasonable predictions of breakdown *in situ*. First, my *in situ* experiment showed rates similar to those in the lab; with all leaf types losing about 20% DM over 6 weeks (Figure 12). A prior *in situ* experiment done in winter 2009-10 revealed loss of nearly 50% DM over 6 months (Gordon Wolfe, personal communication), suggesting breakdown continues over longer time periods. Furthermore, even though BSL is highly convective, leaf litter settles to sediments and I observed pine litter in the lake to be concentrated near shore sediments by wind-driven water movement (Figure 20), which tends to push material towards the northern end of the lake (Siering et al. 2013).

Fig. 20. Pine needles observed on 10/20/2010, concentrated near the shoreline by daily wind-driven water convection. Picture courtesy of Gordon Wolfe.

I found that fresh and dried cedar needles showed similar leaching and decay rates *in vitro* (Figure 8), suggesting that breakdown is not especially dependent on the age of leaves. This is surprising, since other studies have found difference in the decay of old and fresh leaves (Boulton & Boon 1991). O'Connell and Menage (1983) found that weight loss rate of eucalyptus leaves declines in old leaves compared to fresh ones. Boulton and Boon (1991) also criticized leaf litter bag studies that dried deciduous leaves before immersing, which artificially increased leaching rates. However, the tough waxy cuticles on coniferous and ericaceous leaves may reduce this difference. This is supported by my observations that broken (cut) pine needles leached much faster than uncut (Figure 9), suggesting that breakage of the wax barrier is more important than leaf age. This is important because we believe that most leaf material entering BSL is year-old dried leaves deposited on the ground and washed into the lake.

## Leaves Decay Rates in BSL Are Much Faster Than Those in AMD Sites

Waters affected by acid rain or acid mine drainage (AMD), stressed by acidity and dissolved heavy metals, typically show slower leaf decay compared to pristine unaffected sites (Coulson et al. 1960, Carpenter et al. 1983, Bermingham et al. 1996, Schultheis et al. 1997, Niyogi et al. 2001, Siefert & Mutz 2001, Niyogi et al. 2002). It is not clear what factors limit microbial breakdown of leaf material in such systems. Some studies suggest it is not low pH, but rather dissolved zinc and metal oxides (Niyogi et al. 2002), while other studies suggest decrease in shredders and microbial biomass in low pH streams is related to the decrease in leaf decay (Meegan et al. 1996). Studies with fresh waters acidified to simulate acid rain or AMD observed that breakdown rate decrease was

due to low pH and total Al concentrations (Dangles et al. 2004), absence of effective shredder macroinvertebrates (Dangles & Guerold 1998), or decreased microbial activity (Mulholland et al. 1987). Siefert and Mutz (2001) examined leaf litter processing in acidified coal-mine-waste lakes in Lusatia, Germany. These lakes have chemistry somewhat similar to BSL, although typically less acidic (pH 3), and are not thermal (4-20 $^{o}$C). They found that acid did not increase leaching of broadleaf deciduous birch (*Betula*) compared to neutral water, and observed very low *in situ* breakdown rates (0.0013-0.0037 $d^{-1}$) compared to non-acidified systems. They suggested this was due mostly to absence of shredder invertebrates and precipitation of metal oxides (ochre) on leaf surfaces that slow microbial attack.

I know of no other studies of leaf breakdown in geothermal sites, but when pine needles were treated with hot water, fungal colonization increased greatly (Kelly et al. 1984). This suggests the unusual combination of acid and heat in BSL likely may help degrade the wax layer that protects needles against microbial colonization, and makes the coniferous leaf litter more susceptible to microbial colonization and attack. It is also possible that some of the decomposition observed is actually continued chemical attack under low pH and high temperature conditions. As most of the plant material observed entering the lake is pine needles, Table 4 shows the high rates of decomposition in BSL compared to other neutral pH and mesophilic sites. I can conclude that allochthonous pine litter can be a potential C source for microbes in BSL.

Table 4. Comparison of BSL decay rates for ponderosa pine with studies of pine from other natural and acidified freshwater systems

| **Experiment** | **Decay rate ($d^{-1}$)** | **Conditions** |
|---|---|---|
| Unfiltered BSL water | 0.0044 | pH 2, 48 $^{o}$C |
| Webster & Benfield, 1986 | 0.0014 | high pH freshwater ecosystems |
| Albariño and Balseiro, 2002 | 0.0017 | pH 7.7 streams, 1-4.5 $^{o}$C |
| López, Pardo et al. 2001 | 0.0031 | pH 6.4 streams, 5-12 $^{o}$C |

Both Prokaryotes and Eukaryotes Contribute to Leaf Decay

Pine needles lose more DM in unfiltered BSL water than in filtered BSL water, whether or not the needles were cut (Figure 9). This suggests that microbes indigenous to BSL water contribute to leaf decay. At the same time, though, decay was almost as rapid in filtered water; suggesting that spores and microbes on leaves also contribute, or suggests that the heat and acidity contributes to the decay of the needles. I observed fungi growing with pine needles incubated for 28 days at temperature reaching upper 40 $^{o}$C, and the fungus, which was similar to *Penicillium* sp., appeared able to grow in BSL water at that temperature (Figure 10). This temperature is near the upper limit for fungi (Rothschild & Manicinelli 2001), even without the additional stress of very low pH, so this result was surprising, and I did not observe such growth in most incubations. Other studies of micro-eukaryote growth in BSL have observed growth up to 52 $^{o}$C for the heterolobosean *Tetramitus* sp. (Reeder 2011). Bryan Ervin has tested fungi grown from BSL water and leaf material. These include a number of *Penicillium, Aspergillus* and *Cladosporum* sp., which are cosmopolitan taxa that may be accidental or opportunistic members of the BSL community, and other genera that are more often

associated with similar environments: *Philalophora*, *Acidomyces*, and *Ochroconis (Dactylaria),* as well as *Paecilomyces*. Similar fungi have been detected or isolated elsewhere from both natural and acid-mine-drainage influenced environments (Baker et al. 2004, Selbmann et al. 2008, Yamazaki et al. 2010, Yarita et al. 2010). Few appear to be obligate acidophiles or thermophiles, but several grow on complex media at pH 2.2, 35-45 $^{o}$C. However, acidophilic xylanases from *Penicillium* (Kimura et al. 2000) and *Aspergillus* (de Lemos Esteves et al. 2004) were detected via genetic mapping and characterization of lignocellulosic breakdown enzymes. These taxa may be introduced to the lake via leaf-borne spores and then contribute to breakdown.

Alternatively, prokaryotes likely also contribute to lignocellulosic breakdown in BSL. Major prokaryote genes identified in BSL water includes Gram positive taxa such as *Sulfobacillus* and *Acidimicrobium* (Siering et al. 2013). Both are metabolically flexible, but can utilize low levels of heterotrophic substrates. I observed that BSL prokaryotes grew optimally near *in situ* temperatures (Figure 17) on low amounts of yeast extract, which may act as both a C and N source. Geochip data (Siering et al. 2013) indicate $N_2$ fixation genes are highly abundant in the lake, suggesting YE may have acted primarily as a N source. Knowing that BSL prokaruotes were not able to grow as well on glucose as on YE, it can be assumed that nitrogen might be a limiting nutrient. I frequently observed insects that had fallen into the lake and died, which might be an important N source for the microbial community of BSL. However, I was not able to quantify insect N inputs in this study. Bacterial production in oligotrophic lakes can be limited by other nutrients; Vidal et al. (2011) amended samples from 5 oligotrophic lakes in southern Sweden with either glucose (G), phosphorus (P), or G and P together, and

found that bacterial respiration and production was highest when samples were amended with G and P together.

I also observed that oxygen did not appear to affect microbial growth (Figure 16). Given the low solubility of oxygen in high temperatures (Rothschild & Manicinelli 2001), this is not surprising, and both genetic (Wilson et al. 2008) and biogeochemical (Siering et al. 2013) observations suggest that both aerobic and anaerobic processes are important in the lake.

Interestingly, uptake rates of radiolabeled glucose or thymidine were low, while those for acetate were very high (Figure 15), suggesting prokaryote heterotrophic potential varies with substrate. It is possible that breakdown products of polymeric sugars (hexoses) such as starch or cellulose are not favored, while aromatic or phenoxy breakdown products that lead to acetate are. However, this pattern might also be to differential respiration of the substrates, since I measured only cell incorporation and not respired $^{14}CO_2$.

## Constructing a Preliminary C Budget for BSL

My observations suggest that allochthonous plant leaf material can definitely contribute to microbial production in BSL, both prokaryotic and eukaryotic. However, one of my questions was whether this is a major source of BSL secondary production, and how it compares to primary production. Here, I construct a preliminary C budget for BSL, based on observations and assumptions from hydrologic budgets.

Heterotrophic Metabolism Dominates Over Autotrophy, Though Both Are Low

I observed that uptake of radio-DIC was very low, similar to other observations made at other times (Siering et al. 2013). *In situ* DIC is below detection limit; at pH 2, $CO_2$ in equilibrium with the atmosphere is <0.03 ppm. Total inorganic C (TIC) was estimated at 0.5 ppm. Using this range, the uptake rates I observed translate to 0.7-12 μg C $L^{-1}$ $d^{-1}$ for primary production. In contrast, I observed that heterotrophic radiocarbon utilization was variable, but potentially high, as for acetate. Using thymidine incorporation, a standard method for determining bacterial production, I observed turnover of about 0.3% $hr^{-1}$, or 0.03 nM $hr^{-1}$. Assuming 2 x $10^{18}$ cells produced per mol thymidine and 10 fg C per bacterial cell, this translates to about 16 μg C $L^{-1}$ $d^{-1}$secondary production. This is low compared to another oligotrophic (DOC 6.4 mg $L^{-1}$) neutral pH, mesophilic (21$^{o}$C) lake where bacterial secondary production was estimated to be 24.6-88.2 μg C $L^{-1}$ $d^{-1}$ (Chrost et al. 2000). These results suggest that BSL may be a system where secondary production equals or exceeds primary production, similar to that observed in other acidic lakes (Nixdorf et al. 2003). In that study, they found primary production rates to be about 30 μg C $L^{-1}$ $d^{-1}$ in lakes with 1.9 ppm DIC, compared to 137-1145 μg C $L^{-1}$ $d^{-1}$ in freshwater neutral lakes with 15-30 ppm DIC.

Planktonic primary production always results in some loss of fixed C as DOC, although estimates vary widely (Belly et al. 1973, Myklestad et al. 1989). Assuming 20% losses, the measured primary production would fuel only 0.1-2 μg C $L^{-1}$ $d^{-1}$ of secondary production, suggesting allochthonous inputs may provide the majority.

Leaf Litter Input Estimates

Measurements of litterfall to lakes in montane coniferous forests are surprisingly rare. Rau (1976) studied leaf inputs to Findley Lake in Washington state's Cascade Mountains, a setting somewhat similar to BSL. He also observed peaks in leaf litter drop ranging from Sept –Oct for fir and hemlock, the dominant taxa found there, and estimated about 290 g DM $m^{-2}$ $y^{-1}$ for litter traps near the lake shore, similar to other measured values of 300-400 g DM $m^{-2}$ $y^{-1}$ in coniferous forests. Extrapolation from the traps located under trees near BSL suggests annual litterfall to be similar, 100-400 g DM $m^{-2}$ $yr^{-1}$. The lake is topographically situated on a slope, with the southern end below the surrounding forest, and the northern end more level with the surrounding ground. Assuming for first approximation a 10 m 'leaf shed' basin where trees occupy 75% of BSL shore (estimated from Google Earth satellite photos and pictures taken from the site), and modeling the lake as a 1.8 x $10^5$ $m^3$ cylinder of radius 75 m and depth 10 m (Siering et al. 2013), this translates to deposition of about 9.4 µg C $L^{-1}$ $d^{-1}$. However, very little of deposited leaf litter probably reaches the lake during rain and snow run-in; assuming 1-10% reaches the lake, this would equal 0.09-0.94 µg C $L^{-1}$ $d^{-1}$.

Estimating C Inputs/Outputs Based on Hydrologic Budget

Figure 21 shows internal (autochthonous) production and potential allochthonous C sources. Besides leaf litter, the remaining C inputs can be estimated from BSL's hydrologic budget, since DIC/DOC flux = concentration * annual water flux. Figure 22 shows an estimate of hydrologic budget for the lake, based on the measurements and assumptions described in the Methods. Although soil runoff and

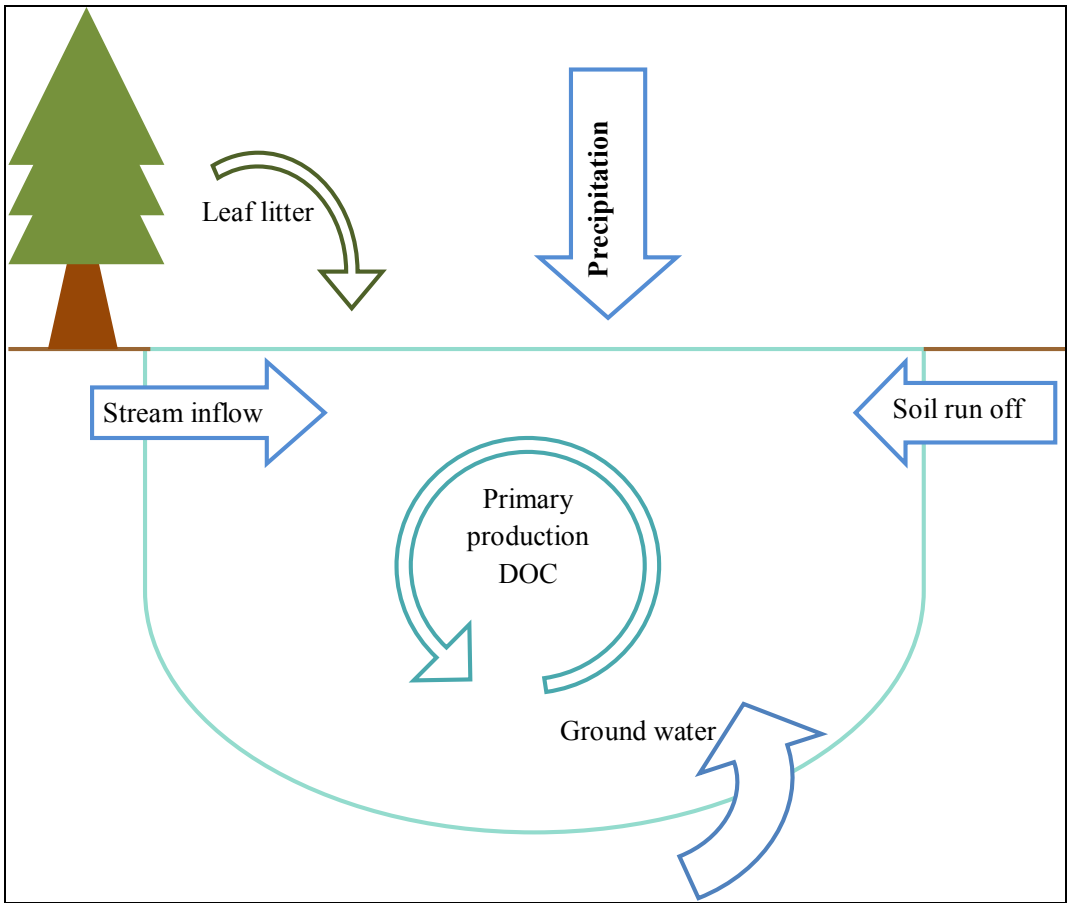


Fig. 21. Contribution of hydrological cycle to the carbon budget in BSL, showing possible autochthonous and allochthonous carbon sources

stream inflow are not known to any precision, fluxes appear to be dominated by evaporation, estimated at >50 mm $d^{-1}$, driven by the gradient between geothermal heating of the water and cool montane air temperatures, and strong daily winds. As the lake level is remarkably stable over the year, varying by less than 10%, this suggests that geothermal upwelling and/or groundwater flux must nearly balance evaporation, suggesting that water has a residence time of about 2 months, and the lake turns over ~5 times each year. Using the estimated hydrological flux and DOC ranges, Figure 23 shows an estimate of C budget for BSL. While numbers are highly uncertain, this suggests that

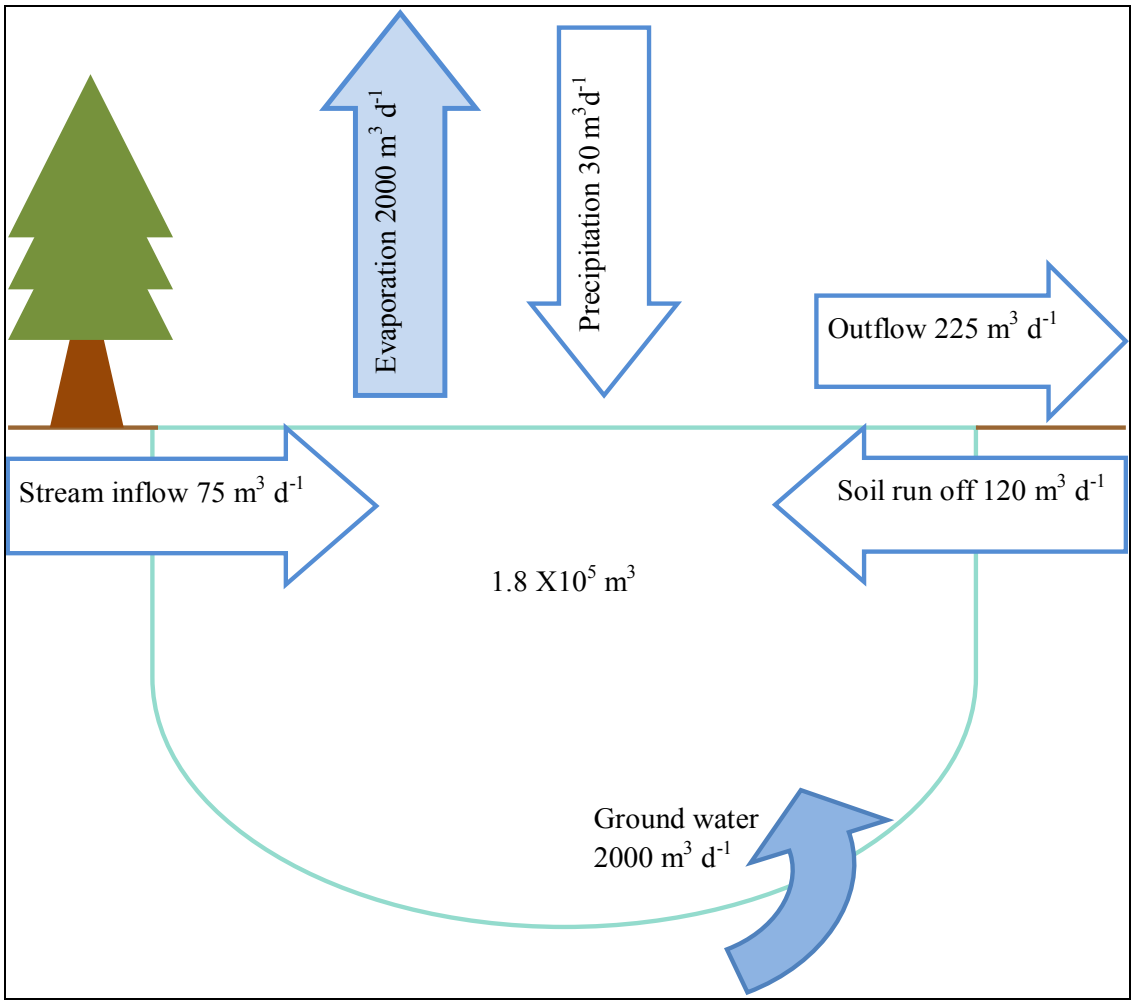


Fig. 22. Estimated hydrological budget in BSL. See text for details

leaf litter contributes little C compared to water sources. However, the contribution of these sources to secondary production also depends on their quality. Labile DOC such as sugars and amino acids supports bacterial secondary production better than refractory DOC (Docherty et al. 2006, Forsström et al. 2013). Although I was unable to measure growth efficiencies of BSL bacteria, bacterial growth efficiencies taken from the literature (Table 5), suggest that allochthonous DOC may supply the majority of BP observed *in vitro*. However, leaf litter contributes negligibly to this total, while soil runoff

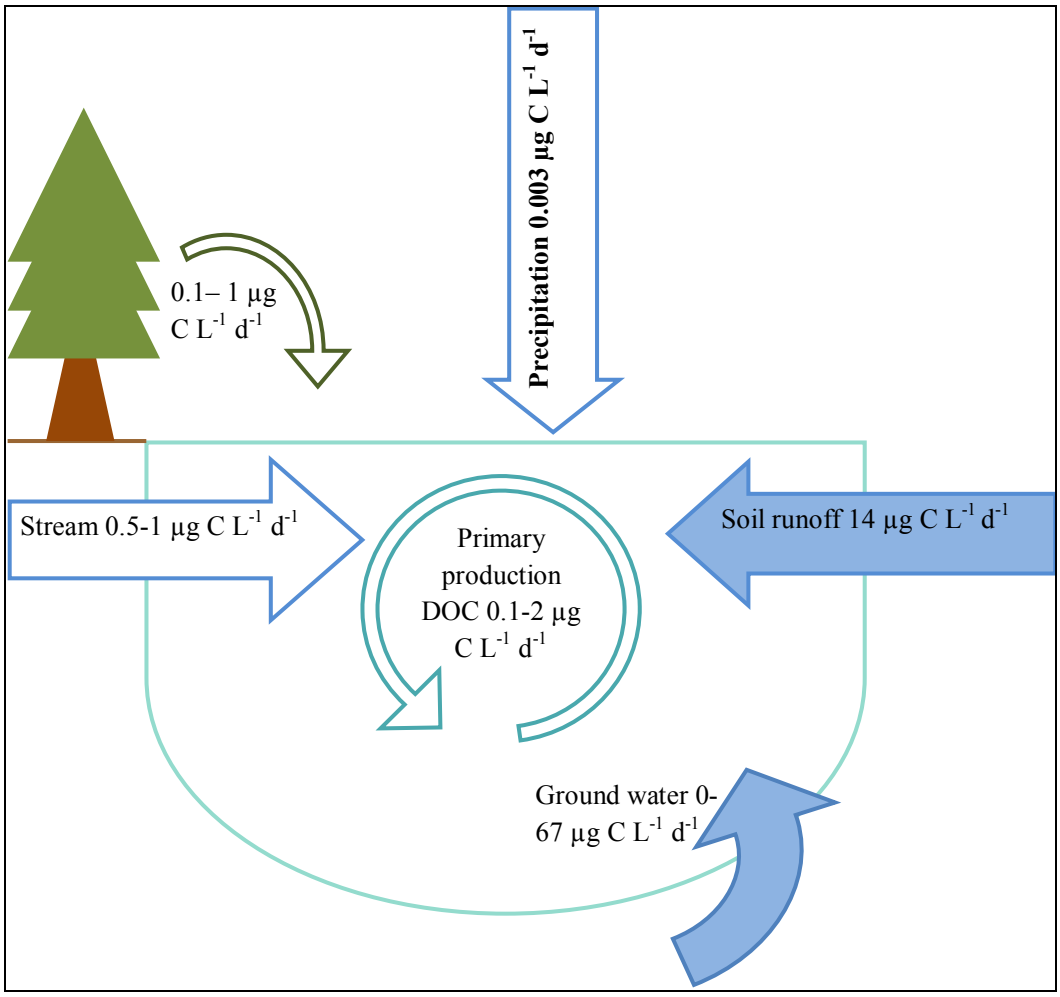


Figure 23. Estimated C budget in BSL. See text for details

is likely the major sources. The total estimated potential BP (Table 5) does not support measured BSL's BP, calculated from thymidine uptake. This suggests that growth efficiency of BSL's bacteria might be higher than what was reported by Kamjunke et al. (2006), or that some of the carbon sources provide more carbon than what is estimated here.

Table 5. Estimates of DOC sources to BSL, growth efficiency of bacteria, and contributions to total secondary (bacterial) production (BP) of 16 μg C $L^{-1}$ $d^{-1}$

| OC source | DOC flux*! | Efficiency (reference) | Potential BP | %BP |
|---|---|---|---|---|
| rain | 0.0 | 10% | 0.0 | 0.0 |
| groundwater | 0.5 | 10% (Kamjunke et al. 2006) | 0.0 | 0.3 |
| leaf input | 0.3 | 10% (Kamjunke et al. 2006) | 0.0 | 0.2 |
| stream | 0.7 | 10% (Kamjunke et al. 2006) | 0.1 | 0.4 |
| PP | 0.6 | 40% (Kamjunke et al. 2006) | 0.2 | 1 |
| soil runoff | 14 | 10% (Kamjunke et al. 2006) | 1.4 | 9 |

## Suggestions for Future Work

There are many areas where this work could be extended and improved. Chemical analysis of leaves and leaf leachates would identify nutrients potentially available to BSL microbes, and further experiments should be done investigating limiting nutrients (N, P, etc.) for bacterial growth. Additionally, although I tried to measure growth efficiencies of BSL bacteria on different C sources (algal byproducts, leaf leachate, or simple carbon sources) using the SYBR green method modified from Martens-Habbena and Sass (2006), the experiment was unsuccessful. Further work is needed to replace the estimates I used from literature with actual growth efficiencies.

Finally, my estimates of allochthonous inputs could be greatly expanded and improved. I did not measure actual leaf infall directly to BSL, and had to assume most was washed in from the surrounding soils. Finally, I frequently observed dead insects in BSL water, which might be a significant nitrogen source for the microbial community. Future work should attempt to quantify these potentially important inputs, as well as hydrologic inputs and DOC burdens.

# LITERATURE CITED

# LITERATURE CITED


Albariño RJ, Balseiro EG (2002) Leaf litter breakdown in Patagonian streams: native versus exotic trees and the effect of invertebrate size. Aquatic Conservation: Marine and Freshwater Ecosystems 12:181-192

Baker BJ, Lutz MA, Dawson SC, Bond P, Banfield J (2004) Metabolically active eukaryotic communities in extremely acidic mine drainage. Applied and Environmental Microbiology 70:6264-6271

Bärlocher F, Oertli JJ (1978) Inhibitors of aquatic hyphomycetes in dead conifer needles. Mycologia 70:964-974

Belly RT, Tansey MR, Brock TD (1973) Algal excretion of 14C-labeled compounds adn microbial interactions in cyanidium caldarium mats. Journal of Phycology 9:123-127

Bermingham S, Maltby L, Cooke RC (1996) Effects of a coal mine effluent on aquatic hyphomycetes. ii. laboratory toxicity experiments. Journal of Applied Ecology 33:1322-1328

Boulton AJ, Boon PI (1991) A review of methodology used to measure leaf litter decomposition in lotic environments: Time to turn over an old leaf? Marine and Freshwater Research 42:1-43

Canhoto C, Graça MAS (1999) Leaf barriers to fungal colonization and shredders (Tipula lateralis) consumption of decomposing Eucalyptus globulus. Microbial Ecology 37:163-172

Carpenter J, Odum WE, Aaron M (1983) Leaf litter decomposition in a reservoir affected by acid mine drainage. Oikos 41:165-172

Chamier AC (1987) Effect of pH on microbial degradation of leaf litter in seven streams of the English Lake District. Oecologia 71:491-500

Chrost R, Koton M, Siuda W (2000) Bacterial secondary production and bacterial biomass in four mazurian lakes of differingtrophic status. Polish J Environ Studies 9:255

Coulson CB, Davies RI, Lewis DA (1960) Polyphenols in plant, humus, and soil. Journal of Soil Science 11:20-29

Dangles, Guerold (1998) A comparative study of beech leaf breakdown, energetic content, and associated fauna in acidic and non-acidic streams. Archiv für Hydrobiologie 144:25-39

Dangles O, Chauvet E (2003) Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. Water Research 37:533-538

Dangles O, Gessner MO, Guerold F, Chauvet E (2004) Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. Journal of Applied Ecology 41:365-378

de Lemos Esteves F, Ruelle V, Lamotte-Brasseur J, Quinting B, Frere J-M (2004) Acidophilic adaptation of family 11 endo-b-1,4-xylanases: modeling and mutational analysis. Protein Sci 13:1209-1218

Diemer GS, Stedman KM (2012) A novel virus genome discovered in an extreme environment suggests recombination between unrelated groups of RNA and DNA viruses. Biology Direct 7:13

Docherty K, Young K, Maurice P, Bridgham S (2006) Dissolved organic matter concentration and quality influences upon structure and function of freshwater microbial communities. Microbial Ecology 52:378-388

Forsström L, Roiha T, Rautio M (2013) Responses of microbial food web to increased allochthonous DOM in an oligotrophic subarctic lake. Aquatic Microbial Ecology 68:171-184

France R, Culbert H, Freeborough C, Peters R (1997) Leaching and early mass loss of boreal leaves and wood in oligotrophic water. Hydrobiologia 345:209-214

Francisco DE, Mah RA, Rabin AC (1973) Acridine orange-epifluorescence technique for counting bacteria in natural waters. Transactions of the American Microscopical Society 92:416-421

Grahn O, Hultberg H, Landner L (1974) Oligotrophication--a self-accelerating process in lakes subjected to excessive supply of acid substances Ambio 3:93-94

Gyure RA, Konopka A, Brooks A, Doemel W (1987) Algal and bacterial activities in acidic (pH 3) strip mine lakes. Applied and Environmental Microbiology 53:2069-2076

Harmon ME, Baker GA, Spycher G, Greene SE (1990) Leaf-litter decomposition in the *Picea/Tsuga* forests of Olympic National Park, Washington, U.S.A. Forest Ecology and Management 31:55-66

Hodkinson ID (1975) Dry weight loss and chemical changes in vascular plant litter of terrestrial origin, occurring in a beaver pond ecosystem. Journal of Ecology 63:131-142

Hoeniger JFM (1985) Microbial decomposition of cellulose in acidifying lakes of south-central Ontario Canada. Applied and Environmental Microbiology 50:315-322

Janik CJ, McLaren MK (2010) Seismicity and fluid geochemistry at Lassen Volcanic National Park, California: Evidence for two circulation cells in the hydrothermal system. Journal of Volcanology and Geothermal Research 189:257-277

Kamjunke N, Bohn C, Grey J (2006) Utilisation of dissolved organic carbon from different sources by pelagic bacteria in an acidic mining lake. Archiv für Hydrobiologie 165:355-364

Kamjunke N, Tittel J, Krumbeck H, Beulker C, Poerschmann J (2005) High heterotrophic bacterial production in acidic, iron-rich mining lakes. Microbial Ecology 49:425-433

Kelly CA, Rudd JWM, Furutani A, Schindler DW (1984) Effects of lake acidification on rates of organic matter decomposition in sediments. Limnology and Oceanography 29:687-694

Kimura T, Ito J, Kawano A, Makino T, Kondo H, Karita S, Sakka K, Ohmiya K (2000) Purification, characterization, and molecular cloning of acidophilic xylanase from *Penicillium* sp. 40. . Bioscience, Biotechnology and Biochemistry 64:1230-1237

Kok CJ, Haverkamp W, Van Der Aa HA (1992) Influence of pH on the growth and leaf-maceration ability of fungi involved in the decomposition of floating leaves of *Nymphaea alba* in an acid water. Journal of General Microbiology 138:103-108

Löhr AJ, Noordijk J, Lrianto K, Van Gestel CAM, Van Straalen NM (2006) Leaf decomposition in an extremely acidic river of volcanic origin in indonesia. Hydrobiologia 560:51-61

López ES, Pardo I, Felpeto N (2001) Seasonal differences in green leaf breakdown and nutrient content of deciduous and evergreen tree species and grass in a granitic headwater stream. Hydrobiologia 464:51-61

Martens-Habbena W, Sass H (2006) Sensitive determination of microbial growth by nucleic acid staining in aqueous suspension. Applied and Environmental Microbiology 72:87-95

Mathews CP, Kowalczewski A (1969) The disappearance of leaf litter and its contribution to production in the river thames. Journal of Ecology 57:543-552

McKinley VL, Vestal JR (1982) Effects of acid on plant litter decomposition in an arctic lake. Appl Environ Microbiol 43:1188-1195

Meegan S, Perry S, Perry W (1996) Detrital processing in streams exposed to acidic precipitation in the Central Appalachian Mountains. Hydrobiologia 339:101-110

Michaelides J, Kendrick B (1978) An investigation of factors retarding colonization of conifer needles by amphibious hyphomycetes in streams. Mycologia 70:419-430

Mulholland PJ, Palumbo AV, Elwood JW, Rosemond AD (1987) Effects of acidification on leaf decomposition in streams. Journal of the North American Benthological Society 6:147-158

Myklestad S, Holm-Hansen O, Vårum KM, Volcani BE (1989) Rate of release of extracellular amino acids and carbohydrates from the marine diatom Chaetoceros affinis. Journal of Plankton Research 11:763-773

Nixdorf B, Kapfer M (1998) Stimulation of phototrophic pelagic and benthic metabolism close to sediments in acidic mining lakes. Water, Air, & Soil Pollution 108:317-330

Nixdorf B, Krumbeck H, Jander J, Beulker C (2003) Comparison of bacterial and phytoplankton productivity in extremely acidic mining lakes and eutrophic hard water lakes. Acta Oecologica 24, Supplement 1:S281-S288

Niyogi D, McKnight D, Lewis W, Jr. (2002) Effects of mine drainage on breakdown of aspen litter in mountain streams. Water, Air and Soil Pollution: Focus 2:329-341

Niyogi KD, Lewis MW, McKnight MD (2001) Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. Ecological Applications 11:506-516

O'Connell AM, Menage P (1983) Decomposition of litter from three major plant species of jarrah (Eucalyptus marginata Donn ex Sm.) forest in relation to site fire history and soil type. Australian Journal of Ecology 8:277-286

Petersen RC, Cummins KW (1974) Leaf processing in a woodland stream*. 4:343-368

Rau GH (1976) Dispersal of terrestrial plant litter into a subalpine lake. Oikos 27:153-160

Rau GH (1978) Conifer needle processing in a subalpine lake. Limnology and Oceanography 23:356-358

Reeder IV WHH (2011) Diversity and activities of phagotrophic micro-eukaryotes in Boiling Springs Lake, Lassen Volcanic National Park. M.S., California State University, Chico, Chico

Rosenberry D, Winter T, Buso D, Likens G (2007) Comparison of 15 evaporation methods applied to a small mountain lake in the northeaster USA. Journal of Hydrology 340:149-166

Rothschild LJ, Manicinelli RL (2001) Life in extreme environments. Nature 409:1092-1101

Rousk J, Baath E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J 4:1340-1351

Schlief J (2004) Leaf associated microbial activities in a stream affected by acid mine drainage. International Review of Hydrobiology 89:467-475

Schneider T, Gerrits B, Gassmann R, Schmid E, Gessner MO, Richter A, Battin T, Eberl L, Riedel K (2010) Proteome analysis of fungal and bacterial involvement in leaf litter decomposition. Proteomics 10:1819-1830

Schultheis A, Sanchez M, Hendricks A (1997) Structural and functional responses of stream insects to copper pollution. Hydrobiologia 346:85-93

Selbmann L, de Hoog GS, Zucconi L, Isola D, Ruisi S, van den Ende AHGG, Ruibal C, De Leo F, Urzi C, Onofri S (2008) Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. Studies in Mycology 61:1-20

Siefert J, Mutz M (2001) Processing of leaf litter in acid waters of the post-mining landscape in Lusatia, Germany. Ecological Engineering 17:297-306

Siering PL, Clarke JM, Wilson MS (2006) Geochemical and biological diversity of acidic, hot springs in Lassen Volcanic National Park. Geomicrobiology Journal 23:129-141

Siering PL, Wolfe GV, Wilson MS, Yip AN, Carey CM, Wardman CD, Shapiro R, Stedman KM, Kyle J, Yuan T, Van Nostrand JD, He Z, Zhou J (2013) Microbial biogeochemistry of Boiling Springs Lake: a physically dynamic, oligotrophic low pH geothermal ecosystem Geobiology in the press

Sigee DC (2004) Freshwater Microbiology: Biodiversity and Dynamics Interactions of Microorganisms in the Aquatic Environment, Vol. John Wiley & Sons, Chichester

Triska FJ, Kennedy VC, Avanzino RJ, Zellweger GW, Bencala KE (1989) Retention and transport of nutrients in a third-order stream in northwestern California: hyporheic processes. Ecology 70:1893-1905

Vidal LO, Granéli W, Daniel CB, Heiberg L, Roland F (2011) Carbon and phosphorus regulating bacterial metabolism in oligotrophic boreal lakes. Journal of Plankton Research 33:1747-1756

Wallis PM, Hynes HBN, Telang SA (1981) The importance of groundwater in the transportation of allochthonous dissolved organic matter to the streams draining a small mountain basin. Hydrobiologia 79:77-90

Webster JR, Benfield EF (1986) Vascular plant breakdown in freshwater ecosystems. Annual Review of Ecology and Systematics 17:567-594

Willey JD, Kieber RJ, Eyman MS, Avery GB (2000) Rainwater dissolved organic carbon: Concentrations and global flux. Global Biogeochemical Cycles 14:139-148

Wilson M, Siering P, White C, Hauser M, Bartles A (2008) Novel archaea and bacteria dominate stable microbial communities in North America's largest hot spring. Microbial Ecology 56:292-305

Witkamp M, Olson JS (1963) Breakdown of confined and nonconfined oak litter. Oikos 14:138-147

Yamazaki A, Toyama K, Nakagiri A (2010) A new acidophilic fungus *Teratosphaeria acidotherma* (Capnodiales, Ascomycota) from a hot spring. Mycoscience 51:443-455

Yarita K, Sano A, Samerpitak K, Kamei K, de Hoog G, Nishimura K (2010) *Ochroconis calidifluminalis*, a sibling of the neurotropic pathogen *O. gallopava*, isolated from hot spring. Mycopathologia 170:21-30-30