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# Using PCR Amplification and Genetic Sequence Analysis of 18S rRNA Genes to Survey the Microbial Diversity and Distribution of Eukaryotic Microbes Inhabiting Two Thermo-acidic Streams in Yellowstone National Park, Wyoming

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# Using PCR Amplification and Genetic Sequence Analysis of 18S rRNA Genes to Survey the Microbial Diversity and Distribution of Eukaryotic Microbes Inhabiting Two Thermo-acidic Streams in Yellowstone National Park, Wyoming

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# Table of Contents

# List of Figures

# List of Tables

## Abstract

A cultivation-independent approach, sequence analysis of 18S rRNA genes PCR-amplified from environmental DNA, was used to explore the diversity and distribution of eukaryotic microbes inhabiting algal mats in two acidic geothermal streams in Yellowstone National Park. The objectives were to: (1) clarify the identity of mat forming algae in Nymph Creek (2) survey microbial species in the Nymph Creek mat over seasonal intervals along a thermal gradient (3) compare microbial species in the Nymph Creek mat with those in Alluvium Creek mats (4) evaluate microbial species in algal mats formed on different substrates in Alluvium Creek. The results show that a novel red alga dominates high temperature regions (~50ºC) of Nymph Creek and two "*Chlorella*-like" algae predominate the cooler regions (<38ºC). The predominant algae in Alluvium Creek were distinctly different from those in Nymph Creek. Several stramenophiles and fungi were detected in each algal mat.



Key words: microbes, cyanidiales, *Cyanidium*, *Galdieria*, *Cyanidioschyzon*, *Chlorella,* algae, stramenophile*,* fungi*,* PCR, extremophile, environmental samples, Yellowstone

# Introduction

*Extremophiles: Practical Significance*

Ecological studies of microorganisms that thrive in extreme environments, such as those of high or low temperature, high pressure, low *pH* or high salinity, have led to the isolation of enzymes that function under extreme conditions. The commercialization of such enzymes has helped stimulate the multimillion-dollar biotech industry and has led to advances in fields including agriculture, biomedical diagnostics, forensics, microbiology, pharmaceuticals and waste treatment (Wilson et al., 2009). Collectively, microorganisms that thrive under harsh environmental conditions are referred to as extremophiles (Satyanarayana et al., 2005). More specific terms for these organisms have been coined. Halophile describes microbes that require high osmotic conditions for growth and acidophile describes microbes that thrive at low *pH*. The terms thermophile and psychrophile (or cryophile) describe microbes that thrive at high (≥ 45°C) and low (≤ 15°C) temperatures, respectively.

Insights gained from basic research aimed at understanding the identity, diversity and ecology of microbial species in extreme environments have led to numerous practical spin-offs, most notably the commercial application of enzymes that remain active at high or low temperatures. One common example is the addition of enzymes, such as proteases and amylases, from thermophiles and psychrophiles in laundry detergents to help remove organic stains from clothing in hot or cold water. An additional benefit is that the replacement of harsh chemicals by enzymes in common household products, and in large-scale

industrial processes, is environmentally responsible. For example, enzymes from extremophiles can replace caustic chemicals used to remove glues and adhesives in the paper pulp recycling industry. Undoubtedly the most notable example of a practical use of a natural product from an extremophile is the heat-stable enzyme *Taq* polymerase (Chein et al., 1976). This DNA polymerase was originally isolated from *Thermus aquaticus*, a thermophile isolated from hot springs in Yellowstone National Park (Brock, 1969). However the practical value of a heat-stable DNA polymerase (Saiki et al., 1988) was not realized until the early 1980's with the development of the polymerase chain reaction (PCR) to amplify minute amounts of DNA (Mullis and Faloona, 1987). Heat stable polymerases made practical the process of DNA amplification since these enzymes tolerate the repeated high temperature cycles used to denature DNA and thus do not have to be added after each cycle, as was the case with the non-heat-stable polymerase used in the original description of PCR (Mullis et al., 1986). Ironically, *Taq* polymerase is perhaps the most useful tool available to microbiologists for the examination of microbial species in hot spring environments (Ward et al., 1998; Woese 1987). More recently, concerns about dwindling supplies of petroleum based fuels, and their contribution to global warming, has prompted scientists to examine Yellowstone's geothermal springs as a source of enzymes to convert cellulose form agricultural products, such as switch grass, into renewable sources of 'green' biofuel energy including hydrogen, methane and ethanol (Inderwildi and King, 2009). With the advent of new, high throughput sequencing technologies, exploring the genetic diversity of microbial

communities (metagenomics) is a burgeoning field. Beginning in 2009, a large scale NSF sponsored genomics project, spearheaded by the Joint Genome Institute, will sequence the metagenomes of twenty of Yellowstone Park's geothermal microbial communities as possible sources of enzymes for biomimetic production of hydrogen as a fuel source **(www.jgi.doe.gov/sequencing/why/99208.html)**.

*Acidic Geothermal Habitats: Yellowstone Park, a Natural Laboratory for Microbial Ecology*

While prokaryotic phototrophic microbial mat communities, that predominate neutral and alkaline hot springs in Yellowstone Park, have been well characterized using molecular methods, eukaryotic algal mat communities that predominate Yellowstone's numerous acidic geothermal springs, have not been extensively studied using cultivation-independent molecular methods. Thus the basic ecology of these mat communities is not understood and their biotechnological potential is unknown. While, the majority of the world's geothermal habitats are found in Iceland, Italy, Japan and New Zealand, many of these springs have been drastically altered by geothermal power projects and human recreational activities. In contrast, Yellowstone National Park's pristine geothermal habitats have suffered little to no anthropogenic destruction, making the park the most well preserved natural laboratory for research on the ecology of extremophiles. The colorful microbial mats that encrust many of Yellowstone's geothermal features capture the attention of visitors, as was the case for early naturalists and scientist who first investigated them. It was geologist Walter H. Reed who first attributed the mats to microorganisms in 1889 (Brock, 1978), and

it was a request to study microbes in these mats that initiated the issuance of the first scientific permit in YNP in 1898 to researcher W. A. Setchell (Varley and Scott, 1998).

Remarkably, despite the hot, acidic conditions, eukaryotic microalgae thrive in low acidic (*pH* ≤3) geothermal springs and form thick (1 to 2 cm), visibly striking blue-green mats that carpet the streambed (Figure. 1). Acidic creeks are created when reduced sulfide ($H_2S$) or sulfide minerals (such as FeS) buried deep within the earth's crust under anaerobic conditions are exposed to oxygen in the atmosphere. Subterranean disturbances such as earthquakes, volcanic activity or human mining activities, can expose reduced sulfur compounds to air and groundwater. Upon contact with water and oxygen in the atmosphere, reduced sulfur compounds are oxidized, resulting in the formation of sulfuric acid ($H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+$) which lowers the *pH* of a stream (Baker et al., 2003; Baker et al., 2004). Enzymatic activities of prokaryotic microbes that use reduced sulfur as a source of electrons to generate metabolic energy can greatly enhance this process (Lopez-Archilla et al., 2001). The acid mine drainage at Iron Mountain in California is an extreme example of this process where the *pH* of some pools is <1 (Baker and Banfield, 2003; Baker et al., 2003; Baker et al., 2004).

*Microbiological and Molecular Methods for Studying Microbes in situ.*

Until the advent of molecular technologies in the late 1980's studies of the ecology of microorganisms in hot springs and other natural environments were constrained because of the limitations of traditional microbiological methods. In

Figure 1. Cyanidiales dominated algae mat.

fact, early scientist believed it was impossible for organisms to survive at high temperatures much less to be part of a thriving micro ecosystem. It wasn't until the 1970's, when microbiologists began to cultivate thermophilic bacteria from Yellowstone's geothermal features, that research on the ecology of extremophiles gained academic recognition. Despite progress in cultivating thermophiles, it had long been suspected that cultivated isolates, and microscopic descriptions of cells, provided inaccurate and incomplete assessment of microbial communities in natural habitats (Ward et al., 1990). In the late 1980's, the development of cultivation-independent, molecular approaches, based on sequence analysis of SSU (small sub unit) rRNA genes PCR-amplified directly from microbial community DNA, revolutionized the fields of microbial ecology and microbial phylogeny (Woese, 1987). Molecular studies proved that there are thousands of novel microbial species in nature, that the simple morphologic characteristics of microbes mask enormous genetic diversity and that cultivated microbes represent only a small fraction of the species in

nature (Pace, 1997). Thus for the past decade, genetic sequence analysis of SSR rRNA genes PCR-amplified from environmental DNA has offered microbial ecologists, for the first time, tools that enable them to identify essentially all microbes in a natural habitat and systematically assess the phylogenetic relationships between them. While many of Yellowstone National Park's geothermal prokaryotic communities have been described using cultivation independent molecular approaches, knowledge of eukaryotic microbial species that predominate Yellowstone's acidic geothermal algal mats is still largely based on traditional methods of microscopy and laboratory cultivation.

*Algal Mat Communities in Acidic Geothermal Streams.*

Previous studies using traditional microbiological cultivation and microscopic methods suggests that high temperature, low *pH* geothermal algal mats are predominated by spherical unicellular thermoacidophilic red algae at high temperatures (~50°C) and that spherical unicellular acidophilic "*Chlorella*-like" algae are present in these mats at cooler temperatures (Brock and Doemel, 1971). However, as mentioned above, cultivation and microscopic descriptions are inadequate for both detecting microbes' *in situ* and identifying microbial species. As is the case for microbes in general, the cellular morphologies of many acidophilic algae are not sufficiently complex to infer phylogenetic relationships, since most lack discernable characteristics and simply appear to be spherical or ovoid cells with very limited internal structures. Thus phycologists have begun to use genetic sequence-based techniques to assess phylogenetic relationships among algae (Albertano et al., 2000; Cinglia et al., 2004; Gross,

1999; Gross et al., 2001; Huss and Sogin, 1990; Huss et al., 1999; Huss et al., 2002; Seckbach, 1998; Wu et al. 2001) and to study them *in situ*, as reviewed below.

*Taxonomic Overview of the Thermophilic Red Algae (Cyanidiales).*

The luxuriant, bright-green algal mats that carpet substrates in high temperature regions of acidic geothermal streams are striking and conspicuous. Not surprisingly, a great deal of research has been focused on revealing the identity of the algae that construct these mats (Albertano et al., 2000; Cinglia et al., 2004; Gross, 1999; Gross et al., 2001; Huss and Sogin, 1990; Huss et al., 1999; Huss et al., 2002; Sechbach, 1998; Wu et al. 2001). Historically, establishing the identity of thermoacidophilic algae using traditional morphologic and biochemical characteristics has been a source of confusion. The thermoacidophilic algae have been variously referred to as, Chlorophyta, Cryptophyta, Cyanophyta, Rhodophyta. They have also been described as a transitional phase between the cyanobacteria and the Chlorophyta (Doemel and Brock, 1971) and as primitive pre-Rhodophyta (Sechbach, 1998). In the early literature, researchers used morphologic and biochemical similarities between thermoacidophilic algal isolates and *Chlorella*, cyanobacteria and protista as the basis for taxonomic assessments (Allen 1959). We now realize that much of the taxonomic confusion stemmed from the fact that thermoacidophilic algae possess an assortment of characteristics that are usually diagnostic of other phyla (Doemel and Brock, 1971) and the thermoacidophilic algae lack a key

characteristic of the Rhodophyta (red algae), the phylum into which modern gene and genomic sequence analyses has them firmly placed (Gross, 2001).

One major source of taxonomic confusion was that thermoacidophilic algal strains do not produce the light harvesting pigment phycoerythrin, which gives rhodophytes their characteristic red color and is diagnostic of the group. Moreover, as do cyanobacteria (photosynthetic, chlorophyll-containing bacteria previously referred to as blue green algae), all red algae produce phycocyanin, a pigment that gives cyanobacteria their blue-green color (Doemel and Brock, 1971). Because thermoacidophilic algae lack phycoerythrin, their cells and the mats they construct, appear blue-green or dark green in color, resembling cyanobacterial cells and mats that form in neutral to alkaline hot springs. Another impediment to taxonomic identification was the fact that thermoacidophilic algae possess Chlorophyll *a* and have simple spherical or ovoid shaped morphologies, as do *Chlorella* spp. thus thermoacidophilic algae are microscopically indistinguishable from *Chlorella* in environmental samples.

Currently, the thermoacidophilic red algae are separated into three genera Cyanidium, Cyanidioschyzon, and Galdieria and are placed within their own order among the Rhodophyta, the Cyanidiales (Ciniglia et al., 2004; Matsuzaki et al., 2004; Lehr and Frank, 2007; Toplin et al., 2008). The Cyanidiales are considered to be among the most primitive of eukaryotes possessing prokaryotic features such as the presence of phycocyanin (Sechbach 1998). Evolutionarily, the Cyanidiales branch outside the cyanobacterial lineage at the base of the Rhodophyta. It is estimated that the Cyanidiales diverged from other

Rhodophyta about 1.3 billion years ago (Ciniglia et al 2004). Yoon has proposed that the Rhodophyta diverged from other eukaryotic lineages after the protozoa but before the divergence of the Fungi, Animalia and Chlorophyta (Yoon et al 2005). Thus the Cyanidales belong to one of the main photosynthetic eukaryotic lineages, they appear to be one of the most ancient groups of algae and they may represent the first evolved eukaryotes. Due to the presences of prokaryotic features in two of the three Cyanidiales genera, *Cyanidioschyzon* and *Cyanidium* and their absence in the third genera, *Galdieria*, it has been proposed that Cyanidiales represent an intermediate between prokaryotic and eukaryotic organisms. However, genetic exchange may account for this observation. It is of note that the genome of *Cyanidioschyzon merolae* is quite small (16Mb) compared to those of other photosynthetic eukaryotes that have so far been sequenced (Suzuki et al., 1995; Matsuzaki et al., 2004).

Thermoacidophilic algae reproduce by the formation of autospores. Based on the number of autospores within sporangia and other morphological characters such as cell shape, plastids, cell walls, vacuoles, six species have been named, *Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria daedala*, *Galdieria maxima*, *Galdieria partita* and *Galdieria sulphuraria* (Sentsova, 1994). However, a phylogenetic study by Cozzolino (2000), based on ribulose-bisphosphate carboxylase gene (rbcL) sequences of thermoacidophilic algal isolates from geothermal habitats in Italy, suggests that *G. partita*, *G. daedala* and *G. maxima* are morphological variants of *G. sulphuraria.* Another phylogenetic study of thermoacidophilic algae by, Gross (Gross et al. 2001)

included 18S rRNA sequences of Cyanidiales isolates obtained from laboratory collections from around the world. The Gross study revealed that there is a bias toward heterotrophic thermoacidophilic algae in culture collections, as evidenced by the high number of isolates of that clade within the heterotrophic *G. sulphuraria* lineage. In the Gross study, the thermoacidophilic algae formed two distinct clades (Gross, et al., 2001). The larger contained the heterotrophic *G. sulphuraria* strains, as well as isolates labeled *G. daedala* and *G. partita.* Thus Gross' study (Gross et al. 2001) supports the conclusion by Cozzolino (Cozzolino et al., 2000), who used rbcL gene sequences, that *G. daedala* and *G. partita* should be included within the species *G. sulphuraria*. In the Gross study (Gross, et al, 2001) a second thermoacidophilic algal clade contained three morphologically and physiologically diverse species: *C. merolae*, *C. caldarium* and *G. maxima* (Gross et al., 2001). The cell morphologies and heterotrophic growth characteristics that distinguish these three species from each other and from *G. sulphuraria* are listed in Table 1. In general, *G. sulphuraria* cells are spherical and indistinguishable from those of *C. caldarium*; however, *G. sulphuraria* strains are distinguished by their ability to grow heterotrophically. *G. maxima* strains also grow heterotrophically but at a much slower rate than *G. sulphuraria*; however, *G. maxima* cells are much larger then those of *C. merolae* which is a strict autotroph distinguished by crescent or club shaped cells and lack of a cell wall.

In 2008, Toplin et al. (2008), performed a much more extensive biogeographic survey of Cyanidiales using rbcL genes and 18S rRNA genes from

isolates and sequences PCR-amplified directly for environmental DNA from habitats in Yellowstone Park, Wyoming, USA, Japan and New Zealand. Toplin et al. (2008) concluded that there is biogeographic patterning within the Cyanidiales, similar to that found for thermophilic cyanobacteria, and that allopatry has led to global speciation. Toplin's phylogenetic analyses of Cyanidiales using rbcL and 18S rRNA gene sequences were in agreement except that the rbcL tree did not show support for separating the Japanese isolates from the New Zealand isolates. This difference may be the result of different evolution rates of chloroplast and nuclear genes. The Toplin (2008) study indicated that the 18S rRNA gene sequences of isolates from Japan and New Zealand were distinctly different from those of other Cyanidiales and were only 91 – 93% identical to their closest GenBank relative, *G. maxima.* Thus these isolates may represent new species. As mentioned above, such discoveries of new thermoacidophilic algal species revealed by assessing biogeographically distant habits were predicted by Ciniglia (2004).

It is speculated that the extreme environmental conditions inhabited by Cyanidiales create strong selection pressures against morphological variation and may explain the lack of morphological features and perhaps the lack of recognized species**.** A recent study by Ciniglia et al. (2004) based on plastid encoded sequences suggests that there is considerable variability within the Cyanidiales and the author speculates that additional molecular studies will reveal previously unrecognized diversity in these algae. In a 2004 study, Ciniglia et al. propose that there are four distinct lineages in the Cyanidiales, a *Galdieria*

| | | |
|---|---|---|
| *Cyanidium caldarium* | Small spherical cell | Strict autotroph |
| *Cyanidioschyzon merolae* | Sickle shaped cell | Strict autotroph |
| *Galdieria sulphuraria* | Small spherical cell | Facultative heterotroph |
| *Galdieria maxima* | Large spherical cell | Weak facultative heterotroph |

Table 1. Currently recognized species of Cyanidiales and identifying characteristics.

lineage (which excludes *G. maxima*), a *C. caldarium* lineage, a novel monophyletic lineage of mesophilic *Cyanidium* spp., and a *C. merolae* lineage that includes *G. maxima* (Ciniglia et al., 2004). With the application of new high throughput sequence technologies and metagenomic analyses to Yellowstone's acidic geothermal microbial mat communities, further revisions to the taxonomy of the Cyanidiales are almost certain.

*Cyanidiales in Nature.*

Early research on thermoacidophilic algae was pioneered by Thomas Brock (1978). In these early investigations, Doemel and Brock (1971) used microscopic and cultivation techniques to study mat forming Cyanidiales in biographically distant acidic geothermal environments. Because of methodological limitations (as discussed in the previous section) based on morphological characteristics and cultivated isolates, Doemel and Brock concluded that there was only one photosynthetic red algal species in warm

(40°C) acidic (*pH* < 5) environments worldwide (Doemel and Brock, 1971). They named this organism *C. caldarium*. Doemel and Brock (1971) were unable to identify temperature strains of *C. caldarium* since cells taken from a variety of temperatures were all found to share the same optimal temperature for photosynthesis (45°-50°C). In their studies, "*C. caldarium" was* only observed at high temperatures (>40°C) in aquatic habitats, however the organism was found in terrestrial habitats at temperatures as low as 10°C.

Doemel and Brock (1971) concluded that the absence of competition from other microbes in terrestrial habitats permits *C. caldarium*'s survival at moderate temperatures. In both terrestrial and aquatic environments, *C. caldarium* was only found in habitats with *pH* range from 1.8 to < 5. Doemel and Brock (1971) noted that the only suitable low *pH*, geothermal habitats that lacked *C. caldarium* were in the geologically young Hawaiian Islands (Doemel and Brock, 1971). Doemel and Brock studied the effects of light intensity on *C. caldarium*. They found that cells grown under high or intermediate light intensity contained low amounts of chlorophyll and photosynthesis was not inhibited upon exposure to high light intensity. However, cells grown under low light intensities contained higher amounts of chlorophyll and phycocyanin and these were inhibited when exposed to higher light intensity. Doemel and Brock found that their thermoacidophilic algal isolates grew heterotrophically in the dark on a number of different carbon sources and heterotrophically in light with the addition of galactose. When "*C. caldarium"* was grown heterotrophically with glucose in the dark, the cells lacked chlorophyll and phycocyanin and the ability to synthesize

these pigments was repressed when exposed to light while maintaining glucose in the culture. We now suspect that Doemel and Brock were actually studying isolates of heterotrophic *G. sulphuraria*, since all know *C. caldarium* isolates are strict autotrophs (Table 1). Doemel and Brock noted that cells transferred from dark heterotrophic culture conditions to autotrophic culture conditions temporarily stopped growing and began to synthesize chlorophyll, since photosynthesis is light-dependent and high concentrations of glucose in culture media inhibits photosynthesis (Doemel and Brock, 1971). The ability of some thermoacidophilic algae to grow either autotrophically or heterotrophically can have distinct advantages since light and nutrient availability vary considerably with depth in the thermoacidophilic algal mats (Ferris et al, 2005).

In other early studies, De Luca et al. (1979) surveyed acidic geothermal habitats in North and Central America in order to determine if more than one species of Cyanidiales existed in these environments. Several sites in YNP were sampled, including areas in the Norris Geyser Basin where my primary study site, Nymph Creek, is located. De Luca found that nearly all of the acidic geothermal hot springs in North and Central America contained two coexisting, morphologically distinct algal cell types, which he described as small (1.5 – 5 μm) cell and large (2.5 – 8 μm). The trophic nature of the isolates was not determined, however based on cell descriptions the taxonomic identity of the cells was evaluated. The smaller alga corresponded to *C. caldarium* based on its size, single mitochondrion, single cup shaped chloroplast bound by a single membrane, lack of vacuoles, and the invariant formation of four autospores

during replication. The larger alga corresponded to *G. sulphuraria* by virtue of a larger cell size, numerous mitochondria, a single cup shaped chloroplast bound by a single membrane, vacuoles, and multiplication by 4-8-16 or 32 autospores (De Luca et al., 1979).

A recent survey by Toplin (Toplin et al., 2008) who used sequence analysis of rbcL and 18S rRNA genes to identify thermoacidophilic algae in YNP reports the presence of several species. The first, and by far the most prevalent and abundant species, has an 18S rRNA gene sequence that is 99-100% identical to that of *C. merolae* strain 10D originally discovered in acidic geothermal habitats in Italy. However, in contrast to all known *C. merolae* strains, which are club shaped, lack cell walls and reproduce by binary fission, the cells of the *C. merolae* strain identified by Toplin are spherical with distinct cell walls, and the organism reproduces by autospore formation.

The second type of alga found by Toplin et al. (2008) has an 18S rRNA gene sequence that is essentially identical to the *C. merolae* mentioned above, but this alga is much less prevalent and also has the typical club or crescent shape *C. merolae* morphology. A third alga discovered in the Toplin study clusters within the *G. sulphuraria* clade described by Gross et al. (2001). These cells grow heterotrophically in the dark on glucose and correspond to the *G. sulphuraria* described by Doemel and Brock (1971). Toplin et al. (2008) made efforts to ensure that his methods did not favor the amplification of *C. merolae* cells that do not have cell walls and thus may more readily lyse and release their DNA during the DNA extraction procedure. Genomic DNA was extracted from

serial dilutions of walled *G. sulphuraria* cells and non-cell walled *C. merolae* cells. The results showed that there was no bias in the extraction and amplification process. Toplin noted that 18S rRNA and rbcL gene sequences may be inadequate for determining the true extent of genetic diversity among isolates from YNP, but microsatellites did show promise for identifying genetic diversity among the isolates (Toplin et al., 2008). Studies using a single-time-point sampling to identify thermoacidophilic algae in mats are prevalent in the literature; however these studies provide only a snapshot of the algal community. Studies of the dynamics of algal populations in these mats based on temporal and environmental factors such as those presented in this thesis are limited but are necessary in order to identify complex interactions at the community level.

*Other Organisms Associated with Thermoacidophilic Algal Mat Communities.*

In contrast to the numerous studies of thermoacidophilic algae that construct mats in acidic geothermal springs, almost nothing is known about the identity and diversity of other eukaryotic organisms that inhabit these mats. Although, there are phylogenetic studies on green algae, fungi and stramenophiles found in acidic environments (Leipe et al., 1994; Redman et al., 1999; Henley et al., 2004; Huss and Sogin, 1992, Huss et al., 2002), their affiliation with eukaryotes found in geothermal environments is superficial at best. In pioneering studies of algal mats in Yellowstone's acidic geothermal environments, Doemel and Brock (1971) reported that fungi and bacteria make up approximately 10% of the cells in mat samples. At temperatures below 40°C, Doemel and Brock report that mat communities included numerous green algae,

including *Euglena sp*, *Chlorella* sp, *Chlamydomonas* sp, *Zygogonium* sp. and stramenophiles (diatoms and photosynthetic flagellates). However, these were microscopic observations and no attempt was made to identify organisms at the species level. Early studies also report the presence of a single fungal species in warm aquatic habitats, referred to as *Dactylaria gallopava* (Tansey and Brock, 1973).

The role of fungi as decomposers suggests they are likely to be important members of algal mat communities. Like algae, concepts about the ecology and the identity of fungi in microbial mat communities are inferred from cultured isolates and microscopic observations. Fungi are thought to exist in greater abundance in geothermal communities than indicated by traditional methods of detection. Due to the development of mycelia, spores and sclerotia, fungi can tolerate prolonged exposure to extremes in temperature and *pH,* allowing them to survive temperature extremes outside their optimal temperature range. In fact, fungi have been isolated from nuclear reactor effluents, Dead Sea Valley soils, desert soils and smoldering log piles (Redman et al., 1999).

There are an estimated 1.5 million species of fungi with only 5% (70,000) described species (Borneman and Hartin, 2000). Of these, fewer than 50 thermophilic fungal species have been described. As is the case for all microbial communities in nature, the lack of taxonomic information about fungal diversity is due, in large part, to the limitations of traditional microbiological methodologies. Thermophilic fungi have been detected at temperatures as high as 55°C (Maheshwari et al., 2000) and many fungal species are acid tolerant (Redman et

al., 1999). In a cultivation-based survey of fungi in YNP, 16 different fungal species were detected in an area near acidic Amphitheater Spring located in the Norris Geyser Basin (Redman et al., 1999). Fungi are considered thermophilic if they grow at temperatures > 50°C and will not grow at temperatures < 20°C. Of the 16 species detected near Amphitheater Spring, where geothermal soils temperatures are as high as up to 70°C, two of the fungal isolates were true thermophiles. These were identified as *Acremonium alabamense* and *Dactylaria constricta* var. *gallopava* (Redman et al., 1999). These species were only found in thermal soils but were never detected in cooler soils. This contrasts with observations of thermoacidophilic algae which are found in acidic soils under a wide range of temperatures. Current literature suggests that there appear to be few thermo-tolerant/ thermophilic fungi whereas acid tolerant (*pH* 3 – 6) species are prevalent in environmental samples (Redman et al., 1999).

*Genomic analysis of the Cyaidiales: Evolutionary Significance.*

There are several hypotheses about the evolutionary history of the Cyanidiales. One is that an Archaean thermo-acidophilic cell incorporated a thermophilic non-acidophilic cyanobacterium into its neutral cytoplasm (Pinto 1993). This association allowed the incorporated organism to serve as a light harvesting organelle or a plastid in *C. merolae.* Subsequently, *C. merolae* evolved autogenously into *C*. *caldarium* and later continued to develop into the more advanced *Galdieria* cells. Of the three thermoacidophilic algal genera, *C. merolae* seems to be the most primitive, being most like cyanobacteria based on morphological aspects. *C. merolae* is the smallest member of this group with a

cell size of 1-2 $\mu$m, it divides by binary fission and has the smallest amount of nuclear DNA (16 Mb). Based on these characteristics Sechbach (1998) hypothesized that *C. merolae* was the most primitive of the Cyanidiales and that the Cyanidiales evolved in a linear fashion towards the more complex *Galdieria* type cells (Sechbach, 1998).

Comparative genomics studies of thermoacidophilic algae (Barbier et al., 2005) are now possible given the recent completion of the *C. merolae* strain 10D genome sequence (Matsuzaki et al., 2004) and the nearly complete sequencing (70%) of the *G. sulphuraria* genome (Weber et al., 2004). Barbier et al. (2005), found that there is a high degree of similarity in the genomes of the two microbes and that 30% of the genome of *G. sulphuraria* is unrelated to that of *C. merolae*. The presence of genes coding for enzymes necessary for heterotrophic growth suggest that *C. merolae*'s strictly autotrophic nature is not ancestral but that it has been lost due to a genetic manifestation. One possible explanation for lack of heterotrophic growth is that *C. merolae* is lacking monosaccharide transporter proteins that are present in significantly higher quantities in *G. sulphuraria.* (Barbier et al. 2005). Toplin et al. (2008), hypothesize that Cyanidiales evolved from a common heterotrophic ancestor with a cell wall much like that of *G. sulphuraria*. *G. sulphuraria* retained the characteristics of the ancestor and can colonize terrestrial habitats while *C. merolae*, lacking a rigid cell wall, is limited to aquatic habitats (Toplin et al., 2008).

*Research Objectives.*

Our current understanding of the ecology of eukaryotic microbial species in acidic geothermal algal mat communities is inaccurate and incomplete due to the limitations of traditional microbiological methods. The use of new, cultivation-independent, molecular methods, namely sequence analysis of 18S rRNA genes PCR amplified from mat DNA, will reveal previously unrecognized species that inhabit algal mat communities.

- Use molecular analyses to identify eukaryotic species in algal mat communities in two acidic streams, Nymph Creek and Alluvium Creek.
- Evaluate the influence of day length on algal species composition in the Nymph Creek mat by sampling at seasonal intervals over an annual cycle.
- Evaluate the influence of temperature on algal species composition in the Nymph Creek mat by sampling at different temperature-defined sites along the natural thermal gradients in the stream.
- Evaluate the species composition of algal mat communities that form on different substrates in Alluvium Creek.
- Compare the identity of eukaryotic microbes inhabiting algal mats in acidic, geothermally extinct Alluvium Creek to those detected in the mat in geothermally active Nymph Creek.

# Methods

*Study sites.*

The study sites for this research were two acidic streams in YNP, Nymph Creek and Alluvium Creek. Nymph Creek is easily accessed from Highway 89 while Alluvium Creek is located in a remote region of the park near the Eastern arm of Yellowstone Lake, approximately 30 miles from Nymph Creek. The locations of the study sites are shown in Figure 2. The most striking feature of Nymph Creek is a lush green algal mat that carpets the stream bead. The Nymph Creek mat is easily viewed from the roadside and is a popular curiosity for researchers and tourists alike. In fact, the Nymph Creek mat is a National Science Foundation-sponsored microbial observatory and it has been the subject of numerous microbiological investigations over the past four decades (Belly and Brock, 1973; Doemel and Brock, 1971; Ferris et al., 2003; Ferris et al., 2005; Revsbech and Ward, 1983, Rothschild, 1994; Sheehan et al., 2003; Toplin et al., 2008).

Nymph Creek has been described in detail in the literature (see references above). Briefly, the creek is an acidic (*pH* 2.7), geothermally heated spring-fed stream ranging from 1 to 2 m wide throughout its length. At its origin, Nymph Creek is fed by several 56 to 60°C hot springs. The depth of the creek from the source springs to a point approximately 10 m downstream is 1 to 2 cm. Further downstream, the overall depth increases and some pools reach depths of 10 cm. The volume of water flowing from the source springs into the upstream region of Nymph Creek varies due to seasonal changes in groundwater levels. Higher


Figure 2. Map of Yellowstone Park. Locations of two study sites, Nymph Creek (Latitude 44.3844 and Longitude -110.2416) and Alluvium Creek, (Latitude 44.75139 and Longitude -110.72694) are indicated.

flow volumes typically occur in the spring during snow melt, while lower flow volumes occur in mid to late summer during times of drought. Since the upstream region of Nymph Creek is shallow, various factors such as increased water flow volumes, sediment deposition, fallen debris, hail, or trampling by animals, often disturbs the flow of hot water and the course of small spring-fed warm water channels changes over time. As a result of these stochastic alterations,

temperatures at any given point in the upstream region can vary dramatically over short intervals of time. It has been noted that temperature gradients from ~52°C to 37°C occur over a distance of as little as 10 cm from the center to the edge of the stream in the upper region of the creek (Ferris et al., 2005). A more stable temperature gradient occurs along the length of Nymph Creek, as the warm source waters cool while they flow downstream for approximately 150 m to the point where Nymph Creek empties into a small lake. Diurnal oscillations in ambient air temperature of 20° C are mirrored by 2°C and 6°C temperature oscillations in upstream and downstream sites at Nymph Creek mat, respectively (Ferris et al, 2005). To avoid sampling microbial populations in the mat that had recently experienced a dramatic temperature shift, temperature probes connected to data loggers were placed in the a warm, upstream and a cool, downstream region of the mat. This provided a measure of assurance that the temperatures at which microbial communities were sampled reflected the temperatures under which the community members normally thrive.

In contrast to the easily accessible, well-studied Nymph Creek mat, the microbes in the extremely remote Alluvium Creek site do not form a continuous mat and have not been extensively characterized. We made one sampling expedition to Alluvium Creek during the course of our studies. Alluvium Creek is an acidic stream that has been thermally inactive for some time. However the *pH* (3.2) and other physico-chemical variables are similar to those present in the low temperature regions of Nymph Creek. The water temperature at all sites in Nymph Creek were 23°C at time we visited the site to collect samples. It stands

to reason that water temperatures along the length of Alluvium Creek are greatly influenced by ambient air temperature; however such data was not collected during our brief sampling expedition.

*Sample Collection.*

Algal mat material was collected and placed into 2 ml micro-centrifuge tubes using a clean weighing spatula. The tubes were kept on dry ice until brought to the laboratory and frozen at -20ºC. Samples were collected at two sites in Nymph Creek, a warm (~50°C) upstream site and a cool (~29°C) downstream site, over a annual seasonal interval in order to explore the influence of seasonal variations in light on the algal species diversity in the mat (Figure 3A and 3B). Samples were collected from these sites at four intervals summer, fall, winter and spring (06/27/00, 08/18/00, 12/07/99 and 04/03/00). To examine the influence of temperature on the algae in the Nymph Creek mat, samples were collected at temperature defined sites (27, 35.5, 37, 38, 40, 44, 46.5, and 49°C) on a single day during the summer (08/18/01). To explore the potential distribution of acidophilic algae and other eukaryotic microbes detected in Nymph Creek in other acidic habitats in the Park, samples were collected from Alluvium Creek on 07/22/02. Due to the inaccessibility of Alluvium Creek it was not possible to return to obtain samples for seasonal analysis. Since there are no temperature variations in Alluvium Creek, it was hypothesized that substrate composition would be the most influential environmental variable on microbial diversity. To evaluate possible affects of substrate on algal species and other eukaryotic microbes in Alluvium Creek, samples were collected from three

surfaces, an algal biofilm coating a log, an algal biofilm covering a rock and a flocculent deposit of algae in the bottom of a calm pool (Figures 3c –3e).

Figure 3A. Upstream high temperature region of Nymph Creek.

Figure 3B. Downstream cooler region (T< 38°C) of Nymph Creek.

Figure 3C.  Alluvium Creek flocculent site.

Figure 3D.  Alluvium Creek rock site.

Figure 3E.  Alluvium Creek log site.

*DNA Extraction.*

DNA was extracted from the environmental samples as previously described for Nymph Creek mat samples (Sheehan, 2003). In this method, 0.5 ng – 3.0 ng of microbial mat material in 0.5 ng increments were mechanically lysed using a mini bead beater (Bio 101, Thermo Savant, Holbrook, NY) at speed 6.5 for 2-45 second cycles and then centrifuged for 3 minutes at 13,200 rpm. After centrifugation, 490µl of 100% isopropanol was added to the supernatant and samples were centrifuged in a cold room for an additional 10 minutes. The samples were allowed to precipitate overnight at 4°C and were centrifuged at maximum rpm for 1 hour, after which the supernatant was discarded and the samples were vacuum dried for 30 minutes at 45°C. Samples were then stored at -20°C until resuspended for PCR amplification. In some cases DNA extracts obtained using the Sheehan protocol did not yield PCR products. This was presumably due to polymerase inhibition caused by impurities in the DNA extracts. Extraction kits designed to remove the impurities that inhibit DNA extraction were used subsequently to ensure that all target DNA was extracted and ultimately of sufficient quality for PCR amplification. Additional extractions were done using the following kits; Mo Bio soil DNA extraction kit, (Mo Bio Laboratories, Inc. Carlsbad, CA), Qiagen DNeasy plant mini kit, and the Qiagen QIA amp DNA Stool mini kit (Qiagen Inc. Valencia, CA).

*PCR Amplification.*

The 18S rRNA gene sequences were PCR amplified using the universal eukaryotic primers CDMF (5'-GTCAGAGGTGAAATTCTTGGATTTA -3') and CDMR (5'-AAGGGCAGGGACGTAATCAACG-3'). This primer set was used in the first PCR-based phylogenetic analysis of 18S rRNA gene sequences in Cyanidiales (Gross et al., 2001) The same primer set has been used in more recent analysis of 18S rRNA genes of Cyanidiales that were PCR amplified directly from environmental samples, including samples from Nymph Creek (Toplin et al., 2008). These primers were originally designed to amplify ~700 bp of the 18S rRNA gene from "all" eukaryotic organisms. The primer set amplifies a region of the 18S rRNA gene from nucleotides 912 to 1593 (Ferris et al., 2005) of *C. caldarium* (GenBank # AB09083) and the sequences of the primers complement the 18S rRNA gene sequences of all thermoacidophilic algae in GenBank. PCR amplifications were performed using *Taq* DNA polymerase and reagents according to the manufacturer's instruction (Promega, Madison, WI). We used 10-100 ng of template DNA per reaction. PCR was performed in an iCycler (Bio-Rad, Hercules, CA) using the following settings; initial 94ºC for 5 minutes, followed by 30 cycles of 94ºC for 30 seconds, 60ºC for 1 minute and 74°C 1 minute, then a final extension step at 74ºC for 10 minutes.

*Cloning.*

PCR products were cloned using a TOPO TA Cloning kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Plasmids were isolated from *E. coli* cells using the QIAprep, Spin Miniprep Kit according to the manufacturer's directions (Qiagen, Valencia, CA). Plasmids were sequenced using a BigDye Ready Reaction Termination Mix (Applied Biosystems, Foster City, CA) according the manufacturers instructions. Sequencing of the cloned 18S rDNA genes was performed by a commercial vendor (Louisiana State University Heath Science Center Genomics Core Facility, New Orleans) using the sequencing primers M13 F (5'CGCCAGGGTTTTCCCAGTCACGAC-3') and M13 R (5'-AGCGGATAACA ATTTCACACAGGA-3').

*Sequence Analysis.*

Sequences were aligned and edited using Sequencher (Gene Codes Corporation, Ann Arbor, MN). Identification of sequences determined using the BLAST algorithm at the NCBI web site (National Center for Biotechnology Information, www4.ncbi.nlm.nih.gov). Phylotypes for this study were defined as 18S rRNA gene sequences that shared ≥ 97% sequence homology. Dendrograms were used to depict 18S rRNA sequence similarity between algal sequences detected in our survey and those of related organisms. Sequence files for use in PAUP* (Swofford, 1998) were aligned in Clustal X (Higgins and Sharp, 1988). Best nucleotide substitution models were selected using Modeltest version 3.06 (Posada and Crandall, 1998). The general time reversible model (GTR) was used for the red algae data with a proportion invariant of (I = 0.5887) and a gamma distribution of (G = 0.5137). The Tamura-

Nei model was used for the green algae data with a proportion invariant of (I = 0.5654) with a gamma distribution of (G = 0.2929). Dendrograms were constructed using the neighbor joining algorithm in PAUP* 4.0b8 software (Swofford, 1998). Bootstrap values were from 500 replicates.

# Results

*Detection and Identification of Eukaryotic Microbes*

The primary aim of this study was to use molecular methods to reassess the diversity of eukaryotic algae and other eukaryotic microbes in the high-temperature, low *pH* algal mat at Nymph Creek. The microbes in this extreme environment had previously been described using only cultivation and microscopic methods of detection and identification. It is now widely recognized that classical microbiological methods provide an incomplete and inaccurate assessment of microbes in natural assemblages (Ward et al., 1992).

According to visual inspection of EtBr stained agarose gels, DNA was obtained from all environmental samples except for one, the flocculent site at Alluvium Creek. All other samples yielded PCR products of the 18S rRNA gene. The same community members at least in terms of algae were detected in samples regardless of the extraction method used, although some specimens did not yield any amplifiable DNA using any method; see Table 2 for extraction protocols used on samples. These were then cloned and identified based on sequence analysis of the 18S rDNA. A total of 215 18S rRNA gene sequences suitable for BLASTn analysis were obtained in this study. Of these, 139 originated from Nymph Creek and 75 from Alluvium Creek. Of the 139 Nymph Creek sequences, BLASTn similarities indicated that 122 were from three different algal species, 15 were from three different fungal species, and 2 were

| Genomic DNA samples | Sheehan protocol | Mo Bio Soil | Quiagen DNeasy Plant | Quiagen QIAamp DNA Stool |
|---|---|---|---|---|
| Alluvium Creek flocculent | - | - | - | - |
| Alluvium Creek log | + | + | + | + |
| Alluvium Creek rock | + | + | + | + |
| Nymph Creek 30°C | + | + | | |
| Nymph Creek 35°C | + | + | | |
| Nymph Creek 38°C | + | + | | |
| Nymph Creek 47°C | + | + | | |
| Nymph Creek 51°C | + | + | | |
| Nymph Creek Apr upstream | + | | | |
| Nymph Creek Apr downstream | + | | | |
| Nymph Creek Jun upstream | + | | | |
| Nymph Creek Jun downstream | + | | | |
| Nymph Creek Aug downstream | + | | | |
| Nymph Creek Dec upstream | + | | | |
| Nymph Creek Dec downstream | + | | | |

Table 2. Genomic DNA extraction methods for Nymph and Alluvium Creek sequences data: + successful extraction attempt, - unsuccessful attempt.

from two different stramenophile (golden algae) species. Of the 75 Alluvium Creek sequences, 22 were from a single algal species, 27 were from 3 different fungal species, and 26 were from 2 different stramenophile species. Based on the results of the BLASTn analyses, a total of 13 operational phylotypes were defined. These phylotypes and their percent sequence similarities to closest

GenBank relatives are summarized (Table 3). As seen in the second column (Table 3) the sequences of some phylotypes, such as ANC1, were essentially identical (99 to 100% BLASTn similarity) to those of known species. Other phylotypes, such as F1ACNC, did not have a high degree of sequence similarity to any described species but were most similar to those of uncultivated environmental clones detected in cultivation-independent studies (Amaral-Zettler, 2002) similar to the study presented here. It is notable that nearly half (6 of 13) of the phylotypes (Table 3) detected in our study were closely related (97-100% similarity) to uncultured sequences from an earlier study in the acidic Rio Tinto in Spain (Amaral-Zettler, 2002). In our analysis, only one sequence, that of a fungus (F1ACNC), was common to both Nymph Creek and Alluvium Creek clone libraries (Table 3). BLASTn analysis indicated that the sequence of Nymph Creek red algal phylotype (ANC1) is identical to that of *C. merolae*, (Table 3).

The dendrogram (Figure 4) illustrates that the Nymph Creek *C. merolae* phylotype clusters within a clade that contains the thermoacidophilic red algal species *C. caldarium* and *G. maxima*. Strains of a fourth red algal species, *G. sulphuraria*, form a second clade of thermoacidophilic red algae distinct from the clade containing *C. merolae*. The thermoacidophilic Cyanidales lineage is distinct from other major lineages in the phylum Rhodophyta. We noted that despite having identical 18S rRNA sequences, the Nymph Creek *C. merolae* is morphologically and physiologically distinct from all other known *C. merolae* isolates. The Nymph Creek strain is spherical, possesses a cell wall, and reproduces by formation of multiple daughter cells while all other known *C.*

*merolae* lack cell walls, are ovoid in shape, and reproduce by binary fission (Ferris et al., 2005).

Several green algae (Chlorophyta) were also detected in the study sites. The 18S rDNA sequence of one Nymph Creek phylotype, (ANC2) is 97% similar to that of *Chlorella protothecoides* var. *acidicola* while the sequence of the other Nymph Creek green alga (ANC3) is 98% similar to that of *Paradoxia multisita* (Table 3). The dendrogram (Figure 5) illustrates the relationship between the Chlorophyta found in Nymph Creek and other Chlorophyta from GenBank. In contrast to Nymph Creek, the predominant phototrophic phylotypes in Alluvium Creek were those of a single *Chlamydomonas* species (AAC1) and two golden algae (SAC1 and SAC2, Table 3). The sequence of AAC1 is 99% similar (only one nucleotide difference) to that of *Chlamydomonas acidophila* (Table 3) an

| Phylotype | closest GenBank relative | GenBank reference# | % sequence identity |
|---|---|---|---|
| Algae | | | |
| AAC1 | uncultured clone RT1n1 | AY082979 | 99% |
| ANC1 | *Cyanidioschyzon merolae* | AB158485 | 100% |
| ANC2 | uncultured clone RT5iin2 | AY082980 | 98% |
| | *Chlorella protothecoides* | AJ439399 | 97% |
| ANC3 | *Paradoxia multisita* | AY422078 | 98% |
| Fungi | | | |
| FAC1 | *Spizellomyces punctatus* | AY546684 | 94% |
| FAC2 | *Acidomyces richmondensis* | AY374300 | 99% |
| F1ACNC | uncultured clone RT3n5 | AY082969 | 99% |
| FNC1 | uncultured clone RT5iin3 | AY082996 | 97% |
| FNC2 | *Geoglossum nigritum* | AY544694 | 89% |
| Stramenophiles | | | |
| SAC1 | uncultured clone RTln9 | AY082970 | 98% |
| SAC2 | *Chrysosphaera parvula* | AF123299 | 98% |
| SNC1 | *Poteriochromas malhamensis* | AB023070 | 98% |
| SNC2 | uncultured clone RT5in36 | AY082999 | 98% |

Table 3. Phylotypes with their nearest GenBank relatives and the percent of similarity between sequences, the Rio Tinto phylotypes are prefaced by the text string "uncultured clone RT" in the second column.

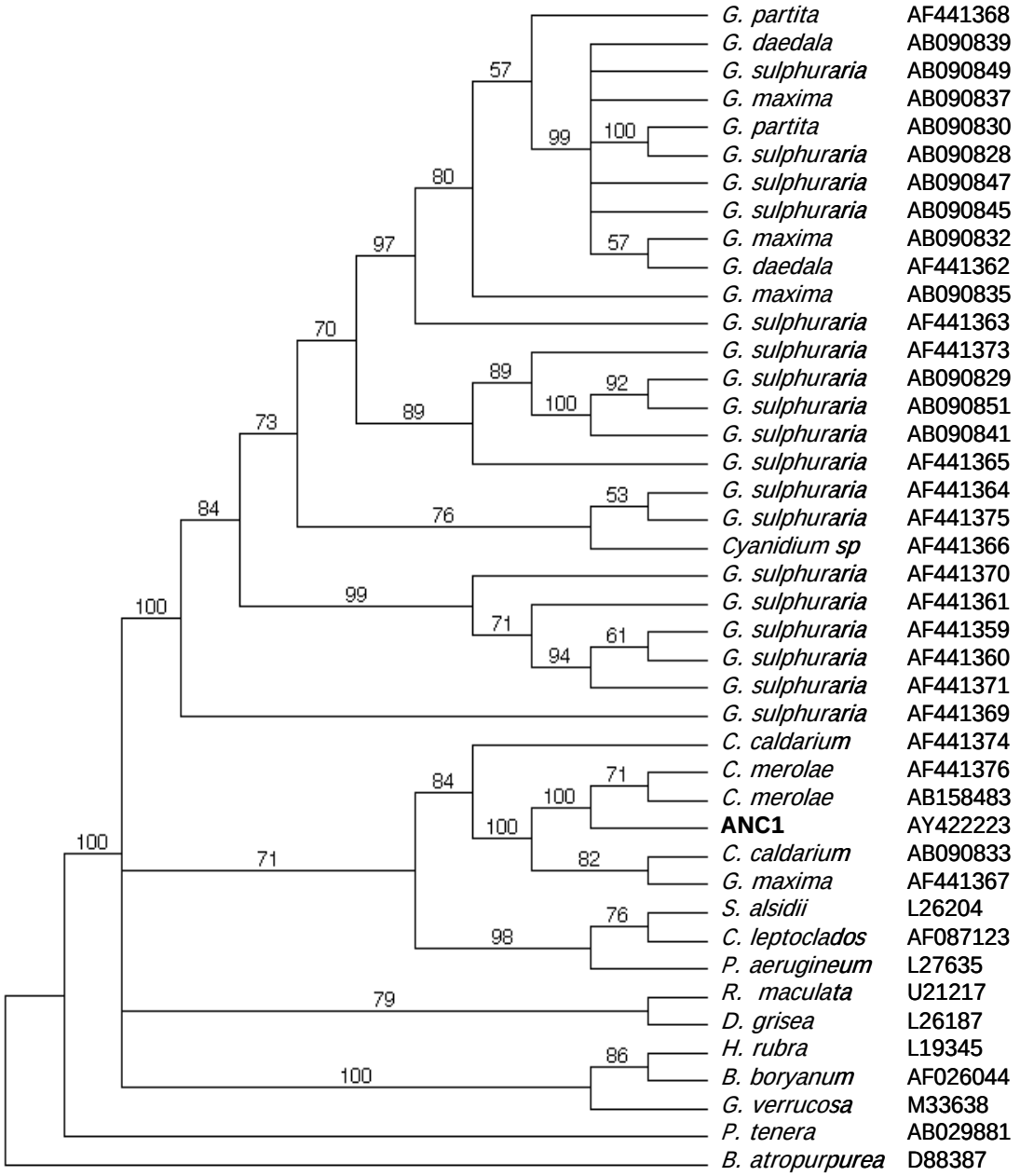


Figure 4. Dendrogram based on ~680 nucleotides of 18S rRNA genes relating the predominant Nymph Creek thermoacidophilic red alga (ANC1) to other red algal sequences. Bootstrap values (>50%) are indicated at nodes. GenBank reference numbers are listed next to species.

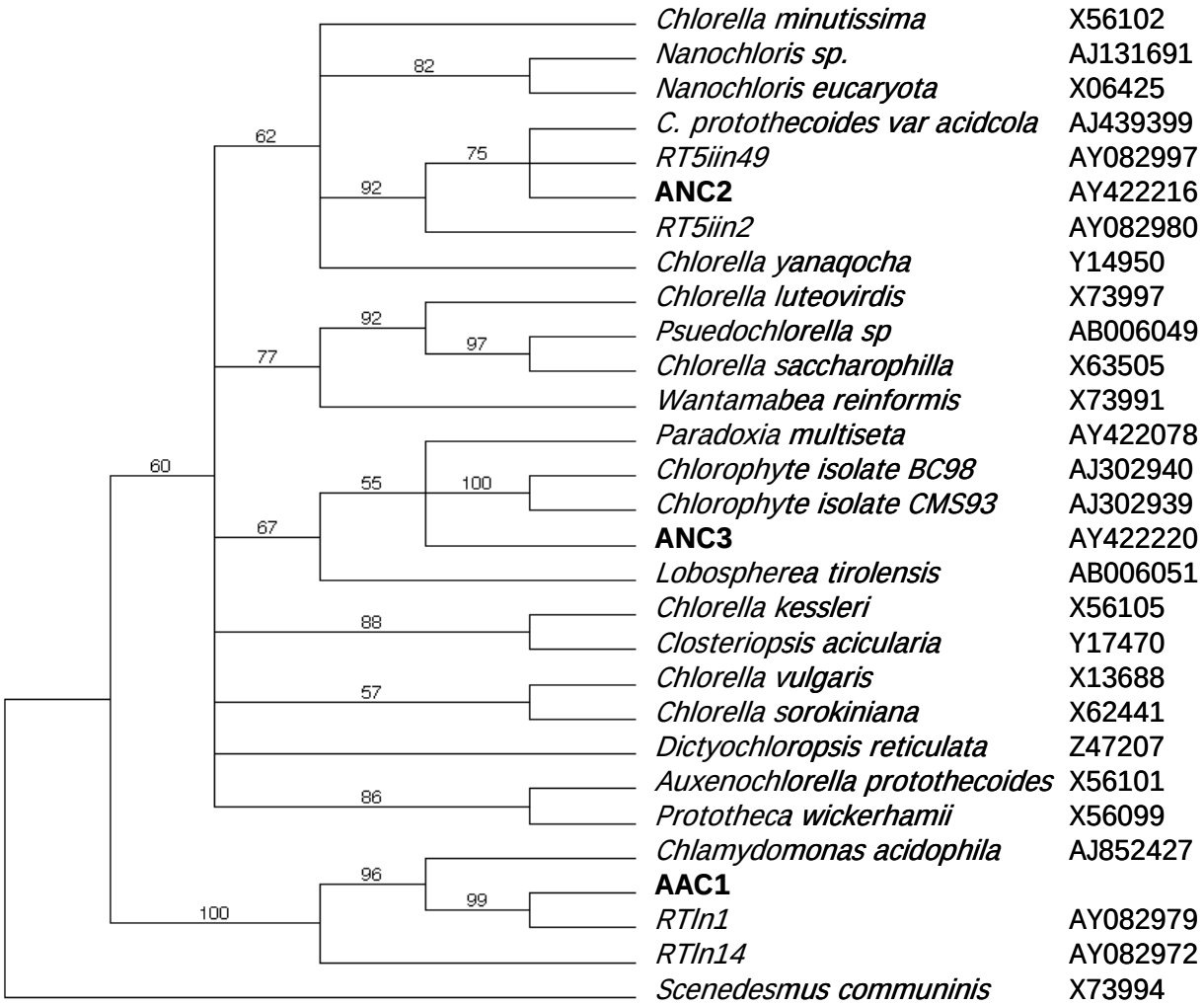


Figure 5. Dendrogram based on ~680 nucleotides of 18S rRNA genes relating green algae detected in Nymph Creek (ANC2 and ANC3) to other green algae sequences. Bootstrap values (>50%) are indicated. GenBank reference numbers are listed next to species.

organism that thrives in acidic aquatic habitats. The Chrysophycaea sequences SAC1 and SAC2 are 98% similar to those of the uncultivated algal clone RT1n9 and to the species *Chrysosphaera parvula* that is known to inhabit Antarctic lakes

(Unrein et al., 2005). There were two Chrysophycaea sequences detected in Nymph Creek. One of them, SNC1 was 98% similar to *Poteriochromas malhamensis* (Table 3) a morphologically nondescript heterotrophic nano-flagellate found in both fresh and marine environments (Berglund et al., 2005). With the exception of clones from the acidic Rio Tinto from the Amaral-Zettler study (2002) the origins of the stramenophiles from GenBank's BLASTn analysis of stramenophile sequences detected in this study are unknown.

Several fungal phylotypes were detected in each study site. One of these (F1ACNC) was the only sequence detected in both Nymph Creek and Alluvium Creek. In addition to F1ACNC, four other fungal phylotypes were detected, two in Nymph Creek (FNC1 and FNC2) and two in Alluvium Creek (FAC1 and FAC2). Two phylotypes from Alluvium Creek, F1ACNC and FAC2, group within the Ascomycota. The closest GenBank relative to the F1ACNC phylotype is the species *Tetrachaetum elegans*, an organism commonly found in aquatic habitats, and the closest uncultivated relative to the F1ACNC phylotype is a sequence from an acidic river in Spain (clone RT3n5, Table 3). The closest relative to the Alluvium Creek phylotype FAC2 is a cultivated fungus, *Acidomyces richmondensis*, which is commonly isolated from aquatic, acid mine drainage habitats. The other Alluvium Creek fungal phylotype, FAC1, clades within the Chytridiomycota. The Chytridiomycota are primitive aquatic fungi that have a motile, flagellated reproductive stage. The remaining fungal phylotypes, which were only found in Nymph Creek, FNC1 and FNC2, have no close acidophilic

relatives, and although their closest relatives are likely in the division Ascomycota, their phylogenetic position within the major fungal divisions is uncertain.

Despite the lack of concordance between phylotypes detected in the Nymph Creek and Alluvium Creek, clone libraries of both study sites were predominated by photosynthetic organisms (Figure 6). In Nymph Creek the predominant phototrophic phylotypes were those of two green (*Chlorophyta*) algae, ANC2 and ANC3, each of which accounted for 39% and 24% of the total clones detected, respectively, and one red (Rhodophyta) alga, which accounted for 24% of the total clones at this site (Figure 8). In Alluvium Creek the predominant phototrophs were a green (Chlorophyta) alga AAC1 which accounted for 30% of the total clones and a golden (Chrysophycaea) alga, SAC2, which accounted for 34% of the total clones (Figure 6).

*Temperature Gradient*

The influence of temperature on the distribution of major phototrophic phylotypes and other non-phototrophic phylotypes in Nymph Creek was evaluated. Temperature probes linked to data loggers were installed in a warm (~50°C) upstream site and a cool (~30°C) downstream site at Nymph Creek and samples were collected from these sites over annual cycles (Ferris et al., 2005). The results show that the thermoacidophilic *C. merolae* phylotype, ANC1, was the predominant (61%) organism in the warm upstream site while the two Chlorophyte phylotypes ANC2 (54%) and ANC3 (37%) were the predominant phylotypes at the low temperature site (Figure 7). One fungal phylotype, FNC2, represented 5% of the clones detected in the upstream site.

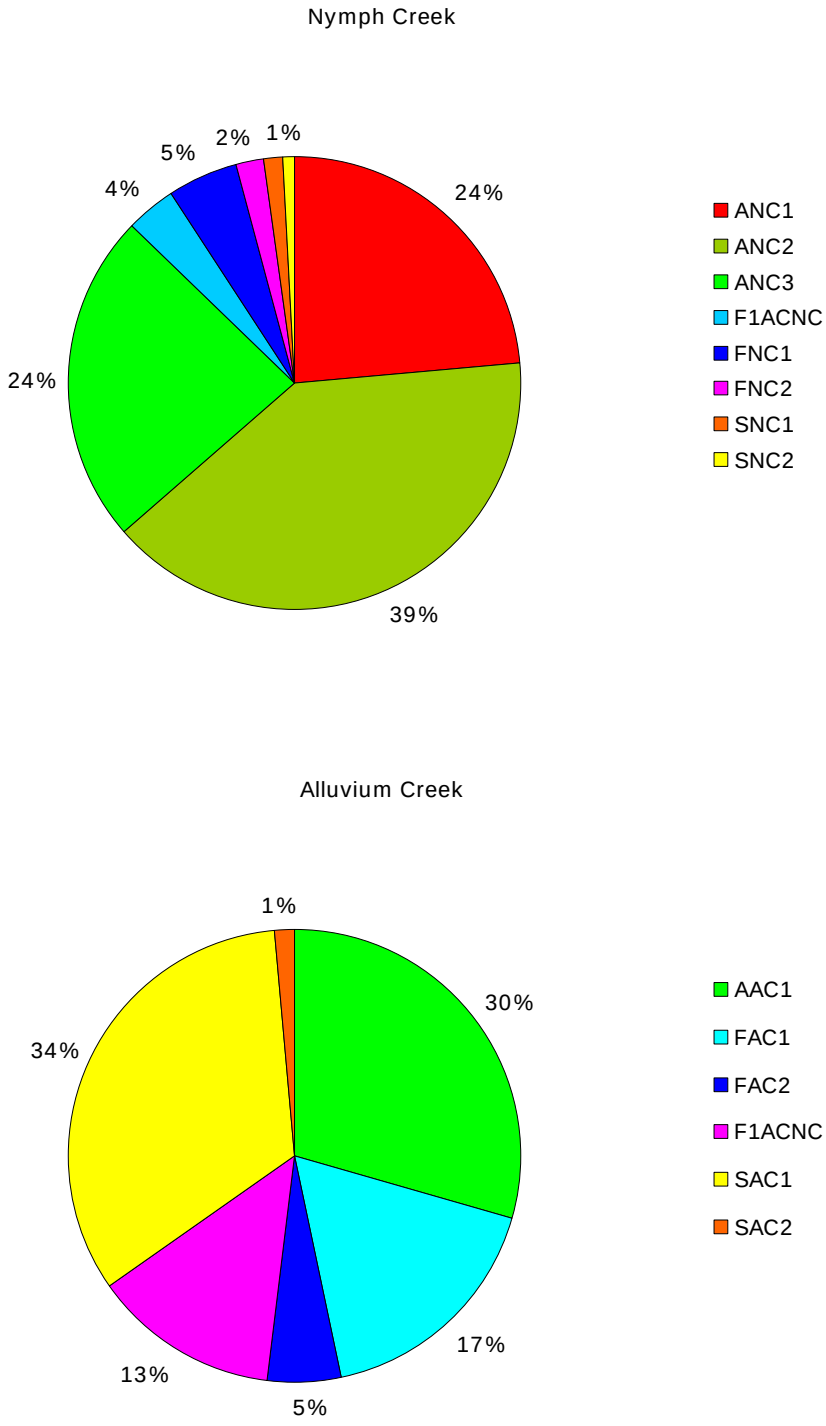


Figure 6. Pie chart showing relative abundance of sequences of each phylotype obtained from cloned PCR products from Nymph and Alluvium Creek.

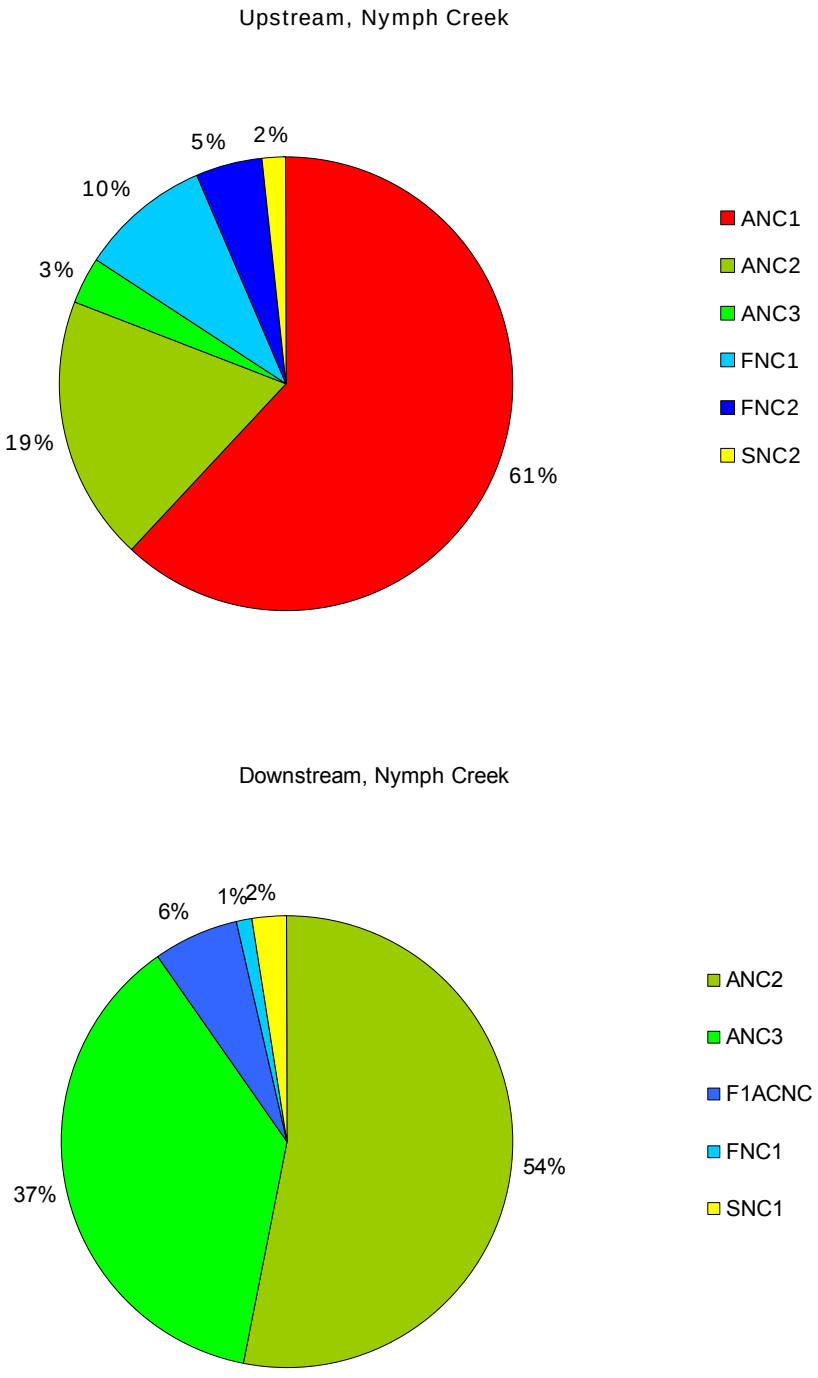


Figure 7. Relative abundance of phylotypes detected in a warm ~50°C upstream site and a cool ~30°C downstream site in Nymph Creek. Includes samples from different seasons.

*Seasonal Analysis*

To examine the hot and cool temperature distribution of these algal phylotypes more critically, samples from the Nymph Creek mat were collected not only at various temperatures along the thermal gradient but at various annual intervals (Figures 8 and 9). Although the sample size is small, a notable observation of both the thermal gradient and seasonal data was that no ANC1 phylotypes were ever detected below 38°C (Figure 8). Nine of the forty sequences obtained from the high temperature region in the seasonal analysis of Nymph Creek were of green algal phylotypes (Figure 9) and thirty of the thirty one sequences detected above 38°C in the temperature gradient data were those of the *C. merolae* phylotype (ANC1) while all algae detected below 38°C where the Chlorophyte phylotypes, ANC2 and ANC3. These three phylotypes were the only algae detected in Nymph Creek throughout the year (Figures 8 and 9).

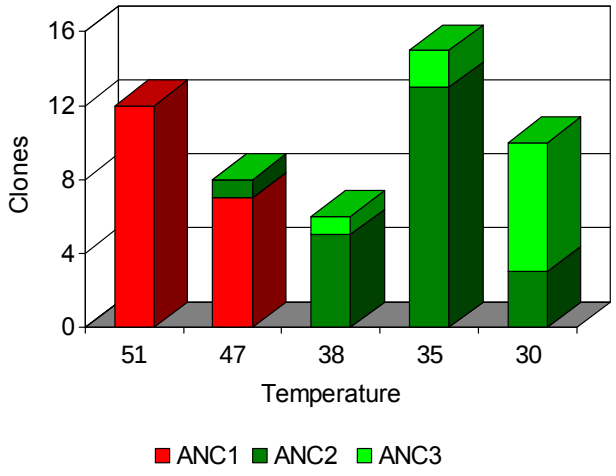


Figure 8. Algae detected along a thermal gradient in Nymph Creek

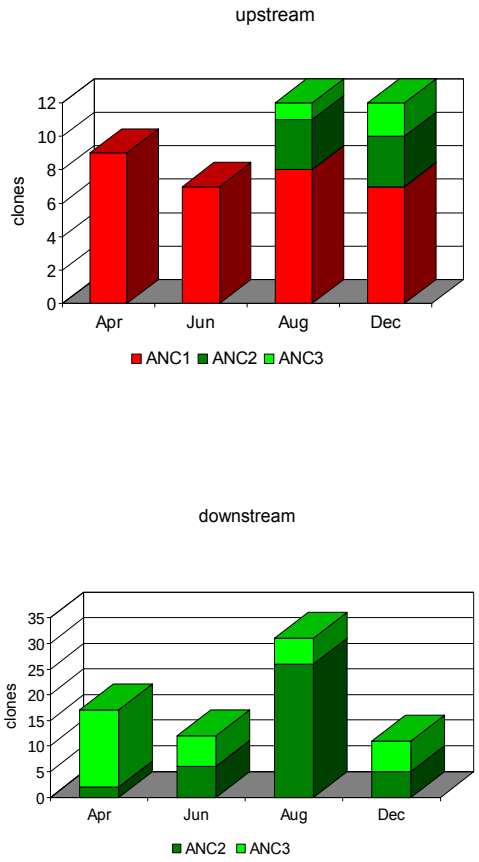


Figure 9. Algae detected at seasonal intervals in Nymph Creek at a warm upstream site and a cool downstream site.

*Alluvium Creek substrate study*

The remote location of Alluvium Creek limited our sampling opportunity to a single day (08/18/01). We noted no thermal gradient in Alluvium Creek and the temperature at all locations was 23°C. There were prominent, green-colored algal biofilms on three distinctly different substrates in alluvium Creek, (1) a

flocculent green mass in the bottom of a quiet pool, (2) a green biofilm on a log that had fallen across the stream with a swift flow of current cascading over it, and (3) a green biofilm on a stone in the stream bed (Fig. 3 C, D and E). We collected specimens at these three sites and refer to them as the flocculent site, the log site and the rock site. No PCR products were obtained from the flocculent site despite repeated efforts to obtain suitable DNA for amplification. However, 75 cloned sequences were obtained from the remaining two Alluvium Creek samples, 42 from the rock and 33 from on the log site. The percent of phylotypes found on each substrate are shown in Figure 10. The results show that the phototroph, *C. acidophila* phylotype, AAC1, was the predominant organism detected in the biofilm at the log site, representing 46% of the clones. The *C. acidophila* AAC1 phylotype was also abundant at the rock site representing 17% of the clones. The golden algal phototroph, SAC1, was the second most abundant organism at the log site, representing 27% of the clones, and it was the most abundant phylotype at the rock site, representing 38% of the clone. The golden algal phylotype, SAC2, represented only 3% of the clones at the log site and was not detected in the rock site library. The fungal phylotype FAC1 was only detected at the rock site where it represented 31% of the clones while the fungus FAC2 was only detected at the log site where it represented 12% of the clones. The fungus F1ACNC, which was detected in a clone library from the cool downstream site in Nymph Creek, was detected in both the log (12%) and rock (14%) libraries at Alluvium Creek.

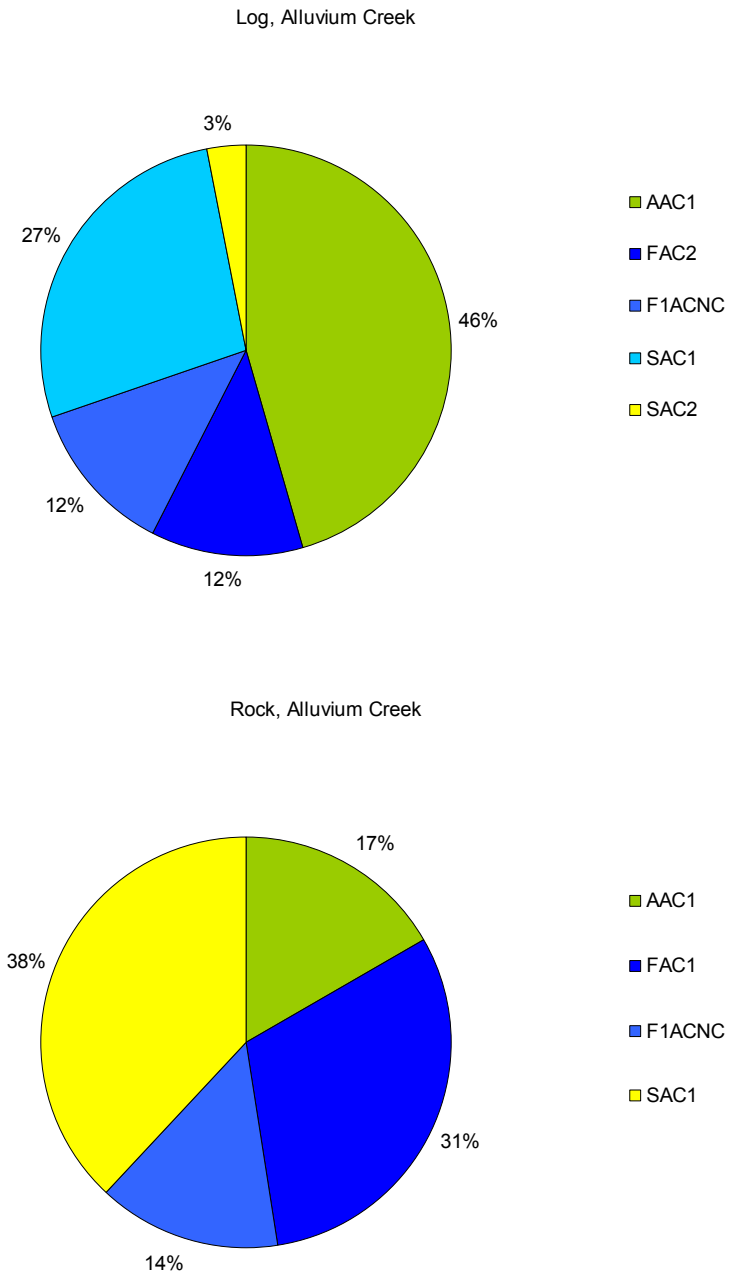


Figure 10. Relative abundance of phylotypes detected on two different substrates, a log and a rock, in Alluvium Creek. Data from a single time point sample collected 08/18/01.

## Discussion

*Characterization of Nymph Creek's Algal Community.*

Our study was the first to apply cultivation-independent molecular methods to unravel the identity of the predominant algae in Yellowstone's acidic geothermal habitats. A notable outcome of our research was the first report of a novel variety of *C. merolae*. Our results, based on sequence analysis of PCR-amplified libraries of cloned 18S rRNA genes, suggest that this single species is the predominant alga in the Nymph Creek mat at temperatures above 38°C. Axenic isolates of this Nymph Creek red alga, referred to in this report as ANC1, and as Type I in our publication (Ferris et al., 2005) were cultivated from the mat and all were found to have the same 18S rRNA gene sequence as the AAC1 sequences cloned directly from the mat. We found that the ANC1 alga has spherical cell morphology, it possesses a distinct cell wall, it has a diameter of 2-4$\mu$m, it does not grow heterotrophically and it reproduces by formation of 4 to 8 daughter cells (autospores) (Ferris et al., 2005). What makes the Nymph Creek *C. merolae* isolate unusual is that although it has the same 18S rRNA sequence as that of other cultivated *C. merolae* isolates in GenBank (AB158483), all known *C. merolae* strains are club or crescent shaped but not spherical, they reproduce by binary fission, not by production of multiple daughter cells and they lack a cell wall. Thus the Nymph Creek strain is truly unique. Recently, a molecular study of thermoacidophilic algae in a microbial mat in a warm, low *pH* stream in Yellowstone Park by Lehr et al. verified our observation (Lehr et al., 2007) of the existence of this unusual *C. merolae* strain in a second Yellowstone Park location,

Dragon Spring. More recently, a study by Toplin et al. (Toplin et al., 2008) show that this unusual *C. merolae* which they referred to as type IA, is by far the most prevalent and abundant thermoacidophilic alga throughout Yellowstone Park. The Toplin et al. (2008) study also suggests that the Yellowstone *C. merolae* is unique to Yellowstone Park, as this organism was not detected in their extensive analyses of similar warm, acidic volcanic habitats in Japan or New Zealand. A comparative genomics study of *C. merolae* and *G. sulphuraria* by Barbier et al. (Barbier et al., 2005) shows that these organisms are distantly related and that the comparatively small genome of C. merolae has retained many genes that have high similarity to those of *G. sulphuraria*. These researchers speculate that *C. merolae* may have evolved from a cell wall-producing ancestor. Thus the Nymph Creek *C. merolae* may represent a "missing link" between the cell walled and cell wall-lacking thermo acidophilic algae. The Nymph Creek strain may make an interesting candidate for genomic sequencing and comparisons.

The Toplin (2008) study also showed that there is a second "*C. merolae*-like" alga in YNP with an ovoid morphology similar to that of known strains and an alga with high 18S rRNA gene sequence similarity to that of *G sulphuraria*, however these were not as prevalent as the unusual *C. merolae*. The observation of *G. sulphuraria* is consistent with early research on high temperature regions (T > 40°C) of the Nymph Creek mat and similar acidic geothermal habitats in YNP, (Doemel and Brock, 1970) which were described as being dominated by a single thermoacidophilic, heterotrophic red alga. Doemel and Brock (1971) referred to the alga as *C. caldarium.* It is currently accepted

that *C. caldarium* and *C. merolae* are not capable of heterotrophic growth and it seems likely that these early studies were biased toward the detection of the rare *G. sulphuraria* described by Toplin (Toplin et al., 2008) since these early studies relied upon enrichment cultures and heterotrophic growth medium under dark growth conditions to obtain and subsequently identify algal isolates. Another early study of thermoacidophilic algae in YNP by de Luca et al. in the 1970's (de Luca et al., 1979) concluded that environments in the Norris Geyser Basin were colonized by two distinct unicellular eukaryotic thermoacidophilic algae, which they referred to as *C. caldarium* and *Protococcus sulphuraria* (now *G. sulphuraria*). The far more abundant alga was reportedly a small (1.5-5 *μ*m), spherical, autospore forming cell which they called *C. caldarium* and the second was a larger (2.5-8 *μ*m) spherical non-dividing cell (Deluca et al., 1979). However, algal cell size can vary widely during various stages of growth and the lack of molecular analyses in these early studies makes direct comparisons to recent studies difficult. However, due to the limited scope of our study, we cannot eliminate the possibility that other thermoacidophilic algae are present in Nymph Creek.

*Analysis of Nymph Creek Algal Mat Populations Along a Temperature Gradient.*

We performed a cultivation-independent analysis of algal diversity in the Nymph Creek mat along a temperature gradient (Figure 8). The results demonstrate that *C. merolae* (ANC1) cells are replaced by "*Chlorella*-like" algae at temperatures below 38°C, and that two different "*Chlorella*-like" algae predominate the Nymph Creek mat at temperatures below 38°C. The two

"*Chlorella*-like" organisms were designated ANC2 and ANC3. We found that the sequence of ANC2 is most closely related to *C. prototheco*ides var. *acidicola*, an acid-tolerant alga originally isolated from low-*pH* soil (Albertano and Taddei, 1984, Huss et al. 2002). This sequence is also nearly identical to a cloned 18S rDNA sequence detected in the acidic Rio Tinto in Spain, an acid mine drainage of prehistoric origin (Amaral-Zettler 2002) located thousands of miles away from Nymph Creek. The ANC3 sequence is 98% similar to that of *Paradoxia multisita* an alga of unknown origin whose 18S rRNA gene was sequenced as part of a phylogenetic analysis of "*Nannochloris*-like" algae (Henley et al., 2004).

*Chlorella* spp. typically do not flourish in high temperature environments nor do they generally form extensive mats in high temperature, low *pH,* aquatic habitats such as those found upstream in Nymph Creek. Rather they are a common component of acidic soils. Their predominance in acidic aquatic environments has rarely been recorded (Huss et al., 2002). The presence of ANC2 and ANC3 in such high abundance in Nymph Creek, and their prevalence in clone libraries in the acidic Rio Tinto in Spain, suggests that the contribution of "*Chlorella*-like" algae to the formation of algal mats in acidic streams and rivers warrants further investigation. Our detection of "*Chlorella*-like" ANC2 and ANC3 in at the warm upstream site in Nymph Creek was likely due to the presence of cooler water at the edge of the creek and to alterations in the course of warm water flow caused by sediment or debris deposition. We noted temperature fluctuations at defined sites in the upstream warm water channels over the course of our seasonal study by placing temperature sensors in the mat attached

to data loggers (Ferris et al., 2005). The lack of detection of *C. merolae* sequences in cooler regions (T<38°C) of Nymph Creek, as shown in our temperature gradient study results, is unexpected. We reasoned that washout of *C*. *merolae* cells from upstream would result in the detection of at least a few sequences downstream. However, our results suggest that the abundance of *C. merolae* is orders of magnitude lower than that of the "*Chlorella*-like" algae downstream, since we failed to detect even a single ANC1 sequence among clones in any mat sample below 38°C. We speculate that the transition from predominantly *C. merolae* to predominantly "*Chlorella*-like" algae occurs abruptly, over an interval of no more than a few feet since the nearest high temperature gradient collection site was only approximately 3 m upstream from the 38°C collection site. Brock noted a sharp line of pigment demarcating the immediate shift from green pigmented "Cyanidium-like" algae to brown pigmented diatoms at 38° in a hot spring in Japan (Brock, 1978). This suggests that transitions in microbial mat populations along temperature gradients can be abrupt rather than gradual.

Lehr et al. (Lehr et al., 2007) point out that there is a lag between cell death and cell decomposition as mats decline. This suggests that non-viable algal cells can persist in the environment and can be mistakenly interpreted as viable members of the community, especially in molecular-based studies of community DNA. This can complicate interpretations of organisms thriving under environmental conditions where they cannot exist, such as the "*Chlorella*-like"

algae we detected in the upstream, high-temperature region of the Nymph Creek mat.

*Seasonal Analysis.*

Algal mats in acidic geothermal springs are known to loose density and to become fragmented at certain times of the year. This phenomenon has been referred to as mat decline (Lehr et al., 2007) In one study of a mat in Dragon Spring decline of the *C. merolae* mat was correlated with increased UV-visible light exposure from increased photo periods in summer months (Lehr and Frank, 2007). In Dragon Spring, this increase in exposure was shown to begin in early May and last until late August. Other declines or disappearances in mats formed by thermoacidophilic algae are attributed to a drop in ambient temperature, and a resulting reduction in water temperature in winter months. Nymph Creek is largely shaded by lodge pole pines (*Pinus contortia*) (Doemel and Brock, 1971) and may be afforded some protection that limits the impact of increased photo period and exposure to high UV and visible light. In fact a recent study by Lehr et al (Lehr and Frank, 2007) suggests the Nymph Creek mat does not experience mat decline in summer months. Although we did not specifically investigate mat decline in Nymph Creek, we noted a degraded, fragmented, Nymph Creek mat (Figure 9) at the warmer upstream sites in August and December 2002 that may be due to increased light intensity and photoperiod. A fragmented mat during winter months may be caused by a reduction in light and a decrease in ambient and water temperature. Despite annual changes in ambient light and temperature, our study of the warm upstream site and a cool downstream site in the Nymph

Creek mat over an annual interval showed that *C. merolae* (ANC1) predominated the upstream site while the two "*Chlorella*-like" algae (ANC2 and ANC3) predominated the downstream site over an annual cycle. No other algal species were detected, suggesting that these three algae predominate the Nymph Creek mat throughout the year.

*Alluvium Creek Comparison.*

Dispersion mechanisms and distribution of mat forming acidophilic algae have not been well studied. To explore the distribution of acidophilic algae, and other eukaryotic microbes, in aquatic acidic habitats in Yellowstone Park, we surveyed eukaryotic microbial populations in algal mats in a non-thermally heated acidic spring, Alluvium Creek, located approximately 30 miles from Nymph Creek, and compared the Alluvium Creek species to those detected in Nymph Creek. We speculated that Alluvium Creek mats were likely to be colonized by at least some of the same low-temperature species found in the Nymph Creek mat, since water temperature, *pH* and chemistry are similar in both systems. Possible mechanisms of dispersion of microbes include transportation by animals, such as waterfowl and bison. For example, green algae and diatoms cling to the feet of migratory birds and acidophilic algal cells can pass through their acidic digestive tracks (Toplin et al. 2008). Wind and rain can also carry algae and other microbes. Presumably these mechanisms can lead to homogenization of acidophilic algal species in aquatic acidic habitats throughout the Park. Since there is no geothermally heated input, and thus no temperature gradient in Alluvium Creek, we sampled the microbial communities in algal mats

that formed on two different substrates, a log and a stone. We found that only one phylotype, a fungus (F1ACNC), was present in Alluvium Creek and Nymph Creek. This fungal phylotype was detected in both the log and the rock biofilms in Alluvium Creek and in the cooler water of the downstream site in the Nymph Creek mat. We did not detect the two chlorella-like algae, ANC2 and ANC3, which predominated the cooler regions of the Nymph Creek mat, in either of the Alluvium Creek mats. Both the log and rock mats in Alluvium Creek contained an abundance of 18S rDNA sequences that were 99% similar to that of *Chlamydomonas acidophila* (AJ852427), an organism known to inhabit acidic lakes (Gerloff-Elias et al. 2005) and to an uncultured clone RT1n1 (AJ783841-44) detected in the Rio Tinto in Spain (Table 3). In addition *C. acidophila*, a golden alga (SAC1) was abundant in both Alluvium Creek mats. Possible reasons that few of the same species were detected in both Nymph Creek and Alluvium Creek may include differences in water chemistry between the two sites that were unknown to us at the time of sampling, or to the small number of clones identified in each stream. Another reason may be that, in contrast to Nymph Creek, microbes in Alluvium Creek are exposed to more extremes in cold temperature due to the fact that Alluvium Creek is not geothermally heated. Even in the downstream portion of Nymph Creek in the middle of winter, water temperatures are near 20°C. Exposure to freezing temperatures in the winter months at Alluvium Creek may prevent the Nymph Creek algae from thriving in Alluvium Creek, as these species would likely be killed or dramatically reduced by freezing temperatures. Despite our failure to detect the same algal species in both the

Nymph Creek and Alluvium sites, our study and other studies show that some microbial species are widely distributed throughout geothermal environments, both within Yellowstone and in more distant geothermal locations. For example, many of the sequences detected in our study matched those detected in the Rio Tinto, an acidic river in Spain. Their detection in such geographically distant locations suggests that these microbes may be widespread in low-*pH* habitats, and that microbial communities in Yellowstone are not isolated from colonization events. In fact, molecular surveys have shown that microbial population-homogenizing events do occur within geothermal habitats in Yellowstone Park. For example, the novel Nymph Creek *C. merolae* strain, initially reported in the high-temperature region of the Nymph Creek mat, has been shown to be widely distributed throughout Yellowstone Park's acidic geothermal habitats (Toplin et al., 2008). Also, unicellular mat-forming cyanobacteria (*Synechococcus* spp.) and "*Chloreflexus*-like" green non-sulfur bacteria are widely distributed throughout Yellowstone Park's high temperature alkaline springs (Ward, 1998). The amoeba, *Neagleria fowleri* and *Legionella*-like bacteria, both found in Nymph Creek, have been shown to exist in warm geothermal springs throughout Yellowstone Park (Sheehan et al., 2003).

Fungal populations in geothermal soils in Yellowstone Park have been described (Redman et al. 1999, Cullings and Makhija 2001). In our analyses, fungi accounted for 11% of the total sequences detected in Nymph Creek and 35% of those detected in Alluvium Creek (Figure 6). In Nymph Creek, fungal sequences were most abundant in the upstream mat, accounting for 17% of

sequences detected (Figure 7) and were most abundant in the Alluvium Creek rock mat, accounting for 45% of sequences (Figure 10). However, none of the algal mat fungal sequences were related to those detected in a study of fungi in Yellowstone's geothermal soils (Redman et al. 1999, Cullings and Makhija 2001). Almost certainly, fungi play a role as decomposers in algal mat communities and contribute to nutrient cycling. Perhaps fungi contribute to the phenomenon known as mat decline described above. Future surveys of all the eukaryotic microbes that thrive in the extreme environments of acidic and geothermal mats are necessary to paint a more complete picture of the diversity and distribution of species in these unique extreme habitats.

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# Vita

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