1. RATIONALE:

Aquatic microbial food webs and loops including prokaryotes, protists, and their viruses have been the subject of numerous investigations over many decades. These assemblages dominate marine and freshwater systems, contribute over half the world's primary production, and have complex control and regulation (12, 33, 57, 69, 84, 89). In the past decade, there have been a number of excellent studies documenting prokaryotic and geochemical diversity in a variety of neutral pH thermal (8, 11, 45, 48, 61, 66, 67, 72, 74, 80, 93, 97), and acidic thermal (3, 13, 18, 28, 29, 36, 47, 51, 55, 60, 98, 100) systems. While there have also been some notable investigations of microbial eukaryotes (4, 5, 23, 28, 83, 94), and viruses (15, 54, 73, 90) in extreme environments, we lack a comprehensive integrated understanding of the basic ecological parameters of many extreme habitats. For example, what are the primary producers in these environments, and what are the relative contributions of chemosynthesis and photosynthesis to primary production? What are the relative effects of resource availability (bottom-up), predation and parasitism (top-down), and physical/chemical controls on the growth, abundance and diversity of microorganisms in extreme environments? In recent years, the study of microbial food webs in extreme environments has garnered attention from ecologists and microbiologists interested in answering these questions and uncovering new ecological paradigms through the examination of species-poor food webs (56, 58, 62, 63, 105). Each habitat provides a unique combination of conditions that will contribute to our understanding of food web ecology. Here, we combine expertise in prokaryotic, eukaryotic and viral biology to propose a Microbial Observatory (MO) in Boiling Springs Lake (BSL), a unique acidic (pH 2), low metal, low chloride, hydrothermal lake. All three domains of life are present in the lake, but the absence of metazoans suggests BSL as a good model system for the study of food web ecology. This MO proposal seeks to develop an understanding of the abundance, distribution, and diversity of prokaryotes, eukaryotes and viruses in two thermally distinct regions of BSL, and to examine the stability of these populations over time. Additionally, we will examine the contributions of photosynthesis and chemosynthesis to the productivity of BSL, and initiate studies on the relative importance of top-down vs. bottom-up controls on the diversity and function of this microbial community.

2. RESUBMISSION-RESPONSE TO REVIEWER'S

The reviews for our first submission were very favorable and indicated strengths in terms of site selection, investigation of the diversity of all members of the microbial community (prokaryotes, eukaryotes, and viruses), probability of discovering novel life forms, and our educational and broader impacts. In this resubmission, we address criticisms about poor referencing of literature, inadequate connection of overall goals to our methods, and over-statement of the uniqueness of our study. In this resubmission, we have referenced more of the relevant studies on extreme environments (though we have undoubtedly missed some). We also incorporated additional methods that will better address how viruses impact prokaryotic and eukaryotic diversity (top-down controls), and we are examining productivity and autotrophic potential to begin to evaluate bottom-up controls in this system. We have begun cultivation efforts (Summer 2005), and expanded the proposed cultivation studies for all members of the community.

3. SITE DESCRIPTION:

Lassen Volcanic National Park (LVNP) in northern California is situated at the southern end of the Cascade Range. Within park boundaries are several high-elevation, acid-sulfate, low chloride springs characteristic of vapor-dominated hydrothermal systems (50). The park is relatively understudied, and very few studies of microbes at LVNP have been published (16, 38, 85, 107). Preliminary work by the PIs indicate abundant novel biological and geochemical diversity in a number of high-temperature (T), acidic geothermal features in LVNP (40, 43, 85-87, 110) (see Preliminary Results).

Boiling Springs Lake Thermal Area (BSLTA) is a pristine, high-elevation (1805 m) designated wilderness area of LNVP that includes a large, acidic (pH 2), hot (52˚C) lake known as **Boiling Springs Lake (BSL),** several weakly pressurized fumaroles, and small hot springs and mud pots of variable T and pH **(Fig. 1).** BSL occurs in an area of weakened rock along a fault zone, and is geologically and

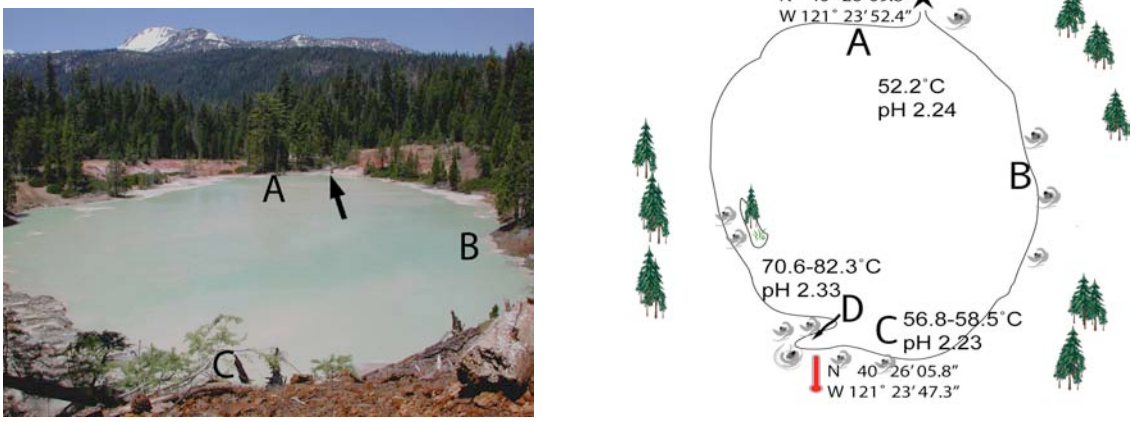

Fig. 1. Photograph and schematic of BSL. (a) view from cliff above S end of the lake; proposed sampling sites A, B, C, and outflow (arrow) are indicated (b).The surface T and pH of several locations around the lake periphery are constant (~52˚C, pH 2.24), except for warmer and more variable temperatures at sites C and D. Several high T springs and mudpots on the periphery are indicated by grey swirls. GPS positions and pH/T data from July 17, 2004.

chemically distinct from caldera-type volcanic lakes typified by the Uzon Caldera (Kamchatka, Russia) and Frying Pan Lake (New Zealand), and from high chloride, lower sulfate features characteristic of the liquid-dominated geothermal systems in Yellowstone National Park (YNP) (24, 52). The BSLTA was extensively studied by USGS geologists from the 1980's to mid-1990's (50, 65, 92, 99). In 1988, the lake area covered approximately 12,000 m^2(92), and the entire BSLTA covered approximately 14,000 m^2. Therefore, BSL is approximately 1/3 the size, and significantly more acidic than the world's largest hot spring - Frying Pan Lake (pH 3.5, 50˚C).

Since the summer of 1999, we have determined approximate lake area, T and pH along lake margins throughout the summer months (June-August), and these values agreed with previous estimates suggesting this is a relatively stable feature. BSL surface water has an average summer T of ~52 +/- 3˚C, and an average pH value of 2.0 +/- 0.4. Winter water temperatures may be significantly cooler. At the northeast end of the lake is a small outflow stream that is typically dry by mid-July. The southern end of the lake appears to be the hottest region of BSLTA, where actively bubbling springs and mud pots are continually present, and T ranges from 65-95˚C, even though many of these features are in direct contact with lake water. On the west and east sides of the lake are a variety of springs ranging in T from 25-93˚C, pH from 1-4.5.

4. PRELIMINARY RESULTS:

Geochemistry: While different thermal features within the same thermal region vary widely in chemical composition (52, 85, 87, 99), conductivity and total dissolved solids (3520 S cm-1, and 1071 ppm, respectively) fall within the middle range for other LVNP sites analyzed. Geochemical analyses of BSL water (from 2004) indicate a fairly high SO42- concentration of 636 ppm (~8 mM), and low Cl- concentrations of 0.32 ppm (~9 µM) (85-87). These data are similar to those reported for this site in the mid-1980's (99). Fe, Mg, and Mn are present within BSL water at 29.8 ppm, 4 ppm, and 16ppm, respectively. All other metals (with the exception of Al and Si) were found at fairly low concentrations (data not shown). NH4 is present at 0.8 ppm (~44 µM), while NO3- is less than 0.02 ppm. Dissolved organic carbon (DOC) was measured at 1.2 mg/L (summer 2004). Gas analyses of features within the BSLTA from the mid-1990's indicate that mole percent dry weight of the volcanic gases emitted from various sites within the BSLTA are ~88% CO2, 6% N2, 4.7% H2S, 0.28% H2, 0.12% Ar, 0.072% CH4, 0.0015% NH3, 0.008%O2, 0.0007% He, with a gas/steam molar ratio of 0.0024 (52).

Microbiology: All PIs and their students have coordinated efforts to conduct joint visits to BSL (and other areas within LVNP) and obtain preliminary data on microbial diversity. Since 1999, 57 undergraduate research students have been involved in cultivation, cloning and sequencing efforts in LVNP projects. (see RUI Impact Statement).

i. Prokaryotes – Siering & Wilson, and undergraduates in their laboratories, have generated 16S rRNA gene libraries from BSL site A (Fig. 1) sediment using three different primer sets (Table 1). In each of the 3 clone libraries, the most numerous clones exhibit low sequence identity (< 88%) with cultured organisms. The A1116R and the U1406R libraries primarily contain Archaeal phylotypes that share <85% identity with the closest described isolates. Some of these are more closely related (97-98% identity) to environmental sequences retrieved from a 60°C, pH 3 sulfate-chloride spring in YNP (51). The P1525R library contained primarily Bacterial phylotypes that share <88% identity with described isolates. Both the 1406R and 1525R libraries have a few clones that show high similarity (≥97%) to common soil/water bacteria that are unexpected in the conditions prevailing at BSL. There was overlap between the detected phylotypes with the different primer sets, but each library contains sequences not detected in the other libraries, reflecting the biases inherent in this approach. Rarefaction analyses indicate that the A1116R and 1406R libraries were sampled sufficiently to determine the majority of predominant phylotypes in the library; analysis of the 1525R library is continuing. An 18S rRNA gene library created from Site A water using the primer set Ek82F/U1200R contained exclusively fungal sequences that shared 96-98% identity with described fungal species. 16S rRNA gene libraries have also been constructed from DNA extracts of site A and site D water samples, and sequencing of these libraries is in progress.

Table 1. Common Prokaryote Phylotypes (PT) in BSL clone libraries

PT	Closest Cultured Match in databases	A1116R library	U1406R library	P1525R library
1	*Thermofilum pendens* (83%)	74/80	11/165	
2	*Staphylothermus marinus* (84%)		75/165	
3	*Desulfotomaculum* sp. (88%)		1/165	64/169
4	*Picrophilus oshimae* (85%)		35/165	
5	*Hydrogenobaculum sp.* (96%)		2/165	12/169
6	*Thermoplasma acidophilum* (98%)		10/165	
7	*Sulfobacillus acidophilus* (92%)			9/169
8	*Fervidobacterium islandicum* (82%)			8/169
9	*Comamonas acidovorans* (99%)		1/165	7/169
10	*Sulfobacillus thermosulfidoxidans*(93%)			7/169

16S rRNA genes were amplified from environmental DNA extracts, cloned, and sequenced in both directions. Libraries were constructed using U515F forward primer and three different reverse primers (7, 71). Percent identity was determined by gapped BLAST analyses (2).

Crude estimates of diversity in Site A water and sediments were also made by studying Terminal Restriction Fragment Length Polymorphisms (T-RF). The sequence data from the clone libraries allows us to gauge the most effective restriction enzymes for discriminating between predominant phylotypes, and it also allows us to assign T-RFs of a particular fragment length to a tentative phylotype. The observed T-RF chromatograms were reproducible and similar to those predicted from the clone library analysis (data not shown). Fragments derived from the novel phylotypes detected in the clone library were also detected in the T-RF chromatograms, but peak height could not be predicted based on clone library abundance, presumably due to cloning biases and PCR specificity changes associated with the use of labeled primers in T-RF studies. The 515F/1406R T-RF profile produced using DNA extracted from the overlying water was distinct from but comparable to that produced using sediment DNA. Of the fourteen predominant fragments detected in the sediment sample, six were also detected in the water sample. One of the two remaining fragments in the water sample was predicted from the sediment clone library. The relative abundance of shared fragments differed between the profiles obtained with the two samples.

Acridine orange direct counts (AODC) indicate approximate cell concentrations of 2.1×10^8 cells/ml sediment, and 1.2×10^6 cells/ml in water (2002 data) (85, 87). A Humboldt State University (HSU) undergraduate student previously obtained isolates from BSL that were identified (16S rDNA sequence) as a *Geobacillus* spp., *Sulfobacillus* spp. and *Alicyclobacillus* spp.. Only the *Sulfobacillus* sp. had been identified by the cloning efforts. The Stedman lab has cultured *Sulfolobus*-like organisms from high-temperature, acidic springs on the east side of BSL, but none from the lake itself. During the summer of 2005, Siering and 2 undergraduates (supported by HHMI and REU summer research fellowships) initiated efforts to cultivate heterotrophic prokaryotes from BSL sediments and waters. Liquid and solid

media were prepared using filtered, autoclaved, pH-adjusted site waters. Gelrite-solidified media were prepared at pH 2.9, 5, and 7. Agarose-solidified media were prepared at pH 2. Liquid media were also prepared at pH 1, 2, and 3. Incubations (+/- O_2) were done at 30, 50, and 70˚C. We used 3 different concentrations of organic amendments: peptone and tryptone at 5, 25, 250 mg/L; yeast extract and glucose at 10, 50, 500 mg/L. Vitamins and trace minerals were also added to all media. We prepared frozen stocks of 98 putative isolates representing all isolation conditions, and are presently determining the identity (16S rRNA gene sequence), and initiating physiological studies on a subset of these in the Genetics Lab and Bacteriology classes taught by Wilson and Siering, respectively at HSU.

ii. Eukaryotes –Wolfe's lab has been characterizing eukaryotic SSU rRNA diversity in acidic, high T samples from various areas within LVNP, including the BSLTA, over a wide variety of temperatures. After optimizing nucleic acid extraction and amplification protocols to maximize apparent eukaryotic diversity (by 18S rRNA gene-targeted DGGE), we found autotrophic protists to be the dominant eukaryotes; heterotrophic protists and fungi are also present, especially at lower temperature sites. Most sequences have high (>95%) similarity to other acidophilic chlorophycean and stramenopile taxa, with alveolates and cercozoa also represented (17).

At BSL, we believe there is a significant, if limited, protist community. SSU rRNA analysis also suggests the presence of chlorophytes (*Chlorogonium*) and chrysophytes (*Poteriochromonas*) at BLSTA, but we believe the dominant alga is the acidophilic rhodophyte *Cyanidioschyzon* and its close relative *Cyanidium*. We have detected these in 18S rRNA clone libraries from lake waters and side pools, observed them in micrographs, and cultured them following enrichments. Curiously, we have not found Cyanidiaceae at other LVNP sites using 18S rRNA methods, despite prior reports of their enrichment from the park (16, 37).

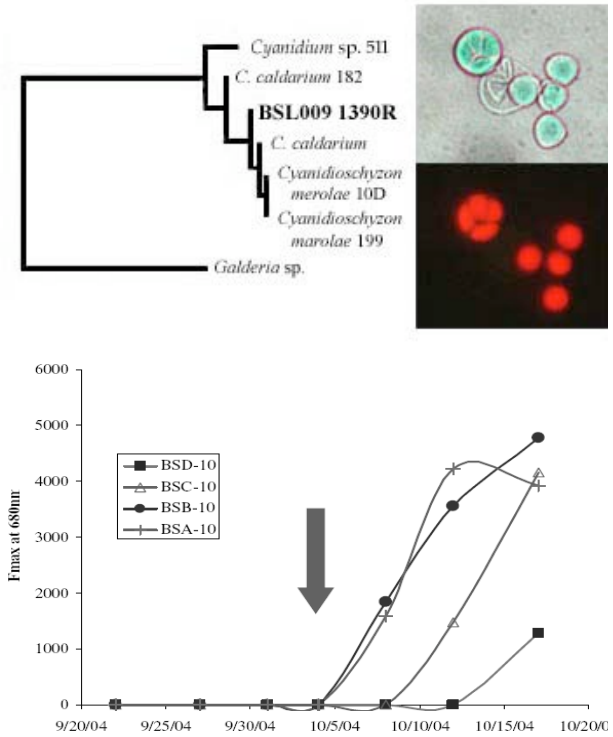

Fig 2. Enrichment of *Cyanidium* from BSL. Top left: 18S rRNA phylogeny of DNA extracted from BSL water column. Top right: brightfield and chlorophyll autofluorescence of *Cyanidium*-like cells from enrichments. Bottom: chlorophyll fluorescence of lake water inoculated into *Cyanidium* medium (pH 2.2) and incubated at 54 °C in 16:8 light:dark cycle. Arrow shows time of bicarbonate addition.

While we suspect the Cyanidiales are likely the main photosynthetic eukaryotic taxa at BSL, it is not clear if they predominate as planktonic or benthic, what limits their productivity, and the extent to which they contribute to food web dynamics. While light penetration is low due to turbidity, BSL has several shallow near-shore shelves that have visible benthic photosynthetic mats, and acidic mining lakes can support considerable benthic primary production (59). Enrichment studies suggest possible CO_2 limitation (Fig. 2), well documented for other acidic lake environments (76), and although most members of this group appear to grow well both auto- and heterotrophically (37), temperature likely imposes a strong limitation on their distributions as well. We suspect that populations may be higher during cooler water column temperatures presumably present in winter months.

Although Cyanidiales physiology, genetics, and taxonomy have been well studied (23, 81, 82), their ecology, and especially their interactions with prokaryotes, grazers, and viruses are still virtually unknown. We have not yet identified any heterotrophic protists in BSL waters. However, cloning has revealed 18S rRNA genes for acidophilic and thermophilic ciliates and cercozoans at other sites in LVNP

that have similar pH and T ranges to BSL (17), and cooler acidic mine lakes can support populations of ciliates and other heterotrophic protists (68). The Cyanidiales can also excrete large amounts of organic matter to fuel acidophilic prokaryote or fungal growth (9). Both Wolfe's and Siering & Wilson's groups (43) have detected fungal SSU rRNA sequences at BSL, and speculate these may be fueled by both algal excretion and DOC from allochthonous forest leaf litter. In preliminary studies, fungi dominate enrichments from leaf and wood litter in BSL water incubated at room temperature, while *in situ* temperatures favor prokaryotes.

iii. Viruses. Stedman's lab has sampled extensively at high T (>70 ˚C) and low pH (<4) locations elsewhere in LVNP as part of the MO-grant "Viruses from Yellowstone Thermal Acid Environments". Recently they have isolated *Sulfolobus*-like organisms and have fusellovirus-like (SSV-like) sequences from peripheral springs at BSL (unpublished). The vast majority of BSL is too cold for *Sulfolobus* (<70˚C), so other viruses are likely to be found there. We have collected ca. 30 L samples of BSL water for microscopic analysis. Algal Phychodnaviruses or their relatives should be easy to identify because of their large icosahedral morphology (102, 103). Direct virus counts in BSL water have been low (10^3 ml^{-1}, Stedman and Rohwer, unpublished) but similar to other high-T low pH systems.

5. GOALS AND OBJECTIVES

The temperature, pH, and geothermal inputs of BSL make it conducive for interactions among active communities of Bacteria, Archaea, microbial Eukarya, and viruses, while almost certainly excluding metazoans. BSL has the advantages of being accessible (~3 km from trailhead), and tractable for research, since we can safely sample from various locations around the entire lake margin. The existence of pH, T, and geochemical gradients created by hot springs that are physically connected to this large, high temperature, acidic lake make BSL an exciting potential MO site.

A. Overall Project Goals and Basis for Collaboration.

Prior studies of acidic, warm lakes and hot springs have largely focused on organismal autecology or biogeography, examining specific environmental adaptations, but not on interactions between organisms. We propose to combine our expertise in a multidisciplinary team to explicitly study the synecology of the unique, probably exclusively microbial ecosystem that BSL provides. **This MO proposal seeks to develop an understanding of the abundance, distribution, and diversity of prokaryotes, eukaryotes and viruses in two thermally distinct regions of BSL, and to examine the stability of the populations over time. Additionally, we will examine the contributions of photosynthesis and chemosynthesis to the productivity of BSL, and initiate studies on the relative importance of top-down vs. bottom-up controls on the diversity and function of this microbial community**.

To address these goals, we will use a combination of field, molecular, and culture-based studies. All PIs involved in this project are interested in factors that control microbial ecosystems, yet each researcher has a different, complementary, primary expertise. Siering and Wilson (HSU) both provide strengths in prokaryotic molecular ecology, isolation, characterization and microscopy; however, each of them is expert in various parts of these larger activities and will integrate the project into different courses at HSU. Wolfe (Chico State University, CSUC) is experienced in limnology and oceanography, eukaryotic molecular ecology, and isolation of protists and characterization of their grazing. Stedman (PSU) provides strengths in viral genetics, enrichment and cultivation of thermophilic and acidophilic Archaea. The specific tasks are detailed below and in the Management Plan of this proposal (see Supplementary Documents).

We are all educators and consider research and education to be inseparable. **Our RUI project will involve numerous undergraduate students in both independent research and class activities. It will provide direct research training of an estimated 100 undergraduates, and impact an additional 700 undergraduates through incorporation of this work into class activities and projects.** The specific educational goals and impacts are detailed below and in the RUI Impact Statement (see supplementary documents).

B. Scientific Objectives and Hypotheses.

1. <u>Characterize site</u>: What are the physical and chemical characteristics of BSL, and how do they vary over time and space?

We speculate that T may increase with depth and there will be multiple subsurface inputs to the lake, although the location, depth and temperature of these inputs are unknown. We also hypothesize that the geochemistry will vary widely between the hot vs. the warm sites, but that it will be consistent at the temporal scales at which we are sampling. We expect light penetration to be limited to the top 0-few meters; prior reports of acidic lakes have found Secchi depths of as little as 10 cm (112).

2. <u>Determine genetic and organismal diversity</u>: What are the numerically dominant prokaryotes, eukaryotes and viruses in BSL identified by culture independent efforts, and how does the diversity vary along temporal and spatial scales? Can we devise culturing strategies to target dominant or novel prokaryotes, eukaryotes, and viruses identified by our culture-independent methods?

We expect uncultivated Archaea and Bacteria to dominate the warm site, and Archaea to dominate the hot site. Several lithoautotrophic prokaryotic genera are expected to be present in BSL water and sediment, while *Cyanidium*-like rhodophytes and acidophilic chlorophytes will likely be the dominant eukaryotes.

We expect microbial abundance and diversity to decrease in the hot vs. the warm portion of the lake, and we hypothesize a greater abundance of all microbial players (prokaryotes, eukaryotes, and viruses) in the sediments (vs. the water samples). We do not expect to find temporal variations on a daily scale; however, we do expect to find monthly, seasonal, and annual variations in diversity. For example, monthly variations in virus diversity have been observed in small high-T low pH springs in YNP (91). We predict that viruses will be the only mechanism of top-down control in BSL despite their low abundance.

3. <u>Cultivate novel organisms</u>: Devise culturing strategies for dominant or novel prokaryotes, eukaryotes, and viruses identified by our culture-independent methods.

We predict we that we will isolate novel microorganisms and viruses from BSL using the approaches described in the Methods section of this proposal (also see Preliminary Results). We predict we will isolate species that are not expected to be present in an acidic lake by using 'non-extreme cultivation conditions indicating that some organisms may survive the conditions of BSL without actively metabolizing or growing.

***4. <u>Determine the main resources in the BSL ecosystem (bottom-up controls)</u>*:** Who are the primary producers in BSL? What are the relative contributions of chemosynthesis vs. photosynthesis, and how does primary production vary spatially? What are seasonal and annual rates of productivity, and is BSL's net productivity autotrophic or heterotrophic? What fuels heterotrophic production: autochthonous primary production, or allochthonous leaf litter?

We predict a significant contribution to primary production from chemosynthetic Archaea and Bacteria since BSL's temperature, turbidity, and acidity likely combine to create a challenging environment for phototrophs. We expect photoautotrophy will be light-limited due to high lake turbidity, and it will therefore be restricted to the lake surface water and to shallow margins of the lake where *Cyanidiales*-type algae are microscopically detectable. Based on pH-limited CO_2, autotrophy may also be C-limited since *Cyanidium* enrichments respond strongly to bicarbonate additions (see preliminary results). BSL's summer temperature is at the upper limit for *Cyanidium*; however, freshwater inputs during early spring snow melt may moderate conditions enough to sustain blooms that contribute to annual autotrophy.

Because of fairly low DOC levels and expected primary producer excretion, we expect heterotrophic productivity to be fueled primarily by allochthonous leaf litter. Depending on substrates, heterotrophic productivity may well exceed primary, making the lake net heterotrophic on a yearly basis. However, viral lysis may provide considerable DOC for heterotrophic growth.

5. <u>Determine if viruses control abundance and/or diversity of ecosystem members (top-down controls)</u>: What is the relative effect of viruses on prokaryotic diversity in a low vs. high diversity ecosystem? Are primary and secondary production controlled by viruses or grazers?

We expect that if both viral and protist populations are low, then top-down control of prokaryotic populations is less likely. Assuming that the overall diversity is greater in the warm vs. hot portion of the lake (see above), we hypothesize that virus amendments will more strongly affect prokaryotic abundance and diversity changes in the hot (less diverse sample) vs. the warm (high diversity) sample. Total cell counts (AODC) from both sites indicate approximately twice as many cells/ml in the warm (vs. hot) site (August 2004 samples). In the high diversity site, each individual species might be below the threshold concentration to support a viral population depending on virus stability. In the low diversity site, species could conceivably get to a high enough density to support a viral population.

C. Educational and Outreach Goals:

In the preliminary phases of this work, the PIs have involved nearly 210 undergraduates in LVNP research and class projects. We expect an additional 800 students to be involved during the project period (see RUI Impact Statement, Supplementary Documents). The major educational goals of this RUI-MO project are to (a) fully immerse undergraduates in all phases of the endeavor, and (b) integrate this research into a broad range of educational experiences, materials and products targeted at undergraduates and the general public. To achieve these goals, we will:

1. Engage a large number of undergraduates in the full spectrum of research activities, from experimental design, field sampling and sample processing, to statistical analyses, interpretation, and presentation of results.

2. Integrate the research into courses offered by the four PIs at their three institutions, including courses in Microbiology, Microbial Ecology, Genetics and Recombinant DNA Labs, a seminar course "Life in the Extremes", and summer courses for elementary and secondary school teachers.

3. Host an annual Research Symposium at LVNP where student participants in the project interact and share their research with each other and interested park personnel and community members. Students will also present their research at regional and national scientific meetings.

4. Have students create a pamphlet for LVNP visitors designed to educate the general public about microbial life in the park, microbial diversity and ecology, microbial observatories, and the work described in this proposal.

5. Have students help design a website that disseminates the results and data obtained from this project and link this website with electronic products from other microbial observatories.

6. Expand opportunities for culturally and ethnically diverse undergraduates to develop as scientists and critical thinkers.

6. EXPERIMENTAL PLAN AND METHODS:

A. Timeline of Activities:

In order to map the crude temporal and spatial variability at the site, we plan three major site visits per year for the duration of the project. BSL is readily accessible from late May to late September, and our visits will be timed to capture this entire period. During the winter, this region receives substantial snowfall, and the unpaved road that leads into the park is not plowed. Motorized vehicles are currently restricted from off-road areas in the park except for emergency. Therefore, winter access requires ~15 miles of snowshoeing/skiing with our equipment to reach BSL. In the future, we may be able to gain winter access to BSL. However, we will focus our current efforts on sampling from late spring to late

summer. Throughout the 5-year project, we will focus on enrichment and cultivation of organisms, and characterization of basic microbiology and geochemistry (Table 2). We will install two VERITEQ SP-1000 dataloggers to collect daily temperature data at sampling locations A and D. These data will be downloaded during physical visits. During longer visits, the dataloggers will be reprogrammed to sample hourly. Geochemical samples will be taken monthly during the first sampling season, and if there is no change, they will be taken yearly. Clone libraries (prokaryotes, eukaryotes, virus, and C-fixation genes) will be made during the first year, and sequencing and analysis will continue throughout the proposed funding period. Additional clone libaries will be generated if microbial dynamics investigations indicate major shifts in community structure. Depending on the data obtained in years 1-3, we will target later cultivation efforts toward dominant taxa or potential functional guilds identified by our molecular studies.

Table 2. Proposed Timeline of Activities

YEAR	2006	2007	2008	2009	2010
Site Characterization:					
remote bathymetry					
remote depth profiling					
geochemistry					
data logger (T), and pH					
Biotic Characterization:					
cell/virus enumeration					
clone libraries					
microbial dynamics					
enrichment and isolation					
virus impacts on diversity					
primary production					
Products and Meetings:					
MLVP symposium	N/A	1	1	1	1
web site development					
park brochure					
large effort	moderate effort		minor effort		

B. Site Characterization

i. Lake Bathymetry and Physical Structure: BSL has gently sloping shelves around the perimeter, but its depth and volume are unknown. The southern end has vents that are much hotter than the lake, but there are likely other thermal sources beneath the surface, as convection pools are visible throughout the lake. The temperature and acidity of BSL prohibit safe sampling from a boat and make bathymetry a technical challenge. We propose instead to use an unmanned survey vehicle (USV) to determine the lake's depth and bathymetry, and to map temperatures and pH across and below the lake surface. In later years of the project, it will be modified to be able to take samples at the surface and at depth. Such a vessel is being designed and built as part of the award-winning senior capstone project in the Mechanical Engineering department at Portland State University (PSU) (see letter). It will be equipped with a depth sounder, temperature and pH probes and GPS, and is operated by radio control. In case this vessel does not function as planned, an alternate vessel will be leased from SeaRobotics Corp. (see letter and brochure, Supplementary Documents).

ii. Sample Collection: Initial sampling will focus on BSL's north and south ends (sites A and D) (Fig. 1). Samples will be collected on three successive days in late May or early June (depending on snow levels), early August, and 1-2 successive days in late September for the first three years of this proposal funding period (2006-2008).

At each sampling time, near surface water and underlying sediment samples will be obtained for geochemical analyses, cell counts, nucleic acid extractions, and enrichments. Unless otherwise indicated, all biological and chemical analyses will be done on aliquots of the same samples. All sampling site locations will be determined by GPS and photographically documented. Water and sediment samples will be collected by pumping or poured into a large container with sterile, wide-mouth, 1L Nalgene containers clamped to a modified telescoping (to 5 m) rod. For virus samples, at least 100 liters of lake water will be

filtered through a 0.45 μm filter to remove most Bacteria, Archaea, and Eukarya, and then concentrated at least 1000 fold in a tangential flow filtration unit with a 100kDa cutoff membrane (15). Filtrate will be returned to BSL. Exogenous organism introduction will be minimized by surface-sterilizing the outside of the collection device with several rinses of 80% EtOH, followed by a rinse in sterile distilled, deionized water. Values for temperature, pH, redox potential, and dissolved O2 will be recorded on site.

Upon completion of sampling at a given site, we will immediately process samples on site for counts of microbial cells and viruses, and for nucleic acid extractions (see below). Funds are requested for generators, vacuum and peristaltic pumps for this purpose. Processed samples will be immediately frozen in liquid N_2 for return to our universities. All frozen samples will be transferred to a -80˚C freezer until nucleic acids are extracted. Ideally, extractions would also be done on-site (without sample freezing), but this is currently not feasible.

iii. Geochemical Analyses: Samples for geochemical analyses will be aseptically collected in polypropylene bottles during sampling trips outlined for the first three years of the proposed funding period. Water analysis will be done using inductively-coupled plasma spectrometry (ICP) for major cations (e.g. Si, Al, Fe, Mg, Ca, K, Na), trace elements (e.g. As, B), and anions (CO3, SO4, Cl). Total Organic Carbon (TOC) and Dissolved Organic Carbon (DOC) will also be measured. TOC, DOC, total S (and in the future, trace gases and S speciation), will be measured at PSU by Dr. Ron Jones (see letter of support). ICP will be performed by Energy Labs, Billings, Montana.

C. Genetic and Organismal Diversity

i. Microbial Abundances: All samples collected for AODC of prokaryotic cells will be fixed in 3.7% formaldehyde on site, and stored on ice (until refrigeration access). Quantitative direct counts of total microbial numbers in sediment and water samples will be performed using previously described AODC methods, and counts will be reported as cells per gram dry weight, or cells per ml. Eukaryote cell abundances will be estimated by collecting DAPI-stained water on membrane filters, and examining these under blue excitation for chlorophyll autofluorescence, and under UV for DAPI. We will also fix 20 – 200 ml samples with Lugol's iodine and settle for total protist counts. Virus-like particles will be counted either directly by staining with SYBR-Gold, (Molecular Probes) (15) or flash frozen and stored in liquid N_2 for later staining and analysis (106). Virus-like particle counts in BSL are very low ($< 10^3$/ml F. Rohwer, personal communication and K.S. unpublished) so larger volumes may be required. Water and sediment samples will be collected and stored anaerobically for enrichment cultures (114).

ii. Genetic Diversity Assessments: We will initially estimate microbial diversity in water and sediment samples by generating SSU rRNA clone libraries for prokaryotes (Siering and Wilson) and eukaryotes (Wolfe), and metagenomic and DNA-pol libraries for viruses (Rohwer and Stedman) from environmental nucleic acid extracts from sites A and D (Fig. 1). Prokaryotic libraries have already been generated for water from sites A and D, and sequencing of clones is currently underway. Crude estimates of diversity will be generated by applying T-RF (prokaryotes), DGGE (eukaryotes), and PFGE (viruses) to environmental nucleic acid extracts from different aliquots of the same samples (see Microbial Dynamics, below)

Prokaryotes: Planktonic cells will be filtered onto 0.2 μm Durapore Millipore filters (typically 50-500 ml/filter), and sediments will be dewatered by filtration or centrifugation, prior to nucleic acid extraction; this processing will be done on site. Nucleic acids from pH-neutralized water and sediment samples will be isolated and purified by previously described methods (85-87). 16S rRNA clone libraries were prepared from pooled, mass-normalized aliquots of environmental DNA from water collected at sites A and D (summer, 2004) using universal primers U341F/U1406R (6, 41, 71) and standard methods. Cloning and sequencing of purified plasmids from the confirmed clones is being done using standard procedures. Sequencing of confirmed clones is underway. Several clone libraries made from site A sediments are currently being analyzed (see preliminary results). A library from site D sediments has been constructed using nucleic acid extracts from 2005 samples. Sequences will be assigned into phylotypes ($\geq$97%

identity) using the program DOTUR (78). Phylotypes will be analyzed using previously described methods (46, 79). Phylogenetic trees will be generated to elucidate evolutionary relatedness among BSL organisms and known clades using distance matrix, parsimony, maximum likelihood, and Bayesian methods (85, 86).

Eukaryotes: Samples will be filtered or dewatered onto membrane filters, then neutralized with sodium hydroxide to pH 7. Samples will be extracted with both CTAB-chloroform (Wizard cleanup) and with the UltraClean Soil kit (Mo-Bio Labs) to avoid bias by one method; preliminary tests showed similar PCR fingerprints were generated from both extraction methods. Nucleic acids will be quantified by PicoGreen (Molecular Probes) and pooled extracts are used at 1-10 ng/ul for PCR. 18S rRNA clone libraries will be created using the eukaryote 82F primer and a universal 1390 reverse, similar to Dawson (26). Cloning, and sequencing of purified plasmids from the confirmed clones will be done using standard procedures

Viruses and virus-like particles: Virus DNA will be prepared by treating virus concentrates with DNAse to degrade free DNA followed by SDS and Proteinase K treatment, phenol extraction and ethanol precipitation (75). Virus DNA will be cloned into Lucigen pSMART vectors to make one clone library for the first year at both sites A and D (Lucigen). The first library will be made from sediments taken at site A by F. Rohwer (see letter of support). Since universal primers are not available for virus genomes, specific viral replicase genes will be targeted with degenerate primers. PCR will be performed with primers specific for DNA polymerase from Phycodnaviruses (103). Reverse-transcriptase PCR will be performed with primers specific for the RNA-dependent RNA polymerase found in novel environmental Picornaviruses (25). In future work, PCR will be performed with primers identified from the viral metagenomics data (Rohwer, letter of support). Purified virus DNA will be labeled with fluorescent dyes and queried against the microarray of the metagenomic data (Rohwer, letter of support).

iii. Microbial Population Dynamics: Using a combination of microbial counts and molecular fingerprinting, we will attempt to identify the temporal and spatial scales at which the microbial communities change. Microbial counts will provide information on stability of the community with respect to total numbers of microorganisms and viruses, while genetic fingerprinting of community nucleic acids will allow us to identify major changes in community structure that occur over temporal or spatial scales.

Prokaryotes: In order to assess variation along temporal or spatial scales, we will first obtain baseline data on the level of variation that exists among independent sampling events, and within the lake itself. During summer 2004, we collected near-surface water samples from four different locations within the lake (sites A, B, C, and D, Fig. 1). At each location, three separate samples were collected at 1 m intervals. For each of the 12 samples, multiple aliquots were processed for nucleic acid extraction. Nucleic acids were extracted from 3 aliquots from each sample, and the resulting 36 nucleic acid extracts will be compared by T-RF to assess the number and relative abundances of different prokaryotic phylotypes within samples (10, 77). Based on preliminary studies, we will initially use the primer set U341F/U1406R in conjunction with the restriction enzyme *Hae III* to generate T-RF profiles. To assess temporal variation in the prokaryotic community, additional T-RF data will be generated from nucleic acids extracted from samples collected on three successive days in late early June, early August, and 1-2 successive days in late September for the first three years of this proposal funding period.

The lengths and relative intensities of fluorescently labeled terminal restriction fragments will be analyzed using RFLPscan Plus v. 3.0 software. Several statistical analyses will be used in order to compare T-RF microbial assemblage profiles to each other, and to look for correlations between community composition and environmental variables. Dr. Michael Camann (HSU Dept. of Biological Sciences) will assist us with statistical analyses (see letter of support). Non-metric multidimensional scaling (NMS) and cluster analysis will be used to describe environmental response gradients and to identify groups within and between samples. Multiple response permutation procedure (MRPP) will be used to test hypotheses regarding differences between groups. Standard community metrics will be used

to compare abundance, species richness, community diversity, and community evenness. Community diversity will be evaluated using the Brillioun index, and community evenness will be evaluated using the Carmargo index. Rarefaction analysis will be used to look for sample size effects. Regression analysis will be used to look for correlations between community composition and environmental variables. Additionally, we will utilize indicator species analyses to assess whether specific taxa are characteristic to specific groups. The software that will be used are PC-Ord and TCE (19, 64).

Eukaryotes: Comparison of eukaryotic DAPI counts will give us a crude indication of the stability of algal populations, and indicate whether populations exhibit seasonal abundance patterns within the summer sampling period. Eukaryote rRNA diversity will be screened by denaturing gradient gel electrophoresis (DGGE), a method that works well for the relatively simple communities (22, 27, 34) expected in this environment. Bands of interest are excised, DNA is extracted by freeze-thawing in water, SSU rRNA genes are re-amplified and electrophoresed to confirm a single band of correct mobility, and then sequenced. We will excise and sequence PCR bands that appear in some, but not all, samples to detect ephemeral populations. Following analyses of our clone libraries, we will assess whether additional primer sets are necessary to target particular groups (14).

Viruses: To assess viral diversity we will perform Pulsed Field Gel Electrophoresis (PFGE) on DNA isolated from virus concentrates and compare them to other samples to monitor gross changes over time as reported previously (54). We will also examine viral concentrates by TEM. To determine the stability of virus-like particles in BSL, virus concentrates will be fluorescently labeled and incubated together with lake water *in situ* and sampled during three-day sampling trips. Virus labeling and detection will be as described previously (15). When we have virus host organisms or consortia, we will test viruses for their infectivity over time. Preliminary results indicate that *Sulfolobus* viruses are stable for >24hrs at room temperature (unpublished). If PCR products with DNA or RNA polymerase genes are found and when virus sequences are available from metagenome data, we will clone the products, and analyze the sequences as described above for prokaryote sequences.

iv. Enrichments and Isolation of Novel Microorganisms.

Prokaryotes: Our preliminary sequence analyses of BSL sediment clones indicated a variety of Bacteria and Archaea in BSL, including a number of uncharacterized genera (**Table 1**). Cultivation of heterotrophic organisms from BSL is already underway (see Preliminary data). Additional cultivation efforts will include enrichment and extinction dilution culturing of lithotrophs (sulfur/sulfide oxidizers, iron, hydrogen oxidizers, and arsenite oxidizers), and anaerobes (S°-reducing, nitrate reducing, and fermentative) using filtered, autoclaved site water as the base.

All unique isolates (as determined by 16S rRNA RFLP analyses) will be subject to basic physiological analyses (cell and colony morphology, T & pH optima and limits, O_2 requirements, gram reaction, etc.). Isolates will also be tested for their susceptibility to infection by virus concentrates by spot-on-lawn procedures or introduction of virus concentrates to enrichment cultures. These physiological studies will be conducted by HSU undergraduates in Siering's BIOL 412 Bacteriology class. The identity of unique isolates will be determined by 16S rRNA gene sequencing which will be incorporated into Wilson's BIOL 440 Genetics Laboratory. Culture stocks will be made for future nutritional and physiological studies, and will be maintained in the HSU culture collection.

Eukaryotes: We will establish enrichment cultures of Cyanidiaceae from sites A-C (**Fig. 1**) using appropriate T and increased CO_2 levels (16, 37). We will construct a T-light gradient incubator (101) to cover the range of environmental conditions found at the site. Enrichment cultures will be examined microscopically and individual cells will be picked for isolation. We will characterize pH, T, and photosynthetic optima of the isolates, and also examine their release of DOC (that might fuel prokaryote or fungal metabolism) by $H^{14}CO_2$ labeling (9). We will characterize prokaryote populations associated with algal isolates by DGGE or T-RF fingerprinting (31). Supernatants of positive enrichment cultures will be analyzed for viruses by TEM (42). Finally, we will examine the ability of isolates to grow heterotrophically on a variety of organic carbon sources. If clone libraries or DGGE studies indicate the

presence of heterotrophic protists at BSL, we will establish enrichments in the dark with organic carbon sources, or with prokaryote enrichments. We will also enrich for fungi using various complex carbon sources (cellobiose, etc.) as well as leaf litter from the BSLTA.

Viruses: Viruses concentrates from sites A and D will be screened by electron microscopy. Fractions containing virus-like particles will be separated by CsCl gradient centrifugation and screened for virus production by spot-on-lawn procedures or addition to enrichment cultures and/or isolates from BSL (42). Initially we will spot virus concentrates on lawns of *Sulfolobus*, *Sulfobacillus*, *Alicyclobacillus, Geobacillus, Thermoplasma,* and *Picrophilus*, organisms whose relatives have already been found in BSL (see above). As new isolates from BSL become available they will be screened as potential hosts. When virus sequences are generated from our clone libraries, we will design PCR primers and amplify these sequences from enrichment cultures and samples. In future work, we plan to use FISH and flow cytometry to screen samples and enrichment cultures for both viruses and proviruses.

D. Main resources in the BSL ecosystem (bottom-up controls)

Productivity measurements. Initial efforts (Years 1 and 2) will focus on comparing primary and secondary production in several water and shallow nearshore shelf sediment samples. Based on bathymetry and T data, we will design more extensive surveys in years 3-4 to try and estimate basin-scale productivity and variations over space and time.

i. Primary production will be estimated via standard $H^{14}CO_3^-$ techniques (39). If possible, incubations will be done in situ, but if not, we will return samples to Wolfe's Chico lab immediately for simulated in situ incubation. All vials will be muffle-heated to remove all traces of formaldehyde (113). We will examine light vs. dark production for photo- vs. chemosynthesis, and also determine the effects of addition of inhibitors such as DCMU (photosynthesis) or cycloheximide (eukaryotes).

ii. Determining identity of active chemoautotrophs in BSL. Because many of the C-fixation genes of Archaea remain unknown, amplifications/phylogeny of genes involved in the Calvin cycle (type I,II, and III) and the reverse TCA cycle (20, 21, 23, 30, 32, 35) may not be a useful approach for determining the identity of chemosynthetic producers in BSL. We will initiate efforts to determine the identity of active chemoautotrophs in BSL samples by the use of stable isotope probing (49, 53, 70, 108). We will establish a series of microcosms (+/- light and O_2) from unamended site water incubated at *in situ* temperatures with headspace containing $^{13}CO_2$. After 90% loss of CO_2 has been demonstrated (by GC/MS, time to be determined empirically), vials will be flushed with air, and DNA will be extracted. ^{13}C-DNA will be separated by CsCl ultracentrifugation, and domain-specific primers will be used to amplify SSU rRNA genes, which will then be cloned and screened by RFLP, T-RF or sequencing. We acknowledge that methods development of stable-isotope probing is not a trivial process (49, 53, 70, 108); therefore, we propose to develop these methods toward the end of the proposed funding period, and seek additional funding for these efforts after we have the methods working.

iii. Heterotrophic production will be measured using ^{3}H-thymidine incorporation (39). We will also test the use of allochthonous complex carbon sources (cellulose, etc.) by monitoring uptake and mineralization rates, and by the increase in cell numbers following addition of particulate material from leaf litter surrounding the lake.

E. Viral controls on abundance and/or diversity of ecosystem members (top-down controls):

i. Impact of virus amendments on prokaryotic and eukaryotic diversity (years 3-5).

a. Microcosms with virus addition: One liter samples will be collected from the high and low temperature sites in BSL. These samples will be split into two polypropylene bottles. Virus concentrates (equivalent to viruses from 50 liters of BSL water) from each of the sites will be added to the subsamples and incubated at in situ temperatures (52 and 80˚C) in BSL. After 1,2, 4, 8, 16, 24 hours and 2 days, 50 ml samples will be removed and DNA prepared from these samples and analyzed as above (Genetic

Diversity Assessments) for prokaryotic and eukaryotic diversity. Multiple studies have employed similar methods to assess relative impacts of viral predation on microbial diversity (1, 44, 104, 111), and have shown distinct changes in abundance and/or diversity patterns after virus amendment, including the identification of individual operational taxonomic units (assigned by T-RFLP) that were sensitive, or immune to viral effects (111).

b. Microcosms without viruses: Samples similar to above will be collected and filtered through 0.2 μm filters. Organisms that are retained on the filters will be washed off with the filtrate of the 100kDa filtration (see Sample collection above). These samples will be incubated as above (microcosms with virus addition) and analyzed similarly.

ii. The impact of eukaryotic grazers. The role of grazers will be determined in a manner similar to that of Simek et al. (88). Samples from BSL will be size-segregated by filtration (<0.8 μm, <5 mm, unfiltered) to remove specific grazer size fractions. These samples will be incubated at in situ temperatures in BSL and analyzed as above (Genetic Diversity Assessments) for prokaryotic and eukaryotic diversity and cell numbers.

7. COLLABORATION AND MANAGEMENT – See Management Plan for details.

The research team leader for the proposed project will be Dr. Patricia Siering. She will deal with management details and facilitate information exchange among participants. All of the PIs will be responsible for supervising undergraduate research projects related to this work, and for integrating this research into courses at their institutions (see RUI Impact Statement, Supplementary Documents). Siering and Wilson will be responsible for the characterization of the prokaryotic component (and SIP-probing) of the BSL communities, overseeing the construction of the LVNP brochure, and coordinating the annual research meeting held at LVNP. Stedman will be responsible for characterization of the viral component of the community, collecting/processing samples for geochemical analyses, supervising survey vessel construction and design, and directing the web site creation. Wolfe will be responsible for conducting lake bathymetry, productivity measurements, and characterization of the eukaryotic component of the community.

Cooperation with other MO Projects.

Five currently and recently funded MO projects offer complementary collaboration opportunities. The Soap and Mono Lakes MOs are performing (or have performed) similar studies to ours in cool alkaline lakes that contain metazoan grazers. In the short term we will compare data and sampling techniques with these groups, and in the future we will make compatible mutually accessible databases (see letters). The MO on Viroplankton Ecology studies viruses in marine environments, but uses similar techniques to those proposed here and will assist in our methods development, analyses and will share data (see letter). One of our PIs (Stedman) is Co-PI on "MO:Viruses from Yellowstone Thermal Acidic Environments", where he studies the sequence and diversity of *Sulfolobus* viruses and other hyperthermophiles found at temperatures ≥ 80˚C. This work is complementary to the lower temperatures, bacterial and eukaryal viruses we expect to find in BSL. The Viruses MO has established an Access based database for biological, physical and geochemical data on acidic hot springs that will be the basis for our database, facilitating collaboration and meta-analyses (see letter).

8. SIGNIFICANCE AND OUTCOMES:

Scientific Outcomes:

This project will add to our understanding of the microbial diversity and function in LVNP, a little-explored but major U.S. National Park. It will also develop a unique microbial observatory in an extreme, limnetic ecosystem. **Our comprehensive approach will provide essential information about this ecosystem such as the:**

(a) Identification of the prokaryotes, eukaryotes, and viruses in BSL, and determination of how their numbers and diversity change across temporal and spatial scales.

(b) Identification of the organic carbon inputs, the potential primary producers, and the relationship between lake bathymetry and the abundance /distribution of primary producers within BSL.
(c) Identification of potential top-down control mechanisms for prokaryotes and eukaryotes, and the role of viruses in controlling the abundance and diversity of prokaryotes in BSL.
(d) Isolation of novel (and unexpected) organisms identified by culture-independent approaches?

By collecting basic information on the microbial players (prokaryotes, eukaryotes, and viruses), primary production, and lake bathymetry, this study will ultimately allow us to address larger questions of ecosystem function. This project will establish a foundation on which a detailed long-term study of this microbial ecosystem can be developed. Microbial community composition and function are controlled by resource availability (bottom-up controls), by predation and parasitism (top-down controls), and by physical/chemical parameters within a given habitat. The data obtained from this project will lead to the design of testable hypotheses on the relative effects of these controls, and on the mechanisms by which these controls act on the microbial community in Boiling Springs Lake. The information gained from this proposed project will contribute to our understanding of other limnetic ecosystems in which microorganisms are the sole inhabitants, and potentially many other habitats where microbes dominate.

Educational Outcomes:

Three of the four investigators on this proposal are at RUI institutions, and all of us are committed to integrating this research into undergraduate courses and independent research projects. We have already integrated this research project into several courses at HSU, PSU, and CSUC, and into curricular materials developed for a wider audience (see RUI Impact Statement, Supplementary Documents). In the preliminary phases of this work, we have involved approximately 150 undergraduate students in this LVNP research by integrating it into class activities, and approximately 50 students have directly contributed to the research through participation in independent undergraduate research projects. During the proposed funding period, we anticipate impacting an additional 700 students through class activities, and we anticipate training another 100 undergraduate research students through direct involvement in research projects. Several undergraduates will also contribute to outreach activities (described below). This proposed project will also fund a Ph.D student (PSU) and a M.S. student (CSUC), and provide research supplies for additional graduate students at all three institutions. See RUI Impact Statement for details.

Electronic Products and Park Brochure:

The main interface for MO PIs, their students and the general public will be via a WWW site hosted at PSU (see letter) that will provide access to our relational database. The database will be updated by PIs and students involved in the project and maintained beyond the term of this proposal (see letter). The database will be compatible with the viruses in YNP MO database. In addition to DNA sequence data, we will include geochemical data, physical data, sample site location, micrographs, and diversity assessments (DGGE gels, PFGE gels, T-RF traces).

Other components of the WWW site will include: a lay introduction to the research; pictures and site description; links to other MO sites and the official LVNP site; short CVs and photos of all of the PIs and links to their lab and institutional sites; a project summary; drafts and final versions of the LVNP brochure, a scientific overview of the expected data and methods; publications related to the MO project. The WWW site will be constructed in year 1 of the project and updated every 6 months.

Results will also be made available to the general public via publication of a pamphlet to be produced and distributed in collaboration with LVNP. This will contain color photographs and information on the biology, limnology, and geology of the BSLTA, but will not contain extensive scientific data. An undergraduate biology student at HSU developed an outline and began writing the introductory text in Spring 2005, and plans to continue work on it during the 05/06 academic year. The cross-disciplinary nature of this task is attractive to many students, and we plan to solicit a

minimum of four students of diverse academic backgrounds to participate in its creation. We will seek at least two students majoring in Natural Resources and Interpretation, and at least one majoring in Biology (emphasis in Microbiology). A high quality product should be generated at little cost beyond printing. Copies will be delivered to the park by summer 2008.

9. RESULTS FROM PREVIOUS NSF SUPPORT:

Kenneth Stedman:

MCB-0132156 Microbial Observatories Program "Viruses from Yellowstone Thermal Acid Environments" 9/02 - 10/07: Dr. Stedman's subcontract on this grant with Montana State University is to discover and characterize new viruses from *Sulfolobus* and other hyperthermophiles. This work is mostly done in LVNP in comparison to YNP. Comparative genomic studies on viruses from YNP and Kamchatka, Russia were recently published including an undergraduate co-author from PSU (109). We have also detected SSV-like viruses and SSV-virus-like sequences in samples from Lassen. Geochemical and full genomic analyses are in progress. Two sampling trips to Lassen have been covered in the press. One was broadcast on National Geographic Television on 10 June 2003. Another, profiled in U.S. News and World Report, was entitled "Viruses from Hell" on 17 June 2002.

DBI-0352224: Aberration-Corrected Photoelectron Microscope: Opening the Nanometer Scale for Organic Matter Microscopy. R. Konenkamp, G, Renfer, and K. Stedman 15/6/2004-31/5/2006: This project is in the design and construction phase. SSV-K1 and *Sulfolobus* have been imaged with a non-aberration corrected photoelectron microscope as controls. This microscope will be used for virus characterization..

Gordon Wolfe:

DUE-0126618: CCLI - Decoding Life: Unifying Undergraduate Biology Education by Incorporating DNA Sequencing. G. Wolfe and J. Bell, CSU Chico, 2/02 to 1/04. The goal of this proposal was to introduce undergraduate students to modern gene-based biology by incorporating hands-on sequencing and database exploration. We created a modern automated DNA sequencing facility and integrated sequencing into a wide variety of undergraduate courses. The sequencer funded in this project has been used in the following CSUC Classes: Biol 6A, Introductory Biology; Biol 11, Microbiology; Biol 275, Microbial Ecology; Biol 331, Advanced Cell & Molecular Biology; Biol 199, independent undergraduate research; Biol 398, 399, graduate research. It has contributed to 5 master's theses and 3 meeting presentations by student authors.

DBI-0010098. RUI - NSF Multi-user Equipment and Instrumentation Resources for Biological Sciences. G. Wolfe, J. Day, and J. Pushnik, CSU Chico, 9/01 to 8/03.This grant supplied 2 advanced fluorescence microscopes for research and teaching. Departmental funds were used to purchase a digital camera used for fluorescence imaging. The microscopes have been used for a variety of faculty and student research, and in courses ranging from introductory to graduate, including: Biol 11, Microbiology; Biol 275, Microbial Ecology; Biol 269, Neurophysiology; Biol 199, independent undergraduate research; Biol 398, 399, graduate research. This project has contributed to 4 peer-reviewed papers and 5 meeting presentations with student authors.

OCE-9730952: Chemical defense by marine phytoplankton against protozoan grazers, S.L. Strom, Western Washington Univ., and G. Wolfe, CSU Chico. 3/98 to 2/01. Eight undergraduates conducted research related to this project; four of these were participants in Shannon Point's Minorities in Marine Science Undergraduate Program. Six of the undergraduates presented their research findings at national meetings (ASLO, Ocean Sciences), and were coauthors on 3 peer-reviewed publications (95, 96, 113). The grant also provided partial support for three graduate students and one M.S. thesis (Olson & Strom, 2002).

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