Amphibian Risk Assessment in Montana: An Evaluation of Chytridiomycosis in Columbia spotted frogs (Lithobates luteiventris)

Alex Kurtz
Carroll College, Helena, MT
This thesis for honors recognition has been approved for the

Department of Life and Environmental Science

Brandon A. Martin
Director

5/10/17
Date

Reader

5/10/17
Date

Reader

5/10/17
Date
Amphibian Risk Assessment in Montana: An Evaluation of Chytridiomycosis in Columbia spotted frogs (*Lithobates luteiventris*)

**Investigator:** Alex C. Kurtz

**Contact Information:**
Alex C. Kurtz  
1601 N. Benton Ave  
Helena, MT 59625  
Phone: (406) 939-3628  
alex.kurtz@me.com

**Host Institution:** Department of Life and Environmental Sciences, Carroll College, Helena, MT
Abstract

The fungus, *Batrachochytrium dendrobatidis*, has been found to negatively impact amphibian populations around the world. This fungus can have multiple effects on frog physiology, including changes in osmotic regulation that may lead to death. *B. dendrobatidis* has been shown to be the driving force for many amphibian population crashes and extinctions around the world. The purpose of this project was to build a predictive model of *B. dendrobatidis* infection, one that would be used to assess population susceptibility in order to identify populations of amphibians at risk of infection. This was accomplished by statistical analyses of several components that contribute to infection vulnerability, including amphibian antimicrobial peptide production, cutaneous bacterial colony structure, infection status for each frog and water nutrient composition. This project collected baseline data that will allow us to establish meaningful relationships between susceptibility factors and disease, which will permit the identification of populations at risk. Overall, it was found that variability within these nutrient factors, such as levels of phosphorus and manganese, as well as differences in AMP production and bacterial communities may have contributed to variations in infection status between lakes.
Introduction

Chytridiomycosis is an amphibian skin disease caused by the fungal pathogen *Batrachochytrium dendrobatidis (Bd)*. This fungus has caused a worldwide decline in amphibian populations (Crawford et al. 2010). However, susceptibility to infection by *Bd* seems to vary between species and within populations (Woodhams et al. 2007).

Beginning in the late nineties, many anuran populations worldwide began to face decline, endangerment, and extinction, especially in the rain forests of Australia and Central America (Berger et al. 1998). This was observed in pristine and protected environments and, therefore, was likely not due to pollution or habitat destruction (Berger et al. 1998). Many proposed etiologies were examined and hypothesized for the observed declines. None was shown to contribute to the cause of death for these amphibian populations until July of 1998, when organisms of the fungal Phylum Chytridiomycota were identified as the root cause of this amphibian mortality (Berger et al. 1998).

Berger et al. (1998) proposed and supported the hypothesis that these fungi parasitize the amphibian and cause changes in epidermal structure that result in death. Whole frogs were preserved and skin samples from these frogs were frozen. Examining these samples by electron microscopy, they consistently showed a large number of fungal sporangia in the epidermis (Berger et al. 1998). These formations were found consistently in the “drink patch,” which is located on the ventral side of the pelvic region and is important in osmoregulation, or the regulation of water and solute uptake (Berger et al. 1998). DNA was extracted from these scrapings and sequenced for identification (Berger et al. 1998). These analyses showed that the parasite genome most closely matched that of *Chytridium confervae*, which is a member of the Order Chytridiales, a sister Order to
Rhizophydiales, the Order to which *Bd* belongs (Berger *et al.* 1998). Finally, to confirm that this parasitic fungus was the true cause of the amphibian decline in these study areas, a transmission experiment was performed where frogs were exposed to unfiltered skin scrapings from deceased frogs (Berger *et al.* 1998). All six of these test frogs displayed a terminal decline in health, strongly suggesting that this chytrid fungus was the cause of death in amphibian populations (Berger *et al.* 1998).

This fungus has a unique lifecycle consisting of two main life stages: a mobile zoospore with a single posterior flagellum, and the sessile, reproductive thallus (zoosporangium) where new zoospores are produced (Piotrowski *et al.* 2004). The lifecycle is easily observed in culture and a very similar lifecycle is observed on amphibian skin in nature (Berger *et al.* 2005). This lifecycle begins with a zoospore that adheres through fibrillar projections to the epidermis of the amphibian’s skin (Baldo *et al.* 2012 & Berger *et al.* 2005). Final colonization of the host skin begins through growth of a germ tube into the epidermal cell (Greenspan *et al.* 2012). The distal end of the tube develops further into a new internal thallus, which matures through usage of epidermal nutrients, while simultaneously moving toward the surface through the migration of differentiating epidermal cells (Piotrowski *et al.* 2004 & Berber *et al.* 2005). Finally, the plug dissolves and new zoospores are released to propagate the infection (Piotrowski *et al.* 2004).

This propagation of the fungus impacts the function of the frogs’ skin (Campbell *et al.* 2012). Much of this physical disruption directly impairs the functionality of the skin and results in ion imbalances and a lowered ability of the frog to osmoregulate and hydrate (Campbell *et al.* 2012). Through these compromised abilities, the ultimate cause
of death for these frogs appears to be cardiac arrest resulting from irregular cardiac electrical stimulation from hypokalemia, a lack of potassium in the blood stream (Campbell et al. 2012).

Variations in immunological characteristics between individuals affect the likelihood of an individual contracting Chytridiomycosis (Woodhams et al. 2007). Two of these immunological characteristics include the microbial communities associated with the amphibian’s cutaneous mucosa and the anti-microbial peptides (AMPs) produced from granular glands in the amphibian skin (Krynak et al. 2015).

Variation in bacterial colony structure plays a role in susceptibility to *Bd* infection. Frogs infected with *Bd in vivo* that survive infection have cutaneous metabolite profiles and bacterial skin community structures that differ from those frogs that do not survive the infection (Becker et al. 2015). Probiotic treatments, using bacteria that produce a strong inhibition of *Bd in vitro*, have been used to test whether, when introduced to frogs, they produce an inhibitory affect (Bletz et al. 2013). Bioaugmentation of this kind can facilitate the repatriation of susceptible amphibian species into a *Bd* endemic area (Bletz et al. 2013). Additionally, bacterial community profiles of frogs treated with probiotics that cleared a *Bd* infection differed significantly from those that retained infections. These studies indicate that the bacteria used in probiotic treatments for the selection of *Bd*-protective bacterial communities are often selective in its host surface (Becker et al. 2015). This suggests that specific bacteria colony structures contribute to an amphibian’s ability to resist infection by *Bd*.

Mixtures of anti-microbial peptides are capable of *in vitro* growth inhibition of *Bd* (Woodhams et al. 2007, Sheafor et al. 2008). Variations in both the type and quantity of
AMP production by species, as well as between individuals in a population, result in differing mortality outcomes after amphibian infection by *Bd* (Woodhams *et al.* 2007).

AMP production and bacterial microbiome structure may be influenced by environmental factors, such as water nutrient composition and temperature (Krynak *et al.* 2015). I predict that AMP production and bacterial community structure will vary between ponds due to differences in host genetics and variation in environmental characteristics.

The objective of the current research is to determine how relationships between antimicrobial skin secretions, cutaneous microbial communities, and environmental water quality affect the susceptibility of amphibians to Chytridiomycosis (Figure 1). These factors could potentially interact to provide resistance to infection by *Bd*. By understanding how these protective mechanisms work with one another and the surrounding environment, insight may be gained into what specific factors allow an amphibian to effectively resist Chytridiomycosis. While several of these immunological parameters have been studied individually, as discussed above, none have looked into interactions between all three factors while simultaneously examining infection load. My

![Figure 1](image-url). A diagram of the hypothetical interactions between immunological and ecological parameters that have the potential to impart resistance to Chytridiomycosis in anuran amphibians.
hypothesis is that each of the factors discussed above (host immune response, the hosts’ symbionts, and the environment) interact together to affect disease outcome (Figure 1).

With this pilot study, the individual factors that best explain variation in infection status will be used to create a risk assessment model for the Columbia spotted frog in Montana. This model can be used to analyze and assess the risk of a given amphibian population and its likelihood of infection by *Bd*. A model such as this can be useful for both the prediction of risk, as well as for the treatment of infected population through environmental manipulation of whichever factors tested best explain the variation in infection status within a population.

**Materials and Methods**

Field location and collection of amphibians: The sampling locations were selected for their previous positive infection status with *Bd*, as well as for their proximity to the laboratory location (Carroll College, Helena, Montana). The Columbia spotted frog (*Lithobates luteiventris*) was chosen for this study due to its abundance in Montana, as well as its ability of some members of the population to persist with chytrid infection (Russell *et al.* 2010). The gradation of infection status allows for the possible construction of an infection model that can predict the likelihood and intensity of infection. Columbia spotted frogs (*Lithobates luteiventris*) were examined at each of four sites in central Montana. These are Park Lake, Gipsy Lake, Jones Pond at the Lubrecht Experimental Forest, and Doney Lake (Figure 2). These sites were identified as containing *L. luteiventris* and have tested positive for Chytridiomycosis in previous seasons (Global *Bd* mapping project, Sheafor, unpublished data). Ten frogs were
collected by hand at each of these sites for a total of 40 samples, with one additional sample from a deceased frog at Jones Pond. An additional eight samples of the boreal toad (*Anaxyrus boreas*) were collected at three sites (Doney Lake n=3, Jones Pond n=3, and Gipsy Lake n=2). Boreal toads have faced significant decline in many areas of its range and are considered a “near threatened” species (Pilliod *et al.* 2010). For these reasons, including its higher susceptibility to infection, the boreal toad was included in this study.

**Figure 2.** Map of the sampling locations of Gipsy Lake, Park Lake, Doney Lake, and Jones Pond surrounding Helena, MT in the Rocky Mountains. (Gipsy Lake, 6356 ft, 21,356 m², 46°30’05” N 111°12’39” W; Park Lake, 6138 ft, 152,416 m², 46°26’48” N 112°09’45” W; Doney Lake, 6273 ft, 181,414 m², 46°26’50” N 112°57’12” W; Jones Pond, 4048 ft, 21,174 m², 113°53’42” N 113°26’26” W)
**Sampling:** Each individual frog or toad was swabbed with sterile foam sampling swabs 10 times on a randomly chosen ventral surface (left or right side) to collect metabolites produced by skin microbes. This was done using Fisher brand polyurethane foam-tipped swabs that had been cleaned in methanol to remove any methanol-soluble impurities (Umile *et al.* 2014). Following the collection of metabolites, these amphibians were rinsed using a sterile Provasoli media to remove transient bacteria. They were then swabbed an additional 10 times on the previously un-swabbed ventral side using rayon swabs to collect bacterial and fungal samples for DNA extraction. A final total-body swab (5x each, dorsal and ventral) was completed to collect bacteria that were then plated on a 1% tryptone/agar plate. Disposable nitrile gloves were changed after obtaining samples from each amphibian. Metabolite and DNA sampling swabs were placed in sterile tubes and stored on dry ice (metabolites) or ice (DNA) until they could be placed in a -80°C freezer pending analysis.

Following the swabbing procedure, amphibians were placed in 50 mL of sterile collection buffer (50 mM NaCl, 25 mM CH$_3$COONa, pH=7.0) containing 500 µl of 20 mM norepinephrine hydrochloride for 15 minutes to obtain skin secretions (Sheafor *et al.* 2008). After removal of the amphibian, the collection buffer was acidified with 500 µl of trifluoroacetic acid (TFA), passed over a C-18 Sep-Pak cartridge, a C-18 filter that binds to proteins, (Waters Corporation, Milford, Massachusetts, USA), and stored on ice until deposition in a freezer. All animals were released at the site of sample collection.

Three amphibian metabolic rates were obtained using a Field Metabolic System (Sable Systems) at each sampling location. Metabolic rates were calculated from rates of...
oxygen consumption and carbon dioxide production. This data was used to test for correlations between metabolic rate and infection status.

In addition, three water samples from geographically separate areas of the lakes were collected in 50 mL Falcon tubes at each site and acidified with 500 µL of trace metal grade HNO₃ for preservation. These samples were used to determine the concentrations of several important elements (Fe, Mg, Mn, Na, Ca, & K) in the water at each site using an ICP-MS (inductively-coupled plasma mass spectrometry) at Carroll College. Additional samples were sent to Energy Labs (Helena, MT) for total Nitrogen, Phosphorus, and Sulfur analyses. Similarly, three additional 50 mL water samples were obtained at each site and filtered using Whatman 0.7 micron filters (GE Life Sciences, Pittsburgh, Pennsylvania) to collect chlorophyll in order to determine the primary productivity of the pond.

**Bd and bacterial community analyses:** MoBio PowerSoil DNA isolation kits (MoBio Labs, Carlsbad, California) were utilized to isolate fungal and bacterial DNA from rayon swab samples. Quantitative PCR was used to determine the amount of Bd present in the extracted DNA, following the protocol of Annis et al. (2004). Quantitative PCR analysis of Bd was carried out by Andrew Loudon at the University of British Columbia. To determine bacterial community composition, Mr. Loudon amplified the 16S rRNA from the extracted DNA (Loudon et al. 2014). Finally, these amplicons were sequenced on an Illumina HiSeq 2000. The resulting reads were clustered into operational taxonomic units using the October 2012 release of Greengenes and assigned to reference OTUs using this Greengenes taxonomy. From these data, a phylogenetic tree was created with FastTree
using the standard procedures within QIIME, and alpha and beta diversity analyses were conducted on these resulting data to determine effects on alpha diversity indices, such as richness and Shannon diversity index (Loudon et al. 2014). The resulting principle coordinate plots compare similarity between bacterial communities using a unifrac distance metric. A similarity matrix places these data in n-dimensional space to place linear lines through the n-dimensions, creating several axes that best explain the most variation. This method creates a visual representation of the variation in community structure and its diversity. Animals that tested negative for chytridiomycosis were considered to be control samples, whereas those that tested positive were considered experimental samples.

Skin secretion analyses: Peptides on the C-18 sep-paks were eluted using a solution of 70% acetonitrile, 29.9% water, and 0.1% TFA (v/v/v). These Sep-Pak cartridges are used to ensure that the antimicrobial chemicals in the final sample were largely the peptides. These eluted peptides were next concentrated down to approximately 1 mL using a Biotage TurboVap 500 (Biotage, Uppsala, Sweden). The concentration of eluted peptides was determined via a micro BCS assay using bradykinin as a standard (Sheafor et al. 2008). The effectiveness of the secretions against *Bd* was determined using a variation of the bioassay utilized in Sheafor et al. (2008). In short, zoospores from a cultured plate of *B. dendrobatidis* (strain PTH02 obtained from Dr. Joyce Longcore, University of Maine) were collected and diluted with 2% tryptone broth to a concentration between $5 \times 10^4$ and $5 \times 10^5$ zoospores in a volume of 50 µl. Concentrations were determined using a hemocytometer. Three replicates of each zoospore dilution (50 µl each) were plated in a
96-well microtiter plate and mixed with 50 µl of peptide dilutions ranging from 5 to 200 µg/mL for each animal. Growth over 10 days was measured daily as the change in absorbance at 492 nm using a Titertek Multiskan plate reader (Titertek Instruments Inc., Huntsville, Alabama). A minimal inhibitory concentration (MIC), the lowest concentration of crude peptides needed where no growth is detectable, will be utilized to determine which peptides from a specific frog are most effective in combatting *Bd* infection (Sheafor *et al.* 2008).

**Bacterial metabolite analyses:** Dr. Kevin Minbiole and Dr. Tom Umile performed the metabolite analyses at Villanova University. Metabolites were extracted by placing swabs in a centrifuge tube, adding 1.0 mL of methanol, vortexing for 5 seconds, allowing to rest for 10 minutes, and vortexing for an additional 5 seconds. Next, the extract was filtered into a new centrifuge tube using a 13 mm syringe filter with a 0.2 µm PTFE membrane. These extracts were then concentrated using a DNA120 SpeedVac with the heating function off. Finally, these dried extracts were resuspended in 100 µl of methanol with 1ppm naphthalene and analyzed by HPLC. (Umile *et al.* 2014). This reversed-phase, high performance liquid chromatography used a Shimadzu LC-20 liquid chromatograph with an ACE C18 column, a Shimadzu SPD-M20A diode array detector, and an Applied Biosystems SCIEX API 2000 triple quadrupole mass spectrometer (Umile *et al.* 2014). All compounds were subsequently separated with a binary mobile phase flowing at a rate of 0.4 mL/min. This phase consisted of acidified water (0.1% formic acid) and acidified acetonitrile (0.1% formic acid). All compounds were eluted and characterized by retention time and total wavelength chromatograms (Umile *et al.* 2014).
Water sample analyses: A portable Vernier LabQuest unit (Vernier Software & Technology, Beaverton, Oregon) was used on-site to measure water temperature, dissolved oxygen levels, pH, and conductivity at three geographically separate areas of each lake. A Perkin Elmer Optima 2000DV atomic emission spectrometer (Perkin Elmer, Waltham, Massachusetts) was utilized to measure concentrations of K, Mg, Fe, Ca, and Mn, which are important elements for bacterial growth. First, a 2% (wt/wt) nitric acid blank was run, then a combination standard containing each of the five study elements in 1.0 ppm concentrations. This was used to establish a baseline and calibration curve for each element. The acidified water samples were analyzed using the standard operating protocol for this instrument. Some elements, such as calcium, were often in too high of a concentration to be accurately measured, as the detector was saturated with photons outside of the linear range of the instrument. To correct this, 1:100 dilutions of each sample were used to measure Ca levels. Energy Labs in Helena, MT analyzed total nitrates, phosphates, and sulfates.

Primary productivity, or the rate at which photosynthetic organisms produce organic compounds in an ecosystem, was assessed via a fluorescence analysis of chlorophyll filtered from water samples. Fifty mL samples of water (three from each water body) were filtered using 0.7 micron glass microfiber filters (GE Healthcare/Whatman, Maidstone, United Kingdom). The filters were rinsed with 90% acetone in a Falcon tube (25 mL) and placed at -20°C for 18-20 hours. These samples were then thawed, centrifuged, and analyzed using a fluorometer measuring fluorescence at 750 nm before and after the addition of a few drops of 10% HCl.
Results

*Bd* and bacterial community analysis: Using qPCR, *Bd* infection status was determined for each of the sampled frogs. These experiments determined copy number, giving an indication of the relative intensity of the individual amphibian’s infections (Table 1).

**Table 1.** *Bd* copy number for each tested Columbia spotted frog at Park Lake, Gipsy Lake, Doney Lake, and Jones Pond. A larger copy number signifies a greater infection by a larger amount of cutaneous *Bd*.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Park Lake</th>
<th>Gipsy Lake</th>
<th>Jones Pond (Greenough)</th>
<th>Doney Lake</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL LI 1</td>
<td>142.36</td>
<td>GL LI 1</td>
<td>813.50</td>
<td></td>
</tr>
<tr>
<td>PL LI 2</td>
<td>0.0</td>
<td>GL LI 2</td>
<td>36.72</td>
<td></td>
</tr>
<tr>
<td>PL LI 3</td>
<td>8371.11</td>
<td>GL LI 3</td>
<td>1627.99</td>
<td></td>
</tr>
<tr>
<td>PL LI 4</td>
<td>0.0</td>
<td>GL LI 4</td>
<td>96.15</td>
<td></td>
</tr>
<tr>
<td>PL LI 5</td>
<td>0.0</td>
<td>GL LI 5</td>
<td>1081.20</td>
<td></td>
</tr>
<tr>
<td>PL LI 6</td>
<td>0.0</td>
<td>GL LI 6</td>
<td>913.24</td>
<td></td>
</tr>
<tr>
<td>PL LI 7</td>
<td>257.03</td>
<td>GL LI 7</td>
<td>Inconclusive</td>
<td></td>
</tr>
<tr>
<td>PL LI 8</td>
<td>5590.56</td>
<td>GL LI 8</td>
<td>5102.50</td>
<td></td>
</tr>
<tr>
<td>PL LI 9</td>
<td>0.0</td>
<td>GL LI 9</td>
<td>9734.63</td>
<td></td>
</tr>
<tr>
<td>PL LI 10</td>
<td>217.71</td>
<td>GL LI 10</td>
<td>2282.73</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td>50%</td>
<td>% Positive</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>GR LI 1</td>
<td>69.34</td>
<td>DL LI 1</td>
<td>174755277.8</td>
<td></td>
</tr>
<tr>
<td>GR LI 2</td>
<td>0.0</td>
<td>DL LI 2</td>
<td>177.99</td>
<td></td>
</tr>
<tr>
<td>GR LI 3</td>
<td>0.0</td>
<td>DL LI 3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GR LI 4</td>
<td>0.0</td>
<td>DL LI 4</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GR LI 5</td>
<td>0.0</td>
<td>DL LI 5</td>
<td>16.42</td>
<td></td>
</tr>
<tr>
<td>GR LI 6</td>
<td>46.78</td>
<td>DL LI 6</td>
<td>53.33</td>
<td></td>
</tr>
<tr>
<td>GR LI 7</td>
<td>0.0</td>
<td>DL LI 7</td>
<td>442.33</td>
<td></td>
</tr>
<tr>
<td>GR LI 8</td>
<td>13.80</td>
<td>DL LI 8</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GR LI 9</td>
<td>0.0</td>
<td>DL LI 9</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GR LI 10</td>
<td>0.0</td>
<td>DL LI 10</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td>30%</td>
<td>% Positive</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>
Bacterial communities were analyzed by Andy Loudon at the University of British Columbia. A principle coordinate analysis plot was generated through the compression of the data into two axes that, together, explain the majority of the variation in community composition. These two axes take into account nearly 55% of the total variation among bacterial communities among the tested frogs (Figure 3). Multiple regression analyses show that the 1st and 2nd principle coordinates were statistically significant in explaining 15% of the variation within *Bd* infection status (1st coordinate: p=0.015, 2nd coordinate: p=0.05). Further multiple regression analyses using these principle components, *Bd* copy number, and other environmental variables will be used in determining potential for predicting infection by *Bd*. The plated, bacterial samples will be used for future analysis in a bioassay to determine efficacy against *Bd*.

**Figure 3.** Principle coordinate analysis comparing bacterial communities among amphibian samples at each tested lake. The closed triangles represent cutaneous bacterial communities on Columbia spotted frogs (CSF). The open diamonds represent the aquatic, environmental bacterial communities. The open circles represent the cutaneous bacterial communities on boreal toads (ABT). Grouping among these communities indicates some selective pressure exerted upon the bacteria from factors associated with the mucosa of the amphibian or the pond chemistry.
Skin secretion analysis: The average AMP concentration was determined for each frog using a BCA assay and a bradykinin standard (Table 2). T-tests were completed to check for significant variations within AMP levels between frogs at each lake. Frogs from Jones Pond had significantly lower AMP concentrations than frogs sampled from the other three bodies of water.

Table 2. Average concentration of AMPs per gram of Columbia spotted frog in each water body (n=10 per lake).

<table>
<thead>
<tr>
<th>Water ID</th>
<th>Average [Protein] (µg/mL)/g frog w/ SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Park Lake</td>
<td>484.29 ± 112.68</td>
</tr>
<tr>
<td>Gipsy Lake</td>
<td>499.21 ± 111.78</td>
</tr>
<tr>
<td>Jones Pond*</td>
<td>148.16 ± 42.65</td>
</tr>
<tr>
<td>Doney Lake</td>
<td>241.76 ± 73.99</td>
</tr>
</tbody>
</table>

*Indicates a significant difference in protein production between Jones Pond and Park Lake (p=0.007), as well as Jones Pond and Gipsy Lakes (p=0.008).

These AMPs will be utilized for future analyses in a bioassay to determine efficacy against *Bd* for each frog.

Bacterial metabolite analysis: A metabolite profile was created for each lake, containing the unique metabolites for each frog sample (Figures 4, 5, 6, & 7).
Figure 4. HPLC retention diagram of metabolites collected from cutaneous bacteria on Columbia spotted frogs (n=6) at Park Lake, Montana. Each peak corresponds with a single metabolite. The larger peaks represent overall greater abundance of a specific metabolite.
Figure 5. HPLC retention diagram of metabolites collected from cutaneous bacteria on Columbia spotted frogs (n=10) at Gipsy Lake, Montana. Each peak corresponds with a single metabolite. The larger peaks represent overall greater abundance of a specific metabolite.
**Figure 6.** HPLC retention diagram of metabolites collected from cutaneous bacteria on Columbia spotted frogs (n=9) at Jones Pond, Montana. Each peak corresponds with a single metabolite. The larger peaks represent overall greater abundance of a specific metabolite.
Figure 7. HPLC retention diagram of metabolites collected from cutaneous bacteria on Columbia spotted frogs (n=10) at Doney Lake, Montana. Each peak corresponds with a single metabolite. The larger peaks represent overall greater abundance of a specific metabolite.
Comparing these four lakes, there is a visual difference in diversity of specific metabolites present. T-tests show a significant difference in metabolite richness (number of metabolites identified per frog) between Jones Pond (Figure 6) and all other tested lakes (Doney Lake/Jones Pond: p=0.006, Gipsy Lake/Jones Pond: p=0.002, Park Lake/Jones Pond: p=0.03). No correlation is present when comparing metabolite richness and *Bd* copy number.

**Water sample analysis:** After analysis by ICP-MS at Carroll College, as well as at Energy Labs, the levels of important nutrients were compiled (Table 3). Several unique characteristics stand out in Gipsy Lake in particular. This body of water, with its highest infection status, contains no phosphorus or manganese, two potentially important nutrients for bacterial growth. Furthermore, it had higher levels of potassium and magnesium.

<table>
<thead>
<tr>
<th>Lab</th>
<th>N</th>
<th>P</th>
<th>S</th>
<th>Fe</th>
<th>Mg</th>
<th>Mn</th>
<th>Na</th>
<th>Ca</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab</td>
<td>0.01</td>
<td>0.59</td>
<td>0.0</td>
<td>4.77</td>
<td>0.18</td>
<td>1.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Park Lake</td>
<td>0.2</td>
<td>0.02</td>
<td>2.1</td>
<td>4.21</td>
<td>0.74</td>
<td>0.32</td>
<td>2.59</td>
<td>0.22</td>
<td>1.16</td>
</tr>
<tr>
<td>Gipsy Lake</td>
<td>0.2</td>
<td>0.0</td>
<td>3.3</td>
<td>0.02</td>
<td>3.31</td>
<td>0.0</td>
<td>1.72</td>
<td>4.37</td>
<td>7.82</td>
</tr>
<tr>
<td>Jones Pond</td>
<td>1.6</td>
<td>0.08</td>
<td>6.7</td>
<td>0.25</td>
<td>1.27</td>
<td>0.01</td>
<td>11.2</td>
<td>0.4</td>
<td>4.12</td>
</tr>
<tr>
<td>Doney Lake</td>
<td>1.2</td>
<td>0.04</td>
<td>0.7</td>
<td>0.15</td>
<td>3.1</td>
<td>0.03</td>
<td>1.1</td>
<td>20.83</td>
<td>0.29</td>
</tr>
</tbody>
</table>
**Table 4.** Water environmental variables obtained using a Vernier LabQuest portable station.

<table>
<thead>
<tr>
<th>Water ID</th>
<th>Water Temp (°C)</th>
<th>DO (mg/L)</th>
<th>pH</th>
<th>Conductivity (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab</td>
<td>15.7</td>
<td>9.39</td>
<td>7.24</td>
<td>40.3</td>
</tr>
<tr>
<td>Park Lake</td>
<td>10.1</td>
<td>8.89</td>
<td>6.95</td>
<td>33.3</td>
</tr>
<tr>
<td>Gipsy Lake</td>
<td>12</td>
<td>9.14</td>
<td>8.08</td>
<td>126.9</td>
</tr>
<tr>
<td>Jones Pond</td>
<td>25.6</td>
<td>12.31</td>
<td>10.84</td>
<td>88.6</td>
</tr>
<tr>
<td>Doney Lake</td>
<td>21.6</td>
<td>7.55</td>
<td>7.65</td>
<td>109.1</td>
</tr>
</tbody>
</table>

Analysis of primary productivity based upon chlorophyll concentrations using fluorometry came up inconclusive and below detectable limits. This indicates low primary productivity in each of the lakes.

**Discussion**

*Bd* and bacterial community analysis:

Gipsy Lake, the highest mountain lake at approximately 6400 ft in elevation, had the highest tested *Bd* infection percentage among the four lakes, at 100%, if the inconclusive frog is considered positive. Park and Doney Lakes (6138 ft and 6273 ft respectively) followed at 50% infection, with Jones Pond (4048 ft) the lowest, at 30%. Infection status seems to follow an elevation gradient. Infection status could be influenced, in part, along this gradient by average daily temperature. *Bd* is not able to live above 25°C, and lakes exposed to periodic temperatures above this threshold may have lower *Bd* concentrations (Piotrowski *et al.* 2004).

The principle coordinate analysis shows that the aquatic bacterial communities at each lake are spread along the 2nd principle coordinate axis (Figure 3). The bacterial community analysis from the swabbed frogs are grouped at the top of this PCO2 axis, but
spread among the PCO1 axis, in which most of the variation among cutaneous bacterial communities is explained. Looking within this spread, bacterial communities taken from Gipsy and Doney Lakes are more spread out, meaning there is more variation within the cutaneous bacterial communities on any single, tested frog. Those for Jones Pond are grouped into a more concentrated area, with less variation among them, indicating a potential selective pressure limiting bacterial diversity. The AMPs, water factors, and/or bacterial metabolites may apply this pressure and result in the selection of specific variations of these that confer resistance to infection by *Bd*.

**Skin secretion analysis:** The anti-microbial peptide analysis will be continued at Carroll College using bioassays. An effective procedure for conducting these assays was developed and detailed in the Materials and Methods section. The overall, average concentration of AMPs produced by frogs at Jones Pond differed significantly from those produced from frogs at the other, tested lakes (Table 2). The frogs at Jones Pond may be producing more specific AMPs that help to direct which infection-preventative bacteria can reside on the skin, as well as contribute to the direct inhibition of *Bd* growth (Woodhams *et al.* 2007). A lack of necessary water nutrients, bacteria/metabolites at Gipsy Lake may have influenced AMP production. Without the ability to produce protective AMPs, the frog population at Gipsy could be more susceptible to infection, as indicated by the 100% infection status.

**Bacterial metabolite analysis:** Bacterial metabolites, classified by retention time, are displayed in Figures 4, 5, 6, & 7. Each peak represents a unique metabolite, with the
height of the peak denoting its relative intensity compared to all metabolites present. Among all four lakes, the metabolite richness of Jones Pond was significantly different than that of the other three locations. When comparing Jones Pond, with its low infection rate, and Park Lake, Doney Lake, and Gipsy Lake, differing patterns of metabolite expression on the retention characterization diagram can be observed. Large metabolite peaks can be observed at approximately 12.55, 14.08, and 15.9 seconds for Jones Pond. These peaks are largely absent in the other lakes, with the exception of the metabolite at 14.08, which is seen at much lower concentrations. Furthermore, the metabolite richness (number of metabolites per frog) at Jones Pond is significantly less than that of the other lakes (GR/DL p=0.006, GR/GL p=0.002, GR/PL p=0.029). These differences may contribute to the variations in infection status among the four tested lakes and their frog populations. Jones Pond, with its given aquatic nutrients, may allow for specific bacteria to survive that are, in turn, able to survive on the mucosa of the frog. These bacteria may produce metabolites, that when coupled with specific AMPs, allow for any individual frog to better resist infection by *Bd* (Becker *et al.* 2015). Lower bacterial community diversity may lead to increased selection for and production of bioactive metabolites that contribute to overall chytrid resistance (Bell *et al.* 2013). Metabolite production by cutaneous bacteria seems to be important in determining this ability for resistance.

**Water sample analysis:** Several unique factors stick out among the nutrient levels in the tested water bodies. Gipsy Lake, which had the highest infection load, had no detectable levels of phosphorus or manganese, two nutrients necessary for many bacterial species to survive. Gipsy also had higher levels of potassium when compared to the other water
bodies. Furthermore, Jones Pond, which had the lowest infection load, had high levels of sodium and sulfur. Bacterial metabolites and AMPs can be protective against infection by *Bd*. Without the necessary nutrients, some bacteria may not survive and thrive in a given aquatic environment. In addition, AMPs that help to select for and determine the cutaneous bacterial community may not be produced without these nutrients. Overall, variations in water nutrient levels appear to be important in determining bacterial community, AMP production, and resistance to infection (Kynak *et al.* 2015).

**Conclusion:** Given these data, several unique traits stand out among the tested parameters when compared to *Bd* infection status. Gipsy Lake, with its lack of detectable phosphorus and manganese, low levels of other nutrients, its unique spread of bacterial communities, and high metabolite richness, may have a greater susceptibility to infection by *Bd* (Table 1). When compared to the variables measured within Jones Pond, differences stand out that may influence its lower infection status. Jones Pond has the least variability within its cutaneous bacterial communities sampled from the frogs, the fewest metabolites present, as well as the lowest average AMP concentration per gram of frog. Each of these may suggest that this frog population has an optimal environment for the more specific production of AMPs and metabolites, and the directed determination of bacterial communities to confer a resistance to infection. Overall, these factors may be important in determining overall potential for infection and will be studied in the future. In summary, this study has worked to identify key variables that help to explain most of the variation within infection status. In the future, multiple regression analyses will be used for this analysis and to determine which specific variables can be thrown out for their
lack of any statistical significance in explaining variation in infection status. For future studies, these factors will be focused upon in the evaluation and potential treatment of frog populations in Montana that are susceptible to infection by the fungus, 

*Batrachochytrium dendrobatidis.*
References


Global Bd mapping project; http://www.bd-maps.net/surveillance/s_country.asp?country=US


