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Characterization of winter microbial communities in the purple pitcher plant (*sarracenia purpurea*)

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Characterization of winter microbial communities in the purple pitcher plant (*Sarracenia purpurea*)

by

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ABSTRACT

Carnivorous pitcher plants trap insects in cone-shaped leaves and digest them to gain vital nutrients. For digestion to occur, plants in the genus *Sarracenia* require mutualistic microorganisms living in their leaves. Few studies have examined how these communities change over time. This study specifically examines the bacterial composition in the most widely distributed species, *Sarracenia purpurea,* in the winter. The leaves of this plant species live for several years, and it is unknown whether microbes overwinter in pitcher fluid or if community structure must be reestablished each spring. This study aims to characterize the winter microbiome in two population of *Sarracenia* collected between the months of November 2012 and January 2014 in two different ways: DNA from the fluid of 57 pitchers in one population was extracted and amplified using ARISA-PCR, and metabolic substrate usage was measured in 36 pitchers in two populations. Bacteria from eight phyla were recovered. The number of unique genera identified within one leaf ranged from 27-60, and the number of unique phylotypes per sample ranged from 59-186. Metabolic usage dropped drastically from summer/fall levels in December, only to rebound in January. Results indicate a large, diverse, and dynamic community of microbes present throughout the winter that are capable of using a wide variety of carbon substrates.

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Chapter 1: Introduction and Background

1.1 Plant-microbe Interactions

Mutualistic interactions between plants and microbes are common occurrences and many of these interactions have been extensively studied. In these partnerships, both organisms receive benefits they could not reap individually. Classic examples include interactions between roots and microorgaisms. It is believed that more than 80% of land plant families participate in arbuscular mycorrhizal symbiosis with fungi alone (Kawaguchi and Minamisawa, 2010). The mutualism between legumes and *Rhizobium* bacteria is another well-known case of such an interaction. In this example, the root systems of several species of legumes act as hosts for the bacteria, providing a safe environment conducive to growth and reproduction (Kiers et al., 2003). In return, rhizobia fix atmospheric nitrogen, converting it to an accessible form for the plants, providing them with essential nutrients. Sphagnum mosses growing in peat bogs use recycled methane that is oxidized by symbiotic bacteria (Raghoebarsing et al., 2005). These interactions are scientifically interesting because they highlight the interdependence of the evolutionarily divergent species involved. The mutualistic interaction between pitcher plants and the microbes inhabiting their pitchers is the focus of this thesis.

Plants in the genus *Sarracenia* harbor bacterial communities in the fluid of their pitchers. The carnivorous pitcher plant *Sarracenia purpurea* is found in bog and fen habitats and is a species native to Michigan. Carnivorous pitcher plants trap insects in cone-shaped leaves and digest them in order to gain vital nutrients that the plants cannot access in sufficient quantities from the soil alone. It is unclear if the plants in the genus *Sarracenia* are capable of producing effective digestive enzymes (Gray et al., 2012), but it is believed that

the plants do require the aid of mutualistic microorganisms living in their pitchers to fully digest their prey. While the bacteria gain a relatively stable, contained habitat with a food source of insect prey being continually replenished, the plants benefit from the decomposing activity of the bacteria (Gray et al., 2012). Though relatively little is known about this complex mutualistic relationship, it is likely that if the right composition of microorganisms is not present in the pitchers, the plants may exhibit limited nutrient uptake, and/or reduced growth.

1.2 Microbial Communities in Pitcher Plants

Sarracenia pitchers are sterile upon first opening (Peterson et al., 2008), and bacterial communities must be established from the surrounding environment. Bacteria may be introduced in a variety of ways such as through the insect prey species of the plants, animal inhabitants of the pitchers, or from other plant matter (like falling leaves). It is important to characterize the bacteria inhabiting the pitcher fluid of *Sarracenia purpurea* in order to gauge the taxonomic richness of this unique microbial community. Bacteria in this species of plant (*S. purpurea*) have for the most part only been identified using culture-dependent methods (Siragusa et al., 2007; Whitman et al., 2005), or have been studied with the intent of characterizing only a small segment of the bacterial community (i.e. methanogens or fecal indicator bacteria) (Krieger and Kourtev, 2012a; Whitman et al., 2005). These studies identified 13 (Siragusa et al., 2007), 2 (Whitman et al., 2005), and 3 (Krieger and Kourtev, 2012a) microbial phylotypes (the term phylotype is used here in place of species) respectively in the pitcher fluid of *Sarracenia purpurea* (Table 1).

*Table 1. Summary of the methods employed and species richness results of previous studies characterizing the microbial communities of the genus Sarracenia. Those using culture independent methods are **bolded**.*

Study	**Peterson et al. 2008**	**Siragusa et al. 2007**	**Whitman et al. 2005**	**Krieger and Kourtev 2012 a, b (2 studies)**	**Koopman et al. 2010**	**This Study**
Organism	*S. purpurea*	*S. minor*	*S. purpurea*	*S. purpurea*	*S. alata*	*S. purpurea*
Culture Independent/Dependent	**Independent**	Dependent	Dependent	**Independent**	**Independent**	**Independent**
Number of Phylotypes	133	13	2	3; 27	485	186

Only two other studies with the purpose of characterizing the microbial community of *S. purpurea* as a whole have utilized culture-independent techniques (Krieger and Kourtev, 2012b; Peterson et al., 2008). In one, the sampled pitchers were located in Massachusetts and a T-RFLP fingerprinting method was used (Peterson et al., 2008). In this study 133 unique peak lengths (each corresponding to a unique phylotype) were isolated (Table 1), but little was reported in regard to taxonomic identification through the use of databases (Peterson et al., 2008). The researchers also found very few peaks that were present in multiple pitchers (Peterson et al., 2008). The other study focused on characterizing bacteria in three specific zones of *S. purpurea* pitchers, identifying a maximum of 27 phylotypes in the liquid of one pitcher (Krieger and Kourtev, 2012b).

More microbial richness was detected through culture-independent techniques in the related species *Sarracenia alata* (Koopman et al., 2010). This study found 485 unique peak

lengths, many of which were present in multiple pitchers (Koopman et al., 2010). Because previous studies involving *S. purpurea* have identified less microbial richness than Koopman et al. (2010), further study of *S. purpurea* using culture-independent techniques is needed in order to determine if bacterial taxonomic richness in *S. purpurea* has been underestimated due to limited methodology, or if there is an inherent difference between the microbial communities harbored by the two plant species. In addition to assessing microbial taxonomic richness, further analysis is needed to identify the bacteria comprising the communities. This will further our understanding of the ecology of the system.

1.3 Pitcher Plants as "Digestive Systems"

The bacterial communities in the guts of mammalian species – particularly humans - have been extensively studied using culture-independent methods and have been more thoroughly classified than the communities inhabiting pitcher plants (Carey et al., 2013; Guinane et al., 2013; Hayashi et al., 2002). The nature of the mutualism between these seemingly divergent systems and the roles played by the bacterial communities in each are potentially very similar as both the (mammalian or human) gut and pitcher plant leaves facilitate digestion. In fact, certain phylotypes that are commonly found in the human gut have also been identified in *Sarracenia* pitchers, such as *Escherichia coli* and *Enterococcus faecalis* (Koopman et al., 2010; Whitman et al., 2005). Although these bacteria have been found in several locations other than mammalian guts (Sehgal et al., 2008), it is possible that a more extensive characterization of the pitcher community in *S. purpurea* will reveal that there are more bacterial phylotypes present in pitcher fluid that are also shared with human gut habitats. Even methanogenic archaea, known to be key mutualistic anaerobes in the

human gut (Bäckhed et al., 2005), have been identified in the bottom portions of *S. purpurea* pitchers (Krieger and Kourtev, 2012a).

While some features of these two systems are similar, there are key differences that must be noted. Mammalian guts represent a more closed environment; pitcher plants are open to the environment, making it easier for microbes to colonize than the gut. While *Sarracenia* pitchers appear capable of providing varied microhabitats, such as anaerobic zones in the deeper regions of the fluid, mammalian guts are much larger and presumably more complex. Additionally, mammalian guts are comprised of more anaerobic zones and are known to harbor several strictly anaerobic microbial phylotypes (Hayashi et al., 2002), which would likely not be able to survive in pitcher fluid.

Despite these differences, identifying similarities between pitcher and human gut microbial communities may lead to a better understanding of the interaction between *S. purpurea* and its symbiotic microbial community because the human gut microbiome has already been extensively characterized. This might in turn provide insight into the digestive process in animal and plant systems.

1.4 Winter Ecology of Microbial Communities

Temporal factors have been identified as a driving force in determining the taxonomic richness of mutualistic bacteria in pitcher fluid (Koopman et al., 2010). From a previous study on a closely related plant species in the same genus we know that bacterial richness is highest in the hottest months of summer (Koopman et al., 2010). Little is known, however, about the bacterial composition in the winter in the genus, as the leaves of the species (*S. alata*) used in Koopman et al. (2010) died back each winter and were replaced by new leaves each spring. Individual *Sarracenia purpurea* pitchers, in contrast, are present on the plant

throughout the entire year, remain open to the environment during the winter, and can last for up to two years (Adlassnig et al., 2011).

Previous studies have focused on freezing patterns in *S. purpurea* and the effect of these conditions on other pitcher inhabitants such as pitcher plant mosquito (*Wyeomyia smithii*) larvae (Kingsolver, 1979). It was concluded that the fluid in the pitchers is insulated by the high thermal capacity of the wet peat soil surrounding the plant. As a result, pitcher fluid does not generally freeze until the bog surface freezes - generally in late November - even if there are short periods of unusually cold weather earlier in the fall (Kingsolver, 1979). Similar studies have never been conducted that focus on the effects of these freezing patterns on bacteria in pitcher fluid.

Freezing conditions pose physiological challenges to the survival and continued metabolic activity of microbial populations. In general these challenges consist of reduced enzymatic rates, limited availability of nutrients, and reduced fluidity of cell membranes (Margesin and Miteva, 2011). At the individual level, a microbial cell's ability to survive freezing depends in part on the chemical makeup of the pitcher fluid. Certain fluids known as cryoprotectants prevent bacteria from being killed during freezing by stabilizing cell membranes and inhibiting protein denaturation (D'Amico et al., 2006). Glycerol, for example is sometimes used to preserve bacterial cultures until they are thawed and used for experiments (Postgate and Hunter, 1961). Although the chemical composition of the fluid in *Sarracenia* pitchers has not been extensively studied, and likely varies from pitcher to pitcher, it has been reported to differ very little from pure water (Adlassnig et al., 2011). It is unknown if it contains cryoprotectants that would aid in the prevention of cell lysing due to freezing. However, certain bacterial phylotypes are known to produce their own

cryoprotectants such as glycine betaine (Chattopadhyay, 2002), trehalose, and exopolysaccharides (Phadtare, 2004).

Other common protective adaptations of organisms to freezing include increasing membrane fluidity by producing more unsaturated fatty acids (Finegold, 1996) and cold-adapted enzymes with high specific activities at reduced temperatures (D'Amico et al., 2006; Georlette et al., 2004; Russell, 2000). The ability of microbes to maintain cell integrity and sustain enzymatic activity may determine which ones survive in the winter. Without these adaptations, most bacterial growth would be halted, and cells would likely be damaged or destroyed by freezing conditions. Because little is known about the microbial composition in this species of pitcher plant, it is not known which, if any, microbial inhabitants of *S. purpurea* possess these cell membrane attributes and whether, if present, they are active in the winter.

Conditions repeatedly cycling between freezing and thawing tend to be especially harmful to microbial cells. Several freeze-thaw cycles will damage cell membranes and result in the lysis of most bacterial cells; techniques involving freeze-thaw cycles are often used by microbiologists to break open bacterial cells and extract DNA (Moré et al., 1994). Bacterial cells in *Sarracenia* pitchers would likely be damaged by the multiple freezing and thawing cycles which occur during the course of a typical Michigan winter. Interestingly, freeze-thaw cycles have also been reported to cause increases in nitrogen and carbon mineralization in soils, largely attributed to more available decomposable microbial biomass and a subsequent burst of respiration (Herrmann and Witter, 2002; Schimel and Clein, 1996). These bursts of metabolic activity are usually confined to the first freeze-thaw cycle

and are inhibited by a significantly reduced microbial population following successive cycles (Herrmann and Witter, 2002; Schimel and Clein, 1996).

It is important to note that in most years, between the months of mid-November and mid-March in southeastern Michigan, there are days where ambient temperatures rise above freezing and small bodies of water may thaw. Research conducted in northern Ohio, which focused on the physical properties of the pitcher fluid of *S. purpurea* throughout the year reported that during the winter months (undefined by the study), pitcher fluid was only completely frozen 20% of the time (Hamilton IV, 2010). The amount of time that pitchers remain frozen in Michigan would not be identical to those in Ohio, and changes would depend upon the severity of the winter, but it is likely during any given winter that pitcher fluid will not remain continuously frozen for the entire season. It is possible that the likely freeze-thaw cycles taking place in pitcher fluid would impact bacterial populations similarly to those in soil. However, given that the contents of pitchers and thus nutrient availability can be quite different depending on the pitcher, metabolic responses to freeze-thaw cycles might vary greatly.

At the community level, the populations of bacteria present in pitcher fluid would be predicted to respond to the onset of winter in a number of ways. It would be reasonable to predict that the populations of some phylotypes are severely reduced when temperatures drop and pitcher fluid eventually freezes during the course of the winter. This response has been observed in *Pseudomonas paucimobilis,* a phylotype inhabiting prairie soil (Morley et al., 1983), and a member of a genus that has been identified in *Sarracenia* fluid. *P. paucimobilis* cultures displayed a 99.9% survival rate when exposed to a single freezing event at -9^{o}C but 40-60% mortality when exposed to multiple freeze-thaw cycles at the same temperature

(Morley et al., 1983). This shows that resistance to freezing can be reduced when temperatures fluctuate above and below the freezing point. When exposed to a single severe freezing event at -27^{o}C, *P. paucimobilus* experienced 40-60% mortality (Morley et al., 1983). Other types of bacteria present in the fluid may be much better adapted to survive, but halt the process of metabolism when pitcher fluid approaches or reaches the freezing point (Holt and Leadbetter, 1969). The bacterial phylotypes in the pitchers that are best suited to live in cold environments may even be able to grow and thrive at freezing temperatures. *Psychrobacter cryohalolentis* exhibits increased cellular ATP and ADP concentrations as incubation temperatures decrease from 22^{o}C to -80^{o}C, indicating the ability of some microbes to conduct metabolism at extremely low temperatures (Amato and Christner, 2009). Overall, the severity of the winter, the number of freeze-thaw cycles taking place in the pitcher fluid, and the adaptations of the microbes living in the fluid will be the biggest factors determining which microbes survive the winter.

1.5 Bacterial Activity

Another question that arises from this study is whether or not bacteria in the pitchers are active during the winter months. Psychrophilic and psychrotolerant bacteria are capable of surviving and growing at temperatures below 0^{o}C (Finegold, 1996). While psychrophiles cannot survive temperatures that reach much above 20^{o}C, psychrotolerant microbes are much more resilient to heat and can grow at temperatures of up to 40^{o}C (Finegold, 1996). It is therefore likely that if there are any psychrotolerant bacteria inhabiting the pitcher fluid of *S. purpurea*, they would be well-adapted to survive and even metabolize during any month of the year. The generally accepted lower limit of observed metabolism and cell division in microbes has been minus 12^{o}C (Finegold, 1996). A more recent article, however, has

reported microbial metabolism at temperatures as low as minus 39^{o}C by detecting CO_2 formation in Alaskan soil (Panikov et al., 2006). While this level of extreme cold adaptation is probably rare and not found in a climate as temperate as Michigan, active microbial communities have been detected in the soil of ecosystems in Michigan during the winter (Aanderud et al., 2013). The presence of the psychrotolerant genus *Pseudomonas* has also been detected in the pitcher fluid of *S. alata* (Koopman et al., 2010), and it is possible that there is an active community of bacteria inhabiting *S. purpurea* pitchers throughout the winter. However, it is still unknown whether any dormant bacteria in thawed or partially thawed pitchers immediately, or ever, become active again, and what their role in plant digestion may be. If such a community does exist, freeze-thaw cycling may determine the level of activity as it has been shown to do in soil microbial communities (Herrmann and Witter, 2002; Schimel and Clein, 1996).

1.6 Research Goals

- **The first goal of this study is to characterize the microbial community in one population of *S. purpurea* collected throughout winter months.**
- **The second goal of this study is to determine the level of metabolic activity and preferred substrates of microbes in two populations of *S. purpurea.***

1.7 Hypotheses

<u>In order to address Research Goal 1)</u> (Characterize the microbial community in one population of *S. purpurea* collected throughout winter months) I hypothesized:

1. Bacteria will be present in the pitchers sampled in the winter.

2. Species richness of the winter community in *S. purpurea* will be less than the sampled communities of the southern species *S. alata* because of the shorter summers, colder winters, and less abundant prey associated with the northern range of *S. purpurea.*
3. Species richness will peak at the start of the winter then decline as winter progresses and temperature consistently decreases. Longer, more extreme periods of freezing typically experienced in mid and late winter combined with multiple freeze-thaw cycles will likely reduce, or even eliminate microbial taxa inhabiting pitcher fluid.
4. Psychrophilic and/or psychrotolerant taxa will be identified in pitchers sampled in winter using molecular data. Members of these taxa will make up a larger proportion of identified bacteria than previous studies on the genus indicate, given that the samples characterized here are the first to be collected in the winter for the genus.

<u>In order to address Research Goal 2)</u> (Determine seasonal potential metabolic activity and substrate use of microbes in two populations of *S. purpurea*) I hypothesized:

1. A wide variety of substrate types will be utilized, given the expectation of a relatively diverse microbial community.
2. Metabolic substrate usage will be lowest during the coldest months (January and December) and highest during the warmest months (July and August). Microbes will be forced to adapt to changing temperatures as summer and fall progress into winter. Many microbes will die or become dormant during the winter, and those that do remain active will have significantly reduced growth. This will be reflected in the carbon usage as indicated by changes in EcoPlate coloration; slower bacterial growth will mean a lower rate of respiration and less carbon consumption.

Chapter 2: Methods

2.1 Sample Collection

The contents of 78 *Sarracenia purpurea* pitchers were collected monthly from November of 2012 to March of 2013 and July of 2013 to January 2014 for ARISA PCR and EcoPlate experiments. Fluid from these pitchers was collected from two separate bogs in southeast Michigan: Waterloo Recreation Area in Chelsea, MI (ARISA data and EcoPlate experiment) and the Fish Lake Bog in Lapeer, MI (EcoPlate experiment only). During each month's collection ten pitchers were plucked at the base of the stem. The liquid or frozen contents of the pitchers were poured into sterile 50 mL vials. During collection only the outsides of the pitchers were handled in order to avoid contaminating the fluid. Fluid collections were kept on ice when transported from the collection site. EcoPlate plating was performed immediately upon returning to the laboratory. Remaining pitcher fluid was frozen until DNA extractions were performed.

2.2 DNA Extractions

DNA extractions were performed on thawed fluid samples as quickly as possible after collection. Approximate volume of samples was recorded and then up to 600 μl of well-vortexed pitcher fluid from each sample was used. If a pitcher sample did not contain at least 600 μl of fluid, all of the fluid was used for the extraction. Fluid extractions were done using a 2mL bead-beating tube containing beads from a Powersoil DNA isolation kit (MoBio, Madison, WI). All microbial DNA was isolated from each pitcher sample according to the manufacturer's directions.

2.3 ARISA PCR

ARISA (automated ribosomal intergenic spacer analysis) PCR is used to estimate microbial richness in fluid and soil samples (Fisher and Triplett, 1999; Ranjard et al., 2001). ARISA targets the bacterial rRNA operon 16S – 23S internal transcribed spacer (ITS) for amplification as the region shows a high level of heterogeneity in length and nucleotide sequence among taxa (Fisher and Triplett, 1999). PCR is performed using a fluorescence-tagged oligonucleotide primer, and an automated electrophoresis system is used to detect the length of DNA fragments (Fisher and Triplett, 1999). Fragment lengths are then referenced to a database where they can be matched to their corresponding organism and identified.

ARISA PCR was conducted on a total of 57 fluid samples from all sampled winter months. This comprises two winters and portions of seven months. 16S rRNA was amplified with ITSF and 5'-end phosphoramidite dye HEX-labeled ITSReub primers (Cardinale et al., 2004). This procedure aims to simultaneously amplify DNA from any microbes present in the pitcher fluid samples. PCRs were run in triplicate and pooled in order to minimize PCR bias. Samples were genotyped at the Sequencing facility at the University of Georgia. Peak Scanner (Waltham, MA) software was used to separate and calculate the sizes of amplified fragments. ARISA peaks were visualized and identified using ADAPT (Kent et al., 2003). ADAPT is a web-application that takes the amplified Internal Transcribed Spacers (ITS) from ARISA genotyping and blasts them against NCBI and SEED databases to assess taxonomic, pathogenic, and trophic composition of samples. Peaks between 150 and 500 base pairs in length were targeted and identified by ADAPT. Taxonomic Richness (species/phylotypes, genus, family, order, class and phylum) of microbial communities were compiled for each sample, compiled by month, and compared

among months. Richness was assessed by pooling data for all relevant samples in a variety of bins (e.g. all samples for total species richness, all samples of that month for monthly species richness). All duplicate peaks at the given taxonomic rank were removed, and the remaining peaks were counted in order to determine taxonomic richness.

2.4 EcoPlate Metabolism Experiments

In order to determine if microbial communities are potentially active during the winter in *S. purpurea* pitchers, Biolog EcoPlates were inoculated with winter samples of pitcher fluid. EcoPlates are 96-well plates with 31 different organic substrate in each well (each replicated 3 times) (Choi and Dobbs, 1999). In addition to the substrate, each well also contains a colorless tetrazolium dye (Stefanowicz, 2006). Pitcher fluid is pipetted into each well and microorganisms in the fluid will oxidize the substrates depending on their mode of metabolism. As the substrates are oxidized, the colorless tetrazolium dye is reduced to a violet formazan (Stefanowicz, 2006). The intensity of the resulting violet color is gauged using a spectrophotometer. In addition to giving an indication of overall activity during the winter months one can potentially learn what substrates or general substrate groups (i.e. carbohydrates, amino acids, carboxylic acids, amines, amides, or polymers) are highly utilized. EcoPlates are regularly used in experiments to measure microbial activity in soils and study the impacts of soil contamination on community activity (Derry et al., 1998; Garau et al., 2007; Widmer et al., 2006). While the use of EcoPlates in this study will not tell us which resources were being utilized by bacteria in the fluid at the time of collection, it will reveal the resources that could potentially be utilized by bacteria living in the pitchers.

The fluid from three pitchers was collected from Waterloo every month from July 2013 to January 2014 for EcoPlate analysis. Three pitchers were also sampled monthly from

the Fish Lake population, but only from August 2013 to December 2013. After 600µl of fluid were removed from the samples for DNA extraction the remaining fluid was poured into a sterile reservoir and loaded into 96-well Biolog EcoPlates (Hayward, CA) using a multi-channel pipet (150µl per well). Individual samples were plated in duplicate. Initial color intensity of wells was recorded using a Biotek Synergy microplate reader (Winooski, VT). Color of wells was then recorded at 24, 48, and 72 hours. All samples were incubated at 23^{o} C for the duration of the experiment. Additional plates were loaded and incubated at 4^{o} C in October (Waterloo samples) and November (Waterloo and Fish Lake samples). These plates were not used for statistical analysis, but they confirmed that microbes in the fluid were capable of respiration when incubated at a temperature more similar to that experienced in the pitcher at the time of collection.

Final absorbance readings of replicate wells were averaged for each substrate. Only data from time point 3 (72 hours) were used. Average Well Color Development (AWCD) measures average color change across all substrates for each sample. AWCD was calculated according to Garland and Mills using the formula $AWCD = \Sigma (C-R)/n$ where C is the absorbance value for each well and R is the absorbance of the control well (Garland and Mills, 1991). Each substrate was also grouped into one of the following categories: carbohydrates, polymers, carboxylic acids, amino acids, and amides/amines according to (Jacinthe et al., 2010).

2.5 Statistical Analysis

Primer-E software (Clarke, 1993), was used to run analyses of similarity (ANOSIM) among samples for community composition and activity. ANOSIM tests the similarity within groups of samples against the similarity among groups of samples for statistical

significance. Each group is individually compared to every other group. Each comparison results in an R value indicating the level of difference between the groups. R values range from -1 to 1. The higher the value of R, the greater the level of difference between groups. Presence/absence data were compiled for each sample at five taxonomic levels (Phylum, Class, Order, Family, and Genus) and the month of collection was the tested factor to determine if there were significant temporal changes in community composition throughout the winter. For community activity, the factors tested were month of collection and population.

Chapter 3: Results

3.1 ARISA Data

The contents of 57 pitchers from Waterloo Bog were characterized using ARISA techniques throughout seven winter months from November 2012-March 2013 and November 2013-December 2013.

A. Taxonomic richness. A total of 186 unique phylotypes, 60 genera, 49 families, 25 orders, 12 classes, and 8 phyla were identified from the sampled pitchers. Species richness of individual samples ranged from 22 to 186 (Fig.1). Monthly averages for species richness ranged from 88.1 (+/- 46.6) in November 2013 to 165.5 (+/- 13.3) in February 2013 (Fig. 2, Table 2), with an overall average of 126.6 (+/-43.3) phylotypes per sample. The average number of unique microbial genera and families per month ranged from 37.0 (+/-13.6) and 30.8 (+/-10.3), respectively, in November 2013 to 55.4 (+/-3.1) and 44.3 (+/-1.9) in February 2013 (Fig. 3, Table 2), with overall averages of 45.9 (+/-10.9) genera and 37.5 (+/-8.3) families. The average number of unique microbial orders, classes, and phyla identified per month ranged from 13.4 (+/-5.3), 8.4 (+/-2.6), and 5.7 (+/-1.9), respectively, in November 2013 to 20.3 (+/-1.6) (February 2013), 10.5 (+/-0.76) (February 2013), and 7.8 (+/-1.5) (December 2012) (Fig. 4, Table 2).

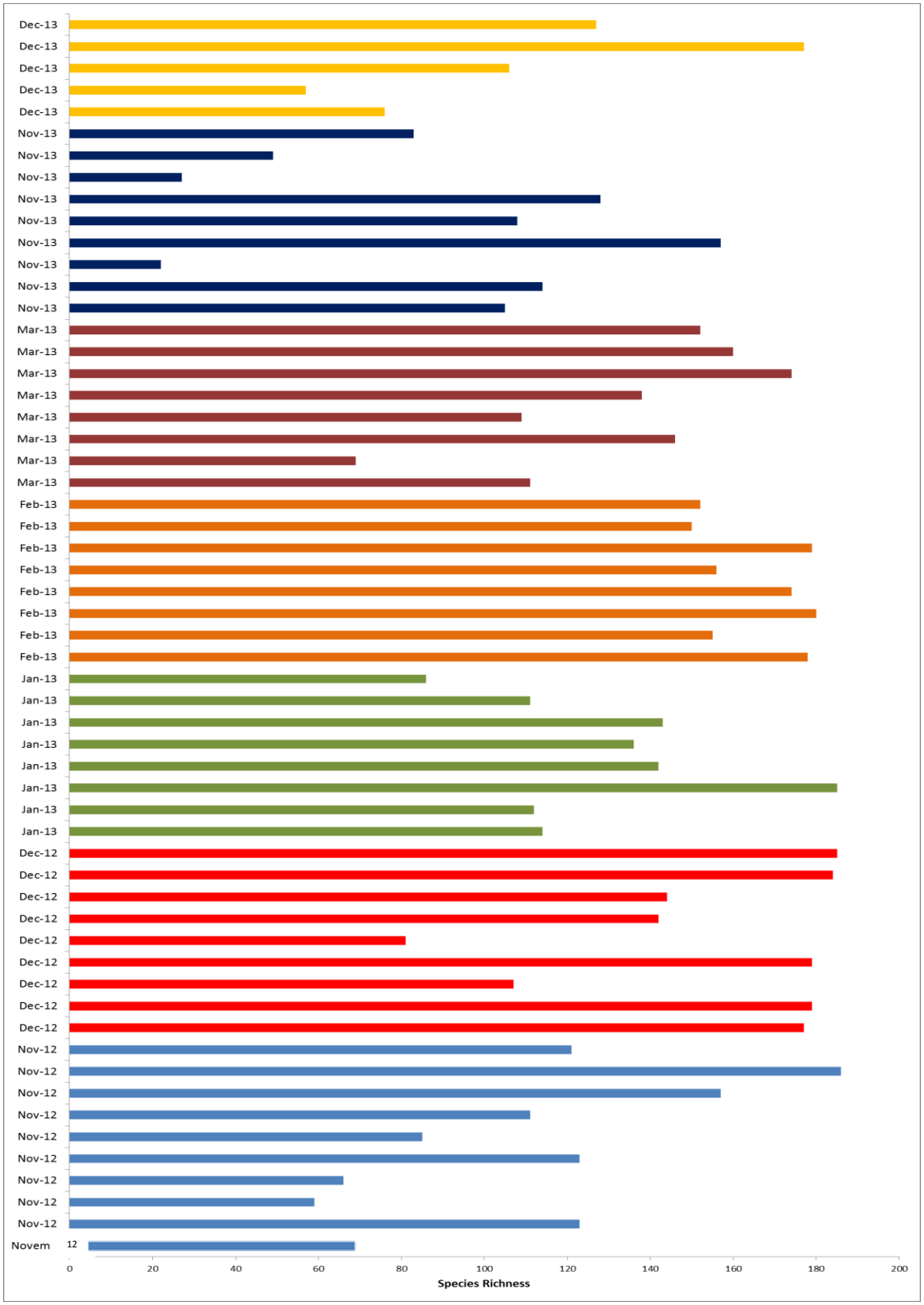

Figure 1. Species richness of each individual sampled pitcher. Bars of the same color are samples collected on the same day.

Table 2. Average monthly taxonomic richness of pitcher fluid samples at each taxonomic level.

	Phyla	Classes	Orders	Families	Genera	Phylotypes
Nov-12	6.2	9.5	16.1	33.2	40.7	109.7
Dec-12	7.8	11.0	20	42.7	52.8	153.1
Jan-13	6.75	10.0	18.38	38.13	45.88	128.6
Feb-13	7.5	10.5	20.25	44.25	55.38	165.5
Mar-13	6.75	10.1	18.75	38.5	47.13	132.4
Nov-13	5.7	8.4	13.4	30.8	37	88.1
Dec-13	6.2	10.2	17.4	34.8	42.2	108.6
Overall Average	6.7	10.0	17.8	37.5	45.9	126.6

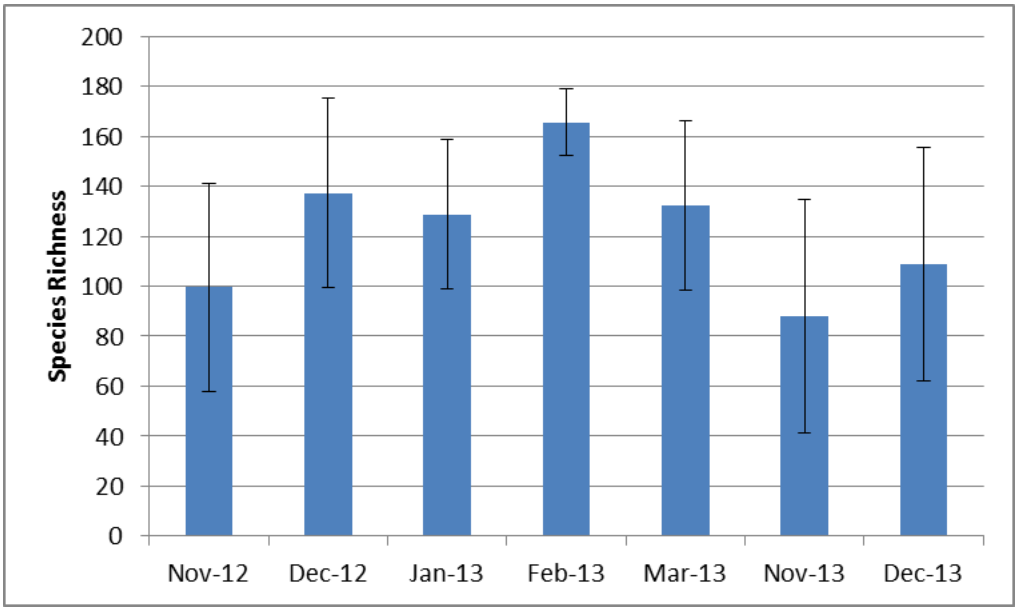

Figure 2. Average species richness identified per month of sampling. Error bars represent standard deviations.

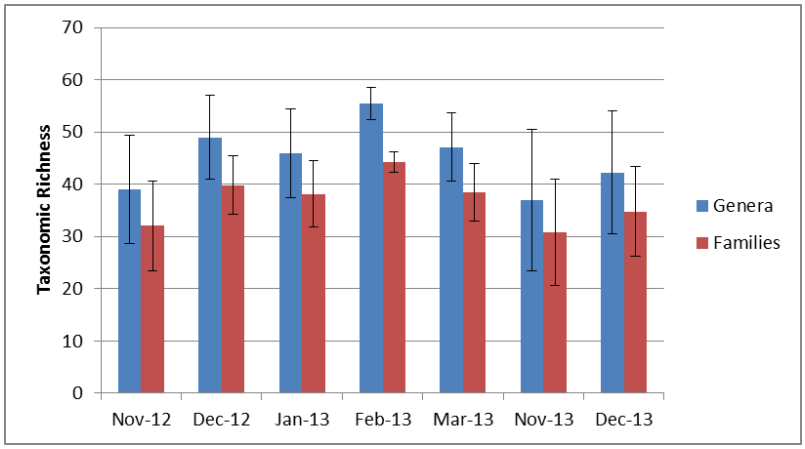

Figure 3. Average number of unique genera and families identified per month. Error bars represent standard deviations.

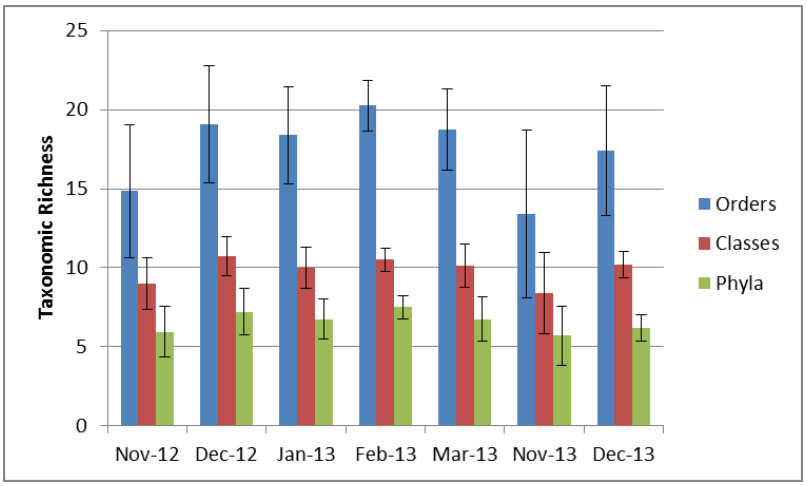

Figure 4. Average number of unique orders, classes, and phyla identified per month. Error bars represent standard deviations.

B. Community composition. Three phyla were present in every sampled pitcher: Actinobacteria, Firmicutes, and Proteobacteria. Firmicutes was the most abundant phylum, making up 63.17% of the 35,519 identified peaks (Fig. 5, Table 3). Actinobacteria was the second most prevalent phylum (21.96%), followed by Proteobacteria (13.06%) (Fig. 5, Table 3). The remaining five identified phyla comprised less than 2% of the identified peaks (Fig. 5). The bacterial classes Actinobacteria, Bacilli, Betaproteobactera, Deltaproteobacteria, and Gammaproteobacteria were present in every sample. Class Clostridia was identified in 56 of the 57 sampled pitchers.

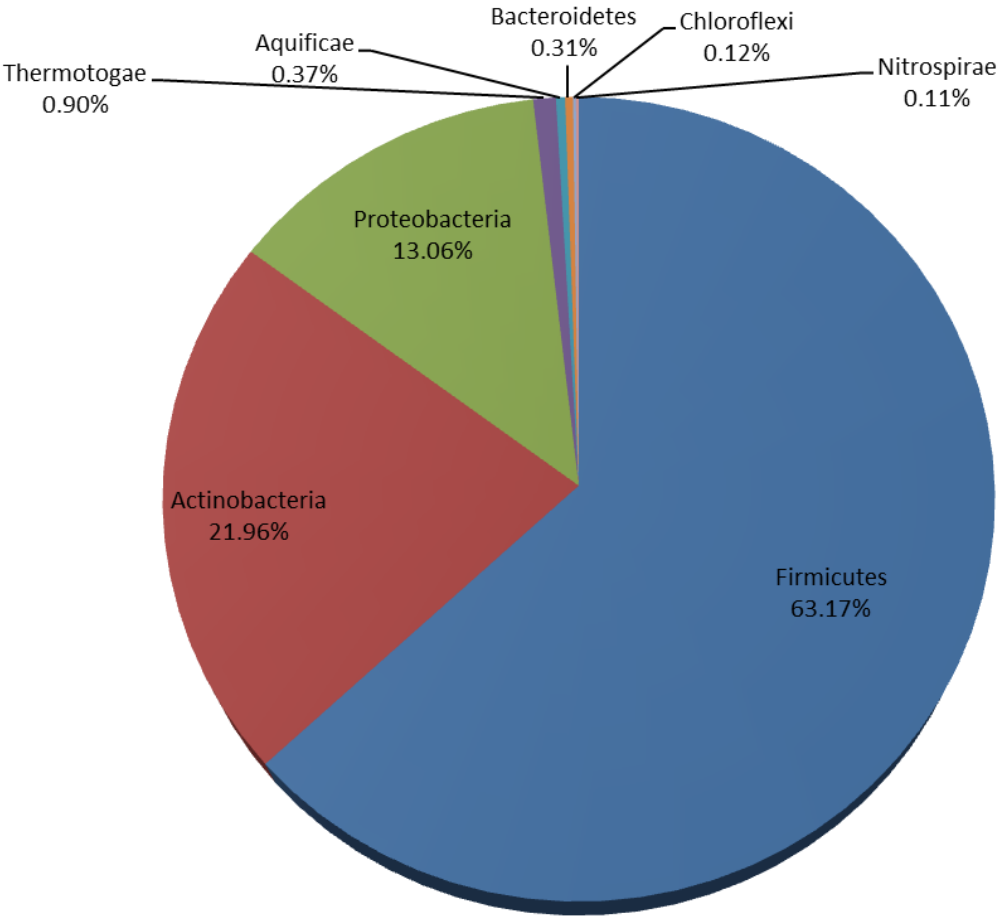

Figure 5. Relative abundance of the phyla identified in winter ARISA samples. Percentages indicate the percentage of the total identified peaks belonging to each phylum.

Table 3. Taxa that were present in at least 90% of the winter samples.

Phylum	**Class**	**Order**	**Family**
Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae
			Corynebacteriaceae
			Micrococcaceae
			Nocardiaceae
			Nocardiopsaceae
			Streptomycetacea
Firmicutes	Bacilli	Bacillales	Bacillaceae
			Enterococcaceae
			Staphylococcaceae
			Streptococcaceae
		Lactobacilliales	
	Clostridia		
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae
		Nitrosomonadales	Nitrosomonadaceae
	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae
	Gammaproteobacteria		

Presence/absence data were collected and analyzed at five taxonomic ranks (phylum, class, order, family, and genus). The taxa present in each month were significantly different from one another at each of the five taxonomic levels over the course of all the months (Table 4). However, the taxa in each individual month were not significantly different from every other month (Table 4). Even though several taxa are present in all or nearly all of the samples throughout the winter (Table 3), the overall composition of microbial taxa was more similar among samples collected in the same month than among those collected in other months.

Nine individual monthly comparisons were not significantly different from one another at any taxonomic rank (Table 4). Six comparisons (Dec13-Nov13, Dec12-Dec13, Feb13-Nov13, Feb13-Dec13, Mar13-Nov13, and Mar13-Dec13) were significantly different from one another at all five taxonomic ranks. Two comparisons (Dec13-Jan13 and Feb13-Mar13) were significantly different from one another at four of the five taxonomic ranks, and one comparison (Jan13-Feb13) was significantly different at three ranks. Most months were significantly different from one another at the class and ordinal taxonomic levels (10 out of the 21 month-month comparisons).

Table 4. ANOSIM results testing for significant differences in presence/absence of organisms at each taxonomic rank. Highlighted cells signify p-values less than 0.05, indicating differences that are significant between the given month-month comparison.

	Genus		**Family**		**Order**		**Class**		**Phylum**	
Months	R statistic	P value	R statistic	P value	R statistic	P value	R statistic	P value	R statistic	P value
Nov12, Dec12	0.019	0.252	0.026	0.193	-0.026	0.654	0.05	0.206	0.036	0.193
Nov12, Jan13	-0.09	0.961	-0.083	0.997	-0.11	0.987	-0.117	0.967	-0.093	0.966
Nov12, Feb13	0.016	0.288	0.039	0.197	0.025	0.281	0.068	0.152	0.026	0.197
Nov12, Mar13	-0.093	0.986	-0.061	0.893	-0.074	0.931	-0.023	0.49	-0.04	0.893
Nov12, Nov13	0.106	0.051	0.1	0.054	0.204	0.014	0.198	0.017	0.242	0.054
Nov12, Dec13	0.085	0.256	0.051	0.360	0.127	0.189	0.32	0.009	0.187	0.36
Dec12, Jan13	0.015	0.309	0.016	0.302	-0.014	0.516	0.038	0.25	0.05	0.302
Dec12, Feb13	-0.029	0.744	-0.031	0.758	-0.007	0.485	-0.028	0.607	-0.024	0.758
Dec12, Mar13	0.03	0.194	0.025	0.274	0.042	0.21	0.029	0.272	0.068	0.274
Dec12, Nov13	0.198	0.003	0.201	0.002	0.28	0.004	0.288	0.015	0.373	0.002
Dec12, Dec13	0.447	0.003	0.501	0.005	0.419	0.013	0.633	0.002	0.391	0.005
Jan13, Feb13	0.103	0.012	0.117	0.016	0.029	0.254	0.028	0.346	0.01	0.016
Jan13, Mar13	-0.064	0.891	-0.01	0.548	0.028	0.3	-0.051	0.712	-0.051	0.548
Jan13, Nov13	0.112	0.063	0.136	0.052	0.234	0.014	0.322	0.007	0.495	0.052
Jan13, Dec13	0.208	0.076	0.283	0.023	0.364	0.01	0.604	0.002	0.399	0.023
Feb13, Mar13	0.202	0.003	0.243	0.002	0.116	0.046	-0.035	0.598	0.01	0.002
Feb13, Nov13	0.261	0.005	0.279	0.004	0.354	0.002	0.381	0.003	0.5	0.004
Feb13, Dec13	0.696	0.002	0.7	0.002	0.751	0.002	0.887	0.001	0.674	0.002
Mar13, Nov13	0.211	0.011	0.231	0.005	0.327	0.004	0.265	0.01	0.457	0.005
Mar13, Dec13	0.391	0.009	0.436	0.004	0.522	0.003	0.575	0.007	0.432	0.004
Nov13, Dec13	-0.04	0.554	0.013	0.391	-0.032	0.534	-0.08	0.707	-0.092	0.391
Global	0.088	0.006	0.101	0.001	0.12	0.002	0.159	0.001	0.164	0.001

3.2 EcoPlate Experiments

In the waterloo population, overall metabolic activity, assessed as Average well color development (AWCD) ranged from an average of 0.50 in the December 2013 samples to 1.16 in the January samples (Fig. 6). The Fish Lake population's AWCD ranged from 0.53 in the December 2013 samples to 1.39 in the October samples (July and January not sampled) (Fig. 6).

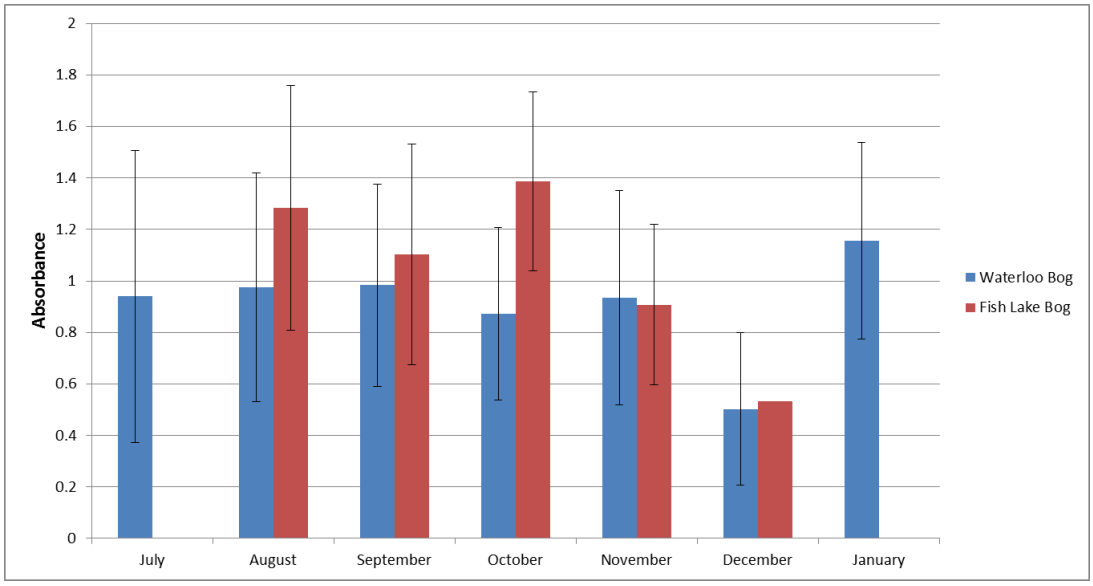

Figure 6. Average well color development per month of Waterloo (blue) and Fish Lake (red) samples. Sampled months range from July 2013 to January 2014. Error bars represent standard deviations.

A. Monthly Analysis. AWCD of pitcher fluid samples in both populations changed significantly by month (Global R = 0.135, p-value = 0.038) (Table 5). Six out of 21 month-month comparisons were significantly different; all of them involved the month of December 2013. Every monthly comparison involving December was significant.

Table 5. ANOSIM results testing for significant differences in AWCD of EcoPlates among the sampled months (both populations pooled). Highlighted cells indicate significance at p<0.05.

Groups	R Statistic	p-value
JUL, AUG	0.111	0.238
JUL, SEP	-0.108	0.619
JUL, OCT	-0.235	0.988
JUL, NOV	-0.265	0.988
JUL, DEC	0.951	0.012
JUL, JAN	0.185	0.2
AUG, SEP	-0.1	0.851
AUG, OCT	0.013	0.344
AUG, NOV	0.028	0.32
AUG, DEC	0.643	0.011
AUG, JAN	0.309	0.101
SEP, OCT	-0.022	0.461
SEP, NOV	-0.059	0.667
SEP, DEC	0.587	0.015
SEP, JAN	0.228	0.131
OCT, NOV	-0.152	1
OCT, DEC	0.315	0.013
OCT, JAN	-0.167	0.836
NOV, DEC	0.28	0.015
NOV, JAN	-0.08	0.536
DEC, JAN	0.383	0.06
Global	0.135	0.038

B. Population comparisons. AWCD between the two collection sites was not significantly different (R = -0.017, p-value of 0.55).

C. Substrate comparisons. The substrates in the EcoPlate wells were broadly grouped into five chemical types: carbohydrates, polymers, carboxylic acids, amino acids, and amines/amides. For the Waterloo samples the average absorbance of wells containing carbohydrates was 1.04, absorbance of polymers was 1.32, carboxylic acid absorbance was 0.82, amino acid absorbance was 0.75, and amine/amide absorbance was 0.58 (Fig. 7). The average absorbance of these substrate types in the Fish Lake samples were 1.19, 1.15, 0.95, 0.99, and 0.70 respectively (Fig. 7). The monthly absorbance values for each substrate type for both populations combined can be viewed in Table 6. Polymers and carbohydrates were

the most heavily used substrate types in each month that was sampled. The absorbance values for each substrate type were reduced by approximately half from November 2013 to December 2013, with the exception of polymers, which display a more limited reduction in absorbance.

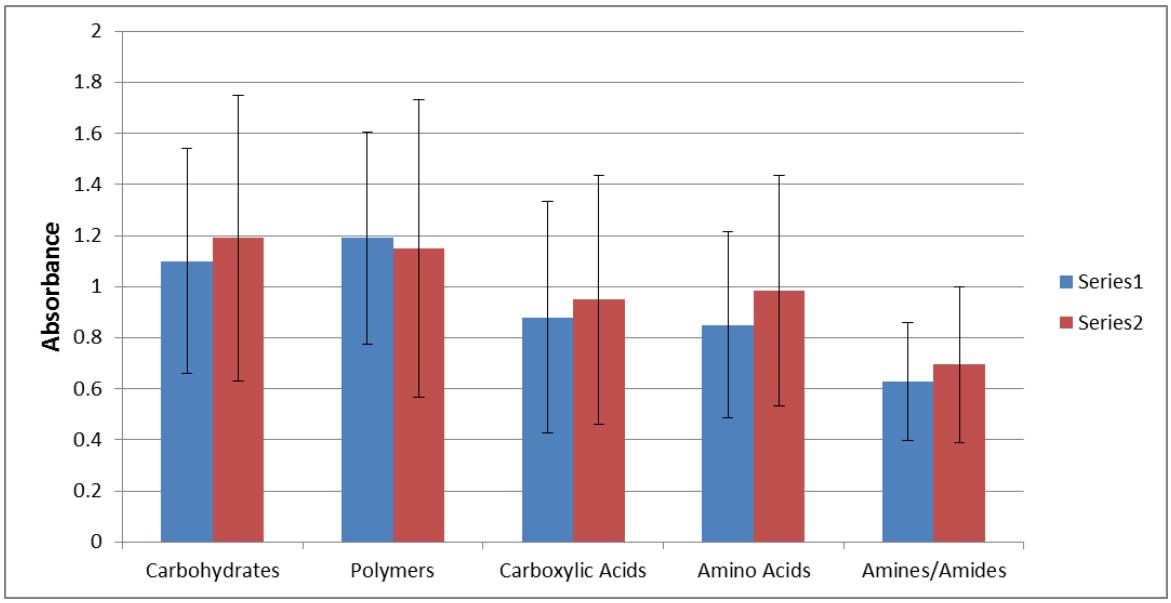

Figure 7. Average absorbance value for EcoPlate wells containing each grouped substrate.

Table 6. Average monthly absorbance values of wells containing each grouped substrate type (both populations, Fish Lake population not sampled in July or January).

	July	August	September	October	November	December	January
Carbohydrate	1.21	1.28	1.23	1.30	1.01	0.55	1.26
Polymer	1.20	1.56	1.16	1.34	1.05	0.71	1.47
Carboxylic acid	0.84	1.02	0.94	0.98	0.88	0.47	0.99
Amino Acid	0.65	0.95	1.09	1.05	0.84	0.47	1.10
Amines/amides	0.37	0.58	0.71	0.81	0.69	0.33	0.94

There was no significant difference between any of the substrate types across all samples (Global R = 0.03, p-value = 0.284). However, analysis of individual substrate type usage over time showed significant changes in the usage of several of the substrate types between months (Table 7). Carbohydrates, carboxylic acids, and amino acids usage all changed significantly over the course of the winter. Carboxylic acids had significant differences between eight of the 21 month-month comparisons. Amines/amides had the fewest, with only three significantly different month-to-month comparisons. Every significant comparison except for two (Aug13-Nov13 and Aug13-Jan13) in carboxylic acids involved Dec13.

Table 7. ANOSIM results testing for significant differences in substrate-type usage between months (both populations). Highlighted cells indicate significant p-values at p<0.05.

	Carbohydrate		**Polymer**		**Carboxylic Acid**		**Amino Acid**		**Amines/Amides**	
	R Statistic	P value	R Statistic	P value	R Statistic	P value	R Statistic	P value	R Statistic	P value
Jul13, Aug13	-0.179	0.76	0.123	0.202	0.272	0.131	0.29	0.119	0.198	0.131
Jul13, Sep13	-0.006	0.44	-0.216	0.845	-0.062	0.595	0.117	0.262	0.198	0.179
Jul13, Oct13	-0.179	0.869	-0.167	0.774	-0.012	0.464	-0.16	0.786	0.006	0.405
Jul13, Nov13	-0.08	0.56	-0.111	0.714	-0.074	0.619	0	0.452	-0.074	0.583
Jul13, Dec13	0.698	0.012	0.537	0.024	0.994	0.012	0.494	0.036	0.346	0.083
Jul13, Jan14	0.222	0.1	0.185	0.2	0.333	0.2	0.185	0.2	0	0.5
Aug13, Sep13	-0.043	0.654	0.035	0.238	0.004	0.398	-0.107	0.922	-0.124	0.935
Aug13, Oct13	-0.043	0.504	0.056	0.203	0.091	0.126	0.015	0.34	-0.083	0.835
Aug13, Nov13	0.078	0.139	0.087	0.156	0.163	0.035	0.126	0.121	-0.026	0.502
Aug13, Dec13	0.583	0.011	0.713	0.004	0.87	0.002	0.631	0.004	0.47	0.013
Aug13, Jan14	0.241	0.119	0.321	0.071	0.549	0.024	0.228	0.143	0.105	0.31
Sep13, Oct13	-0.061	0.645	-0.176	0.985	-0.063	0.699	-0.069	0.686	-0.109	0.955
Sep13, Nov13	0.041	0.262	-0.087	0.792	-0.037	0.543	0.054	0.262	-0.026	0.489
Sep13, Dec13	0.557	0.011	0.209	0.078	0.665	0.004	0.435	0.017	0.62	0.006
Sep13, Jan14	0.309	0.095	-0.154	0.75	0.321	0.083	0.167	0.19	0.099	0.274
Oct13, Nov13	-0.059	0.703	-0.069	0.688	-0.135	0.991	-0.054	0.561	-0.083	0.784
Oct13, Dec13	0.32	0.026	0.187	0.063	0.289	0.006	0.202	0.041	0.387	0.006
Oct13, Jan14	-0.056	0.563	-0.259	1	-0.13	0.702	-0.185	0.821	-0.142	0.75
Nov13, Dec13	0.354	0.006	0.248	0.065	0.181	0.032	0.081	0.173	0.085	0.193
Nov13, Jan14	-0.037	0.488	0.019	0.357	-0.13	0.738	-0.123	0.738	-0.062	0.476
Dec13, Jan14	0.377	0.06	0.457	0.036	0.383	0.06	0.204	0.202	0.401	0.071
Global	0.124	0.035	0.093	0.077	0.188	0.033	0.111	0.04	0.096	0.068

Chapter 4: Conclusions

4.1 ARISA Data

This study characterized the winter pitcher community in the leaves of *Sarracenia purpurea* for the first time. The ARISA results indicated that bacterial communities in the pitcher fluid from *S. purpurea* throughout the winter are rich. Taxonomic richness detected was equal to or higher than previous studies in the same plant species conducted in summer. A total of 11 bacterial phyla have now been recovered in the fluid of genus *Sarracenia,* though Proteobacteria is the only phylum to be recovered in all studies (Table 8). In four out of five of the studies, the phyla Actinobacteria, Bacteroidetes, and Firmicutes were also recovered (Table 8). The most abundant phylum represented in the previous studies was Proteobacteria (Koopman et al., 2010; Krieger and Kourtev, 2012b; Peterson et al., 2008; Siragusa et al., 2007); however, the most abundant phylum identified in this study was Firmicutes. Results from this study found all four of the phyla identified in two studies using the same plant species (*S. purpurea*) (Peterson et al. 2008; Siragusa et al. 2007); however, the ARISA method used here recovered four additional bacterial phyla, 173 more bacterial phylotypes than the culture-dependent methods used by Siragusa 2007, and 53 more phylotypes than the culture-independent methods used by Peterson 2008 (Table 1). A third study on *S. purpurea* identified the phyla Cyanobacteria and Candidate Division TM7 (Krieger and Kourtev, 2012b), which were not found in this study. In a study of microbial richness in a different pitcher plant species (*S. alata*), using the same fingerprinting methods, 7 phyla and 485 unique phylotypes were recovered (Koopman et al. 2010), (Table1).

Table 8. Phyla identified in the studies aimed at characterizing the microbial diversity of Sarracenia pitcher fluid. Highlighted cells indicate phyla recovered in that study.

	Koopman et al. 2010	Krieger and Kourtev 2012	Peterson et al. 2008	Siragusa et al. 2007	Whitman et al. 2005	This Study
Actinobacteria						
Aquificae						
Bacteroidetes						
Candidate Division TM7						
Chlorobi						
Chloroflexi						
Cyanobacteria						
Firmicutes						
Nitrospirae						
Proteobacteria						
Thermotogae						
Unknown						

As was hypothesized, fewer phylotypes were recovered from *S. purpurea* fluid in the winter (this study) than from *S. alata* fluid in the summer using ARISA data (Koopman et al., 2010). This is not surprising given that the climatic conditions that the pitcher ecosystems experience are vastly different. The *S. alata* sampled in the Koopman et al. study grow in the southern United States and presumably have access to a more abundant and diverse supply of prey insects. Microbes in that study were also only collected during the summer. These factors would be expected to translate to a greater number of unique microbial phylotypes being identified in the *S. alata* system than in the *S. purpurea* collected during the winter.

In the study on *S. alata* (51 pitchers collected over 5 months), the number of unique phylotypes found each month ranged from a minimum of 182 in April to a maximum of 284 in July (out of the 485 total) (Koopman et al., 2010). In this study (57 pitchers collected over 7 months), the individual pitcher with the highest level of species richness each month ranged from 157-186 (out of 186 total). Even though the species richness of the average *S. purpurea* pitcher displayed variability depending on the time of winter, most of the total bacterial phylotypes identified in the study were accounted for in each month. In *S. alata* the number of unique phylotypes found each month changed drastically, and was never near the

total number of phylotypes identified (485). This indicates that many bacterial taxa inhabit the *S. alata* pitchers transiently. The number of unique phylotypes found in April by Koopman et al. (2010) (182) was also quite similar to the 186 found in this study. Perhaps the winter *S. purpurea* pitcher and the newly opened *S. alata* pitcher in April are both relatively nutrient-poor environments with similar carrying capacities. The winter composition of microbes in *S. purpurea* might only be made up of a key set of organisms that are adapted to survive in the winter pitcher fluid and/or aid in the digestion of the insect prey. Microbial richness increased in *S. alata* as temperatures and prey availability increased (Koopman et al., 2010); summer sampling in *S. purpurea* would be expected to display a similar trend.

Taxa most frequently identified by Koopman et al. (2010) and this study were quite different. Proteobacteria was the most commonly identified phylum in *S. alata* and previous studies on *S. purpurea* in the summer (Koopman et al., 2010; Krieger and Kourtev, 2012b; Peterson et al., 2008; Siragusa et al., 2007). Firmicutes was the most frequently identified phylum in the winter *S. purpurea* samples. One notable difference between the two phyla is the presence of several species capable of endospore formation in Firmicutes. Bacteria undergo endospore formation to go dormant, which protects against low nutrient availability, desiccation, and extreme temperatures (Bueche et al., 2013). Perhaps bacteria capable of endospore formation are more successful at surviving during the winter.

The most frequently identified phylotype by Koopman et al. was *Nitrosomonas eutropha*. This was identified in this study on *S. purpurea* in 46 of the 57 winter pitcher samples. The genus *Pseudomonas* was also commonly identified in both studies. Surprisingly, the family *Enterobacteriaceae* was the most prevalently identified by Koopman

et al. (2010) (Table 9), but was completely absent in the winter *S. purpurea* samples. *Herbaspirillum* is another example of a common genus found in *S. alata* that was not identified in *S. purpurea* (Koopman et al., 2010). Overall, there appear to be major differences in the microbial taxa that comprise these two systems. However, the microbial communities inhabiting both of these plant species are likely functionally similar. The differences in species composition might be the result of climatic differences as discussed in the following section.

Table 9. Comparison of the most abundant taxa found at each rank in Koopman et al. 2010 and this study.

	Plant Species	Most Common Phylum	Class	Order	Family	Genus
Koopman et al. 2010	*S. alata*	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
This Study	*S. purpurea*	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus

4.2 Bacterial Activity

As was hypothesized, several known psychrotolerant taxa were identified in the winter pitcher fluid sampled here; these species would be expected to have the ability to survive and possibly remain active at low temperatures. These include, but are not limited to *Listeria monocytogens*, *Staphylococcus aureus*, *Bacillus sp.*, *Streptococcus sp.*, *Pseudomonas sp.*, and *Burkholderia sp.* (Afshin and Saeid, 2011; Männistö and Häggblom, 2006). These taxa were found in 38, 56, 55, 57, 48, and 54 samples respectively out of a total of 57 pitchers; although it is unclear whether they are able to grow and reproduce when the pitcher fluid is frozen solid, these taxa were present in each month of sampling. In addition to being present in nearly all of the samples, *Bacillus, Staphylococcus,* and *Streptococcus* were three of the most abundant genera in the winter samples. In the analysis of fluid collected in *S. alata*, these taxa were not identified as being particularly prevalent (Koopman et al., 2010).

Perhaps resistance to cold is a primary factor influencing the different microbial compositions of the two plant species.

4.3 Similarities in Microbial Communities across 'Digestive Systems'

Bacterial species known to inhabit mammalian guts were also found in pitchers in this study. *Enterococcus faecalis*, a fecal indicator bacterium, was found in 54 of the 57 sampled pitchers. Two phylotypes in the genus *Desulfovibrio* (*D. vulgaris* and *D. desulfuricans*) were identified in 48 pitchers. *Desulfovibrio* is a genus of anaerobic sulfur-reducing bacteria that have been found in human feces, and are known opportunistic pathogens (Beerens and Romond, 1977; Goldstein et al., 2003). Members of the genus *Lactobacillus* were identified in 50 pitchers. *Lactobacillus* species are common in the human gut, and known for their probiotic properties (Walter, 2008). Notably absent from the pitcher samples were species of *Bacteroides,* a dominant genus in the human intestinal tract that aids in the breakdown of polysaccharides (Ravcheev et al., 2013).

The presence of taxa found in the human gut therefore, does not signify a community-level structure in pitcher fluid uniquely shared with the human gut. Nor does it necessarily mean that these taxa are playing the same role in pitcher fluid as they do in the human gut. The aforementioned identified microbial taxa in fluid samples have been found in other locations outside the human gut and since *S. purpurea* pitchers are constantly open to the environment, microbial taxa may have arrived from a wide variety of sources. The microbes could have been brought to the pitcher in the guts of insects. The animals that are trapped as prey in the pitchers may be strongly influencing the bacterial community of the fluid when they are digested. Like mammals, insect guts harbor mutualistic microbial communities. Species richness and composition of the communities are highly dependent on the species of

insect host (Engel and Moran, 2013). Some species of insect are known to contain taxa of bacteria widespread in other environments, such as *Pseudomonas* and *Bacillus* (Broderick et al., 2004) (found in this study). Studies on the guts of other insect species have found taxa of bacteria more commonly associated with mammalian guts, like *Enterococcus* and *Lactobacillus* (Chandler et al., 2011; Christopher R Cox, 2007) (also found in this study).

Despite the fact that several taxa of bacteria likely arrive in each pitcher by happenstance, the substantial set of bacterial taxa identified in 90% or more of the pitchers sampled in this study is evocative of the selection of the human and insect gut microbiome by the host (Arumugam et al., 2011; Engel and Moran, 2013). Perhaps the pitchers in turn are exerting pressures selecting for the microbes that were present in nearly all of the samples.

In addition to taxonomic identification, the ADAPT output also reports the trophic and pathogenic composition of the sampled communities. All pitchers were at least 65% heterotrophic. The balance between pathogens and non-pathogens was more balanced, although most of the samples were identified as being more than 50% non-pathogenic.

4.4 Microbial Community Changes in Winter

It was hypothesized that microbial richness would peak in November, the designated start of winter defined in this study, and decrease as the season progressed. Temporal ANOSIM analyses of presence-absence data resulted in a significant global R-statistic among months at every taxonomic level that was tested (Table 4). While this does not mean that each winter month was significantly different from every other winter month, it means that over the course of the entire winter, the compounded differences in the taxa present in Waterloo samples was significant over time. This indicates that the bacterial community composition in *S. purpurea* is quite variable over the winter months. The month of November

2013 was significantly different from most other months at each taxonomic level (Table 4), likely making a large contribution to the observed significant global R-value. For instance, at the rank of class, November 2013 presence/absence data were significantly different from November 2012, December 2012, January 2013, February 2013, and March 2013 - five of the other six months sampled (Table 4).

November sampling is particularly noteworthy for other reasons; microbial richness in November of 2012 and 2013 was lower than every other month at each taxonomic level (Fig. 2, 3, 4). This may be caused by the physiological stress that initial freezing bouts put on microbes. In both 2012 and 2013, for example, mid-late November was the earliest point during which air temperatures were consistently below 0^{o}C and freezing of pitcher fluid likely took place for the first time (Table 10). As discussed in the introduction one freeze can cause high levels of microbial mortality and reduced metabolic activity. Low temperatures can cause microbial lipid membranes to solidify, ice crystals to rupture membranes, and decreased oxygen diffusion (Schimel et al., 2007). In order to survive these conditions, microbes must acclimate by shifting biochemical pathways, altering membrane lipids, or producing protective molecules (Schimel et al., 2007). Not all bacteria are equipped to undergo these necessary changes, and those that are must expend a great amount of energy to do so. Those that cannot successfully acclimate to freezing temperatures will most likely die.

Table 10. Average weekly minimum, mean, and maximum temperatures (in degrees Fahrenheit) of the winter sampling period in 2012, 2013 and 2014 (and weeks immediately preceding it) in Ann Arbor, MI (The Weather Channel LLC, 2014).

Week	Ave. Max	Ave. Mean	Ave. Min	Week	Ave. Max	Ave. Mean	Ave. Min
21-Oct-12	68	56	44	20-Oct-13	52	41	32
28-Oct-12	45	40	36	27-Oct-13	56	46	36
04-Nov-12	49	37	25	03-Nov-13	49	42	34
11-Nov-12	50	39	26	10-Nov-13	46	36	25
18-Nov-12	52	40	28	17-Nov-13	45	38	30
25-Nov-12	42	33	24	24-Nov-13	32	25	18
02-Dec-12	49	41	33	01-Dec-13	45	35	24
09-Dec-12	41	34	26	08-Dec-13	24	17	11
16-Dec-12	41	37	32	15-Dec-13	31	24	18
23-Dec-12	31	24	16	22-Dec-13	32	27	21
30-Dec-12	29	19	9	29-Dec-13	22	16	10
06-Jan-13	43	35	27	05-Jan-14	23	13	2
13-Jan-13	38	29	21	12-Jan-14	30	25	20
20-Jan-13	22	14	6	19-Jan-14	20	8	-4
27-Jan-13	37	28	19	26-Jan-14	23	13	4
03-Feb-13	28	20	11				
10-Feb-13	36	30	23				
17-Feb-13	32	25	17				
24-Feb-13	34	29	24				
03-Mar-13	36	28	19				
10-Mar-13	43	36	29				

In light of the connection between November 2013, its low numbers of taxa present, and its deviation in taxa composition from the other months sampled, it should also be noted that November 2012 did not completely follow a similar pattern. While November 2012, like November 2013, had lower levels of taxa present than every other month at nearly every taxonomic level (Fig. 2, 3, 4) the *composition* of the taxa present in its samples did not significantly deviate from the other months. In fact, other than being significantly different from November 2013 at the rank of class, November 2012 only significantly deviated from December 2013 (Table 4). This suggests that the number of taxa in the November 2012

samples was low, but the taxa that were present were similar to those present in the other winter months. Thus it appears that in November of both sampling years taxonomic richness decreased, though only the November 2013 samples were both lower than any other month *and* different than the other months sampled in terms of taxonomic richness. This may be a result of the greater amount of time between the collection of the November 2013 samples and the previous winter, which could be an indicator of changing community makeup from one year to the next. This divergence of November richness from the rest of the winter months was not predicted.

These drastic changes in physiological tolerance could explain the decreased species richness of the November (2012 and 2013) samples, but does not account for the rebound seen in December (2012 and 2013) and January (2013) (Fig. 2, 3, 4). Increased richness in these same samples might be the result of recovering populations of bacteria that were too reduced to be identified in the November samples. These new taxa could also have arrived in the pitchers through dispersal over the course of the winter, though we suspect this would be minimal.

Another interesting temporal pattern in taxonomic richness was seen in the winter of 2012-2013. Microbial richness rebounded from its November drop off in December, then proceeded to drop off in January and rebound again in February (Fig. 2, 3, 4). This could be because of the unusually warm January that resulted in several freeze-thaw cycles, followed by a more stable February in which temperatures remained below freezing (Table 10). As discussed earlier, multiple freeze-thaw cycles may have diminished microbial populations and/or activity which has been shown to happen in *Pseudomonas* species (Morley et al., 1983). *Pseudomonas*, and likely many bacterial taxa, are adept at surviving a single freezing,

but die off in much larger numbers after repeated freezing events (Morley et al., 1983). It is possible that *Pseudomonas,* and other bacterial players, spend so much energy and resources in surviving a single freezing event that they cannot survive subsequent freezing events. The transitory period between fall and winter is likely a time when fewer bacteria can thrive. Those that can acclimate do so at an energetic cost and those that cannot die. Taxonomic richness in January 2013, like November 2012 and November 2013, may have been low for this reason.

4.5 EcoPlate Data

It was hypothesized that a wide variety of EcoPlate substrates would be utilized by the microbial communities in the pitcher fluid. During experimentation each of the 31 wells produced color on the plates, indicating that all of the substrates were metabolized by microbes in the pitchers. Results of the EcoPlate experiments show levels of activity comparable to other studies utilizing a variety of environmental fluid samples (Choi and Dobbs, 1999). At 72 hours after loading our samples AWCD values ranged from 0.50 to 1.39 (Fig. 6). These values were quite similar in range to the 72-hour readings of EcoPlates loaded with pond water samples in another study (Choi and Dobbs, 1999). It was hypothesized that microbial activity would be the highest during the warmest months, July and August. The lowest activity levels were hypothesized to occur during the coldest months, December and January, and coincide with the period of predicted lows in richness. AWCD in the Waterloo population was highly variable from month to month. It was similar from July until November (slight dip in October and November). In December, AWCD plummeted to approximately half of November, then jumped in January to the highest level of any month sampled (Fig. 6). Although the Fish Lake population was only sampled from

August 2013-December 2013, the October samples produced the highest AWCD (Fig. 6). The December samples in both populations produced the lowest AWCD (Fig. 6). It was predicted that AWCD would be lowest during the coldest two months (December and January). This was supported by the low activity of the December samples, but the high level of activity in the January samples was unexpected.

ANOSIM tests indicated that metabolic activity in the month of December differed significantly from every other month that was tested (Table 5). One possible explanation for the drastic changes seen in the winter may be that lowered activity was a result of a reduced population (seen in November), but was not detected until December (see section 4.6 for more on this link).

Polymers and carbohydrates were the two substrate-types that experienced the most activity among all samples (Fig. 7). Amines /amides were used the least. ANOSIM testing resulted in no significant difference in substrate use among all samples (Global R = 0.03), but substrate use did change over time (Table 6, Table 7). The severe drop off across most substrate usage in December 2013 is likely responsible for much of the detected significance in temporal variation. Interestingly enough, polymer activity was the one type that was not reduced as severely in December 2013. While the AWCDs of the other substrates were approximately cut in half in December 2013, polymer use declined to a lesser extent. It seems that the bacteria that were responsible for metabolizing polymers were not affected as much by cold-induced cell death or reduced activity as observed in other members of the community.

4.6 Linking EcoPlates to ARISA

Both EcoPlate data and ARISA data show a drastic "dip" month (Fig. 2, 8, Table 6). That month is November for the ARISA data (richness) and December for the EcoPlate data (carbon usage). It appears that the weather may influence these dips; the decrease in richness of the community was seen at an earlier point in the winter (November) and subsequently resulted in activity changes of the community (seen in December) (Fig.8). The number of organisms present and the number of phylotypes present decreases in November and then the activity of the community decreases in December. This is followed, in both datasets, by a rebound in values: December for richness and January for activity (Fig. 8). The color development seen in the EcoPlates appears to be directly altered by the composition of the microbial community in a delayed response. More frequent sampling than once per month might reveal exactly how closely the pattern of community change and level of activity follow one another. It is possible that freeze-thaw cycling was the cause of reduced richness in November and activity in December. If this was the case, cells that lysed during a freeze may have provided the nutrients necessary for the pulse in activity witnessed in January during a thaw.

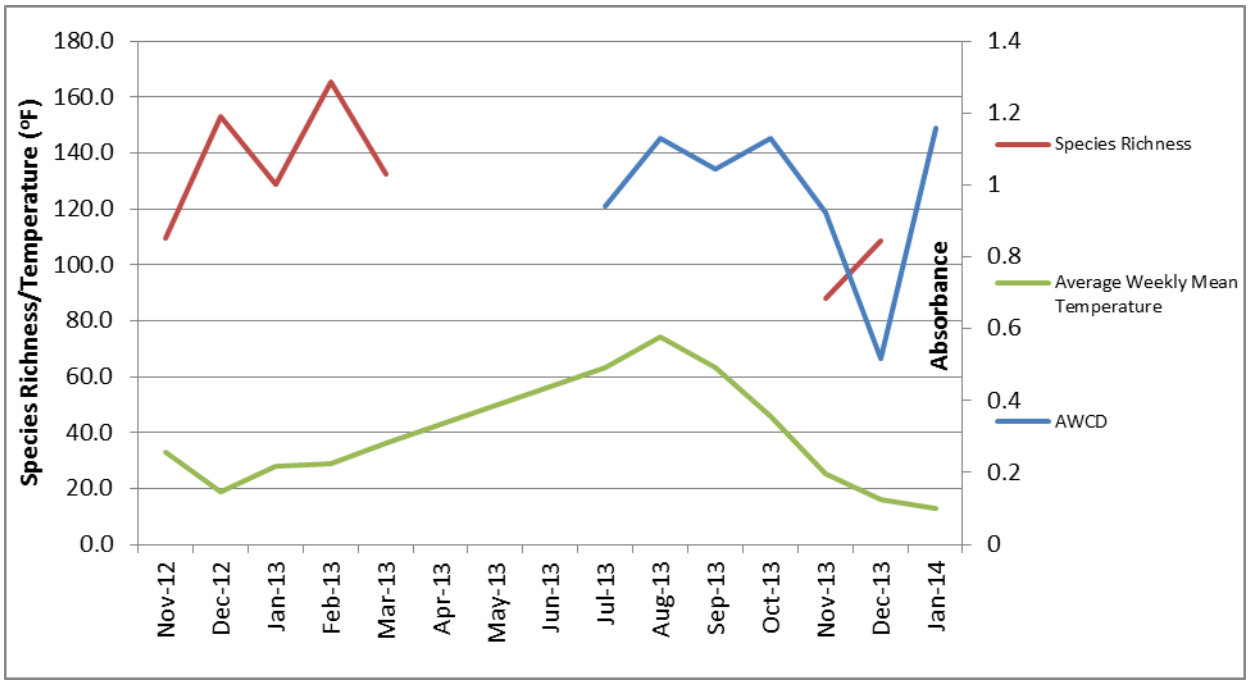

Figure 8. This figure displays trends in species richness, AWCD, and temperature over the course of the entire sampling period. Temperatures indicate the mean average temperature during the week that monthly sampling was conducted.

4.7 Limitations of this Study

One limitation of this study in regard to the subject of bacterial activity is that it is impossible to measure the *actual* activity of the bacteria in the pitchers with the resources at my disposal. Furthermore, the ARISA technology used to identify bacteria does not distinguish between living and non-living fragments of DNA; it must only be intact to be amplified and thus identified. As a result these methods can potentially introduce bias into these experiments (V. Wintzingerode et al., 1997), (Fisher and Triplett, 1999), (Ranjard et al., 2001). What can be gathered from these data is a collection of DNA that is present in the pitcher (DNA that has not decomposed), which, given the fact that the pitcher fluid acts as a sort of digestive system for the plant, may be a good indicator that that organism was alive at the time of collection.

Using EcoPlates to assess microbial activity supplements the DNA identification first by validating that the fluid samples contain living microbes, and not just intact DNA. Secondly the EcoPlates confirm that the microbes present are capable of metabolic activity at room temperature shortly after being thawed. Of course this does not mean that the microbes in the pitchers were active at the time of collection. EcoPlates that were incubated at 4^{o}C (but not used for statistical analysis) displayed drastically delayed color change compared to the same fluid samples incubated at room temperature. Even though their metabolism was slowed down, these plates did display substantial color change across all substrates, eventually achieving color development on par with plates stored at room temperature. The time scales for these reactions were weeks instead of days, but this demonstrated that the microbes in the samples are capable of not only surviving, but metabolizing at cold temperatures. Some of the microbes present in the pitcher fluid are likely inactive during the winter, but it is also possible that some of the more well-adapted microbes are still facilitating digestion, albeit at a much reduced rate.

4.8 Future Work

This study confirms that the winter microbial community of *S. purpurea* is abundant, diverse, and capable of metabolic activity. The next logical step in this line of work is to characterize and compare the summer microbial community in the Waterloo population to the winter community. This will determine if there is a general decrease in taxonomic richness in the months leading up to November, or if richness remains stable until freezing takes place. Characterizing the summer microbial community in *S. purpurea* may also help to identify key seasonal players. If known psychrophilic/psychrotolerant bacteria are notably absent in the summer, it might help to identify the members of the winter community that are

highly specialized to thrive in the winter. The winter community characterized here in *S. purpurea* has a much reduced number of unique phylotypes to the pitcher community characterized in *S. alata* (Koopman et al., 2010) and it will be interesting to see how that might change with summer sampling in *S. purpurea*. A careful comparison of the microbial taxonomic richness of each of these plant species will enable the assessment of just how similar these communities are and might help address how cold weather affects the *S. purpurea* system. Analysis of nutrient levels in pitcher fluid thorough the year might also help to explain fluctuations of microbial activity.

Further research into the microbial composition and activity in the pitchers of *Sarracenia purpurea* will also lead to a greater understanding of the mutualistic relationship between the microbes and their plant hosts. Although known gut microflora have been identified in *Sarracenia* pitchers in this and previous studies, it is still unknown precisely what roles they play in the digestion of prey.

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