The relationship between ecosystem diversity and function focused on the leaves of the northern pitcher plant (S. purpurea) as a model system

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Abstract

Pitcher plants have been the focus of many ecological studies involving food webs as of late. Pitchers of *Sarracenia purpurea* contain a unique habitat within the liquid contained in the leaves of the plant. We hypothesized that the enzymatic activity of extracellular bacteria increased as bacterial species richness in pitchers increased. Denaturing gradient gel electrophoresis (DGGE) was used to characterize bacterial populations in the liquid of pitchers from the same bog. DGGE revealed that individual pitchers contain unique, diverse bacterial populations. We conclude that pitchers of *S. purpurea* maintain high bacterial diversity and also possess variation between individual pitchers within the same location.

Introduction

A central idea in ecological research is the relationship between species diversity and ecosystem function. Previous scientific literature has sparked two very important questions. One is based on how ecosystem function and diversity interact and the second is based on understanding the quantity of various species involved. Years of research has shown that the amount of a particular species doesn't always indicate how significant of an effect that species will have on the ecosystem at hand, however; the identity of the species can notably alter the effect (Hooper *et al*., 2005). The diversity of microbial communities and their resilience has been studied in a similar fashion, but did not explore the idea of diversity and function (Bengtsson, 1998; Coleman & Whiteman, 2005). Microbial communities are vastly understudied, especially in aquatic

environments, but are very important in their role in nutrient cycling and decomposition. Understanding these ambiguous environments on a deeper level requires us to examine the diversity of trophic levels within microbial communities.

The northern pitcher plant, *Sarracenia purpurea,* is an ideal study system in which to study the relationship between diversity and ecosystem function. This carnivorous plant thrives in areas that are lacking in quality nutrients, such as a bog. Northern pitcher plants produce leaves that open to form pitchers that collect rainfall throughout the spring and summer seasons to act as insect traps (Juniper et al., 1989). The pitchers on the plant are sterile when they first open, and microbes colonize the pitchers through dust deposition and through coming in with other organisms (Peterson et al., 2008; Krieger & Kourtev, 2012). Insects (flies, mosquitos, midges, bees, mites, etc.) fall into the pitchers, are unable to get out, and drown. The microbes inside the pitchers secrete enzymes and the pitcher has glands to absorb the nutrients from the decomposing insects. Over time, as microbes, insects, and other organic debris fall into the pitchers, the composition and diversity of the microbial community inside each pitcher changes. The pitcher plants are an ideal study system to track succession in a microbial community, beginning with a sterile to pitcher and becoming a complex and functional microbial community. The bacterial community inside the pitcher plant decomposes the drowned insects or other organic debris by releasing extracellular enzymes, which are measureable using molecular techniques. The DNA from the bacteria can also be extracted and used to identify the composition and richness of each of the pitchers.

In this study, the relationship between ecosystem function, as measured by enzyme activity, and bacterial diversity was explored using two hypotheses:

Hypothesis 1: As the bacterial species richness in pitchers increases, so does the activity of extracellular bacteria-produced enzymes. When the diversity of microbial species increases, the enzyme activity is expected to increase as well. Eventually this relationship will plateau as all the niches within the community are occupied. Hypothesis 2: Pitchers with similar bacterial species richness, but different species composition, differ in the activity of extracellular bacteria produced enzymes due to functional differences between species. Although it is almost certain that the specific species have a significant effect on the ecosystem properties, it has not been tested at a microbial level in this system.

Methods

This research was conducted in a bog located on the Shultz farm, which was 5 miles away from Pierce Cedar Creek Institute in Barry County, Michigan. The area of the bog used was accessible only by canoe. This nutrient-deficient environment provided the necessary conditions for the pitcher plants to develop. Actively growing pitcher plants were first identified and marked. Unopened, new growth pitcher plants were the main focus of this study, as these pitchers provided a sterile environment to begin a microbial studies of colonization. The first day involved marking 16 unopened pitchers at the stem of the pitcher leaf as well as a stake near the plant for identification upon return to the site each day.

Over the course of 21 days, a canoe was used to access the pitchers, avoiding peat mat destruction. On the first day that the pitchers opened, 20-50 ml of sterilized mineral water was added to each pitcher. This water was purchased, diluted, and then autoclaved to emulate rain water. Throughout the study, the same procedure was performed in order to maintain consistency. The canoe was brought to the edge of the mat and a sterile, automated pipette was used to extract precisely 2.5 ml of liquid from within the pitcher, while avoiding contact with the walls of the pitcher to avoid sample contamination. Once the liquid was extracted, 2.0 ml were placed in a sterile tube while the other 0.5 ml was placed in a similarly labeled, separate tube. Once collected, the two tubes were put on ice in the cooler. The temperature was then measured with a digital thermometer by first sterilizing the tip with an alcohol pad and then placing it in the remaining liquid of the pitcher.

Once the temperature was collected and recorded, a new sterile pipette tip was used to replace the 2.5 ml of extracted liquid with 2.5 ml of “rain water.”. After samples were collected from the remaining pitchers following the procedure described above, the samples were transported back to the lab at Pierce Cedar Creek Institute for analysis.

All of the 0.5 mL samples were pulled aside to be combined with 150 µl of formaldehyde for preservation. These were placed in the refrigerator to be sent back to Central Michigan University for analysis. Each sample with 2.0 ml was centrifuged. The bacteria that settled to the bottom of the tube were frozen to be used later in DNA extraction. The rest of the liquid was then split up equally among 4 different 2.0 ml tubes. All of the samples were then combined with 500 µl of a substrate: para-Nitrophenylphosphate (pNPP), Beta-galactosidase (BG), or N-acetylglutamate (NAG).

The fourth tube with sampled liquid was used to measure pH using pH strips. The tubes used to test pH were then combined with 750 µl of acetate to be used as a control. The addition of the substrates was significant in the fact that it allowed for the activity of enzymes from the microbial community to be measured. Initially the pNPP was incubated for two hours but as enzyme activities increased, the incubation time was adjusted to fifteen minutes for some samples. The samples with BG and NAG were always incubated for four hours. After the proper incubation time had passed, the samples were pipetted from the tube and placed into a cuvette with 200 µl of sodium hydroxide. The sodium hydroxide raised the pH of the sample, stopping the enzyme reactions. The cleaved products from the reaction turned yellow according to the pH, so samples with different enzyme concentrations turned different shades of yellow. These different shades were measurable by testing absorbance at certain wavelengths on a spectrophotometer. A spectrophotometer set at 410 nm was blanked (zeroed) and then each sample was placed inside in order to record the absorbance. Once these values were recorded, the solutions and cuvettes were safely discarded. Supplies were replenished for the following day and sample absorbance, temperature, pH and incubation times were all recorded on a digital spreadsheet for later referral.

The remaining unprocessed samples were then transported back to the lab at Central Michigan University for further analysis. The first step was to extract the DNA from the samples collected. Bacteria were removed and processed using a DNA extraction kit (ZR Soil Microbe DNA kit, ZymoResearch) while following the manufacturer's instructions. The 16S rRNA gene in the bacteria was amplified using

the universal primers 338f with a GC clamp attached at the 5' end, and 519r (Morgan et al., 2002). The end result was 20 μl of pure DNA from each original sample.

After DNA extraction, the next step was DNA quantification. This process allowed us to obtain amounts of different nucleic acids within the DNA using a nanospectrophotometer. Once quantified, the samples were ready to undergo polymerase chain reactions (PCR). A standardized PCR mix was used (Promega, Madison, WI) to which $MgCl_2$ was added. The products of the PCR were analyzed using denaturing gradient gel electrophoresis (DGGE). The DGGE was performed using 8% polyacrylamide gels and both 30% and 52.5% denaturing gradients (Krieger & Kourtev, 2011). After electrophoresis, gels were stained with SYBR® Green I and imaged using the Image Station 2000R (Kodak Eastman Company, Rochester, NY). The DGGE separated the DNA into bands typically assumed to represent bacterial community members that make up more than 1% of the population in a sample (Muyzer et al., 1993).

Results

Using pictures from the DGGE data regarding the number of species in each pitcher was collected. Since each gel contained days 1-17 for each pitcher, it was possible to count the bands for each time to indicate the amount of species in each pitcher on each day. An average of the band count was calculated and graphed vs. the time in days that data was collected (Figure 1). This trend showed that as time increased, the microbial community increased. Using the absorbance and the DNA count, enzyme activity was also calculated. Results for ACP, BG, and Nag are shown in the following figures. After

further analysis observing the concentrations of DNA versus band counts, no statistically significant relationship between the amount of DNA and enzymatic activity was found.

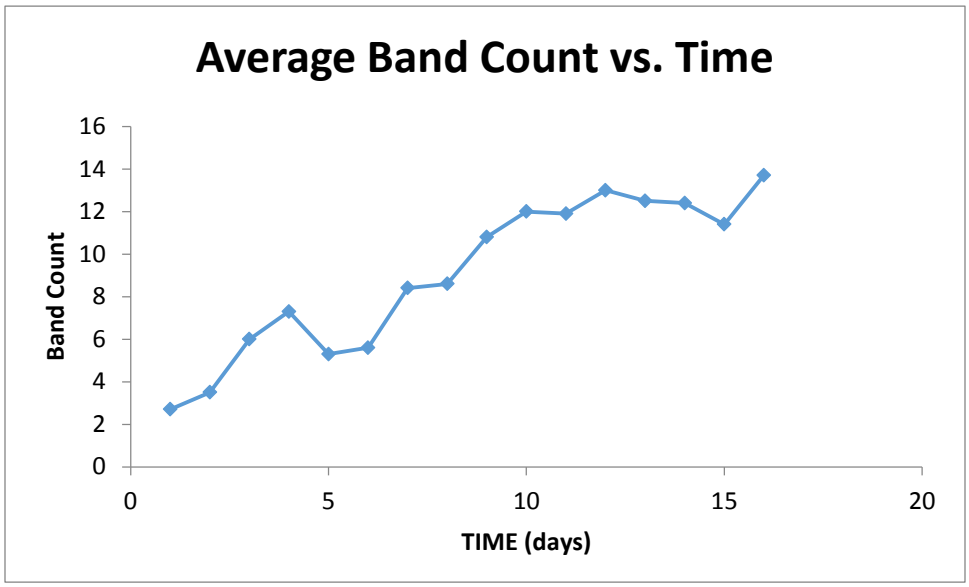

Figure 1: The DGGE band count for each time was averaged and graphed.

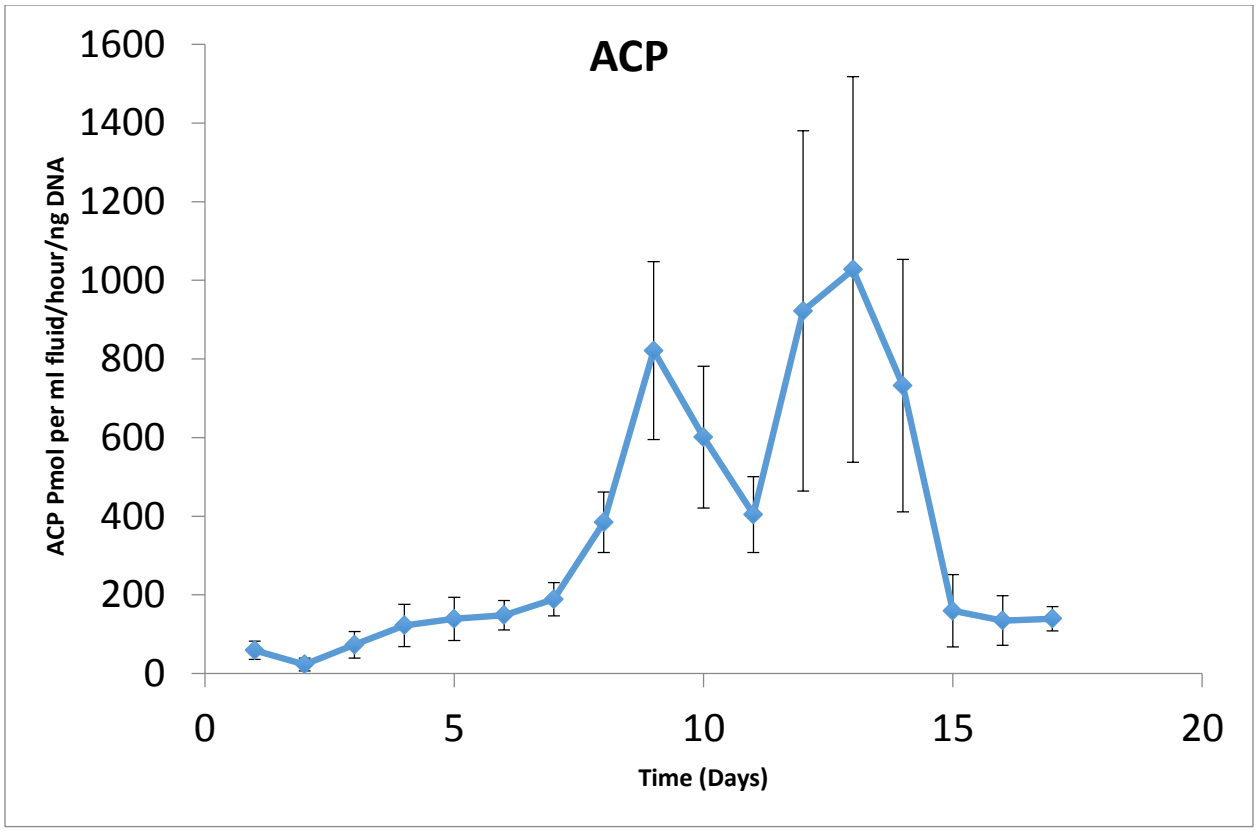

Figure two displays the amount of ACP averaged over the days.

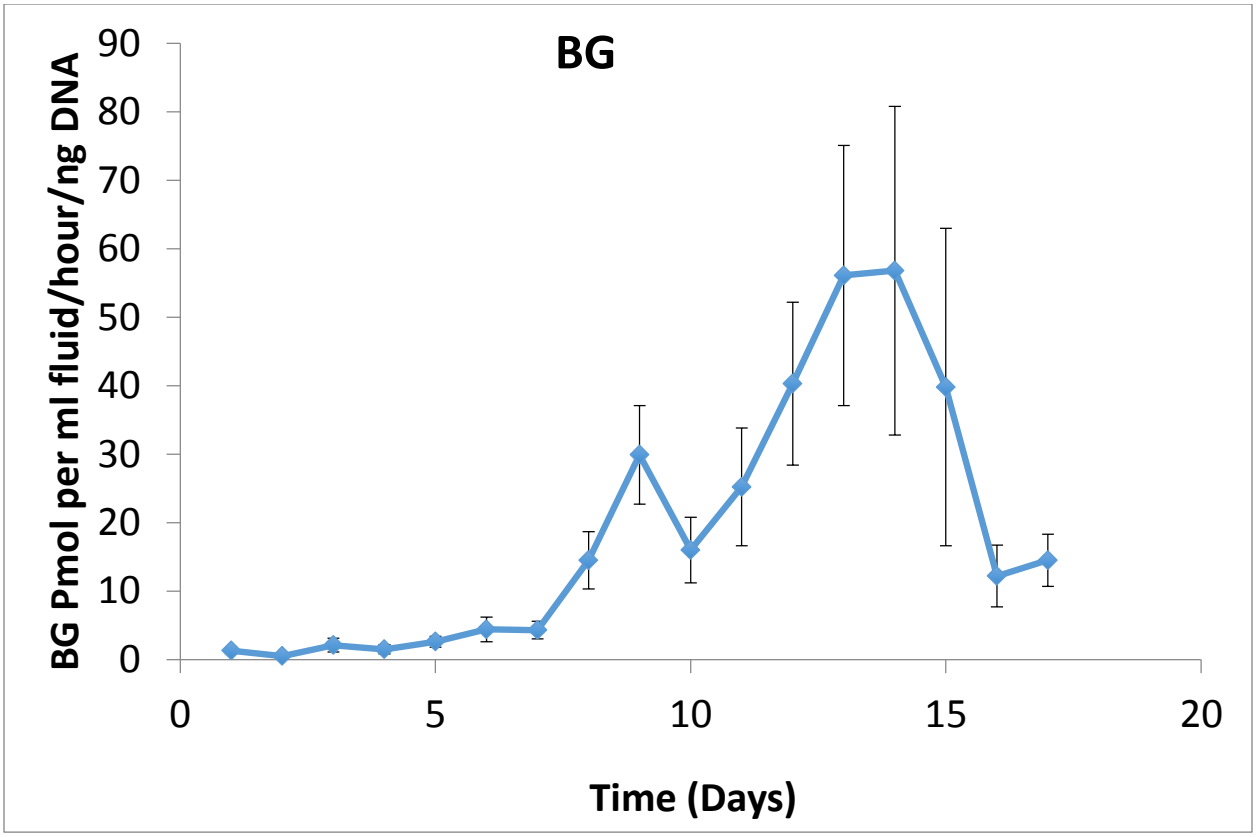

Figure 3 compares the average amount of BG present in the pitcher plants per day.

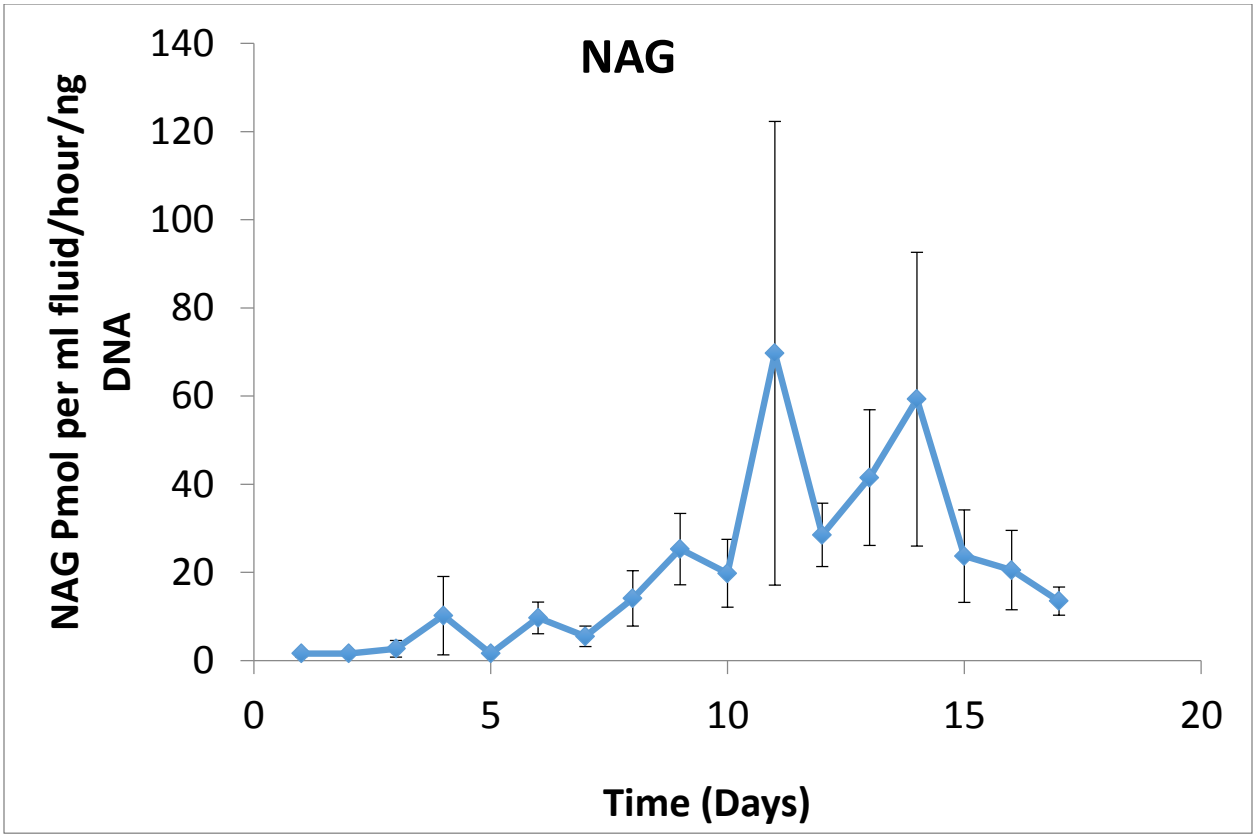

Figure 4 demonstrates the amount of NAG present in each pitcher based on the days.

These above graphs measured the function of the pitchers throughout the course of the study.

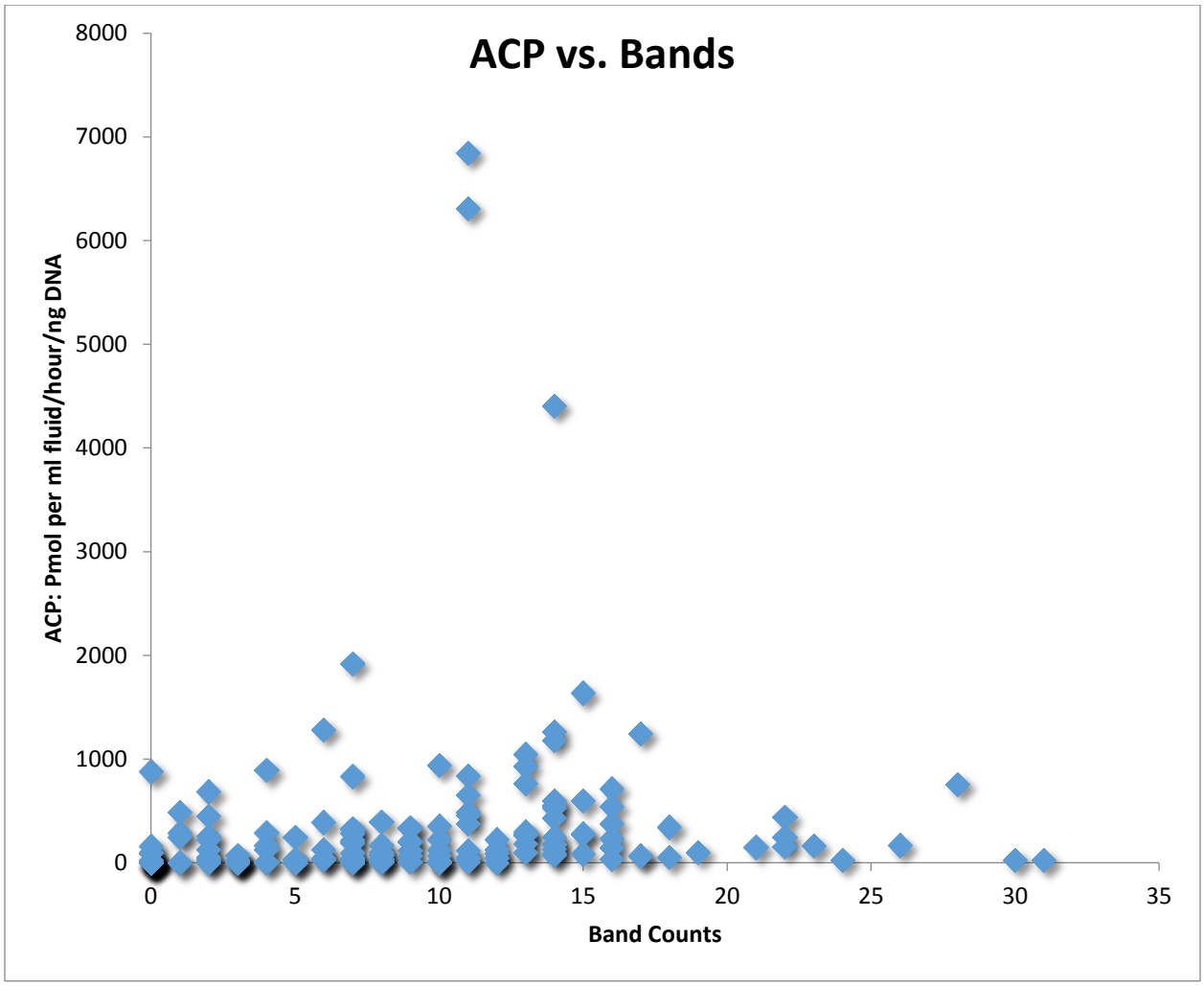

Figure 5 attempts to compare diversity via band count, to function via amount of ACP present.

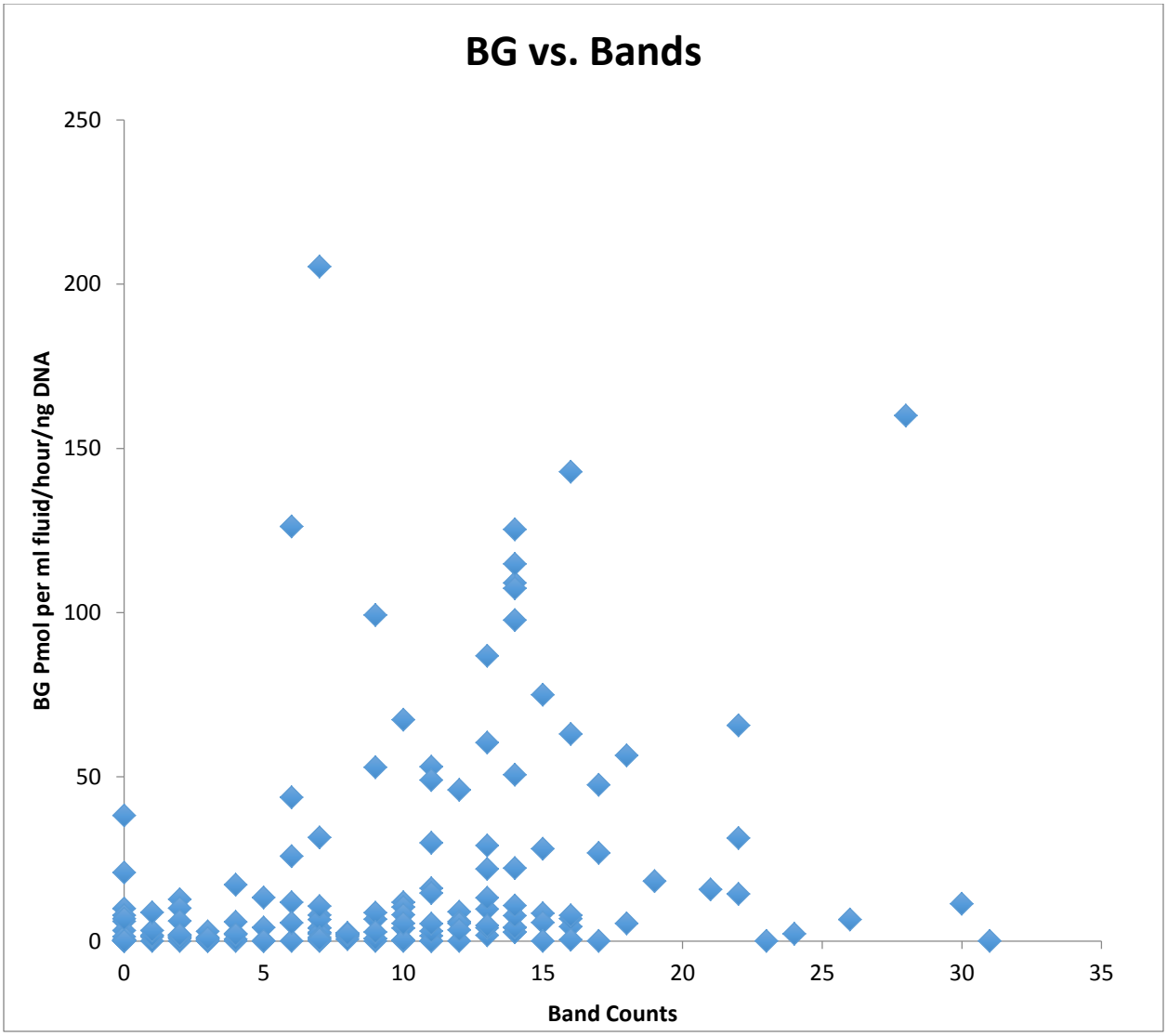

Figure 6 attempts to compare diversity via band count, to function via amount of BG present.

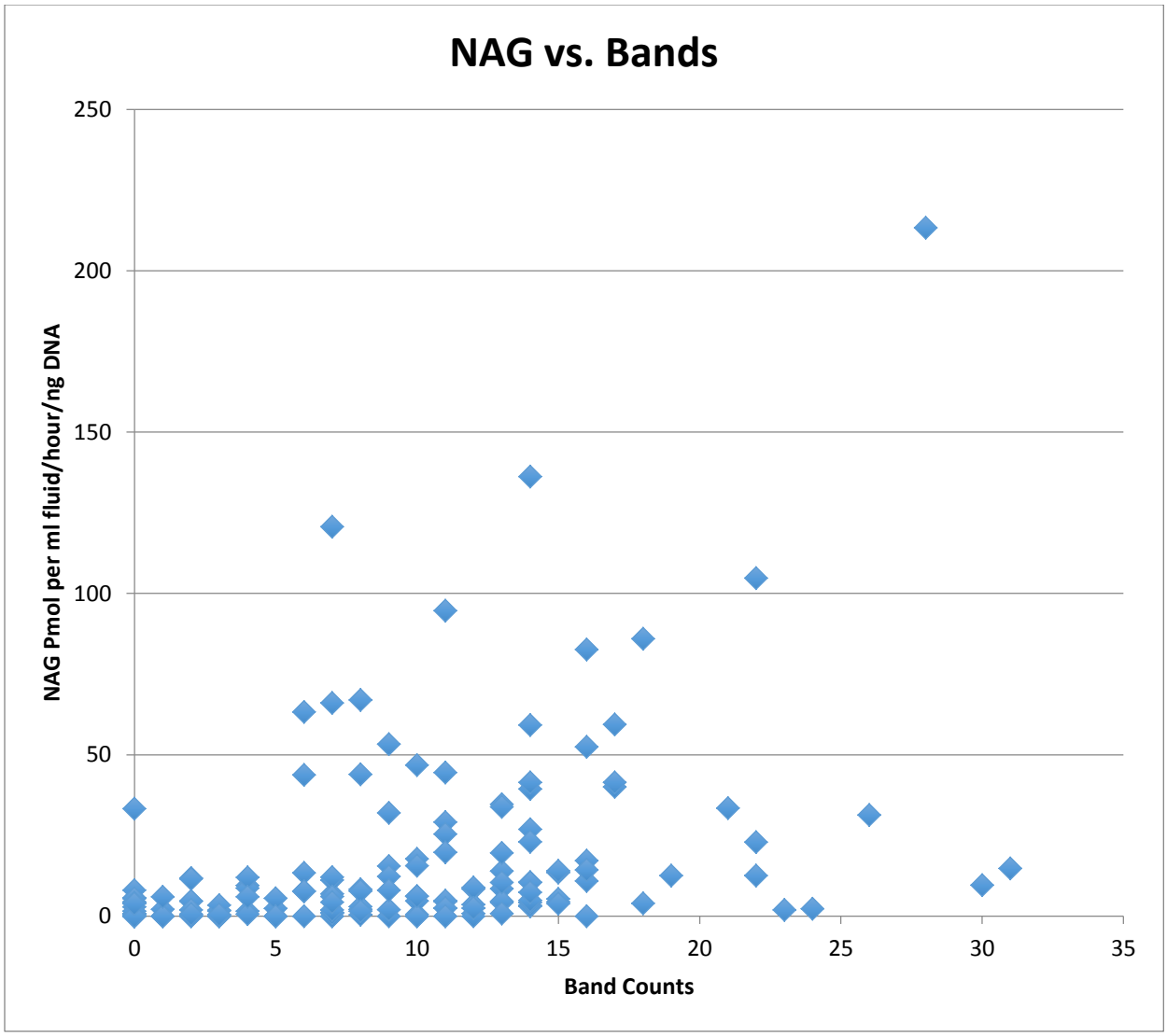

Figure 7 attempts to compare diversity via band count, to function via amount of NAG present.

Discussion

We studied the bacterial community of the habitat associated with pitcher liquid in the leaves of the Northern pitcher plant, *S. purpurea*. We hypothesized that (1) as the bacterial species richness in pitchers increases, so does the activity of extracellular bacteria-produced enzymes, and (2) pitchers with similar bacterial species richness, but

different species composition differ in the activity of extracellular bacteria-produced enzymes due to functional differences between species.

Our results indicate that as time elapsed, bacterial communities within the pitcher increased. DGGE profile analysis suggested that individual pitchers contain unique, diverse bacterial populations. Variability in bacterial populations of pitchers may be the result of multiple factors including: (1) variation of food webs within the pitcher, (2) variations in physical-chemical parameters developing within the pitcher, (3) variation in prey trapped within the pitcher, and (4) genetic variation between different pitchers at a given site (Krieger & Kourtev, 2011).

Additional studies are necessary in order to interpret such findings in the diversity of bacteria within the Northern pitcher plant. This diversity continues to be exceedingly understudied to a large extent, yet the *S. purpurea* progressively captures our interest due to the variability expressed between individual pitchers in such a close proximity. Our study suggests that *Sarracenia* pitchers display inter-pitcher variation. This trend is supported by similar studies with pitchers involving molecular techniques (Morgan et al., 2002; Muyzer et al.,1993). Additional research is required in order to grasp a better understanding of the variability observed within our study.

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