DEGRADATION AND NITROGEN CYCLING IN THE CONTEXT OF BIODIVERSITY-ECOSYSTEM FUNCTION RELATIONSHIPS IN THE INQUILINE BACTERIAL COMMUNITY OF *DARLINGTONIA CALIFORNICA*

By

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ABSTRACT

Biodiversity-ecosystem function (BEF) research aims to explain how species and their environments interact with each other. Microbial communities engage in vital biogeochemical pathways in a variety of natural ecosystems, and yet there are large knowledge gaps about the specific metabolic pathways in which they are involved. Degradation specifically contributes to nitrogen cycling globally through the breakdown of large organic nitrogen compounds into small inorganic nitrogen that is necessary for the survival of many other organisms. In this study, I focused on the degradative function of the inquiline microbial communities found within the carnivorous pitcher plant, *Darlingtonia californica*. *Darlingtonia* grows in nitrogen poor soils and relies on the microorganisms inside of its pitcher to break down insect prey into bioavailable nutrients. The purpose of this study was to identify if specific nitrogen metabolic pathways are driven by *Darlingtonia* bacterial diversity. Fourteen known bacterial isolates were grown in monoculture as well as in mixed cultures of 2-5 species. Additionally, bacteria were collected from *Darlingtonia* pitchers and acclimated in the lab, and serial dilution was performed to produce a diversity gradient. These lab communities were also compared to samples collected from Shasta County, Plumas County, and Del Norte County in California to define the scope of natural diversity observed in this experiment. Communities were given fruit flies as food to compare degradation over 11 days using the broad degradation metric of fly mass loss, and the specific nitrogen function metrics of enzymatic activity of chitinase and protease, and solubilized protein, ammonia, and

nitrate concentrations. While I found increases in degradation potential of higher diversity cultured communities, these positive relationships were not seen in the more complex serial dilution communities. Additionally, nitrogen processing may not be driving insect degradation, as nitrogen metrics could not describe the loss of fly mass observed in this study. Redundant and overlapping functions in this system may allow *Darlingtonia* to maintain insect prey consumption at a range of microbial diversity levels. The benefits of biodiversity on nutrient cycling are commonly discussed, citing positive relationships between the two, however expanding our understanding of redundant relationships between microorganisms and degradation will also strengthen our understanding of the drivers of global biogeochemical cycling and interactions between bacteria and their hosts.

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INTRODUCTION

The relationship between species diversity and ecosystem function is important in global biogeochemical systems, and yet the processes that drive these interactions are still not fully understood (Davies et. al. 2016, Naeem et. al. 2009). Hundreds of biodiversity-ecosystem function (BEF) experiments reveal situations where species diversity leads to higher ecosystem function (Hagan et. al. 2021). This research also shows that a loss of biodiversity leads to a loss in ecological function, which includes the cycling of nutrients and production of biomass (Roger et. al. 2016). Overall, BEF relationships rely on combined impacts of environment and species interactions, including species diversity, richness, abundance, and identity (Hagan et. al. 2021). Importantly, efforts to synthesize the field have revealed a variety of relationships ranging from positive to negative to nonexistent (Hagan et. al. 2021, Schwartz et. al. 2000). Because of the variety of relationships in BEF experiments, it is important not to conflate positive BEF relationships with a healthier system; rather, we must define what drives biodiversity and ecosystem function and what this link means in the context of the system being explored.

While research suggests that biological diversity increases function and stability in ecosystems, there are many complimentary and competitive mechanisms that make it challenging to identify the relationship networks between biodiversity and ecosystem function (Maynard et. al. 2017, Naeem et. al. 2009). Theory predicts three central mechanisms through which diversity can contribute to ecosystem function. First, diversity can increase the chance that highly functional species acquire community

membership (Loreau and Hector 2001). Second, diversity can allow for more efficient use of resources, and therefore more biomass production, through functional complementarity (Loreau and Hector 2001). Third, species use of resources can also overlap, resulting in functional redundancy (Roger et. al. 2016). The broad effects of these mechanisms can shape BEF relationships (Figure 1). In a B-type relationship, highly functional species all contribute equally to the system function, whereas in an A-type relationship, a few species contribute heavily to function, and most species provide small, overlapping contributions (Shwartz et. al. 2000). The C-type relationship depicts the situation where function peaks intermediately and additional species lead to a reduction in ecosystem function. The drivers behind this negative relationship are even less understood than the drivers behind positive relationships, but this type of relationship can occur when highly functional species are outcompeted, resulting in lower ecosystem function in higher diversity communities (Hagan et. al. 2021, Roger et. al. 2016, Steudel et. al. 2016). Diversity metrics remain a practical approach to predictive BEF relationships, yet we need to better understand when we should expect to find each of the specific underlying mechanisms.

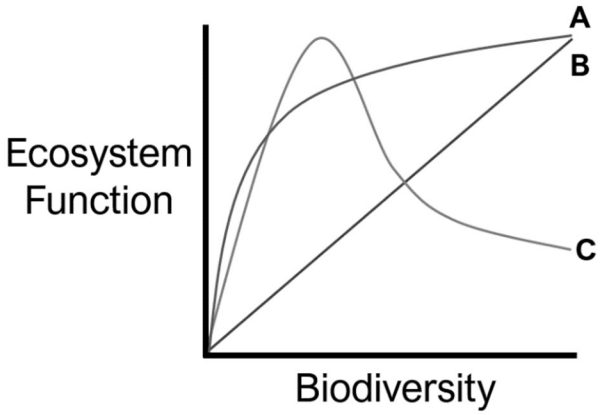

Figure 1. The hypothetical relationships between biodiversity and ecosystem functions (adapted from Strong et. al. 2015).
Relationship A represents redundancy, where many species have overlapping functions. Relationship B represents complementarity, where all species contribute to the ecosystem function. Relationship C depicts an overall negative relationship between increased biodiversity and ecosystem function.

The term biodiversity can encompass many metrics for measuring the variety of life in a system, and it is important to define which aspects of biodiversity impact ecosystem function. For example, higher species richness may indicate that there are more species present in a given system, but it may also be important to account for species relative abundances, as common species may have a more weighted impact on overall function than rare species based on numbers alone (Roger et. al. 2016). Additionally, some species may be more functionally unique than others, and while their functions may be highly quantifiable on very specific metabolic tests, in terms of broad function such as biomass production, their impacts may be overlooked (Lefcheck et. al. 2015). Even though overall biodiversity can be a good measure in understanding the

health of an ecosystem, it is also important to consider the impact of species richness, abundance, and identity on the particular functions in the system being explored.

The majority of BEF research has explored the effects of biodiversity in organisms such as plants and animals, focusing on primary productivity and biomass accumulation, while much less is known about BEF relationships in microorganisms and their key roles in the ecosystem (Roger et. al. 2016). Host-associated microbial communities present a unique opportunity to explore BEF relationships because they have a direct impact on host organism health and survival, both on the individual level and on a species level (Cuellar-Gempeler 2021; Laforest-Lapointe et. al. 2017; Miller et. al. 2018; Vorholt et. al. 2017). The microbial community associated with plants produce hormones and enzymes that process and produce bioavailable nutrients (Compant et. al. 2019), and this community can include bacteria, archaea, fungi, small protists, and in some cases viruses or phages (Berg et. al. 2020) Plant host-associated microbial communities generally are not randomly assembled, rather they tend to have consistent patterns year by year (Vorholt et. al. 2017). Since microbe-mediated traits such as plant protection, nutrient acquisition, and weather resistance aid host success, it would be expected that plants and their associated microbiome co-evolve to maintain consistent microbial communities (Miller et. al. 2018; Theis et. al. 2016; Vorholt et. al. 2017). New species are introduced into these communities through dispersal and evolution, but community composition is also influenced by ecological selection from the host and the environment, such as through pH, temperature, and precipitation (Fitzpatrick et. al. 2020). For a microbial species to contribute to host survival, that species must also be

able to compete well with other microbial species in that community. Host-associated systems rely on the microbial community formed within them for survival, and yet we do not fully understand how host-microbial systems assemble and function.

The microorganisms that live within carnivorous *Darlingtonia* pitcher plants play a crucial role in the health and success of the plant host. The drivers of diversity in this microbial community may be especially fascinating because the pitcher leaves start out closed, opening as they grow in a downward orientation (Figure 2), and the source of microbial input in is currently undetermined. While plants tend to acquire crucial nutrients, especially nitrogen, from the soil, *Darlingtonia* grows in serpentine fens, characterized by nutrient poor soils (Oline 2006), and instead relies on carnivory and luring prey to supplement nutrients from the soil. *Darlingtonia* is not known to produce its own digestive enzymes (Ellison and Farnsworth 2005), but its associated microbial community diversity and biomass have been shown to improve rates of prey decomposition and nitrogen uptake (Armitage 2016, Cuellar-Gempeler et. al. in preparation). In this project, I focused on bacterial diversity as much of the research on degradative function reveals bacteria play a large role in this relationship.

Figure 2. Darlingtonia californica.
The pitchers open downward, in contrast to many of the species within the same family.

Because microbial diversity is known to aid in plant survival in host-associated systems, and *Darlingtonia* grows in nitrogen-poor soils, it is important to understand how microbial diversity affects nitrogen cycling in this system. While microbial diversity can improve prey breakdown and nitrogen uptake (Armitage 2017), the pathways utilized in this relationship are not currently known. The enrichment of metabolic pathways that contribute to the breakdown of amino acids are found during mid-succession in many bacterial communities, such as on corpses (Metcalf et. al. 2016), in host-associated systems (Koenig et. al. 2011), and aquatic environments (Teeling et. al. 2012). Armitage (2017) additionally found that insect degradation in the field changes in *Darlingtonia* pitcher leaves throughout the year, peaking mid-successionally in July, and that these rates were positively associated with bacterial diversity and detritivore abundance. In contrast, laboratory work at Cal Poly Humboldt has shown negative BEF relationships

between *Darlingtonia* bacteria and insect breakdown (Cuellar-Gempeler et. al. in preparation). Nitrogen cycling in plant soils and roots has been well explored, but since pitcher plants are a small subset of the plant community, the interactions between microorganisms and nitrogen acquisition within pitcher plants are not well defined in the context of BEF relationships.

Evaluating the nitrogen metabolic pathways involved in these relationships can reveal drivers behind microbial community composition and diversity. There are many functional steps in nitrogen degradation, including organic nitrogen degradation, denitrification, nitrate reduction, and nitrification (Figure 3, Wang et. al. 2020). While the distribution of bacterial taxa performing these tasks may vary from system to system, it is abundantly clear that nitrogen processing is partitioned amongst different species (Figure 3). Nitrate and ammonia concentrations have been linked to nitrogen metabolism, including impacts on the enzymatic activity of chitinases and proteases in decomposition (Wang et. al. 2020). This is important in the pitcher plant system, where insect prey is composed of a chitin outer shell. Activity of chitinases and proteases will impact breakdown of the insect. As I evaluated the pathways of nitrate, ammonia, and amino-acid breakdown, I also included chitinase and protease activity due to their evident link to the functional steps of nitrogen cycling.

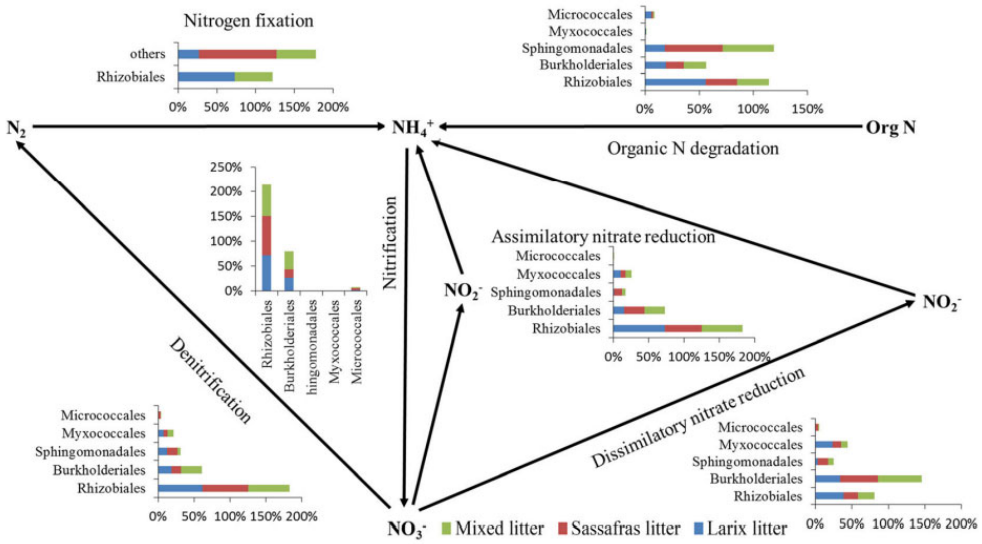

Figure 3. Nitrogen cycling in plant soils (Wang et. al. 2020). Microbial species contributions to soil nitrogen cycling produce complex webs, with different species driving different steps of the relationship.

My goals in this study were to examine the role of *Darlingtonia*-associated bacterial community dynamics on prey insect degradation. To capture different aspects of the diversity-function relationship, I used lab-grown communities of various complexity: bacterial isolates grown in monoculture and bacterial isolates grown in mixed cultures of 2-5 species randomly assorted (Chapter 1), and communities collected from greenhouse pitcher plants grown using a dilution to extinction approach (Chapter 2). I subjected each group to a degradation experiment to evaluate the broad function of insect consumption, or degradation rate, as well as measuring specific metrics within this broad function to evaluate enzymatic activity of chitinase and protease, and solubilized protein, nitrate, and ammonia. I additionally collected samples from three field sites across Northern California to evaluate how the lab-grown communities represent communities that naturally assembled in *Darlingtonia* fens (Chapter 3). No previous work on bacterial

composition and diversity has described the link between nitrogen metabolism mechanisms and insect degradation across the full range of bacterial community complexity, and this project builds a foundation for exploring this in the context of the model plant *Darlingtonia*.

Specifically, with these three approaches I aimed to establish the functional contribution of specific taxa to degradation and their role within mixed communities assembled through artificial mixing, dilution to extinction, as well as natural occurrences across the range of the plant. Bacteria grown in monoculture allowed me to explore how the specific metrics of chitinase, protease, protein, nitrate, and ammonia correlated with the broad function of degradation rate on an individual bacterial species level. Mixed cultures of these same species allowed me to explore how community coexistence impacts the broad and specific functions of insect degradation. Finally, the serial dilution communities allowed me to evaluate if we can use the small-scale results of the mono and mixed cultures to predict how bacterial species will interact in more complex communities, and if these dynamics correlate with ecosystem function. The overall purpose of this work was to identify how community dynamics contribute to ecosystem function in the context of nitrogen provision within *Darlingtonia* pitcher plants.

I addressed two central hypotheses. First, based on high levels of bacterial diversity within pitcher plant leaves, I hypothesized that if species provide redundant nitrogen processing functions, higher diversity would not have an impact on broad and specific nitrogen functions. I also hypothesized that variations in composition would not impact function, as most species are functionally redundant and can perform similar

metabolic tasks. Alternatively, function could increase with bacterial richness as observed by Armitage (2017) over successional gradients, or decrease, as observed in dilution to extinction experiments on carbon substrate usage (Cuellar-Gempeler et. al. in preparation). Secondly, I hypothesized that if the specific nitrogen functions are driving the broad function of degradation, chitinase and protease activity will be positively correlated with fly mass loss as they are the enzymes actively engaged in nutrient breakdown, and protein, nitrate, and ammonia content will be positively correlated as they are the nutrients the plant would need to absorb. My findings have applications in general understanding of the microbial role in the health and survival of this pitcher plant species, and more broadly, to expand this system as a model to understand other host associated microbial community functions and BEF relationships.

METHODS

1. Pitcher Plant Fluid Sample Collection

Bacterial samples were collected from *Darlingtonia* leaves using this protocol at all locations. Sterilization of all equipment was performed *in situ* with 70% ethanol between each sampling. Target leaf was located, and care was taken to cut leaf at the base, closest to the plant rosette. The base of the leaf was pinched closed while cutting with scissors to prevent any loss of fluid or insect matter. The base of the snipped leaf was brought over a 50mL Falcon tube and all fluid was released; once fluid stopped flowing, the leaf was cut vertically and any insect matter was scraped into the tube. Tube was capped and placed on dry ice until they could be frozen at -20º C upon return to Cal Poly Humboldt for DNA extraction.

2. Cultured Community

I revived fourteen cryopreserved bacterial isolates using LB broth. Isolates were previously obtained by Dr. Cuellar-Gempeler from greenhouse and field collections of *Darlingtonia* fluid and maintained in cryovials using 2% DMSO. Once isolates were acclimated to lab conditions grown to sufficient density, they were streaked on LB agar media to confirm purity and then plated to quantify abundance. Samples were diluted with LB broth to 10^7 cells/mL. They were then inoculated into duplicate monocultures by adding 5mL of 10^7 cell/mL culture to 40 mL LB broth for a total of 28 samples, plus two media controls, and incubated at room temperature for 48 hours. Five groups each of 2, 3,

4, and 5 mixed cultures were randomly assembled in equal amounts. For mixed cultures with 2 species, 2.5mL of each species was added, for mixed cultures with 3 species, 1.67 mL of each species was added. For mixed cultures with 4 species, 1.25mL of each species was added. For mixed cultures with 5 species, 1mL of each species was added. This equated to adding a total of 5mL of 10^7 cells/mL culture to 40mL LB broth for a total of 20 samples, plus two media controls, and incubated at room temperature for 48 hours. Samples were plated once more to calculate cell density at the start of the degradation. Bacterial species and composition of groups, as well as abundances, are detailed in Table 1 and Table 2.

Because *Bacillus mycoides* did not produce single colonies upon plating (it formed filamentous mats), counts for this species have been converted from a percentage of plate coverage into colony counts by taking that percent of coverage and multiplying it by the maximum species abundance counted, that being of *Leucobacter sp.* ZYXR1 at an abundance of 1.2×10^{11} cells/mL, as they visually looked the most similar at max coverage. To calculate growth rates, bacterial counts upon culture inoculation were subtracted from bacterial counts at the beginning of the degradation experiment. To calculate end of degradation growth rates, bacterial counts at the beginning of the degradation experiment were subtracted from bacterial counts at the end of the degradation experiment. Isolates in Table 1 were numbered based on their mean degradation rate, with 1 having the highest rate and 14 having the lowest rate.

Table 1. Bacterial isolate identities.

Isolate Number	Scientific Name	Percent Identity	Acquisition Number
1	*Bacillus mycoides*	99.10%	MN022603.1
2	*Leucobacter sp. 3*	98.63%	MH671536.1
3	*Arthrobacter gandavensis 2*	89.83%	MT534559.1
4	*Staphylococcus equorum*	87.03%	KC513844.1
5	*Acinetobacter junii*	94.76%	MF462967.1
6	*Leucobacter sp. 4*	93.75%	MH671536.1
7	*Arthrobacter gandavensis 1*	88.89%	MT534559.1
8	*Leucobacter sp. 2*	98.14%	MH671536.1
9	*Bacillus pumilus*	81.56%	AJ494732.1
10	*Microbacterium oxydans*	96.78%	MT733954.1
11	*Staphylococcus lugdunensis*	97.40%	KU977140.1
12	*Leucobacter sp. ZYXR1*	97.02%	AB847936.1
13	*Leucobacter sp. 1*	98.59%	MH671536.1
14	*Leucobacter sp. dR13-9*	96.51%	HQ436424.1

Table 2. Composition of mixed culture communities.

Group Name	Bacteria Included (By isolate number)	Group Name	Bacteria Included (By isolate number)
Mix 1	7, 13	Mix 11	3, 4, 6, 8
Mix 2	4, 7	Mix 12	1, 6, 11, 14
Mix 3	8, 9	Mix 13	1, 3, 6, 11
Mix 4	11, 14	Mix 14	1, 5, 11,14
Mix 5	3, 9	Mix 15	7, 10, 11, 12
Mix 6	8, 10, 13	Mix 16	1, 7, 8, 10, 11
Mix 7	9, 12, 14	Mix 17	3, 8, 10, 13, 14
Mix 8	3, 6, 13	Mix 18	1, 6, 9, 10, 13
Mix 9	5, 7, 14	Mix 19	5, 8, 12, 13, 14
Mix 10	10, 12, 14	Mix 20	5, 7, 11, 13, 14

3. Serial Dilution Community

Samples were collected on November 15, 2021 from six (6) *Darlingtonia* leaves at the Dennis K. Walker Greenhouse, two (2) leaves from each of three (3) plants following methods from section 1, and samples were combined into one flask producing 10mL of combined sample. Sterile insect broth was made by grinding *Drosophilia* fruit flies, autoclaving ground flies, and combining with sterile DI water at a concentration of 5mg/mL, for a total of 580.5mL. Broth controls were then separated by adding 45mL of this broth to each of three (3) 50mL Falcon tubes. DI water controls were produced by adding 45mL of sterile DI water to each of three (3) 50mL Falcon tubes. The remaining broth was inoculated with the collected *Darlingtonia* fluid sample, and this was

maintained for 8 days, with 1mg/mL feeding every 3 days. This mixture was incubated at room temperature for four (4) days. After incubation, sample was separated into three (3) beakers of 148.5mL to create separate series, and each of these series was then serially diluted at a ratio of 1:10 by taking 13.5mL inoculated broth and adding it to 121.5mL sterile DI water four (4) times, for a total of 5 levels of dilution per series, or 15 communities total. Each community was then fed sterile fruit flies at a level of 1mg/mL and incubated for four (4) days at room temperature. After incubation, each community was separated into three (3) 50mL Falcon tubes of 45mL each. This produced a total of 45 inoculated samples (3 replicate dilution lines of 5 cultures each, with 3 replicated samples each), 3 broth controls, and 3 water controls. 10mL was collected from each sample prior to further experimentation and frozen at -20º C for DNA extraction before degradation experiment.

Dilution level 0 was omitted from analyses. This group started with 5mg/mL fly broth and was fed 1mg/mL sterile ground insect twice before separating and diluting; the amount of unconsumed and consumed fly material was not quantified, and therefore the potential for this dilution level to skew the data due to inconsistent levels of insect food was high.

4. Field-Sampled Bacterial Communities

Pitcher plant fluid samples were collected in July 2021 from 3 sites in California following methods from section 1. Six (6) samples were collected from Gasquet, Del Norte County; five (5) samples were collected from Mt. Shasta, Shasta County; and five

(5) samples were collected from Quincey, Plumas County (Figure 4). Field sites had unique characteristics, allowed for a latitudinal gradient, and encompassed differing conditions that may harbor a range of microbial taxa. The Gasquet site was a bog which was shaded by many trees. The Mt. Shasta site was a grassy meadow characterized by two small creeks. The Plumas site was a rocky mountainside with cold flowing water. These samples were subjected to bacterial extraction.

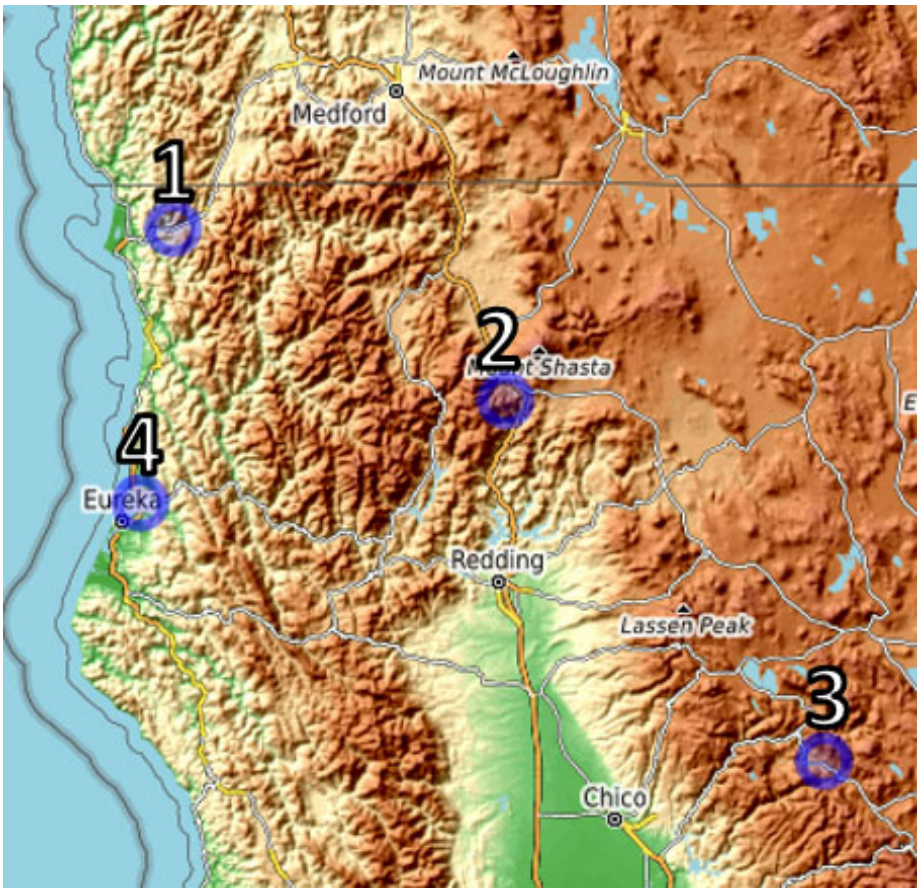

Figure 4. Location of samples in California.
1- Gasquet. 2- Shasta. 3- Plumas. 4- Dennis K. Walker Greenhouse, Arcata.

5. Fruit Fly Preparation for Degradation Experiments

Drosophilia fruit flies were purchased from Arcata Pet Supply in Arcata, CA and grown in lab until maximum capacity of fruit fly culture. Culture was then frozen over night at -20º C to sacrifice flies. Flies were then collected and dried for 48 hours at 60º C and autoclaved to sterilize. These were then transferred into 1.5mL cages, produced by drilling 24 holes into a 1.5mL centrifuge tube to allow for flow of fluid and bacteria, for a total of 12-15mg of flies per 1.5mL cage. Exact mass per cage was recorded by recording each cage without flies and with flies.

6. Degradation Experiment

The cultured community and serial dilution community were subjected to the following degradation experiment. Following acclimation of the communities, fruit fly cages were placed in each sample and samples were loosely capped. Samples were then incubated at room temperature for 11 days in still conditions to simulate the still conditions in which *Darlingtonia* leaves live. Samples were inverted once daily to encourage flow of bacteria in and out of the fruit fly cages for the duration of the degradation experiment. At the end of the 11 days, samples were collected. For both cultured and serial dilution communities, 1.5 mL fluid was collected for each solubilized protein, protease, and chitinase quantification, and 10 mL was collected for nitrate and ammonia quantification. For the serial dilution community, 10mL was collected for DNA extraction after degradation experiment. For the cultured community, samples were

plated to quantify. Any excess sample was preserved in 1.25% DMSO for future use, unrelated to this project.

7. Relevant Measures of Nitrogen Function Assays

Following degradation, samples were collected for measurement of relevant metabolic functions and frozen at -20ºC until measurement. Degradation rate, enzymatic activity of protease and chitinase, solubilized protein, nitrate, and ammonia were measured. Degradation rate was measured by drying fly cages at 60º C for 3 days and subtracting from pre-degradation mass to calculate fly mass loss. Chitinase activity was quantified using the Chitinase Microplate Assay Kit from MyBioSource (catalog #MBS8243204) and read in Spectra iMax at 585 nm. Protease activity was quantified using the Protease Assay Kit from ThermoScientific (catalog #23263) and read on the Spectra iMax at 450 nm. Solubilized protein was quantified using a Bradford assay and reading on a Nanodrop 1000 at 595 nm. Solubilized protein and enzymatic activity of chitinase and protease were measured in duplicate to verify accuracy of spectrophotometric quantification. Ammonia and nitrate assays were performed using the Orion^{TM} High Performance Ammonia Electrode. Bacteria were quantified and identified in the serial dilution and field sample groups by extracting DNA using Qiagen DNeasy PowerWater Kit (catalog #14900-100-NF) and submitted to Argonne Labs for 16S rRNA Illumina sequencing.

8. Bioinformatics and Data Analysis

Cultured Community

First, I calculated degradation and growth rates from the monocultures to establish relationships between these parameters. I ranked the strains according to their degradation rates, with the strongest performing species being rank 1 and the weakest performing species being rank 14. To calculate bacterial growth rates at the start of degradation, I subtracted bacterial cell concentration upon inoculation from bacterial cell concentration at the start of the 11 day degradation experiment. To calculate end of degradation growth, I subtracted cell concentration at the start of the degradation experiment from cell concentration at the end of the experiment. Significance of correlation was tested using a linear model with a significance test for linear regression using the function lm. All analyses and plots were performed and produced using the R statistical environment (R Core Team, 2020; version 4.1.1). Relationships were plotted and visualized using the *ggplot2* package (version 3.3.6, Wickham 2016). *P*-values were adjusted using the Benjamini and Hochberg method degradation rate was the dependent variable and specific nitrogen functions were the explanatory variables (Jafari and Ansari-Pour, 2018).

To establish the biodiversity-function relationship in the mixed community experiments, I used a linear regression model with diversity metrics as the explanatory variables and function metrics as the dependent variable. I used function lm which included a significance of correlation test.

Serial Dilution Community

Bacterial sequences were demultiplexed using idemp (Blostein et. al. 2020). All subsequent steps were performed using the R statistical environment (R Core Team, 2020; version 4.1.1). I used the *dada2* package to trim, quality filter (truncLen=c(160, 160); maxN=0; maxEE=c(5,7); trunQ=3), denoise, merge, and remove chimeras form the sequences (version 1.22.0, Callahan et. al. 2016). I generated the ASV table using the dada function and established taxonomic identity by comparing sequences at the 97% threshold with the Green Genes dataset (McDonald et. al. 2011). Data was further processed and explored using the *phyloseq* package (version 1.38.0, McMurdie and Holmes, 2013), *microbiome* package (version 1.16.0, Lahti et. al. 2017), and *dplyr* package (version 1.0.9, Wickham et. al. 2022). Reads were removed when taxonomic affiliation indicated “Chloroplasts”, “Mitochondria”, or “Archaea”. Figures and plots were produced using the package *ggplot2* (version 3.3.6, Wickham 2016). *P*-values were adjusted using the Benjamini and Hochberg method degradation rate was the dependent variable and specific nitrogen functions were the explanatory variables (Jafari and Ansari-Pour, 2018).

To assess the relationship between diversity and function, I calculated diversity metrics of richness (number of species), Pielou’s evenness (Pielou, 1959), and Shannon-Weiner diversity using the package vegan (version 2.6.2, Oksanen et al., 2020). I confirmed diversity gradient produced in the dilution to extinction experiment with a linear model using the glm function with a Gaussian distribution. I included richness and

series as explanatory factors to assess inter-series variation in diversity gradients. I repeated this model with Shannon-Weiner index as a metric of diversity.

I used the serial dilution data to establish the role of dilution and series in generation of (1) BEF relationships on the broad degradation and specific functions, and (2) community composition patterns. To evaluate BEF relationships, I used a general linear model with Gaussian distribution using function glm from the package *car* (Fox et. al., 2013). The model included diversity metrics and series as the explanatory fixed factors and function as the dependent variables. I used the function Wald test to determine significance of these models by implementing the function ANOVAfrom the *car* package (version 3.1.0, Fox, Friendly, and Weisberg, 2013). I repeated this analysis for the specific functions. I also implemented this analysis with Shannon-Weiner diversity indexes as the diversity metric to assess the role of community structure in driving specific functions.

The effect of dilution and series on community composition relationships were tested with a perMANOVA using the function adonis from the package *vegan* (version 2.6.2, Oksanen et al., 2020). An ordination was used to represent community composition patterns using the function metaMDS in the package *vegan*, and differences in community similarity across groups were evaluated by calculating distance to centroid with the function betadisper (version 2.6.2, Oksanen et al., 2020).

Field Communities

Diversity between field sampled sites was evaluated using richness (number of species), Pielou's evenness (Pielou, 1959), and Shannon-Weiner diversity produced with

the package *vegan* (version 2.6.2, Oksanen et al., 2020). An ordination was used to represent community composition patterns using the function metaMDS in the package *vegan* (version 2.6.2, Oksanen et al., 2020). To evaluate the similarities between the field samples and the serial dilution samples and test for significance, I used the function betadisper for a distance to centroids test in the package *vegan* (version 2.6.2, Oksanen et al., 2020).

RESULTS

Chapter 1: Cultured Community

The cultured communities discussed in this chapter are used to evaluate the correlations between abundance, richness, and broad and minor nitrogen functions on a species level. While this small group of culturable bacteria cannot fully represent the functions of the complex communities observed in the field, the aim is to understand how individual species grow and impact nitrogen cycling, and how these trends are disrupted when these species coexist with one or more other species. Specifically, we aimed at testing these three hypotheses: 1) isolates' degradation rate is inversely correlated with growth rates because enzymes required for prey processing have a fitness cost, 2) isolate degradation rate correlates with their performance in specific nitrogen cycle functions, and 3) mixed communities will show redundant relationships between diversity and functions.

The 14 species grown in monoculture have a wide range of functioning regarding degradation rate (Figure 5) and specific nitrogen processing steps. *Bacillus mycoides* cultures performed the highest degradation (11.2 mg), with 8 species resulting in fly mass retention compared to the control average of 5.3 mg, with the lowest recorded average degradation rate being -2.3 mg. Higher levels of protein were found to accumulate in *Bacillus mycoides*, *Acinetobacter junii*, and *Bacillus pumilus* cultures. High levels of nitrate accumulated in *Arthrobacter gavadensis* 1 cultures. Ammonia accumulated highly

in *Leucobacter sp.* 1 cultures and slightly in *Bacillus pumilus*, *Leucobacter sp.* dR13-9, *Bacillus mycoides*, and *Leucobacter sp.* 3 cultures.

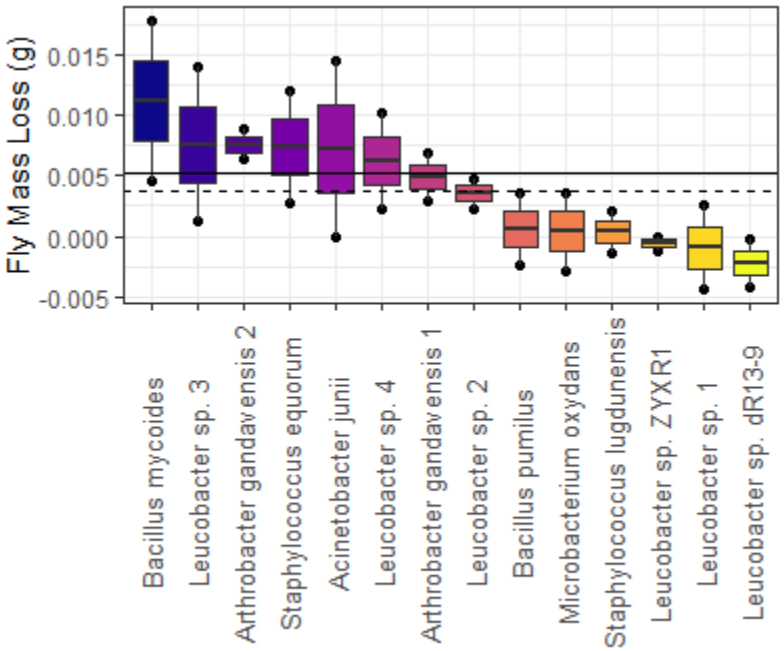

Figure 5. Monoculture Degradation Rates.
The 14 bacteria grown in monoculture performed degradation at varying rates, with Bacillus mycoides being related to highest degradative function and Leucobacter sp. dR13-9 being related to lowest degradative function. The solid black line indicates control average, the dash line indicates average of all isolates.

In the monocultures, degradation rate correlated negatively with growth rates (Figure 6, df = 26, $R^2 = 0.17$, $F = 5.50$, $p = 0.027$), but no other specific function correlated with growth rate (Table 3). There was no correlation between degradation rate and end of degradation bacterial growth (Appendix 3). No specific nitrogen function correlated with degradative function (Table 4). No trend was found between bacterial

abundance and the specific nitrogen functions at the start of degradation and at the end of degradation (Appendix 4, Appendix 5).

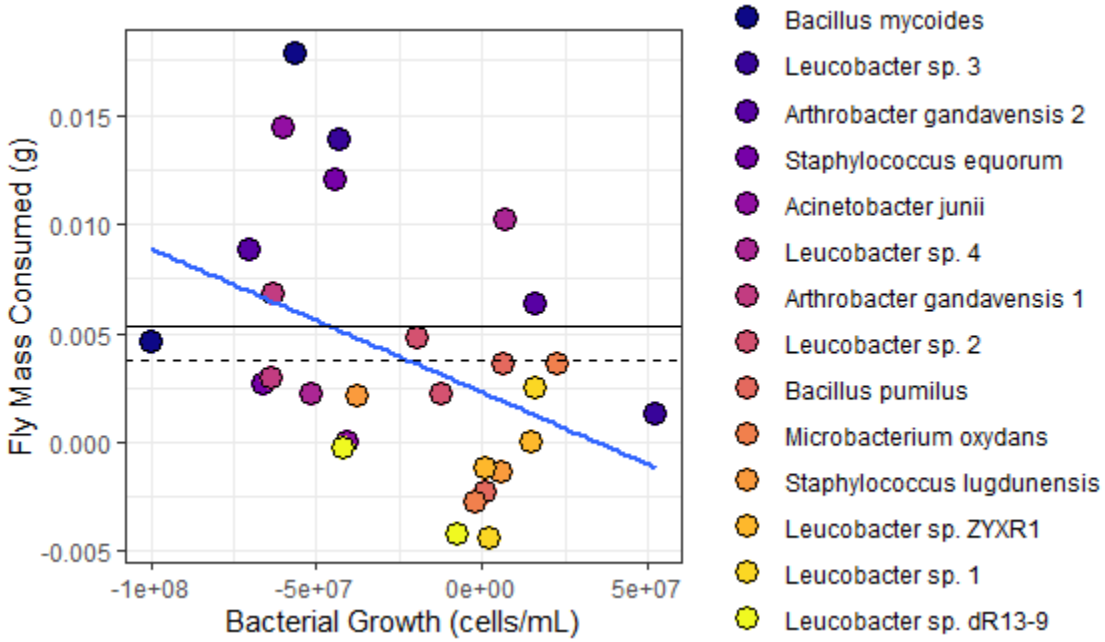

Figure 6. Monoculture bacterial growth vs. degradation rate.
Species with higher growth rates had lower degradation rates at the end of the experiment. The blue line represents the linear model fitted to fly mass loss and bacterial abundance. The dash line indicates sample average, the solid line indicates control average. Colors are used to visualize bacterial species.

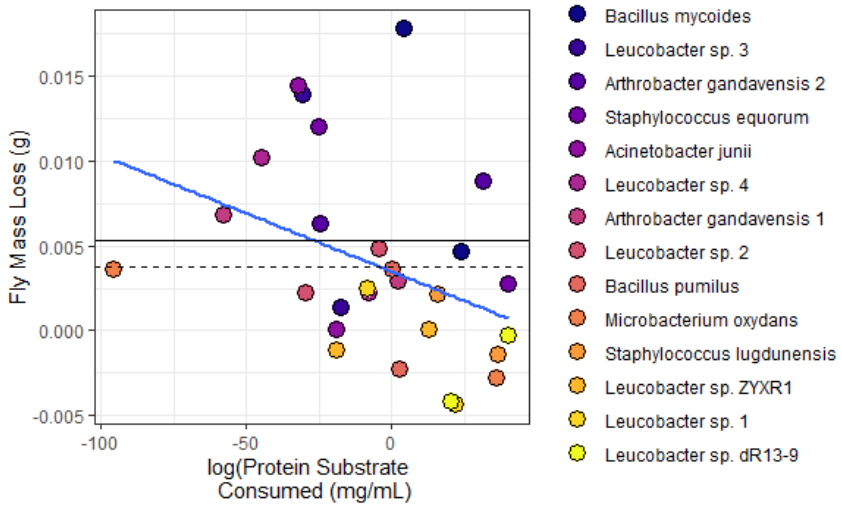

Figure 7. Monoculture degradation rate vs. protease activity.
Protease activity correlated negatively with degradation rate in the monocultures. The dash line indicates sample average, the solid line indicates control average. Colors are used to visualize bacterial species.

Table 3. Monoculture bacterial growth rate vs. function
*Results of linear regression significance test of bacterial growth rate compared with each functional assay. Degrees of freedom for all tests were 26. Significance results are highlighted in bold. *Represents significance to the $p<0.05$.*

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	**0.1429**	**5.501**	**0.02691***
Bradford	0.01209	1.33	0.2592
Nitrate	-0.02331	0.385	0.5403
Ammonia	0.02776	1.771	0.1948
Chitinase	-0.02756	0.2758	0.6039
Protease	0.01138	1.311	0.2627

Table 4. Monoculture degradation rate vs. function
Results of linear regression significance test of degradation rate compared with the specific nitrogen functions. Degrees of freedom for all tests were 26.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Bradford	-0.2247	0.4068	0.6322
Nitrate	-0.02918	0.2346	0.6322
Ammonia	-0.02518	0.3367	0.6322
Chitinase	0.0303	1.844	0.6322
Protease	0.1158	4.534	0.6322

In mixed culture, degradation rate correlated positively with species richness at the beginning of the degradation (Figure 8, df = 18, $R^2 = 0.31$, $F = 8.14$, $p = 0.011$). In contrast, no other correlation was found between other nitrogen metrics and species richness at the beginning of degradation (Table 5), at the end of degradation (Appendix 6), or between degradation rate compared with specific nitrogen assays (Appendix 7).

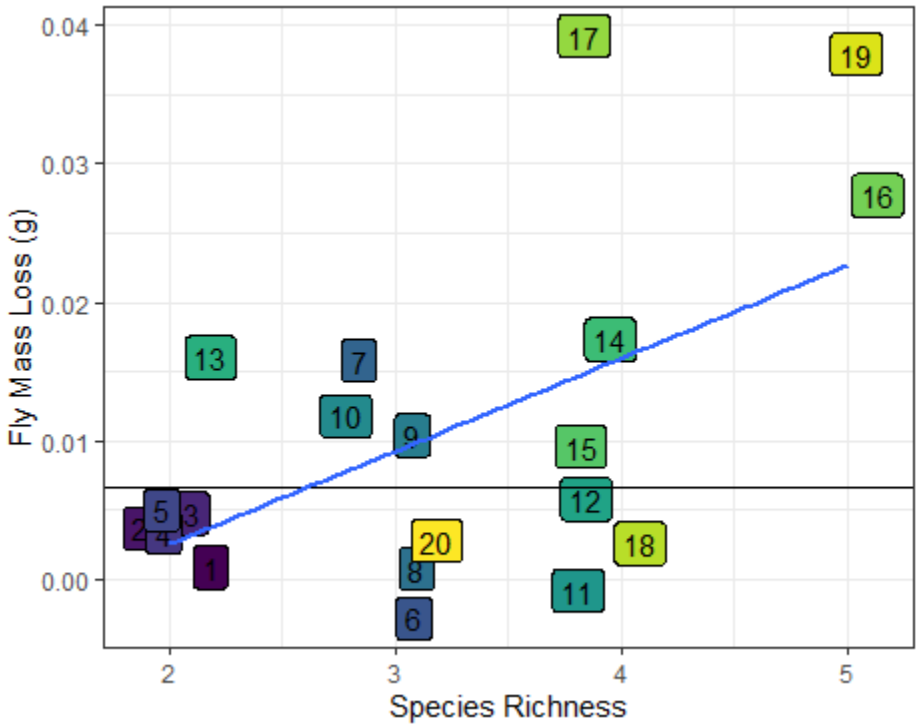

Figure 8. Mixed culture degradation rate vs. species richness.
Degradation rate correlated positively with species richness. Color is used to visually distinguish groups and has other no meaning; numbers represent groupings described in Table 2. The blue line represents the linear regression model fitted to fly mass loss and species richness.

Table 5. Mixed culture species richness vs. function
*Results of linear regression significance test of species richness compared with each functional assay. Degrees of freedom for all tests were 18. Significance results are highlighted in bold. *Represents significance to the p<0.05.*

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	**0.2731**	**8.14**	**0.01056***
Bradford	-0.05516	0.006743	0.9355
Nitrate	0.02025	1.393	0.6333
Ammonia	-0.03524	0.3533	0.9328
Chitinase	0.04893	1.978	0.6333
Protease	-0.04992	0.09655	0.9355

Importantly, diversity treatments did not always result in consistent richness for the duration of the experiment. While 5 groups were inoculated with 5 species, only Group 16 and 19 continued to maintain this richness level by the start of the experiment. Group 17, 18, and 20 reduced to 4, 4, and 3 species respectively. Group 16 and 19 also had two of the three highest fly loss measures.

Chapter 2: Serial Dilution Community

A microcosm dilution to extinction experiment was used to evaluate if complex inoculates that more closely represent pitcher plant microbial communities reflected the patterns found in cultures of mixed isolates. Specifically, I aimed to evaluate the following hypotheses: 1) changes in diversity and variations in composition will not impact function as most species are functionally redundant and 2) degradation rates will correlate with specific nitrogen functions.

The dilution to extinction approach generated a diversity gradient from dilution level 1 (with a dilution factor of 1/10) to 4 ($1/10^4$, Figure 9, df = 34, $R^2 = 0.33$, $F = 18.48$, $p = 0.0001$). It is important to note that all series in the experiment showed a significant diversity gradient, even though series 1 had a much shallower gradient (Appendix 10, df = 35, t-value = 5.19, $p < 0.0001$). However, these clear diversity gradients were unrelated to degradation rates (Table 6). While combined data of the three different series showed no correlation with degradation rate and other specific nitrogen functions, when separated into the three separate functions, BEF relationships took slightly different shapes (Figure 10).

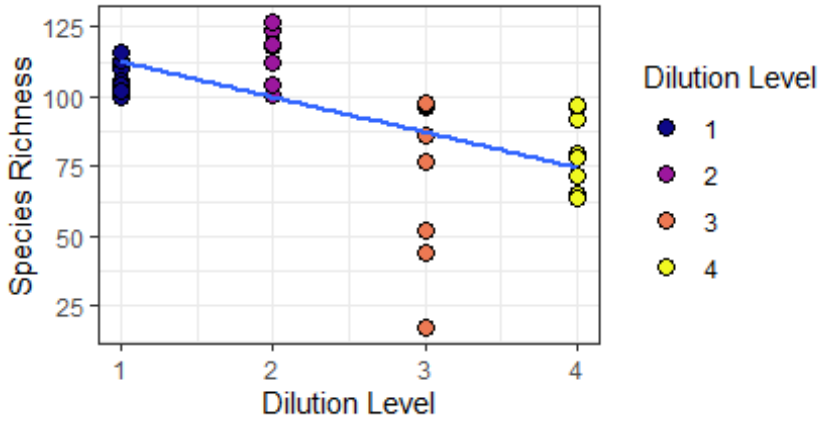

Figure 9. Species richness by dilution level.
The blue line indicates the linear regression model fitted to species richness by dilution level. Colors further visualize the different dilution levels.

Table 6. Serial dilution species richness vs. function
*Results of ANOVA and Wald test on richness compared with functional assays. Degrees of freedom for all tests were 1. Significance results are highlighted in bold. *Represents significance to the $p<0.05$.*

Functional Assay	Chi^2	*p*-value
Degradation Rate	2.4492	0.1176
Bradford	3.636	0.05654
Nitrate	14.388	**0.0001488***
Ammonia	0.67402	0.4117
Chitinase	4.3019	**0.03807***
Protease	1.6376	0.2007

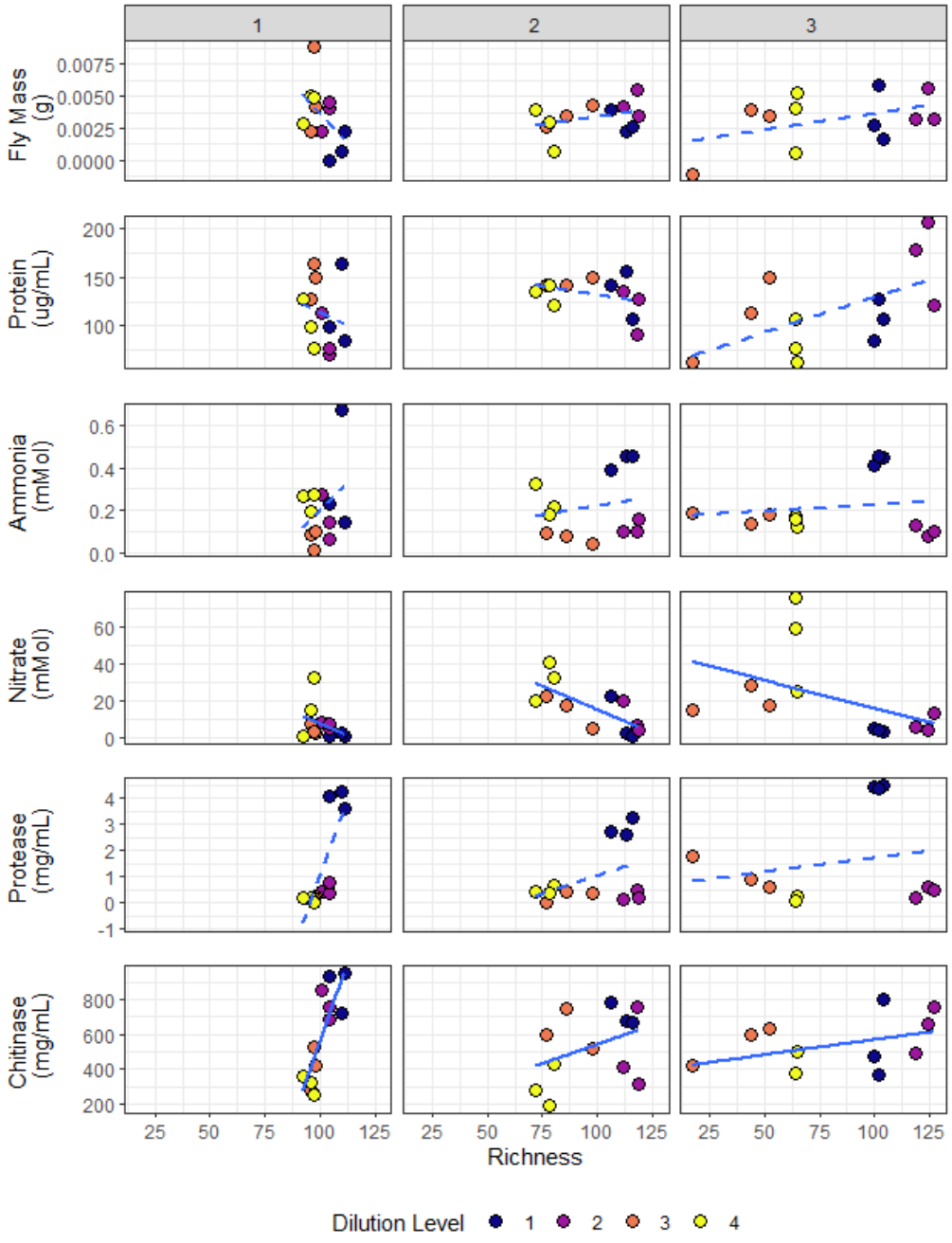

Figure 10. Serial dilution degradation rate vs. function
Degradation rate and nitrogen functions were visualized against richness, separated by series. All lines depict the linear regression model fitted to richness and functional assay. The solid lines depict relationships that were significant, the dash lines indicate relationships that were not significant. Chitinase and nitrate displayed significant trends (Table 6).

In the serial dilution experiment, only two specific nitrogen assays had significant relationships with richness (Table 6), and three specific nitrogen assays had significant relationships with Shannon-Weiner diversity (Table 7). Nitrate trended negatively with bacterial richness (Figure 11A, df = 1, *Chi sq* = 14.39, $p < 0.001$) and Shannon-Weiner diversity (Figure 11D, df = 1, *Chi sq* = 12.82, $p < 0.001$). Chitinase activity trended positively with bacterial richness (Figure 11B, df = 1, *Chi sq* = 4.30, $p = 0.038$) and Shannon-Weiner diversity (Figure 10E, df = 1, *Chi sq* = 8.01, $p = 0.005$). Protease trended positively with Shannon-Weiner diversity (Figure 10F, df = 1, *Chi sq* = 9.07, $p = 0.003$), but not richness (Figure 10C). Note that in all these cases there is a large effect of the dilution level, and that more complex statistical models would likely describe those patterns better. Here, I kept a consistent model that was appropriate for the hypotheses and goals of this study and allowed for straight forward comparisons across tests.

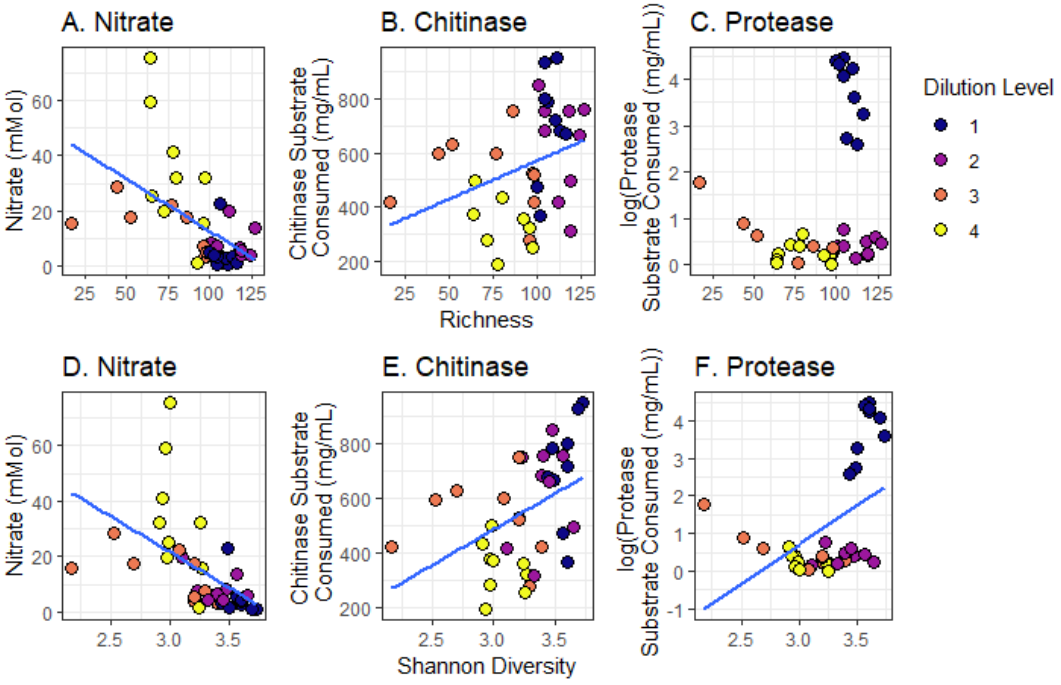

Figure 11. Serial dilution nitrogen metrics correlated with diversity. Plot titles describe the nitrogen function plotted against either richness (A-C) or Shannon-Weiner diversity (D-F). Blue lines represent the linear regression model fitted to diversity and function measure and are only shown for significant results.

*Table 7. Serial dilution Shannon-Weiner diversity vs. function. Results of ANOVA and Wald test on Shannon-Weiner Diversity compared with functional assays. Degrees of freedom for all tests were 1. Significance results are highlighted in bold. *Represents significance to the $p<0.05$.*

Functional Assay	Chi^2	*p*-value
Degradation Rate	0.4377	0.5082
Bradford	1.3031	0.2536
Nitrate	12.819	**0.0003432***
Ammonia	3.837	0.05013
Chitinase	8.0066	**0.004661***
Protease	9.069	**0.0026***

When looking at trends between fly mass loss and the specific nitrogen functions, ammonia (Figure 12A, df = 1, *Chi sq* = 4.75, *p* = 0.073) and protease activity (Figure 12B, df = 1, *Chi sq* = 4.91, *p* = 0.073) trended negatively, but after P-value adjustment due to multiple testing, these were nonsignificant. No other specific nitrogen functional assays significantly correlated with fly mass loss (Table 8).

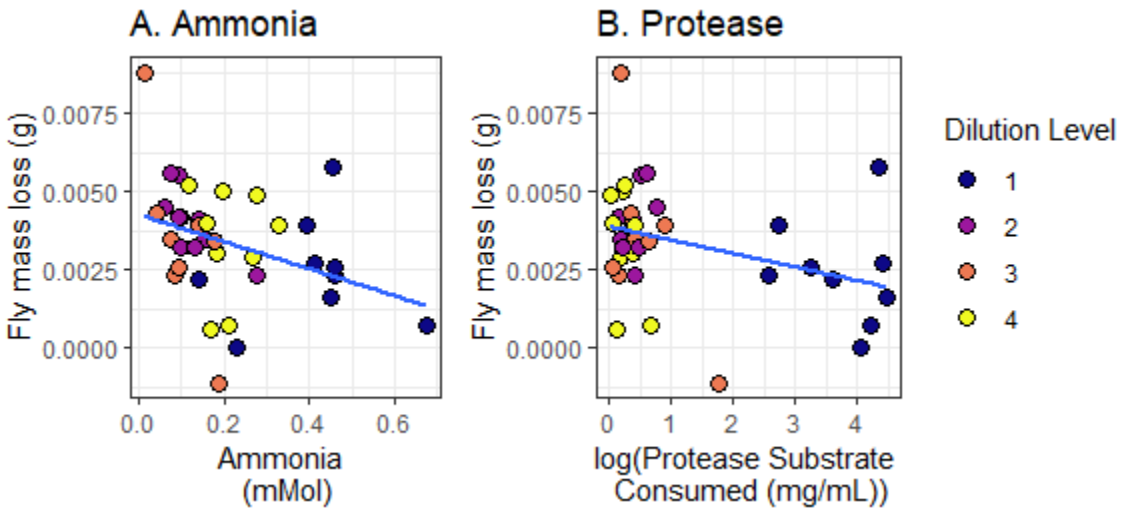

Figure 12. Serial dilution degradation vs. function
A. Ammonia trended negatively with degradation rate. B. Protease activity trended negatively with degradation rate. Blue lines represent the linear regression model fitted to degradation rate and specific nitrogen metric.

Table 8. Serial dilution degradation rate vs. function
Results of ANOVA and Wald test on degradation rate compared with each specific nitrogen function assay. Degrees of freedom for all tests were 1.

Functional Assay	Chi^2	*p*-value
Bradford	2.0099	0.2605
Nitrate	0.18109	0.6704
Ammonia	4.7494	0.0733
Chitinase	0.90268	0.4276
Protease	4.9139	0.0733

Community similarity did not vary across series (Figure 14, df = 2, Sum sq. = 0.33, $F = 0.40$, N.Perm = 999, $p = 0.66$) but did vary across dilutions (Figure 15, df = 3, Sum sq. = 144.91, $F = 4.23$, N.Perm = 999, $p = 0.01$). Dilution seems to be a strong driver of community composition, with most series moving from the least diluted at a central point in the x-axis (Figure 13, dilution 1 orange points) to the most diluted in the far right, midway in the y-axis (Figure 13, dilution 2 pink points to dilution 4 blue points). The main exception to this trajectory is the intermediate dilution in series 3 that departs towards the upper left quadrant of the graph. These trajectories describe the major changes in community composition according to ANOVA upon Distance to centroid (Figure 14, df = 3, Sum sq. = 30.44, $F = 0.3688$, $p = 0.6944$; Figure 15, df = 3, Sum of Sq = 144.91, $F = 4.23$, $p = 0.013$).

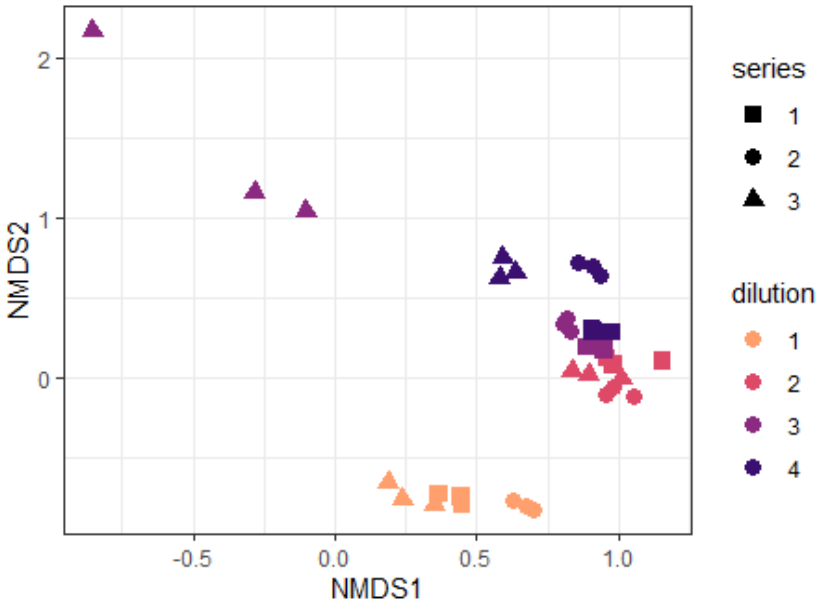

Figure 13. Serial dilution bacterial composition NMDS.
Samples within dilution and series remain grouped closely. Shapes indicate the series each sample was a part of and color indicates with dilution each sample was a part of.

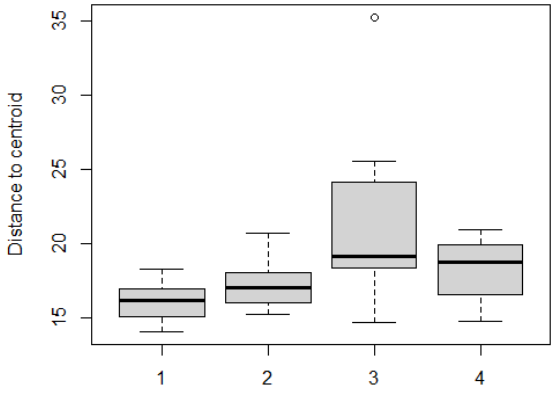

Figure 14. Distance to centroid of dilution level.
Variations in community composition differed.

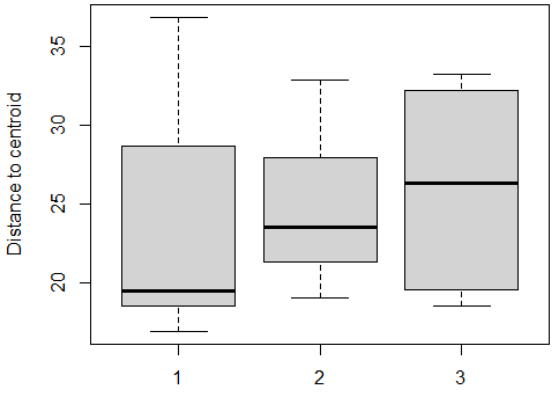

Figure 15. Distance to centroid of series.
Variations in community composition differed.

Chapter 3: Field Communities

The diversity of the field sites was used to evaluate if the cultured communities and serial dilution communities were representative of diversity that is observed across the range of *Darlingtonia* plants. Specifically, I aimed to evaluate the following hypothesis: diversity in the serial dilution experiment represents taxa that are observed across these three *Darlingtonia* sites.

Richness, Shannon-Weiner diversity, and evenness were similar across the three field sites (Figure 16). The highest unique ASV reads were found in leaves from Gasquet with 249 and Shasta with 284; the lowest ASV reads were found in a sample from Plumas with 21. Differences between sites were nonsignificant in richness (df = 2, Sum sq. = 19, $F = 1.56$, $p = 0.25$), Shannon-Weiner diversity (df = 2, Sum sq. = 1.38, $F = 0.80$, $p = 0.47$), and evenness (df = 2, Sum sq. = 0.038, $F = 0.90$, $p = 0.43$). The field sites displayed higher richness than the serial dilutions, where the highest unique ASV reads were found to be 127 in series 3 (Figure 9).

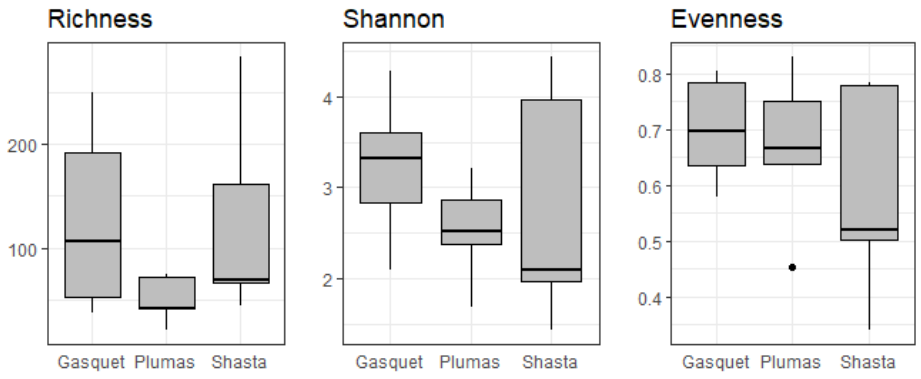

Figure 16. Diversity Metrics Across the Field Sites.
There was no significant difference between the three sites in each diversity metric.

Species found in the serial dilution communities represent many of the Phyla found in the field samples (Figure 17). The diversity of the three different field sites grouped much like the diversity of the three different series in their respective dilution levels, visualized in the NMDS plot (Figure 18). Distance to centroid was calculated to evaluate variances and compositional differences (Figure 19). Compositional variations differed from one another (df = 5, Sum sq. = 90.96, $F = 8.23$, N.Perm = 999, $p < 0.001$), as well as distance to the centroid (Figure 19, df = 5, Sum sq. = 57.01, $F = 7.37$, $p < 0.001$).

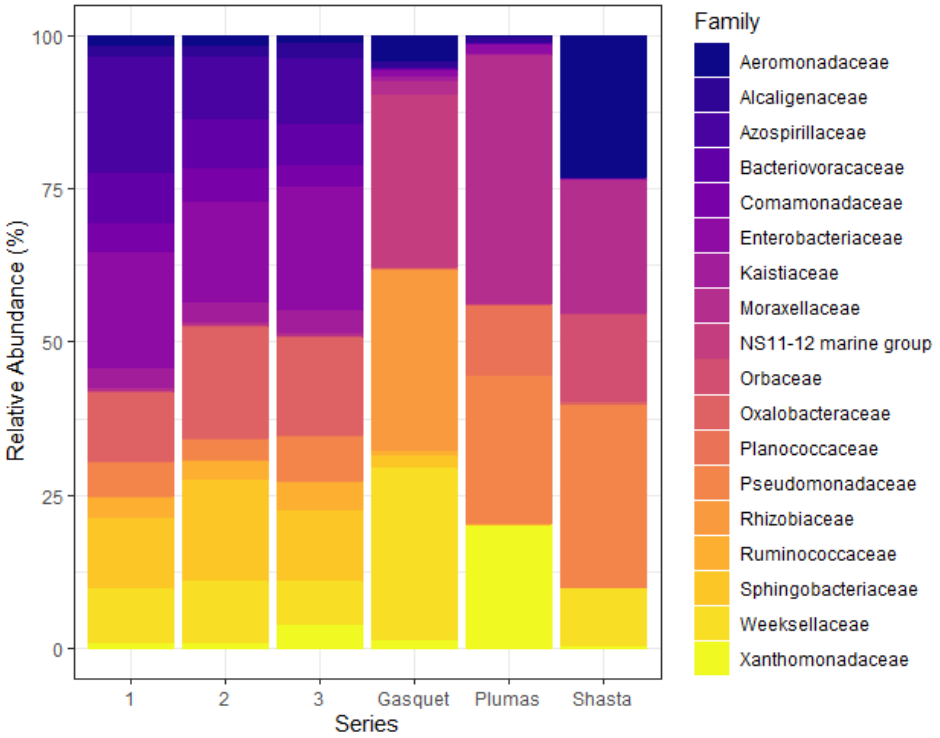

Figure 17. Relative Abundance of Series and Field Sites.
While these groups share many taxa, groups variation of composition was different.

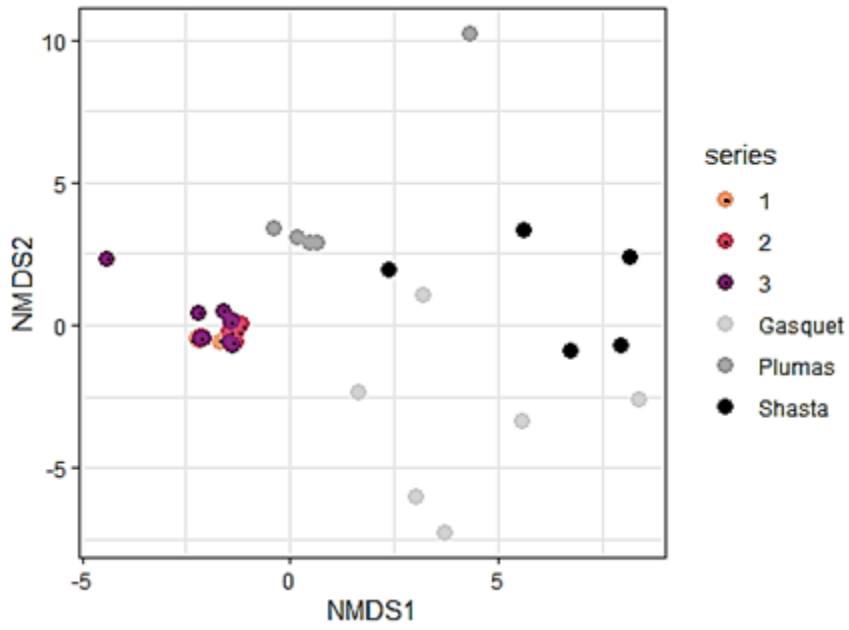

Figure 18. Serial and field NMDS plot.
Variations and compositions between sites were further verified by a distance to centroid test and ANOVA (Figure 18). The 1, 2, and 3 series are visible to the left of 0, while the Gasquet, Plumas, and Shasta series are visible to the right of 0.

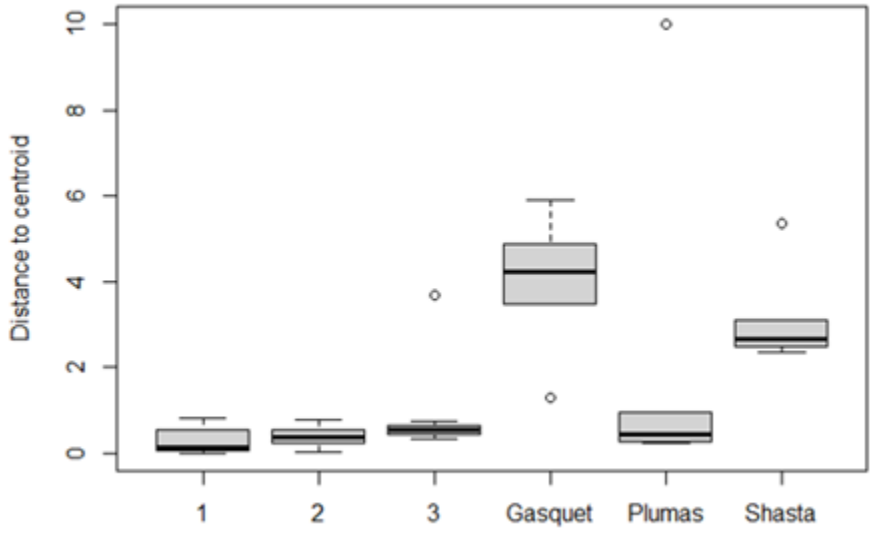

Figure 19. Distance to centroid plot of series and field sites.
Significance test results are in Appendix 14. Variances between series 1, 2, and 3 were similar, and variances between Gasquet, Plumas, and Shasta were similar. Variance within Plumas was similar to series 1, 2, and 3.

DISCUSSION

The relationship between biodiversity and ecosystem function (BEF) can take different forms, yet the drivers of this dynamic relationship are poorly understood. This study aimed to fill this gap by exploring pitcher plant bacterial communities of increasing complexity and establishing the relationship between their diversity, composition, and function in the context of insect degradation. My results revealed BEF relationships of diverse shapes that highlight the role of system complexity, community dynamics, and function. First, I found positive BEF relationships in experiments with mixed cultures, where communities with higher richness had higher degradative potential. Because these highly performing assemblages lacked the highest performing isolates, this positive BEF relationship is likely driven by complementarity between species that perform lower in monoculture but better in coexistence. Second, I found functional redundancy in bacterial nitrogen metabolism and degradation potential, as evidenced by the non-significant relationships between richness and degradation rate in the dilution to extinction experiments. Third, I highlighted several instances of negative BEF relationships, as observed in nitrate and species richness in the serial dilution (Figure 10B) and between degradation rate and species richness in series 1 of the serial dilution (Figure 12). This suggests that unique species may contribute to community function in complex communities but not compete well in diverse assemblages.

Isolate degradation rate was inversely correlated with growth rate, indicating important links between functional contributions and coexistence in microbial communities. For example, *Bacillus mycoides* was ranked 1 in degradation but ranked 14

in mean growth, while *Microbacterium oxydans* was ranked 10 in degradation but ranked 1 in mean growth. This relationship is observed even within species, such as in the case of *Leucobacter sp. 3,* where each of the samples grown in duplicate follow this shape. One sample of the duplicate drives the lower end of the degradation rate-bacterial growth relationship when it displays a higher growth rate, and the other sample drives the upper end of the linear regression when it has a lower growth rate (Figure 6). Trade-offs between growth rates and performance are not uncommon and highlight the cost of investing in metabolic or structural tools (Freilich et. al. 2009). This is an important observation that is otherwise not apparent in either mixed cultures or serial dilution communities.

In mixed community cultures, degradation rate deviates from the connection observed with lower growth rates in monoculture. Higher richness is associated with high degradation rates in these samples. The observation of this increase of degradation rate as species richness increases from 2 to 5 aligns with the hypothetical early increases of either complementary, redundant, or negative BEF relationships (Figure 1). Research on marine benthic macrofauna found that 66% of traits observed at a richness of 151 were also observed when there were only 6 unique taxa present (Strong et. al. 2015). While this research indicates redundancy in marine habitats, when low numbers of species are present, it increases the chance that each taxa brings a unique functional trait, having the potential to increase ecosystem function. Therefore, when observing the low overall species richness of the mixed community cultures, we would expect to see an increase in

function as richness increases, and this may align with redundant, complementary, or negative BEF relationships.

Cultured communities with the highest functional performance were not necessarily associated with single isolates with the strongest degradation potential, suggesting that complex metabolic traits result in fitness costs that compromise the species' abilities to maintain viable populations in mixed cultures. For example, *Bacillus mycoides,* the strongest monoculture degrader, was only found in Group 16 of the 3 high functioning mixed cultures. In the low diversity communities of the mixed culture experiment, coexistence of a few species of varying degradation rates may yield the same overall rate as a community with a strong degrader. The comparable function achieved by mixed communities suggests complementarity plays a role in these microbial communities, yet the functional outcome cannot be predicted from the performance of each species in monoculture. Previous research in microbial colonization of plant roots has likewise found that bacterial isolates behave unpredictably in multiple pairwise cultures, sometimes outcompeting other isolates and sometimes living in balanced coexistence depending on environmental conditions (Tovi et. al. 2021). Importantly, these results indicate complex interactions between functional metabolic activity and other ecological traits.

Cultured communities cannot represent the full extent of bacterial community diversity observed in *Darlingtonia*, which can number in the hundreds of species based on ASV observations from the serial dilution and field communities (Figure 9, Appendix 18). Therefore, the results of the serial dilution expand our understanding of community

diversity and function. In the complex communities of the serial degradation, there was no correlation found between the diversity metrics of richness, abundance, or Shannon-Weiner diversity with degradative rate, contrasting with the results from the mixed cultures. When explored separately, each series had different BEF shapes: series 1 had a negative BEF relationship with degradation, series 2 had no BEF relationship, while series 3 had a slightly positive trend with degradation. However, after correcting for multiple sampling, differences across series were not significant, suggesting overall functional redundancy. Metabolic redundancy is a key mechanism in many systems, including glacial systems (Trivedi et. al. 2020), tropical forests (Reed et. al. 2010), and activated sludge (Chen et. al. 2020). Functional redundancy would not be surprising in diverse microbial communities (Philippot et. al. 2013), and some studies suggest that this redundancy may allow differing community compositions to maintain nutrient processing.

In addition to redundancy, BEF relationships in the serial dilution experiment also displayed negative trends. While degradation visually appeared to have a negative relationship with diversity in series 1, solubilized nitrate correlated significantly negative with increased species richness (Figure 10). It is not surprising that increases in diversity lead to lower production of solubilized nitrate. Nitrate is an abundant source of nitrogen in natural systems, therefore a large number of bacterial taxa have the genes to utilize it for energy, a nitrogen source, or a signaling molecule (O'Brien et. al. 2016), quickly converting it through denitrification or nitrate reduction (Wang et. al. 2020). This is detrimental to highly functional species abundances as they tend to be poor competitors

(Jiang et. al. 2009), so diverse communities may be dominated by lower functioning species (Livingston et. al. 2012).

Field samples revealed higher richness compared to serial dilutions (Figure 9, Figure 16), distinct community composition across sites (Figure 17), and higher compositional variability in Gasquet and Shasta (Figure 18). As expected, lab microcosms can be described as a simplified model of the more diverse and variable microbial communities inhabiting wild pitcher plants. However, Plumas represents an interesting deviation from this pattern, given its high community similarity, likely resulting from the harsh mountain habitat of this fen. These differences in community composition and diversity could influence the degradation potential and specific metabolic functions. Although I can only speculate, higher richness should not result in increases in function, based on the overall redundancy patterns from the serial dilution experiment. However, compositional differences across sites could indicate variation in degradation potential and nitrogen cycling. Based on the NMDS (Figure 18), microbial communities recovered in the Plumas site were more similar to lab microcosms than samples from Gasquet or Shasta, suggesting Plumas functional rates could be the most comparable to observed degradation in the microcosms. Further exploration of the most functional microcosms from the experiment could reveal specific functional taxa not represented in the isolates, that may provide stronger predictions as to functional performance of field samples. *Acinetobacter* and *Leucobacter* genera were detected in both field and serial dilution samples, while *Arthrobacter* and *Microbacterium* were detected in only field samples, and *Bacillus and Staphylococcus* were detected in only

serial dilution samples. Not surprisingly, microbial communities within pitcher plants in the wild vary in composition beyond what can be captured with laboratory experiments, and previous work on pitcher plants has had difficulty linking bacterial diversity to geographic location, weather, or prey availability (Yourstone et. al. 2021). Although the functional consequences of this variability are currently unknown, these plant populations seem to be maintaining a moderate growth (USDA 2022), suggesting microbial activity is sufficient for plant fitness.

Because carnivorous plants rely on nitrogen supply from captured prey to supplement low nitrogen levels in the soil (Ellison & Farnsworth, 2005), and nitrogen is commonly a limiting factor in aquatic microbial habitats (Ågren et. al. 2012), we expected insect breakdown to be driven by pathways that maximize nitrogen cycling. However, nitrogen metabolic pathways in this study were not sufficient to explain prey breakdown. Specifically, microbial activity that results in prey mass loss is not correlated with nitrogen release in the form of solubilized protein, ammonia, or nitrate, or enzymatic activity. Perhaps it is important to consider that nitrogen is not the majority of the mass in these prey items, which are comprised of approximately 10% nitrogen by weight, in the form of mostly protein and chitin (Behie and Bidochka 2013). Chitin degradation not only releases other forms of nitrogen, but also because chitin comprises the exoskeleton of the prey, degradation of this compound potentially increases microbial access to internal organs. Regardless, we found that activity of chitinase did not result in faster degradation rates. These results indicate that degradation is a complex process driven by combined metabolic requirements and species interactions within the microbial

community, and unrelated to the nitrogen requirements of the plant. These results also highlight the challenge of identifying specific functions and pathways underlying broad functions like degradation.

Throughout this study, we failed to find a significant link between function and community metrics taken at the end of the experiment, including richness, diversity, or growth rates. This aligns with studies that focus on initial and end of experiment (realized) diversity, which find that initial diversity drives ecosystem function, whereas the relationship of realized diversity depends on the initial diversity supplied and community assembly in between seeding and functional measurements (Hagan et. al. 2021, Rychteka et. al. 2014). However, my study differs from most of these studied because diversity was explicitly measured at the beginning of the experiment instead of relying on how many species were added. During my results and discussion, I thus focused on how community composition at the start of degradation related to fly mass loss and specific nitrogen metabolism processes. Yet this result highlights how the degradation process itself results in community re-assembly, equivalent to nutrient pulses (Miller et. al. 2019) and, perhaps, how performing a function may result in microbial rearrangement that includes community composition and gene expression components.

CONCLUSION

This is the first study evaluating *Darlingtonia* bacterial diversity and function across a complexity gradient, from single species isolates to samples across the range of the plant. For functional attributes, my study focused on bacterial degradation and nitrogen pathways, which were found to be unrelated. Bacterial diversity was related to function only at very low diversity achieved with mixed cultures, and serial dilution experiments support my hypothesis that function in the *Darlingtonia* bacterial community is driven by redundant traits. Based on these findings, I propose that insect degradation relies on unidentified functions that are redundantly distributed among community members, and that community functional performance results from a combination of redundant functional traits and coexistence mechanisms, the latter defining which species maintain membership. Overall, this study highlights the dynamic range of BEF relationships and hopefully motivates further inquiry into integrating BEF into our broader understanding of community assembly processes.

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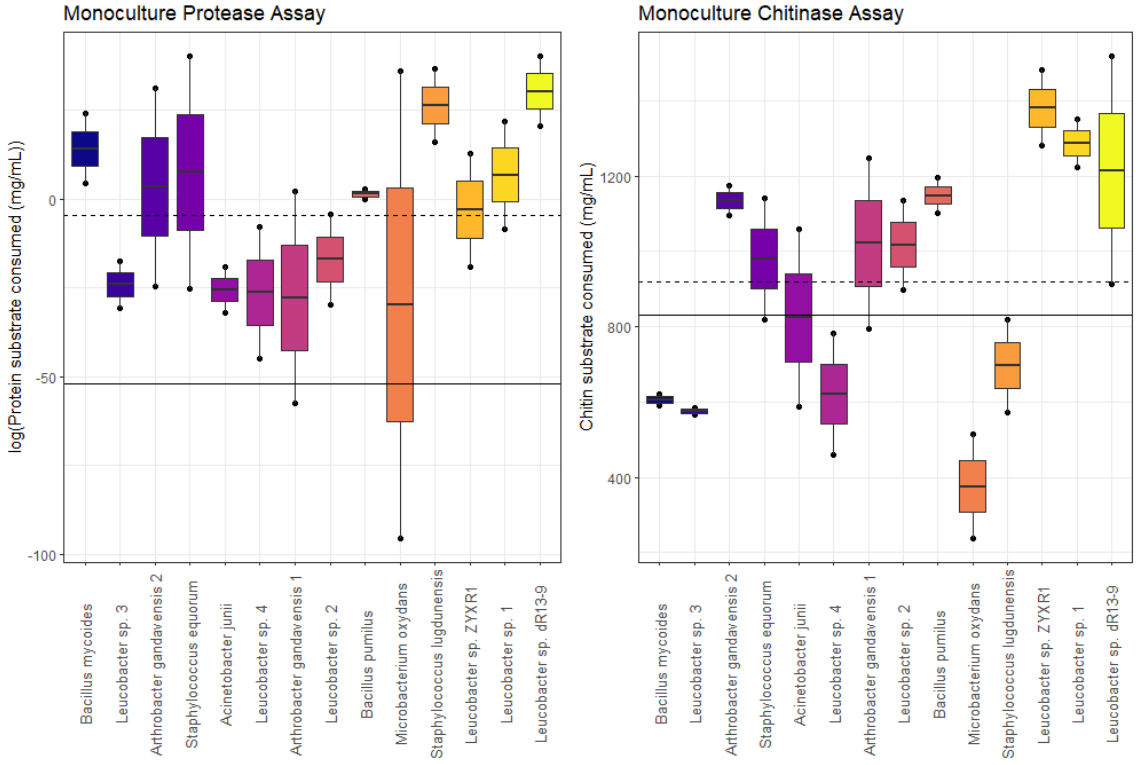
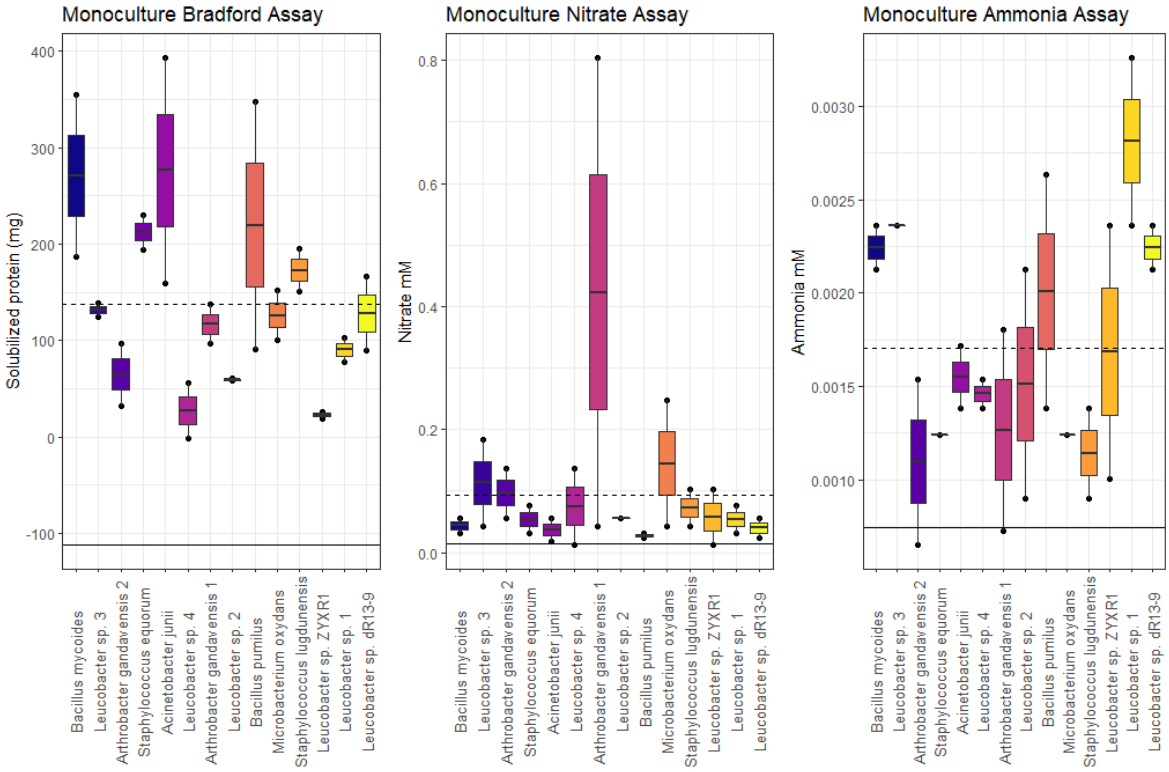
APPENDICES

Appendix A. Enzymatic activity of the monocultures.
Colors are used to visually differentiate each isolate. Solid lines indicate control sample average; dashed lines indicate isolate sample average.



Appendix B. Solubilized nutrients produced in monocultures.
Colors are used to visually differentiate each isolate. Solid lines indicate control sample average; dashed lines indicate isolate sample average.



Appendix C. Monoculture end of degradation growth rates vs. function. Results of linear regression significance test on monoculture end of degradation bacterial growth rates compared with each functional assay. Degrees of freedom for all tests were 26. No significance results produced $p > 0.05$.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	-0.01754	0.5346	0.4712
Bradford	0.06956	3.019	0.09415
Nitrate	0.01064	1.29	0.2663
Ammonia	0.02357	1.652	0.2101
Chitinase	-0.03812	0.008561	0.927
Protease	0.004219	1.114	0.3008

Appendix D. Monoculture abundances vs. function.
Results of linear regression significance test on monoculture total bacterial abundance at start of degradation compared with each functional assay. Degrees of freedom for all tests were 26. No significance results produced $p > 0.05$.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	0.01555	1.426	0.2431
Bradford	0.01209	1.33	0.2592
Nitrate	0.02331	0.385	0.5403
Ammonia	0.02776	1.771	0.1948
Chitinase	-0.02756	0.2758	0.6039
Protease	0.01138	1.311	0.2627

Appendix E. Monoculture end of degradation abundance vs. function.
Results of linear regression significance test on monoculture bacterial abundance at the end of the degradation compared with each functional assay. Degrees of freedom for all tests were 18. No significance results produced $p > 0.05$.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	-0.0192	0.6421	0.4334
Bradford	-0.02723	0.4964	0.4901
Nitrate	-0.0206	0.6162	0.4427
Ammonia	0.1001	3.113	0.09465
Chitinase	-0.0551	0.006384	0.9372
Protease	0.0107	1.206	0.2867

Appendix F. Mixed community end of degradation abundance vs. function. Results of linear regression significance test on mixed community group abundance at the end of the degradation compared with each functional assay. Degrees of freedom for all tests were 18. No significance results produced $p > 0.05$.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	-0.009681	0.8178	0.3778
Bradford	0.02143	1.416	0.2495
Nitrate	-0.02311	0.5709	0.4597
Ammonia	-0.03588	0.3418	0.566
Chitinase	-0.05555	7.00E-05	0.9934
Protease	0.009681	0.8178	0.3778

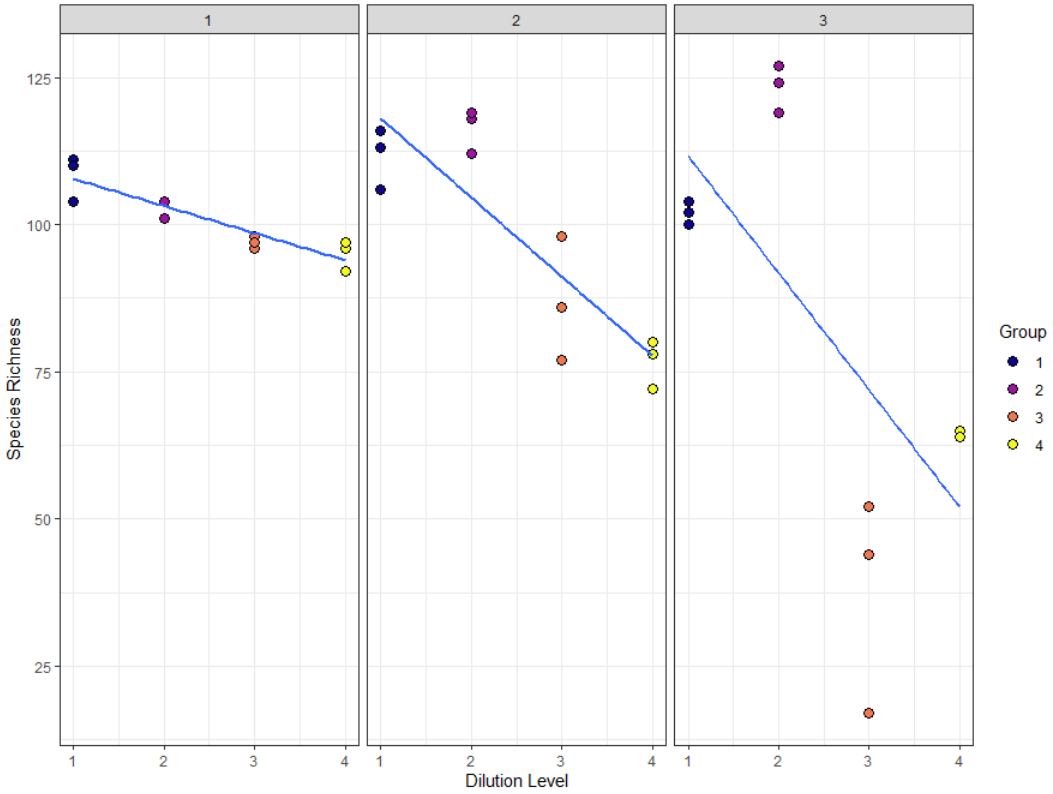
Appendix G. Mixed culture degradation rate vs. function.
Results of linear regression significance test on mixed community group abundance at the end of the degradation compared with each functional assay. Degrees of freedom for all tests were 18. No significance results produced $p > 0.05$.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Bradford	-0.04253	0.2249	0.8756
Nitrate	0.0006859	1.013	0.8756
Ammonia	-0.05381	0.02981	0.8756
Chitinase	-0.05408	0.0252	0.8756
Protease	-0.01312	0.754	0.8756

Appendix H. Serial dilution end of degradation richness vs. function. Results of ANOVA and Wald test on serial dilution species richness at the end of the degradation compared with each functional assay. Degrees of freedom for all tests were 18. No significance results produced $p > 0.05$.

Functional Assay	*R*-squared	*F*-statistic	*p*-value
Degradation Rate	-0.006768	0.8723	0.3627
Bradford	-0.04279	0.2203	0.6444
Nitrate	-0.005494	0.8962	0.3563
Ammonia	-0.05026	0.0908	0.7666
Chitinase	-0.05211	0.05898	0.8109
Protease	-0.03119	0.4254	0.5225

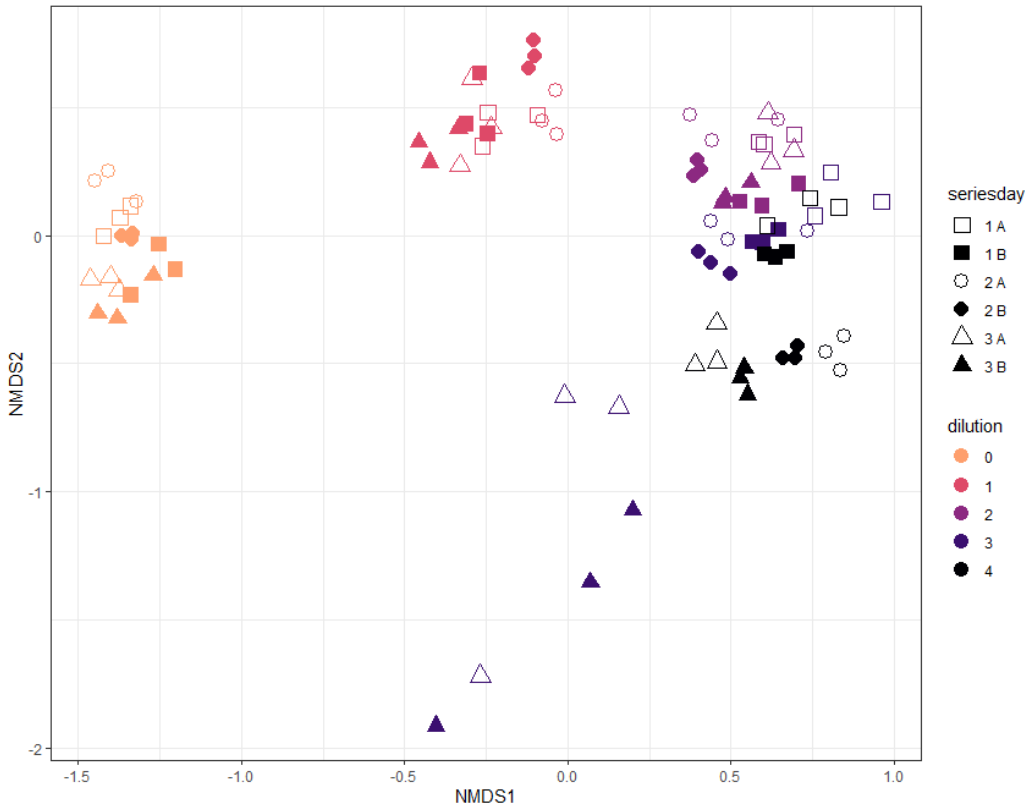
Appendix I. Serial dilution richness gradient by series. Richness gradient across dilution level, separated into the three series. Color is used to further visualize each dilution level.



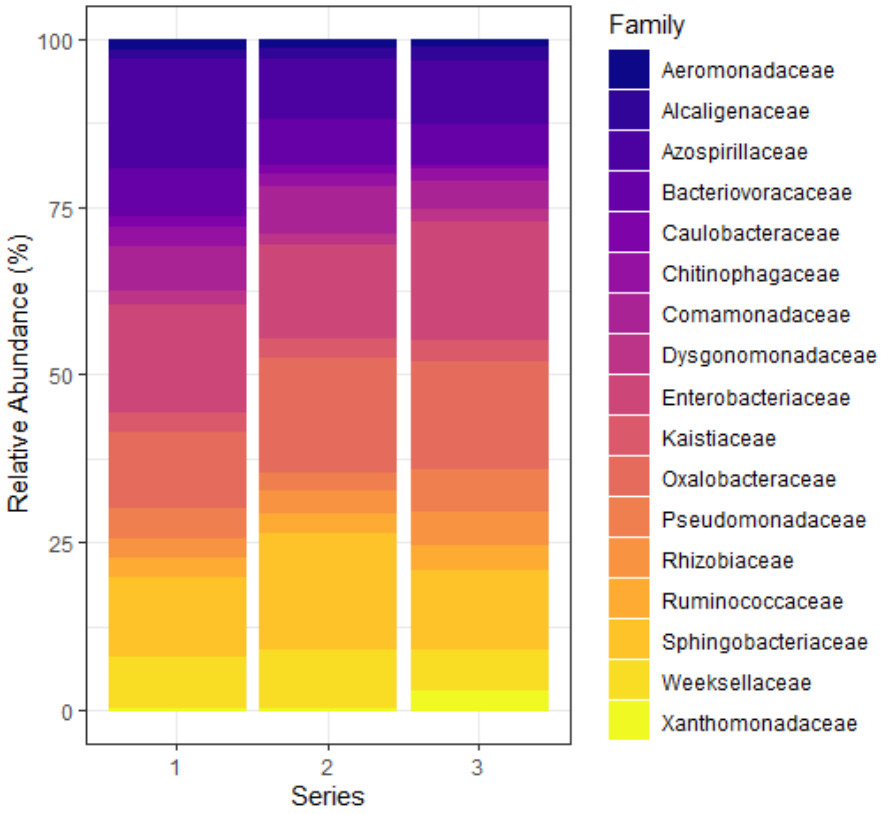
Appendix J. Serial dilution species richness vs. function.
Results of ANOVA and Wald test on serial dilution functions compared with species richness that displayed nonsignificant trends. Degrees of freedom for all tests were 18. No significance results produced $p > 0.05$.

Functional Assay	*Chi sq*.	*p*-value
Fly mass loss	2.18831	0.1391
Bradford	3.7217	0.05371
Ammonia	0.79208	0.3735
Protease	2.6652	0.1026

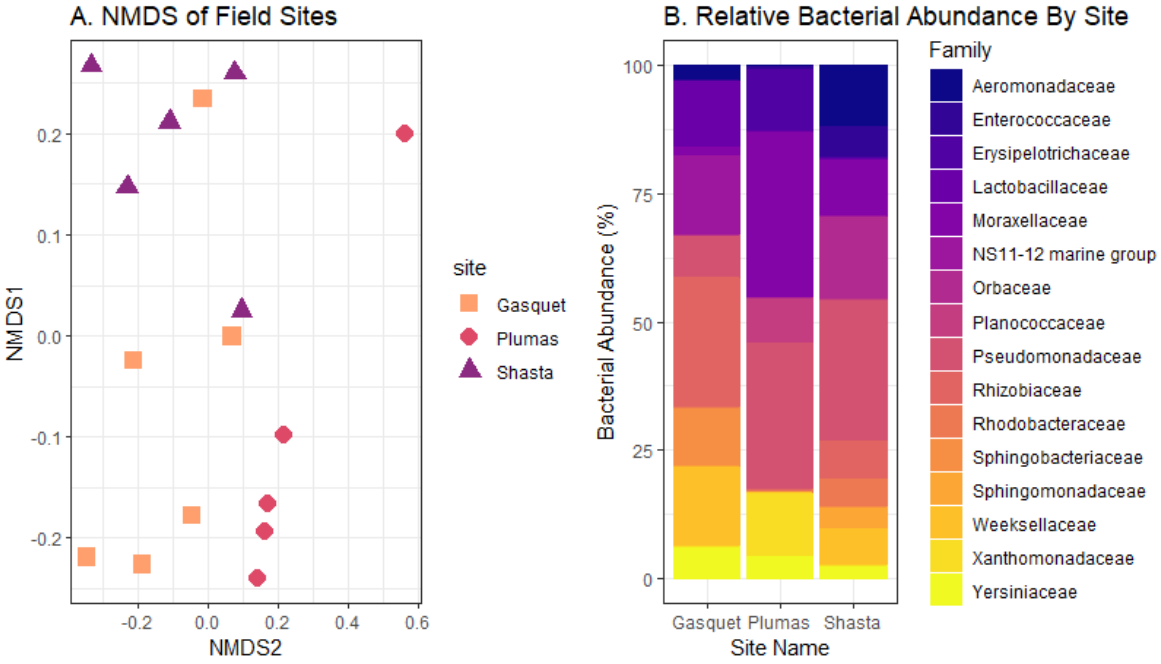
Appendix K. Before and after degradation NMDS plot.
Before samples (1B, 2B, 3B) are indicated by black filled in points, and after samples (1A, 2A, 3A) are indicated by empty points. The way they continue to remain group would indicate that they do not trend toward some specific community composition. Their end of degradation compositions are related to initial composition variation.



Appendix L. Relative abundance of the dilution experiment. Compositional variations were significantly different.



Appendix M. NMDS plot and relative abundance of field sites.
Variance of community composition was similar between the three field sites.



Appendix N. Field site distance to centroid.
Variance of community composition within sites did differ between sites (DF = 1, Sum Sq. = 1.0705, R^2 = 0.69114, F = 31.328, p = 0.001)