COMMUNITY ECOLOGY AND RESTORATION OF DESERT SPRINGS

by

ELIZABETH L. PAULSON

B.A., Mills College, 2006

M.A., University of Colorado, 2012

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This thesis entitled:
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written by Elizabeth L. Paulson
has been approved for the Department of Ecology and Evolutionary Biology

_____

Dr. Andrew P. Martin

_____

Dr. Kendi Davies

_____

Dr. Noah Fierer

_____

Dr. Nolan Kane

_____

Dr. Roseanna Neupauer

Date_____

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Paulson, Elizabeth L. (Ph.D., Ecology and Evolutionary Biology)
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Freshwater ecosystems are among the most important and most imperiled of ecological resources, especially in arid landscapes where aquifer-fed surface waters are biodiversity hotspots that can harbor high levels of endemic and often endangered biota. These desert springs are threatened by water mining, land-use change, biological invasions, and other global change phenomena. My dissertation describes the community ecology of a desert spring system (Ash Meadows, Nevada), including the environmental parameters that drive community composition, the effects of ecological restoration, and spatiotemporal dynamics in stable and restored springs. I used a combined environmental DNA - metagenetic survey method to assess the composition of whole eukaryotic communities from environmental samples of algal mats, the water column, and benthic sediments. Spring size, water temperature, and invasive species (red swamp crayfish or largemouth bass) were all correlated with community composition. This has important implications for conservation management of desert springs, which could decrease in size and increase in temperature with aquifer pumping and climate change. Next, I conducted two chronosequence studies to assess the effects of ecological restoration and habitat creation on community composition and temporal variation. The restoration of a low-flow, high-temperature spring showed remarkable success: after prolonged desiccation and structural modification, the spring community exhibited a successional trajectory towards a historic, natural composition, suggesting environmental filtering during community assembly. The second chronosequence study compared a natural habitat to its constructed analog. The natural habitat, Devils Hole, is a small opening into a deep aquifer, the surface of which comprises the entire range of the endangered Devils Hole pupfish (*Cyprinodon diabolis*). Federal management agencies

constructed an artificial refugium for $4.5 million to harbor a backup population of the pupfish; the 380,000 L refuge tank was designed to exactly replicate the Devils Hole environment. Despite seeding protocols intended to recreate the biotic community found in Devils Hole, and controls over water temperature, insolation, and other abiotic parameters, the refuge tank community differed from the natural habitat in composition and seasonal variability. The community outcomes revealed by these chronosequence studies highlight the importance of monitoring to gauge progress towards ecological goals in managed systems.

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INTRODUCTION

Freshwater ecosystems support extensive biodiversity, and are also a vital human resource. The tight coupling between humans' need for water and that of thousands of other species makes freshwaters ecosystems of high conservation concern (Vörösmarty *et al.* 2010). Freshwaters are currently threatened by development, eutrophication, biological invasions, and water withdrawal (Jackson *et al.* 2001; Dudgeon *et al.* 2006). Climate change also poses a threat to freshwater ecosystems, as changes in temperature and precipitation can have direct consequences for community composition and habitat size, with cascading effects on ecosystem structure and function (Woodward *et al.* 2010b).

Freshwater resources in arid landscapes are of particular conservation concern because they are limited but essential for the maintenance of human population centers, while also supporting isolated communities of organisms with unique evolutionary histories (Shepard 1993; Murphy *et al.* 2013). Desert freshwater ecosystems exist because of geologic fractures that allow water held below ground in aquifers to reach the surface. In the American Southwest, aquifers are mined for municipal, agricultural, and commercial use. Aquifer recharge is dependent on precipitation, which has been diminished during the ongoing drought in this region, and is predicted to continue to decrease with climate change (Seager *et al.* 2007; Cayan *et al.* 2010). Reduced aquifer volume has direct effects on desert spring flow rates, which in turn affects spring habitat size and ecology (Zektser *et al.* 2005). In addition to threats related to aquifer sources, desert

springs have been degraded through biological invasions, channelization, water diversion, grazing of livestock, and commercial and residential development (Deacon & Williams 1991; Shepard 1993). These forces have induced changes to the communities of organisms found in desert springs, led to local extirpations, and also extinction of endemic species (Deacon & Williams 1991).

Desert springs present an opportunity to study the effects of global change phenomena on freshwater ecosystems, including the effects of climate change: many desert springs are geothermally influenced, which makes them ideal for studying the effects of temperature on ecological communities (Woodward *et al.* 2010b; O'Gorman *et al.* 2014). Research on geothermal systems has revealed that increased water temperatures can affect body size of top predators, population sizes, community composition, alpha diversity, and food chain length (Friberg *et al.* 2009; Woodward *et al.* 2010a). Increased water temperature can also cause changes to rates of ecosystem processes such as decomposition (Dossena *et al.* 2012) and nutrient cycling (O'Gorman *et al.* 2012).

Ash Meadows is a geothermally influenced spring system in the Mojave Desert. Located in southwestern Nevada, Ash Meadows has the highest richness of endemic species in the United States (Soltz & Naiman 1978), and is managed as a National Wildlife Refuge. The springs and seeps of Ash Meadows vary in size (~0.1-200 L/s) and temperature (~13-34° C), and were impacted by a variety of anthropogenic activities prior to the area's designation as a Refuge in 1984 (Deacon & Williams 1991). Anthropogenic impacts in Ash Meadows included water diversion, channelization, springhead excavation, livestock grazing, and introduction of non-native species (i.e., crayfish, mosquitofish, bullfrogs, sailfin mollies, largemouth bass, and green sunfish), many of which invaded surface waters across the refuge and remain established today.

Restoration of degraded spring habitats has been ongoing in Ash Meadows since the 1980s, with the purpose of conserving endemic and endangered species. Restoration actions include restoring natural hydrologic regimes, springhead and channel structure, native ecological communities, and also eradication of invasive species.

Adjacent to Ash Meadows is a hydrologic feature known as Devils Hole, which has been the focus of conservation action since the 1970s. Devils Hole is an opening into the aquifer, located at the bottom of a limestone collapse on a hillside above Ash Meadows. This small pool of water comprises the entire natural range of the endangered Devils Hole pupfish (*Cyprinodon diabolis*). Aquifer pumping in the 1960s-1970s caused a decline in water level in Devils Hole, partially exposing the shallow shelf where the pupfish breed and feed (James 1969; Dudley & Larsen 1976). This was one of several times in the 1900s when the pupfish population declined severely, spurring efforts by management agencies to establish an offsite refuge population to buffer the species against extinction. Multiple refuge attempts failed due to environmental differences or mechanical problems (Karam 2005). A new refuge was constructed in 2012, with a multi-million dollar federal grant. This refuge seeks to precisely mimic the abiotic and biotic environment of Devils Hole, and as of April 2016 it maintained a population of roughly 50 Devils Hole pupfish (pers. comm., Corey Lee, 4 April 2016). The natural Devils Hole pupfish population was approximately 130 individuals in September 2015, according to surveys by the Devils Hole Pupfish Incident Command Team (Fall 2015 counts).

With current and future threats to desert springs and the ecological communities they support, it is imperative to gain a thorough understanding of these ecosystems. This includes biodiversity information, as well as environmental drivers of species assemblages. In addition, understanding how these systems respond to restoration and habitat creation practices will

inform future management actions aimed at conserving desert springs and the species they support. Previous research on desert spring community ecology mostly focused on organisms that can be surveyed using traditional, visual methods, such as plants, animals, macro-algae, macroinvertebrates, and some protists. Recent advances in molecular technology and bioinformatics facilitate biodiversity surveys that are largely indiscriminate to body size or taxonomic identity: DNA extracted from environmental samples can provide a rich census across the tree of life.

My dissertation research utilized an environmental DNA-metagenetic survey method to study community ecology in Ash Meadows, Devils Hole, and the constructed Devils Hole refuge environment. In Chapter 1, I describe the correlation between community composition and environmental variables such as temperature, spring size, and invasion status, for 23 springs in Ash Meadows. This chapter also addresses patterns of alpha diversity in Ash Meadows. Chapter 2 follows the fate of a spring restoration project, delimiting community change over time in the restored spring compared to adjacent, reference springs. Chapter 3 is a quantitative comparison of the ecological communities in Devils Hole and the artificial refuge environment, and how they vary across seasons. Research describing the environmental variables correlated with desert spring community composition, as well the ecological outcome of management practices such as restoration and habitat creation, provides important information for evidence-based management interventions and conservation actions in a future with undiminished or increasing global change phenomena.

CHAPTER 1

COMMUNITY ECOLOGY OF A DESERT SPRING SYSTEM[1]

Abstract

Global change phenomena are threatening freshwater ecosystems across the globe. In order to predict the ecological response of freshwater systems to environmental change, it is important to understand the environmental drivers of community composition. The existing literature describes important environmental variables for subsets of taxa, specific habitats, and meso- or microcosm experiments; we surveyed all eukaryotic taxa in a naturally replicated desert spring system using a combined environmental DNA (eDNA) - metagenetic approach. Sequences of 18S rDNA were used to estimate whole eukaryotic community composition in 23 springs that vary for multiple abiotic and biotic variables, including temperature, spring size, and invasion status (presence or absence of red swamp crayfish or largemouth bass). Matrix correlation tests were used to determine the best subset of environmental predictors of community composition, and partial mantel tests were used to assess the relative contribution of environmental and geographic distance. Temperature had a strong effect on community composition. Spring size, invasion status, and landscape position also correlated with community composition. Geographic distance (maximum of 12.5 km between springs) had no effect on community composition. Temperature, spring size, and invasion status were also significant predictors of alpha diversity (species richness and phylogenetic diversity). In addition, temporal variation was quantified over

[1] Co-author: Andrew P. Martin

one-year and half-year time gaps, and was found to be considerable across both time gaps for low abundance taxa (4% shared OTUs), and lower for high abundance taxa (72% shared OTUs). The results of this research have important implications for freshwater communities under pressures from global change phenomena, especially in regards to the significant effects of water temperature, spring size, and invasive species on community composition. In addition, our research lends support to the Baas Becking hypothesis: the communities of organisms in the springs of Ash Meadows comprise dynamic assemblages of taxa, where most detected organisms are ephemeral and few are stable community members. This indicates substantial immigration or passive dispersal into the springs combined with low rates of long-term persistence, possibly due to environmental selection.

Introduction

Freshwater ecosystems are among the most important - and most imperiled - ecological resources on Earth (Jackson *et al.* 2001; Dudgeon *et al.* 2006; Vörösmarty *et al.* 2010). Of particular importance are groundwater systems. There are tens of thousands of springs and seeps of varying size scattered across western North America, and each is connected in some manner to an aquifer (Meinzer 1923; Stevens & Meretsky 2008). Many of these isolated surface waters harbor high levels of biodiversity and endemic taxa (Soltz & Naiman 1978; Shepard 1993). There are approximately 30,000 springs and seeps in the state of Nevada, 700 of which provide habitat for 165 of the state's 173 endemic species (Abele 2011). Despite recognition of the intrinsic value of groundwater systems, there are a number of threats to these systems, most notably water extraction, altered flow and temperature regimes due to climate change, destructive land use practices, and the spread of invasive species (Deacon & Williams 1991; Shepard 1993; Deacon *et al.* 2007). Of these threats, groundwater exploitation for agriculture and

municipal uses is the most pressing concern, in part because of a clear link between water extraction and loss of vegetation communities, land subsidence, and declines in surface water and stream flows (Zektser *et al.* 2005; Elmore *et al.* 2006).

Despite looming threats, our knowledge of freshwater spring community diversity and structure remains inadequate. Most surveys of freshwater community diversity focus on macrophytes (McCreary 1991; Lougheed *et al.* 2001; Hansel-Welch *et al.* 2003; Meerhoff *et al.* 2007); animals such as mollusks, insects, and vertebrates (Schlosser 1982; Agostinho & Zalewski 1995; Delong & Brusven 1998; Haag & Warren 1998; Milner *et al.* 2008; Vaughn *et al.* 2008); or some combination of these taxa (Welborn *et al.* 1996; Friberg *et al.* 2009; Ruhí *et al.* 2014). Yet, these groups include only a small fraction of the organisms that comprise freshwater ecosystems. Moreover, ecosystem function depends on a large suite of interacting species that includes an immense diversity of microbial and protistan producers, consumers, and decomposers (Finlay *et al.* 1997; Hahn 2006). Ideally, studies of freshwater systems utilize methods that provide exhaustive assessment of alpha and beta diversity in a way that enables estimation of the effects of physical and biotic factors on community composition and structure. With estimates of the effects of hypothesized drivers of community composition, we can begin to predict the fate of biodiversity and ecosystem function in the face of climate change, isolation and fragmentation of habitat, and the cascading effects of invasive species.

In this study, we utilized DNA technology that permits exhaustive surveys of biodiversity across all eukaryotes for describing the alpha and beta diversity of a suite of hydrogeological surface waters in an arid environment of the southwestern United States. Our study system was a set of springs and seeps in the Mojave Desert, within Ash Meadows National Wildlife Refuge, Nevada. The springs are fed by a regional deep carbonate aquifer. The deep aquifer is

geothermally influenced, and temperature varies among springs depending on subsurface mixing with the water table as well as subterranean distance traveled before reaching the surface. Springs located nearest to the aquifer source are ~34° C throughout the year, whereas more distant springs have lower temperatures (~15° C). Additionally, Ash Meadows springs vary in size due to differences in discharge rates, which range from 0.1 L/s to over 200 L/s, and the spring system as a whole emits 40,500 liters of water per minute (Walker & Eakin 1963). Water chemistry is similar across springs, with few exceptions (Dudley & Larsen 1976).

In addition to temperature, spring size, spring flow, and elevation, the springs vary for a number of biological and physical features that may impact diversity, including the presence or absence of invasive species such as crayfish (*Procambarus clarkii*) and largemouth bass (*Micropterus salmoides*).

Our study of the eukaryote ecological communities was motivated by two major questions. 1) What is the biological diversity within and among springs? And, 2) what physical and biological factors best explain variation in alpha and beta diversity among springs? Research addressing these questions will provide a baseline for evaluating how projected environmental changes, including mining of water from the aquifer, will influence biological diversity, and may also inform future restoration efforts.

Methods

Site description and sampling

Research was conducted at Ash Meadows National Wildlife Refuge (AMNWR), Nevada (Figure 1.1, Table 1.1), hereafter "Ash Meadows." Ash Meadows is located in the Mojave Desert, and comprises approximately thirty springs and seeps, and associated wetland and riparian habitats. Surface water is derived from a deep carbonate aquifer, which receives drainage from approximately 12,000 km^2 to the northeast of Ash Meadows (Winograd & Thordarson 1975).

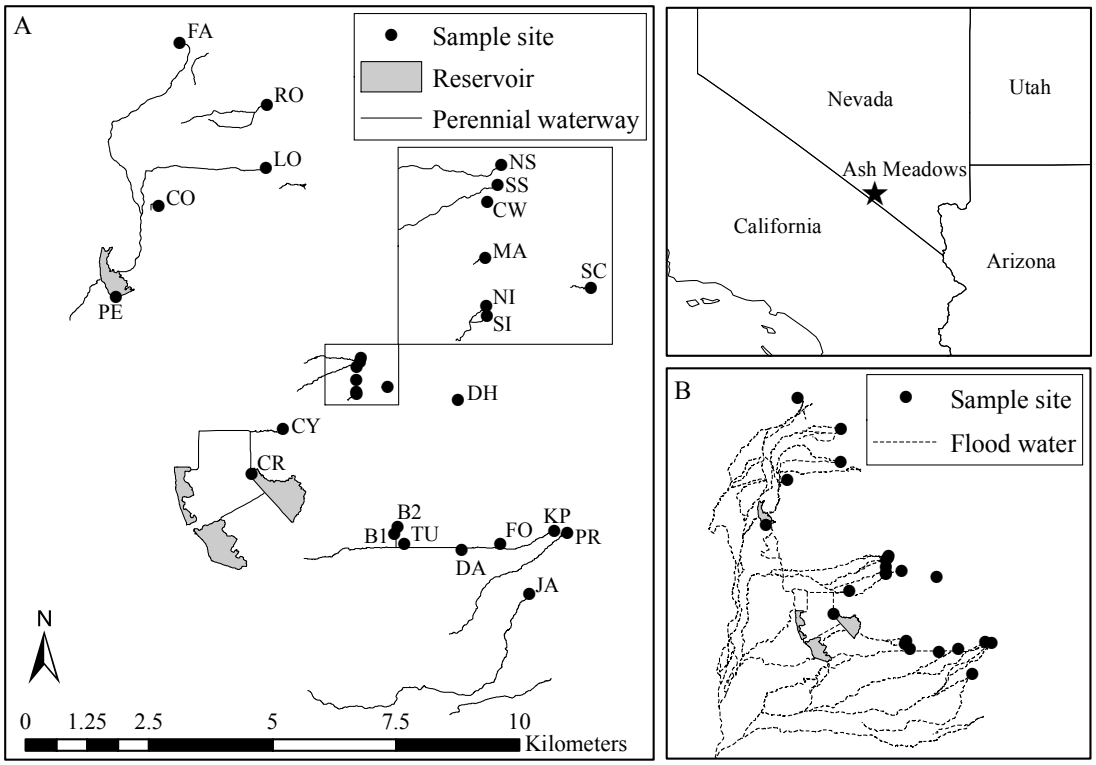

Figure 1.1. Map of the study area in Ash Meadows National Wildlife Refuge, Nevada (A). Abiotic and biotic characteristics of each spring are reported in Table 1.1. Rare flooding events can lead to increased connectivity between otherwise isolated waterways (B).

The deep aquifer is intersected by a fault at the northeast edge of the Ash Meadows system, marked by a line of Paleozoic rocky outcrops (Dudley & Larsen 1976). The fault system provides pathways for water to flow upwards to the land surface. The deep aquifer is geothermally influenced, thus discharge points in Ash Meadows that are located nearest to the fault maintain a constant temperature of ~34° C. Discharge points located further away from the fault emit water with lower temperatures, due to both cooling over subterranean distance traveled and subsurface mixing with local groundwater. The entire system discharges approximately 40,500 L/m, or just under 21,000,000 m^3 per year (Walker & Eakin 1963). Individual springs vary in discharge from 0.1 L/s to nearly 200 L/s (Dudley & Larsen 1976). Episodic flooding

Table 1.1. Characteristics of springs sampled in Ash Meadows National Wildlife Refuge, Nevada. Note: South Scruggs was restored after sampling in 2012, and currently does not have invasive crayfish. Longstreet also underwent restoration after 2013 sampling, though the restoration process focused on habitat structure and not eradication of invasive animals.

Spring	Abr.	Lat.	Long.	Invasive species	Temp (C)	Elevation (m)	Distance to aquifer (m)	Surface area (m^2)	Log (surface area (m^2))
Bradford 1	B1	36.401166	-116.3028057	Crayfish	18	683.56	2635.71	68.19	1.83
Bradford 2	B2	36.402167	-116.3024522	Crayfish	20	683.22	2520.63	69.68	1.84
Cold	CO	36.460790	-116.3459333	Crayfish	18	681.79	2586.81	1.30	0.14
Cottonwood	CW	36.431621	-116.3097668	None	32	705.36	708.22	0.09	-1.05
Crystal	CY	36.420127	-116.3233199	Crayfish	32	670.61	2208.88	136.75	2.14
Crystal Reservoir	CR	36.412053	-116.3284355	Bass	16	664.98	3015.98	627271.95	5.80
Davis	DA	36.397998	-116.2895900	Crayfish	23	692.65	1685.67	456.62	2.66
Devils Hole	DH	36.425342	-116.2914326	None	33.5	742.47	107.41	13.94	1.14
Fairbanks	FA	36.490436	-116.3421221	Crayfish	28	692.10	3472.98	210.05	2.32
Forest	FO	36.399170	-116.2836293	Crayfish	26	695.78	1140.97	677.45	2.83
Jackrabbit	JA	36.390043	-116.2784375	Crayfish	28	694.33	1526.52	60.94	1.78
Kings Pool	KP	36.401535	-116.2738525	Crayfish	31.5	704.30	237.6	66.52	1.82
Longstreet	LO	36.467514	-116.3264313	Crayfish	27	703.31	912.41	250.56	2.40
Marsh	MA	36.429059	-116.3100024	None	31	700.63	870.21	0.58	-0.24
North Indian	NI	36.426914	-116.3098409	None	31	696.62	853.84	0.21	-0.68
North Scruggs	NS	36.433120	-116.3091260	None	34	708.00	602.21	0.21	-0.68
Peterson Reservoir	PE	36.444840	-116.3539505	Crayfish	15	661.58	3709.78	120969.65	5.08
Point of Rocks	PR	36.401248	-116.2715388	Crayfish	33	707.17	142.13	32.89	1.52
Rogers	RO	36.479191	-116.3262426	Crayfish	29	694.63	1588.81	40.88	1.61
School	SC	36.427719	-116.3042623	None	32	713.45	349.27	0.37	-0.43
South Indian	SI	36.426480	-116.3099131	None	31	695.77	865.48	0.09	-1.05
South Scruggs	SS	36.432279	-116.3091465	Crayfish	33	706.66	629.71	0.37	-0.43
Tubbs	TU	36.399084	-116.3011726	Crayfish	21	685.94	2658.16	33.17	1.52

occurs across the system, temporarily connecting isolated springs (Figure 1.1B). Flooding provides a passive dispersal mechanism for aquatic organisms to move from higher to lower positions in the watershed, and also creates temporary connectivity for active dispersal between springs. Water chemistry is highly similar across sample sites with the exception of Jackrabbit Spring, which has higher nitrate levels (Dudley & Larsen 1976).

The springs of Ash Meadows vary in level of past disturbance. Prior to the area's designation as a National Wildlife Refuge in 1984, the springs were used for a variety of purposes: agriculture, cattle ranching, peat farming, crayfish aquaculture, and recreational fishing of introduced sport fish (Deacon & Williams 1991). Pumping, digging out of springheads, and channelization of outflows occurred at many springs. Introduced invasive animals such as red swamp crayfish, American bullfrogs, largemouth bass, mosquitofish, sailfin mollies, screw snails, and plants such as tamarisk and cattails became established during the 1900s.

We sampled 23 sites throughout Ash Meadows (Figure 1.1A) that varied in water temperature, size, elevation, distance from the carbonate aquifer source, and presence or absence of an invasive omnivore: red swamp crayfish or largemouth bass (Table 1.1; sampling dates Table A1). Temperature data were obtained from measurements collected by AMNWR personnel (unpublished data). Elevation data for sample sites was obtained from a 10 m digital elevation model (DEM). Spring size was estimated by surface area, measured from aerial imagery. All spatial, geographic, and elevational data were collected in ArcGIS 3.1 (ESRI, Redlands, CA). Samples were collected from algal mats, benthic sediments, and the water column at each site in an effort to estimate community composition for the whole ecosystem in each spring. A large-bore pipette was used to collect approximately 300 mL of material from

multiple points (3-5) within each habitat type. A 10 μm net was used to sample the water column in larger springs.

Laboratory processing

DNA extractions were performed using MO BIO PowerWater DNA isolation kits (MO BIO Laboratories, Carlsbad, CA), per the manufacturer's protocol with two modifications. First, water samples were centrifuged at 4000 g for 8 minutes, and the pellet was used for extraction. Second, samples were heated at 65° C for 10 minutes after addition of the PW1 reagent. Samples collected from the larger springs in 2013 were extracted in triplicate to ensure that maximum taxonomic scope was encompassed per habitat type, per site, in order to thoroughly explore the diversity present in comparative analyses. Samples from the smaller springs were extracted singly, but the same total number of extractions was produced: one from each habitat type at the springhead in addition to two points downstream. The low-flow springs of Ash Meadows lack the deep semi-spherical springheads found in the larger springs, and were thus treated as linear features. This yielded a total of 9 extractions per spring, for alpha and beta diversity analyses.

A short, variable region of the 18S rDNA gene region was amplified and sequenced to broadly target all eukaryotic taxa. DNA amplification followed the Earth Microbiome Project protocol for 18S Illumina library preparation (http://www.earthmicrobiome.org/emp-standard-protocols/18s/), with the exception of using 2 μL template DNA per reaction volume. The forward Illumina Euk 1391f and reverse Illumina EukBr primer set was used to amplify and barcode ~200 base pairs of the hypervariable V9 region of the 18S rDNA locus (Amaral-Zettler *et al.* 2009). PCR was performed in triplicate, and triplicate reactions were pooled per sample. Pooled amplicons were quantified to normalize pooling per plate, and pooled plate amplicons were quantified to normalize further pooling into a single library. The library was sequenced on

an Illumina MiSeq platform with a V2 300 cycle kit (Illumina, San Diego, CA). Library preparation and sequencing was performed in two rounds, on samples collected in 2012, and those collected in 2013 and 2014.

Data processing

Sequence data were processed using the pipeline described at github.com/leffj/data-tutorials/blob/master/amplicon_data_processing_tutorial/amplicon_data_processing-16S.md, modified for 18S data. This pipeline employs a combination of USEARCH 8 (Edgar 2010) and QIIME 1.9 (Caporaso *et al.* 2010) scripts. First, adaptors were trimmed, sequences were demultiplexed, and paired ends were merged. Quality filtering was conducted with a maximum e rate of 0.005, sequences were dereplicated, and singletons were removed. Next, a *de novo* database was assembled at 97% clustering similarity, using the USEARCH algorithm. The database was then filtered using the SILVA 119 reference set (Quast *et al.* 2013), so that only sequences with at least 75% similarity to those found in the reference set were retained. Last, the raw demultiplexed sequences were mapped to the filtered *de novo* database, with a 97% similarity cutoff, to assemble the final database. Taxonomy assignment was performed with the RDP classifier (Wang *et al.* 2007). An OTU table was constructed from this database for downstream analyses. A phylogeny of representative sequences was inferred using QIIME scripts: a representative set of sequences were picked and aligned, the alignment was filtered using an entropy threshold (e) of 0.10 and a gap filter threshold (g) of 0.80, and the filtered alignment was used to estimate a phylogeny using the FastTree algorithm (Price *et al.* 2009).

Statistical analyses

Alpha and beta diversity were characterized using a subset of the data that included all samples collected in November 2013, South Scruggs samples from November 2012, and Devils

Hole samples from December 2014. South Scruggs was undergoing restoration at the time of 2013 sampling and was completely desiccated, thus the 2012 samples were used for these analyses. Site access to Devils Hole was not granted until 2014. Samples from Davis spring were excluded from alpha diversity analyses, due to problematic site access that resulted in low sampling effort.

All analyses were performed in R (R Core Team 2015). The OTU table was rarefied to 2,257 sequences per sample for beta diversity analyses. Bray Curtis community dissimilarities were calculated on square-root transformed abundances, and visualized in non-metric multidimensional scaling (NMDS) plots. Community similarity across sites was assessed using K-means clustering of NMDS coordinates to estimate the most likely number of clusters for whole communities (combined data for algal mat, benthic sediment, and water column habitats), as well as those associated with individual habitat types. Drivers of community assembly were analyzed using the BIOENV function in vegan (Clarke & Ainsworth 1993; Oksanen *et al.* 2011) to determine the subset of environmental variables that best correlated with community distances (environmental variables are listed in Table 1.1). Partial mantel tests were used to assess the independent contributions of environmental distance and geographic distance to community distance. Overall environmental distance for each community type was obtained through BIOENV analysis results. Although some taxa in this study may actively move between springs, most taxa were microbial and likely disperse passively, by fluvial or aerial vectors. Accordingly, we used Euclidean distances between sample sites as the geographic distance metric, rather than network distances which are sometimes employed for active aquatic dispersers within dendritic stream or river systems (e.g., Grenouillet *et al.* 2008).

The relationship between community composition and water temperature has important implications in the Desert Southwest, where climate change forecasts include increased temperatures and decreased precipitation (Seager *et al.* 2007). Accordingly, the taxonomic basis for differences in community composition between spring temperature categories was assessed for low (15-23° C, n=7), mid (26-29° C, n=5), and high (31-34° C, n=11) temperature springs.

Turnover of OTUs was estimated using data from November 2012, November 2013, and May 2014 sampling time points. OTU tables were split at three commonly used cutoffs: high abundance OTUs were those occurring at greater than 1% across all sequences, mid abundance OTUs fell between 0.01-1%, and low abundance OTUs occurred at less than 0.01% (Pedrós-Alió 2006; Galand *et al.* 2009; Mangot *et al.* 2013). Annual and bi-annual turnover was estimated for each abundance category, and was calculated as the proportion of shared taxa across time points. Annual turnover was estimated from November 2012 to November 2013, and bi-annual turnover was estimated from November 2013 to May 2014. Analysis of variance (ANOVA) was used to compare turnover between abundance categories, and Tukey's HSD was used to determine which pairs were significantly different.

For alpha diversity analyses, sequence data were rarified to 2,257 sequences per sample, samples were pooled per site, and then an additional rarefaction was performed to 11,285 sequences per site. Alpha diversity was estimated using observed OTUs as a species richness estimate, and Faith's PD (Faith 1992) as a measure of phylogenetic diversity. GLM multiple regression and stepwise AIC analysis were used to assess the effect of water temperature, spring size, and invasive species on alpha diversity for whole spring communities. The relationship between water temperature and alpha diversity was further investigated using the following function to test for a unimodal Gaussian distribution:

$$f = ae^{(-0.5(x-x0)^2/b^2)}$$

Results

A total of 6,525,028 sequences from samples across 23 sites and all time points yielded 10,939 OTUs. A small number of OTUs had high relative abundances (>1%, 15 OTUs), while the vast majority of OTUs occurred at lower abundances throughout the system (0.01–1%, 685 OTUs; < 0.01%, 10,239 OTUs).

In this study, environmental variables were significantly correlated (Table A2). For example, warmer springs in Ash Meadows are located at higher elevations, closer to the aquifer source, and are generally smaller. In addition, invaded springs were generally larger, located at lower elevations, had lower temperatures, and were further from the aquifer source (Table A3). Despite these correlations, the results of our analyses indicate the importance of considering all variables in community structuring and diversity: for example, matrix correlations between environment and community structure were improved by the inclusion of water temperature, elevation, spring size, and invasion status.

Drivers of community composition

Whole community composition in the springs of Ash Meadows was best predicted by temperature, spring size, latitude, invasion status, and elevation (Table 1.2 and Figure 1.2). Algal mat and water column communities had the same set of environmental predictors. Benthic sediment communities also had the same predictors, except for the inclusion of longitude rather than latitude. All community types showed no effect of geographic distance when environmental distance was controlled; thus all community types were predominantly structured by environmental variables. K-means analysis of sample clustering in NMDS space revealed four clusters for whole communities, three clusters for algal mat and benthic sediment communities,

Table 1.2. Environmental variables that best predict structuring of whole communities as well as those associated with individual habitat types (algal mat, benthic sediment, and water column). The correlation (Pearson's *r*) between the overall environmental distance matrix and the Bray Curtis community dissimilarity distance matrix was determined with the BIOENV function in vegan (Oksanen *et al.* 2011). Partial mantel tests (999 permutations) were used to assess the isolated effects of environmental distance and geographic distance on community distances.

Community	**BIOENV variables**	**Per variable contribution to overall correlation**	**Environmental distance**	**Geographic distance**
Whole	Temperature Spring size Latitude Invasive species Elevation r = 0.48	0.42 + 0.04 + 0.01 + 0.006 + 0.005 = 0.48	r = 0.46, p = 0.001	r = -0.06, p = 1
Algal mat	Temperature Spring size Elevation Latitude Invasive species r = 0.45	0.38 + 0.03 + 0.02 + 0.01 + 0.007 = 0.45	r = 0.43, p = 0.001	r = -0.05, p = 0.94
Benthic sediment	Temperature Invasive species Elevation Longitude Spring size r = 0.53	0.45 + 0.04 + 0.04 + 0.004 + 0.003 = 0.53	r = 0.50, p = 0.001	r = 0.009, p = 0.40
Water column	Temperature Spring size Elevation Invasive species Latitude r = 0.55	0.48 + 0.06 + 0.01 + 0.001 + 0.001 = 0.55	r = 0.53, p = 0.001	r = -0.07, p = 0.98

and two clusters for water column communities (Figure 1.3). High abundance OTUs at RDP classification level 6 showed differences between low, mid, and high temperature springs (Figure 1.4). Warmer springs had higher relative proportions of golden algae (Chrysophyceae) and green algae (Streptophyta), and lower relative proportions arthropods, diatoms, and conthreep ciliates.

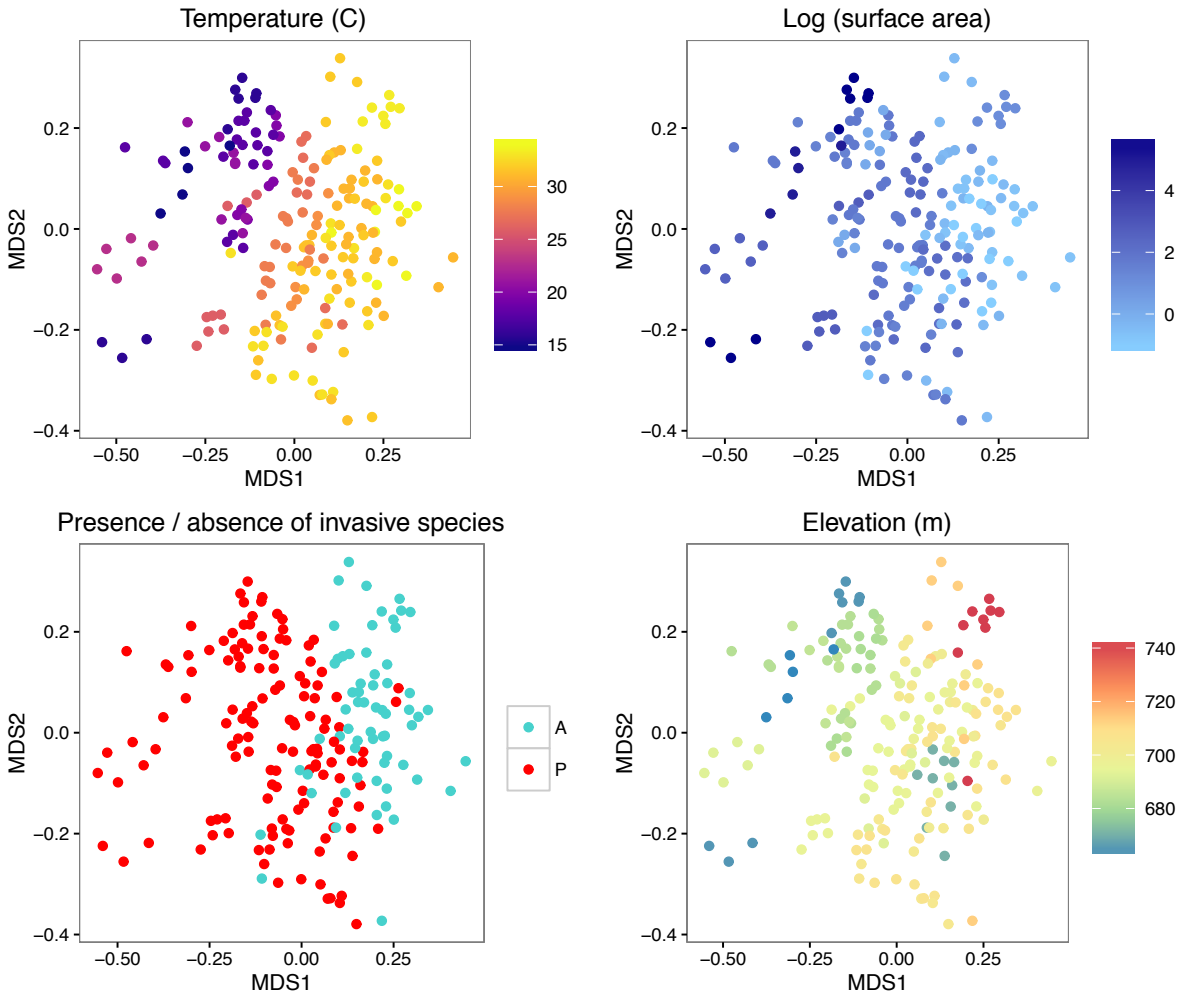

Figure 1.2. Multidimensional scaling plots of Bray Curtis distances for whole community data across all springs. Matrix correlations between community distances and environmental distances showed that whole communities were significantly structured by multiple abiotic and biotic variables, including (clockwise from top left) water temperature, spring size, elevation, and the presence or absence of invasive species. Relative contributions of individual environmental variables to overall environmental correlation are listed in Table 1.2.

Temporal variation

Community shifts over time varied between abundance categories (one-year gap: $p < 0.001$, $F = 337$, $p < 0.001$ for all pairs; half-year gap: $p < 0.001$, $F = 156$, $p < 0.001$ for all pairs), but no difference in shared OTUs was found between one-year and half-year sampling time points within abundance categories ($p > 0.05$ for all pairs). High abundance OTUs had the highest

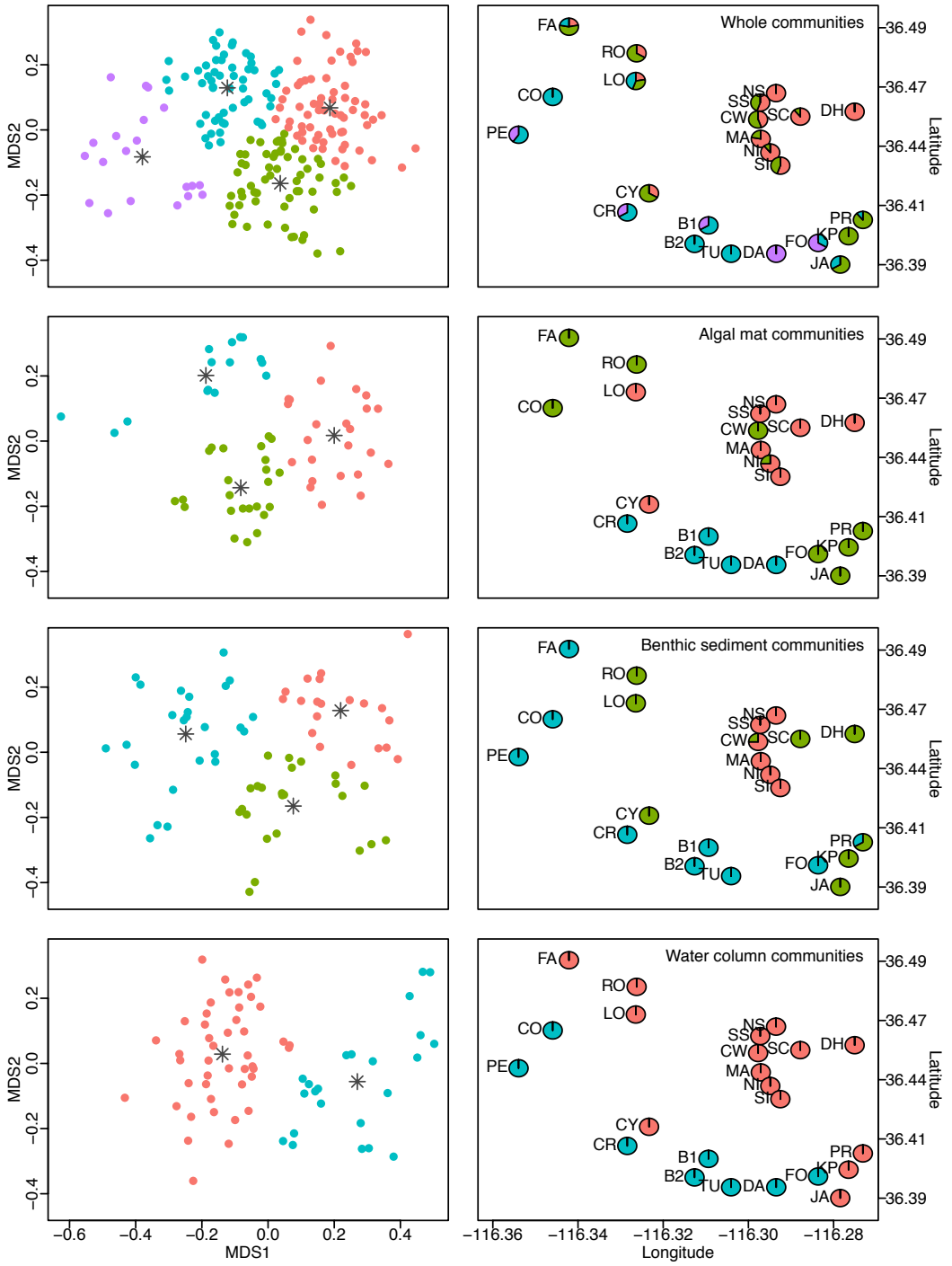

Figure 1.3. Multidimensional scaling plots of whole, algal mat, benthic sediment, and water column community data (left column), colored by K-means clustering assignment. Stars indicate centroids of K-means clusters. Corresponding maps adjacent to each MDS plot (right column) show the spatial configuration of clusters, with pie charts at each spring colored by the proportion of samples assigned to each cluster. Positions of springs were adjusted to fit into the mapping space, but relative geographic relationships are accurate.

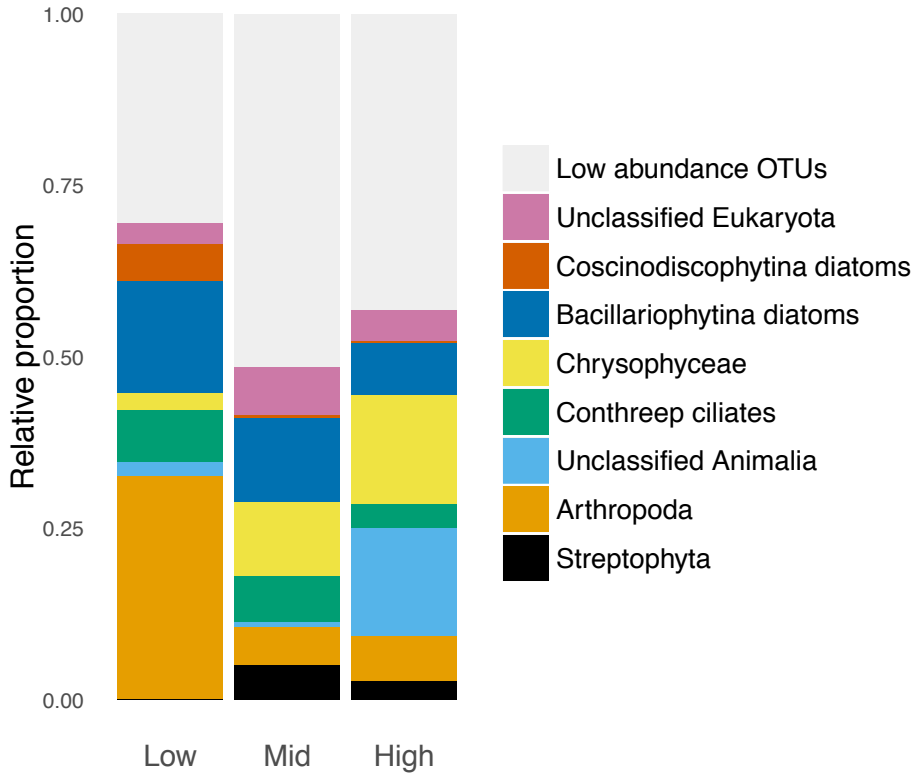

Figure 1.4. Relative proportions of high abundance OTUs at RDP classification level 6, for low (15-23° C, n=7), mid (26-29° C, n=5), and high (31-34° C, n=11) temperature springs. Golden algae (Chrysophyceae) and streptophytes had higher relative proportions in warmer springs. Arthropods, conthreep ciliates, and diatoms had high relative proportions in cooler springs.

proportion of shared OTUs between sampling time points (mean = 0.72), followed by mid-abundance OTUs (mean = 0.32), and low abundance OTUs (mean = 0.04) (Figure 1.5).

Alpha diversity

The best multivariate model for species richness included invasive species, temperature, spring size, and two of the three possible pairwise interaction variables: invasive species and spring size, and temperature and spring size (Table 1.3). Phylogenetic diversity was best predicted by the same variables, but also included the third interaction pair: invasive species and temperature (Table 1.4). Invasive species did not have a significant *p* value in either model, but improved the model fit for both richness and phylogenetic diversity. Species richness modeled

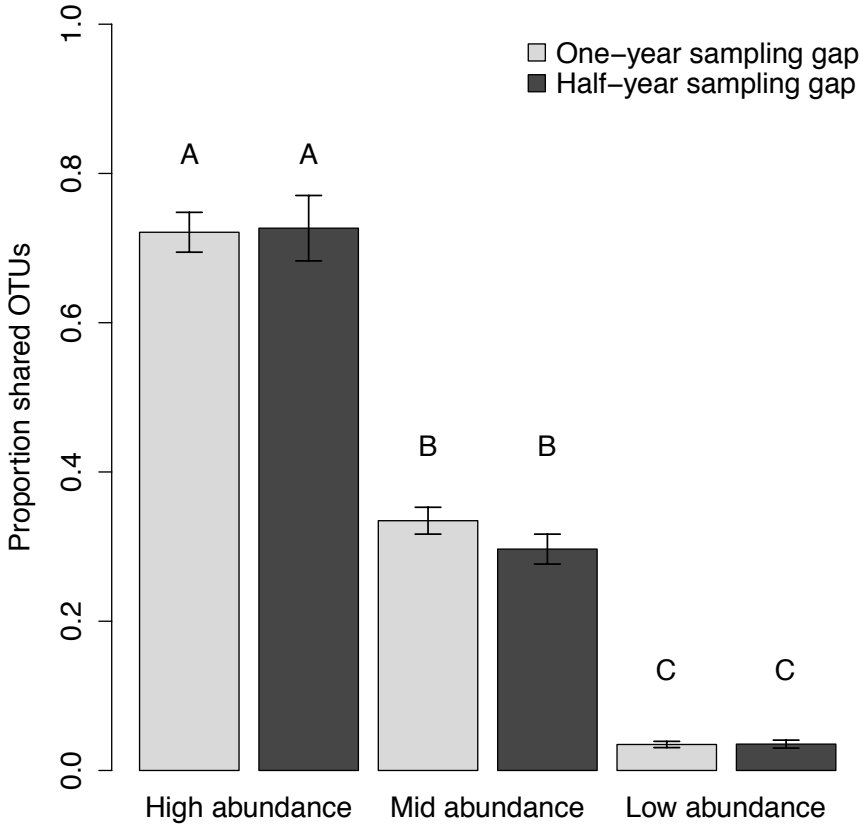

Figure 1.5. Proportion of shared OTUs for high (> 1%), mid (0.01-1%), and low (< 0.01%) abundance OTU categories, across one-year and half-year sampling gaps. Turnover was highest for low abundance OTUs, with an average of 4% shared OTUs detected between timepoints. Mid abundance OTUs had an average of 32% shared OTUs between timepoints, and high abundance OTUs showed the highest temporal stability, with an average of 72% OTUs shared between timepoints. All pairs of abundance categories are significantly different ($p < 0.001$, one-year $F = 337$, half-year $F = 156$), but there is no difference between sampling gaps within abundance categories ($p > 0.05$).

Table 1.3. Results of GLM multiple regression analysis of the relationship between richness (observed OTUs) and invasive species, temperature, spring size, and pairwise interaction variables. AIC model selection resulted in the elimination of the invasive species x temperature interaction variable from the model, though the decrease in AIC value was less than two points.

Coefficient	**Estimate**	**Std. error**	***t***	***p***
Intercept	1403.6	229.8	6.1	< 0.001 ***
Invasive species	-101.9	87.3	-1.2	0.26
Temperature	-25.4	7.0	-3.6	0.002 **
Spring size	-342.5	103.4	-3.3	0.004 **
Invasive species x spring size	182.3	68.8	2.6	0.02 *
Temperature x spring size	6.3	2.8	2.3	0.04 *

Table 1.4. Results of GLM multiple regression analysis of the relationship between phylogenetic diversity (Faith's PD) and invasive species, temperature, spring size, and pairwise interaction variables.

Coefficient	**Estimate**	**Std. error**	***t***	***p***
Intercept	456.9	156.4	2.9	0.01 *
Invasive species	-287.2	158.7	-1.8	0.09
Temperature	-11.0	4.8	-2.3	0.04 *
Spring size	-38.6	14.3	-2.7	0.02 *
Invasive species x temperature	8.2	4.9	1.7	0.12
Invasive species x spring size	20.9	9.7	2.1	0.05 *
Temperature x spring size	0.8	0.4	2.2	0.05 *

by temperature fit a unimodal Gaussian distribution that peaked between 20-25° C (randomization test $p = 0.009$, Gaussian model -ln(L) = 134.33, Gaussian model AIC = 274.66, linear model -ln(L) = 138.19, linear model AIC = 280.38; Figure A1).

Discussion

There is a growing body of literature describing microbial eukaryotes in freshwater systems using NGS techniques (Nolte *et al.* 2010, Bråte *et al.* 2010, Monchy *et al.* 2011, Charvet *et al.* 2012, Bradford *et al.* 2013, Mangot *et al.* 2013, Stoeck *et al.* 2014, Debroas *et al.* 2015, and others). To the best of our knowledge our research is the first study to characterize entire eukaryotic communities in a freshwater ecosystem, without bias towards taxonomy, organism size, or habitat. In addition, this is the first study to characterize whole community eukaryotic diversity of an aquifer-fed spring system. These spring systems are imperiled habitats in the American West due to the combined effects of aquifer pumping, long-term drought, and climate change. The use of eDNA-metagenetic surveying of the three major habitat types in the springs of Ash Meadows allowed us to examine the taxonomic composition of entire spring pools, yielding complete characterization of these communities which may serve as a baseline by which

to gauge the effects of a declining aquifer, changing climatic conditions, and the ecological effects of degradation of springs and spring outflow habitats.

Biodiversity and environmental variables

Water temperature, spring size, and invasion status were significantly correlated with alpha and beta diversity. These results have important implications for biodiversity in desert springs, which may become warmer and smaller due to the direct effects of aquifer pumping (Deacon *et al.* 2007) and the indirect effects of climate change driving increased temperature and decreased precipitation (Seager *et al.* 2007), and more severe droughts (Cayan *et al.* 2010). Spring temperature in Ash Meadows and other geothermally influenced systems is primarily a result of the amount of mixing between heated water from the deep carbonate aquifer and cool water from local basin-fill aquifers. Reduction of local aquifers through pumping, decreased precipitation, or increased evaporation with higher air temperatures, may result in decreased cooling of geothermal waters and consequent increases in spring water temperatures. These processes may also reduce total spring discharge, concurrently decreasing spring size.

Two important goals of this research were to infer possible drivers of whole community composition for freshwater eukaryotes, and patterns of alpha diversity along environmental gradients. This has been done for taxonomic subgroups (e.g., ciliates: Plebani *et al.* 2015), and habitats within freshwater ecosystems (e.g., water column: Charvet *et al.* 2012), but not for whole freshwater communities. Community composition in Ash Meadows was correlated with water temperature, spring size, invasive species, geographic position (latitude and longitude), and elevation. Alpha diversity also varied significantly with temperature, spring size and invasion status. The implications for the association between biodiversity and these environmental variables are discussed below.

Temperature

Temperature had the greatest contribution to overall environmental correlation with community composition in the springs of Ash Meadows. This aligns with a growing body of literature describing the effects of temperature change on community composition of freshwater systems, which has direct and significant implications in the face of climate change. Significant shifts in community structure with changes in water temperature have been documented for fish (Woodward *et al.* 2010a), macroinvertebrates (Woodward *et al.* 2010a; O'Gorman *et al.* 2012), and meiofauna (O'Gorman *et al.* 2012). Dossena *et al.* (2012) found changes in community size structure for benthic invertebrates, with cascading effects on ecosystem functions such as decomposition and nutrient cycling. Our work shows that shifts in community composition with changing temperature are also significant when considering whole freshwater communities comprising thousands of species of micro- and macroorganisms. This indicates that all trophic levels in a freshwater ecosystem may be impacted by changes in water temperature.

Taxonomic differences between community compositions in low, mid, and high temperature springs included higher abundances of golden and green algal OTUs, and lower abundances of diatoms and arthropods in the warmer springs. These differences likely indicate differential thermal adaptions for those taxa, and may drive differences in ecosystem functioning across spring temperatures. Arthropods can have top-down and bottom-up effects on aquatic communities, playing important roles in nutrient cycling and decomposition (Wallace & Webster 1996). Prevalence of primary producers such as diatoms over green and golden algae in the cooler springs, and the opposite pattern in the warmer springs, may drive bottom-up community effects based on differential trophic interactions of consumers with these producer taxa.

In addition to its effects on community composition, we found that temperature also relates significantly to alpha diversity. We found a unimodal pattern of species richness along the

temperature gradient, with peak richness occurring between 20-25° C. This finding for eukaryotic taxa aligns closely with similar work on bacterial communities in geothermal systems, which were found to peak in alpha diversity at 24° C (Sharp *et al.* 2014).

Spring size

The effect of spring size on community composition and alpha diversity may be due to altered interactions such as predation and competition in springs of different sizes. Mesocosm experiments have demonstrated the effects of freshwater habitat size on food web structure (Spencer & Warren 1996) and predation (Maly *et al.* 1981; Hairston 1988; Pearman 1995), and additional studies have found effects of freshwater habitat surface area on taxonomic groups such as diatoms (Katoh 1991) and macroinvertebrates (Dodson 1987). All springs in the study support populations of fish, snails, and macroinvertebrates. The larger spring pools - though able to support larger populations of higher trophic organisms - may have more predator-free space and dispersed grazing pressure. Populations of the same macrobiota in smaller springs occupy proportionally more area, intensifying the spatial distribution of predation and grazing pressure. This could significantly reduce or extirpate populations of primary producers and consumers in the smaller, low-flow springs, resulting in differences in alpha and beta diversity.

Invasive species

Our results contribute to a large body of literature describing the ecological consequences of invasive crayfish and largemouth bass: these species showed a negative correlation with species richness and phylogenetic diversity in the springs of Ash Meadows, and significantly impacted community compositions. Invasive crayfish and largemouth bass can have direct and indirect effects on ecosystems. As omnivorous grazers, red swamp crayfish have direct effects across trophic levels (Gutiérrez-Yurrita *et al.* 1998; Correia 2002). They can also alter their

environment through increasing rates of nutrient cycling (Angeler *et al.* 2001), and engaging in behaviors such as macrophyte clipping and burrowing that alter habitat structure and destabilize banks, resulting in increased water turbidity (Rodríguez *et al.* 2003). The combined feeding and engineering behaviors of invasive crayfish can have direct and indirect effects across trophic levels (Creed 1994). Largemouth bass are gape-limited predators that can have major effects on populations of fish, crayfish, and macroinvertebrates (Hickley *et al.* 1994; Nowlin *et al.* 2006), with associated cascading effects across associated trophic levels.

Geographic position

The effects of latitude and longitude on community composition likely reflect local conditions in Ash Meadows. The springs in Ash Meadows are clustered latitudinally, with spring outflows connecting clusters of northern, central, and southern springs. This may explain community similarity by latitude for whole, algal mat, and water column communities. Benthic sediment communities were correlated with longitude, not latitude, which may be due to the soil types in the area changing along an east-west gradient (Dudley & Larsen 1976). The importance of soil properties in eukaryote community structure has recently been described by Geisen *et al.* (2015) and Tedersoo *et al.* (2015), and has been recognized for soil bacterial communities for some time (Fierer & Jackson 2006; Lauber *et al.* 2009; and many others).

We found no effect of geographic distance on community composition, when environmental distance was controlled. A recent study conducted at a similar spatial scale (< 10 km between sample sites) produced the same results, with freshwater microbial eukaryote community distance explained by environment rather than geographic distance (Simon *et al.* 2015a). At larger spatial scales, geographic distance may play a stronger role in patterns of community structuring: Lepère *et al.* (2013) found a significant effect of geographic distances on small

protist community composition in lakes that were on average 133 km apart, and up to 400 km apart. Similarly, a meta-analysis of unicellular eukaryotes and metazoans found decreasing community similarity along a geographic distance gradient of 10 km to over 10,000 km (Hillebrand *et al.* 2001).

Elevation

The springs of Ash Meadows occur along a shallow elevational gradient of about 50 m, excluding the higher-elevation Devils Hole, which never connects through surface hydrology to the other springs. We found significant effects of elevation on community composition, which may indicate the importance of position along a flow regime in structuring communities at the scale of a watershed (Hart & Finelli 1999). However, temperature and spring size were significantly correlated with elevation, and may be driving the observed effects. This latter explanation is probable, given the shallowness of the elevational gradient.

Temporal variation

Spring communities exhibited high turnover of low abundance taxa, which is consistent with the findings of Nolte et al. (2010). These results can be explained within the framework of the Baas Becking hypothesis, that everything is everywhere, but the environment selects (Baas Becking 1934). If microbial eukaryotic taxa easily disperse at the scale of the study system, there may be a constant, diverse source of immigrants into every spring. However, these immigrants may not achieve stable, long-term populations due to environmental pressure, competition, or both. It is possible that observed turnover rates were also influenced by detection probability (i.e., low abundance OTUs were less likely to be encountered in subsequent sampling bouts).

Conclusions

Water resources are an important conservation issue globally, and are an especially prominent issue in the American West. As mega population centers such as Las Vegas, Nevada and Los Angeles, California experience the effects of extensive drought, anticipation of a future with even less water and greater numbers of people is driving unsustainable decisions by policymakers and landowners. The current, unrestricted pumping of aquifers in California will likely result in far-reaching ecological consequences (Deacon *et al.* 2007), some of which have already been documented (Elmore *et al.* 2006; Morrison *et al.* 2013). Cascading effects of water removal from surface sources such as the Colorado River, in addition to pumping of aquifers, and climate change effects such as decreased precipitation and increased temperatures, may result in the loss of major biodiversity centers such as Ash Meadows, or at least changes to freshwater community structure and function. Our research showing the importance of water temperature, spring size, and invasive species in community composition and alpha diversity contributes to a growing body of literature that delineates the possible outcomes of global change phenomena for freshwater biodiversity, which has intrinsic consequences for ecosystem functions and services.

Acknowledgements

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CHAPTER 2

ECOLOGICAL RESILIENCE IN A RESTORED DESERT SPRING[2]

Abstract

The success of ecological restoration is often gauged by the change in community composition in relation to a target state. Community trajectory during succession, and the associated ecological outcome, can be influenced by abiotic and biotic variables, including environmental filtering, interspecific competition and predation, priority effects, and habitat partitioning. We conducted a twelve-month chronosequence study in a desert spring to follow community change over time during and after restoration, using environmental DNA and metagenetic surveying (18S rDNA) to estimate whole eukaryotic communities in the restored spring, and three adjacent reference springs that defined the restoration target. The restored spring exhibited rapid recovery of alpha diversity, and a community trajectory directed towards the composition found in the reference springs. Many of the organisms targeted for recovery after restoration were detected prior to intentional reintroduction, possibly due to seeding from the groundwater system, high dispersal from adjacent sources, or *in situ* persistence in microrefugia during restoration. Our results suggest environmental selection of the ecological community in this restored desert spring, highlight the importance of nearby sources of dispersing individuals in restored or disturbed freshwater systems, and demonstrate the applicability of molecular methods for restoration monitoring. The observed trajectory of

[2] Co-authors: Hal Fairfield, Andrew P. Martin

community succession indicates that desert springs are resilient ecosystems amenable to restoration actions that eradicate invasive species, restore physical structure, and seed native species.

Introduction

Directional change in community composition over time, or community trajectory, is a major focus of restoration ecology (Hobbs & Harris 2001; Suding *et al.* 2004; Ruiz-Jaen & Mitchell Aide 2005; Capon *et al.* 2015). The successional model of restoration ecology posits that the engineering of natural habitat structure, reinstatement of historic abiotic conditions, and reintroduction of native species will result in the establishment of a desirable community of organisms, with its associated ecosystem functions and services. However, the trajectory of community development during succession can be redirected by abiotic and biotic variables resulting in an unexpected community outcome, or an alternative state (Suding *et al.* 2004). Understanding the drivers of community trajectories in restored systems is essential for achieving restoration goals that include a specific assemblage of organisms or functional traits.

After natural disturbances or restoration activities, community outcomes after assembly and succession are the result of dispersal, environmental filtering, and emergent biotic interactions. Community trajectories may be redirected by limited dispersal, altered environmental conditions, or differences in biotic interactions due to the order of introduction or the presence of a novel community member (Chase 2003; Myers & Harms 2009; Fukami 2010; Fukami *et al.* 2010; Mergeay *et al.* 2011; Bogan & Lytle 2011; Cañedo-Argüelles *et al.* 2015). Ecological theory provides a framework for predicting when a restored ecosystem will follow an expected trajectory: communities that experience strong environmental filtering through harsh abiotic conditions, in habitats that are equally accessible by a regional species pool, may follow a

deterministic trajectory to an expected community outcome due to a limited pool of adapted species (Fukami 2010). In contrast, community development in less harsh environments, with a wider diversity of potential colonizers, may exhibit historical contingency or priority effects: the successional trajectory may be redirected based on the order of colonization driving differential patterns of competition, with cascading effects for subsequently arriving species. Dispersal limitation or the introduction of a novel community member could also lead to alternative community outcomes in either scenario.

Although much attention has been paid to restoration ecology theory in recent years, there are few detailed studies that document community trajectory as a means of assessing the effectiveness of ecological restoration (Capon *et al.* 2015). Here, we describe the community trajectory of a freshwater spring system during and after ecological restoration. Freshwaters are recognized as one of the most important and most threatened natural resources (Jackson *et al.* 2001; Dudgeon *et al.* 2006; Ormerod *et al.* 2010; Vörösmarty *et al.* 2010). In addition, freshwater systems in arid landscapes are of particular conservation concern due to their tenuous persistence under pressures from global change phenomena, and also because they tend to support high biodiversity in generally species-poor regions (Shepard 1993; Stevens & Meretsky 2008). In the western United States, desert springs are threatened by pumping of water for agricultural, commercial, and municipal uses, which can reduce aquifer levels, leading to decreases or cessation in spring flows (Deacon *et al.* 2007), and loss of phreatophytic habitats (Elmore *et al.* 2006; Patten *et al.* 2008). In addition, desert springs in this region are threatened by climate change: climate models predict increased temperatures and decreased precipitation, which may result in decreased recharge of local basin aquifers, increased water loss through evapotranspiration, and more severe droughts (Seager *et al.* 2007; Cayan *et al.* 2010). Many

springs in the Desert Southwest currently exist in a degraded state due to invasion by non-native species, eutrophication, habitat degradation, or altered hydrologic regimes (Shepard 1993; Deacon *et al.* 2007).

Ash Meadows is a spring system in the Mojave Desert that has the highest richness of endemic species in the United States (Soltz & Naiman 1978); at least 24 endemic plants and animals are found in just 30 springs and seeps, across approximately 9,300 ha. Multiple anthropogenic disturbances in Ash Meadows have occurred over the last century, including the introduction of invasive species, structural modifications and diversions of spring pools and outflow channels, the development of agricultural fields that used flood irrigation, construction of roads and reservoirs, regional aquifer pumping that reduced spring flows, and intensive local pumping that caused temporary desiccation of individual springs (Deacon & Williams 1991). These threats spurred the establishment of Ash Meadows as a National Wildlife Refuge in 1984, with the goals of habitat and biodiversity conservation. Over the past few decades, restoration projects in Ash Meadows have aimed to restore the springs to their natural and historic physical structure, and eradicate invasive species when feasible.

We followed the fate of spring restoration in Ash Meadows to study freshwater community response to restoration practices that included habitat desiccation, structural modification, invasive species removal, and seeding of native organisms. The goals of our research were to assess the restored spring community trajectory given environmental conditions, restoration practices, and both managed reintroduction and natural dispersal of spring recolonizers. We conducted a year-long chronosequence study to address the following research questions: 1) how did the restored spring community change over time in relation to adjacent reference spring communities? 2) When did species richness and phylogenetic diversity recover? 3) Did

reintroduction of targeted species influence the restoration trajectory? We employed an environmental DNA-metagenetic surveying method to quantify community composition for all eukaryotic taxa. Microbial and meiofaunal organisms make up a substantial and functionally important portion of the biodiversity in freshwater ecosystems (Finlay & Esteban 1998; Sherr & Sherr 2002; Cardinale *et al.* 2011); restoration monitoring that goes beyond simple diversity measures and traditional visual surveys of vegetation, macroinvertebrates, and macroalgae to include microbiota provides a more informative synopsis of the whole ecosystem. Recent advances in molecular technology make this degree of monitoring a feasible task: environmental samples of water, algal mats, and benthic sediments provide a rich snapshot of the vast community of microorganisms as well as macrobiota and protists (Bradford *et al.* 2013; Mangot *et al.* 2013; Bazin *et al.* 2014), all of which contribute to freshwater community composition and ecosystem function as a whole. The results of this research have direct implications for conservation management and restoration practices in freshwater systems.

Methods

Study system

The Warm Springs Complex (WSC) in Ash Meadows National Wildlife Refuge (hereafter 'Ash Meadows') comprises six high-temperature, low-flow springs. The springheads emit water at constant temperatures between 31-34° C, and spring discharge is between 30-380 L/m. The most distant springheads in the WSC are less than 1 km apart, and the closest are within 0.06 km (Figure 2.1). The WSC encompasses the entire range of the federally endangered Warm Springs pupfish (*Cyprinodon nevadensis pectoralis*), which has been threatened in recent decades by two invasive animals, red swamp crayfish (*Procambarus clarkii*) and mosquitofish (*Gambusia affinis*), and over a longer time period by habitat degradation from human modification of

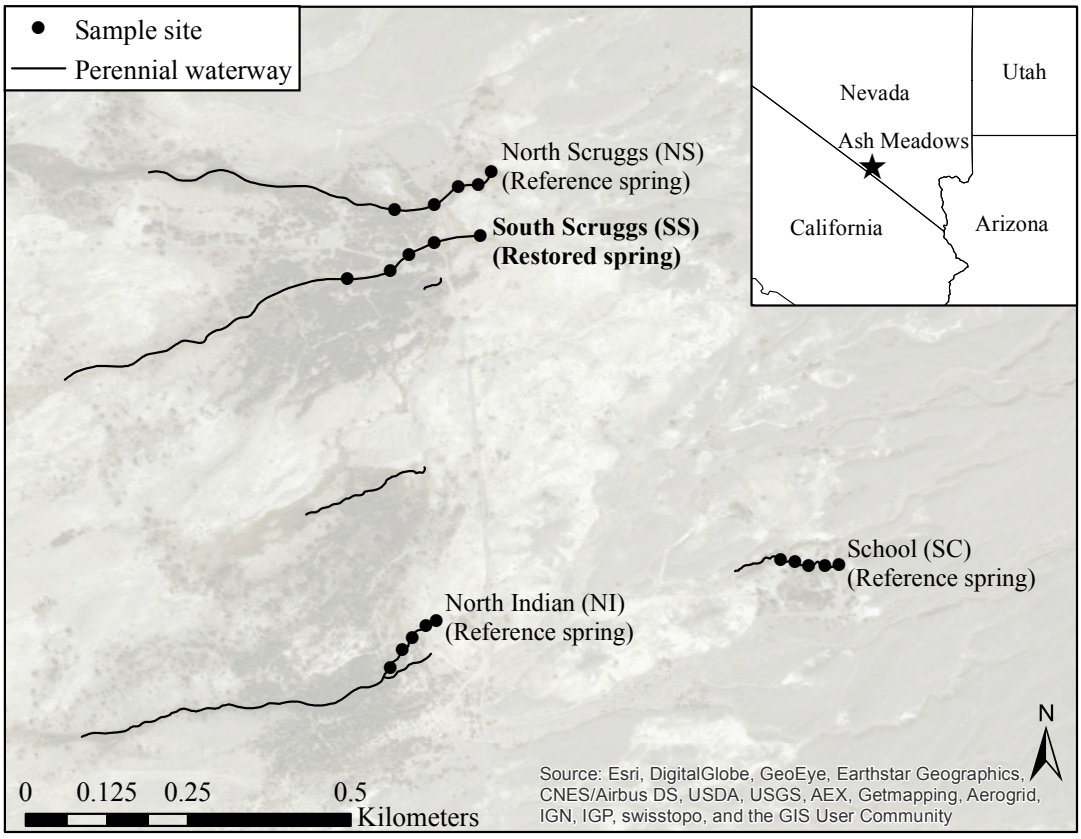

Figure 2.1. The study area was located in the Warm Springs Complex (WSC) of Ash Meadows National Wildlife Refuge, Nevada, USA. Three of the Warm Springs were used as reference springs (NS, NI, and SC) for comparison to the restored spring (SS, bold). SC was restored in 2009, and NI was restored in 2011. NS has not been restored. Samples were collected from the springhead of each spring and four sites along the outflow (black points). These springs maintain temperatures of 31-34° C and flow rates between 30-380 L/m throughout the year.

springheads and outflow channels. The WSC also provides habitat for endemic naucorids and riffle beetles. Restoration was implemented for three WSC springs between 2009-2011, to eradicate targeted invasive species, reconstruct the natural springhead and channel structure, and seed native species. Two additional WSC springs were never invaded by crayfish or mosquitofish, and have not been restored. The sixth WSC spring, South Scruggs Spring (SS), was restored between 2012-2015, and is the focus of this study.

Table 2.1. Dates and counts of organisms that were reintroduced to the restored spring, and the dates of first possible detection after reintroduction. Taxonomic designations were provided by AMNWR personnel. The organisms in gray rows belong to taxonomic groups that were detected prior to reintroduction by management personnel.

Taxon	**Date(s) of reintroduction (2015)**	**Total number of individuals**	**Date of first possible detection** (detected taxon in parentheses)
Elmidae larvae (riffle beetle)	2/10 + 3/12	25	12/3/14 (non-chironomid Insecta)
Microcylloepus (riffle beetle)	2/10 + 3/12	1,202	
Stenelmis (riffle beetle)	2/10 + 3/12	125	
Ambrysus relictus (Naucorid)	5/12	42	
Hyallela (amphipod)	2/10 + 3/12	574	1/2/15 (Amphipoda)
Chironomid larvae (midge)	2/10 + 3/12	40	12/3/14 (*Chironomous* sp.)
Ostracod (seed shrimp)	2/10 + 3/12	13	12/3/14 (Ostracoda)
Oligochaete (aquatic worm)	2/10 + 3/12	5	12/3/14 (Annelida)
Nematode (round worm)	2/10 + 3/12	2	12/3/14 (Nematoda)
Dugesia (flatworm)	2/10 + 3/12	239	12/3/14 (Seriata)
Tryonia (springsnail)	2/10 + 3/12	1,850	6/5/15 (Caenogastropoda)
Pyrgulopsis (springsnail)	2/10 + 3/12	749	
Physa (snail)	2/10 + 3/12	7	1/2/15 (Heterobranchia)
Cyprinodon nevadensis pectoralis (Warm Springs pupfish)	3/24 - 3/26	227	4/7/15 (Teleostei)

Restoration procedure

The restoration goals for SS included restoration of historic vegetation communities, channel structure, and eradication of targeted invasive plants (cattails) and animals (crayfish and mosquitofish). Eradication of aquatic invasive animals was achieved with 23 months of habitat desiccation. Taxa of interest (Table 2.1) targeted for reintroduction post-restoration were salvaged in November 2012 and held in refugia. A combination of French drains and 4-inch PVC pipes with valves and tees was installed at the end of November 2012 to conduct 50% of the flow from the springhead to two 400-gallon tanks. The tanks served as temporary refugia for taxa of conservation concern, including pupfish; an adjacent spring was used as an additional refugium. Outflow from the tanks was diverted along 740 m of PVC pipe into two previously dry washes located below the WSC. In December 2012, the remaining spring-flow was captured and diverted through the established PVC pipe system to the lower washes (total volume: 227 L/m). In March 2013, water in the marsh surrounding the springhead was collected with additional French drains and PVC pipe to further desiccate the area around the springhead and channel, increasing the total drainage volume to 284 L/m. The drainage process facilitated complete desiccation of the springhead and outflow channel, but some marshy areas near the springhead remained for the duration of the restoration project. Crayfish were captured with traps between November 2012 and June 2013; crayfish traps were maintained in the area after June 2013, but no additional individuals were captured. Cattails were manually cleared from the spring area during spring 2013.

In October 2014, a 365 m meandering channel was created with a mini-excavator in the middle reach of the spring outflow, and was subsequently seeded with native vegetation including *Carex nigra*, *Juncus balticus*, *Schoenoplectus* spp. and *Anemopsis californica, Distichlis spicata*, *Sporobolus aeroides,* and *Oryzopsis hymenoides*. The lower channel was

modified with hand tools to increase the depth and width. Native substrata including tufa, gravel, and sand, were collected and placed along the length of the spring channel from October 2014 - January 2015 to improve microhabitat structure.

Spring-flow diversion continued until October 2014 (approximately 23 months). The PVC system allowed re-watering of the spring channel to occur in segments: flow was restored to the lower portion of the spring channel in October 2014, a segment above this reach on November 17, 2014, and to the springhead in January 2015, which yielded full springhead-to-outflow connectivity.

During January 2015, *Spirogyra* algae was collected from lower locations along the outflow and seeded into the upper reaches of the spring. In addition, native *Eleocharis* and *Schoenoplectus* species, and other native wetland plants, were collected as plugs from the marsh surrounding the springhead and planted within and along the margins of the springhead and outflow channel. Although measures were taken to prevent translocation of undesirable organisms (i.e., invasive *Melanoides* snails), these transplant events likely provided the first dispersal vectors for reintroduction of protists and microbial eukaryotes occurring in the *Spirogyra* algal mats and sediments attached to the wetland plant plugs. Targeted native organisms were reintroduced on February 10, March 12, March 24 and 26, and May 12, 2015 (Table 2.1).

Sample collection and processing

Parallel chronosequence sampling was conducted for the restored spring (SS) and three reference springs, two of which had been restored in 2009 and 2011 (SC and NI, respectively) and one unmodified spring (NS) (Figure 2.1). The three reference springs provided comparative data for evaluating ecological community development in the restored spring. The reference

springs also provided an ecological target for restoration as they exhibit natural, historic physical structure and lack invasive fish or crayfish. Each spring was sampled monthly over seven months post-restoration, beginning December 3, 2014, approximately two weeks after water was returned to the restored spring channel. Sampling continued for the restored spring and one of the reference springs (SC) in August, early October, and late November 2015. Each spring was sampled at the springhead and four additional downstream locations (hereafter 'sample sites') approximately 20 m apart. At each sample site, three habitat types were sampled: the water column, algal mats, and benthic sediments. Multiple collections (3-5) were made for each habitat type, to reach approximately 300 mL of sample material. Samples were frozen until DNA extractions were performed. Water samples were centrifuged for eight minutes at 4000 rpm, and DNA extraction was performed on the pellet. Sediment and algal samples were extracted using two 300 µL sweeps through the sample pouch. DNA extractions were performed with MO BIO PowerWater kits (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's protocol, including the additional step of 10 minute lysis at 65° C prior to bead-beating.

Laboratory and sequence data processing followed the methods described in Chapter 1. Briefly, a ~200 bp segment of the 18S rDNA V9 gene region was amplified using a barcoded Illumina primer set (forward Euk 1391f and reverse EukBR) designed by Amaral-Zettler *et al.* (2009). PCR was performed in triplicate, and amplicons were normalized for each pooling step, across samples and then plates. The barcoded library was sequenced on the Illumina MiSeq platform using a V2 300 cycle 150 PE kit (Illumina, San Diego, CA).

Data processing

Sequence data were processed using the pipeline described at github.com/leffj/data-tutorials/blob/master/amplicon_data_processing_tutorial/amplicon_data_processing-16S.md,

modified for 18S data. See Chapter 1 for a full description. *De novo* clustering was performed at 97% sequence similarity on quality filtered (max e=0.005), paired-end data, with a combination of USEARCH 8 (Edgar 2010) and QIIME 1.9 (Caporaso *et al.* 2010) scripts. Taxonomy was assigned from the SILVA 119 database (Quast *et al.* 2013) using the RDP classifier (Wang *et al.* 2007), and an OTU table was built listing all detected taxa and their abundances. A phylogeny was inferred for phylogenetic diversity analyses using the FastTree algorithm (Price *et al.* 2009) in QIIME.

Analyses

All statistical analyses were conducted in R (R Core Team 2015). Community differentiation over space and time, in the restored and reference springs, was quantified using the Bray Curtis dissimilarity metric. Bray Curtis dissimilarities were calculated on rarefied, square-root transformed sequence abundance data. Community variation in the restored and reference springs was analyzed using ANCOVA to test for significant differences in rates of community change over time and space. Ordination of the Bray Curtis distance matrix in NMDS space was used to visualize community clustering by sample, as well as whole community estimates generated by compilation of samples per time point, per spring. Community differentiation by time and sample site was tested using PERMANOVA (adonis) with the mctoolsr R package (Leff 2015), and FDR corrections of all pairwise comparisons were reported.

To test for patterns of community assembly that deviated from a null model, we compared C-scores from the observed community at each timepoint to those found in random assemblages of the same data, over 1000 simulations after a 500 simulation burn-in (oecosimu function in vegan, Oksanen *et al.* 2011). The simulated communities maintained the same number of taxa

per time point, and the same number of occurrences per taxon across all time points, by using fixed row and column sums (the 'quasiswap' null model method).

Recovery of alpha diversity was analyzed using species richness (observed OTUs) and phylogenetic diversity (Faith's PD, Faith 1992). Linear models were used to test for significant changes in alpha diversity over time for the restored and reference springs.

The fate of reintroduced targeted metazoan taxa was assessed through counts of detected DNA sequences for each taxon over time for the restored and reference springs. This census method was used to determine the dates of first detection in the restored spring, and for comparison of abundances per time point between springs. Higher classification levels were used for the taxa that were not explicitly assigned in the OTU table (e.g., Seriata for *Dugesia* sp., and Caenogastropoda for the *Tryonia* sp. and *Pyrgulopsis* sp. springsnails; Table 2.1).

Results

Community composition

Patterns of community variation differed between the restored and reference springs. Rates of community change over time and space were significantly different between springs (ANCOVA, time*spring $p < 0.001$, $F = 176.4$; distance* spring $p < 0.001$, $F = 395.2$). Compared to the reference springs, the restored spring showed a greater increase in community dissimilarity with increasing time between sampling time points, and a lower increase in dissimilarity with increasing distance between sample sites within the spring (Figure 2.2).

Clustering of sample communities differed between the restored and reference springs: generally, the restored spring communities clustered by date, and the reference spring communities clustered by site (Figure 2.3). All corresponding R^2 values for the following results are reported in the Appendix (Tables A4-A11). Samples collected from the restored spring

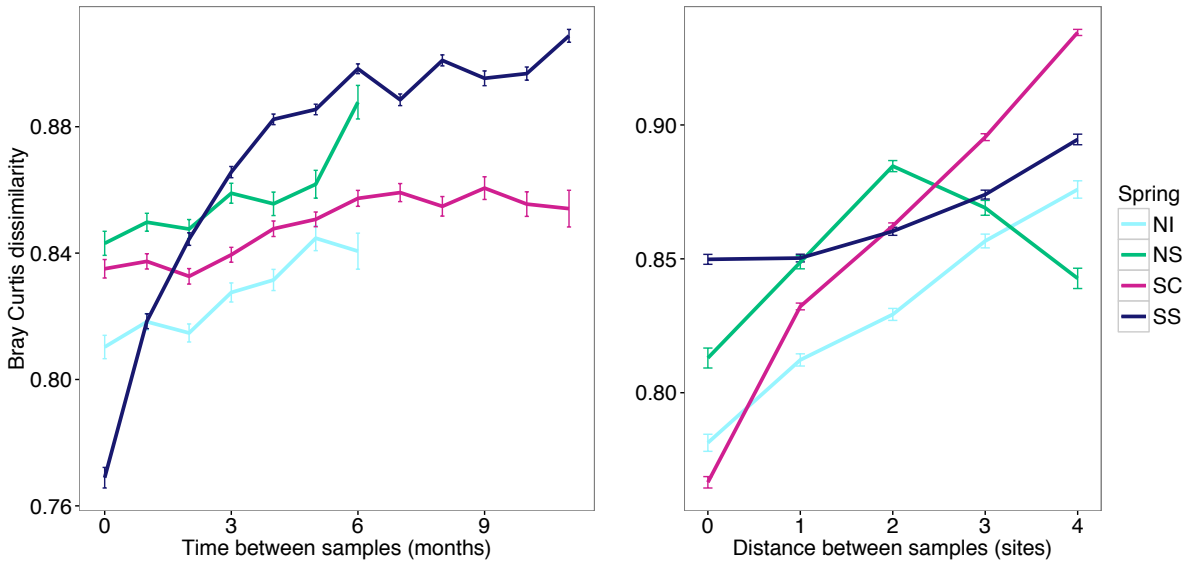

Figure 2.2. Mean Bray Curtis dissimilarities between all within-spring sample pairs across time (left) and distance (right), with standard error. Rates of change over time and distance were significantly different between springs (ANCOVA, time*spring $p < 0.001$, $F = 176.4$; distance* spring $p < 0.001$, $F = 395.2$). Samples from the restored spring (SS) showed the greatest increase in community dissimilarity with increasing time between sample collection (left), and also had the highest community similarity within each time point (when months between samples = 0). The restored spring showed less community differentiation between sample sites than the reference springs (right).

showed community clustering by time point ($p = 0.002$ for all pairs, except May-June $p = 0.02$; Table A4); all outflow site communities were different from that found at the springhead ($p = 0.004$; Table A5), and the lowest and highest outflow site communities differed from each other ($p = 0.006$; Table A5). The reference springs overall showed differences in community composition between sampling sites within each spring, and few differences between time points. SC communities differed for all sites ($p = 0.002$), and no time points (Tables A6-A7). NI communities differed for all sites except the two lowest outflow sites ($p = 0.002$, except the two highest outflow sites $p = 0.007$), and for many inter-seasonal pairs ($p < 0.02$), and for one intra-seasonal pair (December-February $p = 0.02$; Tables A8-A9). NS communities differed for all site

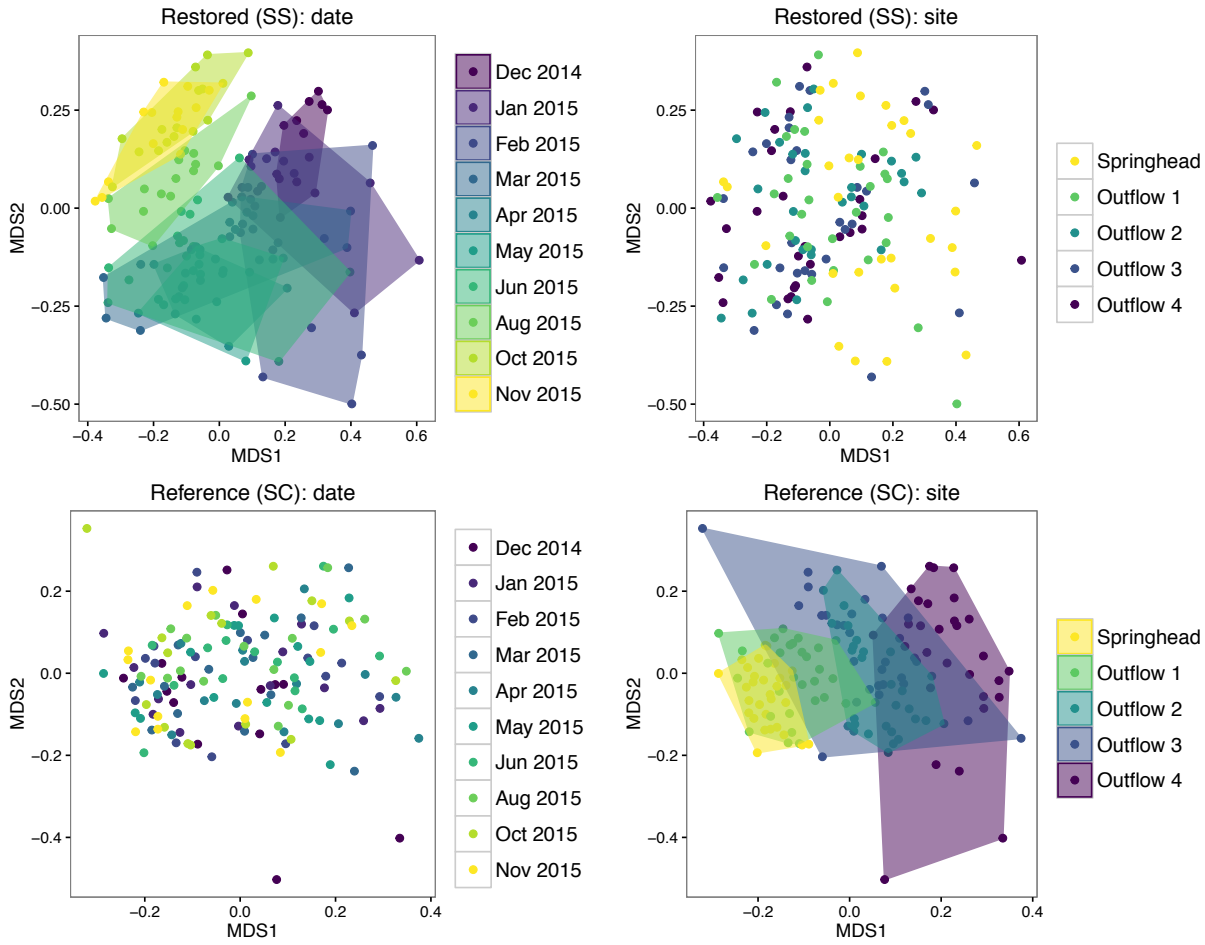

Figure 2.3. NMDS ordinations of Bray Curtis dissimilarities per sample, colored by date (left) and site (right), for the restored spring (top) and one of the reference springs (SC, bottom). Hulls indicate the predominant clustering regime. Samples clustered for all dates in the restored spring ($p = 0.002$ for all pairs, except May-June $p = 0.02$, top left) and for all sites in the reference spring ($p = 0.002$ for all pairs, bottom right).

pairs other than the two highest outflow sites ($p = 0.002$, except the two lowest outflow sites $p = 0.03$), and for the December-June time points ($p = 0.02$; Tables A10-A11).

The restored spring community showed high divergence from the reference springs at the beginning of the chronosequence, but became more similar to the reference spring communities over time (Figure 2.4). The trajectory of the restored spring changed sharply towards the reference springs after the reintroduction of pupfish in late March 2015.

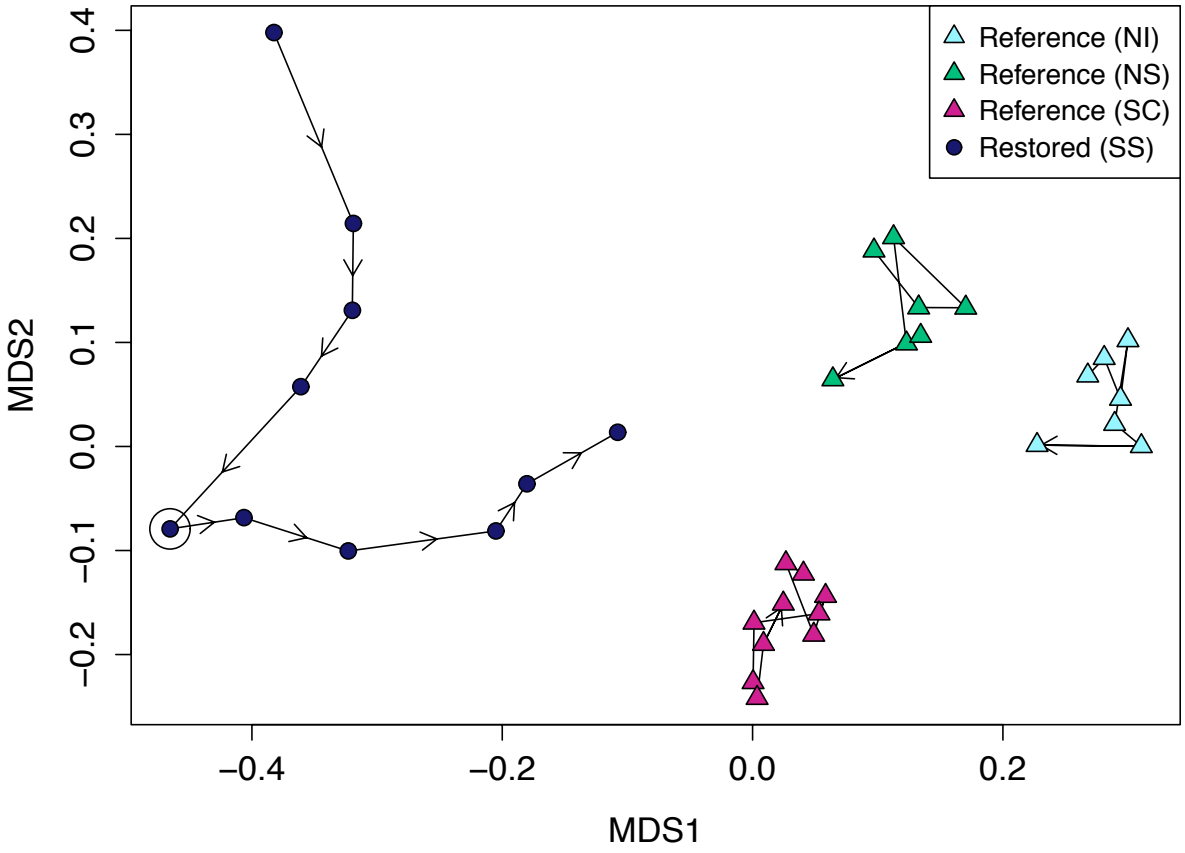

Figure 2.4. NMDS ordination of Bray Curtis dissimilarities for whole community compositions per spring throughout the chronosequence (December 2014 - November 2015). Samples were rarefied to 676 sequences, and then compiled per spring by adding OTU counts. Compiled samples per spring were rarefied to 155,523 sequences. Triangles denote the reference springs, and circles correspond to the restored spring. The black circle shows the first sample date (April 7) after the reintroduction of 227 pupfish into the restored spring (March 24-26). The restored spring community trajectory changed direction after the reintroduction of pupfish, and subsequently became more similar to the reference springs.

A nonrandom pattern of co-occurrence was found for the restored spring during succession ($p < 0.001$, SES = 38.55, for 1000 simulations after 500 burn-ins). More checkerboard pairs were observed between time points in the restored spring (C-score = 2.2007) than were found in the random, null model communities (mean C-score = 2.1201), indicating species segregation during succession.

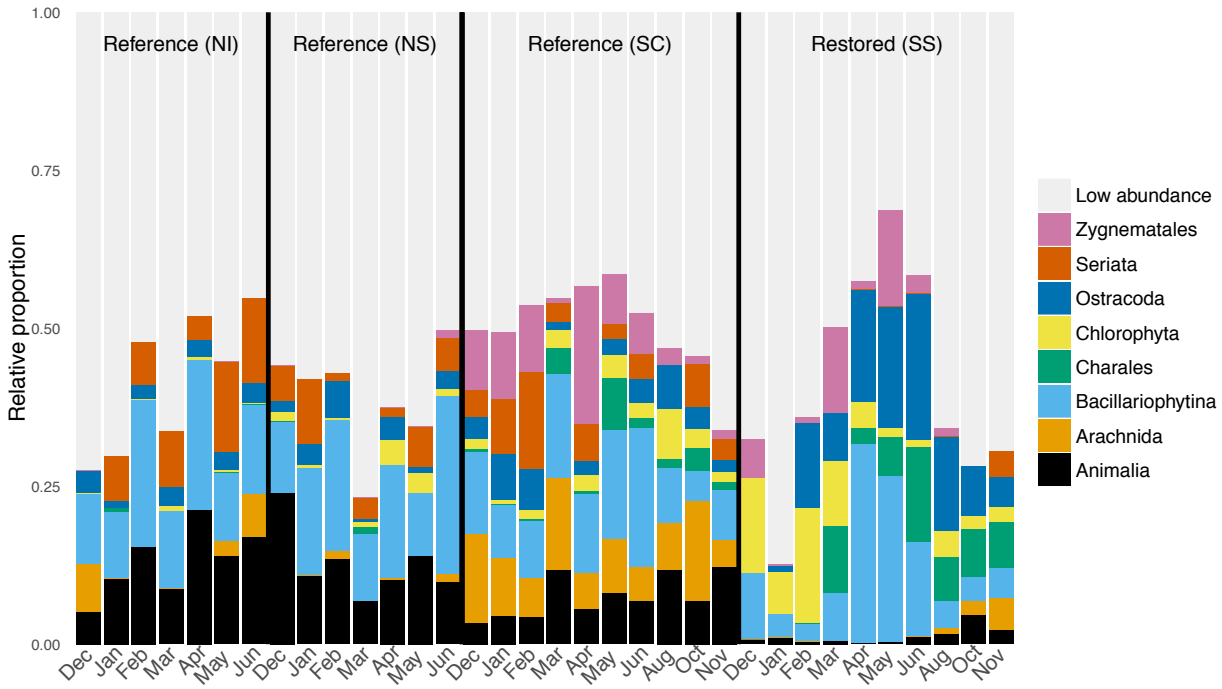

Figure 2.5. Relative proportions of eight high abundance taxonomic groups for the three reference springs (NI, NS, and SC) and the restored spring (SS) throughout the chronosequence (December 2014 - November 2015). Lower abundance taxa are grouped under the 'Low abundance' category.

The restored spring showed a few major differences in high-abundance taxa in comparison to the reference springs (Figure 2.5). The restored spring had higher proportions of algal taxa (Chlorophyta and Charales) and ostracods, particularly compared to NI and NS springs. Water mites (Arachnida) were a high abundance taxon in the reference spring samples, especially SC; this taxon began showing up at comparable abundances in the restored spring later in the chronosequence, in August. A similar pattern was observed for an unclassified Animalia taxon, which had comparatively low relative abundances in the restored spring in the early months of the chronosequence, but increased in August and October. Additionally, Seriata flatworms were found at high relative abundances in the reference springs throughout the chronosequence, but did not reach similarly high abundances in the restored spring until November. Bacillariophytina

diatoms were present in all springs, but showed wide temporal variation in relative abundance in the restored spring. Last, the restored spring had higher proportions of low abundance taxa in the early months of the chronosequence than the reference springs.

Alpha diversity

Alpha diversity in the restored spring was within the range of the reference springs from the first time point in the chronosequence – just two weeks after water was returned to the spring channel (Figure 2.6, species richness; Figure A2, phylogenetic diversity). The total observed OTUs in the restored spring at the first time point (December 2014) was 1,886, while the reference springs had 2,510, 1,628, and 2,236 (NI, NS, and SC, respectively). A weak increase in species richness over the chronosequence was observed in the restored spring ($p = 0.06$, $R^2 = 0.37$), but phylogenetic diversity showed no directional change (Figure A2). The reference spring sampled across the entire chronosequence (SC) did not exhibit directional change in species richness or phylogenetic diversity. Alpha diversity comparisons between the other two reference springs (NI and NS) were not informative, as data for those springs only comprised the first six months of the chronosequence.

Effects of seeding and reintroductions

The strongest observed effect of reintroduced taxa was the change of community trajectory after 227 pupfish were reintroduced into the restored spring in late March 2015 (Figure 2.4). Prior to the reintroduction of pupfish, the restored community trajectory was diverging away from the reference spring communities; after pupfish reintroduction, the restored spring trajectory altered course towards the reference springs. Most of the other metazoan taxa reintroduced into the restored spring, or their higher-order clades, were detected in samples

collected prior to the dates of reintroduction (Table 2.1 and Figure 2.7), with the exception of the *Tryonia* sp. and *Pyrgulopsis* sp. springsnails (Caenogastropoda).

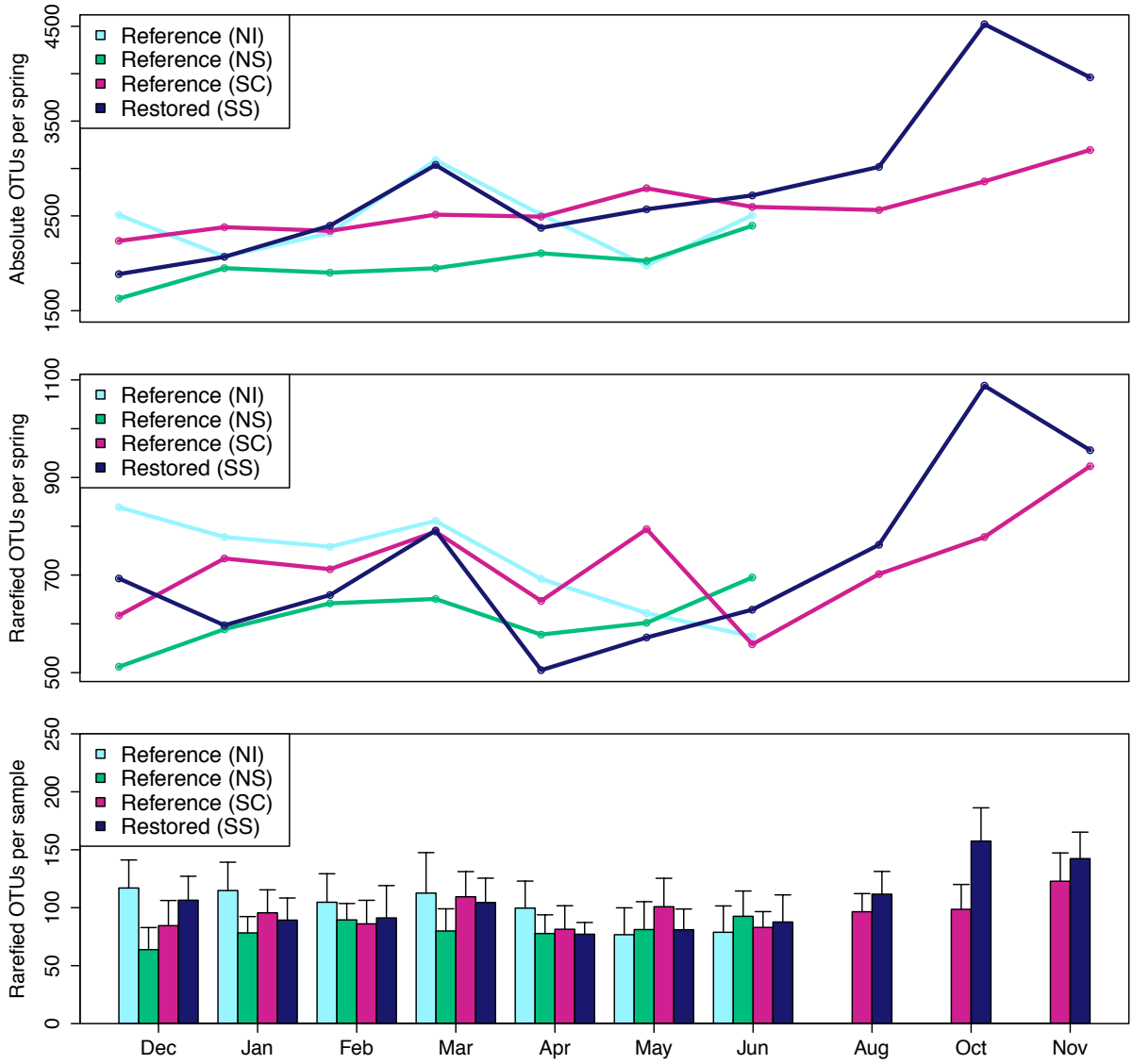

Figure 2.6. Absolute species richness for the four springs from December 2014 to November 2015 (top), rarefied species richness per spring (middle), and rarefied species richness per sample (bottom). Samples were rarefied to 676 sequences. Compiled samples per spring were rarefied to 8878 sequences. SS richness increased weakly over time ($p = 0.06$, $R^2 = 0.37$), and NI richness decreased ($p = 0.004$, $R^2 = 0.84$). SC and NS showed no directional change in species richness over time.

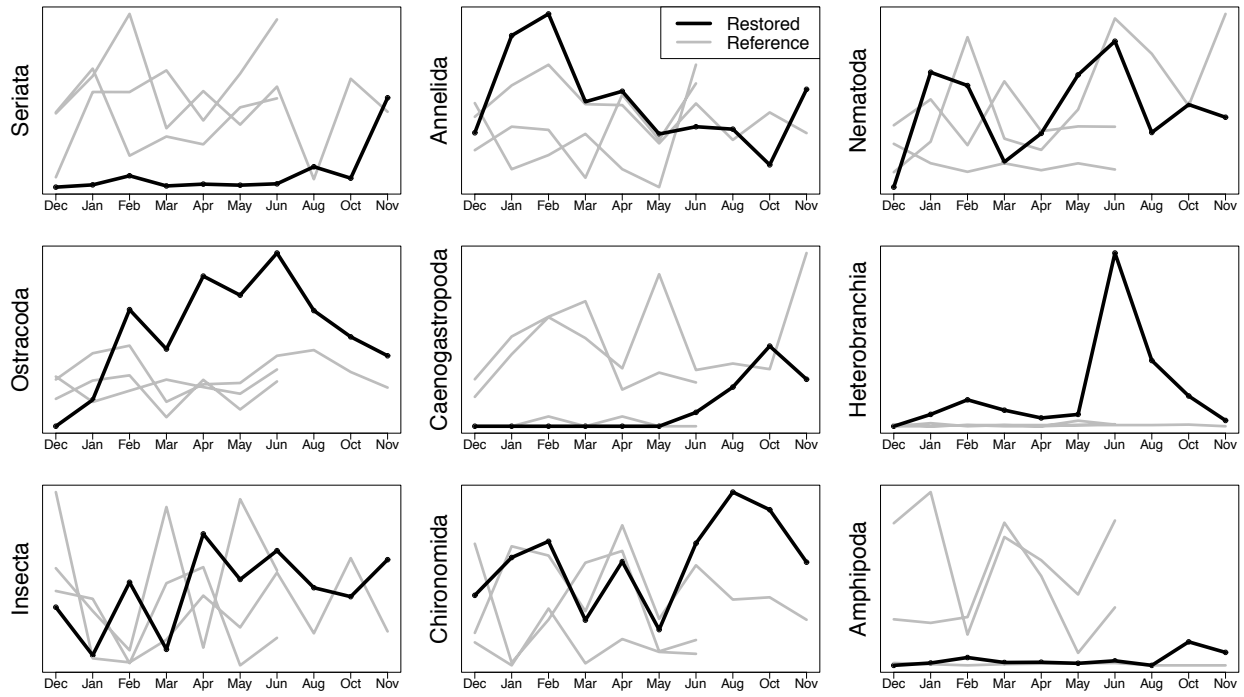

Figure 2.7. Changes in abundances of taxa targeted for reintroduction from December 2014 to November 2015 in the restored spring (black) and the reference springs (gray). Most taxa were reintroduced on February 10 and March 12, except naucorids, which were reintroduced on May 12. The Seriata plot (top left) may include *Dugesia* sp. flatworms. The Insecta plot (bottom left) shows data for non-chironomid insects, which may include Elmidae riffle beetles and naucorids. The Amphipoda plot (bottom right) may include *Hyallela* sp. amphipods.

Discussion

An important goal of restoration ecology is to engineer ecologically damaged or disturbed systems so that they follow a successional trajectory towards a desirable community composition (Hobbs & Norton 1996). We followed the fate of a freshwater spring through successional changes during and after ecological restoration. Restoration was primarily implemented to eradicate invasive crayfish, which were causing a decline in the population size of an endemic endangered pupfish. Crayfish eradication was achieved through prolonged desiccation: flow was diverted from the springhead and channel for nearly two years. During this time, the springhead and channel underwent modification to return the structural state to a more historic, natural

configuration. When water was returned to the spring after 23 months of desiccation, the system began a trajectory of successional change towards a highly diverse community characteristic of the nearby reference springs.

Community composition over time

Two interesting patterns emerged from analysis of community composition over time and across sample sites along the lengths of the restored and reference springs. First, there was a strong effect of date for the restored spring that was largely lacking for the reference springs. This suggests a high degree of species turnover during the chronosequence in the restored spring, a phenomenon characteristic of early ecological succession in a variety of systems (Fisher *et al.* 1982; Drake 1990; Metcalf *et al.* 2016; and many others). The observed high turnover (Figures 2.2 and 2.3, Table A4) reflects colonization of the system by a wide variety of organisms that failed to establish, possibly due to abiotic environmental conditions, or interspecific interactions such as competition or predation. The lesser turnover observed across larger time gaps in the reference springs (Tables A6, A8, A10) is characteristic of later successional stages (Horn 1974), or seasonal variation in species composition (Rosemond *et al.* 2000; Nolte *et al.* 2010; Simon *et al.* 2015b). It is likely that the reference springs were also subject to opportunistic colonization by a wide variety of taxa, but because they were rare relative to the established community members they did not have a strong influence on temporal community structuring.

Second, we observed community variation among sites within springs; this pattern was stronger in the reference springs than the restored spring (Figures 2.2 and 2.3, Tables A5, A7, A9, A11). This is likely due to environmental heterogeneity along the transect from the springhead to downstream sites, which can drive spatial structuring of communities (e.g., Seabloom *et al.* 2005). Variation in environmental conditions such as solar input, water depth, and flow rate may

drive community differentiation among sites within springs. Lack of spatial structuring between site pairs in two of the reference springs (NI and NS, Tables A9, A11) may have been caused by environmental similarities between those pairs of sample sites: both pairs were located in narrow, deep channels shaded by overgrown vegetation. Niche partitioning may account for differences in community composition among sample sites, and this phenomenon may be time-dependent given its lesser appearance in the restored spring, which was in early stages of community development compared to the reference springs. During early succession, the biotic component of an organism's niche (i.e., interactions with other species, and environmental conditions generated by other species) may be more stochastic given higher rates of species turnover. This could result in less well-defined spatial structuring within an ecosystem.

The observed pattern of community differentiation over time in the restored spring reflects both stochastic and deterministic processes. Evaluation of the predictions of stochastic effects on community assembly revealed that species co-occurrence was lower than expected under a null, niche-equivalence model, indicating that species segregation occurred between time points. Evidence for species sorting has been found in other microbial communities (reviewed by Horner-Devine *et al.* 2007), including during succession (Koenig *et al.* 2011). Competitive interactions, habitat partitioning, and environmental selection may have acted together in the early stages of succession in preventing the establishment of many taxa; this hypothesis is also supported by the high proportion of low-abundance taxa observed in the early months of the chronosequence (Figure 2.5).

One notable aspect of community change over time in the restored spring was the marked shift in the community trajectory following the reintroduction and establishment of fish. Prior to the introduction of fish, the successional trajectory was drifting away from the community

composition that was characteristic of the reference springs; after pupfish were reintroduced, the community trajectory changed direction towards the compositional state of the reference springs (Figure 2.4). While we cannot say for certain that the demonstrable shift in succession was due to the presence of pupfish, which are the largest and most abundant omnivores in this system, there are two primary reasons to expect pupfish to have an effect on community composition. First, studies of other aquatic systems have revealed strong effects of fish density on aquatic community diversity (Diehl 1992), and changes in community composition when fish were stocked in previously fishless systems (Eby *et al.* 2006). Second, pupfish are larger and have substantially higher metabolic demands than any other aquatic species in the restored spring, and are therefore likely to significantly influence the relative abundances of other species and the stability of the food web (Otto *et al.* 2007). Thus, the notable shift in succession towards the reference springs was plausibly driven by pupfish reintroduction, and underscores the important effect of pupfish - a top predator in the system (Doucett *et al.* 2007) - on the eukaryotic species composition of these springs.

Overall, the analysis of community composition suggests that there is a relatively stable pool of species that inhabit the warm, low-flow springs of Ash Meadows, and that restoration coupled with the reintroduction of fish has the potential to quickly drive a spring community towards a putative historic, native condition. Moreover, succession occurred relatively rapidly, over a similar time period observed in other desert water bodies (Fisher *et al.* 1982). Over the course of one year, the restored spring community changed from a distinctly different composition compared to the three reference springs, to a community that was as similar to the reference springs as each reference spring was to each other (Figure 2.4). These results highlight that

restoration of freshwater springs is a viable option for the conservation of their ecological communities.

Alpha diversity

The restored spring exhibited a rapid increase in alpha diversity from the earliest stages of the chronosequence. Species richness was similar to the reference springs only two weeks after flow was returned to the restored spring, and most of the major taxonomic groups targeted for recovery were detected prior to their reintroduction. The high alpha diversity at the earliest time point may be evidence for organismal seeding from the groundwater system into the springs of Ash Meadows (Hahn & Matzke 2005; Bradford *et al.* 2010). Analysis of well water from a source near the WSC revealed diverse eukaryotic OTUs, including ciliates, nematodes, and algal taxa (Table A12). Although these taxa may not all be permanent residents of the aquifer, they may be introduced to the groundwater system by water seeping down from the land surface, and subsequently carried by subterranean flows to spring outlets. Resilience of the restored spring to extended desiccation and structural disturbance may also be attributable to rapid dispersal of most eukaryotes at a small spatial scale (Finlay 2002; Bohonak & Jenkins 2003), and the availability of a dormant seedbank of eukaryotes established at the onset of the desiccation phase (Incagnone *et al.* 2015). Dispersing individuals may have reached the restored spring actively, or passively by wind, water, or phoretic vectors such as birds (Figuerola & Green 2002; Green *et al.* 2008), or insects (e.g., ectoparasitic water mites on their hosts; Smith & Cook 1991). Episodic flooding appears to be another avenue for dispersal: we observed a spike in alpha diversity in October 2015, immediately after a flooding event associated with high local precipitation (4,522 observed OTUs, Figure 2.6).

Specific organisms were targeted for reintroduction after restoration (Table 2.1). With the exception of caenogastropod snails (springsnails) and pupfish, reintroduction of many of the organisms may have been unnecessary because many of the taxa, including amphipods, chironomids, ostracods, oligochaetes, nematodes, flatworms, and *Physa* snails were present in the restored spring prior to reintroduction (Figure 2.7). Nonetheless, deliberate reintroduction of putatively key species - especially those of conservation concern - should remain an essential part of restoration efforts, especially since colonization and establishment of native species is likely to vary among systems.

Caveats

There were limitations to identification of taxa using short DNA sequences. In many cases, we inferred the presence of particular species in the absence of direct DNA matches to the taxonomy database. For example, pupfish were the only fish in the system, and were thus presumed to be represented by teleosts identified in the data. Similarly, sequences identified as Caenogastropoda were likely *Tryonia* sp. and *Pyrgulopsis* sp. springsnails, and the Heterobranchia snail identified was most likely *Physa*, as *Physa* is the only heterobranch snail in the system.

This lack of taxonomic resolution was most problematic in monitoring abundances of insect species of interest (two riffle beetles and the naucorid), and the amphipod *Hyallela* sp., which is an important food source for the pupfish. Insect taxa and amphipods were detected throughout the chronosequence, and these broader taxonomic groups may have included the taxa of interest. Although dispersal by *Microcylloepus* and *Stenelmis* riffle beetles from adjacent springs into the restored spring prior to reintroduction is possible, the Elmidae species in Ash Meadows are apterous or brachypterous, and thus considered to be flightless (Shepard 1992); however, there

can be variability in flightedness between individuals within riffle beetle species (Elliott 2008). Aerial dispersal of naucorids, on the other hand, is not well understood, but they have fully developed wings and have been observed flying to lights (Polhelmus 1979), and Miller *et al.* (2002) found genetic evidence for terrestrial dispersal between isolated populations. As for the amphipod taxon of interest (*Hyallela* sp.), dormant stages or burrowing individuals may have persisted *in situ*. Alternatively, adult aquatic insects and amphipods may have lived in microrefugia in the spring area throughout the desiccation phase, negating dormancy or dispersal from adjacent spring source populations.

Conclusions

Using high-throughput sequencing of environmental samples, we observed ecological resilience in a restored desert spring. At the massive taxonomic scale of all eukaryotes, the restoration of South Scruggs Spring was successful: invasive species were eradicated, the natural spring structure was restored, and the spring community changed towards a composition similar to the reference springs that defined the restoration goal. This community outcome may indicate the predominance of environmental filtering over priority effects in this high-temperature, low-flow, oligotrophic system (Fukami & Lee 2006), as the order of colonization by thousands of eukaryotes was likely not identical in all four springs. The return of natural environmental conditions and the availability of recolonizers from the aquifer, seedbank, or nearby springs enabled assembly and succession along a trajectory towards the target composition of adjacent reference springs.

Community recovery after restoration or major disturbances such as drought can be influenced by a variety of factors, including dispersal probability of possible colonizers. The restoration success described here may be less likely to occur in a more isolated system, where

dispersing individuals may not reach the habitat so rapidly, if at all (Heino *et al.* 2015). The combined effects of groundwater pumping and drought in the American Southwest may increase desert spring isolation through desiccation of intermediate refugia across the arid landscape. In addition, periodic spring drying may become more prevalent with changes in groundwater levels. These two forces combined may result in disturbances to spring ecosystems where dispersal limitation drives community trajectories towards alternative states (Bogan & Lytle 2011). Although we have shown that restoration can be successful in these environments, conservation of groundwater is imperative for the persistence of desert spring ecosystems at a larger scale.

Acknowledgements

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CHAPTER 3

COMMUNITY ECOLOGY OF DEVILS HOLE AND ITS CONSTRUCTED ANALOG, AN ARTIFICIAL REFUGE FOR THE DEVILS HOLE PUPFISH (*CYPRINODON DIABOLIS*)[3]

Abstract

Ecological restoration, habitat creation, and artificial refugia are increasingly essential for conservation, particularly for species that are threatened by habitat loss or degradation. The Devils Hole pupfish is one such species, thought to have the smallest range of any vertebrate. Multiple attempts at propagating backup populations in artificial refugia have been made since the 1970s, but all failed due to mechanical issues or environmental differences. Recently, a new refuge was constructed for $4.5 million, designed to replicate the Devils Hole environment. We conducted a comparative analysis of the ecological communities in Devils Hole and the constructed environment, and found significantly different community compositions and temporal variation between the two sites. Community differentiation was likely driven by environmental differences, and dispersal limitation into the constructed environment. In addition, the two systems showed different responses to disturbance (flash flooding). Last, environmental DNA surveys revealed far greater diversity in Devils Hole than had previously been detected through traditional visual survey methods. Our results highlight the importance of monitoring to track progress towards a desired ecological community outcome, as seeding of targeted taxa may not always result in the expected community composition.

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[3] Co-authors: Ambre L. Chaudoin, Andrew P. Martin

Introduction

Habitat loss and degradation are among the top threats to biodiversity (Ehrlich 1988; Dobson *et al.* 1997; Wilcove *et al.* 1998; Dudgeon *et al.* 2006), particularly for species with small isolated ranges (Cincotta *et al.* 2000). Conservation practices that involve habitat restoration or expansion will be increasingly vital as we move into a future with largely undiminished alteration of natural landscapes (Dobson *et al.* 1997). One practice for species conservation that goes beyond restoration of a natural habitat is construction of new habitat, either through modification of a natural landscape or engineering of an artificial structure. This has been implemented for endangered brackish water damselflies in Japan by converting rice patty fields to an artificial habitat of reeds with brackish water inlets (Watanabe 2015); artificial reefs have been constructed with various manmade objects such as concrete structures and ships to restore marine habitats (e.g., Ambrose & Anderson 1990; Clark & Edwards 1999); and fish hatcheries propagate threatened or endangered species such as the Gila trout (Fenn 2015).

The construction of an artificial habitat refugium has been implemented multiple times for the endangered Devils Hole pupfish (*Cyprinodon diabolis*). The Devils Hole pupfish has the smallest range of any vertebrate species (Miller 1948), existing in a 22 m by 3.5 m pool of water, 15 m below the land surface at the bottom of a rocky crevasse in the Mojave Desert. Known as Devils Hole, this pool of water is actually the surface of a deep aquifer: divers mapped the flooded caverns to a depth of 130 m without reaching the bottom (Szabo *et al.* 1994). The aquifer is geothermally-influenced, resulting in a near-constant temperature of 33.5° C at the surface. The Devils Hole environment is highly oligotrophic (Hausner *et al.* 2012) and primary production is also limited by light: direct sunlight reaches the water for less than fours hours per day during the summer and not at all during the winter (Wilson & Blinn 2007). The pupfish are

primarily found over a ~3.5 m by 5 m rocky shelf submerged under 0.3 m of water at the southwest end of the pool, though they can be found at depths up to 15 m in summer months (Baugh & Deacon 1983). This shallow shelf supports a diverse ecosystem including algae, aquatic insects, and an endemic snail (Shepard *et al.* 2000; Herbst 2003; Wilson & Blinn 2007), and provides spawning and feeding habitat for the pupfish (James 1969; Andersen & Deacon 2001).

Beyond the challenges of surviving as a small population in a harsh environment (Gaston 1994), the Devils Hole pupfish has faced anthropogenic threats over the past century that have caused population bottlenecks and habitat loss. The first major documented loss for the population occurred in 1930 when Joseph Wales and George Myers removed 60 individuals to study the morphology of the species (Wales 1930). At the time, they estimated the total population to be approximately 200 individuals; this is likely an underestimate, but underscores that the population was not terribly large. Next, in the late 1960s and early 1970s, extensive groundwater mining in southern Nevada caused a decline in the aquifer, lowering the water level in Devils Hole below the shallow shelf (Dudley & Larsen 1976). This reduction in the pupfish's already small habitat corresponded with a decrease in population size to approximately 100-200 individuals (Andersen & Deacon 2001). The Devils Hole pupfish was listed as endangered under the Endangered Species Act in 1973, with subsequent habitat protections to prevent further water mining that could lower the aquifer again. The population increased to over 500 individuals between the mid-1970s to the mid-1990s, but then began to decline again. In 2004, approximately one third of the population was lost when a flash flood washed a set of larval traps into the aquifer, killing at least 74 individuals (Manning & Wullschleger 2004). By 2013, the population was estimated at 35 individuals. The direct cause of the Devils Hole pupfish's decline

in recent years has not been determined, though multiple population bottlenecks may have amplified the genetic load of deleterious mutations (Martin *et al.* 2012).

Conservation of the Devils Hole pupfish as an enigmatic and endangered species has been a priority for management agencies since the 1970s. Conservation efforts have included attempts to increase larval survival, food supplementation, which still occurs biweekly, and also the construction of artificial refugia designed to harbor and propagate backup populations. A total of 126 pupfish were translocated from Devils Hole to ten sites between 1970-1994, for attempted propagation (Karam 2005). Three of these sites were designed to replicate the Devils Hole environment; the others were either natural springs or aquaria. All attempts at propagation eventually failed, though some refuge populations managed to survive over 20 years despite multiple bottlenecks that reduced the population below ten individuals (Karam 2005). The refuge failures were largely due to mechanical issues related to regulation of water temperature and input volume (Karam 2005), and lack of monitoring personnel. One refuge population was invaded by a small number of fish from a sister taxon (*Cyprinodon nevadensis mionectes*), which resulted in a hybridized population (Martin 2005; Martin *et al.* 2012). Multiple refuge populations developed genetic and phenotypic characteristics that differed markedly from the natural Devils Hole pupfish population, likely due to genetic drift and the effects of different environmental conditions on development (Karam 2005; Wilcox & Martin 2006).

In 2012, the U.S. Fish and Wildlife service was funded approximately $4.5 million through income from federal land sales to construct a new refuge located about one kilometer from Devils Hole, in Ash Meadows National Wildlife Refuge. The Ash Meadows Fish Conservation Facility design took into account some of the sources of error that led to the failures of previous refuge attempts. A ~380,000 L tank was constructed to mimic the cavernous setting and geologic

Figure 3.1. Devils Hole (left) and the Ash Meadows Fish Conservation Facility refuge tank (right), in June 2015.

contours of Devils Hole, with a shallow shelf and a 6.7 m deep end (Figure 3.1). In order to replicate the insolation regime found in Devils Hole, the tank is oriented along the same directional axis and is in a structure that shades the pool on the southeast and northwest, with windows on the southwest end (Figure 3.2). In addition, the ceiling has adjustable louvers to control the amount of light entering the facility from above. Water is pumped from the same aquifer source that feeds Devils Hole, and is conditioned prior to entering the tank to mimic the water quality of Devils Hole, with two exceptions intended to improve conditions for the refuge pupfish: water temperature is maintained between 29.5-30.5° C (compared to 33.5° C in Devils Hole), and dissolved oxygen ranges from 4.0-5.0 mg/L (compared to 2.5 mg/L in Devils Hole).

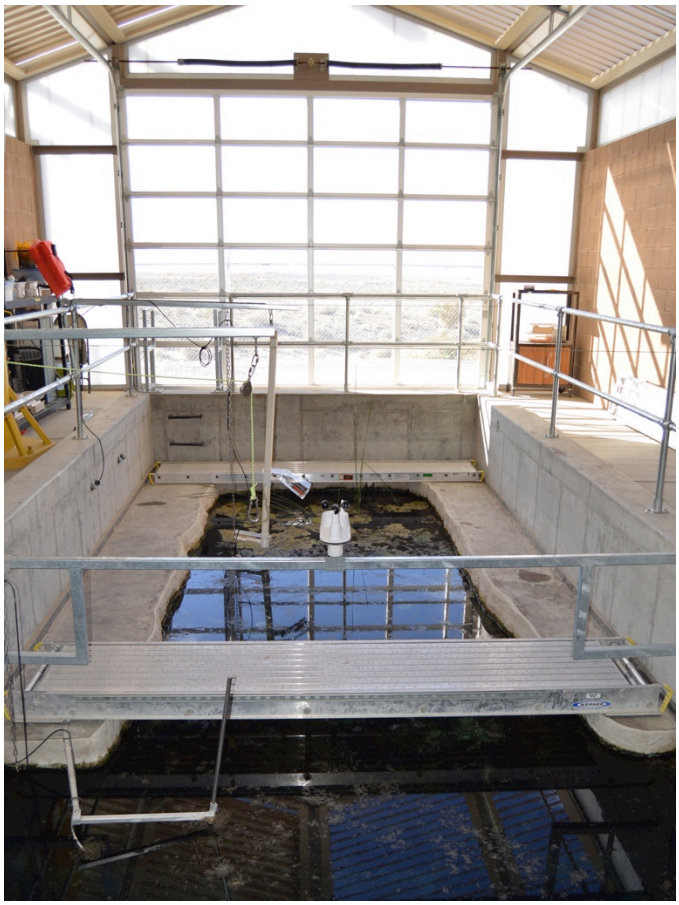

Figure 3.2. The refuge tank is shaded on the southeast and northwest sides, and has a wall of windows to the southwest to mimic the aspect and exposure of Devils Hole. An adjustable louvered ceiling was designed to further replicate the insolation regime of Devils Hole.

Alarm systems and backup measures are in place in the event of mechanical failures, and anti-contamination protocols are implemented to prevent invasion by non-Devils Hole taxa and pathogens.

In addition to the replication of abiotic conditions, the new refuge was also designed to harbor the biotic community of organisms found in Devils Hole in order to fully replicate the Devils Hole environment. The tank community was first seeded with algae and invertebrates from Devils Hole on June 28, 2013. Organisms were introduced into the tank following stringent propagation methods. Biotic material was harvested from Devils Hole, sorted using visual

methods and microscopy, and propagated in aquaria in another part of the facility. The taxonomic identity, estimated abundance, and dates of introduction of targeted taxa are listed in Table 3.1. Endemic Hydrobiidae snails are an important component of the Devils Hole ecosystem, comprising approximately 80% of invertebrate mass (Wilson & Blinn 2007); snails were intentionally excluded from the refuge tank based on concerns about clogging the filtration system, and also due to their hypothesized role as competitors for food resources used by the pupfish.

Community assembly theory provides a framework for predicting community outcomes in different environments (Fukami 2010). It is hypothesized that assembly will follow a deterministic path in environmentally filtered communities: given an available species pool, harsh environmental conditions will select the community members. In contrast, community composition in amenable environments can be more structured by competitive interactions. This can lead to alternative community outcomes depending on the order of introduction or colonization, known as historical contingency or priority effects. The high-temperature, low-oxygen, oligotrophic environment of Devils Hole likely imposes environmental filtering on the community. If this is the case, we would expect the artificial refuge community to reach the same composition, assuming the environmental conditions were the same, and the available species pool included the same taxa found in Devils Hole.

We conducted research to compare the ecological communities in Devils Hole and the artificial refuge. If the abiotic regime of the refuge tank replicated the conditions of the Devils Hole environment, and seeding efforts led to the establishment of the taxa found in Devils Hole, we would expect the refuge tank community to have similar relative abundances of the same species, and the community would exhibit seasonal fluctuations parallel to the community in

Table 3.1. Dates and approximate abundances of all taxa intentionally seeded into the refuge tank. *Spirogyra* and cyanobacteria are listed by weight (g).

Inoculation Date	Rotifers	Paramecia	Other Ciliates	Nematodes	Copepods	Ostracods	Amphipods	Oligochaetes	*Spirogyra* (g)	Cyanobacteria (g)
6/28/2013	63,000	780,000	30,000	.	199	.	.	.	25	.
7/12/2013	20,000	100,000	10,000	.	100	.	.	.	25	.
8/12/2013	.	.	.	.	.	.	400	.	.	.
8/16/2013	28,000	41,250	.	.	2,500	.	175	.	25	.
8/30/2013	10,000	30,000	15,000	.	500	.	200	.	.	.
9/18/2013	570	.	.	.	.	.	.	570	23	.
9/20/2013	40,000	70,000	20,000	.	100	.	40	.	.	.
11/19/2013	.	.	.	.	.	.	106	.	.	.
11/30/2013	.	.	.	.	500	.	200	.	.	.
12/5/2013	22,200	6,000	3,000	9,000	.	.	.	.	60	20
1/7/2014	60,000	600	114,000	.	.	.	.	.	.	12
2/25/2014	56,750	50,250	35,000	19,500	.	.	.	126,000	.	.
3/6/2014	4,500	.	.	.	.	.	.	.	10	.
3/21/2014	.	.	.	.	.	.	.	.	5	.
10/15/2014	.	.	.	.	.	150	.	.	.	.
2/18/2015	.	.	.	.	.	3,504	.	2,280	.	.
2/23/2015	.	.	.	.	.	2,952	.	2,496	.	.
3/16/2015	.	.	.	.	.	2,652	.	2,142	.	.
6/29/2015	.	.	.	.	54	6,195	.	.	.	.
11/4/2015	10,000	.	25,000	3,000	.	6,500	.	.	.	.
TOTALS	**315,020**	**1,078,100**	**252,000**	**31,500**	**3,953**	**21,953**	**1,121**	**133,488**	**173**	**32**

Devils Hole. Alternatively, if abiotic and biotic differences exist between the two systems, the refuge tank community may show significantly different community composition and temporal variation. Finally, the timing of this research enabled observation of the effects of two disturbance events - moderate and severe flooding. This allowed us to assess the effects of a disturbance regime on the natural and constructed systems.

Methods

Sampling was conducted in Devils Hole and the refuge tank bimonthly (every other month) from December 2014 to November 2015 to survey community composition and variation over time. Parallel sampling was conducted in a nearby spring (School Spring in Ash Meadows National Wildlife Refuge) for use as a comparative 'outgroup.' We targeted all eukaryotic taxa using an environmental DNA - metagenetic approach. Approximately 300 mL collections were made from algal mats, the water column, and benthic sediments at five locations across the shallow shelf in each system, for a total of 15 samples per site, per sampling bout. One sample was also collected directly from the well that provides water to the refuge tank, to assess possible contributions of DNA to the refuge tank environment from organisms occurring in the groundwater (Hahn & Matzke 2005; Bradford *et al.* 2010). Although the filtration system prevents seeding of organisms from the groundwater system into the refuge tank, detection of their DNA in the refuge tank environment may influence the comparison between the refuge tank and Devils Hole ecological communities.

Laboratory processing

All samples were frozen until DNA extractions were performed. DNA was extracted from 600 µL of a representative aliquot of each sample. Water samples were centrifuged for eight minutes at 4,000 rpm prior to extraction. MO BIO PowerWater DNA extraction kits were used

(MO BIO Laboratories, Carlsbad, CA), following the manufacturer's protocol, including the additional cell lysis step (ten minutes at 65° C with PW1, prior to bead-beating).

Laboratory and sequence data processing followed that described in Chapter 1. Briefly, the V9 region of the 18S rDNA gene was amplified in triplicate with barcoded primers. Triplicate amplicons were pooled per sample, samples were pooled per 96-well plate, and plates were pooled into a single library. DNA quantitation was performed at each pooling step, to normalize the amount of DNA per sample in the final library. The DNA library was sequenced on the Illumina MiSeq platform, using a 300 cycle V2 kit (Illumina, San Diego, CA), resulting in ~150 bp paired-end reads.

Data processing

Sequence data were processed following the protocol outlined at github.com/leffj/data-tutorials/blob/master/amplicon_data_processing_tutorial/amplicon_data_processing-16S.md, modified for 18S data. See Chapter 1 for details. In short, paired-end reads were merged and processed to assemble a *de novo* database, and clustered at 97% similarity with the UCLUST algorithm (Edgar 2010). Taxonomy was assigned with the RDP classifier (Wang *et al.* 2007) using the SILVA 119 database (Quast *et al.* 2013).

Analyses

Bray Curtis distances from square-root transformed abundance data were used for statistical analysis of community data. Samples were rarefied to 1,640 sequences prior to analysis. All analyses were conducted in R (R Core Team 2015). Community differentiation between the two sites, and between habitat types within each site, was tested using the pairwise PERMANOVA (adonis) function in mctoolsr (Leff 2015). Community differentiation between the two sites was also analyzed with OTUs removed from the refuge tank data set that were detected in the well

water sample. Variation in community composition across the chronosequence was analyzed using the betadisper function in vegan (Oksanen *et al.* 2011). The similarity in community composition of the two sites was further assessed through comparison to the nearby 'outgroup' spring, using UPGMA clustering of whole community compositions. Whole community composition was estimated through summing OTU data for all samples per time point, per site; these collapsed data points were rarefied to 4,920 prior to analysis. The top taxa contributing to overall Bray Curtis dissimilarities were identified using the simper function in vegan. Simper analysis can be misleading, as it identifies taxa with wide variation in abundance within sites as well as between sites. Accordingly, the taxa identified in simper analysis were verified through reported abundance observations in the OTU table: taxa were maintained that showed variation between sites, and those that only varied within a site were discarded.

Changes in alpha diversity over the chronosequence, and after disturbance events, were visualized using rarefied abundance data per sample and per site, and absolute abundance data per site. Species richness and phylogenetic diversity (Faith's PD) were estimated. Because of observed differences in solar input to the two systems, we also tracked abundances of three major primary producer taxa: Chlorophyta, Charophyta, and Diatomea.

Results

Devils Hole and the refuge tank had significantly different community compositions for the duration of the chronosequence ($p = 0.001$, $R^2 = 0.13$, Figure 3.3). A total of 81 OTUs were detected in the well water sample (Table A12), 49 of which were also detected in the refuge tank during the chronosequence. The two communities maintained significant dissimilarity with the well OTUs removed from the refuge tank data set ($p = 0.001$, $R^2 = 0.15$). Within both sites there was differentiation among habitat-associated communities, though algal mat and benthic

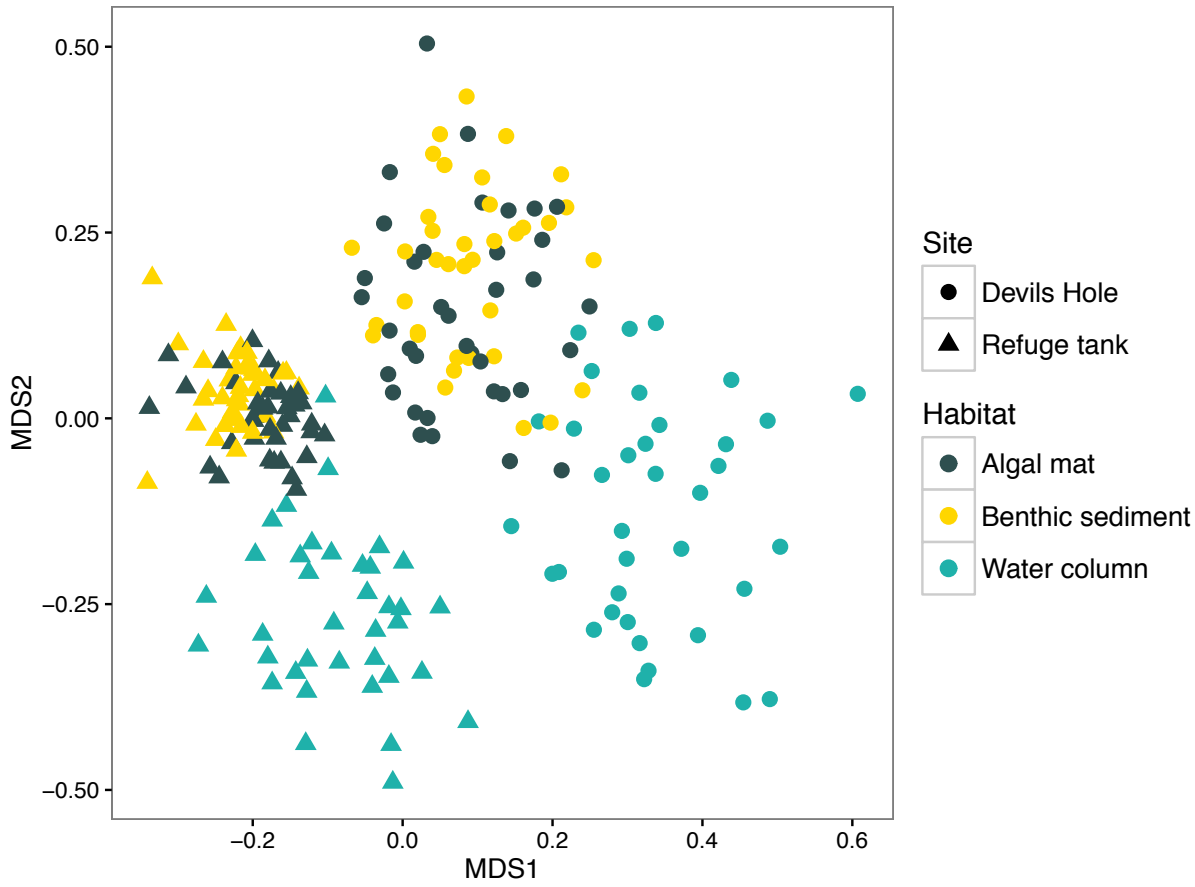

Figure 3.3. NMDS ordination of algal mat, benthic sediment, and water column samples collected between December 2014 and November 2015. The refuge tank and Devils Hole had significantly different community compositions ($p = 0.001$, $R^2 = 0.13$). Habitat-associated communities differed within sites, with greater overlap between benthic sediment and algal mat communities. Across all samples, greater variation in community composition occurred over the chronosequence in Devils Hole than the refuge tank (ANOVA $p < 0.001$, $F = 25.028$).

sediment communities showed more overlap with each other than with water column communities (refuge tank: $p = 0.002$ for all pairs, $R^2 = 0.19$ for algae and sediment vs. water column, $R^2 = 0.09$ for algae vs. sediment; Devils Hole: $p = 0.002$, $R^2 = 0.12$ for algae and sediment vs. water, and $p = 0.004$, $R^2 = 0.03$ for algae vs. sediment). In addition, greater variation in community composition was observed in Devils Hole than the constructed environment over the course of the chronosequence (ANOVA $p < 0.001$, $F = 25.028$). Although the two sites had significantly different community compositions, UPGMA clustering showed that Devils Hole

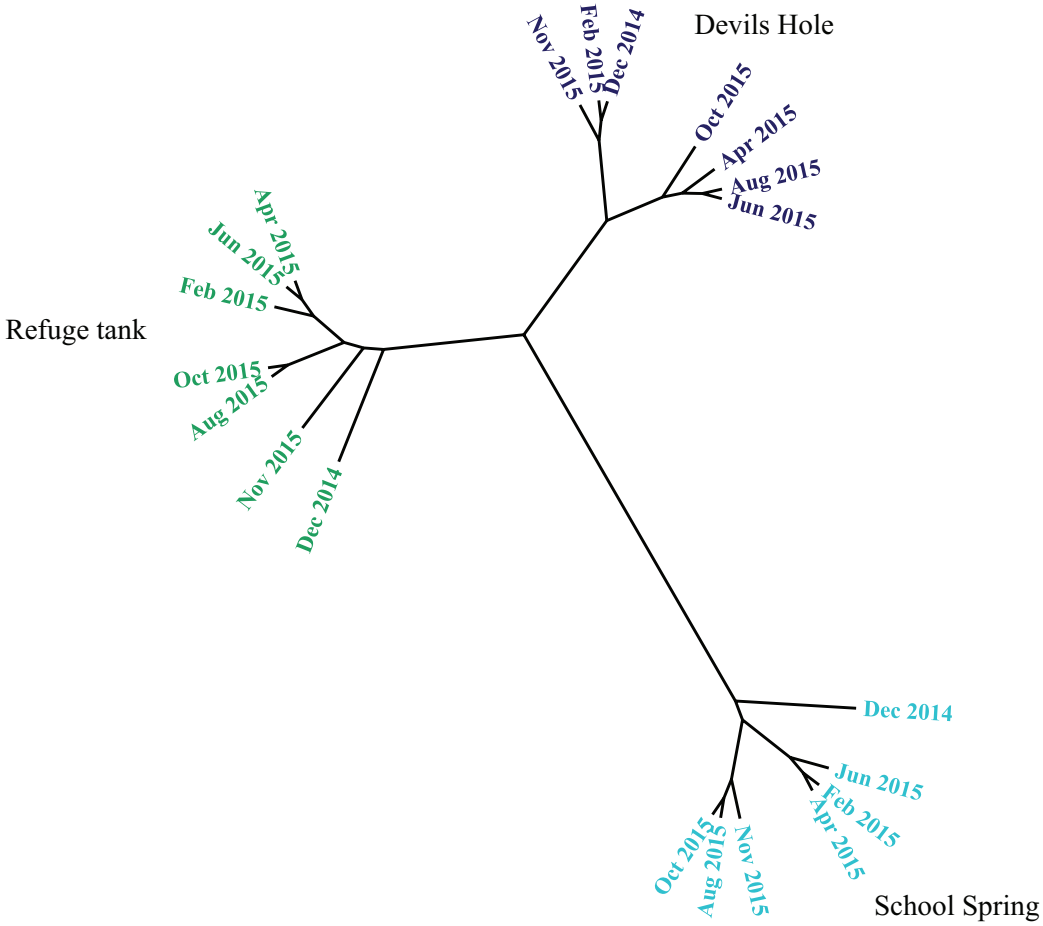

Figure 3.4. UPGMA clustering of whole community estimates per date, per site. Devils Hole is in navy, the refuge tank is in green, and School Spring is blue. Branch lengths are proportional to Bray Curtis dissimilarities. The refuge tank communities were more similar to those found in Devils Hole than School Spring.

and the refuge tank communities were more similar to each other than to those found in the outgroup comparison habitat, School Spring (Figure 3.4). Community composition exhibited temporal variation in both sites (Figure 3.5), however the trajectories did follow parallel paths, and the refuge tank community did not appear to be increasing in similarity to the Devils Hole community over the chronosequence.

The top organisms contributing to Bray Curtis dissimilarities were an annelid taxon in the refuge tank (98.2%), an unclassified animalia taxon in Devils Hole that had high sequence

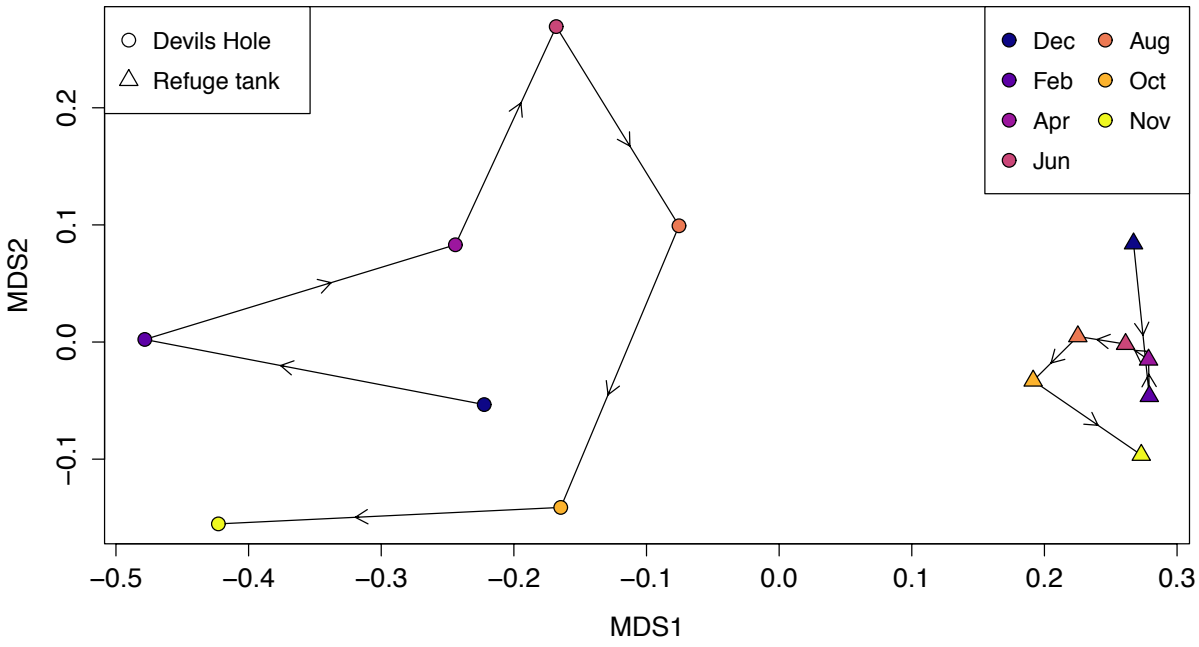

Figure 3.5. NMDS ordination of whole community estimates per site, per sample date, from Bray Curtis dissimilarities. The two sites changed along differing trajectories from December 2014 to November 2015, and the Devils Hole community showed wider variation over the chronosequence.

similarity to snails (96.5%), an annelid taxon in Devils Hole (92.8%), a dexiotrica ciliate taxon in Devils Hole (89%), two nematode taxa in the refuge tank (88% and 87%), a euplotes ciliate taxon in the refuge tank (85%), and a fungal taxon in the refuge tank (83%). These taxa contributed to over 80% of the community dissimilarity, as measured by Bray Curtis distances. Two of the taxa with comparatively high abundances in the refuge tank (the annelid and one of the nematodes) were also detected in the well water sample.

Between December 2014 and November 2015, 2,745 OTUs were detected across both sites. 758 OTUs were detected in both systems, 1,444 were only detected in Devils Hole, and 542 were only detected in the refuge tank. Most OTUs were rare, and few were detected at high abundances (Figure 3.6). Species richness and phylogenetic diversity showed more variation

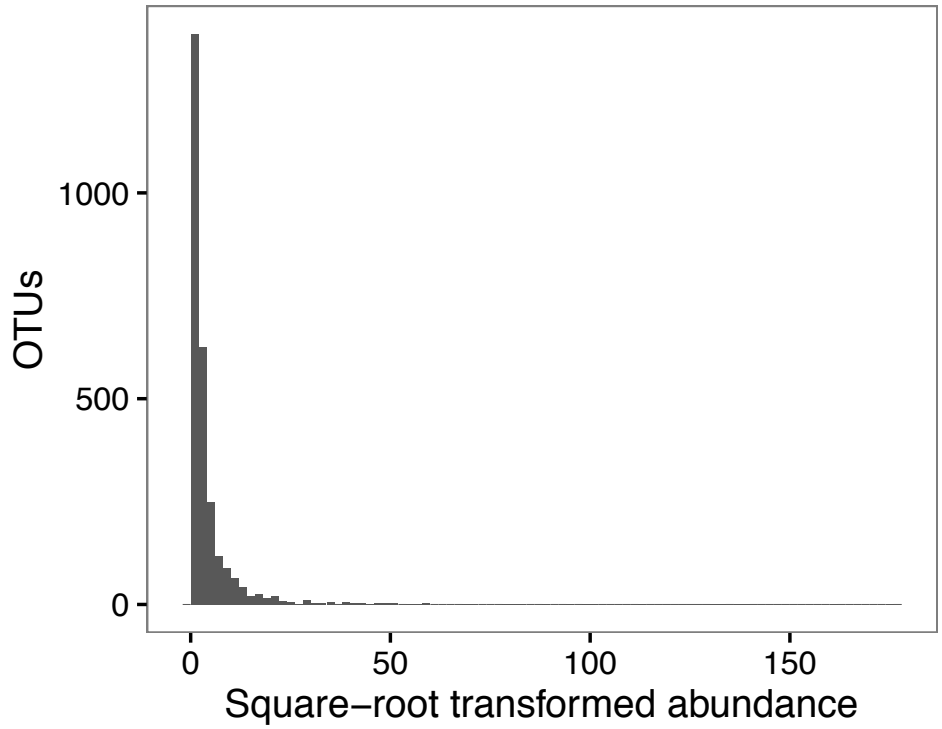

Figure 3.6. Histogram of square-root transformed abundance data for OTUs detected from December 2014 to November 2015 in Devils Hole and the refuge tank. Most OTUs were rare, and few were detected at high abundances.

between time points in Devils Hole than the refuge tank (Figure 3.7, species richness; Figure A3, phylogenetic diversity). Devils Hole alpha diversity had two peaks, in February and October, and reduced diversity in June. The refuge tank followed a similar pattern though with less pronounced variation.

Abundance of primary producers in Devils Hole peaked in June, while abundances of the same taxa in the refuge tank showed a less distinct pattern, with two low peaks in February and August, and overall lower abundances of charophyceaen and chlorophyceaen taxa (Figure 3.8). Charophyceaen algae (dominated by *Spirogyra*) peaked in abundance in June in Devils Hole, and in August in the refuge tank. This taxon maintained higher abundances in fall and winter months in the refuge tank than in Devils Hole.

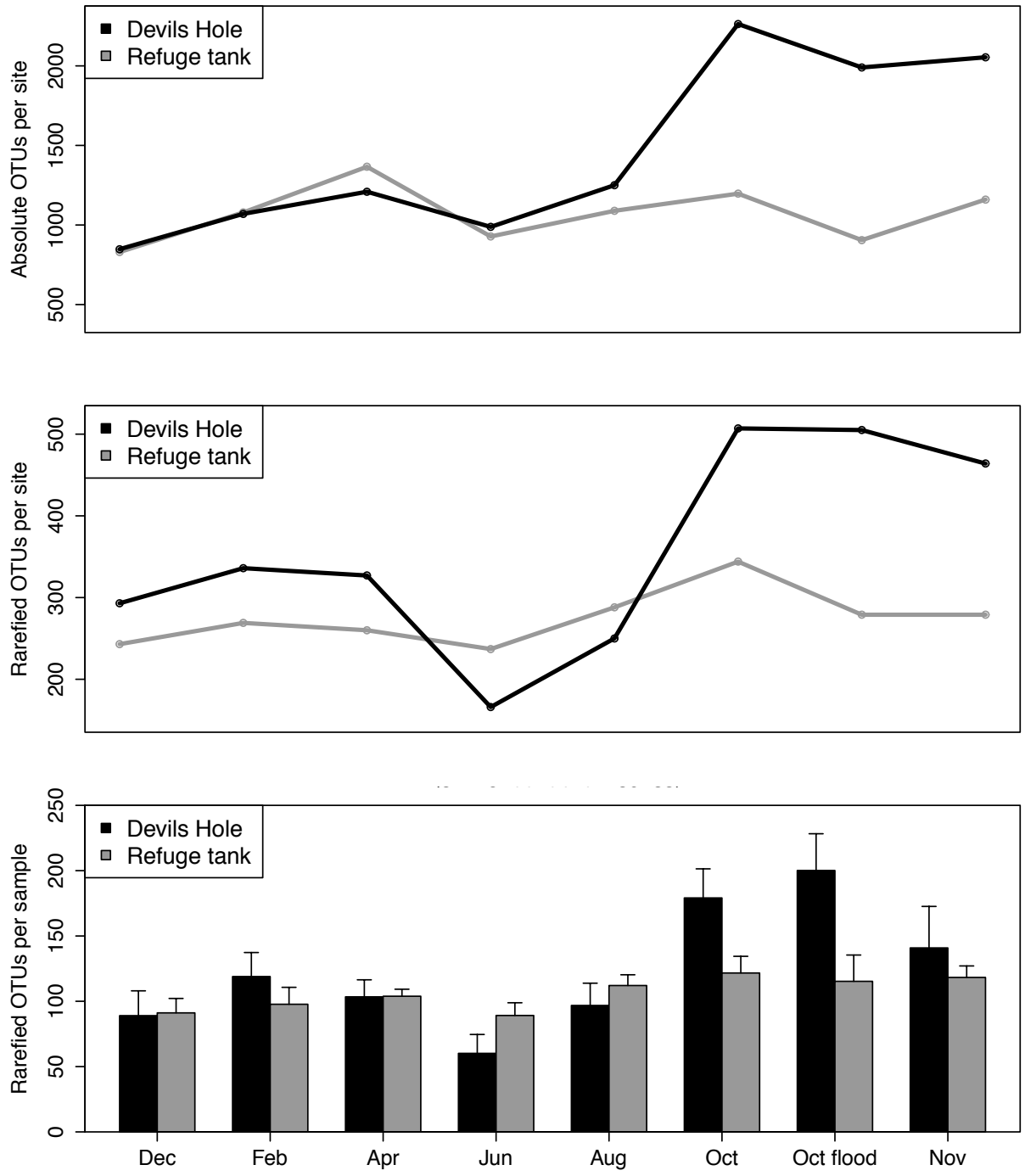

Figure 3.7. Species richness (observed OTUs) in Devils Hole (black) and the refuge tank (gray), between December 2014 and November 2015, including additional samples collected after the second major October flooding event ('Oct flood'). Sample data were rarefied to 1,640 sequences per sample prior to alpha diversity calculations (bottom). Site data were rarefied to 4,920 sequences per site (middle). The top plot shows species richness of non-rarefied data.

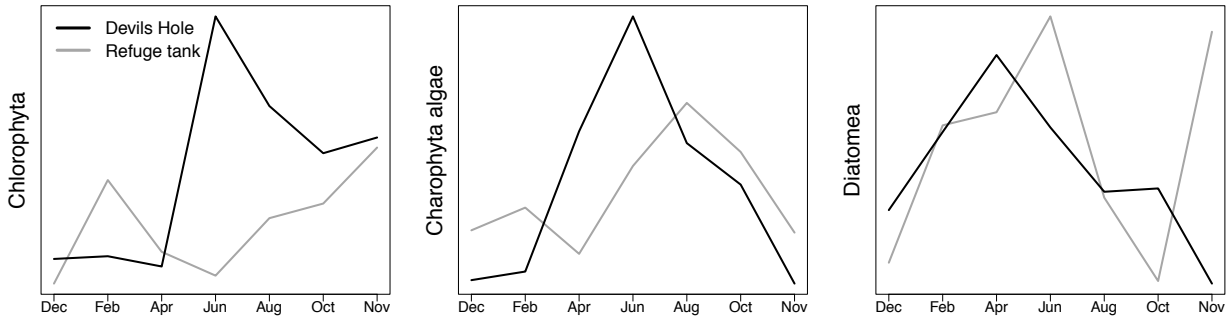

Figure 3.8. Square-root transformed abundances of Chlorophyta, Charophyta algae, and Diatomea in Devils Hole (black) and the refuge tank (gray), from December 2014 to November 2015. *Ankistrodesmus* was a numerically dominant chlorophyceaen taxon. Charophyta predominantly comprised *Spirogyra*.

Discussion

We conducted research to assess the similarity of the ecological communities in Devils Hole and its artificial analog, and found that the two sites differed in community composition, community variation over time, and patterns of alpha diversity. However, the community in the artificial habitat was more similar to that of Devils Hole than to a nearby 'outgroup' spring with similar water temperature and chemistry. The divergence in community composition between the natural and constructed habitats may be due to differences in environmental conditions and selective filtering of colonizers in the refuge tank.

The Devils Hole and refuge tank environments differed in deliberate ways for two parameters, and unintentionally for a third. The refuge tank had lower water temperature and higher dissolved oxygen to improve conditions for pupfish. In addition, the artificial system was designed to mimic the solar regime of Devils Hole, however mechanical problems with the louvered ceiling and greater exposure to the south through a glass wall resulted in direct sunlight reaching the refuge tank throughout the year. For example, direct solar radiation reached the surface of the pool for three hours and twenty-five minutes on December 22, the day after the

shortest day of the year (pers. comm., Luke Oliver, Great Basin Institute). In contrast, Devils Hole receives no direct sunlight on any day in December. In addition to these quantifiable differences, the two systems also differed in overall complexity. Devils Hole is connected to a deep aquifer with geologic and hydrologic complexity, and is located 15 m below the land surface at the bottom of a rocky crevasse with walls that have been shaped over geologic time. It is open to the surrounding landscape, and flooding events can result in streams and cascades of water that collect debris along the surrounding land surface and crevasse walls and pour into Devils Hole. Owls and bats roost in the cavern at the north end of Devils Hole, and vertebrate fecal pellets have been documented as an important source of nitrogen in the system (Wilson & Blinn 2007). In contrast, the refuge tank at the Fish Conservation Facility is enclosed in a manmade structure on top of the land surface. High precipitation events may wash debris from the louvered ceiling into the tank, but flooding across the surface of the surrounding landscape does not reach the tank.

These differences in environmental conditions may impose variable filters on possible colonizing organisms, potentially driving differences in community composition. The differences in solar input likely have consequences for patterns of primary production throughout the year (Diehl 2002; Tirok & Gaedke 2007), which may affect consumer population dynamics. We found differences in abundances of primary producers, particularly chlorophycean and charophycean algae, between Devils Hole and the refuge tank (Figure 3.8). Devils Hole algal taxa peaked in abundance in June, when insolation is highest, whereas the same taxa in the refuge tank peaked in the spring and fall. Diatoms in the two systems showed closer overlap of abundance patterns, with a peak in April in Devils Hole, and June in the refuge tank. However, diatom abundance in Devils Hole continued to decline after the summer months through the fall,

and it increased in the refuge tank between October and November. These differential patterns of primary producer population dynamics may also be influenced by available nutrients. Devils Hole is highly oligotrophic (Hausner *et al.* 2012), and nutrient cycling by macroinvertebrates may provide an important source of nitrogen: snails and benthic insects may contribute as much as 15-70% to algal nitrogen demand in oligotrophic desert systems (Grimm 1988). Snails were intentionally excluded from the refuge tank during the time period when our research was conducted, which may have caused differences in nutrient cycling. The peak in algal abundance in Devils Hole may have been facilitated by the combined effects of increased solar input and nitrogen availability from macroinvertebrate nutrient cycling, while nitrogen limitation in the refuge tank may have limited algal growth despite increased solar input during summer months. Allochthonous carbon is also an important nutrient source for Devils Hole (Wilson & Blinn 2007); the landscape position and relative containment of the refuge tank likely results in reduced allochthonous carbon inputs.

In addition to environmental differences between the two systems, community composition may have differed in the refuge tank due to stringent filtering of possible colonizers. Many taxa were targeted for propagation and introduction (Table 3.1), but these did not encompass the total biodiversity we detected in Devils Hole (2,202 OTUs). Traditional visual methods used to survey biodiversity in Devils Hole revealed only 15 macroinvertebrate species and approximately 80 algal taxa (Shepard *et al.* 2000; Herbst 2003; Wilson & Blinn 2007). This is a gross underestimate of the biodiversity in Devils Hole compared to the number of taxa detected with molecular methods, described here. This discrepancy suggests the existence of vast microbial, cryptic, or rare diversity in Devils Hole. Replicating this diversity in the refuge tank could be attained through more liberal seeding measures, and expansion of targeted taxa beyond those that

can be visually identified. Less stringent seeding may increase the probability of introduction of pathogens and parasites from the Devils Hole system into the refuge tank, but research has shown that the latter can be an important prey item in freshwater food webs (Lafferty *et al.* 2006; Johnson *et al.* 2010; Thieltges *et al.* 2013).

Finally, we were able to observe the effects of two heavy precipitation events - one moderate and one severe - on the natural and constructed habitats. The first event, on October 4-5, 2015, resulted in a small stream of water that ran into the southwest end of Devils Hole, and light cascades off the southern cliff walls. At the Fish Facility, the rain washed over the louvered ceiling into the refuge tank. This first moderate flooding event in October corresponded to an observed increase in alpha diversity in both systems (Figure 3.7), with a greater increase observed in Devils Hole. The second flooding event on October 18 was severe. Water flooded into Devils Hole in a torrent, depositing a mound of rocky debris on the shallow shelf that rose above the surface of the water. This event imposed a moderate to severe disturbance on the Devils Hole ecosystem, resulting in reduced algal cover on the shallow shelf due to sediment deposition. In contrast, this second, severe flooding event did not have an equivalent, severe impact on the refuge tank. Although a greater volume of water from the higher level of precipitation likely passed through the louvered ceiling into the tank, it was not magnified by accumulation across the land surface as was the case for Devils Hole. Samples were collected in both systems approximately ten days after this second flooding event; these samples showed higher alpha diversity per sample in Devils Hole than the samples collected earlier in the month (though the difference was not significant). The same increase was not observed for the refuge tank, where alpha diversity actually decreased after the second flood. The different effects of these disturbance events on the two systems may have provided an additional source of

variability between the natural and artificial environments, which could have led to differences in community composition either due to the force of the disturbance (Sousa 1984; Resh *et al.* 1988; McCabe & Gotelli 2000), or due to differential fluvial dispersal of organisms into the two systems.

Conclusions

Observed differentiation in the communities of the two systems was likely due to differences in environment and limitations on colonization of the refuge tank by taxa that were present in Devils Hole. Similarity in community composition between the two systems may be increased by limiting or eliminating the differences in water temperature, dissolved oxygen, and solar input in the refuge tank, and facilitating colonization of the tank community by a more complete set of taxa that are present in Devils Hole. Although the persistence of the refuge pupfish population may depend on more than the biotic community of which they are apart (i.e., genetic issues may be a major factor), past refuge attempts highlight the importance of the total Devils Hole environment in conservation of the phenotype and genotype of the Devils Hole pupfish (Wilcox & Martin 2006).

Without monitoring, ecological community differentiation between Devils Hole and the refuge tank may not have been detected. As is also the case for ecological restoration projects, monitoring of community change over time provides quantitative feedback about progress towards ecological goals, as seeding of targeted taxa does not always yield the expected community outcome (Suding *et al.* 2004). The methods described here facilitated rapid, broad surveys of thousands of species, providing a time- and cost-effective tool for informative and quantitative ecological monitoring. Finally, efforts to conserve the Devils Hole pupfish are an example of the importance of establishing goals for management actions. If the goal was to

propagate an offsite population of this species, and genetic and morphological identity were not imperative, then fewer resources would have been needed to create a suitable environment, as exact replication of the natural habitat would not be necessary. However, if the goal was to propagate and harbor a backup population of Devils Hole pupfish with the same genetic and morphological characteristics as the natural population, then the environment of the refuge tank should replicate that of Devils Hole as closely as possible given the available infrastructure. While the refuge tank environment is clearly similar to Devils Hole, there are still many differences that may ultimately result in the emergence of pupfish that are demonstrably different from the Devils Hole pupfish.

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CONCLUSION

Freshwater ecosystems are of high conservation concern due to their dual importance for biodiversity and as a human resource (Dudgeon *et al.* 2006; Vörösmarty *et al.* 2010). Understanding community ecology of freshwater systems, and how they can be restored, will inform conservation and management practices into the future. In the first chapter of my dissertation, I described environmental variables that were correlated with community composition in the springs of Ash Meadows, Nevada, as well as patterns of alpha diversity and species turnover rates. I found that water temperature, spring size, and invasion status (presence or absence of invasive crayfish or largemouth bass) were significantly correlated with community composition across springs, and were also significantly related to species richness and phylogenetic diversity. These results have direct implications for freshwater ecosystems experiencing the effects of global change phenomena, including climate change, biological invasions, and groundwater extraction. Projected increases in temperature and decreased precipitation in the Desert Southwest (Seager *et al.* 2007), in conjunction with aquifer pumping, may drive shifts in desert spring community compositions with consequences for ecosystem functions and services (Aylward *et al.* 2005; O'Gorman *et al.* 2012; Griebler & Avramov 2015). Additionally, the results of the research described in Chapter 1 provide further evidence of the

utility of springs as sentinel systems (O'Gorman *et al.* 2014); observed changes to spring communities in desert landscapes may be an early indicator of aquifer over-draft or other direct environmental changes, highlighting the need for immediate management actions.

In Chapter 2, I described community composition change over time during and after restoration of a low-flow, high-temperature spring. I found that the community followed a trajectory of change first away from and then back towards the pre-restoration community composition. In addition, the trajectory of community change in the restored spring was directed towards adjacent, environmentally similar springs with desirable community qualities. The final sampling date revealed a restored community composition that was as similar to the reference springs as those springs were to each other. This successful restoration outcome may have been due to the combined effects of environmental selection of the ecological community, availability of colonizers through natural and managed dispersal, and the reintroduction of pupfish, a key omnivorous species in these systems. These results indicate the utility of the implemented restoration protocol for this type of system, provided that community members - including microbial and protistan species - are able to reach the restored habitat.

In Chapter 3, I described the community ecology of a natural habitat - Devils Hole - and its constructed analog, a refuge tank at the Ash Meadows Fish Conservation Facility. The refuge tank was a multi-million dollar endeavor designed to precisely mimic Devils Hole, including the biotic community of organisms, to harbor a backup population of the endangered Devils Hole pupfish (*Cyprinodon diabolis*). I found significant differences between the two ecological communities, both in species composition and seasonal variation. Divergence between the two communities may have been caused by environmental differences, dispersal limitation into the refuge tank caused by stringent seeding protocols, or overall differences in the inherent

complexity of the natural and manmade habitats. The different environment of the refuge tank may result in genetic and phenotypic divergence of the pupfish population being propagated there (Wilcox & Martin 2006).

The results of my dissertation research highlight the importance of identifying goals and monitoring in conservation work. If the goal for conservation of the Devils Hole pupfish is only to propagate fish and harbor an offsite population, then differences in the two habitats may not matter. However, if the goal is to maintain the genetic and phenotypic identity of the species, then environmental differences in the two habitats should be addressed. Ecological monitoring of the refuge tank environment provided quantitative evidence of differences in the two communities that may not have been detected through visual observation, or day-to-day operations: the refuge tank did not follow the same community trajectory across seasons as the natural habitat, and targeted seeding of Devils Hole biota into the refuge tank did not encompass the diversity of organisms found in the natural habitat. In contrast, monitoring of community change during succession of the restored spring (Chapter 2) revealed the effectiveness of the implemented restoration practices in achieving the desired ecological outcome. Identification of specific ecological goals, and chronosequence monitoring to quantify progress towards those goals, are important components for successful implementation of habitat restoration or creation projects.

Conservation of freshwater ecosystems is important for obvious reasons, but do desert springs matter? What is lost when a desert spring dries up, and the ecological community it supported blinks out? The loss of a desert spring ecosystem has local and regional implications, as it could involve the extinction of endemic species found nowhere else in the world, and it also results in one less stepping stone for migratory birds or dispersing organisms across an arid

landscape. The loss of desert springs also represents a lost opportunity to learn about evolution: years of spatial isolation has resulted in unique evolutionary histories of desert spring species and communities. Finally, desert springs have cultural value beyond the biodiversity they support, in the sense of amazement about the natural world elicited in human visitors. Standing at the edge of a deep, clear pool of water in an otherwise desolate landscape is an experience that incurs wonder and curiosity, especially upon discovery of bright blue pupfish darting across the algae-clad aquatic landscape. This experience can inspire scientific inquiry, conservation action, or even change the daily motions of a person newly aware of the importance of water to beings other than themselves. The conservation of desert springs means preservation of biodiversity, as well as scientific and educational opportunity that reaches beyond the span of the desert.

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APPENDIX

Table A1. Sampling dates for all sites utilized in Chapter 1.

Spring	Abr.	Nov. 2012	Nov. 2013	May 2014	Dec. 2014
Bradford_1	B1		X		
Bradford_2	B2	X	X		
Cold	CO		X		
Cottonwood	CW		X	X	
Crystal	CY		X		
Crystal Reservoir	CR		X		
Davis	DA		X		
Devils Hole	DH				X
Fairbanks	FA	X	X		
Forest	FO		X		
Jackrabbit	JA	X	X	X	
Kings Pool	KP	X	X	X	
Longstreet	LO	X	X	X	
Marsh	MA	X	X	X	
North Indian	NI	X	X	X	
North Scruggs	NS	X	X	X	
Peterson Reservoir	PE		X		
Point of Rocks	PR	X	X		
Rogers	RO	X	X	X	
School	SC	X	X	X	
South Indian	SI		X	X	
South Scruggs	SS	X			
Tubbs	TU		X		

Table A2. Results of simple linear regressions between environmental variables.

Variables	*p*	R^2
Temperature - Elevation	< 0.001	0.53
Temperature - Size	< 0.001	0.40
Temperature - Distance to aquifer	< 0.001	0.66
Size - Elevation	0.004	0.30
Size - Distance to aquifer	0.002	0.35

Table A3. Results of Welch's t-tests on environmental characteristics of invaded springs.

Variable	***p***	***t***
Size	< 0.001	-5.5
Elevation	0.02	2.8
Temperature	< 0.001	4.4
Distance to aquifer	< 0.001	-4.3

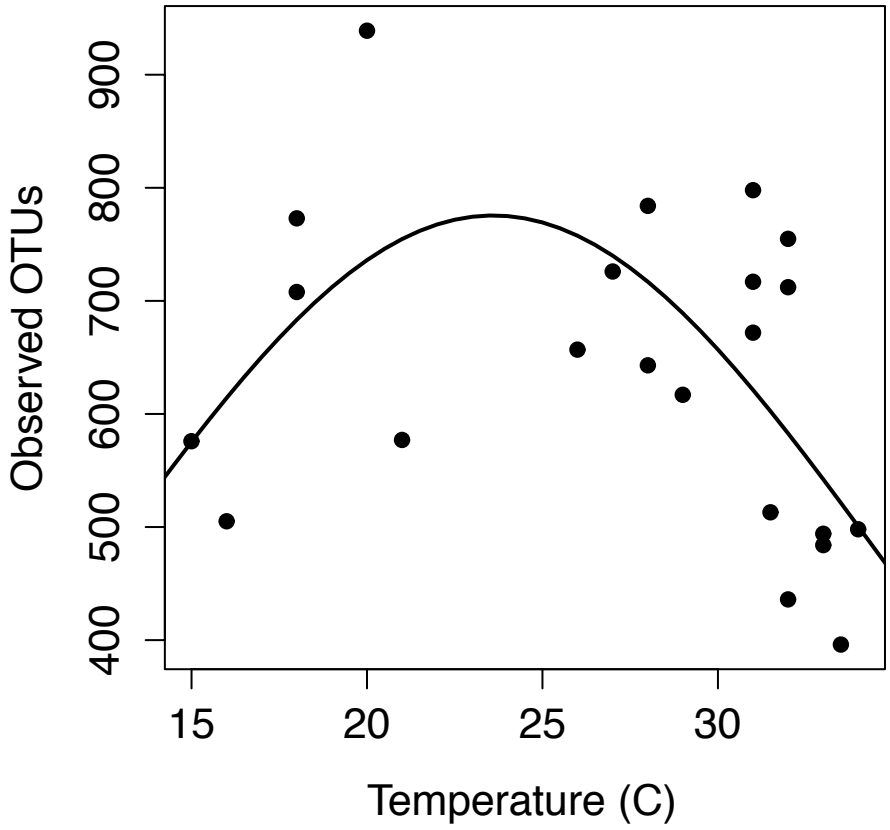

Figure A1. Species richness (observed OTUs) modeled by temperature for sites described in Chapter 1 (randomization test $p = 0.009$, Gaussian model -ln(L) = 134.33, Gaussian model AIC = 274.66, linear model -ln(L) = 138.19, linear model AIC = 280.38). The line shows the predicted relationship under the Gaussian model (see Chapter 1 Methods for the equation).

Table A4. Results of PERMANOVA pairwise comparisons between time points for the restored spring (SS). FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

SS	Jan 2015	Feb 2015	Mar 2015	Apr 2015	May 2015	Jun 2015	Aug 2015	Oct 2015	Nov 2015
Dec 2014	**$p = 0.002$, $R^2 = 0.16$**	**$p = 0.002$, $R^2 = 0.17$**	**$p = 0.002$, $R^2 = 0.19$**	**$p = 0.002$, $R^2 = 0.26$**	**$p = 0.002$, $R^2 = 0.24$**	**$p = 0.002$, $R^2 = 0.21$**	**$p = 0.002$, $R^2 = 0.24$**	**$p = 0.002$, $R^2 = 0.22$**	**$p = 0.002$, $R^2 = 0.22$**
Jan 2015		**$p = 0.002$, $R^2 = 0.09$**	**$p = 0.002$, $R^2 = 0.12$**	**$p = 0.002$, $R^2 = 0.19$**	**$p = 0.002$, $R^2 = 0.19$**	**$p = 0.002$, $R^2 = 0.15$**	**$p = 0.002$, $R^2 = 0.15$**	**$p = 0.002$, $R^2 = 0.16$**	**$p = 0.002$, $R^2 = 0.16$**
Feb 2015			**$p = 0.002$, $R^2 = 0.07$**	**$p = 0.002$, $R^2 = 0.15$**	**$p = 0.002$, $R^2 = 0.15$**	**$p = 0.002$, $R^2 = 0.11$**	**$p = 0.002$, $R^2 = 0.14$**	**$p = 0.002$, $R^2 = 0.14$**	**$p = 0.002$, $R^2 = 0.14$**
Mar 2015				**$p = 0.002$, $R^2 = 0.13$**	**$p = 0.002$, $R^2 = 0.12$**	**$p = 0.002$, $R^2 = 0.10$**	**$p = 0.002$, $R^2 = 0.14$**	**$p = 0.002$, $R^2 = 0.15$**	**$p = 0.002$, $R^2 = 0.15$**
Apr 2015					**$p = 0.002$, $R^2 = 0.08$**	**$p = 0.002$, $R^2 = 0.09$**	**$p = 0.002$, $R^2 = 0.18$**	**$p = 0.002$, $R^2 = 0.21$**	**$p = 0.002$, $R^2 = 0.21$**
May 2015						**$p = 0.02$, $R^2 = 0.06$**	**$p = 0.002$, $R^2 = 0.17$**	**$p = 0.002$, $R^2 = 0.19$**	**$p = 0.002$, $R^2 = 0.20$**
Jun 2015							**$p = 0.002$, $R^2 = 0.09$**	**$p = 0.002$, $R^2 = 0.13$**	**$p = 0.002$, $R^2 = 0.13$**
Aug 2015								**$p = 0.002$, $R^2 = 0.09$**	**$p = 0.002$, $R^2 = 0.11$**
Oct 2015									**$p = 0.002$, $R^2 = 0.08$**

Table A5. Results of PERMANOVA pairwise comparisons between sample sites for the restored spring (SS). FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

SS	Outflow 3	Outflow 2	Outflow 1	Springhead
Outflow 4	$p = 0.73$, $R^2 = 0.01$	$p = 0.14$, $R^2 = 0.02$	**$p = 0.006$, $R^2 = 0.03$**	**$p = 0.004$, $R^2 = 0.06$**
Outflow 3		$p = 0.70$, $R^2 = 0.02$	$p = 0.10$, $R^2 = 0.02$	**$p = 0.004$, $R^2 = 0.05$**
Outflow 2			$p = 0.23$, $R^2 = 0.02$	**$p = 0.004$, $R^2 = 0.05$**
Outflow 1				**$p = 0.004$, $R^2 = 0.03$**

Table A6. Results of PERMANOVA pairwise comparisons between time points for SC, a reference spring. FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

SC	Jan 2015	Feb 2015	Mar 2015	Apr 2015	May 2015	Jun 2015	Aug 2015	Oct 2015	Nov 2015
Dec 2014	$p = 0.75$, $R^2 = 0.03$	$p = 0.45$, $R^2 = 0.04$	$p = 0.31$, $R^2 = 0.04$	$p = 0.15$, $R^2 = 0.05$	$p = 0.17$, $R^2 = 0.05$	$p = 0.11$, $R^2 = 0.06$	$p = 0.07$, $R^2 = 0.06$	$p = 0.12$, $R^2 = 0.05$	$p = 0.17$, $R^2 = 0.05$
Jan 2015		$p = 0.93$, $R^2 = 0.02$	$p = 0.45$, $R^2 = 0.04$	$p = 0.47$, $R^2 = 0.03$	$p = 0.37$, $R^2 = 0.04$	$p = 0.07$, $R^2 = 0.06$	$p = 0.06$, $R^2 = 0.06$	$p = 0.12$, $R^2 = 0.05$	$p = 0.30$, $R^2 = 0.04$
Feb 2015			$p = 0.48$, $R^2 = 0.04$	$p = 0.47$, $R^2 = 0.04$	$p = 0.36$, $R^2 = 0.04$	$p = 0.15$, $R^2 = 0.05$	$p = 0.07$, $R^2 = 0.06$	$p = 0.14$, $R^2 = 0.05$	$p = 0.22$, $R^2 = 0.04$
Mar 2015				$p = 0.21$, $R^2 = 0.05$	$p = 0.73$, $R^2 = 0.03$	$p = 0.17$, $R^2 = 0.05$	$p = 0.07$, $R^2 = 0.06$	$p = 0.09$, $R^2 = 0.05$	$p = 0.22$, $R^2 = 0.04$
Apr 2015					$p = 0.76$, $R^2 = 0.03$	$p = 0.20$, $R^2 = 0.04$	$p = 0.08$, $R^2 = 0.06$	$p = 0.06$, $R^2 = 0.06$	$p = 0.07$, $R^2 = 0.06$
May 2015						$p = 0.74$, $R^2 = 0.03$	$p = 0.17$, $R^2 = 0.05$	$p = 0.07$, $R^2 = 0.06$	$p = 0.12$, $R^2 = 0.05$
Jun 2015							$p = 0.30$, $R^2 = 0.04$	$p = 0.09$, $R^2 = 0.06$	$p = 0.08$, $R^2 = 0.06$
Aug 2015								$p = 0.36$, $R^2 = 0.04$	$p = 0.17$, $R^2 = 0.05$
Oct 2015									$p = 0.33$, $R^2 = 0.04$

Table A7. Results of PERMANOVA pairwise comparisons between sample sites for SC, a reference spring. FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

SC	Outflow 3	Outflow 2	Outflow 1	Springhead
Outflow 4	**$p = 0.002$, $R^2 = 0.09$**	**$p = 0.002$, $R^2 = 0.10$**	**$p = 0.002$, $R^2 = 0.16$**	**$p = 0.002$, $R^2 = 0.20$**
Outflow 3		**$p = 0.002$, $R^2 = 0.07$**	**$p = 0.002$, $R^2 = 0.11$**	**$p = 0.002$, $R^2 = 0.15$**
Outflow 2			**$p = 0.002$, $R^2 = 0.13$**	**$p = 0.002$, $R^2 = 0.17$**
Outflow 1				**$p = 0.002$, $R^2 = 0.09$**

Table A8. Results of PERMANOVA pairwise comparisons between time points for NI, a reference spring. FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

NI	Jan 2015	Feb 2015	Mar 2015	Apr 2015	May 2015	Jun 2015
Dec 2014	$p = 0.20$, $R^2 = 0.04$	**$p = 0.02$, $R^2 = 0.06$**	$p = 0.08$, $R^2 = 0.05$	**$p = 0.01$, $R^2 = 0.07$**	**$p = 0.009$, $R^2 = 0.07$**	**$p = 0.009$, $R^2 = 0.07$**
Jan 2015		$p = 0.10$, $R^2 = 0.05$	$p = 0.40$, $R^2 = 0.04$	**$p = 0.01$, $R^2 = 0.07$**	**$p = 0.02$, $R^2 = 0.06$**	**$p = 0.01$, $R^2 = 0.06$**
Feb 2015			$p = 0.27$, $R^2 = 0.04$	$p = 0.21$, $R^2 = 0.04$	$p = 0.08$, $R^2 = 0.05$	$p = 0.08$, $R^2 = 0.05$
Mar 2015				$p = 0.08$, $R^2 = 0.05$	$p = 0.46$, $R^2 = 0.03$	$p = 0.08$, $R^2 = 0.05$
Apr 2015					$p = 0.07$, $R^2 = 0.05$	$p = 0.08$, $R^2 = 0.05$
May 2015						$p = 0.42$, $R^2 = 0.04$

Table A9. Results of PERMANOVA pairwise comparisons between sample sites for NI, a reference spring. FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

NI	Outflow 3	Outflow 2	Outflow 1	Springhead
Outflow 4	$p = 0.08$, $R^2 = 0.03$	**$p = 0.002$, $R^2 = 0.07$**	**$p = 0.002$, $R^2 = 0.08$**	**$p = 0.002$, $R^2 = 0.11$**
Outflow 3		**$p = 0.002$, $R^2 = 0.06$**	**$p = 0.002$, $R^2 = 0.06$**	**$p = 0.002$, $R^2 = 0.11$**
Outflow 2			**$p = 0.007$, $R^2 = 0.04$**	**$p = 0.002$, $R^2 = 0.15$**
Outflow 1				**$p = 0.002$, $R^2 = 0.13$**

Table A10. Results of PERMANOVA pairwise comparisons between time points for NS a reference spring. FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

NS	Jan 2015	Feb 2015	Mar 2015	Apr 2015	May 2015	Jun 2015
Dec 2014	$p = 0.12$, $R^2 = 0.05$	$p = 0.11$, $R^2 = 0.06$	$p = 0.27$, $R^2 = 0.04$	$p = 0.08$, $R^2 = 0.07$	$p = 0.25$, $R^2 = 0.05$	**$p = 0.02$, $R^2 = 0.07$**
Jan 2015		$p = 0.36$, $R^2 = 0.04$	$p = 0.27$, $R^2 = 0.04$	$p = 0.11$, $R^2 = 0.05$	$p = 0.28$, $R^2 = 0.05$	$p = 0.08$, $R^2 = 0.06$
Feb 2015			$p = 0.25$, $R^2 = 0.04$	$p = 0.37$, $R^2 = 0.04$	$p = 0.48$, $R^2 = 0.04$	$p = 0.12$, $R^2 = 0.05$
Mar 2015				$p = 0.12$, $R^2 = 0.05$	$p = 0.37$, $R^2 = 0.04$	$p = 0.08$, $R^2 = 0.06$
Apr 2015					$p = 0.48$, $R^2 = 0.04$	$p = 0.49$, $R^2 = 0.04$
May 2015						$p = 0.21$, $R^2 = 0.05$

Table A11. Results of PERMANOVA pairwise comparisons between sample sites for NS a reference spring. FDR p values and R^2 values are reported; bolded text indicates $p < 0.05$.

NS	Outflow 3	Outflow 2	Outflow 1	Springhead
Outflow 4	**$p = 0.03$, $R^2 = 0.04$**	**$p = 0.002$, $R^2 = 0.10$**	**$p = 0.002$, $R^2 = 0.12$**	**$p = 0.002$, $R^2 = 0.09$**
Outflow 3		**$p = 0.002$, $R^2 = 0.09$**	**$p = 0.002$, $R^2 = 0.11$**	**$p = 0.002$, $R^2 = 0.08$**
Outflow 2			$p = 0.34$, $R^2 = 0.03$	**$p = 0.002$, $R^2 = 0.06$**
Outflow 1				**$p = 0.002$, $R^2 = 0.06$**

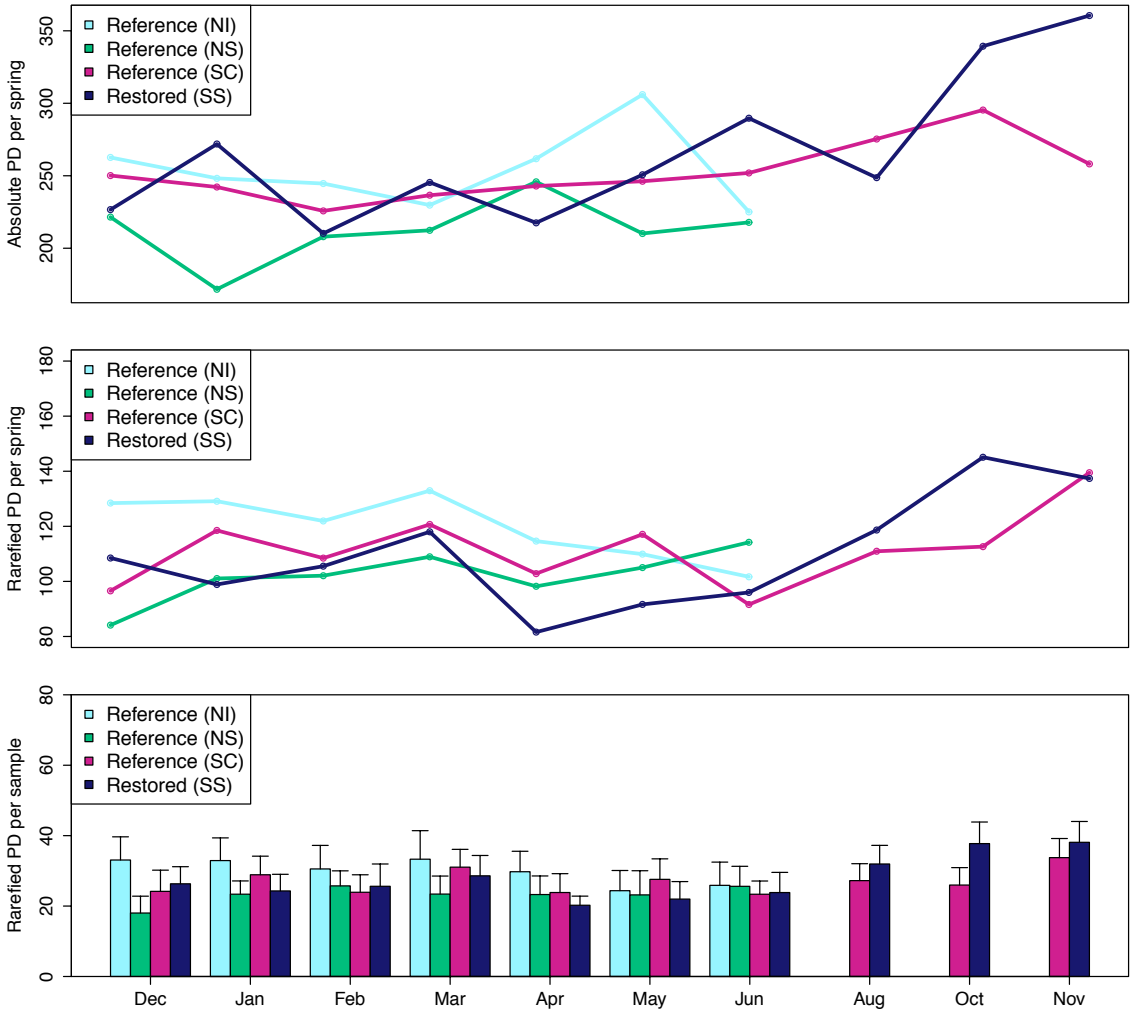

Figure A2. Absolute phylogenetic diversity (Faith's PD) for the four springs from December 2014 to November 2015 (top), rarefied PD per spring (middle), and rarefied PD per sample (bottom). Samples were rarefied to 676 sequences. Compiled samples per spring were rarefied to 8878 sequences. Phylogenetic diversity in NI decreased over time ($p = 0.02$, $R^2 = 0.72$) and NS increased ($p = 0.04$, $R^2 = 0.59$). SC and SS showed no directional change over time.

Table A12. OTUs detected in the well water sample from the Ash Meadows Fish Conservation Facility, with RDP classifications and sequence abundances.

OTU ID	Abundance	Taxonomy
OTU_18	82	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Charales; D_7__Chara
OTU_251	7	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta
OTU_17510	3	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta
OTU_609	33	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta; D_10__Brassicales
OTU_253	136	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta; D_10__Fabales
OTU_20	9	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta; D_10__Fabales
OTU_45	60	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta; D_10__Liliopsida; D_11__Poales; D_12__Zea
OTU_24670	1	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Streptophyta; D_6__Embryophyta; D_7__Tracheophyta; D_8__Spermatophyta; D_9__Magnoliophyta; D_10__Liliopsida; D_11__Poales; D_12__Zea
OTU_4	24	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Charophyta; D_4__Phragmoplastophyta; D_5__Zygnematales; D_6__Spirogyra
OTU_100	20	D_1__Archaeplastida; D_2__Chloroplastida; D_3__Chlorophyta; D_4__Chlorophyceae
OTU_5493	54	D_1__Cryptophyceae; D_2__Goniomonas
OTU_7567	28	D_1__Cryptophyceae; D_2__Goniomonas
OTU_789	6	D_1__Excavata; D_2__Discoba; D_3__Discicristata; D_4__Euglenozoa; D_5__Euglenida; D_6__Heteronematina; D_7__Petalomonas
OTU_423	36	D_1__Excavata; D_2__Discoba; D_3__Discicristata; D_4__Euglenozoa; D_5__Kinetoplastea; D_6__Metakinetoplastina; D_7__Neobodonida;

		D_8__Neobodo
OTU_35458	3	D_1__Opisthokonta
OTU_35747	2	D_1__Opisthokonta
OTU_8	351	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Annelida
OTU_16219	10	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Annelida
OTU_34172	1	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Annelida
OTU_5166	27	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Arthropoda; D_6__Hexapoda; D_7__Insecta; D_8__Gerridae sp.
OTU_540	57	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Craniata
OTU_33	104	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Craniata; D_6__Mammalia
OTU_122	110	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Gastrotricha; D_6__Chaetonotidae
OTU_11	31	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Gastrotricha; D_6__Chaetonotidae
OTU_19	6	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Nematoda; D_6__Enoplea
OTU_85	13	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Nematoda; D_6__Enoplea; D_7__Mononchidae
OTU_238	20	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Platyhelminthes
OTU_363	34	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Platyhelminthes; D_6__Turbellaria; D_7__Catenulida
OTU_149	26	D_1__Opisthokonta; D_2__Holozoa; D_3__Metazoa; D_4__Animalia; D_5__Platyhelminthes; D_6__Turbellaria; D_7__Catenulida
OTU_318	21	D_1__Opisthokonta; D_2__Nucletmycea
OTU_9420	133	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina
OTU_133	1	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina

OTU_32863	1	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina
OTU_4055	5	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina; D_7__Dothideomycetes
OTU_82	193	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina; D_7__Dothideomycetes; D_8__Pleosporales; D_9__uncultured fungus
OTU_21372	14	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina; D_7__Eurotiomycetes; D_8__Eurotiales; D_9__Trichocomaceae; D_10__Aspergillus
OTU_5910	1	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Pezizomycotina; D_7__Sordariomycetes; D_8__Hypocreales
OTU_2797	21	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Saccharomycotina; D_7__Saccharomycetes; D_8__Saccharomycetales
OTU_35552	2	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Saccharomycotina; D_7__Saccharomycetes; D_8__Saccharomycetales
OTU_3210	31	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Saccharomycotina; D_7__Saccharomycetes; D_8__Saccharomycetales; D_9__Incertae Sedis; D_10__Candida
OTU_608	18	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Ascomycota; D_6__Saccharomycotina; D_7__Saccharomycetes; D_8__Saccharomycetales; D_9__Saccharomycetaceae
OTU_6907	6	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Agaricomycotina; D_7__Agaricomycetes
OTU_439	18	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Agaricomycotina; D_7__Tremellomycetes; D_8__Cystofilobasidiales; D_9__Cystofilobasidiaceae
OTU_931	35	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Agaricomycotina; D_7__Tremellomycetes; D_8__Tremellales
OTU_34061	2	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Agaricomycotina; D_7__Tremellomycetes; D_8__Tremellales
OTU_6739	13	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Pucciniomycotina; D_7__Microbotryomycetes; D_8__Sporidiobolales

OTU_2149	69	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Pucciniomycotina; D_7__Microbotryomycetes; D_8__Sporidiobolales; D_9__Incertae Sedis; D_10__Rhodotorula
OTU_19680	1	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Ustilaginomycotina; D_7__Exobasidiomycetes; D_8__Malasseziales; D_9__Incertae Sedis; D_10__Malassezia
OTU_27002	1	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Ustilaginomycotina; D_7__Exobasidiomycetes; D_8__Malasseziales; D_9__Incertae Sedis; D_10__Malassezia
OTU_29137	1	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Ustilaginomycotina; D_7__Exobasidiomycetes; D_8__Malasseziales; D_9__Incertae Sedis; D_10__Malassezia
OTU_27972	60	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Ustilaginomycotina; D_7__Exobasidiomycetes; D_8__Malasseziales; D_9__Incertae Sedis; D_10__Malassezia; D_11__uncultured fungus
OTU_37946	89	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Dikarya; D_5__Basidiomycota; D_6__Ustilaginomycotina; D_7__Exobasidiomycetes; D_8__Malasseziales; D_9__Incertae Sedis; D_10__Malassezia; D_11__uncultured stramenopile
OTU_107	37	D_1__Opisthokonta; D_2__Nucletmycea; D_3__Fungi; D_4__Zygomycota; D_5__Entomophthoromycotina; D_6__Incertae Sedis; D_7__Entomophthorales; D_8__Entomophthoraceae; D_9__Entomophthora; D_10__Entomophthora culicis
OTU_8853	44	D_1__SAR; D_2__Alveolata
OTU_76	1	D_1__SAR; D_2__Alveolata; D_3__Apicomplexa; D_4__Conoidasida; D_5__Gregarinasina; D_6__Eugregarinorida; D_7__Gregarina
OTU_718	48	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep
OTU_31887	6	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea
OTU_707	1	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__CV1-2A-17; D_8__uncultured microeukaryote
OTU_31	3453	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__Hymenostomatia; D_8__Tetrahymena
OTU_32917	8	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__Hymenostomatia; D_8__Tetrahymena

OTU_34799	4	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__Hymenostomatia; D_8__Tetrahymena
OTU_36302	1	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__Hymenostomatia; D_8__Tetrahymena
OTU_19135	201	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__Scuticociliatia
OTU_2417	1	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Intramacronucleata; D_5__Conthreep; D_6__Oligohymenophorea; D_7__Scuticociliatia; D_8__Cyclidium
OTU_47	15	D_1__SAR; D_2__Alveolata; D_3__Ciliophora; D_4__Postciliodesmatophora; D_5__Heterotrichea; D_6__Blepharisma; D_7__invertebrate environmental sample
OTU_1412	32	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Chrysophyceae; D_5__Chromulinales; D_6__JBNA46; D_7__uncultured eukaryote
OTU_1846	90	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Chrysophyceae; D_5__Ochromonadales; D_6__Ochromonas; D_7__uncultured stramenopile
OTU_21	49	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea
OTU_30447	1	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea
OTU_12831	49	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_15572	10	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_6	9	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_28977	1	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_33479	1	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_34077	1	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_37036	1	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae
OTU_71	1	D_1__SAR; D_2__Stramenopiles; D_3__Ochrophyta; D_4__Diatomea; D_5__Bacillariophytina; D_6__Bacillariophyceae

OTU_6381	33	Unclassified eukaryote
OTU_1136	24	Unclassified eukaryote
OTU_66	12	Unclassified eukaryote
OTU_34155	1	Unclassified eukaryote

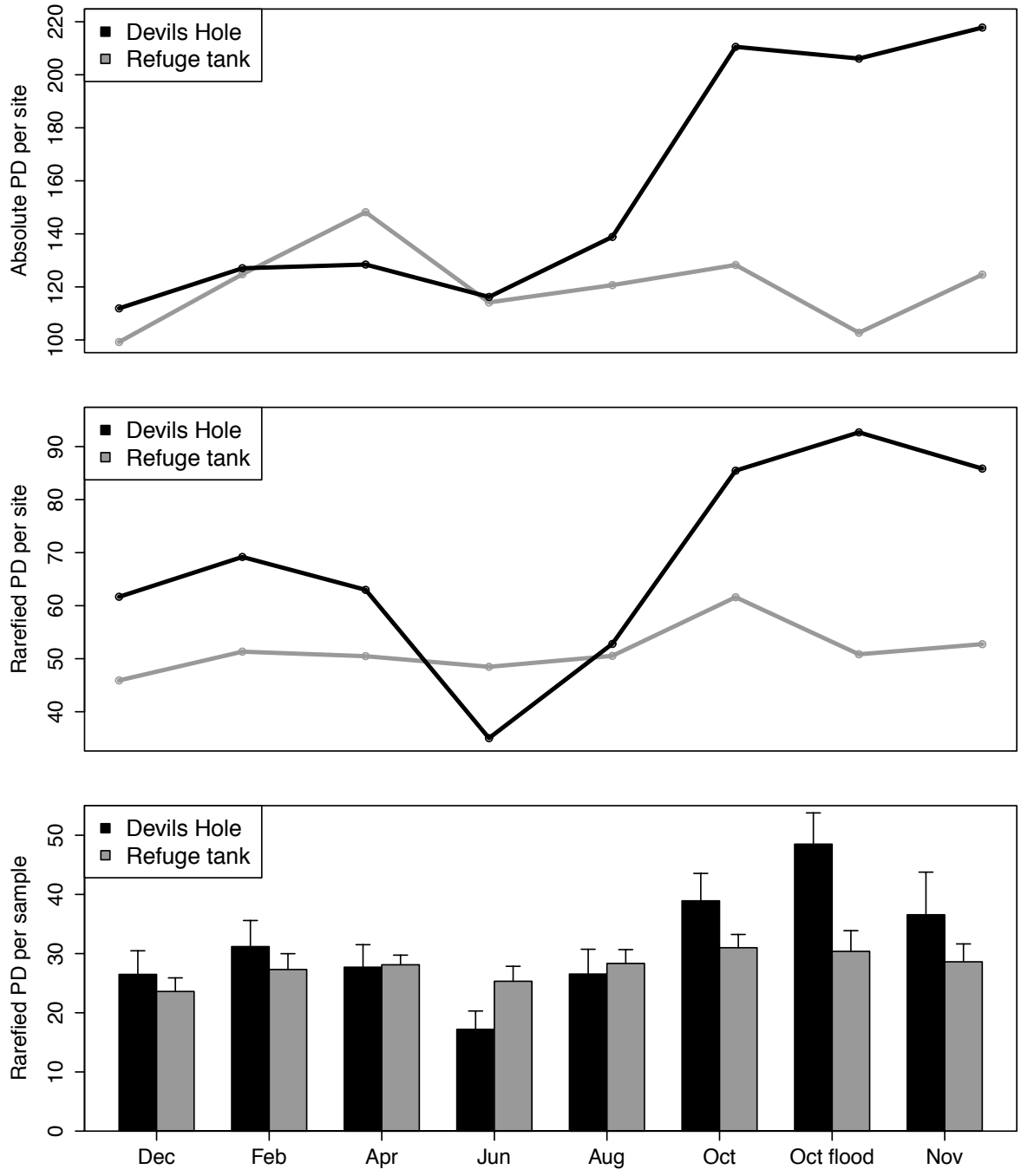

Figure A3. Phylogenetic diversity (Faith's PD) in Devils Hole (black) and the refuge tank (gray), between December 2014 and November 2015, including additional samples collected after the second October flooding event ('Oct flood'). Sample data were rarefied to 1,640 sequences per sample prior to alpha diversity calculations (bottom). Site data were rarefied to 4,920 sequences per site (middle). The top plot shows phylogenetic diversity of non-rarefied data.