AN ASSESSMENT OF THE CORRELATION BETWEEN AMPHIBIAN POPULATIONS, CHYTRIDIOMYCETE COMMUNITIES, AND THE ECOLOGICAL INTEGRITY OF THE HABITAT

By

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ABSTRACT OF THE THESIS

An Assessment of the Correlation between Amphibian Populations, Chytridiomycete Communities, and the Ecological Integrity of the Habitat

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The pathogenic chytrid *Batrachochytrium dendrobatidis* has been implicated in amphibian declines worldwide. Thus far, little is known about the chytridiomycete (zoosporic fungi) communities and the prevalence of *B. dendrobatidis* in New Jersey. The New Jersey Pinelands are an ideal location to look for *Bd* as they are a unique ecosystem, representing an important overlap in the geographic range of many southern and northern species and are experiencing habitat degradation caused by continued development.

In this study 6 sites have been identified using the Pinelands Commission's Comprehensive Management Plan, 3 sites of high ecological integrity (pristine) and 3 sites low of low integrity (impacted). Using current accepted methods, anurans at these sites were collected, swabbed, and processed using PCR to identify the presence of *Bd* on their epidermis. Despite expectations, *Bd* was not found at any site on either anurans or free living in the water, debris, or mud. If *Bd* had been identified relative abundance of

infection as well as species and life-stage of amphibian would have been compared within and between sites.

After the presence of *Bd* was refuted, our work changed to look at general zoosporic fungi (chytrid) communities within the Pinelands and how ecological degradation was altering abundance. Since pollution and nutrient enrichment can alter ecosystems and diversity, we wanted to investigate how fungal abundance changes between pristine and degraded water bodies. Through abundance surveys and molecular analyses, PCR, DGGE, and molecular sequencing, we found that not only was abundance significantly different between sites, zoosporic fungi species were different and sites of differing ecological integrity were dominated by different fungi.

Zoosporic fungi from pristine and degraded sites were then subjected to lab manipulations to observe how changing environmental parameters increased or decreased abundance. pH appeared to be the driving factor in fungi abundance; increasing pH in pristine sites showed a significant decrease in population and decreasing pH found a significant increase in zoosporic fungi in impacted sites. These studies show the potential importance of zoosporic fungi as bioindicators of pollutions and the drastic effect pollution has on ecosystems and biodiversity.

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I would like to dedicate this to my parents for their endless love and support

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Introduction

Chytridiomycetes are a basal branch of true fungi that reproduce via uniflagellate zoospores (Shearer *et al.* 2004). Increasingly referred to as zoosporic fungi in mycological literature, the 1,250 species of chytridiomycetes are distinguished by zoosporic characteristics and divided into 5 orders (Shearer *et al.* 2007). These fungi inhabit aquatic and terrestrial habitats, although many of these zoosporic fungi are determinate in their growth and depend on moisture to disperse their motile zoospores (Shearer *et al.* 2007). These fungi also have the ability to form resting sporangia that can avoid desiccation and unfavorable conditions (Shearer *et al.* 2007), and thus survive in a wide range of ecosystems with varying environmental conditions (Gleason *et al.* 2004). Zoosporic fungi are found on every continent with highest species diversity in temperate regions (Shearer *et al.*2007).

In their ecosystems, chytridiomycetes exist as saprotrophs or parasites feeding on chitin, cellulose, and keratin they obtain from their hosts including algae, fungi, plants, invertebrates, and, recently discovered, vertebrates (Shearer *et al.* 2007; Gleason *et al*., 2008). It is this recently discovered vertebrate parasite, *Batrachochytrium dendrobatidis* that has been implicated in the recent worldwide amphibian declines.

Given their prevalence in temperate zones and that their distribution is determined by environmental conditions rather than geography (Shearer *et al.* 2004), we wanted to investigate the occurrence of zoosporic fungi in the unique ecosystem of the New Jersey pine lands. After an initial investigation of the presence of *Batrachochytrium dendrobatidis* was refuted our work turned to general zoosporic fungal abundance and

how communities respond to varying levels of ecological integrity and changing environmental parameters.

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Chapter I

An investigation into whether the prevalence of *Batrachochytrium dendrobatidis* is correlated with habitat quality in the New Jersey Pinelands.

Abstract

The pathogenic chytrid *Batrachochytrium dendrobatidis* has been attributed to worldwide amphibian declines and although the cause of chytridiomycosis is unknown environmental parameters are believed to be a factor in the recent epidemic. Anuran populations from water bodies of varying ecological integrity in the New Jersey pine barrens were tested for the presence of *Bd.* Initial expectation believed the pathogenic chytrid would be found more frequently in impacted water bodies and less frequent or non-existent in pristine locations. Results however showed that lethal or non-lethal *Bd* was not present at any location during any of the sampling events.

Introduction

Amphibian populations are experiencing a drastic decline with up to 122 species having disappeared since 1980 (Mendelson *et al.* 2006) and approximately one-third of known species severely threatened by extinction (Stuart *et al.* 2004). A 2007 analysis suggests that the current rate of amphibian extinction is 211 times the natural background extinction rate with historical extinction events having coincided with natural disasters (McCallum 2007).

The first recorded amphibian declines began in the 1970s in the western United States, Puerto Rico, and Northeastern Australia (Stuart *et al.* 2004). Subsequent reports showed drastic declines in neo-tropical montane regions such as Central and South

American cloud forests, with many of these areas having similar environmental and ecological characteristics (Stuart *et al.* 2004). These declines were associated with unusually warm/dry conditions that appeared to interact with an "unidentified agent" such as deforestation and an increase in toxic environmental contaminants (Pounds and Crump 1994).

In 1998 a pathogenic chytrid was identified on the epidermis of dead and dying frogs from montane rainforests in Australia and Panama (Berger *et al.* 1998). This chytridiomycosis appeared to be causing declines in amphibians due to mass mortalities among adults; thus, implicating it as the cause of anuran declines in Australia, Central America, and captive poison dart frogs in the United States (Berger *et al.* 1998). This chytrid was described as *Batrachochytrium dendrobatidis* (Longcore *et al.* 1999) and by 2008, *B. dendrobatidis* had been found on every continent except Antarctica (IUCN 2008).

Although the cause and spread of chytridiomycosis is unclear, emerging infectious disease theory suggests that an epidemic is caused when either a novel pathogen enters a geographic region or an endemic disease suddenly increases its host range or increases in pathogenesis (Rachowicz and Vrendenburg 2004). Given that *B. dendrobatidis* has been found on museum specimens dating back to the 1930s (Blaustein and Dobson 2006), chytridiomycosis may have emerged due to either an increase in virulence of the fungus caused by changes in the environment or an increase in susceptibility of the host (Berger *et al.* 1998).

Increases in UV light (Blaustein *et al.* 2005), global warming and temperature (Berger *et al.* 2005, Pounds *et al.* 2006, Di Rosa *et al.* 2007, Rohr *et al.* 2008),

environmental contaminants (Blaustein *et al.* 2005, Di Rosa *et al.* 2007), global amphibian/bait trade and the introduction of exotic species (Blaustein and Dobson 2006, Garner *et al.* 2006), or the interaction of numerous factors (Blaustein *et al.* 2005, Di Rosa *et al.* 2007), as well as vectors such as bird feather (Johnson and Speare 2005) and park visitors (Chestnut *et al.* 2007) have all been attributed to the formation and spread of chytridiomycosis. A leading theory suggests that global climate change has increased cloud cover in Neotropical montane cloud forests creating a thermal optimal environment for chytrids (Rohr *et al.* 2008). Increased nighttime cloud cover traps warm air from escaping into the atmosphere leading to higher average nighttime temperatures and increased daytime cloud cover reduces solar radiation causing lower daytime temperatures (Pounds *et al.* 2006). These changes in daytime and nighttime temperature have created a beneficial thermal environment for *B. dendrobatidis,* which has been shown to grow optimally 23°C (Longcore *et al.* 1999). This loss of direct solar radiation may be also detrimental to amphibians that seek direct sunlight, as exposure to high temperate has been shown to clear chytridiomycosis from amphibians (Andre *et al.* 2008). Alternatively, the Spatiotemporal-Spread hypothesis suggests that the data supports a typical wave-like pattern of infection that, after introduction to South America spread along the Andes (Lips *et al.* 2007).

While most research has focused on mass anuran mortalities in the tropics, research from temperate Australia showed that frogs in these regions had significantly more intense infections than those in the tropics; although these infections were less prevalent (Krieger *et al.* 2007). Characteristics of temperate regions: lower elevations, increased rainfall, and cooler temperature, all contribute to increased chytridiomycosis

intensity (Krieger *et al.* 2007). No chytrid-related mortalities have been documented along the eastern United States or Canada (Ouellet *et al.* 2005, Longcore *et al.* 2006, Rothermal *et al.* 2008); however, infected amphibians have demonstrated *B. dendrobatidis'* presence in temperate locations. It appears that in North America, *B. dendrobatidis* is not limited by geography or host (Pearl *et al.* 2007).

No studies thus far have focused on the prevalence of *B. dendrobatidis* in the Mid-Atlantic region, specifically New Jersey. The New Jersey Pinelands is a unique ecosystem characterized by acidic, nutrient-poor, sandy soils with a pitch pine/black oak dominate canopy and an ericaceous shrub layer. Due to the unique characteristics of the pinelands, they also represent an important overlap in the geographic range of many southern and northern species. The pinelands support 17 amphibian species including three state threatened or endangered salamander species and two tree frog species including the Pine Barrens Tree Frog.

This study focuses on the presence of *B. dendrobatidis* on anuran populations inhabiting bodies of water in the New Jersey pine barrens to determine if changes in these communities correlate with varying degrees of ecological integrity, and if so, does the presence of pathogenic species of chytrids increase as habitat quality decreases. If *B. dendrobatidis* is present in the pine barrens, I expect the community of amphibians and chytrids will change with varying degrees of ecological integrity, that *B. dendrobatidis* will be more abundant in water bodies of lower ecological integrity, and that infected individuals will display varying symptoms among species and life stages.

Materials and Methods

Site Assessment

Sites have been evaluated using the Pinelands Commission's Comprehensive Management Plan, which includes long-term ecological assessment of water bodies. This long-term environmental-monitoring program analyzed water bodies within the Mullica River, Ranacocas Creek, Great Egg Harbor, and Barnegat Bay watersheds for ecological integrity. Each site was identified on a scale of 1 to 5, with 5 being a pristine ecosystem and 1 being a severely impacted system (Zampella *et al.* 2001, 2003, 2005, 2006). Integrity was evaluated through analysis of pH, specific conductance, marginal vegetation, and fish and anuran assemblages. Pristine sites were designated by a low pH, low specific conductance, native and intact marginal vegetation, and native, healthy populations of fish and anuran species. Impacted sites had higher, more neutral pH, high relative specific conductance, a loss of native species, and a high number of invasive species. Since species composition changes with ecological integrity, the same anuran species were not present at all sites, therefore, carpenter frogs (*R virgatipas)*, green frogs (*R clamitans melanota)*, and bullfrogs (*R catesbeiana)* were chosen for sampling due to their range. Carpenter frogs are native to the pinelands and are found at pristine sites, bullfrogs are border entrant species and are found at impacted sites, and green frogs are found at all sites because although native, they have a high tolerance for environmental conditions.

Pakim Pond, Hampton Furnace, and Otter Pond were chosen for their high level of ecological integrity, having typical pineland's water body characteristics, defined as low pH, low specific conductance, minimal invasive species, and healthy native

populations. Indian Mills Lake, Pump Branch Impoundment, and Paradise Lake were chosen due to low levels of ecological integrity defined as high pH, high specific conductance, presence of invasive and border entrant species, and proximity to developed and impervious surfaces.

Preliminary Culturing

Chytrids were baited at sites using cellophane and keratin following instructions provided by Dr. Joyce Longcore (University of Maine). After two weeks, bait was removed from site and grown on PmTG (Shearer *et al.* 2004). Water samples collected from each site were also baited with spruce pollen in lab before being plated. Preliminary cultures showed presence of chytridiomycetes although strain was unidentifiable.

Anuran Sampling

Sites were visited in May 2008 and June 2008 to identify amphibian populations and monitor breeding progress. Each site was sampled three times during July 2008 and August 2008, each time water temperature was recorded and water samples were tested for specific conductance and pH. (Table 1)

Tadpole traps were set overnight but dip-netting was found to be the most effective sampling technique for tadpoles; adult frogs were also sampled when found. Sites were sampled for 2 hours or until 10 individuals had been found. (Table 2) Using Hyatt *et al.* (2007) anurans were swabbed with BD Culture Swabs (Fisher Scientific). Swabbing of tadpoles focused on jaw sheaths since keratin is only found in tadpoles' mouths. Adults were swabbed with special attention to the dorsal pelvic patch and feet. After amphibians

were swabbed, swabs were placed back into sterile tubes and frozen immediately upon return to lab.

Species collection focused on three species that spanned the levels of degradation: Carpenter frogs (*Rana virgatipas)*, Green frogs (*Rana melanota clamitans)*, and Bullfrogs (*Rana catesbeiana*) although Southern Leopard frogs (*Rana sphenocephala*) were also sampled when found. Collected amphibians were recorded for sex, size, and life stage. Nets, buckets, and waders were sprayed with a dilute bleach solution to prevent cross-contamination between sites.

DNA Extraction and PCR

DNA was extracted from culture swabs using the maximum yield protocol from Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories). PCR reactions followed a modified protocol from Boyle *et al.* (2004) and Krieger *et al.* (2006) using primers ITSI-3 Chytr and 5.8S Chytr (GenBank accession # AY598032). The PCR cycling parameters were 5 minute denaturing at 95°C followed by 35 cycles of denaturation at 95°C for 1 minute, annealing for 30 seconds at 51.1°C, and extension for 30 seconds at 72°C. Cycling was followed by final extension time of 10 minutes at 72°C.

PCR products were subsequently run on a gel electrophoresis against a positive control (courtesy of Joyce Longcore) and negative control. After initial PCR reactions for individual samples were processed, extracted DNA was pooled for each site and concentrated using sodium acetate and ethanol, then ran through a PCR reaction and gel electrophoresis.

In late March 2009, water and debris (leaves, twigs, mud), collected from the

submerged bank, were collected from sites. A sample of water was frozen for future analysis and remaining water and debris were baited in lab with chitin and pollen. After 2 weeks DNA was extracted from water and baited debris using Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories). PCR was completed as previously described.

In May 2009, sampling was repeated at Hampton Furnace and DNA processed.

Results

All PCR reactions produced negative results for the presence of *B. dendrobatidis* on the epidermis of anurans as well as water and debris samples from all sites.

Discussion

B. dendrobatidis was not found on any occasion at any of the 6 sites in this study. Despite *B. dendrobatidis*' presence being recorded in several other locations in the United States (Longcore *et al.* 2006, Pearl *et al.* 2007, Rothermal *et al.* 2008) as well as all over the world, at this time it appears to be absent or in quantities too low to detect from the New Jersey pinelands.

Although *B. dendrobatidis* can survive a wide range of environmental conditions, laboratory experiments have showed *B. dendrobatidis* to exhibit slowed growth and limited reproduction at environmental extremes. *B. dendrobatidis* can survive between 4°C to 25°C with maximum growth and reproduction between 17°C and 25°C (Piotrowski *et al.* 2004), and an optimal growing temperature of 23°C (Longcore *et al.* 1999). The majority of spring and summer water temperatures in the pinelands are well within the range of growth and reproduction: 16.6°C to 32°C (Table 1) with water only

exceeding 25°C when in direct sunlight. Changes in temperature between sites and sampling dates was mainly due to exposure to sun, which was dependant on amount and type of vegetation, with impacted sites frequently have less surrounding tree cover.

B. dendrobatidis can tolerate a pH range from 4 to 10, with no growth from pH 3 to 4 and fragmented growth at pH 9 and 10 (Johnson and Speare 2005). The optimal pH for growth and reproduction is 6 to 7.5 with slowed growth and reproduction at 5 to 5.5 and 8 (Johnson and Speare 2005). pH in the pinelands varied drastically between pristine sites from a pH from 3.9 at Otter Pond to 6.8 at Hampton Furnace. The more neutral pH at Hampton Furnace is likely caused by proximity to an old stone building and subsequent limestone runoff. pH was more consistent at degraded sites only varying from 6.3 and 7.0. Although pH may have been too acidic in Otter Pond and Pakim Pond, the pH values of Hampton Furnace and all three of the impacted sites, Paradise Lake, Indian Mills, and Pump Branch Impoundment, were at optimal levels for *B. dendrobatidis* growth.

Nutrients may also be an important component in survival of *B. dendrobatidis*; unfortunately, prior research is limited. Piotrowski *et al.* (2004) tested *B. dendrobatidis* growth on varying media of nitrogen and carbon and found that growth was optimal on tryptone but too high a percentage of nitrogen and/or sugars impaired growth (Piotrowski *et al.* 2004). Johnson and Speare found that *B. dendrobatidis* would grow significantly longer in low-nutrient creekbed sand than in nutrient-deficient water when pH was comparable (2005); however, growth was not compared between the minimal-nutrient sand and a higher-nutrient media. Nutrient composition of water bodies in the pinelands

varies from low nutrients in pristine areas and specific conductance of 34.6 to a specific conductance 321 ppm due to run-off and pollution at degraded sites.

B. dendrobatidis' presence in the New Jersey pinelands is still unclear. It's possible the pinelands are outside of *B dendrobatidis'* geographic distribution or that it is present but limiting environmental conditions have slowed growth and reproduction. Previous research has shown low pH to be a limiting factor but the interaction of pH and nutrients is yet unknown.

Further studies are needed to determine if *B. dendrobatidis* would be able to survive and proliferate in degraded sites where pH is optimal or if nutrient thresholds hinder growth. Also if it is that minimal nutrients are advantageous for *B. dendrobatidis,* would a pristine site like Hampton Furnace, which retains classic pinelands characteristics except for a more neutral pH, be a prime environment for chytridiomycosis?

Future research should focus on pH and nitrogen parameters to determine their effect on *B. dendrobatidis* and what implications this may have on the native species in the unique environment of the New Jersey pinelands. Possible interactions between *B. dendrobatidis*, other chytrid species, and algal communities should also be investigated, as competitive or mutualistic relationships may exist that effect community dynamics.

In October 2009 I submitted my results to the Global Bd Mapping Project (www.spatialepidemiology.net/Bd/) set up by Dede Olson of the USDA Forest Service which tracks positive and negative identification of *Batrachochytrium dendrobatidis* throughout the world. My data was the first for New Jersey.

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Appendix

Table 1: Site assessment at time of sampling. Impacted sites: Pump Branch, Indian Mills, and Paradise Lake have a consistently higher and more neutral pH as well as higher specific conductivity (SC). Pristine sites: Pakim, Otter, and Hampton have a consistently lower pH with the exception of Hampton Furnace as explained above. The SC of pristine sites was low.

Site	Date	Temp (°C)	pH	SC (ppm)	Anurans sampled	Observations
ROUND 1						
Pakim	8-Jul	30.6	5.06	57.6	7	
Pump	8-Jul	30.7	6.32	83.8	2	
Pakim	9-Jul		5.16	34.6	3	
Paradise	14-Jul	26.7	6.641	70.4	10	
Pump	14-Jul	29.7	7.036	70.8	3	
Indian	14-Jul	32.0	6.378	232	11	
Otter	22-Jul	26.7	3.952	47.5	4	
Hampton	22-Jul	18.4	6.125	54.5	8	
ROUND 2						
Pakim	5-Aug	27.5	4.76	41.8	10	
Hampton	5-Aug	17.0	5.88	54.1	11	
Otter	5-Aug					No calling, no tadpoles/adults, lots of fish
Pump	5-Aug	27.5	6.779	77	9	
Indian	6-Aug	24.4	6.425	321	5	
Paradise	6-Aug					No calling, no tadpoles/adults
ROUND 3						
Pakim	26-Aug	24.8	5.256	44.7	10	
Otter	26-Aug	20.2	5.601	37.2	2	
Hampton	26-Aug	16.6	6.852	51.4	10	
Paradise	26-Aug					No calling, no tadpoles/adults
Pump	27-Aug	23.3	6.765	79.6	11	
Indian	27-Aug					no calling, no tadpoles, scarce fish extreme eutrophication, dead turtle

Table 2: Anuran Sampling Data. Carpenter frogs (*R virgatipas)*, green frogs (*R clamitans melanota)*, and bullfrogs (*R catesbeiana)* were chosen sampling species due to their range.

Date	Site	Species	Age	Sex	Size (cm)	PCR Date	Results
8-Jul	Pakim	*R virgatipas*	adult	F		25-Feb	Negative
8-Jul	Pump Branch	unknown	larvae		~2.5	25-Feb	Negative
8-Jul	Pakim	*R virgatipas*	larvae		<2.5	25-Feb	Negative
8-Jul	Pakim	unknown	larvae		~2.5	11-Mar	Negative
8-Jul	Pump Branch	*R catesbeiana*	metamorph			11-Mar	Negative
8-Jul	Pakim	unknown	larvae		~2.5	18-Feb	Negative
8-Jul	Pakim	unknown	metamorph			18-Feb	Negative
8-Jul	Pakim	unknown	larvae		>2.5	18-Feb	Negative
9-Jul	Pakim	*R virgatipas*	larvae			25-Feb	Negative
9-Jul	Pakim	*R melanota*	adult	M		11-Mar	Negative
9-Jul	Pakim	*R virgatipas*	adult	M	~2.5	11-Mar	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		>2.5	25-Feb	Negative
14-Jul	Pump Branch	unknown	larvae		~2.5	25-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		>2.5	25-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>5	25-Feb	Negative
14-Jul	Indian	*R melanota*	larvae		>2.5	25-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>2.5	25-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		<2.5	11-Mar	Negative
14-Jul	Indian	*R catesbeiana*	metamorph		>5	11-Mar	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		~5	11-Mar	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		<5	11-Mar	Negative
14-Jul	Pump Branch	*R catesbeiana*	metamorph			11-Mar	Negative
14-Jul	Pump Branch	*R catesbeiana*	metamorph			13-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		>2.5	13-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		~5	13-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>2.5	13-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>2.5	13-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		>2.5	13-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>2.5	18-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		>5	18-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>5	18-Feb	Negative
14-Jul	Indian	*R melanota*	larvae		~5	18-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		>5	18-Feb	Negative
14-Jul	Paradise	*R catesbeiana*	larvae		<5	18-Feb	Negative
14-Jul	Indian	*R catesbeiana*	larvae		~5	18-Feb	Negative
22-Jul	Otter	*R virgatipas*	larvae		<2.5	25-Feb	Negative
22-Jul	Hampton	*R melanota*	adult	F	>2.5	25-Feb	Negative
22-Jul	Hampton	*R melanota*	adult	F	~2.5	25-Feb	Negative
22-Jul	Otter	*R virgatipas*	larvae		>2.5	11-Mar	Negative
22-Jul	Hampton	*R melanota*	adult	M	~7.5	11-Mar	Negative
22-Jul	Hampton	*R melanota*	adult	F	>5	13-Feb	Negative
22-Jul	Hampton	*R melanota*	adult	F	~2.5	13-Feb	Negative
22-Jul	Hampton	*R melanota*	adult	M	>7.5	13-Feb	Negative

22-Jul	Hampton	*R virgatipas*	adult	F	~4	13-Feb	Negative
22-Jul	Otter	*R virgatipas*	larvae		>2.5	18-Mar	Negative
22-Jul	Otter	*R virgatipas*	larvae		>7.5	18-Feb	Negative
25-Jul	Hampton	*R melanota*	adult	M		11-Mar	Negative
6-Aug	Hampton	*R melanota*	adult	M	>5	25-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	M	>2.5	18-Mar	Negative
6-Aug	Hampton	*R sphenocephala*	adult	F	~5	11-Mar	Negative
6-Aug	Pakim	unknown	larvae		<2.5	11-Mar	Negative
6-Aug	Pakim	unknown	larvae		<2	11-Mar	Negative
6-Aug	Pump Branch	unknown	larvae		~2.5	11-Mar	Negative
6-Aug	Pakim	*R virgatipas*	larvae		<2.5	13-Feb	Negative
6-Aug	Pakim	*R virgatipas*	larvae		>2.5	13-Feb	Negative
6-Aug	Pakim	*R virgatipas*	larvae		~10	13-Feb	Negative
6-Aug	Pakim	*R virgatipas*	larvae		<2.5	13-Feb	Negative
6-Aug	Pakim	*R virgatipas*	larvae		<.2	13-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	M	8	13-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	M	<5	13-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	M	>5	13-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	M	~2.5	18-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	F	<2.5	18-Mar	Negative
6-Aug	Hampton	*R melanota*	adult	F	<2.5	18-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	F	~2.5	18-Feb	Negative
6-Aug	Hampton	*R melanota*	adult	M	>5	18-Feb	Negative
7-Aug	Indian	*R catesbeiana*	larvae		~5	25-Feb	Negative
7-Aug	Pump Branch	Unknown	larvae		~5	25-Feb	Negative
7-Aug	Pump Branch	Unknown	larvae		>2.5	25-Feb	Negative
7-Aug	Pump Branch	Unknown	larvae		>2	11-Mar	Negative
7-Aug	Pump Branch	*R virgatipas*	larvae		~2.5	11-Mar	Negative
7-Aug	Pump Branch	*R virgatipas*	larvae		~2.5	13-Feb	Negative
7-Aug	Pump Branch	Unknown	larvae		~2.5	13-Feb	Negative
7-Aug	Pump Branch	unknown	larvae		~2.5	13-Feb	Negative
7-Aug	Pump Branch	unknown	larvae		~3	18-Feb	Negative
7-Aug	Indian	*R catesbeiana*	larvae		~5	18-Feb	Negative
7-Aug	Indian	*R catesbeiana*	larvae		>5	18-Feb	Negative
7-Aug	Indian	*R catesbeiana*	larvae		>2.5	18-Feb	Negative
7-Aug	Indian	*R catesbeiana*	larvae		~5	25-Feb	Negative
22-Aug	Pump Branch	*R catesbeiana*	larvae		~2.5	25-Feb	Negative
22-Aug	Pakim	*R virgatipas*	larvae		>2	11-Mar	Negative
24-Aug	Otter	*R melanota*	larvae		~9	18-Feb	Negative
26-Aug	Hampton	*R melanota*	adult	F	~4	18-Mar	Negative
26-Aug	Pakim	unknown	larvae		>2	25-Feb	Negative
26-Aug	Hampton	*R melanota*	adult	F	~4	18-Mar	Negative
26-Aug	Pakim	unknown	larvae		>5	25-Feb	Negative
26-Aug	Pakim	unknown	larvae		>2.5	25-Feb	Negative
26-Aug	Pakim	unknown	larvae		>2.5	11-Mar	Negative
26-Aug	Hampton	*R melanota*	adult	M	>7	11-Mar	Negative

26-Aug	Hampton	*R melanota*	adult	M	>5	18-Feb	Negative
26-Aug	Hampton	*R melanota*	adult	M	<9	18-Feb	Negative
26-Aug	Otter	*R virgatipas*	larvae		~8	18-Feb	Negative
26-Aug	Hampton	*R sphenocephala*	adult	M	>8	18-Feb	Negative
26-Aug	Hampton	*R melanota*	adult	F	~2.5	18-Feb	Negative
26-Aug	Hampton	*R melanota*	adult	M	>9	18-Feb	Negative
26-Aug	Pakim	unknown	larvae		<2.5	18-Feb	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~4	25-Feb	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~4	25-Feb	Negative
27-Aug	Pump Branch	*R catesbeiana*	metamorph		~10	11-Mar	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~5	11-Mar	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~4	11-Mar	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~4.5	11-Mar	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~2.5	13-Feb	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		>9	13-Feb	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~5	13-Feb	Negative
27-Aug	Pump Branch	*R catesbeiana*	larvae		~2.5	13-Feb	Negative

Table 3: Geographic coordinates of sites from Pinelands Commission Reports (01, 03, 05, 06)

Site	**Latitude**	**Longitude**
Pump Branch Impoundment	39° 41’50.31	-74°49’43.48
Indian Mills Lake	39°47’44.04	-74°44’24.29
Paradise Lake (Albertson Brook)	39°41’22.44	-74°43’46.64
Otter Pond (Bull’s Run)	39°44’39.05	-74°34’49.95
Pakim Pond (Cooper Branch)	39°52’51.98	-74°31’56.83
Hampton Furnace	39°46’15.58	-74°40’47.53

Chapter II

Changes in the abundance of zoosporic fungi (chytridiomycete) communities with degrading ecological integrity

Abstract

Zoosporic fungi community abundance was surveyed in water bodies of varying ecological integrity over 2 consecutive summers in the New Jersey pine barrens. Of the total 6 sampling sites 3 were of pristine ecological integrity, displaying water chemistry characteristic to that of the pine barrens, and 3 sites were impacted, having low water quality due to agricultural run-off and proximity to impervious surfaces. Zoosporic fungi abundance was consistently and significantly higher in pristine water bodies then in impacted sites. Molecular analysis found that species that were dominant in pristine sites were absent in impacted sites and conversely species that dominated impacted sites were absent in pristine, showing a shift in the fungal community composition based on water quality.

Introduction

Zoosporic fungi (Chytidiomycota) are found in ecosystems from the artic to the tropics in aquatic and terrestrial environments (Powell 1993), with highest species diversity in temperate zones (Shearer *et al.* 2007). Due to their ability to form resting sporangia that can avoid desiccation and unfavorable conditions (Shearer *et al.* 2007), these fungi can survive in a wide range of ecosystems with drastically differing biotic and abiotic parameters (Gleason *et al.* 2004).

Zoosporic fungi feed on chitin, cellulose, and keratin, which they obtain from their environment as parasites or saprotrophs. In aquatic systems zoosporic fungi frequently parasitize phytoplankton; in terrestrials system they form obligate parasitic relationships with vascular plants (Powell 1993, James *et al.* 2006), while the majority of chytridiomycetes inhabit terrestrial systems as saprotrophs (James *et al.* 2006). In each system, a few zoosporic fungal species thrive in large populations, while the majority remain infrequent and in low abundance (James *et al.* 2006).

In the New Jersey Pinelands, urban development and agricultural run-off is affecting the once pristine water quality in sites that abut or are downstream of intensive agriculture or areas designated for urban development. These water bodies, identified by the Pinelands Commission, are exhibiting changes in their water chemistry such as elevated pH, high nutrient content, and an increased specific conductance, caused by run-off, upland land use, invasive species, and habitat loss and degradation. Given the ubiquity of these zoosporic fungi and that their distribution dependant on environmental conditions rather than geography (Shearer *et al* 2007), we wanted to investigate whether zoosporic fungi communities changed as their ecosystem degraded.

Our survey compared the abundance of zoosporic fungi between a site of high ecological integrity: low pH, low nutrients, low specific conductance, against an impacted site: high, more neutral pH, high nitrogen and phosphorus content, and a high specific conductance as designated by the Pinelands Commission. In addition to abundance surveys, preliminary attempts to identify possible changes in zoosporic fungi community composition were conducted.

Materials and Methods

Site Assessment

Sites have been evaluated using the Pinelands Commission's Comprehensive Management Plan, which includes long-term ecological assessment of water bodies. This long-term environmental-monitoring program analyzed water bodies within the Mullica River, Ranacocas Creek, Great Egg Harbor, and Barnegat Bay watersheds for ecological integrity. Each site was identified on a scale of 1 to 5, with 5 being a pristine ecosystem and 1 being a severely impacted system (Zampella *et al* 2001, 2003, 2005, 2006). Integrity was evaluated through analysis of pH, specific conductance, marginal vegetation, and fish and anuran assemblages. Pristine sites were designated by a low pH, low specific conductance, native and intact marginal vegetation, and native, healthy populations of fish and anuran species. Impacted sites had higher, more neutral pH, high relative specific conductance, a loss of native species, and a high number of invasive species.

Pakim Pond, Hampton Furnace, and Otter Pond were chosen for their high level of ecological integrity, having typical pineland's water body characteristics, defined as low pH, low specific conductance, minimal invasive species, and healthy native populations. Indian Mills Lake, Pump Branch Impoundment, and Paradise Lake were chosen due to low levels of ecological integrity defined as high pH, high specific conductance, presence of invasive and border entrant species, and proximity to developed and impervious surfaces.

Zoosporic Fungi Population Surveys

Population surveys assessed the abundance of zoosporic fungi, but did not assess community composition. The initial survey in 2008 was restricted to a comparison between Pakim Pond (pristine) and Indian Mills Lake (impacted). Sites were sampled a total of seven times starting in mid-July and ending in mid-October. In 2009, all six sites were sampled three times from late June to early August.

Sampling consisted of collecting a similar volume of water containing plant debris, or water containing a sample of mud taken from the edge of the lake into a 500 ml Mason jar and returned to the laboratory. Here, a defined volume of pollen grains (approximately 0.5 grams) was added to the surface of the water. After a weeklong incubation period, the total number of zoosporic fungi colonizing pollen grains was counted per 50 or 100 pollen grains using a light microscope at 400 x magnification. This total count of zoosporic fungi per pollen grains was used as an index of zoosporic fungi abundance. Correct observation of zoosporic fungi were was confirmed through correspondence with Dr. Joyce Longcore. Other than identifying the presence or absence of zoosporic fungi on pollen grains, no further taxonomic identification was made (to family, genus or species).

This indirect method of population assessment was adopted for simplicity, as one of the overall aims of the project was to evaluate a simple method of use of potential bioindicators of ecosystem degradation that could be performed by personnel with minimal training.

Statistical analysis

For survey data, difference in abundance of zoosporic fungi between sites and

between sample dates was analyzed by analysis of variance (SAS version 9.1; SA Institute, Cary, NC) with significance between sites or dates being indicated by post-hoc means separation Tukey's Honestly Significant Difference test at an alpha level of 0.05.

Zoosporic Community Analysis

Determination of the community composition between an impacted and pristine site was attempted using denaturing gradient gel (DGGE) analysis on DNA extracted from the zoosporic fungi community. Water samples from Pakim Pond (pristine) and Indian Mills Lake (impacted) were baited with pollen as previously described. Pollen was collected after a week and DNA was extracted from the chytrids on the pollen using the maximum yield protocol from Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories).

The ITS region of the extracted zoosporic fungal DNA was amplified using ITS3 with a 40 bp GC-clamp (5' CGCCCGCCGCGCCCCGCGCCC GGCCCGCCGCCCCCGCCCC GCATCGATGAAGAA CGCAGC 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Nikolcheva *et al.* 2005). The amplification program consisted of an initial denaturation of 2 minute at 95°C followed by 36 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 1 minute and a final extension time at 72°C for 5 minutes (Nikolcheva *et al.* 2005). Using a Dcode Universal Mutation Detection System (Bio Rad) 20ul of the 370 bp amplicon were loaded into an 8% (w/v) polyacrylamide gel with a 30% to 80% denaturation gradient (Nikolcheva *et al.* 2005). The samples were run in a 1x TAE buffer at 55v and 56°C for 16 hours (Nikolcheva *et al.* 2005).

After electrophoresis, the gel was stained with a 5 ug/mL EtBr solution and viewed under UV light. The bands were removed, ran through another PCR and DGGE and sequenced by GeneWiz (South Plainfield, NJ). Sequencing results were then aligned using BLAST from GenBank.

Results

Abundance Survey 2008

During 2008, Pakim Pond and Indian Mills Lake were sampled 7 times from mid July to mid October (Appendix Table 1). Abundance data, number of zoosporic fungi per 100 pollen grains, was analyzed using an ANOVA, with significant differences found between Site, Week, Site*Week (Table 1).

Table 1: Summary of ANOVA of zoosporic fungal abundance (number of fungi per 100 pollen grains) from 2008 abundance survey of Pakim Pond and Indian Mills.

Source	**df**	**P**
Site	1, 44	<0.0001
Week	4, 44	<0.0001
Site x Week	4, 44	<0.0001
Substrate	1, 44	0.6513
Site x Substrate	1, 44	0.4041

Table 2: Mean +/- Standard Error by week for each site during 2008 abundance survey. Pakim Pond is the pristine site and Indian Mills the impacted site.

	Pakim Pond	**Indian Mills**
Week	Mean +/- SE	Mean +/- SE
1	80.3 +/- 13.14	7.82 +/- 1.42
2	75.5 +/- 6.34	8.5 +/- 3.09
3	49.5 +/- 4.91	3.83 +/- 1.01
4	12.83 +/- 2.76	8.5 +/- 1.57
5	83.75 +/- 5.40	6 +/- 2

Pakim Pond was found to have significantly higher zoosporic fungi abundance than the impacted site, Indian Mills (Fig. 1). There was no significant difference in fungi abundance between mud and debris substrates for either site (Table 1; Fig. 2). There was a difference in zoosporic fungi abundance between sampling intervals (weeks in Table 1) with maximal abundance during week 1 and 5 in Pakim Pond and minimal abundance in week 4 (Table 2). Although both sites experienced different population trends during the 5-week sampling, Indian Mills abundance was consistently lower throughout the entire experiment, peaking in week 4 and decreasing in week 5 (Table 2).

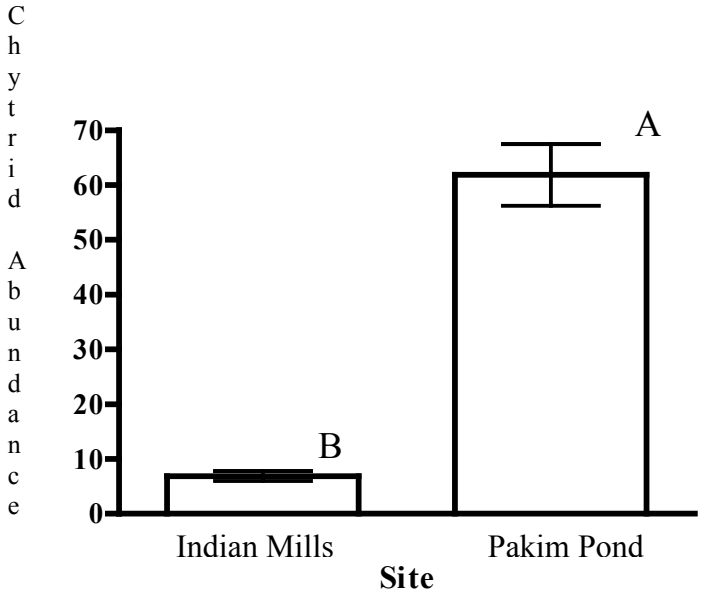

Fig. 1: Zoosporic fungi abundance (number on 100 pollen grains) between Indian Mills (IM) and Pakim Pond (PP) in 2008.

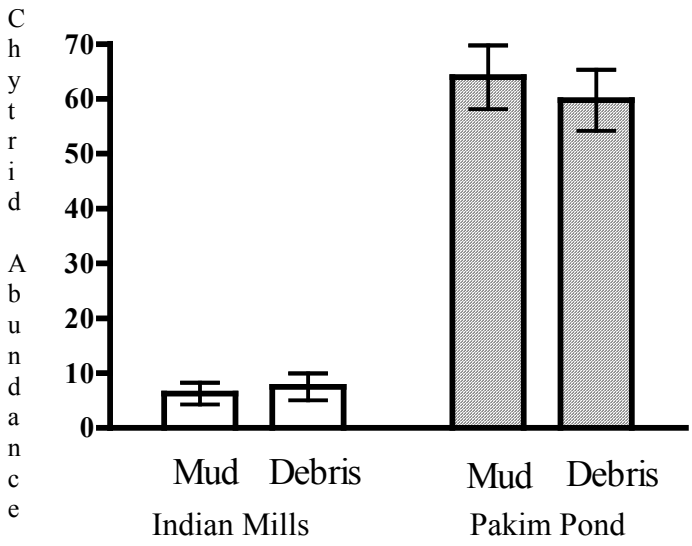

Fig 2: Comparison of substrate and zoosporic abundance (number on 100 pollen grains) between substrate type within Indian Mills (IM) and Pakim Pond (PP).

Abundance Survey 2009

All 6 sites were sampled 3 times during the summer of 2009 (Appendix Table 2). Abundance data was analyzed with an ANOVA using the glm procedure of SAS since three data points were missing due to loss of pollen before time of counting. Significant differences were found for all factors and all levels interactions (Table 3).

Table 3: Summary of ANOVA of zoosporic fungal abundance (number of fungi per 50 pollen grains) from 2009 abundance survey of Pakim Pond, Indian Mills Lake, Otter Pond, Hampton Furnace, Pump Branch Impoundment, and Paradise Lake.

Source	**df**	**P**
Site	5, 72	<0.0001
Week	2, 72	0.0006
Site x Week	10, 72	0.0015
Substrate	2, 72	<0.0001
Site x Substrate	6, 72	0.0004

Table 4: Mean +/- Standard Error by week for each site during 2009 abundance survey. Pristine sites are designated by (P), impacted sites by (I). As previously noted, Pump Branch's Week 1 abundance was taken at time: Week 2, shifting the sampling dates a week later.

Site	Week 1	Week 2	Week 3
Pakim Pond (P)	48 +/- 14.16	68 +/- 14.16	45 +/- 5.70
Hampton Furnace (P)	13 +/- 0.37	45 +/- 5.50	44+/- 4.05
Otter Pond (P)	26 +/- 12.97	41 +/- 3.28	41 +/- 3.60
Indian Mills (I)	28 +/- 2.42	17 +/- 6.48	12 +/- 3.52
Pump Branch (I)	39 +/- 5.4	34 +/- 4.43	18+/- 2.88
Paradise Lake (I)	13 +/- 3.23	33 +/- 6.55	22 +/- 3.66

There were significant differences in zoosporic fungi abundance between sites (Table 3) with Pakim Pond (PP) having significantly higher fungal abundance than any of the other sites and Indian Mills (IM) had the lowest fungal abundance (Fig. 3). Overall pristine sites had significantly higher zoosporic fungi abundance than the impacted sites with all sites experiencing different population curves during the 3-week sampling. (Fig. 4). It should be noted that in week 1 Otter Pond (OP) and Hampton Furnace (HF) had samples of water instead of debris. However, the only significant substrate effect resulted from a significantly lower abundance of fungi in the water samples than in either mud or debris (Table 3); therefore, there is no significant difference in fungal abundance between mud and debris samples, as found in 2008. Week had a significant effect on zoosporic fungi abundance (Table 3) as each site responded differently each week (Table 4), resulting in a significant Site*Week interaction.

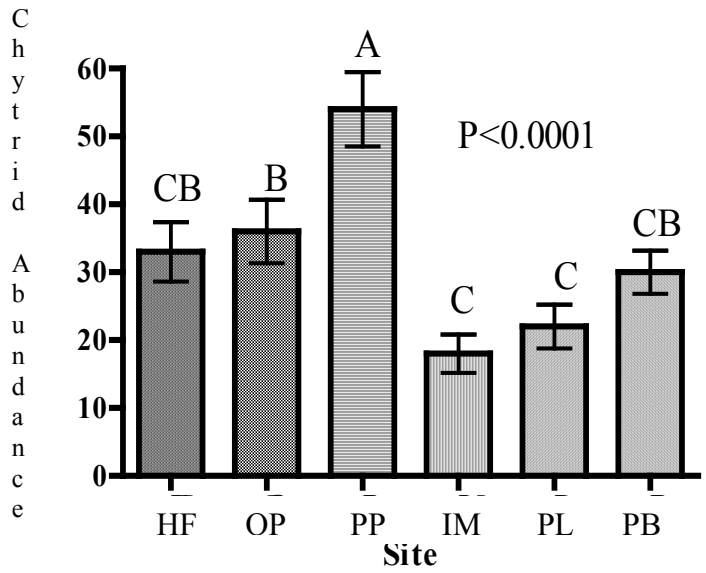

Fig 3: Zoosporic fungi abundance for each site during 2009. The three sites on the left-hand side of the graph, Hampton Furnace (HF), Otter Pond (OP), and Pakim Pond (PP) are pristine sites and the sites of the right-hand side of the graph, Indian Mills (IM), Paradise Lake (PL), and Pump Branch (PB), are impacted

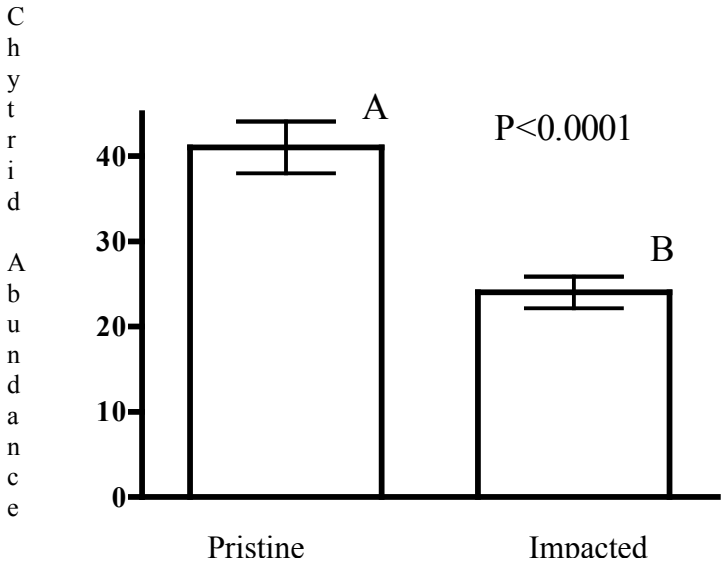

Fig 4: If all pristine and impacted sites are grouped together, there is significantly greater zoosporic fungal abundance in the pristine sites.

DGGE and Sequencing Results

Isolated DNA from pollen in Pakim Pond and Indian Mills found that two species of zoosporic fungi dominated the Pakim community and 1 species dominated the Indian

Mills community (Fig. 5). Both communities were mutually exclusive; species found in Pakim were absent in Indian Mills and species in Indian Mills were absent from the Pakim community.

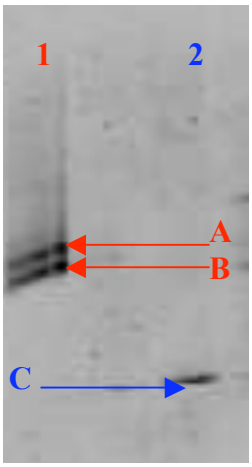

Fig 5: DGGE results from Pakim Pond (Column 1) and Indian Mills (Column 2). Two species of zoosporic fungi dominated the Pakim community (A and B) and 1 species dominated the Indian Mills community (C).

Molecular sequencing of these bands and alignment using BLAST from GenBank found that band B from Pakim Pond was significantly similar to an uncultured soil fungus (Accession #: DQ421295.1). Sequencing of band A produced a low quality score during both sequencing processes and when aligned using BLAST found no significant matches. Band C from Indian Mills was also found low quality after sequencing results and when aligned and ranked by E-Value, *Penicillium resedanum* (Accession #: AF033398.1) was ranked highest with an E-Value of 2e-152.

Discussion

Zoosporic fungi abundance surveys from 2008 and 2009 found that in non-ecologically impacted water bodies, these fungi were consistently more abundant than in sites with higher pollution content and lower water quality. Lower zoosporic fungi

abundance in degraded sites furthers evidence that pollution changes community structure and lowers diversity (Tan & Lim 1983, El-Sharouny 1992, Tsui *et al.* 2001, Hafez *et al.* 2009). Previous studies have found that native microflora in soil communities were more sensitive to environmental conditions and died in the presence of contamination (Hafez *et al.* 2009). These changes in fungal communities due to nutrient enrichment pollution were also observed in rivers (El-Sharouny 1992, Tsui *et al.* 2001). Furthermore, as decreases in native species were observed a community shift towards resistant species not previously found in the area were recorded (Tsui *et al.* 2001, Hafez *et al.* 2009). These changes in community structure had an adverse effect on habitat quality as nutrients were depleted and decomposition slowed (Tsui *et al.* 2001, Hafez *et al.* 2009).

This shift in community structure was validated with the results of the DGGE. The two dominant species in Pakim Pond were absent in Indian Mills and appeared to have been replaced by one novel dominant species.

The results from the molecular sequencing were not as successful. The high quality band B from Pakim Pond was found significantly similar to an uncultured soil fungus that was collected from soil north of Minneapolis MN by Waldrop *et al.* in 2006.

Although band C aligned with *Penicillium resedanum,* the molecular sequencing did not pass Genewiz's quality control and therefore we are not confident in this match. *Penicillium*, an ascomycete, would not have been selected with spruce pollen baiting. Therefore, given the low quality of the DNA from band A (Pakim Pond) and band C (Indian Mills) further DNA isolation and sequencing has to be done to confirm BLAST results. Future research to further identify the species in each community and confirm this

possible shift from endemic to invasive chytrid species is necessary. Other methods of community sampling should be investigated as pollen baiting may be selective and not present a complete representation of the zoosporic fungal community.

Although, the concept that environmental pollution has adverse effects on all levels of an ecosystem is hardly novel, new evidence has suggested just how far-reaching its impact can be. Hafez *et al.* (2009) suggests that environmental conditions can affect the utilization of resources, increase or decrease a community's stress tolerance, limit available ecosystem services, as well as increase a community's susceptibility to biological invasions lowering biodiversity, and endangering native organisms.

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Appendix

Table 1: Pakim Pond and Indian Mills Lake sampling dates for 2008

Site:	Sampling dates
Pakim Pond	7/14/08
	7/28/08
	9/9/08
	9/16/08
	9/24/08
	10/7/08
	10/14/08
Indian Mills Lake	7/14/08
	7/28/08
	9/9/08
	9/16/08
	9/24/08
	10/7/08
	10/14/08

Table 2: Dates of sampling for each site during the summer of 2009. Pump Branch was first sampled at the time that the rest of the sites were sampled for the second time; however, an analysis of variance of the data put this sample time as time 1. The final sample data of Pump Branch was one week later than the rest.

Time period	Site sampled (Pristine/Impacted)	Actual sample date
Time 1	Hampton Furnace (pristine)	6/25/09
	Otter Pond (pristine)	6/25/09
	Pakim Pond (pristine)	7/3/09
	Indian Mills Lake (impacted)	7/3/09
	Paradise Lake (impacted)	7/3/09
	Pump Branch Imp. (impacted)	7/9/09
Time 2	Hampton Furnace	7/8/09
	Otter Pond	7/8/09
	Pakim Pond	7/8/09
	Indian Mills Lake	7/8/09
	Paradise Lake	7/8/09
	Pump Branch Imp.	7/23/09
Time 3	Hampton Furnace	7/30/09
	Otter Pond	7/30/09
	Pakim Pond	7/30/09
	Indian Mills Lake	7/30/09
	Paradise Lake	7/23/09
	Pump Branch Imp.	8/5/09

Chapter III

Environmental conditions and freshwater zoosporic fungi (chytridiomycete) communities in the New Jersey Pinelands

Abstract

Zoosporic fungi (chytridiomycete) abundance, as captured on pollen bait, was found to be significantly higher in pristine sites than impacted sites in a survey of six water bodies of varying ecological integrity (as categorized by the NJ Pinelands Commission) in the New Jersey pine barrens (USA). Using a series of laboratory manipulations of water from a pristine and an impacted site, altered pH appeared to the primary driver of zoosporic fungi abundance in the field. Increasing pH of the naturally acid water of the pine barrens, significantly reduced zoosporic fungal populations and a reduction of pH of impacted water ameliorated conditions and increased abundance. The addition of nitrogen or phosphorus to, or an increase in specific conductance of pristine water reduced zoosporic fungal abundance, but a reduction in these parameters in impacted water did not induce restoration of zoosporic fungi. These results show zoosporic fungi as potential as bioindicators of pollution.

Introduction

Zoosporic fungi (Chytidiomycota) are found in ecosystems from the artic to the tropics in aquatic and terrestrial environments (Powell 1993), with highest species diversity in temperate zones (Shearer *et al.* 2007). Due to their ability to form resistant structures that can avoid desiccation and unfavorable conditions, zoosporic fungi can survive in a wide range of ecosystems with drastically differing biotic and abiotic

parameters (Gleason *et al.* 2004). Thus their distribution appears to be dependant on environmental conditions rather than geography (Shearer *et al.* 2007).

Due to their saprotrophic and parasitic nature, zoosporic fungi have an important, although often overlooked, role in community dynamics (Kagami *et al.* 2007). Aside from hosting on a wide variety of organisms ranging from algae to invertebrates (Shearer *et al.* 2007), Kagami *et al.* (2004) found that the zoospores of these fungi are a significant food source for grazing for zooplankton. Zoosporic fungi feed on chitin, keratin, and cellulose they obtain from their hosts including algae, fungi, plants, invertebrates, and, recently discovered, vertebrates (Shearer *et al.* 2007; Gleason *et al*., 2008). In aquatic systems zoosporic fungi frequently parasitize phytoplankton; in terrestrial systems some genera form obligate parasitic relationships with vascular plants (Powell 1993; James *et al.* 2006), although the majority of chytridiomycetes inhabit terrestrial systems as saprotrophs (James *et al.* 2006). The high host-specificity of many of these species of pathogenic zoosporic fungi (Van Donk & Bruning 1995) also lends them to have a significant effect on their host's population dynamics as well as the ecosystem food-web (Kagami *et al.* 2007). In each system, a few species thrive in large populations, while the majority remain infrequent and in low abundance (James *et al.* 2006). A comparison of zoosporic fungal species in soil between four sites in Virginia found although the majority of species were found at all sites there was still a distinct pattern of distribution (Letcher & Powell 2001), but they did not relate community composition with edaphic variables.

Numerous studies have investigated the effect environmental parameters have on zoosporic fungi populations, specifically related to zoosporic fungi-phytoplankton

interactions, and epidemics (Bruning 1991; Van Donk & Bruning 1995; Kagami *et al.* 2007; Wakelin *et al.* 2007). In a study of parasitism of the alga *Asterionella formosa* by the zoosporic fungi *Rhizophydium planktonicum,* high water temperatures and low nutrient content (particularly phosphorus) favored zoosporic fungal abundance due to rapid sporangial development at the expense of algal growth (Van Donk & Bruning 1995). In another study low water temperature favored algal growth, whereas at temperatures of 5 – 20° C favored zoosporic fungal growth, resulting in algal suppression by the zoosporic fungi pathogen (Van Donk & Ringleberg 1983). Temperature, pH, nutrients, along with light, turbulence, and zooplankton grazing, have all been shown to impact zoosporic fungi related infections on phytoplankton and eutrophication, land-use changes, increasing temperature, and UV radiation may effect how frequently these epidemics occur (Kagami *et al.* 2007). In soil, Gleason *et al*. (2004; 2005) showed that zoosporic fungi had differential survival between taxa to increased temperature with some tolerating higher temperatures than others.

Freshwater systems are highly susceptible to changes in environmental conditions. These conditions can be natural changes in the ecosystem or human induced (Beldon & Harris 2007) such as pollution, land-use changes, increasing UV radiation, temperature fluctuations, and habitat loss or degradation. These factors can change water conditions to the point where the entire community may become altered. This was found in acidified mountain streams of West Virginia where acidic conditions decreased zoosporic fungal species and highest diversity was observed at a more neutral pH (Dubey *et al.* 1994*)*.

In the New Jersey pine barrens, urban development and agricultural run-off is

affecting the once pristine water quality in sites that abut or are downstream of intensive agriculture and urban development. These water bodies, identified by the Pinelands Commission, are exhibiting changes in their water chemistry such as elevated pH, high nutrient content, and an increased specific conductance, caused by run-off, upland land use, invasive species, and habitat loss and degradation. Given the ubiquity of zoosporic fungi and that their distribution is dependant on environmental conditions rather than geography (Shearer *et al.* 2007). To our knowledge there have been no studies on zoosporic fungi in the NJ pine barrens so our aims were twofold (i) are zoosporic fungi present in NJ pine barrens waters and (ii) are zoosporic fungal populations changed as their ecosystem degraded.

We conducted a survey of pine barrens water bodies that had been categorized as either pristine or impacted (Pinelands Commission designation) with the null hypothesis that water quality, as defined in these categorizations, would not affect zoosporic fungal abundance. Through a series of laboratory manipulations, we investigated the effect of altered pH, specific conductance and nitrogen and phosphorus loading have on zoosporic fungi populations from impacted and non-impacted water bodies to identify possible causal agents in changes of zoosporic fungi abundance.

Materials and Methods

Site Assessment

Sites were selected using the New Jersey Pinelands Commission's long-term ecological assessment of water bodies. This long-term environmental-monitoring program analyzed water bodies within the Mullica River, Rancocas Creek, Great Egg

Harbor, and Barnegat Bay watersheds for ecological integrity. Each site was identified on a scale of 1 to 5, with 5 being a pristine ecosystem and 1 being a severely impacted system (Zampella *et al* 2001, 2003, 2005, 2006). Integrity was evaluated through analysis of pH, specific conductance, marginal vegetation, and fish and anuran assemblages. Pristine sites were designated by possessing a low pH, low specific conductance, native and intact marginal vegetation, and native, healthy populations of native fish and anuran species. Impacted sites had higher, more neutral pH, high relative specific conductance, a loss of native species, and a high number of invasive species.

Three sites (Pakim Pond, Hampton Furnace, and Otter Pond) were chosen as pristine sites based on their high level of ecological integrity, having typical pineland's water body characteristics, defined as low pH, low specific conductance, minimal invasive species, and healthy native populations. In contrast, Indian Mills Lake, Pump Branch Impoundment, and Paradise Lake were chosen as impacted water bodies, defined by high pH, high specific conductance, presence of invasive and border entrant species, and proximity to developed and impervious surfaces.

Laboratory Manipulations of Water Chemistry

In an attempt to determine environmental factors that my cause changes in zoosporic fungi abundance, a non-impacted site (Pakim Pond) and an impacted site (Indian Mills Lake) were chosen as extremes of water quality. Water samples from each of these sites were compared and manipulated in the laboratory to bring the Pakim Pond water to an equivalent, degraded, state of the Indian Mills and to improve the quality of Indian Mills to the state of Pakim Pond water through appropriate manipulation of water

chemistry. The abundance of zoosporic fungi was measured by baiting the samples with pollen and counting the number of fungi colonizing 50 or 100 grains as previously described. The changes in abundance as a result of these manipulations was taken as an index of importance of the pollutant in likely causing the observed differences in zoosporic fungi abundance in the field surveys. All laboratory studies were conducted in the same room at ambient temperature. A summary of water quality at each of the two sites along with target changes in water quality in the manipulation experiments are given in Table 1.

Manipulation of Nitrogen

Nitrogen concentration of water samples from both Pakim Pond and Indian Mills Lake (Table 1) were determined colorimetrically using phenate method for ammonia and hydrazine for nitrite/nitrate with an Astoria-Pacific AutoAnalyzer 3 (Clackamus OR) (Allen 1998). Ambient nitrogen levels (µg L-1) in Pakim Pond were 0.305 (NO^3) and 0.31 (NH4); ambient levels in Indian Mills were 0.57 (NO^3) and 0.367 (NH^4).

Sediment and leaf debris from the edge of the water body was collected from Pakim Pond and divided among 24 500 ml Mason jar. All jars received debris from Pakim Pond so as to provide the same zoosporic fungi inoculum to each system. Twelve jars were filled with approximately 250 ml Pakim water and the remaining 12 jars were filled with 250 ml of Indian Mills water. Nitrogen addition was calculated using base levels of NO^3-N and NH^4-N from Pakim Pond. In replicates of three the jars from each site were divided into 4 levels: Control with no additional N loading, in Level 1 NH^4 and NO^3 equal to 0.5 the initial concentration in Pakim were added, Level 2 received 1.5

times the NH^4 and NO^3, and Level 3 received twice the ambient Pakim level of NH^4 and NO^3 (Table 1). Jars were baited with pollen and every 3 days nitrogen levels were adjusted.

One week after baiting, pollen was mounted on microscope slides and the total number of zoosporic fungi on 50 or 100 pollen grains was counted at 400 x magnification. Afterwards nitrogen was readjusted, and deionized distilled H_20 was added to refill jars to original volume. The jars were then bubbled with an aerator for 30 minutes and re-baited with pollen and assessed after another week of incubation.

Manipulation of pH

Water and debris samples collected in April 2009 from Pakim Pond and Indian Mills had initial pH of 4.16 and 7.42 respectively. The experiment was set up as described above using 3 replicates of each of a control and 2 manipulations for a total of 18 jars. The nine Pakim jars were divided into 3 control with no change in pH, 3 jars that received an NaOH solution to raise pH to a median of both sites (5.76), and 3 jars that were raised to the pH of Indian Mills (7.42). The Indian Mills jars were divided similarly with 3 jars receiving an HCl solution to lower to the median level (5.76), and 3 unamended jars as controls and 3 receiving the acidic solution to lower the pH to the initial Pakim level of 4.16 (Table 1). NaOH and HCl were used in the alkaline and acidic solutions as per Piotrowski *et al.* 2004. All jars received the same treatment as previously described of pollen baiting, counting, and aerating. The pH was checked 3 to 4 times a week prior to re-baiting and adjusted accordingly.

Manipulation of Phosphorus

Water and debris samples were obtained in June 2009 from Pakim Pond and Indian Mills Lake. Ambient phosphorus levels for Pakim Pond was approximately 0.1 mg/L and Indian Mills was approximately 0.2 mg/L. Phosphorus concentration was determined colorimetrically using the ascorbic acid method (Allen 1998). Manipulation was set-up identically to the pH experiment with a control, and 2 levels of manipulation based on initial PO_4 levels of Pakim and Indian Mills with a median level target concentration of 0.15 mg/L (Table 1). Phosphorus content was monitored every 3 days and adjusted with KH_2P0_4. Zoosporic fungal abundance on pollen was counted weekly for 4 weeks as above.

Manipulation of Specific Conductance

Water and debris samples were collected from Pakim Pond and Indian Mills Lake in June 2009. Specific conductance was analyzed with Pakim Pond having 48.9 s/cm and Indian Mills having 184.5 s/cm. The experiment followed the previous set-ups with a control and 2 levels of manipulation with a median level target concentration of 116.7 s/cm (Table 1). Specific conductance of each sample was modified through addition of NaCl or deionized distilled water to increase or decrease conductivity. Specific conductivity was adjusted every 3 days and pollen was counted weekly for 4 weeks.

Table 1: Ambient water quality measurements of Pakim Pond and Indian Mills Lake and target concentrations for laboratory manipulations of pH, phosphorus, specific conductance, and nitrogen loading.

Manipulation Level	**Pakim Pond**			**Indian Mills Lake**		
	Control (Ambient)	1 (Median)	2 (Opposite)	Control (Ambient)	1 (Median)	2 (Opposite)
pH	4.16	5.76	7.42	7.42	5.76	4.16
Phosphorus (mg/L P)	0.1	0.15	0.2	0.2	0. 15	0.1
Specific Conductance (s/cm)	48.9	116.7	184.5	184.5	116.7	48.9
Nitrogen Addition (ug/L)	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
	0.153 NO_3, 0.155 NH_4	0.305 NO_3, 0.31 NH_4	0.457 NO_3, 0.465 NH_4	0.153 NO_3, 0.155 NH_4	0.305 NO_3, 0.31 NH_4	0.457 NO_3, 0.465 NH_4

Statistical analysis

For water chemistry effects, each manipulation was considered separately as a factorial designed experiment (site * treatment * time) and analyzed by ANOVA or GLM (missing data) procedures with Tukey's *post hoc* means separation test.

Results

Nitrogen

Zoosporic fungi abundance had a strong response to increasing nitrogen content (Table 2). The increase of nitrogen in Pakim Pond caused a steady decrease in abundance as habitat quality decreased (Fig. 1). Increasing nitrogen in Indian Mills did not show a strong trend in abundance and instead zoosporic fungi levels fluctuated, being at their highest at Level 1and lowest at Level 2 (Fig. 1). ANOVA results for both sites showed zoosporic fungi abundance significantly different between the sites (P= 0.0016) with Pakim having a higher abundance (Table 2). Pakim and Indian both experienced a loss of abundance over time (Table 3), although the rate of loss was significantly

different between the sites (Table 2). Treatment was also found to be highly significant between the sites (Table 2), with zoosporic fungi abundance decreasing as Treatment increased for Pakim Pond and no change over Treatment level for Indian Mills (Table 3). The significant Site*Treatment interaction is interpreted as increasing N loading of Pakim Pond reduces zoosporic fungi abundance, but the attempted cleaning of Indian Mills water did not affect zoosporic fungi abundance.

Table 2: Summary of ANOVA of zoosporic fungi abundance (number of fungi per 50 pollen grains) from nitrogen manipulation for Pakim Pond and Indian Mills.

Source	**df**	**P**
Site	1, 61	0.0016
Treatment	3, 61	<0.0001
Site x Treatment	3, 61	<0.0001
Week	3, 61	<0.0001
Site x Week	3, 61	<0.0001
Treatment x Week	9, 61	<0.0001
Site x Treatment x Week	9, 61	<0.0001

Table 3: Mean and Standard Error of zoosporic fungi abundance for each site from nitrogen manipulation by week.

	Pakim Pond	**Indian Mills**
Week	**Mean +/- SE**	**Mean +/- SE**
1	53.67 +/- 2.72	52.83 +/- 3.13
2	25.0 +/- 4.86	28.82 +/- 4.33
3	18.6 +/- 3.13	31.5 +/- 5.19
4	2.57 +/- 0.65	1.5 +/- 0.34

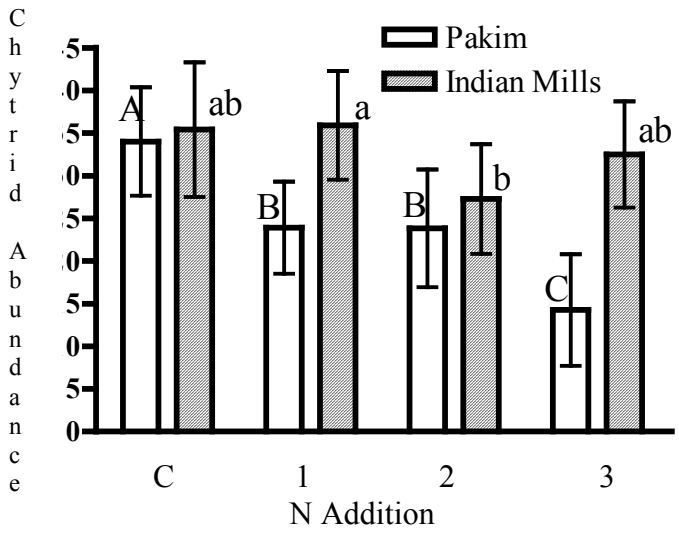

Figure 1: Zoosporic fungi abundance in the presence of increased nitrogen loading. Increasing nitrogen in Pakim Pond (P) resulted in a steady and significant decline ($P<0.0001$), while decreasing nitrogen caused a less distinct change in fungi abundance in Indian Mills (IM) ($P<0.05$)

pH

Altering pH had significant effects on both Pakim Pond and Indian Mills at every level and interaction (Table 4). Increasing pH in the Pakim samples simulated an increase in ecological degradation and caused a significant decrease in zoosporic fungi abundance (Fig. 2). As pH was lowered back to optimal Pinelands levels in Indian Mills samples, fungi abundance increased (Fig. 2). ANOVA results for the entire experiment including treatments and weeks found zoosporic fungi abundance between the sites significantly different ($P= 0.0033$) with Pakim constantly having a higher abundance. If an ANOVA was run for each site exclusively it showed that Treatment, Week, and Treatment *Week all had strong effects on fungi abundance (Table 4). pH was the only parameter where both sites, despite a loss during week 2 in Indian Mills, had overall increases in zoosporic fungi abundance overtime (Table 5).

Table 4: Summary of ANOVA of zoosporic fungi abundance (number of zoosporic fungi per 50 pollen grains) from pH manipulation individually for Pakim Pond and Indian Mills

Pakim Pond	**df**	**P**	**Indian Mills**	**df**	**P**
Treatment	2, 23	<0.0001	Treatment	2, 23	0.0180
Week	3, 23	0.0010	Week	3, 23	0.0095
Treatment*Week	6, 23	<0.0001	Treatment*Week	6, 23	0.0446

Table 5: Means and standard errors of pH manipulation for each site by week. Overall, both Pakim Pond and Indian Mills increased in fungi abundance overtime.

Week	**Pakim Pond** **Mean +/- SE**	**Indian Mills** **Mean +/- SE**
1	34.63 +/- 4.37	29.67 +/- 5.41
2	47.89 +/- 2.18	18.88 +/- 4.43
3	86.33 +/- 27.27	34.44 +/- 6.58
4	70.11 +/- 15.27	72.89 +/- 24.19

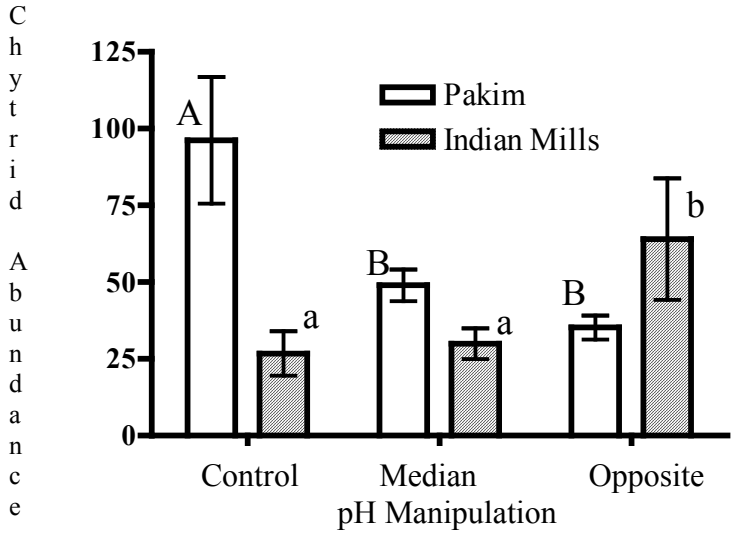

Figure 2: Zoosporic fungi abundance of Pakim Pond and Indian Mills in response to changes in pH. Fungi populations in Pakim Pond (PP) steadily decreased as pH became more neutral ($P<0.0001$) and Indian Mills (IM) populations steadily increased as pH was lowered ($P<0.05$).

Phosphorus

Pakim Pond showed a steady decline in zoosporic fungi abundance as phosphorus content increased and habitat quality decreased. In Indian Mills, as phosphorus decreased there was no significant effect on fungi abundance (Fig. 3). ANOVA results for the entire experiment including treatments and weeks found zoosporic fungi abundance between the sites significantly different (P= 0.0027) with Pakim constantly having a higher abundance. When site specific ANOVAs were conducted Treatment and Week had a significant impact on Pakim Pond while only Week was significant for Indian Mills (Table 6). Site*Week interactions were not statistically significant (P=0.0706) as both sites decreased in abundance over time (Table 7).

Table 6: Summary of ANOVA of zoosporic fungi abundance (number of fungi per 50 pollen grains) from phosphorus manipulation for Pakim Pond and Indian Mills. Summary is based on glm procedure of SAS due to missing data points.

Pakim Pond	**df**	**P**	**Indian Mills**	**df**	**P**
Treatment	2, 23	0.0255	Treatment	2, 15	0.8852
Week	3, 23	<0.0001	Week	2, 15	<0.0001
Treatment*Week	6, 23	0.6265	Treatment*Week	3, 15	0.0954

Table 7: Means and standard errors of phosphorus manipulation for each site by week. Although at different rates, Pakim Pond and Indian Mills both decreased in zoosporic fungi abundance overtime.

Week	**Pakim Pond**	**Indian Mills**
	Mean +/- SE	**Mean +/- SE**
1	101.56 +/- 15.96	No data
2	32.25 +/- 9.47	53.33 +/- 8.82
3	61.78 +/- 14.72	33.89 +/- 3.95
4	6.88 +/- 4.59	3.57 +/- 1.11

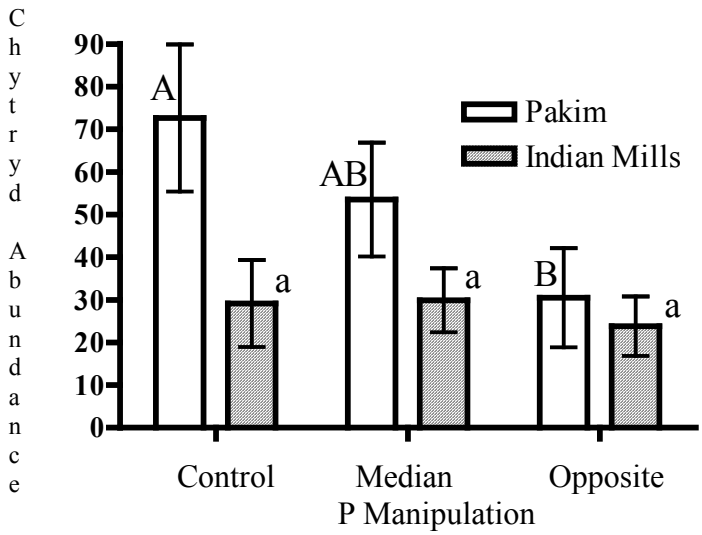

Figure 3: Zoosporic fungi abundance of Pakim Pond and Indian Mills in response to changes in phosphorus concentration. Pakim Pond (PP) abundance displayed a steady decline (P=0.0255) as phosphorus increased while Indian Mills (IM) was unaffected by P concentration (P=0.885).

Specific Conductance

Zoosporic fungi abundance in Pakim Pond decreased as specific conductance increased. As with the other manipulations, fungal abundance decreased as habitat quality decreased (Fig. 4). Zoosporic fungi abundance in Indian Mills did not respond to a decrease in specific conductance. Week had significant effects on zoosporic fungi abundance in Pakim Pond and Indian Mills (Table 8). Treatment was significant in Pakim Pond (Table 8); however, the extremes were significantly different only from each other and not the median so the means did not separate (Fig. 7). ANOVA results for the entire experiment including treatments and weeks found zoosporic fungi abundance between the sites significantly different (P= <0.0001) with Pakim constantly having a higher abundance. Site*Week interactions were statistically significant (P=0.0014) as both sites decreased in abundance over time (Table 9).

Table 8: Summary of ANOVA of zoosporic fungal abundance (number of fungi per 50 pollen grains) from specific conductance manipulation individually for Pakim Pond and Indian Mills. Summary is based on glm procedure of SAS due to missing data points.

Pakim Pond	**df**	**P**	**Indian Mills**	**df**	**P**
Treatment	2, 21	0.0334	Treatment	2, 17	0.4599
Week	3, 21	<0.0001	Week	3, 17	0.0002
Treatment*Week	6, 21	0.1567	Treatment*Week	3, 17	0.8684

Table 9: Means and standard errors of specific conductance manipulation for each site by week. Although Pakim Pond and Indian Mills both decreased overtime only Indian Mills exhibited a steady trend.

	Pakim Pond	**Indian Mills**
Week	**Mean +/- SE**	**Mean +/- SE**
1	150.0 +/- 13.1	63.33 +/- 5.24
2	55.38 +/- 19.07	59.0 +/- 6.45
3	128.25 +/- 24.78	38.56 +/- 4.94
4	25.88 +/- 6.64	8.6 +/- 2.96

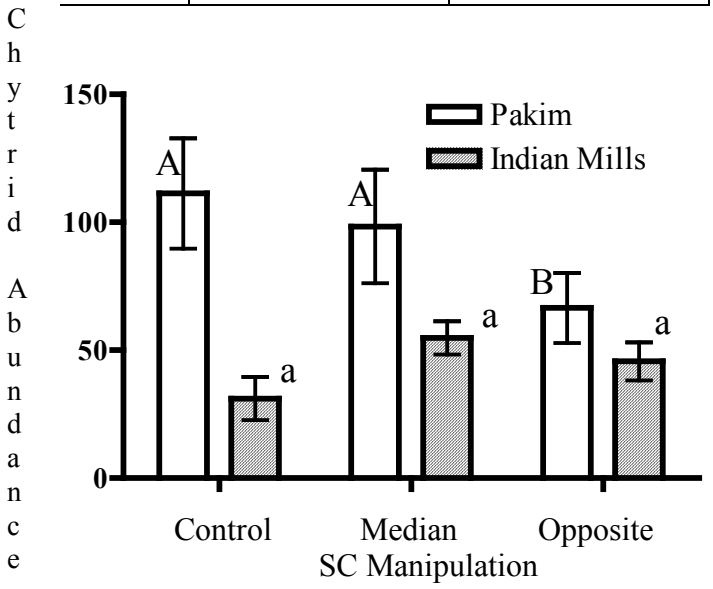

Figure 4: Zoosporic fungi abundance of Pakim Pond and Indian Mills in response to specific conductance. Pakim Pond (PP) displayed a steady decline ($P<0.05$) while Indian Mills (IM) lacked a response to lowered specific conductance.

Discussion

The NJ Pinelands Commission use a suite of water quality parameters to determine the degree of degradation of water bodies in the NJ pine barrens. Of these, pH

and specific conductivity are the main chemical indicators with low pH and conductivity indicating pristine conditions, where an abundance of indigenous plants and animal species exist to the exclusion of invasives (Zampella *et al.* 2001; 20003; 2005; 2006). We elected to use these parameters along with nitrogen and phosphorus as potential nutrient pollutants to use in water chemistry manipulation experiments to determine possible causal effects of these parameters on zoosporic fungal abundance.

In all of the environmental manipulations when Pakim Pond water was degraded through increasing nitrogen, pH, phosphorus, and specific conductance zoosporic fungal abundance decreased significantly (Fig. 1). These results were expected since endemic microflora are frequently sensitive to environmental conditions (Hafez *et al.* 2009) and the total abundance and species of fungi decreases in the presence of pollution (El-Sharouny 1992).

The response from Indian Mills was not as clear. When nitrogen, phosphorus, and specific conductance were lowered simulating increasing water quality, Zoosporic fungal abundance was not significantly different from the control level (Fig. 1). If the zoosporic fungal communities in degraded waters, such as Indian Mills, were endemic then we would have expected increasing abundance as water quality improved during experimentation. However, since there was no clear trend in zoosporic fungal abundance it may be that the community in Indian Mills has shifted from native to invasive species or the sites were so degraded that the remediation attempted could not restore zoosporic fungal abundance. This scenario seems likely when compared to Tsui *et al.* (2001) where in heavily degraded water common species of fungi were absent and had been replaced with species that had not previously been seen in that area. Hafez *et al.* (2009) also found

that in pollution-contaminated soils, fungal communities shifted from native populations to invasive species that were resistant to pollution; this change in community also had an adverse effect on soil quality as nutrients were depleted.

Of the effects in zoosporic fungi population response to altered water conditions, the response to changes in specific conductance was smallest. The difference in zoosporic fungal abundance between specific conductance levels in Pakim pond were only just significant and there was no response in Indian Mills. This may be that the manipulation of other parameters is more important to zoosporic fungi, or that the actual specific conductance in water is dependent on the balance of a number of chemical ions which may not be truly represented by changes in NaCl concentration alone. Of all water quality parameters, pH was the one that had most marked effects on zoosporic fungal abundance and was shown to have some degree of remediation in the impacted Indian Mills site. This is appears contradictory to the findings of Dubey *et al.* (1994) who found higher zoosporic fungi diversity has been observed at more neutral pH. However, when pH is viewed in the context that these native fungi are adapted to the highly acidic waters of the pine barrens, it seems probable that increasing pH it would have adverse effects on the natural community.

Lower zoosporic fungal abundance in degraded sites furthers evidence that pollution changes community structure and lowers diversity (Tan & Lim 1983, El-Sharouny 1992, Tsui *et al.* 2001, Hafez *et al.* 2009). Previous studies have found that native microflora in soil communities were more sensitive to environmental conditions and died in the presence of contamination (Hafez *et al.* 2009). These changes in fungal communities due to nutrient enrichment pollution were also observed in rivers

(El-Sharouny 1992, Tsui *et al.* 2001). Furthermore, as decreases in native species were observed, a community shift towards resistant species, not previously found in the area, were recorded (Tsui *et al.* 2001, Hafez *et al.* 2009). These changes in community structure had an adverse effect on habitat quality as nutrients were depleted and decomposition slowed (Tsui *et al.* 2001, Hafez *et al.* 2009).

As our surveys did not include taxonomic identity of the fungi we found, it would be a logical and necessary next step to obtain information on community compositional changes in these sites to identify changes due to level of impaction. This work should be conducted using both morphological (Chen *et al*. 2000; Letcher & Powell, 2001) and molecular means of identification (James *et al.* 2006; Euringer & Lueders, 2008. Some functional aspect of potential community changes could also be made, such as rates of leaf litter decomposition (Gleason *et al*. 2008) and the interaction between fungal and algal abundance (Van Donk & Bruning 1995; Van Donk & Ringleberg 1983) In this regard, recent evidence has suggested just how far-reaching pollutant impacts can be. Hafez *et al.* (2009) suggests that environmental conditions can affect the utilization of resources, increase or decrease a community's stress tolerance, limit available ecosystem services, as well as increase a community's susceptibility to biological invasions lowering biodiversity, and endangering native organisms.

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