

ABSTRACT

ROSSI, RYANN ELIZABETH. The Role of Multiple Stressors in a Mangrove Die-off: A Case Study in The Bahamas Archipelago. (Under the direction of Dr. Craig A. Layman).

Mangroves are foundation species in coastal ecosystems providing an estimated US \$1.6 billion in ecosystem services worldwide. Unfortunately, mangrove forests are declining because of myriad factors, many related to human activity. Although human activities are the driving cause of mangrove loss globally, natural factors also result in mangrove loss. Here, I present a case study from Abaco, The Bahamas, in which a die-off of dwarf red mangroves (*Rhizophora mangle*) was reported by local fishermen in 2011. Initial data suggest that prior to death these dwarf red mangroves are stressed by multiple factors, including a fungal pathogen, herbivory, and altered abiotic conditions (e.g., hyper-salinity). I used publicly available LANDSAT satellite imagery to map the die-off region and to determine the approximate year die-off began. I compared mean greenness across the die-off region and found a drastic decrease in mean greenness between 2008 and 2010 that did not appear to be associated with a hurricane event, suggesting other factors could be at play. To understand the potential role of herbivory and plant disease in this die-off, I used a combination of field surveys and experiments. First, I conducted a field survey in the die-off region to determine incidence of herbivory and disease. I found that grazed leaves were positively correlated with disease incidence in this region. Next, I conducted a simulated grazing experiment to test the interaction between grazing and disease incidence on leaves. I found that grazed leaves had greater disease severity than control leaves and found a positive correlation between grazed leaves and disease incidence. To help identify the foliar pathogen, I conducted more disease surveys in red mangroves across two islands in The Bahamas. First, I designed and implemented a disease presence survey for the public to assist in documenting signs of disease in red mangroves on Abaco. Despite low participation by the

public, I found that disease was present in mangroves across Abaco. Following this survey, I conducted more robust disease incidence surveys at select mangrove sites and found that disease incidence was greatest in the die-off region compared to other mangrove sites. I also found that *Pestalotiopsis* sp. were the most commonly isolated fungus from infected red mangrove leaves. Isolates from The Bahamas do not appear to be closely related to known *Pestalotiopsis* sp. suggesting novel strains or species of *Pestalotiopsis* in The Bahamas. Taken together this work suggests that disease and herbivory likely contributed to the mangrove die-off on Abaco, and that more work should be done to identify herbivore and disease communities in mangrove ecosystems.

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The Role of Multiple Stressors in a Mangrove Die-off: A Case Study in The Bahamas Archipelago.

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BIOGRAPHY

I grew up in upstate New York near the heart of the Adirondacks. Vacationing at the Jersey shore solidified my love for the ocean and I decided to pursue a BS in marine biology at the University of Rhode Island. While at the University of Rhode Island, I fell in love with coastal ecosystems and have since studied them in Puerto Rico, Rhode Island, Massachusetts, and The Bahamas. When not tromping around in mangroves or salt marshes, you can find me cooking, running, hiking with my dog and partner, or searching for fungi.

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CHAPTER 1: Introduction

Coastal ecosystems are subject to various natural and anthropogenic stressors that often act concomitantly (Crain et al. 2008, Halpern et al. 2008). The systems are often characterized by habitat-forming foundation species that provide the framework for the entire coastal community (Dayton 1972, Bruno et al. 2003, Ellison et al. 2005). As such, understanding how stressors affect these species is especially important. For example, multiple stressors resulted in the extensive loss of salt marsh habitat along the East and Gulf Coasts of the U.S (Silliman and Newell 2003, Silliman et al. 2005). The combination of biotic stressors (a snail grazer and fungal pathogen) and physical stress (drought) interacted to drive marsh die-off. In this tri-partite interaction, snails do not consume large quantities of live plant tissue, instead they injure the plant and facilitate a fungal infection leading to tissue death. Climate-driven drought stress amplified the effect of snail grazing such that fungal-induced die-off was often more severe in areas with high porewater salinities. These three stresses interacted, which lead to more marsh loss than would have any stressor alone. Given the numerous threats facing coastal ecosystems, understanding how multiple stressors may interact is essential to attempting to mitigate impacts to these systems.

As with salt marsh grasses, mangroves are important coastal foundation species. Mangrove forests cover ~150,000km² of coastal habitat (Giri et al. 2011) and provide an estimated US \$1.6 billion in ecosystem services worldwide (Costanza et al. 1997). These services include essential nursery habitat for important reef and commercial marine organisms, carbon sequestration, and protection of coast lines from storms (Barbier et al. 2011). Since the 1970s, mangroves have declined by about 35% worldwide (FAO 2003F, Barbier et al. 2011, Barbier 2016). Anthropogenic activity has caused global mangrove loss with aquaculture,

agriculture and urban land use being some of the most destructive activities, however, sea level rise remains the greatest threat to mangroves in general (Alongi 2008, Gilman et al. 2008, Alongi 2009, Barbier et al. 2011, Friess and Webb 2014). Natural biotic stressors, e.g., herbivory and plant pathogens, also can drive mangrove loss. For example, herbivores such as the mangrove tree crab (*Aratus pisoni*), locusts and hemipteran insects have been documented to cause leaf damage, defoliation and stem death (Feller 2002, Reef et al. 2012, He and Silliman 2016). Likewise, disease has been documented to cause mangrove loss in Africa, Puerto Rico and Australia (Pegg et al. 1980, Wier et al. 2000, Osorio et al. 2016).

Here, I evaluate if herbivory and plant disease, or their interaction, are associated with a mangrove die-off in The Bahamas. First, I characterize the current state of the die-off area, using geospatial analysis to determine the onset of die-off and whether storm events were the initial cause. Next, I investigate if grazing facilitates fungal infection in dwarf mangroves using a simulated grazing experiment. Third, I identify the foliar pathogen present in the die-off region using disease incidence surveys by the public and scientists, DNA sequencing, morphological characterization and Koch's Postulate trials. I also include appendices documenting an herbivore exclusion experiment in addition to the outreach and education activities I have developed. The culmination of this work will shed light on different drivers of mangrove loss and provide important information for Bahamian resource managers and other stakeholders.

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CHAPTER 2: Mapping a mangrove die-off on a local scale: A case study from The Bahamas

Abstract

Mangrove forests and the ecosystem functions they perform are threatened by several stressors, both abiotic and biotic. We used Landsat 5 and 7 annual NDVI composite imagery to elucidate the beginning and duration of a localized red mangrove die-off in The Bahamas. To determine whether a storm event was linked to the initial stages of the die-off, we constructed two time series: maximum annual NDVI and live vegetation cover in the die-off area. We then compared these values to historical NOAA hurricane tracks. Our results show that this die-off began in 2008, four years after the most recent hurricane passed over the region. Hurricanes passed over the region in 2011 and 2012, when live vegetation cover was at its lowest. Annual maximum NDVI and live vegetation cover slightly increased after 2012 suggesting recovery or perhaps a shift to another mangrove or marsh species. Enabled by Landsat imagery, we found that other stressors were likely responsible for initiating the mangrove die-off, but that hurricanes may have exacerbated the mangrove loss.

Keywords: red mangrove, die-off, multiple stressors, NDVI, Bahamas, Google Earth Engine

Introduction

Mangroves provide a myriad of ecosystem services ranging from furnishing habitat for marine organisms important for both commercial and recreational fisheries to carbon sequestration and coastal buffering (Costanza et al., 1997; UNEP, 2006; Barbier et al., 2011; Veitayaki et al., 2017). Mangrove forests are responsible for 10-15% of coastal sediment carbon storage, yet compose just 0.7% of the world's tropical forests (Giri et al., 2011b; Alongi, 2014), and mangrove forests are declining throughout the world (Duke et al., 2007; Giri et al., 2011b). Much of this decline is a result of anthropogenic activities (Alongi, 2002; Friess and Webb, 2014).

While mangrove forests are viewed as resilient to many natural stressors, reports of die-offs have come from The Bahamas, Cuba, Australia, and other locations (Duke et al., 2005; Polidoro et al., 2010; Duke et al., 2017). Sea level rise and other natural events, such as hurricanes, are the suspected drivers of many of these die-offs (Gilman et al., 2008; Ward et al., 2016; Duke et al., 2017). For example, a recent mangrove die-off recently reported in Australia was likely caused by drought and hypersalinity (Duke et al., 2017). Documenting mangrove die-offs and identifying the drivers of declines can give us insights into how to stem the loss of this immensely important ecosystem.

Mangroves cover approximately 17,877 km² throughout Central America and the Caribbean (Giri et al., 2011b; Ward et al., 2016). In The Bahamas, mangroves and other wetland habitats cover an estimated 4,286 km² (Ecological Gap Analysis, 2014). Mangroves and wetlands are important in the Bahamas as they provide critical habitat for flats fishing species such as the Bonefish (*Albula vulpes*), an activity which brings \$141,000,000 into The Bahamas annually (Fedler, 2010). As such, preservation of healthy mangrove forests in The Bahamas is essential.

Monitoring of mangroves is an essential part of an effective management strategy as it provides advanced warning of die-offs and stressors in nearshore environments (Giri et al., 2011a; Long et al., 2014). Recently, there have been reports of mangrove die-offs and diebacks across large scales (Duke et al., 2017; Lovelock et al., 2017). With monitoring plans in place, land managers may be able to identify stressors and initiate management measures to avoid, or curtail the extent of, die-offs. Unfortunately, many mangrove forests are in difficult to access areas making direct *in situ* monitoring difficult and often cost-prohibitive. The use of geospatial imagery has improved monitoring of mangrove distribution and changes in land cover (Giri, 2008; Giri et al., 2011b). Many nations have been able to map mangrove distributions to facilitate future monitoring (Long et al., 2014; Kanniah et al., 2015).

Here, we demonstrate the utility of geospatial imagery in documenting a mangrove die-off in a remote region of The Bahamas. We use publicly available Landsat imagery transformed to Normalized Difference Vegetation Index (NDVI) to determine the beginning of this localized mangrove die-off. We then corroborate these findings with high resolution imagery in conjunction with field observations. Finally, we compare timing of hurricane events to a time-series of annual mangrove cover to determine if a storm event was associated with this localized mangrove die-off.

Methods

Study Area

A mangrove die-off was reported by local fishing guides on Abaco Island, The Bahamas in 2011. The die-off area is located on the west side of the island in the Abaco Marls National Reserve (Fig. 1). This area is composed primarily of red mangroves (*R. mangle*) with some black mangrove (*Avicennia germinans*) and salt marsh grass (*Spartina* sp.) present. The shallow waters

are composed of submerged seagrass (primarily turtle grass, *Thalassia testudinum*), sponge grounds, and sand flats. Most of the area is isolated from direct human impacts, as it is extremely shallow and can only be accessed with specialized boats by those knowledgeable of the seascape. The die-off area is $\sim 1.2\text{km}^2$ and at present has not spread into nearby dwarf red mangrove stands. In this area, skeletons of dwarf red mangroves remain with sporadic live dwarf red mangroves at the edge of the die-off and water's edge. Those dwarf red mangroves that are living have severely damaged leaves due to herbivory and disease (Rossi, 2017). Additionally, peat collapse has occurred due to loss of live mangroves trapping and accreting sediment.

Data and analysis

Landsat 5 and 7 annual NDVI composites from 1989 to 2013 were acquired from the Google Earth Engine website (<https://earthengine.google.com/>). Years with severe cloud cover were removed from the dataset resulting in the exclusion of 1990 and 2003. Images were transformed to Normalized Difference Vegetation index (NDVI) following Chander et al. (2009) by USGS. Handheld GPS units (Garmin etrex 20) were used to outline and ground truth the mangrove die-off area in June 2014. Historical hurricane tracks were acquired from the National Oceanic and Atmospheric Administration (NOAA) Digital Coasts website (<https://coast.noaa.gov/hurricanes/>). Non-publicly available data used in this study can be found here: <https://www.bco-dmo.org/project/653797>.

We used coordinates from handheld GPS units to create an outline of the die-off area as a shapefile using ArcMap 10.3.1. This shapefile was checked against a basemap of the Caribbean produced by the U.S Geological Survey (USGS) and high-resolution imagery from WorldView-2 (<http://www.satimagingcorp.com/satellite-sensors/worldview-2/>). Annual NDVI composites from each year were clipped to the shapefile in ArcMap 10.3.1. NDVI was reclassified into five

categories (Table I). Area of each reclassified NDVI category was calculated for every year in the die-off region using R (version 3.4.1). NDVI values categorized as yellow and pale yellow (0.2-0.5, 0.5-0.7, respectively) were considered as live mangrove. Historical hurricane tracks were filtered such that only storms that directly passed over Abaco Island between 1984 and 2013 were included in our study (Table II). We constructed a time series of live mangrove vegetation cover and of maximum annual NDVI from 1989-2013 in R (version 3.4.1). Maximum annual NDVI was chosen to ensure minor cloud cover did not affect the overall values and reflects the maximum NDVI value over the entire die-off area. We then superimposed hurricane events on the time series to determine if a pattern between storm events and changes in mangrove cover and maximum NDVI were evident.

Results and Discussion

Maximum annual NDVI ranged from 0.10- 0.69 in the die-off region between 1989 and 2013 (Fig. 2). During this period, storm events occurred ranging in strength from a tropical storm to a category 4 hurricane (Table II). Mangrove vegetation and maximum NDVI responded accordingly to hurricane events with notable declines in both NDVI and cover, particularly in 2011-2012 (Fig. 2). Prior to 2008, mangrove vegetation covered 0.67-0.82 km² on the die-off region while barren area covered ~0.2km² out of a total area of 1.2km² (Fig. 2). Following 2008, there is a drastic drop in maximum annual NDVI through 2010 even though no hurricanes occurred during that time (Fig. 2). Simultaneously, mangrove vegetation declined from 0.62km² in 2008 to 0.10km² in 2010 (Fig. 2, 3). Maximum annual NDVI increased following the lowest values in 2010, however, live mangrove vegetation coverage dropped to ~0km² in 2011-2012 and increased slightly to 0.09km² in 2013 (Fig. 2).

Our results show that the die-off began in 2008 and was further exacerbated in 2010-2012 (Fig. 3). The last documented hurricane prior to 2010 was in 2004 which suggests that a storm event was likely not responsible for the initial stage of this die-off. However, hurricanes did occur in 2011 and 2012 which could have expanded the damage that began in 2008 despite increases in maximum annual NDVI (Fig. 2). Hurricanes may lead to mangrove loss through defoliation and mortality in addition to several other stressors (Gilman et al., 2008). For example, storm activity may impact soil elevation and biochemistry introducing abiotic stressors such as erosion, compaction and sulfide toxicity (Gilman et al., 2008).

In our study site, we noted severely damaged leaves on remaining live mangroves and investigated the role that herbivory and disease could play in the die-off. We found that herbivory alone was likely not the cause of the die-off, but, that it could facilitate disease and therefore could contribute to further stressing the mangroves (Rossi, 2017). Biotic stressors such as plant disease and herbivory have been reported in mangroves elsewhere. For example, a localized mangrove die-off in Puerto Rico was attributed to a fungal disease *Cytospora rhizophorae* which lead to cankers and dieback in red mangroves (Wier et al., 2000). In Australia, a locust outbreak resulted in a significant amount of leaf area loss from black mangroves which contributed to a reduction in stem growth (Reef et al., 2012).

Other abiotic factors such as sea level rise, hyper-salinity and drought could also cause mangrove die-offs (Gilman et al., 2008; Duke et al., 2017; Lovelock et al., 2017). Sea level rise is likely the greatest threat to mangrove forests in the future (Gilman et al., 2008). However, recent events in the Gulf of Carpentaria, Australia suggest that drought and hyper-salinity are also important factors in mangrove loss (Duke et al., 2017). Precipitation in the Abaco region varied greatly between 2008-2013 (Table A1) and regions in The Bahamas were under drought conditions in

2009-2010 (Herrera and Ault, 2017). However, the precipitation data are from a short time period and a neighboring Island and therefore may not provide a full picture of precipitation in the die-off region. Porewater salinities in the die-off region ranged from 47-51 ppt (seawater is ~35 ppt) and surface water temperatures ranged from 26-42°C in 2014 (Rossi, 2017). Porewater salinities in the die-off area were not different than surrounding areas of live mangroves on Abaco (26-50ppt) (Rossi, 2017). Additionally, *R.mangle* is considered to have relatively high salinity tolerance which suggests salinity may or may not be an important factor, depending on the physiological limits of these particular dwarf *R. mangle* (Reef and Lovelock, 2015).

We found an increase in maximum annual NDVI after 2012 which suggests some recovery, or perhaps, replacement of red mangrove with another species, such as the black mangrove (*A. germinans*) (Rossi, personal observation). While some damaged red mangroves remain in the area, we observed black mangroves primarily colonizing regions in the die-off area, particularly along the edge and in the region where some red mangrove with leaves remain. However, we do not know the historical density of black mangroves in this area. While the geospatial methods used here precludes distinguishing the difference between mangrove type (i.e., red vs. black mangrove), future monitoring could incorporate the use of high resolution and multispectral imagery to better determine if shift to another species is taking place. If black mangroves are indeed colonizing the area, this could suggest that abiotic factors such as elevated salinities are at play in the region because black mangroves have a higher tolerance for increased salinities (Cintron et al., 1978; Pezeshki et al., 1990). Furthermore, black mangroves are less preyed upon by grazers and are potentially less susceptible to foliar diseases because of salt excretion through leaves (Gilbert et al., 2002; Erickson et al., 2003).

We find little evidence that a storm event was responsible for the initial stages of mangrove loss which suggests other factors, either abiotic or biotic may have been the initial cause of mangrove loss. Specifically, we point to mild drought, herbivory and plant disease as potential causal agents for this localized mangrove die-off. The use of geospatial imagery in our study filled knowledge gaps regarding the initial stages of the die-off in addition to providing evidence that the die-off has slowed and may be in recovery. Our study, along with others, demonstrates the importance of using remote sensing to monitor mangroves, particularly those in remote regions.

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Tables

Table 1: Normalized Difference Vegetation index (NDVI) classifications. Sparse vegetation such as shrubs and dense vegetation indicate mangrove cover in this study.

NDVI Category	Color Classification	Vegetation Classification
-0.5-0.0	Dark Brown	Barren substrate
0.0-0.1	Brown	Barren substrate
0.1-0.2	Mustard Yellow	Very sparse vegetation
0.2-0.5	Yellow	Sparse vegetation such as shrub or grasslands
0.5-0.7	Pale Yellow	Dense vegetation

Table 2: Historical storm tracks that crossed Abaco, The Bahamas between 1984 and 2014. Strength categories presented reflect the strength of the storm as it passed over Abaco. Data adapted from NOAA historical hurricane track database (<https://coast.noaa.gov/hurricanes/>).

Storm	Year	Strength
Floyd	1987	TS
Floyd	1999	H4
Jeanne	2004	H3
Irene	2011	H2
Sandy	2012	H1

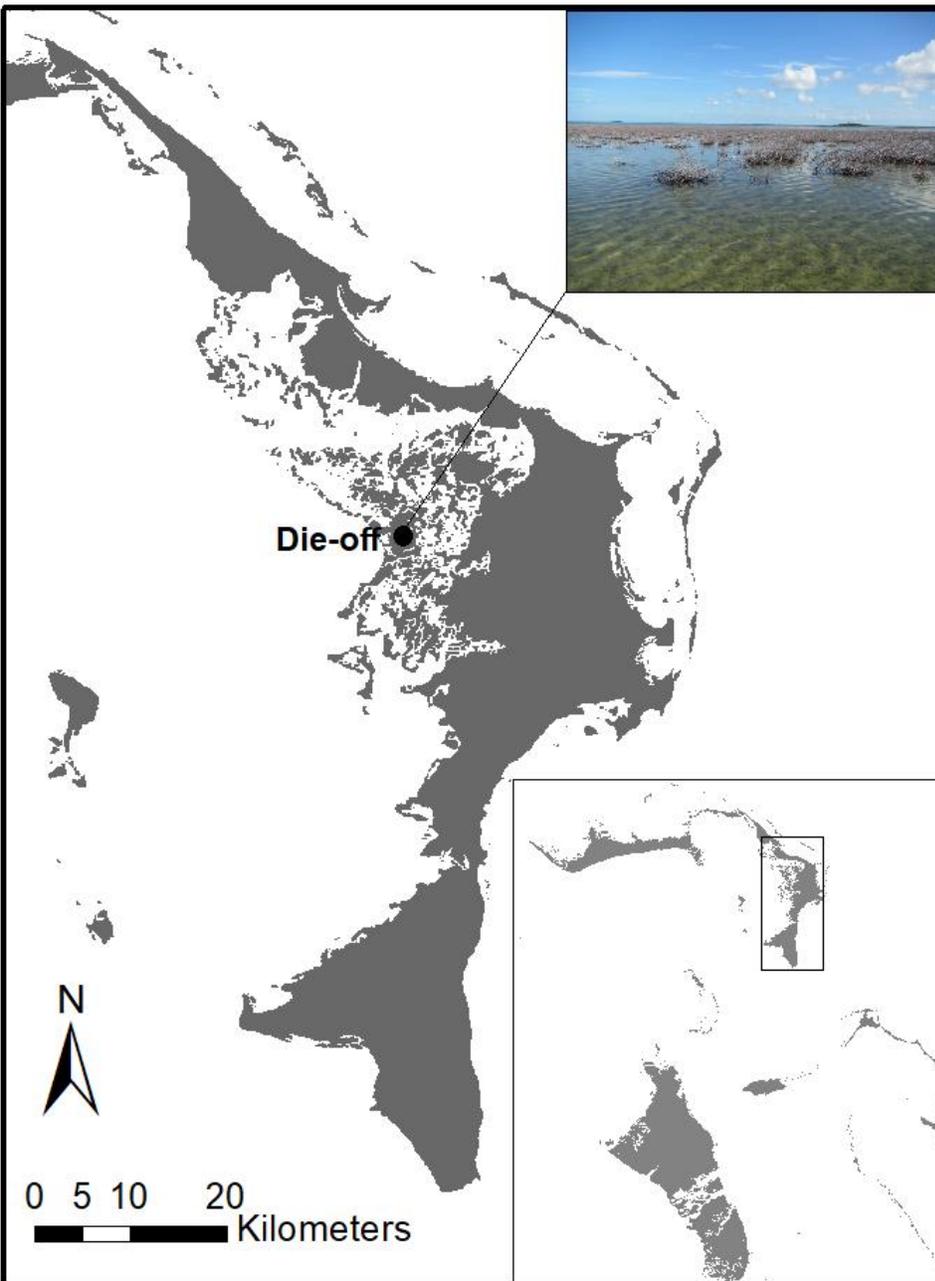
Figures

Figure 1: General location of mangrove die-off on Abaco Island, The Bahamas. The image in the upper right-hand corner depicts the die-off area.

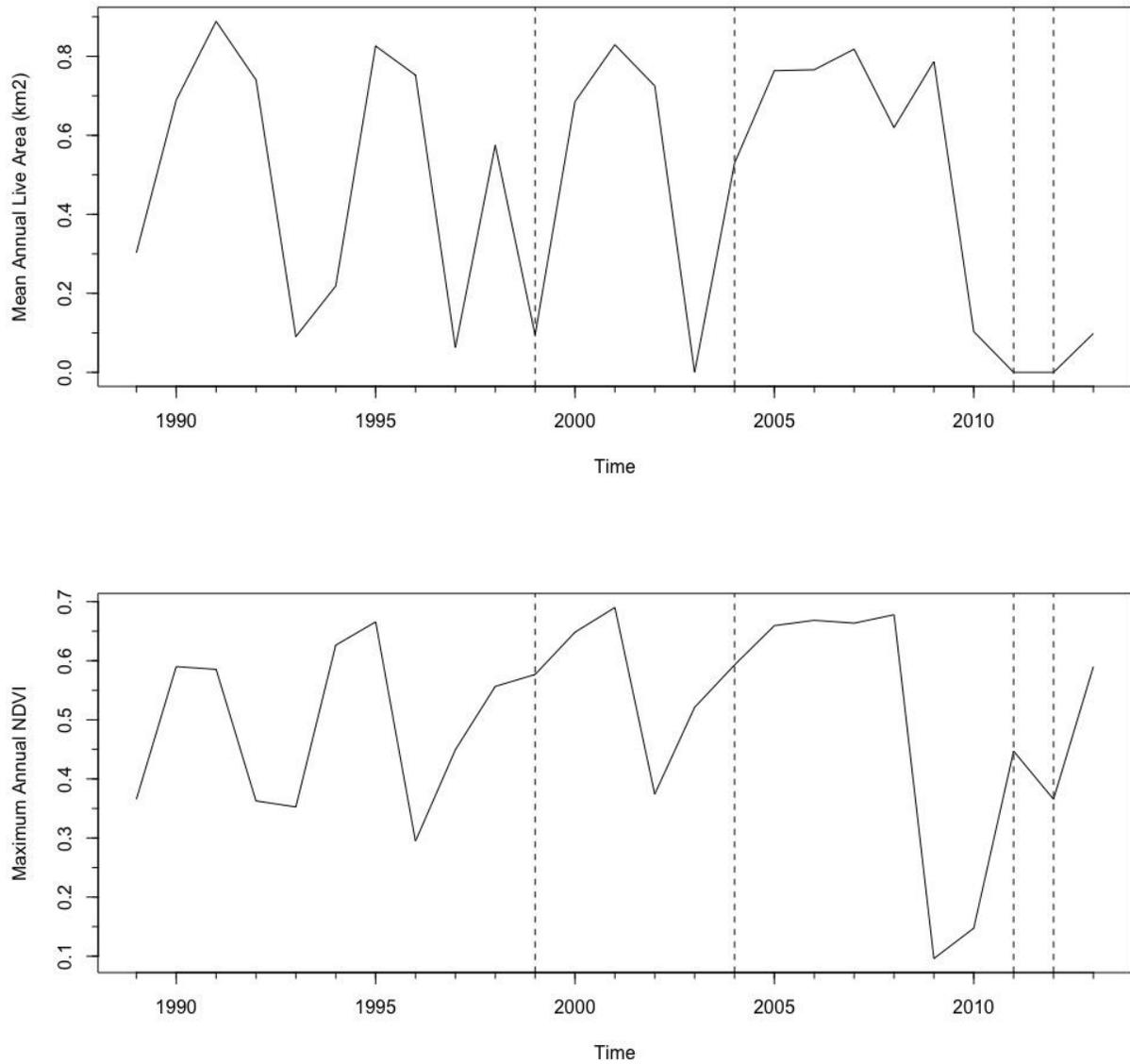


Figure 2: Top panel: Time series of annual mangrove cover from the die-off region between 1989-2013. The dashed lines indicate years hurricanes hit Abaco based on Table II. Bottom panel: Time series of maximum annual NDVI from the die-off region between 1989-2013. The maximum value is the greatest NDVI value from the entire die-off area. The dashed lines indicate years hurricanes hit Abaco based on Table II.

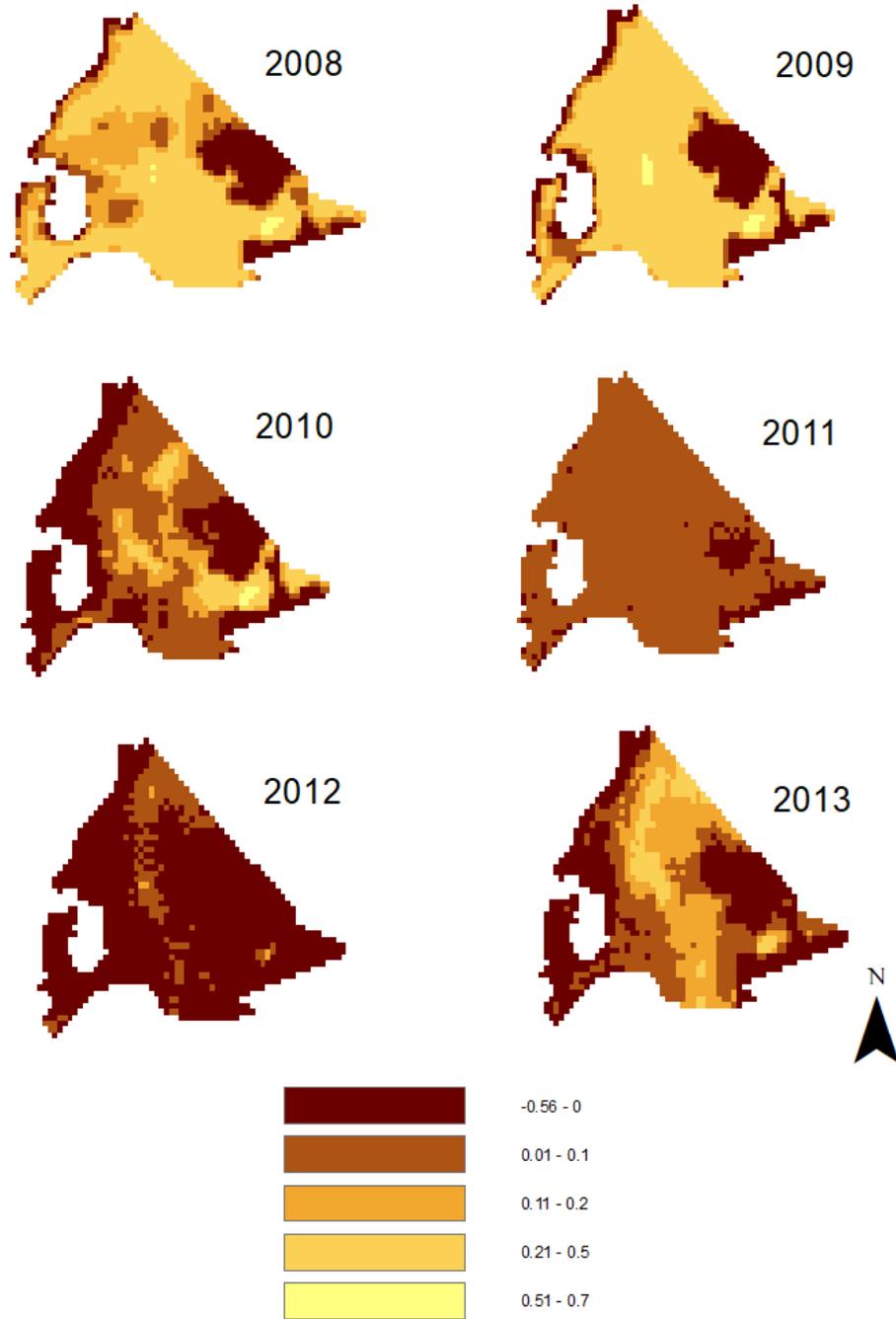


Figure 3: Landsat 7 images of the die-off region from 2008-2013 when peak die-off occurred with NDVI classification based on those mentioned in Table I. Note that the color scheme reflects barren substrate (dark brown) to dense vegetation (pale yellow).

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CHAPTER 3: Herbivory increases incidence of disease related leaf damage associated with a dwarf red mangrove (*Rhizophora mangle*) die-off

Abstract

Mangroves are habitat-forming foundation species that provide the framework for entire coastal communities. They provide a range of ecosystem services, such as carbon sequestration and nursery habitat for many commercially important species. Unfortunately, mangrove forests are in decline because of myriad factors, often related to exposure to multiple simultaneous stressors. We present a report of a recent mangrove die-off on Abaco Island, The Bahamas, which appears to be the result of multiple stressors. We examined the role of herbivory and how it interacts with plant disease to impact the health of adult red mangroves (*Rhizophora mangle*) using field experiments. Preliminary observations in the die-off area suggested grazing by leaf feeding organisms and disease were both be present at high levels. First, we surveyed individual mangrove trees to determine intensity of herbivory on mangroves in the die-off area. We found that herbivory ranged from 0 to 79% with a mean of 29% per tree and disease incidence ranged from 0% to 97% with a mean of 47% per tree. Second, we conducted a simulated grazing experiment to test if herbivory could facilitate disease infection in mangrove plants. Experimental opening of leaf tissues showed that grazing can increase disease severity in mangrove leaf tissue. Taken together, these results suggest herbivory may facilitate disease infection of mangrove leaves and thereby contribute to mangrove die-off.

Keywords: mangrove, disease, herbivory, multiple stressors, *Rhizophora mangle*

Introduction

Mangroves are habitat-forming foundation species whose forests provide the framework for entire coastal communities (Dayton, 1972; Bruno et al., 2003; Ellison et al., 2005). Mangrove forests provide a range of ecosystem services, such as carbon sequestration and nursery habitat for many organisms including commercially important species (Costanza et al., 1997; UNEP, 2006; Barbier et al., 2011). Unfortunately, mangrove forests are declining at a rapid rate because of various factors, often related to human activities such as aquaculture, development and overexploitation (Alongi, 2002; Duke et al., 2007; Giri, 2008; Barbier et al., 2011; Giri et al., 2011; Friess and Webb, 2014). Mangrove die-offs have been documented throughout the globe and have been attributed to bottom-up factors such as relative sea-level rise, drought and extreme salinities (Gilman et al., 2008; Duke et al., 2017; Lovelock et al., 2017).

Historically, bottom-up factors, e.g., nutrients and salinity, were thought of as the primary regulators of coastal plant community structure and function (Teal, 1962; Morris et al., 2002). However, top-down factors such as grazing can play a major role in structuring coastal wetland ecosystems. Snow geese denude salt marsh plants in North American salt marshes (Smith and Odum, 1981; Srivastava and Jefferies, 1996). Marine arthropods, such as crabs, consume roots of marsh plants and contribute to salt marsh dieback in Atlantic salt marshes (Alberti et al., 2007; Alberti et al., 2008; Coverdale et al., 2012; Bertness et al., 2014). In a recent meta-analysis of consumer control studies in coastal wetlands, 412 grazing experiments have been completed in salt marsh systems while only 31 experiments have been completed in mangroves - most of which were focused on seedlings and propagules (He and Silliman, 2016). He and Silliman (2016) summarize the examples when herbivores have been shown to affect survival and reproduction of mangroves. For example, wood-boring, stem girdling, and leaf feeding insects

were documented to destroy between 44-67% of *Rhizophora mangle* canopies in 10m x 10m plots in Belize (Feller and Mathis, 1997; Feller, 2002). Mangrove canopies are also extensively grazed by *Aratus pisonii* (Mangrove Crab) (Erickson et al., 2003). Recently, a boring beetle and moth have resulted in dieback of *Sonneratia alba* mangroves in Kenya (Jenoh et al., 2016). Root-boring and stem-boring beetles have resulted in mortality of mangrove seedlings (Sousa et al., 2003; Alongi, 2009) and crab species such as *Goniopsis cruentata* (Spotted Mangrove Crab) and *Ucides cordatus* (Mangrove Land Crab) consume mangrove propagules (Lee, 1998).

Multiple stressors have driven extensive loss of coastal habitats in many areas. For example, the combination of biotic stressors (a snail grazer and fungal pathogen) and physical stress (drought) interacted to drive salt marsh die-off in the Southeastern U.S (Silliman and Bertness, 2002; Silliman and Newell, 2003; Silliman et al., 2005). In addition to herbivory, another potential stressor in mangroves is plant disease, which has been reported in mangroves across the globe (Osorio et al., 2014). For example, a fungus *Cytophora rhizophorae* caused stem dieback and resulted in mangrove loss in Puerto Rico, while an oomycete, *Phytophthora* sp., infected roots of mangroves in Australia resulting in death (Pegg et al., 1980; Wier et al., 2000).

We examined the role of herbivory and how it may interact with plant disease in adult red mangroves (*Rhizophora mangle*) on Abaco Island, The Bahamas. First, we surveyed individual mangrove trees to explore the incidence of herbivory and disease in the die-off area. Second, we conducted a simulated grazing experiment to test if there is an interaction between herbivory and plant disease. We hypothesized there would be an interaction between herbivory and plant disease such that grazed leaves would have greater incidence of lesions.

Materials and Methods

Site descriptions

Recently, a 1.23km² mangrove die-off was reported on Abaco Island, The Bahamas by local fisherman. The die-off is located on the west side of the island in an area commonly referred to as The Marls. This area is a complex mosaic of red mangroves (*R. mangle*), seagrass (primarily turtle grass, *Thalassia testudinum*), sponges, and sand flats. Most of the area is isolated from direct human impacts, as it is extremely shallow and can only be accessed with flats boats by those knowledgeable of the seascape. Four different mangrove creek sites were used for the simulated grazing experiment: Camp Abaco (CA), Hills Creek (HC), Snake Cay (SC) and Twisted Bridge (TB) (Figure 1). These tidal creeks are fringed by dwarf mangroves (predominately *R. mangle*), with primary benthic habitats being submerged seagrass (primarily *T. testudinum*), benthic macroalgae (e.g., *Halimeda discoidea*, *Caulerpa* spp., *Batophora oerstedii*, *Acetabularia* spp.) and sand flats.

Definitions

Two terms will be used to refer to different aspects of the quantification of disease these are: disease incidence and disease severity. Disease incidence is here defined as the number of lesions on a leaf or the number of leaves on an individual plant with signs of disease. Disease severity is defined as the amount of leaf surface area covered by putative disease lesions.

Herbivory and Disease Survey

A previous cage exclusion experiment suggested that grazers alone did not contribute to the die-off (Table AX). As a result, individual *R.mangle* trees (n=48) in the die-off area were tagged in May 2014 and monitored until January 2016. The total number of leaves, incidence of putative

disease phenotypes and grazing incidence (number of leaves with grazed edges) were recorded every six months for each tree (Figure A1).

Simulated Grazing Experiment

In June-July 2015, we conducted an experiment intended to determine if grazing resulted in increased frequency of putative disease phenotypes, specifically lesions. Prior to establishing the experiment, disease incidence surveys were conducted at each site. Disease incidence surveys consisted of sampling all mangrove trees along a 50m transect and recording disease incidence, DBH, number of shoots missing, grazing incidence, number of leaves, presence/absence of canker disease, number of shoots, presence/absence of snails, presence/absence of leaf galls, presence of dead matter (number of dead prop roots and branches) (Layman, 2016a, b). These sites varied in their disease incidence, with TB having the highest disease incidence followed by CA, HC and SC (Figure 2).

At each site, leaf pairs that showed no signs of damage (i.e., no disease and no grazing) were selected (n=150 pairs/site), and each pair was from the same leaf shoot. One leaf from the pair was cut, simulating grazing, using crafting scissors. The other leaf was marked as the control using permanent marker and flagging tape. Leaf pairs were monitored for lesion development and senescence of paired leaves over a 28-day period (Figure A2). If leaves senesced prior to the end of the 28-day period, they were removed and photographed for digital analysis. All other leaves were collected at the end of the 28-day period and photographed for digital analysis of lesion density using ImageJ (Schneider et al., 2012).

Analysis

Combining all observations from the herbivory and disease survey, linear regression was used to determine if there was evidence of a relationship between grazing pressure, disease, and

the health of the mangroves. Specifically, the relationships between disease incidence and proportion grazed, the number of dead branches and disease incidence, and the number of damaged shoots and disease incidence were determined using individual linear regressions. The number of dead branches was logged transformed and the number of damaged shoots was cube root transformed to meet assumptions of normality and heterogeneity of the residuals. After visual inspection of the data there was evidence that the relationship between disease incidence and the proportion of grazed leaves differed seasonally. As a result, this relationship was examined independently with data collected in the summer and winter sampling periods. The simulated grazing data were analyzed using a split-plot ANOVA with leaf pair as the block, site as a whole-plot factor and type (cut vs uncut) as the split-plot factor. All analyses were completed in R (version 3.3.2 R Core Team (2016)).

Results

Herbivory and disease survey

A wide range of herbivory was observed (0% to 79% leaves grazed, mean= 29%). Likewise, a wide range of disease damage was also observed (0% to 97% leaves diseased, mean=47%). In the summer, there was no significant relationship between disease incidence and grazing ($t_{94} = 1.57$, $p=0.12$, $R^2=0.02$), however, in the winter there was a strong positive relationship ($t_{45} = 7.28$, $p<0.001$, $R^2=0.53$) with higher grazing levels resulting in higher levels of disease incidence (Figure 3). Disease incidence was positively correlated with both the number of dead branches ($t_{94} = 3.59$, $p<0.001$, $R^2=0.11$) and the number of damaged shoots ($t_{94} = 4.24$, $p<0.0001$, $R^2=0.15$) in the entire die-off area.

Simulated Grazing Experiment

There was no interaction between site and type (cut vs uncut) ($F_{3,594}=0.10$, $p=0.96$, Table 1). Yet, sites differed in disease severity ($F_{3,594}=3.62$, $p=0.01$, Table 1) as did type ($F_{1,594}=5.55$, $p=0.02$, Table 1) with simulated grazed leaves having a significantly higher proportion of leaf area (severity) covered in lesions than control leaves (Figure 4). There was not a significant difference in the total number of lesions between simulated grazed and control leaves ($F_{1,595}=2.86$, $p=0.09$) however, there was a trend towards cut leaves having more lesions at two sites, CA and TB, which have greater disease incidence than SC and HC.

Discussion

Our study suggests that herbivory may facilitate disease infection of mangrove leaves, ultimately decreasing mangrove health. In both the survey and the simulated grazing experiment, we found a positive correlation between grazed leaves and disease. This relationship was stronger in winter months suggesting some seasonality, but this should be investigated further before strong conclusions may be drawn. Further, disease incidence was positively correlated with indicators of distress in mangroves, specifically damaged shoots and dead branches. Disease severity was significantly greater in grazed leaves than control leaves. Overall our study demonstrates how chewing grazers could contribute to mangrove loss through facilitation of plant disease.

Surveys in the mangrove die-off area suggest that grazing is strongly correlated with disease in this system. Grazing and mechanical damage are linked to disease in other coastal systems. For example, grazing by a butterflyfish, *Chaetodon capistratus*, increased the rate of infection of black-band disease of corals such as *Montastrea faveolata* (Aeby and Santavy, 2006). Simulated grazing on the red macroalga *D. pulchra* facilitated disease infection by a

bacterial pathogen, *Nautella* sp. that lead to bleaching of the alga (Campbell et al., 2014).

Grazing by the green turtle, *Chelonia mydas*, was found to increase the likelihood of the seagrass *Thalssia testudinum* being infected with *Labyrinthula* (Bowles and Bell, 2004). In U.S South Atlantic salt marshes, snail grazers injure plant tissue enabling the growth of *Fusarium* fungi that kill plant tissue which snails subsequently consume (Silliman and Zieman, 2001; Silliman and Newell, 2003). This combination of grazing and fungal infection lead to greater loss of salt marsh grass than that of any single stressor alone (Silliman et al., 2005). Similarly, grazing scars by burrowing crabs and Hemiptera insects were found to facilitate microbial infection in salt marsh plants in South America (Freitas et al., 2015).

The simulated grazing experiment further supports the idea that grazing facilitates disease infection. We found that grazing resulted in increased disease severity, regardless of the initial disease incidence found at each site. In our case, we found that grazed leaves had a greater area of leaf covered by lesions when compared to control leaves. The severity of disease on a leaf has direct implications on photosynthesis- lesions reduce area for photosynthesis to occur (Shtienberg, 1992; Roloff et al., 2004; Agrios, 2005). The more severe a foliar pathogen, the greater the potential reduction in photosynthesis. When considered at the whole plant level, increased foliar disease severity could have major effects on the health of the overall plant or organism by creating Carbon deficits and impact water use efficiency (Grimmer et al., 2012). For mangroves in particular, increased severity of foliar pathogens could have major implications as mangrove leaves are fairly long lived (mean leaf life span is 16 months) and have a thick, waxy cuticle both of which require more investment than short life span and thin leaves (Reich et al., 1997; Reef et al., 2010). Additionally, foliar pathogens could have an impact on water use

efficiency in mangroves because altering leaf size, succulence and angle is a common response to changes in salinity (Reef and Lovelock, 2015).

Such interactions between herbivores and plant disease are common in many ecosystems and may be additive, antagonistic or synergistic. A recent meta-analysis by Hauser et al. (2013) demonstrated that the combined impact of herbivore and pathogen interactions were mostly additive. However, this metaanalysis was composed of 35 studies, and other work demonstrates that interactions between herbivores and pathogenic organisms are extremely variable (Hatcher, 1995; Stout et al., 2006; Hauser et al., 2013). For example, feeding by the threecornered alfalfa hopper (*Spissistilus festinus*) on plants infected with *Fusarium oxysporum* increased disease severity on alfalfa (Moellenbeck et al., 1992). Other studies demonstrate that feeding by arthropods has no effect or decreases plant resistance to pathogen infection (Stout et al., 2006). Tobacco plants that were exposed to feeding by caterpillars or mites did not result in increased resistance to Tobacco Mosaic Virus (Ajlan and Potter, 1992). Yet, some studies suggest that feeding by an herbivore increases a plants resistance to infection by a pathogen, particularly by arthropods that are sap sucking (Stout et al., 2006). For example, when rice plants were subjected to white-backed planthoppers (*Sogatella furcifera*), their resistance to rice blast (*Magnaporthe grisea*) was increased. However, our study suggests a potentially synergistic interaction between herbivory and disease.

Conclusions

In summary, our study suggests that herbivory facilitates disease infection in a mangrove ecosystem in The Bahamas. However, caution should be taken when making generalizations as this is likely highly dependent on the causal pathogen and type of insect grazing. Although biotic stressors have received relatively little attention in mangrove ecosystems (He and Silliman,

2016), our study suggests they may contribute to mangrove die-off. While many studies have demonstrated the role bottom-up factors such as relative sea-level rise, drought and extreme salinities play in controlling mangrove ecosystems, more work is needed to understand the role of biotic stressors such as herbivory and plant disease. Future studies should incorporate multiple factors, both abiotic and biotic, to understand how these factors may interact to control mangrove ecosystems.

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Tables

Table 1: Split-plot ANOVA table for herbivory survey and simulated grazing experiment.

	Degrees of Freedom		F-value	p-value
	Numerator	Denominator		
<i>Herbivory survey</i>				
Location	2	13	0.66	0.53
Treatment	2	26	0.96	0.4
Location*Treatment	4	26	0.86	0.5
<i>Simulated grazing experiment</i>				
Site	3	594	3.62	0.013
Type	1	594	5.55	0.02
Site*Type	3	594	0.1	0.96

Figures

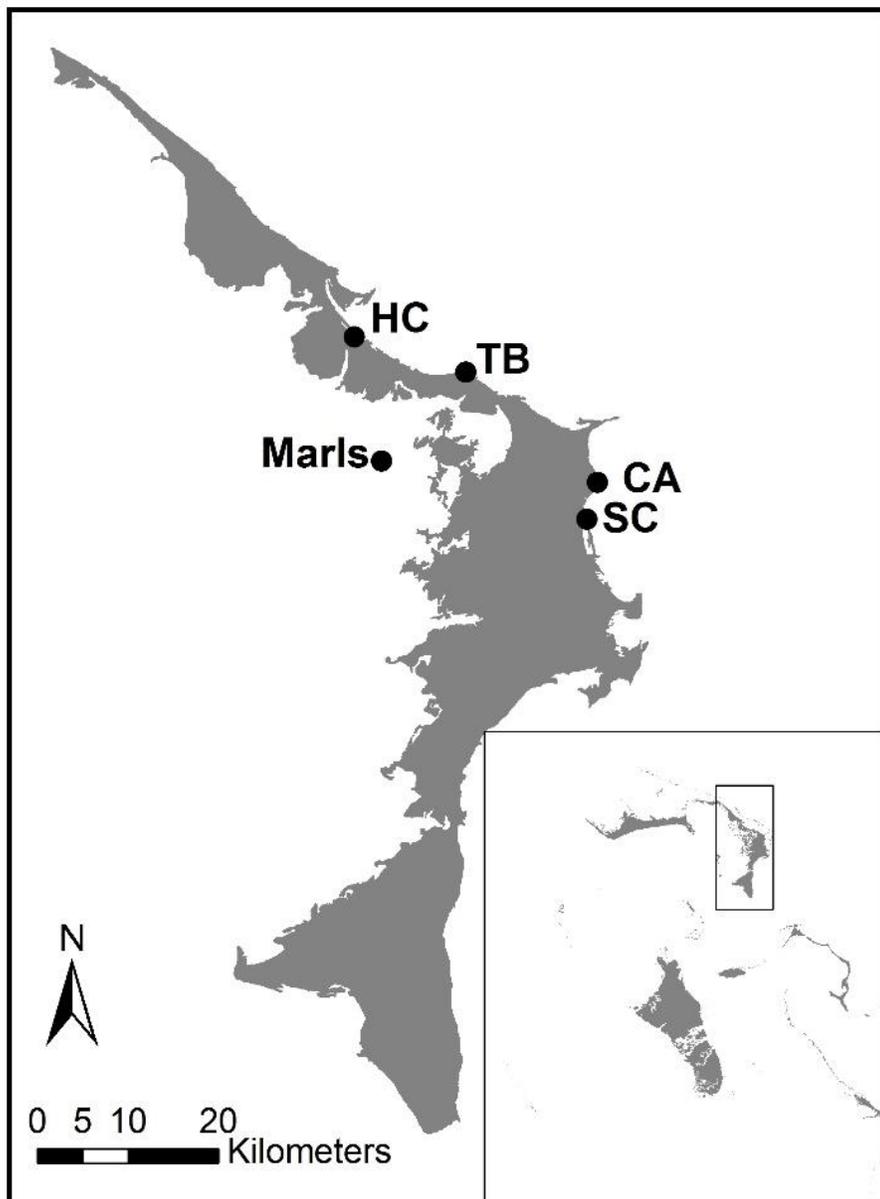


Figure 1: Map of experiment sites on Abaco Island, The Bahamas. The herbivory and disease survey was conducted in the Marls site and the simulated grazing experiment was conducted at all other sites: CA, HC, SC, TB.

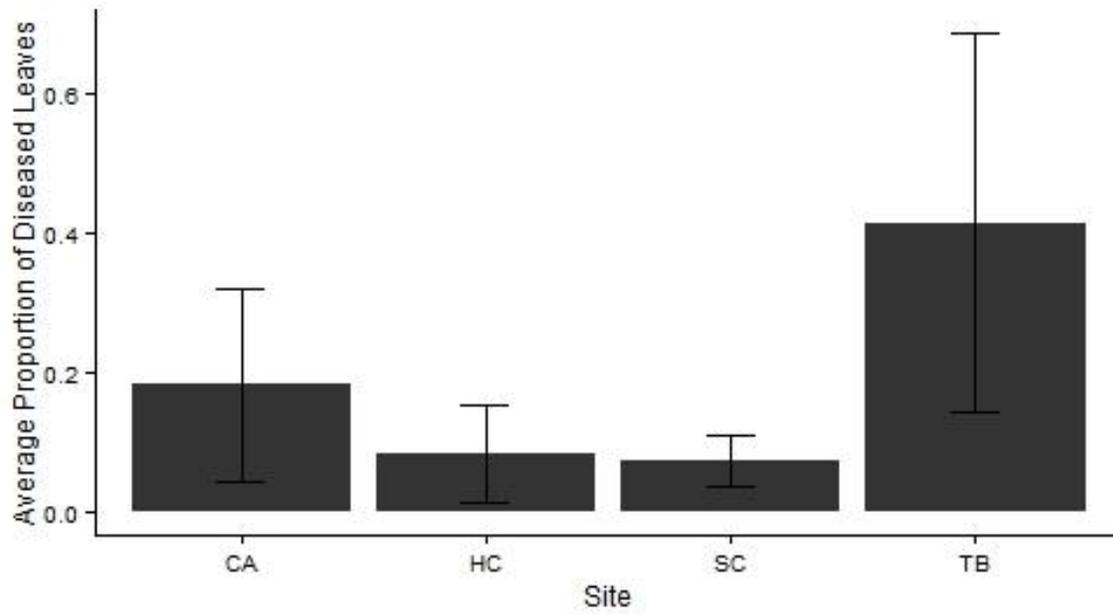


Figure 2: Disease incidence across sites from simulated grazing experiment based upon the number of diseased leaves by tree along 100m transects. Error bars are standard deviations.

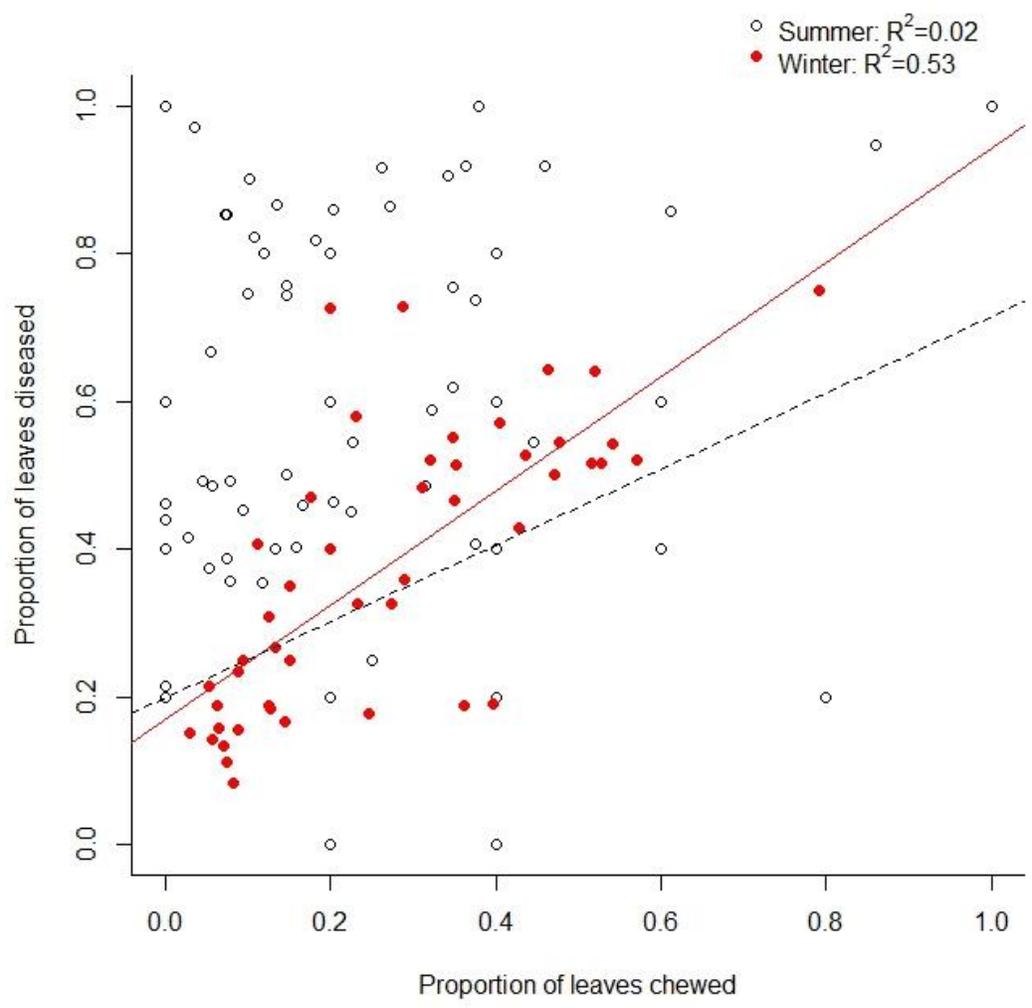


Figure 3: Linear regression of disease incidence by proportion of grazed leaves from herbivore exclusion experiment. Data points in white are from summer ($R^2=0.02$) and those in red are winter ($R^2= 0.53$).

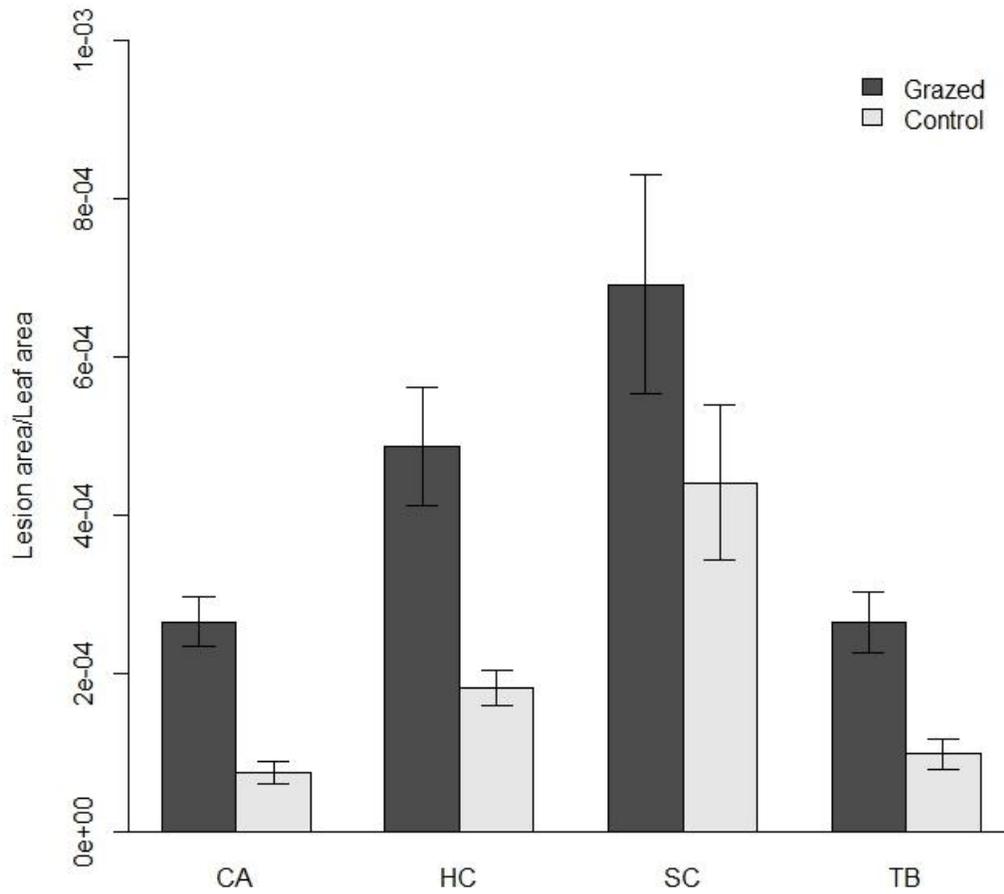


Figure 4: Disease severity across sites from simulated grazing experiment. Dark gray represents grazed and light gray represent control leaves. Error bars are standard deviations.

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CHAPTER 4: Identification of *Pestalotiopsis* sp. infecting Red Mangrove (*Rhizophora mangle*) in The Bahamas

Abstract

Mangroves are foundation species in coastal ecosystems providing an estimated US \$1.6 billion (from 1997) in ecosystem services worldwide. These services range from essential nursery habitat for marine organisms to land accretion and carbon sequestration. Unfortunately, mangrove forests are declining because of myriad factors, many related to human activity. Here, we present a case study from Abaco, The Bahamas, in which dwarf red mangroves (*Rhizophora mangle*) are dying in a ~1.25km² area. Initial data suggest that these dwarf *R.mangle* are stressed by multiple factors, including a fungal pathogen and herbivory. Disease incidence surveys were completed in the die-off area and at four other sites on the island. We found disease present across the island and disease incidence varied from 7 to 44%. We also sampled a neighboring island, Grand Bahama, for presence of the pathogen. Infected leaf samples were collected, and fungi were isolated. *Pestalotiopsis* sp. were the most commonly identified fungi from tissue samples. DNA was sequenced using an internal transcribed spacer, elongation factor 1-alpha, and beta-tubulin genes. When compared to several known *Pestalotiopsis* sp., the isolates from The Bahamas were distinct and not closely related to the subset of known species. Koch's postulate trials indicate that some isolates of *Pestalotiopsis* sp. from The Bahamas caused disease over a 20-day period. Although not all isolates elicited disease symptoms, these tests confirm *Pestalotiopsis* sp. do infect red mangroves in The Bahamas and this is parallel to reports of *Pestalotiopsis* sp. infecting red mangroves in Cuba and Panama.

Introduction

Mangrove ecosystems are important foundation species in the coastal environment. They provide myriad ecosystem services, yet they are in decline worldwide (Duke et al., 2007, Giri et al., 2011). Anthropogenic activity is often the cause of much mangrove loss; however, natural events including storms, drought, and sea level rise are also important drivers (Gilman et al., 2008, Feller et al., 2017). For example, recent die-offs of mangroves in Australia were attributed to drought, high water temperatures and fluctuating sea levels because of the El-Niño Southern Oscillation (ENSO) (Duke et al., 2017, Lovelock et al., 2017). Biotic stressors such as disease are also known to contribute to mangrove loss. For example, the canker causing fungus, *Cytospora rhizophorae* J. & E. Kohlmeyer, was documented to cause stem dieback in red mangroves in Puerto Rico (Wier et al., 2000). Additionally, an oomycete, *Phytophthora* sp. was found as the causal agent of a localized die-off of mangroves in Australia (Pegg et al., 1980). Foliar pathogens such as *Pestalotiopsis* and *Colletotrichum* species have been reported to infect seedlings of red mangroves, e.g., in Panama (Gilbert et al., 2002).

Although there are many individual causes of mangrove die-off, mangrove forests are often subjected to multiple stressors. It is likely that there are interactions between multiple stressors that may facilitate mangrove loss. For example, disease and herbivory are often understudied in mangrove ecosystems (He & Silliman, 2016, Osorio et al., 2014) yet there may be strong interactions between disease and/or herbivory with other stressors such as drought or sea level rise. Interactions such as these have been observed in salt marsh ecosystems (Silliman et al., 2005, Bertness et al., 2008) and forests (Sturrock et al., 2011), thus warranting exploration in mangrove ecosystems.

Plant diseases are generally affected by changes in the environment and, as a result, interactions between disease-causing organisms and hosts are often context dependent. For example, water, light, temperature, and nutrient stress could all contribute to changes in a microbe-host relationship (Newton et al., 2010). Many microbes may have resting endophytic stages that are followed by pathogenesis because of some change in host physiology or additional stress (Newton et al., 2010, Arnold, 2007). Since climate change is projected to impact some of these stressors (e.g., temperature, water), it is predicted that climate change will facilitate spread and severity of plant diseases in many ecosystems (Garrett et al., 2006, Anderson et al., 2004).

Identifying potential pathogens infecting mangroves is important, especially if there are interactions between multiple stressors and disease symptoms. Here, we document *Pestalotiopsis* sp. Steyart, 1949 in red mangrove (*Rhizophora mangle* L.) die-off regions on two Bahamian islands. To our knowledge, this is the first report of *Pestalotiopsis* sp. found infecting red mangroves in The Bahamas, following reports of *Pestalotiopsis* sp. on red mangrove in Cuba and Panama (Gilbert et al., 2002) and knowledge that it is a general pathogen and endophyte of many tropical plants (Maharachchikumbura et al., 2011).

Methods

Site Description

A mangrove die-off was reported by local fishing guides on Abaco Island, The Bahamas in 2011. This die-off is rather remote and therefore anthropogenic activity likely had little to do with the die-off (Figure A1). The area is composed of dwarf red mangroves (*R. mangle*), seagrass (primarily turtle grass, *Thalassia testudinum* Banks & Sol. ex K.D. Koenig), sponge grounds, and sand flats. During 2014-2016, leaves with disease symptoms (i.e., necrotic tissue)

were sampled at several locations including red mangroves adjacent to the die-off area (Figure 1). To increase sample size and determine incidence of disease, red mangroves were randomly sampled in Abaco and Grand Bahama.

Disease Surveys

We performed disease incidence surveys in the die-off region in addition to four sites on Abaco: Camp Abaco, Hills Creek, Snake Cay and Twisted Bridge (Table 1) to better understand presence of disease on Abaco where the die-off occurred. These sites were selected because they were composed of dwarf red mangroves with ~168 leaves/tree on average. Surveys consisted of 50m transects with all red mangrove trees with intact leaves sampled within 2m of the transect. Transects were started on the landward side leading to open water. For each tree sampled, the number of leaves exhibiting disease symptoms was recorded, in addition to the total number of leaves on the tree regardless of disease damage (Table A1). Necrotic leaf tissue was the focal disease symptom throughout these surveys, however grazing scars were also noted. The proportion of disease symptoms per tree and site was calculated.

Sampling and DNA extraction

Diseased leaves were collected from several locations on Abaco and Grand Bahama, including the die-off region. Infected leaves were surface disinfested using a 0.05% bleach solution for 30 seconds, then rinsed in deionized or distilled water and dried on a sterile paper towel. Disinfested leaf pieces were then plated onto acidified potato dextrose agar (APDA) media. Cultures were re-isolated to ensure no contamination. Only pure cultures were examined for morphological characteristics and subsequent DNA extraction and sequencing. DNA extraction was performed from cultures using the CTAB method (White et al., 1990, Lee & Taylor, 1990).

DNA Amplification and Sequencing

All pure cultures were first sequenced using ITS (1 and 2 or 4 and 5) primers to obtain genus identities. Multilocus sequence typing was done using ITS (1 and 2), elongation factor 1-alpha (EF728f and EF2) and beta-tubulin (bt2a and bt2b) primers (Table A2) on the most commonly isolated fungus. PCR was performed in 50- μ l volumes for each sample following the reaction protocol described by Saville et al. (2017). The PCR program followed that used in Ristaino et al. (2001). PCR products were visualized on 1% agarose gels to verify amplification and products were purified for sequencing using ExoSAP-IT (Affymetrix, Santa Clara, CA). Purified products were submitted to the NCSU Genomic Sequencing Lab where Sanger sequencing was performed using each set of forward and reverse primers. PCR and sequencing were repeated twice for each isolate per primer pair.

Pathogen Identification and Morphology

Both morphological characters and ITS sequence data were used to determine genus identities from 70 leaf samples. ITS sequence data were submitted to NCBI BLAST to determine genus identities. Samples of pure cultures were isolated and wet mounted onto microscope slides with lactophenol for further observation of conidia and hyphae. Slides with spores were examined with a Zeiss Axioimager microscope and photographs were taken for spore measurements in imageJ (Schneider et al., 2012). Only cultures with consistent identities determined by both morphological characters and sequence data were considered when determining the most common fungus isolated.

Gene Sequence, PCA and Network Analysis

A subset (n=8) of known sequences of *Pestalotiopsis* species and the outgroup, *Seridium* sp., reported by Maharachchikumbura et al. (2014) were downloaded from GenBank and

analyzed along with *Pestalotiopsis* sequences from The Bahamas (Table A3). This subset was chosen to ensure each isolate had been sequenced with the same genes used in this study.

Sequences were aligned using BioEdit (version 7.2.5) (Hall, 1999). Multiple sequence alignment was performed using CLUSTAL W (Thompson et al., 1994) in BioEdit. All statistical analyses of the nucleotide sequences were completed in SNAP Workbench, version 2.0 (Monacell & Carbone, 2014). SNAP Map was used to collapse sequences into unique haplotypes (Aylor et al., 2006).

Principal components analysis was completed in SNAP Map (Aylor et al., 2006) on concatenated sequences and visualized in R Studio using the package ggplot2 (RStudio, 2016, Wickham, 2009). A median joining network was constructed using concatenated sequences in Network (v. 5.0.0.3; Fluxus Technology, Sudbury UK).

Koch's Postulate Trials

Eight different isolates of *Pestalotiopsis* sp. were prepared for Koch's postulate trials to confirm pathogenicity. Spore solutions were prepared by pouring approximately 10mL of 0.01% Tween 80 (Sigma) onto sporulating cultures. Conidia were then removed from the surface by agitating with a bacterial loop. Liquid contents of the plate were then filtered through four layers of cheesecloth into a 50 mL Falcon tube to remove large pieces of mycelium. The concentration of spore solution collected in the 50 ml Falcon tube was quantified using a hemocytometer (Bright-Line Hemocytometer; Reichert Scientific Instruments, Buffalo, NY) and subsequently diluted to 10,000 spores/mL. The diluted solutions were sprayed onto detached red mangrove seedling leaves on water agar plates that were rinsed with deionized water. Leaves were examined daily over 20 days for lesion development. Reisolations were conducted on leaves that developed lesions to confirm identity of the lesion-causing agent.

Results

Disease Surveys

At specific sites, mean diseased leaves per tree ranged from 7 to 44% along 50m transects (Table 1). The die-off site had the most disease symptoms on leaves per tree (mean=44%) despite 19 completely defoliated trees on the transect (Table 1). Across all sites, mean disease symptoms per tree was 24%. Nearly all trees at each site had symptomatic leaves (90-100%).

Pathogen Identification and Morphology

Pestalotiopsis was the most consistently isolated fungus from leaf samples in The Bahamas (Table A4). Cultures were grown on PDA and produced white aerial hyphae with black fruiting structures (Figure 1, A2). *Pestalotiopsis* isolates contained conidia with two and three appendages and a mean length of 19.5 μM and 19.6 μM respectively (Table 2). All conidia contained three central cells that ranged from concolorous to versicolorous independent of number of appendages (Figure 1, A3).

Gene Sequence Analyses

In total, 1,019 nucleotides were analyzed consisting of 190 nucleotides for the ITS locus, 460 nucleotides for Tef1a, and 369 nucleotides for the beta-tubulin locus. When indels were removed from the alignment, Tef1a had the largest number of haplotypes (h=26) followed by beta-tubulin (h=21) and ITS (h=13) (Table A5). When all loci were concatenated, 24 haplotypes were found.

PCA and Network Analyses

Both PCA and Network analyses support the presence of several distinct haplotypes from the concatenated sequences. The biplot of PC1 and PC2 demonstrate distinct clusters of the outgroup, known species, and those from The Bahamas (Figure 2). This is also mirrored in the

median joining network where the outgroup is clearly separated from all *Pestalotiopsis* sequences (Figure 3). The remaining haplotypes clustered into two groups, one consisting of all known species, and the other consisting of isolates collected from both Abaco and Grand Bahama (Figure 2). Only one shared haplotype was observed in the median joining network, composed of two Abaco isolates and one Grand Bahama isolate (Figure 3).

Koch's Postulate Trials

We performed Koch's postulate experiments with eight isolates. Four out of eight resulted in successful lesion development (Table A6). Lesion development ranged between two and 13 days on after inoculation (Figure A4). All isolates that elicited disease symptoms were confirmed to be infected by *Pestalotiopsis*.

Discussion

Disease symptoms were present on nearly all trees sampled, suggesting that foliar pathogens are likely present at endemic densities in red mangrove forests throughout Abaco and likely Grand Bahama. Differences in the proportion of symptomatic leaves could be attributed to abiotic or biotic stressors present in these different sites. For example, the mangrove die-off site is impacted by a chewing insect, as well as potential thermal and salinity stress (Rossi, 2017). On the other hand, the Camp Abaco site mangroves are exposed to herbivores such as the mangrove tree crab (*Aratus pisonii* Milne Edwards), yet appear to be under less abiotic stressors as that site was recently restored with increased water flow for tidal inundation (Valentine-Rose & Layman, 2011). Differences in multiple stressors, both abiotic and biotic, at each site could explain the variation in the proportion of disease symptoms observed; however, more controlled greenhouse studies would be necessary to tease apart the role of individual stressors.

Cultures and conidia of the most commonly isolated fungus were indicative of described *Pestalotiopsis* sp. (Maharachchikumbura et al., 2011, Maharachchikumbura et al., 2012, Maharachchikumbura et al., 2014). The isolates from The Bahamas had some variation with ranges in mean length and total number of appendages. This variation is supported by gene sequence analyses as 24 haplotypes were found, suggesting a diverse population. Results from the median joining network and PCA also reflect differences in haplotypes from The Bahamas which suggests many distinct sequences rather than several common sequences. These analyses suggest that the *Pestalotiopsis* sp. isolated in The Bahamas may be new species or strains, as they appear to be distinct from the known species used in this study. Increased sampling across Abaco and Grand Bahama are needed to confirm the variation of *Pestalotiopsis*. Additionally, more known *Pestalotiopsis* sequences could be incorporated into future analyses to better elucidate the exact species or strains of *Pestalotiopsis* found in The Bahamas. However, based on the results presented here, it appears that there are multiple species or strains infecting red mangrove leaves that may cause foliar lesions in The Bahamas.

Koch's postulate trials revealed that only some of the *Pestalotiopsis* isolates do cause lesion development. The *Pestalotiopsis* disease cycle involves a resting endophytic stage which could explain why only some isolates elicited lesions (Maharachchikumbura et al., 2011). Because of this resting endophytic phase in the disease cycle, environmental factors play an important role in the transition from endophyte to pathogen for *Pestalotiopsis* (Fail & Langenheim, 1990, Lee et al., 1995). For example, *Pestalotiopsis subcuticularis* (Guba) Wei & Xu is found naturally in the leaves of *Hymenaea courbaril* L., a tree commonly found throughout the Caribbean and South America, and remains in a resting endophytic stage until leaves mature. Once leaves mature, infection by *P. subcuticularis* occurs via mechanical injury (Fail &

Langenheim, 1990). *P. microspora* (Speg.) Zhao & Li was also found to exhibit an endophyte-pathogen relationship with the Florida torryea (*Torreya taxifolia* Arn.) an endangered species in Florida (Lee et al., 1995). In this host, *P. microspora* is present in the inner bark, and after changes in the environment and physiology of the tree *P. microspora* releases phytotoxins which produce disease (Lee et al., 1995). Red mangrove leaves used in the Koch's postulate trials in this study were from seedlings and leaves used may not have been fully mature. If the *Pestalotiopsis* sp. tested were similar to *P. subcuticularis* this design could have affected pathogen development (Fail & Langenheim, 1990). *Pestalotiopsis* sp. have been previously documented as endophytes in *Rhizophora* mangrove species in India (Suryanarayanan et al., 1998, de Souza Sebastianes et al., 2013). Additionally, *Pestalotiopsis* has been reported as a pathogenic fungus on mangroves in Guangxi (Zhou & Huang, 2001) and has been reported to infect *R. mangle* in Cuba and Panama (Gilbert et al., 2002). Symptoms associated with *Pestalotiopsis* infection reported by Gilbert et al. (2002) and Garcia-Lopez et al. (1989) match the symptoms found on red mangrove in The Bahamas. This study demonstrates that *Pestalotiopsis* is a potentially common pathogen in red mangroves and may play an important role in making mangroves less resilient to stressors or interact with stressors to facilitate die-off.

Disease symptoms and *Pestalotiopsis* were first observed in the mangrove die-off region on Abaco where the mangroves are subjected to other stressors such as mechanical damage via herbivory, thermal stress and increased salinity as well as loss soil structure (Rossi, unpublished data). These other stressors could have triggered the transition from endophyte to pathogen in the system (Fail & Langenheim, 1990, Lee et al., 1995). Likewise, if pathogenic *Pestalotiopsis* was present prior to additional stressors, the addition of those stressors could have facilitated the spread of the pathogen by weakening the host plant or through spreading infective propagules

(Sturrock et al., 2011). More work describing the role of other stressors, abiotic or biotic, and interactions with *Pestalotiopsis* are needed to understand the potential of *Pestalotiopsis* to contribute to serious dieback in red mangroves and its other hosts.

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Tables

Table 1: Mean disease incidence across 5 sites on Abaco including the mangrove die-off region. Surveys consisted of sampling live trees at each site within 2m of the 50m transect.

Site Name	Coordinates	No. trees sampled	Mean No. lesions per tree	sd
Camp Abaco (CA)	26°29'25.67"N, 77° 2'32.79"W	37	0.18	0.14
Marls Die-off (Die-off)	26°29'25.54"N, 77°14'12.87"W	20	0.44	0.26
Hills Creek (HC)	26°38'8.40"N, 77°17'9.92"W	33	0.08	0.07
Snake Cay (SC)	26°27'11.29"N, 77° 3'12.11"W	20	0.07	0.04
Twisted Bridge (TB)	26°36'5.08"N, 77°10'31.92"W	62	0.42	0.27

Table 2: *Pestalotiopsis* conidia morphology. Measurements were made using imageJ after photographing conida.

	2 Appendages	3 Appendages
Mean length (μm)	19.5 μm	19.6 μm
Range in length(μm)	13.1-29.6 μm	14.7-27.6 μm
Mean appendage length (μm)	10.7 μm	11.8 μm

Figures

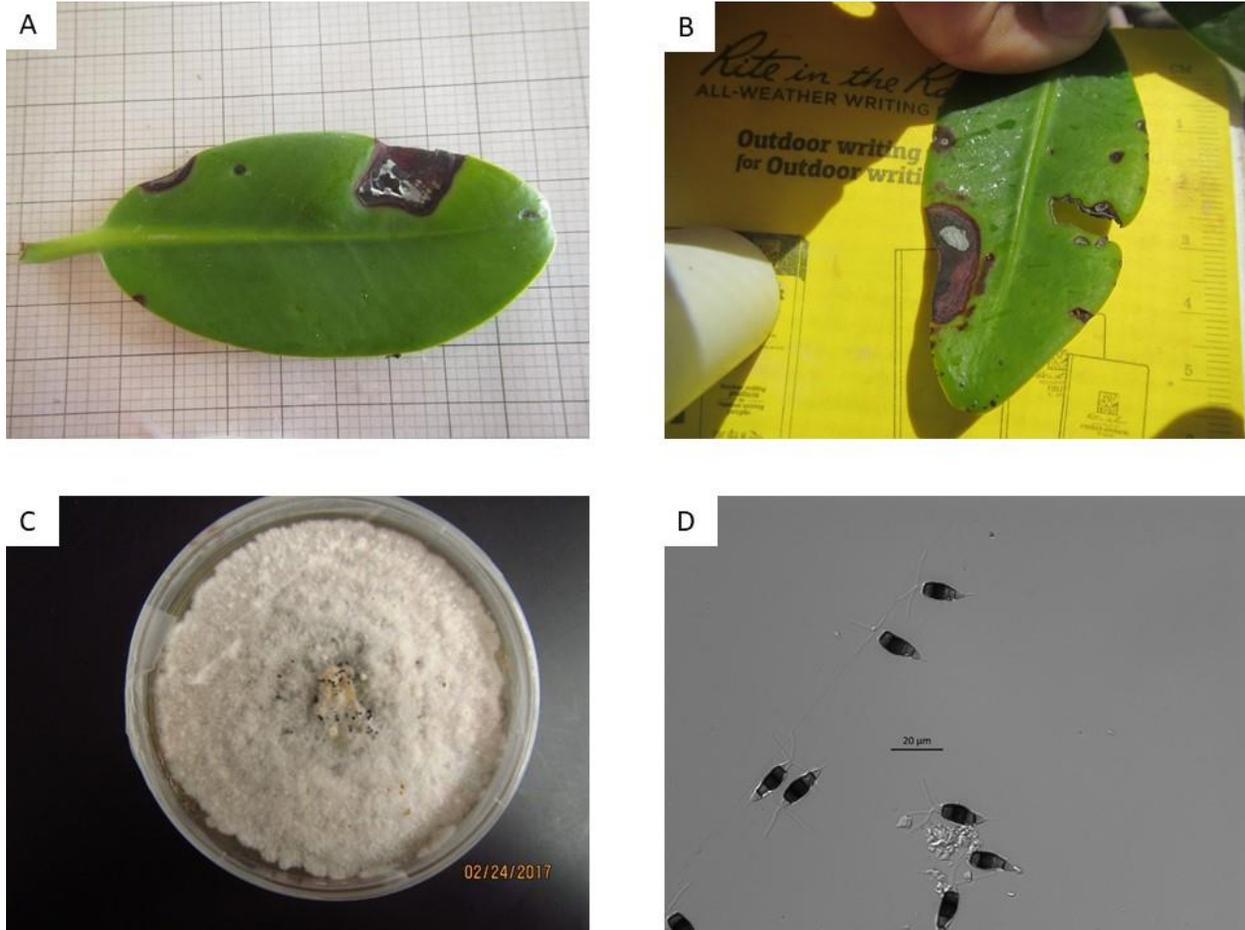


Figure 1: Common leaf symptoms found in the die-off area and on other red mangroves on Abaco and Grand Bahama (A, B). C) Typical culture of *Pestalotiopsis* on acidified PDA after approximately 7 days. Note black pycnidia in center of hyphae. D) *Pestalotiopsis* conidia isolated from pycnidia from cultures grown on acidified PDA.

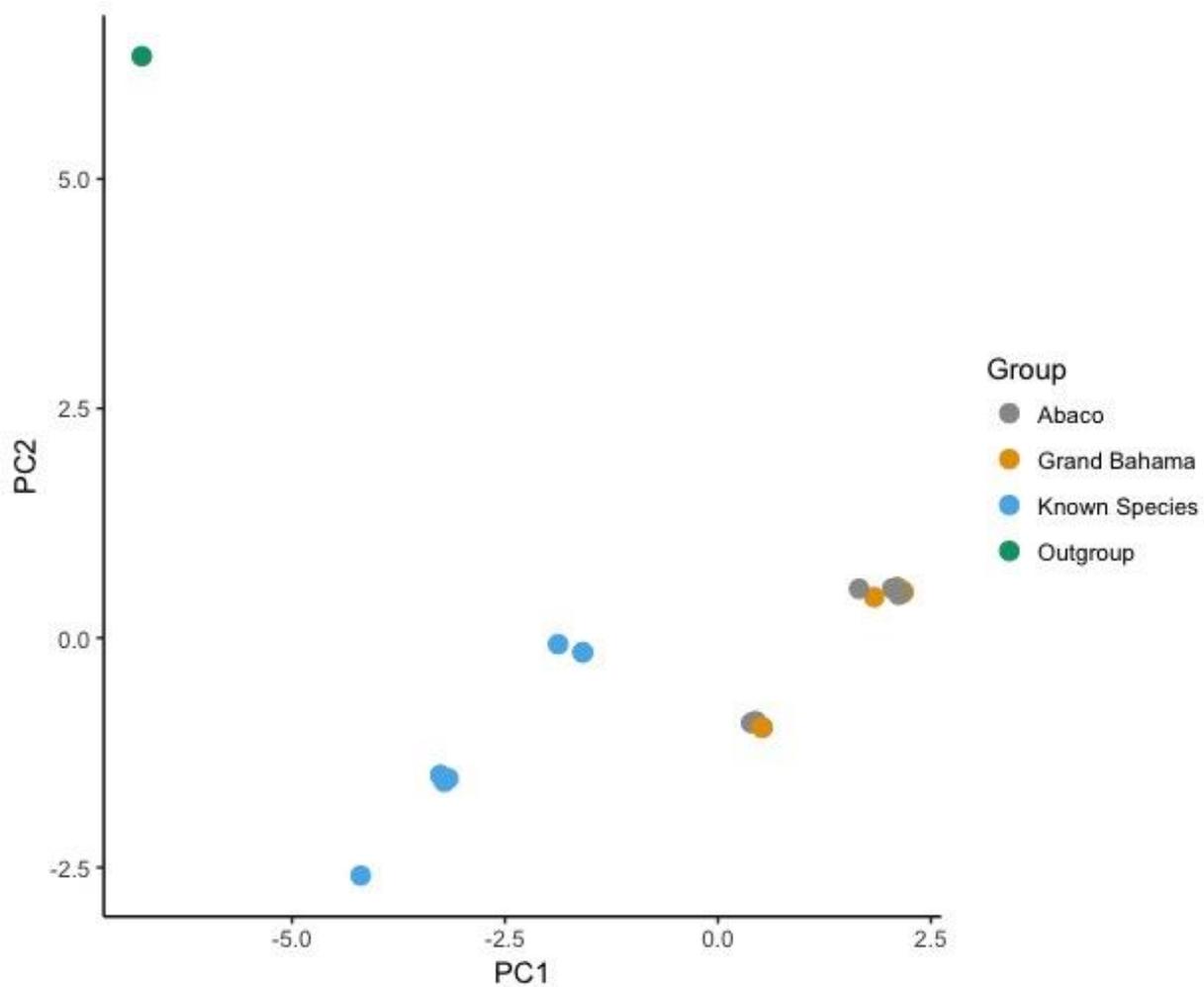


Figure 2: PCA biplot with points corresponding to the locations sampled, based on concatenated sequences for all isolates. PCs refer to genetic diversity. Previously reported sequences are from China, Fiji, and Thailand. New sequences are from Abaco and Grand Bahama in The Bahamas.

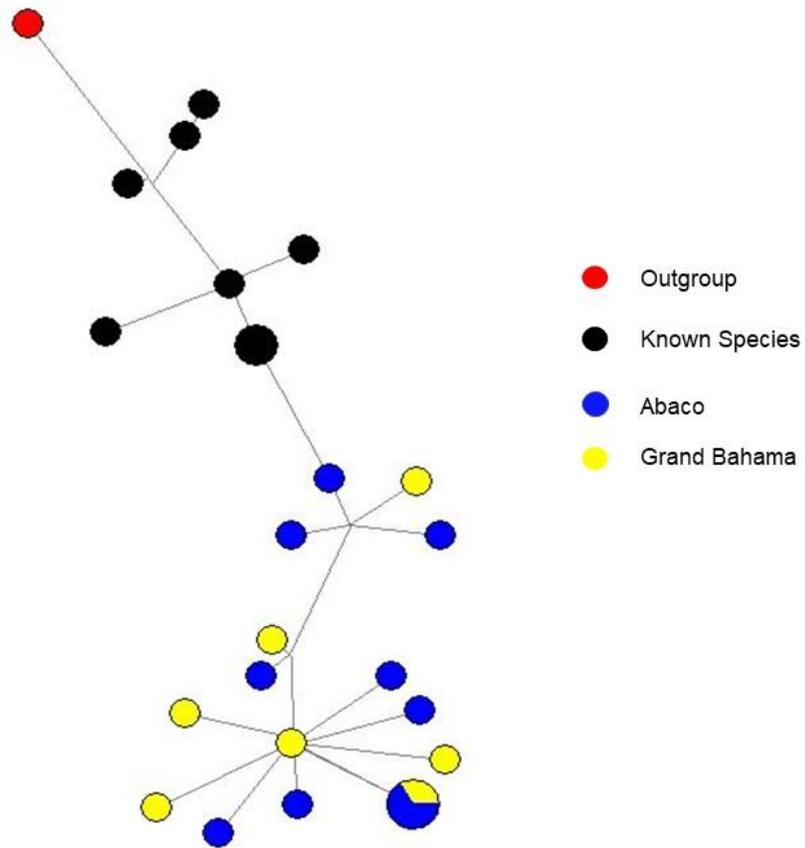


Figure 3: Median joining network based on the concatenation of three loci of *Pestalotiopsis*. Each node is representative of one haplotype with node size proportional to the number of sequences included. Branch lengths are proportional to the number of mutations between haplotypes.

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CHAPTER 5: Mapping prevalence of mangrove disease using citizen science: information gleaned, and lessons learned

Abstract

Citizen science can be useful to answer research questions that involve environmental patterns spanning large areas or over protracted temporal scales. For example, citizen science is a useful tool in documenting invasions and predicting regional disease outbreaks. Here, we designed and implemented a citizen science survey to help identify presence of foliar disease symptoms in red mangrove forests (*Rhizophora mangle*) on Abaco Island, The Bahamas. Our goals were to (1) map disease incidence in mangroves across Abaco and (2) increase awareness of the importance of mangrove ecosystems and the threats they face. Participants were asked to visit a patch of red mangrove and record the number of symptomatic leaves. This survey was disseminated to the public online and through a local non-governmental organization, Friends of the Environment, and data were collected from June to September 2015. Participant turnout was low with only 7 out of 64 observations completed by non-scientists. Despite low turnout, observations by scientists suggested disease was present across Abaco, with more disease symptoms observed in the Northern region (mean= 36 symptomatic leaves/survey patch). Although we achieved our first goal to some degree, our second goal may not have been met due to low participation. We discuss potential shortfalls with recruitment and participation that should be considered in similar future efforts.

Keywords: red mangrove, citizen science, coastal realm, non-governmental organization, foundation species

Introduction

Environmental and conservation research is increasingly utilizing citizen science, the practice of involving the public in the collection of scientific data. It is especially useful when research questions span extended temporal and/or large spatial scales (Bonney et al., 2009, Dickinson et al., 2012, McKinley et al., 2017). For example, citizen science approaches have proven efficacious for documenting disease outbreaks in a number of terrestrial and marine ecosystems (Crowl et al., 2008). Citizen scientists helped document cases of sudden oak death in California, caused by the oomycete *Phytophthora ramorum* (Meentemeyer et al., 2015). Citizen scientists have assisted with documentation of Chagas disease associated with kissing bugs in Texas (Curtis-Robles et al., 2015) and have helped identify acute oak decline in forests in England and Wales (Brown et al., 2017). In marine systems, recreational SCUBA divers have participated in long term monitoring of diseases of coral (Beeden et al., 2014). Data collected from such projects have been used to help managers map incidence of disease, detect new diseases, and improve predictive models (e.g., Holden, 1996, Meentemeyer et al., 2015).

Mangroves are important foundation species that structure entire coastal ecosystems. Mangrove forests cover approximately 150,000km² of coastal habitat (Giri et al., 2011) providing an estimated US \$1.6 billion in ecosystem services worldwide (Costanza et al., 1997). These services include essential nursery habitat for ecologically and economically important marine organisms, shoreline protection, and carbon sequestration (Barbier et al., 2011). In many tropical and sub-tropical systems, including The Bahamas, mangroves provide critical habitat for popular recreational fishery species such as Bonefish (*Albula vulpes*). The flats fishing industry brings approximately \$141 million into The Bahamas annually (Fedler, 2010). In 2011, a die-off of red mangroves (*Rhizophora mangle*) was reported by local stakeholders (primarily fishing

guides) on Abaco Island in The Bahamas. Initial surveys of the die-off region indicated disease symptoms were present on many leaves of live mangroves in the area, suggesting that a foliar pathogen could have contributed to the die-off.

To better understand the potential importance of a foliar pathogen in mangrove die-off, we designed a study to identify incidence of foliar disease symptoms of red mangroves on Abaco Island. We employed an island-wide citizen science survey to determine if leaf disease symptoms were present outside the die-off area. The survey was designed to target citizens who would visit mangrove ecosystems on Abaco (e.g., fishermen, kayakers, bird-watchers). Overall, the goals of the survey were to: 1) map disease incidence across Abaco, 2) use this study to increase awareness of the importance of mangrove ecosystems and the threats they face.

Methods

A group of three scientists (authors of this paper), a web designer and a program coordinator at a local non-governmental organization, Friends of the Environment (FRIENDS), were part of the project development team. A simple survey was developed that consisted of 17 tasks (Table 1, File A1) that could be completed in less than five minutes. Participants were asked to visit any location containing red mangroves, ranging from creeks where shores were completely lined by mangroves to isolated patches adjacent to back roads or towns along the coastline. We defined a patch of mangroves as a cluster of mangroves separated from another patch by water. Clusters of mangroves consisted of at least one mangrove tree. The survey was designed with three possible levels of involvement. At the most basic level, at a mangrove location, participants were asked to record the GPS coordinates and number of diseased leaves using the following categories: 0, 1, 2-10, 11-30, 31-50, >50. The survey pamphlet contained suggested sampling areas and photographs of disease symptoms to look for. Participants could

enhance their involvement by taking photographs of leaves and the mangrove site (involvement level 2) and/or collect samples of leaves that they classified as diseased and healthy (involvement level 3). Participants that collected samples returned them to FRIENDS where samples were verified. Participants were also asked to record whether they observed dead mangrove trees and any other notable observations. Completed surveys could be submitted online or returned to FRIENDS. The survey period was June-September 2015 with the target audience being members of the public who were most likely to access mangrove areas (e.g., fishermen, kayakers, and bird-watchers).

A webpage on the blog Abaco Scientist (<https://appliedecology.cals.ncsu.edu/absci/>) was used as the homepage for this survey. Abaco Scientist received on average 2,656 visits monthly during the initial advertisement (May 2015) and end of the survey (August 2015) period. On this page participants could download the survey pamphlet, view a map of survey sites, and submit data collected from a survey. To help advertise, blog posts were written on the main Abaco Scientist webpage and shared using Twitter (193 followers) and Facebook (455 followers). Additionally, a Facebook page “Abaco Mangrove Survey” (29 followers) was created for the survey where these blog posts were shared, along with links to download the survey pamphlet. Information about the survey was included in the newsletter for FRIENDS, which reaches 2,100 people, and an advertisement to participate in the survey was run in the Abaconian (local newspaper: <http://www.theabaconian.com/>) which has as many as 100,000 monthly page visits and prints 8,000 copies monthly that are distributed on the island, throughout the country and internationally. Additionally, survey packets were provided at FRIENDS for participants who wanted a hard copy and additional supplies for collecting leaf samples. Citizens who were known to frequently visit mangrove areas were contacted directly and given survey materials.

Once surveys were completed and data submitted online or returned to FRIENDS, an email notification was generated for data verification. Verification was done by one author (R. Rossi) and was completed within 1 day. Coordinates entered were confirmed and photos were checked to determine if what the participant noted as disease was indeed an infected leaf. After data were verified, the location of the survey and the number of symptomatic leaves found at that location were updated on the map of Abaco on the project homepage (Figure 2). Identifying information (e.g., name) of participants were not published on the map.

Survey data were used to determine incidence of disease symptoms. Summary statistics of disease incidence and participant turnout were generated on all submissions to determine the mean disease incidence across Abaco. Survey data were grouped into general locations (North, Central, South) based on latitude of sample locations. A one-way ANOVA with a Turkey-HSD post-hoc test was performed to determine if there was a difference in disease incidence among island regions (RStudio, 2016).

Results and Discussion

Of 64 total observations, 14 were from the Central region, 19 from the Northern and 31 from the Southern. Mean disease incidence was highest in the Northern region (36.95 ± 16.1) followed by the South (26.61 ± 15.67) and Central (15.43 ± 14.09) locations (Figure 3). Disease incidence was significantly greater in the Northern region ($F_{2,61}=7.95$, $p=0.001$, Figure 3). Sampling effort differences could be attributed to proximity of mangroves to the roadside and/or ease of access via boat. Seven observations (4 different people) were completed by non-scientists and all were male.

Despite low turnout, the first goal of the survey, to map disease incidence across Abaco was met. Data from this survey indicated that disease symptoms were present in red mangroves

across the island. Difference in disease incidence among sites could be driven by various human impacts adjacent to mangrove sites. For example, in the Northern region, many of the mangroves sampled were adjacent to a road or a town. Proximity to development could be problematic for mangroves because the development activities may have induced abiotic stresses, e.g., reduced tidal flow, altered oxygen dynamics or extreme salinities. Severe abiotic stress in plants often leads to higher disease incidence and severity in other systems (Agrios, 2005, Newton et al., 2010). For example, poplar trees under drought stress and that are infected by a fungal pathogen (*Septoria musiva*) have larger cankers than poplar trees that are infected but not under drought stress (Ramegowda and Senthil-Kumar, 2015, Maxwell et al., 1997).

A popular model for developing and implementing a citizen science project is based on research through the Cornell Lab of Ornithology (e.g., eBird, NestWatch). This model consists of nine steps/tasks that are recommended for a successful citizen science project (Table 2) (Bonney et al., 2009). The main bottleneck in our citizen science project was at steps four and five: recruiting and training participants. Various reasons may have driven low public participation, including understanding participant motivations, ensuring participants were aware of the opportunity, and sustaining interest (West and Pateman, 2016).

We initially designed this survey with the goal of documenting disease symptoms of red mangroves because of the die-off that raised concern with many local stakeholders. Fishermen were especially aware of the die-off, as their livelihoods are to some degree dependent on the health of the local mangrove ecosystems. There was no participation by fisherman in the disease survey, although many remained engaged concerning the data we were generating. Apart from fishermen, knowledge of the mangrove die-off may not have been widespread, limiting more general interest in this environmental issue. The second goal of this survey was to increase

awareness of mangroves and the many threats they face – because of low participant turnout, this goal was not fully achieved.

Another reason for low participation could have stemmed from poor awareness of the project. Advertisement was mostly done online through a blog and Facebook which may not have been utilized by many people who would have participated. A Facebook page was started in May 2015 and the survey was set to begin in June 2015 - more attention to advertising to increase followers on Facebook and other social media platforms may have been useful in generating participation. Additionally, one town, Marsh Harbour, was the main location where other advertisement was focused (e.g., newspaper ads and talking to citizens in person). Since word of mouth is often the best way for information to spread through Bahamian communities, more effort meeting and talking with different communities on the island could have improved turnout (Holdschlag and Ratter, 2016). Furthermore, collaborating with additional NGOs or other organizations (e.g., churches) could have helped recruit participants (Bonney et al., 2009, West and Pateman, 2016, Unell and Castle, 2012). Another issue with participant recruitment could stem from sustaining participation throughout the survey period. We frontloaded recruitment advertising in May 2015, one month prior to the beginning of the survey, and advertised less once the survey began.

Mangroves are often viewed as objectionable habitats, mosquito-ridden and foul smelling - less than ideal locations to spend time (Cormier-Salem, 1999). This was especially problematic because many of those actively involved with environmentally-based volunteer efforts on Abaco are already heavily committed, leaving potential participants faced with a choice of what opportunities to devote time to (West and Pateman, 2016, Penner, 2002). We tried to consider this by ensuring that the survey required little time (<5 minutes), but other environmental

opportunities may still have seemed more attractive than traveling to, and sampling in, mangrove systems. Another potential barrier to participation may have been an unclear protocol. We did not create a specific definition for a “patch” of mangroves which could have confused potential participants. Initially, we thought of having participants use a measuring tape to determine an area to survey, but we thought this would take too much additional time and require an additional piece of equipment. Additionally, we did not explicitly consider demographic characteristics of the participant population when designing the survey - some communities are underrepresented in environmental volunteering and citizen science projects (Ockenden, 2007). We recognize that care should be taken to incorporate more comprehensive, targeted, advertising in future endeavors.

Since the survey occurred over the summer, we missed out on involving students during the regular school year. We attempted to increase participation by training ~30 teachers (from across The Bahamas) in July 2015 through a teacher training workshop hosted by the non-governmental organization Bahamas Reef Environment Educational Foundation. At this workshop, teachers were exposed to activities and lesson plans about mangroves that they could incorporate into their classrooms. We introduced the survey to the teachers in hopes they would utilize them in their classrooms on other islands. One teacher did complete the activity with her students, but many other teachers were unable to do so because mangroves were not located close enough to their schools to make the survey feasible given budget restraints. Additionally, this recruiting technique may have failed because there was no input from school teachers during the development of the survey to ensure its utility as part of classroom curriculum (Bonney et al., 2009).

We found disease symptoms present in red mangroves across Abaco Island using a citizen science survey. However, participation by non-scientists was low and we attribute this to problems with participant recruitment. In the future, more time devoted to advertising and recruitment, particularly by partnering with multiple organizations, may improve participant turnout for citizen science projects. Collaborating with teachers to design citizen science projects that fit into the national curriculum could also improve involvement. Overall, this case study demonstrates the importance of devoting time during the project planning phase to identify participant motivations, ensure means for participants to be aware of the opportunity, and have plans to sustain participant interest.

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Tables

Table 1: List of tasks in the mangrove disease survey pamphlet. See supplemental file for full pamphlet.

Prompt	Description
1	Before you leave make sure you have everything you need. FRIENDS will have prepared bags containing Ziploc bags, flagging tape, permanent marker and a data sheet if you are able to stop by before doing your survey. You will need a GPS, digital camera, 2 Ziploc bags/site, permanent marker, flagging tape, paper, pen/pencil.
2	Choose a location for your survey. For help choosing a location, visit the survey map on the Abaco Scientist website (http://appliedecology.cals.ncsu.edu/absci/).
3	Record GPS point OR mark the area on the map.
4	Record the date and time you start the survey.
5	Take a photo of the site. This should capture multiple trees and show what the site looked like overall.
6	Write down anything you notice about the site. For example, is it located close the road? Near a development? Is it flooded or is it dry?
7	Approach several (at least 3/site) red mangrove patches and perform a visual survey for disease.
8	Record the total number of diseased leaves you see.
9	Tie flagging tape around a tree branch so that it is visible in case we need to return to the site.
10	Take a photograph of what you classified as a healthy leaf.
11	Collect a healthy, non-diseased leaf. Place in Ziploc bag and label the bag with the date, your name, the GPS point and “healthy”.
12	Take a photograph of what you classified as a diseased leaf.
13	Collect a diseased leaf. Place in Ziploc bag and label the bag with the date, your name, the GPS point and “diseased”.
14	Were there any dead mangrove trees at the site? If yes, how many?
15	Record what time you complete the survey.
16	Before leaving the site, be sure that any leaves collected are stored in a Ziploc bag. We do not want to risk spreading disease.
17	Upload your information to the Mangrove Survey form on the Abaco Scientist website (http://appliedecology.cals.ncsu.edu/absci/2015/04/mangrove-citizen-scientist-project/). If you are unable, for any reason, to access the Abaco Scientist website email ryann.rossi@gmail.com or call FRIENDS at 242-367-2721.

Table 2: A common model for developing a citizen science project from Bonney et al. (2009).

Step	Description
1	Choose a scientific question.
2	Form a scientist/educator/technologist/evaluator team.
3	Develop, test, and refine protocols, data forms, and educational support materials.
4	Recruit participants.
5	Train participants.
6	Accept, edit, and display data.
7	Analyze and interpret data.
8	Disseminate results.
9	Measure outcomes.

Figures

Figure 1: A) Image of dead dwarf red mangroves from the die-off location on Abaco, The Bahamas. B) Disease symptoms found on red mangrove leaves in The Bahamas. C) Contents of a survey packet. D) Example of a completed survey.



Figure 2: Participants could access this real-time map displaying survey data collected in summer 2015. Each dot represents the survey location and the color corresponds to the number of leaves with disease. Yellow outlined regions on the map were suggested locations for surveys to be completed.

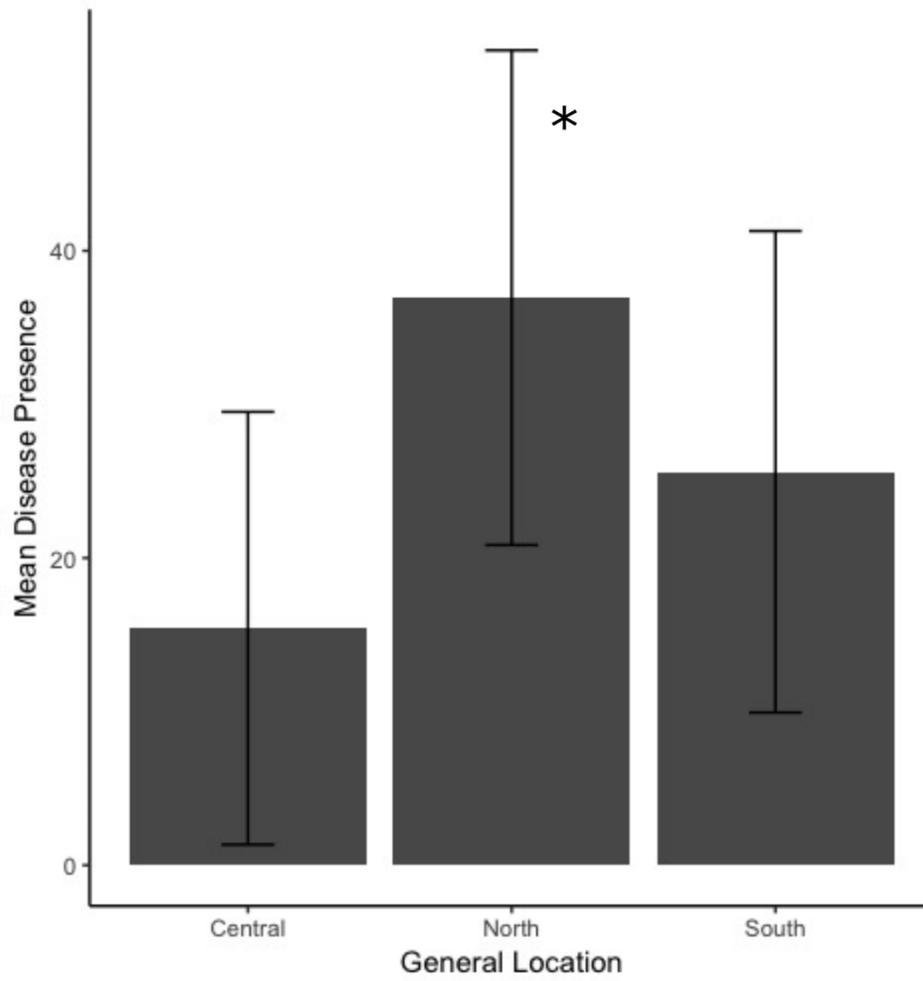


Figure 3: Mean number of diseased leaves in three general regions on Abaco: Central, North and South. The Northern region had significantly higher disease presence.

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CHAPTER 6: Conclusions and Future Directions

Using LANDSAT imagery from 1984-2013 on Abaco, I mapped the change in mangrove cover over time to determine the origin and spread of the die-off. I then examined historical hurricane and tropical storms to determine if die-off spread was correlated with extreme weather events. I found that the die-off likely began in 2008 which was 4 years after the last hurricane and was further exacerbated in 2011-2012 by two hurricane events. This suggests that some other factors may be responsible for the initial stages of this die-off. Initial field surveys suggested that biotic factors such as disease and herbivory may have played a role in the early stages of the die-off.

Based on field surveys in the die-off region, herbivory and foliar disease were noted as very common. I found that disease incidence was positively correlated with herbivory on remaining live trees in the die-off area. Following this survey, I designed a simulated grazing experiment to elucidate whether grazing may facilitate disease infection on mangrove leaves. I found that grazing significantly affected the severity of disease, but not the number of lesions found per leaf, suggesting that grazing does facilitate disease infection. This inspired an effort to document disease in mangroves across Abaco using a citizen science approach. I developed a disease presence survey for the public, but, ultimately, scientists recorded the most data and turnout from local participants was low. Despite low turnout, the survey demonstrated that disease was present in red mangroves across Abaco.

Finally, using molecular techniques in addition to morphological characterization, I identified the foliar pathogen infecting mangrove leaves. This pathogen, *Pestalotiopsis* sp., has a resting endophytic stage prior to becoming pathogenic. Multiple stressors, e.g., herbivory, storm events and potentially a mild drought, may have altered the leaf environment enough to terminate the resting endophytic stage and enable *Pestalotiopsis* sp. to become pathogenic.

Teasing apart the role each of the additional stressors and its interaction with *Pestalotiopsis* is possible through both qPCR techniques and greenhouse experiments.

Overall, my dissertation research suggests that herbivory and disease are important to consider in mangrove ecosystems, especially interactions between them and other stressors. Since relatively little is known about herbivory and disease in mangrove ecosystems, I developed a citizen science project aimed at determining abundance of insect herbivores and plant diseases in mangroves throughout the Caribbean. With funding from National Geographic, I will collaborate with non-governmental organizations (NGOs) and schools throughout the region to implement lesson plans and activities focused on documenting herbivores and diseases present in mangrove ecosystems. This work was developed in collaboration with scientists at NCSU (Craig Layman, Caren Cooper, Jean Ristaino), and educators at two NGOs: Friends of the Environment and Khaled bin Sultan Living Oceans Foundation. From this citizen science project, I hope to better inform knowledge gaps in herbivore and disease abundance in mangroves throughout the Caribbean which will be a first step in better understanding the roles of herbivory and disease in mangroves across broad spatial scales.

APPENDICES

Appendix A: Supplemental material for chapter two

Table A1: Precipitation (mm) from Elbow Cay, Bahamas, a nearby weather station to Abaco Island, The Bahamas. Note that precipitation was recorded daily with several missing days for each year. This data was downloaded from weatherunderground.com.

Year	Total Precipitation (mm)	No. days
2007	1006.2	315
2008	583.2	316
2009	1099.9	362
2010	704.7	353
2011	607.7	302
2012	267.7	335
2013	878.5	355

Appendix B: Supplemental material for chapter three

Herbivore Exclusion Experiment

Experimental plots were arranged in 16 blocks in May 2014. Blocks were placed into distinct habitat types defined as: 1) boundary - a mix of live and dead mangroves, 2) near-boundary (approximately 0.05km from die-off edge), and 3) live (approximately 0.10km from die-off edge) (Fig 1). Eight blocks were placed in the boundary and four blocks were placed into each of the other habitat types (Fig 1). Each block contained three 1 m² plots (total n = 48 plots). Within each block the plots were randomly assigned one of three treatments: cage (exclusion of herbivores), cage control (to control for shading and other factors), and control (no cage). Cage and cage control treatments were constructed from 1mm² mesh galvanized steel hardware cloth and covered a 1m² area encompassing a single dwarf *R.mangle* tree (Fig 2). Height of each cage and cage control was 1m, sufficient to cover trees but allowing space for growth. Cage control treatments had one side removed. The four sides of each cage were inserted approximately 30cm into the surrounding sediment to help exclude burrowing herbivores (e.g., crabs such as *G. cruentata*). Control trees were marked with flagging tape and stakes. During the experiment, tears in the hardware cloth were repaired as needed. HOBO® Data Loggers (Onset Computer Corp.) were deployed in all treatment plots to collect light and temperature data.

The total number of leaves, disease incidence (number of leaves with lesions) and grazing incidence (number of leaves with grazed edges) were recorded every six months within each plot after initiation of the experiment (Fig 3). Tree height and diameter at breast height (DBH) were recorded twice over the duration of the experiment (initial and final sampling). Sticky traps were deployed twice the experiment to confirm effectiveness of herbivore exclusion treatments. Traps were created using sticky tape and hung on mangrove branches within each plot for 24hours.

Analysis

Data from the herbivore exclusion experiment were analyzed using a split-plot ANOVA with location within the die-off as the whole plot factor and treatment as the split-plot factor. Using observations from all sampling periods combined, linear regression was used to determine if there was evidence of a relationship between grazing pressure, disease, and the health of the mangroves. Specifically, the relationships between disease incidence (defined as the proportion of leaves showing evidence of disease) and proportion grazed, the number of dead branches and disease incidence, and the number of damaged shoots and disease incidence were determined using individual linear regressions. The number of dead branches was logged transformed and the number of damaged shoots was cube root transformed to meet assumptions of normality and heterogeneity of the residuals.

Results

The cages were not successful in deterring herbivory on the mangroves, as herbivory was not significantly different among treatments ($F_{2,26}=0.96$, $p=0.40$, Table 1) or locations ($F_{2,13}=0.66$, $p=0.53$, Table 1).

Table 1: ANOVA results from herbivore exclusion experiment.

	numDF	denDF	F-value	p-value
(Intercept)	1	26	0.020675	0.8868
Location	2	13	0.664383	0.5312
Treatment	2	26	0.958021	0.3968
Location:Treatment	4	26	0.862712	0.4993

Figure 1: Experimental design of the herbivore exclusion experiment.

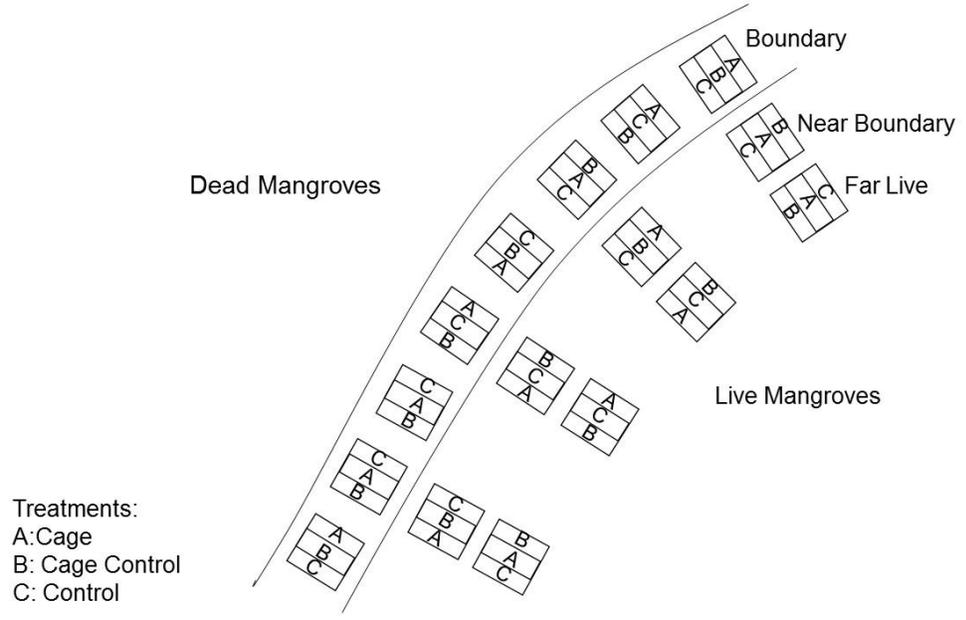
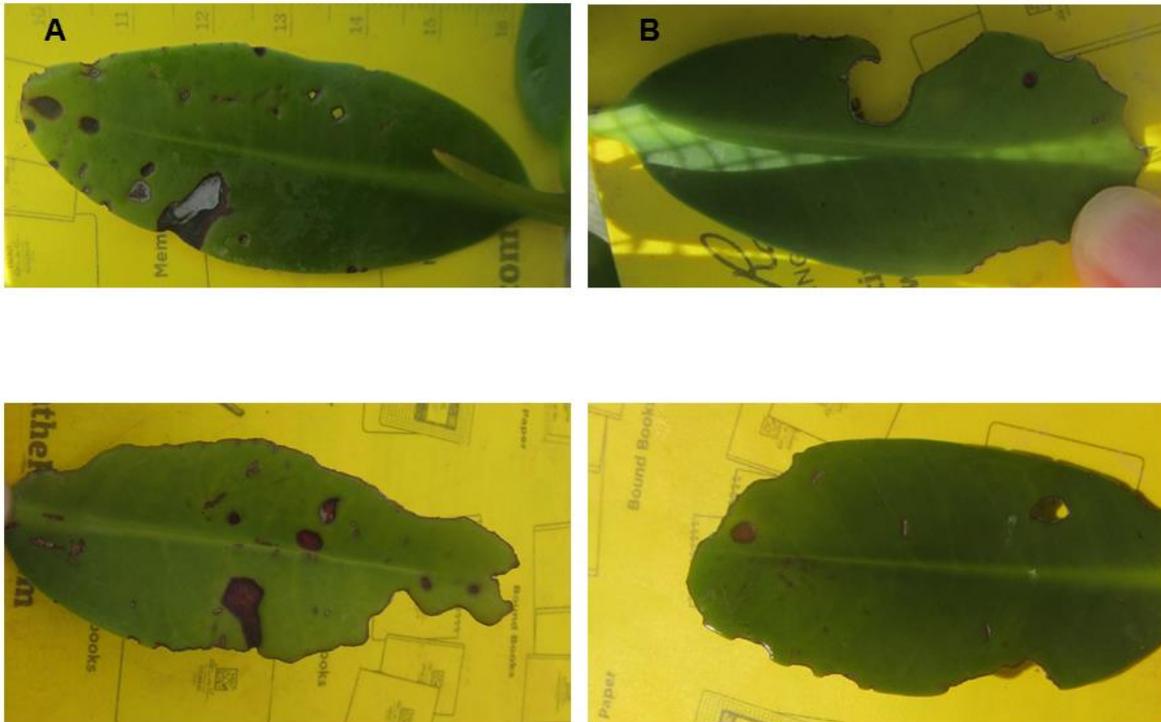


Figure 2: A) Image of fully caged mangrove tree, B) image of exclusion experiment in the field.



Figure 3: Column A depicts what we identified as disease. Column B depicts what we identified as grazed.



Appendix C: Supplemental material for chapter four

Table A1: Summary of items recorded during disease incidence surveys. Surveys consisted of sampling each tree that fell along a 50m transect.

GPS coordinate	No. shoots missing
DBH	Presence of snails
No. leaves with lesions	Presence of leaf galls
No. leaves	Presence of dead matter
No. grazed leaves	No. dead prop root
Presence of canker	No. dead branches
No. shoots	

Table A2: Primer sequences used in analyses.

Primer Name	Sequence	Source
EF728 f	CAT CGA GAA GTT CGA GAA GG	Carbone and Kohn (1999)
EF2	GGA RGT ACC AGT SAT CAT GTT	O'Donnell et al. (1998)
Bt2a	GGTAACCAAATCGGTGCTGCTT TC	Glass and Donaldson (1995)
Bt2b	ACCCTCAGTGTAGTGACCCTTG GC	Glass and Donaldson (1995)
ITS 1	TCCGTAGGTGAACCTGCGG	http://www.fungalbarcoding.org/DefaultInfo.aspx?Page=Primers
ITS 2R2	GCACTCAAACAGGTGTACCTT	http://www.fungalbarcoding.org/DefaultInfo.aspx?Page=Primers

Table A3: Isolate identities and principal component values from SNAP Map analyses based on concatenation of three loci.

Group	Name	PC1	PC2	PC3
Abaco	CR2	2.15261532	0.49758798	0.44848501
Abaco	HCCOC	2.14919405	0.49430513	0.43891291
Abaco	CR4	0.39039208	-0.9259743	-1.9336622
Abaco	594	0.524619	-0.9768048	-1.8900375
Abaco	HCCOC1	2.04549374	0.53764877	0.37482479
Abaco	HC2	2.10475758	0.55160148	0.31884437
Abaco	586	0.44225844	-0.9067211	-1.9187653
Abaco	577	2.12178855	0.46890928	0.37756699
Abaco	1286A	2.14919405	0.49430513	0.43891291
Abaco	1292A	1.65763818	0.53442178	-0.7519313
Grand Bahama	1321A	2.17274849	0.5108784	0.46894993
Grand Bahama	1311A	2.17482188	0.50149556	0.44096446
Grand Bahama	1293B	2.14919405	0.49430513	0.43891291
Grand Bahama	1313B	2.14221198	0.49011156	0.41697298
Grand Bahama	1301A	1.8353937	0.44323055	-0.699876
Grand Bahama	1315A	2.17081694	0.48850007	0.41644218
Grand Bahama	1303A	0.51242349	-0.9728056	-1.9097999
Known Species	Pad1	-1.5884519	-0.157038	1.71412369
Known Species	Pad2	-1.586694	-0.156752	1.70942098
Known Species	Pclavi1	-3.257184	-1.4952934	-0.0986556
Known Species	Pclavi2	-3.257184	-1.4952934	-0.0986556
Known Species	Pdiv	-1.8755069	-0.0678785	1.64543351
Known Species	Pelip1	-4.192931	-2.588936	0.68808174
Known Species	Pfoe2	-3.1637117	-1.5312285	0.00764748
Known Species	Psam	-3.2107386	-1.5690563	0.00793563
Outgroup	Serid	-6.7631595	6.33648103	-1.051049

Table A4: Isolate identities from cultures.

Genus	No. times isolated
<i>Alternaria</i>	2
<i>Cladosporium</i>	3
<i>Collechtrium</i>	1
<i>Curuvlaria</i>	1
<i>Cytospora</i>	10
<i>Diaporthe</i>	3
<i>Diplodia</i>	1
<i>Dothideomycete</i>	2
<i>Neofussicum</i>	3
<i>Penicillium</i>	5
<i>Pestalotiopsis</i>	25
<i>Phlebia</i>	1
Unknown	7
<i>Valsa</i>	8

Table A5: Number of haplotypes present with indels included or removed per locus.

Locus	Indels Included	Indels Removed
Beta-tubulin	27	21
ITS	22	13
Elongation Factor	33	26
Concatenated	26	24

Table A6: Koch's postulate trial results from 8 tested isolates.

Isolate	Lesion Development
NR2	No
HCCOC	No
HCCOC1	Yes
CR4	No
NR1	No
IR1	Yes
1293A	Yes
B4T1	Yes

Figure A1: Map of Abaco and Grand Bahama with sampling locations denoted as black dots. The red square marks the die-off region on Abaco.

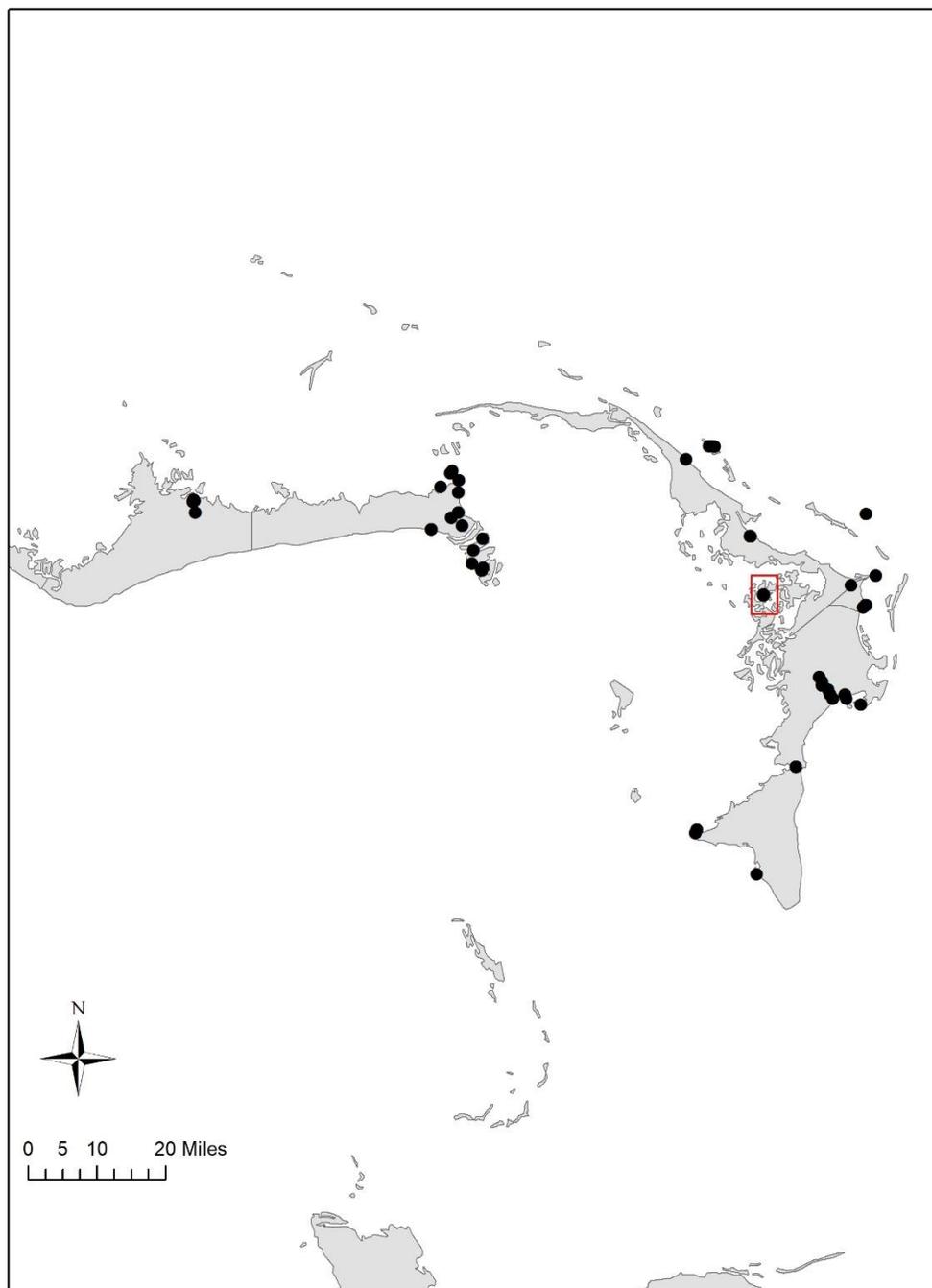


Figure A2: Additional image *Pestalotiopsis* culture with pycnidia on PDA.



Figure A3: Additional images of *Pestalotiopsis* conidia. A) *Pestalotiopsis* conidia with 2 appendages from lab grown culture. B) *Pestalotiopsis* conidia with 3 appendages from lab grown culture.

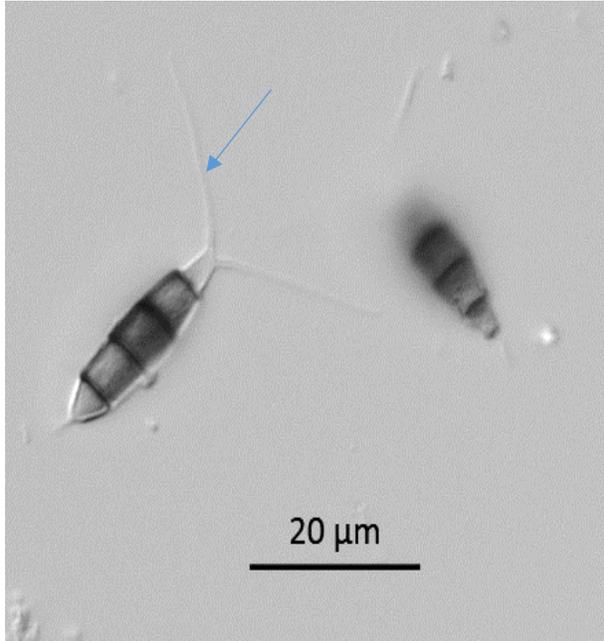
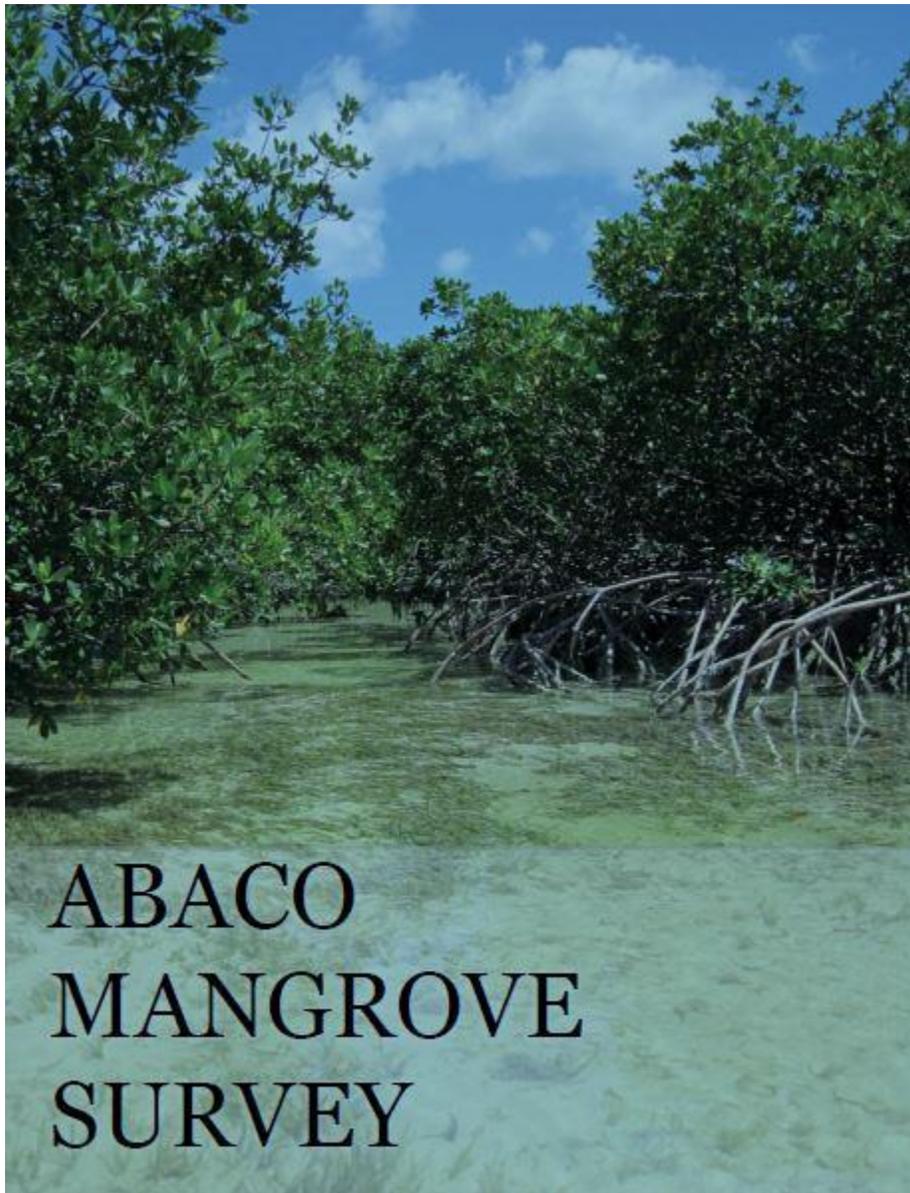


Figure A4: Lesions that developed after inoculation with *Pestalotiopsis* conidia during Koch's postulate trials.



Appendix C: Supplemental material for chapter five

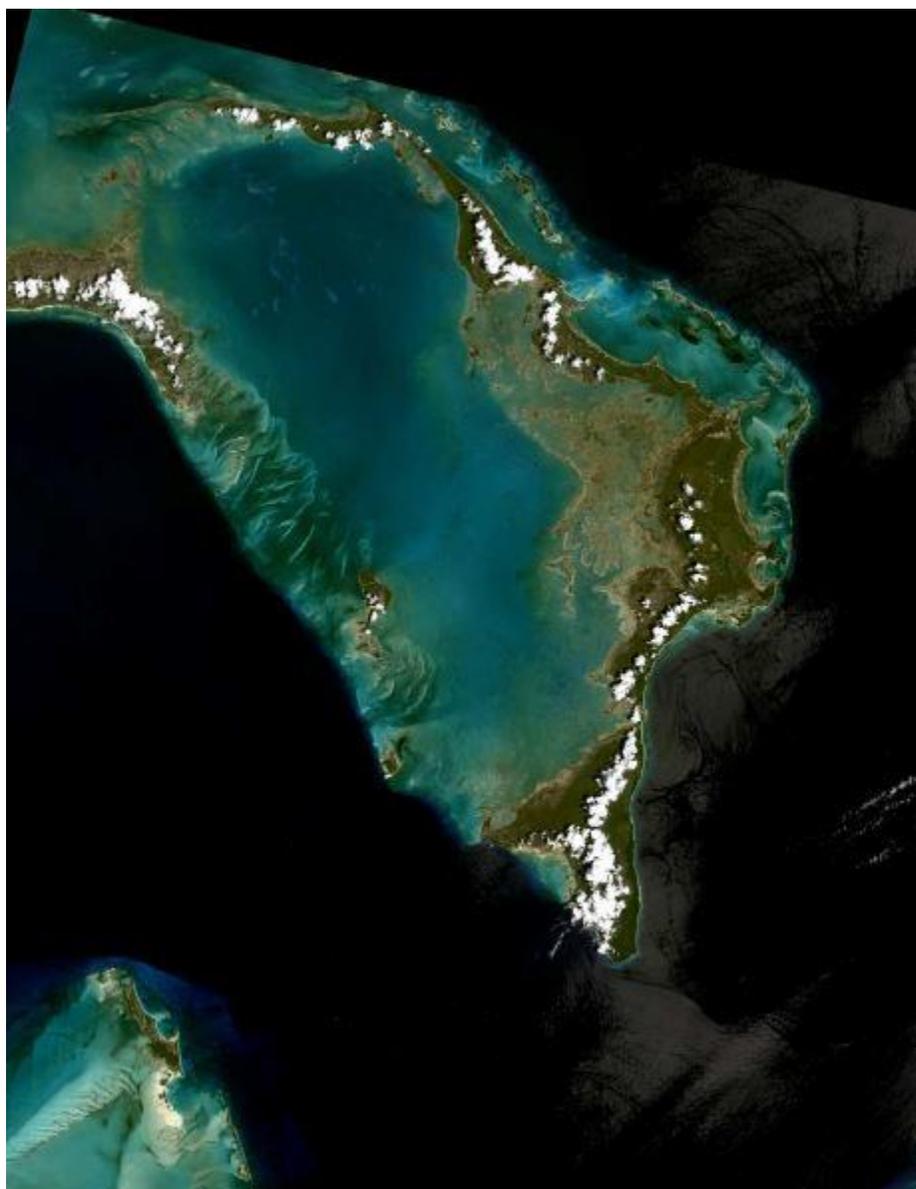
File A1: Copy of survey pamphlet citizen scientists used (7 pages).





Mangrove ecosystems are important coastal habitats. They protect inland areas from storms by breaking waves and capturing sediment. Mangrove creeks also provide excellent habitat for many important species we rely on for food and recreation (e.g., Nassau Grouper, Spiny Lobster, Bonefish). The loss of mangrove ecosystems is detrimental for humans and marine species that rely on them. There are many factors, both abiotic (salinity, hydrology) and biotic (insect grazing, disease) that may contribute to mangrove loss.

We are investigating how plant pathogens may contribute to mangrove loss here on Abaco. This survey will help detect the incidence of plant disease in Red Mangroves across this island.



1) Before you leave make sure you have everything you need. FRIENDS will have prepared bags containing Ziploc bags, flagging tape, permanent marker and a data sheet if you are able to stop by before doing your survey. You will need:

- GPS
- Digital camera (Your cell phone will work)
- 2 Ziploc bags per site
- Permanent marker
- Flagging tape (bright colors preferred)
- Paper
- Pen/pencil

2) Choose a location for your survey. For help choosing a location, visit the survey map on the Abaco Scientist website (<http://appliedecology.cals.ncsu.edu/absci/>).

3) Record a GPS point OR mark the area on the map

Lat: _____ Long: _____

4) Record the date and time you start the survey.

Date: _____ Time: _____

5) Take a photo of the site. This should capture multiple trees and show what the site looked like overall. For example:



6) Write down anything you notice about the site. For example, is it located close to the road? Near a development? Is it flooded or is it dry?

7) Approach several (at least 3/site) Red Mangrove patches and perform a visual survey for disease.

Examples of diseased leaves



8) Record the total number of diseased leaves you see.

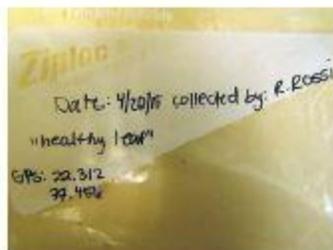
- 0
- 1
- 2-10
- 11-30
- 31-50
- <50

9) Tie flagging tape around a tree branch so that it is visible in case we need to return to the site.

10) Take a photograph of what you classified as a healthy leaf.

Photo Taken? Y N

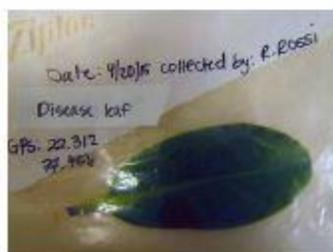
11) Collect a healthy or non-diseased leaf. Place in a Ziploc bag and label the bag with the date, your name, the GPS point and "healthy".



12) Take a photograph of what you classified as a diseased leaf.

Photo Taken? Y N

13) Collect a diseased leaf. Place in a Ziploc bag and label the bag with the date, your name, the GPS point and "diseased".



14) Were there any dead mangrove trees at the site?

Y N

If yes, approximately how many? _____

15) Record what time you complete the survey.

Time: _____

16) Before leaving the site, be sure that any leaves collected are stored in a Ziploc bag. We do not want to risk spreading the disease!

17) Upload your information to the Mangrove Survey form on the Abaco Scientist website (<http://appliedecology.cals.ncsu.edu/absci/2015/04/mangrove-citizen-scientist-project/>). If you are unable, for any reason, to access the Abaco Scientist website email ryann.rossi@gmail.com or call FRIENDS at 242-367-2721.



For more information visit
<http://appliedecology.cals.ncsu.edu/absci/>
<https://www.facebook.com/Abacomangrovesurvey?fref=ts>
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242-367-2721