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Evaluation of the Potential for Ecological Treatment Technologies to Remediate Species of *Phytophthora* from Irrigation Runoff

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EVALUATION OF THE POTENTIAL FOR ECOLOGICAL TREATMENT TECHNOLOGIES TO REMEDIATE SPECIES OF *PHYTOPHTHORA* FROM IRRIGATION RUNOFF

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ABSTRACT

Increased incidences and severity of drought have reduced reliable access to freshwater sources for irrigation purposes by nursery and greenhouse plant producers. Many plant producers are now considering onsite remediation and reuse of water captured from irrigation runoff. However, potential contamination of recycled water with plant pathogens, primarily species of *Phytophthora*, is the primary concern preventing many growers from reusing their water. Species of *Phytophthora* are capable of infecting thousands of host plants and cause some of the most economically important diseases of nursery and greenhouse crops worldwide. *Phytophthora* spp. produce motile, swimming zoospores that often serve as propagules of dispersal and often are the primary infective propagules that initial infections on many plants. While many chemical and physical treatment methods are currently used to disinfest recycled irrigation water, there are many drawbacks to using these technologies. Biological methods for managing *Phytophthora* spp. in waterways, including bioreactors and constructed wetlands, are not as widely implemented and are not well understood.

The overall goal of this dissertation was to assess the potential of passive biological and ecological treatment technologies to remediate *Phytophthora* spp. from irrigation runoff at nurseries and greenhouses, so treated irrigation runoff may be reused on site. Through a series of greenhouse experiments, we determined that the following plant species may be susceptible to the species of *Phytophthora* indicated: *Carex stricta* (*P. cinnamomi* and *P. cryptogea*), *Panicum virgatum* (*P. nicotianae*), and *Typha latifolia* (*P. cinnamomi, P. cryptogea*, and *P. nicotianae*). *Agrostis alba, Iris ensata* ‘Rising Sun’,

and *Pontederia cordata* plants did not appear to be susceptible to the species of *Phytophthora* tested during this study; therefore, they may be suitable for use in constructed wetland systems. Using a controlled model floating treatment wetland (FTW) system, we determined that FTWs established with *Pontederia cordata* plants reduced the flow-through of viable *Phytophthora nicotianae* zoospores as compared to control units containing no FTW at a target hydraulic retention time (HRT) of 4 h. Finally, we determined that laboratory-scale bioreactors containing fir bark reduced flow-through of *P. nicotianae* viable zoospores as compared to control units that did not contain any substrate, during low and high input nitrogen concentration conditions (11.6 ± 0.3 mg/L N and 72.0 ± 3.7 mg/L N, respectively) and at flowrates equivalent to a target 2 h and 8 h HRT.

These are the first studies to evaluate the efficacy of small-scale FTWs and agricultural bioreactors to manage *Phytophthora* species in water and some of the only studies to evaluate ecological technologies for plant pathogen remediation at representative field hydraulic conditions. Future studies should investigate the biogeochemical transformations of nutrients and associated microbial communities within ecological remediation systems to gain further insight into the potential of microbiologically aided removal mechanisms. Interdisciplinary approaches such as this one—which involve teams of agricultural engineers, plant pathologists, plant scientists, and hydrologists—will be crucial for future studies seeking to understand the aquatic ecology of plant pathogens and potentially novel ecological methods for remediation. Increased confidence in and implementation of ecological treatment technologies will

enable producers of greenhouse and nursery crops to safely, economically, and sustainably remediate runoff and drainage waters onsite so that they are able reuse this water for irrigation purposes. Recycling water will help agricultural producers gain access to a reliable water source at a time when access to surface and ground waters is becoming increasingly scarce and contentious due to overuse and increased incidence and severity of droughts.

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CHAPTER 1: INTRODUCTION

Impacts of declining water quality and supply on agriculture

An increasing global population as well as climate change and associated increasing instances and severity of drought have vastly increased our demand of earth's finite supply of freshwater (Christian-Smith et al., 2015; Graffy, 2007; Hess et al., 2016; Pink, 2016). Agriculture, which accounts for approximately 80% of the consumptive water use in the United States (USDA, 2016), is one of the leading primary economic sectors that is affected by drought (Falkenmark, 2013; Kumar and Panu, 1997). Extended periods of drought can have devastating impacts on state and regional economies. In 2011 alone, Texas experienced agricultural losses of an estimated $5 billion as a direct result from the drought that year (Texas Water Resources Institute, 2011). Understandably, reliable access to sources of freshwater for irrigation purposes has become a top priority for agricultural producers (White et al., 2013).

In addition to increasing water scarcity, declining water quality is also an issue of major concern, especially to the agriculture industry. Fertilizers rich in nitrogen (N) and phosphorus (P) are the primary agricultural sources of nutrient pollution in the United States (US EPA, accessed January 2017), with agriculture ranked as the leading source of water quality impairment in rivers and lakes. In the United States, over 20 million tons of fertilizer are applied each year (US EPA, 2014). Excess N and P in water systems stimulate growth of plants and microorganisms, which in turn leads to depletion of dissolved oxygen – a process known as eutrophication (Hasler, 1947; Sawyer, 1966). In some instances, excess nutrients can encourage growth of harmful toxic algal blooms, which can negatively impact ecosystems and human health (Anderson et al., 2002).

Pesticides are another common agricultural contaminant found in aquatic ecosystems, with one or more pesticides or their breakdown products having been detected more than 90% of the time in stream water collected across the US by the United States Geological Survey (Gilliom et al., 2007). The agricultural herbicides atrazine and metolachlor were detected more frequently in agricultural areas as compared to urban areas. Pesticides in water at concentrations as low as 2 micrograms per liter can have detrimental effects on aquatic communities — including zooplankton, algae, and amphibians (Relyea, 2009). Pesticides may accumulate in microorganisms and may subsequently have detrimental impacts on higher trophic levels (DeLorenzo et al., 2001). Pollution and dispersal of plant pathogen inocula in waterways is also of concern when considering agricultural impacts to surrounding environments. Irrigation runoff from agricultural areas has resulted in the dissemination of many common fungal and bacterial plant pathogens (Hong and Moorman, 2005; Steadman et al., 1975). Plant pathogens can negatively impact the structure and evolution of plant communities, which provide critical ecosystem services (Chakraborty, 2013).

The need for improved water quality and more efficient water use throughout the United States has driven the implementation of more restrictive regulations in some states and major watersheds. The Chesapeake Bay Preservation Act was enacted in 1988 to improve water quality in the Chesapeake Bay by requiring the use of effective land management to minimize nonpoint source pollution (Virginia Department of Environmental Quality, 1988). In the Great Lakes region, the United States Environmental Protection Agency (US EPA), in partnership with associated states and local governments, adopted the Water Quality Guidance for the Great Lakes System in 1995 (EPA, 1995). The purpose of the Guidance is to implement programs to reduce toxic chemicals and other pollutants released into the Great Lakes System to “maintain,

protect, and restore water quality throughout the entire basin and preserve the economic foundation of the region" (US EPA, 1995). The Guidance defines minimum levels of protection needed for pollutants that could threaten water quality, with recommended water quality criteria that target hazards specific to the region. In March 2015, California's State Water Resources Control Board adopted an expanded emergency conservation regulation "to safeguard the state's remaining water supplies as California enters a fourth consecutive dry year" (California State Water Resources Control Board, 2015). Measures include restrictions on outdoor irrigation, reporting on monthly water use, and implementation of fines for violations of prohibited activities.

As water supplies decline due to overuse and increased incidence of drought (resulting in a less reliable water supply) and more regulations are passed regarding water-use efficiency and the quality of irrigation runoff, agricultural producers must consider new and nontraditional methods to manage water and meet their irrigation water demands. Agricultural producers must adapt by reducing water use for irrigation, shifting to lower quality water sources, and containing and remediating irrigation runoff onsite so that water may be reused onsite or released with little to no negative impacts on surrounding surface and ground waters.

Nursery and Greenhouse Operations

Nursery and greenhouse crops make up almost 365,000 hectares of the approximately 5.7 million hectares of land devoted to specialty crops in the United States, with a market value of about $19 billion and a workforce made up of about 345,000 individuals (USDA, 2015). The market value of the nursery and greenhouse industry in South Carolina alone is estimated at $165 million (USDA, 2016). As described above, access to reliable sources of freshwater for irrigating these high-value crops has become increasingly limited. An estimated 65% of growers reported

using groundwater wells as a source of irrigation water and about 20 to 30% reported using surface water or city water in a survey of over 50 nursery and floriculture producers and academic, extension, and allied industry professionals in the southeastern US (Fulcher et al., 2016). Commercial nurseries commonly apply irrigation water at rates as high as 2.5 cm per day, which equates to about 47 to 56 million liters per hectare per year of irrigation water (Fulcher et al., 2016; Janick, 2011). When considering leaching fractions as high as 110%, this equates to approximately 93,500 liters per hectare of runoff per day (Janick, 2011).

Because of dwindling supplies of reliable sources of freshwater, perceived negative environmental impacts associated with production runoff, and the potential for more restrictive water use and disposal regulations, growers should consider remediating and recycling irrigation runoff water onsite. However, several issues and concerns currently limit the willingness of some growers to reuse irrigation runoff. These grower-identified issues include potential contamination of recycled water sources with nutrients, pesticides, and plant pathogens as well as the costs associated with implementing necessary treatment technologies (White et al., 2013). Plant pathogens in irrigation water are a significant plant health issue that has garnered much attention over the last several decades (Hong et al., 2014; Gevens et al., 2007); however, little is known about the relationship between concentrations of pathogen inocula in irrigation water and disease incidence (Raudales et al., 2014). Recycled irrigation water may act as a primary source of inoculum and as an effective means of inoculum dispersal (Steward-Wade, 2011). Infective propagules may be produced from susceptible plants in onsite water-holding reservoirs or may be transported from diseased plants in the growing area into onsite reservoirs by way of runoff and leaching. Plant pathogens of concern that have been detected in nursery and greenhouse water resources include species of Oomycetes in the genera *Phytophthora* and *Pythium* as well as

some fungi, bacteria, viruses, and plant parasitic nematodes (Hong and Moorman, 2005; Stewart-Wade, 2011).

Plant Pathogens of Interest: *Phytophthora* spp.

Impacts

Species of *Phytophthora* cause some of the most economically important diseases of nursery and greenhouse crops worldwide (Hwang and Benson, 2005; Leonberger et al., 2013). Over 140 species of *Phytophthora* have been identified, and a number of other potential species are waiting for formal descriptions (Yang et al, 2017). Diseases caused by *Phytophthora* spp. result in root, crown, and fruit rots as well as stem and foliage blight on a multitude of host plants in all climatic zones—including tobacco, vegetables, fruit and ornamental crops, field and forage crops, and trees and shrubs in natural ecosystems (Erwin and Ribeiro, 1996; Yang et al., 2017). Disease in plants occurs when the following three factors are present: a susceptible host, a virulent pathogen, and a suitable environment (Erwin and Ribeiro, 1996). There are species of *Phytophthora* adapted to the wide range of environmental conditions that occur around the world, including the warm humid southeastern US (Erwin and Ribeiro, 1996). Water plays an important role in the life cycle of *Phytophthora* spp. because free water is necessary for the production zoospores (Erwin and Ribeiro, 1996). Due to a limited amount of field data available in the literature, the economic significance of *Phytophthora* spp. has been difficult to ascertain; however, economic damage to crops in the US alone by species of *Phytophthora* is estimated in the tens of billions of dollars (Hong and Moorman, 2005; Tyler, 2002).

Morphological Characteristics and Life Cycle

The genus *Phytophthora*, translated as "plant-destroyer" from Greek, and the closely related genus *Pythium* are Oomycetes and often are referred to as water molds (Erwin and Ribeiro, 1996). Oomycetes are named for their oospores that are sexual, thick-walled, survival spores, but not all Oomycetes naturally produce oospores. Species of *Phytophthora* also produce asexual chlamydospores, which are designed to ensure short-term survival in the absence of a host. In the presence of water, Oomycetes produce zoospores in asexual structures called sporangia, and these motile, swimming spores serve as propagules of dispersal and often are the infective propagules that cause primary infections on many plants (Schumann and D'Arcy, 2010). Each zoospore has two flagella, which allow them to move freely in water. Zoospores are chemotactic (can sense and move toward specific chemicals, like root exudates), negatively geotropic (tend to rise toward the water surface), and are relatively short-lived—if a host is not found within roughly 48 hours, zoospore populations will dramatically decline (Erwin and Ribeiro, 1996; Kong et al., 2012; Porter and Johnson, 2004). Once a zoospore makes contact with susceptible tissue on a host plant, it forms a cyst that then germinates to form hyphae that penetrates host tissue. Once inside the plant, *Phytophthora* spp. grow into plant cells for nourishment and subsequent reproduction (Erwin and Ribeiro, 1996).

Methods of Detection in Water

Baiting and filtration techniques are used to detect *Phytophthora* spp. in water. Fruits (including apples, pears, lemons, and avocados), whole leaves, wounded leaves, or leaf pieces typically are used in baiting bioassays (Erwin and Ribeiro, 1996; Hong et al., 2014; Rollins et al., 2016). If water samples are filtered to trap zoospores, filters can be inverted and directly placed on selective growing media in the lab for quantification of colony forming units (CFUs) or can

be treated with chemicals for DNA extraction and subsequent detection and quantification of *Phytophthora* spp. through quantitative real-time polymerase chain reaction (qPCR). The detection threshold concentration, or the minimum number of propagules that baiting or filtration methods can detect, differs by detection method and is not well characterized across all detection methods (Hong and Moorman, 2005; Rollins et al., 2016). Additionally, the biological threshold of *Phytophthora* spp. required for infection of a host plant has not been well characterized (Hong and Moorman, 2005). During their study comparing five detection and quantification methods for *Phytophthora ramorum* in stream and irrigation water, Rollins et al. (2016) determined that filtration and qPCR were the most sensitive methods at detecting low levels of zoospores. However, qPCR methods detect both living and dead genetic material, which could result in overestimation of viable propagule density (Raith et al., 2014). Additionally, both filtration and qPCR methods give no indication of whether these low levels of inocula are capable of actually causing infection on susceptible hosts. Rollins et al. (2016) found that baiting methods had a wider detection threshold than filtration and qPCR methods, and that filtration, qPCR, and leaf disk methods were reliable methods of quantification of *P. ramorum* zoospores. However, filtration and qPCR methods may not be a reliable method of quantification, as zoospores may pass through the filter pores during filtration (Rollins et al., 2016).

Traditional Water Treatment Technologies

Several physical and chemical methods exist to remediate *Phytophthora* spp. Physical methods of preventing the spread of propagules of *Phytophthora* spp. in waterways include: a) installation of barriers, such as mats and films; b) sedimentation and electro-coagulation; c) filtration, including slow sand filtration or membrane filters; d) heat treatment; and e) ultraviolet

(UV) light (Hong and Moorman, 2005; Steward-Wade, 2011). Irrigation mats, which typically consist of layers of polyethylene, polypropylene, and acryl that are placed on the bottom of ebb-and-flow benches or floors, appear to inhibit movement of inoculum from or to the bottoms of pots. During electro-coagulation, an electric current is produced that attracts charged contaminants in water, such as bacteria, viruses, metals, and suspended solids. These contaminants precipitate out of solution, forming a sludge. While physical methods of removal are typically simple and relatively safe (no chemical additions to water system), they may not be practical for some growers due to high installation and maintenance costs as well as their inability to process large quantities of water.

There are a number of chemical treatments that have been or currently are used to disinfest recycled irrigation water—including chlorine and chlorine-related compounds, bromine, iodine dosing and removal through anion-exchange resin, ozone, hydrogen peroxide, surfactants, acidic electrolyzed oxidizing water, ionization, antimicrobial compounds, peroxyacetic acid, nutrient amendments, such as calcium nitrate or calcium chloride, bubbling of carbon dioxide, and fungicides (Hong and Moorman, 2005; Steward-Wade, 2011). Drawbacks of chemical treatment of irrigation water include sensitivity to solids and water pH (which render them ineffective if not properly managed by growers), potential for formation of harmful byproducts, and potential for technical malfunctions or breakdowns that may require maintenance by technical specialists.

Biological and Ecological Treatment Technologies

Biological and ecological methods of managing *Phytophthora* spp. in water systems are not widely implemented and not well understood. These methods include biological control

agents, such as nitrogen stabilizing chemical formulations; biofiltration, which consists of a porous filter matrix that supports active microbial populations that may be antagonistic to pathogens; and vegetated channels and constructed wetlands, which allow surface or subsurface flow-through of irrigation runoff (Hong and Moorman, 2005; Steward-Wade, 2011).

Plants as Filters: Vegetated Channels and Floating Treatment Wetlands

The use of vegetated channels as an ecological remediation technology has not been investigated for removal of propagules of *Phytophthora* spp. Vegetated channels—also known as vegetative filter strips, vegetative buffers, riparian buffers, and bioswales—have been shown to remediate a host of runoff contaminants from agricultural, industrial, and residential land areas. Vegetated channels are broadly defined as conveyance systems with dense vegetation that intercept and filter surface runoff water from developed areas before that water is released to receiving water bodies. As compared to other common treatment technologies, vegetated channels are relatively inexpensive to install, do not require intensive training, and are safe and easy to maintain (Dabney et al., 2006; Qiu, 2003). Vegetated channels have been shown to reduce sediment loss from agricultural fields by up to 90% (Blanco-Canqui et al., 2004; Daniels and Gilliam, 1996; Yuan et al., 2009) and to reduce agricultural inputs from leaving fields in runoff water—e.g., total phosphorus and nitrogen by up to 95% (Daniels and Gilliam, 1996; Vought et al., 1995) and herbicides and other pesticides by up to 100% (Arora et al., 1996; Patty et al., 1997; Syversern and Bechmann, 2004). Vegetated channels also serve to increase the diversity of flora and fauna in the landscape, stabilize stream banks, and improve habitat for fish and invertebrates within receiving waters (Vought et al., 1995).

The use of vegetated channels to remediate pathogens from runoff is an emerging field of research. Tate et al. (2006) found that vegetative buffers effectively reduced animal agricultural inputs of waterborne *Escherichia coli* into surface waters. Atwill et al. (2002) showed that vegetated buffer strips effectively removed waterborne *Cryptosporidium parvum*, a parasite of mammals, from surface and shallow subsurface flow. However, studies have not been conducted to investigate the potential for vegetated channels to remediate plant pathogens in nursery and greenhouse runoff. In addition, almost no information exists regarding the susceptibility of common wetland plant species to species *Phytophthora*.

Constructed wetlands are water treatment systems that utilize naturally-occurring processes involving vegetation, soils, and associated microbes to improve water quality. There are three general types of constructed wetlands: surface-flow (free-water surface), subsurface-flow (horizontal or vertical flow), and floating treatment wetlands (FTWs) (White et al., 2011). Constructed wetlands have been used for decades to remediate contaminants from industrial, domestic, and agricultural wastewaters (Vymazal, 2011). Constructed wetlands installed at nurseries and greenhouses have been shown to effectively remediate nutrients, particularly nitrogen, from irrigation runoff (Taylor et al., 2006; White, 2013; White, 2018; White and Cousins, 2013). Though constructed wetlands have been shown to remediate bacterial pathogens, the remediation of plant pathogens in constructed wetlands has not been widely investigated (Beutel et al., 2013; Stewart-Wade, 2011; Vacca et al., 2005; Vymazal, 2011). Gruyer et al. (2013b) demonstrated that model horizontal subsurface-flow constructed wetlands removed greater than 99% of *Pythium ultimum* and *Fusarium oxysporum* propagules. The potential for constructed wetlands to remediate *Phytophthora* spp., however, has not been investigated.

A FTW consists of emergent vegetation established upon a buoyant structure that floats on the water surface. Microbial communities colonize the roots suspended below the FTW. These roots and microbial communities serve as natural filters by absorbing and processing nutrients and other pollutants, slowing the flowrate, and enhancing the settling of suspended solids (Khan et al., 2013; Tanner and Headley, 2011). FTWs are a relatively new type of constructed wetland. Most research on FTWs has been conducted at the laboratory- or model-scale, with field-scale implementations being used to remediate municipal sewage, urban runoff, river and lake water, and aquaculture effluent (Pavlineri et al., 2017). Since FTWs can be readily established within existing ponds and channels, they may be a more readily applicable best management practice for nursery and greenhouses growers as compared to traditional constructed wetland systems. Though FTWs have been shown to remediate nutrients, sediment, and metals, their potential to remediate plant pathogens has not been investigated (White and Cousins, 2013).

Agricultural bioreactors

Agricultural bioreactors, as defined herein, are subsurface trenches filled with carbon material (usually wood chips) that intercept runoff water from the growing area before being released into receiving water bodies. For over 20 years, agricultural bioreactors have been extensively studied and shown to effectively remediate nutrients, namely nitrate, from agricultural runoff through the processes of nitrate-reducing bacteria (Blowes et al., 1994; Christianson et al., 2012; Chun et al., 2009; Greenan et al., 2006; Jaynes et al., 2008; Robertson and Merkley, 2009; Schipper et al., 2010). Bioreactors also are known to remediate herbicides and pesticides from agricultural runoff. Celis et al. (2008) reported effective biodegradation of

the herbicides isoproturon and 2,4-dichlorophenoxyacetic acid in sequencing batch reactors, and Gonzalez et al. (2006) reported degradation of selected priority acidic pesticides MCPP, MCPA 2,4-D and 2,4-DP in fixed-bed bioreactors. Bioreactors have also been extensively used to remediate acid mine drainage by sulfate-reducing bacteria (Neculita and Zagury, 2008; Zagury et al., 2006). The use of agricultural bioreactors to treat plant pathogens is an emerging field. Gruyer et al. (2013a) conducted the only known study with carbon-based bioreactors being used to treat water-borne plant pathogens (*Pythium ultimum* and *Fusarium oxysporum*). The lab-scale 3.5-liter bioreactors—which contained a mixture of maple wood chips, sawdust, poultry manure, maple leaf compost, and sand—effectively reduced up to 99.99% of the influent pathogen densities. Currently, the efficacy of carbon-based agricultural bioreactors to remediate species of *Phytophthora* from irrigation runoff has not been studied, representing a substantial knowledge gap in this field.

Research Objectives and Hypotheses

The overall goal of this project is: To assess the potential of passive biological and ecological treatment technologies to remediate *Phytophthora* spp. from irrigation runoff at nurseries and greenhouses, so treated irrigation runoff may be reused on site. Specific research objectives are listed below.

Objective 1:

Assess the potential susceptibility of six aquatic plant species (*Agrostis alba, Carex stricta, Iris ensata, Panicum virgatum, Pontederia cordata,* and *Typha latifolia*) to infection by five species of *Phytophthora* (*P. cinnamomi, P. citrophthora, P. cryptogea, P. nicotianae,* and *P. palmivora*)

commonly found at ornamental plant nurseries in the southeastern US. Treatment groups to be assessed include 'plant only', 'plant+*Phytophthora* spp.', and '*Phytophthora* spp. only'.

Alternative hypotheses:

- Potential susceptibility will differ by plant species
- Potential susceptibility will differ by species of *Phytophthora*

Objective 2:

Determine the effects of the presence of immune plants (as identified in Objective 1) and hydraulic retention time (HRT) on the efficacy of model FTWs deployed in simulated water channels to reduce flow of viable zoospores of *P. nicotianae* through the channels

Alternative hypotheses:

- The flow of viable zoospores through the channels will differ by HRT treatment (1 hour and 4 hours)
- The flow of viable zoospores through the channels will be affected differentially by the species of immune plants established within the FTW

In other words, the presence of immune plants, identified in Objective 1, in a FTW deployed in a water channel may have a detrimental effect on the transport of viable zoospores of *P. nicotianae* through the channel. The efficacy of the FTWs to reduce the flow of viable zoospores through the channels will likely vary by plant species and HRT.

Objective 3:

Determine the efficacy, including the effects of HRT and nutrient concentration, of laboratory-scale bioreactors containing woody substrates to reduce the flow of viable zoospores of *P. nicotianae* through the bioreactors.

Alternative hypotheses:

- Zoospore movement through and survival in bioreactors will differ among substrate types—fir bark, pine bark, plastic, or no substrate
- Zoospore movement through and survival in bioreactors will differ between HRT treatments — 2 and 8 hours
- Zoospore movement through and survival in bioreactors will differ between two input nitrogen concentrations in the bioreactors — 'Low' and 'High' (~10 and 100 mg/L-N, respectively) — because differing nutrient availability in the bioreactors will likely impact the microbial communities present in the bioreactors

In other words, physical filtration capabilities and biochemical conditions created by microbial communities within a bioreactor cell containing a woody substrate may have a detrimental effect on the transport and survival of viable zoospores of *P. nicotianae*. The physical filtration capacity and biochemical conditions created by microbial communities within bioreactors will likely vary by substrate type, HRT, and nutrient concentrations.

Research Significance

The research described in the following chapters seeks to fill several critical gaps in research in the fields of agricultural and ecological engineering, horticulture, and plant pathology. Namely, the use of established ecological treatment technologies (phytoremediation and carbon-based bioreactors) to remediate *Phytophthora* spp. from irrigation runoff and

drainage at ornamental plant production sites has not been previously explored. Currently, data on biological and ecological water treatment options to remove pathogen contaminants from water at nurseries and greenhouses is scarce (Hong and Moorman, 2005; Stewart-Wade, 2011). Results from this project will add to existing literature in the field and serve to ultimately increase knowledge and grower confidence in future efforts to implement biological and ecological treatment strategies. Increased implementation of biological and ecological treatment technologies will enable growers to safely, economically, and sustainably remediate their runoff water onsite, so they can reuse this water for irrigation purposes. Recycling water will help growers gain access to a reliable source of water at a time when ready access to surface and ground waters is becoming increasingly scarce and contentious due to overuse, demand, and increased incidence and severity of droughts.

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CHAPTER 2: POTENTIAL SUSCEPTIBILITY OF SIX AQUATIC PLANT SPECIES TO INFECTION BY FIVE SPECIES OF *PHYTOPHTHORA*

Abstract

The susceptibility of aquatic plants to species of *Phytophthora* has not been investigated. The objective of this study was to assess the potential susceptibility of six aquatic plant species, which could be used in vegetated channels or constructed wetlands, to infection by five species of *Phytophthora* commonly found at nurseries in the southeastern US. In a greenhouse experiment, roots of six plant species (*Agrostis alba*, *Carex stricta*, *Iris ensata* 'Rising Sun', *Panicum virgatum*, *Pontederia cordata*, and *Typha latifolia*) were exposed to each of five species of *Phytophthora* (*P. cinnamomi, P. citrophthora, P. cryptogea, P. nicotianae,* and *P. palmivora*). Zoospore presence and activity in solution were monitored using a standard baiting bioassay with rhododendron leaf disks as baits. Experiments were initiated in 2016 and repeated in 2017 and 2018. During 2016 trials, *Phytophthora* spp. were not isolated from the roots of any of the plants, but, during trials in 2017 and 2018, some roots of *C. stricta, P. virgatum*, and *T. latifolia* were infected with multiple species of *Phytophthora*. Plant presence significantly reduced the percentage of rhododendron leaf disks infected by four of the species of *Phytophthora* but not those infected by *P. cinnamomi*, which suggested that these plants negatively affected the presence or activity of zoospores of four of the five species of *Phytophthora* in the aqueous growing solution. Results from this study demonstrated that certain aquatic plant species may serve as sources of inoculum at ornamental plant nurseries if these plants are used in constructed wetlands in receiving reservoirs or are present naturally in waterways, which could be of concern to plant producers who recycle irrigation runoff water.

Introduction and Background

Plant pathogens in irrigation water are a significant crop health issue that has received much attention over the last several decades (Hong and Moorman 2005; Hong et al. 2014; Gevens et al. 2007). Recycled irrigation water may act as a primary source of inoculum and as an effective means of inoculum dispersal (Steward-Wade 2011). Infective propagules may be produced on susceptible plants growing in onsite water-holding reservoirs or may be transported from diseased plants in the growing area into onsite reservoirs in runoff water. Plant pathogens of concern that have been detected in nursery and greenhouse water sources include species of *Oomycetes* in the genera *Phytophthora* and *Pythium* as well as fungi, bacteria, viruses, and plant parasitic nematodes (Hong and Moorman 2005, Hong et al. 2014; Stewart-Wade 2011). Species of *Phytophthora*, in particular, cause some of the most economically important diseases of nursery and greenhouse crops worldwide (Jones and Benson 2001; Dreistadt 2001; Hwang and Benson 2005; Leonberger et al. 2013). Diseases caused by *Phytophthora* spp. result in root, crown, and fruit rots as well as stem and foliage blights on a multitude of host plants in all climatic zones—including tobacco, vegetables, fruit and ornamental crops, and field and forage crops, and trees and shrubs in natural ecosystems (Erwin and Ribeiro 1996). *Phytophthora* spp. produce zoospores in asexual structures called sporangia, and these motile swimming spores often are the infective propagules that cause primary infections on many plants (Erwin and Ribeiro 1996; Schumann and D'Arcy 2010).

Constructed wetlands have been used for decades to remediate contaminants from industrial, domestic, and agricultural wastewaters (Vymazal 2011). Constructed wetlands installed at nurseries and greenhouses have been shown to effectively remediate nutrients, particularly nitrogen, from irrigation runoff (Taylor et al. 2006; White 2013; White 2018; White

and Cousins 2013). Though constructed wetlands have also been shown to remediate human and mammalian pathogens of concern, the remediation of plant pathogens in constructed wetlands has not been widely investigated (Beutel et al. 2013; Stewart-Wade 2011; Vacca et al. 2005; Vymazal 2011). Gruyer et al. (2013) demonstrated that pilot-scale horizontal subsurface-flow constructed wetlands removed greater than 99% of *Pythium ultimum* and *Fusarium oxysporum* propagules. The potential for constructed wetlands to remediate *Phytophthora* spp., however, has not been studied. The susceptibility of common aquatic plants to *Phytophthora* spp. in ditches and ponds receiving irrigation runoff and drainage water from production areas represents a significant knowledge gap and must be determined before investigation of constructed wetlands as a viable bioremediation option. Therefore, the objective of this study was to evaluate the potential susceptibility of six commonly occurring aquatic plant species to infection by five species of *Phytophthora* frequently found at plant nurseries in the southeastern US (S. N. Jeffers, *personal communication*).

Materials and Methods

Production of inocula

For this greenhouse experiment, five species of *Phytophthora* commonly associated with ornamental plants in the southeastern US were selected: *P. cinnamomi, P. citrophthora, P. cryptogea, P. nicotianae,* and *P. palmivora*. Three isolates of each species were selected for use, and all isolates had been recovered from diseased plants in nurseries and landscapes in South Carolina and Georgia and are maintained in a permanent collection by S. N. Jeffers at Clemson University (Table 2.1). All isolates except for the three isolates of *P. palmivora* were used and

described in a previous study (Ridge et al. 2014). The three isolates of *P. palmivora* were characterized and identified using the same techniques described by Ridge et al. (2014).

Active cultures of each isolate were maintained in long-term storage in glass vials containing 5% clarified V8 Juice agar (Jeffers 2015b) at 15°C in the dark). Before the start of each annual series of experimental trials, isolates were transferred from long-term storage to PAR-V8 selective medium (Ferguson and Jeffers 1999; Jeffers 2015a) and maintained at 20°C in the dark for up to 3 weeks, during which time hyphal growth was observed and identities of cultures were confirmed. Isolates then were transferred to 10% clarified V8 juice agar (cV8A; Jeffers 2015b) to ensure culture purity and maintained at 25°C in the dark for 3 to 4 days. Finally, the isolates were transferred to 10% V8A (Jeffers 2015b) and maintained at 25°C in the dark for 3 to 4 days. Agar plugs from these cultures were used to produce inocula—see below.

Experimental Design

Six wetland plant species were evaluated for potential susceptibility to *Phytophthora* spp.: *Agrostis alba* (redtop), *Carex stricta* (tussock sedge), *Iris ensata* 'Rising Sun' (Japanese iris), *Panicum virgatum* (switchgrass), *Pontederia cordata* (pickerelweed), and *Typha latifolia* (broadleaf cattail). For each trial, small (5 or 10 cm in diameter) rooted plants were received from commercial suppliers located in New Jersey (2016 trials), Maryland (2017 trials), and Georgia (2018 trials), except for *I. ensata* plants, which were received as rhizomes from Georgia (2016 and 2017 trials). Upon arrival, plants were placed in the greenhouse and watered daily for 2 to 3 weeks. Two weeks before each trial began, plant roots were rinsed with running tap water, submerged in a 1:50 ratio of insecticidal soap solution (Safer Insect Killing Soap, Woodstream Corp., Litiza, PA) for 10 min, thoroughly rinsed in running water, dipped in a 1:250 ratio of

quaternary ammonium chloride disinfestant solution (KleenGrow, Pace Chemicals Inc., Burnaby, BC Canada) for 1 min to eliminate potential pathogens on root surfaces, and then thoroughly rinsed again with running water. Each plant was placed in a 2.1-liter plastic containers containing Milli-Q water (EMD Millipore Corporation, Billerica, MA) amended with 10 mg/liter nitrogen from a 24-8-16 (N-P-K) water-soluble fertilizer (Soluble Fertilizer Plus Minors, Southern Agricultural Insecticides, Inc., Hendersonville, NC). During this time, a standard baiting bioassay (see below) was used to monitor each container to ensure that the plants were not contaminated by naturally-occurring species of *Phytophthora*. The day before each trial began, the plants were weighed (wet mass, g) and lengths (cm) of roots and shoots were measured.

The experiment was conducted as a completely randomized block design with six replicated blocks. Each block contained three different treatments: Containers that only had a plant ('Plant only'), containers that only had inoculum ('*Phytophthora* only'), and containers that had a plant and inoculum ('Plant+*Phytophthora*'). Plants were exposed to inoculum for the first 14 days of each trial. A trial refers to one plant species pairing with one *Phytophthora* species. Inoculum for each species of *Phytophthora* was composed of three agar plugs (5 mm in diameter), containing actively growing hyphae (cut from the advancing edge of a V8A culture), from each of the three isolates of that species. Therefore, nine total agar plugs were placed in the bottom of each container of a treatment receiving inoculum. The V8A plugs produced sporangia in the aqueous solution, and sporangia then released zoospores into solution (Erwin and Ribeiro 1996). All six plant species were evaluated for susceptibility to each of the five species of *Phytophthora* in trials conducted in 2016 (April to July) and again in 2017 (March to June). In trials conducted in 2018 (March to May), *C. stricta, P. virgatum*, and *T. latifolia* were evaluated

again for susceptibility to *P. cinnamomi, P. cryptogea*, and *P. nicotianae* because of inconsistent results in the first two years of these plant-pathogen combinations. During all trials, sensors from three HOBO® U12 4-External Channel Data Loggers (Onset Computer Corp., Bourne, MA) were arbitrarily placed in containers to measure and record water temperature every 30 min.

Monitoring pathogen activity

One day after inoculum was added to the containers, a standard baiting bioassay (Ferguson and Jeffers 1999; Ridge et al. 2014) was used to monitor zoospore activity in each container. Ten 5-mm-diameter leaf disks cut from leaves collected from a pesticide-free *Rhododendron maximum* plant were floated on the surface of the aqueous solution in each container. After 3 days, leaf disks were removed, blotted dry with paper towels, embedded in PARPH-V8 selective medium (Ferguson and Jeffers 1999; Jeffers 2015a) in a 10-cm-diameter petri plate, and held at 25°C in the dark for 3 days. Leaf disk perimeters were examined microscopically (20 to 70×) for hyphae of *Phytophthora* spp. Leaf disks from which hyphae of *Phytophthora* spp. grew were judged to be colonized. Activity of zoospores was quantified using a scale from 0 to 100% based upon the numbers of leaf disks out of 10 that were colonized (Ridge et al. 2014). Each time leaf disks were removed from a pot, 10 fresh leaf disks were added; this process was repeated three more times over the 14-day exposure period. The term "exposure period" refers to the first 14 days of each trial when inocula were present in some containers.

On Day 14 of each trial, the plants were removed from containers, thoroughly rinsed under running water, dipped in a disinfestant solution (KleenGrow) for 1 min to eliminate potential propagules on root surfaces, and then thoroughly rinsed under running water again.

Plants were then placed in new 2.1-liter plastic containers containing a solution of Milli-Q water amended with water-soluble fertilizer and no inocula to begin a period of non-exposure to inocula. Five days later (Day 19), leaf disks were placed in each container and removed 3 days later, as previously described. This process was repeated four more times for a total of five sequential bait-exposure periods over the course of 15 days for all trials conducted in 2016 and 2017. For trials conducted in 2018, the baiting process was repeated 11 more times (for a total of 12 sequential baiting events over the course of 36 days). The term "non-exposure period" refers to the time period of each trial when inocula were not present in containers (from Day 14 to the end of the trial).

Effects of Phytophthora spp. on aquatic plants

After the final baiting event in the non-exposure period, plants were removed from solutions, rinsed under running water, dipped in disinfestant solution (KleenGrow) for 1 min to eliminate potential propagules on root surfaces, thoroughly rinsed under running water again, and then blotted dry with clean paper towels. The plant wet mass and lengths of the roots and shoots of each plant were measured and recorded to determine if inocula had a detrimental effect on plant growth. Even though plants for each trial were the same species and age, they naturally varied in size. Therefore, normalized changes in wet mass, root length, and shoot length of each plant were calculated as percentages using the formula:

$$\text{Normalized change} = ((\text{Final Measurement} - \text{Initial Measurement}) / (\text{Initial Measurement})) \times 100$$

Roots were examined for symptoms of disease (e.g., area of discoloration or decay), and roots exhibiting such symptoms were targeted for isolation. If no symptoms were observed, roots

were selected arbitrarily for isolation from throughout the root system. Selected roots were cut into 1- to 2-cm-long pieces, 10 root pieces were combined to make one bundle, and five bundles were embedded into a plate of PARPH-V8 medium. Isolation plates were placed in the dark at 20°C for 3 to 7 days and monitored for colonies of *Phytophthora* spp. If colonies developed, the roots were designated as infected.

Statistical Analyses

All data analyses were conducted using JMP Pro statistical software (Version 14.1.0, SAS Institute, Cary, NC). Data were analyzed using factorial analysis of variance (ANOVA) to determine if the main effects and interactions between *Phytophthora* spp. and time (days) after inocula were added had significant effects ($\alpha = 0.05$) on zoospore activity—i.e., the percentage of leaf disk colonization. Significant effects of the treatments Plant+*Phytophthora* and *Phytophthora* only for each *Phytophthora* spp. on percentage of leaf disk colonization for each aquatic plant species were also analyzed. Additionally, significant effects of the treatments Plant only and Plant+*Phytophthora* for each *Phytophthora* spp. on wet mass and lengths of the roots and shoots of each aquatic plant species were analyzed. ANOVAs were adjusted for random blocking effects and repeated measures over time. Tukey's honest significant difference (HSD) was used to separate treatment means when main or simple effects were found to be significant.

Results

Over the 3-year period of this study, 66 trials were conducted to expose six different aquatic plants to each of five species of *Phytophthora* to evaluate potential susceptibility (Table 2.2). During the 2016 trials, *Phytophthora* spp. were not isolated from root bundles from any of

the plants; however, when the experiment was repeated in 2017, roots of *C. stricta, P. virgatum*, and *T. latifolia* were infected with multiple species of *Phytophthora* (Table 2.2). Only those plant and *Phytophthora* species combinations that resulted in root infection in 2017 were repeated for a third time in 2018, and, during these trials, only roots from *T. latifolia* were found to be infected with *P. cinnamomi*—similar to results in 2017.

Colonization of leaf disks served as a direct measure of zoospore presence and activity. As expected, for all trials throughout 2016, 2017, and 2018, leaf disks in containers with only plants were never found to be colonized by *Phytophthora* spp. (data not shown). However, when inoculum was present in containers during the exposure period, the percentage of leaf disks colonized in containers with plants was consistently and significantly less than that in containers without plants for four of the species of *Phytophthora* (*P. citrophthora*, *P. cryptogea*, *P. nicotianae*, and *P. palmivora*) in trials conducted in all three years: 48 out of 52 trials = 92% (Table 2.3 and Figs. 2.1, 2.2, and 2.3) (data are also presented as tables in Supplementary Tables S2.1-S2.18). Mean leaf disk colonization was generally between 75 to 100% during the exposure period for containers with only inoculum, but leaf disk colonization was generally less than 50% in containers that had both plants and inoculum. The exception was plants exposed to *P. cinnamomi*, which resulted in leaf disk colonization percentages typically greater than 75%. Mean leaf disk colonization by *P. cinnamomi* in containers with plants and inoculum was significantly less than that in containers with only inoculum in only six out of 14 trials (43%) conducted over three years (Table 2.3). *P. cinnamomi* zoospore presence and activity was not affected by the aquatic plants in eight (57%) of the trials.

During the non-exposure period for trials conducted in 2016, mean leaf disk colonization was <1% in containers that had each combination of a plant and a species of *Phytophthora* (Fig.

2.4 and Supplementary Tables S2). During the 2017 trials, mean leaf disk colonization was less than 1% for *A. alba* and *P. cordata* that had been exposed to all species of *Phytophthora.* However, considerably more leaf baits were colonized during the non-exposure period in containers that held the other four aquatic plants that had been exposed to several species of *Phytophthora* spp. in 2017 (mean leaf disk colonization in parentheses): *C. stricta* with *P. cinnamomi* (20.0%) and *P. cryptogea* (2.3%); *I. ensata* with *P. cryptogea* (4.6%); *P. virgatum* with *P. nicotianae* (36.3%); and *T. latifolia* with *P. cinnamomi* (94.2%). All of these plant-pathogen combinations resulted in visible root rot symptoms at the end of the non-exposure period for each trial except for *I. ensata* plants exposed to *P. cryptogea*, which did not have any visible symptoms of root infection. Though mean leaf disk colonization was 0.0% in containers with *T. latifolia* plants that had been exposed to both *P. cryptogea* and *P. nicotianae*, some of these plants had symptoms of root infection at the end of the non-exposure period.

Three growth parameters were measured at the beginning of each trial, before plants were exposed to inoculum, and the end of each trial—after the non-exposure period was over. The changes in these parameters were normalized to account for natural variation in size among the plants used in each trial. There were no significant differences among the normalized growth parameters for the six aquatic plants exposed and not exposed to inocula of the five species of *Phytophthora*; therefore, these data are not presented. The presence of zoospore inoculum of *Phytophthora* spp. did not affect growth of any of the aquatic plants used in this study during the experimental period.

Minimum, optimum, and maximum temperature ranges for the five *Phytophthora* species used in this experiment are presented in Table 2.4 (modified from Erwin and Ribeiro, 1996). During the 2016 trials, maximum water temperatures exceeded 33°C for 80% of the

experimental period, with maximum water temperatures exceeding 37°C for 20% of the experimental period (Fig. 2.5A). During the 2017 experimental trials, maximum water temperatures exceeded 33°C for only 13% of the time, and never exceeded 37°C (Fig. 2.5B). During the 2018 experimental trials, maximum water temperatures exceeded 33°C for 36% of the time and never exceeded 37°C (Fig. 2.5C).

Discussion

In this study, six aquatic plant species that are commonly used in constructed wetlands or vegetated channels or are naturally present in waterways at ornamental plant nurseries were tested for potential susceptibility to five species of *Phytophthora* that are frequently associated with ornamental plants in the southeastern US. The purpose of this study was to identify plants to avoid when installing constructed wetland and vegetated channels at ornamental plant nurseries to help remediate irrigation water. Plants susceptible to one of more species of *Phytophthora* should not be deployed in constructed wetlands because they might serve as reservoirs for these plant pathogens and constantly disseminate inoculum into irrigation water that could be recycled and returned to plants in the nursery.

Though plant root infection varied greatly from year to year over the 3-year study period, results indicate that the following aquatic plant species are potentially susceptible to the *Phytophthora* spp. indicated: *C. stricta* (*P. cryptogea* and *P. cinnamomi*), *P. virgatum* (*P. nicotianae*), and *T. latifolia* (*P. cinnamomi*, *P. cryptogea*, *and P. nicotianae*). This variation in root infections by year could be attributed, at least in part, to temperature differences in the aqueous solution from year to year. Maximum daily water temperatures exceeded 33°C, the maximum reported growing temperature for most species of *Phytophthora* used in this

experiment, for 80% of the experimental period for trials conducted in 2016. During the 2017 and 2018 trials, however, maximum daily water temperatures exceeded 33°C for only 13% and 36% of the experimental period, respectively. High daily water temperatures during the 2016 trials may have inhibited the ability of zoospores to infect root tissue; however, for all 3 years, leaf disk colonization generally remained well above 75% for cases where inoculum was present but plants were not, indicating that the ability of *Phytophthora* species to colonize leaf disks was not affected by extreme water temperatures. The leaf disks used in this experiment had wounded perimeters (by nature of cutting them from rhododendron leaves), but the roots on plants in the containers were not wounded. Studies have shown the occurrence of disease is significantly higher in wounded plants as compared to non-wounded plants (Granke and Hausbeck, 2010, Salas et al., 2000, Tooley et al., 2014. Additionally, plants were intentionally sourced from a different nursery and from different regions of the country each year (New Jersey, Maryland, and Georgia) to ensure plant response to *Phytophthora* species was consistent across plant genotype. Though the plant species evaluated remained consistent from year to year, the genotype and production conditions of plants likely differed from one nursery to the next, and subsequently may have contributed to differences observed in plant root infection from year to year.

Colonization of leaf disks served as a direct measure of zoospore presence and activity throughout this experiment. Leaf disk colonization percentages were generally higher in cases where inoculum was present in the absence of a plant as compared to cases where inoculum was paired with a plant. Except for cases where plants were exposed to *P. cinnamomi*, the presence of plants seemed to have a negative effect on the ability of zoospores to colonize the floating leaf disks. It is possible that zoospore activity was either chemically inhibited by exudates released from plant roots or biologically inhibited by competition with microbial communities associated

with plant roots; studies have shown that the presence of certain microorganisms can promote or suppress disease development caused by species of *Phytophthora* (Frey-Klett et al., 2011; Hong and Moorman, 2005; Hong et al. 2014; Kong and Hong, 2016). Zoospores could have also been physically obstructed by the plant roots in solution, and thus unable to swim up toward leaf disks. Zoospores were constantly being released from agar plugs in the bottom of containers during the exposure period (Ridge et al., 2014). Since plant roots were closer to the plugs, zoospores may have preferentially colonized and encysted upon plant roots, and in some cases were able to penetrate the root tissue, causing infection.

Colonization of leaf disks varied over time by *Phytophthora* species, with colonization of leaf disks decreasing over time by all *Phytophthora* species except *P. cinnamomi*. *P. cinnamomi* is known to be one of the most virulent species of *Phytophthora* and it has the largest host range of any species of *Phytophthora* (Hardham and Blackman, 2018). The ability of *P. cinnamomi* to survive for long periods of time over a wide range of conditions has made eradication of disease very difficult, and may explain its significantly higher level of activity as compared to the other species of *Phytophthora* in this study.

Symptoms of plant infection were not apparent on roots and shoots of trialed plants. Further, the presence of *Phytophthora* spp. did not seem to negatively impact plant growth, even for those plants deemed infected. Observed asymptomatic responses of infected plants was unusual, given that infection by *Phytophthora* species typically results in negative growth response of host plants and their fruit (Davis et al., 1978; Pozo et al., 2001).

Of the six plants used in this study, *Agrostis alba, Iris ensata* 'Rising Sun', and *Pontederia cordata* were not susceptible to any of the five species of *Phytophthora* to which they were exposed under the experimental conditions in the study. Additional field studies are needed

to validate the potential immunity of these three species. These three species of aquatic plants may be suitable for use in constructed wetland systems, as they do not appear to serve as sources of inoculum. In fact, the presence of these plant species may have actually decreased zoospore activity and virulence within the water column, as indicated by the lower percentages of leaf disk colonization in the presence of plants as compared to the absence of plants. Data have shown that *Phytophthora* species are less likely to be observed on roots of plants growing in later stages of constructed wetland systems (Ridge et al., 2019, in press), which suggests that viable propagules were likely eliminated via physical or biological filtration as infested water moved through the constructed wetland system. *Typha latifolia* is one of the most commonly used plant species in constructed wetlands around the world due to their ability to remove high levels of nutrients and heavy metals (Vymazal, 2013). Our results demonstrated that *Typha latifolia* plants might be susceptible to several species of *Phytophthora* that occur in ornamental plant nurseries in the southeastern US. Avoidance of *Typha latifolia* within constructed wetlands receiving agricultural runoff and drainage that contains *Phytophthora* spp. may be warranted, especially if runoff and drainage waters are reused for irrigation purposes, as *Typha latifolia* may actually serve as a source of inocula within the constructed wetland system. However, further investigations of these and other plant species should be carried out within the optimum temperature ranges for *Phytophthora* species to confirm their susceptibility or resistance to infection by species of *Phytophthora*.

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Tables

TABLE 2.1. Sources of 15 isolates of five species of *Phytophthora* used in this study

Species	Isolate no.	Host plant	Location	Substrate	County[a]
P. cinnamomi	02-0912	*Itea virginica* 'Little Henry'	Landscape	Roots	Pickens
	02-1054	*Rosa banksiae*	Landscape	Roots	Lexington
	10-0053	*Viburnum obovatum*	Landscape	Roots	Hampton
P. citrophthora	07-0303	*Heuchera* hybrid 'City Lights'	Nursery	Crown	Aiken
	07-0248	*Rosa* hybrid 'Home Run'	Nursery	Roots	York
	S.lat 3.5	*Sagittaria latifolia*	Nursery	Roots	Grady, GA
P. cryptogea	05-0491S	*Sedum spurium* 'Dragon's Blood'	Nursery	Stem	York
	03-0222	*Dicentra* hybrid 'King of Hearts'	Nursery	Roots	York
	06-0989	*Euphorbia amygdaloides*	Nursery	Roots	Aiken
P. nicotianae	05-0690	*Hibiscus paramutabilis* ×*syriacus* 'Lohengrin'	Nursery	Stem	Edgefield
	06-0496	*Perovskia* sp.	Nursery	Roots	York
	07-1391	*Rosa* hybrid 'The Fairy'	Nursery	Roots	Berkeley
P. palmivora	97-0367	*Hedera helix*	Landscape	Roots	Aiken
	98-2589	*Fatsia japonica*	Nursery	Roots	Berkeley
	00-2137	*Hedera helix*	Landscape	Roots	Charleston

[a] All counties are in South Carolina except one; Grady Co. is in Georgia.

TABLE 2.2. Incidences of infection on roots of six aquatic plant species inoculated with five species of *Phytophthora* in trials conducted each year over a 3-year period[a]

	2016[b]					2017[b]					2018[b]				
Plant species	*P. cin*	*P. cit*	*P. cry*	*P. nic*	*P. pal*	*P. cin*	*P. cit*	*P. cry*	*P. nic*	*P. pal*	*P. cin*	*P. cit*	*P. crp*	*P. nic*	*P. pal*
Agrostis alba	0	0	0	0	0	0	0	0	0	0	…	…	…	…	…
Carex stricta	0	0	0	0	0	**1**	0	**1**	0	0	0	…	0	…	…
Iris ensata ‘Rising Sun’	0	0	0	0	0	0	0	0	0	0	…	…	…	…	…
Panicum virgatum	0	0	0	0	0	0	0	0	**2**	0	…	…	…	0	…
Pontederia cordata	0	0	0	0	0	0	0	0	0	0	…	…	…	…	…
Typha latifolia	0	0	0	0	0	**2**	0	**1**	**2**	0	**2**	…	0	0	…

[a] For each plant-pathogen combination, six replicate plants were used during each trial of each year. Some treatment combinations were not evaluated (…) in 2018 because results from 2016 and 2017 trials were similar and consistent.

[b] Species of *Phytophthora*: *P. cin = P. cinnamomi, P. cit = P. citrophthora, P. cry = P. cryptogea, P. nic = P. nicotianae, P. pal = P. palmivora*).

TABLE 2.3. Activity of zoospores of five species of *Phytophthora* in aqueous solution when one of six aquatic plant species is present (Plant + *Phyt*) or absent (*Phyt* only) during a 14-day exposure period for trials conducted each year over a 3-year period[a]

Plant species	*Phytophthora* species[b]	2016[c]			2017[c]			2018[c]		
		Plant+*Phyt*	*Phyt*	*P* > |t|	Plant+*Phyt*	*Phyt*	*P* > |t|	Plant+*Phyt*	*Phyt*	*P* > |t|
Agrostis alba	*P. cinnamomi*	93.7	87.5	0.513	98.3	95.0	0.734	-	-	-
	P. citrophthora	77.9	74.2	0.694	44.6	98.8	**<0.001**	-	-	-
	P. cryptogea	75.0	87.1	0.209	37.9	100.0	**<0.001**	-	-	-
	P. nicotianae	74.2	99.2	**0.011**	15.4	100.0	**<0.001**	-	-	-
	P. palmivora	92.5	100.0	0.433	10.0	99.2	**<0.001**	-	-	-
Carex stricta	*P. cinnamomi*	89.6	87.5	0.782	96.3	87.9	0.52	79.6	100.0	**0.011**
	P. citrophthora	42.5	74.2	**<0.001**	34.6	99.2	**<0.001**	-	-	-
	P. cryptogea	44.2	87.1	**<0.001**	51.7	87.9	**0.007**	92.9	100.0	0.344
	P. nicotianae	37.1	99.2	**<0.001**	43.3	95.8	**<0.001**	-	-	-

	P. palmivora	55.0	100.0	**<0.001**	45.8	97.1	**<0.001**	-	-	-
Iris ensata 'Rising Sun'	*P. cinnamomi*	81.7	66.7	0.079	93.8	87.9	0.383	-	-	-
	P. citrophthora	24.6	76.3	**<0.001**	10.0	99.2	**<0.001**	-	-	-
	P. cryptogea	1.7	82.5	**<0.001**	10.8	87.9	**0.007**	-	-	-
	P. nicotianae	3.3	89.4	**<0.001**	8.3	95.8	**<0.001**	-	-	-
	P. palmivora	2.1	90.8	**<0.001**	17.1	97.1	**<0.001**	-	-	-
Panicum virgatum	*P. cinnamomi*	55.4	95.4	**<0.001**	95.8	95.0	0.956	-	-	-
	P. citrophthora	27.1	73.8	**<0.001**	44.2	98.8	**<0.001**	-	-	-
	P. cryptogea	28.3	64.6	**<0.001**	45.8	100.0	**<0.001**	-	-	-
	P. nicotianae	29.2	92.9	**<0.001**	65.0	100.0	**0.023**	0.0	96.7	**<0.001**
	P. palmivora	35.4	99.2	**<0.001**	45.0	99.2	**<0.001**	-	-	-
Pontederia cordata	*P. cinnamomi*	75.4	97.9	**<0.001**	83.8	100.0	**<0.001**	-	-	-
	P. citrophthora	0.0	96.3	**<0.001**	0.4	100.0	**<0.001**	-	-	-
	P. cryptogea	0.8	99.2	**<0.001**	0.0	94.6	**<0.001**	-	-	-

	P. nicotianae	0.0	94.6	**<0.001**	0.0	100.0	**<0.001**	-	-	-
	P. palmivora	0.8	96.3	**<0.001**	0.4	100.0	**<0.001**	-	-	-
	P. cinnamomi	81.3	97.9	**<0.001**	94.2	100.0	0.267	87.5	100.0	**0.018**
	P. citrophthora	2.1	96.3	**<0.001**	27.9	100.0	**<0.001**	-	-	-
Typha latifolia	*P. cryptogea*	5.8	99.2	**<0.001**	32.1	94.6	**<0.001**	24.6	100.0	**<0.001**
	P. nicotianae	1.7	94.6	**<0.001**	27.9	100.0	**<0.001**	26.7	96.7	**<0.001**
	P. palmivora	0.4	96.3	**<0.001**	39.6	100.0	**<0.001**	-	-	-

[a] Two to three trials were conducted for each plant-pathogen combination over the 3-year period with six replicates used for each treatment in each trial.

[b] Data are the mean percentages of leaf disks in a container that were colonized by a species of *Phytophthora*.

[c] Data were analyzed by analysis of variance with block as a random effect and repeated measures over time ($\alpha = 0.05$), and the two treatments in each plant-pathogen combination were compared by Student's t-test. Bold values represent significant differences between the two treatments.

TABLE 2.4. Minimum, optimum, and maximum temperature ranges for the five species of *Phytophthora* used in this study (modified from Erwin and Ribeiro 1996)

Species	Minimum (°C)	Optimum (°C)	Maximum (°C)
P. cinnamomi	4 - 5	24 - 28	32 - 36
P. citrophthora	<5	24 - 28	32 - 33
P. cryptogea	<1	22 - 25	31 - 33
P. nicotianae	5 - 7	27 - 32	37
P. palmivora	11	28 - 30	35

Figures

FIGURE 2.1. Colonization of leaf disks that were floating in containers by five species of *Phytophthora* with (Plant+*Phytophthora*, left) or without (*Phytophthora* only, right) one of six species of aquatic plants for 13 days. Containers contained nutrient-amended Milli-Q water (EMD Millipore Corporation, Billerica, MA) artificially infested with a single species of *Phytophthora*, and leaf disks were replaced every 3 days. Data are means ± standard errors (n = 6) from trials conducted in 2016.

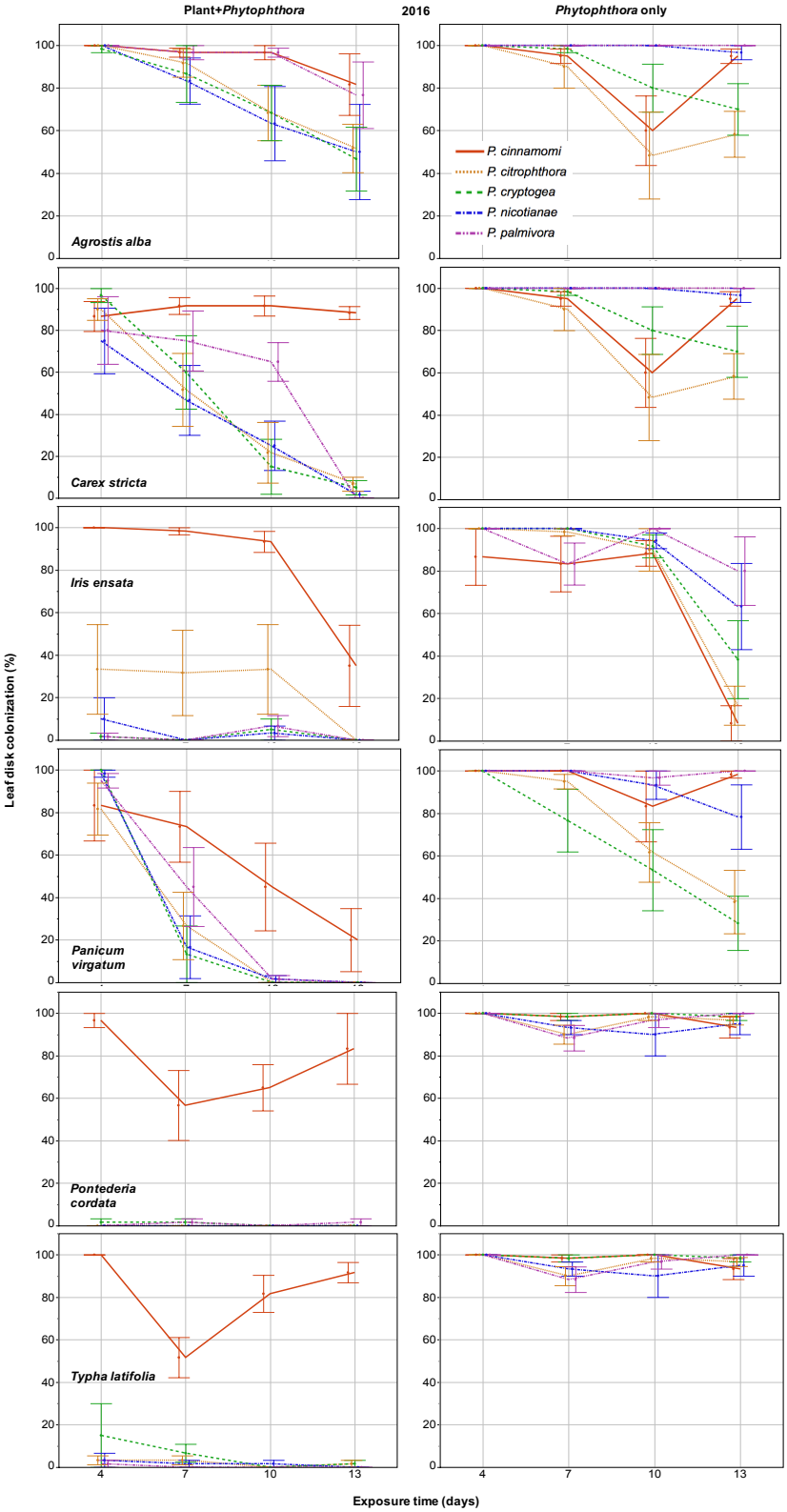

Plant+Phytophthora
2016
Phytophthora only
P. cinnamomi
P. citrophthora
P. cryptogea
P. nicotianae
P. palmivora
Agrostis alba
Carex stricta
Iris ensata
Panicum virgatum
Pontederia cordata
Typha latifolia
Leaf disk colonization (%)
Exposure time (days)

FIGURE 2.2. Colonization of leaf disks that were floating in containers by five species of *Phytophthora* with (Plant+*Phytophthora*, left) or without (*Phytophthora* only, right) one of six species of aquatic plants for 13 days. Containers contained nutrient-amended Milli-Q water (EMD Millipore Corporation, Billerica, MA) artificially infested with a single species of *Phytophthora*, and leaf disks were replaced every 3 days. Data are means ± standard errors (n = 6) from trials conducted in 2017.

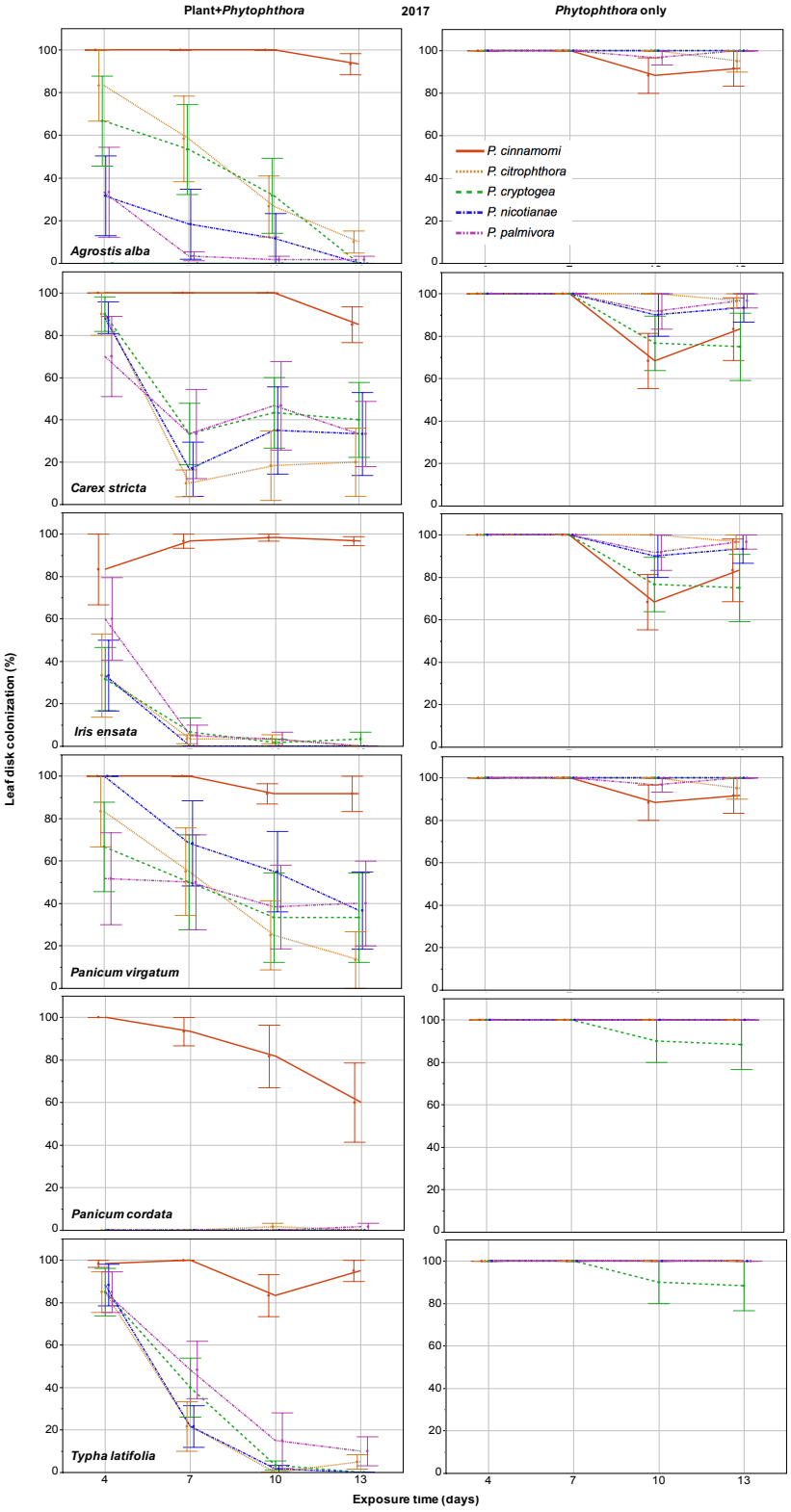

Plant+Phytophthora
2017
Phytophthora only
P. cinnamomi
P. citrophthora
P. cryptogea
P. nicotianae
P. palmivora
Agrostis alba
Carex stricta
Iris ensata
Panicum virgatum
Panicum cordata
Typha latifolia
Leaf disk colonization (%)
Exposure time (days)

FIGURE 2.3. Colonization of leaf disks that were floating in containers by three species of *Phytophthora* with (Plant+*Phytophthora*, left) or without (*Phytophthora* only, right) one of three species of aquatic plants for 13 days. Containers contained nutrient-amended Milli-Q water (EMD Millipore Corporation, Billerica, MA) artificially infested with a single species of *Phytophthora*, and leaf disks were replaced every 3 days. Data are means ± standard errors (n = 6) from trials conducted in 2018.

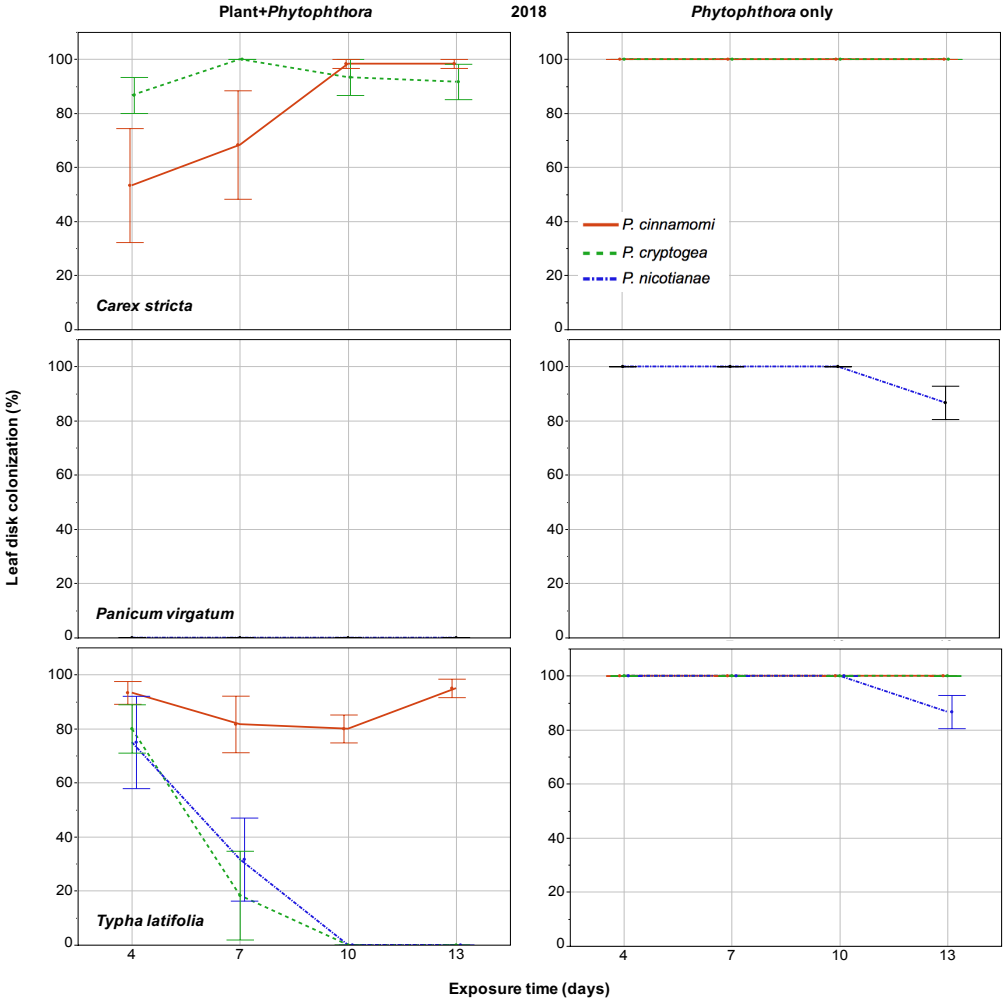


FIGURE 2.4. Colonization of leaf disks that were floating in containers during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial. On Day 14, plants were removed from containers, thoroughly rinsed under running water, dipped in a disinfestant solution (KleenGrow) for 1 min to eliminate potential propagules on root surfaces, and then thoroughly rinsed under running water again. Plants were then placed in new 2.1-liter plastic containers containing nutrient-amended Milli-Q water (EMD Millipore Corporation, Billerica, MA) and no inocula on Day 19, during which time leaf disks were replaced every 3 days. Data are means ± standard errors (n=6) from trials conducted in 2016 (left), 2017 (center), and 2018 (right).

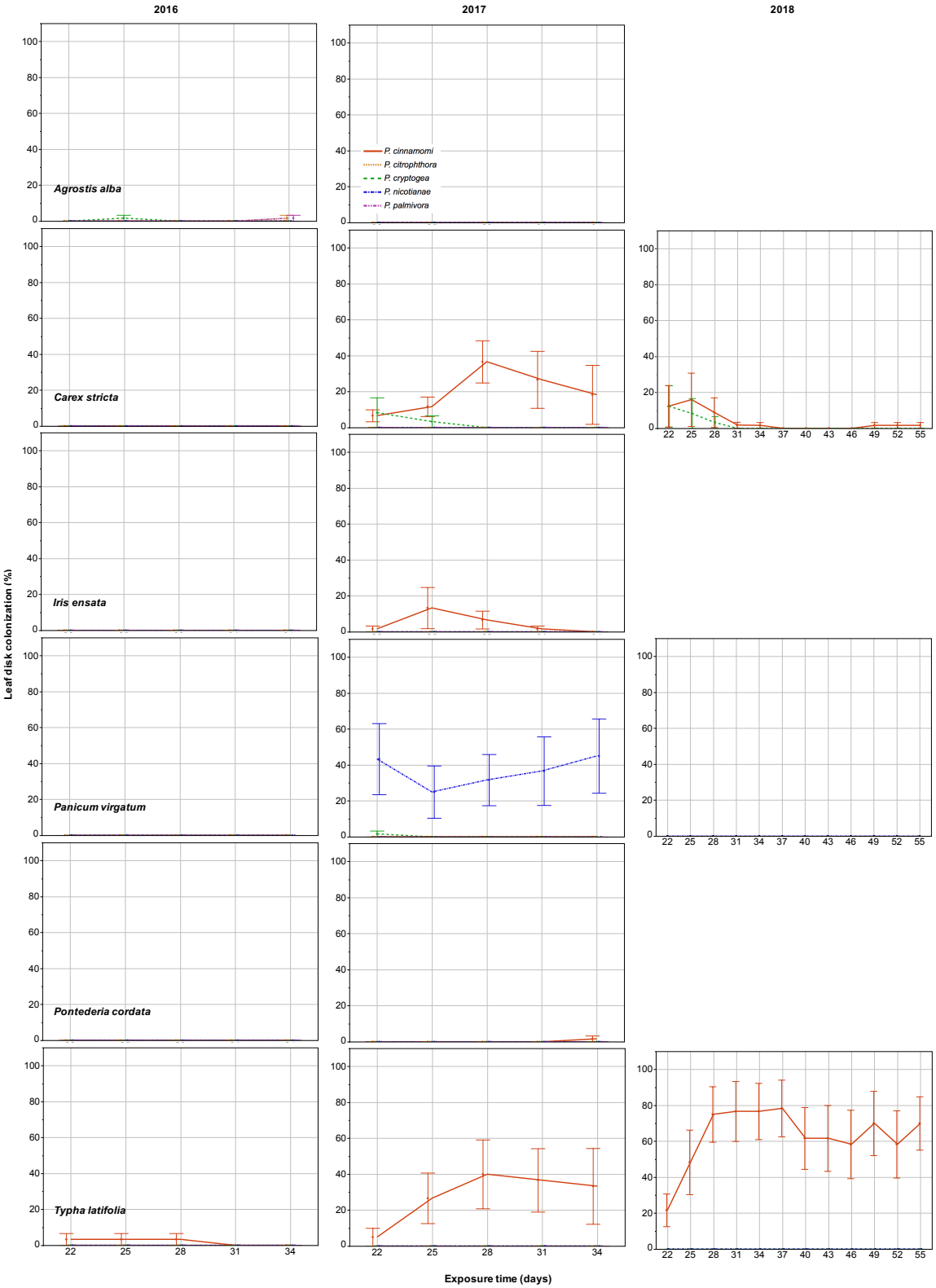
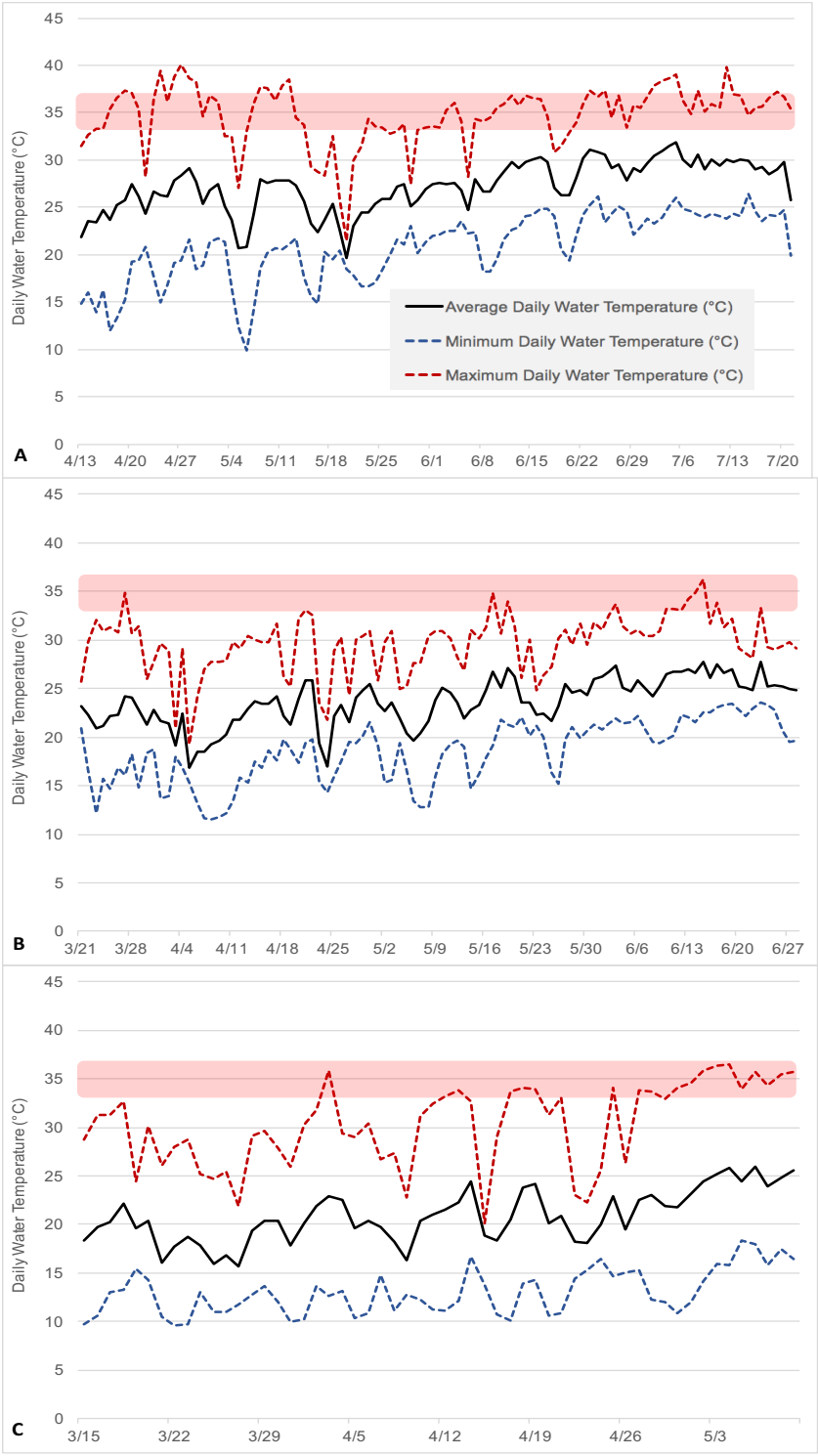

2016
2017
2018
P. cinnamomi
P. citrophthora
P. cryptogea
P. nicotianae
P. palmivora
Agrostis alba
Carex stricta
Iris ensata
Panicum virgatum
Pontederia cordata
Typha latifolia
Leaf disk colonization (%)
Exposure time (days)

FIGURE 2.5. Daily average, minimum, and maximum water temperatures during three trials, each conducted in a different year, to evaluate the potential pathogenicity of six aquatic plant species to five species of *Phytophthora (P. cinnamomi, P. citrophthora, P. cryptogea, P. nicotianae,* and *P. palmivora)*: **A**, 2016; **B**, 2017; and **C**, 2018. Semi-transparent red boxes on each graph represent reported maximum survivable temperatures by these five species of *Phytophthora*.


Daily Water Temperature (°C)
45
40
35
30
25
20
15
10
5
0
Average Daily Water Temperature (°C)
Minimum Daily Water Temperature (°C)
Maximum Daily Water Temperature (°C)
A
4/13 4/20 4/27 5/4 5/11 5/18 5/25 6/1 6/8 6/15 6/22 6/29 7/6 7/13 7/20
B
3/21 3/28 4/4 4/11 4/18 4/25 5/2 5/9 5/16 5/23 5/30 6/6 6/13 6/20 6/27
C
3/15 3/22 3/29 4/5 4/12 4/19 4/26 5/3

Supplementary Tables

TABLE S2.1. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Agrostis alba* (*Phytophthora*+Plant) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Agrostis alba *(Phytophthora*+Plant*)*	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	93.7 a	…	98.3 a	…
P. citrophthora	77.9 a	…	44.6 b	…
P. cryptogea	75.0 a	…	37.9 b	…
P. nicotianae	74.2 a	…	15.4 bc	…

P. palmivora	92.5 a	…	10.0 bc	…
None	0.0 b	…	0.0 c	…
HSD	31.1	…	36.9	…
Time (days)				
4	83.1 a	…	52.5 a	…
7	75.8 ab	…	38.9 ab	…
10	65.6 b	…	28.6 bc	…
13	51.1 c	…	17.5 c	…
HSD	11.6	…	13.7	…
ANOVA (N = 144)				
Phytophthora spp. (df = 5)	…	**<0.001**	…	**<0.001**
Time (df = 3)	…	**<0.001**	…	**<0.001**
Phytophthora spp. X time	…	0.062	…	**0.005**

TABLE S2.2. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Carex stricta* (*Phytophthora*+Plant) during a 14-day exposure period for trials conducted each year over a 2-year [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Carex stricta *(Phytophthora*+Plant*)*	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	89.6 a	…	96.3 a	…	79.6 a	…
P. citrophthora	42.5 b	…	34.6 bc	…	-	…
P. cryptogea	44.2 b	…	51.7 ab	…	92.9 a	…
P. nicotianae	37.1 b	…	43.3 bc	…	-	…
P. palmivora	55.0 b	…	45.8 b	…	-	…

None	0.0 c	…	0.0 c	…	0.0 b	…
HSD	21.1	…	46.5	…	21.3	…
Time (days)						
4	71.4 a	…	73.1 a	…	46.7 a	…
7	54.2 b	…	40.6 b	…	56.1 a	…
10	36.4 c	…	35.3 b	…	63.9 a	…
13	16.9 d	…	32.3 b	…	63.3 a	…
HSD	15.0	…	12.7	…	17.3	…
ANOVA (N = 144, 2016 and 2017, N = 72, 2018)						
Phytophthora spp. (df = 5, 2016 and 2017, df = 2, 2018)	…	**<0.001**	…	**<0.001**	…	**<0.001**
Time (df = 3)	…	**<0.001**	…	**<0.001**	…	**0.038**

Phytophthora spp. X time	…	**<0.001**	…	**<0.001**	…	**0.024**

TABLE S2.3. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Iris ensata* (*Phytophthora*+Plant) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

***Iris ensata* 'Rising Sun'** *(Phytophthora*+Plant*)*	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	81.7 a	…	93.8 a	…
P. citrophthora	24.6 b	…	10.0 b	…
P. cryptogea	1.7 bc	…	10.8 b	…
P. nicotianae	3.3 bc	…	8.3 b	…
P. palmivora	2.1 bc	…	17.1 b	…
None	0.0 c	…	0.0 b	…

HSD	24.3	…	18.6	…
Time (days)				
4	24.4 a	…	40.3 a	…
7	21.7 a	…	18.6 b	…
10	23.6 a	…	17.8 b	…
13	5.8 b	…	16.7 b	…
HSD	11.5	…	12.4	…
ANOVA (N = 144)				
Phytophthora spp. (df = 5)	…	**<0.001**	…	**<0.001**
Time (df = 3)	…	**<0.001**	…	**<0.001**
Phytophthora spp. X time	…	**0.001**	…	**0.005**

TABLE S2.4. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Panicum virgatum* (*Phytophthora*+Plant) during a 14-day exposure period for trials conducted each year over a 2-year period [data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Panicum virgatum *(Phytophthora*+Plant*)*	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	55.4 a	…	95.8 a	…	-	…
P. citrophthora	27.1 b	…	44.2 ab	…	-	…
P. cryptogea	28.3 b	…	45.8 ab	…	-	…
P. nicotianae	29.2 b	…	65.0 a	…	0.0	…
P. palmivora	35.4 ab	…	45.0 ab	…	-	…

None	0.0 c	…	0.0 b	…	0.0	…
HSD	20.2	…	58.3	…	ns	…
Time (days)						
4	76.4 a	…	66.9 a	…	0.0	…
7	29.2 b	…	53.9 ab	…	0.0	…
10	8.1 c	…	40.6 bc	…	0.0	…
13	3.3 c	…	35.8 c	…	0.0	…
HSD	15.2	…	13.9	…	ns	…
ANOVA (N=144, 2016 and 2017, N = 48, 2018)						
Phytophthora spp. (df = 5, 2016 and 2017, df = 1, 2018)	…	**<0.001**	…	**0.001**	…	…
Time (df = 3)	…	**<0.001**	…	**<0.001**	…	…
Phytophthora spp. X time	…	**<0.001**	…	**0.023**	…	…

TABLE S2.5. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Pontederia cordata* (*Phytophthora*+Plant) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Pontederia cordata *(Phytophthora*+Plant*)*	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	75.4 a	…	83.8 a	…
P. citrophthora	0.0 b	…	0.4 b	…
P. cryptogea	0.8 b	…	0.0 b	…
P. nicotianae	0.0 b	…	0.0 b	…
P. palmivora	0.8 b	…	0.4 b	…

None	0.0 b	…	0.0 b	…
HSD	17.8	…	15.1	…
Time (days)				
4	16.4 a	…	16.7 a	…
7	10.0 b	…	15.6 ab	…
10	10.8 ab	…	13.9 ab	…
13	14.2 ab	…	10.3 b	…
HSD	5.9	…	6.4	…
ANOVA (N = 144)				
Phytophthora spp. (df = 5)	…	**<0.001**	…	**<0.001**
Time (df = 3)	…	**0.020**	…	0.054
Phytophthora spp. X time	…	**<0.001**	…	**<0.001**

TABLE S2.6. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Typha latifolia* (*Phytophthora*+Plant) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Typha latifolia *(Phytophthora*+Plant*)*	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	81.3 a	…	94.2 a	…	87.5 a	…
P. citrophthora	2.1 b	…	27.9 b	…	-	…
P. cryptogea	5.8 b	…	32.1 b	…	24.6 b	…
P. nicotianae	1.7 b	…	27.9 b	…	26.7 b	…
P. palmivora	0.4 b	…	39.6 b	…	-	…

None	0.0 b	…	0.0 c	…	0.0 c	…
HSD	11.8	…	18.0	…	16.8	…
Time (days)						
4	20.6 a	…	73.6 a	…	62.1 a	…
7	10.6 b	…	38.6 b	…	32.9 b	…
10	13.9 b	…	17.2 c	…	20.0 b	…
13	15.8 ab	…	18.3 c	…	23.8 b	…
HSD	6.0	…	11.0	…	14.8	…
ANOVA (N = 144, 2016 and 2017, N = 96, 2018)						
Phytophthora spp. (df = 5, 2016 and 2017, df = 3, 2018)	…	**<0.001**	…	**<0.001**	…	**<0.001**
Time (df = 3)	…	**<0.001**	…	**<0.001**	…	**<0.001**
Phytophthora spp. X time	…	**<0.001**	…	**<0.001**	…	**<0.001**

TABLE S2.7. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the absence of *Agrostis alba* (*Phytophthora* only) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Agrostis alba (*Phytophthora* only)	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	87.5 ab	…	100.0	…
P. citrophthora	74.2 b	…	100.0	…
P. cryptogea	87.1 ab	…	99.2	…
P. nicotianae	99.2 a	…	98.8	…
P. palmivora	100.0 a	…	95.0	…
HSD	21.6	…	ns	…

Time (days)				
4	100.0 a	…	100.0	…
7	96.7 a	…	100.0	…
10	84.0 b	…	97.3	…
13	77.7 b	…	97.0	…
HSD	11.1	…	ns	…
ANOVA (N = 120)				
Phytophthora spp. (df = 4)	…	**0.011**	…	0.066
Time (df = 3)	…	**<0.001**	…	0.240
Phytophthora spp. X time	…	**<0.001**	…	0.632

TABLE S2.8. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the absence of *Carex stricta* (*Phytophthora* only) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Carex stricta (*Phytophthora* only)	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	87.5 ab	…	87.9	…	100.0	…
P. citrophthora	74.2 b	…	99.2	…	-	…
P. cryptogea	87.1 ab	…	87.9	…	100.0	…
P. nicotianae	99.2 a	…	95.8	…	-	…
P. palmivora	100.0 a	…	97.1	…	-	…
HSD	21.6	…	ns	…	ns	…

Time (days)						
4	100.0 a	…	100.0 a	…	100.0	…
7	96.7 a	…	100.0 a	…	100.0	…
10	77.7 b	…	85.3 b	…	100.0	…
13	84.0 b	…	89.0 b	…	100.0	…
HSD	11.1	…	10.6	…	ns	…
ANOVA (N = 120)						
Phytophthora spp. (df = 4, 2016 and 2017, df = 1, 2018)	…	**0.011**	…	0.287	…	…
Time (df = 3)	…	**<0.001**	…	**<0.001**	…	…
Phytophthora spp. X time	…	**<0.001**	…	0.352	…	…

TABLE S2.9. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the absence of *Iris ensata* 'Rising Sun' (*Phytophthora* only) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

***Iris ensata* 'Rising Sun'** (*Phytophthora* only)	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	66.7 b	…	87.9	…
P. citrophthora	76.3 ab	…	99.2	…
P. cryptogea	82.5 ab	…	87.9	…
P. nicotianae	89.4 ab	…	95.8	…
P. palmivora	90.8 a	…	97.1	…

HSD	22.8	…	ns	…
Time (days)				
4	97.3 a	…	100.0 a	…
7	93.0 a	…	100.0 a	…
10	92.9 a	…	85.3 b	…
13	41.3 b	…	89.0 b	…
HSD	14.8	…	10.6	…
ANOVA (N = 120)				
Phytophthora spp. (df = 4)	…	**0.026**	…	0.287
Time (df = 1)	…	**<0.001**	…	**<0.001**
Phytophthora spp. X time	…	**0.004**	…	0.352

TABLE S2.10. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the absence of *Panicum virgatum* (*Phytophthora* only) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Panicum virgatum (*Phytophthora* only)	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	95.4 ab	…	95.0	…	-	…
P. citrophthora	73.8 bc	…	98.8	…	-	…
P. cryptogea	64.6 c	…	100.0	…	-	…
P. nicotianae	92.9 ab	…	100.0	…	96.7	…
P. palmivora	99.2 a	…	99.2	…	-	…

HSD	24.0	…	ns	…	.	…
Time (days)						
4	100.0 a	…	100.0	…	100.0 a	…
7	94.4 a	…	100.0	…	100.0 a	…
10	77.6 b	…	97.0	…	100.0 a	…
13	68.7 b	…	97.3	…	86.7 b	…
HSD	13.8	…	ns	…	12.5	…
ANOVA (N=120, 2016 and 2017, N = 24, 2018)						
Phytophthora spp. (df = 4, 2016 and 2017, df = 1, 2018)	…	**<0.001**	…	0.066	…	…
Time (df = 3)	…	**<0.001**	…	0.240	…	**0.017**
Phytophthora spp. X time	…	**0.001**	…	0.632	…	…

TABLE S2.11. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the absence of *Pontederia cordata* (*Phytophthora* only) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Pontederia cordata (*Phytophthora* only)	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	97.9	…	100.0	…
P. citrophthora	96.3	…	100.0	…
P. cryptogea	99.2	…	94.6	…
P. nicotianae	94.6	…	100.0	…
P. palmivora	96.3	…	100.0	…

HSD	ns	…	ns	…
Time (days)				
4	100.0 a	…	100.0	…
7	93.7 b	…	100.0	…
10	97.0 ab	…	98.0	…
13	96.7 ab	…	97.7	…
HSD	4.9	…	ns	…
ANOVA (N = 120)				
Phytophthora spp. (df = 4)	…	0.704	…	0.426
Time (df = 3)	…	**0.013**	…	0.398
Phytophthora spp. X time	…	0.250	…	0.458

TABLE S2.12. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the absence of *Typha latifolia* (*Phytophthora* only) during a 14-day exposure period for trials conducted each year over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Typha latifolia (*Phytophthora* only)	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	97.9	…	100.0	…	100.0 a	…
P. citrophthora	96.3	…	100.0	…	-	…
P. cryptogea	99.2	…	94.6	…	100.0 a	…
P. nicotianae	94.6	…	100.0	…	96.7 b	…
P. palmivora	96.3	…	100.0	…	-	…
HSD	ns	…	ns	…	3.0	…

Time (days)						
4	100.0 a	…	100.0	…	100.0 a	…
7	93.7 b	…	100.0	…	100.0 a	…
10	97.0 ab	…	98.0	…	100.0 a	…
13	96.7 ab	…	97.7	…	95.6 b	…
HSD	4.9	…	ns	…	3.9	…
ANOVA (N = 120, 2016 and 2017, N = 72, 2018)						
Phytophthora spp. (df = 4, 2016 and 2017, df = 2, 2018)	…	0.704	…	0.426	…	**0.014**
Time (df = 3)	…	**0.013**	…	0.398	…	**0.006**
Phytophthora spp. X time	…	0.250	…	0.458	…	**<0.001**

TABLE S2.13. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Agrostis alba* (*Phytophthora*+Plant) during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Agrostis alba *(Phytophthora*+Plant*)*	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	0.0	…	0.0	…
P. citrophthora	0.3	…	0.0	…
P. cryptogea	0.3	…	0.0	…
P. nicotianae	0.0	…	0.0	…

P. palmivora	0.3	…	0.0	…
None	0.0	…	0.0	…
HSD	ns	…	ns	…
Time (days)				
22	0.0	…	0.0	…
25	0.4	…	0.0	…
28	0.0	…	0.0	…
31	0.0	…	0.0	…
34	0.6	…	0.0	…
HSD	ns	…	ns	…
ANOVA (N = 180)				
Phytophthora spp. (df = 5)	…	0.700	…	…
Time (df = 4)	…	0.262	…	…
Phytophthora spp. X time	…	0.547	…	…

TABLE S2.14. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Carex stricta* (*Phytophthora*+Plant) during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Carex stricta *(Phytophthora*+Plant*)*	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	0.0	…	20.0 a	…	3.8	…
P. citrophthora	0.0	…	0.0 b	…	-	…
P. cryptogea	0.0	…	2.3 b	…	2.0	…
P. nicotianae	0.0	…	0.0 b	…	-	…

P. palmivora	0.0	…	0.0 b	…	-	…
None	0.0	…	0.0 b	…	0.0	…
HSD	ns	…	16.1	…		…
Time (days)						
22	0.0	…	2.5	…	8.2 a	…
25	0.0	…	2.5	…	8.1 a	…
28	0.0	…	6.1	…	4.1 a	…
31	0.0	…	4.4	…	0.6 a	…
34	0.0	…	3.1	…	0.6 a	…
37	-	…	-	…	0.0 a	…
40	-	…	-	…	0.0 a	…
43	-	…	-	…	0.0 a	…
46	-	…	-	…	0.0 a	…
49	-	…	-	…	0.5 a	…
52	-	…	-	…	0.6 a	…
55	-	…	-	…	0.6 a	…
HSD	ns	…	ns	…	10.2 a	…

ANOVA (N=180, 2016 and 2017, N = 288, 2018)						
Phytophthora spp. (df = 5, 2016 and 2017, df = 2, 2018)	…	…	…	**0.003**	…	0.509
Time (df = 4, 2016 and 2017, df=11, 2018)	…	…	…	0.427	…	**0.026**
Phytophthora spp. X time	…	…	…	**0.018**	…	0.905

TABLE S2.15. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Iris ensata* 'Rising Sun' (*Phytophthora*+Plant) during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

***Iris ensata* 'Rising Sun'** *(Phytophthora*+Plant*)*	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	0.0	…	4.7	…
P. citrophthora	0.0	…	0.0	…
P. cryptogea	0.0	…	0.0	…
P. nicotianae	0.0	…	0.0	…

P. palmivora	0.0	…	0.0	…
None	0.0	…	0.0	…
HSD	ns	…	ns	…
Time (days)				
22	0.0	…	0.3	…
25	0.0	…	2.2	…
28	0.0	…	1.1	…
31	0.0	…	0.3	…
34	0.0	…	0.0	…
HSD	ns	…	ns	…
ANOVA (N = 180)				
Phytophthora spp. (df = 5)	…	…	…	0.154
Time (df = 4)	…	…	…	0.314
Phytophthora spp. X time	…	…	…	0.264

TABLE S2.16. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Panicum virgatum* (*Phytophthora*+Plant) during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Panicum virgatum *(Phytophthora*+Plant*)*	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	0.0	…	0.0 b	…	0.0	…
P. citrophthora	0.0	…	0.0 b	…	0.0	…
P. cryptogea	0.0	…	0.3 b	…	0.0	…
P. nicotianae	0.0	…	36.3 a	…	0.0	…

P. palmivora	0.0	…	0.0 b	…	0.0	…
None	0.0	…	0.0 b	…	0.0	…
HSD	ns	…	25.9	…	ns	…
Time (days)						
22	0.0	…	7.5	…	0.0	…
25	0.0	…	4.2	…	0.0	…
28	0.0	…	5.3	…	0.0	…
31	0.0	…	6.1	…	0.0	…
34	0.0	…	7.5	…	0.0	…
37	-	…	-	…	-	…
40	-	…	-	…	-	…
43	-	…	-	…	-	…
46	-	…	-	…	-	…
49	-	…	-	…	-	…
52	-	…	-	…	-	…
55	-	…	-	…	-	…
HSD	ns	…	ns	…	ns	…

ANOVA (N=180, 2016 and 2017, N = 288, 2018)						
Phytophthora spp. (df = 5, 2016 and 2017, df = 1, 2018)	…	…	…	**<0.001**	…	…
Time (df = 4, 2016 and 2017, df=11, 2018)	…	…	…	0.671	…	…
Phytophthora spp. X time	…	…	…	0.946	…	…

TABLE S2.17. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Pontederia cordata* (*Phytophthora*+Plant) during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Pontederia cordata *(Phytophthora*+Plant*)*	2016		2017	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.				
P. cinnamomi	0.0	…	0.3	…
P. citrophthora	0.0	…	0.0	…
P. cryptogea	0.0	…	0.0	…
P. nicotianae	0.0	…	0.0	…

P. palmivora	0.0	…	0.0	…
None	0.0	…	0.0	…
HSD	ns	…	ns	…
Time (days)				
22	0.0	…	0.0	…
25	0.0	…	0.0	…
28	0.0	…	0.0	…
31	0.0	…	0.0	…
34	0.0	…	0.3	…
HSD	ns	…	ns	…
ANOVA (N = 180)				
Phytophthora spp. (df = 5)	…	…	…	0.421
Time (df = 4)	…	…	…	0.410
Phytophthora spp. X time	…	…	…	0.468

TABLE S2.18. Differences in the activity of zoospores of five species of *Phytophthora* in aqueous solution in the presence of *Typha latifolia* (*Phytophthora*+Plant) during the non-exposure period (the time period of each trial when inocula were not present in containers) by five species of *Phytophthora* that were present during the first 14 days of each trial over a 2-year period [(data are means, those that share a letter in common are not significantly different based upon Tukey's honest significant difference, with α= 0.05; bold values represent significant differences among treatments in the analysis of variance (ANOVA)].

Typha latifolia *(Phytophthora*+Plant*)*	2016		2017		2018	
Factor, Level	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)	Leaf disk colonization (%)	Two-way ANOVA (P>F)
Phytophthora spp.						
P. cinnamomi	2.0	…	28.3 a	…	63.1 a	…
P. citrophthora	0.0	…	0.0 b	…	-	…
P. cryptogea	0.0	…	0.0 b	…	0.0 b	…
P. nicotianae	0.0	…	0.0 b	…	0.0 b	…

P. palmivora	0.0	…	0.0 b	…	-	…
None	0.0	…	0.0 b	…	0.0 b	…
HSD	ns	…	21.2	…	26.3	…
Time (days)						
22	0.6	…	0.8	…	5.4 b	…
25	0.6	…	4.4	…	12.1 ab	…
28	0.6	…	6.7	…	18.8 a	…
31	0.0	…	6.1	…	19.2 a	…
34	0.0	…	5.6	…	19.2 a	…
37	-	…	-	…	19.6 a	…
40	-	…	-	…	15.4 ab	…
43	-	…	-	…	15.4 ab	…
46	-	…	-	…	14.6 ab	…
49	-	…	-	…	17.5 a	…
52	-	…	-	…	14.6 ab	…
55	-	…	-	…	17.5 a	…
HSD	ns	…	ns	…	12.0	…

ANOVA (N = 180, 2016 and 2017, N = 288, 2018)						
Phytophthora spp. (df = 5, 2016 and 2017, df = 3, 2018)	…	0.435	…	**0.001**	…	**<0.001**
Time (df = 4, 2016 and 2017, df = 11, 2018)	…	0.410	…	0.288	…	**0.008**
Phytophthora spp. X time	…	0.468	…	0.217	…	**<0.001**

CHAPTER 3: A CONTROLLED MODEL SYSTEM TO EVALUATE THE POTENTIAL OF FLOATING TREATMENT WETLANDS TO MANAGE *PHYTOPHTHORA* SPECIES IN IRRIGATION RUNOFF WATER

Abstract

Increased incidences and severity of drought have reduced reliable access to freshwater sources for irrigation purposes by nursery and greenhouse plant producers. Many plant producers are now considering onsite remediation and reuse of water captured from irrigation runoff. However, potential contamination of recycled water with plant pathogens, primarily species of *Phytophthora*, is the primary concern preventing many growers from reusing their water. Floating treatment wetlands (FTWs) consist of plants established on a buoyant structure that floats on the surface of a water body with roots extended down into the water column. FTWs effectively remediate mineral nutrients in agricultural runoff, but little is known about their potential to manage plant pathogens. Therefore, our objective was to investigate the potential efficacy of FTWs to manage *Phytophthora* species in irrigation runoff. The research was conducted using a controlled model system that consisted of 3-m-long plastic troughs that each contained one of four treatments: no FTWs (i.e., an empty trough), FTWs without plants, FTWs with *Agrostis alba* plants, or FTWs with *Pontederia cordata* plants. Water continuously flowed through each trough at a calculated hydraulic retention time (HRT) of 1 or 4 h. A standard density of zoospores of *P. nicotianae* in aqueous suspension was added to influent water entering each trough, and zoospore activity in effluent water leaving each trough at predetermined time intervals was monitored with a baiting bioassay. Results

from this study demonstrated that controlled model FTWs containing *P. cordata* plants have the potential to reduce the movement of viable *P. nicotianae* zoospores through a channel of water at a target HRT of approximately 4 h. Movement of viable zoospores through the troughs was not reduced at the higher flowrate of 1 h HRT or for FTWs containing *A. alba* plants. The mechanism by which FTWs containing *P. cordata* plants reduced zoospore activity is not known; however, it may be due to interception of zoospores by plant roots, which may involve interactions with plant root exudates or the microbiome associated with the roots. This work is one of the first studies to evaluate the potential efficacy of floating treatment wetlands to manage plant pathogens.

Introduction

Increasing global population, climate change, and increasing instances and severity of drought have vastly increased demand for the finite supply of freshwater resources on earth (Graffy, 2007; Hess et al., 2016; Pink, 2016). Agriculture accounts for approximately 80% of the consumptive water use in the United States (USDA, 2018) and is one of the leading economic sectors affected by droughts (Falkenmark, 2013; Kumar and Panu, 1997). State and regional economies can be devastated by extended periods of drought. Understandably, reliable access to freshwater sources for irrigation purposes is a high priority for plant producers, particularly within the nursery and greenhouse industries. Many greenhouse and nursery producers are considering recycling irrigation runoff, after onsite treatment to facilitate its safe reuse (White et al., 2013). However, potential contamination of recycled water with plant pathogens, primarily species of

Phytophthora, is one major concern that prevents some growers from reusing runoff water for irrigation (White et al., 2013).

Species of *Phytophthora* cause some of the most economically important diseases of nursery and greenhouse crops worldwide (Hong et al., 2014; Hwang and Benson, 2005; Jones and Benson, 2001; Leonberger et al., 2013). *Phytophthora* spp. are capable of infecting thousands of host plants—including field, forage, fruit, ornamental, and vegetable crops as well as trees and shrubs in natural ecosystems (Erwin and Ribeiro, 1996). *Phytophthora* spp. produce motile, swimming zoospores that often serve as propagules of dispersal and often are the primary infective propagules that initial infections on many plants (Erwin and Ribeiro, 1996; Schumann and D'Arcy, 2010). While many chemical and physical treatment methods are currently used to disinfest recycled irrigation water, drawbacks of these technologies include sensitivity to solids and water pH (which render them ineffective if not properly managed by growers), potential for formation of harmful byproducts, potential for technical malfunctions or breakdowns that may require maintenance by technical specialists, and inability to process large quantities of water (Hong and Moorman, 2005; Steward-Wade, 2011). Biological methods for managing *Phytophthora* spp. in waterways, including biofilters and constructed wetlands, are not as widely implemented and are not well understood (Hong and Moorman, 2005; Hong et al., 2014; Majsztrik et al., 2017; Steward-Wade, 2011; Vymazal, 2011).

Constructed wetlands, which allow surface or subsurface flow-through of irrigation runoff, effectively remediate nutrients, particularly nitrogen, in irrigation runoff

(Taylor et al., 2006; White, 2013; White, 2018; White and Cousins, 2013). One such type of constructed wetland, floating treatment wetlands (FTWs), consist of plants established on a buoyant structure that floats on the surface of a water body with plant roots extended down into the water column below the buoyant structure. These roots with their established microbiome serve as natural filters by absorbing and processing nutrients and other pollutants, slowing the flowrate of water through the system, and enhancing the settling of suspended solids (Khan et al., 2013, Tanner and Headley, 2011). Since FTWs can be readily established within existing ponds and channels, they may be a more applicable best management practice for nursery and greenhouse plant producers than are traditional constructed wetland systems. However, little is known about the potential of FTWs to manage plant pathogens in water. Therefore, the objective of this was study was to evaluate the potential of FTWs to reduce the number of propagules of *Phytophthora* species in irrigation runoff using a controlled model system.

Materials and methods

Experimental layout and operation

The experiment was conducted within an outdoor controlled FTWs model system located in the Water Treatment Technology Laboratory at the South Carolina Water Resources Center of Clemson University in Pendleton, SC, USA (34°38'N, 82°46'W). During each trial, pond water amended with 6.3 ± 1.5 (mean ± standard error) mg/L N from a 24-8-16 (N-P-K) water-soluble fertilizer (Southern Agricultural Insecticides, Inc., Hendersonville, NC) was continuously pumped from three 7,900-L tanks into one

proximal end (head) of each of nine plastic LDPE troughs (Priefert, Mount Pleasant, TX, USA) measuring 3 m × 0.6 m × 0.2 m (Fig. 3.1). Each trough was randomly assigned one of three treatments (n = 3, for each treatment): FTWs with no plants, FTWs with *Agrostis alba* plants, and FTWs with *Pontederia cordata* plants. These plant species were selected for use in this controlled model FTWs system because they do not appear to be susceptible to several different species of *Phytophthora (*including *P. nicotianae)*, based on results in a prior study (see Chapter 2). Each trough containing a FTWs, consisted of three approximately 0.6 m × 0.6 m × 1.3 cm polyvinyl chloride flexible plastic floating mats in series, with each mat containing 12 polypropylene plastic 7.6-cm-diameter aerator plant containers (for a total of 36 plants in each trough that contained either *Agrostis alba* (redtop bentgrass) or *Pontederia cordata* (pickerelweed) plants. Polypropylene plastic baffles (30.5 × 22 cm) were installed along the sidewall of each trough on either side of each plastic floating mat, for a total of six baffles per trough, to direct flow toward the FTWs and prevent preferential flow along sidewalls.

Nutrient-amended water continuously flowed through each trough at a calculated target hydraulic retention time (HRT) of 1 or 4 h (herein referred to as high and low flowrate, respectively) during each trial, which lasted either 4 h (four complete cycles of flow-through for 1 h target HRT trial) or 8 h (two complete cycles of flow-through for 4 h target HRT trial). These relatively low HRTs (quick flowrates) were chosen to reflect typical flow conditions in channels receiving agricultural runoff (Dollinger et al., 2015; Knox et al., 2007; Moore et al., 2011). A suspension of *P. nicotianae* zoospores (see below) was introduced to the proximal end of each trough at the beginning of each trial

after one complete flow-through cycle of nutrient-amended water had been pumped through the FTWs system. Concentrations of *Phytophthora* spp. inoculum recovered from nursery and greenhouse runoff collection reservoirs have been reported within the range of 0 to 10^1 zoospores/mL; however, concentrations are typically less than 1 zoospore/mL (Bush et al., 2003; Kong et al., 2003; Loyd et al., 2014; Stewart-Wade, 2011). During exploratory preliminary trials, enough zoospore suspension was introduced to each trough to bring the total zoospore concentration within the trough to approximately 11 to 57 zoospores/mL; however, these extremely high concentrations of zoospores appeared to overload the model FTWs system, resulting in continuous release of high concentrations of zoospores in the effluent from each trough (these data did not differ by FTWs type, and are presented in Supplementary Table S3.1). Therefore, a target concentration of 5.7 zoospores/mL was used throughout the experiment. To achieve this, 3 L of zoospore suspension (500 zoospores/mL) was introduced into each trough for each trial. One trial was completed each week for six consecutive weeks (three randomly selected trials at low flowrates, three randomly selected trials at high flowrates) from Aug. 24 to Sept. 28, 2018, for a total of six trials (n = 9 per FTWs treatment type).

Production of inocula

An isolate of *Phytophthora nicotianae* (isolate no. 05-0690; originally recovered in 2005 from the stem of a *Hibiscus paramutabilis* ×*syriacus 'Lohengrin'* plant from a nursery in South Carolina), which was maintained in a permanent collection in the laboratory of Dr. S. N. Jeffers at Clemson University, was used in this study. This isolate

was characterized and identified in a previous study by Ridge et al. (2014). An active culture of the isolate was maintained in 10-cm-diameter disposable petri dishes containing PARPH-V8, a medium selective for species of *Phytophthora* (Jeffers 2015b), at 15°C in the dark). Before the start of each experimental run, cultures were transferred onto 10% clarified V8 agar (cV8A; Jeffers, 2015c) in 10-cm-diameter disposable petri dishes and incubated at 20°C in the dark for 3 days. Concentrated suspension of zoospores was produced from mycelium mats using a procedure reported previously (Drechsler et al., 2014; Nyberg et al., 2014). The concentrations of zoospores in suspensions prepared throughout this experiment were quantified using a hemacytometer and ranged from 1.2 to 4.0 x 10^5 zoospores/mL. This concentrated suspension was diluted to prepare a standard zoospore suspension with a concentration of 500 zoospores/mL. A fresh stock of zoospore suspension was prepared before each trial of this study. Detailed methods describing how large volumes (several hundred milliliters) of concentrated zoospore suspension for this experiment were created and are described in Appendix A.

Pathogen monitoring and analyses

Water samples of approximately 250 mL were collected across the width of each trough from just below the water surface from the proximal of the trough where nutrient-amended pond water continuously entered (influent) and from the distal end of the trough where water continuously exited (effluent). Samples were collected every 30 min during the trials at high flowrates (8 samples/trough), and every 1 h during trials at low flowrates (8 samples/trough). Samples were collected from the pond source water and from within

each trough before each trial began to confirm that viable zoospores were not present. Zoospore viability was evaluated using a standard leaf disk baiting bioassay described by Ridge et al. (2014). Ten 5-mm leaf disks were punched from leaves of a pesticide-free *Rhododendron maximum* plant and floated on the surface of each water sample. After 3 days, leaf disks were removed, blotted dry with paper towels, embedded in PARPH-V8 selective medium in a 10-cm-diameter petri dish, and held at 25°C in the dark for 3 days. Leaf disk perimeters were examined microscopically (20 to 70×) for characteristic hyphae of *P. nicotianae*. Activity of zoospores was quantified using a scale from 0 to 100% based on the number of leaf disks that appeared to be colonized out of 10 (Ridge et al., 2014). Zoospore activity was plotted over time to create a zoospore activity curve. Area under the zoospore activity curve (AUZAC) was calculated using the same method used to estimate the area under a disease progress curve (AUDPC) so comparisons of zoospore activities among treatments could be made (Madden et al., 2007):

$$AUZAC = \sum_{n=1}^{n_i-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where t is the time of each observation, y is the percent leaf disk colonization, i is the order index for the times, and n_i is the number of readings.

A scaled version of the area under the zoospore activity curve (sAUZAC) was calculated, so comparisons could be made between trials over different time durations:

$$sAUZAC = \frac{AUZAC}{(t_F - t_0)(100)}$$

where ($t_F - t_0$) is the time duration: t_F is the final observational time point and t_0 is the initial observational time point.

Water quality monitoring and chemical analyses

Dissolved oxygen (DO, mg/L), temperature (°C), and pH of the pond water and effluent water were recorded during each trial using calibrated, handheld water quality probes (YSI ProPlus, Yellow Springs, OH, USA). Pond water samples were collected at times 0 h and 1 h, and effluent samples were collected at times 2 and 3 h for high flowrate trials and at times 4 and 5 h for low flowrate trials. These samples were evaluated using a Dionex ICS-1600 ion chromatograph (Thermo Scientific, Waltham, MA, USA) for nitrate, nitrite, and phosphate ions, with a lower detection limit of 0.2 mg/l. All analyses were conducted according to US EPA protocol method 6010B (US EPA, 1997), and calibration standards and quality control points were placed intermittently throughout sample analyses for quality assurance and control.

Plant physical features

At the initiation of the experiment, root length (cm) and wet mass (g) of six randomly selected plants from each trough containing plants were measured (N = 36).

Root length and wet mass of these same plants were measured at the conclusion of the experiment to measure plant growth over the experimental duration.

Statistical analyses

All data analyses were conducted using JMP Pro statistical software (Version 14.1.0, SAS Institute, Cary, NC, USA). Data were analyzed using factorial analysis of variance (ANOVA) to determine if the main effects and interactions of FTWs type (no plants, *Agrostis alba* plants, and *Pontederia cordata* plants) and flowrate (high and low) had significant effects ($\alpha = 0.05$) on the responses of sAUZAC (as determined by percentage of leaf disk colonization) and water quality parameters. If interactions were determined to be significant, simple effects of individual treatments were evaluated. Fisher's least significant differences (LSD) was used to separate treatment means when main or simple effects were found to be significant.

Results

Viable zoospores did not carry over from one trial to the next. Samples taken from troughs at the beginning of each trial, before zoospores were introduced, did not contain viable zoospores based on no infection of the leaf disks used to bait these samples. For the low flowrate treatment, percent leaf disk colonization over time from effluent samples from troughs containing FTWs with *Pontederia cordata* plants was lower than for troughs containing FTWs with *Agrostis alb*a and FTWs with no plants

(Fig. 3.2). However, leaf disk colonization over time from effluent samples during the high flowrate trials was much more variable.

When considering the main effects of FTWs type and flowrate for all trials combined (Table 3.1), the main effects of FTWs type and flowrate were significant predictors of sAUZAC (i.e., transport of viable zoospores) in the ANOVA model (p <0.001 for both). The transport of the highest number of viable zoospores (highest mean sAUZAC) was associated with FTWs containing no plants, while transport of the lowest number of viable zoospores was associated with FTWs containing *Pontederia cordata*. Transport of viable zoospores through FTWs containing *Agrostis alba* was lower than FTWs containing no plants, but higher than FTWs containing *Pontederia cordata*. Trials conducted at the low flowrate were associated with transport of less viable zoospores through the FTWs system than trials conducted at the high flowrate.

When comparing transport of viable zoospores among FTWs types for each individual trial, FTWs containing *Pontederia cordata* reduced flow-through of viable zoospores in two out of three trials during low flow conditions as compared to FTWs containing no plants (Fig. 3.3). Transport of viable zoospores did not differ by FTWs type during high flow conditions. Relative AUZAC mean values were extremely variable from one trial to the next for these high flowrate trials, during which water was being pumped at a flowrate four times higher than for the low flowrate trials.

Root lengths and wet masses of *Pontederia cordata* were greater ($p < 0.001$) than those of *Agrostis alba* at the experiment initiation and conclusion (Table 3.2, Fig. 3.4).

Root lengths and wet masses of both plant species were higher ($p < 0.001$) at the experiment conclusion as compared to the initiation of the experiment.

Average water temperature throughout the experiment was 29.1 ± 1.82°C. DO concentrations and pH were similar in source water and FTWs containing plants, and were generally higher in FTWs containing no plants as compared to source water and FTWs containing plants (Table 3.3). Floating treatment wetlands that did not contain plants appeared to contain the highest density of algae, as observed visually. When comparing DO concentration and pH among FTWs types and source water for each individual trial, FTWs containing *Pontederia cordata* were associated with lower DO concentration and pH as compared to source water and FTWs containing no plants in two out of three trials during low flow conditions (Fig. 3.5). Dissolved oxygen concentrations and pH for trials conducted at the high flowrate were much more variable among source water and FTWs type. Average ammonium-N, nitrite-N + nitrate-N, and phosphate-P concentrations are reported in Supplementary Table S3.2 and Supplementary Fig. S3.1. During high flow conditions, nutrient concentrations generally did not substantially vary. During low flow conditions, effluent from FTWs contained lower concentrations of nutrients as compared to the source water in two out of three trials, with greatest reduction in nutrient concentration occurring in FTWs containing *Pontederia cordata*.

Discussion

Model FTWs containing *P. cordata* plants reduced the flow of viable *P. nicotianae* zoospores through 3-m-long troughs in two out of three trials at the slow

flowrate when compared to troughs containing a FTWs without plants. Therefore, there appears to be potential for FTWs to manage *Phytophthora* spp. at ornamental plant nurseries. However, at the high flowrate, the flow of *P. nicotianae* zoospores through the model FTWs was not impeded. High flowrates and turbulent flow conditions may explain this variability in zoospore activity, especially for troughs containing no plants. In the only published study to evaluate the efficacy of a field-scale reed bed constructed wetland system to remediate *Phytophthora* species, Headley et al. (2005) reported non-detectable levels of *P. cinnamomi* in effluent samples at a HRT of at least 1.3 d. These results indicate that a longer HRT or increased contact time between zoospores and plants (i.e., in a longer channel or trough and slower flowrates) may be necessary to significantly reduce the flow of zoospores through a FTWs in the field.

Although root lengths and wet masses of plants significantly increased from the beginning to the end of the experiment, with *Pontederia cordata* having longer roots and higher wet masses in both instances, an associated decrease in transport of viable zoospores over time was not observed. Troughs containing *P cordata* plants reduced zoospore activity in effluent samples as compared to troughs containing *Agrostis alba* at the low flowrate. The mechanism by which FTWs containing *Pontederia cordata* reduced flow-through of *P. nicotianae* zoospores is not known. However, it could be attributed to the physical obstruction of zoospores by plant roots as well as negative effects of plant root exudates or the root-associated microbiome on zoospores (Kong and Hong, 2016; Kong et al., 2010).

Recorded water temperatures throughout the experiment were within the optimal temperature range (27 to 32°C) reported for *Phytophthora nicotianae* (Erwin and Ribeiro, 1996). The aquatic ecology of plant pathogens is an emerging field, optimum DO concentrations or pH for survival by most species of *Phytophthora* has not been studied (Hong and Moorman, 2005; Hong et al., 2014; Kong and Hong, 2014; Kong et al., 2009). Kong and Hong (2014) reported that *Phytophthora* species generally favored DO concentrations from 5.3 to 5.6 mg/L and that *P. nicotianae* was more sensitive to extreme fluctuations in DO compared to the other species of *Phytophthora* they studied. An optimum pH of 7 has been reported for survival of isolates of *P. nicotianae*, with an approximately 50% die-off of *P. nicotianae* zoospores reported at pH of 5 (Kong et al., 2009). During our study, average DO concentrations in the water column were lower in FTWs that contained plants, especially *Pontederia cordata* (7.1 ± 0.1 mg/L), as compared to FTWs that contained no FTWs (7.6 ± 0.1 mg/L). Average pH values were also typically lower in FTWs that contained *Pontederia cordata* (6.1 ± 0.1) as compared to FTWs that contained no plants (6.4 ± 0.1). Changes in water quality parameters in FTWs containing plants may have contributed to the decline in activity of zoospores as observed in effluent water samples. The increases in DO and pH values in FTWs that did not contain plants may have been due to the presence of algae in these systems; these effects have been observed in other wastewater treatment systems (Tadesse et al., 2004). Effects of algal populations on survival of *Phytophthora* species has not been reported, and it is unclear how algae may have affected survival and transport of *P. nicotianae* zoospores in this study.

Few studies have explored the potential for constructed wetlands to remediate pathogens from agricultural runoff waters. Diaz et al. (2010) reported removal efficiencies of up to 87% and 97% for *E. coli* and enterococci, respectively, in four constructed wetlands in an agricultural watershed in California. HRT was determined to have the greatest effect on the efficiency of bacteria removal. VanKempen-Fryling and Camper (2017) reported higher levels of attachment of *E. coli* on the biofilm of *Carex utriculata* and *Schoenoplectus acutus* plants as compared to a nylon string control surface in model wetland reactors. Gruyer et al. (2013) evaluated the effectiveness of model constructed wetlands planted with *Typha latifolia* to remove *Pythium ultimum* and *Fusarium oxysporum* pathogens, which are typically found in greenhouse wastewater. They observed removal efficiencies of up to 99% at an estimated HRT of 5 d; however, these flow conditions may not be representative of typical field conditions.

Conclusions

This study is one of the first studies to evaluate the efficacy of FTWs, a modified constructed wetland, to manage *Phytophthora* species in a controlled model system, and one of very few studies to evaluate the potential efficacy of constructed wetlands to remediate plant pathogens at representative flow conditions found in the field. Results from this study demonstrated that model FTWs established with *Pontederia cordata* plants reduced the flow of viable *P. nicotianae* zoospores through the system compared to control units containing FTWs without plants at a target HRT of approximately 4 h. The flow of zoospores through these controlled model systems was not reduced at the

higher flowrate of 1-h-target HRT or for FTWs planted with *Agrostis alba plants* at either flowrate. The mechanisms by which model FTWs reduced the flow of zoospores are unknown; however, plant root density, changes in water quality parameters as a result of plant root exudates, and potential interactions with root-associated microbiomes are possibilities. Further investigations on the potential for FTWs to reduce the movement of zoospores of *Phytophthora* species in flowing water are needed—including studies using different species of plants, various species of *Phytophthora*, and varying flowrates, as well as investigation into the mechanisms involved in restricting movement. Eventually, though, studies in the field at ornamental plant nurseries will be needed to demonstrate the actual efficacy of FTWs to manage *Phytophthora* spp. in irrigation water under varying environmental conditions.

The area under the disease progress curve (AUDPC) is a method commonly used by plant pathologists to quantitatively summarize disease development over time in plants. It is worth noting that, to the authors' knowledge, these data represent the only published study whereby the AUDPC method (herein called area under the zoospore activity curve or AUZAC) has been used to evaluate the efficacy of a treatment system to reduce the flow of zoospores of *Phytophthora* spp. in moving water. Interdisciplinary approaches such as this one, that involve teams of agricultural engineers, plant scientists, and hydrologists, will be crucial for future studies seeking to understand the aquatic ecology of plant pathogens and potential novel ecological methods for their remediation.

Acknowledgements

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Tables

TABLE 3.1. Differences among floating treatment wetland type and flowrate on zoospore activity, shown as scaled area under the zoospore activity curve, in a model outdoor system.

Factor, Level	sAUZAC[a]	Two-way ANOVA (P > F)[b]
FTWs type		
No plants	0.41 a	…
Agrostis alba	0.32 b	…
Pontederia cordata	0.21 c	…
LSD	0.08	…
Flowrate		
High	0.37 a	…
Low	0.26 b	…
LSD	0.06	…
ANOVA		
FTWs type	…	<0.001
Flowrate	…	<0.001
FTWs type X Flowrate	…	0.334

[a] Mean values of the scaled area under the zoospore activity curve (sAUZAC) were compared using analysis of variance (ANOVA). Means for each treatment factor with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

[b] Results for six trials were combined, with 3 replicates of each floating treatment wetland (FTWs) type per trial. Three trials were carried out at a high flowrate (1-h-target hydraulic retention time, HRT) and three trials were carried out at a low flowrate (4-h-target HRT).

TABLE 3.2. Plant growth parameters for *Agrostis alba* and *Pontederia cordata* plants within a model floating treatment wetland system.

Plant, Level	Root length (cm)[a]	Wet mass (g)[a]
Agrostis alba		
Initial	10.5 ± 0.3 d	29.9 ± 2.1 c
Final	29.9 ± 2.1 b	120.5 ± 12.5 b
Pontederia cordata		
Initial	20.4 ± 1.4 c	50.4 6.0 c
Final	41.4 ± 1.2 a	176.1 ± 20.0 a

[a] Data are means ± standard errors (n = 18), and were analyzed using analysis of variance. Six randomly selected plants from each of three replicate troughs were measured at the experiment initiation and conclusion (6 weeks later). Means within a column with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

TABLE 3.3. Differences among source water and floating treatment wetland type and flowrate on water quality parameters in a model outdoor system.

Factor, Level	Dissolved oxygen (mg/L)[a]	P > F[b]	pH[a]	P > F[b]
Sample Location				
Source water	7.2 b	...	6.2 b	...
FTWs with no plants	7.6 a	…	6.4 a	…
FTWs with *Agrostis alba*	7.2 b	…	6.2 ab	…
FTWs with *Pontederia cordata*	7.1 b	…	6.1 b	…
LSD	0.2	…	0.2	…
Flowrate				
High	7.3	…	6.1 b	…
Low	7.3	…	6.3 a	…
LSD	ns	…	0.1	…
ANOVA				
Sample Location	…	<0.001	…	0.007
Flowrate	…	0.951	…	0.029
Sample Location X Flowrate	…	<0.001	…	0.073

[a] Mean values of the dissolved oxygen (DO) and pH were compared using analysis of variance (ANOVA). Means for each treatment factor with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

[b] Results for six trials were combined, with 3 physical replicates of each floating treatment wetland (FTWs) type and two samples collected per source water and floating treatment wetland

type per trial. Three trials were carried out at a high flowrate (1-h-target hydraulic retention time, HRT) and three trials were carried out at a low flowrate (4-h-target HRT).

Figures

FIGURE 3.1. Schematic (left) of a trough containing a model floating treatment wetland (FTWs) with floating mats (grey rectangles), plants in aerator containers (green circles), baffles to direct water flow (blue lines), and direction of water flow (arrows), and photograph (right) of two troughs containing model FTWs side-by-side—one with plants and one without plants.

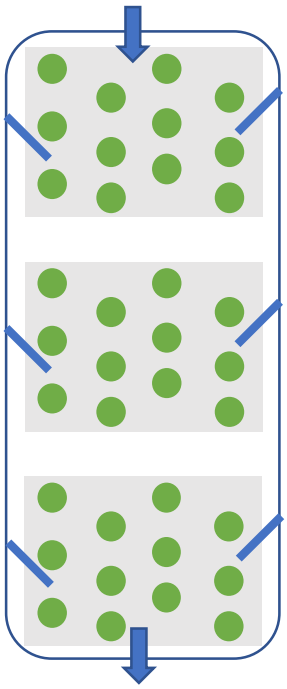


FIGURE 3.2. Differences among floating treatment wetland type and flowrate on transport of viable zoospores in a model outdoor system, with low flowrate trials (at 4-h-target hydraulic retention time, HRT) on the left, and high flowrate trials (at 1-h-target HRT) on the right. Data are mean percentages ± standard errors (n = 3).

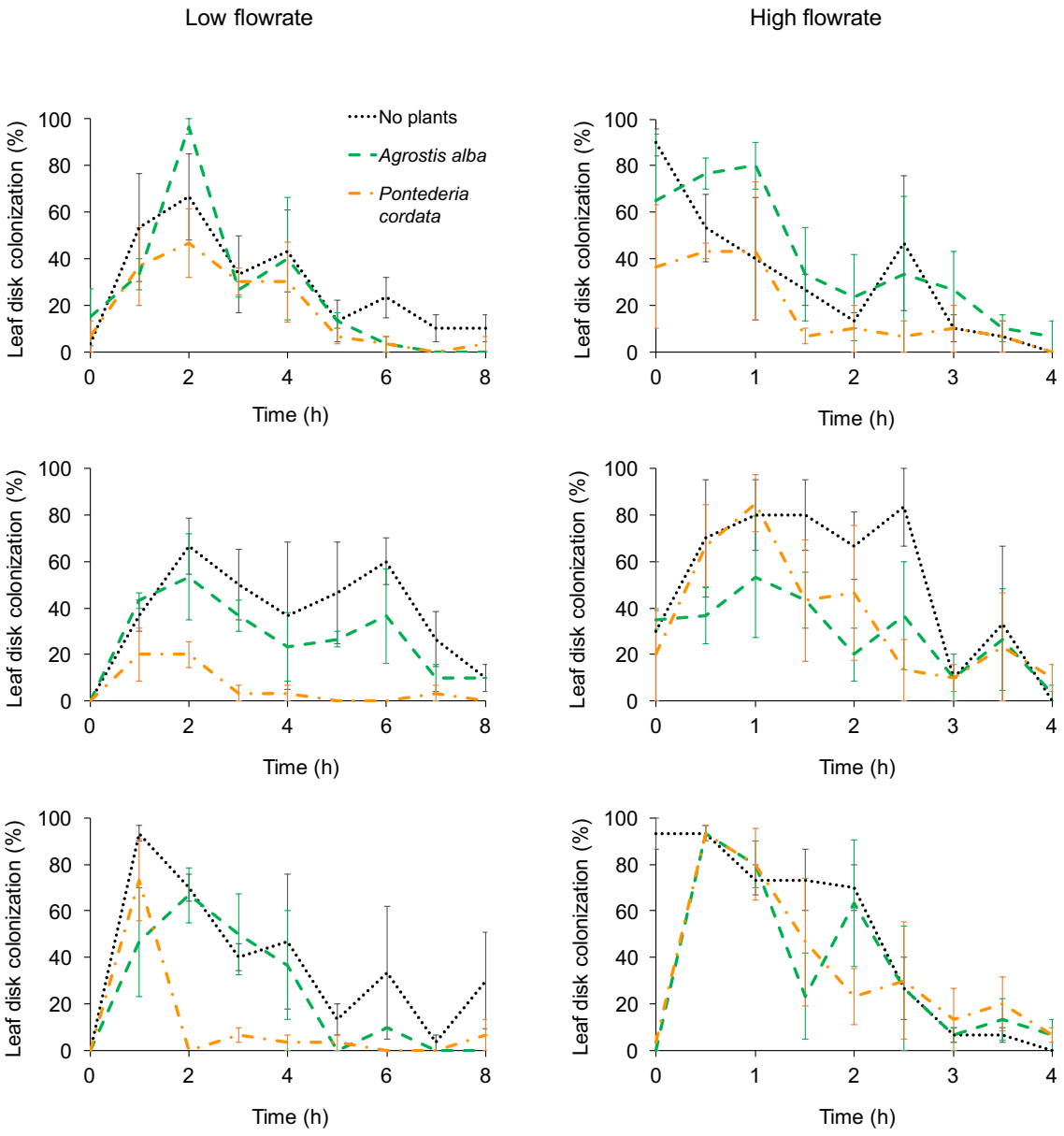


FIGURE 3.3. Differences on transport of viable zoospores, shown as scaled area under the zoospore activity curve (sAUZAC), for each trial (shown as dates) at low (left) and high (right) flowrates for floating treatment wetlands (FTWs) containing *Agrostis alba,* no plants, or *Pontederia cordata* in a model outdoor system, with three replicates of each FTWs treatment type per trial. Data are means ± standard errors, and were analyzed by analysis of variance (ANOVA). Means associated with different letters within a trial mean sAUZAC significantly differed among FTWs type, as determined by Fisher's least significant difference test (LSD; $\alpha = 0.05$).

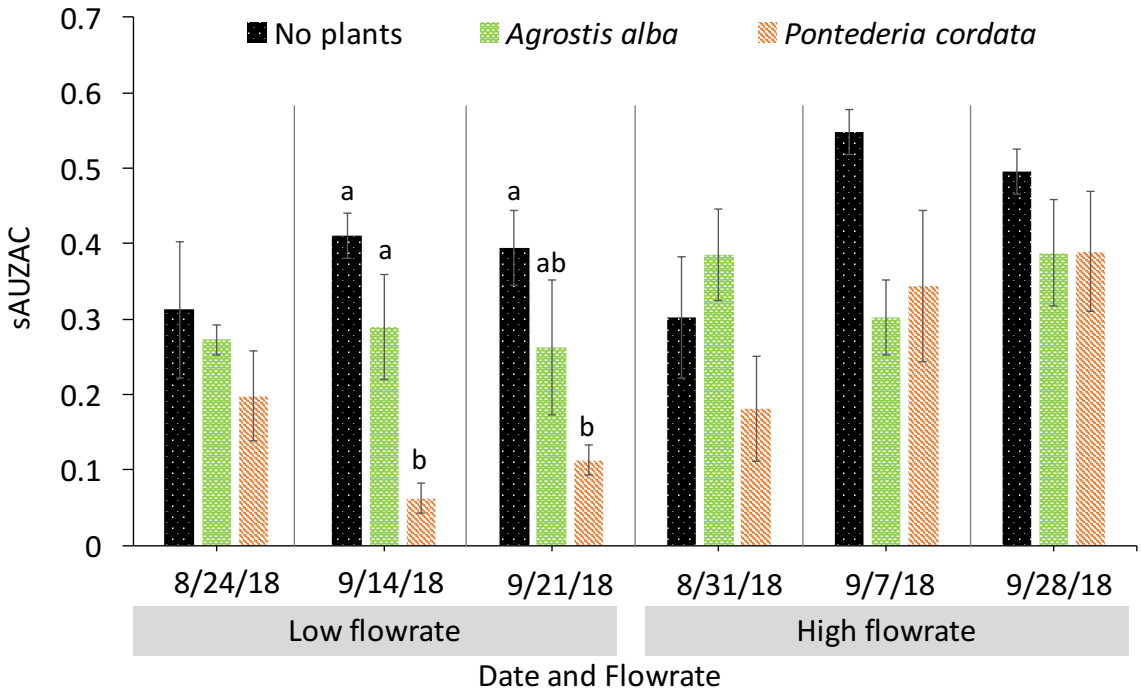
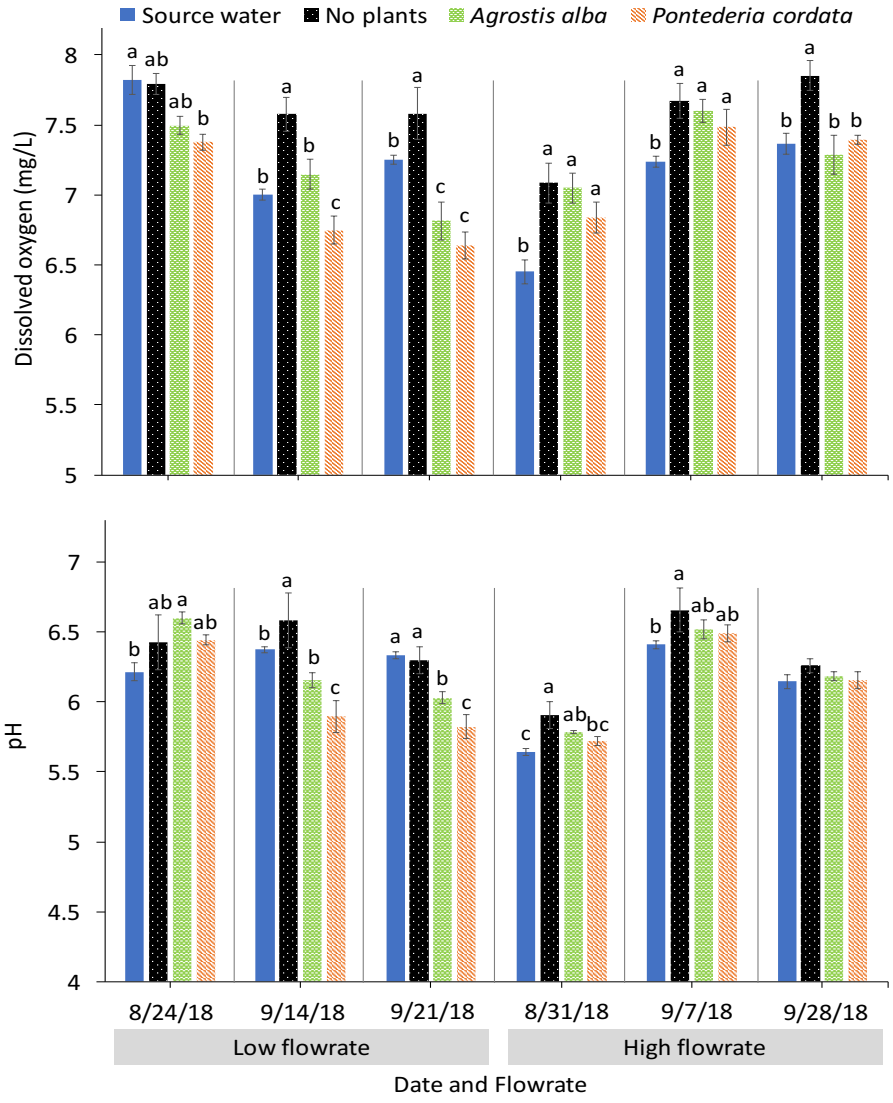


FIGURE 3.4. Root scans of representative plants of *Agrostis alba* (top) and *Pontederia cordata* (bottom) before initiation of experiment (left) and at the experiment conclusion (right).

FIGURE 3.5. Dissolved oxygen concentrations (top) and pH values (bottom) during low (left) or high (right) flow conditions of effluent from each floating treatment wetland (FTWs) treatment type (No plants, *Pontederia cordata*, and *Agrostis alba*) and source water. Data are means ± standard errors during each trial, and were analyzed using analysis of variance. Values within each trial that share the same letter or that do not contain letters are not significantly different as determined by Fisher's least significant difference test (LSD; $\alpha = 0.05$).



Supplementary Tables

TABLE S3.1. Differences among floating treatment wetland type and flowrate on zoospore activity, shown as scaled area under the zoospore activity curve, in a model outdoor system during exploratory pre-experimental trials.

Factor, Level	sAUZAC[a]	Two-way ANOVA (P > F)[b]
FTWs type		
No plants	0.88	…
Agrostis alba	0.79	…
Pontederia cordata	0.85	…
LSD	ns	…
Flowrate		
High	0.90 a	…
Low	0.78 b	…
LSD	0.10	…
ANOVA		
FTWs type	…	0.367
Flowrate	…	0.017
FTWs type X Flowrate	…	0.864

[a] Mean values of the scaled area under the zoospore activity curve (sAUZAC). Means for each treatment factor with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$; ns = no significant difference).

[b] Results for four trials were combined, with 3 replicates of each floating treatment wetland (FTWs) type per trial. Two trials were carried out at the high flowrate (1-h-target hydraulic retention time, HRT) and two trials were carried out at the low flowrate (4-h-target HRT).

TABLE S3.2. Differences among source water and floating treatment wetland effluent and flowrate on nutrient concentrations in a model outdoor system.

Factor, Level	NH_4-N (mg N/L)[a]	P > F[b]	NO_2-N + NO_3-N (mg N/L)[a]	P > F[b]	PO_4-P (mg P/L)[a]	P > F[b]
Sample Location						
Source water	2.6 a	...	3.7	...	1.2	...
FTWs – no plants	2.1 b	…	3.5	…	1.2	…
FTWs – *A. alba*	2.4 ab	…	3.4	…	1.1	…
FTWs – *P. cordata*	2.2 b	…	3.3	…	1.1	…
LSD	0.3	…	ns	…	ns	…
Flowrate						
High	2.1 b	…	3.3	…	1.1 b	…
Low	2.5 a	…	3.5	…	1.2 a	…
LSD	0.2	…	ns	…	0.08	…
ANOVA						
Sample Location	…	<0.001	…	0.067	…	0.085
Flowrate	…	<0.001	…	0.145	…	0.001

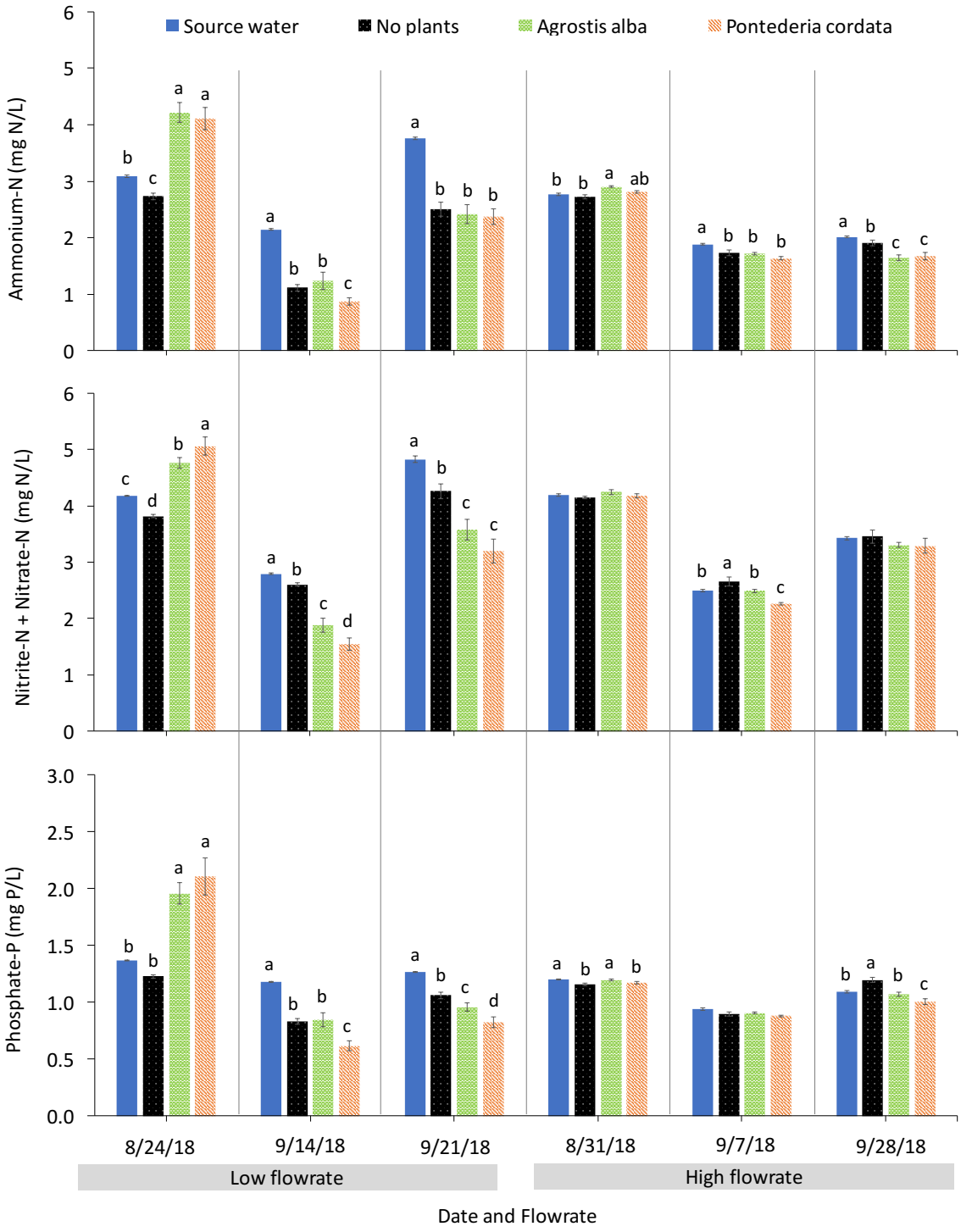
Sample	…	0.034	…	0.198	…	0.098
Location X Flowrate						

[a] Mean values of ammonium-N (NH_4-N), nitrite-N + nitrate-N (NO_2-N + NO_3-N) and phosphate-p (PO_4-P) were compared using analysis of variance (ANOVA). Means for each treatment factor with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

[b] Results for six trials were combined, with 3 physical replicates of each floating treatment wetland (FTWs) type and two samples collected per source water and floating treatment wetland type per trial. Three trials were carried out at a high flowrate (1-h-target hydraulic retention time, HRT) and three trials were carried out at a low flowrate (4-h-target HRT).

Supplementary Figures

FIGURE S3.1. Ammonium-N (top), nitrite-N + nitrate-N (middle) and phosphate-P values (bottom) during low (left) or high (right) flow conditions of source water and effluent from each floating treatment wetland (FTWs) treatment type (No plants, *Pontederia cordata*, and *Agrostis alba*). Data are means ± standard errors during each trial (shown by date), and were analyzed using analysis of variance. Values within each trial that share the same letter or that do not contain letters are not significantly different as determined by Fisher's least significant difference test (LSD; $\alpha = 0.05$).


Source water
No plants
Agrostis alba
Pontederia cordata
Ammonium-N (mg N/L)
Nitrite-N + Nitrate-N (mg N/L)
Phosphate-P (mg P/L)
8/24/18
9/14/18
9/21/18
8/31/18
9/7/18
9/28/18
Low flowrate
High flowrate
Date and Flowrate

CHAPTER 4: POTENTIAL OF SUBSURFACE BIOREACTORS TO REMEDIATE *PHYTOPHTHORA* SPECIES IN AGRICULTURAL DRAINAGE WATER

Abstract

The potential contamination of recycled water with plant pathogens is the primary concern preventing many nursery and greenhouse crop producers from recycling irrigation runoff and drainage water onsite. Subsurface bioreactors are low-cost, low-maintenance ecological treatment technologies that effectively reduce nitrate from agricultural drainage; however, their potential to remediate plant pathogens has not been investigated. The objective of this study was to evaluate the potential of laboratory-scale subsurface bioreactors containing different bark substrates to restrict passage of zoospores of *Phytophthora nicotianae* in water passing through the bioreactors. Results from this study demonstrated that laboratory-scale bioreactors containing fir bark reduced ($P < 0.001$) transport of viable zoospores when compared to control units that did not contain any substrate during low and high input nitrogen concentration conditions (11.6 ± 0.3 mg/L N and 72.0 ± 3.7 mg/L N, respectively) and at flowrates equivalent to target 2-h and 8-h hydraulic retention times (HRT). The highest total nitrogen concentration reduction reported (~31% removal) occurred in fir bark bioreactors during low flow (8-h HRT) and low input N conditions. Microbial activity in bioreactors containing fir bark was likely higher—as evidenced by high dissolved organic carbon concentrations, high C:N ratios, and low dissolved oxygen concentrations. Though the mechanisms by which fir bark bioreactors prevented flow-through of pathogen propagules are unknown, potential interactions with naturally-occurring microbial communities likely contributed

to remediation of *P. nicotianae* zoospores. To the authors' knowledge, this is the only reported study to evaluate the potential of agricultural bioreactors to manage species of *Phytophthora* in irrigation runoff and drainage. Future studies with bioreactors to remediate plant pathogens should investigate varying types of woody substrate and should focus on understanding microbial community dynamics to gain further insight into remediation mechanisms.

Introduction

Reliable access to sources of freshwater for irrigation purposes has become a top priority for agricultural producers, particularly those growing ornamental plants in the nursery and greenhouse industries (White et al., 2013). Many growers are now considering remediating irrigation runoff so they can capture, recycle, and reuse this water onsite. However, several factors currently prevent some agricultural producers from reusing runoff for irrigation. These grower-identified barriers include potential contamination of recycled water with salts, pesticides, and plant pathogens as well as the costs associated with implementing necessary treatment technologies (White et al., 2013). Plant pathogens in irrigation water are of particular concern because economic damage caused by plant pathogens to crops in the US alone is estimated to be in the tens of billions of dollars (Hong and Moorman, 2005; Gevens et al., 2007; Tyler, 2002; Zappia et al., 2014). Infective propagules of plant pathogens may be transported from diseased plants in the production area into onsite reservoirs by way of runoff and leaching. Some of the most economically important plant pathogens of concern that have been detected in

nursery and greenhouse runoff and irrigation waters include species of *Phytophthora*—which cause root, crown, and fruit rots as well as stem and foliage blights on a multitude of host plants, including agricultural crops, ornamental crops, and urban and forest trees (Hong and Moorman, 2005; Hwang and Benson, 2005; Leonberger et al., 2013; Stewart-Wade, 2011). *Phytophthora* spp. produce motile, swimming zoospores that often serve as propagules of dispersal and often are the primary infective propagules that initial infections on many plants (Erwin and Ribeiro, 1996; Schumann and D'Arcy, 2010).

Drawbacks to chemical and physical treatment methods to disinfest recycled irrigation water include agrichemical sensitivity to turbidity and water pH (which render chemicals ineffective if not properly dosed and managed by growers), potential for formation of harmful chemical byproducts, potential for technical malfunctions or breakdowns that may require maintenance by technical specialists, and limited capacity to process large volumes of water (Hong and Moorman, 2005; Steward-Wade, 2011). Biological methods for managing *Phytophthora* spp. in water systems are not widely implemented and not well understood. These methods include biofiltration (including carbon-based bioreactors and slow sand filters) and constructed wetlands that allow surface or subsurface flow-through of irrigation runoff (Hong and Moorman, 2005; Steward-Wade, 2011). Subsurface agricultural bioreactors are trenches filled with carbon material (usually wood chips) that intercept runoff water from the growing area before it is released into receiving water bodies. For over 20 years, subsurface bioreactors have been studied extensively and shown to effectively remediate nitrate in agricultural runoff through the activities of naturally-occurring nitrate-reducing bacteria (Bell et al., 2015;

Blowes et al., 1994; Christianson et al., 2012a; Chun et al., 2009; Greenan et al., 2006; Jaynes et al., 2008; Robertson and Merkley, 2009; Schipper et al., 2010). Bioreactors have also been shown to remediate herbicides and pesticides in agricultural runoff (Celis et al., 2008; Gonzalez et al., 2006) as well as heavy metals in acid mine drainage (Neculita and Zagury, 2008; Zagury et al., 2006). Bioreactors require little to no modifications to existing infrastructure, do not require land to be taken out of production, are inexpensive to install, and require little to no maintenance (Christianson et al., 2012b; Robertson, 2010).

Currently, the efficacy of subsurface bioreactors to remediate species of *Phytophthora* in irrigation runoff and drainage has not been reported, representing a substantial knowledge gap in this field. Therefore, the objective of this study was to assess the potential of fir and pine bark in laboratory-scale bioreactors to prevent viable zoospores of *P. nicotianae* in water from passing through these systems.

Materials and methods

Experimental layout and operation

The experiment was conducted in the Water Treatment Technology Laboratory at Clemson University's South Carolina Water Resources Center in Pendleton, SC, USA (34°38'N, 82°46'W). Twelve laboratory-scale bioreactors were constructed using 17-L plastic containers (Sterilite Corporation, Townsend, MA, US) measuring 44 cm × 31 cm × 23 cm (Fig. 4.1) Three replicate bioreactors were randomly assigned to each of four treatments: No substrate, K1 polyethylene plastic filter medium (Cz Garden Supply,

Southfield, MI, US), Douglas fir bark nuggets (Rexius, Eugene, OR, US), and pine bark nuggets (Nature's Choice, Inc., Glennville, GA, US). Bioreactors containing no substrate served as controls. Plastic medium was used to represent physical filtration without the biological filtration attributes expected of the bioreactors containing woody substrates. Bark was chosen as a woody substrate because it is easily accessible to greenhouse and nursery crop growers. Pine bark is a typical component in the potting media used to grow plants throughout nurseries and greenhouses in the eastern US while fir bark is a typical component in the potting media used to grow ornamental plants throughout the western US (Gomez and Robbins, 2011). Substrate was gently tamped intermittently to increase packing density within each bioreactor, which also contained two polycarbonate baffles (30.5 cm × 7.5 cm × 1.5 mm, Fig. 4.1) to prevent preferential flow along sidewalls. All outer sidewalls of bioreactors were painted black to limit light penetration to simulate in-field subsurface bioreactor conditions.

Before trials were initiated, pond water amended with 24.7 ± 2.4 mg/L N (mean ± standard error) from a 24N-8P-16K water-soluble fertilizer (Southern Agricultural Insecticides, Inc., Hendersonville, NC) was continuously pumped for 8 weeks (start-up period) at a calculated target hydraulic retention time (HRT) of 12 h through peristaltic pumps from one 1,135-L tank into one proximal end (head) of each bioreactor to establish microbial communities and stabilize effluent dissolved organic carbon (DOC) concentrations within bioreactors containing woody substrates (Hoover et al., 2015; Maxwell et al., 2018; Fatehi-Pouladi et al., 2019). During all trials, deionized water amended with fertilizer at either 11.6 ± 0.3 mg/L N (referred to as low N) or 72.0 ± 3.7

mg/L (referred to as high N) continuously flowed through each bioreactor at a calculated target HRT of 2 or 8 h (high or low flowrates, respectively) during each experimental run, which lasted either 8 h or 12 h, respectively. One trial was completed each week for 8 weeks from March to May 2018. Each trial consisted of randomly selected combinations of input N concentration (high and low) and flowrate (high and low) treatment factors: High flowrate and low input N concentration, high flowrate and high N concentration, low flowrate and low N concentration, low flowrate and high N concentration). Two trials of each of these four combinations of input N concentration and flowrate were carried out. These relatively low HRTs (i.e., fast flowrates) were chosen to reflect typical flow conditions in agricultural bioreactors and channels receiving agricultural runoff and drainage (Christianson et al., 2012b; Dollinger et al., 2015; Knox et al., 2007; Moore et al., 2011). A suspension of *Phytophthora nicotianae* zoospores was introduced to the proximal end of each bioreactor at the beginning of each trial after two pore volumes (see below) of nutrient-amended water had been pumped through each bioreactor. Flowrates corresponding to target HRTs of 2 and 8 h were calculated as follows:

$$Q = \frac{V \times \theta}{HRT}$$

where Q = flowrate, V = bioreactor container volume, and θ = effective porosity.

Rhodamine WT (Bright Dyes, Kingscote Chemicals, Miamisburg, OH, USA), a fluorescent dye commonly used in environmental tracer studies, was introduced to each bioreactor at a target HRT of 2 h to characterize internal flow dynamics (Sabatini and Austin, 1991; Dierberg and DeBusk, 2005). The tracer study was performed at the conclusion of the experiment to avoid potential detrimental effects of rhodamine dye on *P. nicotianae* zoospores or naturally-occurring microbial communities, as genotoxic effects of rhodamine WT have been reported (Behrens et al., 2001).

Substrate properties

Fir and pine bark substrates were washed, oven dried, and passed through a series of sieves with pore diameters measuring 5.1, 3.8, 2.5, 1.9, and 1.3 cm. Particles larger than 5.1 cm and smaller than 1.3 cm were discarded, a particle size distribution curve was created, and physical characteristics of substrates were calculated (Table 4.1). Both bulk density and carbon:nitrogen (C:N) ratio values were larger for fir bark compared to pine bark. Porosity of bark and plastic substrates were determined similarly to methods described by Christianson et al. (2010): substrate was packed in 1-L bottles, pore volume was filled with water, bottles were capped and allowed to sit for 24 h (for water to be absorbed by the woodchips), and then more water was added to refill the container to 1-L. Effective porosity was calculated as the sum of the total volume of water added divided by 1 L.

Zoospore production

The isolate of *Phytophthora nicotianae* (isolate no. 05-0690) used in this study was recovered in 2005 from the stem of a *Hibiscus paramutabilis* × *syriacus* 'Lohengrin' plant growing in a nursery in South Carolina, and it is maintained in a permanent collection in the laboratory of Dr. S.N. Jeffers at Clemson University. This isolate was characterized and identified in a previous study (Ridge et al., 2014). An active culture of the isolate was maintained in 10-cm-diameter disposable petri dishes containing PARPH-V8 agar, a medium selective for species of *Phytophthora* (Ferguson and Jeffers, 1999; Jeffers, 2015b), at 15°C in the dark. Before the start of each experimental run, cultures were transferred onto 10% clarified V8 agar (cV8A; Jeffers, 2015c) in 10-cm-diameter disposable petri dishes and incubated at 20°C in the dark for 3 days. Zoospores were produced from mycelium mats growing in 10% cV8 broth (Jeffers, 2015c) following methods described previously (Drechsler et al., 2014; Nyberg et al., 2014). The concentration of zoospores in the suspension was quantified using a hemacytometer and ranged from 1.7 x 10^5 to 4.8 x 10^5 zoospores/mL. This concentrated suspension was diluted to prepare 38 L of zoospore suspension with a standard concentration of 5,000 zoospores/mL, which was distributed equally to all bioreactors at the beginning of each trial. Laboratory-scale experiments evaluating the disease potential of *Phytophthora* spp. on ornamental plants previously have used suspensions with concentrations of up to 10^4 zoospores/mL (Granke and Hausbeck, 2010; Kong and Hong, 2010); however, concentrations of *Phytophthora* spp. inoculum recovered from nursery and greenhouse runoff collection reservoirs have been reported within the range of 0 to 100 zoospores/mL (Bush et al., 2003; Kong et al., 2003; Loyd et al., 2014; Stewart-Wade,

2011). A fresh stock of zoospore suspension was prepared before each trial of this study. Detailed methods describing how a large volume (i.e., several liters) of concentrated zoospore suspension was prepared for this experiment are included in Appendix B.

Plant pathogen monitoring and analyses

Effluent water samples of approximately 3 L were continuously collected from each bioreactor throughout each trial (i.e., all effluent was collected throughout the experiment duration). During trials conducted at the high flowrate, 16, 13, 10, and 9 effluent samples were collected from bioreactors containing no, plastic, pine bark, and fir bark substrate, respectively. During trials conducted at the low flowrate, 8, 7, 5, and 5 effluent samples were collected from bioreactors containing no, plastic, pine bark, and fir bark substrate, respectively. Twelve influent water samples of approximately 250 mL were collected at the beginning of each trial to ensure pathogen inoculum was introduced to each bioreactor, and another two 250-ml samples of zoospore suspension were collected to ensure the pathogen remained viable over the course of the trial. Effluent samples also were collected before zoospores were introduced to each bioreactor at the beginning of each trial to ensure viable zoospores were not already present. Zoospore viability was evaluated using a standard leaf disk baiting bioassay described by Ridge et al. (2014). To conduct the bioassay, ten 5-mm-diameter leaf disks were punched from leaves of *Rhododendron maximum* that were free of chemical pesticides, and leaf disks were floated on the surface of each water sample. After 3 days, leaf disks were removed, blotted dry with paper towels, embedded in PARPH-V8 selective medium in a 10-cm-

diameter petri dish, and held at 25°C in the dark for 3 days. Leaf disk perimeters were examined microscopically (20-70×) for hyphae of *P. nicotianae*. Presence and activity of zoospores in a water sample was quantified using a scale from 0 to 100% based upon the number of leaf disks that appeared to be colonized out of 10 (Ridge et al., 2014). Zoospore activity of effluent samples was plotted over time to create a zoospore activity curve. Area under the zoospore activity curve (AUZAC) was calculated using the same method used to estimate the area under a disease progress curve (AUDPC) so comparisons of zoospore activities among treatments could be made (Madden et al., 2007):

$$AUZAC = \sum_{n=1}^{n_i-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where *t* is the time of each observation, *y* is the percent leaf disk colonization, *i* is the order index for the times, and n_i is the number of readings.

A scaled version of the area under the zoospore activity curve (sAUZAC) was calculated, so comparisons could be made between trials over different time durations:

$$sAUZAC = \frac{AUZAC}{(t_F - t_0)(100)}$$

where ($t_F - t_0$) is the time duration: t_F is the final observational time point and t_0 is the initial observational time point.

Water quality monitoring and chemical analyses

Dissolved oxygen (DO, mg/L), temperature (°C), pH, and the oxidation reduction potential (ORP, mV) of influent and effluent samples, as well as samples collected from a port within the middle of each bioreactor, were recorded during each trial using calibrated, handheld water quality probes (YSI, Yellow Springs, OH, USA; OAKTON Instruments, Vernon Hills, IL, USA). Additionally, influent and effluent samples were collected and analyzed using a Dionex ICS-1600 ion chromatograph (Thermo Scientific) for ammonium (NH_4), nitrate (NO_3), and nitrite (NO_2) ions, with a lower detection limit of 0.2 mg/L. Samples were also analyzed using a Shimadzu TOC-VCPH total organic carbon analyzer (Shidamzu Scientific Instruments, Kyotok Japan) for dissolved (non-purgeable) organic carbon (DOC) and total nitrogen (TN), with lower detection limits of 1.0 mg DOC/L and 0.9 mg TN/L, respectively. Analyses were conducted according to US EPA protocol methods 9056A and 9060A (US EPA, 2004; US EPA, 2007) and calibration standards and quality control points were placed intermittently throughout sample analyses for quality assurance and control. Influent samples were collected at times 0, 2, and 4 h after trials began, and effluent water samples were collected at 2, 4, and 6 h for the 2-h target HRT trials and at 8, 10, and 12 h for the 8-h target HRT trials.

Statistical analyses

All data analyses were conducted using JMP Pro statistical software (Version 14.1.0, SAS Institute, Cary, NC, USA). Data were analyzed using three-way factorial analysis of variance (ANOVA) to determine if the main effects and interactions of input nitrogen concentration (high and low), flowrate (high: 2-h-target HRT; low: 8-h-target HRT), and substrate type (none, plastic medium, fir bark, and pine bark) had significant effects ($\alpha = 0.05$) on the responses of sAUZAC (as determined by percentage of leaf disk colonization) and water quality parameters. If interactions were determined to be significant, simple effects of individual treatments were evaluated. Fisher's least significant differences (LSD) was used to separate treatment means when main or simple effects were found to be significant.

Results and discussion

Results from the tracer study confirmed that replicate bioreactors of a given substrate (none, plastic, fir bark, or pine bark) performed similarly, as peak tracer recovery occurred at similar times across bioreactors (Fig. 4.2). Peak tracer recovery occurred earlier than the predicted one pore volume for ideal plug-flow conditions for bioreactors containing no substrate (none), fir bark, and pine bark. Other studies of the internal hydraulics of bioreactors have reported similar results and attributed these deviations from ideal conditions to possible dispersion and short-circuiting (presence of 'dead zones') within the bioreactor (Christianson et al., 2013; Christianson et al., 2016; Hoover et al., 2015).

Percent leaf disk colonization and calculated sAUZAC was lowest for bioreactors containing fir bark at all combinations of flowrate and input N concentration experimental treatment factors (Figs. 4.3 and 4.4, Table 4.3). In other words, bioreactors containing fir bark reduced the greatest amount of flow-through of viable zoospores. In most cases, bioreactors containing fir bark released less than 20% of viable zoospores leaving bioreactors containing no substrate. Bioreactors containing pine bark released similar levels of viable zoospores as compared to bioreactors containing plastic medium for all treatment factors combinations of flowrate and input N concentration, except for the case of high flowrate and high input N concentration, where bioreactors containing pine bark reduced greater amounts of viable zoospores as compared to bioreactors containing plastic medium (i.e., lower mean sAUZAC value; Fig. 4.4). Control bioreactors containing no substrate consistently released the highest levels of viable zoospores as compared to bioreactors containing plastic or woody substrate. All bioreactors generally appeared to release lower amounts of viable zoospores during high input N and low flowrate conditions as compared to low input N and high flowrate conditions (Table 4.3).

The use of agricultural bioreactors to remediate plant pathogens is an emerging field. Gruyer et al. (2013) conducted the only study, to the authors' knowledge, of agricultural bioreactors to treat waterborne plant pathogens (*Pythium ultimum* and *Fusarium oxysporum*). The lab-scale 3.5-L bioreactors – which contained a mixture of maple wood chips, sawdust, poultry manure, maple leaf compost, and sand – effectively reduced up to 99.99% of the influent pathogen concentrations. However, a HRT of 5 d

was used during this study, which may not represent typical flow conditions in a nursery or greenhouse setting. Several studies have investigated the efficacy of woodchip bioreactors to remove bacterial and viral mammalian pathogens of concern. Soupir et al. (2018) reported removal efficiencies of up to 96% and 94%, respectively, for *E. coli* and *Salmonella,* as well as 96% nitrate and 85% dissolved reactive phosphorus removal, in column woodchip bioreactors at a 24 h HRT at 21.5 °C. Rambags et al. (2016) reported 2.9 $\log_{10}$ and 3.9 $\log_{10}$ removals of *E. coli* and F-specific RNA bacteriophage, respectively, as well as up to 99.9% reduction of nitrate, in full-scale denitrifying woodchip bioreactors operating at a HRT of 8 d in New Zealand. Zoski et al. (2013) reported removal efficiencies of *E. coli* of up to 98% in laboratory-scale bioreactors containing wood shavings and P-immobilizing reactive aluminum and iron oxides (water treatment residuals) at a flowrate of 2 mL/s (HRT not reported).

Dissolved organic carbon effluent concentrations were higher from bioreactors containing bark substrate as compared to no or plastic substrate during the start-up period (Fig. 4.5), with effluent concentrations reaching up to 170 mg C/L for bioreactors containing fir bark. After several weeks of continuous operation, DOC levels from bioreactors containing bark substrate decreased to levels similar to bioreactors containing no or plastic substrate. This initial spike and subsequent leveling off of DOC effluent concentration from bioreactors containing woody substrate has been reported during the start-up period for several other studies, and can likely be attributed to initial release of labile carbon and small particles from woody substrate (Bell et al., 2015; Christianson et al., 2012a; Christianson and Schipper, 2016; Hoover et al., 2015).

Influent ammonium-N and nitrite+nitrate-N concentrations from bioreactor influent as compared to bioreactor effluent for each substrate type are shown in Fig. 4.6. Nitrite+nitrate-N concentrations were generally lower in effluent from bioreactors containing bark, particularly fir bark during low input N and low flowrate conditions, as compared to concentrations of influent or effluent from bioreactors containing no substrate. Total nitrogen removal occurred only during high flowrate (2 h HRT) and high input N conditions in fir bark bioreactors (4% average TN concentration reduction), and during low flowrate (8 h HRT) and low input N conditions in fir and pine bark bioreactors (Fig. 4.7, Table 4.2), with average TN concentration reductions of 31% and 26%, respectively. The soluble fertilizer used during this experiment contained 24 parts N (by weight), of which 5% was in the form of nitrate, 5% as ammonium, and 14% as urea. While denitrification has been reported as the main N removal mechanism in woody substrate bioreactors receiving nitrate, in cases of high ammonium input, such as in this study, ammonium volatilization may have also contributed to total N removal (Greenan et al., 2006; Healy et al., 2012; Hoover et al., 2015). In future studies, one form of N (e.g., nitrate for agricultural studies or ammonium for wastewater treatment) should be utilized to permit better characterization of the microbial processes and transformations of nutrients occurring within the bioreactor, as the dominance of certain naturally-occurring microbial populations could directly impact survival of introduced pathogens.

During experimental trials, DOC concentrations were highest in effluent samples collected from bioreactors containing fir bark for all treatment combinations of flowrate and input N concentration except for the high flowrate and high input N treatment

combination (Fig. 4.8). Carbon:nitrogen ratio and bulk density values were also higher for fir bark as compared to the pine bark substrate (Table 4.1). Access to more readily available labile carbon within fir bark bioreactors likely contributed to higher levels of microbial activity, which may explain the high level of *P. nicotianae* remediation that occurred within these bioreactors as compared to the others. Measured ORP of effluent and samples collected from the middle of the bioreactor were lower as compared to influent conditions for only the fir bark bioreactors operating at low input N and low flowrate (8 h HRT) conditions (Fig. 4.9), which corresponded with the highest N removal reported (Table 4.2; Figure 4.6). ORP did not appear to reach low enough levels for denitrification to occur (-50 to +50 mV; YSI Environmental, 2008); however, since redox conditions were not measured in-situ, we cannot definitively conclude denitrification did not occur. Dissolved oxygen concentrations in effluent and middle samples were lower than influent samples for fir and pine bark in all cases, and for plastic medium in all cases except high flowrate and low input N treatment factor combination (Fig. 4.9). These decreases in DO were likely due to enhanced microbial activity in the bioreactors, regardless of the presence of a supplemental carbon source.

Conclusions

This is the first published study, to our knowledge, that evaluated the potential of agricultural subsurface bioreactors to manage species of *Phytophthora*, and one of very few studies to evaluate bioreactors for plant pathogen remediation at representative field hydraulic conditions. Results from this study demonstrated that laboratory-sale

bioreactors containing fir bark reduced ($p < 0.001$) flow-through of viable *Phytophthora nicotianae* zoospores as compared to control units that did not contain any substrate during low and high input nitrogen concentration conditions (11.6 ± 0.3 mg/L N and 72.0 ± 3.7 mg/L N, respectively) and at flowrates equivalent to 2 h and 8 h HRTs. The highest total nitrogen concentration reduction reported (~31% removal) occurred in fir bark bioreactors during low flowrate (8 h HRT) and low input N conditions.

Dissolved organic carbon concentrations were consistently highest in effluent samples collected from bioreactors containing fir bark substrate, which also had the highest C:N ratio and bulk density as compared to pine bark substrate. Though the exact mechanisms by which bioreactors diminish flow-through of plant pathogens are unknown, access to more readily-available, labile carbon within fir bark bioreactors likely contributed to high levels of microbial activity (evidenced by lower effluent dissolved oxygen concentrations). Potential interactions with naturally-occurring microbial communities likely contributed to the remediation of *P. nicotianae* reported. Further studies of bioreactor capacity to reduce flow-through of plant pathogens should investigate varying types of woody substrate and should focus on understanding the biogeochemical transformations of nutrients (and associated microbial communities) within the bioreactor to gain further insight into potential microbiologically-aided removal mechanisms.

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Tables

TABLE 4.1. Physical characteristics of the three substrates evaluated for use in laboratory-scale bioreactors for remediation of infective propagules of *Phytophthora nicotianae* in water.

Substrate	Effective porosity[a]	Particle Diameter[b]			Particle diameter x height	Coefficient of gradation[c]	Uniformity coefficient[d]	Bulk density	Carbon:Nitrogen ratio
		D_{10} (cm)	D_{30} (cm)	D_{60} (cm)	(cm)	(C_c)	(C_u)	(kg/m^3)	(C:N)
Fir bark	0.55	1.55	1.95	2.30	n/a	1.07	1.48	225	149:1
Pine bark	0.65	2.15	2.75	3.30	n/a	1.07	1.53	185	93:1
K1 plastic[e]	0.84	n/a	n/a	n/a	1.00 x 0.70	1	1	158	n/a

[a] Effective porosity was calculated as the sum of the total volume of water added to the pore volume of substrate within a 1-L container divided by container volume

[b] D_{10}, D_{30}, and D_{60} are the particle diameters for which 10%, 30%, and 60% of the substrate are finer (by weight), respectively

[c] Coefficient of gradation (C_c) is calculated as $(D_{30}^2) / (D_{10} \text{ X } D_{60})$

[d] Uniformity coefficient (C_u) is calculated as D_{60} / D_{10}

[e] K1 plastic = polyethylene plastic filter medium

TABLE 4.2. Differences among substrate type, flowrate, and input nitrogen (N) concentration on average percent total nitrogen (TN) concentration reduction within laboratory-scale bioreactors.

Factor, Level	TN removed (%)[a]	Three-way ANOVA (P > F)[b]
Substrate type		
None	-6.6 b	…
Plastic	-2.4 b	…
Fir bark	12.3 a	…
Pine bark	9.3 a	…
LSD	4.6	…
Flowrate		
High	-1.4 b	…
Low	7.7 a	…
LSD	3.3	…
Input N concentration		
High	2.0	…
Low	4.3	…
LSD	ns	…

ANOVA		
Substrate type	…	<0.001
Flowrate	…	<0.001
Input N concentration	…	0.152
Substrate type X Flowrate	…	<0.001
Substrate type X Input N concentration	…	0.102
Flowrate X Input N concentration	…	<0.001
Substrate type X Flowrate X Input N concentration	…	0.497

[a] Percent concentration reduction was calculated as the difference between average influent and effluent TN concentrations divided by average influent concentration, multiplied by 100. Negative values indicate TN was not removed from the system. Mean values were compared using analysis of variance (ANOVA). Means for each treatment factor with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$; ns = no significant differences).

[b] Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates).

TABLE 4.3. Differences among substrate type, flowrate, and input nitrogen (N) concentration on average zoospore activity, shown as scaled area under the zoospore activity curve, within laboratory-scale bioreactors.

Factor, Level	sAUZAC[a]	Three-way ANOVA (P > F)[b]
Substrate type		
None	0.78 a	…
Plastic	0.52 b	…
Fir bark	0.20 c	…
Pine bark	0.46 b	…
LSD	0.07	…
Flowrate		
High	0.53 a	…
Low	0.46 b	…
LSD	0.05	…
Input N concentration		
High	0.33 b	…
Low	0.66 a	…
LSD	0.05	…

ANOVA		
Substrate type	…	<0.001
Flowrate	…	0.011
Input N concentration	…	<0.001
Substrate type X Flowrate	…	0.207
Substrate type X Input N concentration	…	0.014
Flowrate X Input N concentration	…	<0.001
Substrate type X Flowrate X Input N concentration	…	0.058

[a] Mean values of the scaled area under the zoospore activity curve (sAUZAC) were compared using analysis of variance (ANOVA). Means for each treatment factor with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$; ns = no significant differences).

[b] Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates).

Figures

FIGURE 4.1. Laboratory-scale bioreactors: Each substrate—fir bark (a), plastic medium (b) and pine bark (c)—was placed in a plastic tub with black-painted exterior walls and containing baffles (d). A side view schematic (e) illustrates baffle placement and the blue arrows represent expected bulk flow direction.

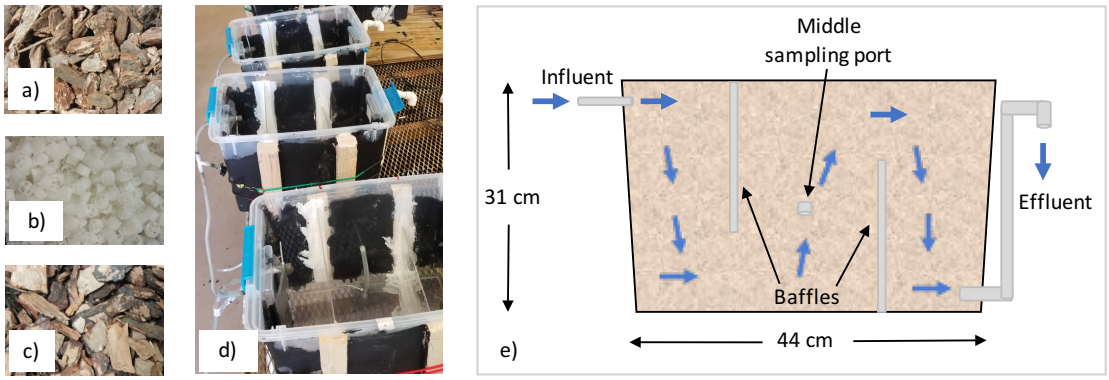


FIGURE 4.2. Recovery of fluorescent dye in the effluent of each substrate treatment (n = 3) during post-experiment tracer studies at a target hydraulic retention time of 2 h. Rhodamine concentrations are normalized to the highest recovered effluent concentration.

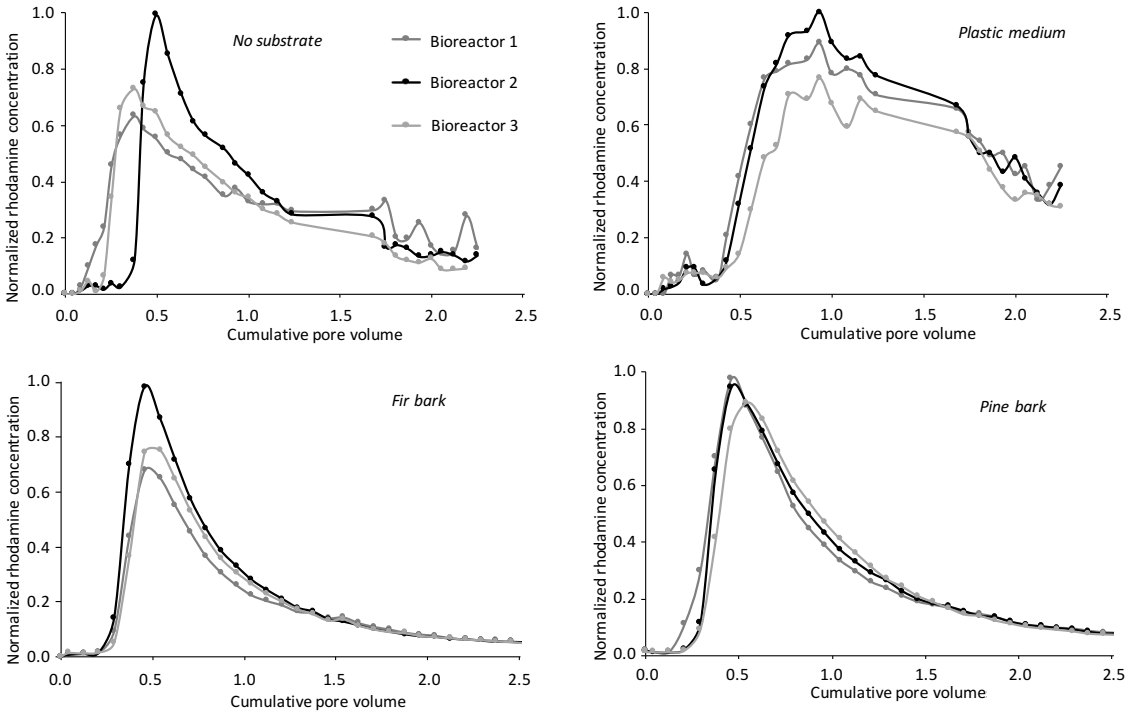


FIGURE 4.3. Percent leaf disk colonization over time for each level of two experimental treatment factors: Low and high input N concentration; low and high flowrate. Data in each graph are means ± standard errors for two trials (n = 6).

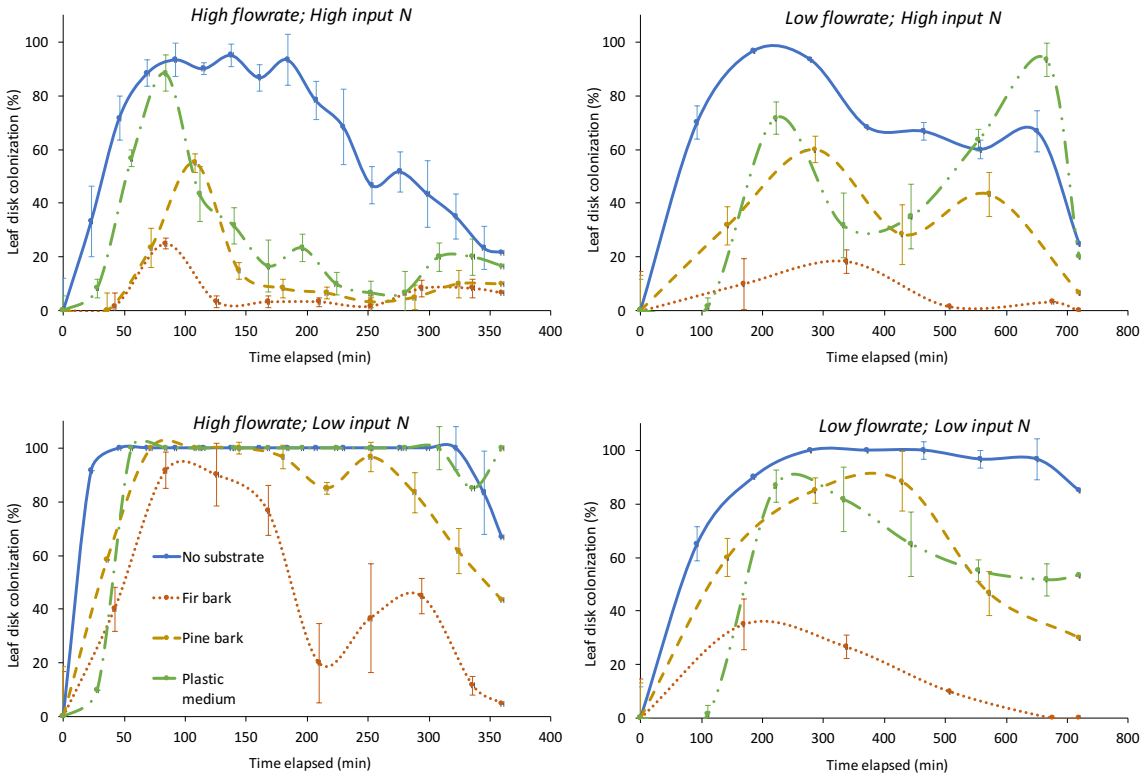


FIGURE 4.4. Comparison of scaled area under the disease progress curve (sAUZAC) using analysis of variance (ANOVA). Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates). Means for each treatment factor combination with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

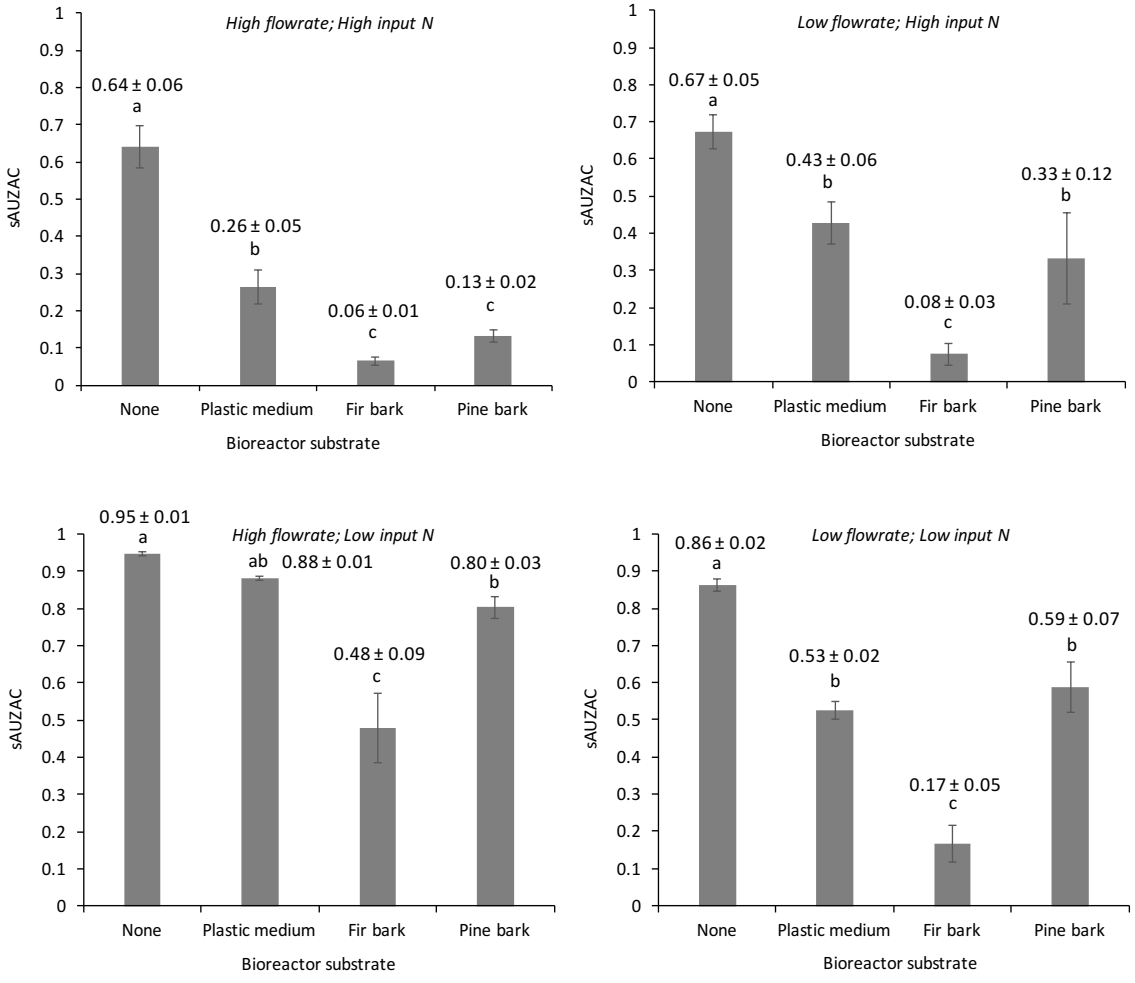


FIGURE 4.5. Mean dissolved organic carbon concentrations from laboratory-scale bioreactors during the 8 weeks before experimental trials were initiated (start-up period). Data are means (n = 3) ± standard errors.

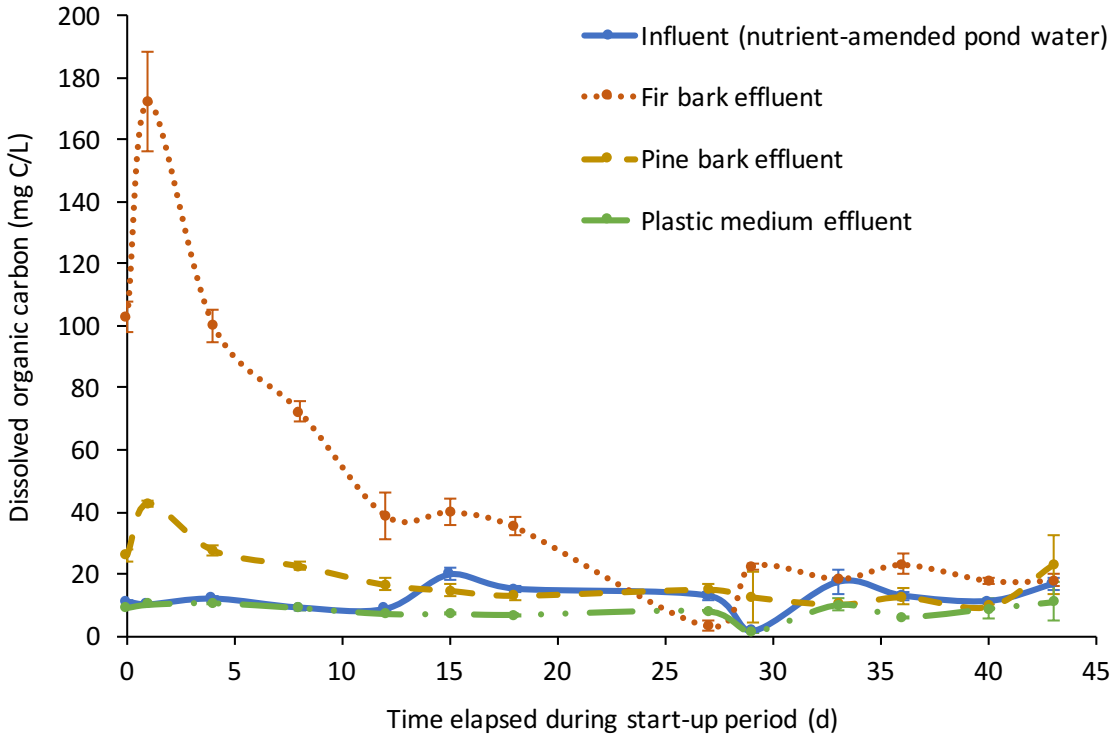


FIGURE 4.6. Average ammonium-N (NH_4-N) and nitrate-N + nitrite-N (NO_2-N + NO_3-N) concentrations from influent as well as effluent from laboratory-scale bioreactors containing no substrate, plastic medium, fir bark, and pine bark. Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates). Means for each treatment factor combination with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

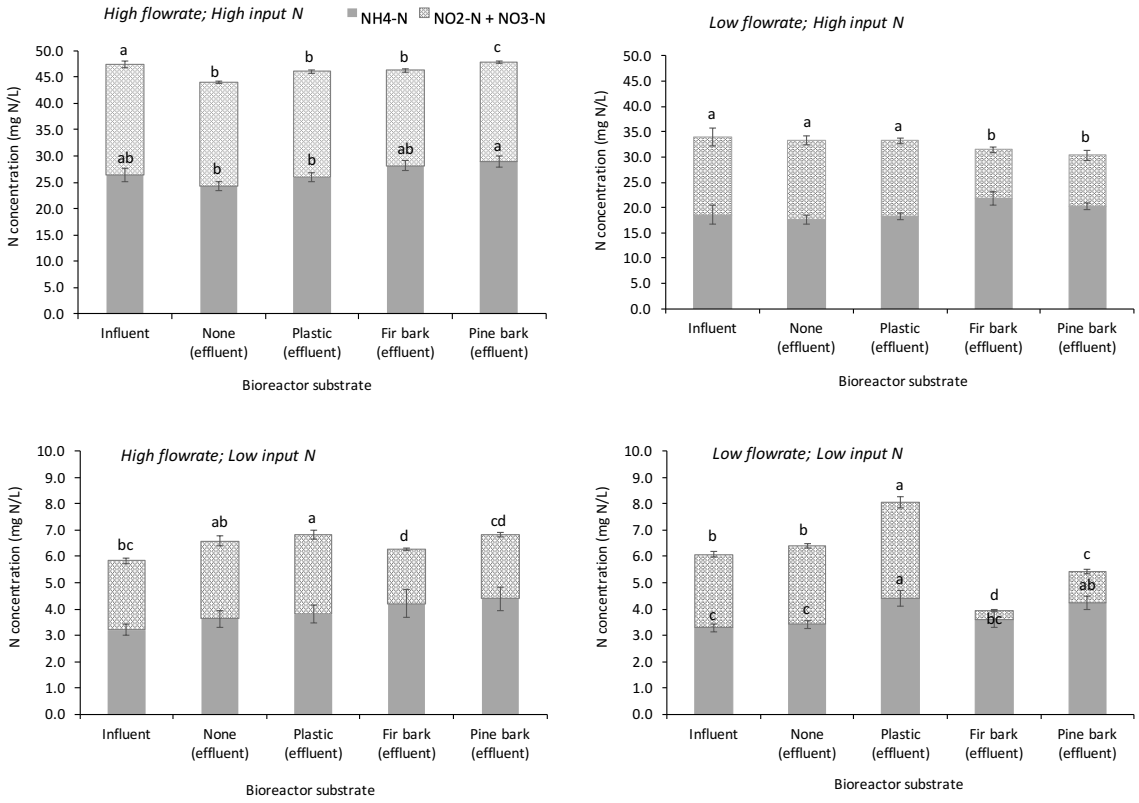


FIGURE 4.7. Average total nitrogen (TN) concentrations from influent as well as effluent from laboratory-scale bioreactors containing no substrate, plastic medium, fir bark, and pine bark. Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates). Means for each treatment factor combination with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

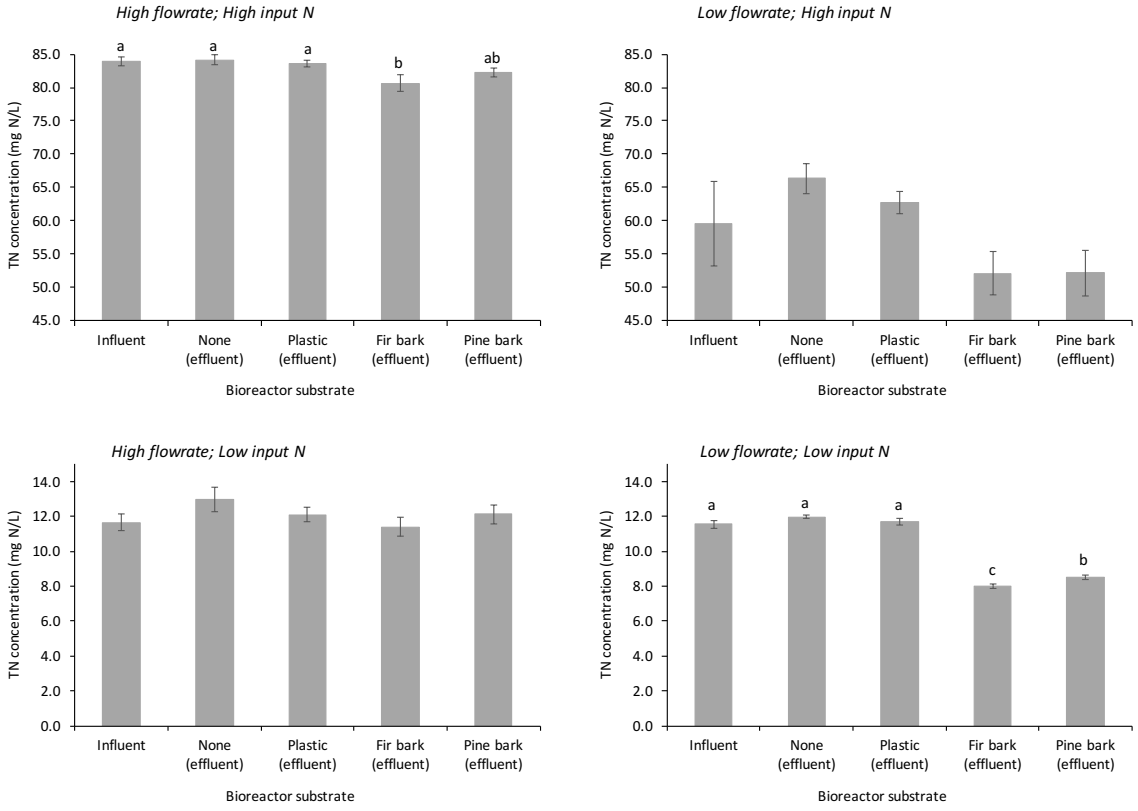


FIGURE 4.8. Average dissolved organic carbon (DOC) concentrations from influent as well as effluent from laboratory-scale bioreactors containing no substrate, plastic medium, fir bark, and pine bark. Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates). Means for each treatment factor combination with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).

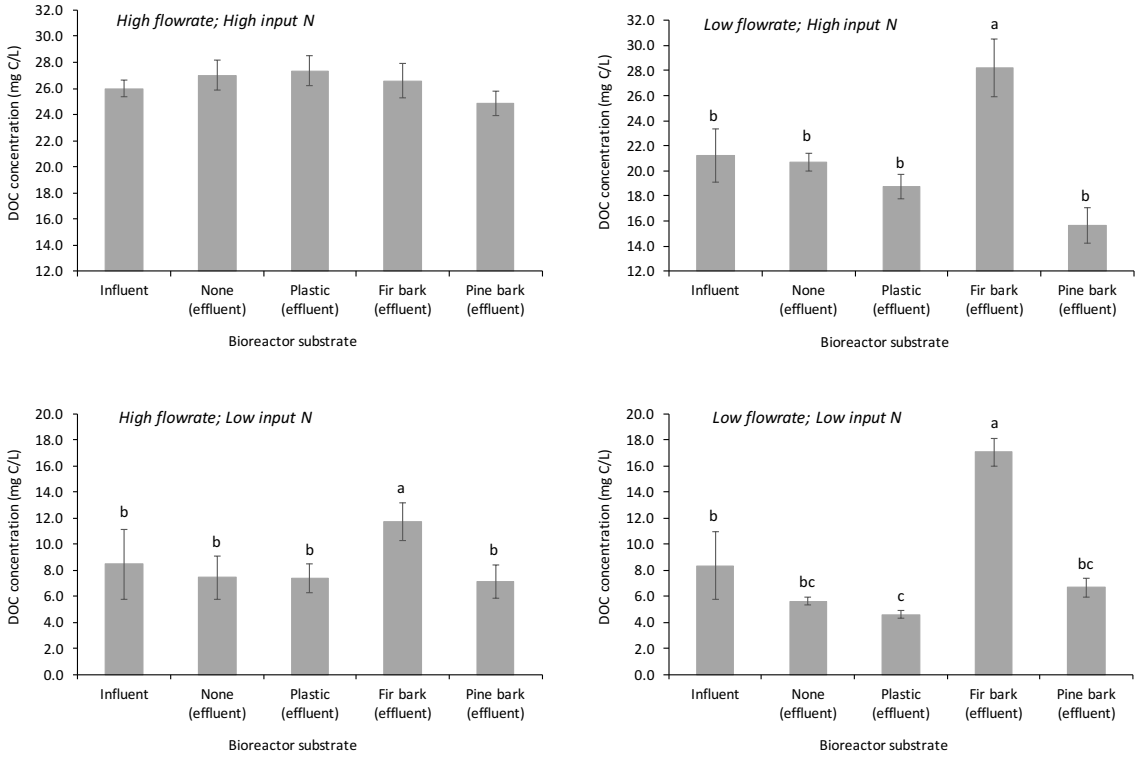
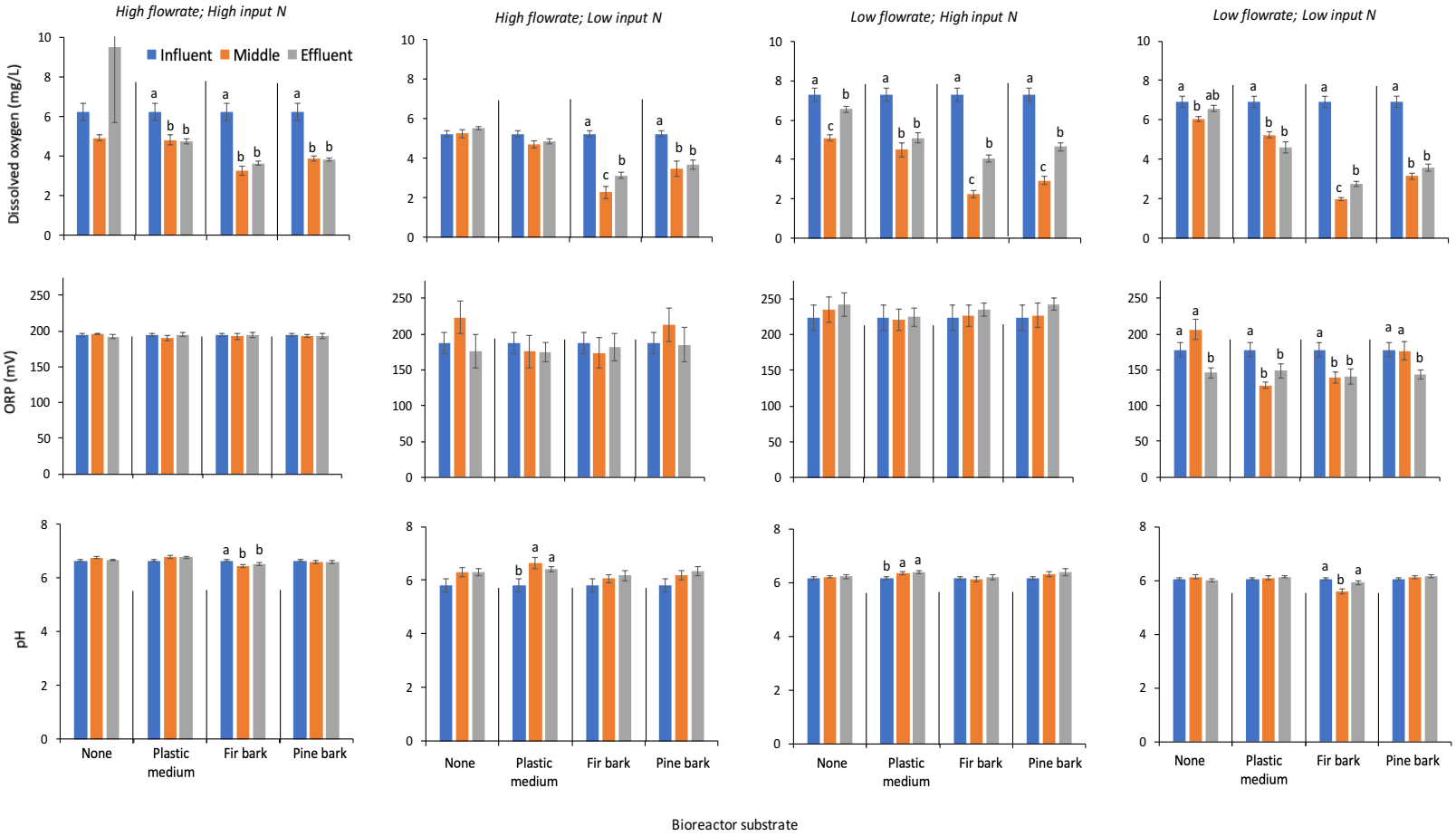


FIGURE 4.9. Average dissolved oxygen (DO) concentration (top row), oxidation reduction potential (ORP, middle row), and pH (bottom row) from samples collected from the influent, middle, and effluent of laboratory-scale bioreactors containing no substrate (none), plastic medium, fir bark, and pine bark. Data are means ± standard errors for two replicated trials for each level of two experimental treatment factor combinations (flowrate and input N concentration) across each substrate type (of which there were 3 physical replicates). Means within each substrate type for each treatment factor combination with different letters are significantly different based on Fisher's least significant difference (LSD; $\alpha = 0.05$).


High flowrate; High input N
High flowrate; Low input N
Low flowrate; High input N
Low flowrate; Low input N
Influent
Middle
Effluent
Dissolved oxygen (mg/L)
ORP (mV)
pH
None
Plastic medium
Fir bark
Pine bark
Bioreactor substrate

CONCLUSIONS

This research demonstrated that:

- The following plant species may be susceptible to the species of *Phytophthora* indicated:
    - *Carex stricta – P. cinnamomi* and *P. cryptogea*
    - *Panicum virgatum – P. nicotianae*
    - *Typha latifolia – P. cinnamomi, P. cryptogea*, and *P. nicotianae.*
- *Agrostis alba, Iris ensata*, and *Pontederia cordata* were not susceptible to *P. cinnamomi, P. citrophthora, P. cryptogea, P. nicotianae,* and *P. palmivora* under the experimental conditions used in this study; therefore, they may be suitable for use in constructed wetland systems because they do not appear to serve as sources of inoculum.
- Each year, plants used in susceptibility trials were purchased from different nurseries located in different regions of the country in an attempt to ensure plant response to *Phytophthora* species was consistent across plant species. Though the plant species evaluated remained consistent from year to year, the genotype and production conditions of plants likely differed from one nursery to the next, and subsequently may have contributed to differences observed in plant root infection from year to year.
- The presence of plant roots seemed to have a negative effect on the ability of zoospores to colonize floating leaf disks for *P. citrophthora, P. cryptogea, P. nicotianae,* and *P. palmivora*. It is possible that zoospores were physically

obstructed by plant roots, chemically inhibited by exudates released from plant roots, and/or biologically inhibited through competition with microbial communities associated with plant roots.

- The presence of *Phytophthora* spp. did not seem to negatively impact aquatic plant growth, even for plants determined to be infected. Additionally, symptoms of root rot were not observed on plants determined to be infected. These seemingly asymptomatic responses of infected plants were unusual, given that infection by *Phytophthora* spp. typically results in negative growth response and root rot of host plants when *Phytophthora* spp. are exposed to plant roots.
- *Typha latifolia* is one of the most commonly used plant species in constructed wetlands around the world due to its ability to remove high levels of nutrients and heavy metals; however, our results demonstrated that *Typha latifolia* plants may be susceptible to multiple species of *Phytophthora*. Therefore, *Typha latifolia* probably should not be used in constructed wetlands receiving agricultural runoff water if treatment of *Phytophthora* species is desired because this plant species may actually serve as a source of inoculum within the constructed wetland system. Obviously, these results need to be confirmed in actual functioning constructed wetlands at nurseries in various locations.
- Pilot-scale floating treatment wetlands (FTWs) established with *Pontederia cordata* reduced the flow-through of viable *Phytophthora nicotianae* zoospores compared to control units containing no FTW at a target hydraulic retention time (HRT) of 4 h. Reductions of zoospores of *P. nicotianae* were not observed for

high flowrate conditions (1 h HRT) or for FTWs planted with *Agrostis alba* at either 1 h or 4 h target HRTs.

- Laboratory-scale bioreactors containing fir bark reduced flow-through of *P. nicotianae* viable zoospores as compared to control units that did not contain any substrate, during low and high input nitrogen concentration conditions (11.6 ± 0.3 mg/L N and 72.0 ± 3.7 mg/L N, respectively) and at flowrates equivalent to a target 2 h and 8 h HRT.
- Laboratory-scale fir bark bioreactors removed ~31% of total nitrogen, which occurred during low flow (8 h HRT) and low input nitrogen (11.6 ± 0.3 mg/L N) conditions.
- High dissolved organic carbon concentrations, carbon:nitrogen ratio, and bulk density of fir bark substrate likely provided access to more readily available labile carbon, which may have contributed to high levels of microbial activity (as evidenced by low effluent dissolved oxygen concentrations) and subsequent remediation of *P. nicotianae*.
- Because results from these studies were generated using small-scale model systems, results will need to be verified by conducting similar experiments under field conditions.

This research provided insight into the susceptibility of common aquatic plant species to species of *Phytophthora* commonly found in nurseries in the southeastern US – information that was previously not known. These findings have great implications for

nursery and greenhouse operations that recycle irrigation water, as viable zoospores may be introduced to susceptible plants growing in onsite water-holding reservoirs through irrigation runoff and drainage water. Infected plants within these reservoirs could then serve as an effective means of inoculum dispersal. Additionally, our results demonstrated that not only are certain aquatic plants not susceptible to selected species of *Phytophthora*, but these plant species may actually be capable of preventing flow-through of and suppressing the infective capabilities of *Phytophthora* spp.

These are the first studies to evaluate the efficacy of small-scale FTWs and agricultural bioreactors to manage *Phytophthora* species in water and some of the only studies to evaluate ecological technologies for plant pathogen remediation at representative field hydraulic conditions. This is the first study to adapt the area under the disease progress curve (AUDPC) method, used by plant pathologists to quantitatively summarize disease progress over time, to quantify zoospore activity over time and, therefore, evaluate the efficacy of a remediation system.

Future studies should investigate the biogeochemical transformations of nutrients and associated microbial communities within ecological remediation systems to gain further insight into the potential of microbiologically aided removal mechanisms. Interdisciplinary approaches such as this one—which involve teams of agricultural engineers, plant pathologists, plant scientists, and hydrologists—will be crucial for future studies seeking to understand the aquatic ecology of plant pathogens and potentially novel ecological methods for remediation. Increased confidence in and implementation of ecological treatment technologies will enable producers of greenhouse and nursery crops

to safely, economically, and sustainably remediate runoff and drainage waters onsite so that they are able reuse this water for irrigation purposes. Recycling water will help agricultural producers gain access to a reliable water source, at a time when access to surface and ground waters is becoming increasingly scarce and contentious due to overuse and increased incidence and severity of droughts.

APPENDICES

A. Preparation of zoospore suspension for controlled model floating treatment wetland system

Methods for preparing zoospore suspension were adapted from those reported by Drechsler et al. (2014) and Nyberg et al. (2014). Isolate #05-0690 of *P. nicotianae* was grown on cV8A. Approximately 100 5-mm-diameter agar plugs were excised from the colony margin and transferred into one 38.1 cm X 25.4 cm X 5.08 cm sterile Pyrex glass baking dishes and about 350 mL of sterile 10% clarified V8B (cV8B = 100 ml of buffered and clarified V8 Juice [Ferguson and Jeffers, 1999] and 900 ml of distilled water) were added to each Pyrex dish. Dishes were then kept at 25°C in the dark for 72 h. Mycelium mats were then strained from each Pyrex dish through metal strainers to remove cV8B, and mats were rinsed twice with about 500 mL of distilled water. About 500 mL of non-sterile soil extract solution (NSSES; Jeffers and Aldwinckle, 1987) were added to each Pyrex dish and cultures were returned to 25°C in the dark for 48 h for sporangia to form. To stimulate zoospore release, colonies were placed at 15°C for approximately 25 min and then moved to room temperature (22 to 25°C) for approximately 50 min. Zoospore suspension from multiple dishes were combined in a beaker and gently mixed. The density of zoospores in this concentrated suspension was quantified using a hemacytometer. A calculated volume of the concentrated suspension was diluted with enough distilled water to make a standard zoospore suspension with a total volume of 38 L and a final density of zoospores of approximately 500

zoospores/mL. About 3 L of this standard zoospore suspension was poured into each experimental unit at the beginning of each trial. A fresh stock of standard zoospore suspension was prepared before each trial of this study.

B. Preparation of zoospore suspension for laboratory-scale bioreactor study

Methods for preparing zoospore inoculum were adapted from those reported by Drechsler et al. (2014) and Nyberg et al. (2014). Isolate no. 05-0690 of *Phytophthora nicotianae* was grown on 10% clarified V8 agar (cV8A). Approximately 100 5-mm-diameter agar plugs were removed from the colony margin and transferred into 38.1 cm × 25.4 cm × 5.1 cm sterile, glass Pyrex baking dishes and about 350 mL of sterile 10% clarified V8 broth [cV8B = 100 ml of buffered and clarified V8 Juice (Ferguson and Jeffers, 1999) and 900 ml of distilled water] were added to each dish. Dishes were held at 25°C in the dark for 72 h. Mycelium mats then were strained from each Pyrex dish through metal strainers to remove cV8B, and mats were rinsed twice with about 500 mL of distilled water. About 500 mL of non-sterile soil extract solution (NSSES; Jeffers and Aldwinckle, 1987) were added to each dish and cultures were returned to 25°C in the dark for 48 h for sporangia to form. To stimulate zoospore release, colonies were placed at 15°C for approximately 25 min and then moved to room temperature (22 to 25 °C) for approximately 50 min. Zoospore suspensions from multiple dishes were combined in a beaker and gently mixed. The density of zoospores in this concentrated suspension was quantified using a hemacytometer. A calculated volume of the concentrated suspension was diluted with enough distilled water to make a standard zoospore suspension with a total volume of 38 L and a final density of zoospores of approximately 5,000 zoospores/mL. This standard zoospore suspension was pumped into each laboratory-scale bioreactor at the beginning of each trial. A fresh stock of standard zoospore suspension was prepared before each trial of this study.