Detection of *Pythium* spp. in Golf Course Irrigation Systems

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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Abstract

Many *Pythium* spp. are causal agents of disease on creeping bentgrass putting greens. Phytopathogenic *Pythium* spp. are known to disseminate through irrigation systems in agricultural settings, and this study provides evidence that *Pythium* spp. also disseminate through golf course irrigation systems. Water samples were collected from irrigation heads and water sources at ten golf courses in Missouri and Kansas. Samples were collected from 2018 to 2019 in April, July, and October. Phosphorus, nitrogen, and chloride concentrations were quantified for irrigation head samples taken each sampling month to determine if these parameters influence *Pythium* frequency. *Pythium* spp. were detected in samples through baiting and membrane filtration. Cultures were isolated on PARP media and DNA was extracted from putative *Pythium* isolates. ITS regions were PCR amplified, sequenced, and compared with Genbank accessions. Phylogenetic trees were constructed using representative sample sequences, sequences from seven morphologically identified reference isolates, and similar Genbank accessions. Detected species include *Lagenidium giganteum, Pythium biforme, P. insidiosum, P. marsipium, P. plurisporium,* and *Saprolegnia hypogyna*. Twenty-one clades lacked species-level resolution, and 14 of these clades were associated with *Pythium* species. Clades A, C, D, E, I, and M contain pathogenic *Pythium* species that cause root and crown rot on creeping bentgrass. Detected *Pythium* communities were dependent on the detection method used and sampling source. *Pythium* frequency and diversity were highest in April 2019. Sample temperature, sampling site, chloride, and nutrient concentrations did not influence *Pythium* frequency in samples. All irrigation systems that utilized surface water sources contained at least three *Pythium* spp. over the course of two years.

Chapter 1:

Literature Review

Putting greens in the transition zone

The transition zone of the United States is a region characterized by high humidity, hot summers, and cold winters. The northern boundary of this region spans from northern Missouri to Maryland and the southern boundary passes through Tennessee and North Carolina (Dunn & Diesburg 2004). The northern climate of this region is conducive for growing cool season grasses while the southern climate is conducive for growing warm season grasses. The space in between experiences both temperature extremes and few grasses used in the turf industry are well adapted to the transition zone climate (Dunn & Diesburg 2004).

In 2010 there were approximately 352 golf facilities in Missouri with an estimated economy of 888.6 million dollars (SRI International 2011). The job security of superintendents who manage these facilities relies on their ability to sustain the health of many hectares of land in a cost-efficient manner. A substantial amount of resources are allocated to putting green maintenance due to the excessive attention required to nurture highly stressed plants and satisfy the high expectations of playability by golfers. Many superintendents have developed management practices specific to their putting greens due to variations in climate and topography within the transition zone, but construction of most putting greens remains consistent throughout.

Most putting greens are built on specifications designed by the United States Golf Association with slight variations. The average putting green is 465 – 697 m^2 (Beard 1982). The USGA recommends that construction should begin by establishing a compact subgrade approximately 41 cm below the desired surface grade (USGA 2015). Drainage trenches should be cut into the subgrade in a gridiron or herringbone pattern with five

meter spacing (Beard 1982). The main drainage pipe should run along the line of maximal fall. Pipes made of perforated plastic are then installed in trenches at a 0.5 – 4% slope (Beard 1982). The water drainage site varies but many locations will drain excess water into sewers, natural bodies of water, or collection ponds. The ideal soil profile for putting greens will maximize drainage through use of large soil particles (USGA 2015). Pea gravel should be used near drainage pipes and sand particles (0.25 – 1 mm diameter) should be used in the root zone (USGA 2015). Turfgrass selection and establishment are often left to the expertise of the superintendent and developer.

Creeping bentgrass (*Agrostis stolonifera L.*) is a cool season grass originating from central Europe and is the predominant species used on putting greens in the transition zone (Dernoeden 2012, Dunn & Diesburg 2004). The advantages of using creeping bentgrass for putting green construction include fine blade morphology, rapid stolon growth, shade tolerance, close mowing tolerance, cold tolerance, and moderate heat tolerance if enough water is provided (Brede 2000). Some disadvantages of creeping bentgrass include high organic matter accumulation, slow establishment, slow spring greenup, and susceptibility to disease (Brede 2000). Optimal temperatures for creeping bentgrass growth are 10 – 21°C. Roots will cease growth as soil temperature exceeds 27°C and heat stress will begin when ambient temperature reaches 30°C (Dernoeden 2012). Many varieties of this species have been produced with favorable adaptations for different environments. In 1956, the University of Pennsylvania released the variety "Penncross" which became the most common turfgrass variety used to establish putting greens due to its resilience and aggressiveness when compared to other cultivars (Dernoeden 2012, Dunn & Diesburg 2004). High quality putting greens are considered

uniform, firm, resilient, and mowed close to the crown (Beard 1982). Nearly all putting greens are mowed daily at or below 3.2 mm to maintain this standard (Dunn & Diesburg 2004). However, creeping bentgrass is more susceptible to infection by various pathogens when exposed to hot summer temperatures and low mowing heights.

The evapotranspiration requirement of a putting green varies based on available sunlight, temperature, and humidity. An average putting green requires 2.54 – 5.08 cm of water application a week from rainfall or irrigation (Dernoeden 2012). Golf courses either purchase municipal water for irrigation or utilize water bodies such as on-site retention ponds, lakes, or effluent sources. Historically, superintendents maintain soil at field capacity or wait for the first signs of wilt to irrigate (Fu & Dernoeden 2009). Soil maintained at field capacity will result in consistently greener turf but creates a conducive environment for fungal, algal, and oomycete pathogen development (Dernoeden 2012, Fu & Dernoeden 2009). Irrigation applied after the first signs of wilt will use less water and help adapt plants to drought, but waiting too long can result in high canopy temperatures and potentially damage turf (Dernoeden 2012, Fu & Dernoeden 2009). Tools such as soil moisture probes are now available to measure volumetric water content in real time to aid in adapting specific watering regiments (Gatlin 2011, Moeller 2012). Putting greens should be maintained at lower volumetric water content if waterborne pathogens become prevalent. Low soil moisture inhibits pathogen development and can potentially decrease the chance of pathogen dissemination.

Pythium taxonomy and life cycle

Oomycota is a phylum of water molds within the kingdom Chromista. For many years the organisms in the phylum were considered true fungi due to similar lifestyle

characteristics such as heterotrophic carbon assimilation, absorptive nutrition, vegetative propagation through mycelium, and production of spores. Evidence from molecular identification methods indicate that Oomycetes are phylogenetically closer to diatoms and brown algae than fungi (Schroeder et al. 2013). Oomycetes differ from true fungi in that their hyphae are coenocytic, nuclei are diploid, and cell walls are composed of cellulose and β-glucans. The order Peronosporales is within the phylum Oomycota and contains plant pathogenic families such as Albuginaceae, Peronosporaceae, and Pythiaceae. The Pythiaceae family contains causal agents of well-known diseases such as late blight of potato and damping off of seedlings. The former disease, caused by the genus *Phytophthora*, contributed to the Irish potato famine while the latter disease, most often caused by the genus *Pythium*, results in major crop losses worldwide (Hendrix & Campbell 1973). *Pythium* and *Phytophthora* share many of the same hosts but *Pythium* is isolated much more frequently from soil and greenhouse irrigation systems (Hendrix & Campbell 1970, Hong et al. 2002).

The genus *Pythium* was first established in 1858 and currently has more than 100 described species (Hendrix & Campbell 1973, Kageyama 2014, Schroeder et al. 2013). *Pythium* distribution is ubiquitous and species occupy a wide range of ecological niches in soil and fresh water (Gill 1970, Parkunan & Ji 2013, Pittis & Colhoun 1984, Schroeder et al. 2013, Shokes & McCarter 1979). Nearly all *Pythium* spp. have the potential to infect one or many hosts across a wide range of taxa including animals, fungi, and plants (Gerbore et al. 2014, Hendrix & Campbell 1973, Mendoza et al. 1993). However, these organisms can survive in soil without a host through a saprophytic lifestyle (Schroeder et al. 2013).

All species of *Pythium* propagate through mycelium and most species are homothallic (Middleton 1943). Most species produce mitotic xenospores as biflagellate zoospores and meiotic memnospores as oospores. Optimal conditions for growth and spore development vary among species (van der Plaats-Niterink 1981). However, one or both spore stages are rare or absent in some species (Sideris 1932). An aqueous environment stimulates mycelium to form sporangia, in which motile zoospores take approximately twenty minutes to form. When the sporangia matures it releases a vesicle, which deteriorates to discharge encapsulated zoospores into solution (Middleton 1943). Zoospores are kidney shaped and exhibit chemotaxis in an aqueous environment (Jones et al. 1991, Raftoyannis & Dick 2006). Zoospores that come in contact with host tissue will retract flagella, encyst, and form a germ tube to initiate infection (Gold 1983, Jones et al. 1991). Oospore development is stimulated by environmental factors such as temperature and sterol availability (van der Plaats-Niterink 1981). Oospore formation is initiated by conjoining male (antheridia) and female (oogonia) gametangia (Middleton 1943). A mature oospore is spherical and encased by a thick cell wall that facilitates constitutive dormancy (Martin & Loper 1999). Zoospores and oospores near host tissue will germinate, produce mycelium, and attempt host infection.

Pythium spp. are traditionally identified through morphological characterization. The *Monograph of the genus Pythium* (van der Plaats-Niterink 1981) is a commonly used reference to identify species based on morphology. The list of traits used for identification is extensive and there are often overlaps between phenotypes of different species (Kageyama 2014). Isolates can exhibit variations in morphology depending on which culture medium is used (Kageyama et al. 1997). Some isolates are impossible to

morphologically identify because sexual structures cannot be produced (Kageyama 2014). Old cultures should not be used in morphological identification due to alterations in behavior and morphology such as loss of hyphal compatibility in heterothallic species and potential thinning of oospore cell walls (Kageyama 2014, Martin & Loper 1999). The difficulty associated with consistent morphological identification has generated a need to explore molecular methods for additional species confirmation.

Techniques like isoelectric focusing of proteins have been used to identify six different *Pythium* species (Adaskaveg et al. 1988). This method establishes a banding pattern for proteins in an immobilized pH gradient gel based on their isoelectric point. Banding patterns for each species is consistent between isolates produced from different geographic regions and culture media. However, isoelectric focusing of proteins was not efficient enough to distinguish between different varieties of the same species (Adaskaveg et al. 1988). Other electrophoretic analyses rely on PCR amplification of DNA and visualization of products in a gel stained with ethidium bromide.

A commonly used locus for PCR identification is the internal transcribed spacer (ITS) region. This region of genomic DNA is situated between small and large-subunit rRNA sequences and is highly conserved throughout taxa. An oomycete specific ITS primer, ITSOo, has been designed to produce a 1 kb amplicon when paired with the universal primer ITS5 (Nikolcheva & Bärlocher 2004). Species-specific ITS primers are available for nearly all *Pythium* species (Schroeder et al. 2013). Primers have also been designed to amplify partial ITS sequences of five causal agents of Pythium root rot and function in a multiplex PCR (Asano et al. 2010). ITS regions can be digested with restriction enzymes to produce restriction fragment length polymorphisms (RFLP) with

distinct banding patterns (Chen 1992, Kageyama et al. 2005). RFLP analysis has been used to identify *Pythium* spp. causing carrot cavity spot, decline of reed stands, and ginseng root disease (Schroeder et al. 2013).

DNA sequences can be used to distinguish between PCR amplicons of equal length and detect different species within a mixed *Pythium* culture. Some *Pythium* spp. have identical ITS sequences but other loci such as cytochrome oxidase (*cox*) genes can be used for greater resolution and identification (Bala et al. 2010, Choi et al. 2015). Sanger sequencing methods are capable of producing clear sequences if only one species is present during DNA extraction. Unidirectional sequences are adequate for identification if they are long and clear (Schroeder et al. 2013). Next generation sequencing methods such as pyrosequencing assays have the potential to produce clear sequence results from multiculture DNA extracts of oomycetes (Vettraino et al. 2012). The ITS sequences of numerous *Pythium* spp. have been uploaded to Genbank for comparison using the Basic Local Alignment Search Tool (BLAST, Levesque & De Cock 2004). Phylogenetic trees have been constructed using ITS, *cox*, and large nuclear ribosomal subunit genes (Kageyama 2014, Kageyama et al. 2005, Levesque & De Cock 2004, Martin 2000, Matsumoto et al. 1999). The clade system applied by Lévesque and De Cock (2004) is considered a standard reference for subsequent phylogenetic studies and suggests that shape of sporangia is the best morphological characteristic for distinguishing clades (Kageyama et al. 2005, Levesque & De Cock 2004). Evidence from large-subunit ribosomal DNA D1/D2 and *cox* sequencing has caused many traditional *Pythium* spp. to be reclassified into the genera *Elongisporangium, Globisporangium, Ovatisporangium,* and *Pilasporangium* (Uzuhashi et al. 2010).

Pathogenicity of *Pythium* spp.

Pythium spp. are known causal agents of a variety of plant diseases such as damping off of seedlings, blight, and root rot. Root exudates initiate chemotaxis of nearby zoospores (Donaldson & Deacon 1992, Jones et al. 1991). Zoospores often infect near the zone of elongation but have also been observed infecting mature root cells and root hairs (Jones et al. 1991, Kraft et al. 1967). Zoospores will aggregate near the first site of infection and high zoospore counts are associated with increased disease severity (Kraft et al. 1967, Raftoyannis & Dick 2002). Species such as *P. graminicola* cannot generate enough pressure to penetrate creeping bentgrass roots directly, suggesting host-degrading enzymes are necessary for infection (MacDonald et al. 2002).

Disease symptoms will differ depending on the infecting *Pythium* species. Non-pathogenic species like *P. torulosum* produce no disease after infecting creeping bentgrass (Feng & Dernoeden 1999, Kerns & Tredway 2008). Pathogenic species can infect creeping bentgrass and cause diseases such as Pythium root rot, Pythium blight, and Pythium root dysfunction (Kerns & Tredway 2010, Smiley et al. 2005). Putting greens that exhibit a *Pythium* disease do not provide an ideal putting experience for golfers. Management practices vary between *Pythium* spp. and proper identification and sometimes speciation of causal agents can be crucial to controlling *Pythium* diseases.

Pythium diseases are diagnosed through symptomology, signs of the pathogen, and identification of the infecting *Pythium* species. Signs of *Pythium* infection include the presence of oospores in symptomatic tissue, production of zoospores from symptomatic tissue, and, in the case of Pythium blight, mycelia observed on the leaf surface. Pythium foliar blight shares causal agents with Pythium root rot but is primarily caused by *P.*

aphanidermatum and *P. graminicola* (Smiley et al. 2005). Pythium blight develops most in hot and humid weather. Symptoms include brown patches of turf and a greasy texture on the leaf surface (Smiley et al. 2005).

Pythium root rot is caused by a variety of *Pythium* and *Globisporangium* spp. such as *G. ultimum, P. aphanidermatum, P. graminicola, P. vanterpoolii,* and many other species (Abad et al. 1994, Hsiang et al. 1995). This disease can develop quickly in saturated soils and symptoms may present at any point in the growing season (Smiley et al. 2005). Symptoms include root necrosis and irregularly shaped areas of chlorotic turf ranging 4 – 8 cm in diameter. At high temperatures the smaller chlorotic patches can coalesce to form large areas of symptomatic turf (Smiley et al. 2005). Pythium root dysfunction is caused by *P. volutum, P. aristosporum,* and *P. arrhenomanes* (Feng & Dernoeden 1999, Kerns & Tredway 2008). Pythium root dysfunction develops most in warm and dry conditions (Kerns & Tredway 2010). Symptoms include patches of orange or yellow turf ranging 10 – 50 cm, stunted roots that lack root hairs, and a tan discoloration of root tissue (Hodges & Coleman 1985, Kerns & Tredway 2008).

Disease severity is influenced by many factors relating to the pathogen, environment, and host. Some *Pythium* spp. are considered more aggressive than others (Abad et al. 1994, Hsiang et al. 1995). However, isolates of the same species can vary in aggressiveness and in rare cases even *P. torulosum* can be pathogenic (Nelson & Craft 1991). A single plant can host more than one species at a time. Disease severity may be influenced when multiple causal agents are present, but most combinations have not been tested. Temperature and soil moisture are considered important environmental factors that influence *Pythium* pathogenicity (Biesbrock & Hendrix 1970, Hodges & Campbell

1994, Hsiang et al. 1995, Raftoyannis & Dick 2002, Thomson et al. 1971). High transpiration rates caused by summer heat will exacerbate the chlorotic symptoms of Pythium root rot and Pythium root dysfunction due to inadequate water uptake by infected roots. Constant soil saturation will promote zoospore production and increase the chance of spreading disease. Lastly, creeping bentgrass is more susceptible to infection by pathogenic species such as *P. volutum* and *P. arrhenomanes* during the first 10 years of establishment (Kerns & Tredway 2010).

Water currents are the primary vector for zoospore dissemination. Therefore, disease progression will often move down slopes and putting greens in low-lying areas are at higher risk for infection. However, species such as *G. ultimum* var. *ultimum* do not produce zoospores and must rely on mycelia for natural dissemination. Mycelia fragments and zoospores can also disseminate through irrigation systems in agriculture and greenhouse settings (Bush et al. 2003, Hong & Moorman 2005, Pittis & Colhoun 1984, Pottorff & Panter 1997). However, *Pythium* dissemination through golf course irrigation systems has not been reported.

Pythium disease management

Fungicide programs to control *Pythium* disease on putting greens can cost approximately $6,100 per hectare each year (Tim Burch, personal communication, 12/11/2019). Fungicide efficacy varies among *Pythium* diseases due to differences in fungicide tolerance between causal agents. Pythium blight is best controlled with applications of cyazofamid, azoxystrobin, fosetyl-Al, mefenoxam, and propamocarb (Vincelli et al. 2017). Pythium root rot and Pythium root dysfunction are best controlled with cyazofamid. However, Pythium root dysfunction is also controlled with Qol and Qil

fungicides such as pyraclostrobin, fluoxastobin, and azoxystrobin (Kerns & Tredway 2010).

Many cultural options are available to suppress *Pythium* disease on putting greens such as resistant cultivars, restricting excess soil moisture, and increasing beneficial microbe populations. Resistant cultivars such as Crenshaw and SYN-96 provide some resistance to Pythium root dysfunction (Kerns & Tredway 2008). Excessive irrigation has been shown to increase disease severity of Pythium root rot of highbush blueberry (Bryla & Linderman 2007). Therefore, deep and infrequent irrigation methods should be practiced to reduce excess soil moisture. Beneficial microbes such as heterotrophic bacteria and *Trichoderma harzianum* have been shown to suppress *Pythium* growth and infection (Martin & Loper 1999, Nelson & Craft 1992).

Compost applied through topdressing may also suppress *Pythium* growth by introducing beneficial microbes (Craft & Nelson 1996). Biological control agents like *Pythium oligandum* are capable of suppressing pathogenic *Pythium* spp. through a variety of mechanisms such as competition for niche space, competition for nutrients, antibiosis, initiation of host plant defenses, and hyperparasitism (Gerbore et al. 2014, Martin & Hancock 1987, Martin & Loper 1999). However, *P. oligandum* has not been studied for biological control effects in a turf setting.

Other cultural practices for *Pythium* management involve avoiding initial colonization and preventing dissemination. Putting greens should not be established in low-lying areas near water drainage sites because these areas are prone to accumulation of zoospores in soil solution. Irrigation systems should be established with fresh pipes because mycelium may adhere to old pipes (Al-Sa'di et al. 2008). Pathogen free water

sources should be selected to prevent dissemination through irrigation systems. A Colorado greenhouse study showed well and city water did not contain *Pythium* (Pottorff & Panter 1997).

If no pathogen free water sources are available then there are a variety of water treatment methods such as heat, UV sterilization, and chemical additives such as chlorine, ozone, and fungicides (Hong & Moorman 2005, Stewart-Wade 2011). Other methods of water treatment such as slow filtration, pressure, and sonication are available but these lack efficiency or supporting evidence (Stewart-Wade 2011). UV-C (~254nm) sterilization systems have the capacity to disrupt DNA and RNA, resulting in disinfestation of plant pathogens in irrigation water (Hong & Moorman 2005, Stanghellini et al. 1984). A dosage of 250 mJ/cm^2 is required to kill nearly all pathogens but only 80 – 100 mJ/cm^2 is required to eliminate *Pythium* from irrigation water (Newman 2004, Zhang & Tu 2000). The required dosage is greater if the water is turbid or nutrient rich (Hong & Moorman 2005, Sutton et al. 2000). Water heat-treatments of 95°C for 30 seconds will kill nearly all plant pathogens in solution (Newman 2004). Heat-treatments of 51°C for 15 seconds is sufficient to kill *P. aphanidermatum* (Amsing & Runia 2000).

Numerous chemical additives are available to treat irrigation water. These treatments should be selected with care because some lack efficacy data or pose phytotoxicity risk (Stewart-Wade 2011). Many additives increase oxidative reduction potential (ORP) of water to strip cell membranes of electrons, causing subsequent death of waterborne organisms (Lang et al. 2008). A variety of compounds can be added to increase ORP such as hypochlorite, calcium hypochlorite, chlorine, chlorine dioxide, or

ozone (Newman 2004, Raudales et al. 2014). Specific modes of action differ slightly between additives and the required dosage to kill *Pythium* is dependent on pH, structures of *Pythium* present, and type of additive (Lang et al. 2008, Newman 2004, Raudales et al. 2014). Phytotoxicity is associated with a high chlorine dosage but this threshold is higher than the required dose to kill *Pythium* (Raudales et al. 2014). Fungicide additions to irrigation water are well studied but this treatment method is expensive and potentially contributes to fungicide tolerance in waterborne pathogens (Stewart-Wade 2011).

Pythium detection

Pythium detection methods in host tissue include baiting, culturing with selective media, and cultureless methods using molecular techniques such as PCR amplification or enzyme-linked immunosorbent assays (ELISA). Baiting techniques exploit zoospore chemotaxis by spatially separating healthy and infected tissue in shared water. Zoospores develop on infected tissue and colonize healthy bait tissue. The subsequently infested bait can then be used for isolation with culture media. *Pythium* spp. can be isolated from host and bait tissue with corn meal agar amended with 10 mg/L pimaricin, 250 mg/L ampicillin, 10 mg/L rifampicin, and 100 mg/L pentachloronitrobenzine (Bush et al. 2003, Jeffers & Martin 1986). *Pythium* spp. can be detected in host tissue without culturing through DNA extraction and PCR amplification using selective primers. ELISA kits have been designed to detect *Pythium* in symptomatic host tissue, but these kits may result in false positives when *Phytophthora* spp. are present (Pringsh 1990). Similar methods are available to detect *Pythium* in irrigation water.

Methods used for detecting *Pythium* in irrigation water include baiting, membrane filtration, ELISA, and zoospore trapping immunoassays (ZTI). Viable zoospores can be

baited directly from irrigation water and subsequently colonized tissue can be used for isolation. Membrane filters can be used to separate motile zoospores, encysted zoospores and mycelial fragments from irrigation water. A comparison of different materials and pore sizes demonstrated that Durapore filters (five µm pores) are the most efficient for filtering *Pythium* from water samples (Hong et al. 2002). *Pythium* can be isolated directly from membrane filters by plating the filter on selective media or indirectly by washing membrane filters with a .09% agar solution and spreading the solution on selective media (Pettitt et al. 2002). *Pythium* DNA can also be directly extracted from filter membranes and bait tissue to bypass culture production. Polyclonal antibodies are used in detection methods such as ZTI and ELISA (Pettitt et al. 2002). ELISA tests are commercially available to screen irrigation water for *Pythium* but these tests lack specificity and are unable to differentiate between living and dead zoospores in water samples (Ali-Shtayeh et al. 1991, Wakeham et al. 1997). A ZTI binds zoospores to a membrane, initiates germ tube development, and polyclonal antibodies are bound to developing germ tubes to facilitate dye staining. Germinated zoospores can then be visualized and counted using a microscope (Pettitt et al. 2002).

Each detection method is associated with specific advantages and disadvantages. Isolation techniques provide live cultures but select for the fastest growing species in a sample. Cultureless methods are less time consuming and more likely to detect slower growing species. ELISA tests are user-friendly but not reliable for species-specific detection. A ZTI is a sensitive method for quantifying the number of viable zoospores in a sample but other methods such as direct plating of membrane filters are more consistent (Pettitt et al. 2002). Previous studies have compared detection methods based on positive

detection of *Pythium* in a sample (Pringsh 1990). The species diversity of *Pythium* spp. in a sample can be quantified through Simpson and Shannon diversity indices (Redekar et al. 2019, Weiland 2011). Detection methods can be compared using average species diversity but these values will be influenced by primer selection (Esmaeili Taheri et al. 2017) and number of sequences used during analysis.

Research objectives

Pythium root rot is one of the most common biotic issues found on creeping bentgrass putting greens in Missouri. *Pythium* spp. are known to spread locally through zoospore movement. However, *Pythium* dissemination to putting greens is not fully understood when putting greens are newly established, highly elevated, or isolated from areas of *Pythium* incidence. *Pythium* has been introduced to agriculture and greenhouse settings through irrigation systems and this study focuses on *Pythium* dissemination through golf course irrigation. This research compares methods used to detect *Pythium* spp. in irrigation water, evaluates the influence of temperature, chloride, and macronutrients on *Pythium* frequency, and establishes golf course irrigation systems as a source of *Pythium* inoculum. Specific research objectives are:

1. Detect *Pythium* spp. dissemination through golf course irrigation systems in Missouri and Kansas
2. Determine if baiting and membrane filtration detect identical *Pythium* communities, determine if *Pythium* communities differ between sampling sources, and assess seasonal changes of *Pythium* communities.

3. Determine if *Pythium* frequency differs between sampling site and month, and determine if temperature, macronutrients, or chloride influence total *Pythium* frequency.

Literature cited

Abad, Z. G., Shew, H. D., & Lucas, L. T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology, 84(9), 913-921.

Adaskaveg, J. E., Stanghellini, M. E., Gilbertson, R. L., & Egen, N. B. 1988. Comparative protein studies of several *Pythium* species using isoelectric focusing. Mycologia, 80(5), 665-672.

Al-Sa'di, A. M., Drenth, A., Deadman, M. L., Al-Said, F. A., Khan, I., & Aitken, E. A. B. 2008. Potential sources of *Pythium* inoculum into greenhouse soils with no previous history of cultivation. Journal of Phytopathology, 156(7-8), 502-505.

Ali-Shtayeh, M. S., MacDonald, J. D., & Kabashima J. 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Disease, 75(3), 305-311.

Amsing, J. J., & Runia, W. T. 2000. Lethal Temperatures of Soilborne Pathogens in Recirculation Water from Closed Cultivation Systems. World Congress on Soilless Culture: Agriculture in the Coming Millennium, 554, 333-340.

Asano, T., Senda, M., Suga, H., & Kageyama, K. 2010. Development of multiplex PCR to detect five *Pythium* species related to turfgrass diseases. Journal of Phytopathology, 158(9), 609-615.

Bala, K., Robideau, G. P., Désaulniers, N., De Cock A. W. A. M., & Lévesque, C. A. 2010. Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada. Persoonia: Molecular Phylogeny and Evolution of Fungi, 25, 22.

Beard, J. B. 1982. Turf management for golf courses, John Wiley & Sons, Hoboken, NJ

Biesbrock, J. A., & Hendrix, F. F. 1970. Influence of soil water and temperature on root necrosis of peach caused by *Pythium* spp. Phytopathology, 60(5), 880-882.

Brede, D. 2000. Turfgrass maintenance reduction handbook: sports, lawns, and golf. Ann Arbor Press, Chelsea, MI

Bryla, D. R., & Linderman, R. G. 2007. Implications of irrigation method and amount of water application on *Phytophthora* and *Pythium* infection and severity of root rot in highbush blueberry. HortScience, 42(6), 1463-1467.

Bush, E. A., Hong, C., & Stromberg, E. L. 2003. Fluctuations of *Phytophthora* and *Pythium* spp. in components of a recycling irrigation system. Plant Disease, 87(12), 1500-1506.

Chen, W. 1992. Restriction fragment length polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. Phytopathology-New York and Baltimore then St Paul, 82, 1467-1467.

Choi, Y. J., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., Ploch, S., Shin, H. D., Shivas, R. G., Telle, S., Voglmayr, H., & Thines, M. 2015. Towards a universal barcode of oomycetes - a comparison of the cox1 and cox2 loci. Molecular Ecology Resources, 15(6), 1275-1288.

Craft, C. M., & Nelson, E. B. 1996. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium* graminicola. Applied and Environmental Microbiology, 62(5), 1550-1557.

Dernoeden, P. H. 2012. Creeping Bentgrass Management, CRC Press, Boca Raton, FL.

Donaldson, S. P., & Deacon, J. W. 1992. Role of calcium in adhesion and germination of zoospore cysts of *Pythium*: a model to explain infection of host plants. Microbiology, 138(10), 2051-2059.

Dunn, J., & Diesburg, K. 2004. Turf Management in the Transition Zone, John Wiley & Sons.

Esmaeili Taheri, A., Chatterton, S., Gossen, B. D., & McLaren, D. L. 2017. Degenerate ITS7 primer enhances oomycete community coverage and PCR sensitivity to *Aphanomyces* species, economically important plant pathogens. Canadian Journal of Microbiology, 63(9) 769-779.

Feng, Y., & Dernoeden, P. H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Disease, 83(6), 516-520.

Fu, J., & Dernoeden, P. H. 2009. Creeping bentgrass putting green turf responses to two irrigation practices: Quality, chlorophyll, canopy temperature, and thatch–mat. Crop Science, 49(3), 1071-1078.

Gatlin, R. 2011. A giant leap forward: How soil moisture meters are changing the game of putting green irrigation. USGA Green Section Record, 49, 1-3.

Gerbore, J., Benhamou, N., Vallance, J., Le Floch, G., Grizard, D., Regnault-Roger, C., & Rey, P. 2014. Biological control of plant pathogens: Advantages and limitations seen through the case study of *Pythium* oligandrum. Environmental Science and Pollution Research, 21(7), 4847-4860.

Gill, D. L. 1970. Pathogenic *Pythium* from irrigation ponds. Plant Disease Reporter, 54(12), 1077-1079.

Gold, S. E. 1983. The effect of temperature on Pythium root rot of spinach grown under hydroponic conditions. M.S., University of Arizona.

Hendrix, Jr, F. F., & Campbell, W. A. 1970. Distribution of *Phytophthora* and *Pythium* species in soils in the continental United States. Canadian Journal of Botany, 48(2), 377-384.

Hendrix, F. F. & Campbell, W. A. 1973. *Pythiums* as plant pathogens. Annual Review of Phytopathology, 11(1), 77-98.

Hodges, C. F., & Campbell, D. A. 1994. Infection of adventitious roots of *Agrostis palustris* by *Pythium* species at different temperature regimes. Canadian Journal of Botany, 72(3), 378-383.

Hodges, C. F., & Coleman, L. W. 1985. *Pythium*-induced root dysfunction of secondary roots of *Agrostis palustris*. Plant Disease, 69(4), 336-340.

Hong, C. X., & Moorman, G. W. 2005. Plant pathogens in irrigation water: challenges and opportunities. Critical Reviews in Plant Sciences, 24(3), 189-208.

Hong, C., Richardson, P. A., & Kong, P. 2002. Comparison of membrane filters as a tool for isolating pythiaceous species from irrigation water. Phytopathology, 92(6), 610-616.

Hsiang, T., Wu, C., Yang, L., & Liu, L. 1995. Pythium root rot associated with cool-season dieback of turfgrass in Ontario and Quebec. Canadian Plant Disease Survey, 75(2), 191-195.

Jeffers, S. N., & Martin, S. B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease, 70(11), 1038-1043.

Jones, S. W., Donaldson, S. P., & Deacon, J. W. 1991. Behaviour of zoospores and zoospore cysts in relation to root infection by *Pythium* aphanidermatum. New Phytologist, 117(2), 289-301.

Kageyama, K. 2014. Molecular taxonomy and its application to ecological studies of *Pythium* species. Journal of General Plant Pathology, 80(4), 314-326.

Kageyama, K., Nakashima, A., Kajihara, Y., Suga, H., & Nelson, E. B. 2005. Phylogenetic and morphological analyses of *Pythium* graminicola and related species. Journal of General Plant Pathology, 71(3), 174-182.

Kageyama, K., Ohyama, A., & Hyakumachi, M. 1997. Detection of *Pythium ultimum* using polymerase chain reaction with species-specific primers. Plant Disease, 81(10), 1155-1160.

Kerns, J. P., & Tredway, L. P. 2008. Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Disease, 92(6), 862-869.

Kerns, J. P., and Tredway, L. P. 2010. Pythium root dysfunction of creeping bentgrass. Plant Health Progress, 11(1), 40.

Kraft, J. M., Endo, R. M., & Erwin, D. C. 1967. Infection of primary roots of Bentgrass by zoospores of *Pythium aphanidermatum*. Phytopathology, 57(1), 86-90.

Lang, J. M., Rebits, B., Newman, S. E., & Tisserat, N. 2008. Monitoring mortality of *Pythium* zoospores in chlorinated water using oxidation reduction potential. Plant Health Progress, 10, 1094.

Levesque, C. A., & De Cock, A. W. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Research, 108(12), 1363-1383.

MacDonald, E., Millward, L., Ravishankar, J. P., & Money, N. P. 2002. Biomechanical interaction between hyphae of two *Pythium* species (Oomycota) and host tissues. Fungal Genetics and Biology, 37(3), 245-249.

Martin, F. N. 2000. Phylogenetic relationships among some *Pythium* species inferred from sequence analysis of the mitochondrially encoded cytochrome oxidase II gene. Mycologia, 92(4), 711-727.

Martin, F. N., & Hancock, J. G. 1987. The use of *Pythium oligandrum* for biological control of preemergence damping-off caused by *P. ultimum*. Phytopathology, 77(7), 1013-1020.

Martin, F. N., & Loper, J. E. 1999. Soilborne plant diseases caused by *Pythium* spp.: ecology, epidemiology, and prospects for biological control. Critical Reviews in Plant Sciences, 18(2), 111-181.

Matsumoto, C., Kageyama, K., Suga, H., & Hyakumachi, M. 1999. Phylogenetic relationships of *Pythium* species based on ITS and 5.8 S sequences of the ribosomal DNA. Mycoscience, 40(4), 321-331.

Mendoza, L., Hernandez, F., & Ajello, L. 1993. Life cycle of the human and animal oomycete pathogen *Pythium insidiosum*. Journal of Clinical Microbiology, 31(11), 2967-2973.

Middleton, J. T. 1943. The taxonomy, host range and geographic distribution of the genus *Pythium*. Memoirs of the Torrey Botanical Club, 20(1), 1-171.

Moeller, A. 2012. Identify soil moisture status more accurately than ever before. Michigan State Univ Green Section Rec, 50(9), 1-5.

Nelson, E. B., & Craft, C. M., 1991. Identification and comparative pathogenicity of *Pythium* spp. from roots and crowns of turfgrasses exhibiting symptoms of root rot. Phytopathology, 81(12), 1529-1536.

Nelson, E. B., & Craft, C. M., 1992. A miniaturized and rapid bioassay for the selection of soil bacteria suppressive to Pythium blight of turfgrasses. Phytopathology, 82(2), 206-210.

Newman, S. E. 2004. Disinfecting irrigation water for disease management. 20th Annual Conference on Pest Management on Ornamentals, 970, 1-10.

Nikolcheva, L. G., & Bärlocher, F. 2004. Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. Mycological Progress, 3(1), 41-49.

Parkunan, V., & Ji, P. 2013. Isolation of *Pythium litorale* from irrigation ponds used for vegetable production and its pathogenicity on squash. Canadian Journal of Plant Pathology, 35(3), 415-423.

Pettitt, T. R., Wakeham, A. J., Wainwright, M. F., & White, J. G. 2002. Comparison of serological, culture, and bait methods for detection of *Pythium* and *Phytophthora* zoospores in water. Plant Pathology, 51(6), 720-727.

Pittis, J. E., & Colhoun, J. 1984. Isolation and identification of pythiaceous fungi from irrigation water and their pathogenicity to *Antirrhinum*, tomato and *Chamaecyparis lawsoniana*. Journal of Phytopathology, 110(4), 301-318.

Pottorff, L. P., & Panter, K. L. 1997. Survey of *Pythium* and *Phytophthora* spp. in irrigation water used by Colorado commercial greenhouses. HortTechnology, 7(2), 153-155.

Pringsh, P. 1990. Comparison of serological and culture plate methods for detecting species of *Phytophthora*, *Pythium*, and *Rhizoctonia* in ornamental plants. Plant Disease, 74(9), 655.

Raftoyannis, Y., & Dick, M. W. 2002. Effects of inoculum density, plant age and temperature on disease severity caused by pythiaceous fungi on several plants. Phytoparasitica, 30(1), 67-76.

Raftoyannis, Y., & Dick, M. W. 2006. Zoospore encystment and pathogenicity of *Phytophthora* and *Pythium* species on plant roots. Microbiological Research, 161(1), 1-8.

Raudales, R. E., Parke, J. L., Guy, C. L., & Fisher, P. R. 2014. Control of waterborne microbes in irrigation: A review. Agricultural Water Management, 143, 9-28.

Redekar, N. R., Eberhart, J. L., & Parke, J. L. 2019. Diversity of *Phytophthora*, *Pythium*, and *Phytopythium* species in recycled irrigation water in a Container Nursery. Phytobiomes Journal, 3(1), 31-45.

Schroeder, K. L., Martin, F. N., de Cock, A. W., Lévesque, C. A., Spies, C. F., Okubara, P. A., & Paulitz, T. C. 2013. Molecular detection and quantification of *Pythium* species: evolving taxonomy, new tools, and challenges. Plant Disease, 97(1), 4-20.

Shokes, F. M., & McCarter, S. M. 1979. Occurrence, dissemination, and survival of plant pathogens in surface irrigation ponds in southern Georgia. Phytopathology, 69(5), 510-516.

Sideris, C. P. 1932. Taxonomic studies in the family Pythiaceae II. *Pythium*. Mycologia, 24(1), 14-61.

Smiley, R. W., Dernoeden, P. H., & Clarke, B. B. 2005. Compendium of turfgrass diseases, American Phytopathological Society, St. Paul, MN

SRI International. 2011. The Missouri Golf Economy. Golf 20/20

Stanghellini, M. E., Stowell, L. J., & Bates, M. L. 1984. Control of root rot of spinach caused by *Pythium aphanidermatum* in a recirculating hydroponic system by ultraviolet irradiation. Plant Disease, 68(12), 1075-1076.

Stewart-Wade, S. M. 2011. Plant pathogens in recycled irrigation water in commercial plant nurseries and greenhouses: their detection and management. Irrigation Science, 29(4), 267-297.

Sutton, J. C., Yu, H., Grodzinski, B., & Johnstone, M. 2000. Relationships of ultraviolet radiation dose and inactivation of pathogen propagules in water and hydroponic nutrient solutions. Canadian Journal of Plant Pathology, 22(3), 300-309.

Thomson, T. B., Athow, K. L., & Laviolette, F. A. 1971. The effect of temperature on the pathogenicity of *Pythium aphanidermatum, P. debaryanum*, and *P. ultimum* on soybean. Phytopathology, 61(8), 933-935.

USGA 2015. USGA Recommendations for a Method of Putting Green Construction, United States Golf Association.

Uzuhashi, S., Tojo, M., & Kakishima, M. 2010. Phylogeny of the genus *Pythium* and description of new genera. Mycoscience, 51(5), 337-365.

van der Plaats-Niterink, A. J. 1981. Monograph of the genus *Pythium*, Centraalbureau voor Schimmelcultures Baarn

Vettraino, A. M., Bonants, P., Tomassini, A., Bruni, N., & Vannini, A. 2012. Pyrosequencing as a tool for the detection of *Phytophthora* species: Error rate and risk of false molecular operational taxonomic units. Letters in Applied Microbiology, 55(5), 390-396.

Vincelli, P., Clarke, B., & Munshaw, G. 2017. Chemical control of turfgrass diseases 2017, University of Kentucky, Kentucky, USA.

Wakeham, A. J., Pettitt, T. R., & White, J. G. 1997. A novel method for detection of viable zoospores of *Pythium* in irrigation water. Annals of Applied Biology, 131(3), 427-435.

Weiland, J. E. 2011. Influence of isolation method on recovery of *Pythium* species from forest nursery soils in Oregon and Washington. Plant Disease, 95(5), 547-553.

Zhang, W., & Tu, J. C. 2000. Effect of ultraviolet disinfection of hydroponic solutions on Pythium root rot and non-target bacteria. European Journal of Plant Pathology, 106(5), 415-421.

Chapter 2

Influence of detection method, sampling location, and month on communities of *Pythium* spp. pathogenic to *Agrostis stolonifera*

Abstract:

Many *Pythium* spp. are causal agents of disease on creeping bentgrass putting greens. Phytopathogenic *Pythium* spp. are known to disseminate through irrigation systems in agricultural and greenhouse settings, and this study expands those findings to golf course irrigation systems. Water samples were collected from irrigation heads and water sources at ten golf courses in Missouri and Kansas. Samples were collected from 2018 to 2019 in April, July, and October. Each sample was probed for *Pythium* spp. through baiting and membrane filtration. Cultures were isolated on PARP media and DNA was extracted from mycelium, bait tissue, and membrane filters. ITS regions were PCR amplified, sequenced, and compared with Genbank accessions. Phylogenetic trees were constructed using representative sample sequences, seven morphologically identified isolate sequences, and Genbank accessions. Detected species include *Lagenidium giganteum, Pythium biforme, P. insidiosum, P. marsipium, P. plurisporium,* and *Saprolegnia hypogyna.* None of these species are known pathogens of creeping bentgrass. Clades A through U lacked species-level resolution. Clades A, C, D, E, I, and M contain pathogenic *Pythium* species that cause root and crown rot on creeping bentgrass. Membrane filtration captured a wider variety of *Pythium* spp. than baiting, and sampling from irrigation heads captured a wider variety of *Pythium* spp. than sampling from the water source. Both detection methods resulted in the highest species diversity in April of 2019. This research shows year-round dissemination of *Pythium* through golf course irrigation systems and highlights the importance of using multiple detection methods when probing for *Pythium* species.

Introduction:

Phytopathogenic *Pythium* spp. are known to cause diseases on creeping bentgrass (*Agrostis stolonifera L.*) putting greens such as Pythium root rot, Pythium root dysfunction, and Pythium blight (Abad et al. 1994, Kerns & Tredway 2008, Smiley et al. 2005). Symptoms from these diseases result in a reduction in playability and aesthetics, and if left unchecked considerable turfgrass loss. Causal agents of *Pythium* disease produce zoospores that disseminate through water and saturated soil (Schroeder et al. 2013). Phytopathogenic *Pythium* spp. have been detected in agricultural and greenhouse irrigation systems (Bush et al. 2003, Hong & Moorman 2005). Retention ponds and other open water bodies used as an irrigation source on golf courses may provide a habitat for *Pythium* spp. and potentially serve as a point source of *Pythium* inoculum. A variety of methods are available to detect Oomycetes in irrigation water (Ali-Shtayeh et al. 1991, Hong et al. 2002, Pettitt et al. 2002, Sanchez et al. 2000). The objectives of this study were: i) to determine if baiting and membrane filtration detect identical *Pythium* communities in irrigation water samples ii) to determine if *Pythium* communities differ between samples taken from irrigation heads and water sources and iii) assess seasonal changes of *Pythium* communities in water samples.

Pythium spp. are often detected using baiting methods (Hendrix & Campbell 1973). Host tissue is used to bait zoospores in solution and the subsequently colonized bait is used for isolation. Infested bait tissue is plated on *Pythium* selective media to facilitate mycelial development and suppress growth of contaminants (Jeffers & Martin 1986). Putative *Pythium* spp. are then isolated and identified through morphology and/or DNA sequencing (Robideau et al. 2011, van der Plaats-Niterink 1981). Another isolation-

based method used to detect *Pythium* spp. in water samples is membrane filtration. Water samples are passed through a membrane filter by vacuum and filters are either plated directly on selective media or washed with agar prior to solidification (Pettitt et al. 2002). A variety of filters have been tested for the efficacy of capturing *Pythium* spp. (Hong et al. 2002).

Isolation-based detection methods have specific advantages and disadvantages. These methods produce live cultures, which can be used for morphological identification and pathogenicity experiments demonstrating the detected propagules are viable. Membrane filtration methods are advantageous since mycelia fragments can be captured if zoospores are not easily produced by the present *Pythium* species. Bait selection and bait trap incubation temperature influence baiting efficacy due to differences in host preference and optimal temperature for development among *Pythium* species (Raftoyannis & Dick 2006, van der Plaats-Niterink 1981, Watanabe et al. 2008). Both isolation-based detection methods can impose selection bias through media selection and incubation temperature of cultures. The fastest growing species on a specific media or at a specific temperature will outcompete slower growing species. Therefore, species with slow mycelium development are more difficult to detect using these methods.

There are a variety of cultureless methods available to detect *Pythium* species. DNA can be extracted directly from bait tissue and membrane filters prior to culturing. The extracted DNA can be PCR amplified and sequenced to provide evidence of present species. The efficacy of this method relies on the sequencing method used. Sanger sequencing may not produce a clear sequence if multiple species are present in a sample. Next generation sequencing methods such as pyrosequencing or paired-end sequencing

can provide clear sequences when multiple species are present (Redekar et al. 2019, Vettraino et al. 2012). Methods such as zoospore trapping immunoassays (ZTI) and enzyme-linked immunosorbent assays (ELISA) utilize antibodies to detect present species. ZTIs are a sensitive method for quantifying the number of living zoospores in a sample, but isolation-based methods with membrane filters are more consistent (Pettitt et al. 2002). ELISA tests are commercially available to detect *Pythium* but do not provide reliable species level resolution (Ali-Shtayeh et al. 1991).

Sample source and time of sampling should be considered when probing for *Pythium* species. The predominating species in an irrigation system may differ between seasons due to temperature or other environmental differences, and detected species have also been shown to differ between water reservoirs in a recycled irrigation system (Redekar et al. 2019). Irrigation systems constructed with infested materials or exposed to soil (e.g. irrigation leaks) may contribute to microbial community differences between water sources and water near the irrigation head (Al-Sa'di et al. 2008). Irrigation systems should be probed at multiple times and locations to detect all present species.

Reliable detection of *Pythium* spp. is beneficial for assessing the risk of using different water sources for irrigation. Detection methods should capture a wide range of *Pythium* species if the target species is unknown. Zoospore baiting and membrane filtration were selected for evaluation in this study because they are more reliable then ZTIs, more accurate then ELISAs, and compatible with Sanger sequencing methods (Ali-Shtayeh et al. 1991, Pettitt et al. 2002). Evidence of pathogenic *Pythium* spp. in irrigation systems may help improve disease management practices available for golf courses.

Materials and methods:

Sample collection. Eight golf courses in Missouri and two in Kansas with a history of disease caused by *Pythium* root-infecting species were selected for this study (Table 2.1). Clear oblong glass baking dishes (2.8 liters, 22.9 x 33.0 cm, Corelle Brands LLC, Rosemont, IL) were placed on putting greens and used to collect three L of water from multiple irrigation heads. Baking dishes were triple-rinsed with deionized (DI) water and autoclaved prior to use. A Van Dorn water sampler (Flinn Scientific Inc., Batavia, IL) was used to collect two L of water from open water sources (Table 2.1). The Van Dorn sampler was positioned near irrigation intakes approximately 0.6 m from the bottom of each water source. The Van Dorn sampler was sterilized with 10% NaClO and triple rinsed with DI water prior to each use. Irrigation head samples (IHS) and water source samples (WSS) were consolidated in separate glass media bottles and sealed with polybutylene terephthalate caps (Corning Inc., Corning, NY). Media bottles were washed with powdered precision cleaner (Alconox Inc., White Plains, NY), triple-rinsed with DI water, and autoclaved prior to use. Water samples were transported and stored at 15°C for up to two weeks. Irrigation head and water source samples were processed separately.

Zoospore baiting. Six-week-old creeping bentgrass (*Agrostis stolonifera L.* var. 'Penncross') leaves were used for baiting zoospores in water samples. Plants were established in a greenhouse and sown in 10.2 cm diameter plastic pots containing an autoclaved sand medium. Pots were placed in greenhouse trays without drainage and plants were flood irrigated with DI water as needed. Plastic pots and greenhouse trays were sterilized with 10% NaClO and triple rinsed with DI water prior to use. Bentgrass

leaves were cut to six cm, suspended in 150 ml of DI water, and autoclaved prior to baiting.

A 500 ml aliquot from each water sample was partitioned into two sterile 250 ml beakers. An autoclaved wire mesh tea infuser was used to suspend 10 creeping bentgrass leaves in each beaker. Autoclaved aluminum foil was used to cover each bait trap. Bait traps were incubated on a lab bench at ~23°C for 48 hr. After incubation, the bait tissue was dried on a sterile paper towel and cut in half. Half of the bait tissue was plated onto Petri dishes containing PARP media (Jeffers & Martin 1986). PARP medium contained 20 g corn meal agar (Becton, Dickinson and Company, Franklin Lakes, New Jersey), 250 mg ampicillin sodium salt (Fisher Scientific, Waltham, MA), 10 mg pimaricin (Fisher Scientific, Waltham, MA), 10 mg rifampicin (Fisher Scientific, Waltham, MA) dissolved in 1 ml dimethyl sulfoxide (Fisher Scientific, Waltham, MA), and 5 ml 5% pentachloronitrobenzene (Sigma-Aldrich, St. Louis, MO) per L of DI water. The other half of bait tissue was placed in two screwcap vials and stored at -20°C until direct DNA extraction. Autoclaved creeping bentgrass leaves that were not used in bait traps were plated onto PARP media to ensure complete sterilization. No cultures developed from these autoclaved leaves. Petri dishes were sealed with Parafilm (Bemis Company, Neenah, WI), stored on a lab bench at ~23°C, and checked daily for mycelia growth. Developing colonies were examined under a microscope and cultures with white coenocytic hyphae were isolated on fresh PARP media.

Membrane filtration. Two 500 ml aliquots from each water sample were vacuum filtered through two Durapore filters with five µm pores (MilliporeSigma, Burlington, MA) to capture zoospores and mycelial fragments. Filters were held in place

by an autoclaved 47 mm magnetic filter funnel (Pall Corporation, Port Washington, New York). One filter was cut into 16 equal pieces and plated on PARP media. Petri dishes were sealed, stored on a lab bench at ~23°C, and checked daily for mycelia growth. Developing colonies were examined under a microscope and cultures with white coenocytic hyphae were isolated on fresh PARP media. The second filter was cut into 1 x 1 mm squares, split into four screwcap vials, and stored at -20°C until DNA extraction.

Propagating mycelium. Mycelium from putative *Pythium* spp. and morphologically characterized isolates of *G. ultimum* var *ultimum, P. aphanidermatum, P. aquatile, P. dissotocum, P. graminicola, P. irregulare,* and *P. oligandrum* from the collection of J. Mihail (University of Missouri, Columbia) were propagated by transferring hyphae to V8 media overlaid with cellophane (Gel Company, San Francisco, CA). V8 media contained 15 g granulated agar (Fisher Scientific, Waltham, MA), 100 ml original V8 juice (Campbell, Camden, NJ), and 0.75 g calcium carbonate (Fisher Scientific, Waltham, MA) per L of DI water. Mycelium was scraped off cellophane using a flame sterilized scalpel and stored in screwcap vials (Thermo Fisher Scientific, Waltham, MA) at -20°C.

DNA extraction and PCR. DNA was extracted from mycelium, bait tissue, and membrane filters using the protocol and reagents in the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA). DNA concentrations were quantified with a NanoDrop OneC Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and extracts were diluted with nuclease free water (Qiagen, Hilden, Germany) to a concentration of 50 ng/µl. PCR mixtures contained 4 µl of DNA template (200 ng of DNA), 25 µl of DreamTaq Hot start PCR master mix (Thermo Fisher Scientific,

Waltham, MA), 1 µl (200nM) of the Oomycete specific primer ITS4Oo, 1 µl (200nM) of the universal primer ITS5, and 19 µl of nuclease free water (Nikolcheva & Barlocher 2004, White et al. 1990). Thermocycler (Eppendorf, Hamburg, Germany) settings were 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 49°C for 30 sec, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. Successful amplifications were confirmed through electrophoresis, cleaned using ExoSAP-IT PCR product cleanup reagent (Applied Biosystems, Foster City, CA), and Sanger sequenced by the DNA Core Facility at the University of Missouri.

Phylogenetic analysis. Contiguous sample sequences were assembled and representative sample sequences were selected using SeqMan Pro (14; DNASTAR Inc., Madison, WI). The Basic Local Alignment Search Tool (megaBLAST) was used to compare representative sample sequences with Genbank accessions. The top two or three similar sequences based on maximal percent identity match were used in phylogenetic analysis. The ITS sequences of *Pythium* spp. described in Abad et al. (1994) were downloaded from Genbank using accessions from Levesque et al. (2004) and Robideau et al. (2011) and included in the phylogenetic analysis. All sequences were aligned using ClustalW in MEGA (7, Pennsylvania State University, State College, PA) and truncated within 25 base pairs from the outermost nucleotides conserved between 70% of all sequences. Two phylogenetic trees were constructed with the neighbor-joining algorithm using the Kimura two-parameter model. One tree utilized *Clarireedia homoeocarpa* (GQ386985) as an outgroup and was constructed with sequences associated with *Globisporangium* and *Pythium. Globisporangium* spp. were included in the phylogenetic analysis of *Pythium* spp. because some causal agents of *Pythium* disease, such as *Pythium*

ultimum var. *ultimum,* have been reclassified into *Globisporangium* (Uzuhashi et al. 2010). The tree containing Oomycetes other than *Pythium* spp. utilized *P. aphanidermatum* (AY598622) and *P. arrhenomanes* (AY598628) as an outgroup and was constructed using sequences associated with other Oomycetes. Bootstrap values on branch nodes are based on 1000 random samples of the data set.

Statistical analysis. The following hypotheses were tested: Species diversity and average number of *Pythium* sequences obtained per sample is identical between i) detection methods, ii) sampling site, iii) and months sampled. In total, 72 attempts from each detection method, 72 attempts from each sampling source, and 36 attempts from July 2018, April 2019, July 2019, and October 2019 were used to compare diversity indexes and the average number of sequences obtained per sample (Table 2.2). The number of sequences obtained per clade was used as a proxy measurement of species richness in each sample. The diversity of species in each sample was evaluated using Shannon-Wiener and Simpson's diversity index (Redekar et al. 2019, Shannon 1948, Simpson 1949, Spellerberg & Fedor 2003, Weiland 2011). Arithmetic mean of diversity indices was evaluated for detection method, sampling source, and month. Standard error of the mean was added/subtracted from each mean value. Means were considered different when mean values did not overlap based on standard error. Differences in the average number of *Pythium* and off-target Oomycete sequences obtained per sample were evaluated using PROC GENMOD in SAS 9.4 (SAS Institute). *Pythium* data followed a zero-inflated Poisson distribution while off-target Oomycete data followed a standard Poisson distribution. To create a balanced dataset, data from April 2018, October 2018, and those from sample site four were excluded from statistical analysis (Table 2.1).

Samples from April 2018 were excluded because WSS were not collected. Samples from October 2018 were omitted because samples were not collected from five sites. Sample site four was irrigated from municipal, chlorinated water collected from the Missouri River, and no *Pythium* spp. were detected in samples from this site.

Results

Detected *Pythium* clades. In total, 511 ITS sequences were obtained using the isolation-based methods described above, with 453 sequences identified as a *Pythium* or *Globisporangium* spp. Cultureless methods were mostly unsuccessful due to the inability of Sanger sequencing to resolve the multiple species present in many samples. Eleven *Pythium* and four off-target Oomycete sequences were obtained through direct DNA extraction of bait tissue and zero sequences were obtained from direct DNA extraction from membrane filters. Detected *Pythium* spp. include *P. biforme, P. insidiosum, P. marsipium,* and *P. plurisporium* (Figure 2.1). Clades A through N could not be resolved to the species level. Clades A, C, D, E, I, and M contain causal agents of root and crown rot on creeping bentgrass (Table 2.3; Abad et al. 1994). However, these clades also contain nonpathogenic species or species with unknown pathogenicity to creeping bentgrass. Clades G, H, J, and L contain phytopathogenic *Pythium* spp. but it is unknown if they infect creeping bentgrass (Allain-Boulé et al. 2004, Bouket et al. 2015, Li et al. 2014, Mankin 1969, Sprague 1950, Weiland et al. 2013).

Off-target Oomycetes. Detected off-target Oomycete species include *Lagenidium giganteum* and *Saprolegnia hypogyna* while clades O through U could not be resolved (Figure 2.2). None of the detected off-target Oomycetes are known phytopathogens (Table 2.4). Clades Q and S were only detected through baiting while

Saprolegyna hypogyna and clades P, R, and T were only detected though membrane filtration. *Saprolegnia hypogyna* and clade S were only detected in water sources. Clades P, Q, R, and T were only detected in irrigation heads. Clade P was only detected in April 2019. Clade S was only detected in July 2018. *Lagenidium giganteum* and clade T were only detected in July 2019. Clade Q was only detected in October 2018. *Saprolegnia hypogyna* and clade R was detected in two different months. Clade V was detected in three different months. Clade O was detected in five different months. There was no significant difference observed in the number of off-target Oomycetes detected in each sample regardless of detection method, sampling source, and month sampled (Table 2.5).

Differences between isolation methods. *Pythium* species detected only through baiting include *P. biforme* and *P. insidiosum* (Figure 2.3). *Pythium* species detected only through membrane filtration include *P. marsipium, P. plurisporium,* and clades A, D, I, J, K, M, and N. Clade H was detected most frequently and comprised 52% and 36.7% of total sequences obtained through baiting and membrane filtration, respectively. There was no statistical difference between methods when measuring the average number of *Pythium* sequences obtained per sample (Table 2.5). Most samples produced a range of one to seven sequences per sample (Figure 2.4). Baiting methods detected up to three species per sample while membrane filtration methods detected up to five (Figure 2.5). Membrane filtration resulted in higher diversity index values (Table 2.6). Excluding site four, 8.8% of all samples contained *Pythium* spp. only detected through baiting, 15.4% of samples contained *Pythium* spp. only detected through membrane filtration, 31.9% of samples contained *Pythium* spp. detected through both methods, and 43.9% of samples

did not contain detectable *Pythium* spp. or clear sequences could not be obtained through sanger sequencing.

Difference between sampling source. *Pythium biforme, P. plurisporium,* and clades A, I, and N were only detected in WSS (Figure 2.6). *Pythium marsipium, P. insidiosum,* and clades D, J, K, L, and M were only detected in IHS. Clade H was the most abundant clade in both WSS and IHS, and produced 62.5% and 33.1% of total sequences, respectively. No statistical difference was observed between sampling sources when measuring the average number of *Pythium* sequences obtained per sample (Table 2.5). Samples from water sources contained up to four species while IHS contained up to five species in a single sample (Figure 2.5). *Pythium* diversity was greater in IHS and more samples contained detectable *Pythium* spp. compared to WSS (Table 2.6). Every clade detected in April and October 2018 was detected in IHS (Figure 2.7).

Difference between months. Clades C and H were detected every sampling date (Figure 2.7). Clade D was only detected in April 2018. Clades F, I, J, and K were only detected in April 2019. *Pythium insidosum, P. plurisporium,* and clades A and N were only detected in July 2019. *Pythium marsipium, P. biforme,* and clade M was only detected in October 2019. Clades B and E were detected in two different months. Clade L was detected in three different months. Clade G was detected in five different months.

A statistical difference was observed between months when measuring the average number of *Pythium* sequences obtained per sample (Table 2.5). April 2019 had the highest number of samples with detectable *Pythium* spp. regardless of detection method (Table 2.6). April 2019 had the highest diversity while July months had the lowest. October 2019 had a diversity index similar to April 2019 and July months. Clade

H was the most abundant in July 2018, April 2019, and July 2019 (Figure 2.7). Clade C was the most abundant in April 2018 and October 2018. Clade G was the most abundant in October 2019. Samples with more than three *Pythium* species were only collected in April 2019 (Figure 2.5).

Discussion

Pythium and other Oomycetes disseminate through irrigation systems (Bush et al. 2003, Redekar et al. 2019). This research demonstrates that dissemination also occurs in a golf course setting except if treated municipal water is used as an irrigation source, corresponding to previous research (Pottorff & Panter 1997). Overall, the number of clades detected in this study is comparable to previous experiments that probe irrigation systems for Oomycetes using isolation-based detection methods (Parke et al. 2014). Recently developed primers, such as ITS3oo, may increase the success rate of PCR from DNA extracts of putative *Pythium* isolates (Riit et al. 2016). Species identification solely through ITS sequencing has been shown to leave some clades unresolved due to similarities in ITS sequences between species (Levesque & DeCock 2004, Redekar et al. 2019).

The two species only detected through baiting and nine species only detected through membrane filtration supports the assumption that different detection methods will impose a selection bias (Bush et al. 2003, Redekar et al. 2019). The size of plated bait tissue and membrane filters may have influenced the detected diversity. If plated materials are cut into smaller pieces before placing onto media, then it could further minimize the chance of developing colonies outcompeting others before isolation, thereby increasing diversity estimates. Next generation sequencing methods could also be

used when multiple species are present in a culture, or to bypass culturing steps entirely (Redekar et al. 2019, Vettraino et al. 2012).

A thorough sampling scheme is necessary to fully characterize *Pythium* populations in irrigation systems. The higher diversity indicies observed in irrigation heads may suggest that conditions within the irrigation system are a conducive enviornment for *Pythium*. The higher diverisity values of IHS may also be attributed to the relative increase of water sample size in relation to total volume of water in an irrigation system as opposed to a water source. The seasonal changes of *Pythium* frequency may be due to the influence of temperature on individual *Pythium* spp. or the influence of microbe community differences throughout the year. The prevalence of clade H throughout the study was likely due to the pathogenic nature of *P. adhaerens* to algal species that may be present in surface water sources, but the increase of clade G in October was likely due to the colder (5 – 18°C) optimal temperatures for *P. monospermum* zoospore development (van der Plaats-Niterink 1981). Baiting zoospores at higher temperatures may have increased the chances of detecting some highly aggressive causal agents of Pythium root rot, such as *Pythium aphanidermatum* and *P. myriotylum* (Watanabe et al. 2008). Mammal pathogens, *Pythium insidiosum* and *Lagendium gigantium,* were detected during the summer, which may have been due to higher optimal temperatures for zoospore development (Mendoza & Prendas 1988). The increase of detectable populations in April 2019 has also been observed previously when measuring the number of colony forming units from January through May (Pettitt & Skjøth 2016). Other studies have reported *Pythium* dissemination throughout the year and

variation associated with the frequency of *Pythium* spp. (Bush et al. 2003, Redekar et al. 2019).

Future experiments that utilize current findings and next generation sequencing methods will be key in elucidating the profile of microbes present in irrigation systems. A study of *Pythium* and *Phytophthora* detection in a greenhouse irrigation system through paired end sequencing illustrates the power of next generation sequencing methods in detecting a wide range of microbes (Redekar et al. 2019). Next generation sequencing data paired with culturing methods could further estimate how many species may go undetected in studies utilizing culture-based methods, while also demonstrating which species are viable at the time of sampling. Species level resolution of a microbial community facilitates risk assessment of various water sources. Testing the pathogenicity of viable propagules will allow researchers to expand the list of problematic species and possibly establish minimum economic thresholds (Abad et al. 1994, Kerns & Tredway 2008). Water sterilization techniques, such as UV-C treatments, heat, or chemical additives can then be optimized for problematic species (Hong & Moorman 2005).

This study lays groundwork for future investigations of microbial communities in irrigation systems for the golf industry and establishes irrigation water as a potential point source of *Pythium* inoculum on putting greens. None of the sequences resolved to the species level are known pathogens of creeping bentgrass. Clades containing known pathogens of creeping bentgrass vary between slightly (clades C, D, I, and M), moderately (clade D), and highly (clade A) aggressive causal agents of crown and root rot (Abad et al. 1994). The presence of these potentially pathogenic clades highlights a possible risk of utilizing surface water as a source for putting green irrigation.

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Literature Cited:

Abad, Z. G., Shew, H. D., & Lucas, L. T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology, 84(9), 913-921.

Al-Sa'di, A. M., Drenth, A., Deadman, M. L., Al-Said, F. A., Khan, I., & Aitken, E. A. B. 2008. Potential sources of *Pythium* inoculum into greenhouse soils with no previous history of cultivation. Journal of Phytopathology, 156(7-8), 502-505.

Ali-Shtayeh, M. S., MacDonald, J. D., & Kabashima J. 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Disease, 75(3), 305-311.

Allain-Boulé, N., Lévesque, C. A., Martinez, C., Bélanger, R. R., & Tweddell, R. J. 2004. Identification of *Pythium* species associated with cavity-spot lesions on carrots in eastern Quebec. Canadian Journal of Plant Pathology, 26(3), 365-370.

Bisht, G. S., Joshi, C., & Khulbe, R. D. 1996. Watermolds: Potential biological control agents of malaria vector *Anopheles culicifacies*. Current Science, 393-395.

Bouket, A. C., Arzanlou, M., Tojo, M., & Babai-Ahari, A. 2015. *Pythium kandovanense* sp. nov., a fungus-like eukaryotic micro-organism (Stramenopila, Pythiales) isolated from snow-covered ryegrass leaves. International Journal of Systematic and Evolutionary Microbiology, 65(8), 2500-2506.

Bush, E. A., Hong, C., & Stromberg, E. L. 2003. Fluctuations of *Phytophthora* and *Pythium* spp. in components of a recycling irrigation system. Plant Disease, 87(12), 1500-1506.

Ellis, M. L., Paul, P. A., Dorrance, A. E., & Broders, K. D. 2012. Two new species of *Pythium*, *P. schmitthenneri* and *P. selbyi* pathogens of corn and soybean in Ohio. Mycologia, 104(2), 477-487.

Hanlin, R. T. 1978. Plant disease index for maize in the United States, Part 1: Host Index. University of Georgia, Georgia, USA

Hendrix, F. F. & Campbell, W. A. 1973. *Pythiums* as plant pathogens. Annual Review of Phytopathology, 11(1), 77-98.

Hong, C. X., & Moorman, G. W. 2005. Plant pathogens in irrigation water: challenges and opportunities. Critical Reviews in Plant Sciences, 24(3), 189-208.

Hong, C., Richardson, P. A., & Kong, P. 2002. Comparison of membrane filters as a tool for isolating pythiaceous species from irrigation water. Phytopathology, 92(6), 610-616.

Jeffers, S. N., & Martin, S. B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease, 70(11), 1038-1043.

Jiang, R. H. Y., de Bruijn, I., Haas, B. J., Belmonte, R., Löbach, L., Christie, J., van den Ackerveken, G., Bottin, A., Bulone, V., & Díaz-Moreno, S. M. 2013. Distinctive expansion of potential virulence genes in the genome of the oomycete fish pathogen *Saprolegnia parasitica*. PLoS Genetics, 9(6).

Kawamura, Y., Yokoo, K., Tojo, M., & Hishiike, M. 2005. Distribution of *Pythium porphyrae*, the causal agent of red rot disease of *Porphyrae* spp., in the Ariake Sea, Japan. Plant Disease, 89(10), 1041-1047.

Kerns, J. P., & Tredway, L. P. 2008. Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Disease, 92(6), 862-869.

Lee, S. J., Jee, B. Y., Son, M. H., & Lee, S. R. 2017. Infection and cox2 sequence of *Pythium chondricola* (Oomycetes) causing red rot disease in *Pyropia yezoensis* (Rhodophyta) in Korea. Algae, 32(2), 155-160.

Levesque, C. A., & De Cock, A. W. 2004. Molecular phylogeny and taxonomy of the genus Pythium. Mycological Research, 108(12), 1363-1383.

Li, Y. P., You, M. P., & Barbetti, M. J. 2014. Species of *Pythium* associated with seedling root and hypocotyl disease on common bean (*Phaseolus vulgaris*) in Western Australia. Plant Disease, 98(9) 1241-1247.

Mankin, C. J. 1969. Diseases of grasses and cereals in South Dakota. Agricultural Experement Station, South Dakota State University, Brookings, SD.

Mendoza, L., & Prendas, J. 1988. A method to obtain rapid zoosporogenesis of *Pythium insidiosum*. Mycopathologia, 104(1), 59-62.

Nikolcheva, L. G., & Bärlocher, F. 2004. Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. Mycological Progress, 3(1), 41-49.

Osman, A., Ali, E., Hashem, M., Mostafa, M., & Mekkawy, I. 2010. Genotoxicity of two pathogenic strains of zoosporic fungi (*Achlya klebsiana* and *Aphanomyces laevis*) on erythrocytes of Nile tilapia *Oreochromis niloticus niloticus*. Ecotoxicology and Environmental Safety, 73(1), 24-31.

Parke, J. L., Knaus, B. J., Fieland, V. J., Lewis, C., & Grünwald, N. J. 2014. *Phytophthora* community structure analyses in Oregon nurseries inform systems approaches to disease management. Phytopathology, 104(10), 1052-1062.

Pettitt, T., & Skjøth, C. 2016. A simple model describes development of early peaks in oomycete zoospore inoculum detected in southern UK outdoors horticultural reservoirs. Acta Mycologica, 69(2), 1-7.

Pettitt, T. R., Wakeham, A. J., Wainwright, M. F., & White, J. G. 2002. Comparison of serological, culture, and bait methods for detection of *Pythium* and *Phytophthora* zoospores in water. Plant Pathology, 51(6), 720-727.

Pottorff, L. P., & Panter, K. L. 1997. Survey of *Pythium* and *Phytophthora* spp. in irrigation water used by Colorado commercial greenhouses. HortTechnology, 7(2), 153-155.

Raftoyannis, Y., & Dick, M. W. 2006. Zoospore encystment and pathogenicity of *Phytophthora* and *Pythium* species on plant roots. Microbiological Research, 161(1), 1-8.

Redekar, N. R., Eberhart, J. L., & Parke, J. L. 2019. Diversity of *Phytophthora*, *Pythium*, and *Phytopythium* species in recycled irrigation water in a Container Nursery. Phytobiomes Journal, 3(1), 31-45.

Riit, T., Tedersoo, L., Drenkhan, R., Runno-Paurson, E., Kokko, H., & Anslan, S. 2016. Oomycete-specific ITS primers for identification and metabarcoding. MycoKeys, 14, 17.

Robertson, G. I. 1973. Pathogenicity of *Pythium* spp. to seeds and seedling roots. New Zealand journal of agricultural research, 16(3), 367-372.

Robideau, G. P., de Cock, A. W., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D. W., Désaulniers, N., Eggertson, Q. A., Gachon, C. M. M., Hu, C. H., Küpper, F. C., Rintoul, T. L., Sarhan, E. S., Verstappen, E. C. P., Zhang, Y., Bonants, P. J. M., Ristaino, J. B., & André Lévesque, C. A. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Molecular Ecology Resources, 11(6), 1002-1011.

Romansic, J. M., Diez, K. A., Higashi, E. M., Johnson, J. E., & Blaustein, A. R. 2009. Effects of the pathogenic water mold *Saprolegnia ferax* on survival of amphibian larvae. Diseases of aquatic organisms, 83(3), 187-193.

Schroeder, K. L., Martin, F. N., de Cock, A. W., Lévesque, C. A., Spies, C. F., Okubara, P. A., & Paulitz, T. C. 2013. Molecular detection and quantification of *Pythium* species: evolving taxonomy, new tools, and challenges. Plant Disease, 97(1), 4-20.

Schurko, A. M., Mendoza, L., Lévesque, C. A., Désaulniers, N. L., de Cock, A. W. and Klassen, G. R. 2003. A molecular phylogeny of *Pythium insidiosum*. Mycological research, 107(5), 537-544.

Shannon, C. E. 1948. A mathematical theory of communication. The Bell system technical journal, 27(3), 379-423.

Simpson, E. H. 1949. Measurement of diversity. Nature, 163(4148), 688-688.

Smiley, R. W., Dernoeden, P. H., & Clarke, B. B. 2005. Compendium of turfgrass diseases, St. Paul, MN: American Phytopathological Society

Spellerberg, I. F., & Fedor, P. J. 2003. A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener'Index. Global ecology and biogeography, 12(3), 177-179.

Sprague, R. 1950. Diseases of cereals and grasses in North America. Diseases of cereals and grasses in North America.

Tojo, M., Watanabe, K., Kida, K., Li, Y., & Numata, S. 2007. Mottle necrosis of sweet potato caused by *Pythium scleroteichum* in Japan and varietal difference in susceptibility to the disease. Journal of General Plant Pathology, 73(2), 121-124.

Uzuhashi, S., Tojo, M., & Kakishima, M. 2010. Phylogeny of the genus *Pythium* and description of new genera. Mycoscience, 51(5), 337-365.

van der Plaats-Niterink, A. J. 1981. Monograph of the genus *Pythium*, Centraalbureau voor Schimmelcultures Baarn

van der Plaats-Niterink, A. J. 1972. The occurrence of *Pythium* in the Netherlands. III. *Pythium flevoense* sp. n. Acta botanica neerlandica, 21(6), 633-639.

Veterano, S. T., Coffua, L. S., Mena-Ali, J. I., & Blair, J. E. 2018. *Pythium yorkensis* sp. nov., a potential soybean pathogen from southeastern Pennsylvania, USA. Plant Pathology, 67(3), 619-625.

Vettraino, A. M., Bonants, P., Tomassini, A., Bruni, N., & Vannini, A. 2012. Pyrosequencing as a tool for the detection of *phytophthora* species: Error rate and risk of false molecular operational taxonomic units. Letters in Applied Microbiology, 55(5), 390-396.

Watanabe, H., Kageyama, K., Taguchi, Y., Horinouchi, H., & Hyakumachi, M. 2008. Bait method to detect *Pythium* species that grow at high temperatures in hydroponic solutions. Journal of General Plant Pathology, 74(6), 417.

Weiland, J. E. 2011. Influence of isolation method on recovery of *Pythium* species from forest nursery soils in Oregon and Washington. Plant Disease, 95(5), 547-553.

Weiland, J. E., Beck, B. R., & Davis, A. 2013. Pathogenicity and virulence of *Pythium* species obtained from forest nursery soils on Douglas-fir seedlings. Plant disease, 97(6), 744-748.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, 18(1), 315-322.

Zitnick-Anderson, K. K., & Nelson Jr., B. D. 2015. Identification and pathogenicity of *Pythium* on soybean in North Dakota. Plant Disease, 99(1), 31-38.

Table 2.1. Distribution of samples taken from golf course irrigation systems in Missouri and Kansas and types of water sources used by each site. Striped cells indicate dates when samples were only collected from irrigation heads. Grey cells indicate dates when samples were collected from irrigation heads and water sources. Empty cells indicate dates when no sample was taken.

Location	Site	Water source (ha)	Irrigation sampling dates (Month-Year)					
			Apr-18	Jul-18	Oct-18	Apr-19	Jul-19	Oct-19
Columbia, MO	1	Surface water (0.6)						
	2	Surface water (3.1)						
	3	Well						
St. Louis, MO	4	Municipal						
	5	Surface water (0.8)						
Cape Girardeau, MO	6	Surface water (4.9)						
Springfield, MO	7	Surface water (3.1) & Well						
Branson, MO	8	Surface water (1.0)						
Olathe, KS	9	Surface water (62.7)						
	10	Surface water (9.6)						

Table 2.2. The number of *Pythium* detection attempts and statistically analyzed attempts from water samples taken from Missouri and Kansas golf course irrigation systems.

		Number of detection attempts	Number of statistically analyzed detection attempts
Detection method	Baiting	96	72
	Filtration	96	72
Sample source	Irrigation head	110	72
	Water source	82	72
Month	April 2018	20	0
	July 2018	38	36
	October 2018	20	0
	April 2019	38	36
	July 2019	38	36
	October 2019	38	36

Table 2.3. Pathogenicity of *Pythium* spp. and unresolved clades isolated from golf course irrigation systems in Missouri and Kansas between 2018 and 2019.

	Species	Disease	Host	Reference
	Pythium plurisporium	Nonpathogenic	Creeping bentgrass	(Abad et al. 1994)
	Pythium biforme	-	-	-
	Pythium insidiosum	Pythiosis	Mammals (Humans)	(Schurko et al. 2003)
	Pythium marsipium	-	-	-
Clade	Species	Disease	Host	Reference
A	*P. myriotylum*	Root and crown rot	Creeping bentgrass	(Abad et al. 1994)
	P. zingiberis	Nonpathogenic	Creeping bentgrass	(Abad et al. 1994)
	P. scleroteichum	Mottle necrosis	Sweet potato	(Tojo et al. 2007)
B	*Pythium sp.*	-	-	-
C	*P. torulosum*	Nonpathogenic	Creeping bentgrass	(Abad et al. 1994)
	P. rhizo-oryzae	-	-	-
	P. catenulatum	Root and crown rot	Creeping bentgrass	(Abad et al. 1994)
D	*P. afertile*	Root and crown rot	Creeping bentgrass	(Abad et al. 1994)
	P. kashmirense	Damping off of seedlings	Soybean	(Zitnick-Anderson et al. 2015)
E	*P. aquatile*	Damping off of seedlings	Tomato	(Robertson 1973)
	P. dissotocum	Root and crown rot	Creeping bentgrass	(Abad et al. 1994)
	P. coloratum	-	-	-
F	*P. pectinolyticum*	-	-	-
	P. flevoense	-	-	-
	P. capillosum	-	-	-
G	*P. monospermum*	Root rot	Barley	(Sprague 1950)
	P. monospermum	Root rot	Switchgrass	(Sprague 1950)
	P. monospermum	Root rot	Sacaton grass	(Mankin 1969)
	Pythium sp.	-	-	-
H	*P. adhaerens*	Root rot	Corn	(Hanlin 1978)
	P. porphyrae	Red rot	Seaweed	(Kawamura et al. 2005)
	P. chondricola	Red rot	Red algae	(Lee et al. 2017)

Table 2.3. (*cont.*) Pathogenicity of *Pythium* spp. and unresolved clades isolated from golf course irrigation systems in Missouri and Kansas between 2018 and 2019.

Clade	Species	Disease	Host	Reference
I	*P. yorkensis*	Damping off of seedlings	Soybean	(Veterano et al. 2018)
	P. perplexum	Damping off of seedlings	Cucumber	(Veterano et al. 2018)
	P. nodosum	-	-	-
	P. multisporum	Root and crown rot	Creeping bentgrass	(Abad et al. 1994)
J	*G. intermedium*	Root and hypocotyl disease	Common bean, Parsnip, and Parsley	(Li et al. 2014)
	P. attrantheridium	Cavity spot	Carrot	(Allain-Boulé et al. 2004)
K	*Pythium sp.*	-	-	-
L	*P. rostratifingens*	Damping-off	Douglas-fir	(Weiland et al. 2013)
	P. rostratum	Cavity spot	Carrot	(Allain-Boulé et al. 2004)
	P. kandovanense	Pathogenic	Perennial rye	(Bouket et al. 2015)
M	*P. longisporangium*	-	-	-
	P. selbyi	Seedling and root rot	Corn and soybean	(Ellis et al. 2012)
	P. carolinianum	Crown and root rot	Creeping bentgrass	(Abad et al. 1994)
N	*P. takayamanum*	-	-	
	P. parvum	-	-	-
	P. pleroticum	-	-	-

Table 2.4. Pathogenicity of Oomycetes and unresolved clades isolated from golf course irrigation systems in Missouri and Kansas between 2018 and 2019.

Speices		Disease	Host	Reference
Lagendium gigantium		Lagenidiosis	Mammals	(Mendoza et al. 2016)
Saprolegnia hypogyna		Saprolegniosis	Atlantic salmon, frogs, stoneflies	(Sarowar et al. 2013)
Clade	*Species*	Disease	Host	Reference
O	*Saprolegnia sp.*	-	-	-
P	*Saprolegnia parasitica*	Saprolegniosis	Salmon	(Jiang et al. 2013)
	Saprolegnia sp.	-	-	-
Q	*Leptolegnia caudata*	Larval death	Mosquito	(Bisht et al. 1996)
	Saprolegnia sp.	-	-	-
R	*Saprolegnia litoralis*	-	-	-
	Saprolegnia ferax	Embryo death	Amphibians	(Romansic et al. 2009)
S	*Aphanomyces laevis*	Nuclear lesions	Tilapia	(Osman et al. 2010)
	Aphanomyces repetans	-	-	-
T	*Pythiogeton sp.*	-	-	-
U	*Lagenidium sp.*	-	-	-

Table 2.5. *P*-values associated with the average number of *Pythium* and off-target Oomycete sequences obtained per sample of irrigation water. Samples were collected from Missouri and Kansas golf courses in July of 2018, April of 2019, July of 2019, and October of 2019. *Pythium* data follows a zero-inflated Poisson distribution and off-target Oomycetes follow a standard Poisson distribution. *P*-values of *Pythium* are based on a Chi-Square statistic. *P*-values of off-target spp. are based on an *F* statistic.

Goodness of Fit		*Pythium*	Off-target
Pearson Chi-Square / DF		0.949	1.38
Predictor variable	DF	*Pythium*	Off target
Month	3	<.0001	1.0000
Sample source	1	0.5529	0.9954
Detection method	1	0.0937	0.9950

Table 2.6. Average *Pythium* diversity indices in regards to detection method, sampling source, and month. Evaluated samples were taken from nine Missouri and Kansas golf course irrigation systems.

		Shannon-Wiener	Simpson	N[a]
Detection method	Baiting	0.2010 ± 0.0642[b]	0.1278 ± 0.0407	31
	Membrane filtration	0.3673 ± 0.0725	0.2230 ± 0.0409	39
Sampling source	Water source	0.1711 ± 0.0642	0.1084 ± 0.0396	31
	Irrigation head	0.3093 ± 0.0655	0.1970 ± 0.0402	39
Month	July 2018	0.1100 ± 0.0601	0.0751 ± 0.0416	23
	April 2019	0.4015 ± 0.1002	0.2449 ± 0.0582	16
	July 2019	0.1635 ± 0.0709	0.1059 ± 0.0456	20
	October 2019	0.2819 ± 0.1233	0.1901 ± 0.0812	11

[a] N – Number of samples.
[b] ± Standard error

Figure 2.1. Phylogeny of morphologically characterized isolates and representative sequences obtained from putative *Pythium* spp. isolated from golf course irrigation systems based on ITS (1 & 2) and 5.8S genes of ribosomal DNA. Unresolved clades are denoted A through N. Bootstrap values are based on 1,000 resamplings of the data set and displayed near branch nodes.

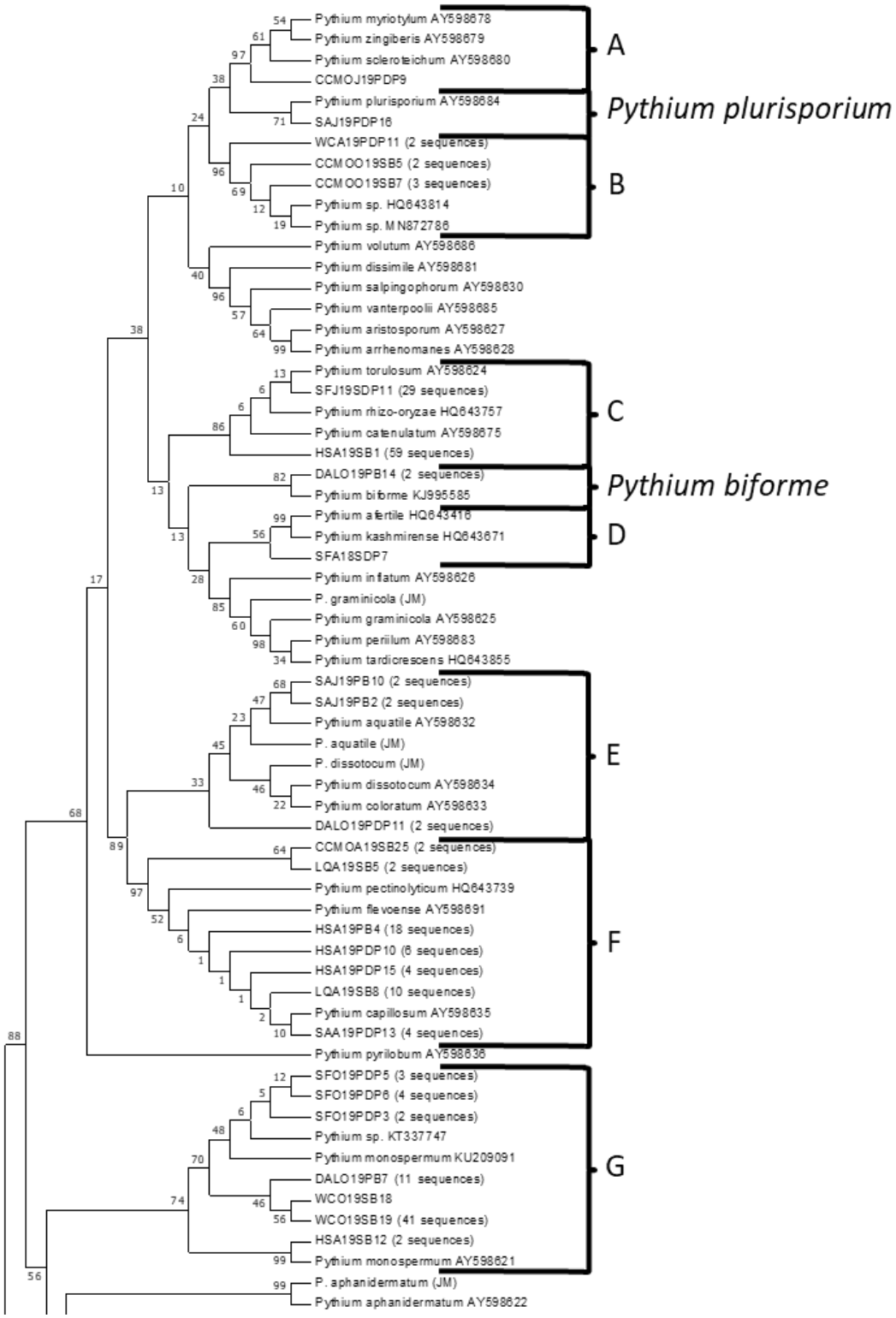


Figure 2.1. (*cont.*) Phylogeny of morphologically characterized isolates and representative sequences obtained from putative *Pythium* spp. isolated from golf course irrigation systems based on ITS (1 & 2) and 5.8S genes of ribosomal DNA. Unresolved clades are denoted A through N. Bootstrap values are based on 1,000 resamplings of the data set and displayed near branch nodes.

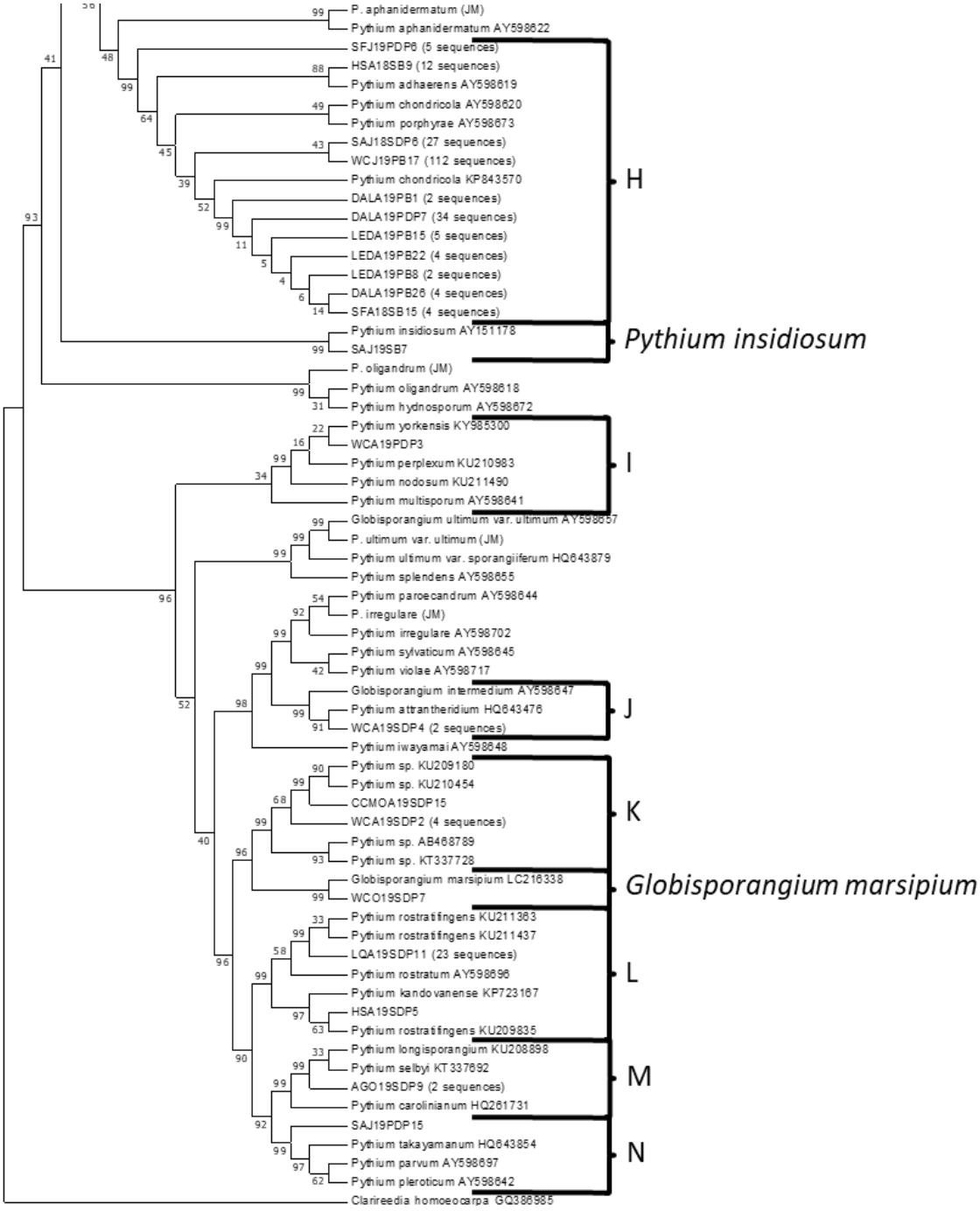


Figure 2.2. Phylogeny of representative sequences obtained from off-target Oomycetes isolated from golf course irrigation systems based on ITS (1 & 2) and 5.8S genes of ribosomal DNA. Unresolved clades are denoted O through U. Bootstrap values are based on 1,000 resamplings of the data set and displayed near branch nodes.

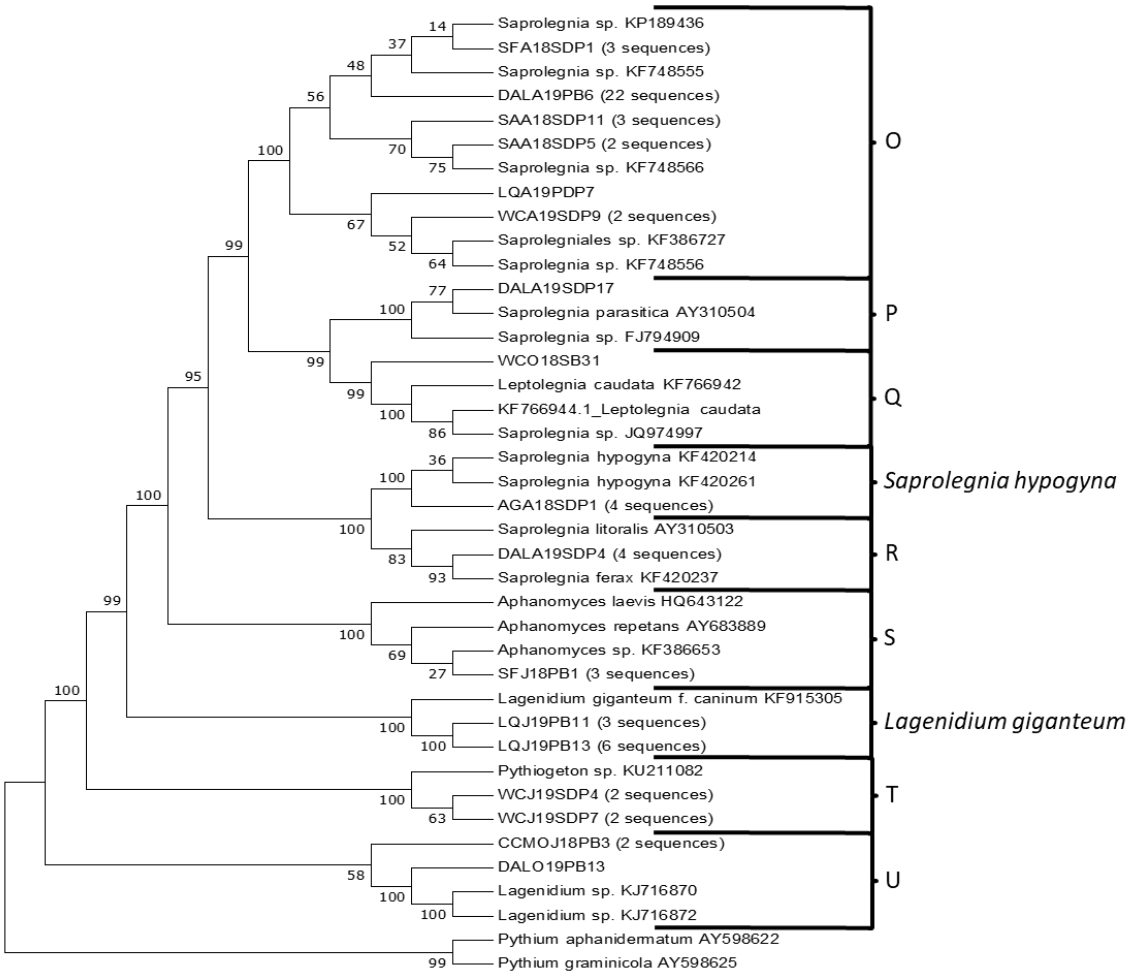


Figure 2.3. Relative distribution of *Pythium* clades detected through baiting and membrane filtration in Missouri and Kansas golf course irrigation water samples. Samples were collected in April, July, and October of 2018 and 2019. Unresolved species are denoted A through N. Striped cells indicate clades that were detected through both methods.

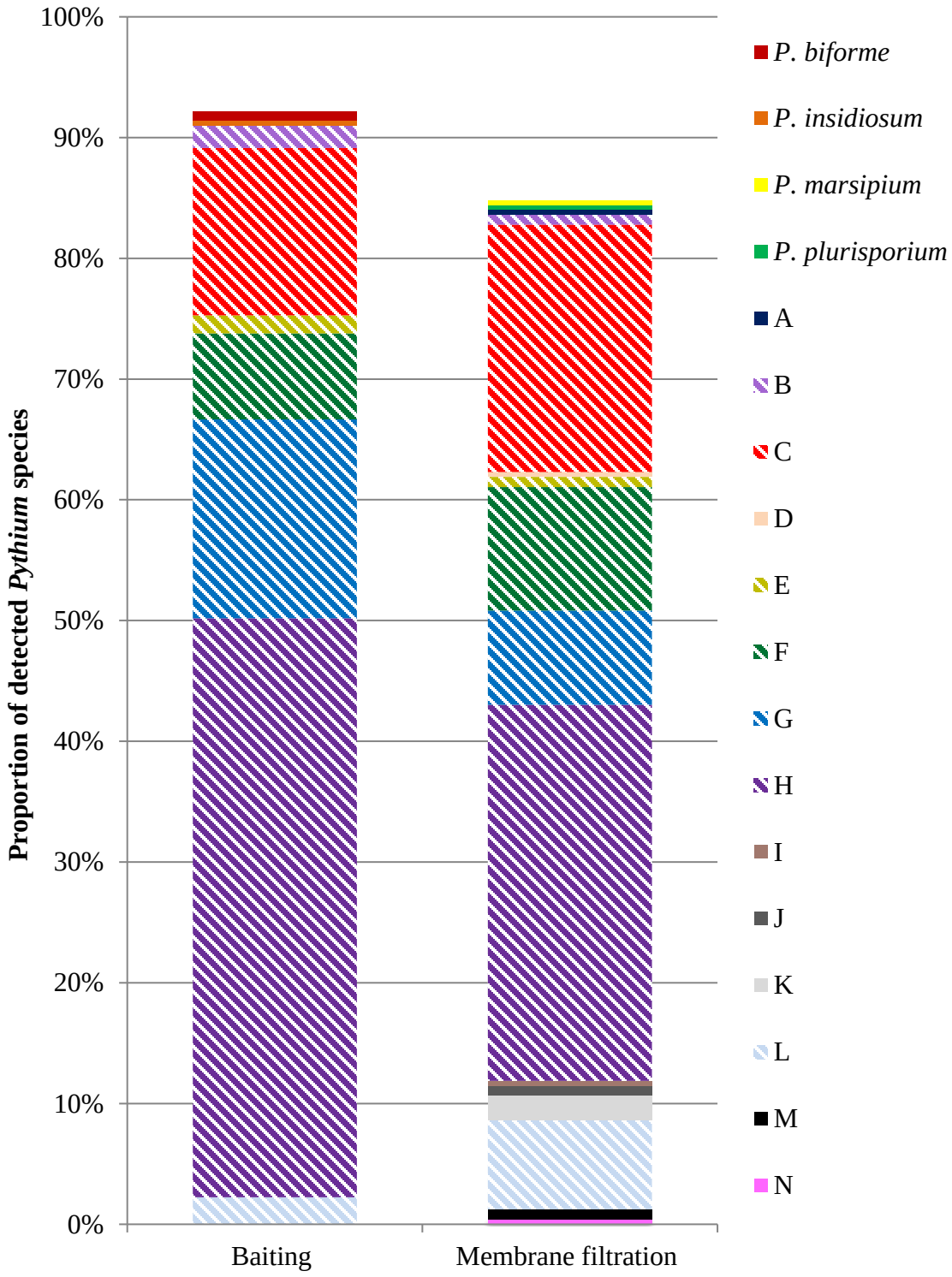


Figure 2.4. Average number of *Pythium* sequences obtained from water samples collected from Missouri and Kansas golf course irrigation systems in July of 2018, April of 2019, July of 2019, and October of 2019. Error bars are based on standard error.

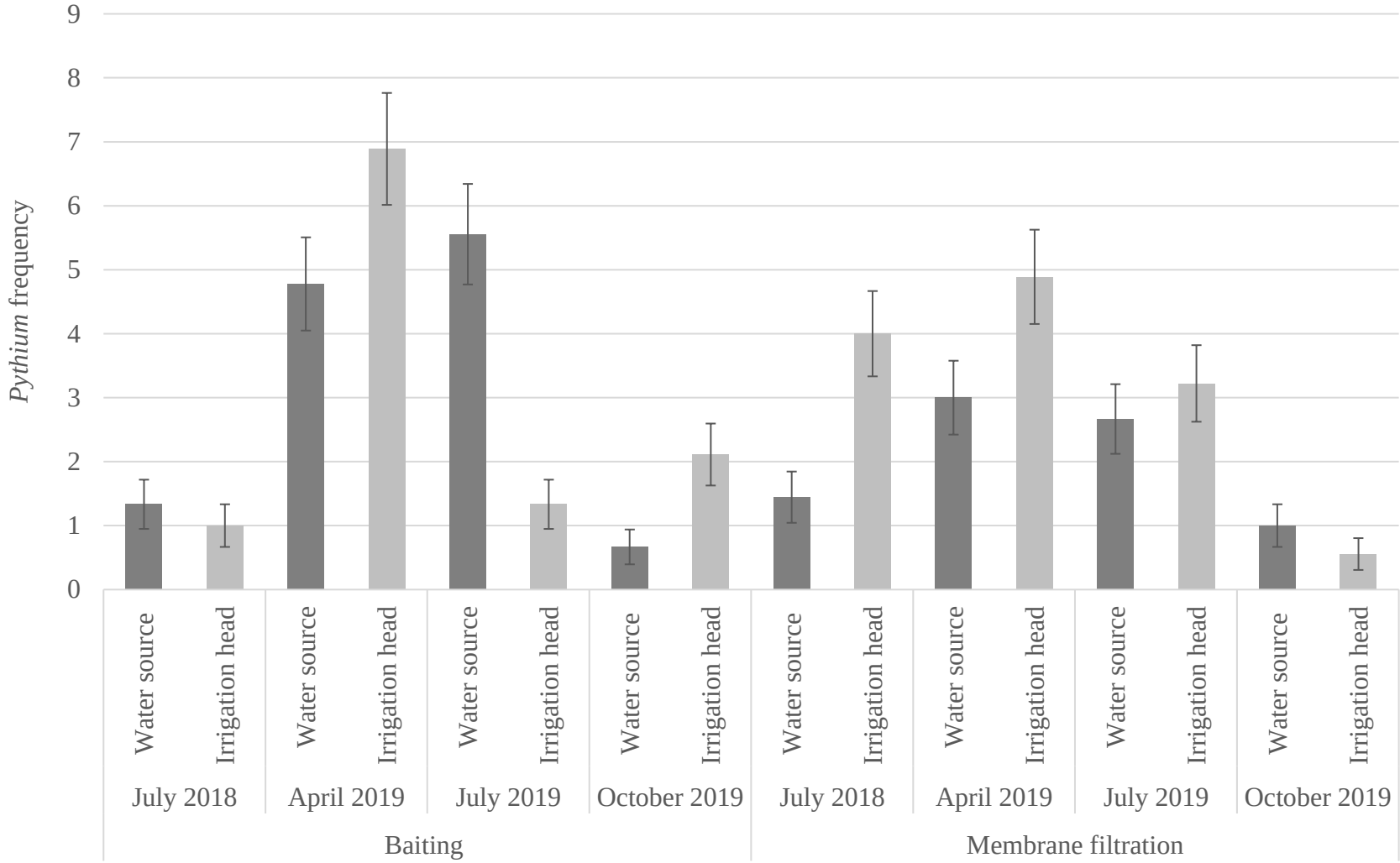


Figure 2.5. Proportion of samples based on the number of detectable species. Samples were collected from nine Missouri and Kansas golf course irrigation systems.

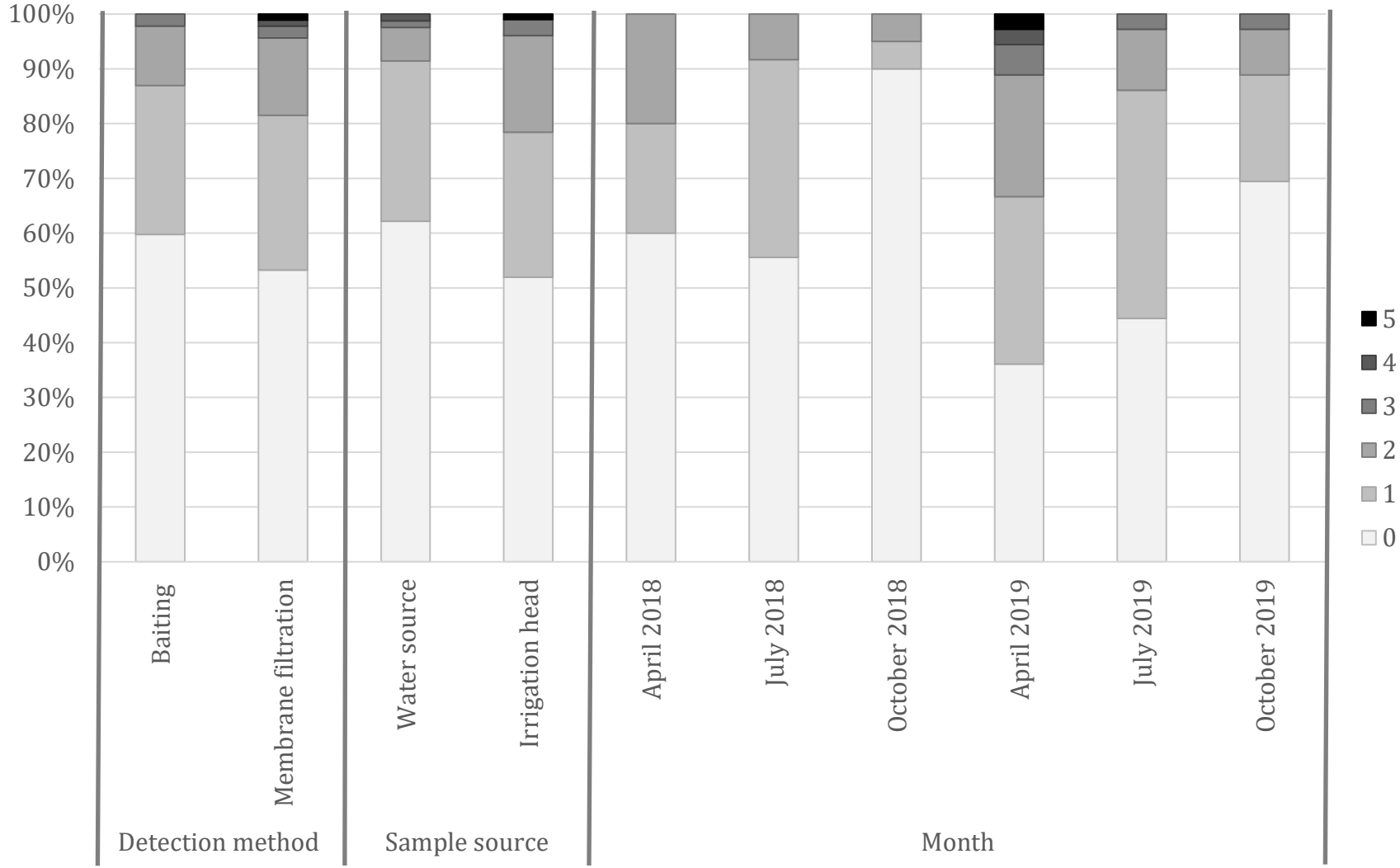


Figure 2.6. Relative distribution of *Pythium* clades detected in water samples from irrigation heads and water sources from Missouri and Kansas golf course irrigation systems. Samples were collected in April, July, and October of 2018 and 2019. Unresolved species are denoted A through N. Striped cells indicate clades that were detected in both sampling sources.

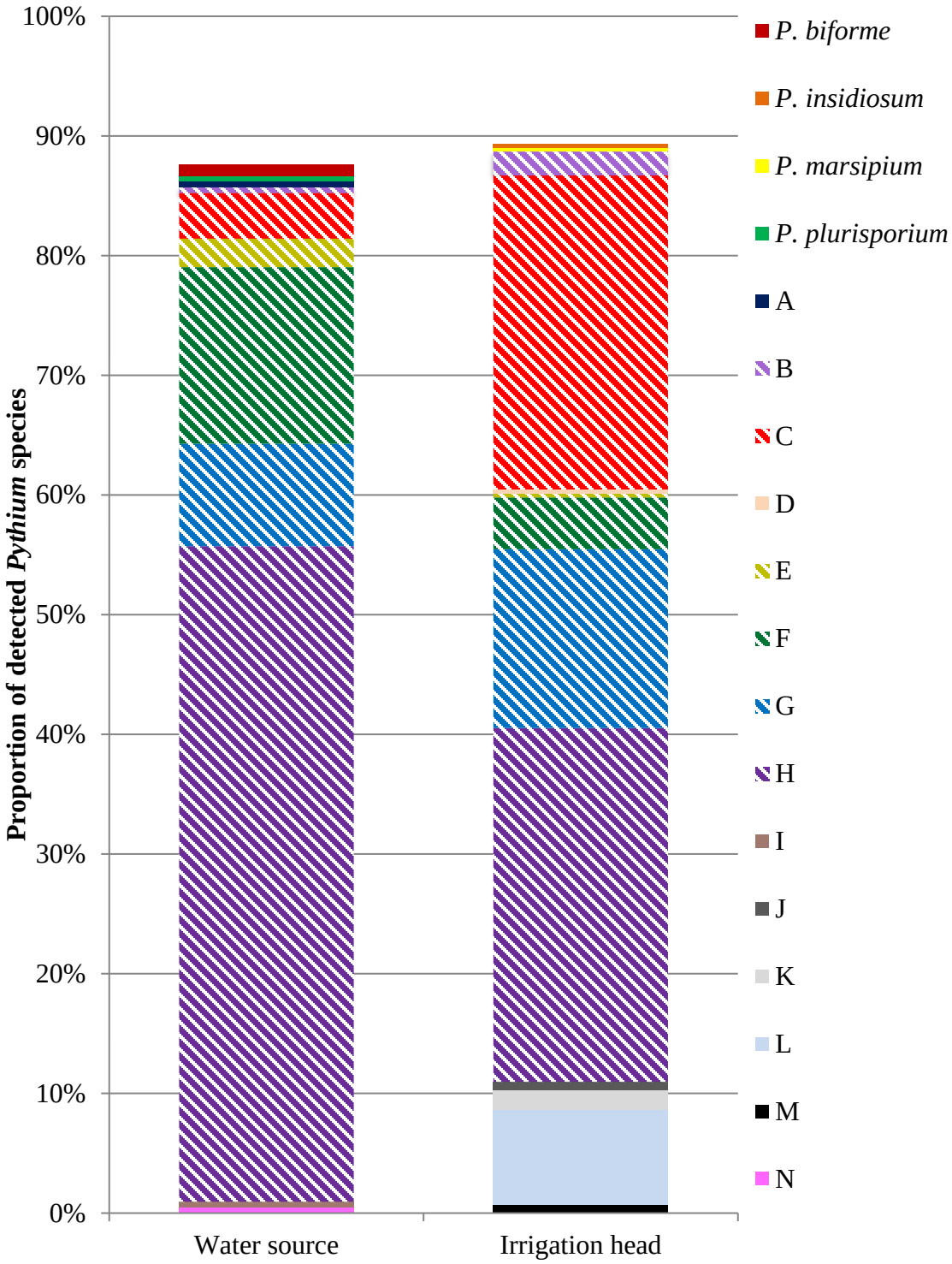
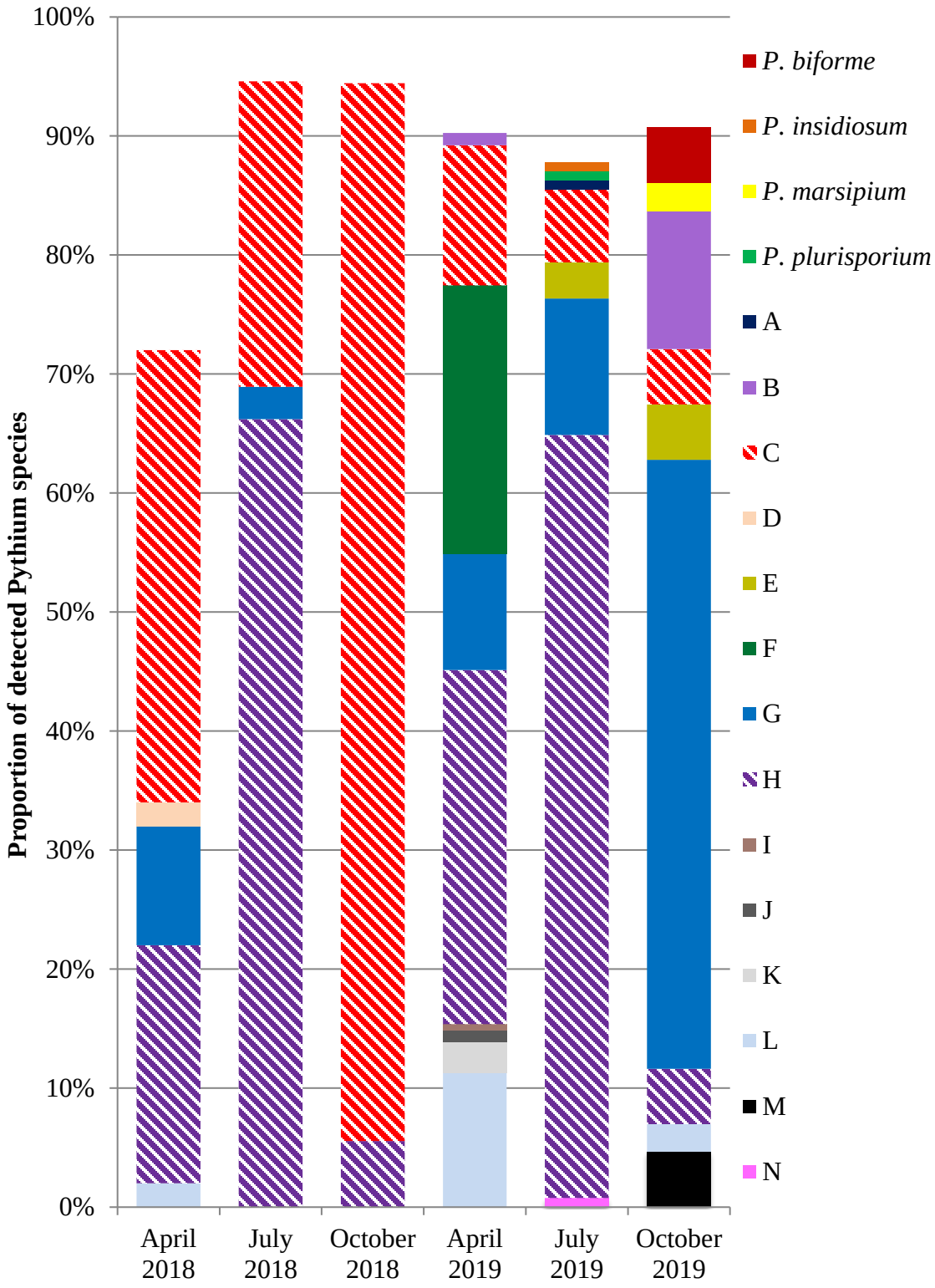


Figure 2.7. Relative distribution of *Pythium* clades detected in Missouri and Kansas golf course irrigation water samples in April, July, and October of 2018 and 2019. Unresolved species are denoted A through N. Striped cells indicate clades that were detected every month.



Chapter 3

Influence of sampling site, month, and nutrient concentrations on *Pythium* frequency in golf course irrigation samples.

Abstract: The genus *Pythium* contains causal agents of several diseases on creeping bentgrass putting greens. *Pythium* spp. are known to disseminate through irrigation system but the influence of macronutrients and chloride on *Pythium* in water is not well understood. Water samples were compared between eight golf courses in Missouri and Kansas. Samples were collected from 2018 to 2019 in April, July, and October. Each sample was probed for *Pythium* spp. through baiting and membrane filtration, and samples were assayed for temperature, total phosphorus, total dissolved phosphorus, total dissolved nitrogen, and chloride. Cultures were isolated on PARP media and DNA was extracted from mycelium. ITS regions were PCR amplified, sequenced, and compared with Genbank accessions. Phylogenetic trees were constructed using representative sample sequences, morphologically identified isolate sequences, and Genbank accessions. Detected species include *Pythium biforme*, *P. insidiosum, P. marsipium*, and *P. plurisporium.* None of these species are known pathogens of creeping bentgrass. Clades A through N lacked species-level resolution. Temperature, sampling site, total phosphorus, total dissolved phosphorus, total dissolved nitrogen, and chloride did not influence *Pythium* frequencies in samples. However, sampling month did significantly influence the frequency of *Pythium* detection. Overall, all irrigation systems that utilize surface water sources contained at least three *Pythium* spp. over the course of two years. This study establishes irrigation water as a consistent source of potentially pathogenic *Pythium* inoculum on putting greens, and expands the list of *Pythium* spp. that come in contact with putting greens in Missouri and Kansas.

Introduction:

Creeping bentgrass (*Agrostis stolonifera L.*) is a common turfgrass used to establish golf course putting greens in the transition zone. These plants are placed under intense abiotic stress due to frequent mowing and temperature extremes. These conditions predispose putting greens to infection by *Pythium* species that cause diseases such as Pythium root rot and Pythium root dysfunction (Abad et al. 1994, Kerns & Tredway 2008, Smiley et al. 2005). *Pythium* spp. are known to disseminate through irrigation systems in agricultural and greenhouse settings (Bush et al. 2003, Hong & Moorman 2005). Most *Pythium* spp. produce zoospores at different temperatures, which influences species abundance in irrigation systems throughout the year (Redekar et al. 2019, van der Plaats-Niterink 1981). Oomycota, the phylum containing *Pythium*, is closely related to diatoms and brown algae (Schroeder et al. 2013). Given there is enough silica, diatom abundance in marine waters is influenced by macronutrient concentrations, but this has not been observed in *Pythium* (Bruland et al. 2001). The objectives of this study were to i) detect *Pythium* spp. disseminating through golf course irrigation systems in Missouri and Kansas ii) determine if total *Pythium* frequency differs between sample site and month and iii) investigate if temperature or water quality influences total *Pythium* frequency.

Pythium root rot is caused by several *Pythium* spp. that vary in pathogenicity (Abad et al. 1994). Some of the most aggressive species include, but are not limited to, *G. ultimum, P. aphanidermatum, P. graminicola,* and *P. vanterpoolii* (Abad et al. 1994). Symptoms of Pythium root rot include root necrosis and patches of chlorotic turf that coalesce into large areas during the summer (Smiley et al. 2005). Pythium root

dysfunction is primarily caused by *P. volutum* and *P. arrhenomanes* (Kerns & Tredway 2008). Symptoms of Pythium root dysfunction include tan discoloration of roots, absence of root hairs, and patches of chlorotic foliage. Both diseases are unsightly and adversely disrupt the playing surface. While cultural practices such as maintaining proper greens drainage and utilizing more tolerant turfgrass species or cultivars may help to mitigate damage from Pythium diseases, preventive fungicide treatment on greens with a history of the disease is often necessary (Kerns & Tredway 2010). Fungicide treatments can cost superintendents 2,300 to 6,100 dollars per hectare treated each year (Tim Burch, personal communication, 12/11/2019). Understanding and elucidating point sources of *Pythium* inoculum can potentially improve or expand these cultural practices and minimize fungicide use.

The influence of nutrient concentrations on *Pythium* populations in water is not well studied. Water bodies that supply golf course irrigation systems are often surrounded by maintained turfgrass or agriculture and may collect fertilizer runoff. The subsequent influx of macronutrients could potentially influence *Pythium* populations in the water. The influence of macronutrients and basic water quality parameters on *Pythium* needs to be investigated to determine if nutrient rich water bodies are more likely to introduce *Pythium* spp. to irrigation systems. This information when coupled with geographic and temporal *Pythium* distributions will allow for more accurate risk assessment of water bodies used to irrigate putting greens.

Materials and methods:

Sample collection. Eight golf courses in Missouri and two in Kansas with a history of disease caused by *Pythium* root-infecting species were selected for this study.

Clear oblong glass baking dishes (2.8 liters, 22.9 x 33.0 cm, Corelle Brands LLC, Rosemont, IL) were placed on putting greens and used to collect three L of water from multiple irrigation heads. Baking dishes were triple-rinsed with deionized (DI) water and autoclaved prior to use. Water temperature at the irrigation head was determined by averaging three readings taken by an infrared (IR) temperature meter (Spectrum Technologies, Bridgend, United Kingdom). A Van Dorn water sampler (Flinn Scientific Inc., Batavia, IL) was used to collect two L of water from open water sources. The Van Dorn sampler was positioned near irrigation intakes approximately 0.6 m from the bottom of each water source. The Van Dorn sampler was sterilized with 10% NaClO and triple rinsed with DI water prior to use. Irrigation head samples (IHS) and water source samples (WSS) were consolidated in separate glass media bottles and sealed with polybutylene terephthalate caps (Corning Inc., Corning, NY). Media bottles were washed with powdered precision cleaner (Alconox Inc., White Plains, NY), triple-rinsed with DI water, and autoclaved prior to use. A direct soil electrical conductivity (EC) meter (Spectrum Technologies, Bridgend, United Kingdom) was used to measure temperature of WSS. High-density polyethylene containers (Thermo Fisher Scientific, Waltham, MA) were used to store 60 ml aliquots from IHS at -20°C prior to water quality analysis. The remaining sample was transported and stored at 15°C. Irrigation head and water source samples were probed for *Pythium* spp. separately.

Zoospore baiting. Leaves from six-week-old creeping bentgrass (*Agrostis stolonifera L.* var. 'Penncross') plants grown in a sterile greenhouse setting were used for baiting zoospores in water samples. Bentgrass leaves were cut to six cm, suspended in 150 ml of DI water, and autoclaved prior to baiting. A 500 ml aliquot from each water

sample was partitioned into two sterile 250 ml beakers. An autoclaved wire mesh tea infuser was used to suspend 10 creeping bentgrass leaves in each beaker, and covered with foil. These bait traps were incubated on a lab bench at ~23°C for 48 hr. After incubation, bait tissue was dried on a sterile paper towel and cut in half. Half of the bait tissue was plated onto PARP media (Jeffers & Martin 1986). PARP medium contained 20 g corn meal agar (Becton, Dickinson and Company, Franklin Lakes, New Jersey), 250 mg ampicillin sodium salt (Fisher Scientific, Waltham, MA), 10 mg pimaricin (Fisher Scientific, Waltham, MA), 10 mg rifampicin (Fisher Scientific, Waltham, MA) dissolved in 1 ml dimethyl sulfoxide (Fisher Scientific, Waltham, MA), and 5 ml 5% pentachloronitrobenzene (Sigma-Aldrich, St. Louis, MO) per L of DI water. The other half of bait tissue was placed in two screwcap vials and stored at -20°C until direct DNA extraction. Autoclaved creeping bentgrass leaves that were not used in bait traps were plated onto PARP media to ensure complete sterilization. Petri dishes were sealed with Parafilm (Bemis Company, Neenah, WI) stored on a lab bench at ~23°C, and checked daily for mycelia growth. Developing colonies were examined under a microscope and cultures with white coenocytic hyphae were isolated on fresh PARP media.

Membrane filtration. Two 500 ml aliquots from each water sample were vacuum filtered through two Durapore filters with five µm pores (MilliporeSigma, Burlington, MA) to capture zoospores and mycelial fragments (Hong et al. 2002). Filters were held in place by an autoclaved 47 mm magnetic filter funnel (Pall Corporation, Port Washington, New York). One filter was cut into 16 equal pieces and plated on PARP media. Petri dishes were sealed, stored on a lab bench at ~23°C, and checked daily for mycelia growth. Developing colonies were examined under a microscope and cultures

with white coenocytic hyphae were isolated on fresh PARP media. The second filter was cut into 1 x 1 mm squares, split into four screwcap vials, and stored in -20°C to await DNA extraction.

Propagating mycelium. Mycelium from putative *Pythium* spp. and morphologically characterized isolates of *G. ultimum* var *ultimum, P. aphanidermatum, P. aquatile, P. dissotocum, P. graminicola, P. irregulare,* and *P. oligandrum* from the collection of J. Mihail (University of Missouri, Columbia) were propagated by transferring hyphae to V8 media overlaid with cellophane (Gel Company, San Francisco, CA). V8 media contained 15 g granulated agar (Fisher Scientific, Waltham, MA), 100 ml original V8 juice (Campbell, Camden, NJ), and 0.75 g calcium carbonate (Fisher Scientific, Waltham, MA) per L of DI water. Mycelium was scraped off cellophane using a flame sterilized scalpel and stored in screwcap vials (Thermo Fisher Scientific, Waltham, MA) at -20°C.

DNA extraction and PCR. DNA was extracted from mycelium, bait tissue, and membrane filters using the protocol and reagents in the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA). DNA concentrations were quantified with a NanoDrop OneC Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and extracts were diluted with nuclease free water (Qiagen, Hilden, Germany) to a concentration of 50 ng/µl. PCR mixtures contained 4 µl of DNA template (200 ng of DNA), 25 µl of DreamTaq Hot start PCR master mix (Thermo Fisher Scientific, Waltham, MA), 1 µl (200nM) of the Oomycete specific primer ITS4Oo, 1 µl (200nM) of the universal primer ITS5, and 19 µl of nuclease free water (Nikolcheva & Barlocher 2004, White et al. 1990). Thermocycler (Eppendorf, Hamburg, Germany) settings were

10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 49°C for 30 sec, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. Successful amplifications were confirmed through electrophoresis, cleaned using ExoSAP-IT PCR product cleanup reagent (Applied Biosystems, Foster City, CA), and Sanger sequenced by the DNA Core Facility at the University of Missouri.

Phylogenetic analysis. Contiguous sample sequences were assembled and representative sample sequences were selected using SeqMan Pro (14; DNASTAR Inc., Madison, WI). The Basic Local Alignment Search Tool (megaBLAST) was used to compare representative sample sequences with Genbank accessions. The top two or three similar sequences based on maximal percent identity match were used in phylogenetic analysis. The ITS sequences of *Pythium* spp. described in Abad et al. (1994) were downloaded from Genbank using accessions from Levesque et al. (2004) and Robideau et al. (2011) and included in the phylogenetic analysis. All sequences were aligned using ClustalW in MEGA (7, Pennsylvania State University, State College, PA) and truncated within 25 base pairs from the outermost nucleotides conserved between 70% of all sequences. Two phylogenetic trees were constructed with the neighbor-joining algorithm using the Kimura two-parameter model. One tree utilized *Clarireedia homoeocarpa* (GQ386985) as an outgroup and was constructed with sequences associated with *Globisporangium* and *Pythium*. *Globisporangium* spp. were included in the phylogenetic analysis of *Pythium* spp. because some causal agents of *Pythium* disease, such as *Pythium ultimum* var. *ultimum,* have been reclassified into *Globisporangium* (Uzuhashi et al. 2010). The other tree utilized *P. aphanidermatum* (AY598622) and *P. arrhenomanes* (AY598628) as an outgroup and was constructed using sequences associated with other

Oomycetes. Bootstrap values on branch nodes are based on 1000 random samples of the data set.

Water quality analysis. Ten ml aliquots were taken from the 60 ml of IHS stored in high-density polyethelene containers and used to evaluate the concentration of total phosphorus (TP), total dissolved phosphorus (TDP), total dissolved nitrogen (TDN), and chloride. Water samples were filtered through glass microfiber filters (0.7µm pores, Whatman plc, Maidstone, United Kingdom) prior to TDN and TDP analysis (Crumpton et al. 1992). Total dissolved nitrogen concentrations were analyzed using a 2nd derivative spectroscopy method following persulfate digestion (Crumpton et al. 1992). Total phosphorus and TDP were analyzed using an ascorbic acid colorimetric method following persulfate digestion (Eaton 1996). Nutrient concentrations were quantified with a Spectronic Genesys 2 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Chloride was analyzed using a mercuric thiocyanate method on a Lachat Flow Injection Analyzer (Hach company, Loveland, CO, Diamond 1994). Each analysis was processed in triplicate and the average of the three values was used for statistical analysis. Detection limits of nitrogen, phosphorus, and chloride are 2.50 µmol of nitrogen per L, 0.03 µmol of phosphorus per L, and 1.41 µmol of chloride per L.

Statistical analysis. The following hypotheses were tested: the number of total *Pythium* sequences obtained per sample is identical between i) sites ii) months and iii) nutrient concentrations. Temperature measurements were compared between 91 water samples taken from irrigation heads and water sources. Total phosphorus, TDP, TDN, and chloride concentrations were compared between sites that utilize surface water as a source of irrigation. Water quality data were tested for normality using the Shapiro-Wilk

test ($p < 0.05$). When possible, non-normally distributed data was transformed (common log or square root) to achieve normality. Differences between water quality measurements of each sample were evaluated using PROC GLIMMIX in SAS 9.4 (SAS Institute).

The number of *Pythium* sequences obtained per clade was used as a proxy measurement of species richness in each sample. *Pythium* counts were pooled between IHS and WSS prior to analysis. Differences in the number of *Pythium* sequences obtained per sample were evaluated using PROC GENMOD in SAS. Total *Pythium* frequency followed a negative binomial distribution and linear models were used to predict *Pythium* frequency. A negative binomial distribution was used due to the unbalanced dataset, and the subsequent increase in variance in April and October 2018. Data omitted from statistical analysis include observations from sites that only utilize municipal and well water. Municipal water was taken from the Missouri river and sterilized with chlorine. This site was excluded because no *Pythium* spp. were detected. A site that only utilized well water was excluded because nothing was detected in the water source, and to restrict comparisons to irrigation systems that utilize surface water (Table 3.1).

Results

Detected *Pythium* clades. In total, 511 ITS sequences were obtained using the isolation-based methods described above, with 453 sequences identified as a *Pythium* or *Globisporangium* spp. Cultureless methods were mostly unsuccessful due to the inability of Sanger sequencing to resolve the multiple species present in the sample. Eleven *Pythium* and four off-target Oomycete sequences were obtained through direct DNA extraction of bait tissue and zero sequences were obtained from direct DNA extraction

from membrane filters. Detected *Pythium* spp. include *P. biforme, P. insidiosum, P. marsipium,* and *P. plurisporium* (Figure 2.1). Clades A through N could not be resolved to the species level. Clades A, C, D, E, I, and M contain causal agents of root and crown rot on creeping bentgrass (Table 2.3, Abad et al. 1994). However, these clades also contain nonpathogenic species or species with unknown pathogenicity to creeping bentgrass. Clades G, H, J, and L contain phytopathogenic *Pythium* spp. but it is unknown if they infect creeping bentgrass (Allain-Boulé et al. 2004, Bouket et al. 2015, Li et al. 2014, Mankin 1969, Sprague 1950, Weiland et al. 2013).

Differences between sites. Total phosphorus, TDP, TDN, and chloride concentrations varied between sample sites and both temperature measurements varied between months (Table 3.2). The highest chloride concentration was observed in an irrigation system supplied by lake water (Figure 3.1.A.). The lake is surrounded by urban development and increased chloride concentrations may be attributed to large volumes of salt used to clear surrounding roadways in the winter. The highest TP, TDP, and TDN concentrations were observed in irrigation systems supplied by surface water surrounded by fertilized turfgrass or agriculture (Figure 3.1.B and 3.1.C). July water temperatures ranged from 23.6 – 27.7 while April and October temperatures ranged from 11.4 – 18.9 (Figure 3.2). Location, IR temperature, WSS temperature, TP, TDP, TDN, and chloride concentrations did not significantly influence total *Pythium* frequency (Table 3.3). However, month was shown to significantly influence total *Pythium* frequency. The highest average *Pythium* frequency was observed in April of 2019 and July months (Figure 3.3.B).

Pythium biforme, *P. insidiosum*, *P. marsipium*, *P. plurisporium* and clades A, D, I, J, and N were only detected from one site during a single month (Table 3.4). Clade C, a potential causal agent of Pythium root rot on creeping bentgrass, and clade H, were obtained from all irrigation systems that utilize surface water. Clade C was detected most frequently in site 5 and during the first four sampling months. The lowest frequency of potentially pathogenic *Pythium* clades was obtained from the largest water body. Clade F was only detected in April 2019, but was present in seven sites. Two or three potentially pathogenic *Pythium* clades were detected in five sites and four months. Up to 10 species were detected in a single site and nine species detected in a single month.

Discussion

Prior to this study, it was established that *Pythium* and other Oomycetes disseminate through irrigation systems that utilize recycled or surface water sources (Bush et al. 2003, Hong & Moorman 2005, Redekar et al. 2019). This research demonstrates that *Pythium* dissemination through irrigation systems also occurs in a golf setting. Overall, the number of clades detected in this study is comparable to previous experiments that probe irrigation systems for Oomycetes using isolation-based detection methods (Parke et al. 2014). Total phosphorus concentrations of water samples were similar to previous research that sampled Missouri water bodies (Jones & Knowlton 1993). Sites with higher TP, TDP, and TDN concentrations are likely due to fertilizer runoff from agriculture or surrounding well-maintained turfgrass. The temperature differences observed between months are characteristic of the seasonal changes throughout the transition zone (Dunn & Diesburg 2004).

Exponential and logarithmic models for predicting total *Pythium* frequency based on IR temperature, WSS temperature, TP, TDP, TDN, and chloride did not improve Akaike information criterion values when compared to linear models. The lack of interaction between these parameters and total *Pythium* frequency suggests that other factors may explain the variation of total observed *Pythium* populations such as fluctuations of individual *Pythium* species, presence of alternative hosts, or decomposing host tissue in water. This research supports previous studies that have shown that *Pythium* species in an irrigation system will change throughout the year since many *Pythium* spp. were only observed in a single month and/or location (Redekar et al. 2019). Clade F, a clade only detectable in April 2019, accounted for 25% of total *Pythium* frequency that month, and is likely a cause for the significant increase in *Pythium* frequency. The consistent *Pythium* frequency between locations is likely due to clades H and C. The high frequency of Clade H throughout the study is likely due to *P. adhaerens.* This *Pythium* spp. is pathogenic to green algae, which is present in many open water bodies and supports the assumption that *Pythium* frequency may be dependent on the presence of host tissue (van der Plaats-Niterink 1981). The high frequency of Clade C is likely due to *P. torulosum* and *P. catenulatum. Pythium torulosum* has slow to moderate mycelial growth rates on water agar and PARP media, but is commonly isolated from creeping bentgrass (Abad et al. 1994, Hsiang et al. 1995, Kerns & Tredway 2008, Nelson & Craft 1991). *P. catenulatum* is a moderate to rapidly growing *Pythium* species and likely outcompeted other developing isolates on petri dishes (Feng & Dernoeden 1999). Consistent frequency of sequences within clade C may suggest that isolation-based

detection methods used in this study may have imposed a selection bias, making it difficult to attribute frequency differences to other parameters.

Future experiments should utilize next generation sequencing methods paired with culturing methods to expand the breadth of detected species. Evidence of additional species may elucidate which species are competing for available resources, and provide a more accurate representation of the proportion of pathogenic *Pythium* spp. in water samples. Other experiments should focus on resolving the pathogenicity of *Pythium* spp. detected in this study. Eight *Pythium* clades detected in this study have unknown pathogenicity to creeping bentgrass, and the six clades that contain causal agents of root rot on creeping bentgrass also contain other species with unknown pathogenicity. Even less is known regarding how disease severity is influenced by infection of multiple *Pythium* species. Understanding how each of these *Pythium* species, or a combination of species, influences creeping bentgrass health is essential for assessing their risk.

The exact profile of *Pythium* spp. appears to be a case-by-case basis, so it is difficult to assess the risk associated with all surface water sources. Based on this research, superintendents that utilize surface water for irrigation should expect the presence of at least one *Pythium* species in the irrigation system. The small size of water samples taken in this study in relation to total water use on a putting green suggests that *Pythium* inoculum in irrigation water is much more common than previously assumed. Golf courses that utilize high-risk water sources and want to decrease the inoculum load distributed by an irrigation system may need to consider higher cost management practices such as utilizing municipal water or investing in water sterilization practices.

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Literature cited

Abad, Z. G., Shew, H. D., & Lucas, L. T. 1994. Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina. Phytopathology, 84(9), 913-921.

Allain-Boulé, N., Lévesque, C. A., Martinez, C., Bélanger, R. R., & Tweddell, R. J. 2004. Identification of *Pythium* species associated with cavity-spot lesions on carrots in eastern Quebec. Canadian Journal of Plant Pathology, 26(3), 365-370.

Bouket, A. C., Arzanlou, M., Tojo, M., & Babai-Ahari, A. 2015. *Pythium kandovanense* sp. nov., a fungus-like eukaryotic micro-organism (Stramenopila, Pythiales) isolated from snow-covered ryegrass leaves. International Journal of Systematic and Evolutionary Microbiology, 65(8), 2500-2506.

Bruland, K. W., Rue, E. L., & Smith, G. J. 2001. Iron and macronutrients in California coastal upwelling regimes: Implications for diatom blooms. Limnology and Oceanography, 46(7): 1661-1674.

Bush, E. A., Hong, C., & Stromberg, E. L. 2003. Fluctuations of *Phytophthora* and *Pythium* spp. in components of a recycling irrigation system. Plant Disease, 87(12), 1500-1506.

Crumpton, W. G., Isenhart, T. M., & Mitchell, P. D. 1992. Nitrate and organic N analyses with second-derivative spectroscopy. Limnology and Oceanography, 37(4): 907-913.

Diamond, D. 1994. Determination of chloride by flow injection analysis colorimetry. QuikChem method, 10-117.

Dunn, J., & Diesburg, K. 2004. Turf Management in the Transition Zone, John Wiley & Sons.

Eaton, A. D. 1996. Standard methods for the examination of water and wastewater, American Public Health Association, Washington, DC.

Ellis, M. L., Paul, P. A., Dorrance, A. E., & Broders, K. D. 2012. Two new species of *Pythium*, *P. schmitthenneri* and *P. selbyi* pathogens of corn and soybean in Ohio. Mycologia, 104(2), 477-487.

Feng, Y., & Dernoeden, P. H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. Plant Disease, 83(6), 516-520.

Hanlin, R. T. 1978. Plant Disease Index For Maize in the United States, Part 1: Host Index. University of Georgia, Georgia, USA

Hong, C., Richardson, P. A., & Kong, P. 2002. Comparison of membrane filters as a tool for isolating pythiaceous species from irrigation water. Phytopathology, 92(6), 610-616.

Hong, C. X., & Moorman, G. W. 2005. Plant pathogens in irrigation water: challenges and opportunities. Critical Reviews in Plant Sciences, 24(3), 189-208.

Hsiang, T., Wu, C., Yang, L., & Liu, L. 1995. Pythium root rot associated with cool-season dieback of turfgrass in Ontario and Quebec. Canadian Plant Disease Survey, 75(2), 191-195.

Jeffers, S. N., & Martin, S. B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease, 70(11), 1038-1043.

Jones, J. R., & Knowlton, M. F. 1993. Limnology of Missouri reservoirs: an analysis of regional patterns. Lake and Reservoir Management, 8(1), 17-30.

Kawamura, Y., Yokoo, K., Tojo, M., & Hishiike, M. 2005. Distribution of *Pythium porphyrae*, the causal agent of red rot disease of *Porphyrae* spp., in the Ariake Sea, Japan. Plant Disease, 89(10), 1041-1047.

Kerns, J. P., & Tredway, L. P. 2008. Pathogenicity of *Pythium* species associated with Pythium root dysfunction of creeping bentgrass and their impact on root growth and survival. Plant Disease, 92(6), 862-869.

Kerns, J. P., and Tredway, L. P. 2010. Pythium root dysfunction of creeping bentgrass. Plant Health Progress, 11(1), 40.

Lee, S. J., Jee, B. Y., Son, M. H., & Lee, S. R. 2017. Infection and cox2 sequence of *Pythium chondricola* (Oomycetes) causing red rot disease in *Pyropia yezoensis* (Rhodophyta) in Korea. Algae, 32(2), 155-160.

Levesque, C. A., & De Cock, A. W. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Research, 108(12), 1363-1383.

Li, Y. P., You, M. P., & Barbetti, M. J. 2014. Species of *Pythium* associated with seedling root and hypocotyl disease on common bean (*Phaseolus vulgaris*) in Western Australia. Plant Disease, 98(9) 1241-1247.

Mankin, C. J. 1969. Diseases of grasses and cereals in South Dakota. Agricultural Experement Station, South Dakota State University, Brookings, SD.

Nelson, E. B., & Craft, C. M., 1991. Identification and comparative pathogenicity of *Pythium* spp. from roots and crowns of turfgrasses exhibiting symptoms of root rot. Phytopathology, 81(12), 1529-1536.

Nikolcheva, L. G., & Bärlocher, F. 2004. Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. Mycological Progress, 3(1), 41-49.

Parke, J. L., Knaus, B. J., Fieland, V. J., Lewis, C., & Grünwald, N. J. 2014. *Phytophthora* community structure analyses in Oregon nurseries inform systems approaches to disease management. Phytopathology, 104(10), 1052-1062.

Pottorff, L. P., & Panter, K. L. 1997. Survey of *Pythium* and *Phytophthora* spp. in irrigation water used by Colorado commercial greenhouses. HortTechnology, 7(2), 153-155.

Redekar, N. R., Eberhart, J. L., & Parke, J. L. 2019. Diversity of *Phytophthora*, *Pythium*, and *Phytopythium* species in recycled irrigation water in a Container Nursery. Phytobiomes Journal, 3(1), 31-45.

Robertson, G. I. 1973. Pathogenicity of *Pythium* spp. to seeds and seedling roots. New Zealand journal of agricultural research, 16(3), 367-372.

Robideau, G. P., de Cock, A. W., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D. W., Désaulniers, N., Eggertson, Q. A., Gachon, C. M. M., Hu, C. H., Küpper, F. C., Rintoul, T. L., Sarhan, E. S., Verstappen, E. C. P., Zhang, Y., Bonants, P. J. M., Ristaino, J. B., & André Lévesque, C. A. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Molecular Ecology Resources, 11(6), 1002-1011.

Schroeder, K. L., Martin, F. N., de Cock, A. W., Lévesque, C. A., Spies, C. F., Okubara, P. A., & Paulitz, T. C. 2013. Molecular detection and quantification of *Pythium* species: evolving taxonomy, new tools, and challenges. Plant Disease, 97(1), 4-20.

Schurko, A. M., Mendoza, L., Lévesque, C. A., Désaulniers, N. L., de Cock, A. W. and Klassen, G. R. 2003. A molecular phylogeny of *Pythium insidiosum*. Mycological research, 107(5), 537-544.

Smiley, R. W., Dernoeden, P. H., & Clarke, B. B. 2005. Compendium of turfgrass diseases, St. Paul, MN: American Phytopathological Society

Sprague, R. 1950. Diseases of cereals and grasses in North America. Diseases of cereals and grasses in North America.

Tojo, M., Watanabe, K., Kida, K., Li, Y., & Numata, S. 2007. Mottle necrosis of sweet potato caused by *Pythium sclerotеichum* in Japan and varietal difference in susceptibility to the disease. Journal of General Plant Pathology, 73(2), 121-124.

Uzuhashi, S., Tojo, M., & Kakishima, M. 2010. Phylogeny of the genus *Pythium* and description of new genera. Mycoscience, 51(5), 337-365.

van der Plaats-Niterink, A. J. 1981. Monograph of the genus *Pythium*, Centraalbureau voor Schimmelcultures Baarn

van der Plaats-Niterink, A. J. 1972. The occurrence of *Pythium* in the Netherlands. III. *Pythium flevoense* sp. n. Acta botanica neerlandica, 21(6), 633-639.

Veterano, S. T., Coffua, L. S., Mena-Ali, J. I., & Blair, J. E. 2018. *Pythium yorkensis* sp. nov., a potential soybean pathogen from southeastern Pennsylvania, USA. Plant Pathology, 67(3), 619-625.

Weiland, J. E., Beck, B. R., & Davis, A. 2013. Pathogenicity and virulence of *Pythium* species obtained from forest nursery soils on Douglas-fir seedlings. Plant disease, 97(6), 744-748.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, 18(1), 315-322.

Zitnick-Anderson, K. K., & Nelson Jr., B. D. 2015. Identification and pathogenicity of *Pythium* on soybean in North Dakota. Plant Disease, 99(1), 31-38.

Table 3.1. Distribution of samples taken from golf course irrigation systems in Missouri and Kansas that utilize surface water sources. Striped cells indicate dates when samples were only collected from irrigation heads. Grey cells indicate dates when samples were collected from irrigation heads and water sources. Empty cells indicate dates when no sample was taken.

Location	Site	Water source (ha)	Irrigation sampling dates (Month-Year)					
			Apr-18	Jul-18	Oct-18	Apr-19	Jul-19	Oct-19
Columbia, MO	1	Surface water (0.6)						
	2	Surface water (3.1)						
St. Louis, MO	3	Surface water (0.8)						
Cape Girardeau, MO	4	Surface water (4.9)						
Springfield, MO	5	Surface water (3.1) & Well						
Branson, MO	6	Surface water (1.0)						
Olathe, KS	7	Surface water (62.7)						
	8	Surface water (9.6)						

Table 3.2. Effect of sampling site and month on water quality parameters of samples taken from eight golf courses in Missouri and Kansas. Samples were collected in April, July, and October of 2018 and 2019. Goodness of fit values are derived from Chi-Square values divided by degrees of freedom. *P*-values are based on an *F* statistic.

Response variable	Goodness of fit	*P*-value	
		Site	Month
Temperature – IR[a]	8.41	0.7649	<0.0001
Temperature- WSS[b]	30.94	0.5974	0.0011
Total phosphorus[c]	0.06	<0.0001	0.1306
Dissolved phosphorus[c]	0.10	<0.0001	0.1121
Dissolved nitrate[d]	0.04	<0.0001	0.0618
Chloride[c]	0.04	<0.0001	0.5254

[a] IR – Infrared

[b] WSS – Water source sample

[c] Common log transformed

[d] Square root transformed

Table 3.3. Effect of sampling site, month, temperature, nutrient concentrations, and chloride on total *Pythium* frequency response in samples taken from eight golf courses in Missouri and Kansas. Samples were collected in April, July, and October of 2018 and 2019. Total *Pythium* frequency follows a negative binomial distribution. Goodness of fit values are derived from Chi-Square values divided by degrees of freedom. *P*-values are based on a Chi-Square statistic.

Predictor variable	DF	Goodness of fit	*P*-value
Location	7	0.7674	0.8854
Month	5	0.9774	0.0197
Temperature -IR[a]	1	0.6361	0.9256
Temperature- WSS[b]	1	0.6435	0.5020
Total phosphorus	1	0.6501	0.7262
Dissolved phosphorus	1	0.6336	0.9213
Dissolved nitrate	1	0.6880	0.4898
Chloride	1	0.6148	0.7347

[a] IR – Infrared

[b] WSS – Water source sample

Table 3.4. Frequency of *Pythium* spp. and clades detected in Missouri and Kansas golf course irrigation systems. *Pythium* counts were derived by combining the number of *Pythium* sequences obtained from irrigation head samples and water source samples.

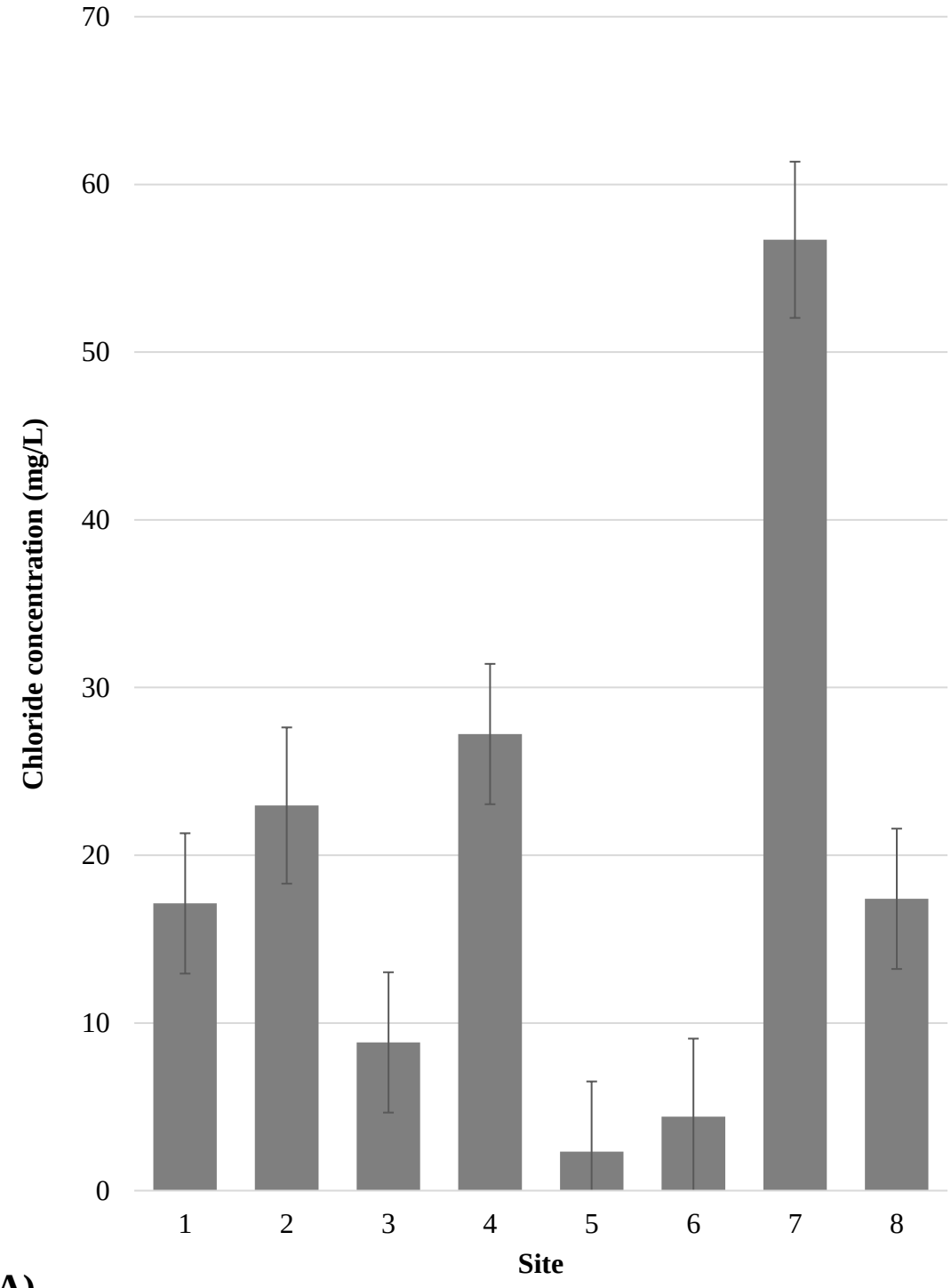
	Site #							
Species/Clade	1	2	3	4	5	6	7	8
P. biforme				2				
P. insidiosum			1					
P. marsipium								1
P. plurisporium			1					
A		1						
B		5						2
C	6	5	1	17	44	7	2	5
D	1							
E			4	1				
F	3	5	5	1	25		4	1
G	11	24	7	8	1		2	10
H	16	32	20	55	10	19	17	33
I								1
J								2
K		1						4
L	1				1	7	13	2
M								
N			1					
Total	38	73	40	84	81	33	38	61

Scale	5	15	25	35	45	55

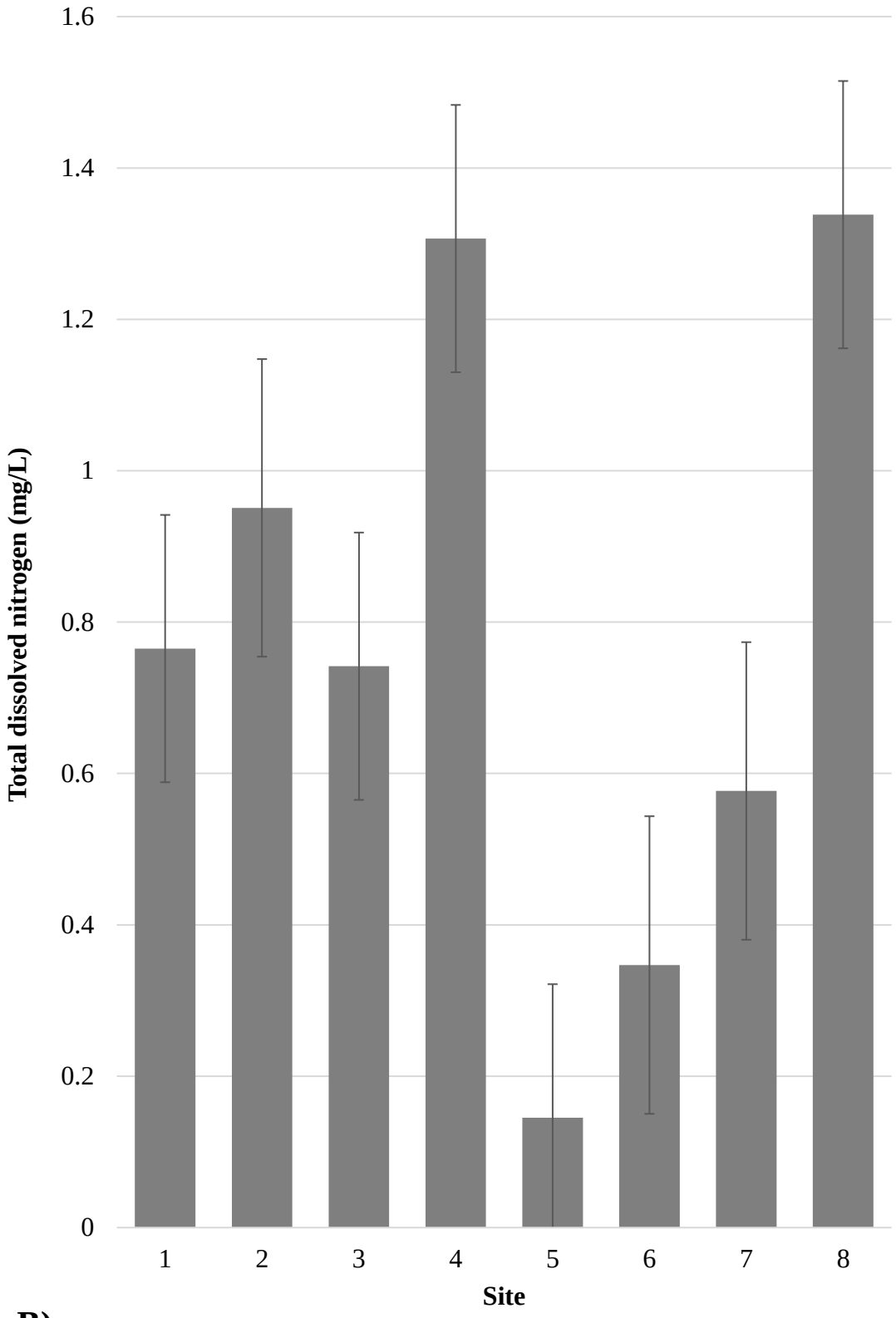
	2018			2019		
Species/Clade	April	July	October	April	July	October
P. biforme						2
P. insidiosum					1	
P. marsipium						1
P. plurisporium					1	
A					1	
B				2		5
C	19	19	16	23	8	2
D	1					
E					4	1
F				44		
G	5	2		19	15	22
H	8	49	1	58	84	2
I				1		
J				2		
K				5		
L	1			22		1
M						
N					1	
Total	34	70	17	176	115	36

Scale	4	24	44	64	84

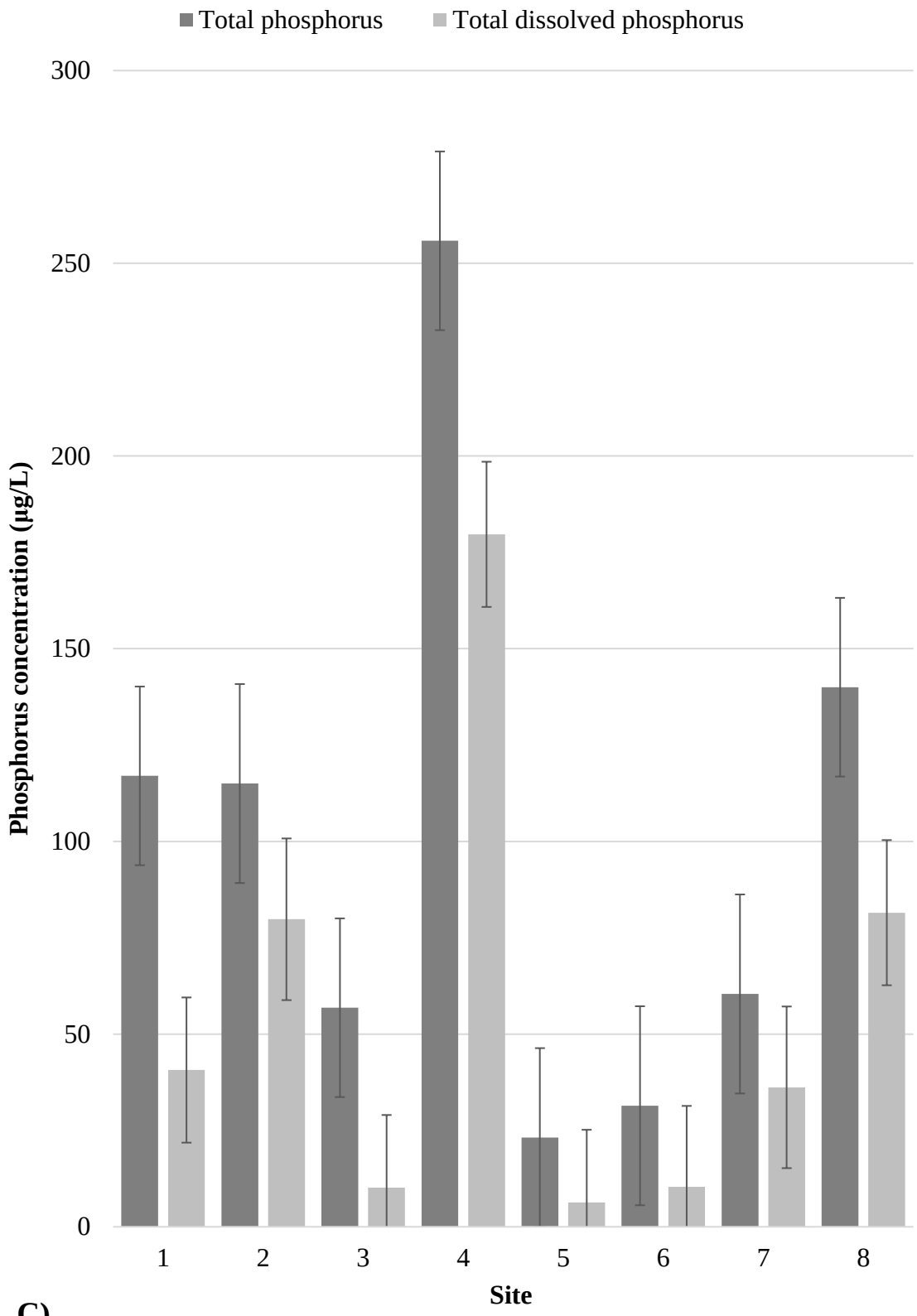
Figure 3.1. Difference between **A)** chloride **B)** total dissolved nitrogen and **C)** phosphorus concentrations of water samples collected from eight golf course irrigation systems in Missouri and Kansas. All transformed data was back transformed to original values. Error bars represent standard error.



A)


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Total dissolved nitrogen (mg/L)
1
2
3
4
5
6
7
8
Site

B)


Total phosphorus
Total dissolved phosphorus
300
250
200
150
100
50
0
Phosphorus concentration (µg/L)
1
2
3
4
5
6
7
8
Site

C)

Figure 3.2. Differences between **A)** infrared and **B)** WSS temperatures. Water samples were collected from eight golf course irrigation systems in Missouri and Kansas. Error bars represent standard error.

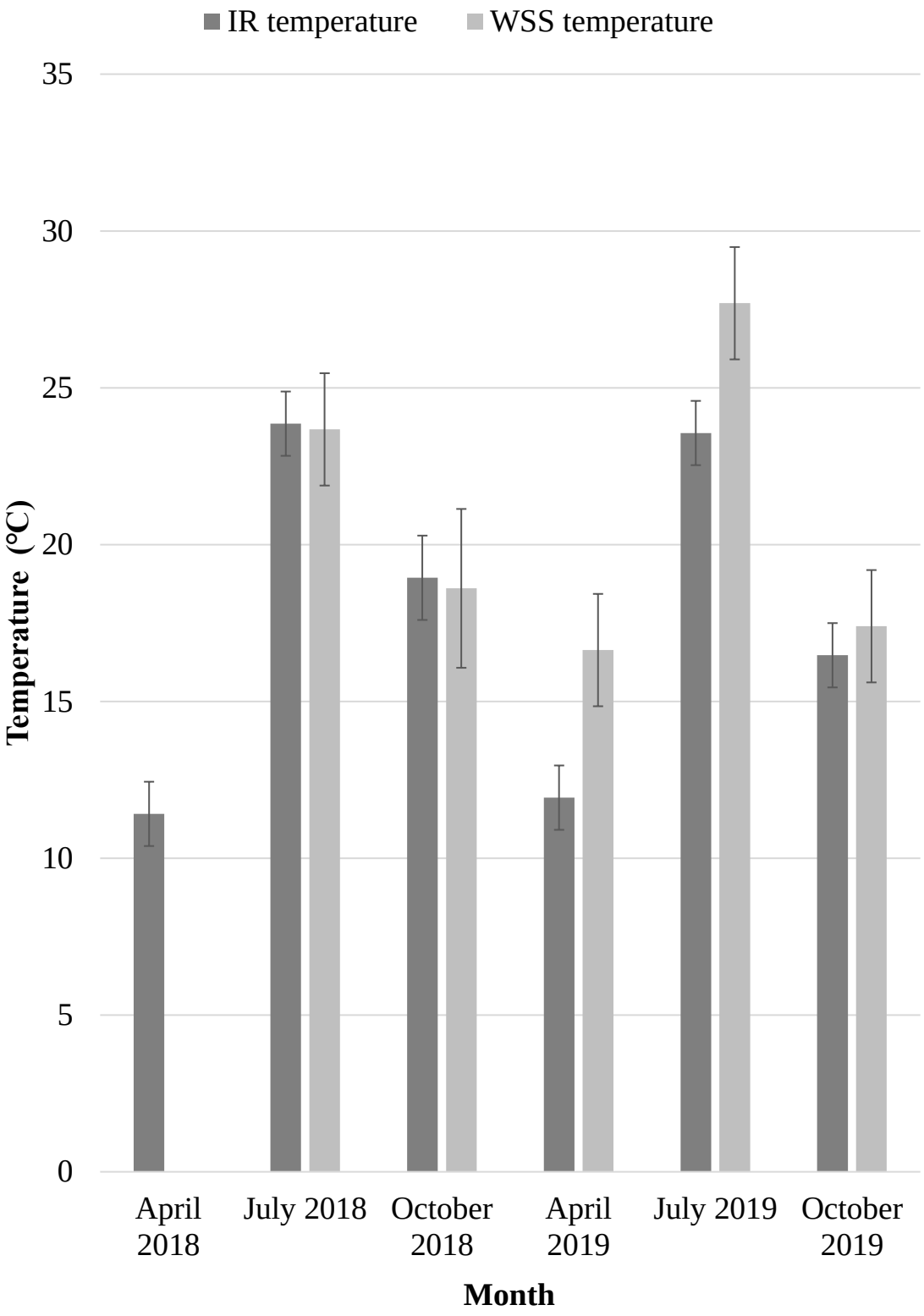
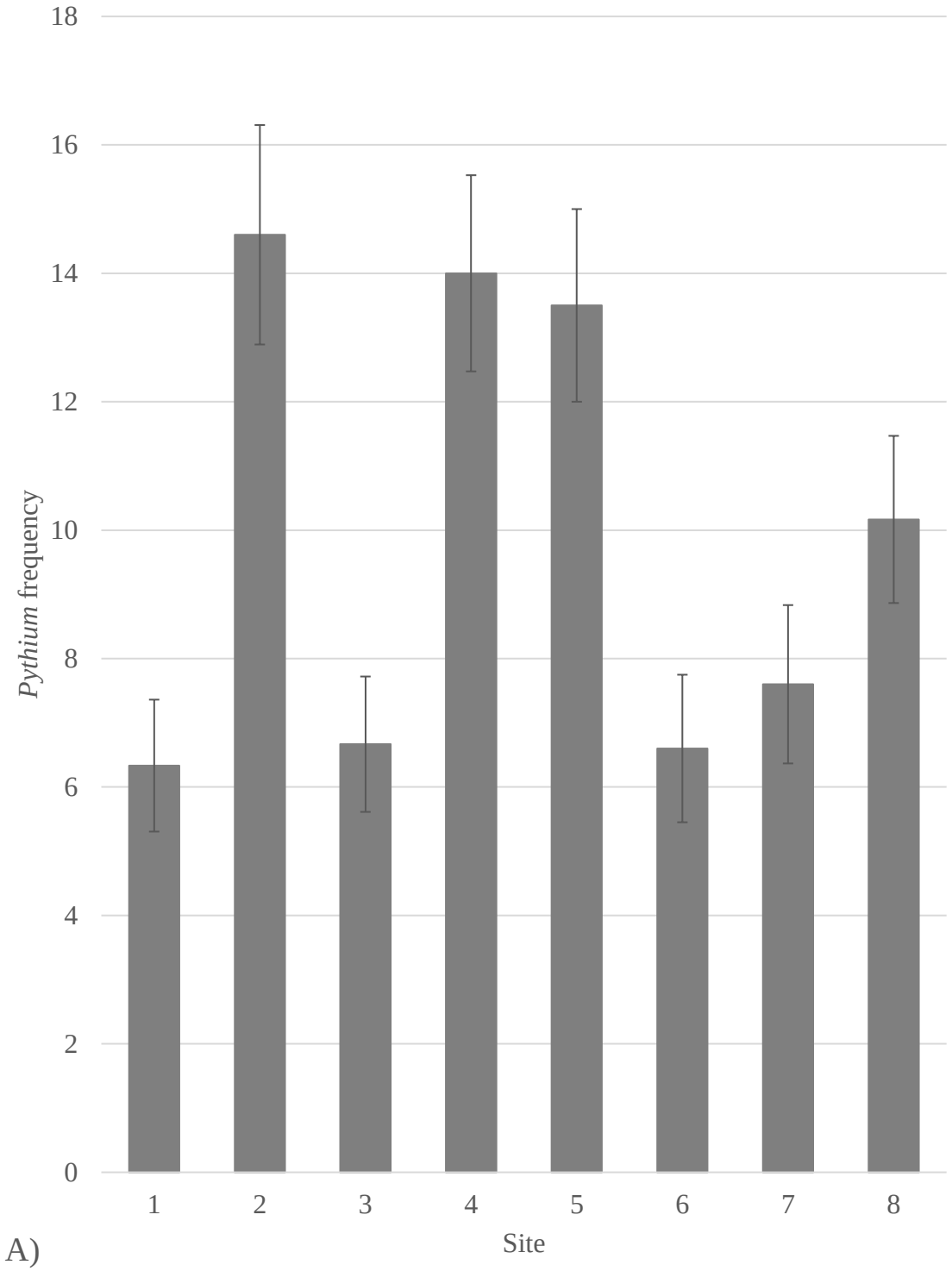
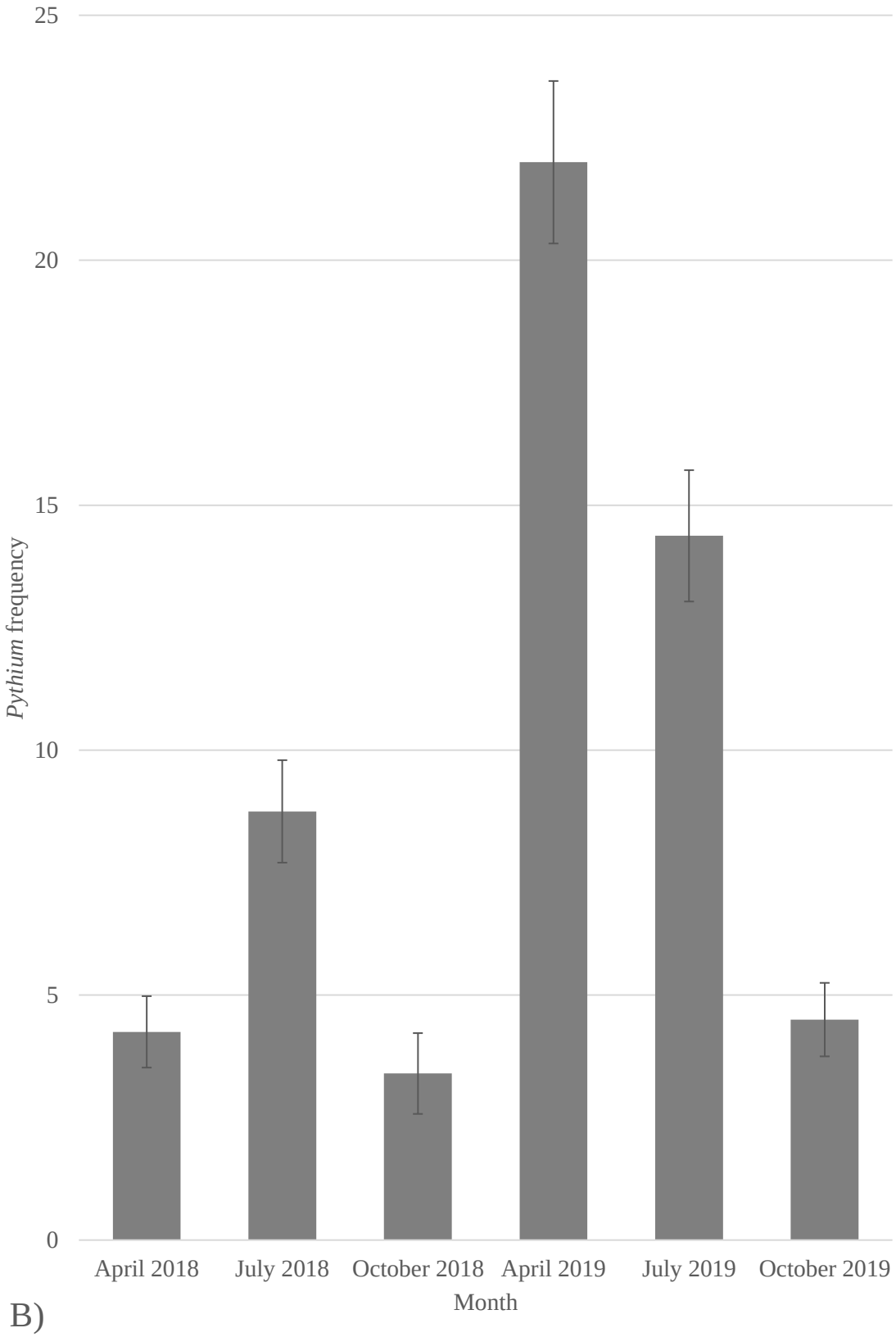


Figure 3.3. Average *Pythium* frequency response in relation to **A)** sampling site and **B)** month. Water samples were collected from eight golf course irrigation systems in Missouri and Kansas. Error bars represent standard error.



A)


25
20
15
10
5
0
Pythium frequency
April 2018
July 2018
October 2018
April 2019
July 2019
October 2019
Month

B)

Addendum

Detection of cyanotoxins in irrigation water and potential impact on putting green health

Submitted to the 2021 International Turfgrass Society Research Journal as a short communication article

Abstract

Creeping bentgrass putting greens require increased irrigation during summer months to meet evapotranspiration requirements. Golf courses that utilize local water bodies for irrigation may potentially spread phytopathogenic waterborne microbes to putting greens. Cyanobacteria associated with yellow spot and black layer have been detected in water bodies and are known to produce cyanotoxins that may hinder plant growth at high concentrations. Water samples were collected from irrigation heads, irrigation intakes, and the top 0.5 m of surface water sources. Anatoxin, cylindrospermopsin, microcystin, and saxitoxin concentrations were analyzed using ELISA kits. Microcystin and saxitoxin were detected in irrigation systems that utilize surface water sources. Anatoxin was detectable in every irrigation system, regardless of water source. Cylindrospermopsin was only detected in one system that utilizes the largest water body in the experiment. Each site with detectable levels of microcystins, cylindropermopsin, and anatoxin-a meet the minimum threshold set by Missouri for recreational water use. However, the effect of cyanotoxin concentrations detected in this study on creeping bentgrass health is not well understood and should be examined in future experiments.

Introduction

Creeping bentgrass (*Agrostis stolonifera*) is a cool season turfgrass used on putting greens throughout the transition zone. During hot summer months, frequent irrigation is required to meet evapotranspiration requirements and maintain turfgrass quality. Many golf courses utilize local water bodies for irrigation sources, potentially creating a dissemination pathway for waterborne pathogens of creeping bentgrass. In Missouri, cyanobacteria, also known as blue-green algae (e.g., *Oscillatoria* and *Nostoc* spp.), have been detected in water bodies and are known invaders of bentgrass putting greens (Baldwin & Whitton 1992, Drouet 1932, Hodges 1987). Approximately 120 different cyanobacteria species produce potentially toxigenic chemicals in water bodies, and nearly half of them, including several *Phormidium*, *Oscillatoria* and *Nostoc* spp. produce microcystin, a cyanotoxin that is hazardous to humans and can inhibit plant development (McElhiney et al. 2001). Other potentially harmful cyanotoxins produced by cyanobacteria include anatoxin, cylindrospermopsin, and saxitoxin (Machado et al. 2017).

Cyanobacteria and associated toxin development can cause turf thinning on bentgrass greens and have been associated with both yellow spot and black layer formation (Hodges 1987, Tredway et al. 2006). The impact of microcystin on plant growth has been documented on a wide variety of crops, but not on turfgrasses (Machado et al. 2017). In most cases, exposure to high microcystin levels (>100 µg/L) in plant experiments decreased seed germination, growth and photosynthesis, and promoted oxidative stress. Irrigation water has not been investigated as a potential source of cyanobacterial toxins, and their impact on bentgrass growth is unknown. The objective of

this study was to obtain data on the levels of cyanotoxins in irrigation water, laying the groundwork for future investigation into assessment of cyanobacteria and cyanotoxins in irrigation sources and their impact on putting green health.

Materials and methods

Water samples were collected in July of 2019 from ten golf courses in Missouri and Kansas, US. July samples were collected assuming that warmer temperatures may cause cyanobacteria blooms and resultant higher cyanotoxin levels (Paerl & Huisman 2008). The sampled locations utilize various water sources for irrigation including retention ponds, wells, a lake, and municipal water. Samples were collected directly from irrigation heads, near irrigation intakes, and from the surface of water sources (*n*=27). Water was collected and consolidated from multiple irrigation heads using Pyrex baking dishes (2.8 liters, 22.9 x 33.0 cm, Corelle Brands LLC, Rosemont, IL). A Van Dorn sampler (Flinn Scientific Inc., Batavia, IL) was used to collect water samples near the irrigation intake. The water surface was sampled with a 0.5 m deep sampling tube. Water samples (10 ml) were stored in amber borosilicate sample vials (Berlin Packaging, Chicago, IL) and sealed with polytetrafluoroethylene-lined caps (Berlin Packaging, Chicago, IL). Vials underwent three freeze/thaw cycles to lyse cells and release stored cyanotoxins. Anatoxin, cylindrospermopsin, microcystin, and saxitoxin concentrations were analyzed using Abraxis ELISA kits (Eurofins, Luxembourg) and quantified using a plate-reading spectrophotometer (Peachtree Corners, GA). The stated detection limits for these tests is >0.02 µg/L, 0.05 µg/L, 0.15 µg/L, and 0.15 µg/L for saxitoxin, cylindrospermopsin, anatoxin, and microcystin, respectively.

Results

Cyanotoxins were detected in every sample (Table 4.1). Anatoxins were detected in 96% of samples, including a site that utilizes municipal water. Microcystin and saxitoxin were detected at sites that utilize surface water sources. The site with the largest water source (62.7 ha – LQ; Table 4.1) was the only site with detectable levels of each toxin, and the only site with detectable levels of cylindrospermopsin. The highest observed microcystin concentrations were between 8.53-8.65 μg/L, found in the surface water and irrigation intake from a large agricultural pond (9.6 ha) that also serves as a golf course irrigation source. The water sample taken from irrigation heads at this golf course (WC; Table 4.1) had a much lower microcystin concentration (0.34 μg/L) than this source pond. Microcystin levels from all other samples averaged 0.38 μg/L. All anatoxin, cylindrospermopsin, and saxitoxin concentrations were ≤ 0.30 μg/L.

Discussion

Missouri has established qualitative thresholds for cyanotoxins in recreational water based on the presence or absence of microcystins, cylindrospermopsin, and anatoxins. Each site with detectable levels of microcystins, cylindrospermopsin, and anatoxins meet the minimum thresholds. At the low, and perhaps ecologically relevant, microcystin concentrations found in our samples, most studies in plant-soil systems indicate no deleterious effects on plant health, and in some cases low concentrations (<10 μg/L) may even accelerate plant growth (Machado et al. 2017). The effect of multiple cyanotoxins and a potential additive deleterious effect on plant growth have not been well studied, however, nor has the potential impact of even small doses as aerosolized irrigation spray on plant surfaces. Future research in this area, and the potential for

irrigation to serve as a deliver mechanism of terrestrially capable cyanobacteria inoculum on golf putting greens, is warranted.

Literature cited

Baldwin, N. A., & Whitton, B. A. 1992. Cyanobacteria and eukaryotic algae in sports turf and amenity grasslands: a review. Journal of applied phycology, 4(1), 39-47.

Drouet, F. 1932. A list of algae from Missouri. Bulletin of the Torrey Botanical Club, 289-300.

Hodges, C. F. 1987. Blue-green algae and black layer. Landscape Management, 26(10), 38-42.

Loftin, K. A., Graham, J. L., Hilborn, E. D., Lehmann, S. C., Meyer, M. T., Dietze, J. E., & Griffith, C. B. 2016. Cyanotoxins in inland lakes of the United States: Occurrence and potential recreational health risks in the EPA National Lakes Assessment 2007. Harmful Algae, 56, 77-90.

Machado, J., Campos, A., Vasconcelos, V., & Freitas, M. 2017. Effects of microcystin-LR and cylindrospermopsin on plant-soil systems: A review of their relevance for agricultural plant quality and public health. Environmental Research, 153, 191-204.

McElhiney, J., Lawton, L. A., & Leifert, C. 2001. Investigations into the inhibitory effects of microcystins on plant growth, and the toxicity of plant tissues following exposure. Toxicon, 39(9), 1411-1420.

Paerl, H. W., & Huisman, J. 2008. Blooms like it hot. Science, 320(5872), 57-58.

Tredway, L. P., Stowell, L. J., & Gelernter, W. D. 2006. Yellow spot and the potential role of cyanobacteria as turfgrass pathogens. Golf course management. PACE Turfgrass Research Institute, San Diego, 83-86.

Table 4.1. Cyanotoxin concentration of water samples taken in July of 2019 from golf course irrigation systems in Missouri and Kansas.

Site (Size and source)	Location	Microcystin concentration (µg/L)	Anatoxin concentration (µg/L)	Saxitoxin concentration (µg/L)	Cylindrospermopsin concentration (µg/L)
CCMO (Pond)	Irrigation head	BDL[x]	0.25	0.04	BDL
	Irrigation intake	0.2	0.26	0.05	BDL
	Surface water	BDL	0.27	0.04	BDL
DAL (Pond)	Irrigation head	0.23	0.29	BDL	BDL
	Irrigation intake	0.84	0.17	BDL	BDL
	Surface water	0.92	0.27	BDL	BDL
LED (Pond)	Irrigation head	BDL	0.19	BDL	BDL
	Irrigation intake	0.42	0.16	BDL	BDL
	Surface water	0.32	BDL	BDL	BDL
SA (Pond)	Irrigation head	0.16	0.22	BDL	BDL
	Irrigation intake	0.26	0.21	BDL	BDL
	Surface water	0.28	0.20	BDL	BDL
SF (Pond)	Irrigation head	0.38	0.25	0.09	BDL
	Irrigation intake	0.54	0.27	0.12	BDL
	Surface water	0.44	0.28	0.10	BDL
WC (Pond)	Irrigation head	0.34	0.30	0.03	BDL
	Irrigation intake	8.65	0.24	BDL	BDL
	Surface water	8.53	0.29	0.03	BDL
HS (½ pond & ½ well)	Irrigation head	BDL	0.15	BDL	BDL
	Irrigation intake	BDL	0.19	BDL	BDL
	Surface water	0.22	0.18	BDL	BDL
AG (well)	Irrigation head	BDL	0.22	BDL	BDL
	Irrigation intake	BDL	0.26	BDL	BDL
LQ (lake)	Irrigation head	0.19	0.24	0.04	0.08
	Irrigation intake	0.28	0.29	0.10	0.09
	Surface water	BDL	0.25	0.11	0.09
STLCC (municipal water)	Irrigation head	BDL	0.18	BDL	BDL

[x]BDL – Below the detection limit of the Abraxis ELISA tests used. These limits are <0.15 µg/L – microcystin, < 0.15 µg/L – anatoxin, < 0.02 µg/L – saxotoxin, and < 0.05 µg/L cylindrospermopsin.