The biogeography, phylogeny, and dispersal of freshwater and terrestrial free-living ciliates in Florida, USA

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ABSTRACT

Microbial ecology: the biogeography, phylogeny, and dispersal of ciliates in Florida, USA

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As organisms at the foundations of food webs, ciliated protozoa are an integral part of the microbial loop and the ecosystems they support. This project was designed to explore the freshwater and terrestrial ciliate populations of sub-tropical Florida, USA, an uninvestigated geographic range with similar environmental characteristics to those found in previously-studied locations in sub-tropical and tropical Africa. Through extensive sample collection covering a wide variety of habitats, morphological and molecular techniques were used to describe the target ciliate taxa present in these environments and to determine their presence/absence and their geographical distribution. Of special interest were the 'flagship' ciliate species found, with some recorded outside of Africa for the first time, and the first records made for the Americas of both freshwater and terrestrial flagships. As a result of major sampling, some ciliate species were found to be new to science, and these are described in detail at both morphological and molecular levels. The 18S rRNA gene sequences were obtained for several species, some for the very first time, and are provided here to investigate phylogeny. Long-term monitoring of four sites produced a large dataset of water parameters and occurrence of target ciliate species, allowing a better understanding of the niche requirements for these ciliates. The development of dynamic models was undertaken to enhance discussions surrounding potential dispersal mechanisms of target ciliate species over large distances. Agent based models were constructed to visualize microcosm interactions of a target ciliate species to various environmental stimuli.

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Declaration

I declare that the work in this thesis was made in accordance with the requirements of the University's Code of Practice for Research Degrees and that it has not been submitted for any other academic award. The work is the candidate's own. Work was done with the assistance of others as indicated.

ABBREVIATIONS

AZM	**Adoral Zone of Membranelles: the long cilia surrounding the oral aperture of some species**
BDM	**Bird Dispersal Model. A model created for this project to visualize dispersal between nodes via agent based birds**
DAPI	**4′,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to adenine–thymine rich regions in DNA**
DI H_2O	**Deionized water**
DIC	**Differential Interference Contrast microscopy: a technique which enhances contrast, also known as Nomarski**
DO	**Dissolved oxygen**
EIE	**Everything Is Everywhere: An often debated theory in microbial biogeography**
EM	**Electron Microscopy**
ENP	**Everglades National Park**
HBOI	**Harbor Branch Oceanographic Institute: The research facility owned by Florida Atlantic University at which all of the research was conducted for this project**
NDM	**Neighbour Dispersal Model: A model created for this project to visualize dispersal between connected nodes**
PCR	**Polymerase Chain Reaction: A technique used to amplify copies of targeted DNA**
PSU	**Practical Salinity Unit: The ratio of salts within a water sample compared to a KCl control. By design, PSU is nearly identical to parts per thousand (ppt)**
rRNA	**Ribosomal Ribonucleic Acid: A component essential for protein synthesis**

Chapter 1. A REVIEW OF THE LITERATURE AND AN OVERVIEW OF THE PROJECT

1.1 Background

Ciliates are single-celled microbial eukaryotes that move with hair-like projections called cilia (Lynn 2008). The phylum Ciliophora is an ancient group, existing on earth for hundreds of millions of years (Bomfleur et al. 2012). Ciliates, along with other groups of free-living phagotrophic protists, are the most important grazers of microbes in aquatic environments (Patterson 1996). Ciliates are known to be the top predators in the microbial foodwebs in some environments (such as Xu et al. 2014) and are therefore of importance in understanding total ecosystem function. Symbiosis allows some ciliates to thrive in habitats varying from anoxic to oligotrophic (Dziallas et al. 2012), which are often habitats that are not accessible to other organisms, i.e. metazoans. The importance of ciliated protozoa in ecosystems is well documented in the literature for their role in the microbial loop (Azam et al. 1983; Esteban et al. 2015; Weisse 2017) and food webs (Finlay and Esteban 1998b; Schmid-Araya et al. 2016). The microbial food web in aquatic environments is driven by the release of Dissolved Organic Carbon (DOC) from algae and photosynthetic bacteria. The microbial loop is the process whereby DOC produced by the phytoplankton through photosynthesis, enters the food web and allows it to develop into several trophic levels within an ecosystem. The dissolved organic carbon then cycles up the food chain through various consumers, starting with the bacteria taking up this DOC and using it for growth. Bacteria are then consumed by larger microbes (i.e. flagellates and ciliates) that also prey upon the phytoplankton (Azam et al. 1983). When the protozoa have digested what they have eaten, they excrete the waste material into the water. This material includes ammonia and phosphorus, which are taken up as nutrients by growing phytoplankton, and the cycle of the microbial loop is completed. Larger zooplankton and small fish graze the larger organisms in the microbial loop and so channel organic matter up the aquatic food chain (Berninger et al. 1991).

Despite their taxonomic diversity, broad functionality, and ability to obtain large population densities in a short period of time (Finlay and Fenchel 1986a), the geographical distribution and structure of ciliate communities is still unexplored in many regions of the world (Finlay 2002; Clamp and Lynn 2017) and poses a barrier for studies of ciliate biogeography. The last two decades in particular have seen biogeography research in ciliates become increasingly dominated by the term "flagship" species (Foissner et al. 2008 and references therein).

Borrowed from the field of wildlife conservation, the term "flagship" refers to ciliates whose morphological distinctiveness is such that their presence should not be missed in any environmental sample. The term as applied to ciliates is used in a unique way distinct from that commonly used in megafaunal conservation; rather than a species selected to raise conservational awareness, flagship ciliates are used to investigate the potential for restricted biogeographic distributions and endemism (Foissner 2006). As such they have been considered the "ultimate proof" for testing biogeographical theories surrounding microbial endemism (Foissner 2006; Foissner 2007; Foissner et al. 2008; Segovia et al. 2017) and can be a useful tool for better understanding taxa with unknown spatial distributions (Andelman and Fagan 2000). The idea that flagship ciliates are 'proof' for microbial endemism is perhaps a flawed concept: when the size of the globe is considered with the astronomical number of niches compared with the number of microbial ecology researchers present in any given area, it is likely that large parts of the planet remain unexplored for microbial diversity, and even for areas which have been studied that effort may not be exhaustive.

Flagship ciliates represent an ideal target when seeking a better understanding of ciliate biogeography. Since Florida had never benefited from an investigation of its flagship ciliates, it represents a significant knowledge gap for this group of organisms when compared to similar warm, tropical and subtropical areas worldwide.

1.2 'Flagship species' as a conservation biology concept

A term used throughout this thesis is 'flagship ciliate' which is unusual with respect to the way in which 'flagship' is traditionally used within biology and ecology. The origin of the term 'flagship' is derived from maritime parlance; the flagship is occupied by the commander and is unmistakably recognizable as it flies their flag (Yezerinac and Moola 2006). In common usage, the term has since evolved to denote conspicuousness and importance, e.g. a

large flagship chain store (Moore et al. 2010). This commonly used term marks the 'flagship' as the most representative or important among a given group.

In biology, the use of 'flagship' comes from the conservation biology arena in which the use of 'flagship species' is implemented to increase species conservation awareness (Heywood 1995; Verissimo et al. 2011). In general, flagship species are those that are easily recognizable amongst others, some may be evident from their large size while others may have a conspicuous morphology. They are often animal and plant species under threat of extinction or somehow endangered. Since its initial introduction into this field (see Mittermeier 1986; Mittermeier 1988) the flagship concept has been consistently used, with occasional modifications to enhance its applicability in conservation biology (for a review, see Verissimo et al. 2011 and references therein). The conservational flagships can be used as a surrogate species for attracting sociopolitical and sociecological interest for a given conservation issue (Caro and O'Doherty 1999) and are selected either with the goal of leveraging public interest, or directly by the public through support of causes they find appealing (Home et al. 2009).

In conservation biology, flagship species help to create awareness and recognition (Caro and O'Doherty 1999; Barua et al. 2011), while promoting topics such as habitat loss and other anthropogenic influences including climate change. As an example, polar bears are classically viewed as a flagship species that anchors a conservation campaign. The same is true for the 'Big 5' species popular on African safaris (Western 1987; Williams et al. 2000). However, this status was established with few criteria other than an appeal to a global public (Caro and O'Doherty 1999; Barua et al. 2011).

The loss of a well-known flagship animal species from a habitat might actually have a greater impact on the public than the loss of the habitat itself, even though the two are related (Simberloff 1998; Home et al. 2009). Monitoring of flagship species linked to climate change (and species loss) is one of the most invoked areas for the use of the term (Barua et al. 2011). Funding is more likely to be obtained for charismatic flagships, allowing for their conservation which, in turn, may benefit communities as a whole (Verissimo et al. 2011; Bennett et al. 2015).

1.3 The term 'Flagship' as it applies to ciliates

In contrast to the conservation of megafaunal flagships and the predominant use of the term in that context, protist conservation is a recent concept (Cotterill et al. 2008; Esteban

and Finlay 2010; Cotterill et al. 2013). Whilst the actual geographical ranges and dispersal patterns of this entire group is still contentious (Foissner 1999; Finlay et al. 2001; Finlay 2002; Foissner et al. 2003; Fontaneto 2011; Bourland 2017), the original use of the term 'flagship ciliate' has been in the biogeographic context and this remains its foremost application (Foissner 1998; Foissner et al. 2008; Foissner 2010; Bourland 2017).

In common with the flagship concept used by conservation biologists, the term 'flagship ciliate' (Foissner 2006) refers to ciliates that could easily be recognized in an environmental sample and that have some kind of morphological distinctiveness. The term is, however, used in a characteristic way by some microbiologists: rather than a species selected to raise conservational awareness and public support (Home et al. 2009; Cotterill et al. 2013; Hines and Esteban 2017) flagship ciliates are used in some ciliate biodiversity and biogeography studies to 'prove' restricted biogeographic distributions and endemism (Foissner et al. 2008). The best examples of flagship ciliates are those that are most obvious (Foissner 2005; Foissner 2006; Foissner 2007; Foissner et al. 2008). As described above, flagship species in conservation biology represent a symbol of the area or habitat where they thrive, and this may represent another basis for the term's use with unicellular organisms such as ciliates. The finding of a large and 'charismatic' ciliate species in a particular region of the world can be an indicator of a successful biogeography study (Esteban et al. 2001), and recording such species can lead to a more complete understanding of an ecosystem, while also enhancing knowledge of global biogeography, dispersal and evolution. For the purpose of this research a 'flagship ciliate' was defined as a large cell (e.g. >1mm) or a cell with a combination of features which make it distinct within a sample. These characteristics include bright coloration or distinct morphological features (e.g. a row of contractile vacuoles), with the most ideal flagships having a combination of all three.

1.4 Is 'everything everywhere'?

A long-standing debate (Foissner 1999; Foissner 2002; Finlay 2002; Finlay and Fenchel 2004) in ciliate biogeography surrounds the statement "everything is everywhere" (EIE) with one side alleging endemic ciliate species are common, and the other stating there are no endemic ciliates as they are globally ubiquitous. A key component, "but the environment selects" is often omitted from the EIE debate. The second part of this phrase is important to consider, as it is the ecological constant that explains the theory. Ciliates can obtain astronomical population numbers (Finlay 2002) and therefore could possibly spread

everywhere, constantly. The areas to which these ciliates spread must, however, be suitable to their survival, and include at least some of the parameters of their preferred ecological niche. For example, species that are adapted to cold climates would be unlikely to thrive if dispersed to a geothermal spring, in a similar way that chlorinated swimming pools are not host to, or overrun by, various creatures. The difficulties of detecting some ciliate species, even the flagships, within a habitat is a continuing issue confounding our understanding of microbial dispersal (Finlay and Fenchel 2004). On the other side of the EIE debate about ciliate dispersal is the 'moderate endemicity model' (Foissner et al. 2008). This states that 30% of ciliate species are endemics (Foissner 2007). This is not a true theory as it is currently unfalsifiable, as it is impossible to prove a species is absent from an area (e.g. North America) with current techniques (Finlay et al. 2004; Caron 2009). In reality the two arguments (Finlay and Fenchel 2004; Foissner et al. 2008) are perhaps one in the same, the 'environment selects' such that species adapted for tropical conditions can be found in such habitats (globally), and would not be found in cold climates (e.g. Northern Europe). The degree to which a species could exist globally may be underestimated due to under sampling (Fenchel and Finlay 2004) to the extent that no records may exist for an entire continent, despite the species potentially thriving commonly. This is the basis for selecting Florida, a previously uninvestigated area, as an environment to test for flagship ciliates known from warm regions and alleged to be restricted to in these areas (e.g. Africa). Future biogeography investigations with detailed molecular analysis might shed deeper light onto the truth of ciliate dispersal. The 18S rRNA gene has been show to be be identical from two populations of a large ciliate species separated by over 1,600km (found in South England and Sweden), which also shared identical morphology (Hines et al. 2018). However, before such comparisons are possible, detailed morphometrics, site ecology, and micrographs must be obtained in order to make comparisons of the molecular data from separated species meaningful. In this respect, this PhD project was designed to investigate these issues and potentially provide the foundation for future research within this field, and will allow other scientists to compare their local ciliates with the Florida species reported here. The implementation of theoretical dynamic modeling is a field of study that has never before been used in the study of ciliate ecology. Dispersal of microorganisms has long been an interest of scientists (Darwin 1859), but mechanisms of action remained elusive. The use of dynamic (computational) modeling for other organisms and systems has looked into ways which species move or are dispersed

through active and passive motion to and from a habitat or region (Lookingbill et al. 2010; Linder et al. 2012). Such methods are here applied to ciliates to enhance discussions into potential dispersal.

1.5 Why was this study needed?

There are numerous published examples of large ciliates (Dragesco and Dragesco-Kernéis 1986 and references therein) that are noteworthy because many have been long since documented in the literature (Dragesco 1970; Dolan et al. 2014), but may remain relatively unstudied: some have not been recorded from outside of their originally documented range (i.e. the discovery site) or even photographed. Most flagship taxa have been reported from the Gondwanaland paleogeographic regions, with this being a basis for their alleged endemicity (Foissner et al. 2008; Bourland 2017).

Since flagship ciliates are large and can be unmistakable within a sample (Foissner et al. 2008) they were traditionally thought of as proof of microbial endemism, such that since they had not been recorded from certain parts of the globe (e.g. North America) they were thought to be absent from that region and were classified as restricted to their discovery site (e.g. Africa). The debate about the extent of microbial biogeographies continues, as it is nearly impossible to prove that any particular species is completely absent from a given geographic area or a continent as it could be present at a low population density and therefore undetected (Finlay et al. 2004; Caron 2009 and references therein). Focusing on 'flagships' from diverse and novel habitats around the world is a relevant addition to global biodiversity and biogeography studies especially when considering that only limited studies have been carried out in large regions such as North America. A major goal of this project was to investigate the hypothesis that flagship ciliates could spread to and be detected within Florida, USA (Chapter 3).

The State of Florida has an area of 170,356 km^2. This is a very large area when considering the vast number of habitats that could be sampled. By comparison England is 130, 279 km^2. Florida is largely flat, with the highest point in the state being only 105 meters above sea level (Britton Hill located in the Florida panhandle). The Florida peninsula is topographically devoid with its highest peak (Sugarloaf Mountain in Lake County) reaching only 95 meters above sea level (Darack 2007). Innumerable water bodies and aquatic habitats exist within the State; many are ephemeral based on weather events. Since much of coastal

and southern Florida is current or former swampland, Florida has major drainage systems including both manmade canals and natural rivers. Florida is surrounded to the East and South by the Atlantic Ocean, and by the Gulf of Mexico to the West, but has ample freshwater systems.

Despite its large size and vast amount of habitats, Florida makes an interesting location in which to test for the presence of previously undocumented 'flagship' ciliates, and potentially species which are new to science. For this PhD research, sampling within freshwater systems (rather than marine habitats) was chosen to maximize the potential isolation of species, such that an ocean would not directly connect potential distant habitat areas. Despite the large distances that exist between the known ranges of some ciliate species (see Chapter 3), it was hypothesized that some flagship ciliates could be detected within Florida if intensive sampling was applied. This was thought possible primarily because ciliates are known to reach vast population numbers (Finlay 2002), with many of them able to form cysts/dormant stages that would help ciliates overcome physical barriers to dispersal. Freshwater systems which appear isolated are actually interconnected in a variety of ways, and to investigate these mechanisms further, theoretical models were created (Chapter 6).

Florida's lakes and freshwater systems are geologically young, having first been formed 10,000 to 8,000 years ago (Kenney et al. 2016). These systems formed once the climate had stabilized after the last ice age, at a time when sea levels and climatic conditions in Florida reached the current state (Watts 1969; Watts 1975). Before this point any freshwater species which may have been dispersed from older habitats would have been destroyed due to unfavorable climate and inundation by seawater. It is therefore clear that any freshwater ciliate species currently found in Florida could not have thrived in this region uninterrupted for more than 10,000 years. The geologically young habitats of Florida are in contrast to the freshwater habitats of Africa which have existed for millennia (Dragesco and Dragesco-Kernéis 1986), and forms the basis of thinking that ciliates may have dispersed to Florida from these much older regions.

Florida has a humid subtropical climate with a defined rainy season from May through to October and an average daytime temperature of 32˚C during this period. This climate is analogous to areas previously investigated for ciliates within Africa (Dragesco and Dragesco-Kernéis 1986) where the majority of the described large freshwater species (e.g. Dragesco 1970) inhabit warm waters. The studies of tropical freshwater ciliate biodiversity

from Africa formed the baseline for investigating such taxa in Florida and helped formulate the hypothesis that some flagship ciliates known from Africa could be found in Florida, even if they were previously thought to have a restricted biogeography (Dragesco 1980; Foissner et al. 2008).

A large knowledge gap in the study of ciliates and flagship biogeography is the absence of molecular data. Many of the flagship ciliates were described (e.g. Dragesco 1970) before the invention of the polymerase chain reaction (PCR) and many of these have remained unseen since their initial discoveries. Consequently, these taxa are not represented in large repositories such as GenBank. The obtaining of molecular sequences for flagship ciliates would ensure that future researchers can make accurate comparisons to species which they may find in other distant or diverse regions.

The most recent examples of described flagship ciliates are from soil (Foissner 2005; Foissner et al. 2006; Foissner and Stoeck 2008; Foissner 2016), which often have noticeable color (Foissner 2004) that aids in recognition despite their small size. This recent avenue of research inspired the investigations of Florida soils to see if any of these flagships could be found in North America (see Chapter 5).

Any microbial diversity study is a snapshot of a habitat in time in a dynamic world. Species that are considered rare perhaps are only actually rarely sampled and recorded (Dunthorn et al. 2008), as predominant populations of even flagship species could remain unnoticed until an area is intensively searched, as occurred for the large continent of Africa (Dragesco and Dragesco-Kernéis 1986). However, if efforts are focused on the flagship ciliate species the resulting findings may provide insight into the divergent theories surrounding microbial global dispersal, especially if underexplored areas are targeted for insufficiently studied taxa (Küppers and Claps 2012; Küppers 2014). Investigations into flagship ciliates may also enhance our understanding of dispersal for other flagship protists (Tyler 1996; Zapata and Fernandez 2008; Foissner and Hawksworth 2009; Lahr and Souza 2011; Neustupa et al. 2011).

1.6 Aim and Objectives

Aim: To document and understand the presence of ‘flagship’ ciliates, and species of ciliates new to science in Florida’s fresh waters and soils.

This was achieved through the following objectives:

1. To investigate Florida's freshwater habitats for ciliates, focusing on flagship species
2. To document, record and describe freshwater ciliate species new to science
3. To investigate Florida soils for presence of flagship ciliates
4. To create dynamic models to explore and understand ciliate dispersal

The Objectives are organized into the following Chapters:

Chapter 3. Flagship freshwater ciliates of Florida

Hypothesis: From the perspective of sampling for microbial eukaryotes, Florida is an unexamined tropical habitat, with freshwater areas analogous to those published in the literature with previously investigated sites in Africa. Despite large distances between known species distribution ranges (e.g. continents), some 'flagship' ciliates could thrive and be found in Florida when intensive sampling efforts are undertaken and the right habitat is investigated.

Despite the vast literature on alleged ciliate endemism (such as Dragesco 1980; Foissner 1999; Foissner 2006; Foissner et al. 2008), previous and conflicting literature suggests the possibility of finding a ciliate flagship species in either a previously sampled or an unsearched area (Esteban et al. 2001; Foissner 2005; Foissner and Stoeck 2006; Bourland 2017). Florida was chosen as a novel analogous habitat to areas previously described as containing 'endemic' flagship ciliates. The aim was to search for target species, to determine whether flagship ciliate species could be detected and thrive within Florida study sites.

Chapter 4. Freshwater ciliate species new to science discovered in Florida

Hypothesis: Species of ciliates which are new to science will be discovered in Florida during intensive sampling and monitoring of 'flagship' ciliates, due to global undersampling of microbial eukaryotes.

The potential to discover species which were new to science was hypothesized at the beginning of the project, as biodiversity studies often reveal new species when novel habitats are first investigated (e.g. Dragesco and Dragesco-Kernéis 1986). The documentation of species new to science from a region such as Florida would further highlight that major areas of the globe are uninvestigated for ciliate biodiversity.

Chapter 5. Flagship Soil ciliates of Florida

Hypothesis: Flagship soil ciliates thrive in Florida and they will be distinct from the freshwater flagship ciliate taxa that have been discovered. Sampling of terrestrial systems will reveal soil ciliate species previously thought to have a geographical distribution restricted to a given continent, due to their ability to encyst, and the similarity of Florida ecosystems to those in their known geographical ranges.

In addition to flagship ciliates being reported in freshwater habitats (Dragesco 1970), soil is another environment in which different flagship ciliates can thrive (Foissner 2016 and references therein). Soil was investigated during this project to investigate the potential for flagship ciliates to be present and detected within Florida soils.

Chapter 6. Combining dynamic modeling with investigations into ciliate biogeography and ecology

Hypothesis: Theoretical modeling is useful in understanding eukaryotic microbial dispersal and behavior; outputs can be generated which enhance understanding beyond experimental observations.

Interdisciplinary dynamic modeling has never been applied to the study of ciliate biogeography and ecology. The project was undertaken to provide proof of concept that this avenue of exploration could provide meaningful discussions for the greater understanding of biogeography beyond that which can be observed experimentally.

Chapter 2. METHODS

2.1. Study sites and sample collection

2.1.1 Site selection

Florida has many wetland areas including natural marsh, natural and man-made ponds, and drainage canals (Figures 2.1, 2.3). Initially sites were selected based on visibility, access, and proximity to the Harbor Branch campus (Figure 2.2) (HBOI). These sites were found by searching aerial images, ground observations, internet searches, word of mouth (e.g. anglers, other scientists), and random exploration. Sites were sampled to investigate the ciliate communities and to determine whether the site would be of value to the project in terms of presence of potential 'flagship' ciliate species. Some of the sites selected were observed to be regularly treated with pesticides, which was evident from the obvious lines of dead terrestrial vegetation, which negatively affect the food webs (e.g. by killing algae) in which ciliates are a key component; such sites were eliminated from the investigation.

Several unmanaged ponds located on isolated property and either forgotten, or uncared for, were found locally. Such sites were an old farmland pond, unmanaged canals, and wild wetland areas. These proved to be extremely productive and, along with man-made canals, formed the focus of this project (Figure 2.1).

Several other targeted sites were selected within protected areas, notably the State Parks. Sampling permits for Florida State Parks were obtained for the project: 12081610A (2016/2017), 12181710 (2018), 04221920 (2019). This allowed free entry and collection in areas which would otherwise be restricted (Figure 2.2).

To investigate the potential geographical ranges of target ciliate species observed in mainland Florida, locations in the north and south of the State were studied. The southernmost area lies within the Everglades National Park (ENP) for which a Federal collections permit was required: permit 2017-18: EVER-2017-SCI-0035, Study# EVER-00553. This was renewed for the remainder of the project (2018/19): EVER-2018-SCI-0043 Study# EVER-00553. The northern point was located on public land in St. Augustine and did not require a collection permit (Figure 2.1).

Four sites located within 4km of HBOI (Figure 2.2) were selected for long-term monitoring (sites 1-4 in Table 2.1). Two of these were in nature reserves (Table 2.1 #2 and

#3), one was a man-made drainage canal (Table 2.1 #1), and one was a wetland habitat located next to a road (Table 2.1 #4).

Site #	Site Name	GPS Coordinates	County	Habitat Type	Distance from HBOI (km)
1	Lakewood Park Canal	27°31'56.3"N 80°23'54.1"W	St. Lucie	Artificial canal	3.66
2	Indrio Savannahs Preserve	27°31'36.6"N 80°21'47.7"W	St. Lucie	Natural wetlands	1.31
3	DJ Wilcox Preserve	27°31'20.0"N 80°21'06.9"W	St. Lucie	Natural pond	2.2
4	Graveyard	27°32'45.9"N 80°21'50.4"W	St. Lucie	Natural swampland	0.95
5	Orange Tree	27°31'53.1"N 80°21'18.3"W	St. Lucie	Natural vegetation	1.1
6	Pinelands Preserve	27°20'16.8"N 80°37'12.7"W	St. Lucie	Artificial canal	33.85
7	Blue Cypress Conservation Area	27°39'48.6"N 80°38'38.5"W	Indian River	Disturbed wetland	31.11
8	Exit 110 canal	27°09'41.6"N 80°30'59.1"W	Martin	Artificial canal	44.56
9	Jonathan Dickinson State Park	26°59'59.3"N 80°06'09.3"W	Palm Beach	Old artificial pond	61.12
10	Farmland Pond	27°09'23.1"N 80°34'17.8"W	Palm Beach	Old artificial pond	47.23
11	Grassy Waters Preserve	26°48'16.0"N 80°09'51.4"W	Palm Beach	Water catchment area	83.86
12	Dreher Park	26°40'10.5"N 80°04'06.4"W	Palm Beach	Artificial pond	100.86
13	Everglades National Park	25°19'24.3"N 80°49'55.7"W	Miami-Dade	Natural wetlands	250.67
14	Saint Augustine	29°54'06.5"N 81°21'40.6"W	St. Johns	Artificial pond	280.3

Table 2.1: Florida sampling sites where target ciliate species were found. Sites 1-4 were long-term monitoring sites (Chapter 3). Sites 13 and 14 were the Southernmost and Northernmost sites sampled during this study. GPS coordinates and distances were determined using Google maps.

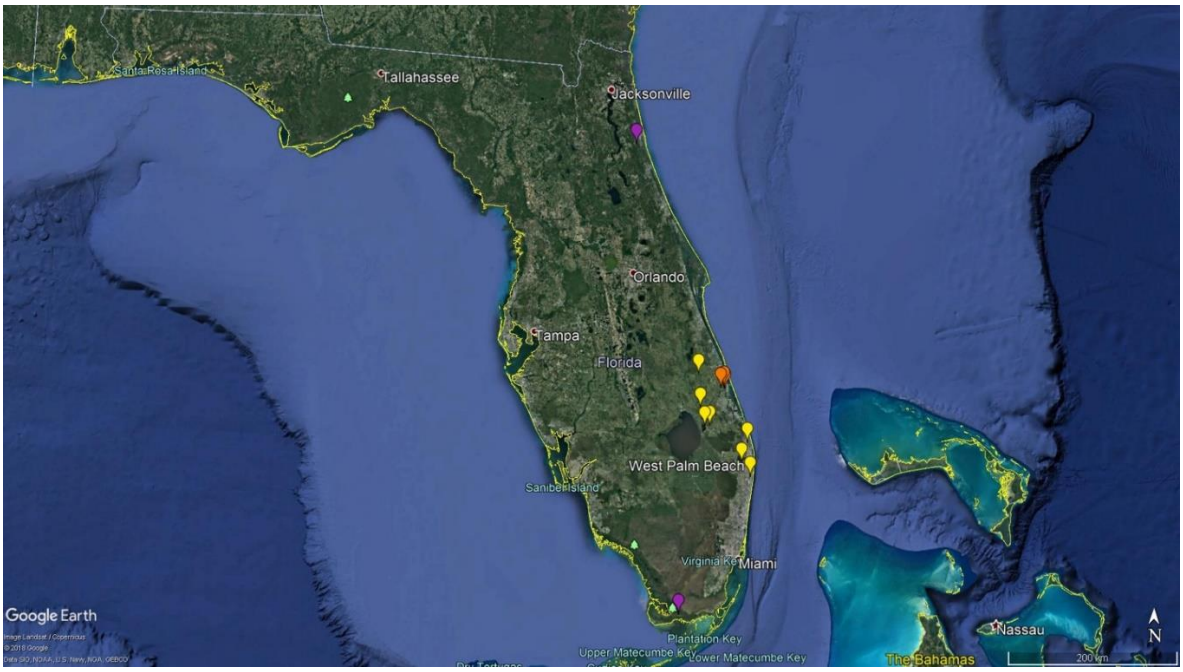

Figure 2.1. Map of the state of Florida. The orange cluster is the local long term monitoring sites (expanded in Figure 2.2). The yellow clusters are sites which were monitored throughout the study. The two purple sites are the northernmost and southernmost sites investigated during this study. Scale bar 200km.

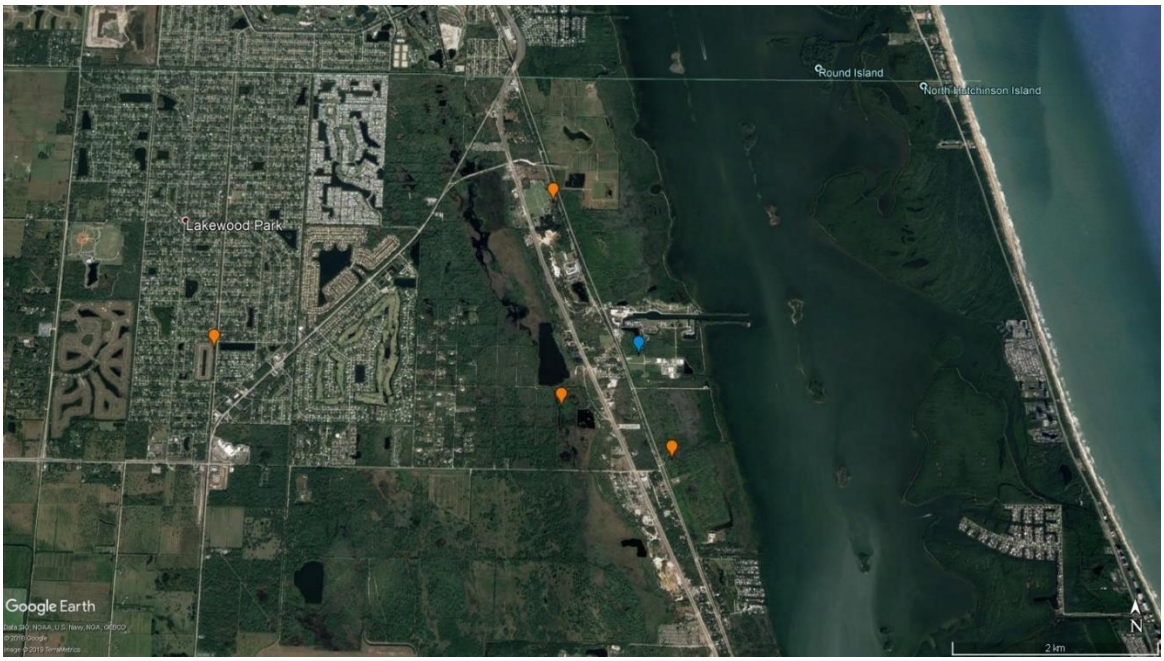

Figure 2.2. The four local long-term monitoring sites are marked in orange. The blue mark is the Orange tree soil site at HBOI. Note the proximity of the Indian River Lagoon (brackish) to the east of sites, with the barrier island, beach, and Atlantic Ocean visible at far right. Scale bar 2km.

Figure 2.3. Examples of sampling sites in Florida.

A: a freshwater pond around Blue Cypress preserve, Indian River county.

B: a wetland in Jonathan Dickinson State Park, St. Lucie county (Table 2.1 #9)

C: an alligator hole in a wetland at Indrio Savannahs Preserve, St. Lucie county (Table 2.1 #2).

D: swamp area at Okeeheelee Nature Preserve, Palm Beach county.

E: the bottle sampler is in air for sampling an artificial canal habitat in Martin county (Table 2.1 #8).

F: a large (~4 meter) wild alligator next to unsampled pond at HBOI, St. Lucie county.

2.1.2 Sample collection

Sampling techniques followed published methods (e.g. Hines et al. 2016). A caged corked bottle sampler (Conbar ep, Monroeville NJ, USA) containing a 500mL glass 'Boston round' bottle (Figure 2.4) with a 10m line. The bottle sampler was a total of 25cm in length. During use, an open bottle was corked using the bottle sampler's stopper. The sampler was deployed on a line into the area of the waterbody being sampled. Once in the water, the cork stopper was removed by a sharp tug on the line allowing the bottle to fill with water at the desired depth. The sampler was then retrieved and the bottle removed and capped for transport to the lab. Sampling of new (previously uninvestigated) sites involved the use of five to fifteen bottles in order to obtain wider representation of the microbial communities present at the site. At long-term monitoring sites three to five bottles were collected per site on each sampling. The device was washed between collections with sterile deionized H_2O, and was rinsed with 70% ethanol after collections on return to the laboratory. Bottles were autoclaved before reuse.

Figure 2.4. Caged sampler with 500mL bottle. This collecting apparatus was used for filling 500mL sample bottles from all sites investigated during the project. This cage opens to allow

the insertion of a bottle. The cork on a line mechanism allowed for it to be pulled within the site, obtaining a sample at the desired depth.

A 2-meter grab (or "dipper"), which was essentially a 500mL plastic cup on a pole (Scienceware BelArt Products, Wayne NJ, USA) was used for shore edge collections and sampling of some deeper areas such as pressed into aquatic vegetation where the bottle sampler would not work. Samples collected using the grab were transferred into sterile plastic 125mL or 500mL bottles.

2.1.3 Measurement of site metadata

A proDSS YSI (four port "Digital Professional Series" by Xylem, Yellow Springs OH, USA) was used to measure site metadata, equipped with probes to obtain pH, Conductivity (measured in PSU), Temperature, and Dissolved Oxygen data. The size of the YSI sensor probe was 35 cm. The probe was deployed so that it would lay submerged horizontal on the sediment substrate. This was closest to the zones sampled using the 500mL bottle sampler. The probe was allowed to settle for at least 30 seconds before a reading was taken. The probe was washed in deionized H_2O between sites.

In most study sites, two readings were taken: first being close to the pond edge, and the second was deeper (~2-5m) into the habitat.

2.1.4 Uhlig ice extraction

For examining interstitial estuarine ciliates, the Uhlig ice extraction was performed (Uhlig 1964). Using a plastic cylinder (diameter 5cm) a sediment core was obtained from a submerged sandy substrate. This was taken back to the lab and processed within 20 minutes. The overlaying water was discarded. At the lab, a 10cm-diameter cylinder with a 200 µm micron mesh at the bottom end opening of ~8cm. The tube was filled half way with the collected sediment. 'Ice sludge' prepared using artificial sea water which was frozen in cube trays at -20˚C two hours before extraction, allowing for easy manipulation rather than solid blocks that are produced from freezing freshwater. This was added to the upper half of the cylinder and allowed to melt through the sediment.

The core was suspended over a glass petri dish with a small amount of the artificial sea water wetting the bottom. As the ice melted it created a downward moving temperature gradient which caused interstitial ciliates to drip out through the filter, falling through to the Petri dish. The majority of the sand remained in the tube held by the mesh, and the dish was

slowly filled with the water containing a high concentration of ciliates. The dish was examined under a dissecting microscope, and subsamples taken and further examined on a Sedgewick-Rafter counting chamber.

2.2 Ciliate and algal cultures

2.2.1 Laboratory cultures of ciliates

Ciliates collected locally could be maintained on the lab bench for weeks to months in untouched 500 mL environmental sample bottles with protection from harsh artificial light being provided by paper towels, foil, or cardboard boxes. For less productive bottles, incubation at 30˚C sometimes allowed cryptic species to excyst and colonize the samples.

These experimental 'cultures' were enriched by adding either axenic *Euglena gracilis*, or boiled and squashed wheat grains (farro, *Triticum* sp.), which allows growth of bacteria, fungi, and protists in the cultures.

In all cases bottles were kept undisturbed to allow an oxygen gradient to develop, allowing the migration of ciliates into defined and often predictable zones.

2.2.2 Monocultures of Loxodes rex

Water collected from an area where the species was known to be present was first filtered to remove particulates (0.45µm, Millipore nitrocellulose filter), and then autoclaved (121˚C, 20 minutes). Sediment was collected from bottles also found to contain *L. rex* from the same site. Large organics were removed from this sediment using a 4mm filter, and then excess water was removed using a 65˚C oven overnight. Sterilized habitat water (10ml per tube) was then pipetted into sterile 16x100mm screw cap tubes. The dried sediment (0.05g) was added to each tube and allowed to settle to the bottom (~1hour). Tubes were then autoclaved (121˚C, 20 minutes). Tubes were cooled and kept for 24 hours at room temperature after which time the water developed a straw to amber color. *L. rex* from a 500ml environmental bottle culture were washed 3x with DI H_2O and inoculated into the prepared tubes at a density of 5 cells per tube. Tubes were fed periodically with *Euglena gracilis*. Tubes were opened for a minimal time during feeding; this reduced the disturbance of the growing cells and reduced the risk of contamination. Growth of the cultures was monitored by eye count, recording the number of ciliates present in the tube. For *L. rex* the feeding cycle was optimized as 2 drops of a dense *Euglena* culture added every two days of the experiment.

2.2.3 Algal culture

A culture of axenic *Euglena gracilis* "Z" was purchased from Carolina Biological Supply Company (Burlington, NC, USA). Cultivation methods followed those described in the literature (James 2012) and as follows:

Each component was added with constant stirring and heat:

DI H_2O	475 mL.
Sodium acetate	0.5g
Beef extract	0.5g
Tryptone	1g
Yeast extract	1g
$CaCl_2.2H_2O$	5 mg

Once dissolved, the solution was brought to a final volume of 500ml with DI H_2O and autoclaved (121˚C, 20 minutes). The broth was cooled and stored at 20˚C.

Sterile 40 mL of broth was added to sterile Nunc EasYFlask 75cm^2 disposable tissue culture flasks (Thermo Scientific, Denmark) and inoculated with the *Euglena*. Cultures were maintained by periodic sub-culturing into fresh growth medium in an environmental chamber maintained at 26˚C. The artificial light source was a row of four 76cm 60W aquarium lights with a diel cycle of 14 hours on and 10 hours off.

2.3 Observations and Microscopy

2.3.1 Microscopy

Individual ciliate cells were picked using a micropipette under an Accu-Scope dissecting microscope for use in PCR, culture, or onto welled slides for examination under higher powered microscopy. Initial observations were made using a 1 mL Sedgewick-Rafter counting chamber which allowed observation, enumeration and photography.

An Olympus BX-53 Olympus microscope, equipped for Fluorescence, Differential Interference Contrast (DIC), Phase Contrast, Brightfield and Darkfield microscopy, was used for detailed observation and photomicroscopy.

2.3.2 Photomicroscopy

An Olympus DP72 camera attached directly to the BX-53 microscope was used to record the images of the ciliates. The camera uses 12.8 Megapixels (4140 x 3096), and is equivalent to ISO1600. TIF files are generated at ~4.0MB (megabit) per image by the cellSense software package.

An iPhone was used to record videos for outreach (e.g. Instagram) allowing both regular video and time lapse (6x speed). In order to attach the iPhone to the BX-53 microscope an ocular was removed and the iPhone was mounted using an iLabcam adaptor (iDu Optics, Detroit MI, USA) with a built-in 10x objective. This adaptor was also used with the Accu-Scope dissecting microscope.

Ciliates were measured using the Olympus cellSens (Standard 1.17, Build 16030) software package which is accurate to 0.1µm. An 'arbitrary line' was drawn between the two points of interest (e.g. the ciliate body) on a live image or a micrograph. This distance was converted to length using the cellSens software package.

2.3.3 Electron Microscopy

Individual cells were hand-picked with glass pipettes into a chamber and washed 3x with 0.5mL sterile site water. Cells were then transferred to a 2mL plastic microcentrifuge tube and fixed in 5% (v/v) glutaraldehyde (EM grade, Electron Microscopy Sciences, Hatfield, PA) in Phosphate Buffered Saline (EM grade, Electron Microscopy Sciences, Hatfield, PA). Tubes were shipped overnight to Dr. Pat Blackwelder, University of Miami for sample preparation and electron microscopy.

The fixed cells were filtered onto a nylon monofilament cloth with a 62µm pore opening mesh in a small filtration set up. Cells captured in this mesh were then rinsed three times for 5 minutes in 0.05M sodium cacodylate buffered seawater and post-fixed for 1 hour in 1% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) in the seawater buffer. The cells were then rinsed in three changes of the seawater buffer, dehydrated in three changes of 20, 50, 70, 95 and 100% ethanol, and dried in three changes of hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA) for 5 minutes each. Care was taken to ensure the cells were continuously immersed during this process.

After drying they were mounted on Al stubs covered with carbon adhesive tabs and coated with Pd in a sputter coater. Cells were then imaged at several magnifications in a Philips XL-30 Field Emission SEM.

2.3.4 Observations

Most of the large ciliates could be manipulated using a glass Pasteur pipette or a sterile 1 mL plastic pipette. Manipulation of smaller ciliates required the use of a micropipette: a glass Pasteur pipette was heated over a Bunsen burner with the middle of the elongated tip in the flame, and tweezers at the tip end. Once the heat had caused the glass to soften, it was delicately pulled, creating an elongated tube with a narrow opening (Dragesco and Dragesco-Kernéis 1986).

Observation of picked cells was performed on a standard well slide or standard microscopy slide, with a cover slip carefully applied to avoid squashing the cells. Welled slides were used for larger species to ensure they did not immediately rupture, but some species were too fast moving when first picked to adequately observe. Up to five slide preparations were made at a time when a target species was found in good abundance. Within one hour many cells slow as water evaporates and oxygen gradients on the slide change, allowing for observation of faster species that have slowed down. Care was taken to ensure observations were made on cells before morphology was distorted. Even after a cell has ruptured, however, it can reveal valuable characteristics, including intracellular components.

2.3.5 Staining

2.3.5.1 Methyl Green Stain

Methyl Green staining (see for example Cedrola et al. 2015) was used to reveal the nuclear apparatus of ciliates. A solution was made using 10 mL 35% formaldehyde solution, 90 mL distilled water, 0.06 g methyl green, and 0.8 g sodium chloride. The stock solution is stable when stored in dark bottles.

One to two drops of sample containing ciliates was added to a welled slide with up to an equal volume of stain. A cover slip was then added to the slide and observed using brightfield microscopy. The amount of stain was varied to provide the best image of the cell: a low amount of stain produced an image with a clear background and a faint blue nuclear apparatus; more dye and a longer fixation time showed greater coloration. This method was also used to record diagnostic characteristics beyond the nuclear apparatus such as ciliary rows. The dye could also be added under the cover slip to prepared wet mount slides which were found to contain an interesting species.

2.3.5.2 Silver impregnation

A classic technique in investigating ciliates is the silver carbonate impregnation (Fernández-Galiano 1994). Silver impregnation can reveal some structures not easily visible in unstained cells. The method used was as described in the literature (Foissner 2014). The working stock reagents were prepared as follows:

1. "Rio-Hortega" ammoniacal silver carbonate solution

   50 mL 10% aqueous silver nitrate solution was added to a flask. 150 mL of 5% aqueous Na_2CO_3 solution was added in small volume under constant stirring. To this 25% NH_3 was added drop by drop with an electric Pipetaid (Drummond) until the precipitate dissolved. DI H_2O was added to bring total volume to 750 mL.
2. Proteose-peptone solution

   Proteose peptone (4g) was added to 96 mL distilled water and allowed to dissolve without stirring. To this 0.5 mL formalin 37% (w/v) was added.
3. Fixative

   0.2 mL formalin 37% (w/v) was added to a flask containing 9.8 mL distilled water.

The production of working stock "Fernández-Galiano's fluid" was as follows. The working stock was prepared immediately before use as the mixture is unstable and ineffective after ~3 hours:

In a fume hood, 0.3 mL C_5H_5N (commercial concentration pyridine) was added to a clear glass flask. 2-4 mL of the Rio-Hortega ammoniacal silver carbonate solution was then added. Proteose-peptone solution (0.8mL) was then added. Finally,16 mL distilled water was added and gently stirred. When done correctly the mixture turns a shiny milky white, at this stage the working stock was stored in a dark brown glass bottle for immediate use.

For silver impregnation target cells were micropipetted and washed 3x in DI water. A small volume of cells was then picked into a welled slide. While working in a fume hood, 1-2 drops of fixative was added to the slide and allowed to fix for 1-3 minutes while swiveling the slide to mix. 1-3 drops of Fernández-Galiano's fluid was then added, swiveling to mix for 10-60 seconds. The slide was then placed on a preheated 60-70˚C slide warmer and moved in a constant circular motion until a color change from milky white to dark cognac brown was observed (~2-4 minutes). A cover slip was then added and the slide observed with brightfield microscopy. As mentioned in the literature (Foissner 2014) this method is

extremely variable, with modifications necessary for each species, and even between preparations.

2.3.5.3 DAPI staining

DAPI (4',6-diamidino-2-phenylindole dihydrochloride) is a classic fluorescent nuclear stain with a high affinity for DNA (Johnson et al. 1982; Fenchel 2013; Nana 2015). A stock DAPI solution (1mg/ml) was prepared in DI H_2O; this was diluted 1:1000 when used. Approximately fifty cells were washed 3x in DI water. Ciliates were then fixed in 2% formalin in Phosphate Buffered Saline for 1 hour. DAPI (200µl) was then added to 1.8ml ciliate suspension and allowed to stain for 15 minutes, the cells were then washed twice in 1x Dulbecco's Phosphate Buffered Saline (PBS, Gibco, Life Technologies).

One drop of SlowFade gold antifade reagent (Invitrogen) was placed on a standard flat glass slide. Two drops of the fixed ciliate solution were then added and covered with a large cover slip. Cells were observed using an Olympus BX53 fluorescent microscope using the DAPI filter cubes $excitation_{max}$ 358nm, $emission_{max}$ 461nm. Images were recorded using the Olympus cellSens software package.

2.4 Molecular biology

2.4.1 Method #1

2.4.1.1 Extraction

DNA extraction was modified from that described by Bourland (Bourland et al. 2012, 2014) using the Qiagen DNeasy Blood & Tissue kit. Individual cells were picked and washed 3x in DI water, then pipetted into a sterile 1.5ml centrifuge tubes. Proteinase K (20uL or 40µL) was added to the tube, mixed, and incubated overnight at 56˚C. The following day, a second batch of cells was collected and washed using the same method, with Proteinase K digestion being reduced to 5 minutes following the manufacturer's directions (Qiagen 2006). The proteinase K time step depended on the type of ciliate sequenced. From this step, DNA extraction followed manufacturer's directions (Qiagen 2006) using a Mini Spin Plus centrifuge (Eppendorf) to elute the DNA.

Samples were cryopreserved in a VWR freezer (Avantor, PA, USA) at -80 °C: Ciliates of interest that were encountered before PCR was optimized, or that could not be immediately worked on, were picked and washed as described here. An individual cell (or group of single

selected cells) was put into a 1.5mL tube with a small volume (0.5mL) of 100% ethanol, which could be stored indefinitely. Cryopreservation was also used for storage of extracted DNA before PCR, as described here, at -20 °C using a freezer (General Electric, MA, USA). Before molecular sequencing, the tubes were put in a dry bath incubator (Fisher Scientific) for ~20 minutes to remove the excess ethanol prior to extraction methods.

2.4.1.2 Amplification

The primers selected for and most often used (Figure 2.2) for amplification of a fragment of the 18S rRNA gene in target ciliates were Euk-82F and EukB (Elwood et al.

Primer	Sequence 5'-3'
Forward: Euk-82F Forward: EukA	GAA ACT GCG AAT GGC TC AAC CTG GTT GAT CCT GCC AGT
Reverse: EukB	TGA TCC TTC TGC AGG TTC ACC TAC

Table 2.2. The primers used in PCR of ciliate 18S rDNA sequences
The technique was optimized with *Sonderia*, and subsequently used throughout the project. Euk-82F and EukB were the most frequently used primer pair.

1985; Medlin et al. 1988). Primers are listed in Table 1 and were purchased from Integrated DNA Technologies (Coralwood, IA, USA).

The PCR method was adapted form that described in the literature (Embley et al. 1992). A master mix for PCR was made as follows, with values multiplied by the number of samples:

DI H_2O	20.475µL
5x Green GoTaq Flexi Buffer (Promega)	11.66 µL
$MgCl_2$ (25mM Promega)	4.66 µL
dNTPs (1µM)	7.29 µL
Primers (10µM)	1.1 µL each
GoTaq® Flexi DNA Polymerase (5U/µl)	0.23 µL

40 µL of master mix was aliquoted into labelled PCR tubes, 10 µL of DNA product was then added. PCR was performed using an MJ Mini Personal Thermo Cycler (Biorad) and the following protocol:

> 10 cycles of denaturation at 94˚C for 40 seconds, hybridization at 55˚C for 40 seconds and extension at 72˚C for 2 minutes. This then was followed by 20 cycles at 92˚C for 30 seconds, 55˚C for 40 seconds and extension at 72˚C for 2.5 minutes. A 6-minute extension at 72˚C completed the program, with the total time step being ~2:32 hours.

This worked for *Sonderia*. For some ciliates this produced only a small amount of product; in these cases, an additional 25 cycles were added to the protocol (See step 8 in HH3 protocol below).

2.4.2 Method #2

2.4.2.1 Extraction

REDExtract-NAmp PCR ReadyMix (Sigma Aldrich) was used as an alternate method combining both extraction and amplification of the sample. The method followed that described in the literature (Kim and Min 2009).

The method followed the 'saliva' protocol as described by the manufacturer, with the reaction scaled down to one tenth volumes as described by Kim and Min (2009). In a single tube 100µl Extraction Solution was mixed with 25µl Tissue Prep Solution. The mixture was then added to each of 10 tubes (12.5µl to each), containing 1 previously selected, picked, and washed ciliate (as described above) and vortexed for 5 seconds. The tubes were incubated for 10 minutes at ambient temperature and then heated in a dry bath incubator (Fisher Scientific) for 3 minutes at 95˚C. Neutralization Solution (10µl) was then added to each tube and thoroughly mixed. Samples were either amplified immediately or stored at -20˚C.

2.4.2.2 Amplification

A Master Mix was prepared by combining 52µl of sterile DI H_2O, 100µl Extract-N-Amp PCR reaction mix and 4 µl of each primer (10µM) in a sterile tube. Mastermix (16µl) was pipetted into each PCR tube to which 4µl of the individual DNA extract (described above) was added. The mixture was mixed thoroughly. Amplification followed the protocol described for Method #1 above. The addition of 30 cycles (to the original 15 at step 8)

significantly improved amplicon yield. This method produced results for the majority of target ciliates.

“HH3” heating block step protocol:

1. 94˚C for 40 seconds
2. 55˚C for 40 seconds
3. 72˚C for 2 minutes
4. GOTO step 1, 9 times
5. 92˚C for 30 seconds
6. 55˚C for 40 seconds
7. 72˚C for 2 minutes 30 seconds
8. GOTO step 5, 45 times
9. 72˚C for 6 minutes
10. 4˚C forever.

2.4.3 Imaging PCR products

Tris/Borate/EDTA (TBE, 5x stock) was prepared by combining to 27g Tris base, 13.75g boric acid and 10mL 0.5mM ethylenediaminetetraacetic acid (EDTA) to a final volume of 500mL in DI H_2O.

1% agarose gels were made by boiling 0.5g agarose (Fisher) in 50mL 0.5x TBE. While still hot, 5µl ethidium bromide (5mg/mL) was added to the flask and stirred. The agar was allowed to cool for several minutes and was then poured into two plastic molds with the desired comb (8 or 16 well) in place. Gels not used immediately were stored in 0.5x TBE buffer.

PCR product (5 µL) was loaded into individual wells on the 1% agarose gel. A 1,000 bp PCR DNA ladder (GeneChoice) was loaded into lane 1 allowing an estimation of the size and quality of the bands. Gels were run in TBE (5x) in a mini horizontal submarine unit (HE33, Amersham Biosciences) at 80V for ~40 minutes using a PowerPac300 (Bio-Rad). After electrophoresis, a Chemidoc MP imaging system (Bio-Rad) was used to image and record the bands.

PCR products were purified using a Gencatch Purification Kit (Epoch Life Sciences) following manufacturer’s directions. DNA concentration were determined using a Nanodrop

2000C (Thermo Fisher Scientific). Purified reactions were transferred into new labelled PCR tubes.

Sanger sequencing was conducted by MCLab (South San Francisco, CA, USA). Since the amplicons were ~1,500bp, sequencing required 20 µl of DNA at a concentration of 10-20ng/µl. The sequences were initially analyzed using the FinchTV software package which allowed a preliminary BLAST search on GenBank. Subsequent analysis was performed using the software packages within the DNAStar Lasergene 12 Core Suite which allowed editing of sequences and the creation of contigs. Sequences were aligned using MEGA version 10.0.5.

2.4.4 Phylogenetic analysis

Evolutionary analyses were conducted in MEGA-X (version 10.0.5) (Kumar et al. 2018). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus trees were inferred from 500 replicates which was taken to represent the evolutionary history (Felsenstein 1985) of the ciliate analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 6 nucleotide sequences. There were a total of 1500 positions in the final dataset. Trees were edited within TreeGraph 2.14.0-771 beta from Newick files generated within MEGA.

2.5 Soil Samples

2.5.1 Collection and observation

Top soil layers down to 1.5cm were collected using a sterile metal scoop and transferred into sterile 125mL Nalgene bottles. These were taken back to the laboratory and cultures were started within 1 hour. Soil cultures were made by placing ~50g soil in sterile 9cm glass petri dishes (Pyrex) and wetting with ~25 mL sterile DI H_2O. The dish was swirled to mix in the overlaying floating soil particles. Wheat grains (farro) were prepared by boiling in DI H_2O for ~15 minutes and then allowing them to cool in for 10 minutes in fresh DI H_2O. Grains were squashed by hand and were added to the cultures with one at the edge and one in the

center of the Petri dish, such that each grain was half submerged and half above water/sediment line to encourage fungal growth. The lid was placed on the Petri dishes and cultures were incubated at 25˚C, 30˚C, and 37˚C. After 24 hours of incubation the enriched cultures were examined every 24 to 72 hours for periods up to several weeks. Water was added as needed as incubation caused drying. New farro grains were added when breakdown (e.g. consumed by bacteria, fungi and worms) had occurred.

In order to sample the enriched cultures, they were held at a slight tilt and a sterile pipette was used to transfer the top runoff water at an edge onto an observation chamber. Due to the relatively low amount of water in these concentrated soil cultures, after observation this liquid was returned to the culture, with additional DI H_2O added as needed.

2.5.2 Soil characteristics

2.5.2.1 Measurement of soil chemistry

Standard methods were followed to obtain soil data (Finlay et al. 2000). Samples were freshly collected from the Orange tree site and left overnight at 60˚C to remove excess water. This material was then sieved (2mm) to remove large organics. A 1:5 soil/water suspension was made with 60g soil to 300mL DI H_2O and stirred for 30 minutes. The sample was allowed to settle for 15 minutes and then the YSI probe was used to determine pH and salinity.
The addition of a filtration step using Whatman GF/F glass fiber filters was also performed; there was no difference in salinity and pH observed between the two methods.

2.5.2.2 Determination of soil type

Soil samples from the Orange Tree site were collected in triplicate and processed for soil characteristics within an hour of collection using the following modified techniques (such as Folk 1974; Dean 1974).

About 60g of sediment from each replicate was dried for 1 hour at 60°C and clumps were broken apart using a mortar and pestle. Samples were then sieved through 2 mm and 0.063 mm sieves to separate the gravel (> 2mm), sand (2mm to 0.063 mm), and fines (< 0.063 mm) into fractions (Folk 1966). The sieves were shaken by hand for ten minutes and each of the fractions was rinsed into separate pre-weighed beakers using deionized water. Samples were dried in a 60°C oven for 48 hours. Each fraction's absolute weight was divided by the total of all three fractions to calculate the percentage.

Water content was determined by placing ~30g of sediment on glass Petri dishes and then drying in an oven for 48 hours at 60° C. The difference in pre- vs post-drying weights were used to determine water content. The dried sediment was ground briefly using a mortar and pestle and sieved to remove the fraction above 2mm which was used for total organic matter analysis: One gram of the fraction was put into ceramic crucibles and placed in a 550° C pre-heated muffle furnace for four hours to burn off the organic matter. Crucibles were allowed to cool for one hour in the furnace, then for another hour in a desiccator, reweighed to calculate the sediment weight loss reported as % Total Organic Matter.

2.6 Data analysis

All data was manipulated within Microsoft Excel; charts were generated using Sigmaplot 14. For data analysis for Chapter 6, please refer to the Material and Methods section in that chapter

2.7 Applications

2.7.1 Language translation

Nearly all the texts of ciliates recorded from Africa used during this project were published in French (e.g. Dragesco 1970). To confirm exact details from relevant sections, the Google Translate app (Version 5.23.0 and previous) on iPhone versions 6 and 7 were used. Sections of interest were scanned using the phone camera, which digitally reads in the French text. This can then be translated into English and read word for word with very high accuracy and clarity. This free app is currently able to auto detect several languages nearly instantly, and was of great use for other texts not published in English encountered throughout the project.

2.7.2 Instagram

The application Instagram was used to create the account microbialecology using Version 81.0. This was run on an iPhone7 (model MN8U2LL/A A1660) using iOS software version 12.1.4 (16D57). The applications were primarily run in the USA.

Chapter 3. FLAGSHIP FRESHWATER CILIATES OF FLORIDA

3.1 Hypothesis

From the perspective of sampling for microbial eukaryotes, Florida is an unexamined tropical habitat, with freshwater areas analogous to those published in the literature with previously investigated sites in Africa. Despite large distances between known species distribution ranges (e.g. continents), some 'flagship' ciliates could thrive and be found in Florida when intensive sampling efforts are undertaken and the right habitat is investigated.

3.2 Introduction

Free-living unicellular eukaryotes make up the foundations of food chains and are an essential component of the microbial loop (Azam et al. 1983; Finlay and Esteban 2015). The sheer number, and therefore biomass, of ciliated protozoa in a given ecosystem can both outnumber and outweigh the other, larger, species present (Fenchel and Finlay 2004). Within the field of microbiology, the implications of global distribution of protozoa and other microorganisms (e.g. bacteria) are important factors to be considered in areas such as disease dispersal and invasive species distribution (Foissner et al. 2008 and references therein). Documenting the biogeography and biodiversity of large (and therefore the most fragile and obvious to detect) ciliates, some outside of their originally described ranges, will help expand the overall knowledge within the field, and shed new light on the dispersal and survival potential for other microorganisms, such as protists. Research into microbes including ciliates are disproportionately biased to sampling areas close to researchers and their institutions, mostly in Northern Europe (e.g. Foissner et al. 2008). Several isolated (some detailed) campaigns have been carried out in parts of Africa (e.g. Dragesco 1966a; Dragesco 1966b; Dragesco 1966c; Dragesco 1966d; Dragesco and Njiné 1971; Dragesco 1972a; Dragesco 1972b; Njiné 1979), and they form the underpinning for the novel research undertaken in Florida during this project.

There are numerous examples of large ciliates (Dragesco 1970; Dragesco 1972b; Dragesco and Dragesco-Kernéis 1986 and references therein) that meet the definition of 'flagship' species (see Chapter 1), and are noteworthy because even though many have been long since documented in the literature (Da Cunha 1914; Dolan et al. 2014) in many instances they remain relatively unstudied, with some not recorded from outside of their originally documented locations or even photographed (Dragesco 1972b). Most flagship taxa are

reported from the Gondwanaland paleogeographic regions, forming part of the basis for their alleged endemicity (Foissner et al. 2008; Bourland 2017). To test this hypothesis, freshwater and terrestrial (Chapter 5) habitats of Florida, USA, a comparatively new region in geologic terms, were examined to explore the diversity of the tropical ciliates present, and to search for some flagship species originally known only from other continents, e.g. Africa. The previous ciliate research in Africa recorded 267 species; at the time this was said to have been half as many as would be expected from similar efforts in Europe (Dragesco and Dragesco-Kernéis 1986). Of the African numbers recorded it was reported that between 23% and 30% were new to science (Dragesco 1973; Dragesco 1980; Dragesco Dragesco-Kernéis 1986), with some endemicity theorized.

3.3 Methods

All materials and methods conducted during the research for this chapter are presented in Chapter 2.

3.4 Loxodes rex

Some data exists on the sampling details and ecology of sites investigated during African ciliate research. *Loxodes rex* was found in some locations with a pH range of 5.1-6.2, and at an oxygen saturation of 0.4-2.9% (Dragesco and Dragesco-Kernéis 1986). *L. rex* was noted as being as commonly encountered and in high numbers from natural habitats as well as species depauperate puddles in Africa (Dragesco 1970; Dragesco 1973). The high biomass and commonality reported from Africa for this ciliate was a key consideration in developing the hypothesis that the species could spread to North America and would be detected in Florida. African freshwater systems were reported as varying from 22 ˚C to over 32˚C, which was believed to be a barrier to greater species diversity (Dragesco 1973; Dragesco and Dragesco-Kernéis 1986), as most ciliates often prefer cooler temperatures (Dragesco 1980).

3.4.1 Loxodes rex in Florida

The class Karyorelictea is a group of mostly marine and brackish water ciliates with elongated and flattened forms, commonly encountered in interstitial habitats (Lynn 2008). The only freshwater representatives are the *Loxodes*, a genus with six known species from habitats worldwide (Xu et al. 2014). The genus has benefited from detailed examinations of its ecology and morphology (Finlay and Fenchel 1986a).

Loxodes are voracious predators of micro eukaryotes and prokaryotes (e.g. cyanobacteria), with a flexible hooked beak at their oral aperture used for feeding. Different species of *Loxodes* can co-exist within the same habitat by feeding on differently sized prey items (Finlay and Berninger 1984).

Loxodes is microaerophilic and prefers oxygen gradients of 5-10%, and can sense this and adjust its positioning in the water column accordingly (Esteban and Finlay 2009). An intriguing organelle unique to Karyorelictea is the Müller vesicle, in *Loxodes* this is a dense barium crystal (the statolith) which acts as a small statocyst-like organelle for the cell (Finlay and Fenchel 1986a). This mechanoreceptor informs the cell which way is up or down, and the cell moves accordingly, even when disturbed (Fenchel and Finlay 1986). The movement of this organelle causes a depolarization of the ciliary membrane which changes the speed and direction of the ciliary beat (Fenchel and Finlay 1986), thus causing a movement behavior after a stimulus. The larger the *Loxodes*, the higher the number of Müller vesicles (Fenchel and Finlay 1986; Esteban et al. 2001 and see below), which inform the ciliate of its position relative to the gravity vector. This organelle allows the ciliate to orient and rapidly move itself throughout the water column and sediment (Finlay and Fenchel 1984) in response to gravity, oxygen levels and light. Reactions to gravity and microgravity in *Loxodes* have been the subject of interdisciplinary studies, including space flight (Hemmersbach and Hader 1999; Häder et al. 2017).

In addition to reacting to gravity, *Loxodes* reacts to both oxygen concentration and light, with studies suggesting both the tactic and kinetic responses to each are related, as light responses were found to change in various oxygen concentrations (Fenchel and Finlay 1986) such that light sensitivity reactions are likely closely related to oxygen toxicity keeping the cell in its preferred ecological niche. A significant, and unusual, adaptation in this ciliate is its ability to respire using nitrate reductase, in which nitrate serves as the terminal electron receptor rather than oxygen, this allows the cell to thrive under anaerobic conditions (Finlay et al. 1983).

The largest species in the *Loxodes* genus is *L. rex;* a tropical ciliate over 1mm in length which was alleged to have a restricted biogeography (such as Foissner et al. 2002; Foissner at al. 2006). This species was the first and most important target for understanding whether flagship species known to thrive in tropical Africa could also be present in Florida, USA.

L. rex is a prime example of a flagship ciliate, and an example of how species alleged to have a 'restricted biogeography' can be challenged when sampling efforts increase. Originally described from Cameroon, and later Uganda, Africa, (Dragesco 1970; Dragesco and Dragesco-Kernéis 1986) it was thought to be endemic to Africa and served as a classic example for proof of microbial endemism (Foissner 2006; Foissner et al. 2008). The species is common in sites investigated in Africa (Dragesco and Dragesco-Kernéis 1986; Finlay et al. 1999 and see below), however the species was not recorded outside of this range and was thought to have restricted biogeography (Foissner 2002; Foissner 2006). Since the cell is so large and easy to identify, it was thought highly unlikely that *L. rex* was overlooked in well-searched areas such as Europe and North America (Green 1994). However, this line of thinking may be flawed: when the vast number of habitats is considered on a global scale, it overshadows even the most intensive biodiversity studies. A record from Thailand (Esteban et al. 2001) suggested that the species can exist outside of Africa.

Subsequent sampling of global areas, with a focus on soil habitats, including Africa (Foissner et al. 2002) and South America (Foissner 2016), did not record *L. rex* (Foissner 2016 and references therein). *L. rex* was also absent from all reliable published species lists from Europe as well as North and South America, until it was discovered in Florida, USA (Hines et al. 2016 and see below).

Despite interest in the evolution and molecular taxonomy of *Loxodes* (Foissner 1996; Andreoli et al. 2009; Xu et al. 2014), *L. rex* had never been sequenced. Such data is imperative for better understanding the evolutionary position of this group of ciliates, as the largest freshwater representative of the Karyorelictea.

3.4.2 Results

A large (>1mm) ciliate was discovered during the preliminary stages of this project. The species was clearly a *Loxodes* based on its occurrence in freshwater systems, being dorsoventrally flattened, and having a large hooked beak-like oral aperture (Figure 3.2). The species was dark in color and, due to its large size, the species was hypothesized to be the flagship ciliate *L. rex*. Further investigation was undertaken, and the morphological data (Table 3.1) matched the *L. rex* descriptions from Africa (Dragesco 1970), confirming the

species identification and making it the first record of this species in the Americas (Hines et al. 2016).

Loxodes rex		
Location	Africa	Florida
Cell length (µm)	500-1,200 (mean 750)	550-1,350 (mean 835)
Cell width (µm)	mean 250	mean 205
Number of macronuclei	132-181 (mean 150)	~80
Number of micronuclei	39-138 (mean 67)	~70
Contractile vacuole	Absent	Absent
Kineties	79-84	~80
Color	Dark brown	Dark brown
Symbiotic algae	Not present	Not present
Molecular sequence	No	Yes
Genus specific diagnostics:		
Müller bodies	~60	45-60

Table 3.1. Morphological data for *Loxodes rex* discovered in Florida, compared with the literature data for *L. rex* from Africa. The large number of Müller bodies and the high number of ciliary rows are the most obvious distinguishing features of the species, along with its large size, with individuals often reaching lengths over 1mm.

Over 500 individual cells were examined during this investigation. The large number of Müller bodies, along with the high number of ciliary rows are the most obvious features to distinguish the species, along with its large size, with individuals often reaching lengths over 1mm. The morphologies described in the two habitats match, confirming the Florida species is the same as that originally described from Africa (Dragesco 1970).

3.4.3 Molecular phylogeny

L. rex was discovered (Dragesco 1970) before the molecular era and had not benefited from DNA sequencing. Several sequences of other *Loxodes* species are available in databases (Xu et al. 2015). The 18S rRNA gene was sequenced from several Florida *L. rex* cells. A phylogenetic tree was constructed to further examine the evolutionary relationship between *L. rex* and other species in this genus, as well as other members in the order Karyorelectida (Figure 3.1). Two sequences were deposited into Genbank for *L. rex* as a result of this project: MK507765 and MK543436. The phylogenetic tree shows that the Florida ciliate was within

the genus *Loxodes* and distinct from those previously deposited in the database. The sequence results coupled with the morphometrics confirms that the identity of this ciliate was *L. rex*.

3.4.4 Laboratory observations

L. rex responds rapidly to light, with tumbling occurring within the first 3 seconds of shining a bright flashlight into a sample bottle. On a welled microscope slide, the ciliate contracts within one second when illumination is increased by swinging the top lens of the microscope condenser into place. This effect can be seen on a larger scale in 500mL sample bottles containing large numbers of *L. rex*: when kept shaded with paper towels they accumulate a thick band of migrated cells; when the paper towels are removed, and ambient light is introduced, the ciliates tumble and migrate downward towards the sediment.

L. rex also reacts to vibrations. When a slide containing a cell is tapped, or bench containing a sample is hit, the ciliate immediately (but slowly) contracts into a shorter fattened form, before continuing its movement, sometimes switching direction. The ciliate will similarly respond to multiple vibrations in a series. The movement of the Müller vesicles are responsible in part for the ciliates movement 'behaviors.' Each Müller body was found to have a total length of ~13µm, with the barium crystal at center approximately 4µm across (Figure 3.3 A, D).

L. rex reacts to oxygen gradients in the same way as it does to vibrations, such that on a welled slide a cell which swims close to the slide edge (e.g. towards a higher oxygen gradient beyond the barrier) will contract, and adjust its movement, sometimes moving up and back into this zone several times before finding its preferred path. When 500mL bottles are left undisturbed a predictable migration and accumulation occurs based on the oxygen gradients present (Finlay and Esteban 2009).

L. rex, like other *Loxodes* (Lynn 2008) reproduces via binary fission (Figure 3.3 B, C). When a *Loxodes* ciliate reaches a certain length, it begins to split in half (bipartition), and a new oral aperture forms for the new daughter cell. The process finishes when the two connected cells (at times swimming in opposite directions) split, with the now approximately half sized 'mother' cell fully intact and ready to continue growth and restart the cycle. The 'daughter' cell continues to develop its oral aperture and grows until the cycle is also again repeated. Given certain environmental conditions, this can occur rapidly, contributing to the large dispersal potentials for this ciliate, and potentially rapid colonization rates (see models in Chapter 6). Conjugation was not observed for *L. rex*, but it was seen in Florida in the

smaller *L. striatus*. The movement of *L. rex* is a steady gliding, but the cell is highly flexible and can twist and twirl when it encounters a barrier. The ciliate also can fold up its sides when gliding (Figure 3.2 B).

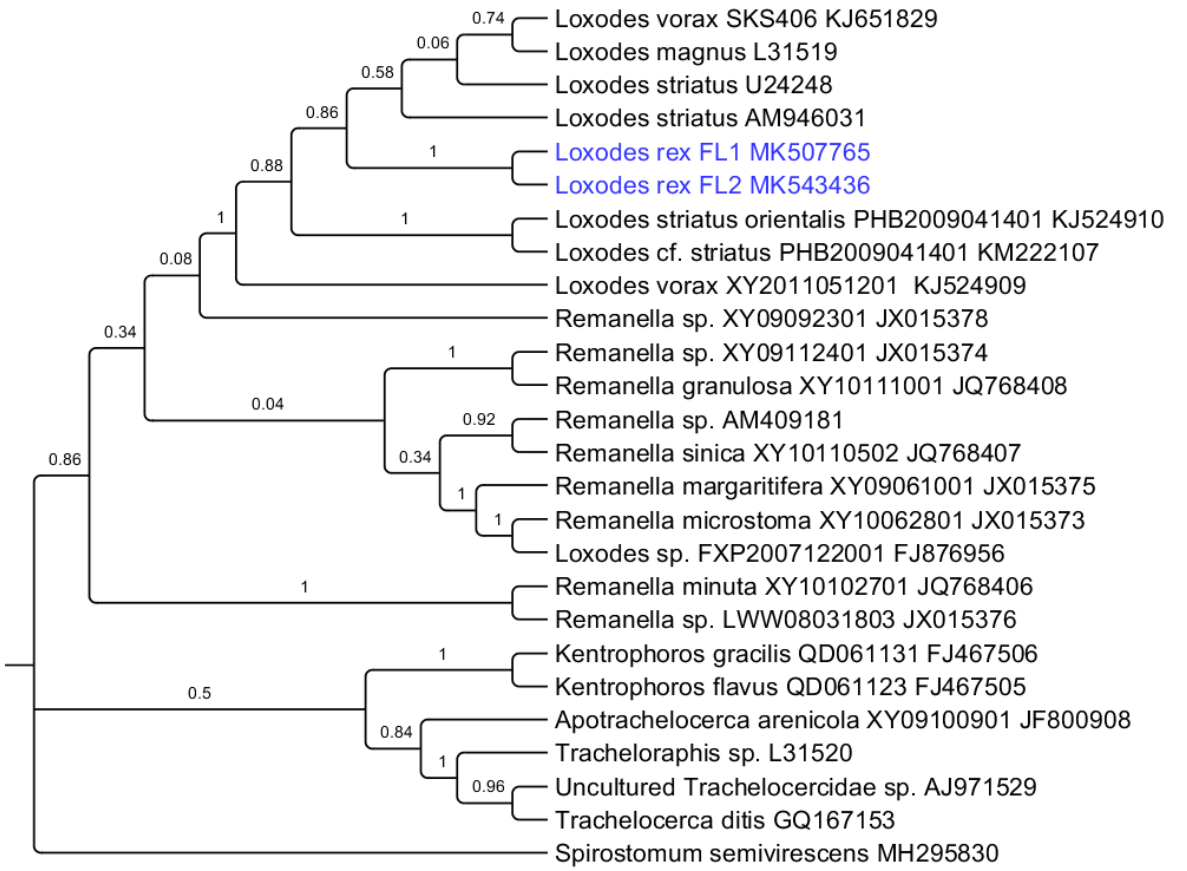

Figure 3.1. Phylogenetic tree of *Loxodes rex.*

The two sequences for *Loxodes rex* generated during this project and deposited into GenBank are colored blue in this tree. These are the first *L. rex* sequences ever recorded. The other species in the tree were selected based on BLAST search, and comparison to literature data. This includes several other Karyorelectea which are ciliates with similar appearance from marine and brackish habitats, often interstitial. All trees created for this project were assembled using standard techniques (Chapter 2). The tree is rooted with *S. semivirescens* from the order Heterotrichida as the outgroup (bottom). GenBank accession numbers FL1 is MK507765 (1,480 bp) and FL2 MK543436 (1,477 bp). Current Genbank provided phylogeny for *L. rex*: Eukaryota; Alveolata; Ciliophora; Postciliodesmatophora; Karyorelictea; Loxodida; Loxodidae; *Loxodes*.

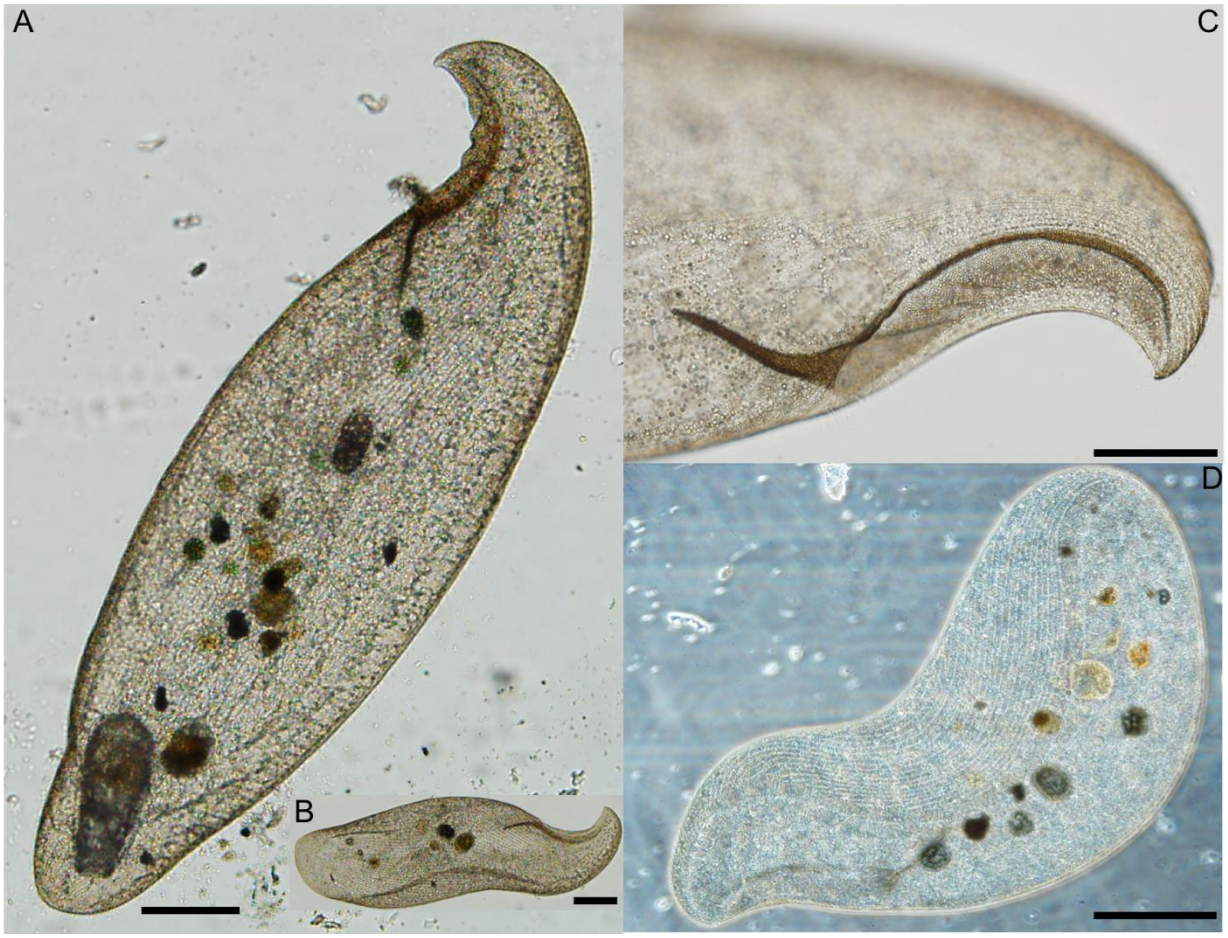

Figure 3.2. The flagship ciliate *Loxodes rex.*

A: *in vivo* image of a swimming cell. The large hooked beak area of the oral aperture is distinct at upper left of image. At the bottom of the cell a large oval testate amoeba has been ingested. Numerous ingested algae are scattered throughout the cytoplasm. Note the dark coloration of the species, and the numerous ciliary rows. Scale bar 100µm.

B: a cell in swimming posture with slightly curved sides. The distinct oral aperture is clearly seen upper right, along with the diagnostic numerous ciliary rows and dark coloration. Scale bar 100µm.

C: close up of the oral aperture (partial side view) with the buccal funnel descending at left. Note the dense line of ciliary rows behind the oral aperture extending across the cell.

D: phase contrast image of the ciliate reveals the rows of cilia descending down the cell, which is slightly squashed on the slide. Numerous algae have been digested in food vacuoles throughout the cytoplasm. Scale bar 100µm.

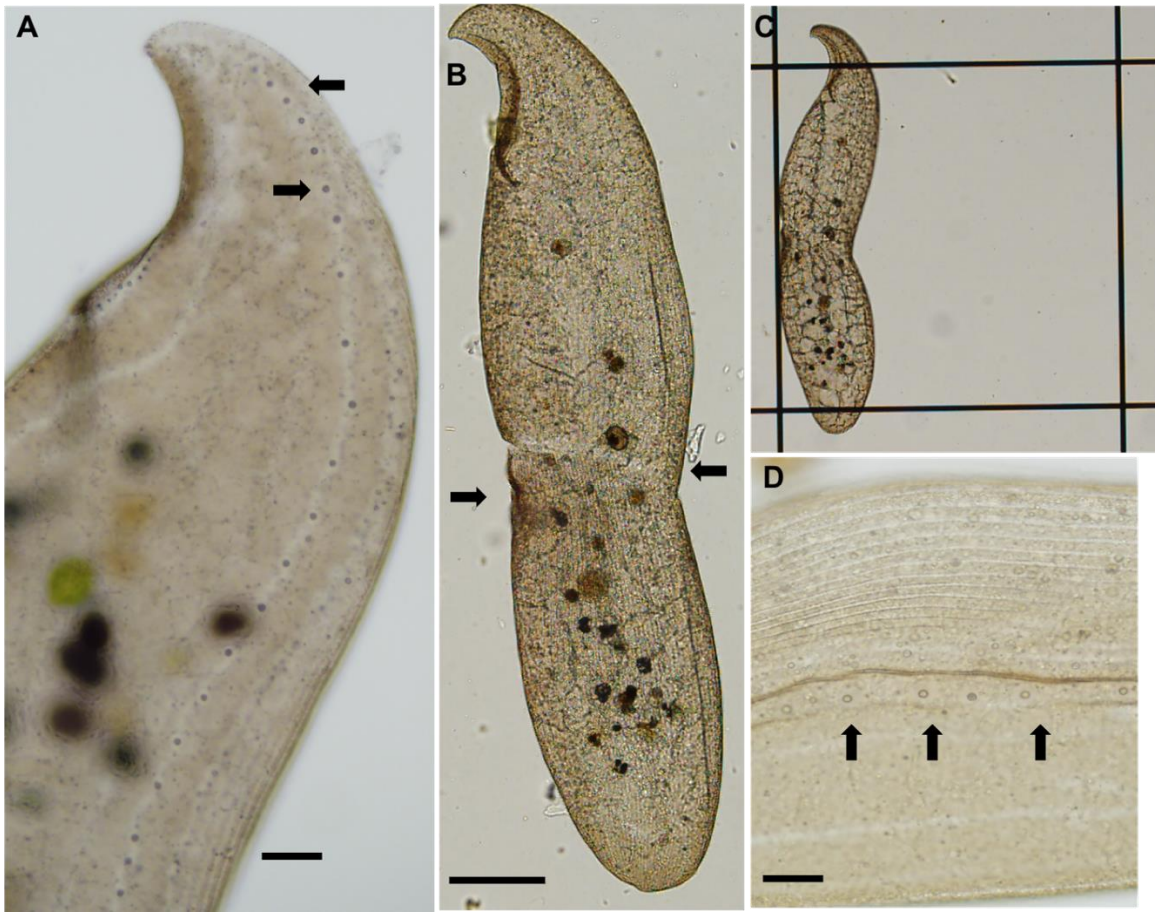

Figure 3.3. Flagship ciliate *Loxodes rex* additional cell morphology.

A: a row of Müller bodies descending down the cell is indicated by the two arrows. The oral aperture at top left runs parallel to the start of this row. Müller bodies (~22) are visible in this image of a squashed *L. rex*, with several unseen out of focus, and more extending down the cell out of frame.

B: *L. rex* undergoing binary fission. The fully formed oral aperture of the mother cell is parallel to the 'B' at top. The forming split across the cell is apparent here (arrow right), and just above the newly forming oral aperture of the daughter cell indicated by the arrow at left. The daughter cell's oral aperture is forming as indicated by arrows at either side of center. The cell will eventually split into two new *L. rex* cells, which will continue to grow and recapitulate the cycle. Scale bar 100µm.

C: *L. rex* on a Sedgewick rafter chamber, during early stage of binary fission. The top of cell is the 'mother' cell, with fully formed oral aperture just below the C. Each square on the grid contains a microliter of water on a 1mL chamber. Each line of the square is 1,000 µm in length, showing the ciliate here is well over 1mm in size.

D: the line of circular Müller bodies in a row is indicated by the 3 arrows, with each circular barium crystal nucleus visible as a distinct darker circle within the round shaped vesicle. The cell is squashed to better reveal this feature. The dense layer of ciliary rows is visible above the line of Müller bodies. Scale bar 30µm.

3.4.5 Long term monitoring

Based on the novelty of the *L. rex* discovery in Florida, and its ability to form large population numbers, it was selected as the main flagship target for investigation during this project. Four distinct sites with close proximity to the laboratory were selected for long term monitoring (Chapter 2). The four sites were monitored for over 1 year, with sample collections made weekly to biweekly. The presence or absence of *L. rex* was recorded, along with the water metadata presented in Figures 3.4-3.7 (and see Chapter 2). These data help to define the niche requirements for this target flagship ciliate. The unusual environmental phenomena which occurred during sampling period make the data set all the more intriguing: An unusual drought period that affected Florida for the first half of 2017 was ended by the flooding rains from Hurricane Irma. From a data perspective, this was fortuitous, as it provides a 'time zero' for investigation of the return of a ciliate population.

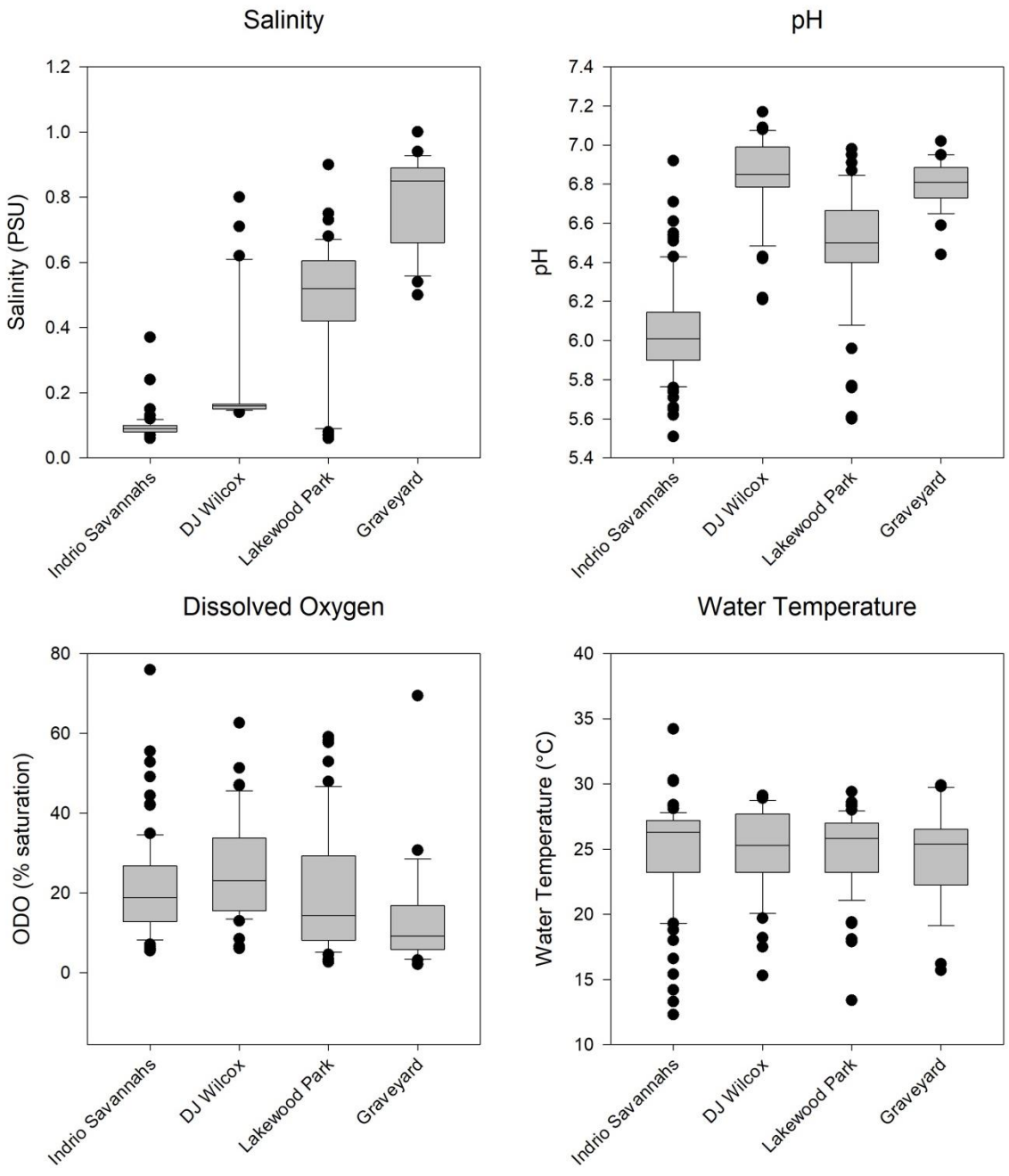

Figure 3.4. Box plots showing the physical parameters of water at the four long-term monitoring sites from which samples were collected.

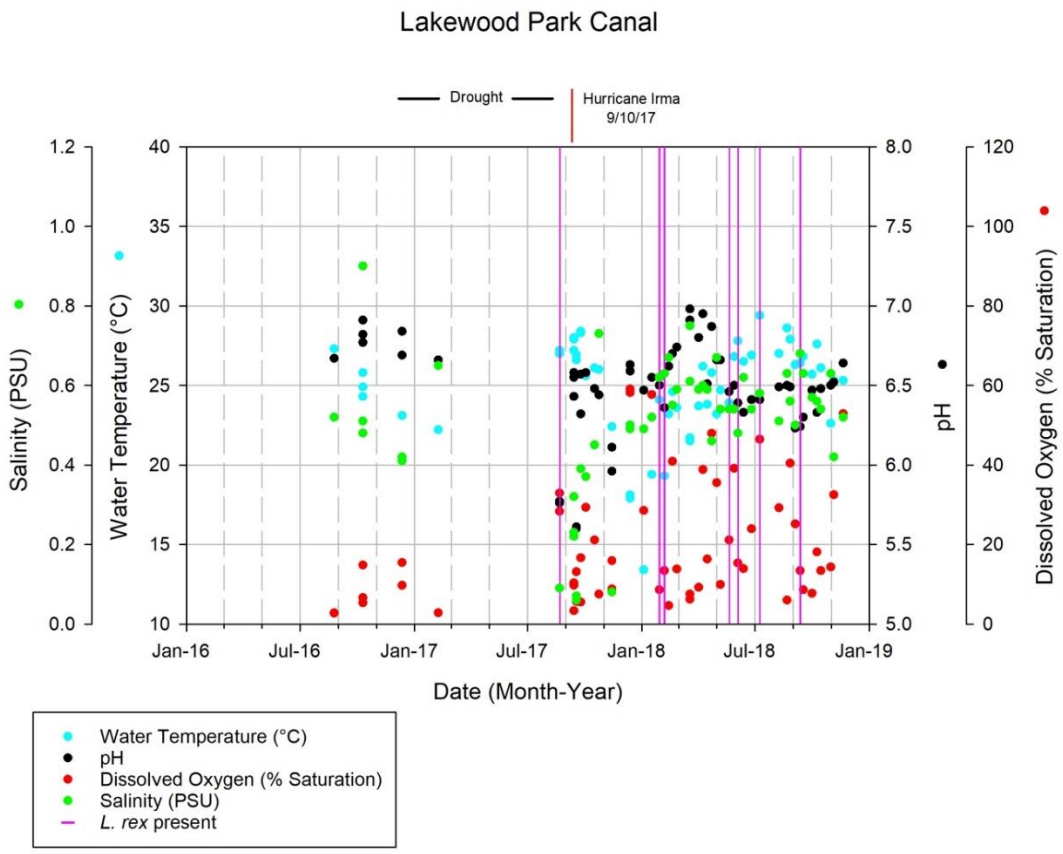

Figure 3.5. Lakewood Park Canal: Metadata recorded from sampling events indicated by the colored dots, and the presence of *L. rex* is indicated by the overlaying purple lines. The same parameters are in Figures 3.6 and 3.7 for the different sites. Note the significant dates at top, 'drought period' followed by the September 10th, 2017 Hurricane Irma. Scale at bottom is month-year.

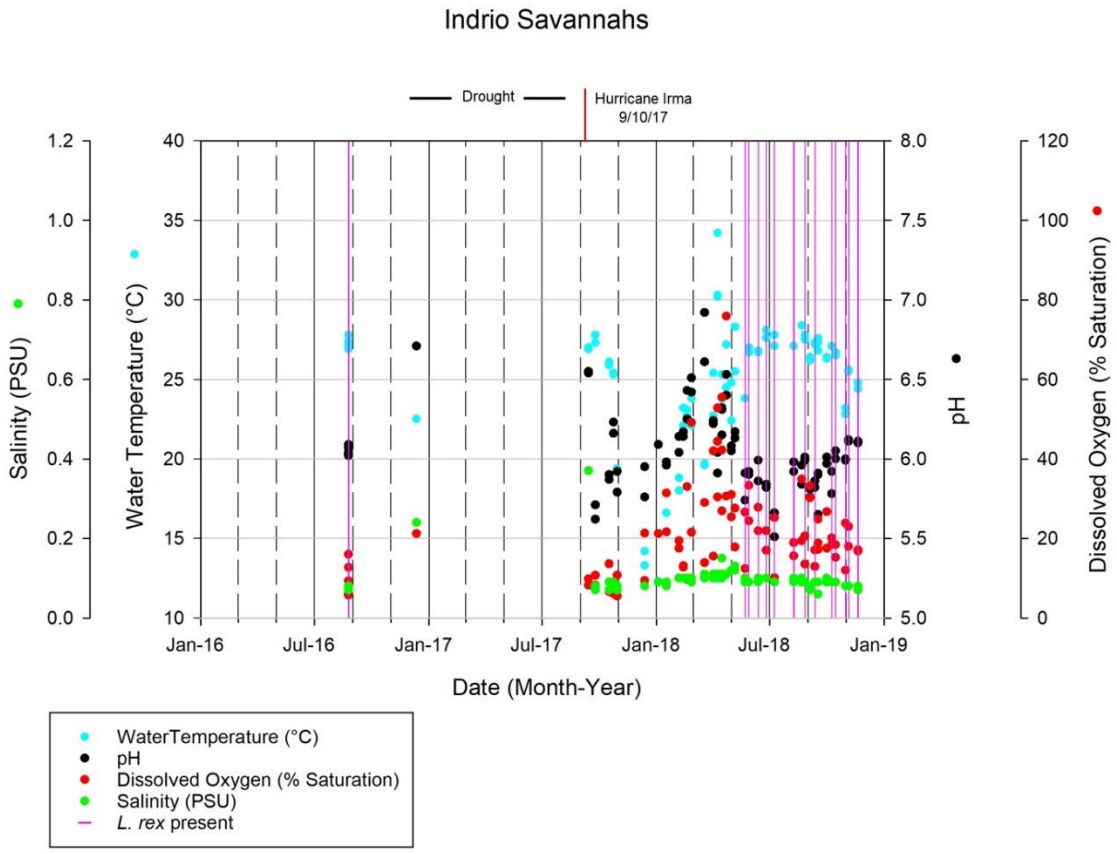

Figure 3.6. Indrio Savannahs Preserve metadata: Lakewood Park Canal: Metadata recorded from sampling events indicated by the colored dots, and the presence of *L. rex* is indicated by the overlaying purple lines. The same parameters are in Figures 3.5 and 3.7 for the different sites. Note the significant dates at top, 'drought period' followed by the September 10th, 2017 Hurricane Irma. Scale at bottom is month-year.

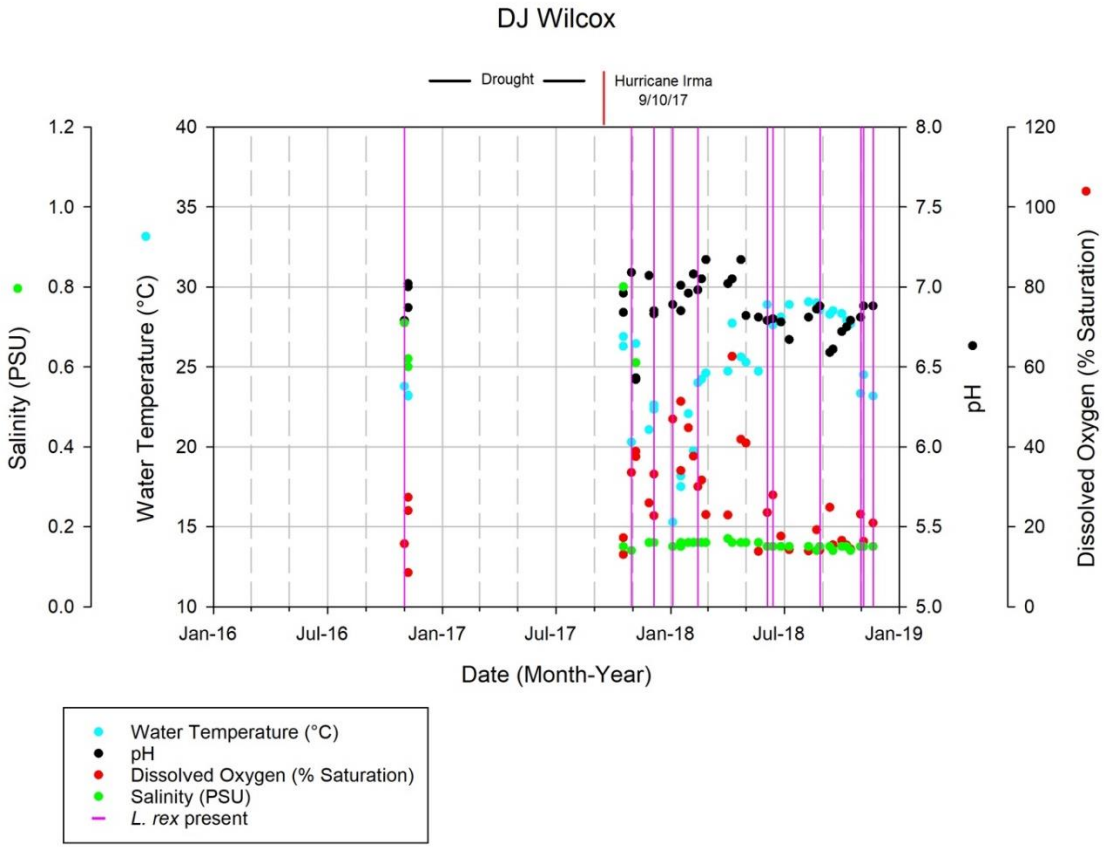

Figure 3.7. DJ Wilcox Preserve metadata: Lakewood Park Canal: Metadata recorded from sampling events indicated by the colored dots, and the presence of *L. rex* is indicated by the overlaying purple lines. The same parameters are in Figures 3.5 and 3.6 for the different sites. Note the significant dates at top, 'drought period' followed by the September 10th, 2017 Hurricane Irma. Scale at bottom is month-year.

3.4.6 Sampling habitats

Four distinct freshwater habitats were studied. Aquatic vegetation densities and types, substrate, nutrient input, and size were different across the sites. Despite this, three out of the four sites periodically contained *L. rex*, under varying environmental conditions. It is clear that *L. rex* has a preferred ecological niche, and is tolerant of variations, including temperature (Hines et al. 2016).

Lakewood Park canal: Figure 3.5. (Site #1 Chapter 2 Table 2.1).

This site was known to contain the ciliate *L. rex*. and was the original discovery site of *L. rex* in preliminary stages of the project. The species was present during the study before sampling methods were optimized, and before the equipment (e.g. YSI) was obtained for sampling and monitoring. Once the long-term study was initiated, the species was not recorded before the drought period. During this time samples were found to be largely devoid of life perhaps due to herbicides used along the canal which may affect the microbial consortia present. After Hurricane Irma sampling resumed and the species was again recorded under conditions in which it was known to thrive (Hines et al. 2016).

Indrio Savannahs: Figure 3.6. (Site #2 Chapter 2 Table 2.1).

This site was selected as it is a very large wetland habitat. *L. rex* was recorded during the first sampling event at this habitat (Figure 3.8) which would undergo the greatest change of all the sites monitored. The drought period removed all standing water from the site, which turned into a grassland (Figure 3.8, C). Hurricane Irma deposited significant water locally (55 cm in Fort Pierce) and, after this, monitoring of the site resumed. Once the site returned to conditions similar to those seen before the drought period, the target species was again regularly recorded as present. The site had the freshest water (i.e. lowest PSU) and lowest pH of the four long-term study sites.

DJ Wilcox Preserve: Figure 3.7. (Site #3 Chapter 2 Table 2.1).

This site was the last to be selected as a long-term monitoring site, but its close proximity to the laboratory, and different habitat type made it a worthwhile addition. The target species was found here on first investigation, and therefore a complete data set was obtained throughout the long-term monitoring project. The Preserve contains a large (90x25m)

freshwater pond ~1 meter in depth. Although water was not completely depleted from the site during the drought period, sampling was halted. The site was sampled immediately after Hurricane Irma, and the target species was recorded there shortly after. The species was often recorded within this site.

Graveyard: Figure 3.4. (Site #4 Chapter 2 Table 2.1).
L. rex was never found at this site, however, this was the source of a ciliate species new to science (Chapter 4). The anaerobic and stagnant nature of this site made it a good candidate for long-term monitoring. The salinity at this site was higher than at other sites and this perhaps precluded some flagships from thriving.

3.4.7 Changes to the Indrio Savannahs site

Indrio Savannahs was found to contain *L. rex* along with other flagships and species new to science (Chapter 4). The site contained highly organic sediments, with samples always containing various microalgae (e.g. *Micrasterias*). The long-term monitoring of this site began before the drought was ended by a major storm event. Hurricane Irma formed in the South Atlantic and devastated several islands. It made landfall in Florida Keys as a Category 4 storm and weakened to a Category 3 storm as it moved north across the state with wind speeds close to 209km per hour at its core. Gale force winds (e.g. 63-87 km/h) spread 350 km across the entire State as the large hurricane affected all parts of Florida. This storm was responsible for adding a significant water input to all local systems, including ~55 cm in Fort Pierce (Roth 2017). Fortunately, the storm passed without destroying the laboratory or access to sites. The hurricane event provided an interesting 'time zero' point for the previously dry Indrio Savannahs site, allowing for the study of ecological succession of a site known to contain a target flagship, and a determination of how rapidly its microbial consortia would respond, including the target flagships, from a previously dry habitat. Once water parameters stabilized, *L. rex* was found again during periods when it was predicted to occur at its highest densities (spring through summer). This evidence further supports the hypothesis that some flagships ciliates are able to spread and colonize new areas rapidly, further expanding their biogeographies.

Figure 3.8. Indrio Savannahs preserve showing the different environmental conditions encountered during long term monitoring.

These images were taken from roughly the same point at the main sampling location for this long term monitored habitat. Note the wooden sign towards middle left of the photos as a reference.

A: the site during a productive sampling period in November 2016. Note the dense aquatic vegetation such as Nymphaeaceae.

B: the site during drought conditions in March 2017, at this stage all surface water had dried, and aquatic vegetation dried.

C: the site in July 2017 during the persistent drought the dried organic mud was covered by grasses. Hines in foreground for scale (1.89 meters). The white grab device was pressed into the plants while searching for any remaining or new water.

D: the site in September 2017, a week after Hurricane Irma brought back significant water to the area. The water returned to previous levels, and swamped all of the existing grasslands. The organic sediments observed microscopically, a key habitat for ciliates, were not as prevalent as before.

E: the site in August 2018 after water levels stabilized, Nymphaeaceae had regrown, and organic sediments returned to the site after the breakdown of vegetation. The physical and chemical parameters along with the microbial consortia had been restored to those observed at the start of the project (A).

3.4.8 The geographic range for the detection of L. rex in Florida

Sampling was carried out in a variety of habitats in addition to the long term study sites and *L. rex* was found at the seven sites listed in Table 3.2; these data help to further define both the environmental tolerance and the preferred ecological niche of the species. The range of *L. rex* across Florida was unknown, but it was hypothesized that the species could thrive wherever it finds its preferred habitat. Two areas at geographical extremes within the State were selected: Everglades National Park (ENP) which is the very southernmost point of the contiguous United States; and the historic town of Saint Augustine, near the northern border with Georgia. The species was recorded from both these habitats; the distance between these two sites is 511 km. The presence of *L. rex* in a site outside of its predicted preferred niche (such as salinity recorded in Everglades National Park) does not necessarily mean the species is thriving, but may further suggest the resilience of the species. Within ENP ~20 sites were sampled, with one being found to contain *L. rex*, amongst dense periphyton assemblages. The

Location	pH	DO (% sat)	Salinity (PSU)	Temp. (˚C)
Grassy Waters Preserve (Site #11 Chapter 2 Table 2.1)	6.29	15.6	0.05	26
Everglades National Park (Site #13 Chapter 2 Table 2.1)	7.74	43.7	1.01	23.5
Farmland Pond (Site #10 Chapter 2 Table 2.1)	7.25	5.1	0.13	30.6
Saint Augustine (Site #14 Chapter 2 Table 2.1)	6.86	5.6	0.13	29
Blue Cypress Conservation Area (Site #7 Chapter 2 Table 2.1)	6.5	3.1	0.11	29
Dreher Park (Site #12 Chapter 2 Table 2.1)	6.96	8	0.25	24
Johnathan Dickinson State Park (Site #9 Chapter 2 Table 2.1)	5.65	9.8	0.07	22

Table 3.2. Additional sampling sites and the water metadata at time of sampling which were found to contain the flagship ciliate *Loxodes rex.*

largest freshwater lake in Florida, Lake Okeechobee (60km from HBOI) was sampled numerous times (including 27°11'44.4"N 80°49'46.9"W). Despite these efforts at the lake and its immediate surrounding habitats, no target ciliates were recorded.

3.4.9 Discussion

Loxodes rex is both very large and one of the most fragile ciliates (Finlay and Esteban 2001a). Although some studies theorize that all ciliates can encyst (Beers 1948) a resistant phase (e.g. cyst formation) for the *Loxodes* life cycle has never been recorded (Lynn 2008), making it an interesting model organism with which to investigate biogeography. The freshwater species is a prime target for testing the concept of endemism within ciliates; dispersal of the large *L. rex* may be less likely than that of a smaller-sized species that readily encyst, e.g. *Colpoda* spp.

L. rex was first found to exist outside of Africa when it was encountered in Thailand (Esteban et al. 2001) in a record which was ignored (Foissner 2006) and challenged (Foissner et al. 2002) in the ciliate biogeography literature. The thriving population in Florida, USA (Hines et al. 2016) confirms the ability of this ciliate to thrive in similar habitats thousands of kilometers apart and in a global distribution. These records expand the biogeography for this species and suggest that it may have a global distribution into locations where it finds its preferred ecological niche (Finlay 2002); tropical freshwaters (Dragesco 1980). *L. rex* is a flagship ciliate because of its large size, and conspicuous morphology which easily distinguishes it from other species in the genus *Loxodes*. *L. rex* was common in ciliate studies in Africa (Dragesco and Dragesco-Kernéis 1986) and is similarly common in favorable habitats of Florida. *L. rex* represents the full cycle of usefulness for a 'flagship ciliate' species whereby it was discovered from a given region and an ecological hypothesis was formed that it was so large and obvious that it must be endemic to Africa, having been recorded only in that location. With increased sampling efforts the species has now been found on three distinct continents. With continued sampling effort this may lead to its discovery in further world regions (Finlay and Esteban 2001a; Finlay and Esteban 2001b). The recording of *L. rex* from Florida is a striking example which has implications for diversity of other protists (Azovsky et al. 2016). Despite not yet being recorded from 'well searched' areas such as the continent of Europe and China (Xu et al. 2015), it may yet be detected as sampling efforts concentrate in the habitats where *L. rex* is likely to thrive, i.e. tropical freshwater, and oxygen-

depleted warm sediments. There is clearly some resilience of the species in being able to disperse to numerous ponds encountered in Florida (Figure 2.1).

Loxodes species inhabit the sediment-water interface of freshwater systems in Florida. Due to it being a microaerophile, if the sediments become completely anoxic the cell migrates out of the sediments into their preferred zones. This niche is readily sampled using the described methods (Chapter 2). The ciliate accumulation within oxygen depleted zones is also observed in 500mL sample bottles. When left undisturbed for 24 to 100 hours the *Loxodes* migrate into a predictable band above the sediment layer, sometimes in dense accumulations. When cell numbers are low, they are difficult to detect while still within the sediments but, given time, they are easy to distinguish by naked eye within water column of sample bottles, and individual cells can be picked.

Accumulation of the ciliates in the oxygen-depleted zone likely provides refuge from predators which avoid this area (Finlay and Fenchel 1986a and see Chapter 6). The photosensitivity of *Loxodes* may also act as a predator avoidance strategy (Finlay and Fenchel 1986b): light avoidance allows the cells to avoid predators such as insect larvae and micro crustaceans (e.g. copepods) (Finlay and Fenchel 1986b), which can either feed on the cells or break them apart via their movements. Light avoidance is also critical in high sunlight environments and this is augmented by the pigment granules that are present in all *Loxodes*. *L. rex* has the darkest color of all the species (Dragesco 1970), which may be an adaptation to its tropical habitat.

Detailed documentation of *L. rex* morphology and molecular biology is important to minimize the potential for future misidentification of the species from global areas. Often ciliates, including *Loxodes*, are poorly described with morphometric overlaps and alleged 'new species' having been identified from observing only several (e.g. >15) cells (such as Xu et al. 2015) which are similar or identical to previous records in the literature. The data here forms the baseline for future research into *L. rex* and should eliminate 'new' species of *Loxodes* being described which are actually *L. rex* simply found from new areas.

Due to its fragility, all attempts at fixation failed, despite having an adequate supply of cells. The *Loxodes* genus is well documented to respond poorly to classical staining techniques (Foissner 2014). Bouin's fixative was also not attempted due to health and safety concerns at the host laboratory due to some of its components including picric acid. Fixation methods (e.g. silver impregnation) either lysed the cell or shrank it into a ball so that no new

details were revealed. *In vivo* microscopy of organisms was sufficient to provide intricate details of the cell and its structure. When left on a welled slide the movement of the species slows as water evaporates and oxygen gradients change. This gives a few minutes for examination in which the species is very slow or still, before noticeable morphological distortion occurs. Often the ciliate was found to return to normal swimming after a slow pause, apparently undamaged.

The smaller and ubiquitous *Loxodes, L. striatus,* is found in Florida and is approximately 150µm in length. It was often encountered in sediments and water column samples, as has been described for other habitats worldwide (Finlay and Berninger 1984; Finlay and Esteban 2009). A coexistence was observed within the Florida samples including those containing *L. rex*. Often the presence of the much more frequent but smaller *L. striatus* was a good indicator that the less prevalent and larger *L. rex* may also be present within a given sample. The two species coexist within the same habitats, *L. rex* feeding on much larger protists with particle sizes too large for *L. striatus*, which feeds on smaller protists.

3.4.9.1 Phylogeny

As expected, the *L. rex* sequences group within the currently available sequences of *Loxodes* in Genbank (all of which are included here). In GenBank FJ876956, labeled as "*Loxodes*", is likely a misidentification based on its position within this tree. It is worth noting that Genbank entries, which in many cases are submitted without a peer-reviewed publication, can easily produce spurious results.

Remanella, the marine representative of the class Karyorelectida is the next closest match based on morphology to *Loxodes*, and this is reflected in the phylogenetic reconstruction and BLAST results observed in Genbank. The genus *Kentrophoros,* also in Karyorelectida is related to the *Loxodes*, as well as the two *Trachelocerca* sequences (Lynn 2008). The sequences generated from this project clearly place *L. rex* within the genus *Loxodes,* and it is distinct from the previously deposited smaller species within the genus.

Morphologically the large number of ciliary rows give *L. rex* a distinct appearance, and clearly distinguishes it from the second largest of the *Loxodes*, *L. magnus*. The ciliate *L. magnus* is far thinner (e.g. half the width) than *L. rex* and its ciliary rows are not obvious under magnifications of 100x or less using light microscopy of *in vivo* samples. Despite some size overlap with a range of up to 800µm recorded (Dragesco and Dragesco-Kernéis 1986) the two are easily distinguished with *L. rex* being darker in color even after division, and the

shape being more ribbon like in *L. magnus*. A more detailed feature is the number of Müller bodies, which is far fewer (10-24) for *L. magnus* (Dragesco and Dragesco-Kernéis 1986), and easily noticeable when observing those parallel to the oral aperture, which are ~5 or less compared to the 10 or more present in the Florida *L. rex.*

3.5 Other Flagship Ciliates found in Florida

The recorded here presence of *L. rex* in North America helped to validate the hypothesis that flagship ciliates can overcome barriers of perceived endemic restrictions. This inspired further investigation into which other species may exist within Florida that were previously considered to be endemic to other continents.

3.5.1 Frontonia vesiculosa

Frontonia vesiculosa (Da Cunha 1914) is the largest species yet described in the free-living freshwater ciliate genus *Frontonia*, which is currently represented by about 40 species found in freshwater, marine and brackish ecosystems (Fan et al. 2011; Chen et al. 2014). *F. vesiculosa* is a freshwater ciliate classified as a 'flagship ciliate' with restricted geographical distribution, and thus serving as another alleged proof for protist endemism (Foissner 2006; Foissner 2008). Even though it was originally described over 100 years ago from Brazil (Da Cunha 1914), little work has been undertaken on this ciliate; the single record from the USA (Bullington 1939, see below) has not been included in the biogeography literature (Foissner 2006; Foissner et al. 2008). The fine structure of *F. vesiculosa* in Africa was described (Yusa 1965), but *in vivo* photomicrographs for the species were never published. Due to its large size (Table 3.3), and conspicuous row of contractile vacuoles, the species is perhaps the second most sought-after flagship ciliate (Foissner et al. 2008). Since it had been described only from South America and Africa, it was thought to provide further proof of a ciliate with a Gondwana distribution (Foissner et al. 2009), but note that the record from the US was excluded/ignored in those papers. *F. vesiculosa* was another main target for this research to document flagship species in North America and provide further details on the ecology of these ciliates ciliate.

3.5.1.1 Results

Originally described in Brazil, South America, (Da Cunha 1914) *F. vesiculosa* was later discovered in Cameroon, Uganda, and Benin, Africa (Dragesco and Dragesco-Kernéis 1986). Despite this species being discussed in the biogeography context (Dragesco and Dragesco-Kernéis 1986; Foissner 2006; Foissner et al. 2008) these are the first photographs of this cell (Figure 3.9). The morphology of the Florida strain matches the literature (Table 3.3), and its large size and conspicuous row of contractile vacuoles make this species easy to identify. Over 500 freshly-collected cells were observed and measured for this study.

The literature morphometrics from Africa, South America and the USA were compared with the new description from Florida (Table 3.3). All of the data matches closely, indicating that they are the same species.

Frontonia vesiculosa				
Location	Africa	South America	USA	Florida USA
Cell length (μm)	370-900 (mean 400)	300-500 (mean 340)	308-660 (mean 463)	415-1,000 (mean 644)
Cell width (μm)	80-190 (mean 119)	120-160 (mean ~140)	110-262 (mean 162)	130-271 (mean 188)
Number of macronuclei	1	1	1	1
Number of micronuclei	2-12	Numerous	3-8	2-10
Contractile vacuoles	3-9 (mean 6)	6-8 (mean 6)	5-8 (mean 6)	5-8 (mean 6)
Kineties	140-250	ND	ND	ND
Color	ND	ND	Clear/ light pink	Clear/ light brown
Symbiotic algae	Not present	Not present	Not present	Not present
Molecular sequence	No	No	No	No
Genus specific diagnostics:				
Contractile vacuole canals	Several	7-10	7-10	7-10

Table 3.3. Morphometrics of the ciliate *Frontonia vesiculosa* from literature and the new record in Florida.

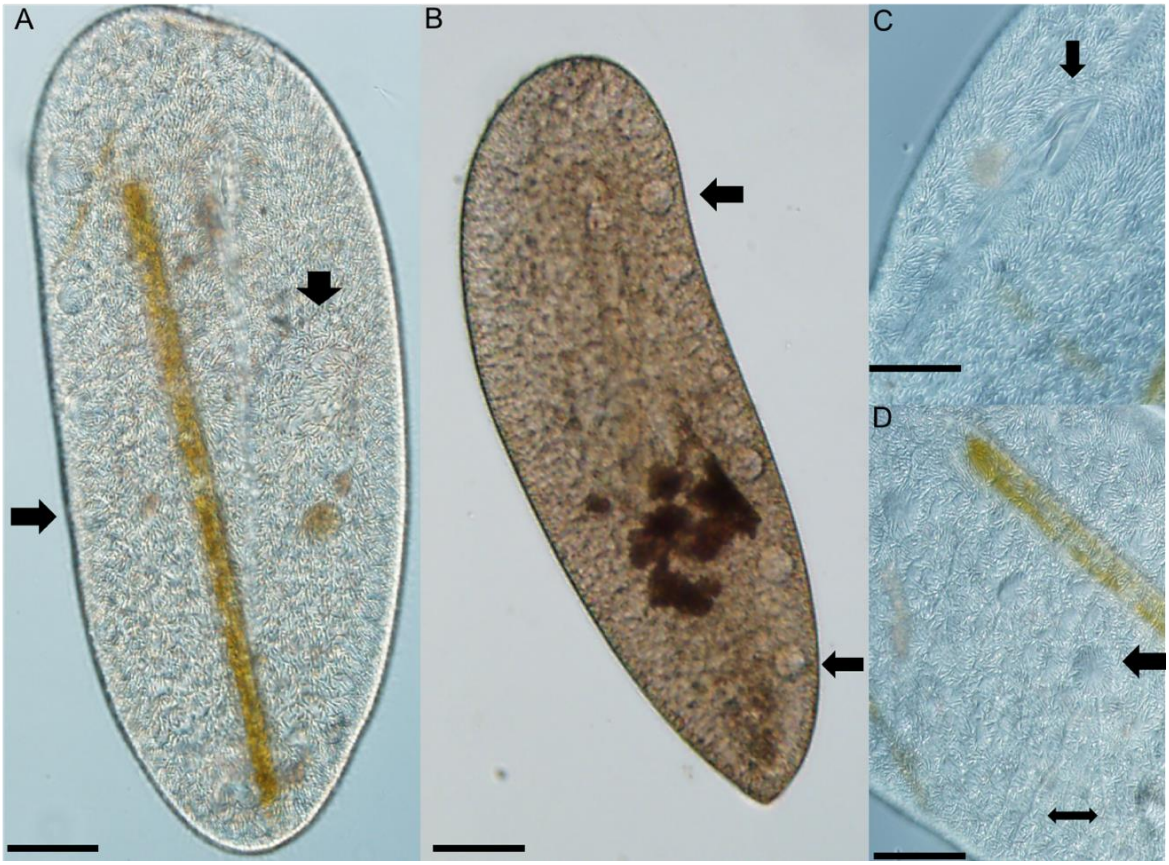

Figure 3.9. The flagship ciliate *Frontonia vesiculosa in vivo.*

A: *in vivo* image of *F. vesiculosa* slightly squashed on a slide with all diagnostic features obvious. The arrow at left indicates the row of contractile vacuoles along the ciliates side. The yellow rectangle is an ingested diatom. The arrow at right shows the position of the clear oval macronucleus. DIC microscopy. Scale bar 100µm.

B: *in vivo* brightfield microscopy of a swimming cell. The two arrows indicate the row of contractile vacuoles which run the length of the ciliate. Note the natural dark coloration. Scale bar 100µm.

C: close-up of the oral aperture indicated by the arrow. Scale bar 20µm.

D: close up of 3 contractile vacuoles, with one indicated by the large top arrow. The long feeder canals are visible and are indicated by the lower double arrow. Scale bar 25µm.

Despite numerous attempts, PCR of the 18S rRNA gene failed. This may have been due to the presence of a polymerase inhibitor. From morphology alone, the species is clearly within the genus *Frontonia*. The large size as well as row of numerous contractile vacuoles identifies the species as *F. vesiculosa*.

3.5.1.2 Ecology of F. vesiculosa in Florida

Common locally, it was first discovered along the edges of a large (325x20m) ~10-year-old artificial pond, (27°31'48.0"N 80°23'57.5"W) which had been constructed for a development of a large housing area which was abandoned for about 10 years until construction resumed halfway through this project. The species has since been discovered in numerous sites (below). The species has been found within hot (27-35°C) shallow waters in samples collected along the shallowest edge of ponds, and in deeper (30cm) bottle collected sediments. It often co-occurs in the oxygen-depleted zones with *L. rex.* *F. vesiculosa* was present in both full sunlight and vegetation-shaded aquatic zones. Large accumulations of 20 cells mL^{-1} were occasionally observed from productive zones. The species was recorded numerous times from the Lakewood Park canal (Table 2.1 #1) in association with *L. rex* as well as at other long-term monitored sites and in some sites only investigated once (results below).

It is worth noting the difficulty of finding this cell even during a productive sampling. Ten samples of 125mL bottles were taken at the same time from the shore of the pond, spaced out from within ~1.8 meters of each other under conditions that appeared identical. Of these 10 sample bottles, only one contained *F. vesiculosa*, and in this bottle it was present in high numbers (~25 per ml). The other nine bottles were devoid of the target species. This illustrates the difficulty of detecting a given ciliate target, even if it is present in high numbers from a selected area.

This species was found in the highest densities at the discovery site where the surface water in the shallowest areas was 35°C. The water temperature dropped to 28°C at 1m depth less than 2m from the edge of the pond. The edge of the pond was very shallow (less than 5cm) and was subject to significant solar heating during hot and humid tropical conditions. It was in this hot, shallow area that *F. vesiculosa* was found in highest densities from the top ~2cm of the water-sediment interface which contained a dense silt and algal substrate.

The range of *F. vesiculosa* is undetermined, but it was regularly encountered during intensive sampling. The northernmost site sampled during this project, Saint Augustine, contained the species. *F. vesiculosa* was not recorded in the southernmost site, the Everglades.

Sites with *F. vesiculosa*	pH	DO (% sat)	Salinity (PSU)	Temperature (°C)
Lakewood Park Canal (Site #1 Chapter 2 Table 2.1)	6.67	2.8	0.52	27
Blue Cypress Conservation Area (Site #7 Chapter 2 Table 2.1)	7.26	3.1	0.17	29
DJ Wilcox Preserve (Site #3 Chapter 2 Table 2.1)	6.67	8.5	0.62	23
Indrio Savanah Preserve (Site #2 Chapter 2 Table 2.1)	5.92	24.4	0.09	26.5
Saint Augustine (Site #14 Chapter 2 Table 2.1)	6.33	5.6	0.15	27

Table 3.4. Additional sites and water parameters where *Frontonia vesiculosa* was recorded in Florida.

3.5.1.3 Discussion

F. vesiculosa has been poorly represented in the literature, with some (Cai et al. 2018) incorrectly listing its size as only 300-400µm and recording an absence of feeder canals; both of these statements are incorrect. Several feeder canals are always present and its size range of 400-900µm has been reported in all other published literature.

The lack of the USA record (Bullington 1939) from the biogeographic literature (Foissner et al. 2008) is also perplexing. The work by Bullington (1939) contains a section on *F. vesiculosa*, and was included in the major works of Dragesco (Dragesco 1970; Dragesco and Dragesco-Kernéis 1986) with the further *F. vesiculosa* descriptions in the records from Africa, but is ignored by later ciliate biogeography theories mentioning the

species (e.g. Foissner et al. 2008). This early work is important, as it contains the first record of this species from North America. The work states that *F. vesiculosa* came out of an old laboratory culture made up from pine needles and *Spirogyra*, cultured from Kings pond, and Railroad pond (Bullington 1939), which is presumably (41°54'35.8"N 70°42'20.0"W) in Massachusetts, Northeastern USA. This represents the first record for North America; our findings of *F. vesiculosa* in Florida are the first from its apparent natural habitat with the natural ecology of this species. Based on near exact morphologies, there is little question that the species studied by Bullington (1939) is identical to the original description by Da Cunha (1914) and the later African records (Dragesco 1970, Dragesco and Dragesco-Kernéis 1986). Since this northern North American site is far outside of the tropics, it is possible to speculate on this organism's true dispersal and survival potentials. A recent alleged record of *F. vesiculosa* from lake Baikal, Russia, (Alekperov et al. 2012) would also greatly expand this species' biogeography. However, the morphological diagnostics from this record vary greatly from the literature, with a much smaller cell size and fewer vacuoles recorded, and is therefore questionable pending molecular taxonomic work within the genus.

F. vesiculosa's distribution in South America and Africa was thought to be evidence of a Gondwana restriction (Foissner 2006), by which populations may have been separated by millions of years due to shifting plate tectonics (Dragesco and Dragesco-Kernéis 1986). The expansion of the biogeography of this species to Florida, USA, is evidence for the potential ease of dispersal, such that ciliates can likely spread out of any original separation caused by events including the separation of continents. Perhaps a contributing factor to this species not being investigated or as yet discovered globally, is that these are the first *in vivo* photos for this species. The formation of the super continent, Pangaea, which would start to break up during the cretaceous period, has been a theory for modern day distributions of protozoa (Foissner 2006). Perhaps, however, with enough time, (i.e. >100 million years) from this split to current continental positioning, would be sufficient to allow for ciliates to interdisperse throughout the entire biosphere and colonize new areas outside of this distribution range. This research provides evidence to support this statement.

Members of the *Frontonia* genus have been reported to form cysts (Foissner et al. 1994), which would increase their dispersal potential. Cysts may have been present in the samples used by Bullington (1939), these may have been dispersed from a distant habitat, and only made viable under his laboratory conditions. If such *Frontonia* cysts, which must

be present in the ciliate seed bank (Finlay and Esteban 1998b), are dispersed to the northern USA, and temperatures rise in the summer, it is probable that shallow sunlit ponds are capable of supporting a thriving community containing this flagship ciliate, when conditions analogous to the tropical environment become available. The ponds of that northern region could hardly be confused at the macro level with a tropical habitat, but within a well-lit shallow pond, the first few millimeters along the shore of water could be heated to temperatures equal to that of the tropics, providing a suitable habitat for *F. vesiculosa* excystment, or colonization from new dispersals. Ciliates perhaps then represent a unique set or organisms which, although some may exhibit stenothermic restrictions, can overcome this when environmental factors become favorable even ephemerally at the micro level. Despite seemingly ideal conditions for growth, some small percentage of ciliate populations still form cysts, which contributes to the cryptic biodiversity in an ecosystem (Esteban and Finlay 2010). This could explain their overall success and constant presence within ecosystems; if a sudden disruption of the ecosystem was to occur, at least some of the population would survive to excyst when favorable factors return. A warm layer of water within the edge of a pond could provide a potential habitat for a dispersed tropical species, even if that pond had been under ice months before. These potential 'survival islands' mimic the required condition of the tropics perhaps well enough to facilitate colonization and therefore further spreading via cyst or random cell dispersal. This adaptability, and relative ecosystem plasticity, must be a driving force in the overall success for this group of organisms.

No cysts of *F. vesiculosa* were recorded during the Florida investigations, but older cultures incubated for one to two weeks at 30°C were sometimes found to have the species in high number, where it had been undetected previously. It is likely that the ciliate existed in low numbers, or was dormant inside cysts in the sediment. Further work is necessary to understand this phenomenon.

Records from Africa showed *F. vesiculosa* ingested a wide variety of organisms including: Diatoms, *Oscillatoria*, *Closterium*, *Spirogyra*, *Trachelomonas, Difflugia,* rotifers, *Vorticella,* and *Arcella* (Dragesco 1970). This is consistent with what was available and observed in the Florida habitats. Observations on the Florida *F. vesiculosa* showed that the beating cilia that help the cell ingest a large prey item could reverse, thereby expelling the item when the cell has perhaps attempted more than it can fit within its body cavity, such as large desmids. Full cells distorted into irregular shapes swam poorly and far slower, and were

much more fragile to pipetting than normal cells. *F. vesiculosa* was never observed to burst due to feeding, despite some instances where the cell was stretched and distorted to accommodate the ingesta.

Given the ubiquity of the smaller *F. leucas*, the dispersal potential for this large flagship representative requires further study. The ability of *F. vesiculosa* to thrive in hot (~35°C), bacteria-filled water shows its resilience to what may seem an unfavorable habitat for other protozoa. The apparent ability of the cell to encyst (Bullington 1939) further allows for a much greater dispersal potential for the species.

This species may also be a good candidate to test the theory of a 'molecular flagship' by which an exact morphology of a species from three distinct biogeographies can be sequenced to see what, if any, meaningful diversity between biogeographically separated strains exists. A potential for investigating and understanding a 'ciliate molecular clock' will lead to deeper understanding of the timing for a ciliate dispersal between populations and the connectivity of habitats. Whether or not a given species is globally dispersed rapidly, or represents distinct populations separated by millions of years can only be investigated with advancing molecular techniques. *F. vesiculosa* failed to yield molecular sequences during investigations for this project, and remains unsequenced globally. There are likely a wide variety of inhibitors that contribute to this failure, but this is unknown, and despite adequate materials and a variety of methods, the species remains unsequenced. Research into the large ciliate *Spirostomum semivirescens* showed that a ciliate separated from distinct areas over 1,600km could have both exacting morphologies and exact 18S rRNA sequences (Hines et al. 2018). It is likely *F. vesiculosa* shares these same features, but future research including sequences from two or more continents (of properly identified material) will be needed to investigate this further.

3.5.2 Bursaria caudata

The large ciliate *Bursaria truncatella* has long interested scientists (Müller 1786). This ciliate can measure over 1mm in length and is essentially a giant baseball-glove-shape mouth, and was the only species within the genus until *B. ovata* (Beers 1952) was described. The species *Bursaria caudata* (Dragesco 1972b) was discovered in Africa at only one location: Lake George in Uganda. The large size of this ciliate (up to 1,500 µm) makes it an interesting candidate as a flagship species. Since this species was not discovered in subsequent African studies (Dragesco and Dragesco-Kernéis 1986), or anywhere else globally (Foissner 1993) it

may be difficult to sample and or detect despite the apparently cosmopolitan distribution of the similar (and equally large) species *B. truncatella* being readily detected globally. *B. catudata* was discovered before the molecular era and had not been observed since.

3.5.2.1 Results

A large ciliate which was clearly a *Bursaria* was encountered from a productive farmland pond (Site #10 Chapter 2 Table 2.1). This species was distinct from the commonly observed species *B. truncatella*. The hourglass like oral aperture and caudal tip matched the original description of the rarely recorded species *B. caudata*. (Table 3.5)

Bursaria caudata		
Location	Africa	Florida
Cell length (µm)	800-1,500	750- 1,100
Cell width (µm)	~400	~400
Number of macronuclei	1 (ribbon-like)	1 (ribbon-like)
Number of micronuclei	ND	ND
Contractile vacuole	1	1
Kineties	>100	>100
Color	Dark brown	Dark brown
Symbiotic algae	Not present	Not present
Molecular sequence	No	No
Genus specific diagnostics:	None	None

Table 3.5. Morphometrics for *Bursaria caudata* with comparison of the African description to the Florida discovery.

Geographic distribution:

Africa, Uganda where it was found from only one location around Lake George. USA, Florida, (this study). Unseen since its original discovery by Dragesco (1972b).

African strain description: Width was not recorded in text, but an inferred size from scale bars in the original drawing was ~400µm (Dragesco 1972b). The cell resembles *Bursaria truncatella.* Large buccal funnel, narrow vestibular opening. Cell highly vacuolated, has a characteristic long tail-like caudal tip of varying length (Dragesco 1980). Macronucleus long and ribbon like (Dragesco and Dragesco-Kernéis 1986). Found only in areas around Lake George (Uganda).

Florida description: The Florida species matched the original African description closely and was identical to the drawings (Dragesco and Dragesco-Kernéis 1986). No mean values were produced due to low species numbers. The overall morphology matches those described for the African strain, e.g. one large macronucleus in the shape resembling a folded ribbon or tube. Cells are highly vacuolized. Long tail-like caudal end. Vestibular funnel folded (Figure 3.10). The structure of oral aperture is hourglass-like, and folded in unlike that seen in *B. truncatella* which appears as an open pocket. The vestibular cleft is folded and more narrow than in other *Bursaria.*

Coloration: Color pigments are present, giving a coloration of medium black/brown. Clearly darker than the local strain of *B. truncatella*.

Movement: The ciliate moves very quickly when freshly picked. Cells move twice as fast as the locally common *B. truncatella*. The cells travel rapidly in a straight line, not within small circles as observed in *B. truncatella* which appears to slowly spin around the slide. Large counterclockwise spirals were observed before the ciliate sped out in a straight line. Avoidance to light stimulus behavior was observed, with the cell rapidly retreating with the addition of light, as has also been observed in other *Bursaria*.

Florida ecology: Collected from an old natural farmland pond (46x20 meters) within the Lake Okeechobee watershed (27°09'23.1"N 80°34'17.8"W) of SW Martin County (Site #10 Chapter 2 Table 2.1). The site was in direct full sunlight with no overhanging or floating aquatic vegetation that might provide shade. Specimens were collected at the water-sediment interface using standard methods (Chapter 2); cells were found at 30cm depth in dark-colored waters at 30°C and 35°C. The organic muck substrate contained a dense ciliate assemblage; abundant megafauna (birds, alligators, turtles) was also observed. Flagships including *Loxodes rex* were frequently encountered with the species. This site was monitored throughout the project, but held this species on only one occasion. Metadata was unrecorded from the sampling event which produced the species. Molecular sequencing was not possible due to low cell number.

Figure 3.10. *Bursaria caudata.* A: *in vivo* image. The oral aperture is conspicuous at right, as well as the tail-like caudal tip to left. Scale bar 100µm. B: brightfield microscopy of a swimming cell. Note the dark coloration. Scale bar 100µm. C: *in vivo* image of a swimming cell, with the details of the large oral aperture and buccal funnel obvious. Scale bar 100µm. D: *in vivo* image of a cell picked and slightly squashed, which lessens the caudal tip morphology. Scale bar 100µm. E: cyst from a large Colpodid ciliate, likely a *Bursaria* sp. Scale bar 100µm.

3.5.2.2 Phylogenetic analysis

The species *B. caudata* had never been sequenced, and remains unrecorded from other global biogeographies. Due to low cell numbers a molecular investigation was not possible for Florida strain. The more common *B. truncatella* alternatively represented a good target to sequence, and matched other strains within the available database (Figure 3.11).

This cosmopolitan ciliate *B. truncatella* matched exactly to the previous morphological literature on the species (e.g. Dragesco and Dragesco-Kernéis 1986 and references therein). The molecular sequence and phylogenetic positioning were as expected.

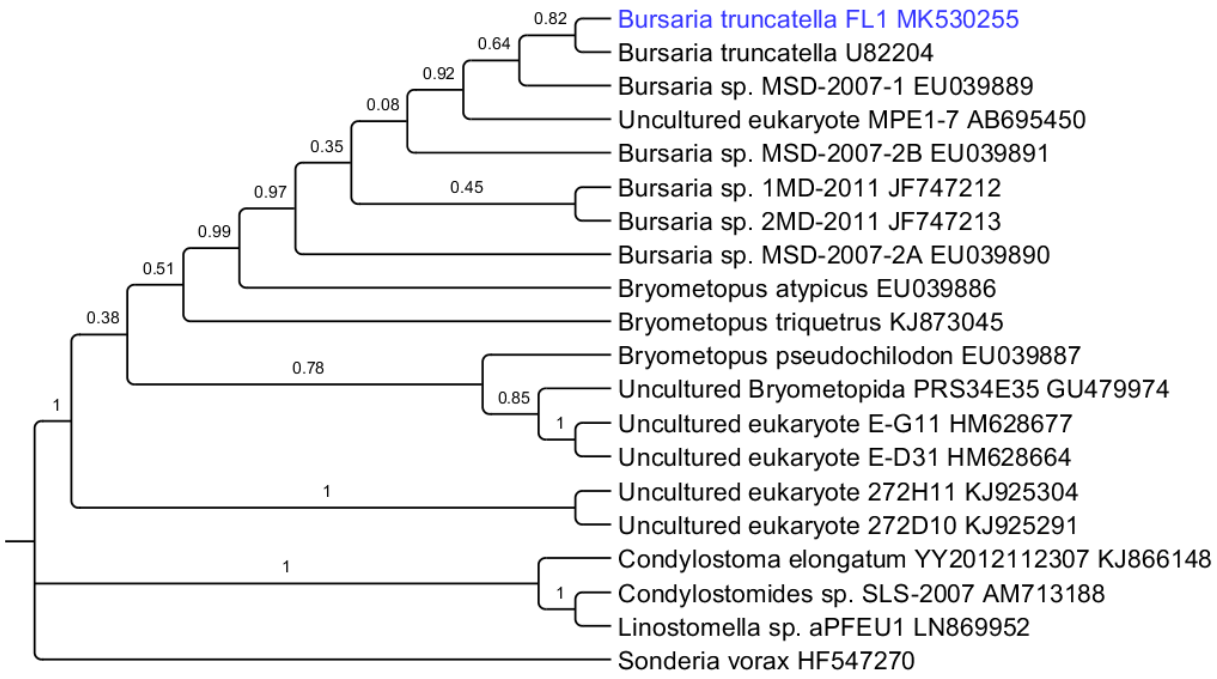

Figure 3.11. Phylogenetic tree for *Bursaria truncatella* sampled from Florida, a likely close relative of the flagship *B. caudata*. The sequence generated and deposited into GenBank during this project is colored blue. *B. truncatella* FL1 with GenBank accession number MK530255 (1,564 bp) match to what has been previously deposited into Genbank. The *Sonderia vorax* sequence is the outgroup for the tree root (bottom). The phylogenetic positioning provided by GenBank for this sequence is: Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Colpodea; Bursariomorphida; Bursariidae; *Bursaria*.

3.5.2.3 Discussion

The discovery of *B. caudata* in Florida, USA, is both the first record for the Americas and the first record for this species outside of Africa. At a distance of over 12,000km between the habitats where this species has been recorded, it appears this species can obtain a global tropical distribution. The dispersal potential is enhanced by its likely ability to form cysts. The large (>1mm) size, and distinct morphology (e.g. with a tail-like caudal tip) add to the potential ease of detection by researchers globally, if they looked for such species. It is unlikely that this species is restricted only to Uganda, Africa, and Florida, USA, and therefore could be monitored in future global studies, particularly in the tropics. *B. caudata* makes a

good candidate of a known species from the historic literature for being a flagship ciliate, with obvious size and morphology. Cysts of *B. truncatella* which had been cold treated (1 month at 15°C followed by 3 months stored at 5°C) were found to excyst readily under laboratory conditions (Beers 1948) when provided with food and incubated at room temperature, suggesting a survival mechanism which would enabled survival during dispersal events; this may also be retained in *B. caudata*. The cyst structure of *B. truncatella* is well documented (Beers 1948 and references therein). Large sticky cysts (375µm) encountered during Florida sampling were not directly observed to excyst and produce *B. caudata* cells, and since the samples containing the target species were mixed with other species of *Bursaria* the presence of *B. caudata* cysts cannot be confirmed here. *B. ovata* has also been reported to form cysts (Beers 1952), and so it is reasonable to assume *B. caudata* can also form cysts.

During microscopy observations the ciliate 'tail' would shrink into the body as the cell flattened before lysing. Observations of this species had to be conducted rapidly to preserve morphology. It is possible that the fragility of this species has resulted in it being so rarely recorded. All fixation methods tried on the African specimens failed (Dragesco and Dragesco-Kernéis 1986) as they did for the Florida population. Because of this, detailed morphometrics remain elusive for this interesting species. No subsequent populations were found that would have allowed further morphological depiction and molecular characterization of this species.

3.5.3 Target flagship ciliates not encountered in Florida

There are several ciliate species that were targets for discovery during sampling of Florida's environments. Not all targets were discovered, which highlights some of the on-going issues with biogeographic studies. Whether or not unseen target species thrive locally is unknown, only that they were not discovered during the limited sampling period of the project.

3.5.3.1 Neobursaridium gigas

The ciliate *Neobursaridium gigas* is of interest as a flagship ciliate with an alleged restricted biogeography (Dragesco and Dragesco-Kernéis 1986, Esteban et al. 2001, Foissner et al. 2008 references therein). This ciliate was a target for investigations in Florida, but this species was not encountered in Florida, and remains unrecorded from North America. Originally discovered in South America, Argentina (Balech 1941) it was later found to be thriving in Africa (Nilsson 1962; Dragesco 1968). Records from Thailand (Esteban et al. 2001) and India (Mahajan and Nair 1971) further expand the range of this ciliate. The ciliate was described from Africa as sometimes obtaining abundances so high as to produce a whitish layer visible (to the naked eye) on the surface of the water (Beadle and Nilsson 1959). The reasons for it not being recorded from Florida are many, most obviously that the state of Florida is vast, and the species' preferred niche was not sampled during a bloom. This lack of data acts as further evidence for the issues surrounding undersampling and detection bias, with so many microniches existing, intensive searches are never exhaustive, and therefore even target species can go unrecorded despite potentially being present. A confounding issue with biogeography is perceived endemism, rather than proof of a given species actually being absent from a given region (Finlay and Fenchel 1999; Finlay et al. 2004; Caron 2009). Further sampling efforts are needed at a global scale in order to understand fully a given ciliate's dispersal and niche.

3.5.3.2 Paramecium jankowski

Paramecium is a well-studied genus and classic 'textbook' example of a ciliate (Fokin 2010). Several tropical species of *Paramecium* (e.g. *Paramecium africanum*) remain unrecorded since their original discovery from Africa (Dragesco 1970). *Paramecium jankowski* (Dragesco 1970; Dragesco-Kernéis 1986) is of particular interest; this species may have been found during this project in Florida (Figure 3.12), but in very low numbers and only on one occasion (Site #10 Chapter 2 Table 2.1), which did not provide enough information to confirm its identity. The ciliate measured ~300µm in length by 130µm in width, which matches the African size range of 250-420µm (Dragesco 1970) and overall basic morphology in the literature (Dragesco and Dragesco-Kernéis 1986). No molecular work and morphometrics were determined since the species was found in small numbers on only one occasion, from a farmland pond in (Table 2.1 site 10).

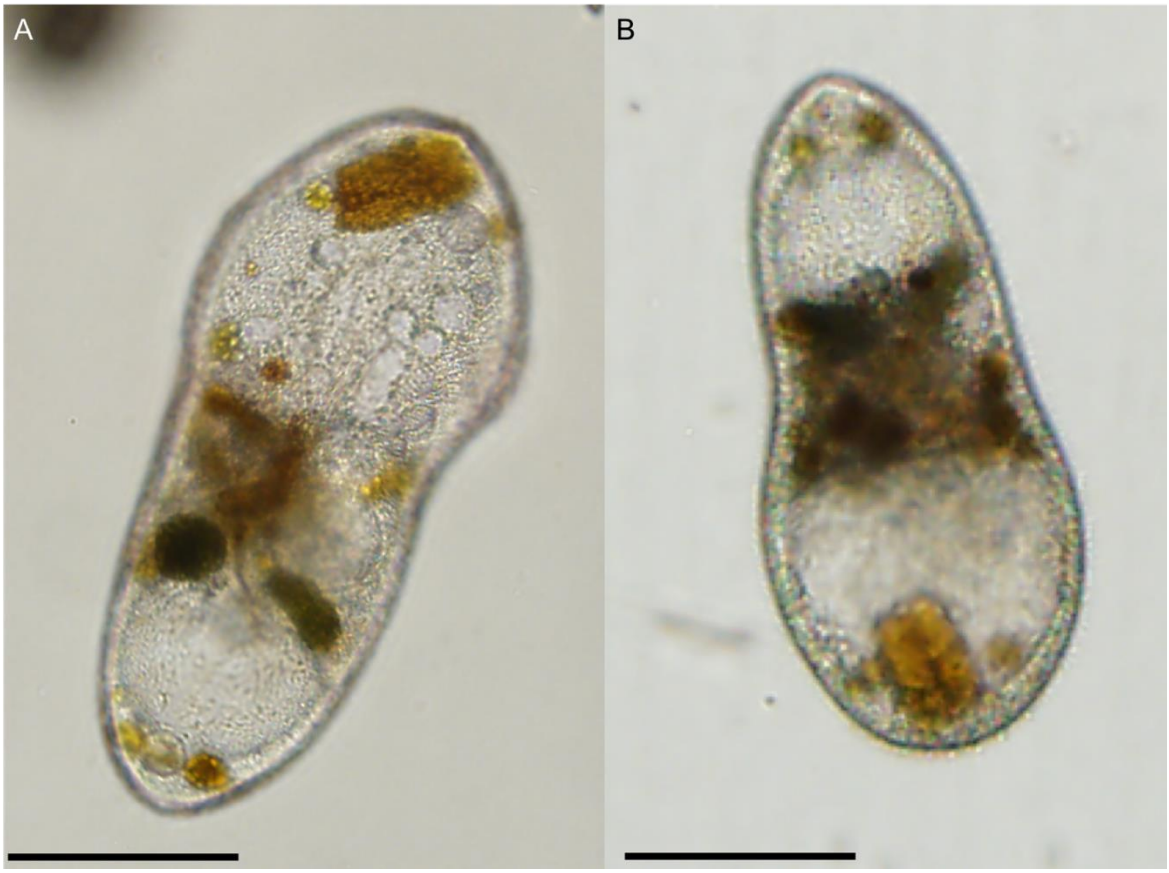

Figure 3.12. An unidentified ciliate discovered in Florida likely in the genus *Paramecium*.
A: the ciliate pictured here resembles in morphology *Paramecium jankowski*, a ciliate unseen since its discovery in Africa (Dragesco 1970). Scale bar 100µm.
B: a reverse view of the unknown ciliate likely a *Paramecium*. Scale bar 100µm.

3.6 Conclusions

Based on the discovery of *Loxodes rex* and other large freshwater flagship ciliate species, the hypothesis that despite large distances between known geographical ranges, some flagship ciliates could thrive and be found in Florida when intensive sampling efforts are undertaken was shown to be valid. Finding these large ciliate species thought to be restricted to one region (e.g. Africa) suggest that Florida is a suitable habitat for a wide range of flagship ciliates to thrive in, provided that the habitat studied contains, at least on some level, their ecological niche requirements (Finlay 2002; Finlay et al. 2004; Finlay and Fenchel 2004). The molecular sequences deposited for *L. rex* will allow future researchers to compare their species and sequences to the data generated from Florida.

Ciliates in the genus *Loxodes* and *Frontonia* were almost always encountered in any samples which contained ciliates. It seems these are 'weedy' genera, which are hardy and able to thrive under a variety of parameters, as described here within Florida samples. The species are also capable of fast growth (Chapter 6). The flagship representatives of these genera were also the most frequently encountered of the flagships in Florida samples.

Intensive sampling conducted during this project has revealed similar environmental parameters in Florida to those described in the African literature; similar ciliate assemblages (Dragesco and Dragesco-Kernéis 1986) were also encountered. Water temperatures were equivalent to those recorded in Africa and consequently species diversity was low, but species richness was, at times, high. In contrast to the literature in which many large ciliates are described as endemic (e.g. Foissner 2006; Foissner et al. 2008 and references therein), this study confirms that distance is not a barrier for dispersal for some species, including the largest (>1mm) ciliates (Esteban et al. 2001; Hines et al. 2016).

Chapter 4. FRESHWATER CILIATE SPECIES NEW TO SCIENCE DISCOVERED IN FLORIDA

4.1 Hypothesis

Species of ciliates which are new to science will be discovered in Florida during intensive sampling and monitoring of 'flagship' ciliates, due to global undersampling of microbial eukaryotes.

4.2 Introduction

Ecological investigations on ciliates in Florida, including biodiversity research with species lists, have been undertaken in the past from marine and brackish environments (such as Noland 1937; Borror 1962; Strüder-Kype and Lynn 2003; Strüder-Kype et al. 2006). The freshwater ecosystems of Florida have been investigated to a lesser extent in lakes of varying chemistry with their planktonic ciliates being the main focus (Beaver and Crisman 1981; Beaver and Crisman 1989). These studies involved the wider community structure of ciliates as an experimental approach to understand microbes forming lake planktonic biomass, including blooms; ciliate taxonomy and ecology was not the focus (Beaver and Crisman 1982) and consequently species lists were not produced. Species new to science, or reports of new biogeographical records, were not reported in previous freshwater Florida studies, and the largest ciliate recorded was *Stentor niger* at ~200µm (Bienert et al. 1991). Although biodiversity was not the subject of the research, ciliates were reported to make up a significant and integral part of lake planktonic biomass, with blooms reported (Beaver and Crisman 1990; Bienert et al. 1991 and references therein). The taxa they did identify were found to be ubiquitous throughout their sampled areas (Beaver and Crisman 1989).

The State of Florida is sufficiently large (see Chapter 1) to provide countless freshwater habitats for the microbial consortia in the form of natural wetlands and swamps, artificial areas (e.g. drainage canals), and ephemeral habitats due to flooding. The warm subtropical climate of Florida is analogous to parts of Africa, and therefore may contain similar microbial communities, particularly with regards to ciliate species. Extensive research conducted in Africa (e.g. Dragesco and Dragesco-Kernéis 1986 and numerous references therein) provides the main (and only detailed) examples for ciliate biodiversity in warm freshwater systems. Ciliate biodiversity in Africa was found to be very low when compared to other areas, such as colder climates found in Europe (e.g. France), which

contained up to twice as many species after the equivalent sampling efforts (Dragesco 1980). It was theorized that the warm water of African sites provided a barrier to some ciliates, which could not survive within the temperature range of 28-35˚C commonly encountered (Dragesco 1973). The overall biodiversity of ciliates in Africa was low (Dragesco 1973), but the biomass of new species (e.g. the large flagships) was very high, such that some of the new species were extremely common (Dragesco and Dragesco-Kernéis 1986). It was reported that many of the ~23% of new species found during African faunistic ciliate surveys were endemic, based on these species being new to science (Dragesco and Dragesco-Kernéis 1986). However, species once thought to be endemic rarely stand the test of time after intensive sampling (Hines et al. 2016 and see Chapter 3). It is likely that there are some species adapted to thrive globally in warm water habitats which still await discovery, with Florida providing an analogous, but as yet unsearched, area to test this hypothesis. Freshwater habitats of Florida investigated in this PhD research were found to fall within the same temperature ranges as those of the African study sites. A hypothesis that Florida would contain species new to science, some large and obvious (e.g. detectable), was formed based on the historical literature.

4.3 Methods

All materials and methods undertaken during research are presented in Chapter 2.

4.4 <u>Sonderia</u> n. sp. a ciliate species new to science

4.4.1 Introduction and background

Species within the genus *Sonderia* are commonly found in marine and brackish water habitats, and are known to have a global biogeography (Dragesco 1968; Borror 1972; Dyer 1989; Al-Rasheid 2001; and Tiffany et al. 2007) to the extent that they are considered ubiquitous within saline environments (~35ppt). These ciliates are important in linking foodwebs to higher tropic levels (Finlay and Esteban 1998a) within the microbial loop. Detailed studies of the genus *Sonderia* exist, (Fauré-Fremiet and Tuffrau 1955; Dragesco 1968; Modeo et al. 2007; Modeo et al. 2013) but despite this, only one 18S rRNA gene sequence, labeled as a *Sonderia vorax*, had been deposited in GenBank (Modeo et al. 2013; Nitla et al. 2018).

Many ciliates are well documented as inhabiting and thriving in anoxic habitats (Fenchel and Riedl 1970; Fenchel and Finlay 1995; Lewis et al. 2018) and oxygen-depleted

anaerobic waters (Finlay and Fenchel 1989). Ciliates are known in some environments to be the top predators in the microbial loop (such as Xu et al. 2014), and are therefore of importance in understanding total ecosystem function. Ciliates in general can also harbor ectosymbiotic bacteria (Esteban et al. 1993; Epstein et al. 1998) this is also true for the genus *Sonderia*, which harbors both ecto- and endosymbiotic prokaryotes (Modeo et al. 2013), as well as archaeal methanogens (Modeo et al. 2013). Sulfide-rich habitats have thriving ciliate populations (Finlay et al. 1991) and these habitats can be encountered globally. Symbiosis allows ciliates to thrive in habitats varying from anoxic to oligotrophic (Dziallas et al. 2012), which are often difficult niches for other organisms to inhabit. Symbiosis further allows these organisms to acquire survival abilities they would otherwise lack, such as photosynthesis (Esteban et al. 2010; Dziallas et al. 2012). Prokaryote symbionts of ciliate hosts are an area of increasing interest (Fenchel and Ramsing 1992; Fokin 2012) but one that is in need of further research. The prokaryotic consortia of ciliates can be the subject of detailed studies after obtaining images by electron microscopy (Esteban et al. 1993), but can also be first observed under basic light microscopy conditions.

Hydrogen sulfide is often considered to be toxic, however, a habitat of sulfureta from microbial mat communities has been shown to harbor *Sonderia* amongst thriving ciliate communities (Dyer 1989; Fenchel 2013). *Sonderia* have been recorded from colorless sulfur bacterial mats on which they also feed (Bernard and Fenchel 1995). Habitats from which ciliates of the genus *Sonderia* have been recorded include the Arctic at a depth of 24m and 33 parts per thousand (ppt) salinity (Azovsky and Mazei 2018) and the anoxic sandy sediments of brackish water habitats in Europe (Fenchel and Jansson 1966) and Africa (Dragesco and Dragesco-Kernéis 1986).

Following extensive sampling and careful examination of local freshwater sites in Florida, a ~300µm-long ciliate was discovered. The ciliate differed from previously observed species in both morphology and habitat. The ciliate was photographed and identified as a potential new species in the genus *Sonderia,* a genus that is common from, and thus far only reported from, the marine environment. Since these samples were not of marine origin, this freshwater discovery was pursued further. The species was initially rare but was later discovered in larger numbers (~50 per mL) at a freshwater site very close to the HBOI campus, allowing for a detailed investigation. The species is novel in that it comes from a freshwater habitat, has a nuclear apparatus ~triple the size of previously described *Sonderia*

species, and the total cell size is the largest described for this genus, being double the length of other species. The sequence of the 18S rRNA gene was also obtained and is distinct from the only other identified *Sonderia* species deposited in Genbank.

4.4.2 Results

Sonderia n. sp. was primarily discovered and studied from a single and unique site, and later from four additional collection sites in Florida, ranging from 0.23 to 0.87 Practical Salinity Units (PSU) (below). This new species was found in novel freshwater habitats, is double the size of previously-described species in the genus and has a distinct 18S rRNA gene sequence. The ciliate was found to fix well using silver carbonate and methyl green (Modeo et al. 2006; Foissner 2014; Cedrola et al. 2015).

Sonderia n. sp.	
Location	Florida
Cell length (µm)	230-375 (mean 305)
Cell width (µm)	130-205 (mean 155)
Macronucleus (µm)	45-75 (mean 60)
Micronucleus (µm)	35-50 (mean 40)
Contractile vacuole	Not present
Ventral kineties	55-60
Dorsal kineties	25-32
Color	Clear
Symbiotic algae	Not present
Molecular sequence	Yes
Genus specific diagnostics:	
Striated band	⅓ cell length
Extrusomes (µm)	~40

Table 4.1. Morphometrics of *Sonderia* n. sp. The measurements were determined from at least 40 individual cells.

4.4.2.1 Cell description

To determine cell size, 40 freshly-collected cells were picked within an hour of sampling from the natural environment and measured *in vivo*: the greatest length for *Sonderia* n. sp. was found to be 375µm (Table 4.1). The cells are often full with a large variety of algal species that have been ingested as food, but that do not distort cell morphology. Size determination of the nuclear apparatus included counting at least 40 individuals of both *in*

vivo and methyl green stained cells, with this technique not altering size. Further diagnostic descriptors such as kinety numbers again followed the technique of counting at least 40 individuals.

Shape: elongated ellipsoid, dorsoventrally flattened. Clearly defined 'lips' at oral aperture. Densely ciliated, ectosymbiotic bacteria present and dense, observable under light microscopy, and clearly observable with DAPI staining (Figure 4.1). Live specimens have no color, but the cytoplasm contains numerous food vacuoles colored by the breakdown of prey.

Movement: flat gliding, much slower in comparison to other ciliates of similar size. Some fixed spiraling counterclockwise and, in deeper water of observation chamber, slow orbital rotation was observed. No phobic response to light or vibrations was observed.

Nuclear apparatus: the macronucleus (Figure 4.1C) is ellipsoid (Table 4.1). No micronucleus was observed.

The clearly ellipsoid shape is different to all the other diagnostic *Sonderia* literature (Modeo et al. 2013) which shows a circular nucleus of half the size or less, of the Florida strain. Extensive examination failed to reveal a micronucleus. The macronucleus was always ovoid, and present below the oral aperture of the cell (Figure 4.1).

Contractile vacuole: Observations made with DIC, multiple fixation techniques, and all other *in vivo* observation techniques revealed no such structure in this *Sonderia*, which was a characteristic observed in species of other ciliates (e.g. *Frontonia*).

Food vacuoles: Desmids, cyanobacteria, diatoms, and various microalgae have all been observed within cells. *Sonderia* sp. contains numerous food vacuoles of varying shape and number, with dense accumulations of ingested items often observed, but not so as to deform morphology (Figure 4.1A).

Extrusomes: Present and often visible after fixation with formalin, or when the cell lyses during observation. The exact type and function of these remains unknown.

Striated band: a diagnostic feature unique in plagiopylids and sonderiids (Lynn 2008) is present. The 'striated band' is a long stripe with short parallel striae running down from the corner of the mouth along the left side of the cell (Figure 4.1B). This band was revealed with silver impregnation and usually ran down approximately ⅓ the length of the cell.

Prokaryotic symbionts: A prokaryotic assemblage was clearly identified on the outer layer of the Florida *Sonderia* cells and also after lysis. The bacterial population was stained with

DAPI and observed using fluorescence microscopy (Figure 4.1D). Scanning electron microscopy showed that the ciliate is densely covered with small rod-shaped bacteria, placed between the ciliary rows (Figure 4.2).

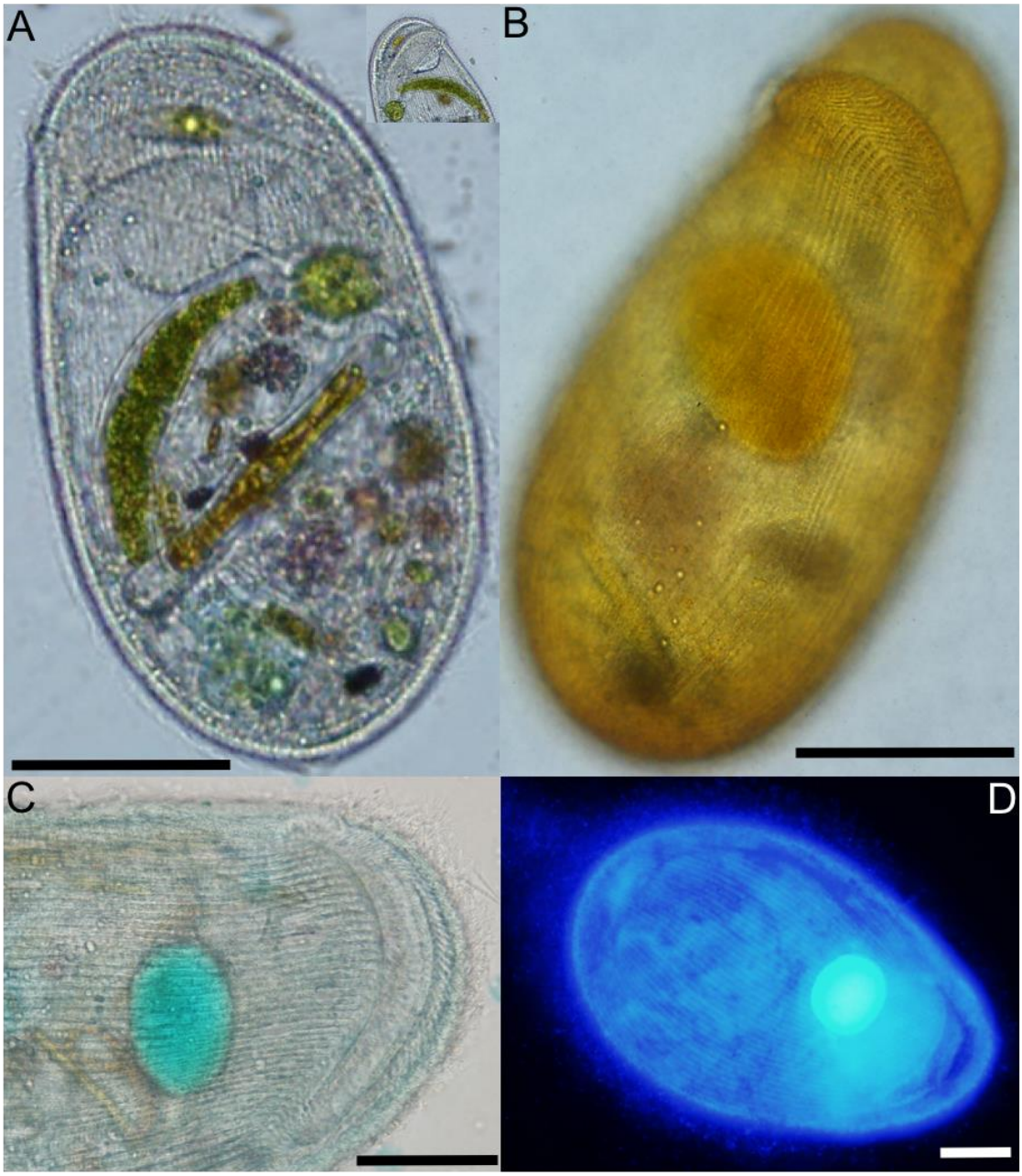
A
B
C
D

Figure 4.1. Images of *Sonderia* n. sp, *in vivo* and after various staining techniques.
A: *Sonderia in vivo*, with a side view (insert) showing the oral aperture (‘lips’ structure). Note the numerous multicolored microalgal food items within the cell including a large green desmid, a large yellow diatom, and various colored digesting microalgae. Scale bar 100µm.
B: *Sonderia* after silver impregnation. Note the ciliary rows running diagonal down the cell, and the darkened oval which is the macronucleus. Scale bar 100µm.
C: *Sonderia* after methyl green stain. Note the blue oval macronucleus, and the detailed structure of the ciliate’s mouth (right). The striated band is faintly visible at the top of the cell. Scale bar 50µm.
D: *Sonderia* after DAPI staining under fluorescence microscopy. Note the brighter white macronucleus and the visible ciliary rows. The cloud around the cell is ectosymbiotic bacteria being released, also visible on the cell surface. Scale bar 50µm.

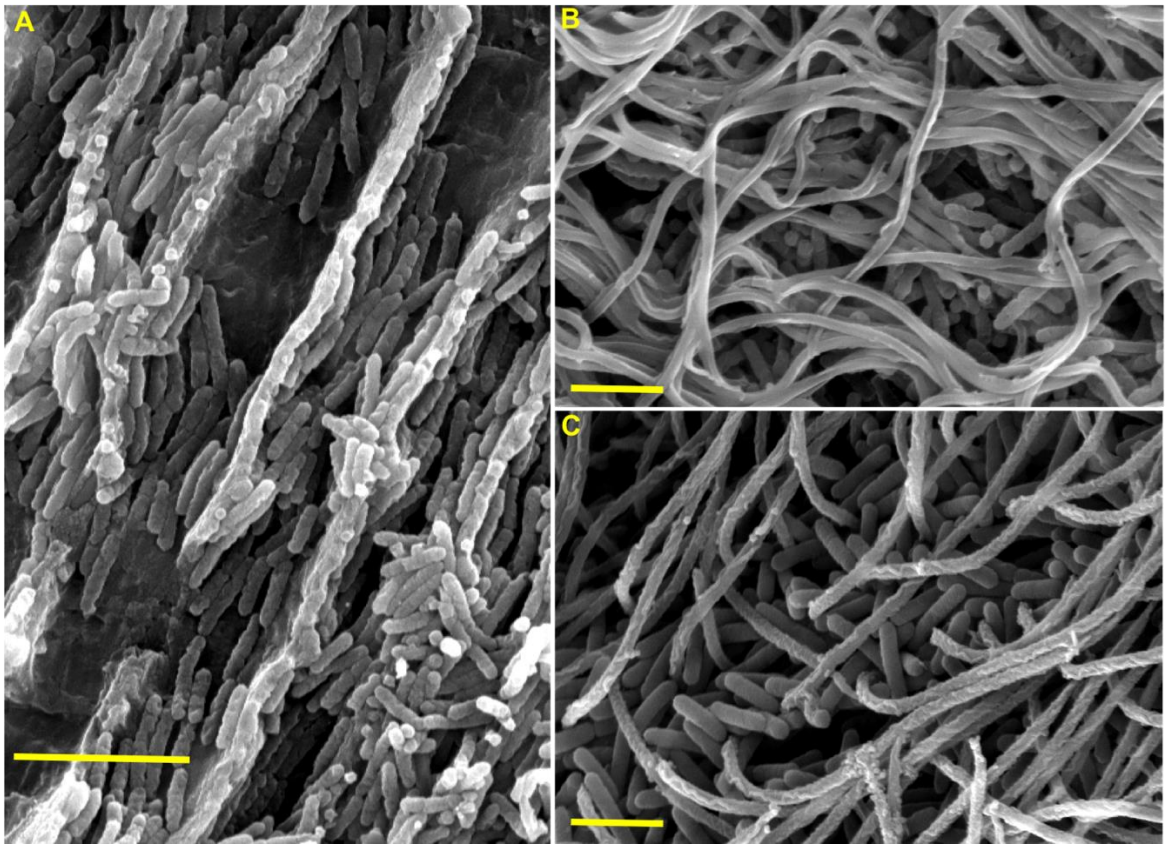

Figure 4.2. Scanning Electron Microscopy of *Sonderia* n. sp.

A: ciliary rows (with cilia broken off during fixation) are distinct running across the image. In between these rows are the numerous rod-shaped bacteria. Scale bar 5 µm.

B: intact cilia are visible above a dense lower layer of symbiotic bacteria. Scale bar 2µm.

C: oval shaped bacteria thrive on the cell membrane of the *Sonderia*. Scale bar 2µm.

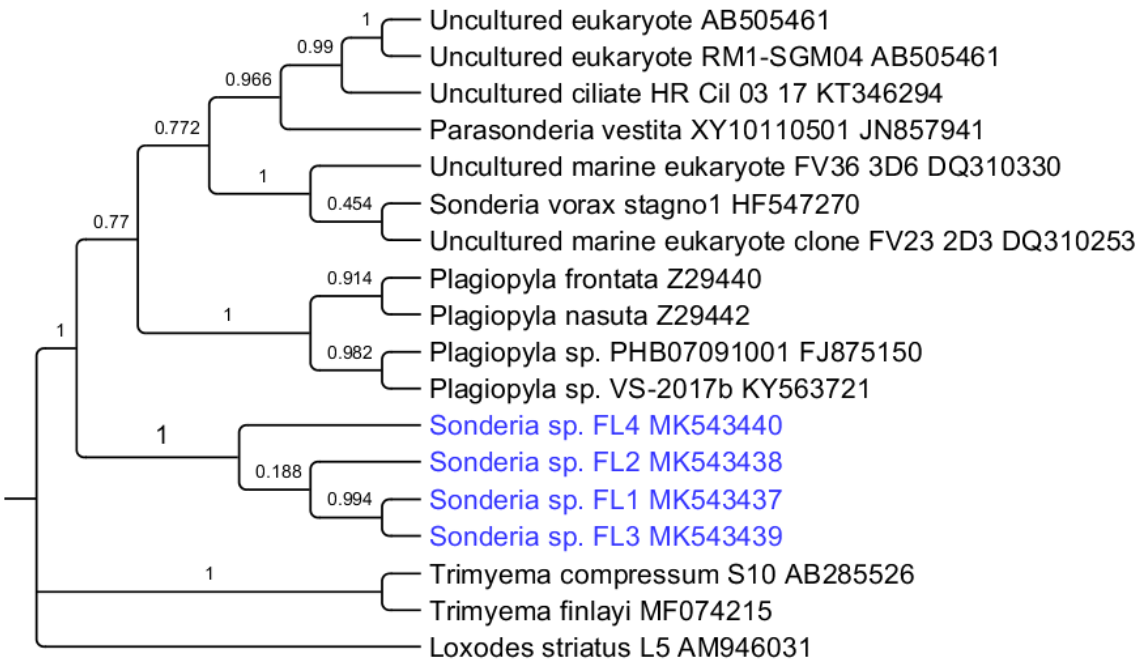

Figure 4.3. Phylogenetic tree of *Sonderia* n. sp.

Four contigs for the new species of *Sonderia* were assembled from sequences obtained for this project and deposited in GenBank (blue on the tree). FL1 MK543437 (1,562 bp), FL2 MK543438 (1,560 bp) FL3 MK543439 (1,560bp), FL4 MK543440 (1,561 bp). The tree was constructed from the closest BLAST matches. The new species forms a distinct branch within the *Sonderia*. The uncultured marine eukaryotes DQ310253.1 and DQ310330.1 are likely a *Sonderia* or closely related as is apparent from their proximity to *S. vorax* sequences. *S. vorax*, the only other sequenced *Sonderia*, is distinct from the Florida sequences. *Loxodes striatus* is the rooting outgroup (bottom). The phylogenetic positioning for the sequence provided by GenBank is: Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Plagiopylea; Plagiopylida; Sonderiidae; *Sonderia*.

4.4.2.2 Molecular phylogeny

Since the species was new to science based on morphology, the 18S rRNA gene from four cryopreserved specimens was sequenced to compare the Florida representative to those deposited in Genbank. The phylogenetic tree shows the relationship of the FL1, 2, 3, and 4 specimens of *Sonderia* to those in the literature; it is related but distinct from other *Sonderia*.

4.4.2.3 Florida <u>Sonderia</u> n.sp. habitat

Type location: 27°32'45.9"N 80°21'50.4"W (Site #4, Table 2.1) Fort Pierce, Florida (USA). A natural freshwater swampy area with dense tree and vegetation cover.

Habitat: Shallow freshwater swamp with anoxic sediment and dense microalgae assemblages both planktonic and on surface of sediments. Shade from surrounding vegetation such as thriving populations of large palms and trees with vines along with invasive plants such as *Schinus terebinthifolia* (Brazilian pepper). Overlaying the water was a thick covering of small aquatic plants (*Lemna*) that were always present when the site contained water. Salinity was always less than 1.0 PSU, even though the location was ~1,200 meters from the brackish Indian River Lagoon, with brackish water bodies in even closer proximity (see maps Chapter 2), but distinct and isolated from these waters, with a railroad track backing the Eastern end of the site and the Western end by a road. The site was periodically flooded with rainwater during storm events, but was constantly wet and mostly stagnant when productive, likely being fed only from storm events. The maximum water depth was 60cm. Cyanobacterial mats (e.g. *Oscillatoria*) were present, together with bacterial mats (e.g. *Beggiatoa*) which were often collected and continued to thrive within the 500 mL sample bottles. The highest densities of *Sonderia* n.sp. were collected in the substrate within the dead organic matter layer from depths of 10 to 30cm. Within the sampled material, *Sonderia* n.sp. was obtained most frequently by pipetting directly from the bottom of the glass bottle within or below a fine sediment layer, most often in the center of the bottle. A strong sulfur smell, at the highest levels encountered during this project, was always present at the site and was released during sampling.

The physical conditions at the collecting site are shown in Table 4.2. The variation in these parameters can be seen in Chapter 3 Fig 3.4.

The type locality was a site of long-term monitoring. The species was recorded from the first sampling event at this habitat. Due to drought conditions at the type location the site became completely dry so sampling ceased during this period. Dry or slightly damp sediments from once submerged areas were periodically collected and rewetted in the laboratory, with no success. It is unknown for how long the site had previously been underwater. The target species was not found after Hurricane Irma provided substantial water

to rewet the site, and over one year later when the site still had water the species was not recorded.

Location	pH	DO (% Sat)	Salinity (PSU)	Temp. (˚C)
Type Location, Graveyard	6.8	2.1	0.46	29.5
Pinelands Preserve, St. Lucie County (Table 2.1 #6)	7.06	4.9	0.36	28
Exit 110 Canal, SW Martin County (Table 2.1 #8)	6.92	8.4	0.23	21.5

Table 4.2. Metadata for sampling locations where *Sonderia* n.sp. was found.

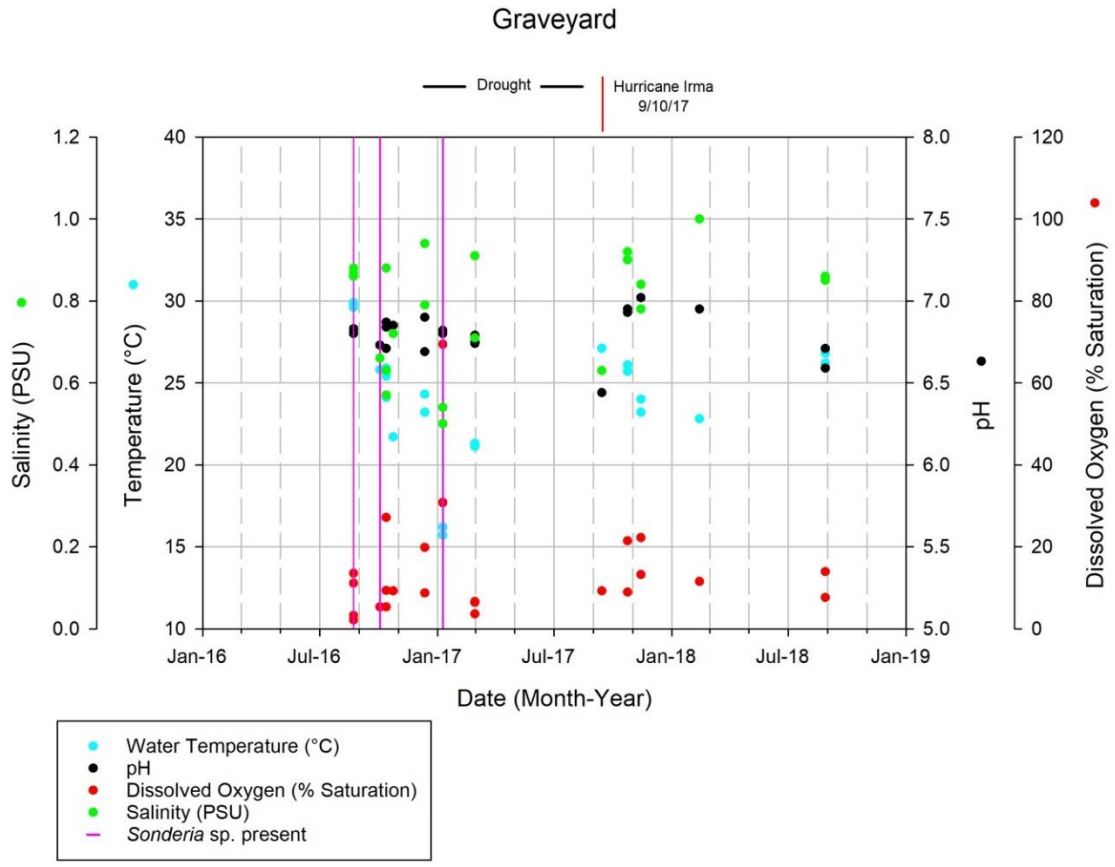

Figure 4.4. Water metadata for type locality "Graveyard" with presence of *Sonderia* n.sp. The pink vertical lines represent the presence of *Sonderia.* One record was obtained during the drought period before the site was totally lost. The influx of water from Hurricane Irma restored the site to conditions similar to those at the start of the project, however, the species was not recorded again.

***Sonderia* additional habitats:**

In addition to the type locality, the new *Sonderia* was encountered in three other freshwater habitats: The Pinelands Preserve in St. Lucie county (Table 2.1 #6); a canal in southwestern Martin county (Table 2.1 # 8); the Lakewood Park canal; and a drainage canal on the Harbor Branch campus. The physical parameters for these two sites (at time of *Sondreia* presence) are shown in Table 4.2.

Lakewood Park Canal: 27°31′56.3″N 80°23′ 54.1″W (Site #1, Table 2.1). The same site as *L. rex* discovery (Chapter 3, Hines et al. 2016). Found sporadically and concurrently with flagship ciliates such as *L. rex* in low densities of always less than 5 per mL on the two occasion of occurrence. Metadata was not recorded from these samplings.

Pinelands Preserve: 27°20'16.8"N 80°37'12.7"W (Site #6, Table 2.1). Located ~39km West (inland) from ocean salinity habitats (e.g. the Indian River Lagoon). Recorded here twice in low densities under 5 per mL. The site is adjacent to a *Citrus* orchard with numerous trees in the family Rutaceae present (e.g. *Citrus reticulata*) and therefore likely received high nutrient runoff as a result of agricultural practices. The canal is also flanked by two fields to the North and West which contain *Bos taurus* (i.e. cattle). Very dark muck and anaerobic sediments, with large *Sonderia* present from 30cm depth below a dense *Lemna* cover (Table 4.2).

Drainage canal: 27°31'51.0"N 80°21'07.7"W. Adjacent to an aquaculture building on the HBOI campus was found to contain the *Sonderia*, this site is close to the type locality being less than 2km away. This canal was treated with herbicides and *Sonderia* was lost after two sampling events.

Exit 110 canal: 27°09'41.6"N 80°30'59.1"W (Site #8, Table 2.1). This is a deep 90cm freshwater canal (Table 4.2) on the road South West towards Lake Okeechobee. The drainage canal is adjacent to a farm with various livestock (often *Bos taurus* in this region). *Sonderia* was found on two occasions and was noted to be smaller (~200µm) and far less frequent than in other localities, but otherwise showed identical morphologies. This site is inland at ~32km from ocean salinity habitats. The species was not found within the nearby long-term monitored farmland area, the site of *Bursaria caudata* discovery (Chapter 3).

The loss of the species at the Type location and the low densities at other sites limited the studies that could be completed. This included electron microscopy; both optimization of

scanning electron microscopy and the description of ultrastructure by transmission electron microscopy.

Figure 4.5. A brackish water Sonderiid species from Florida.

A: *in vivo* partial side view image of the unidentified ciliate from a brackish water habitat. The small arrow indicates the terminal contractile vacuole. Scale bar 10µm.

B: image of the Sonderiid species with ingested yellow and purple algae present. Scale bar 10µm.

4.5 A brackish-water Sonderia

During an experiment to optimize the Uhlig ice extraction method (Uhlig 1964, and see Chapter 2) a brackish water *Sonderia*-like small ciliate (65 to 100µm) was found in interstitial habitats from the Indian River Lagoon on HBOI campus (27°31'58.9"N 80°20'55.7"W). It is possible this brackish Florida species is actually in the *Parasonderia* genus (Xu et al. 2013), but further investigation is required. Observing this brackish species also revealed how easily a contractile vacuole of this type could be observed (Figure 4.5) which confirms its probable absence from the new *Sonderia* species. No sequence was obtained, but this area would benefit from future investigations.

4.6 Novel Colpodea species

A large and distinct ciliate (Figure 4.6) was encountered from within the Indrio Savannahs Preserve wetlands (Site #1, Table 2.1) on only one occasion. Due to morphological characteristics the ciliate is suggested here to belong within the class Colpodea. The Colpodid is probably within or related to the genus *Woodruffides* (Foissner 1993). This ciliate is very likely a new species, and perhaps may even represent a new genus (Dr. Foissner pers. comm.). The large size and intricate oral aperture were of immediate interest (Figure 4.6 A), and surprisingly no such organism of similar size was found within the vast Colpodea literature (such as Foissner 1993; Vďačný and Foissner 2019). Other *Woodruffides* (Lynn 2008, Bourland et al. 2012) are of smaller size, being at, or below, 100µm. At over 10 times this size, the Florida representative may be another large tropical flagship ciliate for this genus and is certainly distinguishable with the naked eye.

Colpodea species	
Location	Florida
Cell length (µm)	865- 1,700 (mean 1,050)
Cell width (µm)	250-385 (mean 350)
Moniliform macronucleus	⅔ cell length
Number of micronuclei	ND
Contractile vacuole	Not present
Kineties	>100
Color	Dark brown
Symbiotic algae	Not present
Molecular sequence	No
Genus specific diagnostics:	
Macronucleus nodules	~16
Macronucleus nodules (µm)	~30

Table 4.3. Morphometrics for the new colpodid species. Measurements were from 15 freshly collected cells.

4.6.1 Cell morphology

The morphology of the species was so much larger than similar species that it was at first difficult to give an exact genus assignment to the organism.

The macronucleus was moniliform, and up to ⅔ of the cell body. Approximately 16 nodes were present. The micronucleus was not observed due to dense cortical pigmentation. No obvious contractile vacuole(s) were observed.

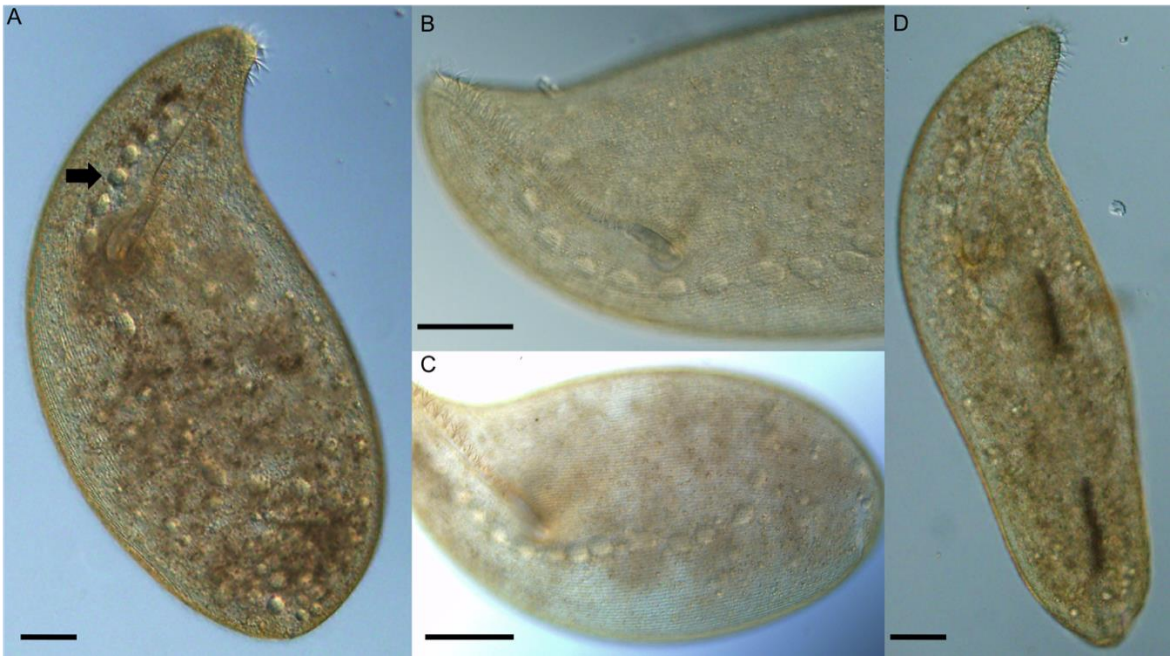

Figure 4.6. A new and giant Colpodea species collected from Indrio Savannahs Preserve.
A: *in vivo* DIC image of the species. Arrow indicates the bead like chain of the macronucleus. This apparatus is parallel to the long oral aperture. Note the natural dark coloration of the cell. Scale bar 100 µm.
B: anterior half of the cell showing the bead like macronucleus and the long track to the oral aperture, forming a structure resembling a beak. Scale bar 100 µm.
C: posterior portion of the cell showing the length of the macronucleus, and the dense ciliary rows. Scale bar 100 µm.
D: swimming form of a freshly picked cell, which is more elongated than in A. Scale bar 100 µm.

With the naked eye this species appears dark yellow to green, and under light microscopy is also quite dark in color but closer to brown and dark yellow. Cortical pigment granules are present, giving the ciliate a darker appearance than *L. rex.*

Several tubes of the cell were cryopreserved at -80˚C in 100% ethanol, however, subsequent PCR was unsuccessful, as it was for many other frozen cells. No sequence was obtained from fresh specimens, due to low cell number and the likely presence of inhibitors.

4.6.2 Type location

Indrio Savanahs Preserve at a site several meters north of the long-term monitoring site (Site #2, Table 2.1) 27°31'40.6"N 80°21'48.7"W. The habitat is within a 297-acre reserve that is largely a nutrient poor, scrubby flatwoods containing a basin and depression swamp which is a conservational restored natural freshwater drainage area (Chapter 3 Figure 3.8). The species was collected from the shore using standard methods (Chapter 2) from waters at ~30cm depth. The species was found here only once but in three simultaneously collected 500 mL sample bottles. It was present in low densities. Water metadata was not recording during this sampling event.

The cell was found swimming vertically in 500 mL bottles three days after collection, in a depleted oxygen zone also inhabited by *L. rex*. This zone is commonly populated with *Loxodes* that migrate into their preferred layer after collection and sediment settling (Fenchel and Finlay 1986; Hines et al. 2016). Movement of the species was very slow, approximately half the speed of *L. rex*. No movement reaction to light or vibrations were detected, but this could be due to the fragile cell being close to lysing as a result of observation manipulation. A hook-like beak in association with the oral aperture was apparent, and movement in a cupped 'U' shape, also as in *L. rex*, was recorded. Large Desmidiales (e.g. ~400µm *Closterium* sp.) and other microalgae were the observed food source. The preferred habitat for this ciliate is unknown, and awaits further discovery.

4.7 Two new species of <u>Prorodon</u>

The size range for ciliates in the genus *Prorodon* is from below 50µm to a maximum recorded length of 400µm (Dragesco 1970). There have been 10 species of *Prododon* described from Africa, which represent the largest sizes previously recorded worldwide (Dragesco 1965; Dragesco 1966d; Dragesco 1970; Dragesco and Dragesco-Kernéis 1986). The largest species, *Prorodon africanus* was described from Cameroon and it also is the largest *Prorodon* described thus far, with a length of 200 to 400µm (Dragesco 1970; Dragesco 1972b; Dragesco and Dragesco-Kernéis 1986). The Florida population of *Prorodon* contains two, large, species with distinct morphologies. It is likely that both represent species which are

new to science. The African literature pre-dates the molecular era, and with no similar work being undertaken since.

Both species were discovered twice from the same location, and co-occurred in large blooms within the same 500mL samples. The species were extremely sensitive to observational manipulation, often swelling and lysing within 90 seconds when picked into a 1mL chamber. Their large size and elliptical morphologies made them problematic for examining in welled slides, as they were often flattened. The cells lysed immediately with the introduction of any fixatives. The tapered body form found in Florida cell types was not reported in other *Prorodon* species from Africa, and is absent from the literature. No molecular sequences had previously been deposited for large *Prorodon*, with the African records (Dragesco 1966d; Dragesco 1970; Dragesco and Dragesco-Kernéis 1986) being the first and only investigations in which large cells of freshwater *Prorodon* had been found.

4.7.1 Prorodon n. sp. 1

The large ciliate (Figure 4.7) was different from those described in the literature due to its tapered body form, and its large size (Table 4.4) which precluded it being one of the often-encountered cosmopolitan *Prorodon*.

4.7.1.1 Cell morphology

The Florida species was densely ciliated and dark in coloration (Figure 4.7). An average of 170 kineties were reported from the large Africa species (Dragesco 1970). Since the Florida cell did not fix well, only an approximate number of kineties could be determined; this was well over 100. The macronucleus is large and singular in both the African and Florida large *Prorodon* species. The ciliate is distinct due to the tapered morphology of the cell, as seen in Figure 4.7.

Prorodon n.sp 1	
Location	Florida
Cell length (µm)	300- 450 (mean 400)
Cell width (µm)	175-250 (mean 220)
Macronucleus (µm)	~ 60
Number of micronuclei	ND
Contractile vacuole	Not present
Kineties	>100
Color	Clear/ light brown
Symbiotic algae	Not present
Molecular sequence	Yes
Genus specific diagnostics:	None

Table 4.4. Morphometrics for *Prorodon* n. sp. 1. Measurements from 30 freshly collected cells.

The ciliate was found to be very light sensitive, having a very fast avoidance movement to light from a flashlight in 500mL bottles and microscope light on a 1mL chamber. Despite the relative fragility of the cell, the tapered morphology was found to be consistent between the observed cells and was not an artifact of slide preparation or lysis. This distinct morphology is therefore likely to be a valid diagnostic feature for this species even though the genus is reported to have variable morphology under observation (Dragesco 1970).

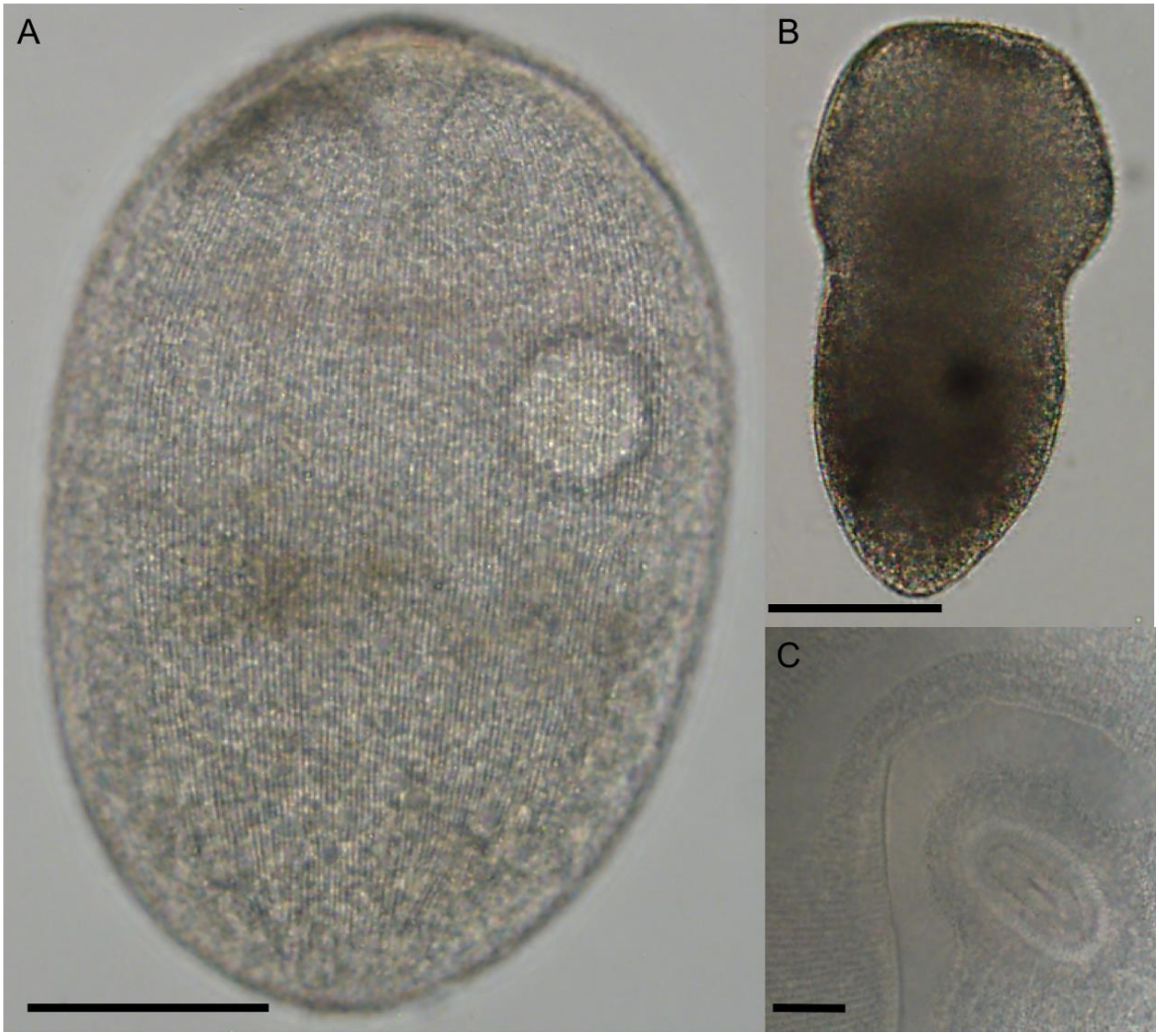

Figure 4.7. A new species in the genus *Prorodon* sp 1.

A: *Prorodon* squashed on a welled slide to reveal the circular macronucleus (clear circle at right) and the numerous ciliary rows. Oral aperture is at top of image. Scale bar 100 µm.

B: the natural tapered body form of a freshly picked moving cell *in vivo*. Scale bar 100 µm.

C: the oral aperture of the ciliate, apical view. Scale bar 10 µm.

4.7.1.2 Molecular phylogeny

The 18S rRNA gene was sequenced for the new species of *Prorodon* sp.1 (Figure 4.8). The closest match in Genbank was to *Prorodon teres* but was sufficiently distinct to indicate that they were not identical species. All *Prorodon* species from Africa remain unsequenced. The

tree created here contains two "uncultured marine eukaryote" sequences and it is likely these are also *Prorodon* despite the absence of morphological data.

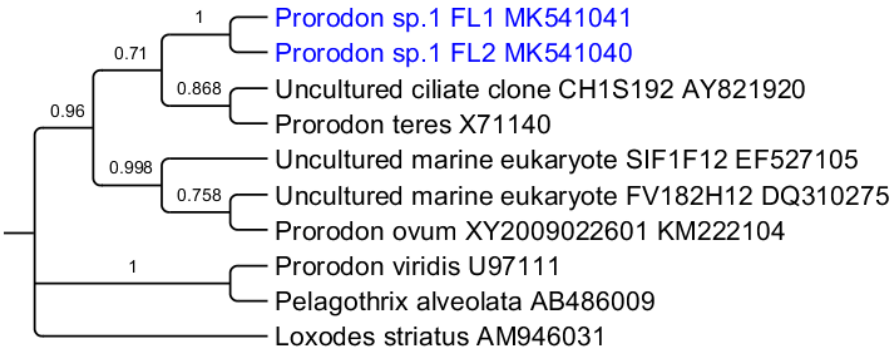

Figure 4.8. Phylogenetic tree for *Prorodon* n. sp. Two samples of the Florida species 1 were sequenced and deposited into GenBank (highlighted in blue). FL1 MK541041 (1,553 bp), and FL2 MK541040 (1,553 bp). The Florida sequences cluster with, but are distinct from, *P. teres* and the other *Prorodon* sequences available in Genbank. It is likely the uncultured marine eukaryotes included in this tree are *Prorodon* or closely related genera. *L. striatus* is the ciliate rooting outgroup for the tree. The phylogenetic positioning provided by GenBank is: Eukaryota; Alveolata; Ciliophora; Intramacronucleata; Prostomatea; Prorodontida; Prorodontidae; *Prorodon*.

4.7.1.3 Type locality

Site #1 (Table 2.1) a bridge over a drainage canal in Lakewood Park. The site was shallow (30cm) and stagnant during time of *Prorodon* detection with no floating aquatic vegetation. The water was in direct sunlight. Large (~2cm) spherical colonial algae were present overlaying and mixing within the dense degraded organic sediment.

The initial discovery of the species pre-dated the optimized collection techniques used later in the project, so no metadata was obtained at that time. The second and only other time the species was observed the following parameters were recorded: pH 6.5, DO 8.6 (% saturation), salinity 0.62 (PSU), temperature 24˚C.

4.7.2 Prorodon n. sp. 2

A second distinct *Prorodon* species which is potentially new to science was sampled along with *Prorodon* n. sp. 1. This cell is larger in both length and width than the previously described *Prorodon* and had a slightly larger single macronucleus (Table 4.5).

Prorodon n. sp. 2	
Location	Florida
Cell length (µm)	480- 590 (mean 550)
Cell width (µm)	210-320 (mean 240)
Macronucleus (µm)	~75
Number of micronuclei	ND
Contractile vacuole	Not present
Kineties	>100
Color	Dark brown
Symbiotic algae	Present
Molecular sequence	No
Genus specific diagnostics:	None

Table 4.5. Morphometrics for *Prorodon* n. sp. 2 from 30 freshly collected cells.

The cells were measured *in vivo*, quickly after picking as shrinking, flattening, and lysing was commonly observed in these cells. Cells were filled with green algae (Figure 4.9), which was suspected to have been mostly ingested rather than symbiotic due to the darker coloration and variations of densities, as seen in another large *Prorodon* (Dragesco 1970). No molecular sequence was obtained for this species, despite adequate starting material and simultaneous attempts with the other *Prorodon*. Inhibitors of an unknown nature may have prevented amplification.

Lower densities were encountered of this *Prorodon* species (4 per mL) compared to species 1 described above, which were both investigated during a bloom, with species 1 being 8 cells per mL. Cell movement consisted of fast equilateral spiraling, with cell capable of fast darting movements in straight lines. *Prorodon* n. sp. 2 was found in a man-made stagnant freshwater canal, on two occasions only (as above *Prorodon* n. sp. 1), despite long-term monitoring of this and several similar sites.

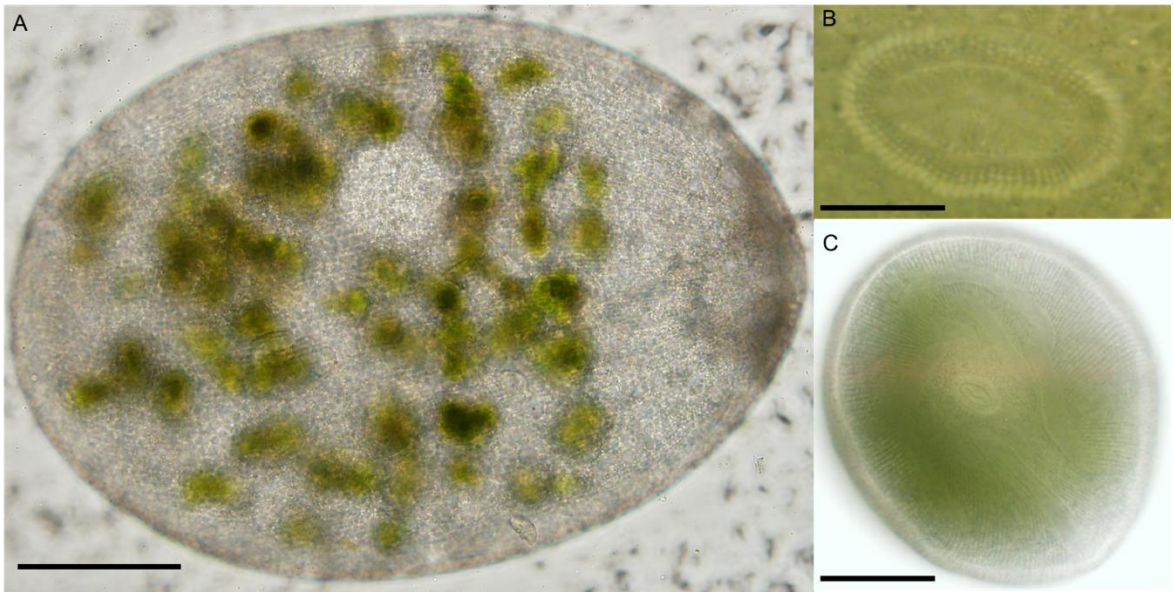

Figure 4.9. *Prorodon* n. sp. 2. A species new to science in the genus *Prorodon*.

A: squashed cell reveals the clear macronucleus at center. Some potentially symbiotic algae alongside ingested algae are present throughout the cytoplasm. Scale bar 100 µm.

B: close up of the oral aperture of the ciliate in anterior view. Scale bar 100 µm.

C: apical view of the ciliate showing oral aperture, and the green tint from the algae within the cytoplasm below. Scale bar 50 µm.

4.8 Pseudoblepharisma tenue var. viride

A ciliate that builds a protective case, or lorica, and contains green symbiotic algae was discovered in three Florida sites. The cell had a long adoral zone of membranelles (AZM) and a single, oval macronucleus. The ciliate was highly contractile, and this ability, along with the symbiotic algae, first pointed to the identification of a ciliate in the genus *Spirostomum*.

The ciliate species *Spirostomum semivirescens* is highly contractile, densely packed with symbiotic green algae, and is known to build a protective coating (Esteban et al. 2009; Hines et al. 2018). The Florida cell though did not have a moniliform macronucleus as described for *S. semivirescens*, and was ⅓ to ¼ the size. Further taxonomic investigation allowed the identification of this ciliate as being either a new species within the little known genus *Pseudoblepharisma*, or the species *Pseudoblepharisma tenue* var. *viride* (Kreutz and

Foissner 2006), a documented but mostly uninvestigated species. This was due to its size and overall shape, along with the presence of dense symbiotic green algae (Figure 4.10). This approximately 300µm Heterotrich had been recorded only from anaerobic sediments in Europe (Germany), but never reported to build a case, and is absent from all reliable detailed species lists published from other geographies. No sequence has been deposited for this genus. The *P. viride* from Europe was sometimes found to be pink after ingesting *Rhodobacteria* (Kreutz and Foissner 2006), which was never observed within the Florida populations, despite some ciliates of other genera exhibiting this color. The original 1926 descriptions for *Pseudoblepharisma* is in Kahl (1932), which is also a description from Germany, of which both match. The previous records do not mention the ciliate's case building behavior, which was commonly observed within Florida samples, with cases observed in each 1mL water-sediment subsamples that also contained the ciliate.

4.8.1 Cell description

The ciliate matched closely to descriptions of the genus *Pseudoblepharisma* based on morphology (Table 4.6).

Pseudoblepharisma tenue var. *viride*	
Location	Florida
Cell length (µm)	310-480 (mean 410)
Cell width (µm)	38-50 (mean 40)
Macronucleus (µm)	~30
Micronucleus (µm)	~6
Contractile vacuole	Present
Kineties	ND
Color	Clear
Symbiotic algae	Present
Molecular sequence	Yes
Genus specific diagnostics:	
Case-building	Present

Table 4.6. Taxonomic investigation in Florida was performed on 30 freshly collected individual cells of *Pseudoblepharisma tenue* var. *viride*.

The ciliate has a nuclear apparatus similar to *Spirostomum teres* (Boscaro et al. 2014). The mouth is long, up to ½ to ⅓ of the ciliate's body length, with an elongated AZM. The terminal vacuole is clear of symbiotic algae and can somewhat deform the cell. No cysts were observed for this species, and are not reported in the literature.

The ciliate's surface was also densely packed with bacteria, perhaps a symbiont, which were always observed during examinations of the species. These were rod-shaped bacteria with a length of ~8µm (Figure 4.10 B). Bacterial cells were observed on the ciliate's surface and were especially visible when cell lysed, perhaps also being present within the cytoplasm. No molecular investigations of the bacteria (e.g. 16S rRNA gene) were undertaken. Bacterial cells were also observed in association with the nuclear apparatus.

The cell was densely packed with symbiotic algae as has been observed in *Spirostomum semivirescens* from Europe (Hines et al. 2018). It is likely that the symbiotic algae species in Florida is *Chlorella* due to morphological similarities. Targeted molecular sequencing of the algal symbiont was not undertaken, and no algal contamination was detected within the ciliate sequences. Electron microscopy was attempted for this cell, due to interest with the dense prokaryotic assemblages that were apparent along with the symbiotic algae. The cells did not fix well for EM, and no usable images were obtained even though ample starting materials was processed.

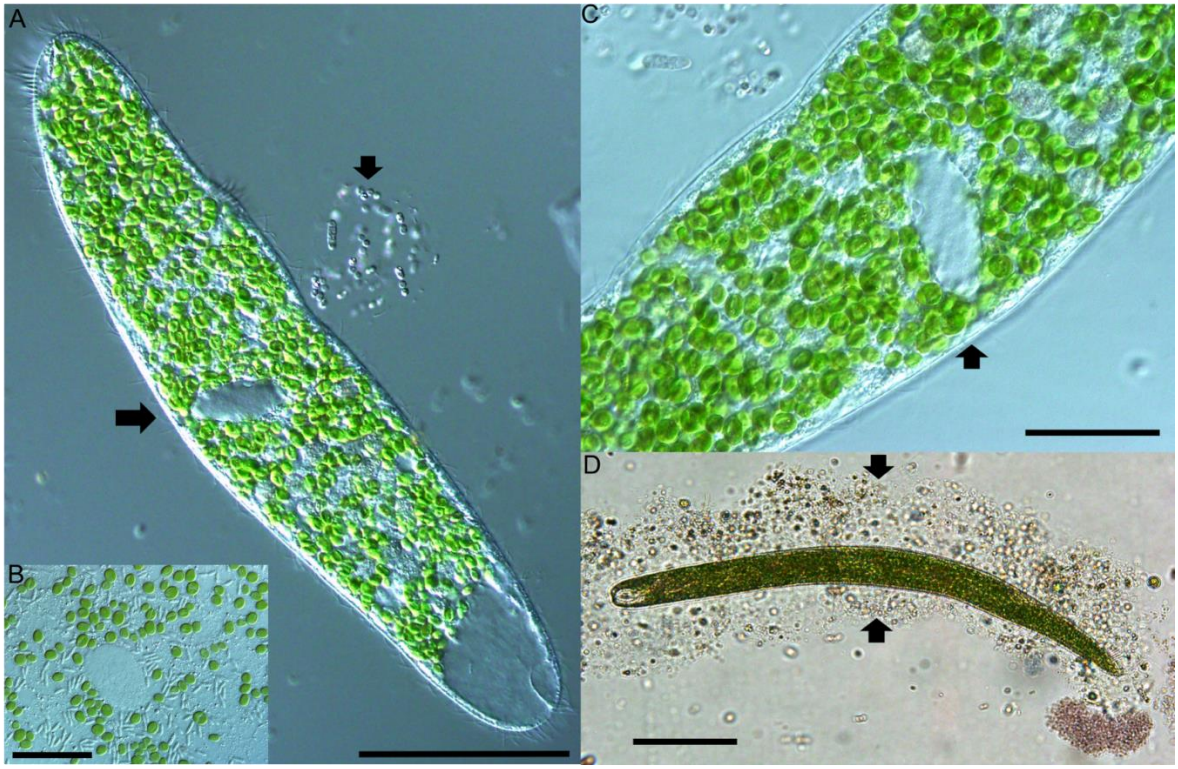

Figure 4.10. *Pseudoblepharisma tenue* var. *viride.*

A: *in vivo* image of the ciliate showing dense assemblage of symbiotic green algae. The arrow at left indicates the oval macronucleus. The small arrow at right shows a cloud of bacteria which has come off the ciliate, potentially symbiotic on the cytoplasm. The large clear contractile vacuole is obvious at the posterior end of the cell (bottom right of image). Scale bar 100 µm.

B: close up of the macronucleus (clear oval) surrounded be symbiotic algae resembling *Chlorella*. Numerous rod shaped bacteria are also clearly observed here. Scale bar 20 µm.

C: close up section of the ciliate with arrow indicating the nuclear apparatus. The circular symbiotic algae are densely packed throughout the cytoplasm. Scale bar 20 µm.

D: *in vivo* image of the ciliate after it was picked and left undisturbed on a slide for ~1 hour. The two arrows indicate either side of a casing the ciliate has made, and rests entirely within. Scale bar 100 µm.

4.8.2 Molecular phylogeny

The genus *Pseudoblepharisma* had never before been sequenced. As predicted, the genus fits with, but is distinct among the species of *Spirostomum*. There is confusion within the tree (collapsed lines) as to the exact placement of this sequence. Species in the genera *Anigstenia* and *Blepharisma* are placed on the tree due to their being in the family Blepharismidae (Lynn 2008); they branch distinctly from *Pseudoblepharisma* on this tree (Figure 4.11) despite being in this same family.

The *Spirostomum* species of *S. ambiguum*, *S minus*, and *S. semivirescens* all have a moniliform macronucleus. *S. teres*, with a singular macronucleus is morphologically most similar, but branches further away. The presence of symbiotic algae and case building are most similar to *S. semivirescens*, which is a close but distinctly separate match on the tree generated from Genbank data.

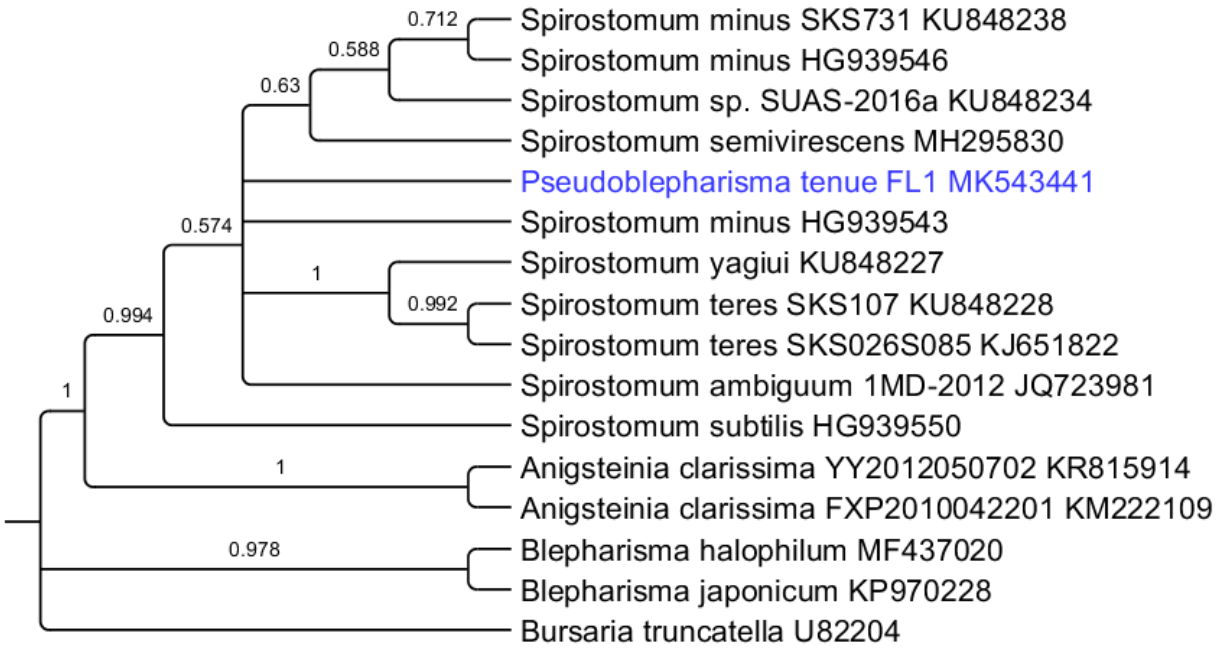

Figure 4.11. Phylogenetic tree for *Pseudoblepharisma tenue* var. *viride*.

A sequence from a single cell was obtained and deposited into GenBank (indicated in blue) FL1 MK543441 (1,597 bp). This is the first sequence deposited for this genus. The Florida

species matches closely to, but distinctly within, *Spirostomum. Bursaria truncatella* is the outgroup for the tree (bottom). The phylogenetic positioning provided for GenBank is: Eukaryota; Alveolata; Ciliophora; Postciliodesmatophora; Heterotrichea; Heterotrichida; Blepharismidae; *Pseudoblepharisma*.

4.8.3 Type locality

Indrio Savannahs Preserve (Site #2, Table 2.1): 27°31'36.6"N 80°21'47.7"W.

Habitat: full sunlight, under the cover of large Nymphaeaceae (lily pads) to 60cm depth. Found within the light ('fluffy') organic sediments common in these habitats. Abundances of 10 to 50 cells per mL were encountered, with higher densities when top layer sediments were subsampled.

This ciliate was always found in rich organic sediment, and when present it is always found in conjunction with other green ciliates (such as *Frontonia*, *Prorodon* spp.). It is likely that this species is mixotrophic in that it filter feeds on bacteria from the water while hidden in its case, and also receives nutrients from its algal symbionts, a relationship well documented within ciliates (Esteban et al. 2010).

The species was discovered in two other Florida locations:

Blue Cypress Conservation Area: 27°39'48.6"N 80°38'38.5"W (Site #7, Table 2.1). This site is a shallow canal area with dense Nymphaeaceae (lily pads) leading to a swamp area. The site is West ~33km inland from the Atlantic Ocean. At the time of sampling the following water paramaters were recorded: pH 7.26, DO 0.24 (% saturation), salinity 0.17 (PSU), temperature 29˚C.

Jonathan Dickinson State Park: 26°5959.3"N 80°06'09.3"W (Site #9, Table 2.1). This site contained the species in similar abundance. At the time of sampling the following water paramaters were recorded: pH 6.79, DO 6.9 (% saturation), salinity 0.5 (PSU), temperature 29˚C.

Both of these locations have loose sediments below Nymphaeaceae (lily pads) and overall appear be very similar to the type locality. The two locations are ~96km apart, but are similar in their microbial consortia, plant and sediment type. Jonathan Dickinson State Park is interesting in that it was the site of ordinance practice during WWII, ecological

succession was then allowed to develop undisturbed, and later the larger site designated a State Park. The pond area sampled here formed in one of the pits created by the detonation of the explosives. The pond and its microbial population has developed likely undisturbed over the last 75 years since the habitat was formed. No flagship ciliates were recorded from this site, despite high ciliate biomass.

The ciliate survives for several days in a closed 500ml bottle at room temperature (~25˚C), shaded from artificial overhead light by paper towels. The ciliate also survived in a 30˚C incubation chamber, however, no population growth was detected. Because of this, along with its common presence in high densities within the study sites, the ciliate was not established in culture.

The ability of this ciliate to contract is readily observed on a 1 mL chamber slide. The lorica is essentially a mucilage-like sediment house which the ciliate uses for protection and feeding. The observed structures were much wider and more open (loose) than those observed in *S. semivirescens* from the UK (Esteban et al. 2009; Hines et al. 2018). The ciliate behaves such that from the lorica it pokes out its anterior end to filter feed, and when disturbed retreats back into the casing which covers the entire cell. The case building was recorded from all three sites. The ciliate is sensitive and quickly reacts to vibrations, by contracting rapidly, either into the lorica or when free-swimming, similar to behavior observed in the European *S. semivirescens*. The Florida species does not obviously react to light, unlike the UK *S. semivirescens* which rapidly contracts with the sudden addition of bright light. The contractions observed in the Florida cell showed it can quickly shrink into a small spiral when threatened or disturbed, as in the genus *Spirostomum*, but it does not do this as readily when protected inside its lorica. The cell is a fast swimmer, but becomes very slow moving when inside or making the lorica. When the lorica are picked and transferred from environmental samples they remain intact; the ciliate remakes a lorica if left undisturbed and this process can be observed microscopically on a 1mL chamber over the course of an hour.

4.9 Discussion

The intensive sampling conducted in Florida's freshwater habitats has revealed the presence of several ciliate species that are probably new to science. These have been examined and compared to related genera and species within the literature. These discoveries highlight

some of the knowledge gaps that still exist in this area of science, in particular microbial ecology and biogeography.

4.9.1 Sonderia n. sp.

This study has identified the first freshwater member of the genus *Sonderia*. The only previous study which mentions a *Sonderia* from an area not clearly defined as saline was from Spain (Sola et al. 1989), but metadata and site parameters were absent from this record. The species described was much smaller and morphologically distinct from the new Florida species, and molecular analysis was not conducted. It is possible that other freshwater representatives of this genus await discovery, as sampling efforts are increased from a wider geographic range, and this genus is targeted. The lack of a previous freshwater *Sonderia* records further highlights how undersampling still remains a key issue in studies of microbial biogeography. This is surprising as the genus is distinct, appears to respond well to all classic protistological staining techniques, and is cosmopolitan.

Since this genus was previously known only from marine and brackish-water habitats, this freshwater representative adds a new ecological niche for this genus. Florida provides unique habitats in which freshwater (den Hartog 1967) to full strength sea water are separated by ~100 meters or less. Laboratory-based experiments which slowly decrease salinity levels over time were found to be successful in revealing cryptic freshwater ciliates (Esteban and Finlay 2003), but culture methods have largely failed for *Sonderia* (Dyer 1989).

Unlike the *Sonderia* type locality, other sites in Florida had dense organic sediments to some extent, all of which showed decay of vegetative material. It is possible that the bacterial communities of the *Sonderia*-habitat were such that little or no breakdown of organic material occurred. The sediment density was very low at this site, with the overall bacterial community apparently distinct from other more common anaerobic habitats sampled in Florida; as breakdown was extremely slow, such that black-stained leaves and seeds would be pulled out whole with little sign of degradation. *In vivo* observations of bacteria in samples were far lower than all other sites investigated. This undoubtedly provides a unique habitat in which the *Sonderia* thrived. From samples with the largest densities of *Sonderia*, no metazoan or other large ciliates were present from this anoxic zone. The *Sonderia* was the top and only predator within this aquatic habitat, and each cell was found to be always densely filled with cyanobacteria, and other algal protists (Figure 4.1A) more so than any other species examined; clearly, *Sonderia* plays a main role in nutrient cycling

for these habitats in which it thrives. Interestingly, several other sites were found to contain a plethora of small anaerobic ciliates, and provided habitats which would be expected to support *Sonderia*, however, this ciliate was not found despite repeated sampling efforts. It is possible that the species has highly specific niche requirements making it susceptible to being overlooked during sampling events. Due to the micro niche partitioning within microbial communities (Finlay and Esteban 1998a), it is possible to have species which prefer high levels of saturated Dissolved Oxygen (DO) to be present within the same 500 mL bottle as those requiring a depleting level, down to anoxic conditions. Aquatic floating plants at the bottle surface provide a steep gradient (as they gave off oxygen), and perhaps act as a natural microcosm of the environment sampled, with sediments at bottom being oxygen deplete due to prokaryotic and eukaryotic respiration and metabolism. The anaerobic *Sonderia* was occasionally discovered within the same 500mL bottles from the *L. rex* discovery site alongside flagship ciliates which prefer a depleted DO level, and that some ciliates which are obligate anaerobes are occasionally encountered in oxygenated waters (Esteban et al. 1995) especially if disturbed and mixed from the microhabitat during sampling.

Prokaryotes are well known to be symbiotic with ciliates, especially in oxygen-depleted habitat zones (Fenchel and Finlay 1991; Lewis et al. 2018). These prokaryotes are amenable to growth in unique habitats, and likely give their host an ecological advantage, and allow for survival in an expanded niche. An estimate of prokaryotic ectosymbiont volume has been made for a marine *Sonderia*, and has been shown to comprise some 10^5 cells, or 20% of the total cell volume (Fenchel 2013). In maintaining a population of this size, the prokaryote assemblage must be critical to the health and function of the host protozoan (Fenchel et al. 1977; Finlay and Fenchel 1989). The ciliate may benefit from these prokaryotes in respect to metabolism, with the prokaryotes improving energy metabolism of the ciliate host. Endosymbiotic archaeal methanogens present in species within the genus *Sonderia* allow for it to thrive within these anoxic zones (Fenchel 2013). Perhaps such archaea are invaluable for survival in areas with limited resources, such as hydrogen (Fenchel 2013), and therefore may also aid in areas with too much of a given substance which could otherwise be toxic to the ciliate host. Such a consortium gives the ciliate an advantage in habitats which other organisms (and potential predators) are unable to persist. The *Sonderia* described here was by far the largest ciliate encountered within its type habitat.

4.9.1.1 Molecular investigation of Sonderia

Many studies and surveys involving only environmental DNA exist which records sequences with no morphological data (Hu et al. 2016), such that only DNA/RNA was extracted and amplified from a given area. A large section of current GenBank results are 'uncultured eukaryote' which often were in the top search results for unusual ciliates sequenced during this project. One such example is with the *Sonderia* sequences obtained from Florida. Sequences in GenBank with close matches to the Florida *Sonderia* sequences come from a study (Takishita et al. 2010) only listing "uncultured eukaryote" (AB505461) with a query cover similar to the only other known *Sonderia* deposited into the data base. This record is interesting in that the sequence was obtained from cold-seep sediments off Japan from a depth of over 1,100 meters (Takishita et al. 2010). The samples were collected from microbial mats at a chemosynthetic cold-seep site. It is likely from matching the new Florida sequences that the uncultured ciliate from this survey was indeed a *Sonderia*. The idea that *Sonderia* thrives in these deep sea habitats is further strengthened by another "uncultured ciliate" (KT346294) in GenBank (Pasulka et al. 2016) which matches to the new Florida *Sonderia* sequences deposited in GenBank for this project. This entry was also interesting being from a Pacific Ocean deep-sea methane-seep ecosystem, sampled from off the West coast of USA (Pasulka et al. 2016). This matching of BLAST results for unknown sequences in GenBank to known species highlights the diversity of habitats in which niches suitable for *Sonderia* can exist. The parallels between a freshwater site existing at 30cm depth in warm Florida verses a cold marine site at over 1,000m depth both containing *Sonderia* with similar 18S rRNA gene are intriguing, but both have anoxic sediments rich in bacteria with hydrogen sulfide likely present. This makes this genus all the more worthwhile for further study, particularly from more readily accessible freshwater habitats.

How far the new freshwater *Sonderia* species thrives within inland areas of Florida remains unknown. However, based on our sequence analysis and the overall morphology, it is clear that the new freshwater Florida *Sonderia* is a close relative of the previously sequenced marine *Sonderia* (Modeo et al. 2013).

4.9.2 Colpodea species

This ciliate species has not been observed since it was originally discovered in Florida. Since this species was recorded from within the same oxygen-depleted zones of the 500mL sample bottle, it is possible this species is microaerophilic, similar to the *Loxodes* with which it was

found. The cells of this species were found to shrink into small ovals and lyse within 5 minutes of being put onto a Sedgewick-Rafter counting chamber, perhaps as a reaction to oxygen. This rapid cell lysis (without introducing chemicals) was observed only in this and the *Prorodon* species, and not in other ciliates throughout the project including the similar sized and shaped *L. rex*. Cells picked onto a full welled slide lasted for longer (~1 hour), but still were much more fragile than *L. rex*. All fixation methods (e.g. stains) failed, as did silver impregnation. This was not surprising, due to the large size and fragile nature of the cell. It is possible that many cells were destroyed during collection and transport back to the laboratory, although care was always taken. There are no similar-sized species within the literature of Colpodea, with the majority being under 500µm, (Foissner et al. 2002; Foissner 2016), with size being the main reason for consideration as a new species, and potential new genus (pers. comm. Prof. Dr. Foissner).

At first the species was believed to be *Rostrophrya regis* due to its overall body shape. This species was described only from Africa, Cameroon (Njiné 1978; Dragesco and Dragesco-Kernéis 1986). That species was only 400µm in maximum length, and has a singular small circular macronucleus. Further investigation pointed instead to the species being related to *Woodruffides metabolicus*, due to similar morphology of oral aperture and macronucleus type, despite the new species being of much larger size (e.g. 10x larger). Many interesting species exist within the Colpodea literature that have not benefited yet from molecular investigations.

The new species was discovered in samples containing *L. rex* and *Pseudoblepharisma*, both had been observed as common from this site. The site continued to be monitored routinely, but never revealed again this target species, despite other flagships often being encountered. Sampling at one-year mark (March 2017) was not possible due to drought conditions in which the site was completely dry. Samples collected throughout the second year mark also did not contain the species, despite the site being continuously sampled when drought conditions ceased. This species was first noticed with the naked eye, and they appeared to be larger cells of *L. rex* and with a darker color, so it is unlikely they were present but overlooked within samples. The huge size, conspicuous morphology (dark color, shape, oral aperture) make it intriguing this species has not yet been described, although this is likely confounded by either its rarity or lack of detection due to undersampling, having been unrecorded again from a site shown to harbor the species.

Lugol's iodine fixative was used primarily during previous freshwater investigations in Florida (such as Beinert et al. 1991), as well as ethanol (Strüder-Kypke and Lynn 2003). Fixation without *in vivo* observations limits the ability to study ciliate diversity, as large ciliates lyse with the introduction of such chemicals (this study), and would therefore be missed in biodiversity surveys. Samples for this study were collected and rapidly first observed without fixation; chemical analysis (i.e. fixation and staining) was undertaken only after a species of interest was selected based on *in vivo* observation. Studies which do not have a detailed component of ciliate observation dedicated to *in vivo* examination of samples (shortly after collection) could result in some fragile ciliate species, which were present, not being detected.

This species represents further evidence for the difficulty of detecting even the largest of ciliates from given samples. The cell nearly reaches 2mm in length, is dark in color, and of conspicuous morphology. It was discovered as thriving (once) in a habitat which was routinely monitored for over one year. Despite all these characteristics, the species was not observed again in Florida. The potential for other giant ciliate (as well as enumerable smaller) species which await detection may be quite high, especially when considering that large regions of the globe remain uninvestigated.

4.9.3 Prorodon species

The site of discovery for the two species of *Prorodon* was the most heavily monitored and sampled habitat throughout this project. Sampling of the site throughout the year and at the one-year point after initial discovery was unproductive; this was probably due to the site having just been treated with herbicides, which likely collapse the microbial foodweb by killing algal prey items. The subsequent drought conditions during this project also negatively influenced the site, and even during periods that were productive for other species these *Prorodon* were not recorded. Sampling at the second year mark was again unproductive for these target *Prorodon*. Despite the site being sampled intensively and producing other target ciliates (e.g flagships) the target *Prorodon* species were recorded only twice.

The species were found in high abundance during two sampling events in the canal site where the original *L. rex* discovery was made and which was the most sampled location during this project. It is surprising that this species was never detected there before, and was recorded only once after first identification at this site, and nowhere else throughout the project. A large bloom in six 500 mL bottles was observed with many thousands of

individuals, such that it was impossible to see through the bottle due to the biomass of *Prorodon* which resulted in its first discovery. Bottles incubated at 30˚C had a rapid increase in species density to 15 cells/ml, before a crash eliminated the population within 48 hours. Samples kept at ~25˚C also survived for approximately two days, but at a lower cell density (3 per mL).

Ciliates are known to obtain large abundances such that they are unmistakable with the naked eye such as water colored red due to high densities of *Blepharisma* (Giese 1973). It is unsurprising then that the *Prorodon* sp.1 bloomed in such a manner to be unmistakable within sample bottles. It is more intriguing that this species was never recorded from any other study sites in Florida; in contrast, other ciliates (e.g. *L. rex*) could often be detected in low densities even during times of unfavorable environmental conditions such as brief cold weather drop to 9˚C (Hines et al. 2016). It is possible then that the preferred niche of *Prorodon* was not adequately sampled, and that they were an accidental introduction via wind or birds and happened to be sampled during a time of a bloom, before an apparent population crash (such that no cells were detected within the site 24 hours later or sample bottles 48 hours later). During long-term sampling in Florida the two species of *Prorodon* were recorded on only two occasions. This rarity allows for the possibility that this species may thrive in other world regions (such as Africa), but they are simply undetected. Due to the potential of forming large population numbers, it is unlikely that this species is endemic to Florida. Future sampling may reveal this species from a wider geographic range.

4.9.4 Pseudoblepharisma tenue var. viride

Documenting *Pseudoblepharisma tenue* var. *viride* from Florida represents another species recorded from one region (Europe, Germany) but found later to thrive in a habitat at a large distance (~7,750 km between sampling areas) from separate continents. The molecular sequence for the Florida species represents the first for the genus, with the original description (Kahl 1932) pre-dating the molecular era and its subsequent recording being based only on morphology. The German records (Kreutz and Foissner 2006) match exactly to the Florida strain, except for the common case building behavior recorded in the Florida representative. In this instance it is intriguing as the German sphagnum ponds of Simmelried are very different from the Florida sites in that they freeze over during the four seasons, whereas the local population thrives in waters around 29˚C without such extreme variation. The Simmelried is a three hectare-sized moorland and sphagnum swamp near the town of

Hegne, off of Lake Constance, in Southern Germany. The area formed after the last ice age some 15,000 years ago, and is interesting in that is has accumulated a large diversity of protists (Kruetz and Foissner 2006) in a relatively short period of time, similar to the timing for formations of freshwater habitats in Florida (Watts 1969; Watts 1975). As the first sequence deposited for this genus, it will allow future researchers to compare their strains to those found in this project.

The issues with GenBank either lacking data and containing misidentified organisms continue to confound species identification, such that had this species not benefited from a detailed morphological investigation, sequencing alone would have placed this cell into the genus *Spirostomum*, rather than fitting it to its new location as a distinct species within a previously unsequenced genus. The sequenced Florida *Pseudoblepharisma* clustered closely but distinctly from *Spirostomum.* This entry into GenBank will help to form the foundations of future investigations within this genus and allow for a comparison to the European strain(s).

Although the ciliates were most often found within their natural habitat of sediments, certain conditions within the 500mL sample bottles would occur that would force the species up into the water column. The reasons for this could be many (Finlay et al. 1987), with reaction to oxygen levels being most likely for this behavior. After some time (usually between 48 and 100 hours) an almost pure green layer of cells would be observed near the top of some sample bottles; this was used for picking for sequencing as the ciliates were more readily sampled and cleaner than when dwelling within the sediments, which could further inhibit PCR reactions.

Some other mixotrophic ciliates containing endosymbiotic algae (such as *Chlorella*), at first counterintuitively, do not congregate at the water surface for direct sunlight, but rather at the oxic-anoxic boundary (Berninger et al. 1986; Finlay et al. 1987; Bienert et al. 1991). Often this zone is at the sediment water interface, with the ciliates accumulating there because diffused nutrients necessary for the cell as well as a low oxygen tension are present, despite being in an opposite direction to sunlight input (Finlay and Fenchel 1986b). This also necessitates the ciliate's case building behavior, which requires a habitat on substrate rather than within a water column. The species has also been listed as an indicator of anaerobic conditions (Kreutz et al. 2012). The ciliate's ability to vertically migrate as observed within 500mL sample bottles is evidence for adaptability to oxygen gradients and light. Whether or

not they can form a cyst like stage is unknown, but likely their protective casing may aid dispersal (e.g. wind, birds) as well as survival during unfavorable conditions such as drought, similar to *Spirostomum semivirescens* (Hines et al. 2018)

4.10 Conclusions

The diversity of ciliate species encountered during sampling in Florida (USA) was in accordance with the literature from Africa (Dragesco and Dragesco-Kernéis 1986), such that overall species number was low in richness but at times high in biomass. The discovery of four potential new species and one rare genus from a new biogeography further highlights the issues of undersampling (Foissner et al. 2006; Caron 2009), and the need to study and protect such habitats for their microbial consortia. Due to climate change and subsequent saltwater intrusion, habitats in Florida may be lost within a human lifetime. As such, species diversity such as research conducted during this PhD should be continued to document organisms before their niches are lost. Preserving given hotspots exclusively for the microbial diversity contained has been undertaken in Europe (such as Hines and Esteban 2017), but does not yet occur in North America. Documenting further microbial diversity is essential to understand the functioning of such unique ecosystems before they are lost.

The Hypothesis was shown to be valid during the sampling for this project, with the discovery of several new species. It is worth noting that these species were obviously unique from basic morphology alone, and at a given time (if only once) were unmistakable within the samples due to large size and high numbers of cells within samples. It is likely that a higher number of less obvious ciliate species (i.e. small or cryptic) exist unnoticed within various local and global habitats, with a wider array of future molecular sequencing perhaps showing an even greater diversity. The new species uncovered from Florida further highlight how much is unexplored within microbial diversity, where species greater than 1mm in size can go undiscovered at a global level.

Chapter 5. FLAGSHIP SOIL CILIATES OF FLORIDA

5.1 Hypothesis

Flagship soil ciliates thrive in Florida and they will be distinct from the freshwater flagship ciliate taxa that have been discovered. Sampling of terrestrial systems will reveal soil ciliate species previously thought to have a geographical distribution restricted to a given continent, due to their ability to encyst, and the similarity of Florida ecosystems to those in their known geographical ranges.

5.2 Introduction

Ciliated protozoa are extremely common in soil environments, despite frequently being in a cryptic state (Esteban et al. 2006). As grazers of small protists and bacteria, ciliates are fundamentally important in healthy soils (Esteban et al. 2006). Ciliates in soil live within the micro water content surrounding soil particles (Finlay et al. 2001). Often these members of the community are undetected due to being encysted. Methods exist to stimulate ciliate excystment in the laboratory and allow for the examination of the communities present (Chapter 2). Rewetting of soil samples reveals a community of ciliates, including cryptic species (Finlay and Fenchel 2001) which emerge as their preferred niche develops. Although a large ciliate population may not at first be readily detected in a given fresh soil sample, when environmental conditions change, a dynamic community may develop, as the ciliates excyst along with the growth of other protist and prokaryotic communities. Ciliates adapted for soil life can form cysts in order to survive adverse conditions, and these cysts may remain viable for many years (Finlay et al. 2001).

Ciliates feeding on bacteria within soils release nitrogen (NH_4^+) which is available as nutrients for plants (Ingham et al. 1985). Ciliates also feed on other protists, regulating the populations and providing additional micronutrients to the community. Ciliates in soil are integral members of the microbial loop (Azam et al. 1983) in both directions of trophic levels, acting not only as consumers but also as food for members of the soil community. Ciliates are also important in the mineralization of nutrients in soil (Griffiths 1986) and are therefore beneficial to plant communities. The rates of carbon and nitrogen cycling in soil are stimulated by the ciliates present as grazers on bacterial communities within soil (Finlay et

al. 2000). It has been suggested that ciliates could be considered a bioindicators of soil health due to their responses to anthropogenic influences (Li et al. 2010).

Ciliates are well documented as inhabiting all states of soil oxygenation, from obligate anaerobes to aerophiles (Lynn 2008). This is in contrast to the pervasive beliefs of amateur gardeners expressed in various blogs and social media outlets (personal observations), that the presence of ciliates in soils is indicative of exclusively anaerobic, and therefore unhealthy, conditions. The community of trophic soil ciliates present in a given area changes over time at small spatial scales and is influenced by factors such as daily fluctuations in water content (Finlay et al. 2000). As such, soil ciliate communities are capable of rapid change, with both total excystment and blooms possible. Ciliates are common within all soils (Bamford 1995; Bates et al. 2013) and are important members of microbial communities in all global regions. Soil ciliates are thought to form cysts more readily in areas that experience dryness (Foissner et al. 2002) rather than rainforest habitats which maintain constant moisture (Foissner 1997). It is possible that more saturated soils act in a similar way to freshwater habitats, such that they should be examined immediately after sampling as their community is more active (i.e. not encysted), and vulnerable to change.

Examples of flagship soil ciliates exist in the literature that are described as endemic to a particular region such as Africa (Foissner et al. 2002) or South America (Foissner 2016). Although a wealth of ciliate diversity is claimed to exist in soils, ciliate biodiversity in general in sparsely recorded (Venter et al. 2018). New species of soil ciliates are still being described from 'well-searched' areas such as Europe (Foissner et al. 2005), which confirms that the extent of soil ciliate biogeography and biodiversity is still undetermined.

The ciliate communities of Florida soils have been largely unexplored. A single report of a sample collected from Everglades National Park revealed a new species, but it is unclear whether this species is limnetic due to the swamp habitat in which it was collected (Foissner 2016).

Due to the vast literature on soil ciliates from global regions (Foissner et al. 2016 and references therein), Florida soil samples were occasionally taken in conjunction with sampling of freshwater habitats during this PhD project. An initial focus was given to sites that reflected the diversity of soil types typical of Florida, including both 'Florida scrubland' (i.e. dry sandy dunes) and denser wooded habitats encountered during collections in Everglades National Park. Ciliates were encountered from these habitats, but no species of

interest as ‘flagships’ were recorded from the few samples investigated. When freshwater sites dried up during the drought conditions that occurred during the project, some sediment from these once aquatic habitats was collected and rewetted. None of the targeted freshwater species were recovered using this technique, however, a different (e.g. soil adapted) community was observed.

As a result of this limited study of Florida soils, one site was found to be very productive: an abandoned natural wooded area on the Harbor Branch Oceanographic Institute campus. This site yielded two ‘flagship’ species: one is the first record of the species outside of Africa, and the other is the first record for North America.

5.3 Methods

All methods used in this study are described in Chapter 2.

5.3.1 Florida soil study site:

The soil surrounding a wild growing *Citrus* tree resembling in appearance and taste of *Citrus aurantium* fruit (known commonly as “bitter orange” or “Seville orange”) was the study site for this project, 27°31'53.1"N 80°21'18.3"W (Site#5, Table 2.1). This tree type was introduced from Spain and now grows wild throughout Florida. This particular tree is on the HBOI campus in the site of an old, unmanaged, wooded area with the fruit falling and rotting back into the ground. Numerous smaller trees were found to be germinating within several meters. The tree is within a densely wooded area (Figure 5.1), and the site has been untouched for at least 50 years. The site is rich with insects of the family Culicidae (Mosquitos) confirming that it is chemically untreated: it is located in an area of HBOI where treatment with insecticides is prohibited due to proximity to an active shrimp aquaculture program. The soil is largely sandy (white ‘sugar sand’) with dense organic material mixed throughout, and some leaf litter present (Table 5.1).

Figure 5.1. The discovery site of the Florida flagship soil ciliates.

A: a close up of the orange tree sampling site top layer of ground soil, which was sampled for this project. Fallen orange fruits are present here, as well as a juvenile tree at left. The soil is covered by dead leaves and is a gray colored sand due to the presence of organic material.

B: the orange tree trunk is at right. This area is densely vegetated and unmanaged. The orange tree was in bloom in this image.

5.3.2 Soil characteristics

Soil characteristics for the study site were determined (Table 5.1). The average water content of soils collected at the sampling site was 18.47%. The remaining solid fraction had an average Total Organic Matter of 8.06%. The average grain size breakdown was: 0.62% gravel, 96.95% sand, and 2.42% fines.

Replicate	Water Content	Total Organic Matter	Total Organic Carbon	Gravel Percentage	Sand Percentage	Fines Percentage
1	20.52	8.42	3.37	0.20	96.7	3.09
2	18.78	9.77	3.90	0.19	97.43	2.37
3	16.10	6.00	2.40	1.46	96.71	1.81
Average	18.47	8.06	3.22	0.62	96.95	2.42
Standard Deviation	2.2	1.90	0.76	0.73	0.41	0.63

Table 5.1. Soil metadata. Three replicates of soil from the study site were analyzed to determine the soil type from which ciliates were cultured.

Soil chemistry was determined in the laboratory (Chapter 2). A 1:5 soil to deionized water suspension was made with fresh sample to determine soil metadata using the YSI. At a temperature of 23°C the pH was 7.60 and the salinity was 0.06 (PSU).

5.4 Condylostomides etoschensis

C. etoschensis was previously described from only Namibia, Africa (Foissner et al. 2002), despite numerous soil investigations from other global habitats (Foissner et al. 2008, Foissner 2016 and references therein) leading to the claim that this species was endemic to that world region. The species is distinct due to its bright gold coloration and large oral aperture which distinguish it from other common soil species. This species encountered within Florida samples matched the description of *C. etoschensis* (Foissner et al. 2002)

5.4.1 Results

A large contractile vacuole in the cell's posterior end was described in the Africa descriptions (Foissner et al. 2002), which deforms the cell when full. This was also observed in Florida cells along with the adoral zone of membranelles (AZM) being long and conspicuous. The oral aperture was wide and occupied nearly 50% of cell length.

The type location, and only site of observation in Africa, was within a "highly saline soil" (although no data were given) from an ephemeral pool in Etosha Pan, Namibia (Foissner et al. 2002). Conjugation was recorded in the African strains in which two cells lock onto each other at the oral aperture and exchange genetic material. Although rarely observed, this was also recorded in Florida samples (Figure 5.2 B). Cells were observed to stay in this state for over 1 hour.

Condylostomides etoschensis		
Location	Africa	Florida
Cell length (µm)	160- 300 (mean 240)	165-310 (mean 225)
Cell width (µm)	70- 150 (mean 110)	70-150 (mean 110)
Moniliform macronucleus	1	1
Number of micronuclei	~21	ND
Contractile vacuole	Present	Present
Kineties	37	~40
Color	Gold	Gold
Symbiotic algae	Not present	Not present
Molecular sequence	No	Yes
Genus specific diagnostics:		
Macronucleus size	⅔ cell length	⅔ cell length
Nodule number	~8	~8
Nodule length (µm)	~25	~25

Table 5.2. Morphometrics for *Condylostomides etoschensis* discovered in Florida compared to the original description recorded in Africa.

Cysts were observed and well documented from the African site. Cysts with a similar appearance were recorded in Florida, however, these were never directly observed to excyst.

Based on morphology alone (Table 5.2) the species was confirmed to be C. *etoschensis* due to its soil habitat, size, and its unusual gold coloration from cortical granules. No molecular sequence was provided from the diagnostic literature (Foissner et al. 2002) and the species had not been recorded since, including from similar sampling campaigns in South America (Foissner 2016 and references therein).

Finding this species in North America is the first record outside of its original African range, at a distance of ~12,000 km from its documented habitat, and suggests that this and other soil ciliate species can overcome barriers to dispersal such as distance.

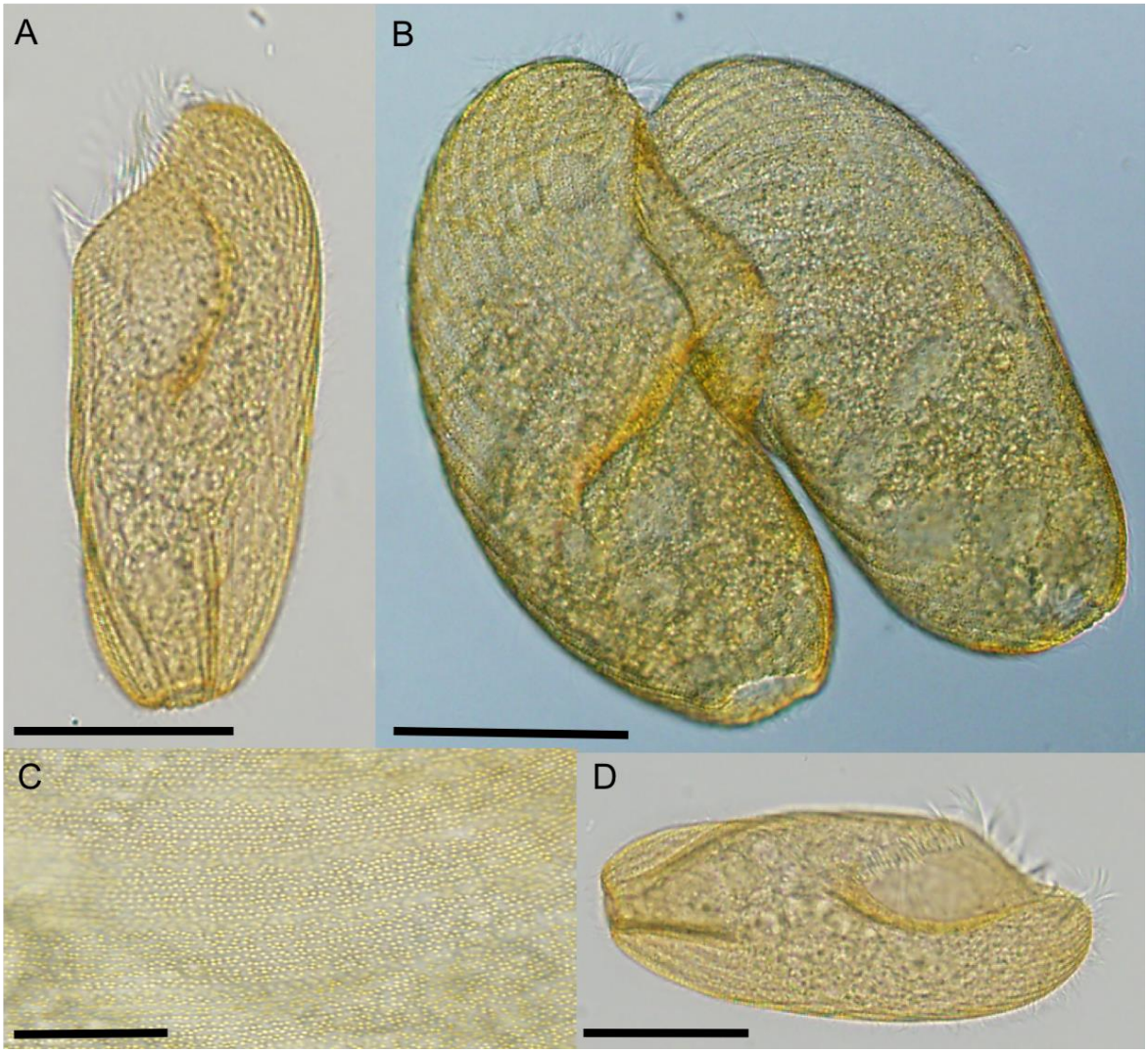

Figure 5.2. Flagship soil ciliate *Condylostomides etoschensis*.

A: *in vivo* image. The ciliate is swimming and the natural gold color is clear in brightfield microscopy. Scale bar 100 µm.

B: the two cells are joined in conjugation at the mouth to exchange genetic material. Scale bar 100 µm.

C: a close up of the cell's cytoplasm showing the ciliary rows and cortical granules which cause the gold coloration. Scale bar 10 µm.

D: the large oral aperture at upper right is conspicuous in this *in vivo* image, as well as the long AZM. Scale bar 100 µm.

5.4.1.1 Molecular phylogeny

Since molecular data for this species does not exist in the literature or in Genbank, the Florida record is the baseline for future work within this genus and for other global biodiversity studies that may encounter this cell. Many Heterotrichs have been sequenced, with several species of *Condylostomides* currently available in Genbank. The Florida cell matches closely to the only sequence available for *C. coeruleus*, a similar but blue species (see below). *Linostomella* sp. clustered closely with *Condylostomides* species as predicted in the diagnostic literature (Lynn 2008). (Figure 5.3).

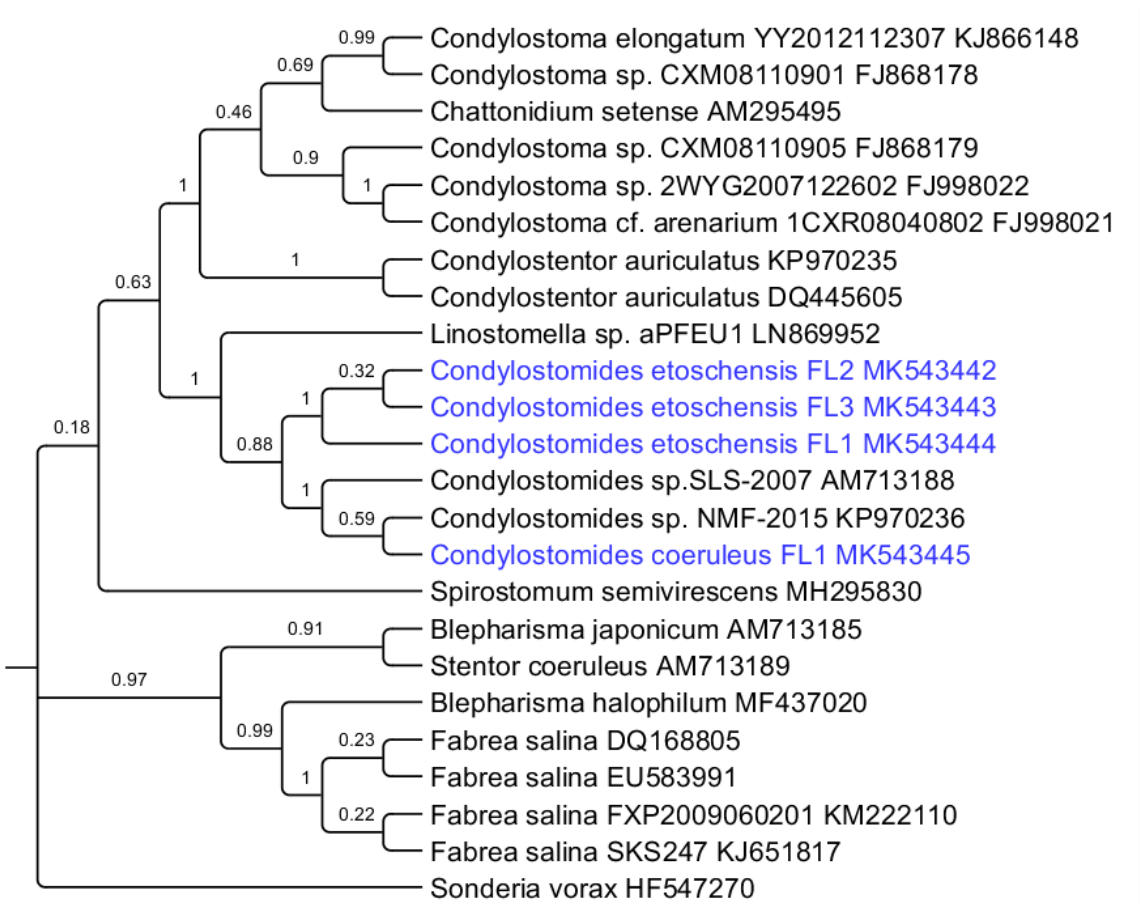

Figure 5.3. Molecular phylogeny of two soil *Condylostomides* ciliate species discovered in Florida. The sequences generated during this project and deposited in GenBank are colored blue in this tree. The ciliate *C. etoschensis* had 3 sequences deposited which are shown on this tree (FL1, FL2, and FL3) with all clustering. GenBank accession numbers: MK543444 (1,505bp), MK543442 (1,513bp), and MK543443 (1,501bp). This is the first sequence

obtained for this species. The ciliate *C. coeruleus* (see text below) (FL1 MK543445) had one sequence entry during this project, and the sequence was only obtained for the forward direction giving an amplicon of 799 bp. The sequence AM713188 on this tree was provided previously from *C. coeruleus* (Foissner 2016). Several heterotrichs are within this tree, as selected from BLAST searches related to the Florida ciliates. The outgroup for the tree (bottom) is *Sonderia vorax*. The phylogenetic data provided by Genbank for theses species: Eukaryota; Alveolata; Ciliophora; Postciliodesmatophora; Heterotrichea; Heterotrichida; Condylostomatidae; *Condylostomides*.

5.5 Condylostomides coeruleus

Investigations of terrestrial ciliates from South America (Foissner 2016) revealed new species, including the conspicuous species *Condylostomides coeruleus*. This species was not recorded from similar soil campaigns in African habitats (Foissner et al. 2002). Due to this apparently restricted biogeography this blue ciliate was recently described as a 'flagship' with a biogeography limited to the previous discovery sites explored in South and Central America (Foissner 2016).

During investigations of the gold soil ciliate *C. etoschensis* in Florida (see above), this morphologically-similar but blue-colored species was found within the same subsamples coming from the same cultures. Based on habitat type, morphology and coloration this species was identified as *C. coeruleus* (Figure 5.4).

5.5.1 Results

5.5.1.2 Morphometrics and habitat

Detailed morphometrics were obtained to compare the Florida species to the diagnostic literature.

Condylostomides coeruleus		
Location	South America	Florida
Cell length (µm)	150-315 (mean 235)	110-220 (mean 160)
Cell width (µm)	85-155 (mean 120)	40-64 (mean 55)
Number of macronuclei	1	1
Number of micronuclei	ND	ND
Contractile vacuole	Present	Present
Kineties	39	~40
Color	Blue	Blue
Symbiotic algae	Not present	Not present
Molecular sequence	Yes	Yes
Genus specific diagnostics:		
Macronucleus size	⅔ cell length	⅔ cell length
Nodule number	~9	~9
Nodule length (µm)	~25	~25

Table 5.3. Morphometrics for *Condylostomides coeruleus* discovered in Florida compared to the original description from South America (Foissner 2016). The Florida cell matches to that described from the literature (Schmidt et al. 2007; Foissner 2016).

Descriptions from South and Central America

Geographic distribution: The species was first discovered in South America, Venezuela (type locality), and later described from 'Central America', Costa Rica and the Dominican Republic (the latter of which is actually within the Caribbean) (Foissner 2016).

Habitat: This was described as "slightly to moderately saline mud and soil from transient grassland puddles". No metadata, such as salinity measurements, were provided in these records.

Florida location:

Orange Tree: 27°31'53.1"N 80°21'18.3"W (Site #5, Table 2.1). This species was always found in the same samples as *C. etoschensis*.

Conspicuously blueish to blueish-green due to cortical granules, with coloration varying depending on the microscopy technique used. The soil characteristics are those provided above, as both flagship species of *Condylostomides* were found together. This is the first record of *C. coeruleus* for North America.

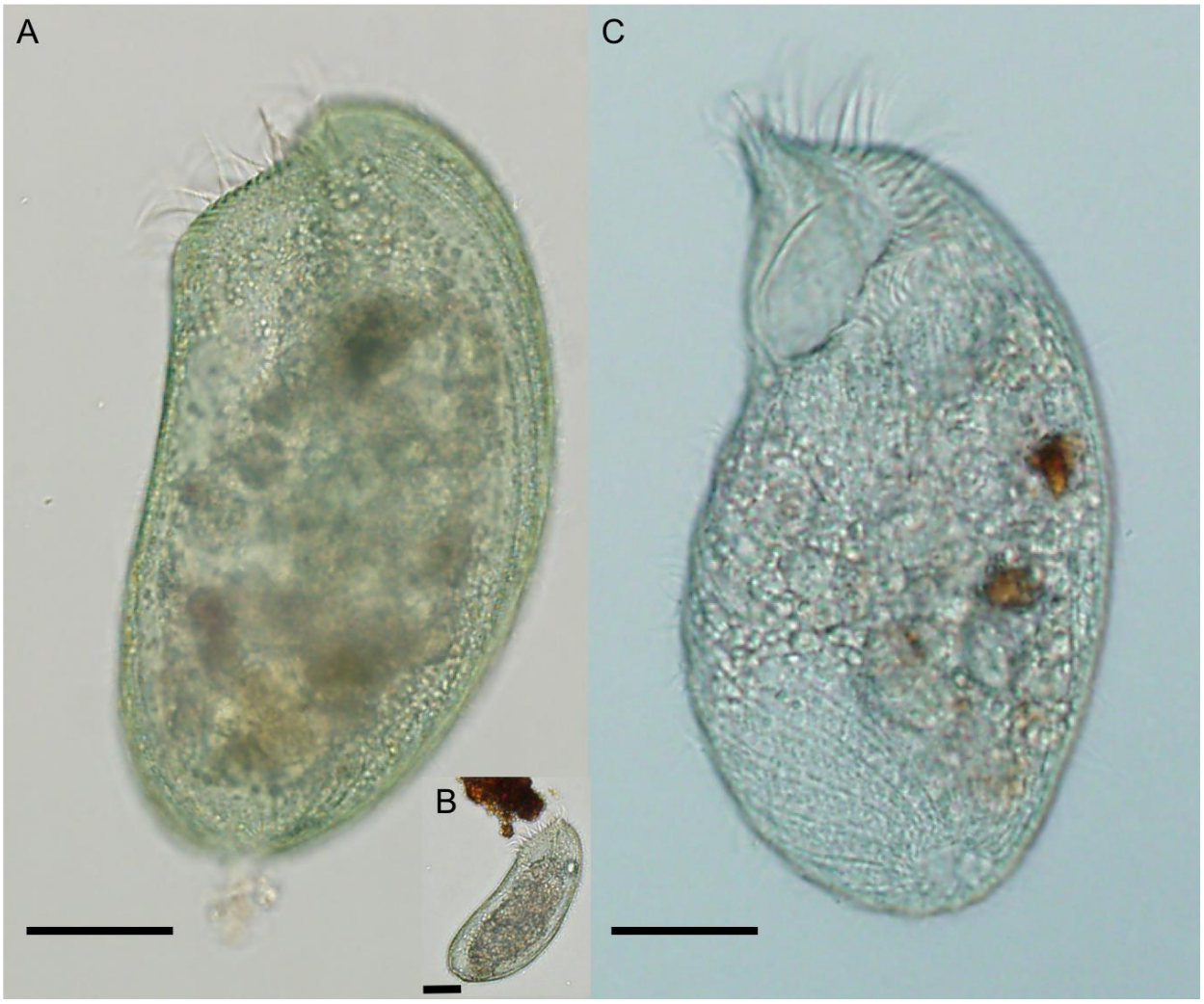

Figure 5.4. *Condylostomides coeruleus in vivo*.

A: brightfield microscopy showing distinct blue green coloration of a swimming cell. Oral aperture at upper left. Scale bar 40 µm.

B: the cell is feeding off bacteria surrounding soil particles. Scale bar 40 µm.

C: view of oral aperture (top) and ciliary rows leading down to terminal vacuole of *C. coeruleus*. Long AZM visible above oral aperture. The blue hue of the cell's coloration is obvious under DIC microscopy. Scale bar 40 µm.

5.5.1.3 Molecular sequence

At a morphological level the two species of gold and blue *Condylostomides* appear closely related, which raises the question of molecular phylogeny. The original and only previously deposited sequences for this species (AM713188; Schmidt et al. 2007) were compared to the Florida samples. PCR of the 18S rRNA gene gave a poor yield of the amplicon, this was only sufficient to sequence in the forward direction giving a 799bp sequence. This Florida sequence matches the previously deposited sequence, with the sequences clustering as expected with the other *Condylostomides* (Figure 5.3).

5.6 Laboratory cultures

Cultures were made from freshly collected soils (Chapter 2). Densities of 5 cells per mL were commonly encountered. These cell cultures were stable for at least six months in natural soil cultures when maintained with water and food at 30°C. Productive cultures yielded densities of 35 cells per mL within the first week.

To test the response of cultures to adverse conditions, triplicate soil cultures were prepared, examined and found to contain the target flagship soil ciliates. These cultures were left to incubate at 30℃ for 3 months. Without water being added, the cultures (in glass Petri dishes, Chapter 2) were completely dry in less than a week. After 3 months the cultures were restarted again and treated as described (Chapter 2) to stimulate excystment. A stable and similar ciliate population developed. This included the population of target flagships at the same densities as previously recorded. A previously productive soil sample ‘forgotten’ in the 30℃ incubator (Curtis Matheson Scientific, TX, USA) was rewetted after being untouched for more than one year, and a similar microbial consortium appeared, including the target C. *etoschensis* despite total desiccation during this time.

The bright pink ciliate *Blepharisma americanum* was also found within Florida soil samples alongside the flagships, *C. etoschensis* and *C coeruleus*. Blooms of *B. americanum* were recorded from several soil cultures, apparently thriving on the bacteria present. The areas surrounding the boiled farro grains resulted in the densest ciliate richness. *B. americanum* was observed to ‘climb’ up the side of the grain and pool at the top, in a pink accumulation visible to the naked eye (Figure 5.5).

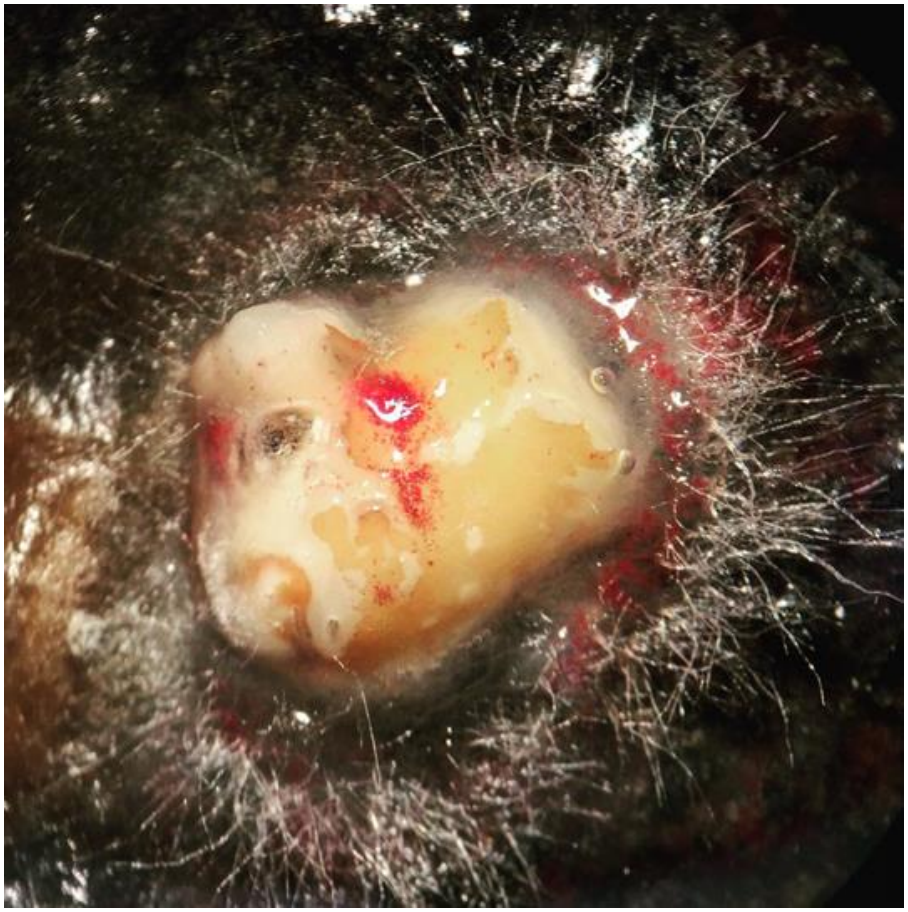

Figure 5.5. Dissecting microscopy image of a farro grain colonized with mold. The pink surrounding layers are the accumulations of the pink-colored ciliate *Blepharisma*, likely *B. americanum*, in densities making the ciliates coloration visible to the naked eye. The ciliates have accumulated at the top of the grain and pool at the center. Grain size is ~1.5cm in length.

5.6.1 Soil bacteria

High levels of bacterial growth can be deleterious to ciliates (Beers 1948) but this was not observed for the target species in the Florida soil cultures, which clearly thrived when large numbers of bacteria (a food source) were present. A purple bacterium was observed to grow on the surface of some cultures in association with the farro grains. It was thought this was a common bacterium *Chromobacterium violaceum*, however, a molecular analysis of the 16S rRNA gene revealed the purple Florida bacterium as a *Massalia* sp., a genus only recently reported as producing the purple pigment violacein (Agematu et al. 2011). The Florida sequence matched 99% at a genetic level to the Japanese strain. The bacterium was sequenced from a sample taken from around a farro grain (soil culture food); the bacterium was not readily amenable to culture.

The purple soil bacterium in the genus *Massilia* has been found to produce violacein (Agematu et al. 2011) as an anti-protozoa mechanism (Matz et al. 2004). Whether or not this is the case in Florida soil cultures is unclear, but certainly there was a stable population of ciliates within samples found to contain the purple *Massilia*. It is worth reporting the expansion of the biogeography of this bacterium from Japan, now to Florida, despite the ubiquity of bacteria being widely accepted in the literature (Finlay 2002; Foissner et al. 2006). Any decrease in ciliate biomass by the presence of purple bacteria in cultures was not noticed.

Molds were a common feature developing in the soil cultures after rewetting and incubation. Farro grains left half way above the sediment layer often resulted in dense fungal growth, which in turn accumulated higher levels of ciliates in the surrounding area, feeding on the bacteria present. The microniches surrounding the grains were the densest in all biomass, and therefore the area best to subsample for examination.

5.7 Discussion

5.7.1 Condylostomides etoschensis

'Flagship' soil ciliates investigated during this project were all isolated from rewetted soil samples and were never found in freshwater samples. Similarly, the freshwater flagships ciliates (Chapter 3) were never recorded in rewetted soils during this project.

The target ciliate cysts for the species described here were apparently always present in soil samples from the discovery site over the course of sampling for over 1 year, as the species were always found after rewetting. Gold colored cysts likely belonging to C. *etoschensis* were found in soil samples, sometimes in numbers of 20 mL^{-1}. Despite numerous attempts these were never directly observed to excyst. The African description of the cysts (Foissner et al. 2002) matches that of the cysts observed in Florida samples. As previously described, encystment is a response to adverse conditions: some cultures found to contain the 'flagships' were left to dry for 3 months, and upon rewetting and feeding a similar stable population developed. A neglected sample was similarly rewetted after one year with the same results, indicating that the Florida cells are equally capable of cyst formation.

Fresh dry soil may have few active ciliates present, but a huge number may be recorded later as the amount of water increases, due to excystment of ciliates. The large

number of cysts present in soils ensures the survival of a stable ciliate population under all environmental conditions, and as such all natural soils contain ciliates.

The soil communities of Florida were found to contain relatively few species when freshly examined, and even after 24 hours only small *Colpodea* were observed. After two days a more diverse community developed following excystment. At a global level, ciliate soil diversity is unresolved due to undersampling (Chao et al. 2006) which confounds ciliate diversity and biogeographies at all levels. It is likely the natural bacterial and small protist community takes time to develop under incubation, and it is only when these levels have increased that ciliate excystment occurs in high enough numbers to be detected (Foissner et al. 2002).

Soil cultures which were over saturated (nearly flooded) and overfed (triple amount of Farro wheat grains) then incubated at 30℃ showed the best results for growth of 'flagship' ciliate targets and overall ciliate biomass (e.g. small Hypotrichts and *Colpodea*). These conditions proved most productive for smaller protists and bacteria to flourish and these serve as the food sources for target ciliates. The literature suggests that the oversaturation of cultures or allowing them to 'spoil' negates ciliate species development (Foissner et al. 2002) which is a rule likely true for most samples. The Florida cultures, however, required larger amounts of water and higher feeding rate to reveal the flagships in greatest density. Standard methods (Foissner 2016) were followed with success, but the two flagship targets were most prevalent when cultures were treated as described (Chapter 2).

5.7.2 Condylostomides coeruleus

This species has been described as an "endemic Gondwana Flagship" (Foissner 2016), this was despite being reported in the same text as Central American areas which were not part of a Gondwana breakup. The new record from Florida, a geologically recently emerged habitat (Watts 1969), disprove the alleged restriction. It is surprising though that the gold *Condylostomides etoschensis* was never recorded in South American investigations, but is likely a result of undersampling of ciliates and known difficulty with detection of species even if present (see Chapter 3).

On average only two *C. coeruleus* cells per mL could be identified from productive samples after thorough searching. This species was always in subsamples found to also contain *C. etoschensis*. Although appearing blue in color under high-power magnification,

when using a dissecting microscope (used for picking of cells and initial observations) they appeared nearly colorless, such that their overall movement type rather than color was used as the indicator for picking cells. No other species of soil *Condylostomides* were observed during these investigations.

The Florida strain of *C. coeruleus* was smaller than that reported in the literature. Florida measurements were made on cells taken from fresh cultures, and this may not have allowed the species to grow to its full size. All other morphological diagnostics match those described in the literature (Foissner 2016).

Molecular comparisons are now possible to further investigate this genus. *Linostomella* sp. was theorized as being the closest relative to *Condylostomides* (Foissner et al. 2002; Lynn 2008). The phylogenetic tree created along with the new sequences generated for *C. etoschensis* during this this project currently supports this.

It is clear from these results that C. *coeruleus and C. etoschensis* can thrive within the same ecological niche. The habitat they require, and the environmental factors that stimulate excystment are evidently present in the Florida soil samples. The two species were always found together in investigations during this project.

No cysts were directly observed that match the Venezuelan description of *C. coeruleus*: bluish and about 100µm in diameter (Foissner 2016). It is possible that even if present in high numbers they were obscured by the soil particles they were attached to and were therefore overlooked during this investigation.

The original description suggests the possibility for this species to be 'common in slightly to moderately saline habitats' (although no data values were given) of South and Central America (Foissner 2016). The species was thought to be a litter or limnetic species based on its blunt shape (Foissner 2016). The Florida soil habitat was found to be mostly sandy with organic material (Table 5.1). This species was never recorded in limnetic samples investigated during this project (e.g. Chapter 3).

5.8 Conclusions

The diversity of ciliates in any habitat is still poorly investigated, with both new species awaiting discovery, and 'flagship' ciliates being recorded from new biogeographies.

The hypothesis that flagship soil ciliates will exist in Florida that are distinct from the freshwater flagship taxa was found to be valid after the discovery of two flagship soil ciliates in Florida, with minimal sampling effort. The first record outside of Africa for

Condylostomides etoschensis is further evidence for the ability of ciliates to disperse globally. The first record for North America of *Condylostomides coeruleus* is additional evidence that species thought to be restricted to South and Central America can overcome this geographic barrier and thrive within Florida, and likely other habitats at a global level. Sequences for flagship ciliates alleged to have restricted biogeography (Foissner et al. 2008) simply do not exist in databases (Schmidt et al. 2007), with only a handful present at the time of writing. Deposition of the three *C. etoschensis* sequences will allow for future research to compare their study sites to the Florida baseline.

The ability of soil ciliates to readily form cysts, as well as exhibit conspicuous coloration makes them good candidates to test for ciliate biogeography. As sampling efforts increase, these and other soil ciliates will probably have their biogeographic distributions expanded.

Soil samples were taken sporadically in addition to intensive sampling of freshwater habitats. As such, these results although novel, are by no means exhaustive, and likely many other flagship soil taxa await discovery in Florida. This investigation of Florida soils suggests Florida is both capable of harboring a diverse ciliate community, and that soil flagships, like freshwater flagships, can spread to global regions wherever they find their preferred ecological niche.

Chapter 6. COMBINING DYNAMIC MODELING WITH INVESTIGATIONS INTO CILIATE BIOGEOGRAPHY AND ECOLOGY

6.1 Hypothesis

Theoretical modeling is useful in understanding eukaryotic microbial dispersal and behavior; outputs can be generated which enhance understanding beyond experimental observations.

6.2 Overview

Theoretical dynamic modeling is a field of study that has never before been applied to ciliate ecology. A core hypothesis of this PhD project, that large, 'flagship' ciliates can disperse to and thrive within Florida, was shown to be valid (Hines et al. 2016 and see Chapter 3). Since it has now been established that ciliates have spread to and within Florida, models do not need to examine 'if' this is possible; however, theoretical modeling of ciliate populations could be useful in understanding some of the ways in which microbes might spread, and how changes to system variables could affect ciliate dispersal. Thus, theoretical dispersal models may enhance our understanding of microbial biogeography in general, and can be applied to this investigation of Florida's freshwater systems.

Field sampling conducted during this PhD project provided the first record for *Loxodes rex* in the Americas (Hines et al. 2016 and see Chapter 3). The species was found frequently and is widely distributed throughout Florida. It was also found to occur in sufficient numbers to allow lab-based studies of its growth rate. *L. rex* was therefore selected as the species upon which theoretical models could be based.

Theoretical modeling was undertaken as a proof of concept in order to understand whether this novel approach could be useful for discussing theories surrounding ciliate dispersal and for the field of microbiology in general. The inclusion of theoretical modeling is a novel approach to understanding complex issues of microbial ecology is an entirely new concept for the field of ciliate biogeography, and one which can be beneficial beyond this project.

Three separate and dynamic models (i.e. they are run and 'play' on a computer) were created for this project. These models are summarized here, and discussed throughout the chapter.

1. Name: The "Neighbors Dispersal Model" (NDM)

Real-world system: A connected system of water bodies transposed onto the State of Florida.

Model Type: Network and Graph Theory.

Purpose: To represent a passive spread of *L. rex* that occurs purely because the water bodies are connected together via the network. This model will have a more uniform spread.

2. Name: "Bird Dispersal Model" (BDM).

Real-world system: A connected system of water bodies transposed onto the State of Florida, superimposed with agent based birds.

Model Type: Network and Graph Theory, Agent based.

Purpose: To represent a passive spread of *L. rex* that occurs in a connected system due to agents (birds) movement on the network. (Even if two ponds are connected on the network, the cell won't be spread unless a bird flies between the two).

3. Name: "Bottle Model".

Real-World System: A 500mL environmental sample.

Model type: Agent based.

Purpose: To simulate behavior of *L.rex* in a closed micocoism system by applying simple rules.

6.3 Introduction

The idea that ciliates can perhaps thrive wherever their preferred ecological niche is realized (Finlay 2002; Fenchel and Finlay 2003) is extremely important to the understanding of global biodiversity and biogeography but, in reality, demonstrating how microbes disperse over large or short geographical distances is difficult to explore for a variety of reasons, such as undersampling, innumerable niches, and detection bias over large biogeographies. The investigation presented in this Chapter set out to examine the phenomenon of local ciliate dispersal, and to provide some theoretical explanations for the ecological observations made on the ciliate species targeted in Florida, USA. The development of models was undertaken to form the basic underpinning by which to extend hypotheses of microbial dispersal beyond that which was observed experimentally.

The mechanisms surrounding long distance dispersals of microbial organisms have long been considered (Darwin 1859); however, detailed practical investigations as to enumeration remain sparse. By constructing models to visualize habitat connectivity within a study system, one can begin to discuss, and potentially better understand, the actual dispersal potential that ciliate species may have. When considering a large study system (e.g. Florida), there are values and data which will always remain unobtainable through fieldwork, particularly when dealing with microbes: theoretical models would help in the examination of a large system (e.g. Florida), and potentially allow the development of meaningful discussions as a platform with which to explore and visualize different scenarios. The models were created with input ready variables such that they can be enhanced should more data become available beyond the scope of this project. It is important to remember the often invoked aphorism ‘all models are wrong, but some are useful' (e.g. Box 1976). With such limitations in mind, the models developed during this PhD project were created to aid in understanding microbial dispersal theories, and to examine how habitat connectivity through a network may affect the spread of the target ciliate. Evocative but simple models are the best suited for this purpose, and suggest future directions within this potentially new interdisciplinary field. Dispersal models are a productive avenue in ecological studies, particularly for understanding range expansion, invasion, and potential colonization of species with metadata factors that are too vast to measure accurately in situ, especially over time. Models for understanding dispersal are found in multiple forms: grid-based dispersal (Berec 2002; Sullivan et al. 2012), dispersal across a network (Jordan et al. 2003; Fortuna et al. 2006; Bodin and Norberg 2006), spatial dynamic models (BenDor and Metcalf 2006), and diffusion-based organism dispersal (Skalski and Gilliam 2003) are some of the most often invoked.

The actual modeling approach used often depends on the species in question and the environment in which it is found, and therefore which variables will be simulated within the model. Dispersal models of land species often use a grid approach, in which the species is capable of walking between any points on a landscape and each grid is assigned suitable or unsuitable for the species to inhabit (Berec 2002). Aquatic species are often modelled using networks where each node is a habitable area and anything in between seen as uninhabitable ground (Lookingbill et al. 2010; Linder et al. 2012).

Attempting to include complex datasets, or expecting to predict results beyond the basic experimental model design would only detract from valuable insights; a complex system can never be completely understood, even with constant direct observation. The models' limitations are thus fully acknowledged, and treated accordingly for extrapolation of meaningful ecological theory and dispersal potentials.

The dispersal potentials for large flagship ciliate species are perhaps far greater than previously thought (Esteban et al. 2001). *Loxodes rex* is a 'flagship' ciliate (Hines et al. 2016 and see Chapters 1 and 3) and by documenting *L. rex* in Florida, USA its biogeography was expanded by over 10,000km from its initial perceived restricted zone of Tropical Africa (Hines et al. 2016). The implications for global biodiversity, disease dispersal, and the actual absence of microbial endemics can be extrapolated by this finding. *L. rex* was chosen as the target species to use within the models for this PhD project, due to the novelty of its discovery in Florida, its interest and 'fame' as a flagship ciliate, its large size and substantial local population numbers, and the potential for novel contributions into dispersal and habitat connectivity. Flagship ciliates are an increasing subject of interest (Hines and Esteban 2017), despite their dispersal being poorly understood.

Unlike models which are created to predict a potential event (e.g. weather such as hurricanes), these models were constructed after the proof of an event (ciliate occurrence in Florida, Chapter 3) of interest was documented. The models can aid in understanding such dispersal mechanisms. For this study, the dispersal of *L. rex* to the Americas (Hines et al. 2016) was the confirmed evidence for the event on which to underpin the models. This dispersal study can help in discussions of network expansion for the species across Florida and, by extrapolation, the discussion can include other microbes at a global scale. The question here is not if this is possible, but in what manner this has occurred, and to what ecological extent insights can be obtained from studying the mechanisms of dispersal, their dispersal rate, and what factors may alter the connectivity through a connected network.

The ability of an organism to populate a large region is controlled by properties of the local habitat alongside regional processes that facilitate dispersal (Havel and Shurin 2004). While studies of local habitat properties investigate controlling factors of population growth rates and niche partitioning such as temperature, salinity, predator population and food availability (Finlay and Berninger 1984; Finlay and Fenchel 1986a), regional studies of dispersal tend to concentrate on the physical mechanisms by which the organisms spread

(Michels et al. 2001; Harvel and Shurin 2004; Green and Figuerola 2005; Wilkinson et al. 2012). When modeling a system, the extent of detail that is required of the model output often heavily determines the extent of detail that goes into model construction. For system dynamic modeling (Abram 2018), and modeling in general, a common practice is to keep the model as simple as possible, expanding sections of the model in stages if it fails to reflect the trends of interest occurring in the study system (Abram and Dyke 2018). With simplicity in mind for an, as yet, unforged association between theoretical system dynamic modeling and microbial ecology, simple models with various adjustable parameters were created (see below). These would help explain and further explore mechanisms of action leading to dispersal of the flagship ciliate *L. rex* through a connected network. Also produced was a model which investigates the observed behavior of *L. rex* in laboratory microcosm in response to environmental stimuli such as light, temperature, oxygen concentration, predator activity, population sizes, and division rate. This type of interactive 'agent based' model had equally never before been applied to the study of ciliate ecology. Several tools were implemented for model production, and are detailed below.

To aid understanding of the dynamic system and to advance discussion of the unknown variables, a cyclic diagram was constructed at the beginning of the model construction phase to visualize the complex nature of these interactions and any potential feedback processes which may be most important in the ability of *L. rex* locally to populate a given habitat (Figure 6.1). This allowed for determining which values might need to be included, and which values could be investigated further. This Figure was used as a reference point throughout the project, particularly concerning the ecological contribution *L. rex* makes towards its local ecosystem. With so many known variables with unknown values, the necessity of a simple model to help explain such interactions was immediately clear, especially when considering a Florida-wide model with countless water bodies, each with different (changing) parameters. As a bird flies, the length of Florida is similar to the length of Great Britain, such that the study site for this project is not small. By understanding the complexity of the habitats and their connectivity, a realistic goal for the models and their contributions was planned. Limiting factors of unknown values (e.g. all metadata across all water bodies in a continent) were considered, and the actual majority of deliverables of the models were formulated prior to their completion. It was clear that a useful model could be constructed with only the input of growth rates, and the known fact of dispersal being

necessary. The models could show theoretical colonization times given certain values, but they could not predict exact time for dispersal within all Florida freshwater habitats.

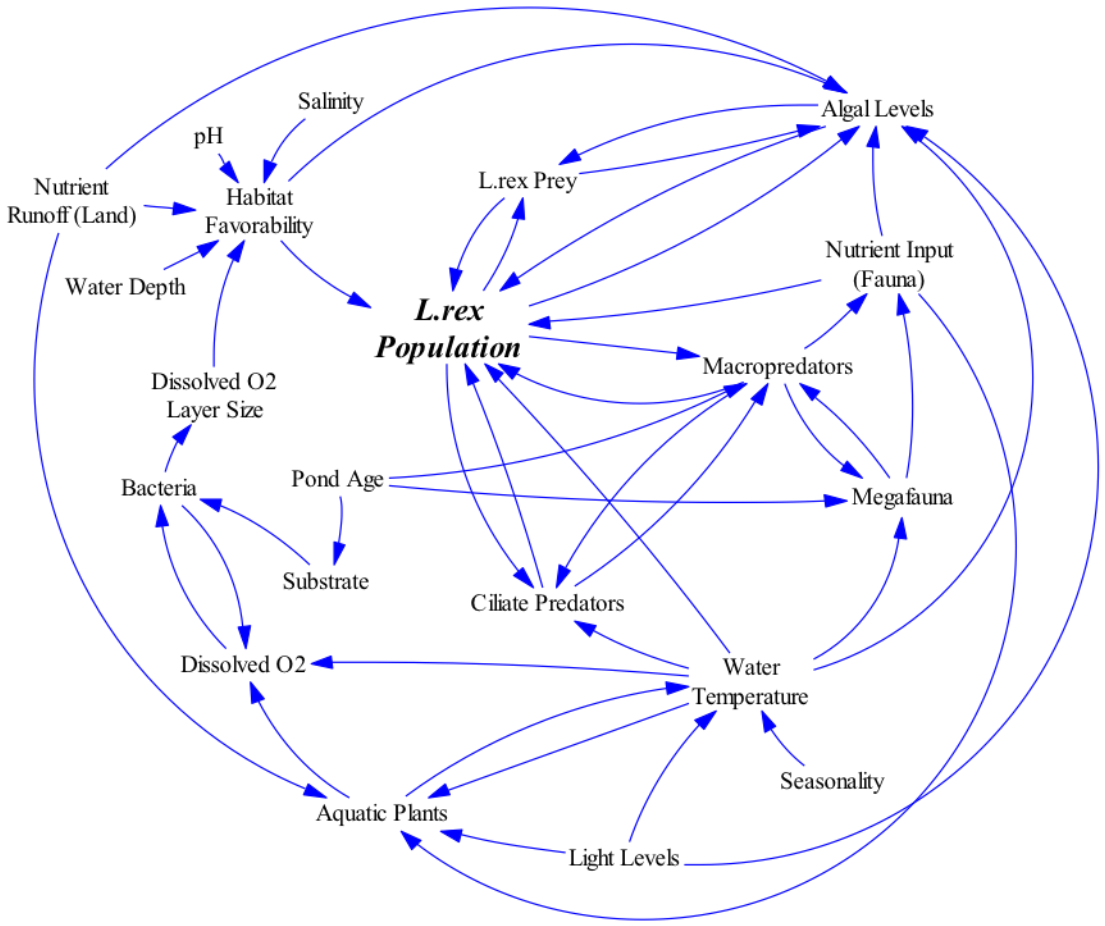

Figure 6.1. Cyclic diagram of some of the major factors affecting the success of a population of *Loxodes rex* in a freshwater Florida habitat.

6.4 Background

When studying the dispersal of *L. rex* across multiple individual habitats, it was important to consider the species niche (Finlay and Fenchel 1986a) in the dynamics of an apparently isolated system (e.g. a freshwater pond) alongside its ability to form a meta community, which are multiple local communities (e.g. ponds) interconnected through habitat connectivity (Leibold et al. 2004; Leibold and Norberg 2004). Community theory focuses on closed interactions of local communities i.e. Lotka-Volterra (Leibold et al. 2004). Selecting

a large and conspicuous ciliate species, with a known habitat only in fresh waters, was a novel approach by which to examine habitat connectivity through dispersal. The ability of the species to colonize should be considered on both a local and regional scale; this is possible to visualize when all isolated water bodies are actually viewed as systems connected within a network through the various dispersal mechanisms here considered. Dispersal limitations have, in the past, been used to understand the differences between local and regional species diversity, finding that species richness of a given taxon across a region was heavily determined by local factors of the habitat, even when found only meters apart (Shurin 2000; Allen 2007; Alfonso et al. 2010; Audet et al. 2013). This suggests then that dispersal limitations could play less of a role than local habitat characteristics, in accordance with the long held (Finlay 2002 and references therein), but often criticized (e.g. Foissner et al. 2008 and references therein), view in microbiology that 'Everything is Everywhere' but that *the environment selects*. These models help to explain how everything (*L. rex*) may get to a preferred habitat everywhere (e.g. to the new range of Florida) through habitat connectivity using known and hypothesized mechanisms of dispersal, which are investigated via the development of theoretical models with programmed and programmable values. The freshwater ponds of Florida are, at first glance, seemingly isolated, certainly when compared to marine habitats that are globally connected through oceans. This is why freshwater systems were a prime choice in which to study species with alleged restricted biogeography. However, when dispersal mechanisms are further examined, an interconnected network of freshwater areas in Florida was revealed, with species apparently able to spread throughout this network (see below and Chapter 3).

Many species' dispersal models hold species population as a limiting factor (Jordan et al. 2003; Sullivan et al. 2012) alongside age and ability to reproduce. Ciliates, however, are theoretically able to fully populate any environment as long as at least one cell survives within an area it finds favorable (Incagone et al. 2015) irrespective of population age. The real-world dispersal and respective time constraint for *L. rex* is unknown, with its preferred ecological niche investigated in Florida during this project (Chapter 3). The novelty of *L. rex* discoveries at a global scale are well documented (Esteban et al. 2001 and see Chapter 1), which highlights the necessity of investigating further the mechanisms of action which allow this species to potentially spread globally.

From a modeling perspective, the presence of *L. rex* in multiple isolated water bodies across a wide region lends itself to a network format, where each water body is a separate node and connections form between nodes via dispersal events. Multiple examples where networks have been successfully implemented in the study of various systems can be found in the literature (such as Jeger et al. 2007 and references therein). Network properties have been explored (Jordan et al. 2003 and references therein) that investigate disease spread across networks and show that the number of nodes and links are one of the most fundamental properties of networks. In reviewing model types for the formation of this chapter (e.g. exploring within Netlogo data bank), it was clear that disease spread was a good analogy for *L. rex* colonization of Florida. In disease models, there is a "pathogen" (here *L. rex*) which "infects" (here spreads to) a "host" (here a pond). Whether or not the host becomes infected (here colonized) is a programmable value in the model, and if it does then this host (pond) will act to further spread the disease (cell dispersal to other ponds). This shows disease spread as a very logical analogy to habitat connectivity across a network, and one that can be followed to show the spread of a target ciliate within interconnected habitats. A similar inspiration came when studying models of wildfire burn patterns throughout a forest. The model (archived in NetLogo data bank) generates a connected network with a given spread rate. Since the network was randomly generated, sometimes the fire would spread rapidly across the modelled connected forest, while in other runs, with a difference in the network connectivity being patchy or lacking, the fire would not fully spread. This inspired an investigation of node connectivity in this projects dispersal network, to investigate the time step variations between spreading of the ciliate given different degrees of connectivity, and using different dispersal proxies (see results below).

6.4.1 Loxodes rex growth rates

An invaluable data point for the creation of these models was to determine growth rates for *L. rex*, a previously unknown value. Growth rates and doubling times for ciliates have been documented, and the growth rates for smaller species of *Loxodes* are known (Finlay and Fenchel 1986a) allowing the extrapolation of population biomass in a given area. Various growth rates were calculated under laboratory microcosm for *L. rex* in consideration of the dispersal model 'time step ticks' (see below) and *L. rex* population growth time (Figures 6.5-6.9). Growth data for *L. rex* was generated in the laboratory at a series of temperatures and at different optimized inoculation levels of both food and ciliate density (Figure 6.4 and see

Methods Chapter 2). Modeling has also been used to calculate population numbers using varying percentages of cell mortality, and the time steps necessary to achieve these values (e.g. Figure 6.8). Since the dynamic total exact in situ site values of a habitat cannot be known, several parameters were examined to see how long it would take to achieve a large number of inferred biomass from a small farmland pond, which was calculated to be approximately 100 billion cells, based on sample observation (see below). The assumption was made that the actual in situ value for any given pond could be less than this level of biomass, but still result in a dispersal event. Due to such astronomically high population numbers of *L.rex* (Figure 6.10) it is possible that these microcosm experiments may actually limit the growth (rather than artificially increase it), and that the environmental parameters that lead to such extreme and rapid growth are common in eutrophic Florida ponds.

Some detailed studies exist which estimate population sizes of *Loxodes* in one well studied habitat (Finlay and Berninger 1984), but these values perhaps vary depending on a multitude of environmental factors and metadata. It is possible then that some ponds act as much higher reservoirs for ciliate populations (e.g. eutrophic ponds), and therefore dispersals, where less productive ponds are stepping stones where the species may exist cryptically (De Meester et al. 2005), 'waiting' to bloom when factors may become favorable. Some species are likely to exist in high enough numbers to be dispersed from a pond but without being detected by researchers during sampling.

Temperature is known to affect ciliate growth rate (Taylor 1978; Dragesco and Dragesco-Kernéis 1986). This value was important to investigate for the target species, allowing a comparison of what is optimal vs what is found naturally in Florida. Growth rates of the tropical (allegedly stenothermically restricted) flagship ciliate *Neobursaridium gigas* from Africa have been studied (Dragesco 1968) revealing large variations in growth rates with changing temperature. Understanding these temperature preferences for *L. rex* in Florida was the starting point for obtaining doubling times for use in the models (see below).

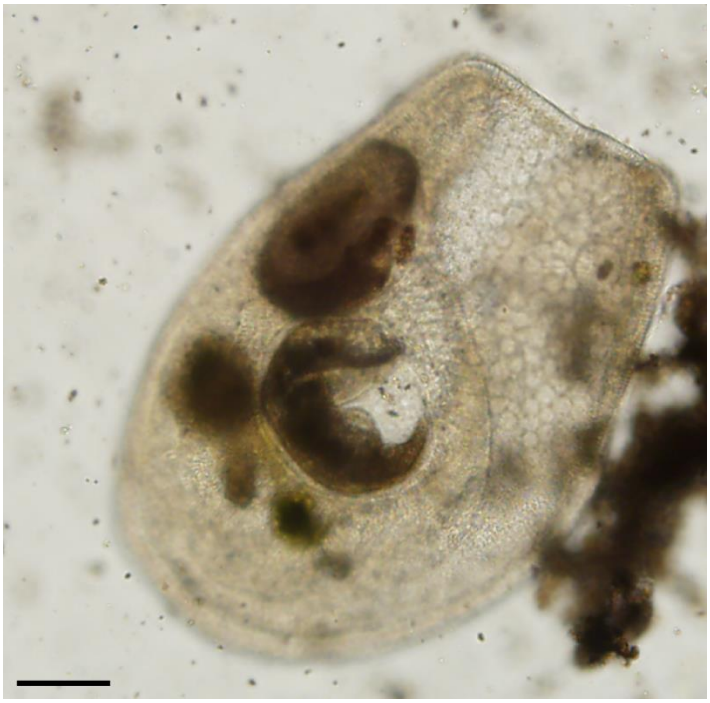

Figure 6.2. *Bursaria truncatella* feeding on *Loxodes rex*. Two cells of *L. rex* are compressed within the ciliate. Scale bar 250µm.

The literature states no predation of *Loxodes* by ciliates, even by large carnivorous *Bursaria truncatella (*Finlay and Berninger 1984). It was surprising then to find in the Florida samples that the largest of the *Loxodes*, *L. rex*, was actively fed upon by the cyst-forming large suspension feeder *B. truncatella* (Figure 6.2). Whether *L. rex* was a targeted prey item or an accidental capture is unknown, as it may be a result of large population densities, especially as the ciliates often inhabit different oxygen layers. This novel ecological finding may highlight another of the many potential unknown environmental and ecological factors which may make any models less precise (i.e. an unknown interaction), but do not affect the accuracy here due to death rate calculated as a proxy for all potentials within the given set values within the model run, even those yet to be documented.

6.4.2 Dispersal Mechanisms

The physical properties of an organism affect its dispersal (Criales et al. 2015) and the dispersal potential changes whether active or passive dispersal is utilized. *L. rex* is incapable of moving itself between macrohabitats without external forces, and therefore its dispersal is controlled by passive mechanism. This is in contrast to a flying insect, for example, which can spread by active (e.g. flying) and passive (e.g. wind blowing) actions. The exact and

complete mechanisms by which *L. rex* spreads are unknown. Our ability to explore multiple scenarios by which *L. rex* undergoes passive dispersal is enhanced by computational models, allowing system parameters to be changed readily and multiple simulations to be run and contrasted side by side. Many factors are known in the spreading of small organisms (such as Finlay 2002; Faurby and Funch 2011; Incagnone et al. 2015). One of the models presented here use the visual output of moving "birds" which can also be a proxy for all dispersal events. In reality, the majority of dispersal events are probably due to birds or wind, but an insect or even a less obvious event such as a large alligator or turtle walking between aquatic locations could lead to a habitat connection, and therefore a spread as seen in the models. Indeed, many large birds are often observed to be feeding and moving during sampling of Florida sites known to contain *L. rex*. When some birds fly away, obvious aquatic plants (e.g. *Lemna)* was observed on their beaks and feet. Along with the organic sediment stuck on these birds, a flight into a neighboring aquatic habitat could actively cause a spread, especially given time and the numerous number of these daily interactions across the state. Passive transport mechanisms for other taxa have been shown to include many animals and attached aquatic macrophytes, waterfowl and aquatic insects (De Meester et al. 2002; Cottenie et al. 2003), dispersal especially through bird flight (Green and Figuerola 2005; Frisch et al. 2007) as well as wind and rain (Cáceres and Soluk 2002). Dispersal is also known to occur via anthropogenic activity including, mainly in marine environments, boats and ballast (Havel and Shurin 2004; Wikinson et al. 2012). Major storm events such as hurricanes clearly also effect the dispersal of species in Florida (see below and Chapter 3).

Although the size of small organisms has been considered to be effected by passive wind transport in some studies (e.g. Wilkinson et al. 2012) others have studied larger organisms (e.g. 2mm marine invertebrates) and found little hindrance to dispersal at this value (Green and Figuerola 2005). This makes the dispersal of *L. rex* all the more intriguing due to its large (550-1,350 µm) size, and having been found on multiple continents (Dragesco 1970; Esteban et al. 2001; Hines et al. 2016) despite its apparent fragility and obligatory freshwater habitat. This brings into question what the actual size barrier of endemism may be for microbes: *L. rex* at a size in excess of 1mm has clearly spread despite its fragility and no resting cyst being observed for any species of the *Loxodes* genus. The extrapolation to disease dispersal (i.e. much smaller organisms like bacteria) is apparent, with connected networks potentially allowing spread of many organisms across phyla.

6.5 Methodology

The models created for this project are intended to act as a platform with which to discuss potential dispersal mechanisms across the State of Florida. The intention of the models is not to confirm methods of transport or predict exact time values for dispersal of the species *Loxodes rex*. Simulations focus on network properties, including network connectivity and initial seeding of the species, and the properties of the ciliate and its niche requirements which may have either accelerated or hindered its dispersal success. The models act as a way to theorize how the species has come to colonize Florida and how, when considering the large observed populations and the demonstrated rapid laboratory doubling times, the progress of a successful colonization can be studied.

The tools implemented for model construction used the free software NetLogo (version 5.3) to formulate and code the usable models (Wilensky 1999). NetLogo is an interactive software that specializes in the construction of network and agent-based models alike. For this project it was used to visualize and experiment with multiple scenarios of ciliate dispersal, as well as to study other types of models and their outputs. All aspects of these models were coded specifically for this project and the desired investigation for this study, and have been adapted specifically to understand more deeply the dispersal mechanisms present in Florida. The software tool Vensim (Ventana Systems Inc. 2006, version 6.0) was used for creating the initial cyclic diagram visualization for the *L. rex* habitat, and the requirements of its survival, creating an active network graph model of the habitat. Vensim was also used for the source sink diagram (Figure 6.3) which further helped to visualize the complexity of the model system. Boxplots and stand alone graphs were created using Excel. All graphs used within the model output figures (such as Figures 6.13, 6.15) were coded into and generated directly by the model, and organized into figures within PowerPoint after model run completion.

Two different models have been created to simulate species dispersal across the State of Florida. These models undergo a simulation and actively move based on the user selected parameters, such that the figures here are merely screen grabs during a playing or completed model, with many parameters changeable and moving which can be watched as it plays out:

> The first model ‘The Neighbors Dispersal Model’ (NDM) utilizes network and graph theory, whereby the species is able to spread from an inhabited node to a clear node, so long as there is a direct connection between them, similar to how a virus may be

modelled spreading through a human population. This model was created with several user friendly interface buttons by which parameters of the model could be adjusted.

The second model 'The Bird Dispersal Model' (BDM) combines network and graph theory with an agent based approach, whereby the dispersal of the ciliate is governed by the random movements of bird agents across the network. Here, the birds act as a proxy for all dispersal mechanisms (e.g. wind) which may occur within the system.

The models generate random networks of connected water bodies dispersed across a map image of Florida. The networks have been constructed in the form of patch dynamics of classic metapopulations (Leibold et al. 2004). Each water body acts as its own self-contained habitat which the species is able to inhabit. These form hubs with other local water bodies, facilitating species dispersal and generating a large and widespread population of the same species through habitat connectivity. Internal interactions of a single water body's metadata components and variables have been visualized in a dynamic source-sink form (Figure 6.3). Many of the values for these variables remain unknown, and were not necessary for the inclusion or completion of the model, or indeed its effectiveness. Here the boxes represent stocks of value which can go up or down in a system. Black arrows are known as 'rates' and they represent the physical movement of material going in or out of a stock. Blue arrows represent an interaction between two components of the system. Clouds represent sources and sinks. Where material within the system originates from and where it ends up respectively. They are represented by clouds because for modeling purposes, the exact places the sources and sink represent are not needed and are irrelevant within this model system. Various feedback loops, both positive and negative can be visualized with such an analysis beyond the scope of this project.

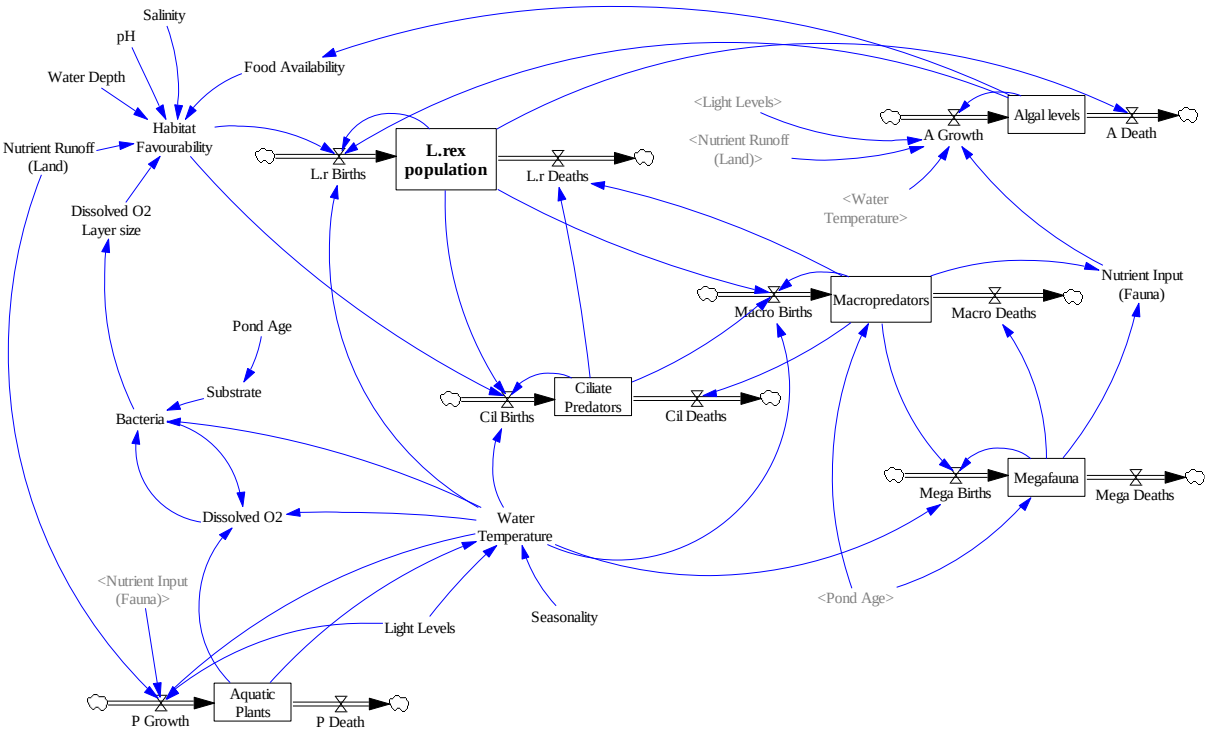

Figure 6.3. A system dynamic diagram is a structural representation of a system which visualizes, and with enough data, is able to simulate all of the interactions that occur. Here the source-sink system dynamic diagram is representative of a theoretical water body which a *Loxodes rex* population could populate, and potentially thrive within given favorable conditions in the actual system. The hourglass shape within the arrows is a tap which represents that the rate of flow can be changed, like a tap, that you can tighten or loosen to decrease or increase the flow of the material respectively.

Parameters affecting dispersal speed and dispersal trends are set homogenously across each water body. These include spreading success probability, colonization success probability, average node degree and population of inhabitants. Many of the parameters can be adjusted by the user to fit the desired simulation.

The use of different modeling techniques allows us to get the most out of exploring the difference between a local vs a regional system, with the ultimate goal of being able to combine the findings of the two to gain a greater understanding of the overall system. The system dynamic diagram (Figure 6.3) is concerned with the local interactions within each aquatic habitat. The network diagrams are concerned with the dispersal between these systems on a regional scale. Overall nine scenarios for each NDM and BDM models were

chosen to highlight different features affecting ciliate dispersal, specifically how water body connectivity vs initial seeding of the *L. rex* species affects the final outcome of dispersal spread capability, trend of the dispersal and how it may change through time, and time taken for dispersal with respect to other simulations. These data are shown in Figures 6.13 and 6.15.

6.5.1 Growth rate of Loxodes rex

L. rex population number: A set of 500mL samples were collected from a productive eutrophic old farmland pond (Table 2.1 # 10 and site described in Chapter 3). A large population of *L. rex* was obvious to the naked eye in (Figure 6.10). A density of 300 mL^{-1} was inferred using measurements from counts on a 1mL chamber (see Chapter 2), which extrapolated to approximately 150,000 cells of *L. rex* contained within a single 500 mL bottle. The (irregular) area of the particular sampled pond (maximum distances 150ft by 65ft) was calculated (13,625 square feet) and this area was calculated in square inches (1,962,000 sq in) was multiplied by the approximate zone (10 inches) *L. rex* was found to inhabit within this pond giving a value of (19,620,000 cubic inches). The exact liter amount for this area was calculated from the potential habitat zone within this pond (321,514 L of water). This yields a cell number of $9.65x10^{10}$. Rounding up for cells inhabiting the sediment, or temporarily out of their favorable zones gives an approximate value of 100 billion cells of *L. rex* thriving in the given sampled pond. This vast number was the basis for the investigation of the *L. rex* growth rates. This number was thought to be so large that dispersal from this pond would be imminent and that, due to sheer density, this number of *L. rex* was close to carrying capacity, clearly thriving on the ample supply of algae and favorable environmental conditions. Graphs were calculated which determined the theoretical potential time, given various survival rates, that would be required to reach this 100 billion population size in a favorable aquatic habitat (see results below).

The growth rate for *L. rex* had never before been investigated. This was an important variable to obtain for building in real data into these models, such that colonization time rate could be inferred based on *L. rex* division time. The experimental inspiration (Dragesco 1968) for the *L. rex* growth investigation used similar parameters, in that a small number of large ciliates was subjected to temperature challenges to determine both optimum growth rate, and tolerance, with growth numbers recorded by eye over time (Figure 6.4 and see Chapter 2).

Ciliate culture methods are detailed in Chapter 2. For this investigation, five *L. rex* cells were picked and washed three times in filtered pond water from the same study site. These were picked and put into labelled 15 mL tubes. The experiment was repeated three times, with triplicate done each time for the temperature variables. Growth rates were recorded, using Equatherm incubators (Curtis Matheson Scientific, TX, USA) at 15˚C, 25˚C, 30˚C, 37˚C, 55˚C. (Figure 6.4). These data were generated for use in both the establishment of model values and also in the development of lab-based cultivation optimization for ciliates. Cells were counted by eye with the aid of a flashlight, this being possible due to the large size of the cells, and necessary so as not to disrupt the cultures with subsampling for microscopy. Population sizes were recorded every 24 hours for 10 days (Figure 6.4). Cultures were not opened so as not to disturb the growing cells or introduce contaminants, and are therefore recorded as number per tube rather than by subsampled mL. Before the optimized triplicate growth study was performed, the optimal feeding regimen had been established as two drops of cultured axenic *Euglena gracilis* every other day.

Calculations of the growth rates built into the model followed the mathematics within the literature (Fenchel 1968; Finlay 1977; Petz et al. 1985). The generation time was calculated from the growth rate constant (k) using the formula $k = \frac{logN_1 - Log\ N_0}{0.301t}$ where N_0 is the number of cells at time 0, N_1 and is the number of cells at time t. The generation time is the inverse of the growth rate constant.

The growth of *L. rex* under laboratory conditions was observed in sealed tubes containing 5 cells at the start of the experiment with the number of cells being counted at 24 hour intervals. The data was generated from three sets of triplicate runs. It was shown that optimal growth occurred at 30°C (Figure 6.4). The amount of growth was reduced at 25°C and 15°C; the cells were killed at both 37°C and 55°C. The variability of the observed growth rate between tubes was high, as shown by the size of the error bars, but still there was some degree of linearity up to six days. Due to this high level of variability and the numerous laboratory observation of single cells dividing within a 24-hour time period, the models were based on a standardized division time of 24 hours. Within all the dynamic models these values can be changed by the user.

6.5.2 The models' parameters

Dispersal modeling of *L. rex* was conducted using inspiration from basic network theory (e.g. Bollobás 1979; Chartrand 1985; Chen 1997) and graph theory (Harary 1969). Graph theory, combined with network modeling can highlight critical areas (patches and corridors) which heavily influence a species distribution and survival capability (Lookingbell et al. 2010). These are important factors to consider, and were implemented in the initial stages of the model building process.

The attributes described below are used for different properties of the models, including: **Water Bodies and Nodes, Connectivity, Time, Dispersal** and **Colonization potential,** and **Agents.** Each section explains how a model component was implemented and what it potentially represents in the real world.

Water Bodies and nodes: Each water body that *L. rex* is able to successfully inhabit and colonize is represented as a single node on the network. A water body which is incapable of successfully housing the species is not included within these models, but variability in colonization success can be changed in the model interface by the user at a run setup.

Across the State of Florida, the total number of water bodies and location of these water bodies in which *L. rex* is able to thrive is unknown. The placement of a node is therefore randomized with each simulation, able to be present anywhere within the Florida state borders.

Every node is set with the same characteristics, creating a homogeneous network across the State. Each water body is discrete and internally homogenous with an uninhabitable surrounding landscape creating species stepping stones (Urban and Keitt 2001; Bodin and Norberg 2006).

For placement, each node is randomly (random number generated by the model) put to a location on the 700x900 (patches) grid containing the Florida State map. The software programs each area of any space as a "patch" such that if an area is set at 10 the software referrers to them as 10 patches in diameter. Each patch is 1.2 pixels in size. If the randomly generated location does not lie within the boundaries, it is randomly reassigned until it is placed in a suitable location. The size of each node is not representative of the real relative size of the water body. The resulting node placement allows for, but is not likely to include

node overlap. Any overlaps that occur simply represent two separate water bodies occupying a very close region of space, and does not represent a merge of two or more water bodies.

In both NDM and BDM models, any node is capable of existing in one of three states at any point in time, visualized by colors on the model interface:

- **Clear** (light blue circle): A water body with no *L. rex* present.
- **Exposed** (yellow circle): a successfully spreading event, *L. rex* has reached the water body. The water body is now able to accept *L. rex* (becoming inhabited) or reject it (returning the water body to a clear state) based on the selected parameters for the model run.
- **Inhabited** (red *L. rex* shaped icon): the water body has accepted *L. rex* allowing it to colonize, and can now act as a new hub for dispersal, e.g. further nodes can be infected from this water body form the connected network.

The model interface was created to have several usable variables to program into a given model run (Figure 6.17). These can be selected, as each run generates a new network, on which the user's preferred variables can be examined. At the beginning of model simulation, each node is automatically set to clear unless otherwise specified by the user. Before simulation begins, inhabited nodes can either be chosen by the user, or selected at random by the model (which generated a random number each time). This feature of the interface allows the user to choose the initial location of inhabited water bodies for varying dispersal scenarios, or by chance. The models do not account for any extinction events across the State of Florida as the water bodies are assumed to be in favorable conditions for the species (e.g. water bodies which are not suitable for *L. rex* growth are simply blank zones between nodes on the network). Once the node has entered the final (red) inhabited state, they stay inhabited for the remainder of the simulation, representing a successful colonization event of the species. The speed that the models run can be adjusted, to show a slow spread, or rapid speed to complete a run sometimes within a fraction of a second in order to understand further the required time ticks and look for patterns. Further variables were omitted so as not to overcomplicate the theoretical models.

Connectivity: The Neighbors Dispersal and Bird Dispersal models take on the properties of randomly generated networks, (see Jeger et al. 2007). Networks are generated based on the number of initial nodes and connectivity set by the user which is calculated via average node degree. The generation of connections between nodes has been based on the networks formed in 'Virus on a Network' from the NetLogo Library (Stonedahl and Wilensky 2008).

Connectivity in the models can be set anywhere from an average node degree of 1 to 20 and is uniform across the network. Average node degree refers to the average number of connections that any one node will have to other nodes within the network. Connections are bi-directional and therefore a connection between two nodes counts as an average node degree of 1 for both nodes. This connectivity degree is a good proxy to actual highly connected water bodies vs those which are more isolated. Such that birds that often utilize one productive pond and fly to another nearby aquatic habitat would make these two habitats very connected, when compared to a more isolated farmland pond with less obvious and frequent means of passive dispersal.

The model first calculates how many connections should be created to satisfy the chosen average node degree, based on the number of water bodies within the network. Connections are then assigned by randomly creating links between nodes, reselecting any that already exist, until the necessary number of connections has been reached. Any individual node is capable of holding more or holding less connections than the average node degree, but across the entire network the average number of connections remains the same.

Time: Models use discrete time steps. The total number of time steps is recorded by the model as 'ticks'. The model runs until all nodes have become successfully inhabited. If a node is not capable of becoming inhabited (i.e. due to a lack of connection to the rest of the network) the simulation will run forever or until manually stopped. Each tick of the model then represents a time step within the model.

Within a single "time step" of the mode the following occurs:

1) +1 to the tick counter
2) In the NDM: species dispersal can occur from any inhabited node to any neighboring clear node where a direct connection exists. In the BDM: agents travel randomly between connected nodes, dispersing the species as they go.

3) Any clear node which has had the species successfully spread to it via the neighboring connection will become exposed and then transition into a clear or inhabited state determined by the colonization probability.

Within the NDM, what each time step of the model represents in real time would be constrained by time it takes for a spreading event to occur and the time it takes a water body to accept *L. rex* and allow for it to colonize, going from a clear to inhabited state.
Within the BDM, the limiting factors of species spread time become the distance a bird is capable of flying in a given time step and the time it takes for a water body to accept and populate the species, going from a clear to inhabited state. Such model time steps though are difficult to apply to an actual spacetime value to the real world the model is simulating.

Dispersal and colonization potential*:* Dispersal and colonization potential are implemented separately within the models and considered as:

Dispersal potential: the chance for a successful dispersal event to occur between an inhabited water body and a clear water body, causing a clear water body to change state from clear to exposed.

Colonization potential: the chance for *L. rex* deposited in a new water body to be able to successfully colonize that new habitat, causing an exposed water body to change to an inhabited water body, or return to a clear state, able to become inhabited in another time step.

Dispersal potential is only implemented into the NDM as the dispersal mechanism within the BDM is controlled through (bird) agents. Colonization potential is clearly implemented in both models
For the purposes of this study and model simplicity, dispersal and colonization probability are implemented as percentages and set homogeneously across all water bodies of the network.
Agents: Agents are implemented into the Bird Dispersal model (BDM) act as the dispersal mechanism between water bodies, unlike the diffusion of the species through the NDM.

The initial number of agents (bird graphic symbol) is chosen by the user on model set-up and can be placed at random by the model or by the user's choice. Each time-step the agents 'fly' from their current location to a new node which is directly connected. If this

movement happens to be between an inhabited node and a clear node, then this is counted as a successful dispersal event, causing the new location to change from clear to exposed (red). Agents are only able to move across connections of the network, and are only able to move once every time step.

For the scenarios described in the results section, the number of agents on the network was kept to 20. This number is not supposed to reflect actual bird numbers in Florida, but it acts as a proxy for the number of potential dispersal events that could occur in any one-time step. The numbers of agents were kept constant as the time for a species to disperse across the network is partially dependent on the number of agents on the network. The number of agents for the simulations was set at 20 so as to generate enough agents to run the simulations at an observable rate, but also to not flood the network while still being able to identify potential patterns and trends within the networks dispersal.

6.6 Dispersal Models

To promote discussion on the extent to which habitat connectivity and initial seeding of a ciliate species impede or benefit its dispersal across a modeled network of freshwater bodies, the following nine scenarios (Table 1) were developed and ran for each of the two models:

	Low Connectivity (Average node Degree = 5)	Medium Connectivity (Average Node Degree = 10)	High Connectivity (Average Node Degree = 20)
1 random seed event	A	B	C
10 random seed events	D	E	F
10 placed seed events along the East Coast	G	H	I

Table 6.1. The variables present in model set up for each of the two 3x3 grids produced for figures created using data after the model runs.

Each scenario was run and recorded up to 100 times. Some model runs, particularly those at low connectivity, often did not finish (end with a complete spread colonization) as the species could never reach every node due to breaks in the network. These events were recorded and examples extracted for discussion. In scenarios whereby many of the 100 runs did not finish,

the scenario was run until at least 30 of the recorded runs included successful dispersal of the species to every node. For NDM: A,G,H,I were only run to 30, and for BDM A,D,H,G,I were only run to 30. This allowed for tick time averages to be calculated for each scenario, based on successful dispersal events of the entire network. The comparison of these tick times have been visualized as box plots (Figures 6.14, 6.16). For each scenario, the number of water bodies was kept at 300 with dispersal probability and colonization probability kept at 100%.

6.7 Bottle Model

The initial novel observation that inspired the creation of the "bottle model" was that ciliates in the real world behave in an analogous way to 'agents' coded into this simple model. Ciliates do not 'think' as they lack a central nervous system and are a single cell. They react to stimuli and move accordingly, 'behavior' which is well documented in *Loxodes* (Finlay and Fenchel 1986a and see Chapter 3). This is essentially how agents within a model 'behave': agents are given a simple set of rules and appear to behave by reacting (e.g. moving) in accordance with their programming, but clearly this is without 'thinking'. If the environmental and habitat parameters are known for a ciliate, along with their reactions to these stimuli, they can be modelled very effectively for their behavior in a small environment (e.g. a closed system).

To further explore the validity of combining agent based modeling with ciliate ecology this microcosm model was created to explore the behavior of *L. rex* in a 500mL sample bottle. The *L. rex* here are entirely agents, as are the copepod predators. Since in the actual environment *L. rex* behaviors are reactionary, the simple rules (e.g. oxygen preference movement rates, reaction observed to light) known from *Loxodes* populations can be programed as rules into the agents in the model that are analogous to real world observations. If model behaviors then match those observed in nature, this 'truths' the model, such that its outputs beyond what can be examined experimentally (e.g. many generations over time, or large population oscillations and crashes, observed rapidly from the model) can be believed more readily, giving deeper insights to *L. rex* populations. This model was created to understand the population dynamics and fluctuations in laboratory microcosm, and by extrapolation *L. rex* populations in the larger environment. The model shed some light onto *L. rex* behavior, particularly with predator avoidance, and movement as a result to light stimuli (see below).

The designed model allows the user to investigate how *L. rex* tend to cluster within their preferred 5-10% oxygenated zones and how this can be achieved through a very simple set of rules. Agents are programmed to move randomly with each time step. The agents are on a 70x70 grid in the model, which gives 70x70 spaces. *L. rex* movement in the unfavorable zones is 1 space per time step (and the predators is 3 spaces). When an *L. rex* enters the favorable zone, its movement is slowed to 10% (0.1 of a space) of its maximum speed while in this zone. By assigning simple rule sets to each agent, their movements and behavior patterns of real ciliates could be replicated with relative ease. The avoidance response of *L. rex* to light was coded into the model also, and many light sources can be added by the users. Predators which come into contact with a ciliate kill it in the model, whether or not it is eaten or just physical breakdown (as in the environment). Factors such as division probability, division time, and *L. rex* lifespan can be set at the model start, and changed throughout the model runs. This allows for the creation of population oscillations. Some chosen parameters lead to a crash, and some are stable indefinitely, and this can be adjusted by the user, who has access to real time population graph outputs generated by the model.

The model is a 70x70 grid in which each patch represents a specific area for the agent. Using the sliding bars in the model interface, the user can select the following variables for the bottle:

Number of *L. rex* (to 600)

Number of predators (to 20)

Sample capacity of *L. rex* (to 600)

Division of *L. rex* possibility (0-100%)

Division timer (0-10). This represents the minimum number of ticks that it takes a *L. rex* to divide.

So if at model start Division-Timer = 6, Division-Probability = 100%, Time for *L. rex* to divide is 6 ticks.

Division-Timer = 6, Division-Probability = 50%, Time for *L. rex* to divide is 6 to ~12 ticks. This means that the divisions between the *L. rex* population will not occur simultaneously, provided the Division-Probability is set to < 100%.

Lifespan of *L. rex* (0-300 ticks)

The real time graph generated by the model during a run plots the population of *L. rex* with its number over time (model ticks). The lines are also coded to show in which area the *L. rex* are present; an interesting variable to track: Red: oxygenated (unfavorable, fast movement). Yellow: 5-10% oxygen zone (the 'preferred' niche for this species, slower movement). Black: anoxic waters and sediment (unfavorable, fast movement).

A white flash of an *L. rex* cell represents a successful division, and two are then present.

A black flash of an *L. rex* represents a light source avoidance behavior. This shows which cells are affected by the addition of a light source when they are added, or when they move into contact with a light (graphic simulated as a sun).

L. rex populations, and total population number are also graphed in order to show as many variables as possible that are present throughout the run.

After model set up, a red ball is at the model center. Press go to start the variable (cell) movement in all directions. The go button can be released at any time to view the assemblage, review the graph output, or add in a light. Most importantly, all the sliders here can be adjusted during the model run, to create further changes within the system in real time. These can be marked within the graph to show their significance to the population.

An orange sun drawing can be added anywhere in the model as the "Add-Light" button, at any time during a model run. At model startup; all ciliates start in a ball at the center, and all predators start in a ball upper left (this top layer is where predators are most likely encountered, as lowering oxygen gradients provide a refuge in ecosystems). Therefore, it is important to allow a few ticks for there to be an expected distribution, similar to the real settling of a 500mL sample bottle.

Copepod predators are coded to only move randomly within the upper (oxygen zone) and cannot enter or affect cells in the lower zones. They do not react to the light source.

Movement: generated by random number with a variable between patches. Movements via direction of 40 degrees is also possible within the tick time.

Agents in the model are programed with given rules, and then behave accordingly within the model constraints once it is played and not controlled. *Loxodes* in the real world acts in the same way, as it has evolutionary rules of behavior without thinking.

6.8 Results

6.8.1 Loxodes growth rates

Species within the genus *Loxodes* are well known to obtain large population sizes (Finlay et al. 1983). Environmental temperature has a direct effect on both cell reproduction and size (Finlay 1977) with natural (found in the habitat) warmer temperatures generally being more favorable than colder temperatures (those less frequently encountered).

The volume of the habitable area of the small water body in which the highest densities (300 mL) of *L. rex* were collected (and used in the temperature experiment) was calculated to contain 321,514L of water. Based on laboratory observations, *L. rex* can achieve a density of 300,000 L^{-1}. This gives an estimate of $10x10^9$ individual *L. rex* within the habitat. Previous studies estimated the wet weight of a single *Loxodes* cell (Finlay and Fenchel 1986a), which was adjusted for the size of *L. rex* and determined the wet weight of an individual cell as ~0.4µg. This gives an estimate for the total wet weight of the species of $4x10^{10}$ µg or 40 kg wet weight in a small farmland pond. The time in which these population numbers can be reached, given a known doubling time and death rate, are explored using the models illustrated in Figures 6.5-6.9. It is possible that given favorable conditions populations containing billions of cells in a pond could be achieved over a period of days.

The growth rates of *L. rex* were determined within the laboratory at set temperatures, allowing the ideal growth conditions to be examined (Figure 6.4). These values were then investigated to see how certain variables (starting number, death rate) could affect the time rate (in days) for *L. rex* to colonize a given theoretical habitat to the population of 100 billion. Figures 6.5-6.9 explore this theoretical population time based on known division rates. They show that the potential to reach this vast number is a matter of days, not months or years. Clearly, a larger seeding event (Figure 6.7) allowed for a faster colonization rate than 1 cell when all other parameters were not considered. Even when the death rate (unknown value) was simulated at 80% (rather than 0%) colonization still occurred rapidly (Figure 6.8) suggesting colonization of *L. rex* is rapid in a favorable habitat.

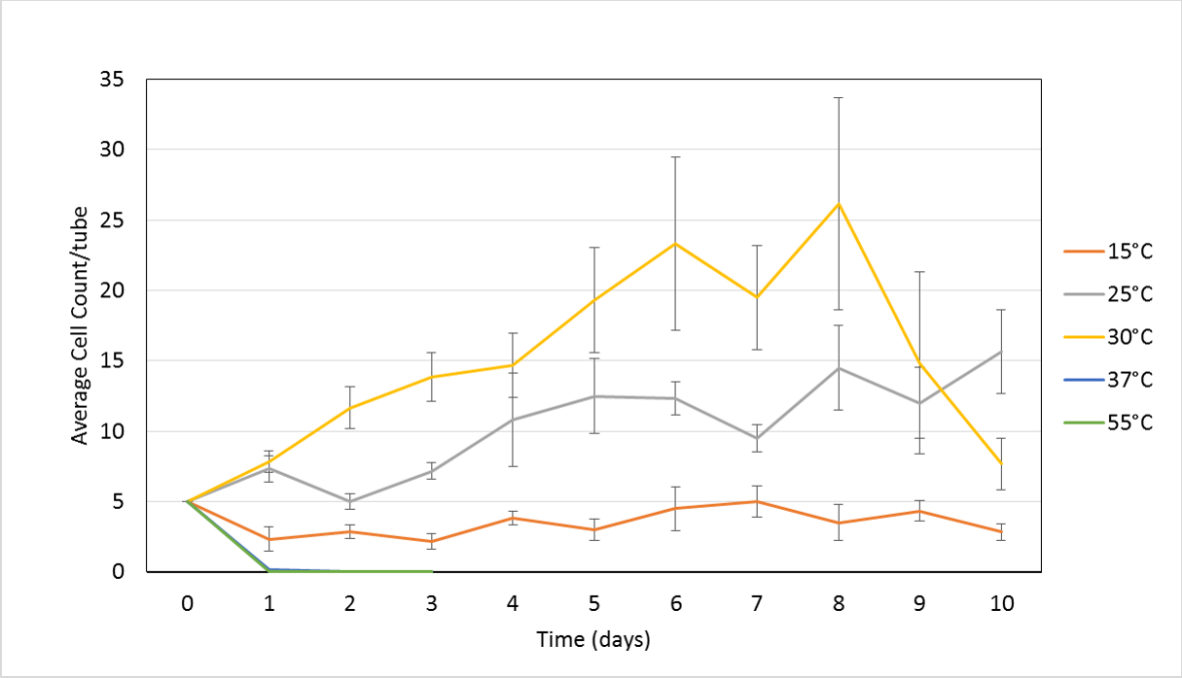

Figure 6.4. Results of the *Loxodes rex* temperature growth rate experiments. The optimum temperature for *L. rex* was found to be 30˚C. From this a doubling time of 24 hours was calculated, with the numbers here shown averaged from three sets of triplicate runs. The high temperature of 37˚C and 55˚C killed all ciliates within 24 hours, and the lines merge here. Populations were stable but not productive at 15˚C. At 25˚C population numbers increased, but more slowly than the optimum 30˚C.

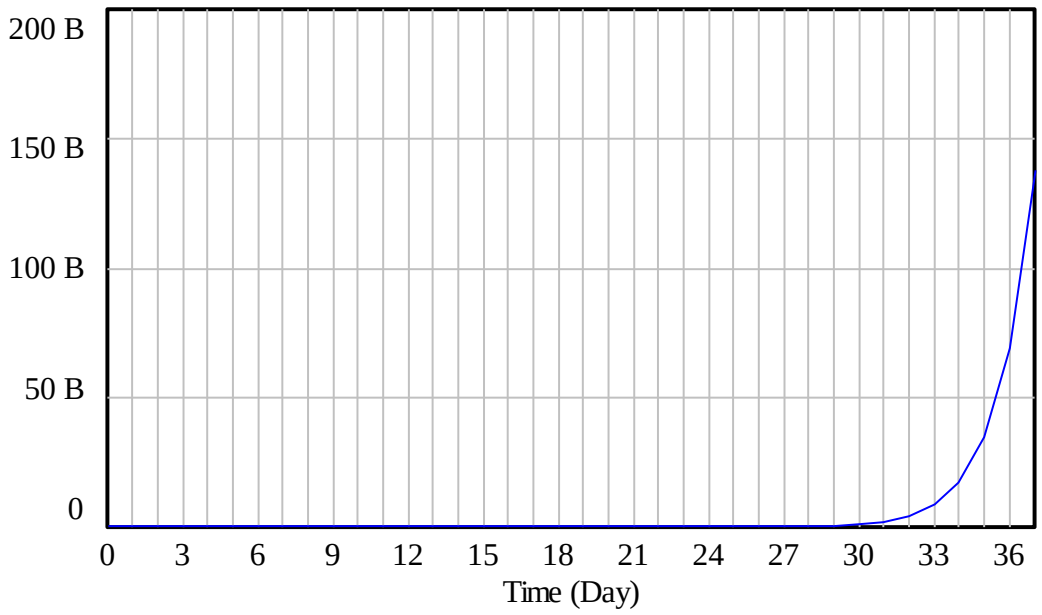

Figure 6.5. This is a theoretical output of potential *L. rex* population using laboratory growth rate data obtained (Figure 6.4). Several graphs were produced to explore potential values of *L. rex* populations using varied parameters (see below). In this theoretical population figure the doubling time was set to 24 hours. The model started with 1 cell. The death rate was set for 0%. Y represents the population number in billion for all graphs below. The graph is in billions as this was the calculated value from a real small farmland pond (see above) for *L. rex* sampled, and is considered to be such a high value that dispersal is inevitable at this population concentration.

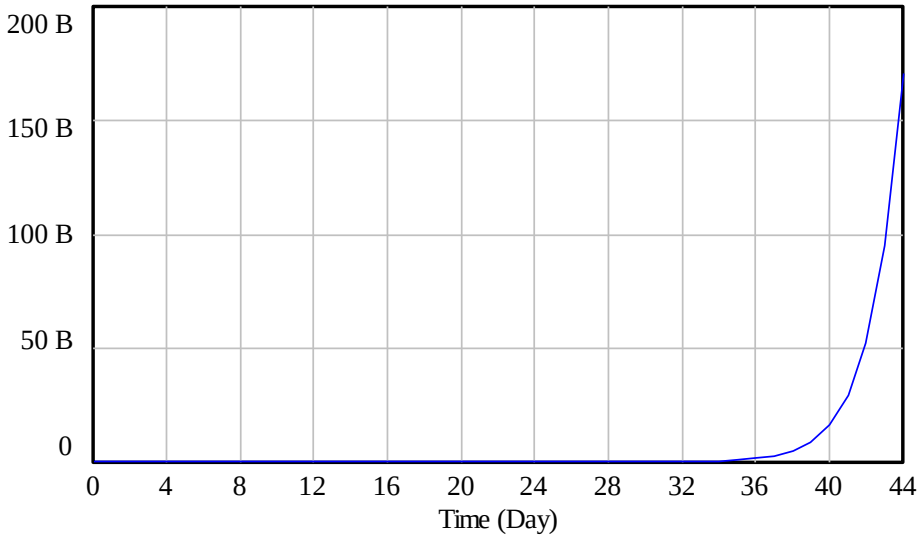

Figure 6.6. The doubling time for this output was set at 24 hours. The model starts with 1 cell. The death rate was set to 20%. Clearly the time to reach 100 billion cells here was longer than in Figure 6.5 with a death rate set to 0%.

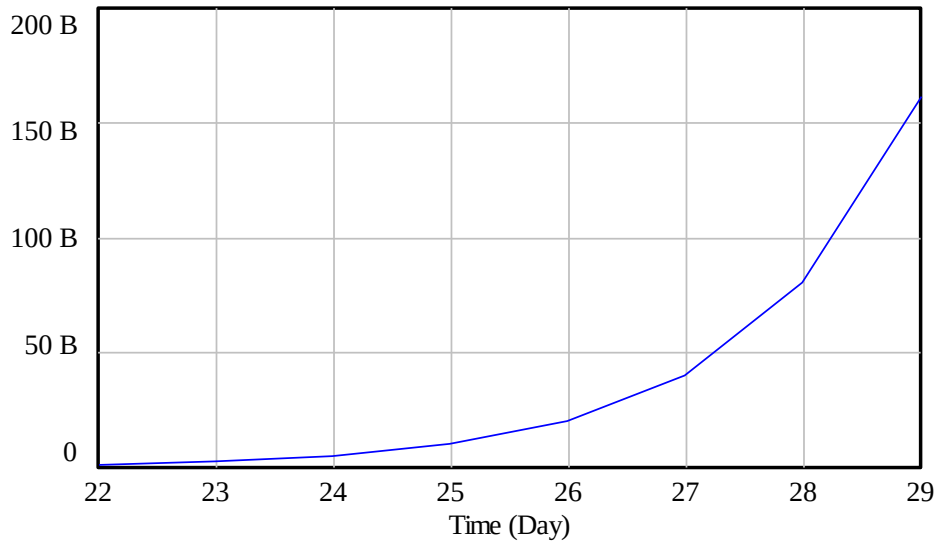

Figure 6.7. Doubling time was set to 24 hours. The starting cell population was 300, and the death rate was set to 0%. Note this graph is the same output as Figure 6.6 but started at day 22 to show how quickly a larger seeding event (here 300) can spread to the 100 billion mark. This shows that a dispersal event containing 1mL worth of dense ciliates can lead to a rapid colonization.

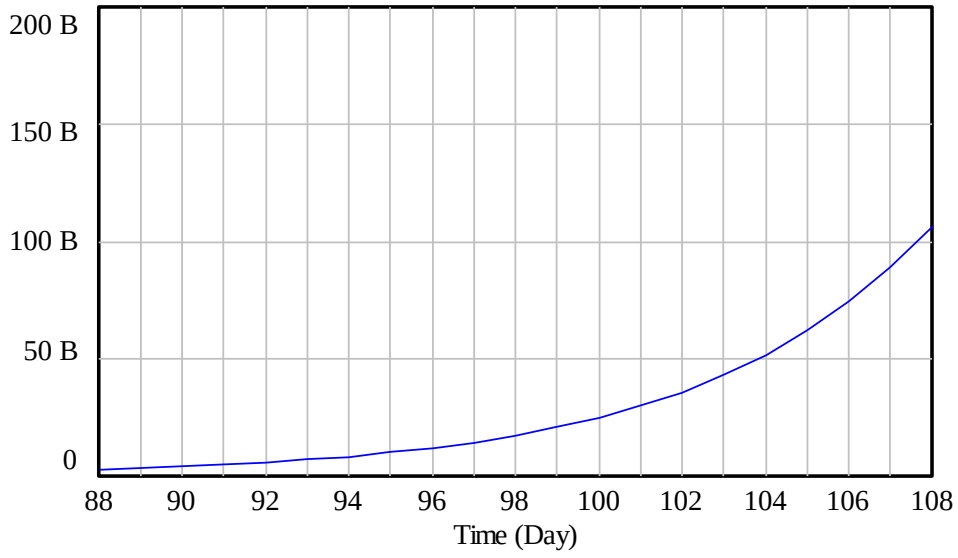

Figure 6.8. The cell doubling time was set to 24 hours. The initial population was 300 cells. The death rate here was set to 80%. Graph starts at day 88 and shows 20 days of growth up to the 100 billion figure. This was calculated to explore, with even a high death rate, the potential time interval needed to reach this population value.

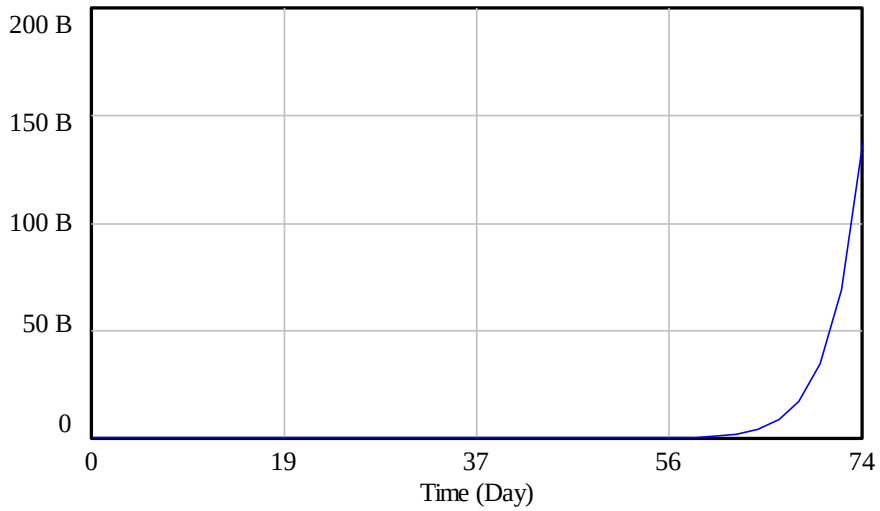

Figure 6.9. The doubling time was set to 48 hours, which was higher than real values found in the laboratory, but potentially present under unfavorable environmental conditions in the real ecosystem for some areas. The death rate was 0%, and the time interval to reach 100 billion was more than in Figure 6.5.

6.8.2 Bottle model results

Based on countless laboratory observations of *L. rex*, a good understanding of their 'behavior' was obtained. The literature on *Loxodes* describes in detail their responses to factors such as vibration stimuli, light, and movement within preferred oxygen layers (Fenchel and Finlay 1986). A novel and unexpected result of the bottle model was that with the coding of a few basic rules, or laws for each agent to follow, similar to exacting model outputs were created comparable to the laboratory finding (Figure 6.12). The accumulation of *L. rex* within the preferred oxygen zone, but at the boundary of avoidance for a light source is exactly as observed in microcosm. A thinking organism (e.g. multicellular) may retreat as far away from the stressor as possible, but these agent based *Loxodes* (lacking a brain in the model and real life) accumulate in this zone closest to the boundary between negative avoidance and favorable slowed movement (Figure 6.11). Similarly, the model predicts the

accumulation of the *L. rex* within the small preferred oxygen layer of the bottle. It also shows how cells can be far outside of this zone due to random movements, some of which fall victim to predation. It can be visualized here how this niche parameter given a significant evolutionary advantage to *L. rex* by being able to thrive in a niche not utilized by predators, and therefore allowing for large populations to thrive. Oscillations of population can also be examined in a matter of minutes at a stable state, rather than days (if ever) in the laboratory. The actual size of the population will remain at much higher numbers than the sample capacity if the division chance is high and time to divide set extremely low. To maintain the actual population size around the sample capacity, Division-Timer must be set high (> 2) and Division-Probability to low (< 50%).

Various ecological concepts can be tested for, and explored by changing the variables within the bottle model.

Example 1: "Thriving species"

In the following simulation population of *L. rex* can be able to maintain itself within the carrying capacity. Predation levels are not high enough to eliminate *L. rex.* Then population of *L. rex* accumulates within a preferred O_2 gradient. (Leave simulation running - % *L. rex* in each zone can be shown on plot).

Population-*L.rex*:	50
Population-predator:	2
Sample-capacity:	200
Division-Probability:	50
Division-timer:	5
Lifespan:	100

Example 2: "Population crash"

Population crash can be simulated by setting the initial population to higher than the carrying capacity of the bottle. This simulates a lack of nutrients to sustain high *L. rex* population numbers (as could be present over time within depleted microcosm), limiting their ability to divide. Numbers must reach the sample capacity limit before the population dies of 'age'. Other reasons for a crash may include a buildup of toxic waste materials or oxygen toxicity (this is what was observed in the laboratory, as a population of >100,000 cells can crash to 0 within hours). Things to look for within a model run are a crash in populations, and possible

rapid recovery once carrying capacity is hit. A population crash is often caused by multiple *L. rex* reaching the age limit simultaneously without being able to divide due to the capacity limit of the bottle being reached.

Population-*L.rex*:	200
Population-predator:	1
Sample-capacity:	100
Division-Probability:	100
Division-timer:	5
lifespan:	100

Example 3: "Light avoidance zone"

Implement a light avoidance zone using the 'Light-draw' button. Ensure it is pressed and use the mouse left click to implement a light source. This can be done post setup or during simulation. Move position of the light source to get an obvious layer of avoidance accumulation (Figure 6.12).

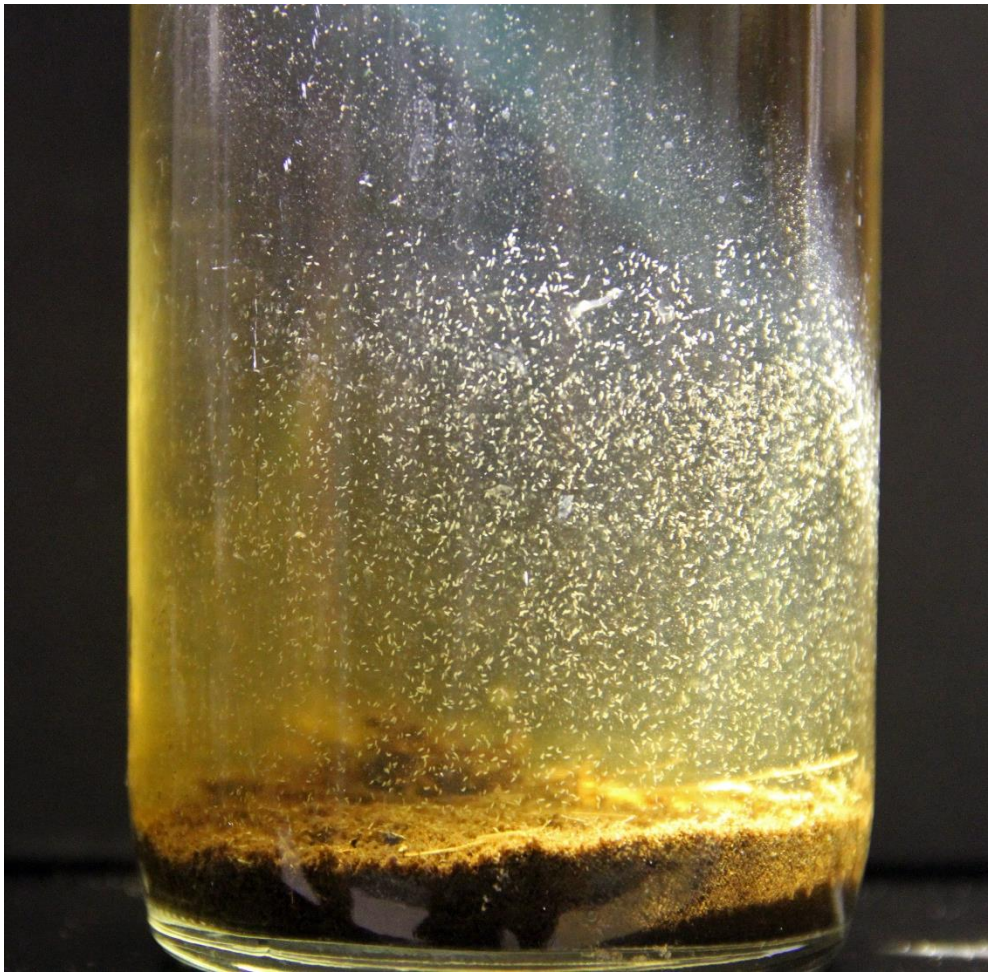

Figure 6.10. 500mL environmental sample of *L. rex* in microcosm.

A non-magnified 500mL natural sample bottle. This environmental sample was collected (Table 2.1, Site #10), is unaltered, and was left to settle for 24 hours. Nearly all the white 'dots' are a single cell of *L. rex*, visible to the naked eye. The focus is on the front of the bottle, but the high cell density continues throughout the bottle. The ciliates have migrated out of the sediment into their preferred oxygen zones. These observations inspired both types of models produced for this project, and behavior here is related exactly to the "bottle model" described.

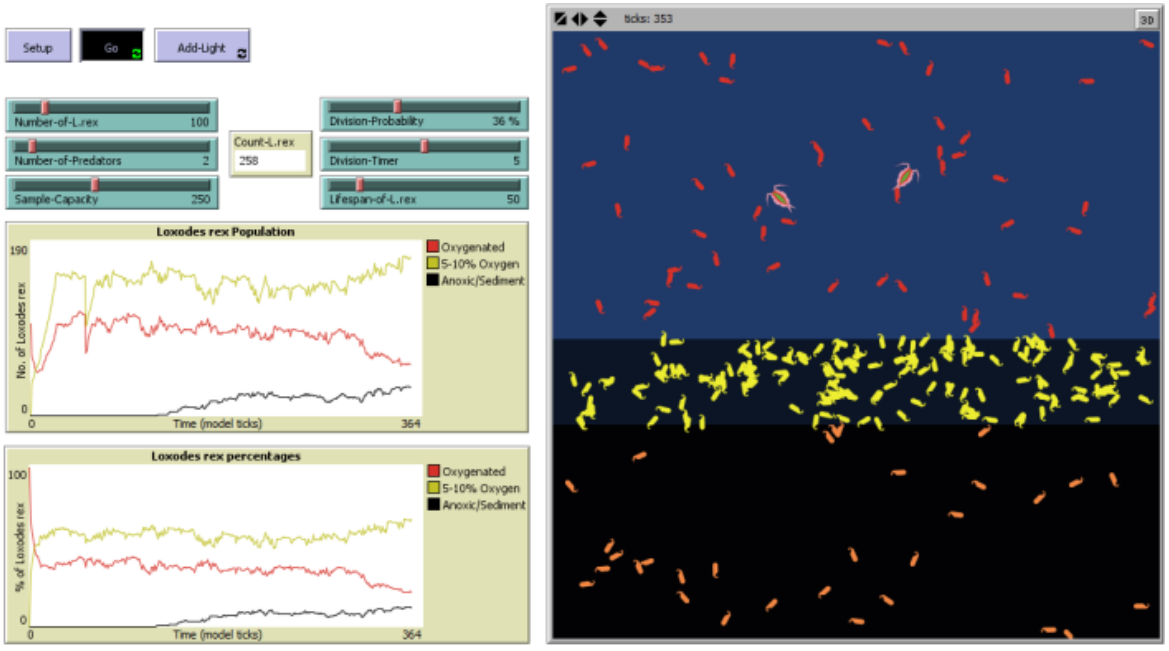

Figure 6.11. The bottle model: showing the behavior of *Loxodes rex* within a 500ml bottle of the laboratory. The zones include oxygenated (light blue with red *L. rex*), 5-10% oxygen (dark blue with yellow *L. rex*) and the anoxic layer (black with orange *L. rex*). Copepod 'Predators' (pink with green crustacean graphics) will only stay within the oxygenated bottle zone leaving a safe zone for *L. rex* to thrive. *L. rex* turns white when dividing into another cell, and black when avoiding a light source (not shown in figure).

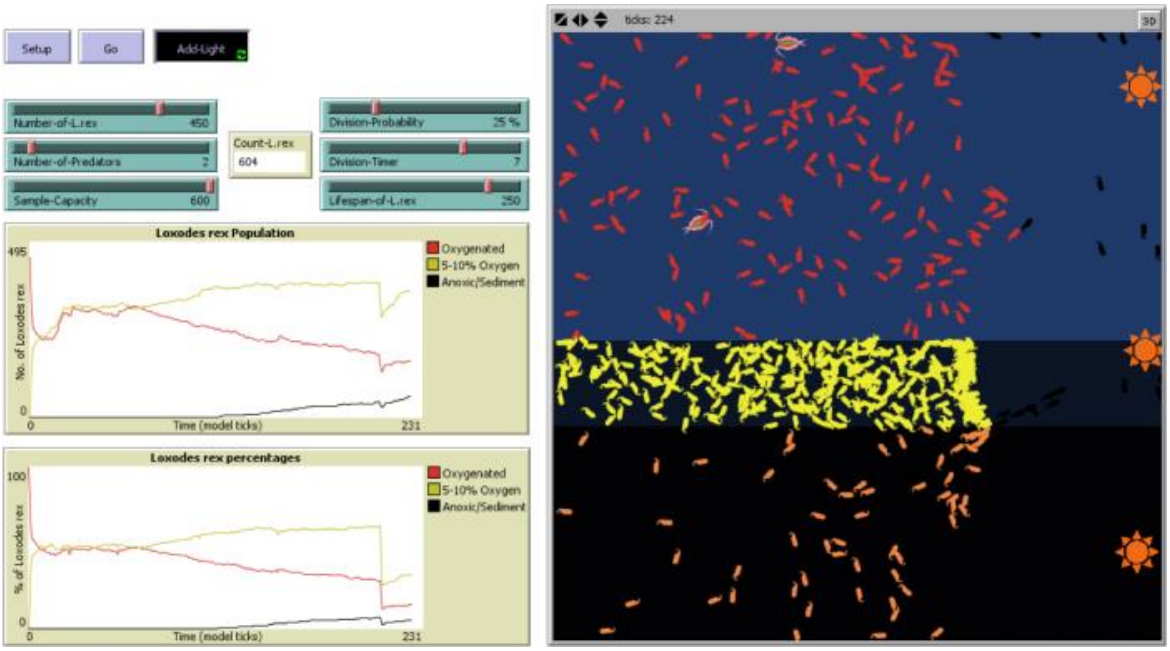

Figure 6.12. The model in motion with the addition of 3 light sources (suns, at right) in the various zones. Note the dense line of accumulation at the light boundary in the yellow zone; this behavior was also observed in the laboratory and it is interesting that this, apparently less-than-ideal positioning, occurs in both the real world microcosm and in the model.

6.8.3 Dispersal model results

Nine scenarios were constructed for the Neighbors Dispersal Model and Bird Dispersal Model exploring low to high habitat connectivity vs initial seeding of the ciliate. From each run of a scenario, a single figure was chosen to form a 3x3 grid for comparison across the scenarios including a plot showing dispersal trend for that figure (Figures 6.13 and 6.15). Boxplots have been generated to show the recorded outputs of ticks times across the multiple scenario runs (Figures 6.14, 6.16).

6.8.3.1 Neighbors Dispersal model results

Results from nine individual scenario runs (at 100 or 30 each) for the NDM are recorded in Figure 6.13 with data for each plotted as a boxplot in Figure 6.14. In the 3x3 grid clear water bodies can be seen as light blue circles, initial seeding events are shown by red *L. rex* shaped nodes representing inhabited water bodies and yellow circles are exposed water bodies which show how much dispersal has occurred after just one-time step.

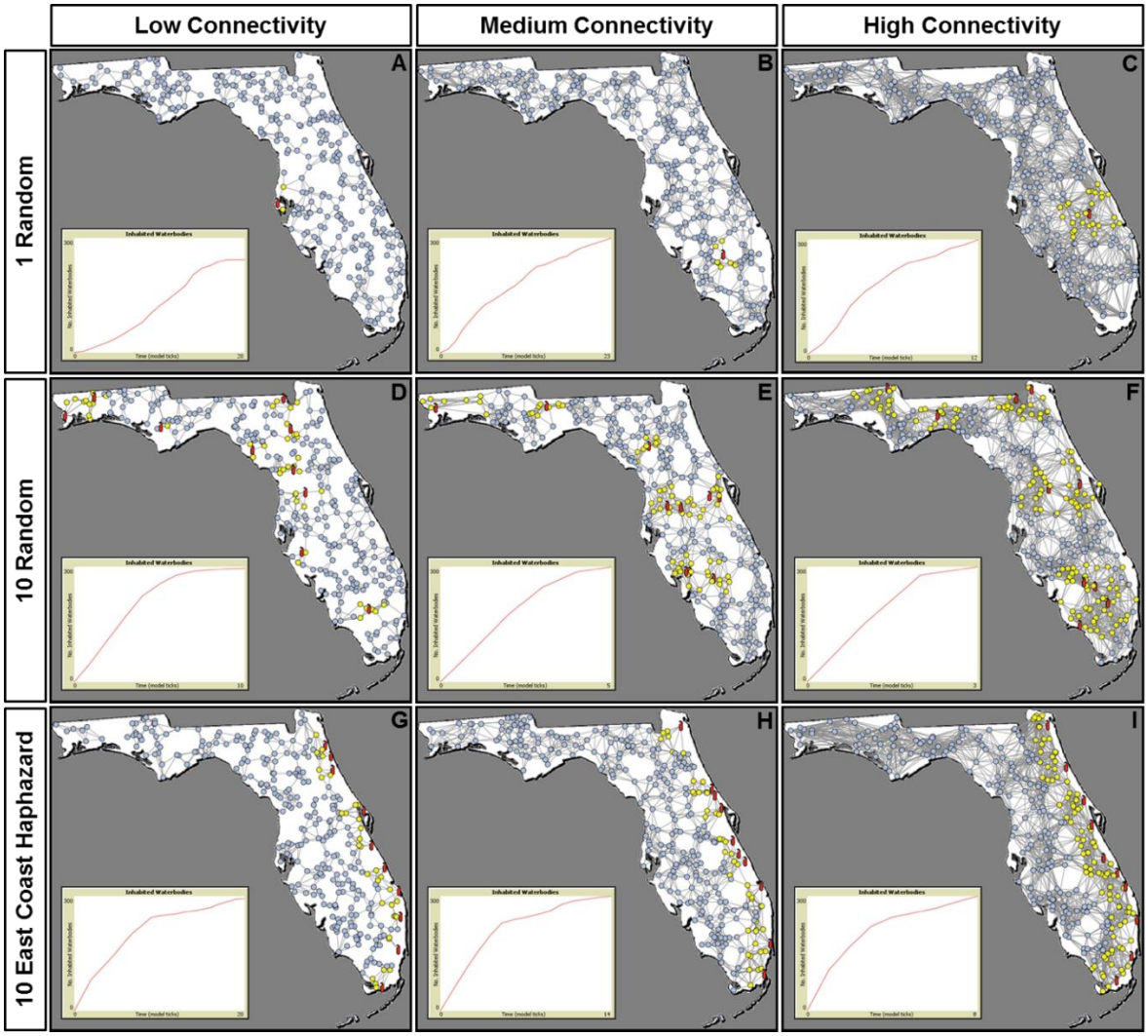

Figure 6.13. A 3x3 grid showing nine scenarios of the NDM. Red *Loxodes rex* shaped nodes show where initial seeding of inhabited nodes took place. Light blue circle nodes show clear water bodies and yellow circles show exposed water bodies. The images are taken during simulation after one model time step has occurred to show the initial spread. The superimposed graphs on the image are produced after the final time step to show the results of the connectivity within the network generated.

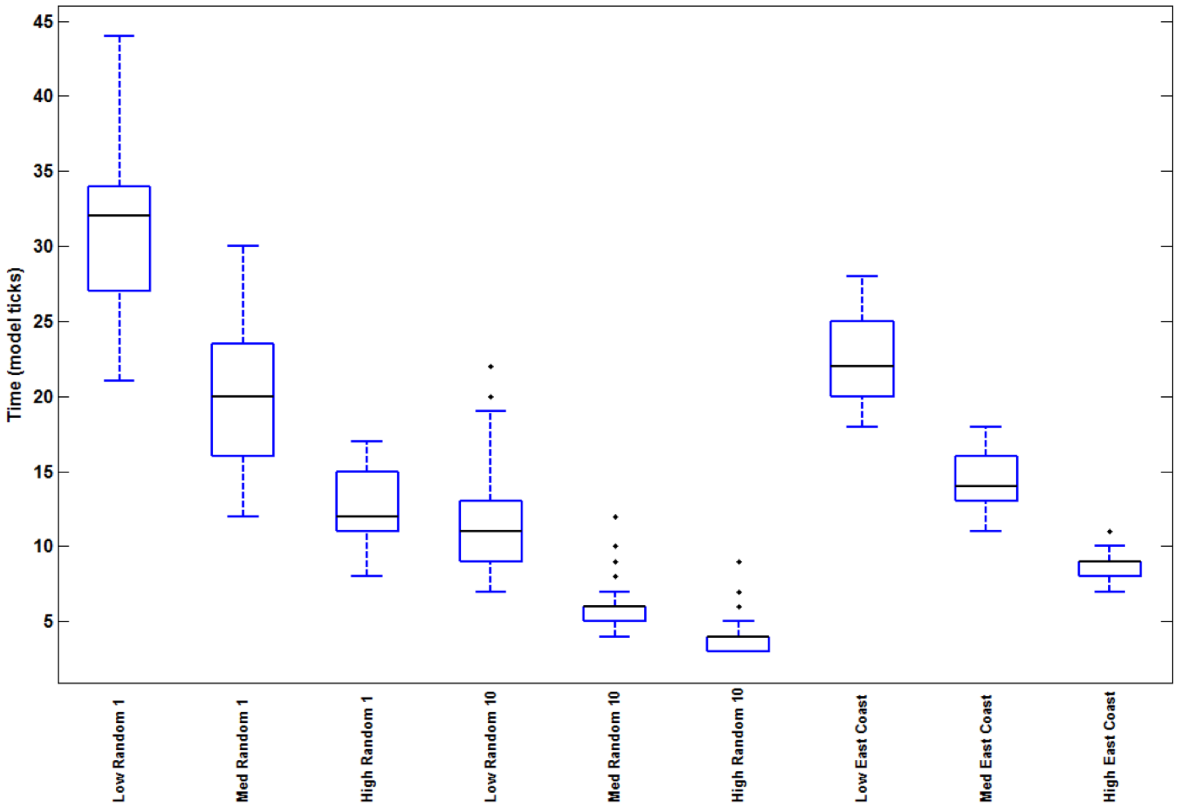

Figure 6.14. Boxplot for the 9 scenarios of NDM. Boxes show the median and the 25 and 75 percentiles of model tick times. Whiskers show any data which sat outside of the inner 25-75 percentiles and outliers are represented by black dots. Boxplots show the tick times for multiple runs of each scenario in the NDM. The tick time is stopped and recorded when every water body has become inhabited by the ciliate species.

6.8.3.2 Bird Dispersal model results

Results from nine individual scenario runs for the model are recorded in Figure 6.15 with data for each plotted as a boxplot in Figure 6.16. In the 3x3 grid clear water bodies can be seen as light blue circles, initial seeding events are shown by red *L. rex* shaped nodes representing inhabited water bodies. The initial placement of bird agents is shown in black. The graphs inserted (Figures 6.13, 6.15) are produced in real time by the models, and these are the final recorded data for the completed run of the given scenario.

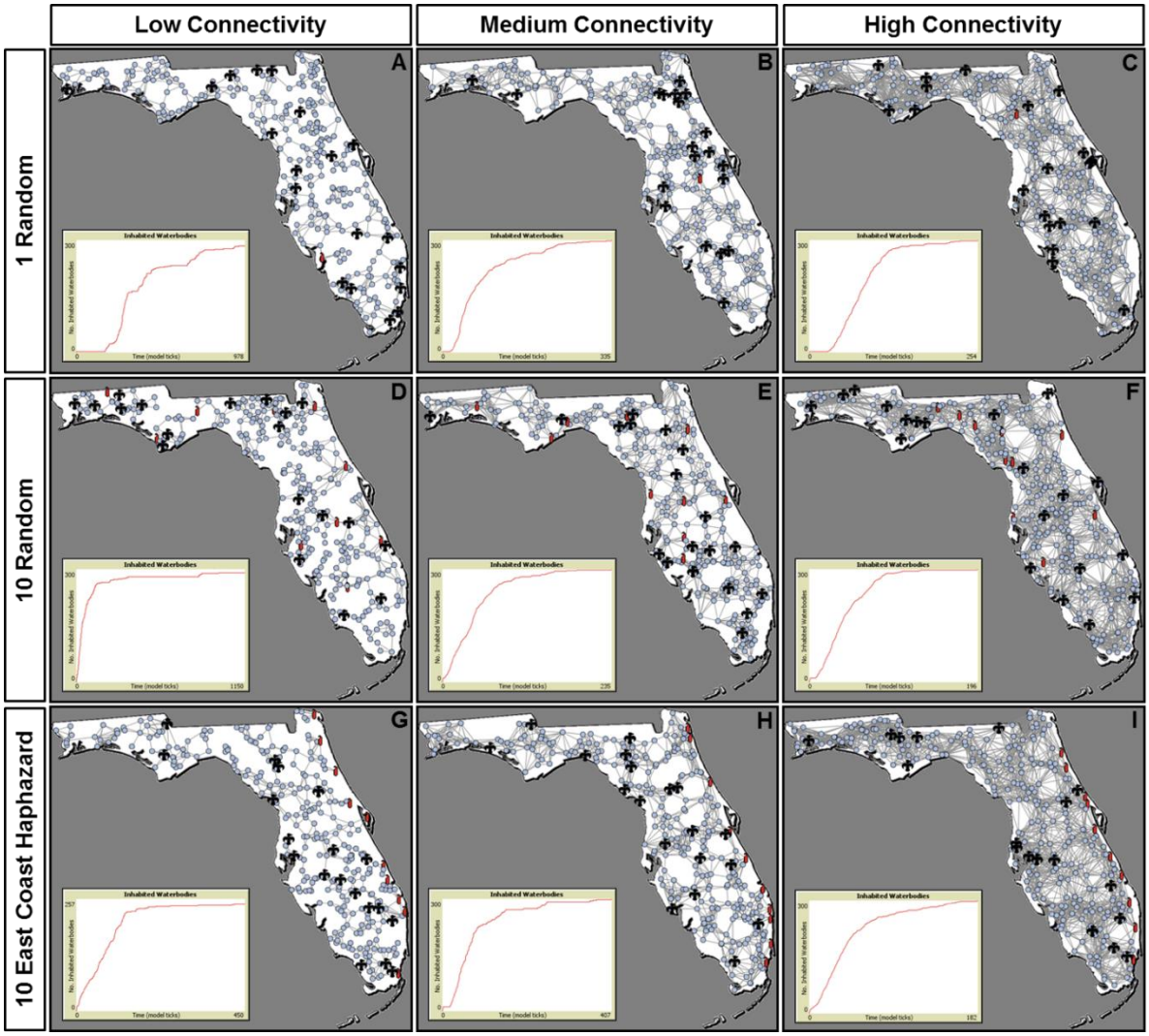

Figure 6.15. 3x3 grid showing nine scenarios of the BDM. Red *L. rex* shaped nodes show where initial seeding of inhabited nodes took place. Light blue circle nodes show clear water bodies and initial placement of bird agents are show in black. The images are taken after model set-up to show an example of the initial scenario set-up. The superimposed graphs were taken after the model has finished its run to produce the data shown. Note the number of ticks decreases from left to right in all scenarios as the connectivity increases in the network (see graphs).

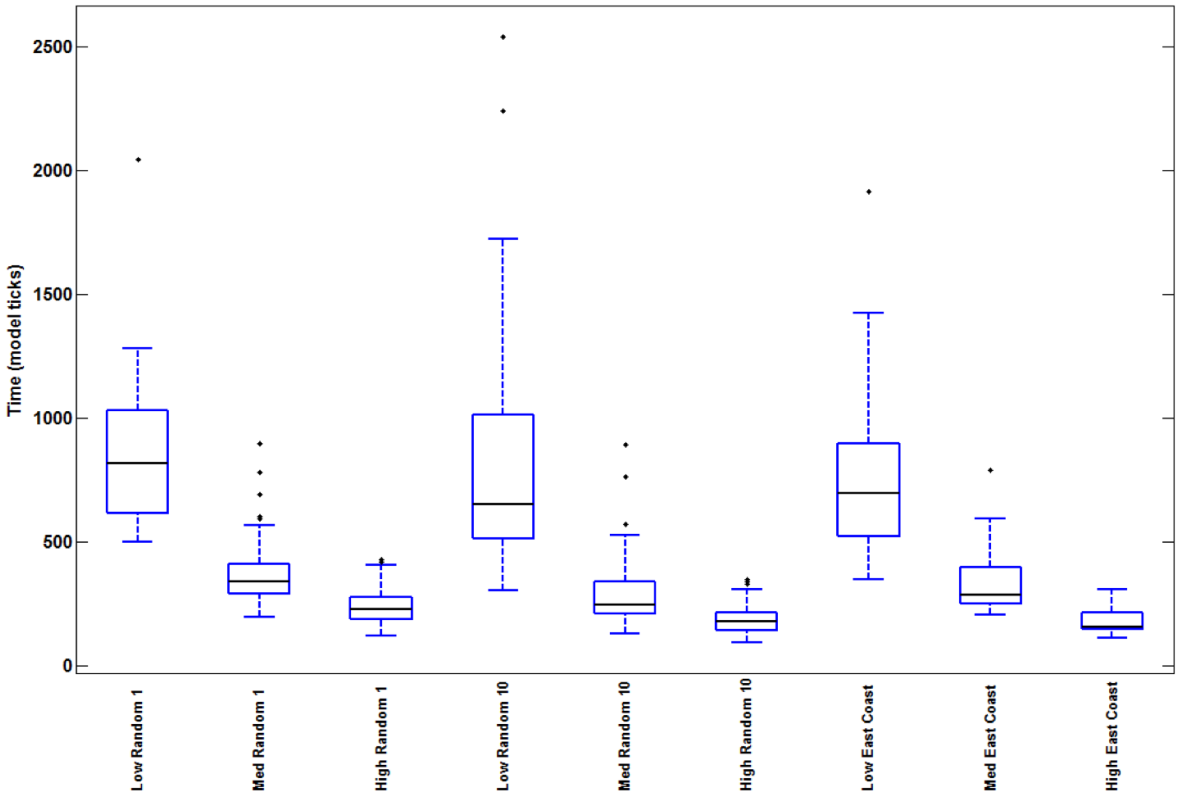

Figure 6.16. Boxplot for the 9 scenarios of BDM. Boxes show the median and the 25 and 75 percentiles of model tick times. Whiskers show any data which sat outside of the inner 25-75 percentiles and outliers are represented by black dots. Boxplots show the tick times for multiple runs of each scenario in the BDM. The tick time is stopped and recorded when every water body has become inhabited by the ciliate species. Each were run 100 times with values recorded.

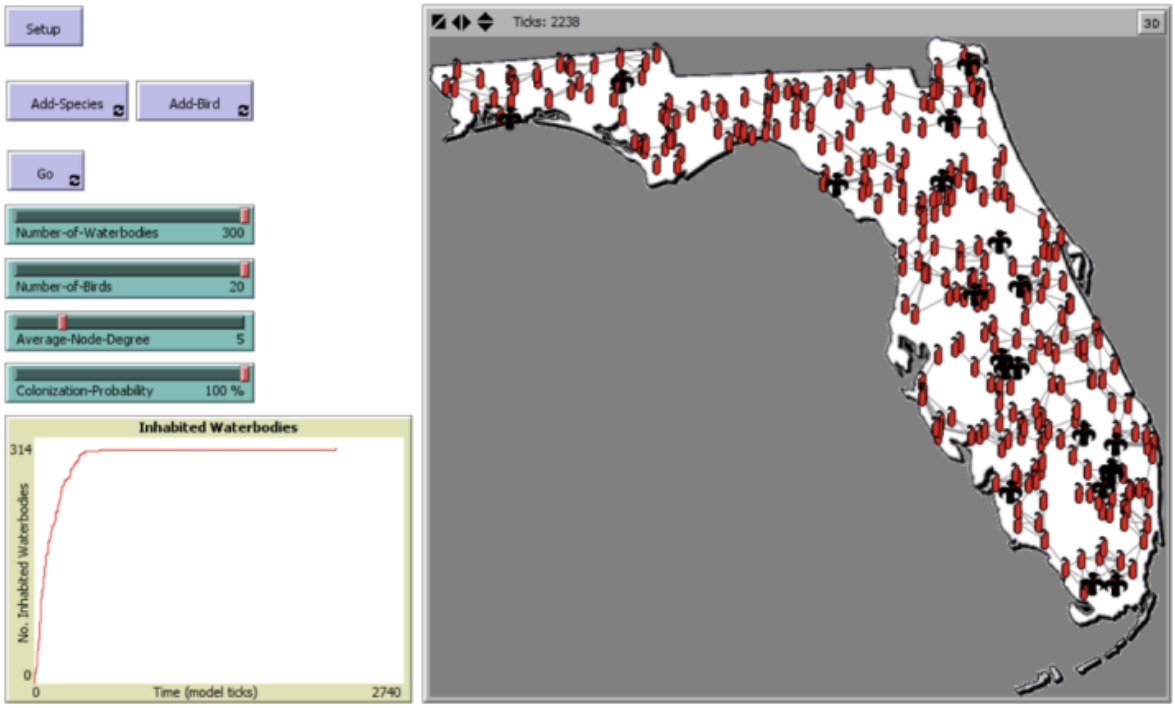

Figure 6.17. Bird model interface. Note the changeable variables (at left) as described in the text. This run was unusual in that it was 99% complete at 501 ticks, but took to 2,238 ticks to fill the last remaining water body. Such outliers became obvious with the additional model runs, and perhaps also mimic actual issues of dispersal in nature.

6.9 Discussion

6.9.1 Loxodes rex cells

Dispersal potentials would be significantly enhanced by the presence of 100 billion cells in a small pond. Of course this number is not necessary for actual dispersal; a very small number of cells could be present and dispersal could still occur especially as the preferred niche of *L. rex* is often disturbed by water birds (feeding and walking), which could transfer the cells necessary for dispersal between ponds. Since real world dispersal rates cannot be determined from theoretical models, here the number of 100 billion cells was used as a way to show that even with the requirement that the population reaches this vast number, producing high probability of a spread, the potential time required for dispersal, based on laboratory results and field findings, is relatively short even on a human scale. It can be inferred then that *L. rex* can spread across Florida, and reach huge population numbers in a matter of days from a large storm, or similarly in days, weeks or months from passive dispersal (e.g. bird flight), rather than taking millennia. When large dispersal events such as storms, and the potential

for groups of birds travelling long distances (kilometers rather than meters) are considered, this time step is of course even shorter. The results of the growth rate and the model dispersal ticks can extrapolate the rapidness of a colonization event occurring. A theory to advance for flagship ciliates then is that even if a spread between continents is rare, given time it could happen, and only one cell would be needed to potentially spread rapidly across Florida. The high number of potential dispersals occurring (i.e. attempts) can be thought of as a lottery, such that the chances of any one ticket winning are very low, but that one will eventually win is inevitable (Finlay et al. 2004; Wilkinson et al. 2012).

L. rex was found to grow well at both 25˚C and 30˚C laboratory setting. It is important to note that the ideal temperatures found for *L. rex* growth (~30˚C) in the laboratory was similar to the water which was frequently encountered during field sampling (Chapter 3). This contributes to the high population numbers of *L. rex* in Florida, and the large number of potentially suitable habitats aids in its dispersal. *L. rex* was shown to survive at both 15˚C in laboratory experiment and at 9˚C in the environment (Hines et al. 2016). Temperatures above 37˚C killed the cells within 24 hours of laboratory incubation (Figure 6.4).

With just 1 cell dispersed into a favorable environment, the models have revealed several scenarios through which large populations can be rapidly reached (Figures 6.5-6.9). The time step of a 24-hour division is likely very close to the one found in nature for other *Loxodes* species, but values of far longer than this were also calculated and still fall within the spectrum of weeks for the population density to reach the high populations where dispersal is likely, rather than the millennia claimed in literature suggesting ciliate endemism via dispersal restrictions (Foissner et al. 2008). The data shows that *L. rex* can theoretically reach billions of cells in a pond within 30 days, and therefore spread across the entire state in a matter of days to months, however, it is likely that this could happen over a shorter time. Artificial ponds were not dug as part of this project due to time limitations, as well as land usage restrictions and safety issues concerning spread of insect-borne diseases. Instead, natural ponds were examined over the course of this PhD project, (Chapter 3) and it was found that a pond can go from uninhabitable (e.g. drought dry) to wet (and favorable) and contain the target *L. rex* in detectable numbers within this relatively short period of time.

6.9.2 Dispersal Models

Dispersal in these models is assumed to happen entirely through passive transport (nothing purposely being dispersed and species not purposely acting in order to travel) and the manor

at which the species is dispersed becomes heavily reliant on critical patches and node centrality (Bodin and Norberg 2006). From literature on disease spread (Gallos and Argyrakis 2003; Brauer 2005; Saraaäki and Kaski 2005), it is assumed that original placement of the organism, along with network connectivity will heavily determine the spreading potential within the first few stages of dispersal and ultimately determine the species' ability to successfully disperse to every water body. This is reflected in the results above for these models. As connectivity increases within the networks, the shortest pathway between any two nodes reduces and separated habitat islands become connected with other islands, meaning the ability for a ciliate species to spread between any two locations becomes more and more likely, in both the environment and the simulated models.

The boxplots created from the model outputs show a regular trend of increasing connectivity leading to lower completion times for each scenario. In the real world, as habitats becomes more connected, the number of neighboring water bodies to an already inhabited water body increases, creating an increased likelihood that a successful dispersal event will occur for at least one of these neighboring nodes. Also within a real ecosystem constant dispersals will add to the net ciliate population, and decrease further its colonization time step.

Upon model set-up, nodes first prioritize connecting to their closest neighbors. Within the actual system it is possible that a given bird could disperse a cell at a great distance, beyond that which may be simulated in a low connectivity model (e.g. flying 100km vs 10m to disperse in the nearest water body). This is, however, overcome by the habitat connectivity of stepping stones in the model, and therefore only closest neighbor connections have been generated for this set of simulations. Clearly the potentials for dispersal in the real world, no matter how unlikely, outnumber those which would be useful to attempt to simulate within the models, but act as a way to say that a faster spread is likely to occur than shown. The models are spatially implicit as movement between nodes is assumed to be equal across the entire network.

A given time step could represent many interactions of all bird's flight within a single day between connected water bodies. Such that in the real world, many birds may be spreading the species simultaneously, which would decrease the necessity of high survival rates for initial colonization to be successful (ex 100% at 1 seed time per tick, vs 50% at 100

seed times per tick). This further shows that few individuals are needed for a new colonization event to occur.

A storm dispersal event can be simulated by selecting 10 water bodies haphazardly (by the user) on a given area, such as the East coast (Figure 6.15 G, H, I) and watching the spread. This is another way in which to study the effects of isolated 'habitat islands' within the network. Such 'islands' can be overcome in the real ecosystem when the mechanisms of action of both models are combined, e.g. a spread from the east coast occurs, and birds are also present. In the real environment, the birds (acting as a proxy also to wind and water flow) may, given time, overcome all barriers of separation, and therefore the only limiting factor for *L. rex* colonization of Florida will be realization of its preferred environmental niche, rather than actual dispersal limitations. Such actual investigations, however, are difficult as a result of sampling bias, and the vastness of the geographic area (e.g. hundreds of kilometers) being studied. The notion in ecology that freshwater ecosystems are isolated habitat islands within a system is not the case for Florida, and these models give a multitude of results suggesting that habitats are in fact interconnected through dispersal events (rather than physically touching). Whether or not a pond is directly connected to another pond via water does not make it an island, and therefore global spread of protists (and therefore microbes in general) may still remain greatly underestimated. Birds are documented to spread species between continents (Hahn et al. 2009), with Florida being an important habitat for tropical species migration flight paths between North and South America (Martell et al. 2004). Whether or not a species is invasive or native can be difficult to determine (Carlton 1996). Despite *L. rex* being originally described in Africa, the ease of which its dispersal potential could spread throughout the tropics suggests that its presence in Florida is natural, despite being undetected for so long due to undersampling. Whether or not *L. rex* originated from Africa is thus far unknown, but due to the comparatively young freshwater age of Florida (Watts 1969; Watts 1975), and the recent ice age mechanisms exposing terrestrial freshwater, it is likely that this state was colonized more recently than more ancient continents such as Africa (Foissner et al. 2008). Future work investigating this question from a molecular perspective is increasingly more possible, as sequences are made available (Chapter 3).

Individual nodes on the network can be "seeded" as colonized at the start of the model rather than locations generated at random. While initial seeding does affect the initial spread

of a species, it will not affect the final outcome on the same network. However, more initial seeding will increase the likelihood for future spreading events to be successful and will reduce the likelihood of species extinction from the area as the initial dispersal is not just reliant on the successful colonization of a single water body.

In low habitat connectivity scenarios within in the models where connections had not formed a single large network, but instead the network was broken into sections, initial seeding heavily determined how much of the network the species was able to colonize. If only one seeding event occurred, then whichever network island contained the water body of that seeding event became fully colonized while all other remained clear. At the same level of connectivity, but with 10 random seeding events, there was a much greater chance that at least one water body on each network island would have been inhabited. This allowed the species to colonize the entire network, without the need for a fully connected habitat system. Although not built into the model, these habitat islands could act as refuge for the species, where a pond with limited or no current connectivity was protected from the network, which could allow for a slower spread of the species after an extinction event, should one ever take place. More likely in Florida, a constant spread of the species occurs, such that as a particular pond oscillated between favorable conditions, there could always be ciliates introduced to it, which would thrive during a favorable conditions cycle, decrease during certain conditions, and bloom once the water body shifted to most favorable conditions.

The models help to explore potential ways in which this spread may have occurred, and continues to occur, throughout Florida. In addition, this information adds to the theory that even large microbes do not have restricted biogeographies; the question after finding a species in a novel area is "How might it have arrived there? And how could it spread?" The models produced for this project provide a mechanism for further investigation beyond that which can be observed experimentally.

Unlike studies of disease spread, the models are not constructed in order to investigate methods of reducing or illuminating dispersal. With a very definitive niche in the food web, it is almost certain that a species such as *L. rex* becomes a vital part of generating stability within an ecosystem, and is a major supporter in the microbial loop as one of the top single celled predators locally.

6.9.3 The bottle model

This model was created to examine microcosm observations, and to introduce a novel concept that apparently complex ciliate behavior is similar to an agent based model, such that complex behavior can be the result of a very few simple rules which the ciliates follow, and can therefore be shown in an appropriately coded model. By the examination of potential different population parameters in the model allows for the study of many variables and their potential effects on a microcosm of *L. rex* in real life. This model was also valuable in outreach lectures which was an initial motivating factor for its creation, as well as understanding system dynamics within predator prey relationships. Since the discovery of 150,000 cells within a 500mL bottle was so interesting to the biodiversity aspects of the project, it seemed logical to model this here so as to better understand the population variability. This also enhances the dispersal models, since it is these large populations, once they are present within a water body, which are dispersed.

The 40kg of wet weight estimated for a small productive Florida pond for *L. rex* is enough to equal and or outweigh many of the macro predator species within the ecosystem including fish. The importance of this is to appreciate that a species unknown from within the ecosystem may contribute more to an ecosystem in terms of its physical weight than many species which are often considered vital for modeling of various systems. Such large populations affect water clarity, e.g. a Secchi disk will show skewed results such that the water is not murky, but full of dark single cells. When this biomass for a small pond is considered, the implications for the unknown microbial biodiversity, at a global scale, can be envisioned, such that other 'predictive' models may potentially be lacking important biodiversity interactions, such as microbial diversity.

6.10 Conclusions

The hypothesis that models could be a useful addition to the field of microbial ecology was shown to be valid. The theoretically connected networks produced here allowed the exploration of habitat connectivity and time steps which were beyond what could be observed experimentally. The doubling times derived from laboratory data combined with the theories of habitat connectivity in the models suggest that *L. rex* can rapidly spread throughout an entire network, obtaining huge populations numbers. The data collected in the field (Chapter 3) during this PhD support this idea.

Underlying ecologic theories of the importance of the bottom (foundation) of the food chain can also be tested: Since *L. rex* is such a large and obvious target that had gone undocumented until now, what else may thrive in Florida that has been undetected? Previous models in the literature of ecosystem dynamics are perhaps flawed in that they omit this predominant section within the microbial loop, thus influencing the food webs. Questions of tropical ecosystem dynamics and health can also be examined using the spread of *L. rex* as a proxy for cryptic ecosystem biodiversity. These three models suggest how important it is to understand a system's complete biodiversity, as a predominant taxon could be missed and therefore skew the results of what could be a robust model.

Additionally, the bottle microcosm model produced unexpected results which mirrored more closely to the real world observations that was coded in the model (response to light and accumulation layers). Both types of models are useful for understanding ecology both across large areas, and microcosm for longer periods of time than can be observed and manipulated experimentally. Future research in the field should consider collaborative efforts between modelers and microbiologists to enhance the outputs of both sides.

Clearly the point of this is not to decide which type of model is "correct" or which time step is the true value for Florida. Such data are impossible to obtain from this study, and hence the need for models to explore an unknown. The hypothesis was shown to be valid, in that the models created here have provided ample areas in which to explore and discuss potential dispersal mechanisms across a network, and are a valid way forward for the field to advance.

The bottle model has been shown to not only be useful for outreach, but for studying the 'behavior' of ciliates vs agent based ciliates, which were found to be strikingly similar. As such, microcosms can be thought of as systems which can be modeled to produce results which can allow for the rapid understanding behavior and ecological phenomena beyond what is immediately observable.

Chapter 7. CONCLUSIONS, DISSEMINATION AND FUTURE DIRECTIONS

The overarching aim of this study was to investigate the biogeography and biodiversity of flagship ciliates by testing for their presence in Florida freshwater and soil sites, and later investigating their habitats as well as their ecology. An additional goal was to record species which could potentially be new to science discovered throughout the study period. To better understand potentials of ciliate dispersal and colonization, as well as investigate ciliate behavior in microcosm, dynamic models were developed to add understanding and complement the discussion. Throughout the project the results have been presented at numerous scientific conferences and within peer-reviewed journals. In order to reach a more general and varied audience, social media was utilized; an Instagram account was created, and the results are discussed within this chapter. All the aims and objectives set up for this PhD research have been achieved.

7.1 Flagship freshwater ciliates of Florida

The investigation of flagship ciliate dispersal during this PhD research included numerous freshwater habitats of Florida (a geologically recent system), which were explored to investigate the presence of target species including some ciliate flagships originally known only from Africa, for which the literature claims endemism (such as Dragesco 1980; Foissner 1999; Foissner 2006; Foissner et al. 2008). The results of long-term monitoring at four sites, as well as numerous collections in various habitats, have revealed a similar microbial assemblage in Florida equivalent to that previously described in the literature from similar habitats (Dragesco and Dragesco-Kernéis 1986; Foissner et al. 2002). Although by no means an exhaustive effort, the Florida sampling has revealed for the first time the presence of several large and distinctive flagship ciliate species, which perhaps indicate that distance is not a barrier for dispersal in even the largest (>1mm) of ciliates (Esteban et al. 2001; Hines et al. 2016; Hines et al. 2018).

Despite literature arguing for endemism among ciliates, only a small fraction of global habitats has ever been investigated (Finlay et al. 1996; Foissner 1999) and those that are investigated often include only single sampling occasions. When considering the vast areas of habitats, and the few taxonomists active in this field (Dunthorn et al. 2014), the

implication that a ciliate is 'endemic' because it has only been found in one location (Foissner 2004; Stoeck et al. 2007) is more likely to be evidence of undersampling (Caron 2009). Rather than further academic debate on this subject, this idea was tested in this PhD project by long term monitoring throughout Florida which revealed the presence of both, flagship ciliates and novel taxa. This shows that from intensive sampling in a previously unsearched region, ciliates previously thought restricted to a given area can be found at a distant site.

The first recording within the Americas of the flagship ciliate *Loxodes rex* (Hines et al. 2016) highlights the extent of undersampling. The species was recorded within long term monitoring sites, and from both the southernmost and northernmost sites within the State (>500 km distance). This discovery validated the hypothesis that Florida was a suitable habitat in which flagship ciliates can thrive. Similarly, the documentation of the flagship ciliate *Bursaria caudata* from Florida fresh waters was a first record outside of Africa for this species, which was previously known from only one site (Chapter 3). Furthermore, recording the flagship ciliate *Frontonia vesiculosa* in Florida further suggests that these and other large flagship ciliates can thrive wherever they find their preferred ecological niche and are likely to be found throughout the tropics, and possibly beyond.

7.2 Freshwater ciliate species new to science discovered in Florida

Sampling sites investigated during this project ranged between the extreme south of Florida, the very end of continental USA (Everglades National Park), and north close to the border with Georgia. Between these distant locations samples were collected from numerous freshwater sites along the east of the State. A variety of habitats were sampled in the search for flagship ciliates, ranging from artificial drainage canals to farmland ponds and natural swamps. Ciliate species which are new to science were uncovered during extensive sampling, and are recorded here along with site descriptions, morphometrics, micrographs, and gene sequences when possible. Four freshwater species that are potentially new to science were discovered and described (Chapter 4). This validated the hypothesis that species new to science could be detected as sampling efforts in a given area increase. One of the most obviously unique, and the largest of the new species encountered (Colpodea sp. Chapter 4) was observed only once from three samples in a long-term monitoring site. Despite this ciliate species being unmistakable when observed (even with the naked eye) in a single sample, regular collections of over one year from the initial long term monitoring habitat (and over 200 other sites sampled throughout Florida) failed to reveal this ciliate again. Similarly, the

two new species of *Prorodon* were recorded only twice, from a long-term site which was the most investigated habitat during this project. This again highlights issues of undersampling and detection bias, such that even conspicuous ciliates can be missed during an intensive project, suggesting that many other species await discovery.

7.3 Soil ciliates of Florida

The soil habitat inhabited by ciliates is interesting from a dispersal perspective as even though soil habitats between continents are often not connected (e.g. Africa and North America), their ciliate consortia are known to readily form cysts, which, for example increases the chance for dispersal via wind and animal movement.

The discovery of two flagship ciliates in Florida soils, validated the hypothesis that flagship soil ciliates would be found in Florida. The discovery of the bright gold flagship ciliate *Condylostomides etoschensis* in Florida was a first record for this species outside of Africa, its alleged endemic range. The recording of the blue flagship ciliate *Condylostomides coeruleus* was a first record for North America, and the first time these two flagship *Condylostomides* species have been recorded on the same continent. None of the soil flagships were discovered in freshwater habitats, and similarly no freshwater flagship ciliates were recovered within soil cultures. The results from the Florida soil study suggest that flagship taxa can be readily discovered in soils despite a given species being an alleged endemic not previously recorded from the study continent. This investigation also demonstrates how two conspicuous species described previously from distinct continents can be found thriving together, from within a single sample, expanding their biogeography to a new continent (North America).

7.4 Combining dynamic modeling with investigations into ciliate biogeography and ecology

The production of dynamic models for the study of ciliate biogeography had never before been attempted. The hypothesis was shown to be valid due to the discussions that are made possible, which result in further investigation using such models. This included being able to investigate trends and outputs beyond that which could be examined experimentally.

By realizing that the ‘behavior’ of *L. rex*, governed by a few simple rules is analogous to agent based models, a robust microcosm dynamic model was created to investigate behavior and population oscillations. The speed with which different variables could be applied and investigated was extremely useful in understanding the dynamic interactions

possible in a 500 mL bottle; this could be extrapolated to larger habitats than could ever be examined experimentally. Using laboratory-obtained growth rates, colonization potentials of *L. rex* were calculated for a small pond, which can be extrapolated to a wider area. When this is discussed in context of the dynamic models, it suggests a theoretical rapid colonization of the target ciliate species through a connected network. This was in line with what was observed from Florida sites; *L. rex* existed across the state (from south to north), and was detected from within habitats which were previously unfavorable (e.g. drought conditions) but became favorable (e.g. wet) within the short time of this PhD.

7.5 Outreach and dissemination of results through Social Media: Instagram

Much of the research presented in this PhD thesis has been presented to peer scientists at many scientific conferences; however, this type of research generated a large amount of information and images that were of interest to a far broader population, and who do not have access to it via traditional scientific outputs. In order to address this issue involving dissemination of outputs, an Instagram account; microbialecology, was created in 2017 to share some of the micrographs and videos generated during this PhD. In the two years since starting the account it has amassed a global audience of over 65,000 followers at the time of writing. The content has, at times, been viewed over 1 million times in a single week.

Social media is an emergent area in science communication, with apps such as Twitter (López-Goñi and Sánchez-Angulo 2017) increasingly being used to communicate science. Instagram is an image-based app which has been shown to be increasingly important in science outreach, particularly with microbiology (Hines and Warring 2019). It was clear that while some viewers had familiarity with classic examples of microbes (e.g. protozoa such as ciliates), most were seeing these organisms as living, moving creatures for the first time. By sharing the microbial images and providing explanations about the organisms, a global audience became actively engaged with the research field examined during this PhD. Followers of the account range from specialists in the field to interested public who have likely never had access to research grade equipment.

Posts can be surprisingly far reaching when they are marked by Instagram algorithms as of interest to a wider viewership: the microbialecology account for example had over 1.4 million impressions in a single week at the end of 2018 (Figure 7.1). The algorithm works like a positive feedback loop (Hines and Warring 2019) such that the more a post is seen and engaged with, the more users it will be shown to. Any number of posts can be picked up by

international media outlets, which further disseminates the research on social media, and also beyond the platform, such as National Geographic (Russia), and The Daily Mail (UK).

The proprietary algorithms used by Instagram are not publicly available. Predicting with great accuracy what will go 'viral' (with the help of the algorithm) is therefore impossible, and one can only create posts based on a feel for the type of content that may be popular. Understanding the audience for one's account and the content which is best received is accomplished through trial and error. Short videos of microbial organisms with a 'cute' appearance (e.g. fat Playthelminthes), a well-known and interesting reputation (e.g. Tardigrade), or a unique form of movement (e.g. the ciliate *Dileptus* or *Bursaria*) often do very well on the microbialecology account. Photos of common ciliates (e.g. *Paramecium*) do poorly compared to less known examples, such as the ciliate *Loxodes rex*, which do quite well.

Posting content related to textbook phenomena is also a productive way to link into the interests of educators and students alike. During sampling in Florida, mixotrophic ciliates were often encountered which clearly show the relationship between an alga and a ciliate host. In general, symbiotic relationships in ecology are well documented but perhaps less so for ciliates in popular media. These posts often receive a large viewership, increased in part because the cells (e.g. *Frontonia vernalis*) were visually appealing.

Image content is constantly created as a result of PhD research. Social media outlets such as Instagram are an easy way in which to share this content with an increasingly global audience. Many of the followers of microbialecology account are from developing nations, and they can freely access the content so long as they have internet. The potential to inspire anyone, anywhere, to investigate something they find interesting within science was the ultimate goal of creating the account and has thus far proven successful.

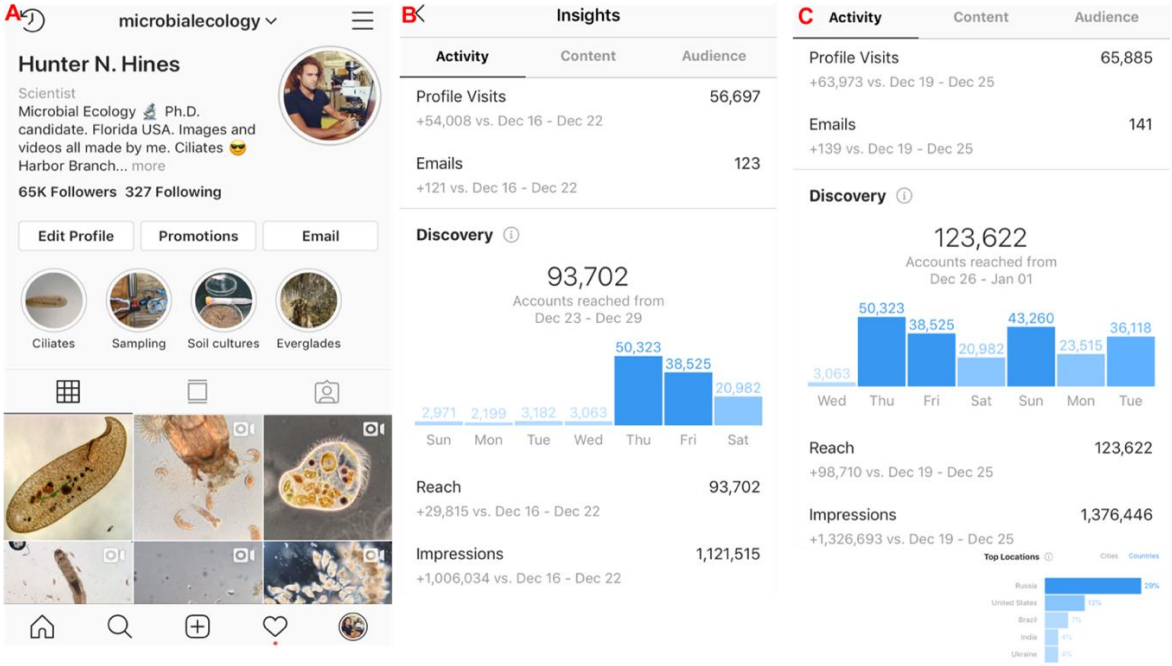

Figure 7.1. Screen shots from the microbialecology Instagram account.

A: screen shot of the microbialecology Instagram profile, taken 22nd March 2019.

B: screen shot from the data generated by Instagram for the microbialecology account during the dates of December 23-29, 2018. The jump in numbers of accounts reached (see bar graph) is typical when a post(s) goes 'viral' e.g. from 3,000 to 50,000 overnight. The 'impressions' on these dates was just over 1 million.

C: data provided by Instagram for the account in the next week continuing from B. The impression numbers would go to over 1.4 million during this week. Note the graph at bottom right which listed the current top 5 countries of followers' locations: Russia, United States, Brazil, India, and Ukraine.

7.6 Overall conclusions

Despite some of these ciliate species having been originally described quite some time ago in the literature (Da Cunha 1914; Dragesco 1970), they lacked basic data necessary for further investigations. Missing data for these interesting species was produced during this PhD. These data include the first ever published micrographs for the flagship ciliate *Bursaria caudata*, which was previously only known from drawings. The first *in vivo* images of *Frontonia vesiculosa*, and first detailed *in vivo* images for *L. rex* are also recorded here, as

well as details of habitat metadata and ecology, which were lacking from the original descriptions.

Overall 14 sequences (of the 18SrRNA gene) were deposited into GenBank during this PhD. These novel 18S rRNA sequences include: the first data for flagship ciliates *Loxodes rex*, and *Condylostomides etoschensis*; the first sequence of *C. coeruleus* from North America; the first sequence from the genus *Pseudoblepharisma*; and sequences for a freshwater *Sonderia* species new to science, closely related to marine congeners, some of which are from the deep sea.

The dynamic models revealed numerous areas to investigate in ciliate biogeography, which suggest times for dispersal are rapid for this group due to large population numbers and rapid division rates. Similarly, the microcosm model of *L. rex* explained some observed behavior (e.g. light reaction) and highlighted the importance of it inhabiting its preferred ecological zone of 5-10% O_2 in respect to predator prey interactions. Population oscillations could be run in the models, with large time steps far beyond that which could be examined in the laboratory.

Flagship ciliates are capable of dispersal to global regions, including North America, despite being previously thought to be restricted to a given area or continent. Undersampling of global habitats must be the main reason for these taxa not previously being recorded. The discovery of numerous freshwater species new to science further suggest that this, and many other areas of the planet, contain suitable habitats for interesting ciliate species that await discovery. Flagship ciliate species were also found to thrive in Florida beyond their previously alleged restricted ranges and are likely to thrive wherever they encounter a suitable habitat. Dynamic modeling was shown to be useful in understanding and visualizing some mechanisms of dispersal, and agent based models are suggested to be useful in the exploration of ciliate populations and behavior, particularly in a microcosm environment.

7.7 Limitations of study

When considering the vast areas of global habitats, and the few taxonomists and ecologists active in this field, the implication that a ciliate species is endemic because it was not (yet) recorded in global samples from any type of region (Foissner 2004; Stoeck et al. 2007) is only evidence for under sampling. Even though 1 million ciliates may be present in a small pond, they may never be detected even after years of sampling (Finlay et al. 2004), and short-

term diversity studies may miss taxa which are extremely common but simply happen to be absent at the time of sampling or not detected within the small subsamples. When considering the large number of potential habitats across a region such as Florida, there is likelihood that many species were missed, especially those that are truly rare in the environment.

Site access was a limiting factor around Florida, as many potentially interesting sites are not accessible due to private property (e.g. restricted land around airports, extensive farmlands). Actual physical access to a given water body in Florida can prove challenging, due to rough terrain and dense thorny and toxic vegetation, particularly in unmanaged areas. Florida also contains numerous venomous snakes, wild boar, large alligators, and biting insects, which all contribute to shortening scouting and sampling trips.

Sequencing of some species was unsuccessful for two major reasons: some ciliates are rarely found and so material could not be used for the isolation of DNA; for other species, even though DNA could be isolated, amplification was not successful. This may have been due to the presence of PCR inhibitors; however, this could not be overcome by the numerous basic changes made to the protocol.

7.8 Future directions for research

The investigation of Florida flagship ciliates raises some further questions: the function of some morphological features, specifically pigmentation; the absence of contractile vacuoles; and the potential for conjugation between cells from a wide biogeography.

The pigmentation observed in Florida *L. rex* and *F. vesiculosa* matches those described in the literature (Dragesco 1970), and is perhaps a defence against UV damage or strong light from the sun. The chemical makeup of these pigments in flagship ciliates is as yet unexplored. The bright gold and blue pigmentations observed in the cortical granules of the soil *Condylostomides* species is of an unknown function, but has clearly evolved with some significance in survival. Further investigation into the functionality and composition of ciliate pigments is a future research avenue to explore, with questions of ciliate survival under environmental challenges being addressed especially if pigmentation can be experimentally manipulated.

Contractile vacuoles are organelles found in ciliates from the marine environment (Modeo et al. 2013) as well as commonly in freshwaters (Dragesco 1970). In order to counteract cellular swelling caused by an inwardly directed osmotic gradient, the contractile

vacuole(s) expels fluid from the ciliate at varying rates (Patterson 1980). The absence of contractile vacuoles in some ciliates, including from some of the large ciliates encountered in Florida (e.g. *L. rex*), raises questions about how they may maintain osmoregulation, and whether it is through a different mechanism than ciliates with conspicuous contractile vacuoles (e.g. *F. vesiculosa*). In the case of *L. rex*, the highly vacuolised cytoplasm and the flatness of the cell may act in a similar way to a traditional contractile vacuole, as could the large oral aperture. It is also possible that the osmotic pressures do not hinder the cell in surviving long enough to reproduce.

With advances in molecular identification of ciliates, an additional question to explore is whether the same ciliate species separated by wide geographical distances, but of exacting morphologies and molecular signatures, would readily conjugate, and what the result of this might be at a morphological and deeper molecular level. The research described in this thesis provides the baseline data for future work from within other biogeographies.

An interesting area to explore beyond this study would be whether or not, due to a potentially large dispersal and seed bank of species, a given site far outside of the tropics that goes above a warm (e.g. 28°C) water barrier, even temporarily, could then harbor an alleged restricted tropical freshwater species. These temporary tropical habitat 'islands' would further facilitate the global distribution of species. Indeed, the studies of *L. rex* have shown that this (thus far only warm habitat) species can withstand cold events down to 9°C in Florida (Hines et al. 2016). Despite thriving in warm habitats, this does not necessarily mean that the species is immediately destroyed when it leaves this preferred ecological zone. Sampling areas of northern North America (e.g. Delaware), which have extreme seasons (ice and snow covering in winter, and >37°C in summer) could yield interesting results for targeting freshwater flagship ciliates in seasons both within and outside of their preferred niches.

With this same idea in mind, flagship soil ciliates can also be investigated outside of their potential range. Since this group readily forms cysts, it is possible a 'tropical' species could disperse to any northern areas, and cultures could be made which mimic the preferred niche to see if they can be recorded from these ranges, despite distances.

Overall the Florida ciliate morphological and molecular data will provide the underpinning for future researchers to compare their ciliates to from a wide biogeography. All the original

ciliate research in Africa predates the molecular era (e.g. Dragesco 1970) and sparse research has been undertaken there since. Obtaining molecular sequences from flagship taxa from their originally documented site, such as *L. rex* from Africa, would provide interesting data to compare with the Florida sequences. In this respect, documenting *L. rex* from South America (e.g. Brazil) would provide data from another continent, allowing these to be compared throughout the tropics at a global level. Several other flagship species are good candidates for this line of investigation, including *Frontonia vesiculosa*, for which the Florida strain is an exact match to the South American and African records, but has yet to be sequenced. Investigating these flagship ciliates at a global level for both morphological and molecular signatures will help to better understand microbial dispersal and biogeography of these interesting ciliates.

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APPENDIX

Papers published during the course of the project:

Hines, H., McCarthy, P. and Esteban, G., 2016. The first record for the Americas of *Loxodes rex*, a flagship ciliate with an alleged restricted biogeography. *Microbial Ecology*, 71 (1), 5-8.

Hines, H. and Esteban, G., 2017. Report of the 2016 Protistology-UK spring meeting. *European Journal of Protistology*, 58, 195-197.

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MICROBIOLOGY OF AQUATIC SYSTEMS

The First Record for the Americas of *Loxodes rex*, a Flagship Ciliate with an Alleged Restricted Biogeography

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Abstract As the foundations of food webs, protozoa are essential to the success of an ecological system. These organisms are often overlooked, and research in the Americas is sparse. Recent samplings conducted in freshwater canals and ponds in Florida, USA, have revealed *Loxodes rex*, an alleged endemic ciliate species. Originally described as endemic to tropical Africa, *L. rex* has been considered a prime candidate for proof of microbial endemism. Our studies have shown this giant, non-encysting ciliate to be thriving in subtropical Florida. Our observations are novel and include both the first record of occurrence for the Americas and the first high-quality in vivo images for this charismatic species.

Keywords Biogeography · Ciliate · Endemism · Florida · *Loxodes rex* · Protozoa

Introduction

Most large animals (e.g., mammals) show restricted biogeographies and endemism. There exists an unknown size range under which all species dispersals are probably ubiquitous (e.g., bacteria). Protists, including the large tropical ciliates, probably lie below this barrier of endemism [1]. If this is the case, wherever the specific environmental parameters for their growth exist, and adequate dispersal time has passed, then any range of ciliates would be found in the system, despite distances of thousands of kilometers between habitats. Microbial populations are so large, and distribution potential at a global level is so prevalent, that protozoan dispersal may not be affected by physical barriers [2, 3].

Loxodes rex (Protozoa, Ciliophora, Karyorelictea) is a giant (>1 mm) non-encysting, unicellular eukaryotic organism that actively moves in a freshwater environment as a voracious predator. The beating of ciliary rows enables the cell to seek out its preferred microhabitat within an ecosystem. An organelle unique to the class is the Müller body, a statocyst-like organelle that informs the cell of its position relative to the gravity vector [4].

L. rex has been listed as a flagship species and identified as one of the ultimate proofs for the theory of microbial endemism [5–7]. This ciliate was originally described as endemic to tropical African countries such as Cameroon and Uganda [8, 9] but later found thriving in Thailand [10].

Methods

Water samples were collected from within a ~2-km stagnant section of a freshwater canal system in the watershed of the Indian River Lagoon, Florida, USA (27°31′56.3″N 80°23′54.1″W). The canal is ~7 m wide, at least 50 years old, and has had little if any management after completion. Samples were collected from the bottom layer sediment interface (~60 cm) of the canal, using a weighted and corked 500-ml glass sample bottle on a line. The samples were taken four times per week during the months of October 2014 to January 2015, with *L. rex* always being found, often in high densities

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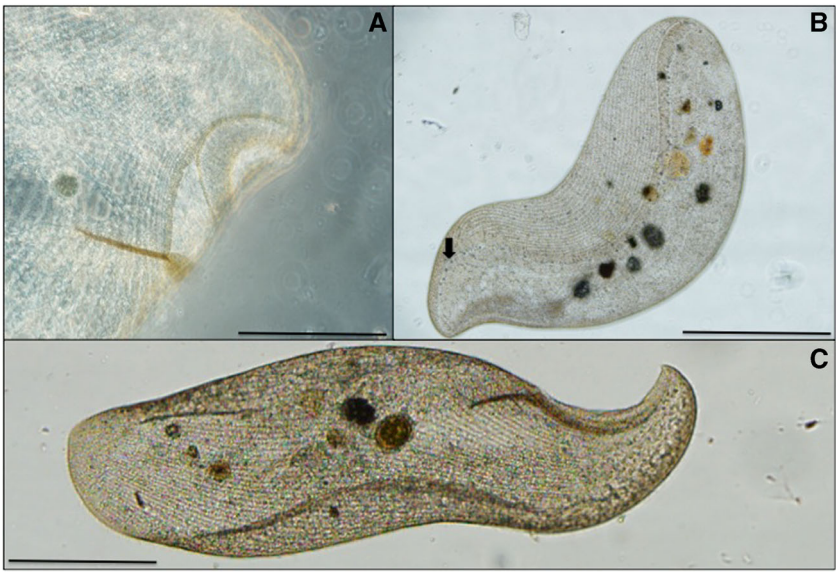

Fig. 1 *Loxodes rex*, a flagship free-living ciliate species. In vivo image of cells found thriving in Florida, USA, greatly expanding the known biogeographic range (see text for further details). **a** Oral aperture of *L. rex*. Scale bar 100 μm. **b** Detail of ciliary rows and the line of Müller bodies down the length of cell (see *arrow*). Scale bar 250 μm. **c** Image of swimming cell, showing a large number of ciliary rows diagnostic to the species. Scale bar 250 μm

of up to 10 cells per 1-mL subsample. Water temperatures ranged between 21 and 29 °C.

For the identification of further habitat characteristics, a handheld YSI 63 sensor was deployed within the system. Temperature, pH, salinity, and conductivity were obtained at various points throughout the water column in the habitat that contained *L. rex*. A second device, a ProODO, was used to measure dissolved oxygen content throughout the water column and was tested in several points in the habitat.

Subsamples from the canal water were taken from the bottles using sterile pipets. These were examined within a 1-mL glass Sedgewick-Rafter counting chamber using an Olympus BX-53 light microscope. Cells were further picked and pipetted onto a welled microscope slide for observations of greater detail. Cell measurements were carried out, and digital images were created and analyzed using the Olympus CellSens software package. More than 500 cells were measured. All aspects of this study were performed on living cells.

Results and Discussion

Initial studies revealed the presence of an abundant ciliate population in the water samples. The giant ciliate observed was flattened, with a concave and anterior oral aperture situated on a rim, forming a beak-like hook so that the ciliate has a clear left and right side (Figs. 1 and 2). The right side is ciliated, with dikinetids arranged in bipolar kineties; the left

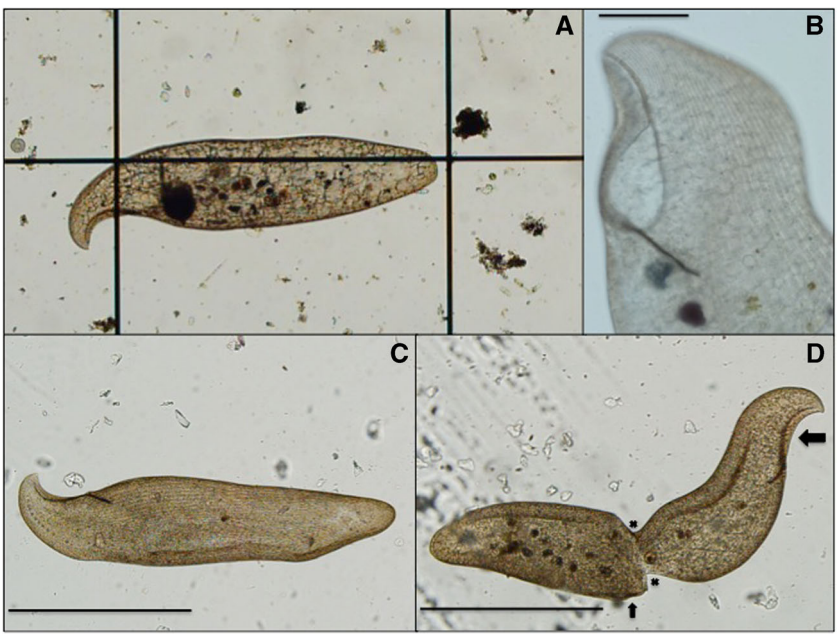

Fig. 2 **a** View of living *Loxodes rex*. *Center vertical lines* of the chamber are 1000 μm apart. **b** Open oral aperture (*top left*) with ciliary row detail. Scale bar 100 μm. **c** Swimming *L. rex* with numerous ciliary rows. Note the row of Müller bodies at the bottom left which runs parallel to the oral aperture. Scale bar 500 μm. **d** Image of a dividing cell. The cell at the *right* is the original cell, and the cell at the *left* is the newly formed cell, with division area indicated by *X*'s. Note difference in oral aperture development from complete at the right (*large arrow*) to new formation at the middle right (*small arrow*). Scale bar 500 μm

Table 1 Comparison from the literature of original *Loxodes rex* description from Africa [8, 9] with our current findings of *L. rex* in Florida, USA. The strain from Florida is larger, with the largest cell recorded. More than 500 cells were measured for this study

	Loxodes rex (Africa)	*Loxodes rex* (Florida)
Number of kineties	79–84	~80
Number of Müller bodies	~60	45–60
Number of macronuclei	132–181 (average 150)	~150
Number of micronuclei	39–138 (average 67)	~70
Length range	500–1200 μm	550–1350 μm
Length average	750 μm	835 μm
Width average	250 μm	205 μm
Color	Dark brown	Dark brown

side bears only two bipolar kineties, and the posterior end is rounded. The Müller bodies can be found in a lengthwise row down the left side of the cell body starting with ~10 opposite the oral aperture, with an additional ~40 throughout the cytoplasm along this line. This is diagnostic to the ciliate *L. rex* [9] (Table 1).

The YSI devices showed that the pH, conductivity, and temperature varied little from the surface to the bottom (<60 cm) of the habitat. Values of pH 6.6, 0.5‰ salinity, and 22 °C were obtained during a normal sample period. Dissolved oxygen content varied dramatically within the vertical system, with the highest values at the surface. The surface zone had levels between 62–65 % dissolved oxygen (DO) saturation and 5.44–5.66 mg/L DO, which was due to the high concentration of aquatic plant life such as *Lemnoideae*. The bottom zone (where *L. rex* is found in the greatest numbers) was confirmed to be nearly anoxic; with dissolved oxygen levels between 2.2–2.8 % DO saturation and 0.19–0.24 mg/L.

L. rex was found to thrive in a freshwater, mostly stagnant, canal within the Indian River Lagoon watershed and also three surrounding ponds. Our findings of *L. rex* living in subtropical Florida are novel and significantly expand the global distribution range for this organism. At a distance of over 10,000 km from the known African habitat range, the Florida *L. rex* strain indicates that dispersal is not a limiting issue for the species, which probably thrives wherever it finds its preferred ecological habitat.

Although sampled from the oxygen-depleted layers of shallow freshwater systems, the true ecological niche for *L. rex* is not yet known, as the ecosystem present in Florida is analogous to the original African sites. We have found other large ciliates with alleged restricted distributions [5, 9] such as *Frontonia vesiculosa* in the same Florida site (work in progress).

Our confirmation that *L. rex* inhabits the oxic/anoxic zone within the water column is consistent with the *Loxodes* literature, stating the cells prefer a habitat with oxygen tensions between 5 and 10 % [4]. In vivo observations revealed that the species is extremely sensitive to vibrations, with cells rapidly contracting in size after a stimulus. Light level increase also appears to affect *L. rex* swimming behavior, as with other *Loxodes* species [4].

Further studies of the US populations of this and other large-sized ciliates will enhance our understanding of the potential distribution for ciliate species, and by extrapolation of other microbes, particularly protists. A 3-day cold weather event in South Florida (air temp ~4 °C) caused water temperatures at the study site to drop to 9 °C. Samples were taken throughout this period and revealed that *L. rex* survived, in low population densities, during this cold period. High abundances rapidly recovered in both the habitat and laboratory cultures once temperatures increased to at least 20 °C. The *L. rex* tolerance range remains unknown, but these initial results suggest that the species can tolerate zones outside of the tropics, thereby expanding its potential global distribution.

The difficulty with which the microbial biome is elucidated from any number of sample areas contributes to the idea that perceived microbial endemism is simply a result of the unexplored—not the nonexistent. As more sites are sampled, more specimens and therefore greater ecological understanding of *L. rex* can be obtained, including its detailed niche and dispersal potentials. As more habitats are examined, additional so-called "flagship" ciliates will have their biogeographic status expanded and changed.

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Conference

Report of the 2016 *Protistology-UK* Spring meeting

The 2016 Spring meeting was a double celebration. First was the change of the Society's name from *British Society for Protist Biology* to the more snazzy (and shorter!) *Protistology-UK*. Second was the actual meeting of the newly-named Society, which was held at Bournemouth University (UK) on 6–8th April and hosted by Genoveva Esteban. The meeting consisted of a plenary lecture and three symposia—one of which was a special workshop sponsored by *PhycoNet* (a Biotechnology and Biological Sciences Research Council [UK] Network in Industrial Biotechnology and Bioenergy). There were 50 scientists and graduate students in attendance; an assemblage of protist enthusiasts from all over the UK and several additional countries from three different continents including Europe, North America and Asia. The meeting was sponsored by *PhycoNet*, the *Microbiology Society* (UK), *Elsevier* and Bournemouth University (UK).

The meeting began not with lectures, but with a field trip to East Stoke Nature Reserve in Dorset (south of England), a freshwater fen habitat which is the subject of ongoing protistological research, and a hot spot of protist diversity. The visit commemorated the announcement that the site will be protected due to its intriguing microbial communities found within, a first for the UK. Visitors were treated to a guided tour by Genoveva Esteban and a conservation officer from the Dorset Wildlife Trust; an information board (Fig. 1) was revealed highlighting the importance of the site for microbial life. Infamous British weather was ever present, so as to give visitors an authentic experience of fieldwork in the UK.

The plenary lecture was delivered on the opening evening by Gill Malin (University of East Anglia, Norwich, UK) which was a timely summary of a changing ocean and the implication in regards to protist ecology. The talk focused on DMSP (dimethylsulphoniopropionate), a zwitterion found within the cells of protists. DMSP is the main precursor for dimethylsulphide (DMS), a 'trace-gas' that plays an important role in the global sulphur cycle via sea-air exchange, transferring sulphur to terrestrial and freshwater environments where it can be a limiting nutrient. DMS is an area of ongoing research involving a way in which the earth's system is cooled via production of aerosol reflective particles.

Fig. 1. Genoveva Esteban and Alan Warren (forefront of the image) unveiling the protists information board at East Stoke Nature Reserve (UK).

The talk explored how such research could predict oceanic change.

Three symposia were organised, the first of which focused on *Eukaryote taxonomy and diversity studies in an High Throughput Sequencing (HTS) era,* chaired by David Bass (Natural History Museum, London, and Centre for Environment, Fisheries and Aquaculture Science [Cefas]).

Presentations in this session began with back-to-back talks by Cedric Berney (CNRS & UPMC, Station Biologique, Roscoff, France) who summarised an international collaborative project proposing an integrative and dynamic database framework, such as *Unieuk* and *Eukref* to bring together and curate the big molecular data that is being produced, working as a common language in protistology. The second talk presented the *Tara Oceans* project and some of its exciting results, including the thousands upon thousands of protist lineages/genetic 'types' being recovered from the world's oceans. The proposed Unieuk/Eukref will hopefully bring understanding and order to this, and we all look forward to it.

Micah Dunthorn (University of Kaiserslautern, Germany) gave an enlightened talk on his molecular diversity studies of

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0932-4739/

soils in Neotropical rainforests—a geographical area that is largely unexplored as far as prostists are concerned. Micah reported a novel approach for examining sequence reads, many found as having little similarity to the known databases. This molecular approach suggests that Apicomplexan parasites dominate the protistan soil communities in the tropical areas, which was equally diverse in the communities thus far examined.

Michael Cunliffe (Plymouth University, UK) spoke on the developing field of HTS, which reveals marine protist and fungi diversity at the molecular level, and what HTS diversity actually means, its use and misuse in understanding microbial eukaryote diversity. In particular, Michael showed a few examples linking HTS-derived diversity data with microscope-derived data, and the value of combining HTS with environmental metadata.

The final talk in the session was delivered by Grant D. Stentiford (Cefas, UK) who spoke from an agency perspective on the importance of a clear taxonomy on issues such as UK food security (e.g. parasite identification and ranges). The use of morphological species identification increased molecular resolution should improve our understanding of the moving target of the anthropogenic species concept. This is important in that UK regulations (as well as global) on import/export based upon a particular parasite must have a clear identifying marker so as to be useful in understanding its presence or absence from a given region, such that policy in place must not be hindered by a changing species identification.

Day two contained the second Symposium entitled *Eukaryote Genome*, chaired by Demetra Andreou (Bournemouth University). This section included invited talks by Ross F. Waller (Cambridge University) who introduced Dinoflagellates as a new and fascinating way in which to study chromatin structure. The talk explored the radically different nuclear organisation in this phylum in regards to other eukaryotes. Ross' lectures are always aesthetically very pleasing indeed—and this one on dinoflagellates was no exception.

Bryony A.P. Williams (Exeter University, UK) spoke about microsporidian parasites, their environmental genomics, providing insights into metabolism and host exploitation. She highlighted the host specificity of some members in the group, as well as the actual locations where they inhabit the host cell, and the importance of sequencing genomes for the identification of molecular signatures of intracellular organisms.

William H. Lewis (PhD student at Newcastle University [M. Embley and R. Hirt] and Bournemouth University [G. Esteban], UK) spoke about his research on anaerobic ciliate genomes to understand endosymbiotic archaea and the diminishing genomes of their ciliate hosts' hydrogenosomes ('anaerobic' mitochondria). Ciliates are an excellent model to study hydrogenesomes from a molecular, evolutionary and ecological perspective. Will uses single-cell genome and transcriptome sequencing of the ciliates, their organelles and their endosymbionts. The talk emphasised the importance of deep sequencing to answer questions of evolution and ecology.

Tom A. Williams (Bristol University, UK) gave insight into the widest to date population genomic survey of *Microsporidium*. High genomic streamlining in ancestors of this group is contrasted with the apparent specialisation from different world regions sampled. The enormity of such a project is obvious while the ending target will be genetic resolution to the population level.

The symposium concluded with '*contributed and students*' talks starting with Gareth Bloomfield (Cambridge University, UK) who discussed his project on the origins of macromycetozoa. These 'slime moulds' exhibit evolution that remains unclear. Gareth looked at fruiting bodies as a way to examine such theories which included an interesting observation of a cannibalistic amoeba interaction. Martin Carr (University of Huddersfield, UK) spoke about Choanoflagellates as an analog to study the evolution of animals, with comparisons of missing genes between these groups and the functional ecological significance for protein synthesis. A higher than expected morphological diversity of the group was reported and interestingly few freshwater–salt water incursions were identified.

The third symposium was on *Protist Ecology* and was chaired by Daniel Franklin (Bournemouth University, UK), which included the invited talk by David J.S. Montagnes (Liverpool University, UK) about potential thermal sensitivities of phagotrophic protists using a study model for consumer–prey dynamics to study how warming environments could affect predator–prey interactions. Important areas such as cyanobacteria blooms and the thermal tolerance of mixotrophic eukaryotes were explored.

Manuela Hartmann (Southampton University, UK) spoke about the importance of the smallest phagotrophic phytoplankton (the picoplankton) in the Arctic, a little-known protist group but one of great ubiquitous ecological significance for the open ocean. CO_2 fixation and bacterivory were examined, with this research highlighting the importance of mixotrophy as an important survival strategy globally, not just in the tropics.

The contributed and student talks concluded the section first with Lars Grossmann, (University of Duisburg-Essen, Germany) who spoke about HTS and the similarities of communities using standardised sampling techniques. The distinct habitat types were found to contain distinct microbial assemblages.

The session concluded with PhD student Hunter N. Hines (Bournemouth University, UK, and Harbor Branch Oceanographic Institute, USA) who gave a lecture on his discovery of flagship ciliates in Florida, many being new records of occurrence. This research further confirms the notion that microbial diversity is unexplored globally, and that biodiversity and biogeography of large ciliate species such as *Loxodes rex* offer to continue research into microbial eukaryote global distribution and dispersal. Hunter won the Protistology-UK prize for best student's presentation.

The third and final day of the conference held an *Applied Phycology Workshop* sponsored by *PhycoNet*. The session was introduced by John Day from the Culture Collection of Algae and Protozoa, Scottish Association for Marine Science, UK. First up was Saul Purton (University College London, UK), who gave an overview of *PhycoNet*—an UK-based network enabling biologists, engineers and industrial partners to consolidate their knowledge and expertise to unlock the industrial biotechnology potential of microalgae. Saul also gave a lecture about the therapeutic proteins synthesised from the model flagellated algae *Chlamydomonas* after genetic engineering. Antibacterial enzymes to target human pathogens, and bio-encapsulated vaccines for oral delivery in aquaculture and poultry industries were discussed.

A sticky business—development of efficient biofilm photobioreactors for microalgae was presented by Björn Podola (University of Köln, Germany), explaining the difficulties in the production of microalgae at industrial scale. Björn illustrated a new technology to achieve exactly that by using porous substrate bioreactors (PSBRs), which achieve great algal yield using vertical surfaces of biofilm cultures and significant reduction of liquid volume.

Brenda Parker (University College London, UK) gave a talk on the exploration of the economic feasibility of protist processing as a biofuel. The marine diatom *Phaeodactylum tricornutum* was examined at all stages for economic recovery, and novel large scale cost-effective production methods were discussed from a biological as well as economic perspective. John Love (Exeter University, UK) gave a talk on algal microfossils and their use as proxies to reconstruct past environments. Love and his group have developed new protocols using Fluorescent Activated Cell Sorting (FACS) that allows rapid analysis and purification of microfossils as well as enabling for molecular sequencing. This method can help to explore past protist communities of water bodies, and compare to modern analogues with regards to environmental change. Alla Silkina (Swansea University, UK) gave an overview on the use of microalgal pigments in industry, e.g. as antioxidants, immune-system boosters and as colourants; Alla also presented novel production-process technologies for a range of high value pigments.

In addition to the lectures, there were poster sessions for the Protistology-UK meeting attendees to view the work of, and interact with students. The meeting concluded with final remarks by David Bass (current President of the Society) on the importance of protists in ecology, taxonomy, biotechnology and science in general, and the announcement that the 2017 meeting will take place during the Annual Conference of the Microbiology Society, 3–6 April, in Edinburgh (Scotland, UK).

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ORIGINAL PAPER

Molecular Investigation of the Ciliate *Spirostomum semivirescens*, with First Transcriptome and New Geographical Records

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The ciliate *Spirostomum semivirescens* is a large freshwater protist densely packed with endosymbiotic algae and capable of building a protective coating from surrounding particles. The species has been rarely recorded and it lacks any molecular investigations. We obtained such data from *S. semivirescens* isolated in the UK and Sweden. Using single-cell RNA sequencing of isolates from both countries, the transcriptome of *S. semivirescens* was generated. A phylogenetic analysis identified *S. semivirescens* as a close relative to *S. minus*. Additionally, rRNA sequence analysis of the green algal endosymbiont revealed that it is closely related to *Chlorella vulgaris*. Along with the molecular species identification, an analysis of the ciliates' stop codons was carried out, which revealed a relationship where TGA stop codon frequency decreased with increasing gene expression levels. The observed codon bias suggests that *S. semivirescens* could be in an early stage of reassigning the TGA stop codon. Analysis of the transcriptome indicates that *S. semivirescens* potentially uses rhodoquinol-dependent fumarate reduction to respire in the oxygen-depleted habitats where it lives. The data also shows that despite large geographical distances (over 1,600 km) between the sampling sites investigated, a morphologically-identical species can share an exact molecular signature, suggesting that some ciliate species, even those over 1 mm in size, could have a global biogeographical distribution.

Key words: Protist; stop codon; RNA-seq; anaerobic respiration; symbiotic algae; Heterotrich.

Introduction

The genus *Spirostomum* Ehrenberg, 1834, currently comprises eight species of ciliates found globally in fresh and brackish water habitats (Boscaro et al. 2014). These single-celled eukary-

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otes can be found in high abundances, and some species can obtain body sizes that are visible to the naked eye, e.g. *S. ambiguum*. The ciliate *S. semivirescens* is a large (usually over 1 mm in length) protist with densely-packed endosymbiotic green algae that resemble *Chlorella* (Esteban et al. 2009a; Kreutz and Foissner 2006). Despite its large size and conspicuous bright green color, it is still largely absent from published global ciliate species lists, with only a few sparse records of the species. This makes the species an ideal candidate in which to investigate its biogeography. Neither the algal endosymbiont nor the ciliate host have benefitted from molecular examinations, even though this is an active area of research for other species of ciliates, especially *Paramecium bursaria* (Hoshina and Imamura 2008; Hoshina and Kusuoka 2016).

Different ways to adapt to anoxic environments have been described among ciliate species (Esteban et al. 2009b; Finlay et al. 1983; Yarlett et al. 1981). Ciliates are also known for their wide diversity of genetic codes, where stop codons are recoded to be translated into amino acids (Knight et al. 2001). To get insight into how such traits have evolved, large-scale data sets, which cover the whole genome content of the species of interest, are needed. In this study we generate such data by RNA sequencing at the single cell level. *S. semivirescens* was specifically targeted, as it has been missing from earlier examinations of this well-studied genus (Boscaro et al. 2014; Shazib et al. 2014). In the research presented here, *S. semivirescens* was isolated from freshwater habitats in the UK and Sweden. Transcriptome data was also generated from another *Spirostomum* species to complement our investigation into *S. semivirescens*. Data generated in this study is a necessary piece for improved understanding of the *Spirostomum* genus and the whole suborder Heterotricha. Molecular data for *S. semivirescens* is provided for the first time, along with the first molecular identification of the symbiotic algae associated with this species.

Results

The *Spirostomum semivirescens* found thriving in the UK's anoxic ditch sediments matched exactly the previously described records of occurrence and morphology from a fen pond ~100 meters away (Esteban et al. 2009a). Densities of up to 15 cells per mL were observed, with cells being maintained in natural samples for one week after collection. When left undisturbed for about one hour the ciliate builds an external case or coating; the ciliate is contractile, and retracts into the casing if disturbed. This could provide protection during a dispersal event (e.g. wind, birds). *S. semivirescens* was not observed to form cysts; however, there are records of other *Spirostomum* species being able to form cyst precursors (Ford 1986, and own observations in cultures of *S. ambiguum*). Cells were always found to be densely packed with bright green endosymbiotic algae (Fig. 1).

S. semivirescens from Swedish study sites was immediately identified from the freshly-collected samples from both locations as being morphologically identical to the UK strain, and the additional diagnostic literature (Esteban et al. 2009a). *S. semivirescens* was found to be 800–1,500 μm in length and 25–45 μm in width with more than 50 cells being measured (Fig. 1). Densities of up to 30 cells per mL were observed, but more often were found to be 5 per mL from both locations, each showing productive ciliate concentrations, with green *Frontonia* reaching up to ~1,000 per mL, especially from an algal mat sampled in Stadsskogen. The *S. semivirescens* cells were observed to build a loose casing, be contractile, and always densely-packed with endosymbiotic green algae. The casing observed in the Swedish specimens of *S. semivirescens* was larger (wider) and less densely packed than observed in the UK, perhaps due to different composition of available sediments and/or to the length of time that the ciliate samples were left undisturbed, allowing them to build a larger protective coat. The samples were collected during a warm period in August 2015, but *S. semivirescens* was later found to thrive during much colder periods in winter, even being regularly recovered from the habitat under a ~15 cm thick layer of ice.

Sequencing and Transcriptome Quality

For all seven transcriptomes (Table 1) a total of 9.3 Gb sequencing data was generated. Low levels of contamination were indicated by MEGAN that assigned less than 5% of the contigs as prokaryotic in each assembly. Less than 4% of the contigs were classified as Viridiplantae, despite the high number of algal endosymbionts in *S. semivirescens*. For 17% of the 23,933 transcripts in the co-assembly more than 10 reads from each of the six *S. semivirescens* mapped and for 49% of the transcripts 10 reads or more from at least three different replicates mapped. Based on this level of consistency between the transcriptomes and the similar relative expression level of transcripts between replicates (Supplementary Material Fig.

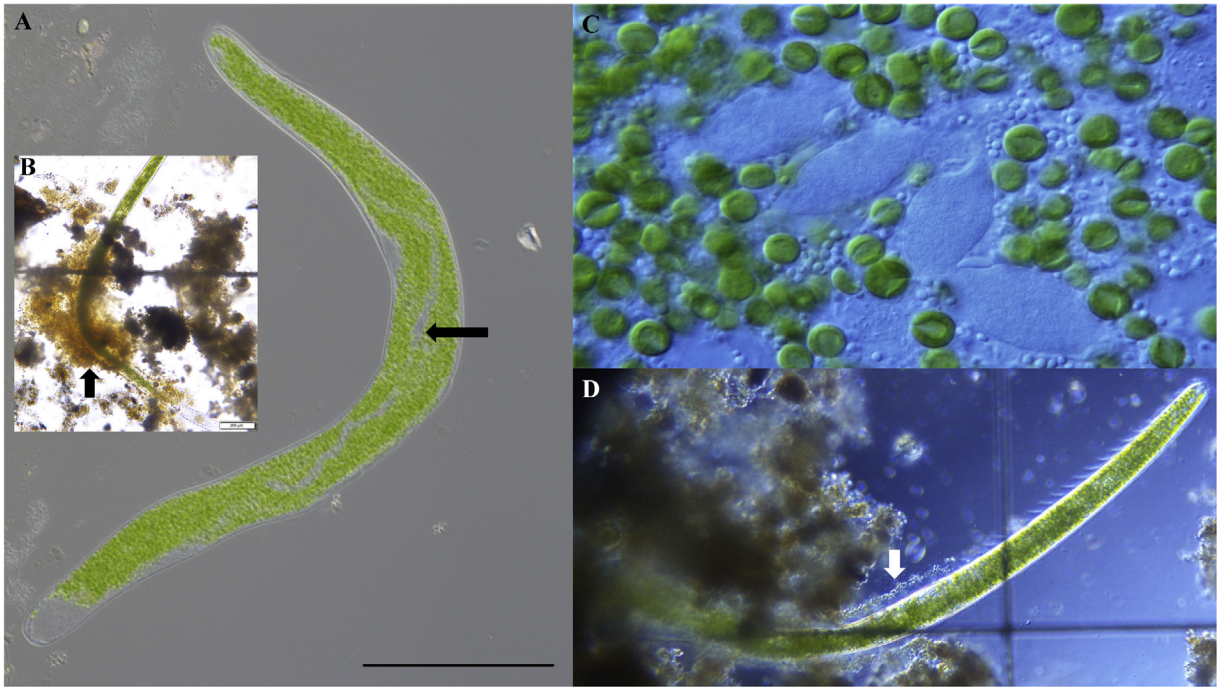

Figure 1. *Spirostomum semivirescens* in vivo micrographs of the specimens collected from UK and Sweden. **A**: *S. semivirescens* collected from Sweden. Note the long moniliform macronucleus (arrow) running along the center of the ciliate. The cell is packed with endosymbiotic green algae, a diagnostic characteristic. Scale bar 200 μm. **B**: *S. semivirescens* collected from UK. The cell is shown here after leaving it undisturbed for a few hours on a counting chamber, as evident by the thick casing (arrow) it has produced. **C**: *S. semivirescens*' nuclear apparatus from a UK cell. Note the nodes of the macronucleus, and the small micronucleus at top of the oval shapes. The densely-packed endosymbiotic green algae are clearly in view in this cell. **D**: *S. semivirescens* collected from the UK. This specimen has begun to build its coating, which is the thin layer (arrow) around the center of the ciliate.

Table 1. Transcriptome data generated. The sum of the pro- and eukaryotic fraction of contigs will not be 100%. This is due to the high number of contigs where diamond could not find any hit in the nr database and therefore no MEGAN assignment could be done.

Species	Sampling site	Contigs*	Prokaryotic contigs (%)**	Eukaryotic contigs (%)**
S. semivirescens	Stadsskogen	22,396	2.6	44
S. semivirescens	Stadsskogen	34,101	2.3	49
S. semivirescens	Oxhagen	25,329	2.5	48
S. semivirescens	Oxhagen	31,377	2.9	51
S. semivirescens	Dorset	17,644	4.3	46
S. semivirescens	Dorset	27,952	2.8	53
Spirostomum sp.	Stadsskogen	35,543	3.2	47

*Number of contigs in assembly after 95% CD-HIT reduction.
**Fraction of contigs in assembly assigned by MEGAN as pro- or eukaryotic.

S1) we decided to use the co-assembly (Table 2) in downstream analysis.

Phylogeny

The phylogenetic analysis of the *Spirostomum* genus was based on a concatenation of 18S rRNA gene, 28S rRNA gene and the internal transcribed spacer region in between the two rRNA sequences. The tree topology showed that members of major *Spirostomum* clades grouped together in the same way as observed in earlier studies (Boscaro et al. 2014; Shazib et al. 2014). However, relationships between these clades changed and *S. teres*

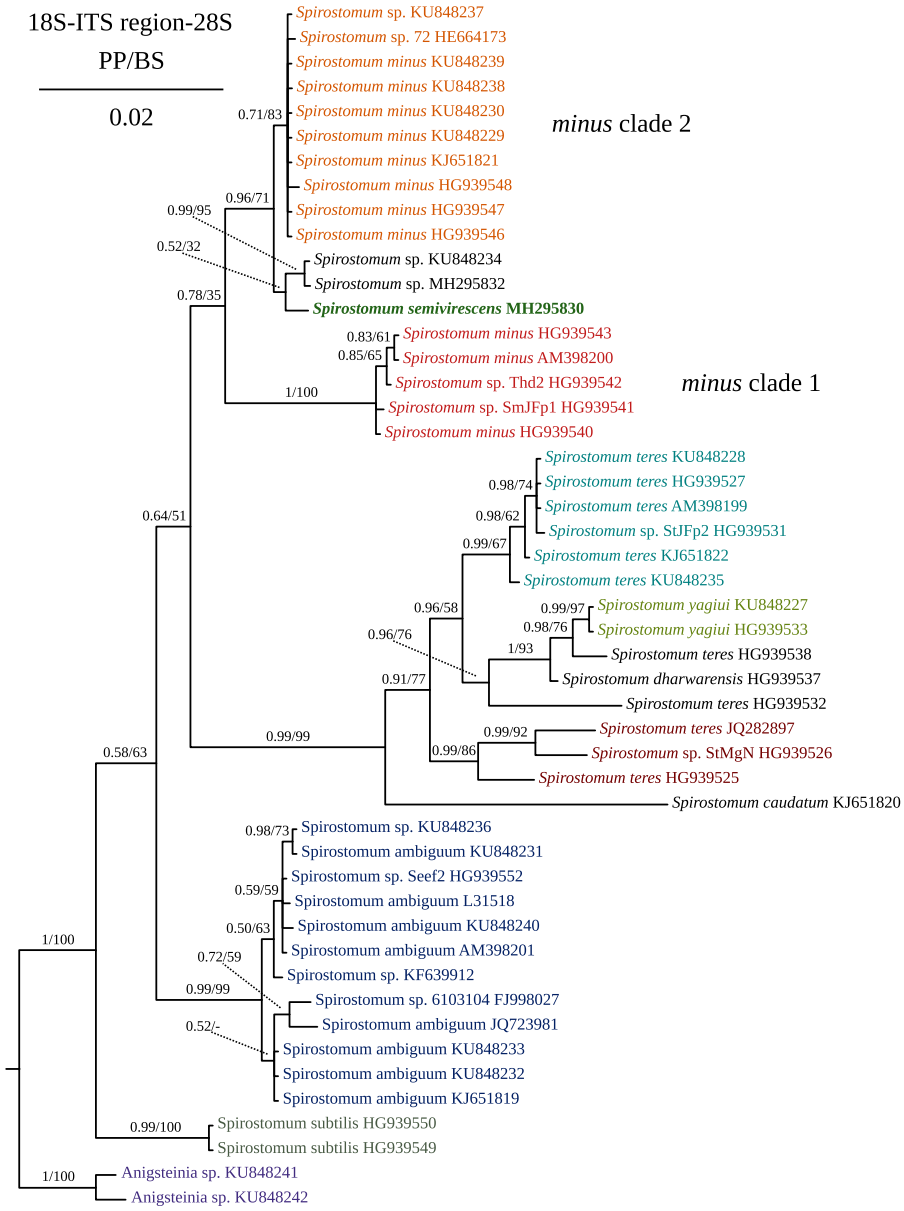

Figure 2. Phylogenetic relationships for the *Spirostomum* genus. Topology is inferred from a concatenation of 18S rRNA gene, 28S rRNA gene and the ITS region with PhyloBayes using the CAT + GTR model, bootstrap values from the maximum likelihood analysis is mapped on the tree as well. The branch length of the outgroup is reduced by ten times for visual purposes. For taxa where the different rRNA sequences have different accession numbers, i.e. are not available as one contig, the accession number for the 18S rRNA gene is displayed. The concatenated rRNA sequences assembled from each of the *S. semivirescens* replicates were identical or within the expected error from polymerase, therefore only one *S. semivirescens* taxon was placed in the tree (see text). The *minus* clade 1 and 2 is marked on the tree based on how these two clades are referred to in earlier literature (Boscaro et al. 2014; Shazib et al. 2014). The sequence for *Spirostomum* sp. MH295832 was also generated by this study (see text).

Table 2. Assembly statistics for the co-assembly of six *S. semivirescens* replicates.

Total number of contigs	23,933
Transcriptome size (Mb)	31.5
Average contig length (nucleotides)	1317
Longest contig (nucleotides)	12,455
Shortest contig (nucleotides)	210
Average GC content (%)	51
Total amount of data assembled (Gb)	6.3

together with *S. yahiui*, *S. dharwarensis* and *S. caudatum* was placed as a sister clade next to *S. minus*. The regions used to infer the phylogeny shared only a total of one mismatch in the 18S rRNA region, no mismatches in the ITS region and three in the 28S rRNA region between the six replicates. Based on the number of PCR cycles used prior to sequencing this is in line with what could be expected from polymerase errors. Therefore, only one *S. semivirescens* taxon is placed in the tree (Fig. 2). The phylogenetic analysis indicates that *S. semivirescens* is most closely related to the members of the clade earlier referred to as "*minus* clade 2" (Boscaro et al. 2014). This clade consists of *Spirostomum minus* and an unnamed species (KU848234) first discovered by Shazib et al. (2014). The unnamed species in "*minus* clade 2" was placed as sister taxon with high support together with the colorless *Spirostomum* species (MH295832) found during this study which was not identified prior to sequencing.

No algal 18S rRNA gene could be found in any of the transcriptome assemblies, despite the high number of algal endosymbionts in *S. semivirescens*. It is possibly that lysis of the algae was inefficient leading to poor transcriptome coverage of the endosymbiont. However, in five of the six *S. semivirescens* transcriptome assemblies a 28S rRNA gene could be found with high identity to *Chlorella vulgaris*. Transcriptome data of another ciliate that harbors similar endosymbiotic algae, *Stentor polymorphus*, has been generated for species from the same pond in Stadsskogen sampled in this study (Onsbring et al. 2018). If the algae observed in the *S. semivirescens* transcriptomes are contamination, the same contamination could potentially be observed in the *S. polymorphus* transcriptome. No 28S rRNA gene identical to the assumed *S. semivirescens* endosymbiont could be found in the *S. polymorphus* data. Instead another 28S rRNA gene with high identity to *Chlorella vulgaris* was found. Except for the 28S rRNA gene with high identity to *Chlorella vulgaris*, no other algae related rRNA sequence was detected more than once in each transcriptome. Both the *Spirostomum* and *Stentor* algal endosymbiont sequences branched together with *Chlorella vulgaris* in the tree (Fig. 3).

Identification of Anaerobic Metabolism

Members of the *Spirostomum* genus are often encountered in the oxygen-depleted sediment layers of waterbodies. Thriving in these habi-

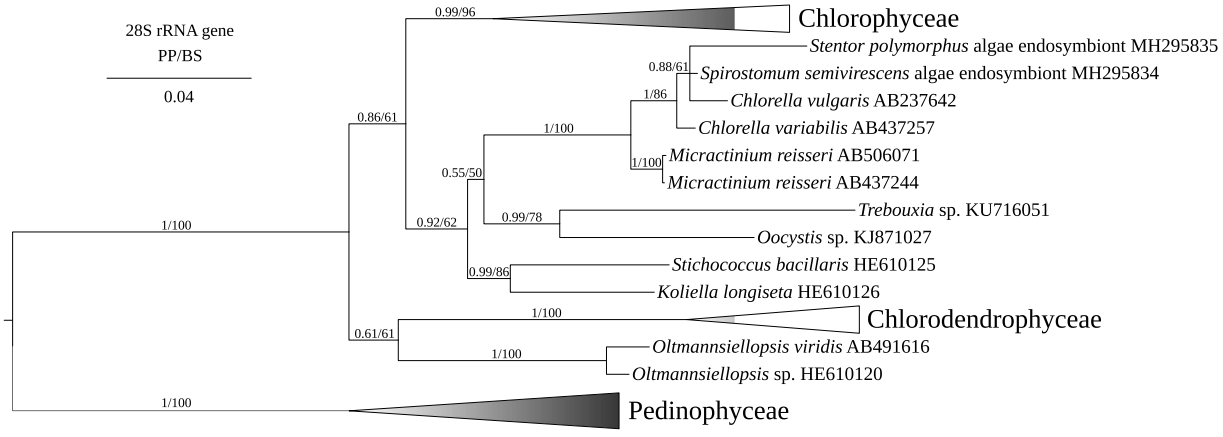

Figure 3. Phylogenetic relationships for green algae species of the class Trebouxiophyceae and other close relatives. The endosymbiont obtained from within the *Spirostomum semivirescens* sequences is in bold. Topology is inferred from the 28S rRNA gene with PhyloBayes using the CAT + GTR model, bootstrap values from the maximum likelihood analysis is mapped on the tree as well. A closely related, but not identical, 28S rRNA gene was found in the algal endosymbiont of *Stentor polymorphus* sampled from the same pond as *S. semivirescens* (Stadsskogen, Sweden).

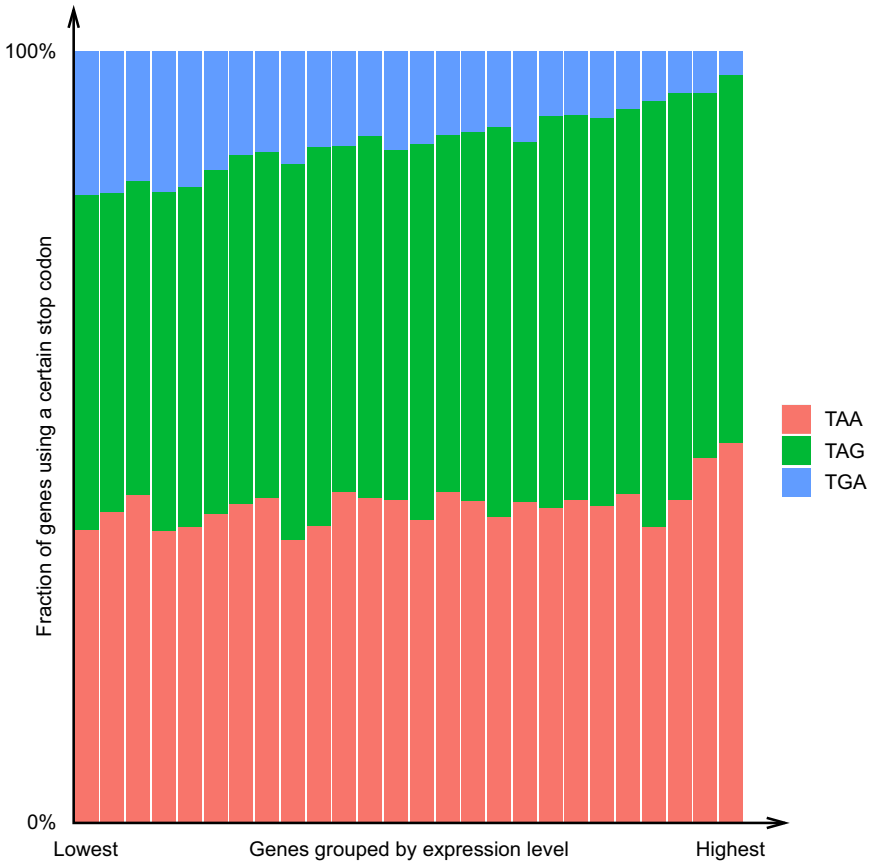

Figure 4. Relationship between stop codon frequency and gene expression levels. *Spirostomum semivirescens* transcripts ranked by expression level and then separated by groups of 500. Each bar displays the fraction of 500 genes using a specific stop codon, except for the lowest expressed group which had a total number of transcripts not evenly divided by 500. A trend is shown such that the fraction of transcripts terminating the translation with TGA is decreasing as the expression level is increasing.

tats would require the ciliate to be able to respire under anoxic conditions. Therefore, proteins involved in previously described anaerobic respiration pathways (Müller et al. 2012; Stairs et al. 2015) were searched for in the *S. semivirescens* transcriptome to get better insight into its anaerobic lifestyle. A tblastn search could identify a match for the rhodoquinone biosynthesis protein RquA of the bacterium *Rhodospirillum rubrum* (WP_011390975). The match had an 86% query coverage and 47% sequence identity. The putative RquA sequence from *S. semivirescens* carried a 21 amino acid mitochondrial targeting sequence at the N terminus with a predicted probability to be targeted to the mitochondrion of 86% (Claros and Vincens 1996). This is consistent with previous reports that have shown eukaryotic RquA has predicted mitochondrial localization (Stairs et al. 2014). The assumption that the identified potential RquA sequence in the *S. semivirescens* transcriptome is indeed a true RquA is further supported by the presence of a 9 amino acid motif (Lonjers et al. 2012). This motif contains glutamine and valine in RquA instead of aspartate and glycine used by UbiE and UbiG at corresponding positions. UbiE and UbiG are methyl transferases involved in the ubiquinone biosynthesis, which has a high sequence similarity to RquA. Based on the tblastn search we could not find any evidence for the presence of hydrogenosomes, pyruvate formate lyase activity or dissimilatory nitrate reduction, which have been found in other microbial eukaryotes (Müller et al. 2012; Stairs et al. 2015).

Codon Usage

The investigation of the codon usage showed that TAA, TAG and TGA are used by *S. semivirescens* as stop codons. A relationship between gene expression levels and TGA frequency could be observed

where TGA was more common among the genes with low expression (Fig. 4). Only 3% of the 500 genes with the highest expression had TGA as a stop codon while 19% of the 500 genes with the lowest expression were terminated with TGA. A similar relationship between TGA stop codon frequency and gene expression level was observed when analyzing the *Spirostomum* transcriptome generated in this study and the *Stentor polymorphus* transcriptome previously generated (Onsbring et al. 2018; data not shown).

Discussion

Specimens of *S. semivirescens* have been recorded from oxygen-depleted, freshwater habitats in the UK before (Esteban et al. 2009a); the isolates used in this study represents a further habitat in the UK, and new records for Sweden from two sites separated by ~30 km. All strains were observed to be morphologically identical. The molecular analysis revealed identical molecular sequence between strains at the highly variable 18S rRNA level, confirming the match between the two sample groups of this large >1 mm ciliate. By investigating this species at a wider global resolution, the geographical distribution of these micro-organisms has been expanded; at a distance of over 1,600 km between the sampling sites investigated in the UK and Sweden, the discovery of a strain with matching molecular sequences supports previous findings that microbial species thrive wherever the right conditions for their growth are found globally (Finlay 2002; Hines et al. 2016). This has wider implications for global microbial dispersal, particularly ciliate biogeography and biodiversity, with this species being a good target to investigate from other world regions (i.e. the tropics) for comparisons at a global level.

S. semivirescens has thus far been recorded from Germany (Kreutz and Foissner 2006), UK (Bradley et al. 2010; Esteban et al. 2009a, and this study), and now Sweden. The *Spirostomum minus viride* investigated by Foissner and Gschwind (1998) in Germany fits the morphological features of *S. semivirescens* and both are probably conspecific. Records from Russia (Boscaro et al. 2014) and Japan have also been reported (Esteban et al. 2009a), which comes to demonstrate that species' biogeography expands as sampling efforts increase.

The phylogenetic relationships found in this study show that the *S. ambiguum* clade was placed differently compared to previously published phylogenies (Boscaro et al. 2014; Shazib et al. 2014). When using RAxML (Stamatakis 2014) instead of IQ-TREE to calculate the phylogeny the same topology as in Shazib et al. (2014) was achieved with 66 in bootstrap support for *S. ambiguum* together with *S. subtilis* branch as sister clade to both groups of *S. minus*. The bootstrap support for *S. subtilis* as sister clade to *S. ambiguum* was 56. Since the IQ-TREE package contains a wider selection of evolutionary models to choose from and is reported to often find topologies with higher likelihoods (Nguyen et al. 2015) than RAxML, the bootstrap values from IQ-TREE were maped on the bayesian tree (Fig. 2). *S. subtilis* was placed as the deepest branching taxa in the *Spirostomum* genus as seen before in Boscaro et al. (2014) but not in Shazib et al. (2014). *S. semivirescens* could be placed with high support in the Bayesian tree as a close relative to *S. minus*. This is consistent with the similar morphology of the nuclear apparatus, where *S. semivirescens and S. minus* share the moniliform macronucleus shape (Shazib et al. 2014). The closest relatives found for the endosymbiotic algae were *C. vulgaris* and *C. variabilis*, both reported as endosymbionts in other ciliate species (Zagata et al. 2016).

The TGA frequency was estimated to 11% of the stop codons in *S. semivirescens*, based on the genes used to investigate the relationship between expression level and stop codon frequency. In another heterotrich, *Stentor coeruleus*, the TGA frequency is 9%, based on the CDS file available from the online database StentorDB. Swart et al. (2016) report 5% and 1% TGA stop codon frequency for *Climacostomum virens* and *Fabrea salina*, respectively. However these estimations for *C. virens* and *F. salina* were only based on 285 and 96 proteins, respectively. Only 38 species out of 283 had a TGA stop codon frequency below 12% (Swart et al. 2016), several of these species could have already had their TGA reassigned since Swart et al. (2016) predicted less than 10 TGA stop codons for 11 of these cases. The relatively low TGA frequency among these heterotrichs indicates that TGA termination could have a higher fitness cost compared to the other stop codons. Therefore, there could potentially be a higher fitness gain in replacing the TGA codon for genes with a high expression level compared to genes with a lower expression level. Such selection pressure could cause the observed bias with fewer TGA in highly expressed genes as in *S. semivirescens*. Since it has been suggested that codon frequency is reduced prior to reassignment of codons (Mühlhausen et al. 2016), this leads to the question if *S. semivirescens* could be in an

early stage of codon reassignment. Close relatives such as *Blepharisma* have already reassigned the TGA stop codon (Lozupone et al. 2001), *Condylostoma magnum* can use all three stop codons, uncluding TGA, as both stop and sense codon (Swart et al. 2016) and *Climacostomum virens* has been suggested to be in a transitory state of stop codon reassignment (Heaphy et al. 2016). Given these observations in other heterotrichs, the connection between stop codon reassignment and stop codon bias with expression level could be worth further investigations. Gene expression levels for different stop codons have been investigated before in model organisms, but have turned out to have no relationship (Sun et al. 2005). Gene expression levels for different codons have mainly been investigated for sense codons before and have been observed in eukaryotes, e.g. *Saccharomyces pombe*, whoes stop codon frequency also correlate with expression level (Hiraoka et al. 2009). However, in *S. semivirescens* for most sense codons the frequency is changed slightly for the top 1,000 highest or lowest expressed genes and for some sense codons the frequency is not affected by expression level at all (Supplementary Material Figs. S2–S5). Interestingly, the TAA frequency, which seems to be affected by mutational biases (Swart et al. 2016), is rather constant for *S. semivirescens* when comparing different expression levels (Fig. 4). In *S. semivirescens* the decrease in TGA frequency with higher expression levels is leading to an increase in TAG frequency, a change that requires the change of two nucleotides, instead of one.

We suggest that *S. semivirescens* uses rhodoquinol-dependent fumarate reduction to respire under anaerobic conditions. This is based on the high sequence identity to RquA found in *Rhodospirillum rubrum*, and the presence of the expected motif and mitochondrial targeting tag. A potential RquA sequence could also be found as well in the *Spirostomum* sp. data generated in this study. In both cases the sequence identity to *R. rubrum* RquA was above 40% and query coverage above 85%, the RquA motif was found and probability of export to mitochondria were over 70%. Since a putative *rqu*A gene was found in the two *Spirostomum* species and this gene has also been reported in several other ciliates from the class Heterotrichea (Stairs et al. 2018), the whole *Spirostomum* genus might use this pathway for anaerobic respiration. The heterotrichs formed a monophyletic group in a phylogenetic analysis of the RquA protein from both prokaryotic and eukaryotic species. The relationship between the heterotrichs in the RquA phylogeny mirrored the topology of a phylogenetic analysis for their respective 18S rRNA genes (Supplementary Material Fig. S6). Additionally, Stairs et al. (2018) located a potential *rqu*A sequence in the *Stentor coeruleus* genome, generated by Slabodnick et al. (2017), which gives further support to that heterotrichs code for *rqu*A within their genome. Therefore we suggest that the identified *rqu*A genes in this study are highly unlikely to be a contamination.

Conclusion

As more data are generated at the genomic level for different species in the *Spirostomum* genus, the relationship between major clades can be resolved. With the rRNA data that is currently available, *S. semivirescens* can be assigned as closest relative to *S. minus* and the endosymbiotic algae was identified as a member of the *Chlorella* genus. Insights into the transcriptome suggest that *S. semivirescens* use rhodoquinol-dependent fumarate reduction for respiration under anoxic conditions, which is likely also used by the other members of the genus since it has been observed in other species from the class Heterotrichea (Stairs et al. 2018) that also thrive in anoxic habitats similar to those where *S. semivirescens* is found. Our observations indicate that *S. semivirescens* could be in an early stage of codon reassignment. Therefore *S. semivirescens* could potentially be a relevant species to study for a better understanding of the evolution of the genetic code. Our results also indicate that it is possible for ciliates with identical morphologies, but from distant geographical areas, to also have identical molecular signatures.

Methods

Study sites: *UK study site.* Ciliates were sampled during June 2015 in Dorset, South England from a fen pond (50°40′44.5″N 2°11′29.5″W) and from a freshwater ditch, both located on the flood plain of the River Frome (50°40′52.0″N 2°11′21.8″W). *Spirostomum semivirescens* had previously been shown to thrive within this area (Esteban et al. 2009a) and this site is known to be a hotspot of ciliate biodiversity, with sampling efforts often revealing the *S. semivirescens* species. The fen habitat is densely wooded and dimly-lit with temporary ponds rich in organic sediment. The ditch had similar parameters, and was about 100 meters away from the fen. Oxygen levels were very low (<5%). The sediment-water interface was sampled using a corked 500 mL caged sample bottle on a line. The corked line was pulled once the apparatus had sunk, to allow water and sediment within the desired oxygen-depleted depths to be collected. The area sampled in the fen pond and the ditch had a depth of less than 30 cm. 1 mL subsamples

were observed in a Sedgewick Rafter chamber. Many cells were encountered and examined, with densities of up to 15 cells per mL of sediment subsample.

S. semivirescens cells collected from this location were hand-picked under a dissecting microscope using a micropipette, and were stored in RNAlater (Thermo Fisher Scientific) for transport to Uppsala University, Sweden for transcriptome analyses. cDNA synthesis (see below) was performed within three days of removal from the UK sampling site and storage.

Sweden study sites. Samples were collected from two freshwater locations (see below) during August 2015. Air temperature was 25 °C in full sun, with water temperature of 18 °C recorded. The first location investigated was Stadsskogen "city forest" – an ancient, densely-wooded and dimly-lit forest area (59°50′19.0″N 17°37′21.4″E). Within this habitat, a small pond location was chosen, and a pH of 6.0 was recorded, with a conductivity of 47 μS/cm. Samples collected ranged from a ∼30 cm depth to shallow ∼4 cm samples obtained by hand along the shoreline and on submerged algal mats. The second location sampled was a shallow eutrophic farmland pond with dense organic sediment, at "Oxhagen" in full sunlight with some aquatic plant coverage (60°5′47.6″N 17°46′4.7″E). Within this location, a pH of 6.6 was recorded, with a conductivity of 292 μS/cm. Samples were taken from 30 cm deep zones from various areas along the middle and edge of the pond.

Sampling methods were identical to the technique used in the UK (see above). Samples were taken back to the laboratory at Uppsala University, with subsamples being analyzed on a 1 mL Sedgewick Rafter chamber. 1 liter of water was taken from the sampling locations for laboratory analysis of the pH using a handheld PW9420 pH meter (Philips). To determine conductivity a Crison conductimeter 522 was also utilized on the removed samples within 2 hours of collection.

Samples were examined within 3 hours of removal, and the ciliates were found to thrive naturally for at least one week in the 500 mL bottles. Both sites were extremely productive for ciliates, with many harboring endosymbiotic algae such as *Stentor polymorphus*, *Frontonia* sp. and *Loxodes rostrum*. Anaerobic ciliates of the genus *Plagiopyla*, *Metopus* and *Caenomorpha* were present, as the sediment layer was largely oxygen depleted.

cDNA generation and sequencing: Both preserved and fresh ciliates were washed twice in double distilled water before single cells were picked in a 0.4 μL volume into a 0.2 mL PCR tube (VWR). cDNA synthesis was done according to the Smart-seq2 protocol (Picelli et al. 2014). Aliquots were diluted to 0.2 ng/μL based on dsDNA concentration measured with a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). The diluted aliquots were prepared for sequencing using Nextera XT DNA Library Preparation Kit (Illumina). Two *S. semivirescens* replicates were sampled from each sampling site, i.e. the fen in Dorset, the pond in Stadsskogen and the pond in Oxhagen. The *Spirostomum* species lacking algae were sampled in Stadsskogen. For all six *S. semivirescens* replicates sequencing was done on Illumina MiSeq, 300 base pair, pair-end reads using v3 chemistry. The unidentified *Spirostomum* species were sequenced on Illumina HiSeq, 250 base pair, pair-end reads.

Transcriptome assembly: Raw reads were trimmed with Trimmomatic v0.35 (Bolger et al. 2014) by first removing primer sequences and DNA library preparation related sequences with the settings ILLUMINACLIP:2:30:10. Also in the following order LEADING:5, TRAILING:5 SLIDINGWINDOW:5:16 and MINLEN:80 were applied. Artificial reads were identified and removed using BLAST v2.2.30+ (Altschul et al. 1990) by a blastn search against the NCBI UniVec database. Transcriptome assembly was both carried out with Trinity v2.2.0 (Grabherr et al. 2011) and SPAdes v3.9.0 (Bankevich et al. 2012). The SPAdes assembly was done with a k-mer size of 99 and only used for the phylogenetic analysis since the rRNA contigs assembled by SPAdes were larger than in the Trinity assembly. In all other analysis, the Trinity assemblies were used. Full transcriptome alignment to NCBI nr database was done with DIAMOND v0.8.37 on sensitive blastx mode (Buchfink et al. 2015). The alignment results were analyzed with MEGAN v5.8.3 (Huson et al. 2007), which contig assignments were used to estimate the fraction of the data originating from the host, algae or prokaryotes.

Identification of anaerobic respiration pathway: Anaerobic respiration proteins previously found in other eukaryotes (Müller et al. 2012; Stairs et al. 2015) were searched for in the transcriptomes via tblastn search. To search for the presence of hydrogenosomes queries with [FeFe] hydrogenase, pyruvate:ferredoxin oxidoreductase and the maturase proteins HydE, HydF and HydG were used. Both pyruvate formate lyase and the enzyme to activate this protein were search for to detect pyruvate formate lyase activity. Also nitrate reductase, fumarase and RquA were used as queries to detect other anaerobic pathways.

Phylogenetic analysis: The rRNA sequences used in the phylogenies were identified with Barrnap (Seemann 2013). The ciliate sequences used to infer the phylogeny (Supplementary Material Table S1) were gathered by downloading all *Spirostomum* sequences available in the SILVA database and all sequences generated by Shazib et al. (2014). The algae sequences were gathered by using the identified 28S rRNA gene from *S. semivirescens* as a seed in a blastn search against NCBI nt database. CD-HIT V4.6.6 (Li and Godzik 2006) was used to remove identical sequences. Multiple sequence alignments were produced by MAFFT X-INS-i (Katoh 2002) where the CONTRAfold algorithm (Do et al. 2006) was used for pairwise structural alignment. The multiple sequence alignments were manually curated. BMGE was used to trim the curated alignment (Criscuolo and Gribaldo 2010). Bayesian inference tree topology was calculated with PhyloBayes v1.5a (Lartillot and Philippe 2004) using the CAT + GTR model. Four chains were used and both trees ran until maxdiff calculated by the PhyloBayes bpcomp-command were below 0.1. Burn-in was selected by monitoring – log likelihood plotted against generation of trees. For the ciliate Tree 13000 generations was generated and the burn-in was set to 1000. For the algae Tree 37000 generations was generated and the burn-in was set to 1000. Maximum likelihood trees were calculated with IQ-TREE (Nguyen et al. 2015) using the TIM + R2 model for the ciliate and TN + R3 model for the algae. The model tester in the IQ-TREE package selected the models in the maximum likelihood tree according to the Bayesian Information Criterion. Two long branches were removed in both the ciliate and the algae phylogeny that could potentially produce artifacts in the tree topology. To rule out that that the identified *rqu*A sequences from the tblastn search were contamination we repeated the phylogenetic analysis by Stairs et al. (2018). Additional sequences added in this phylogeny were the potential *rqu*A sequences identified in this study and a potential *rqu*A sequence from the transcriptome of *Stentor polymorphus* (Onsbring et al. 2018). Multiple sequence alignment done by MAFFT L-INS-i (Katoh 2002) was trimmed with trimal (Capella-Gutiérrez et al. 2009) and the tree topology was calculated with IQ-TREE (Nguyen et al. 2015), using ultrafast bootstrap approximation with the LG + C50 model that was selected by the Bayesian Information Criterion.

Stop codon usage analysis: To analyze codon usage all six *S. semivirescens* replicates were assembled with Trinity v2.2.0 to a single assembly. To this assembly raw reads were mapped using Bowtie 2 (Langmead and Salzberg 2012) with the settings "–end-to-end –k 20 –D 20 –R 3 –N 1 –L 20 –i S,1,0.50 –X 1000". Because of the redundancy, often caused when assembling transcriptomes de novo (Duan et al. 2012), the contigs were clustered to transcripts using Corset v1.06 (Davidson and Oshlack 2014). The longest open reading frame from the longest contig in each cluster where then extracted. A blastp search against NCBI nr database using DIAMOND v0.8.37 (Buchfink et al. 2015) was then used to select all contigs with hits to *Stentor coeruleus*, *Paramecium tetraurelia*, *Oxytricha trifallax*, *Stylonychia lemnae*, *Tetrahymena thermophila*, *Pseudocohnilembus persalinus* and *Ichthyophthirius multifiliis* to discard contamination for downstream analysis. The species used to select contigs for further analysis represented the seven ciliates with the most blast hits. Selecting contigs based on more species would not have changed the outcome of the analysis since potential additional species had few hits and would in most cases have a hit as well to any of the seven mentioned species. The count matrix calculated with Corset during the clustering step was then used to rank the extracted open reading frames based on their expression level. To take different sequencing depth for each library into consideration, the total number of mapped reads for each species were used to normalize the number of mapped reads to each transcript. These values were then added together for each transcript to rank all the transcripts based on their normalized sum of mapped reads. The statistics for stop codon usage and the relationship to expression level was finally collected based on the transcripts selected by the blast search and the ranking of the normalized sum of mapped reads. Additionally, the transcripts were ranked based on expression level for each of the individual replicates, for comparison with the average, to assess the feasibility of averaging out the noise and the consistency between replicates (Supplementary Material Fig. S1). The redundancy-reduced co-assembly used in the codon analysis has been deposited in GenBank under the accession GGNT00000000. An assembly for the unidentified *Spirostomum* species was generated in the same way and deposited in GenBank under the accession GGNU00000000. The first versions for both transcriptome assemblies are described in this paper. The accession number for the raw reads reported in this paper is SRA: SRP145156.

Conflict of interest

None.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.protis.2018.08.001.

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CAREER COLUMN · 04 FEBRUARY 2019

How we use Instagram to communicate microbiology to the public

Social media is a powerful tool for science communication. Instagram's image-focused model is no exception, say Hunter Hines and Sally Warring.

Hunter Hines & Sally Warring

Hunter Hines (left) and Sally Warring (right) sample microbes at a microbiology conference in Vancouver, Canada. Credit: Hunter N. Hines @microbialecology

While in graduate school, we both created Instagram accounts to share photos and video clips of microorganisms that we encountered through our research.

We started using Instagram because we both felt a deep sense of wonder when viewing microbial organisms through the microscope, and thought this was something that could be shared. As PhD candidates in microbiology laboratories, we were viewing microbes daily. When looking for an online platform for sharing this content, Instagram ticked a lot of boxes.

Instagram is an image-based social-media platform on which users share photographs and short videos (of up to one minute) with optional written captions, and interact with other users by liking and commenting on the content they share. Although several social-media platforms exist for the sharing of visual information, Instagram is the simplest to use: you don't need any video- or photo-editing skills beyond the application, for example, which makes it straightforward to post videos and photos directly from your phone to the platform.

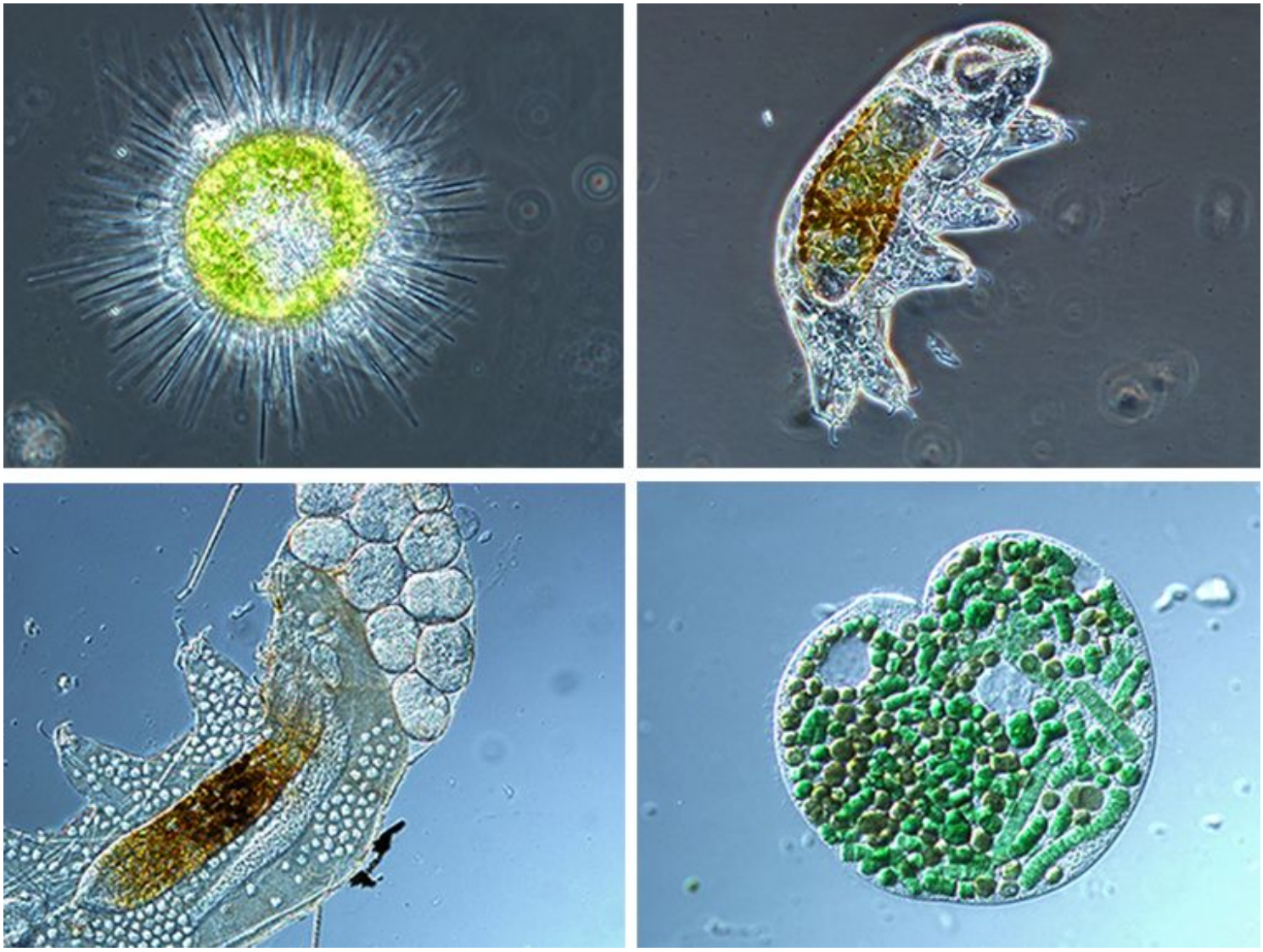
Instagram is also a thriving community. Since its launch in 2010, user numbers have steadily grown, hitting 1 billion in 2018 and making news along the way, including when 50 million people — equal to the population of Spain — 'liked' a picture of an egg. Along with its expansion has come the popularization of the selfie. Many accounts are dedicated to lifestyle and beauty content. A growing body of scientists is there, too, sharing imagery from the lab, field, microscope, data figures and selfies in interesting and informative ways.

Hunter Hines samples microbial mats for organisms such as tardigrades and small ciliates in a freshwater spring. Credit: Hunter N. Hines @microbialecology

Instagram's algorithm can work like a positive-feedback loop. You post an image or video that is seen by some of your followers, and Instagram will monitor how many of those viewers engage with your post by liking or commenting. The more engagement the post gets, the more people Instagram will show it to, either by bumping it to the top of followers' feeds, or by adding it to a curated collection of posts in Instagram's 'Explore' section, which is seen by followers and non-followers alike. We've found that regular posting — once daily or every other day — keeps your audience engaged, which encourages more viewers. Posts can be organized into categories by adding up to 30 hashtags covering topics as broad as #biology, or as specific as #loxodesrex, which helps interested users to find your images in a sea of content.

We've found that predicting what will go 'viral' is nearly impossible (the tardigrade or 'water bear', however, is an invariably popular subject), and instead recommend posting a wide range of content within your niche, and experimenting with different visuals and captions. These approaches have taught us what draws the most interest. To generate new content, we often find ourselves expanding the diversity of organisms we examine far beyond the scope of our projects, which in turn increases our understanding of microbial ecology.

Constructing the captions that accompany each post and keeping track of their impact has given us experience in how to communicate microbiology with a tone that is understandable and relaxed, yet informative. A brief text explainer of the image and its significance is sufficient. The goal is to stimulate interest and conversation, rather than to be conclusive. Followers will soon ask you questions if they want to know more. We've found that questions can lead to exchanges about the basic biology of a cell, the nature of consciousness and intelligent life and the role of climate change and pollution in shaping ecosystems. The more you engage with your followers, the more you'll get back.



Clockwise from top left: a ‘green sun animacule’, likely in the genus *Acanthocystis* (viewed at 400x under phase-contrast microscopy); a tough tardigrade ‘water bear’, collected from a spring in Florida, that has ingested microbial food (phase-contrast microscopy at 200x); a tardigrade moulting eggs into its shed cuticle; and a ciliate contorted into a heart after feeding on various filamentous algae and cyanobacteria (200x under differential interference contrast microscopy). Credit: Hunter N. Hines @microbialecology

These accounts also provide connections outside the lab, and expose us to increasingly diverse communities. Our activities on Instagram have led to ongoing collaborations with artists, film-makers, industry professionals, community groups, start-ups and non-profit organizations from all over the world. S.W.’s account, @pondlife_pondlife, for example, is currently exhibiting images of microbial life at the Brooklyn Botanic Garden, and works with several educational organizations throughout New York City, running workshops on microscopy. Combined, @pondlife_pondlife and H.H.’s account, @microbialecology, have more 100,000 followers from all around the world, and we continually receive positive messages from people who have never had access to a microscope and are fascinated by what they are seeing in our posts.

Our time on Instagram has left us thinking that there is much public interest in the daily workings of science, and that many researchers could find a following for their work, from audiences with specialist-level interest to those with no familiarity with science. Instagram can be seen as a microphone for amplifying newly published research and current projects in real time to a much wider audience than conventional scientific publishing can manage.

We hope that we are also showing microbes and microbiology in a positive light for the general audience, and are increasing public awareness of microorganisms as part of Earth's biodiversity.

As scientists, we produce a tonne of visual data in many different forms. If you're looking for a way to share yours with a wide audience, Instagram is a fantastic place to do it. We find the community on Instagram to be engaged and engaging, and we see the platform as an ideal tool for scientists to share information freely on a potentially unlimited array of subjects.

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