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THE ECOLOGY OF AN INVASIVE TOXIGENIC PROTIST

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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My dissertation is dedicated to my wife Mary Beth Lord, my son, Richard James Zamor (RJ), and my soon to be arriving son, Michael Dixon Zamor.

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Abstract

Humans have facilitated a spread of nonnative biota across the Earth. As nonnative species, their interactions with native species, their surrounding community, and their environment are novel, and thereby provide unique opportunities for studying community assembly and the maintenance of community diversity. The study of nonnative species is also important because some experience population explosions and spread rapidly throughout a new habitat and cause negative environmental and economic impacts. Such nonnative species are often referred to as invasive species.

Freshwater systems are among the most impacted environments by invasive species because they are some of the most disturbed ecosystems on the planet. Disturbances to freshwater ecosystems include impoundments in riverine networks and increased anthropogenic nutrient pollution (i.e., cultural eutrophication). These disturbances facilitate the establishment and spread of invasive species by changing resource availability and altering species interactions, which creates openings for invasive species.

Another result of these disturbances in freshwater ecosystems is the worldwide proliferation and establishment of harmful algal blooms (HABs). Harmful algae are planktonic microbes from multiple taxonomic groups that can produce toxins. Similar to invasive species, these species are infamous for being able to dominate their ecosystems at high densities and produce devastating ecosystem and economic effects. Much of the research on harmful algae ecology focuses on the prevention and control of bloom formation or toxin production, but fundamental questions of community ecology

regarding dispersal, distribution, and ultimately establishment of these microbial invasives have received little consideration.

This lack of attention may be due to the fact that microbial species in general are neglected in the invasion ecology literature. Most concepts and hypotheses in invasion ecology have been derived from the study of macrobial multi-cellular organisms. That microbes have historically not been considered within the field of invasive ecology is at least partially explained by one of the prevailing hypotheses of microbial community ecology: "everything is everywhere, but, the environment selects." This hypothesis suggests that dispersal is not limiting for microbes and thus microbes may not be invasive at all, but rather present in a given environment at low densities at any given time. One implication of this hypothesis for microbial invasion ecology is that our ability to discern a microbial invasion is limited by our ability to detect it. However, another implication of the "everything is everywhere, but, the environment selects" hypothesis is that the environment is the primary determinant of whether a given microbe is abundant enough to be detected (i.e., microbial establishment). Because relatively high detection limits (which are continually being reduced as molecular technology advances) and a lack of traditional taxonomy has severely limited the study of microbial invasives, there are numerous questions that need to be addressed in order to understand the factors governing invasion and successful establishment of microbial species.

The harmful algal species, *Prymnesium parvum* provides an excellent opportunity for ecologists to gain insight into the factors responsible for the successful establishment of invasive microbes. *Prymnesium parvum* is a toxigenic protist in the

class Prymnesiophyceae. It was first characterized from marine systems, but has since invaded freshwater systems worldwide. It is notorious for its negative impacts on ecosystems, chief among them, the killing of fish. Fish affected by *P. parvum* toxins exhibit hemorrhaging of the gills, and *P. parvum* blooms typically cause massive fish kills. *Prymnesium parvum* blooms are also considered to be Ecosystem Disruptive Algal Blooms (EDABs) characterized by their adverse direct effects on fishes and herbivorous invertebrate grazers, as well as their indirect effects on nutrient and food web dynamics, which create feedbacks enabling bloom persistence. In North America, the first record of a *P. parvum* bloom is from a fish kill in 1985 in the Pecos River system of southern Texas, USA. During the subsequent two decades, *P. parvum* has expanded its range and bloomed and caused fish kills in reservoirs and rivers throughout much of the southern United States from California to Florida, and as far north as Wyoming and West Virginia. Numerous studies, in particular laboratory experiments, have been conducted into the autecology of *P. parvum* that suggest attributes that might make it a good invader, although many of these have not been put into a natural context. Similarly, much is known about the distribution of its blooms, although the factors responsible for invasion of a system and subsequent bloom development are not well understood and have not been directly addressed.

In 2004, *P. parvum* invaded and bloomed in Lake Texoma, OK-TX, USA causing a massive fish kill in many of the shallow areas of the reservoir. Including this first bloom, *P. parvum* has bloomed during winter in seven of the nine winters through 2012. This created a unique opportunity for me to use *P. parvum* to investigate questions in invasion ecology using a microbial invader, specifically questions about

microbial establishment. In my dissertation, I address factors important to understanding the success of *P. parvum* invading new systems and the consequences of a *P. parvum* bloom on the fish community. In my first chapter, I modified a recently developed method for detecting *P. parvum* using quantitative polymerase chain reaction (qPCR) and tested its efficacy as an alternative to microscopy for *P. parvum* detection and enumeration in a long-term monitoring program in Lake Texoma. Abundance estimates of *P. parvum* were similar for both methods, but I detected *P. parvum* at multiple sites using qPCR where it previously had gone undetected by microscopy. Using qPCR, I substantially reduced processing time, increased detection limit and reduced error in *P. parvum* abundance estimates compared to microscopy. Thus, qPCR is an effective tool for detecting and monitoring *P. parvum*, particularly at pre-bloom densities, and should likewise prove useful in monitoring programs for the other HAB species for which qPCR methods have been developed.

In chapter two, I sampled fish near and offshore over an annual cycle encompassing a *P. parvum* EDAB event in two coves (i.e., a bloom site and a reference site) of Lake Texoma. My objective was to document the processes of extirpation and recovery of a fish assemblage to the disturbance of an EDAB event. *Prymnesium parvum* bloomed in one cove from mid-December 2008 until May 2009, eliminating all fish during this period. Fish toxicity bioassays indicated no substantial differences in susceptibility across fish species to *P. parvum* toxins. Fish rapidly recolonized the bloom site in May 2009 after the *P. parvum* bloom diminished. Fish assemblages were resilient to the *P. parvum* EDAB, recovering to previous abundance, richness, and composition within six months. My results suggest that the reservoir-wide fish

metacommunity enabled a rapid recovery of local fish assemblages following a spatially heterogeneous EDAB event.

In chapter three, I used a four-year data set from an ongoing monitoring program in Lake Texoma (OK-TX) to construct a predictive model relating *P. parvum* presence or absence to environmental parameters at a local scale. I then tested this model at the regional scale in conjunction with environmental sampling to predict presence and absence of *P. parvum* in the watershed of the Red River, one of two tributaries to Lake Texoma and a neighboring watershed, the Canadian River, as well as a few sites in the Arkansas River watershed. Based on three environmental factors, specific conductance, total nitrogen, and total nitrogen : total phosphorus ratio, my predictive model accurately classified *P. parvum* as present or absent in Lake Texoma for 74% of the samples. Applying this model to the adjacent watersheds also showed strong predictive power, correctly classifying 87% of the sites sampled within the Red River watershed and 81% of the sites sampled in the Canadian River watershed. Sites where the model predicted *P. parvum* but none was detected may be particularly vulnerable to *P. parvum* establishment and should be more closely monitored for future invasion success. Misclassifications by the model of sites in which *P. parvum* was detected suggests that dispersal has occurred, but that the environmental conditions were not conducive to population establishment. Indeed, at these sites, *P. parvum* abundances, when detected, were low. While this study cannot rule out dispersal limitation as a major factor involved in the biogeography of *P. parvum*, my results do indicate that the establishment and spread of this harmful algal species appears to be limited by environmental conditions in the invaded habitat.

In chapter four, I experimentally assessed the roles of community resistance and propagule pressure (i.e., the number of invaders entering a habitat or the frequency of invasions to a habitat) on the establishment success of *P. parvum*. One of the seminal hypotheses in the field of invasion ecology is that more diverse communities should be more resistant to invasion by exotic or non-native species. Similarly, propagule pressure is thought to facilitate an invasive species' establishment success by increasing the ability of an invading population to absorb the challenges of its new environment. Propagule pressure and community resistance to invasion are predicted to interact to affect the probability of community invasion and species establishment in a new community. I show experimentally that regardless of community diversity, establishment success by the microbial invader, *Prymnesium parvum* in an environmentally-compatible habitat, is determined by propagule pressure.

Currently, there are two theoretical possibilities that might explain the rapid range expansion of *P. parvum*. One possibility is that *P. parvum* is an invasive species that has dispersed to and established in new ecosystems. The second possibility is that this range expansion is driven by changes in the environment, particularly relating to salinity as affected by climate change and water resource overexploitation, and that *P. parvum* has always been present in these systems (i.e., "everything is everywhere, but, the environment selects"). My dissertation adds to the literature by showing that indeed both dispersal and environmental selection can play a role in *P. parvum* establishment.

The results from my dissertation are only a starting point for continuing to ask questions about the importance of dispersal and the environment to the outcomes of microbial invasion. Further investigation into the roles of environmental filtering, and

the possibility of prior presence of *P. parvum* should prove to be fruitful avenues of future research. Furthermore, understanding how the environment and *P. parvum* affect community composition should allow us to gain insight into how *P. parvum* interacts with other microbes. Ultimately, all of this information is important not only to our understanding of *P. parvum* specifically, but to our understanding of microbial invasions and harmful algal blooms, in general, and thus will contribute to our ability to mitigate the impacts of HABs and EDABs.

Chapter 1 – Incorporating molecular tools into routine HAB monitoring programs: using qPCR to track invasive *Prymnesium*

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Abstract

Microscopy, a staple of monitoring programs for tracking the occurrence and abundance of harmful algal bloom (HAB) species, is time consuming and often characterized by high uncertainty. Alternate methods that allow rapid and accurate assessment of presence and abundance of HAB species are needed. For many HAB species, such as the toxigenic haptophyte, *Prymnesium parvum*, molecular methods including

quantitative real-time PCR (qPCR) have been developed with the suggestion that they should be useful for monitoring programs. However, this suggestion rarely has been put into action. In this study, we modified a recently developed method for detecting *P. parvum* using qPCR and tested its efficacy as an alternative to microscopy for *P. parvum* detection and enumeration in a long-term monitoring program in a recently invaded subtropical US reservoir. Abundance estimates of *P. parvum* were similar for both methods, but we detected *P. parvum* at multiple sites using qPCR where it previously had gone undetected by microscopy. Using qPCR, we substantially reduced processing time, increased detection limit and reduced error in *P. parvum* abundance estimates compared to microscopy. Thus, qPCR is an effective tool for detecting and monitoring *P. parvum*, particularly at pre-bloom densities, and should likewise prove useful in monitoring programs for the other HAB species for which qPCR methods have been developed.

1. Introduction

Methods for rapidly and accurately processing field samples to assess the presence of harmful algal bloom (HAB) species are vital to any monitoring program attempting to understand HAB species distributions within aquatic systems and networks, their dynamics within those systems, and ultimately preventing or mitigating their harmful affects (HARRNESS 2005). The capacity to detect the formation of blooms early in their development is particularly important if we hope to subvert their establishment. One such species, *Prymnesium parvum*, has invaded plankton communities in coastal and freshwater systems throughout the world, blooming and

killing fish and other organisms (Carter 1937, Edvardsen & Imai, 2006). Since its discovery as the agent responsible for fish kills in southwestern Texas in the 1980s, *P. parvum* has expanded its range dramatically, blooming and causing fish kills in Oklahoma and across the southern United States, from California to Florida (Hambright et al. 2010).

Sampling for *P. parvum* typically involves obtaining a water sample, preserving it, and examining small subsamples under the microscope during which *P. parvum* cells are identified and counted. Microscopy can be time and labor intensive, especially if low error and detection limits are important. In many HAB monitoring programs that depend solely on microscopy, accuracy is often sacrificed for increased sample number, leading to conservatively high limits of detection and reduced capability for early detection, invasion status, or bloom initiation. Additionally, some HAB species are relatively small and fragile and can become distorted during preservation (Galluzzi et al. 2008), making their identification and detection even more difficult. Furthermore, enumeration of HAB species is often conducted amidst a diverse plankton assemblage, especially prior to bloom formation, making cells even more difficult to distinguish from other closely related taxa, thereby making highly trained personnel essential (Humbert et al. 2010). Although microscopy is commonly available in most laboratories, the above-mentioned factors render this method inefficient for accurate routine assessments of HABs. Because of these shortcomings, numerous molecular approaches have been developed for assessing HAB species (Humbert et al. 2010, IOC 2010). Specifically for *P. parvum,* methods for identifying and quantifying specific small subunit rRNA genes, such as dot blot hybridization (Simon et al. 2000), flow

cytometry (Simon et al. 1997), and solid phase cytometry (Töbe et al 2006) have been developed. West et al. (2006) also have develop a method based on solid phase cytometry and monoclonal antibodies. While excellent data can be generated using any of these methods, they can be prohibitively expensive, especially in cases involving numerous samples or continuous on-site monitoring.

Quantitative PCR (hereafter qPCR) is another molecular approach that has become a relatively common tool for monitoring HAB species (Humbert et al. 2010, IOC 2010; Martins and Vasconcelos 2011). Owing to factors such as fast processing times and relatively low cost of initial setup and consumables, qPCR provides a relatively inexpensive alternative for HAB species enumeration and monitoring (IOC 2010). Multiple qPCR methods have been devised for HAB species, including *Pseudo-nitzchia* (Fitzpatrick et al. 2010), numerous dinoflagellates (Bowers et al. 2000, Galluzzi et al. 2004, Kavanagh 2010), various cyanobacteria (Koskenniemi et al. 2007, Al-Tebrineh et al. 2011), and *P. parvum* (Galluzzi et al. 2008, Manning and LaClaire 2010), but most of these methods have not been incorporated into routine monitoring programs. Since *P. parvum* invaded and bloomed in Lake Texoma, OK-TX in 2004, we have been monitoring its population dynamics using microscopy-based methods (Hambright et al. 2010). Beginning in 2008, we began monitoring *P. parvum* using a modified version of the qPCR method developed by Galluzzi et al. (2008). Here we show that not only can this method be used as a viable alternative to microscopy in a routine monitoring program, but that qPCR also offers a lower limit of detection and higher levels of accuracy than microscopy.

2. Materials and Methods

2.1 Study Site

Lake Texoma is an impoundment of the Red and Washita Rivers on the border of Oklahoma and Texas, USA. It was constructed in 1944 for flood control, hydropower generation and recreation. *Prymnesium parvum* first bloomed in Lake Texoma in the winter of 2003-2004, with large populations restricted to the Red River arm of the lake, and blooms to littoral areas (Hambright et al. 2010). A regular monitoring program has been maintained since 2005 and in Jan 2008 we began collecting samples for use in molecular assays of *P. parvum* using qPCR at eight littoral sites (L) and five pelagic sites (P), either on the Red River arm (L1, L2, L3, L4, P1, P2), the main body (L5, L6, P3, P4), or the Washita River arm (L7, L8, P5) of the lake (see Hambright et al. 2010 for further site descriptions).

2.2 qPCR Primer Redesign

Methods for quantifying *P. parvum* via qPCR using primers targeting the ribosomal internal spacer transcribed (ITS2) region were developed by Galluzzi et al. (2008; PrymF, 5'-TGTCTGCCGTGGACTTAGTGCT-3' and PrymR, 5'-ATGGCACAACGACTTGGTAGG-3'). Galluzzi et al. (2008) did not report any aspecific amplicons or primer-dimers in their reactions, but we were unable to repeat their methods without formation of primer-dimers (which produced artificially high cell density estimates) using either *P. parvum* monocultures (UTEX strain LB 2797, UTEX Culture Collection, Colorado River, Texas, USA; maintained in our lab since 2006), natural lake samples, or culture isolates derived from Lake Texoma. Addition of bovine

serum albumin and manipulations of magnesium concentrations (Bustin and Nolan 2004) failed to alleviate the problem. We deduced that the problem might have been caused by self-complementarity in the GGTAGG motif located on the 3' end of PrymR (Rychlik 1993). Therefore, we redesigned the PrymR primer by removing 3 bases from the 3' end (creating PrymR–3, 5'-ATGGCACAACGACTTGGT-3') thereby removing the potential for the two guanosine doublets to contact and form hydrogen bonds (Hardin et al. 1991). We used Primer BLAST (Rozen and Skaletsky 2000) to check for target specificity in GenBank, EMBL, DDBJ, and PDB databases and tested the redesigned primer using varying concentrations of lake and culture samples. Our primers only produced matches to the ITS2 rRNA gene of *P. parvum* (GenBank accession numbers: *P. parvum* AM690999.1, *P. parvum* f. *patelliferum* AF289038.1, *P. parvum* FJ907460.1). The use of the PrymR–3 primer eliminated the appearance of primer-dimers (as determined by the melt curve analysis with a dissociation protocol at the end of each qPCR run) in all subsequent reactions.

Robustness of the modified protocol was assessed using *P. parvum* strains from diverse geographic locations, including field samples and *P. parvum* cultures derived from Dunkard Creek, West Virginia-Pennsylvania, USA (UOBS-WANA 576); Lake Granbury (UOBS-Granbury-506, Texas, USA; Lake Diversion, Texas, USA (UOBS-Diversion 504; Colorado River, Texas, USA (UTEX LB 2797); Florida, USA (UTEX LB 22837); and Norway (ppar 054, Bjerknes 28, Bognefiorden 28, and NIVA-3/89/3 ES). Our method accurately identified *P. parvum* from all locations and cultures.

2.3 Standard Curve

We constructed standard curves using linear plasmid DNA containing the cloned sequence targeted by the primers on the ITS2 region of *P. parvum* strain UTEX LB2797. We purified the plasmid DNA using the UltraClean Standard Mini Plasmid Prep Kit (MoBio Laboratories Inc.). This product was then digested with enzyme ScaI (Invitrogen) inside the ampicillin resistance gene and repurified in linear form using an UltraClean 15 DNA Purification Kit (MoBio Laboratories Inc.). We used linear plasmid DNA to avoid overestimation of gene and cell copy numbers that can result from supercoiling of circular plasmid DNA in the qPCR reaction (Hou et al. 2010). We quantified our linear plasmid using a high sensitivity Quant-iT dsDNA assay kit on a Qubit fluorometer (Invitrogen) and calculated plasmid copy concentration based on the average mass of one base pair. We constructed standard curves using 10-fold serial dilutions ranging from 2 to 2×10^6 copies with three analytical replicates. Data from four independent standard curves with freshly made plasmid standards were used for standard curve calibration for lake samples and data from nine independent standard curves were used for standard curve calibration for culture samples. Different curves were used for quantification of the two sample types because different well factors were used in the determination of sample fluorescence for lake and culture samples during the qPCR reaction (persistent and dynamic well factors, respectively). A standard curve based on linear plasmid DNA and no-template negative controls were included in each run and baseline threshold was held constant between runs to ensure the consistency of standards and quantification between runs.

2.4 Microscope vs. qPCR and Error Comparison

Samples for microscope counts were preserved using Lugol's solution (1% final concentration) and counted within two days from collection (haemocytometer method minimum of six 1-μL subsamples; detection limit = ~333 ± 817 cells mL^{-1} (mean ± SD)) using a stereomicroscope with DIC Nomarsky illumination at 200× magnification. Identification was confirmed at 400× magnification. Although this detection limit generates values that individually are not statistically different from zero, our long-term monitoring program has revealed that such low values, when at multiple sites and times, are biologically significant. Furthermore, this method was used in order to maintain comparability with Texas Parks and Wildlife Department data for *P. parvum* in Lake Texoma during 2004-2006, and to allow timely assessment of *P. parvum* in thirteen lake samples weekly, as standard counts following sedimentation (Lund et al. 1958) would have greatly decreased sample throughput. For qPCR enumeration, we filtered 100-1000 mL (depending on particulate load) of lake water through GF/F glass-fiber filters using gentle vacuum (< 0.17KPa) and then applied methods adapted from Countway and Caron (2006) for processing the material retained on the filter. The filters were folded twice and submerged in 2 mL of lysis buffer (100 mM Tris [pH 8], 40 mM EDTA [pH 8], 100 mM NaCl, 1% sodium dodecyl sulfate), 200 μL of 0.5-mm zirconia-silica beads, in 15-mL Falcon tubes and stored at –20ºC until processing. Frozen filters were thawed in a 70ºC water bath for 5 min and lysed by bead beating on a vortexer set to the highest setting for 30 s. Heating and bead beating was repeated two additional times to ensure complete lysis. The crude lysates were then poured into sterile, 2-mL microcentrifuge tubes and stored at –20ºC until they were analyzed with qPCR. All

samples were analyzed as crude lysates diluted 1:100 in qPCR reactions to remove inhibitory effects of lysis buffer, cellular contents, or other organismal DNA before adding them to qPCR reactions.

For lake samples, we performed qPCR assays in a final volume of 25 μL containing 1× SYBR Green PCR Mastermix (Applied Biosystems), 100 nM PrymF and PrymR-3 primers (Invitrogen), and 3μL of template from the diluted crude lysates. For each sample, we examined at least three replicate reactions using 96-well plates sealed with optical film B (BioRad) on an iQ5 real-time PCR detection system (BioRad) and analyzed using the associated iQ5 optical system software. Each reaction included an initial denaturation at 95°C for 4 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min (Galluzzi et al. 2008). Fluorescence within qPCR reactions for lake samples was determined using persistent well factors. Detections were considered significant for samples that produced a detectable signal in at least two replicates and the mean DNA quantity was significantly different from 0 ($P \leq 0.05$).

Because qPCR quantifies the number of copies of a targeted sequence and there are potentially multiple copies of that sequence in a cell, we needed to determine the copy number of the sequence that we targeted for a *P. parvum* cell before we could translate gene quantities into meaningful cell densities. To determine the mean sequence copy number per cell for lake samples, we conducted three independent experiments in which we created 24 independent samples from log-phase growth batch cultures, dilutions of those cultures, and dilutions of steady-state chemostat cultures from a strain of *P. parvum* isolated from Lake Texoma (site L2; UOBS-LebanonPool-249). Cultures were grown in batch and chemostat in modified COMBO medium (Kilham et al. 1998)

at N:P = 16:1 (800uM N : 50uM P), supplemented with 6 g Instant Ocean™ L^{-1}, and maintained on a 12:12 light:dark cycle at 25ºC. Sample *P. parvum* abundances ranged from ~150 – 15,000 cells mL^{-1}. We used low-cell densities to avoid inhibition of qPCR reactions due to excessive amounts of template DNA. Culture sample cell densities were determined via flow cytometry (BD-FACSCalibur) and culture samples were filtered and processed for qPCR as noted above for lake samples, except we used 200 nM of PrymF and Prym R–3 primers to ensure that enough primer was present to bind to all of the available copies of target DNA. Fluorescence in qPCR reactions for culture samples was analyzed using dynamic well factors within separate runs. Copy number was then defined as the slope of simple linear regression of copies of the targeted ITS2 sequence detected by qPCR versus *P. parvum* abundances detected using flow cytometry (SPSS v19). The copy number regression was forced through the origin, as copy number should directly correspond to cell number (Motulsky & Christopoulos 2004). Although data were not normally distributed, data were not transformed to avoid transformational biases that can occur when returning data to its original format and because the primary goal of the analysis was to define the relationship (i.e., the slope) between cell number and gene copies.

To assess the accuracy of the experimentally-determined copy number, we regressed *P. parvum* cell densities for lake samples determined microscopically with cell densities determined by translating lake sample sequence quantities determined via qPCR. Cell densities in lake samples were estimated by dividing the quantities of the targeted sequence in each sample by the number of sequence copies per cell obtained from the regression analysis described above (i.e., the slope). We limited our analysis to

samples from Jan. 2008 – Mar. 2011 in which *P. parvum* was detected using both methods, N = 177). Four samples with standardized residuals > 4× standard deviations were removed. Three of these samples had substantially higher sequence copies than were found in any other sample and would correspond to *P. parvum* abundances much higher than have ever been reported in nature. The fourth sample had extremely low numbers of sequence copies for the number of cells detected microscopically. The regression of cell density from both methods was also forced through the origin, as cell density determined microscopically should directly correspond to cell densities determined via qPCR (Motulsky & Christopoulos 2004). We also plotted cell density estimates through time from both the microscope and qPCR to further compare the two methods. To estimate and compare the measurement error associated with our qPCR and microscopical methods we determined the coefficient of variation from the analytical replicates and subsamples for each lake sample that produced a significant cell density estimate by either qPCR or microscope count, respectively. These error estimates were plotted against the *P. parvum* cell densities produced from those measurements and we determined the relationship between cell density and error using simple curve fitting (SPSS v19).

3. Results

3.1 Standard Curve

The four independent standard curves for quantifying lake samples based on fluorescence assessed with persistent well factors yielded a linear relationship between the threshold cycle (Ct) and the $\log_{10}$ of the starting quantity of linear plasmid ITS2

rDNA sequence (SQ), Ct = -3.64x 64(SQ) + 34.85, $r^2 = 0.99$, efficiency = 0.88% (Figure 1a). The nine independent standard curves for quantifying culture samples from the copy number experiments based on fluorescence assessed with dynamic well factors yielded the relationship Ct = -3.39(SQ) + 32.45, $r^2 = 0.99$, efficiency = 0.97% (Figure 1b).

3.2 Microscope vs. qPCR and Error Comparison

The average number of copies of the targeted sequence of ITS2 rDNA per *P. parvum* cell based on samples from the strain isolated from Lake Texoma was 11.7 ± 0.6 (slope ± SE) (Figure 2a). Quantities of the targeted sequence obtained through qPCR for the strain isolated from site L2 were also a good fit to cell densities obtained via flow cytometry ($N = 24$, Adj. $r^2 = 0.94$, $P < 0.001$; Figure 2a). When converted to cell densities using this copy number, gene quantities obtained for lakes samples by qPCR were equivalent to cell density estimates determined microscopically (qPCR cell density = 1.1(microscope cell density; $N = 177$, Adj. $r^2 = 0.85$, $P < 0.001$; Figure 2b).

Overall, *P. parvum* abundance estimates by both qPCR and microscopy were similar at each of the 13 monitored sites through time (Figures 3 and 4). The highest abundances by either method were recorded during winter months at sites typically known to experience *P. parvum* blooms (e.g., L2, L3, P1; for detailed site locations see Figure 1 in Hambright et al. 2010). At other sites with mid-range abundances of *P. parvum* we also saw consistent results between qPCR and the microscope (e.g., L1, L4, L5, P2). The lowest cell density that we detected in the lake was 26 cells mL^{-1} (P3), increasing our practical detection limit nearly 13-fold over the detection limit of our

microscope method employing a haemocytometer (~333 cells mL^{-1}). This increase in limit of detection was most evident in sites with the lowest *P. parvum* abundances, particularly those sites in which blooms have never been observed (e.g., L6, L7, L8, P3, P4, P5).

Coefficient of variation plotted against mean *P. parvum* cell densities revealed similar power curve relationships for both qPCR ($CV = 0.59(dens)^{-0.2}$; Adj. $r^2 = 0.26$, $P < 0.0001$, $N = 271$) and microscopy ($CV = 40.2(dens)^{-0.5}$; Adj. $r^2 = 0.90$, $P < 0.0001$, $N = 187$) with measurement error increasing with decreasing cell density (Fig. 5). However, the coefficients of variation for qPCR-determined *P. parvum* densities were generally much lower than those for the microscope method, rising to no more than 0.7 near the minimum level of detection. By contrast, coefficient of variation for the microscope method rose to 2.5 at its detection limit.

4. Discussion

We modified Galluzzi et al.'s (2008) qPCR method by deleting three bases from the 3' end of the reverse primer and tested whether it could be used as an alternative to microscopy in our ongoing lake monitoring program. The modified method eliminated the occurrence of primer-dimers, was robust for multiple *P. parvum* strains from North America and Norway and provided similar cell densities in natural samples compared to those obtained by standard microscopical methods. Relative to microscopy, qPCR had a much lower detection limit with substantially lower error and time investment (see also Fitzpatrick et al. 2010). Furthermore, the copy number of the targeted ITS2 rDNA sequence for the strain isolated from Lake Texoma (11.7 ± 0.6) was consistent with

values obtained for the two different strains assessed by Galluzzi et al. (2008; Strain KAC 39, Norway: 10.0 ± 2.8 and Strain CCMP 708, Scotland: 15.6 ± 1.6). This consistency suggests that our method is also robust to natural variations that could potentially affect copy number including cell division and sex (sensu a haplo-diploid life cycle; Larsen and Edvardsen 1998), and genomic variability. Because PrymR–3 lowered detection limit and removed the potential for self-complementarity and primer-dimers during amplification, it may be more suitable for accurately quantifying the presence of *P. parvum* in natural samples than the original PrymR used by Galluzzi et al. (2008).

The high levels of total suspended solids in Lake Texoma (Atkinson et al. 1999) limited the volume of water that we could filter through a GF/F filter, restricting the amount of algae that we were able to concentrate on a filter, and ultimately, our detection limit. For example, if we were to filter 200 mL of lake water, detection of an average of one sequence copy per reaction would translate to 28 cells mL^{-1}, while the same detection after filtering 1L of lake water would translate to 6 cells mL^{-1} – a major increase in detection limit. However, our actual detection of 26 cells mL^{-1} at P3 (800mL filtered) and other similar findings of low cell densities by qPCR suggests that we have increased our detection limit by at least 13-fold from the detection limit of our haemocytometer and microscope method (26 cells mL^{-1} vs. 333 cells mL^{-1}). Indeed, a 26 cells mL^{-1} detection limit is comparable to the minimum limits of detection that can be obtained using the Ütermohl sedimentation method with extensive subsampling under an inverted microscope (Lund et al. 1958), which is even more time intensive than our haemocytometer method.

While the patterns of *P. parvum* cell densities based on qPCR were similar to those based on microscope enumeration, we were able to detect *P. parvum* on many occasions in which the detection limit of our microscopical methods precluded its detection (e.g., L6, L7, L8, P4, P5). As we approached these low cell densities, measurement error for both methods increased. However, measurement error in the qPCR samples was weakly explained by cell density (26% of the variance) suggesting that something other than cell density is a better predictor of measurement error using qPCR. Measurement error was also substantially lower for qPCR (180% lower at maximum values, i.e., CV = 1.8), which lends credence to values obtained using this method. In late 2009, numerous sites (L1, L2, L3, L4, L5, P1) showed increases in *P. parvum* abundances detected by qPCR that were consistent with bloom formation patterns in previous years, but that went undetected by the microscope even though cell densities were well above its detection limit (up to 50,000 cells mL^{-1}). There are a few instances where *P. parvum* was detected by the microscope, but not by qPCR (e.g., L8 Feb 2008 and Jan 2009). As pointed out above, such low values at the microscope detection limit (~333 ± 817 cells mL^{-1}) are not statistically different from zero when considered individually. Given the vast improvement in detection limit using qPCR it is likely that those two instances are in fact due to microscope error (e.g., misidentification of a morphologically similar cell, etc.). The increase in detection limit, the reduction of measurement error, and the detections of *P. parvum* by qPCR that were not detected by microscopy suggest that using qPCR should increase the ability to detect *P. parvum* earlier during an invasion event or during bloom formation and that the enumeration by

the microscope-based haemocytometer method can miss early bloom formation even when abundances are above its detection limit.

Since Galluzzi et al. (2008) developed the original qPCR method for assessing *P. parvum* densities using SYBR green, Manning and La Claire (2010) developed a new method incorporating multiplex methods and molecular beacons for qPCR using 4 genomic DNA markers. Although this multiplex method is more technologically advanced it is hindered by a reduced reaction efficiency of the probes with increasing geographic and genetic distance. Hence the multiplex method would likely also be useful in lake monitoring programs, but would need to be optimized to a given system and ecotype. Because we targeted a more conserved genetic marker in the small subunit rDNA, our modified method seems to have avoided this limitation as the gene copy number for the targeted ITS2 region is consistent across 4 different strains and two continents and is able to detect successfully 14 different strains across 2 continents. Thus it appears that our modified Galluzzi et al. method may be more generally applicable in monitoring programs than multiplex methods.

Because we assess *P. parvum* densities at 13 sites in the lake approximately 32 times per year (up to 400 samples annually; Hambright et al. 2010), we have chosen to sacrifice detection limit for quantity of samples by using the haemocytometer-based microscopy method for enumeration. At approximately 15 minutes per subsample, and at least six subsamples per sample, monitoring *P. parvum* abundances via microscopy is a significant investment of time (~600 person-hours annually). Using qPCR, we can quantify *P. parvum* densities in 24 samples with triplicate replication every 3 hours. Of these three hours, only one hour is required for setting up the qPCR reactions, while

during the remaining time the machine is running unattended. Thus, we can count the same number of samples (with lower error and higher resolution, precision, and accuracy) for a given year in 8% of the time necessary with microscopy (~50 person-hours).

In conclusion, in this study we show that qPCR can be used successfully to assess and efficiently track *P. parvum* abundances in natural environmental samples. The method is specific, sensitive, and rapid. Since qPCR methods have been and are being developed for many HAB species, it is likely that similar methodology employing qPCR could be expanded in order to help shift the focus from a reactive stance dealing with the harmful effects of *P. parvum* and other HABs after they have bloomed to a more proactive stance aiding in the early detection of blooms or the invasion of new ecosystems. Specifically, detection of *P. parvum* with qPCR could prove useful if combined with routinely collected environmental data to predict *P. parvum* presence in new systems where it has previously gone undetected or in predicting which systems *P. parvum* might invade in the future. This would be particularly salient for newly forming monitoring programs for systems in which *P. parvum* has recently or is currently invading.

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Figures

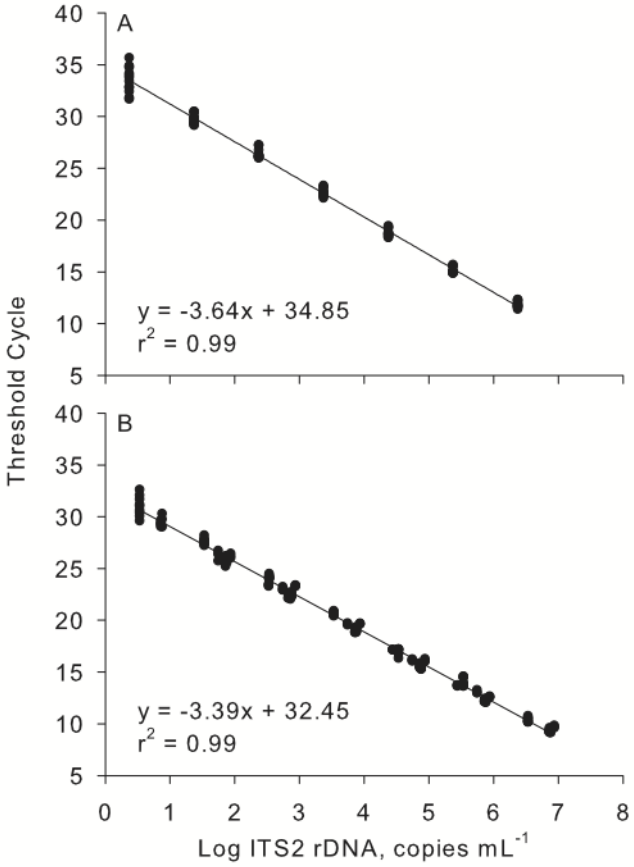

Figure 1. Composite standard curve calibration of *Prymnesium parvum* qPCR method produced via dilutions of linear plasmid containing the ITS2 rDNA sequence using (a) persistent well factors for lake and (b) dynamic well factors for culture samples.

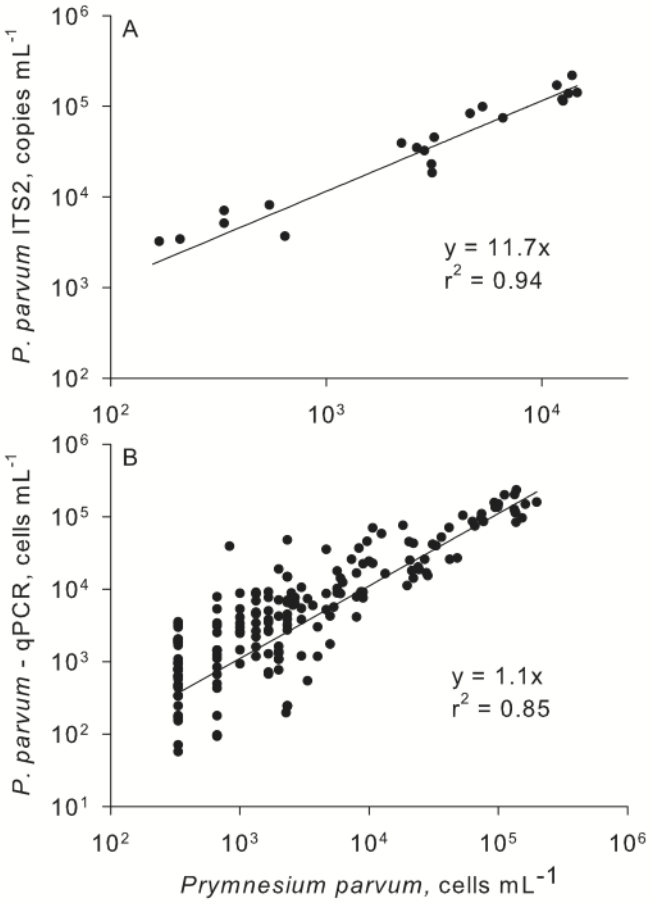

Figure 2. (a) Linear regression of *P. parvum* abundance (cells mL-1) obtained via flow cytometry and ITS2 rDNA (copies mL-1) obtained via qPCR for culture samples to copy number of the targeted sequence per cell. (b) Linear regression of log *P. parvum* abundance (cells mL^{-1}) obtained via microscope counts and *log P. parvum* abundance (cells mL^{-1}) converted from ITS2 rDNA quantities obtained via qPCR from Jan. 2008 – Mar. 2011 that detected *P. parvum* using both methods.

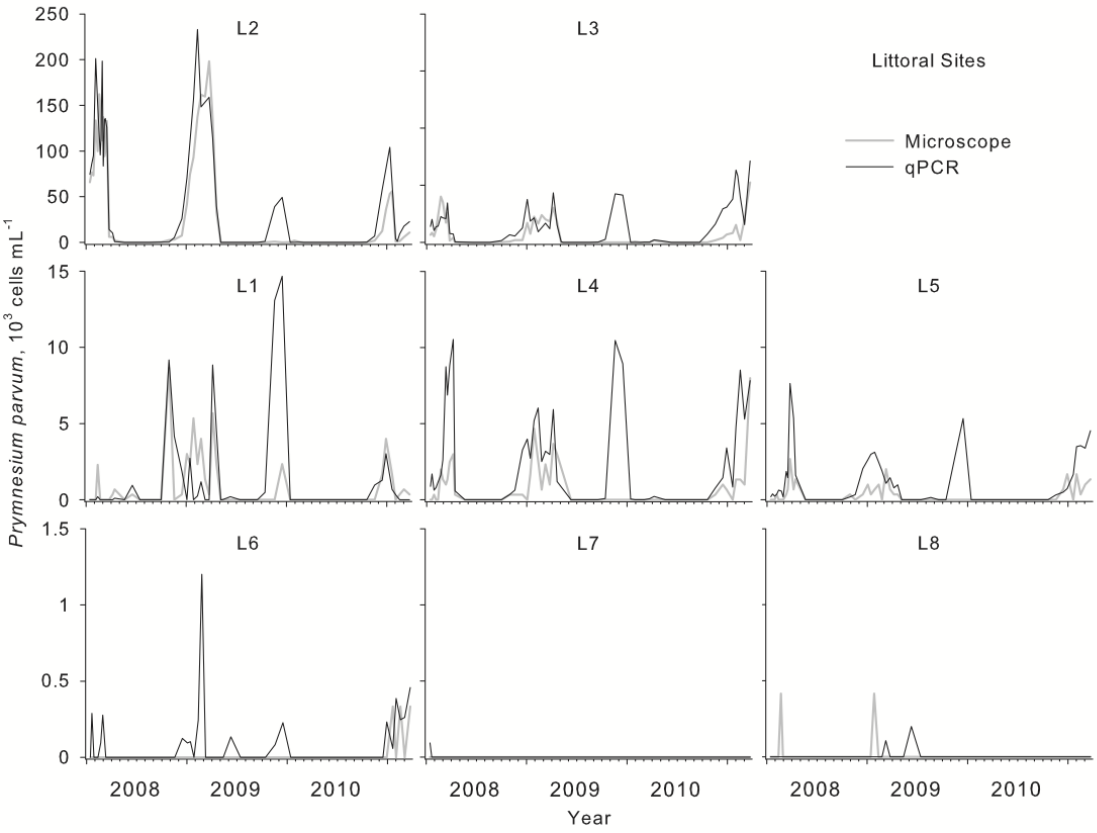

Figure 3. Comparison of *P. parvum* abundance estimates obtained via microscope (black line) and qPCR (red line) at littoral stations from Jan. 2008 – Nov. 2010 in Lake Texoma. See Hambright et al. (2010), for description of monitoring program and sampling station details.

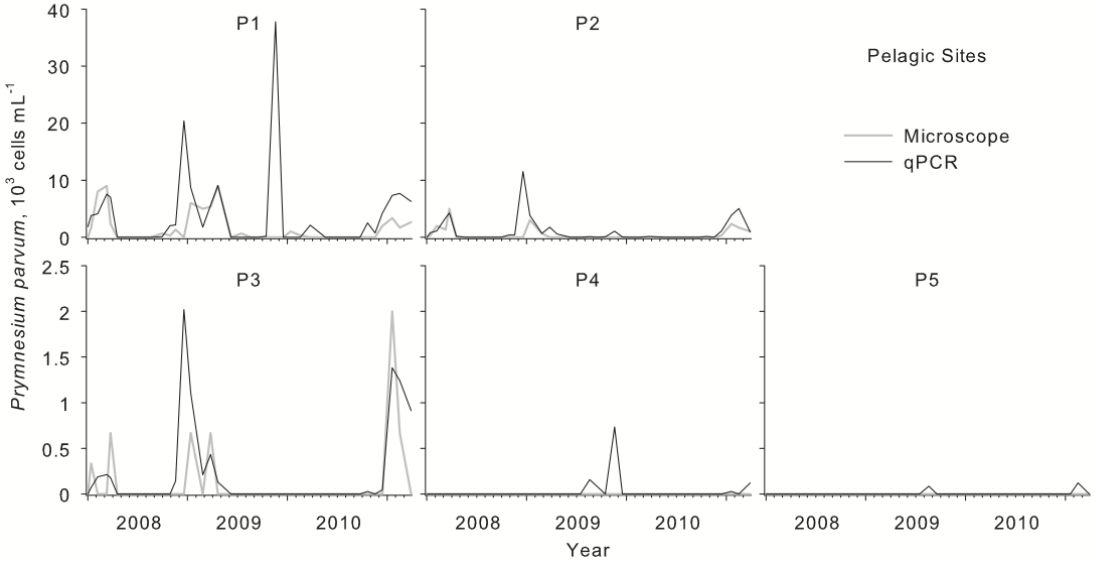

Figure 4. Comparison of *P. parvum* abundance estimates obtained via microscope (black line) and qPCR (red line) at pelagic stations from Jan. 2008 – Nov. 2010 in Lake Texoma. See Hambright et al. (2010), for description of monitoring program and sampling station details.

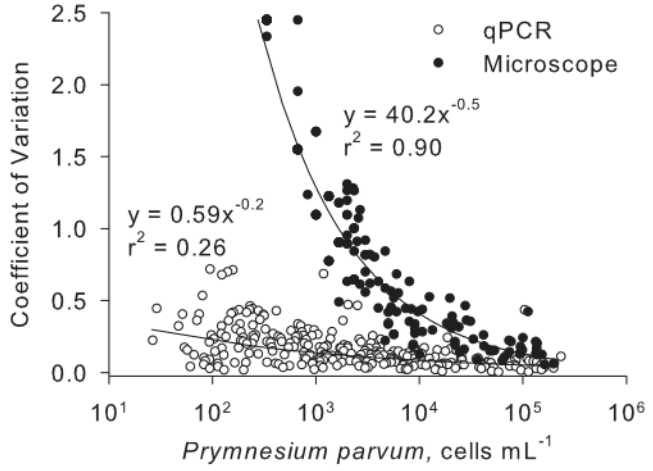

Figure 5. Error estimates (coefficient of variation) for measurements of P. parvum cell density using qPCR (red circles) and microscopy (black circles).

Chapter 2 – Rapid recovery of a fish assemblage following an ecosystem disruptive algal bloom

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Keywords: Ecosystem Disruptive Algal Bloom (EDAB), harmful algae, fish kill, *Prymnesium parvum*, Lake Texoma

Abstract

Disturbance of freshwater ecosystems through cultural eutrophication has resulted in an increased global occurrence of Harmful Algal Blooms (HABs). Ecosystem Disrupting

Algal Blooms (EDABs), as their name implies, are a subset of HABs that produce extensive disturbances across entire ecosystems. *Prymnesium parvum* is one such EDAB species that has invaded freshwater systems worldwide, causing massive fish kills and other negative effects. Fish kills frequently occur during HABs and EDABs, but there has been little study of long-term implications of these fish kills, nor of the resilience and recovery of fish assemblages following kills. We sampled fish near and offshore over an annual cycle encompassing a *P. parvum* EDAB event in two coves (i.e., a bloom site and a reference site) of a Southern Great Plains reservoir, Lake Texoma. Our objective was to document the processes of extirpation and recovery of a fish assemblage to the disturbance of an EDAB event. *Prymnesium parvum* bloomed in one cove from mid-December 2008 until May 2009, eliminating all fish during this period. Fish toxicity bioassays indicated no substantial differences in susceptibility across fish species to *P. parvum* toxins. Fish rapidly recolonized the bloom site in May 2009 after the *P. parvum* bloom diminished. Fish assemblages were resilient to the *P. parvum* EDAB, recovering to previous abundance, richness, and composition within six months. Our results suggest that the reservoir-wide fish metacommunity enabled a rapid recovery of local fish assemblages following a spatially heterogeneous EDAB event.

Introduction

Ecological communities can be influenced profoundly by ecosystem disturbances and environmental perturbations (Hutchinson 1953, Connell 1978, White and Pickett 1985). Natural and anthropogenic disturbances can shape community structure by changing resource availability and creating opportunities that can be used

by newly arriving species or aggressive species within the disturbed community (White and Pickett 1985, Davis et al. 2000, Lockwood et al. 2007). Freshwater ecosystems have experienced extensive anthropogenic disturbances associated with increased nutrient availabilities (Smith 2003, Smith and Schindler 2009), which have caused an increased incidence of Harmful Algal Blooms (HABs) worldwide (Hallegraeff 1993, Smith 2003, Smith and Schindler 2009). Harmful algal blooms are proliferations of algae that cause deleterious effects on other organisms, often via the production of toxins (Hallegraff 1993, Landsberg 2002, Granéli and Turner 2006). The global frequency and severity of HABs has ignited interest in assessing their consequences for human and economic health (Granéli and Turner 2006). Moreover, a subset of HABs have been described as Ecosystem Disrupting Algal Blooms (EDABs) because the species involved not only respond to disturbances, such as increased nutrients, but also become a disturbance themselves by altering ecosystem structure and function (Sunda et al. 2006).

Beyond the negative effects of HABs in general, EDABs are characterized by their adverse direct effects on fishes and herbivorous invertebrate grazers, and their indirect effects on nutrient and food web dynamics, which create feedbacks that can enable bloom persistence (Sunda et al. 2006). One such EDAB species is *Prymnesium parvum* (Carter 1937), a toxigenic marine haptophyte that has invaded freshwater systems worldwide causing widespread fish kills (Edvardsen and Imai 2006, Lutz-Carrillo et al. 2010). In addition to causing fish kills, recent studies have revealed other substantial negative impacts on herbivorous zooplankton, including reduced survivorship, growth rates, and fecundities (Michaloudi et al. 2009, Remmel et al.

2011), primarily through the release of contact glycolipid toxins, which likely evolved to support heterotrophy (Henrikson et al. 2010, Remmel and Hambright 2012). *Prymnesium parvum* blooms also have substantial impacts on abundances and diversity of other unicellular eukaryotes and bacteria (Michaloudi et al. 2009, Jones et al. *in prep*). In North America, the first record of a *P. parvum* EDAB is from a fish kill in 1985 in the Pecos River system of southern Texas, USA (James and De La Cruz 1989). During the subsequent two decades, *P. parvum* gradually expanded its range causing fish kills in reservoirs and rivers throughout much of the southern United States from California to Florida, and as far north as Wyoming and West Virginia (Roelke et al. 2010, Hambright et al. 2010, Zamor et al. 2012).

Although EDAB-related fish kills have been documented numerous times in the literature (reviewed in Landsberg 2002), fish are rarely integrated into analyses assessing bloom dynamics. Currently there is surprisingly little data on fish kills themselves (e.g., species affected and the extent of mortality). Thus, little is known of the longer-term response, e.g., the resilience and recovery, of fish assemblages to EDAB-related fish kills. Our goal here was to document the response of a fish assemblage to the disturbance of an EDAB event. We sampled fishes in two coves of a Southern Great Plains reservoir during a *P. parvum* EDAB: one cove experienced a *P. parvum* bloom, and has experienced previous recurrent *P. parvum* blooms; the other cove experienced *P. parvum* presence, but no blooms. Our study covered an annual cycle, including samples before, during, and after a bloom and provides insight into the extirpation and subsequent reassembly and recovery of a fish assemblage from the devastating effects of a *P. parvum* bloom.

Methods

Study Site

Lake Texoma, constructed in 1944, was formed by the impoundment of the Red and Washita Rivers on the border of Oklahoma and Texas, USA (Fig.1). Its drainage basin encompasses 87,500 km^2 and at normal lake elevation is the 12^{th} largest reservoir in the USA. At least 50 species of fish inhabit Lake Texoma, including many recreationally important species (e.g., striped bass, *Morone saxatilis* (Walbaum); Matthews et al. 2004). *Prymnesium parvum* first bloomed in Lake Texoma in the winter of 2004 causing a massive fish kill in many of the shallow areas of the Red River arm of the reservoir (Hambright et al. 2010). Including this first bloom, *P. parvum* has bloomed during winter in seven of the nine winters through 2012, with the strongest blooms in Lebanon Pool (hereafter LP), a ~130-ha embayment formed by the confluence of Hauani Creek and Lake Texoma (Fig. 1). Extensive sedimentation by the Red River has blocked much of the mouth of LP, for which connectivity to Lake Texoma is usually maintained only through one or two narrow channels to the main reservoir body (Fig. 1) during normal to high water levels, but is often lost seasonally at low water levels, such as during winter and early spring (Fig. 2). *Prymnesium parvum* EDABs in Lake Texoma appear to be fueled by high nutrient concentrations and salinities when LP is disconnected from the reservoir due to low water levels (Hambright et al. 2010). However, blooms of *P. parvum* do not always produce fish kills (e.g., winter 2007-2008), and it is currently unclear what environmental conditions trigger increased *P. parvum* toxicity. The closest reservoir embayment upstream is ~1050-ha Wilson Creek Cove (hereafter WCC; Fig. 1), which can also lose its

connection to the reservoir during seasonally low water levels (Fig. 1) and has similar water chemistry as LP; however, it has never experienced a toxic bloom or fish kill, although *P. parvum* is commonly present in WCC at low abundances during bloom periods (Hambright et al. 2010, Zamor et al. 2012; Fig. 2). We therefore focused on fish in LP as an affected assemblage while WCC served as a reference for unaffected assemblages.

Fish Sampling

We assessed the temporal effects of the *P. parvum* EDAB on fish by sampling nearshore and offshore fish assemblages in LP and WCC, before, during, and after a toxic bloom in LP. We sampled nearshore fish monthly from October 2008 – March 2009 and during June, July, and October of 2009. At each sampling event, we collected nearshore fish by conducting four 25-m seine hauls (1.83m × 4.57m seine, mesh = 3.18mm) along four 100-m reaches of shoreline that were separated from one another by at least 100m (Fig. 1). We surveyed the same four 100-m reaches each month. Seined fish were pooled from the seine hauls within each 100-m survey and were immediately preserved in 10% formalin before returning to the lab for identification. We completed 16 seine hauls per sampling trip (four per 100-m survey) at each site regardless of the number of individuals collected (e.g., no fish during the *P. parvum* bloom at LP). In the lab we identified and measured individual fish (total length; TL) for up to 100 arbitrarily-selected individuals of each species from each reach. Following measurement, we counted any remaining individuals for species numbering over 100 and then stored all fish in 50% isopropyl alcohol.

We sampled offshore fish monthly from November 2008 – October 2009 using gill nets. At each cove in each month, we set two experimental gill nets for a single night. Nets were 61m × 1.8m and composed of eight 7.6-m panels, with mesh ranging from 1.27cm – 10.16cm. Mesh size changed at each panel in 1.27-cm increments (i.e., panel 1 = 1.27-cm mesh, panel 2 = 2.54-cm mesh, etc.). A shallow net was set horizontally with the float line just below the surface, and a deep net was set horizontally, with the lead line along the bottom. Nets were set in the evenings between 16:00 and 19:00 and retrieved the following day between 10:00 and 14:00. Fish were removed from nets, and weighed and measured (TL).

Algae monitoring

As part of a larger ongoing *P. parvum* monitoring project on Lake Texoma (see Hambright et al. 2010), densities of *P. parvum* were assessed monthly in both WCC and LP during the study period using microscope- and qPCR-based counts (Zamor et al. 2012). Because qPCR offers better resolution of cell numbers with lower error rates (Zamor et al. 2012), qPCR results are reported in this paper. We defined blooms as any time *P.* parvum densities exceeded 10,000 cells mL^{-1}, as densities above this level frequently result in fish kills (Roelke et al. 2010).

Fish toxicity bioassays

In order to test for the possibility of species-specific differences in susceptibility to *P. parvum* of Lake Texoma fish, we conducted *P. parvum* toxicity bioassays with four Lake Texoma fishes (striped bass, *Morone saxatilis*; inland silverside, *Menidia*

beryllina (Cope); gizzard shad, *Dorosoma cepedianum* (Lesueur)) and for reference, juvenile and adult fathead minnows (*Pimephales promelas* (Rafinesque)). Because there is little data available in the literature other than anecdotal evidence (see Rhodes and Hubbs 1992) that fish may differ in susceptibility to *P. parvum* toxins, we chose these species to represent the taxonomic and ecological breadth of fishes found in the lake (Matthews et al. 2004). Rather than using standard acute toxicity bioassays (USEPA 2002), we measured time to death of lake fish relative to time to death in 10- to 14- day-old fathead minnow larvae. This procedure allowed us to make useful inference regarding *P. parvum* toxicity to Lake Texoma fish without the high level of sacrifice required in standard LC_{50} bioassays. All fish in each bioassay were exposed to concentrations of laboratory-cultured *P. parvum* (for culturing methods see Zamor et al. 2012) that exceeded a previously determined LC_{50} concentration for fathead minnow larvae by 50-150% (Hambright *unpublished data*) for 24 hours. Each experiment consisted of six test fish and six fathead minnow larvae, half of each received *P. parvum,* while half served as controls (except for two shad experiments in which two and four fish were used). All lake fish used in experiments were collected from the lake by shoreline seining (and presumably individuals younger than one year old) and allowed to acclimate to laboratory conditions for 48 hours prior to use in experiments. Adult fathead minnows were laboratory-reared individuals younger than one year old. Bioassays with lake fish and adult fathead minnows were conducted in one-gallon aerated jars containing three individuals per jar and separate jars for each species. Larval fathead minnow bioassays were conducted in 100-mL jars. *Prymnesium parvum* grown in 15 ppt salinity culture medium was added to half of the jars at final

concentrations of 200,000-400,000 cells mL^{-1}, and the same volume of 15 ppt culture medium without *P. parvum* was added to the other half. The volume of culture used varied with culture density, and bioassay salinities ranged between 1 and 3 ppt. Time to death following the addition of *P. parvum* to jars was recorded for all individual fish in each experiment. Experiments without complete mortality of all fish exposed to *P. parvum* at the end of 24 hours were not used in data analysis to eliminate possible confounding by low-toxicity *P. parvum* cultures. Analyses of normality and relative species-susceptibility were conducted in R (R Development Core Team 2011).

Data Analysis

We assessed the effects of the *P. parvum* EDAB on the temporal dynamics of fish communities by comparing total fish abundances, species richness, and assemblage structure in both coves before, during, and after the bloom. However, due to differences in sampling times and methods, we analyzed the seine and gill net data separately.

Nearshore fish: Abundance and Species Richness

To assess variation in nearshore fish abundance between coves during the sampling period, we used repeated-measures ANOVA. The dependent variable was $\log_{10}$-transformed total fish abundance in each reach (n = 4), time (month) was the repeated measure, and between subjects effect was cove. Effect sizes of independent variables (time and cove) were assessed via partial η^2. Species richness was similarly assessed over time in each cove using repeated measures ANOVA. However, because the number of individuals collected varied amongst reaches, we estimated species

richness in each reach through individual-based rarefaction (Hurlbert 1971) using the rarefy function in R (R Development Core Team 2011). Species richness in each reach was rarefied to 45 individuals (i.e., the smallest number of individuals collected amongst all reaches). ANOVA was conducted in PASW v. 18 (SPSS Inc. 2009) and assumptions of sphericity were met in both analyses. No fish were caught in LP during the bloom period (see Results below); therefore these three months were excluded from analyses.

Offshore fish: Abundance and Species Richness

Two gill nets were set in each cove during a given month, and the fish removed from each were not tabulated separately, therefore we could not assess within-month variation in total fish abundance or species richness. Accordingly, we qualitatively investigated trends in total fish abundance and species richness. However, we did rarefy species richness in each month using eight individuals (i.e., the least number of individuals collected in a cove in one month) to control for the abundance of individuals captured between coves and amongst months.

Fish Assemblage Structure

We were interested primarily in assessing differences in assemblage structure at different spatial and temporal scales. Thus, we tested for differences in assemblage composition before and after the *P. parvum* EDAB between and within the two coves during the sampling period for the nearshore fish assemblage.

We used Nonmetric Multidimensional Scaling (NMDS) based on Bray-Curtis dissimilarities to assess fish assemblage structure for each 100-m survey in both coves, before (October – December 2008) and after the bloom (June, July, and October 2009). Data were $\log_{10}(x + 1)$ transformed to reduce the effects of super-abundant species (e.g., *Menidia beryllina*). Similarly, only species that appeared in > 10% of collections were used in the analysis to avoid a skewing of the analysis by rare species. As in our analyses of abundance and richness, we removed the three months during the bloom (January-March 2009) because no fish were caught in LP (see Results below). Following NMDS ordinations, a multi-response permutation procedure (MRPP) using Euclidean distances and 10,000 permutations was used to assess if groups (i.e., before and after bloom within and between coves) exhibited significant clustering in the two-dimensional NMDS space. Significant clustering was determined through comparisons of the expected and observed MRPP statistic δ (the overall weighted mean of within group means of the pairwise dissimilarities amongst sampling units). Significance of δ is assessed similarly to a *p*-value. Cut-off values for Significance of δ were adjusted using a Bonferroni correction for multiple comparisons ($\alpha = 0.0125$). If MRPP indicated significant clustering of groups, indicator species analysis (ISA) with 10,000 iterations was conducted and species with significant indicator values above 0.50 were determined to be driving group separation. All analyses were conducted in R unless otherwise stated (R Development Core Team 2011). We used the vegan package for NMDS and MRPP (Oskanen et al. 2012) and the package labdsv for ISA (Roberts 2012).

Results

Prymnesium parvum cell densities above 10,000 cells mL^{-1} were first detected on 15 Dec 2008 in LP (25,000 cells mL^{-1}), peaked on 10 Feb 2009 (253,000 cells mL^{-1}) and remained above 10,000 cells mL^{-1} until 1 May 2009. Cell densities never reached 10,000 cells mL^{-1} in WCC (Fig. 2). The decline in cell densities in late April-May 2009 coincided with increases in the lake elevation following spring rains (Fig. 2). Using both sampling methods, a total of 35,660 fish representing 33 species were collected (see Table S1 in Supporting Information).

Nearshore Fish: Abundance and Species Richness

Most individuals, 33,385 fish, were collected in our nearshore samples, representing 22 and 25 species in LP and WCC, respectively. Fish were collected in both coves in October, November, and December 2008. From January – April 2009, fish were caught in WCC but not in LP (Fig. 2). Fish reappeared in the catch in LP during sampling in June 2009.

Time had the strongest significant effect on total fish abundance (effect size = 0.80), followed by cove (0.52), and their interaction (0.32; Table 1). Fish abundance was higher in LP than WCC before the bloom. However, abundances in both coves declined to equivalent levels in December. Following the EDAB, abundances increased in both coves and were greater than levels observed before the bloom by July. Fish abundance was slightly higher in WCC in June, but then returned to a similar pattern seen before the bloom as fish were more abundant in LP on the last two sampling dates (Fig. 3).

Variation in rarefied species richness was dependent on cove (effect size = 0.96), followed by time (0.88) and their interaction (0.79) (Table 1). As with abundance, species richness was higher in LP than in WCC prior to December. However, in December richness was slightly higher in WCC. Following the EDAB, species richness returned to similar levels as observed before the bloom in LP, but increased substantially in WCC in June and July before returning to pre-bloom levels in October (Fig. 3).

Offshore Fish: Abundance and Species Richness

A total of 2,275 fish were collected in our offshore samples with 17 species caught in LP and 18 species caught in WCC. As with the nearshore fish assemblage, fish were collected in both coves in October, November, and December 2008, but from January – April 2009, fish were only caught in WCC. However, both total fish abundance and species richness rebounded rapidly in LP starting in May 2009. Offshore fishes qualitatively demonstrated dynamics similar to nearshore fishes for total fish abundance over the sampling period. However, no noticeable quantitative differences in rarefied species richness between the two coves were evident from June-October 2009 (Fig. 3).

Nearshore Fish: Assemblage Structure

A stable NMDS ordination was obtained for the nearshore fish assemblage (stress = 15.5%; Fig. 4). The nearshore fish assemblage within sites was different before and after the bloom in WCC (MRPP, significance of $\delta = 0.0001$), but not in LP (MRPP,

significance of $\delta = 0.0451$; Bonferroni adjusted $\alpha = 0.0125$; Table 2). Mosquitofish (*Gambusia affinis* (Baird and Girard)) were indicative of the before-bloom assemblage in WCC, but a new suite of species, primarily consisting of species that can obtain larger body sizes, were indicative of the after-bloom assemblage, including: threadfin shad (*Dorosoma petenense* (Gunther)) and gizzard shad, smallmouth buffalo (*Ictiobus bubalus* (Rafinesque)), striped bass and white bass (*Morone chrysops* (Rafinesque)), white crappie (*Pomoxis annularis* (Rafinesque)), and carp (*Cyprinus carpio* (Linnaeus)) (Table 3).

Between site comparisons indicated that the nearshore fish assemblage was different between LP and WCC before the bloom (MRPP, significance of $\delta = 0.0002$), but not after the bloom (MRPP, significance of $\delta = 0.0797$; Table 2). Similar to the within site comparison, mosquitofish were also indicative of the before-bloom assemblage in WCC when compared to LP, while inland silverside, threadfin shad, and red shiner (*Cyprinella lutrensis* (Baird and Girard)) were indicators of the before-bloom assemblage in LP (Table 3).

Fish toxicity bioassays

We examined toxicity in first-year striped bass (Number of experiments (N) = 8, number of test fish (n) = 24, 2.84 ± 1.25g; wet weight ± SD), gizzard shad (N = 8, n = 21, 3.02 ± 2.56g) and inland silversides (N = 9, n = 27, 0.85 ± 0.55g), and similarly-sized adult fathead minnows (N = 7, n = 21, 2.74 ± 1.09g). Because all larger fish survived longer than 10- to 14-d-old fathead minnow larvae, there is an indication that fish size affects toxicity. On average, relative susceptibilities for the four species tested

were similar, with death occurring in ~5-7× the amount of time required for fathead minnow larvae (Fig. 5). Results indicate that for the species tested, there is little difference in the relative susceptibilities of these fishes to *P. parvum* (Kruskal-Wallis, $\chi^2 = 3.317$, df = 3, p = 0.345).

Discussion

Harmful Algal Blooms have received increased societal attention primarily because of their negative anthropocentric impacts. These impacts range from aesthetic concerns including beach fouling and discolored or distasteful water to severe consequences including damage of fisheries or recreational resources, or even human fatality. These negative impacts are primarily known for marine and coastal systems, while freshwater HABs and their impacts have received far less attention. Research on freshwater HABs has primarily focused on cyanobacteria and their aesthetic impacts. Recently however (mid-1980s), the EDAB-forming species, *P. parvum*, has become a source of concern for scientists and resource managers of freshwater bodies of the southern United States, where it is now known as a notorious fish killer.

For nearly three decades, blooms of *P. parvum* and their impacts have been discussed in terms of fish loss, including numbers, biomass, and dollars (Southard et al. 2010). Surprisingly, few studies have quantified the recovery of fish assemblages following the disturbance caused by the invasion, establishment, and proliferation of *P. parvum* populations. As seen in numerous other inland systems worldwide that have experienced *P. parvum* blooms, Lake Texoma experienced a near lake-wide fish kill in 2004, and annually since then local fish kills in isolated coves and backwaters

(Edvardsen and Paasche 1998, Hambright et al. 2010). Since the initial 2004 fish kill, most kill events have been limited to the northern and western shores of Lake Texoma on the Red River arm of the reservoir (Hambright et al. 2010). The winter of 2008-2009 was no different. *Prymnesium parvum* bloomed in LP, reaching densities of up to 232,000 cells mL^{-1} by 10 Feb 2009. Although there were few dead fish observed washed up on the shore, toxicity tests conducted within 1 hour of sampling using water from LP was highly toxic to juvenile fathead minnows, producing complete mortality of three fish within one hour. Intensive seine and gill net sampling confirmed the fish kill as the 2008-09 bloom of *P. parvum* in Lake Texoma severely reduced abundances and species richness of both near and offshore fish assemblages at the bloom site (LP), while the fish assemblages in the reference cove (WCC) showed typical seasonal variation in abundance and richness throughout the sampling period.

Recovery of fish assemblages following EDABs appears to be dependent on spatial heterogeneity of EDAB effects coupled with connectivity to source populations. Although the *P. parvum* EDAB resulted in an apparent complete kill in LP, fish assemblages quickly recovered once the bloom subsided. The reappearance of fish in LP coincided with increased spring rains, water level, and the reconnection of LP and WCC to the main reservoir body. Connectivity and flooding can be instrumental in maintaining fish assemblages as fish can colonize isolated or disturbed habitats when high water removes barriers to movement (Franssen et al. 2006). Generally, fish in connected freshwater systems (rivers and reservoirs) demonstrate high resilience and rapid recovery to both natural and anthropogenic disturbances (Olmstead and Cloutman 1974, Matthews 1986, Peterson and Bayley 1993, Matthews and Marsh-Matthews

2003). In systems in which spatial heterogeneity in environmental conditions and fish assemblages can be extreme, such as was observed in the Pecos River, TX, system, recovery can be delayed substantially (up to 18 months) as nearby habitats and their resident assemblages, which serve as sources of initial immigrants, can be quite different from the habitat that recently experienced a fish kill (Rhodes and Hubbs 1992). However, in our study, with little spatial variation in fish assemblages, but substantial variation in EDAB events across coves, there are many refuge populations that can serve as sources of new immigrants following an EDAB event. Thus a reservoir-wide, homogeneous fish metacommunity (sensu Leibold 2004) enabled a rapid recovery of local fish assemblages following a spatially heterogeneous EDAB event. We suspect that the main channel and other nearby tributaries and coves provided a refuge from complete extirpation of the fish metacommunity, as well as a source of emigration for re-establishing the fish assemblage following the *P. parvum* bloom once connectivity was reestablished.

Because *P. parvum* EDABs affected the fish assemblages in LP in previous winters (2003-04 through 2007-08), it is unclear whether the fish assemblages recovered to their original composition before the first *P. parvum* bloom in the winter of 2003-04. Hence, the LP fish assemblage could potentially always be composed of new immigrants and their offspring following blooms. Both near and offshore fish assemblages in Lake Texoma are relatively stable across years, likely due to their depauperate species richness and dynamic environment created by highly fluctuating water levels (Gelwick and Matthews 1990, Gido et al. 2000, Matthews et al. 2004, Eggleton et al. 2005). However, the fish assemblages in LP and WCC were different

from each other before the bloom and the assemblage in WCC after the EDAB was different from its composition before the EDAB. We suspect the differences relate to previous blooms and a general reset following floods. In 2007, the lake experienced a century flood, with lake levels averaging 194.5 m amsl (conservation pool = 188.1 m amsl) in July and likely resetting all assemblages (Gelwick and Matthews 1990, Gido et al. 2000, Matthews et al. 2004). Though not as dramatic as the 2007 rains and flooding, the water levels rose rapidly in spring 2009, averaging 190.1 m amsl during May (Fig. 2), completely flooding all backwaters and coves, and likely resetting the assemblages as in 2007. Indeed, length-frequency distributions for two nearshore fish species common in both coves before and after the bloom (inland silverside and emerald shiner (*Notropis atherinoides* (Rafinesque))), suggest that young-of-year (YOY) individuals colonized both coves similarly after the bloom and grew during the following months (e.g., Fig. S1, S2). During the winter of 2007-2008, *P. parvum* developed a major bloom in LP with smaller blooms downstream, but not in WCC. Unlike 2007 and 2009, the water level in spring 2008 barely topped the conservation pool level, averaging 188.9 m amsl in April. Hence in 2008 prior to the bloom, the assemblages in LP and WCC were different as a result of local effects and cove-specific differences (e.g., the prior fish kill in LP) and that can cause differences in the structure of littoral assemblages in Lake Texoma (Gido et al. 2002).

Our study is consistent with these previous findings from Lake Texoma and from other studies examining fish responses to disturbance. However, it is unique because, to our knowledge, it is the only study to document recovery of fish assemblages to an EDAB in a reservoir. It provides a mechanism for the maintenance of

this stability in terms of patch-dynamics and mass-effects following a stochastic extinction and an influx of individuals from the reservoir fish metacommunity (Leibold 2004) during periods of high connectivity due to high water levels associated with spring rains which overwhelmed any local effects that caused differences in cove fish assemblages before the bloom. Hence, our results suggest that the general fish community structure is rather resilient to localized *P. parvum* EDABs when connected to viable source populations. However, situations in which an entire lake or reservoir is affected by a bloom merit further study as a lack of nearby source populations could translate into long-term, and potentially dire, consequences to fish assemblages. Little is known about the chronic effects of repeated EDABs on fish assemblages, both in terms of repeated exposure to toxins and the effect of repeated exhaustive fish kills. In extremely disturbed ecosystems that experience chronic blooms, we might expect to find lower species diversity and a community made up of opportunistic (colonizing) individuals (Connell, 1978). Indeed, Lake Texoma, like many other aquatic ecosystems of the Southern Great Plains, is well known for its relatively high levels of disturbance (Matthews 1988, Dodds et al. 2004), and relatively low-diversity assemblages (but not necessarily low richness (Gido et al. 2000, Eggleton et al. 2005)). Hence, investigations into chronic effects of blooms on fish assemblages would be particularly useful in systems where the fauna is not "pre-adapted" to disturbances. Finally, because the rapid loss and then recovery of fishes in response to EDABs could produce significant feedbacks in both grazer and nutrient dynamics that are characteristic of EDABs, we suggest that assessment of both fish and connectivity beyond the cursory mention of

their occurrence should be included when studying EDABs and their implications in affected ecosystems.

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Tables

Table 1. Results from two repeated measures ANOVA with fish abundance (log_{10}-transformed) and rarified species richness as dependent variables. Degrees of freedom are represented by df (Hypothesis, Error). Effect sizes of independent variables (time and cove) were assessed via partial η^2.

Dependent	Source	df	*p*	Effect size
Abundance	Time	5,30	<0.001	0.795
	Cove	1,6	0.042	0.524
	Time × Cove	5,30	0.032	0.321
Richness	Time	5,30	<0.001	0.884
	Cove	1,6	<0.001	0.961
	Time × Cove	5,30	<0.001	0.785

Table 2. Results from multiple response permutation procedure (MRPP) comparing before bloom and after bloom nearshore fish assemblages within sites and before bloom and after bloom assemblages between sites. Significance of δ is equivalent to a *p*-value. Significance of δ is adjusted using a Bonferroni correction for multiple comparisons (α = 0.0125).

MRPP results

Comparison	Expected δ	Observed δ	Significance of δ (α = 0.0125)
Within Sites			
Before bloom LP vs. after bloom LP	0.4825	0.4608	0.0451
Before bloom WCC vs. after bloom WCC	0.6454	0.3998	<0.0001
Between sites			
Before bloom LP vs. before bloom WCC	0.4154	0.3183	0.0002
After bloom LP vs. after bloom WCC	0.5680	0.5422	0.0797

Table 3. Results from indicator species analysis (ISA). The indicator value (IV) and the associated *P*-value indicate species that contributed to discriminating between before bloom and after bloom assemblages within or between sites.

ISA results

Comparison	Species	IV	*p*
Within Sites – WCC			
Before bloom	*Gambusia affinis*	0.8174	0.0001
After bloom	*Dorosoma petenense*	0.8597	0.0003
After bloom	*Dorosoma cepedianum*	0.7218	0.0009
After bloom	*Ictiobus bubalus*	0.6667	0.0010
After bloom	*Morone saxatilis*	0.6667	0.0012
After bloom	*Notropis atherinoides*	0.6554	0.0232
After bloom	*Pomoxis annularis*	0.6365	0.0028
After bloom	*Morone chrysops*	0.5833	0.0043
After bloom	*Menidia beryllina*	0.5496	0.0010
After bloom	*Cyprinnus carpio*	0.5000	0.0143
Between Sites – Before bloom			
LP	*Menidia beryllina*	0.7310	0.0008
LP	*Cyprinella lutrensis*	0.7209	0.0233
LP	*Dorosoma petenense*	0.5556	0.0218
WCC	*Gambusia affinis*	0.9367	0.0001

Figures

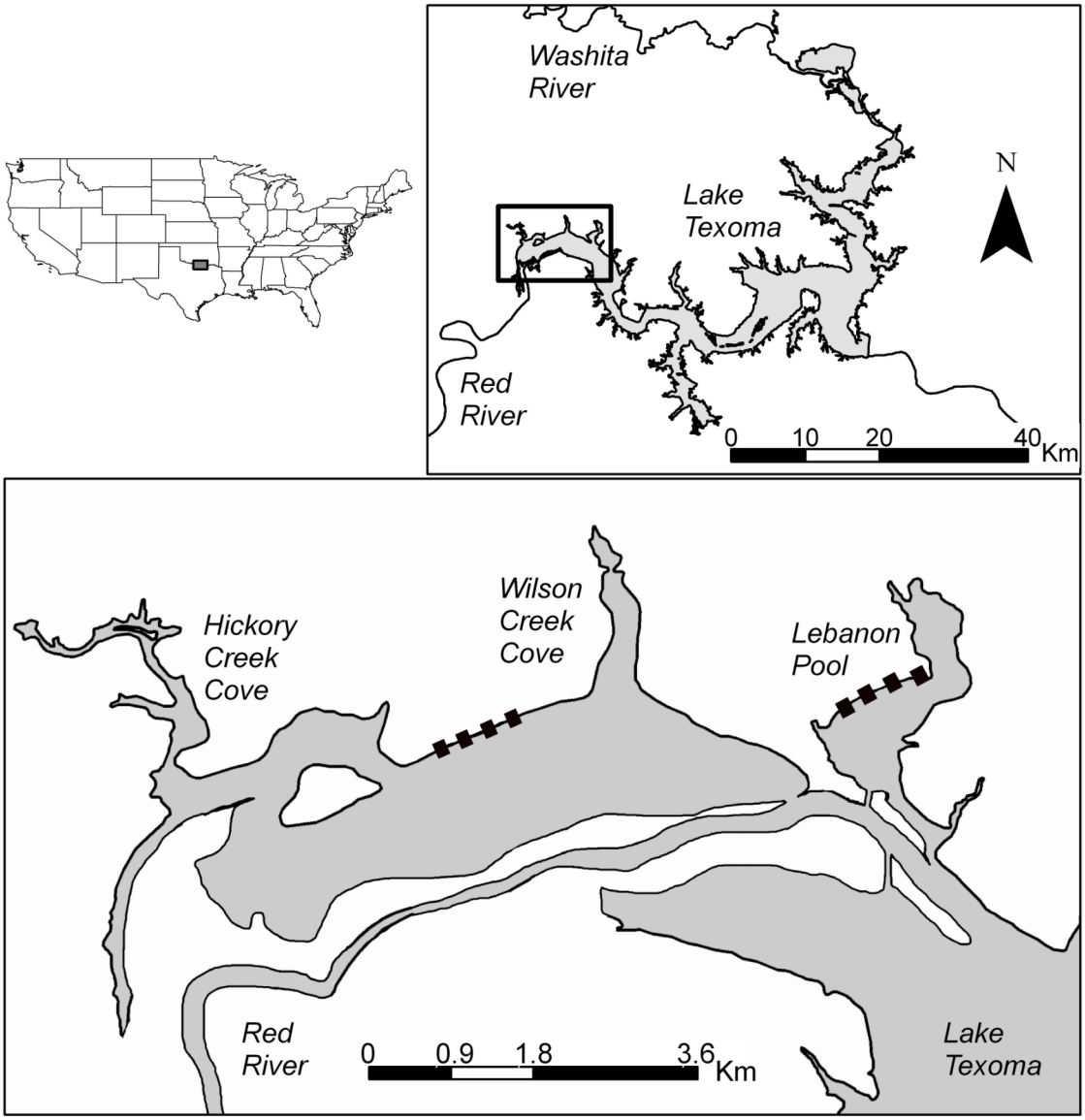

Figure 1. Map of Lebanon Pool and Wilson Creek Cove collection sites in the Red River arm of Lake Texoma on the border of OK-TX, USA. Black bars indicate reaches of beach sampled during nearshore and offshore collections at each site.

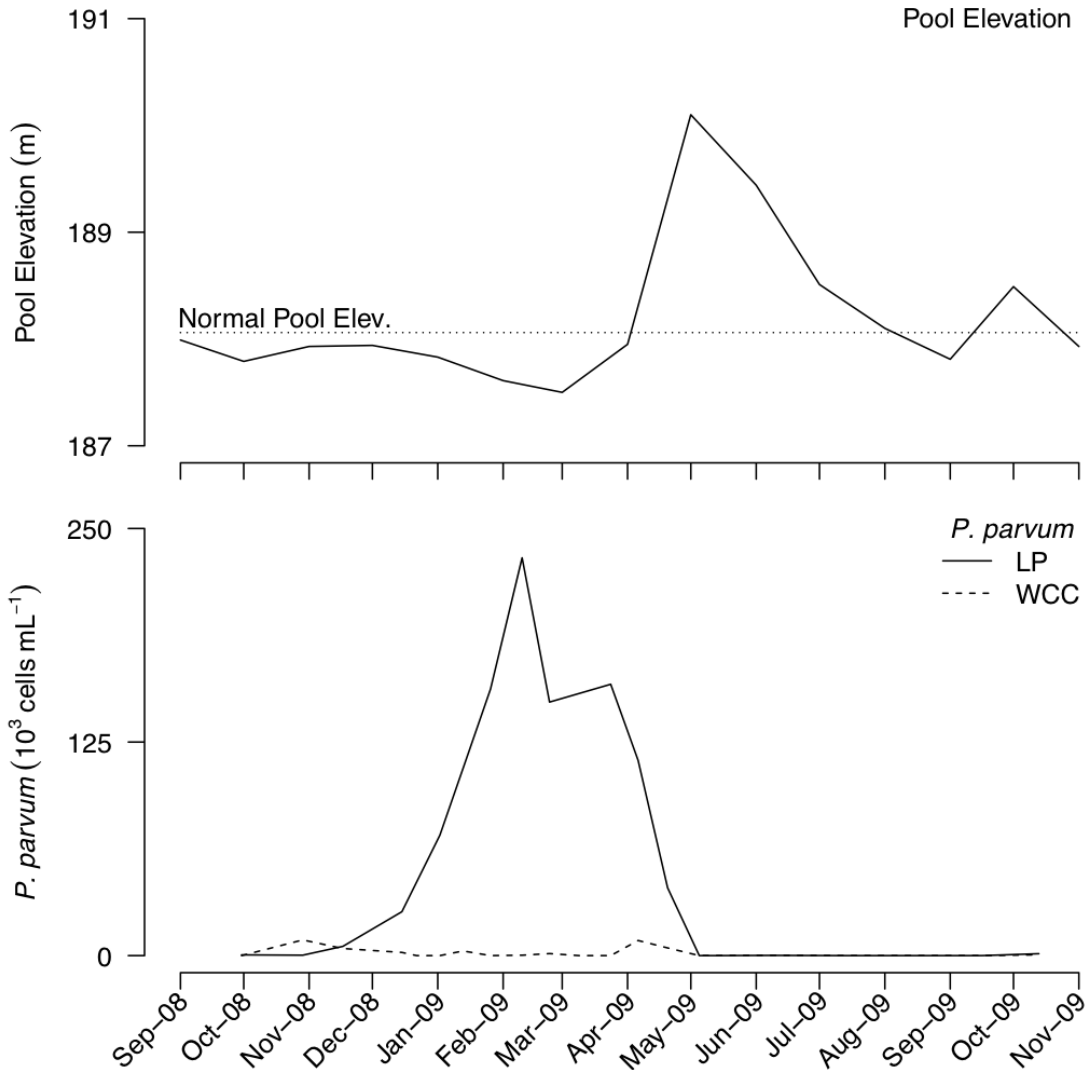

Figure 2. Average lake elevation (top panel) and *P. parvum* cell densities (bottom panel) during the study period.

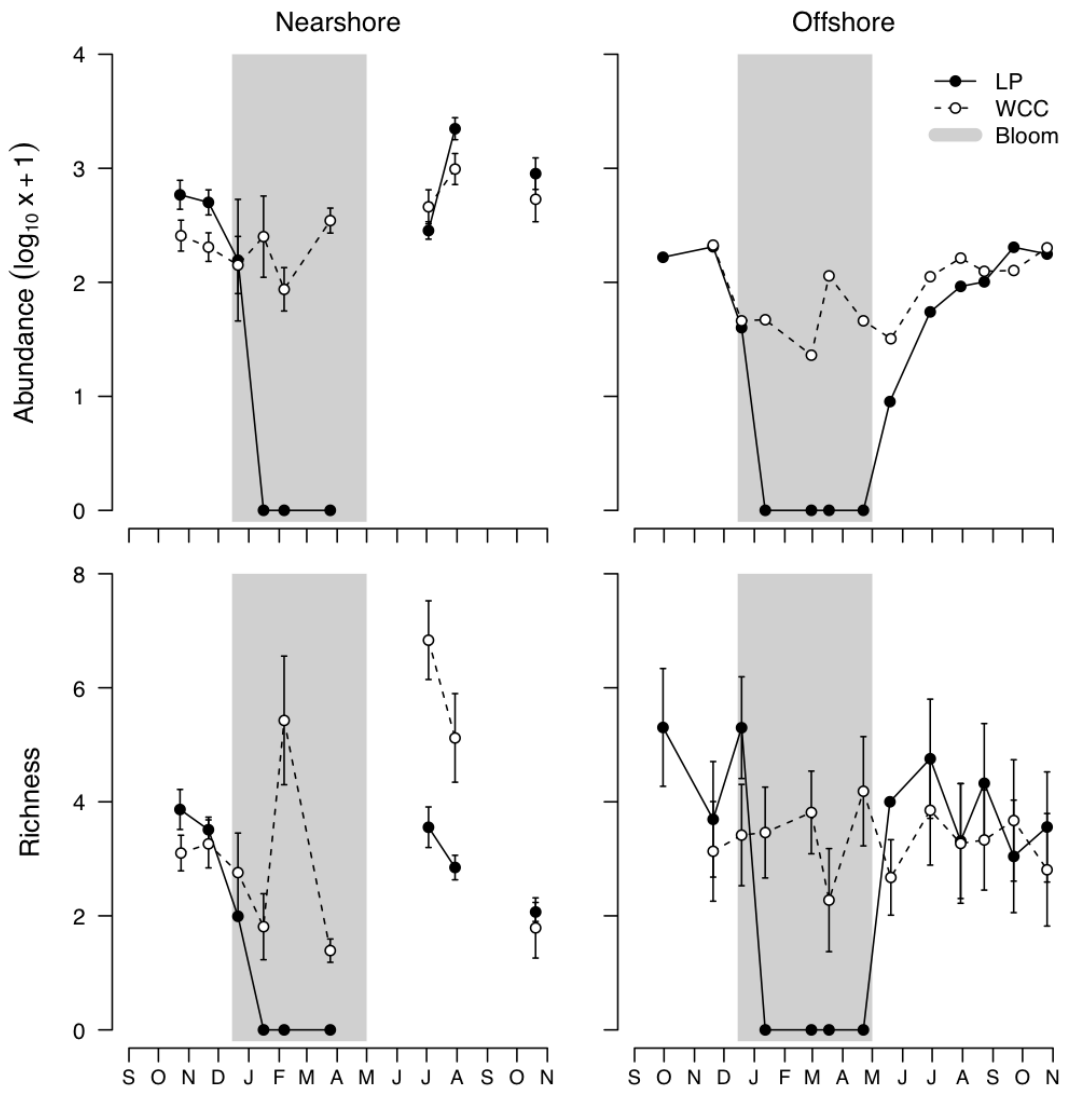

Figure 3. Fish abundance and rarefied species richness in nearshore (left two panels) and offshore samples (right two panels). Abundances are $\log_{10}(x+1)$ transformed for ease of comparison.

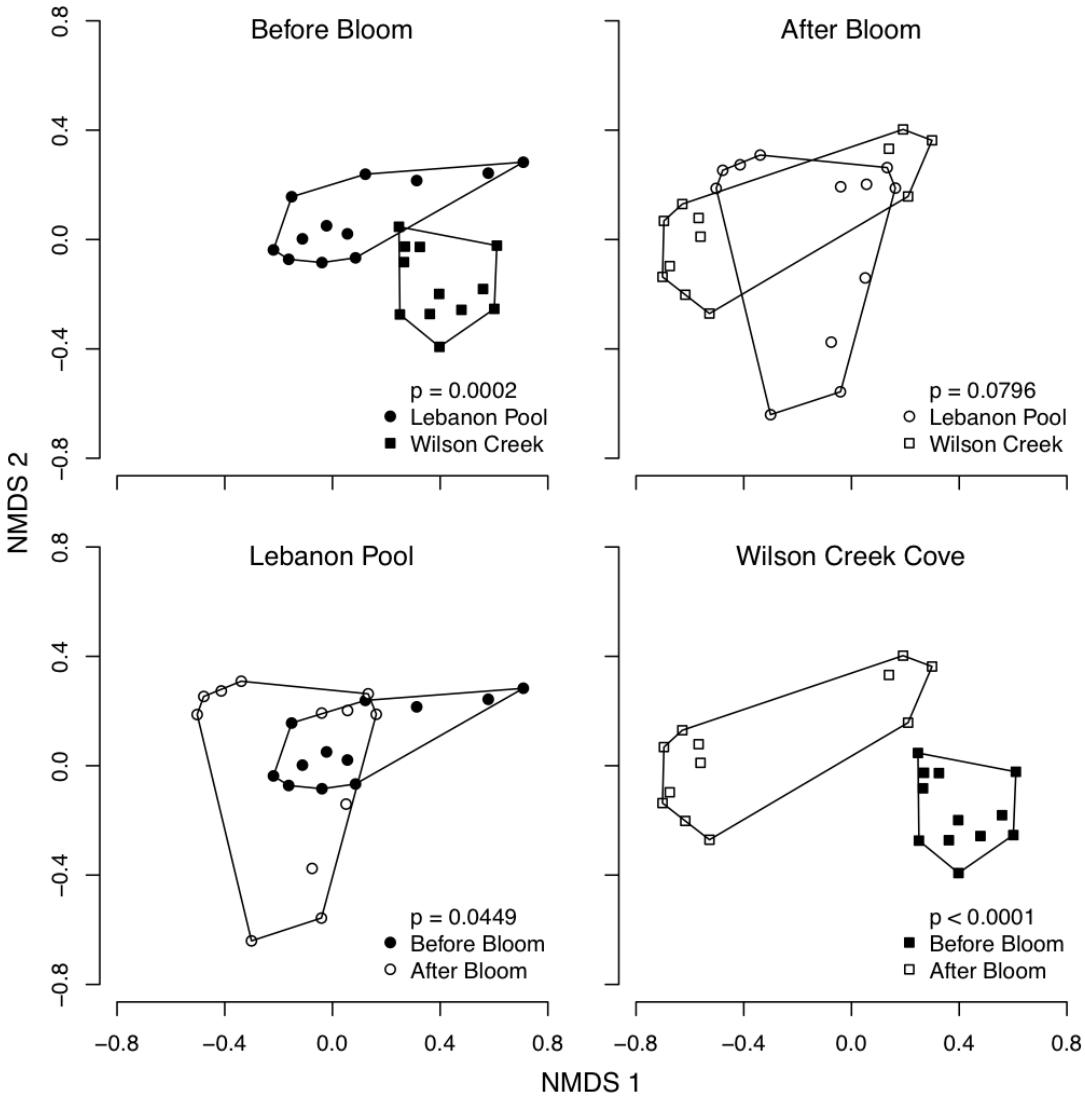

Figure 4. Results of NMDS of the nearshore fish assemblage. Ordinations of the single NMDS are presented as four subsets to allow visualization of comparisons of before bloom and after bloom nearshore fish assemblages within sites (top two panels) and before bloom and after bloom assemblages between sites (bottom two panels).

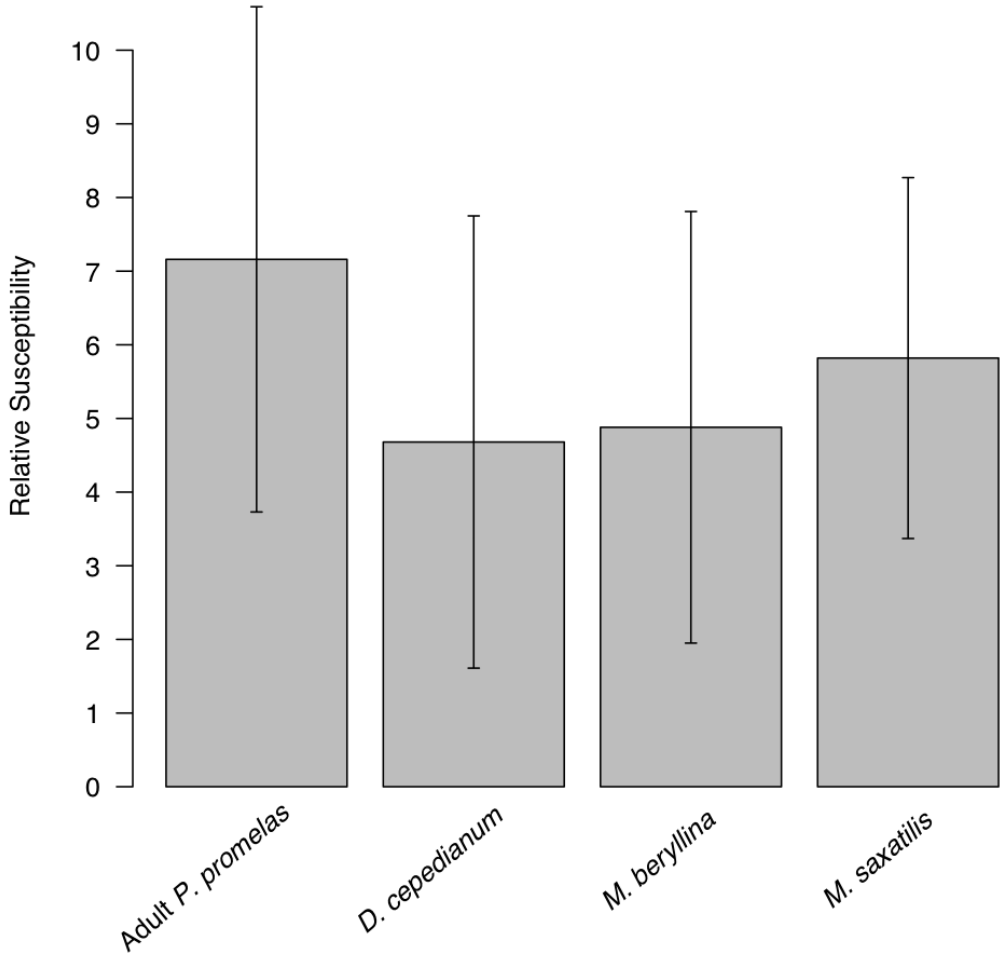

Figure 5. Susceptibility of common Lake Texoma fishes to *P. parvum* in terms of time to death relative to fathead minnow larvae.

Supporting Information

Table S1. Species and total number of individuals collected in each cove by sample type over the entire sampling period.

	Lebanon Pool		Wilson Creek Cove	
Species	Nearshore	Offshore	Nearshore	Offshore
Menidia beryllina	17416	-	10440	-
Dorosoma petenense	822	138	865	119
Notropis atherinoides	505	-	885	-
Cyprinella lutrensis	462	-	44	-
Gambusia affinis	60	-	628	-
Ictiobus bubalus	60	52	426	23
Dorosoma cepedianum	34	430	280	689
Cyprinus carpio	21	3	28	20
Pimephales vigilax	17	-	2	-
Notropis potteri	16	-	58	-
Lepomis megalotis	15	-	2	-
Morone saxatilis	12	78	26	13
Macrhybopsis hyostoma	10	-	-	-
Morone chrysops	7	36	36	25
Lepomis macrochirus	3	-	50	-
Cyprinella venusta	2	-	-	-
Pomoxis annularis	2	40	53	81
Ctenopharyngodon idella	1	-	-	2
Lepomis humilis	1	-	40	-
Lepomis microlophus	1	-	-	-
Hybognathus placitus	-	-	43	-
Ictalurus furcatus	-	12	9	16
Ictalurus punctatus	-	33	1	85
Lepomis gulosus	-	-	1	-
Notropis stramineus	-	-	1	-
Aplodinotus grunniens	-	116	-	72
Atractosteus spatula	-	2	-	5
Carpiodes carpio	-	8	-	5
Hiodon alosoides	-	0	-	3
Ictiobus cyprinellus	-	15	-	3
Lepisosteus oculatus	-	10	-	14
Lepisosteus osseus	-	41	-	25
Lepisosteus platostomus	-	25	-	36

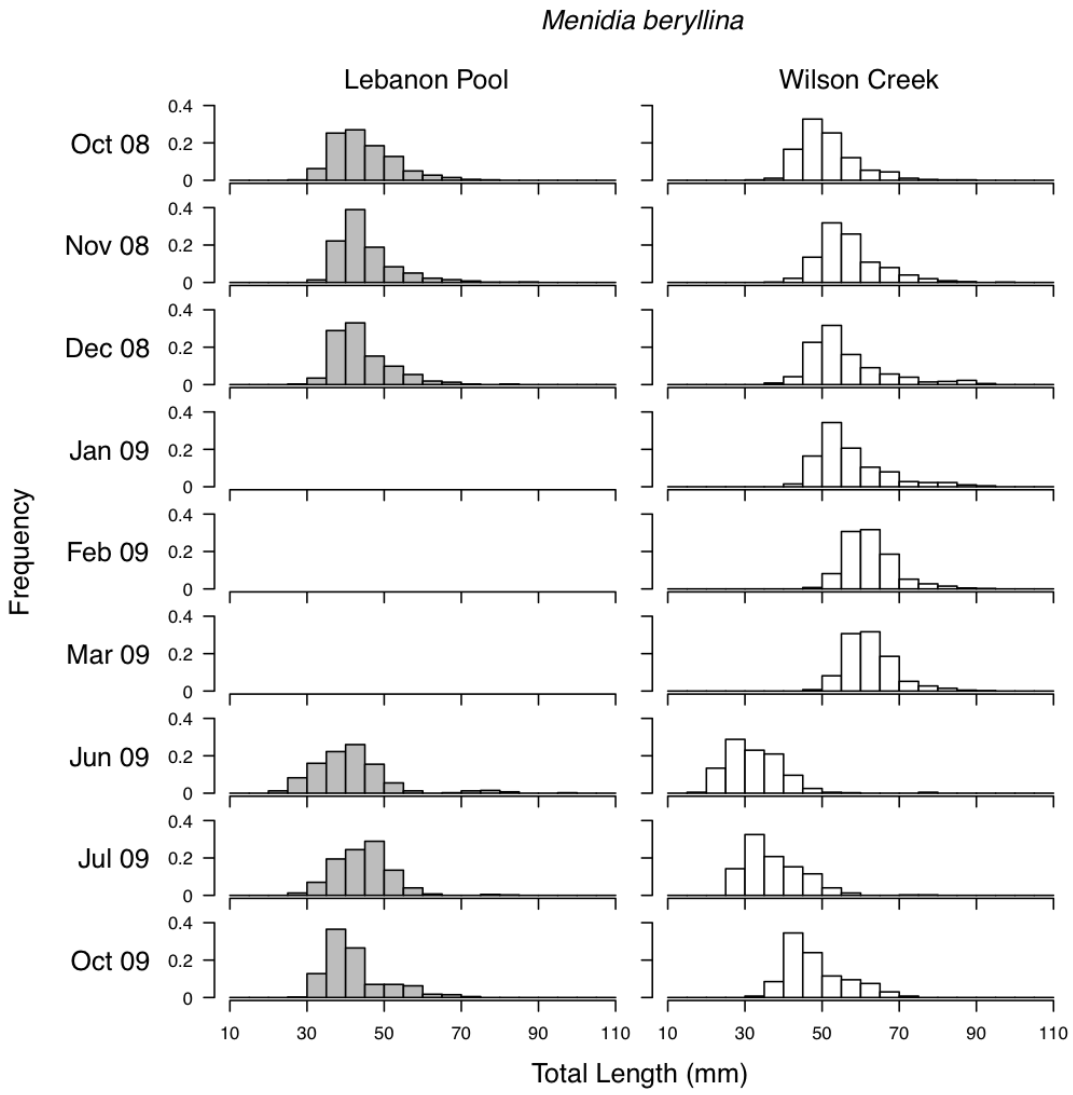

Figure S1. Length-frequency histogram of inland silverside (*Menidia beryllina*) collected in both coves throughout the study period.

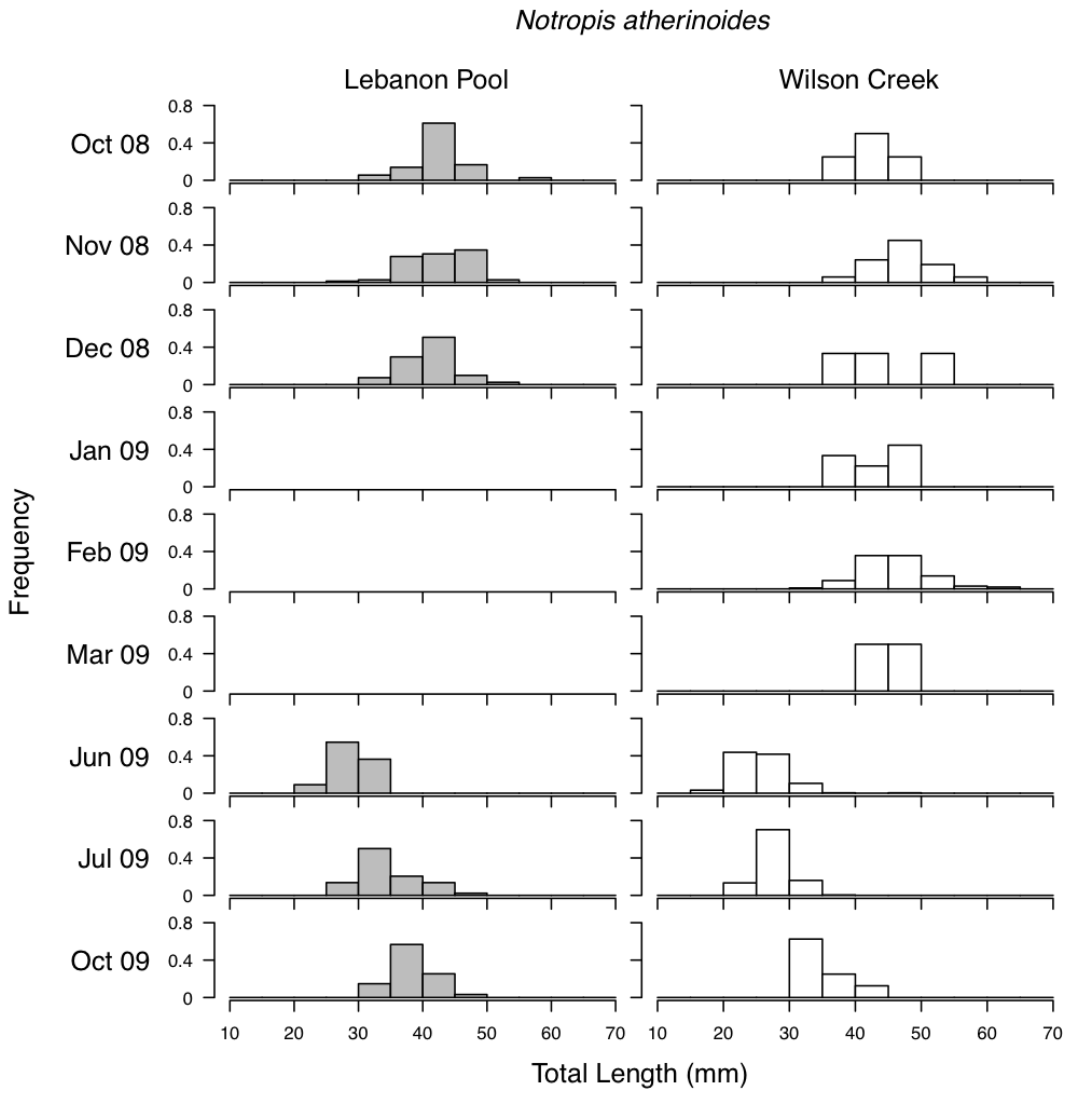

Figure S2. Length-frequency histogram of emerald shiner (*Notropis atherinoides*) collected in both coves throughout the study period.

Chapter 3 – Dispersal alone is not enough: environmental conditions predict the presence of an invasive harmful alga

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Keywords: *Prymnesium parvum*, microbial invasive species, "Everything is everywhere, but, the environment selects", dispersal, microbial invasion process model

Abstract

All invasive species must successfully invade and establish before they can spread. Early detection is key to limiting the spread of invasives, but can be particularly difficult for invasive microbes. The microbiologist's credo 'Everything is everywhere, but, the environment selects,' infers that microbes are continuously being introduced to a system with establishment being dependent on environmental conditions. Therefore, environmental factors should be instrumental in predicting invasion success and establishment for microbial invaders. *Prymnesium parvum*, a toxigenic marine unicellular eukaryote, has invaded many freshwater systems throughout the southern and southwestern US. Like many invading species, *P. parvum* invasions often result in

population explosions (i.e., blooms) in invaded systems. These blooms disrupt the structure and function of the entire system by causing massive fish kills and altering nutrient and food web dynamics. Using a 4-year data set from our ongoing monitoring program in Lake Texoma (OK-TX) we constructed a predictive model relating *P. parvum* presence or absence to environmental parameters at a local scale. We then tested this model at the regional scale in conjunction with environmental sampling to predict presence and absence of *P. parvum* in the watershed of the Red River, one of two tributaries to Lake Texoma and a neighboring watershed, the Canadian River, as well as a few sites in the Arkansas River watershed.

Based on three environmental factors, specific conductance, total nitrogen, and total nitrogen : total phosphorus ratio, our predictive model accurately classified *P. parvum* as present or absent in Lake Texoma for 74% of the samples. Applying this model to the adjacent watersheds also showed strong predictive power, correctly classifying 87% of the sites sampled within the Red River watershed and 81% of the sites sampled in the Canadian River watershed. Sites where the model predicted *P. parvum* but none was detected may be particularly vulnerable to *P. parvum* establishment and should be more closely monitored for future invasion success. Misclassifications by the model of sites in which *P. parvum* was detected suggests that dispersal has occurred but that the environmental conditions were not conducive to population establishment. Indeed, at these sites, *P. parvum* abundances, when detected, were very low. While we cannot rule out dispersal limitation as a major factor involved in the biogeography of *P. parvum*, our results do indicate that the establishment and

spread of this harmful algal species appears to be limited by environmental conditions in the invaded habitat.

Introduction

Recently, the need to address microbial invaders has been brought to the attention of the field invasion ecology (Litchman 2010). Indeed, a recent list of the most damaging invasive species in the US released by the US Department of Agriculture included two microbes (*Phytophthora ramorum* –water molds responsible for Sudden Oak Death, and *Candidatus liberibacter asciatus* – bacteria responsible for Citrus Greening disease; USDA 2013). Because knowledge about microbial invaders is limited, it should be informative to look to concepts derived in macrobial systems when trying to interpret microbial invasions. Macrobial invasion models typically involve understanding transport, establishment, spread, and most importantly impact of invasive species (Lockwood et al. 2007). Implicit within these models is that an anthropogenic component is involved with many of these aspects, especially transport and impact perception. However, applying these invasion models to microbes is not straight-forward within the context of general microbiological knowledge and understanding – captured in the infamous credo of microbiology, "Everything is everywhere, but, the environment selects," first postulated by Baas Becking in 1934 (DeWit and Bouvier 2006). "Everything is everywhere," suggests that either all microbes are always established in any given habitat or, perhaps more appropriately, that any given microbe could be in or could be transported to any given habitat at any given time (de Wit and Bouvier 2006). The apparent ubiquity in microbial taxonomic distributions (Finlay

2002, Sogin et al. 2006, Gibbons et al. 2013) highlights the difficulty in understanding transport and dispersal for microbial species because most microbes are passive dispersers and could be transported by any number of other vectors besides humans. Although it may be possible to estimate of how many invasive microbes are being transported by human vectors it would be supremely difficult to assess most other potential vectors. This difficulty in estimating transport and dispersal for microbes suggests that current macrobial invasion models may not be sufficient for characterizing the early stages of invasion for microbes.

However, even if microbes are "everywhere" this may not matter for invasion ecologists, as we are ultimately concerned with avoiding or mitigating impact (Lockwood et al. 2007), and microbial impacts are often only perceived as harmful once a species has reached high abundance i.e., blooms. Blooms are often controlled by environmental conditions (i.e., "the environment selects"), which determine what species reach bloom densities and subsequently have negative impacts (e.g., cyanobacteria; Smith and Schindler 2009). Additionally, environmental selection, beyond just determining impacts will also control the ability of the microbial invader to spread by determining the number of propagules available for dispersal to other habitats, by limiting source population densities (Martiny et al. 2006, Lockwood et al. 2007, Hanson and Martiny 2010). Hence, if we are to begin to understand microbial invasions we need to focus on what environmental conditions allow that invader to become successful and cause perceptible harmful impacts.

One potential microbial invader that is receiving increased attention, both in the literature and from the general public, is the toxigenic eukaryote, *Prymnesium parvum*,

a.k.a. golden algae. *Prymnesium parvum* was originally classified from marine systems (Carter 1913), but over the past ~25 years has started appearing as blooms in freshwater ecosystems in southern North America causing massive fish kills (Roelke et al. 2010). Its blooms have been a particular problem in Oklahoma and Texas, where *P. parvum* has appeared to follow a northern trend of range expansion from its first discovery in North America in the Pecos River drainage in southern Texas (1985; James and De La Cruz 1989) to the Red (2001) and Canadian River basins (2003) of northern Texas and Oklahoma during this period (Hambright et al. 2010). We sought to address the question of how environmental conditions affect the distribution of a potential microbial invader, by testing if we could predict the distribution of *P. parvum* at a local scale based on environmental parameters. In turn, we hypothesized that if we could predict the distribution of *P. parvum* at a local scale based on environmental parameters, then similar environmental selection should be happening at the regional scale, with the same parameters proving to be good predictors of *P. parvum* presence or absence.

Methods

Study Sites and Site Monitoring

To assess the hypothesis that *P. parvum* presence is predictable by environmental factors at the local scale, we created a model using discriminant function analysis (DFA; see *Data analysis* for details) using data from our ongoing monitoring program on Lake Texoma, an impoundment of the Red and Washita Rivers on the border of Oklahoma and Texas, USA. We have maintained a regular monitoring program on the lake since 2005, assessing physical and nutrient parameters of the lake

as well as *P. parvum* abundances via microscopy at eight littoral sites and five pelagic sites throughout the lake (see Hambright et al. 2010 for monitoring details). We have also incorporated molecular monitoring of *P. parvum* via qPCR since 2008 (Zamor et al. 2012).

To assess the applicability of this model and thus the predictability of *P. parvum* presence by environmental factors at the regional scale, we sampled 62 sites in the Red River Basin located on the southern border of Oklahoma and Texas in the winter and early spring of 2008 (25 January – 01 March). Sites were sampled during this period because *P. parvum* is known to bloom during these seasons in freshwater locations throughout the region (Hambright et al 2010, Roelke et al. 2010). Site locations ranged from Greenbelt Reservoir in the Texas Panhandle (34.9981, -100.90662) to Clear Lake in Southeastern Oklahoma (33.69316, -94.63592; Figure 1). Similarly in the early spring of 2009 (08 April – 10 April) we sampled 24 sites within the South Canadian River Basin from Lake Meredith in the Texas Panhandle (35.70699, -101.55546) to its confluence with the Arkansas River at Robert S. Kerr Reservoir near the Oklahoma – Arkansas border (35.4008, -94.96812; Figure 1). We also sampled 2 sites on Lake Keystone, the impoundment at the confluence of the Arkansas (36.23486, -96.36239) and Cimarron Rivers (36.18546, -96.29608; Figure 1). Lake and reservoir sites ranged in surface area from <0.002 km^2 cattle ponds and oxbows to 360 km^2 reservoirs.

At each site, we measured physical parameters, nutrients, and took samples for *P. parvum* using the same protocols as for littoral sampling in Hambright et al. (2010) and in Zamor et al. (2012). In brief, physical parameters were sampled *in situ* by wading 1-5 m offshore and collecting ~20 L of water in a bucket in which we measured

temperature, dissolved oxygen, specific conductance, pH, and redox using a Hydrolab DS5 sonde. Water samples for nutrient analyses were collected in two 250-mL Nalgene bottles and preserved on ice until we returned to the lab where we froze 50 ml of whole water and 50 ml of GFF-filtered water until processing. We processed these samples for total (whole water samples) and dissolved (filtered samples) phosphorus and nitrogen digested in acid (P) and alkaline (N) persulfate at 120ºC for 1 h via flow injection auto analysis (Lachat Quikchem 8500 FIA).

We also collected 1-L water samples in Nalgene bottles for analysis of *P. parvum* presence via qPCR and transported them on ice until processing them in the lab. With these, we filtered 50 mL – 1000 mL (typically 350mL) onto GF/F filters using gentle vacuum (< 0.17KPa). The volume of sample filtered depended on the amount of sediment and other particulates present in the water samples. Filters were then folded and placed into 15-mL plastic Falcon tubes containing 2 mL of lysis buffer (100 mM Tris [pH 8], 40 mM EDTA [pH 8], 100 mM NaCl, 1% sodium dodecyl sulfate) and 200 μL of 0.5-mm zirconia-silica beads. Samples were then heated in a hot water bath (5 min, 70°C) and vortexed at the highest setting for 30 s. This process was repeated a total of three times to create crude lysates. Crude lysates were then transferred into sterile 2-mL microcentrifuge tubes and stored at –20°C until we analyzed them with qPCR. We examined each sample with at least triplicate analytical replicate qPCR reactions in 96-well plates sealed with optical film B (BioRad) using an iQ5 real-time PCR detection system (BioRad) and analyzed using the associated iQ5 optical system software (see Zamor et al. 2012, for further qPCR reaction methods and details).

Data Analysis

We examined the utility of environmental parameters to predict *P. parvum* presence within Lake Texoma using discriminant function analysis (DFA). *Prymnesium parvum* abundance was measured microscopically and by qPCR analysis, which offers a more accurate and lower detection limit (Zamor et al. 2012). We constructed the discriminant function using environmental data (specific conductance, μS cm^{-1}; chlorophyll-a, μg L^{-1}; total and total dissolved phosphorus, TP and TDP, μg L^{-1}; total and total dissolved nitrogen, TN and TDN, mg L^{-1}; and TN:TP and TDN:TDP, mM) from the Lake Texoma monitoring project from Jan 2008 through Apr 2012 (n = 631). Temperature, dissolved oxygen, pH, and redox were excluded because samples were taken at different times of day, which can greatly affect these parameters.

Neither the raw data nor $\log_{10}(x + 1)$ transformed data were multivariate normal (tested using a generalization of the Shapiro-Wilk test for normality using the "mvtnormtest" package v. 0.1-9 (Slawomir 2012) in R v. 2.15.3 (R Development Core Team 2013)), so multivariate outliers (n=208) were removed from the data set after their identification using the "pcout" function based in the "mvoutlier" package v. 1.9.9 (Filzmoser and Gschwander 2013) in R. Removing outliers did not improve normality, however DFA was still deemed appropriate due to this test's robustness against violations of this assumption as long as violations of this assumption are not due to outliers (Tabachnick and Fidell 2007). Hence, we used the raw data with outliers removed to classify *P. parvum* presence at the local scale. Linear DFA was then conducted in PASW v. 19 (IBM Corp. 2010) based on equal probabilities of the remaining 423 samples using stepwise variable selection to account for effects of

colinearity of variables. Stepwise variable selection was based on Wilks-Lambda with an F value of 3.84 required for entry and 2.71 required for removal. Separate covariance matrices were used during classification to account for heterogeneity of variance-covariance matrices between groups (Box's M, F=11.731, p <0.0001). Following classification, DFA predictions for each sample were categorized as percent correctly or incorrectly predicted for the entire model and for *P. parvum* presence and absence. After this model was constructed using the local scale Lake Texoma data, we applied its classification function to the regional scale data taken from the Red River and other river watersheds.

Results

One significant discriminant function was produced from the local scale (i.e.,Lake Texoma) data ($F_{3, 419} = 50.529$, $p < 0.0001$), based on the environmental parameters specific conductance ($F_{remove} = 109.827$), TN:TP ($F_{remove} = 26.363$), and TN ($F_{remove} = 6.482$). Site classification scores (C) used to predict *P. parvum* presence or absence, were determined from the following classification function:

$$C = 0.0018(SpCond) - .0428(TN:TP) - 1.0255(TN) - 0.9542$$

Classification scores at group centroids were -0.5736 for *P. parvum* absence and 0.626 for *P. parvum* presence resulting in a cut score of 0.0524, above which *P. parvum* presence was predicted. This function correctly classified 74.2% of samples in Lake Texoma (Table 1). Within groups this function correctly classified 79.9% of samples where *P. parvum* was absent and 64.8% of samples where *P. parvum* was present. Sites classified as having *P. parvum* showed higher specific conductance (present 1900 ±

522; absent 1448 ± 535; median ± IQR), higher TN (present 0.75 ± 0.32; absent 0.71 ± 0.30), and lower TN:TP (present 25.9 ± 13.9; absent 33.3 ± 18.2). In Lake Texoma during the 4-year sampling period, *P. parvum* was detected at least once at each sampling site throughout the lake. False negatives (i.e., misclassifications of sites where *P. parvum* was detected, but that were classified as not present) (n = 61) occurred primarily in sites where *P. parvum* abundances were low (i.e., 70% of these misclassifications were from detections of < 1000 cells mL^{-1} and an additional 18% were below what is considered typical bloom levels of 10,000 cells mL^{-1}; Roelke et al. 2010).

Using this local model at the regional scale produced a similarly accurate classification of sites: 87.1% of sites in the Red River and 81.8% of sites in the Canadian and Arkansas River were correctly classified based on the environmental parameters specific conductance, TN, and TN:TP (Table 1). Of the 62 sampled sites within the Red River watershed, we detected *P. parvum* at 12 sites. These sites were primarily located in southwestern region of the watershed. We also detected *P. parvum* in North Lake, OK (33.6761, -95.0545) located near the eastern edge of the sampling area. Within the Red River watershed, 88.0% of sites where *P. parvum* was detected were correctly classified, and 83.3% of sites where *P. parvum* was not detected were correctly classified. As seen at the local scale, the 2 false negatives (North Lake and Lake Wichita, TX) occurred primarily in sites where *P. parvum* abundances were low.

In the Canadian and Arkansas River watersheds, we detected *P. parvum* at 4 sites. These 4 sites were located in the westernmost edge of the sampling area originating at a known bloom site: the Lake Meredith spillway. The remaining 3

detections were downstream from Lake Meredith. Sites within the Canadian Arkansas River watersheds were also well classified with 75.0% and 81.8% of sites where *P. parvum* was present or absent were correctly classified, respectively. As previously, in the sole misclassification of a site where *P. parvum* was found abundance was low.

Discussion

Prymnesium parvum was first detected in Lake Texoma in 2004 when it caused a near lake-wide fish kill. It has since bloomed in 8 of the last 10 years causing fish kills in isolated coves and backwaters (Hambright et al 2010, Zamor et al. *in review*). These blooms occur primarily in the Red River arm of the reservoir and have been previously correlated with high levels of specific conductance and low N:P ratios (Hambright et al. 2010). However, *P. parvum* has been detected at all reservoir sites via qPCR, suggesting that dispersal does indeed occur throughout the reservoir. This, in conjunction with the correlation of blooms and environmental parameters, suggests that environmental factors at least partially determine where *P. parvum* can bloom and thus determines the potential for *P. parvum* to have an impact large enough to draw anthropogenic attention.

We tested this hypothesis at the local and regional scale using a discriminant function analysis, which was able to accurately classify sites at the local level based on the environmental parameters specific conductance, TN:TP, and TN and was able to accurately predict sites where *P. parvum* was present or absent at the regional scale. Sites classified as having *P. parvum* showed higher specific conductance, higher TN, and lower TN:TP, supporting the previous findings that *P. parvum* favors higher

conductivities and lower TN:TP ratios. Additionally, that higher specific conductance and TN would favor *P. parvum* presence is logical due to its marine origin, where nitrogen is a common limiting nutrient (Howarth 1988) and conductivities are much higher than in freshwater. Low N:P ratios are also thought to cause increased toxicity in *P. parvum* (Granéli and Johansson 2003, Hambright et al. *unpublished data*) and thus would theoretically favor *P. parvum* as well (Remmel and Hambright 2012).

Because the classification function produced through the DFA analysis is accurate in predicting *P. parvum* presence at the regional scale it should be a useful tool for resource managers and scientists for focusing monitoring or prevention efforts. Although producing accurate predictions of *P. parvum* presence is desirable, misclassifications by the model offer useful information, both in terms of general theory and for management. A false positive (i.e., a prediction of *P. parvum* presence in which none is detected) indicates that the environment at a site may be conducive to invasion and establishment by *P. parvum*, and thus it should be monitored for future invasion success. In the Red River watershed, false positives were typically located near other sites where *P. parvum* was detected and false positives exhibited either high specific conductance, TN, or low TN:TP values. Since the time of our sampling *P. parvum* has bloomed in one of these sites, Altus-Lugert Reservoir (34.94001, -99.28574; Lehrman, B., Greer County, OK Game Warden, *personal communication*). Similarly in the Canadian and Arkansas River watersheds, the 4 false positives were located in sites adjacent to the 3 sites where *P. parvum* was present and that were downstream from a known bloom site. Since the time of our sampling blooms of *P. parvum* have formed in two of these sites, both in the main body of Lake Meredith and its outflow

(VanLandeghem, M. M., Texas Tech University, *personal communication*). False negatives are also useful in that they suggest that dispersal has occurred, but that the environment is not conducive to population establishment. For example, we detected *P. parvum* in North Lake, which is far removed from any of the other sites where *P. parvum* was detected in the Red River watershed, but it was found in low abundance. A parallel situation exists in Lake Marvin in the Canadian River watershed. Indeed, for the majority of false negatives at both the local and regional scale *P. parvum* abundances were low, suggesting that environmental conditions are playing a role in determining the ability of this microbial invader to impact a system. Further, evidence that dispersal is occurring for this microbial invader is provided by the three detections in the Canadian River watershed that were downstream from a known source population.

Our results suggest that "everything is everywhere, but, the environment selects" is important in understanding how microbial invaders are introduced, established and ultimately cause perceptible impacts. In a typical, macrobial model of invasion, transport and introduction are two hurdles that an invasive species must pass through in order to establish a population (Lockwood et al. 2007). Our results suggest that dispersal is important for microbial invaders. Indeed, studies have addressed "Everything is everywhere" with some showing evidence of dispersal limitation (Martiny et al. 2006, Telford et al. 2006, Ramette and Tiedje 2007). However, the distributions of microbes as defined in all of these studies are constrained by detection limits, which makes "Everything is everywhere" currently impossible to reject as a null hypothesis (Foissner 2006). Our inability to reject the potential for everything to be everywhere is further illustrated by the concept of the microbial rare biosphere (i.e., the

vast number of microbial taxa present in extremely low abundances in natural assemblages; Sogin et al. 2006) and the presence of dormant microbial seed banks (Lennon & Jones 2011, Gibbons et al. 2013). Despite intense sampling efforts in these and other similar studies, they were still unable to estimate the full extent of the phylogenetic diversity present in a given habitat. However, it has been suggested that this limitation may be overcome within three years with continued technological advancement in high-throughput genetic sequencing (Gibbons et al. 2013). It is simply not currently possible to sample enough to determine the true microbial diversity of a given assemblage, and as such, we cannot conclude a given species (such as a microbial invader) is absent from the system. These limits to our detection are indeed important when viewed from larger scale. For example, if we were to scale up from one cell of a given microbial invader going undetected in a gram of soil or a milliliter of water, it would be equivalent to one million undetected cells in a cubic meter of either medium (Fierer and Lennon 2011). However, it is certainly possible to get a coarse estimate of the dispersal of a microbial invader. Currently the best that we can definitively state is that if we detect a species in a habitat we can conclude that it is capable of dispersing there. However, given their ability to passively disperse, persist at low levels, form resting cysts, and our evidence from two watersheds showing dispersal of *P. parvum* of roughly 300-km from known bloom sites suggests that these barriers may not be as difficult to pass for microbes.

Hence, we propose a more specified model for microbial species that removes the hurdle of transport and incorporates the principles of "everything is everywhere, but the environment selects" into the model by accounting for abundance and its resulting

impacts (Figure 3). In this model because of "everything is everywhere" introduction and establishment are equivalent, but, "the environment selects" ultimately determines impact and spread. Introduction and establishment are equivalent because microbial invaders could be continuously dispersing to a given habitat (as indicated from the qPCR detections that make up our local data and regional data),or could always be established at low levels potentially through a persistent microbial seed bank (Lennon and Jones 2011, Gibbons et al. 2013). Regardless of which of these alternatives is true, "the environment selects" will govern impact by determining which species are able to bloom. Generally, environmental filtering has been shown to be important in differences in detected diversity for microbes (Horner-Devine et al. 2004, Fierer and Jackson 2006). If environmental conditions allow a given microbe to bloom, and to thus reduce diversity, many ecosystem functions can be impacted and ecosystem stability is potentially reduced (Tilman 1996, Balser and Firestone 2005, Sunda et al. 2006, Bissett et al. 2010). This impact is important when considering that the vast diversity seen in microbial communities is thought to provide functional redundancy and ecosystem stability (Caron and Countway 2009).

Interestingly however, the alternatives of continuous dispersal or always established have different implications for how the environment will affect spread. In the case of continuous dispersal the environment will control the number and availability of surplus propagules that are passively dispersed to new environments. However, if it is the case that microbes are always established then changes in environmental conditions will determine where established microbes reach population abundances that can be detected as well.

In conclusion, our classification model shows that the presence of a microbial invader is governed by environmental selection. As such we suggest in our qualitative model that environmental selection govern impact of microbial invaders. Since in most cases microbial ecologists are still fairly limited in our ability to determine dispersal, even as detection technologies are becoming more accessible, we should look for instances where suspected invasive microbes are having discernable impacts when trying to test theories of invasion ecology. We also suggest that environmental selection will ultimately be more important in determining where we are able to more consistently detect and predict future invasions and impacts.

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Tables

Table 1. Classification of *P. parvum* presence based on environmental parameters[a,b,c]

			Predicted (DFA)		
			Present	Absent	Total
Observed (qPCR)	Lake Texoma	Present	103 (64.8%)	56 (35.2%)	159
		Absent	53 (16.3%)	211 (79.9%)	264
	Red River	Present	10 (83.3%)	2 (16.7%)	12
		Absent	6 (12.0%)	44 (88.0%)	50
	Canadian & Arkansas River	Present	3 (75.0%)	1 (25.0%)	4
		Absent	4 (18.2%)	18 (81.8%)	22

a. 74.2% of Lake Texoma samples correctly classified.
b. 87.1% of Red River sites correctly classified
c. 80.8% of Canadian River & Arkansas River sites correctly classified

Figures

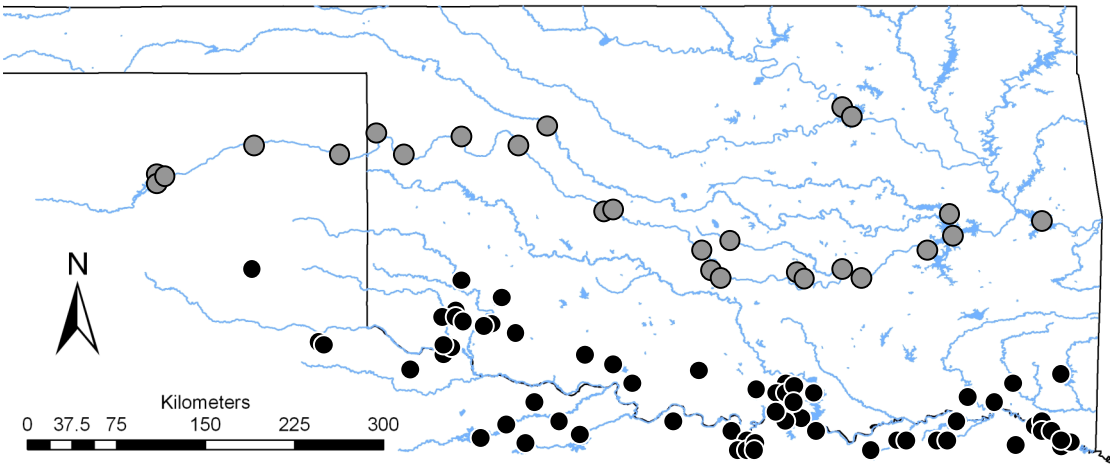

Figure 1. Map of collection sites in Red River watershed (black circles) and Canadian, Arkansas, and Cimarron River watersheds (grey circles).

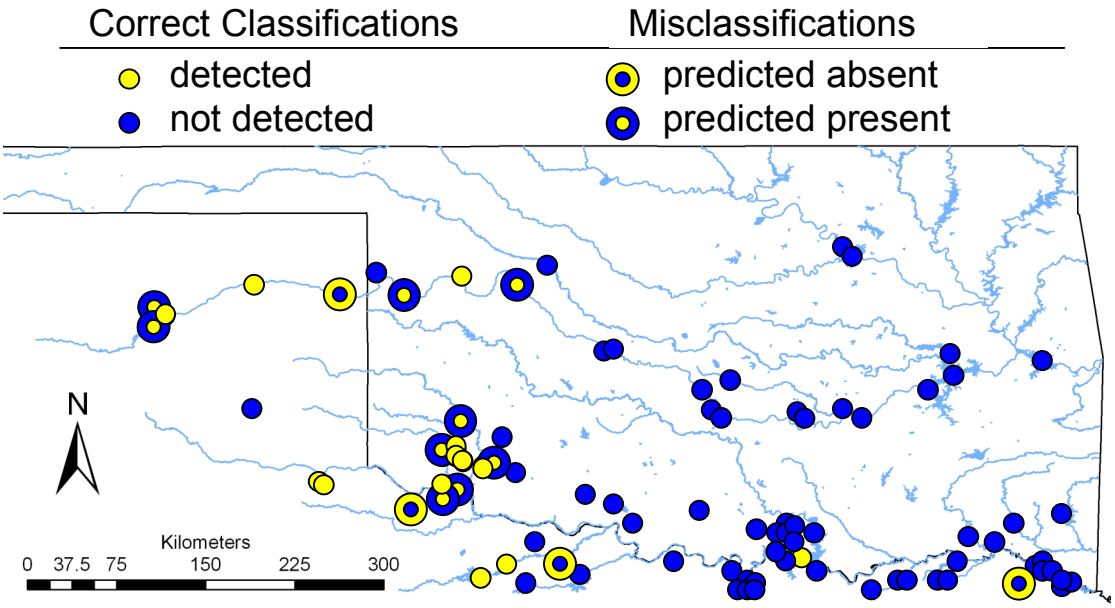

Figure 2. Map of collection sites in Red and Canadian, Arkansas, and Cimarron River watersheds where *P. parvum* was detected correctly classified by the DFA analysis as present (yellow circles) or absent (blue circles). Misclassifications are displayed as their predicted color encircled with a colored ring associated with the actual detection of *P. parvum* at a given site (yellow – present; blue – absent).

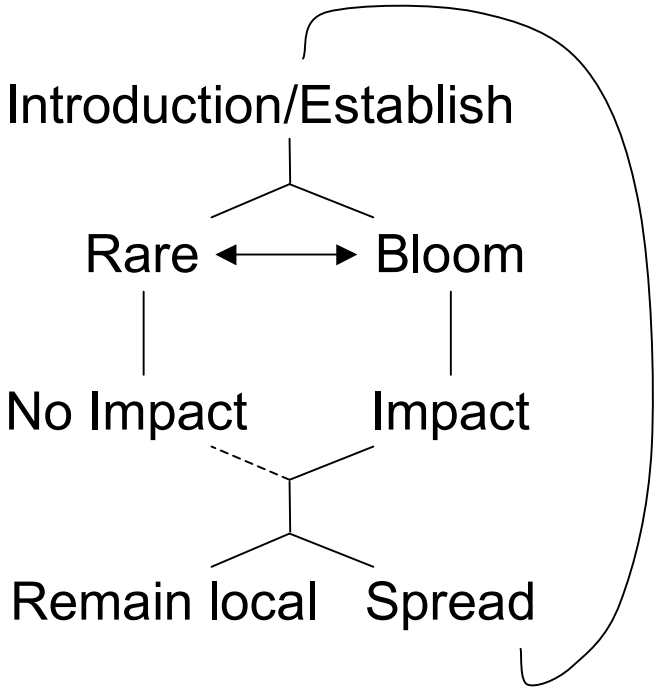

Figure 3. Invasion process model depicting stages of a microbial invasion, including effects of abundance on perceived impacts.

Chapter 4 – Propagule pressure determines establishment of a microbial invader

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One of the seminal hypotheses in the field of invasion ecology is that more diverse communities should be more resistant to invasion by exotic or non-native species[1,2]. Similarly, propagule pressure (i.e., the number of invaders entering a habitat or the frequency of invasions to a habitat) is thought to facilitate an invasive species' establishment success by increasing the ability of an invading population to absorb the challenges of its new environment[3]. Propagule pressure and community resistance to invasion are predicted to interact to affect the probability of community invasion and species establishment in a new community[4]. Our general understanding of these factors in invasion ecology is derived from the study of multi-cellular organisms, but it is unknown how these factors might affect the establishment success of microbial invaders[4]. Here we show experimentally that

regardless of community diversity, establishment success by the microbial invader, *Prymnesium parvum* in an environmentally-compatible habitat, is determined by propagule pressure.

Based on ecological niche theory in which organisms are thought to occupy an *n*-dimensional resource space[5], more diverse communities should use existing resources more extensively and thereby limit the resources available for an invading species. Hence, the idea that disturbance facilitates the establishment of invaders by reducing resistance is widely accepted[3], as disturbances that eliminate one or more species from a habitat should lead to increased resource availability. Some disturbances, such as nutrient pollution, can increase resource availability directly. In either case, disturbances favor species with fast growth and reproductive rates (e.g., *r*-selected species)[6-8] and tend to lead to simplified communities which pose reduced resistance to invasion by exotic species. Dispersal, and more specifically, a component of dispersal, propagule pressure, has also emerged recently as a significant predictor of establishment success by an invading species[9,10].. Together, community resistance and propagule pressure interact to affect establishment success of an invading species in a new community[11]. In theory, all communities are inherently invasible, with diverse communities with high resistance to invasion simply requiring higher propagule pressure in order to be successfully invaded.

Our general understanding in invasion ecology is derived predominantly from the study of multi-cellular organisms. Thus, it is unknown how factors such as community resistance and propagule pressure will impact the establishment success of microbial invaders[4]. Moreover, it is not even known whether the concepts of dispersal

limitation and propagule pressure even apply to microbial communities. The infamous credo of microbiologists, "everything is everywhere, but, the environment selects" posits that microbial species are or have the capacity to be ubiquitously distributed or they are continuously invading new habitats, but that the environment determines which microbes are at densities high enough to be detected[12,13]. Evidence generated through the study of biogeographic patterns in microbes suggests processes beyond environmental filtering, such as community assembly and dispersal limitation, are important determinants of microbial community composition[14-15]. However, tests of the underlying factors (e.g., community resistance and propagule pressure) thought to be driving biogeographic patterns are needed.

Here, we tested the hypotheses that reduced community resistance and increased propagule pressure will increase the establishment success of a microbial invader, *Prymnesium parvum,* in experimental freshwater microbial communities. In the past three decades, *P. parvum*, a harmful algal bloom species, has spread from marine coastal environments into freshwater systems throughout the southern half of the United States causing fish kills wherever it blooms[16].

Eighteen experimental mesocosms contained a mixed freshwater microbial assemblage taken from three sites within an invaded waterbody (Lake Texoma, Oklahoma and Texas, USA). These sites consistently differ in *P. parvum* abundances (the primary bloom site, 10-200 $\times 10^3$ cells mL^{-1}; a medium abundance site, $\leq 10 \times 10^3$ cells mL^{-1}; and zero to low abundance site, 0-0.5 $\times 10^3$ cells mL^{-1}). Salinity was manipulated in all bottles to simulate conditions in natural systems when *P. parvum* is known to bloom (2.3ppt). Temperature was also maintained at 15°C to simulate

conditions of late winter – early spring when *P. parvum* typically blooms in freshwater systems[17]. Community resistance to invasion was manipulated in half of the mesocosms by supplementing nitrogen and phosphorus levels to facilitate an algal bloom and reduce community diversity. Microbial diversity was monitored using a pyrosequencing-based metagenomics approach. After seven days, *P. parvum* was added to both high and low diversity communities at three concentrations simulating three levels of propagule pressure. Establishment success by *P. parvum* was assessed three and seven days after *P. parvum* additions.

Nutrient additions produced the desired bloom and community simplification after the 7-day incubation, with increased chlorophyll concentrations (Fig. 1) and reduced eukaryotic diversity (Fig. 1) in mesocosms receiving nutrient additions. This effect was maintained throughout the experiment, as diversity for eukaryotes remained lower in mesocosms that received nutrients (Fig. 1). However, both eukaryotic richness (Fig. 1) and bacterial diversity and richness were unaffected by nutrient additions (Fig S1). Both eukaryotic and bacterial diversity and richness at the end of the experiment were unaffected by *P. parvum* additions (Fig. S2).

Prymnesium parvum establishment success was unaffected by diversity of the receiving eukaryote community, but was directly proportional to propagule pressure (Fig. 2, Table 1). Following addition of the highest propagule pressure (1.3×10^7 cells; 6,400 cells mL^{-1} mesocosm concentration), *P. parvum* maintained viable populations after seven days. In the medium propagule treatment (1.3×10^6 cells; 640 cells mL^{-1} mesocosm concentration), *P. parvum* was still present in some mesocosms at very low densities after seven days, but would have likely disappeared given more time. In the

low propagule pressure treatment (1.3×10^5 cells; 64 cells mL^{-1} mesocosm concentration), *P. parvum* was detected in some mesocosms on day 3 but by day 7 had become undetectable. Hence, high propagule pressure overwhelmed any effects of reduced resistance (e.g., increased resources or reduced diversity).

Our results suggest that eukaryotic microbial diversity doesn't affect invasion success by an eukaryotic microbial species in a system that is otherwise environmentally favorable. Similar results have been seen in understory forest communities as propagule pressure from a mixed invasive assemblage taken from similar environments overwhelmed any effects of ecological resistance[18]. Although other experimental studies on multi-cellular organisms have shown that at the neighborhood scale resistance conferred by diversity has a negative effect on establishment[19], results from larger scales field studies indicate positive relationships between richness and invasibility[20-22]. This larger scale effect has been attributed to factors that can covary with species diversity such as habitat diversity[20] or even propagule pressure[22]. Hence, in our study it is a possibility that we were unable to sample diversity at a scale small enough to detect neighborhood-scale resistance effects. However, another possibility is that by controlling other environmental factors (e.g., temperature and salinity) in order to directly manipulate resistance in terms of resources and diversity we also altered interactions between the native community and *P. parvum*, as abiotic conditions have been shown to effect species interactions in macrobial communities[19,23]. This may explain how *P. parvum* was able to overcome the effects of increased diversity. By changing salinity and temperature we may also have created more habitat diversity and thus niche space for *P. parvum* that it was able to use despite

the fact that theoretically fewer resources were available. Furthermore, we examined the processes of propagule pressure and resistance in a eutrophic – hyper-eutrophic system. Even though our manipulation of nutrients changed diversity and thus theoretically resistance, this environment might be characterized as low resistance due to the high availability of resources prior to manipulation.

In Lake Texoma, (Oklahoma and Texas, USA) (i.e., where we obtained our microbial communities) blooms of *P. parvum* have occurred in most winters since 2003-2004, but only in the western Red River arm of the reservoir[17]. Even though downstream transport serves as a passive dispersal agent and propagule pressure is likely to be high given that bloom densities can approach 2×10^5 cells mL^{-1}, *P. parvum* has not successfully established populations (defined as recurring winter blooms) beyond Buncombe Creek[24]. Contrary to our experimental results, this pattern supports the hypothesis that environmental conditions are more important than propagule pressure in Lake Texoma. Moreover, this latter hypothesis was further supported by a landscape-scale survey for *P. parvum* across the Red River and Canadian River watersheds in Oklahoma and north Texas. Although *P. parvum* blooms occur upstream in both systems, and dispersal rates downstream are presumably high, *P. parvum* was only detected in sites with elevated salinities (i.e., with specific conductance >1900 μS cm^{-1})[25]. Thus while the latter half of the 'everything is everywhere, but, the environment selects' hypothesis is supported by our field data, the experimental results presented here suggest that dispersal may be a critical component of microbial biogeography.

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Methods & Methods Summary

Experimental mesocosms (n=18) contained a mixed freshwater microbial assemblage taken from three sites in Lake Texoma (L2, L4, and L6[17,24]). Resistance was manipulated in half of the mesocosms by adding nitrogen and phosphorus to levels known to facilitate *P. parvum* blooms (low resistance = 199μg L^{-1} P, 1.25 mg L^{-1} N; high resistance = 69 μg L^{-1} P, 0.79 mg L^{-1} N[17]) and allowing the communities to incubate for 7 days. *Prymnesium parvum* was added to the communities from cultures at three different levels of propagule pressure. Establishment success was assessed 3 days after propagule additions and again at the termination of the experiment (7 days) using a microscope and hemacytometer.

Effectiveness of the nutrient addition at manipulating microbial community diversity (i.e., diversity determined by inverse Simpson diversity index, richness determined by Catchall[26]) was assessed at the addition of *P. parvum* propagule pressure treatments and at the end of the experiment using high-throughput sequencing of the v6[27] and v9[28] hypervariable regions of the 16S and 18S rRNA genes for the bacterial and eukaryotic communities respectively. Operational taxonomic units (OTUs) were defined at a 3% sequence dissimilarity cutoff and classified based on the SILVA rRNA database[29]. All sequence processing, alignments, calling OTUs, and diversity estimation was conducted in the open source software package 'mothur' v.1.27.0[30]. All mesocosm sequence data were subsampled to the lowest number of sequences obtained for any mesocosm prior to estimations of diversity and richness. Effects of nutrients on diversity and richness at Day 0 were only assessed in high nutrient/low propagule pressure, low nutrient/low propagule pressure, and low nutrient/zero propagule pressure

control treatment combinations. Comparisons of all mesocosms were not made at this time point because were unable to sequence the community for all mesocosms. *In-vivo* measurements of chlorophyll-a were also taken at Day 0 and Day 7 using a TD 700 bench-top fluorometer.

All statistical analyses including appropriate tests of normality and variance were conducted in R[31]. All comparisons of the eukaryotic and bacterial community are analyzed separately. Fixed effects of day, nutrient treatment, propagule pressure treatment, the interaction of day and nutrient treatments, and the random effect of mesocosm identity on diversity and richness were compared using linear mixed effects models and the R package "nlme."[32] Post-hoc t-tests were conducted when any significant effects of nutrient treatment was detected. Comparisons of chlorophyll-a between nutrient treatments and day-7 and day-14 of the experiment were also conducted using a linear mixed effects model comparing fixed effects of nutrients, the repeated measure, their interaction, and the random effect of mesocosm identity. Post-hoc t-tests were also conducted for chlorophyll when any significant effects of nutrient treatment were detected. A generalized mixed effects model with a lognormal Poisson distribution[33] was fitted to the *P. parvum* establishment data and then likelihood ratio tests using Chi-square were used to compare fixed effects of nutrients, propagule pressure treatments, the repeated measure, the random effect of mesocosm identity, and the interaction between nutrients and propagule pressure. This model was chosen because the dataset was not normally distributed and suffered from overdispersion. Due to limitations of degrees of freedom we were unable to test interactions between the repeated measure and nutrients or propagule pressure, or the interaction of all three

terms. We used Tukey's test for post-hoc tests for comparing the different propagule pressure treatments.

Figures

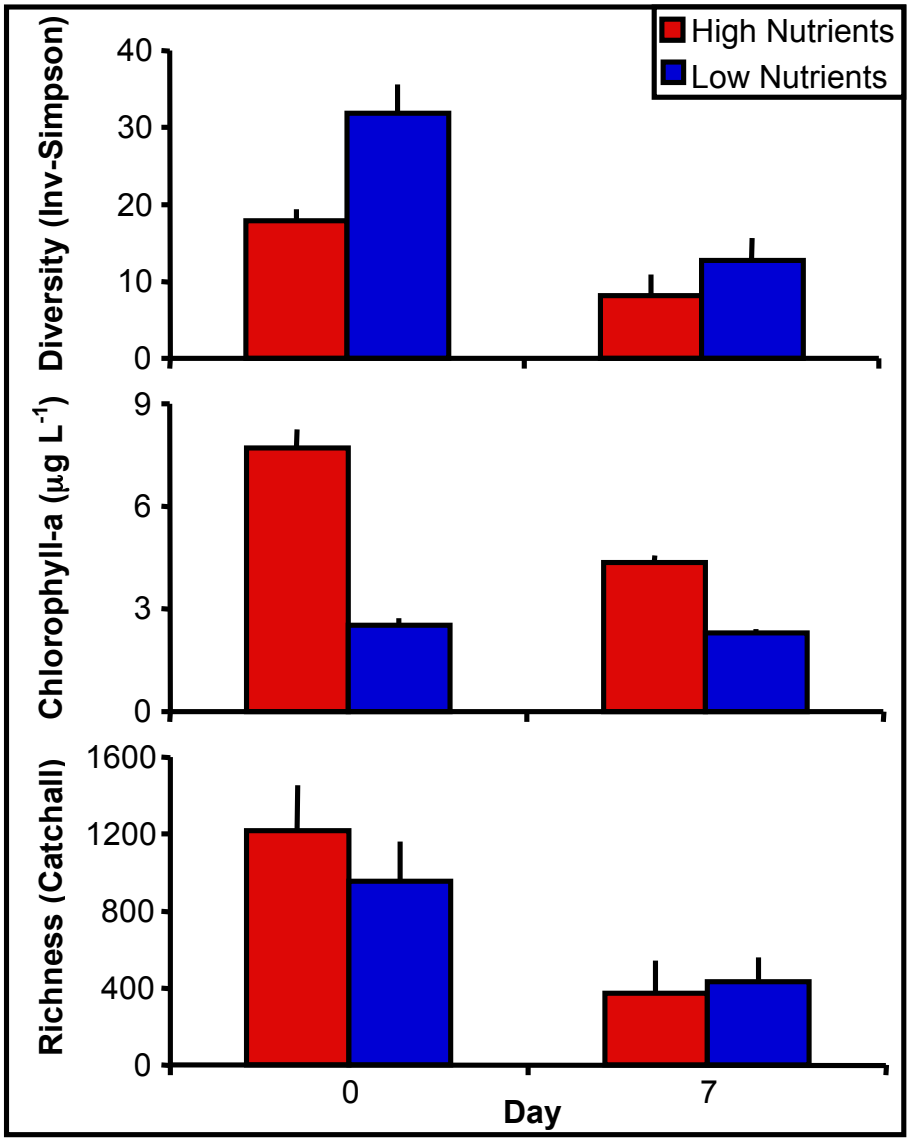

Figure 1. Effects of nutrient addition on the eukaryotic community. Chlorophyll-a (top panel) was increased in the high nutrient treatment (linear mixed effects model, F-nutrients$_{1,16}$ = 1102.21, p < 0.001) at both the addition of *P. parvum* propagule pressure treatments (Day 0: Welch's t-test, $t_{1,7}$=25.66, p<0.0001) and at the end of the experiment (Day 7: Welch's t-test, $t_{1,7}$=25.12, p<0.0001). Eukaryotic diversity (middle panel; inverse Simpson diversity index) was decreased in high nutrient treatments (linear mixed effects model, F-nutrients$_{1,16}$ = 35.78, p < 0.0001; Day 0: student's t-test, $t_{1,7}$=5.91, p<0.001) and this effect was maintained throughout the experiment (Day 7: student's t-test, $t_{1,7}$=3.25, p=0.004). Nutrients did not have an effect on rarified eukaryotic OTU richness (bottom panel; Catchall; linear mixed effects model, F-nutrients$_{1,16}$ = 0.24, p = 0.63). However, chlorophyll (F-day$_{1,16}$ = 1102.21, p < 0.0001), diversity (F-day$_{1,7}$ = 164.45, p < 0.0001), and richness (F-day$_{1,7}$ = 91.53, p < 0.0001) all declined by the end of the experiment.

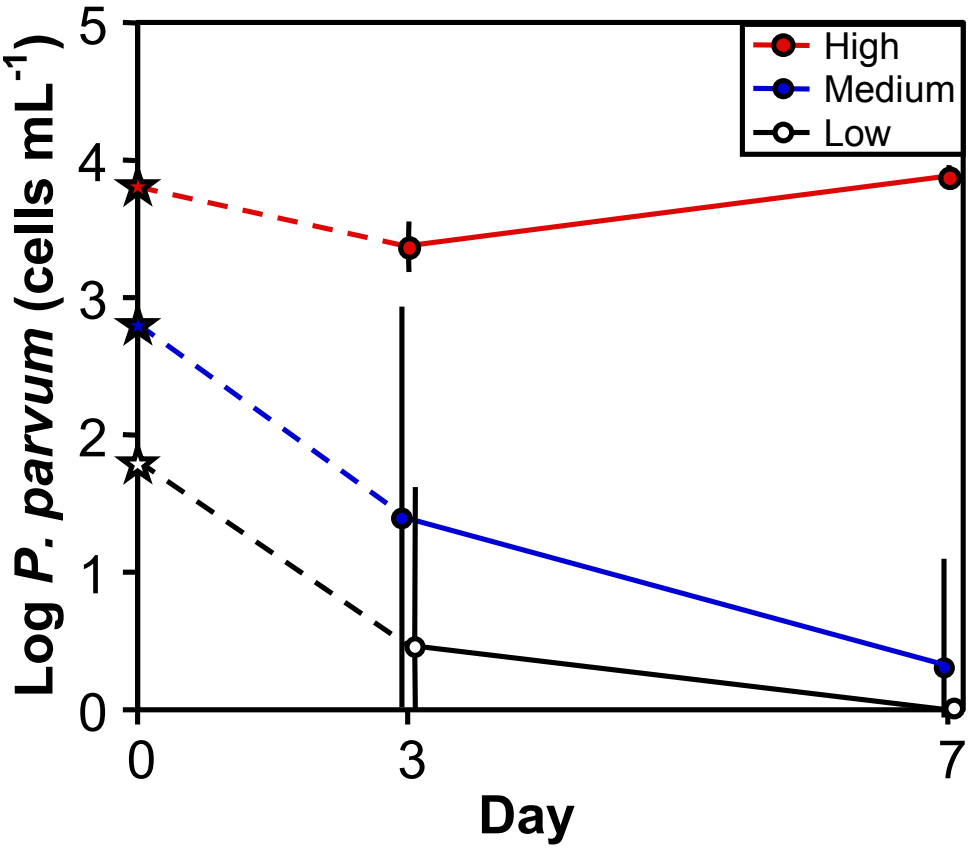

Figure 2. Establishment of *P. parvum* throughout the experiment. Stars depict Day 1 inoculations. Different propagule pressure treatments are depicted by different colored circles (High – red, Medium – blue, and Low – white). Results show a significant of effect of propagule pressure on establishment success (Generalized Linear Mixed Model, χ^2 Likelihood Ratio Test of fixed effects (df=2), p <0.0001). High propagule pressure treatments showed significantly greater establishment than either medium or low propagule pressure treatments (Tukey post-hoc, both p <0.0001). Time of sampling (GLMM, χ^2 LRT, (df=1), p = 0.20), nutrients (GLMM, χ^2 LRT (df=1), p = 0.08), and the interaction of nutrients and propagule pressure (GLMM, χ^2 LRT (df=1), p = 0.27) had no effects on establishment.

Supplementary Information

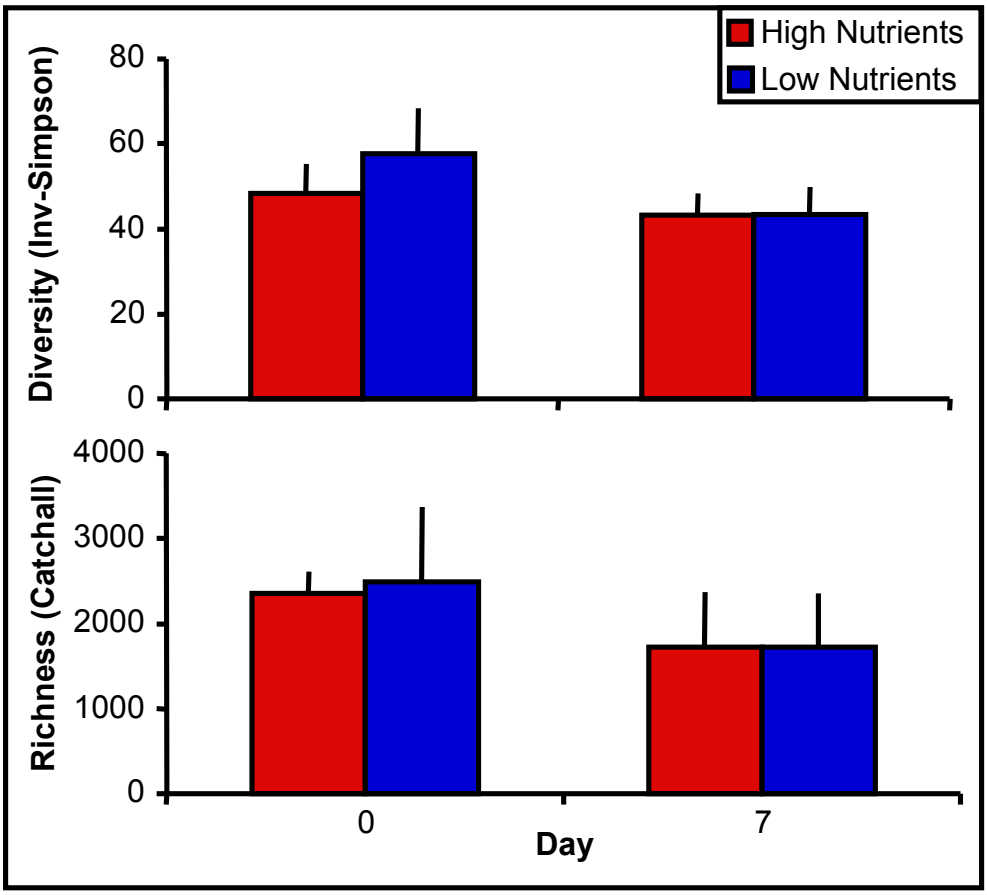

Figure S1. Figure 1. Effects of nutrient addition on the bacterial community. Bacterial diversity (top panel; inverse Simpson diversity index) was unaffected by nutrient additions (linear mixed effects model, F-nutrients$_{1,16}$ = 1.13, p = 0.30). Nutrients did not have an effect on rarified bacterial OTU richness (bottom panel; Catchall; linear mixed effects model, F-nutrients$_{1,16}$ = 0.02, p = 0.88). However, bacterial diversity (F-Day$_{1,7}$ = 16.18, p = 0.005) and richness (F-Day$_{1,7}$ = 6.91, p = 0.03) did decline throughout the experiment.

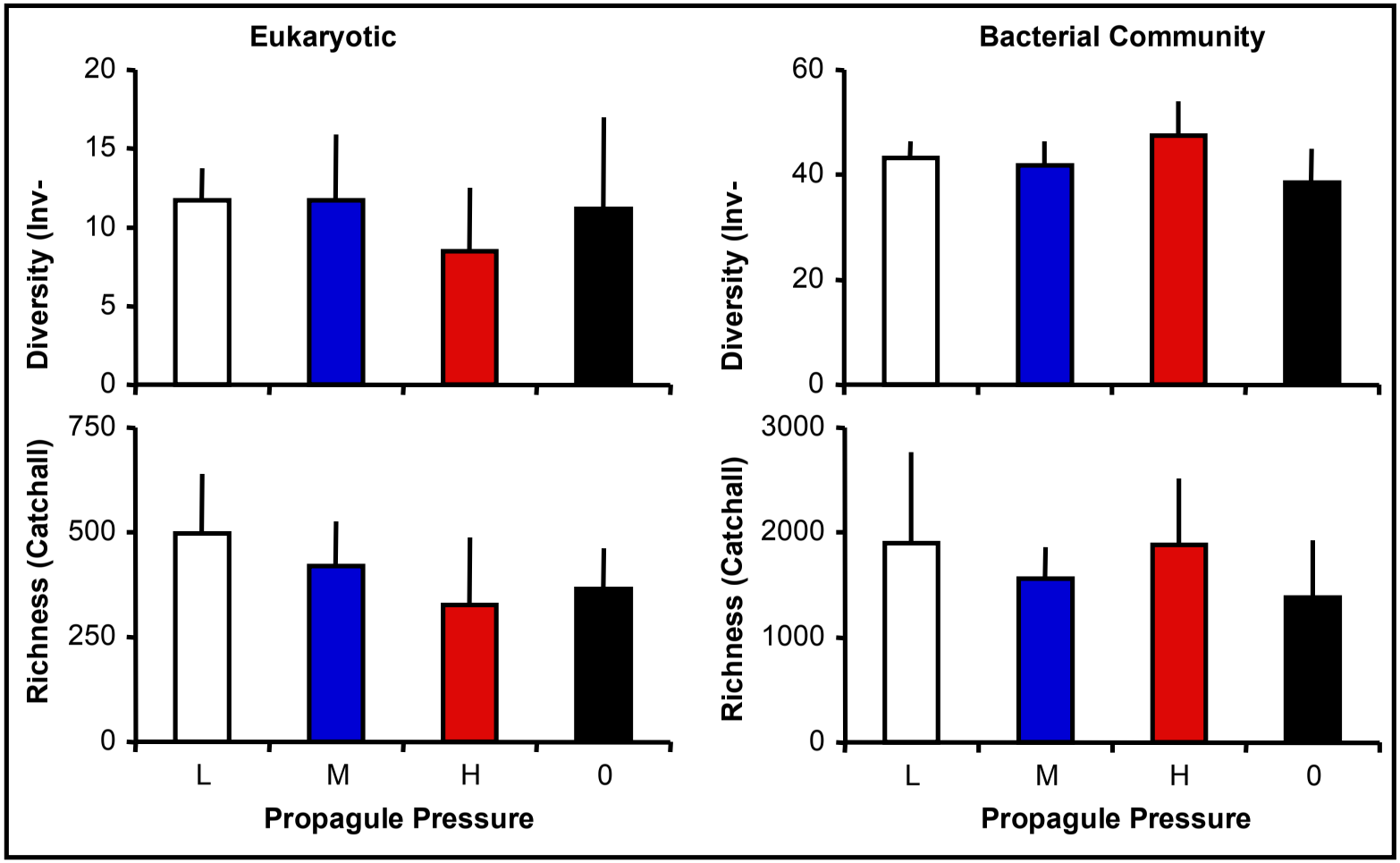

Figure S2. Effects of propagule pressure on the microbial community. Propagule pressure treatments had no effect on either eukaryotic community diversity (linear mixed effects model, F-proppress$_{3,16}$ = 1.13, p = 0.37) and OTU richness (linear mixed effects model, F-proppress$_{3,16}$ = 1.21, p = 0.33) or bacterial community diversity (linear mixed effects model, F-proppress$_{3,16}$ = 1.22, p = 0.33) and richness (linear mixed effects model, F-proppress$_{3,16}$ = 0.47, p = 0.71).

Post Script

The first recorded bloom of *Prymnesium parvum* in fresh water occurred in the Workum See, Holland in 1920. However, it was not until it bloomed in the majority of aquaculture ponds in Israel in the late 1940s that it began to gain serious attention from the scientific community. Since that time *P. parvum* is known to have bloomed on every continent except for Antarctica. In North America the first documented bloom of a *P. parvum* occurred in 1985 in the Pecos River, TX, USA (although it may have been there since the 1960s). From there it steadily migrated north arriving in the Red River, OK, USA in 2000 and Lake Meredith in the Texas panhandle (USA) and the Canadian River, TX in 2003. Since these blooms, its range has rapidly expanded in the past decade causing fish kills in reservoirs and rivers throughout much of the southern United States from California to Florida, and as far north as Wyoming and West Virginia. Hence, worldwide research on *P. parvum* has spanned 9 decades (6 of which were intensive) and intensive research within North America has spanned the past 3 decades.

However, the majority of this research has focused on either autecological characteristics of *P. parvum* and the formation of its toxins, or on documentation of bloom occurrence. Little research has addressed questions relating to what might explain why this species has been so successful at expanding its range. There are two theoretical possibilities that might explain the rapid range expansion of *P. parvum*. One possibility is that *P. parvum* is an invasive species that has dispersed to and established in new ecosystems. The second possibility is that this range expansion is driven by changes in the environment driven by factors such as climate change or overexploitation

of water resources and that *P. parvum* has always been present in these systems (i.e., "everything is everywhere, but, the environment selects"). The hypothesis "everything is everywhere, but, the environment selects" is firmly rooted within microbial community ecology and indeed gradients in environmental characteristics have been shown to be correlated with differences in microbial community composition (e.g., pH and soil microbes). However, biogeographic patterns unrelated to environmental parameters have also been observed, which suggest that dispersal is important to the establishment of microbial species in new environments. What makes addressing these possibilities using the species *P. parvum* more interesting is that there is a need generally for investigations of microbial invasive species and that *P. parvum* has such dramatic negative effects because it is a harmful algal bloom species. In my dissertation, I show that indeed both dispersal and environmental selection can play a role in *P. parvum* establishment. Specifically, results obtained from field data suggest that environmental selection is a primary determinant of where *P. parvum* is detected. However, experimental results show that in environments that favor *P. parvum,* the size of the invading population can determine whether *P. parvum* is able to establish or not.

Future research that would further tease apart factors important to dispersal versus environmental filtering would be to repeat the experiment from Chapter 4 without the additions of salt or the manipulations towards colder temperatures. Because we added salt and manipulated temperature we controlled for the environment so that we could specifically test for factors important to understanding dispersal and ultimately affecting establishment. In essence, the previous research was attempting to address the "everything is everywhere" component of the "everything is everywhere,

but, the environment selects" hypothesis. By removing these environmental controls this experiment would also test for the importance of environmental filtering in determining invasion success. In this scenario I would expect that *P. parvum* would be less capable of invading mesocosms given our field data presented in Chapter 3, which suggests that the environment is a strong predictor of where *P. parvum* is detected. However, if *P. parvum* were capable of establishing in these mesocosms, with less favorable environmental conditions, then I would expect that establishment success would be highest in the highest propagule pressure treatments as demonstrated in Chapter 4.

Another way to address the possibility of "everything is everywhere" directly using *P. parvum* would be to test if *P. parvum* has always been present in Lake Texoma and other systems that have had blooms of *P. parvum*. It has been suggested that *P. parvum* has the ability to form resting cysts. Therefore, it is possible that these cysts could exist in the sediments of lakes where *P. parvum* has previously established. Using a combination of sediment cores taken from the bottoms these lakes and the qPCR method from Chapter 1, one could attempt to detect *P. parvum* in dated slices to determine *P. parvum* presence, thus directly testing "everything is everywhere". Any detections of *P. parvum* in years previous to its first bloom would suggest that indeed *P. parvum* has always been present in the system, just not at easily detectable levels. A further step would be to compare environmental conditions at the time of these detections to the current environment, which, if different, would increase the evidence that indeed environmental filtering is controlling bloom establishment. Any differences in environment would also provide a mechanism showing that indeed the environment

has changed in such a way that has favored *P. parvum* and that this may be occurring in other affected systems.

Although investigating the interplay of dispersal and environmental filtering is interesting it is not the only fascinating aspect of community and invasion ecology that could be addressed using *P. parvum* or that is addressed by my dissertation. In Chapter 4 of my dissertation I ask the question "does increased resistance by the community have a negative effect on the establishment of the microbial invader *Prymnesium parvum*?" In this research I manipulated the microbial community by adding nutrients, which caused a phytoplankton bloom in the experimental mesocosms. This bloom resulted in changes in the eukaryotic diversity and taxonomic evenness. This change in diversity has numerous implications for community ecology, primarily that interactions between taxa, and thus the competitive arena within the mesocosms, was potentially altered. Understanding changes in species interactions (i.e., competition) strikes at the heart of community ecology and has been shown to be important in determining community composition in both multi-cellular organisms and microbes. For example, the idea of invasion resistance is built upon niche theory in that more diverse communities will either use more resources or use them more efficiently, and depending on the degree of use, will exclude invaders. Furthermore, community composition is thought to be important for ecosystem functioning. Thus, the fact that eukaryotic diversity in the mesocosms changed is interesting because how community composition was affected by the manipulations (both the addition of nutrients and the addition of an invader) allows us to investigate how these taxa might be interacting and the importance of the environment in determining outcomes of those interactions.

The next question for this research is "How did the microbial community change with the additions of nutrients and with the addition of *P. parvum*?" Theoretically, I should be able to make predictions about which taxa might be affected by these manipulations. For example, in the eukaryotic community one might expect diatoms to increase as they are often among the first phytoplankton species to increase during spring phytoplankton blooms. They are also potentially less edible by micro-predators such as ciliates that were present in the experiment, or even *P. parvum* (a known mixotroph), due to their siliceous cell wall. Because the raw values for bacterial diversity and richness appeared to be unchanged by the manipulation it would be interesting to investigate if community membership also remained unchanged. The theoretical importance of the microbial rare biosphere (i.e., the presence of numerous taxa at very low abundances) is thought to be that it maintains the functionality of the microbial community even though community membership can change with a given environmental change and this may be potentially occurring in the bacterial community.

An interesting follow-up to this analysis would be to sequence the microbial communities using the DNA samples taken from various sites throughout the state from Chapter 3. These communities could be compared within one another and with community data from Lake Texoma to determine if communities shift in a similar way in response to the presence of *P. parvum*. This comparison could also provide insight into the importance of environment (i.e., the competitive arena) in determining freshwater microbial community composition.

In conclusion, the results from my dissertation are only a starting point for asking questions about the importance of dispersal and the environment to the outcomes

of microbial invasion. Further investigation into the roles of environmental filtering, and the potential for the prior presence of *P. parvum* should prove to be fruitful avenues of future research. Furthermore, understanding how the environment and *P. parvum* affect community composition should allow us to gain insight into how *P. parvum* interacts with other microbes. Ultimately, all of this information is important not only to our understanding of *P. parvum* specifically, but to our understanding of microbial invasions and harmful algal blooms as a whole, and thus our ability to mitigate their impacts.