EXPLORING A CHEMICAL APPROACH FOR THE MITIGATION OF *Prymnesium parvum* BLOOMS AND ECOLOGICAL CONSIDERATIONS

A Thesis

by

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ABSTRACT

Known as Golden Algae in popular media, *Prymnesium parvum* causes harmful algal blooms. When stressed, it secretes increased amounts of toxic chemicals called *prymnesins*, which have resulted in major fish kills in Texas. Although many options exist for mitigation of blooms, a feasible protocol for control of blooms on large-scale impoundments has yet to be identified.

Chemical control of *P. parvum* using six different enzyme inhibiting aquatic herbicides was explored in laboratory experiments. Of the six chemicals screened, one (Flumioxazin) was selected for further study due to a significant decrease in *P. parvum* cell numbers with increasing chemical concentration. It was applied to natural plankton communities during *in-situ* experiments (Lake Granbury, Texas). The first experiment was conducted during a period of *P. parvum* bloom initiation (March) and the second experiment conducted during a post bloom period (April). Experiments were carried out in 20 L polycarbonate carboys covered in 30% shade cloth to simulate natural light, temperature and turbulence conditions. Flumioxazin was additionally screened in the laboratory on the common game/forage fish bluegill sunfish (*Lepomis macrochirus*) for six weeks with weekly re-application of flumioxazin to treatment tanks.

Cell counts via light-microscopy, showed the chemical flumioxazin caused significant decreases in *P. parvum*, but no significant differences in zooplankton abundance during the period of bloom initiation. However, significant decreases in adult copepods were observed during the post bloom period, most likely due to decreased light

penetration and inhibition of the photosensitive mode of action, but no significant decreases in *P. parvum*. No significant effects of flumioxazin were observed on growth, survival or feed conversion ratio for *L. macrochirus*.

DEDICATION

To my Mom and Dad

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Daniel Roelke, who offered me a job early on in my undergraduate career. Even though I didn't quite know what a "phytoplankton" was, he took the time to give me one of the greatest opportunities of my life. For that, and the countless things in the lab that I broke, I am grateful. I am also very indebted to Dr. Fran Gelwick who was a constant mentor throughout my undergraduate and graduate careers. She also was one of the few people who would listen to my stories of catching monster bass on Lake Falcon. I would like to thank Dr. Miguel Mora as well, for his patience and for always being willing to meet with me when I would show up at his office with no appointment. Dr. Michael Netherland was also instrumental in my graduate career by providing funding and unmatched expertise.

I would also like to thank Dr. Manuel Pina and HLAE for giving me the means and motivation to continue my education and for helping me to be a better leader. I also thank my lab mates, classmates and students, all of whom I consider my friends. Of course, I would also like to thank Kelly Haile.

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CHAPTER I

INTRODUCTION*

1.1 Background/Literature Review

The ecology of freshwater ecosystems is complex. Dynamic food web structures, changing abiotic factors and anthropogenic inputs pose major challenges to fisheries managers. Additional stressors including over exploitation, natural flow modification, pollution and habitat degradation (Dudgeon et al. 2006) require comprehensive and innovative approaches to their correction.

Another challenge, the invasion of territories or niches by non-native species, is becoming increasingly common in many aquatic habitats (Rahel et al., 2008). Climate change and anthropogenic activities such as expanded settlement, transport of materials, and habitat disturbance have been linked to an increase in abundance and distribution of alien species (Occhipinti-Ambrogi 2007, Vitousec et al., 1997, Dukes and Mooney 1999). Such invasions can cause changes in species diversity that result in ecosystem alterations (Lodge 1993, Chaplin et al., 2000, Hooper et al., 2005) making fisheries management an even more arduous task. Today estimates of damages caused by non-native aquatic species exceed 1 billion dollars in the US alone (OTA 1993, Pimentel et

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al. 2000).

Invasive algae can subjugate native species and, via unchecked growth, proliferate such that entire aquatic ecosystems are affected, with local extinction of many indigenous populations (Meyer 1971). Presently, harmful algal blooms (HABs) like those caused by cyanobacteria and dinoflagellates are increasing world wide (Hallegraeff 1993, Landsburg 2002, Granéli and Turner 2008). The increase in bloom incidences can often be linked to nutrient enrichment, global climate change and other anthropogenic activities (Paerl et al., 2011, Paerl and Paul 2012, Granéli and Turner 2008, Roelke et al. 2011, 2012) and in many cases, can have lasting consequences in the aquatic environment.

Unlike the problem of invasive-aquatic plants, there are few feasible control options for invasive phytoplankton. For example, physical removal of filamentous algae is a common and effective practice on small ponds but may not be an efficient control measure on a large scale. Similarly, use of clay flocculants to decrease population densities of HABs at sea can be an effective approach in HAB mitigation (Shirota 1989), however the efficacy of clay flocculation on the HAB *Prymnesium parvum* was found effective only under certain conditions, and sediment toxicity was undesirably increased in some instances (Sengco et al., 2005). In addition, inland freshwater lakes where *P. parvum* blooms frequently occur in the southcentral USA are typically shallower than marine systems, and planktonic cells may be more susceptible to re-suspension during mixing events. Chemical control of algae through addition of naturally occurring

chemicals is considered environmentally friendly. However, one of the most popular natural chemical control methods, the use of barley straw as an inhibitor for algae growth (Ridge et al., 1999), has largely had mixed results (Everall and Lees 1996, Kelly and Smith 1996, Grover et al, 2007). There are alternative biological control options for algae. For example, viruses, bacteria, fungi, protozoa and actinomyctes offer avenues of control. Some viruses have shown to be effective at controlling various species (Jackson and Sladecek 1970, Daft et al., 1975) although their production, storage and application can be logistically troublesome (Sigee et al., 1999).

In comparison, registered algaecides have been applied with greater success primarily because of their high efficiency and ease of application. They are widely used in aquaculture settings to control the foul taste and odor causing cyanobacteria *Oscillatoria perornata* in catfish ponds (Schrader et al., 1998, Schrader and Harries 2001) as well as recreational lake and drinking waters (Moore and Kellerman 1905, McKnight 1983). Many of these, widely used, broad spectrum algaecidal chemicals such as copper based compounds can lead to deterioration in water quality and secondary toxicity (Schrader et al., 2004). Recent registrations of some herbicides for aquatic use may provide additional management options. For example, Netherland (2009) showed that some acetolactate synthase (ALS) and phyotene desaturase (PDS) inhibiting compounds are effective against organisms that cause harmful algal blooms. These herbicides target plant specific enzymes, and disrupt carotenoid production (PDS) or amino acid production (ALS) (Senseman 2007). Some algae may be more sensitive than others and in theory, selectivity of aquatic herbicides for particular algal species

could minimize the impact on non-target algae while reducing biomass of the targeted undesirable algae.

As mentioned above, the haptophyte *P. parvum* known as golden algae in the popular media is responsible for harmful algal blooms and is a major concern to biologists, fisheries managers and aquaculturalists. Blooms are characterized by extensive fish kills, foul odor, and a golden coloration of the water (Kaartvedt et al., 1991, Guo et al., 1996, Sager et al., 2008, Southard et al., 2010). According to recent studies, secreted toxins (prymnesins) are believed to create a selective advantage for *P. parvum* through mechanisms such as resistance to grazing, immobilization of prey under heterotrophic conditions, and allelopathy (Roelke et al., 2007, Barreiro et al., 2005, Fistarol et al., 2003, 2005, Granéli & Johanson 2003, Uronen et al., 2005). *P. parvum* occurs worldwide (Collins 1978, Edvarsen and Paasche 1999, Granéli et al., 2012), with a recent expansion into the western hemisphere (Lutz-Carrillo et al., 2010). It was first documented in Texas in 1985 (James and de la Cruz 1989), but has since spread throughout warmer regions of the USA, most recently to some northern states (Sager et al., 2008, Roelke et al., 2011, Brooks et al., 2011).

Several management techniques were explored in the mitigation of toxic *P. parvum* blooms. These include pulsed hydraulic flushing and associated nutrient loading, nutrient fertilization, particle flocculation onto clay particles and addition of barley straw extract (Roelke et al., 2007, Hagstrom and Granéli 2005, Sengco et al., 2005, Hayden et al., *In Press*). Other methods include the application of ammonium sulfate, copper sulfate and potassium permanganate and other broad spectrum herbicides (Barkoh et al.,

2010, Rodgers et al., 2010). Although several management techniques have been successful in controlling *P. parvum* in laboratory settings, in-lake mesocosm experiments, and hatchery ponds, a feasible large-scale management tool has yet to be identified (Southard et al. 2010).

The application of an aquatic herbicide or algaecide selective for *P. parvum* during its early growth phase could decrease its abundance enough and suppress a bloom long enough to by-pass conditions otherwise favorable for bloom initiation. Additionally, in large areas where total chemical treatment may not be feasible, enzyme specific herbicides, when applied to smaller areas, might provide bloom-free refuges for fish and other aquatic life. Such refuges could be natural coves or other protected sites where fish could reside until toxic *P. parvum* blooms are no longer a threat. Proof of this concept is necessary to evaluate the effects of flumioxazin on those fish species predominant in water bodies where *P. parvum* blooms are known to occur.

1.2 Overarching Objective

The overarching objective for this study is to identify a chemical herbicide that may be suitable for control of HABs caused by *P. parvum* in freshwater lakes and rivers where the species has become problematic. This goal will be achieved by accomplishing the following specific objectives:

1) Evaluate the efficacy of six chemical herbicides as possible control agents of *P. parvum* in a laboratory setting.

2) Evaluate the herbicide found most effective in natural assemblages (plankton community) by conducting in-lake mesocosm experiments during periods of *P. parvum* bloom development and post-bloom decline.

3) Screen the herbicide found most effective for its effects on a predominant game fish species (*Lepomis macrochirus*) in waters where *P. parvum* blooms are known to occur.

CHAPTER II

A CHEMICAL APPROACH FOR THE MITIGATION OF *Prymnesium parvum* BLOOMS*

2.1. Purpose

In this study the efficacy of 6 enzyme specific herbicides that were newly registered or in the process of registration for aquatic use were evaluated as control agents for *P. parvum*. To evaluate the efficacy of each chemical, our initial screenings were performed in a laboratory setting with monocultures of *P. parvum*. We then chose the most effective algaecide and evaluated its effect on a natural alga assemblage by conducting in-lake mesocosm experiments during periods of *P. parvum* bloom development and post-bloom decline.

2.2. Methods

2.2.1 Laboratory Screening

Monocultures of *P. parvum* used for our experiments (UTEX LL 2979, Culture Collection of Algae at the University of Texas at Austin) were maintained in f/2 medium (Guillard 1975) under conditions similar to winter in southcentral USA, a period of year

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when blooms occur (4 ppt salinity, 12°C, 12:12 light:dark (L:D) cycle). Our culture maintenance schedule comprised monthly inoculation into fresh media at a 1:10 volume to volume ratio, which allowed the culture to reach a senescent growth phase prior to the next inoculation.

We screened six new herbicides by dosing *P. parvum* cultures and measuring resulting algal growth. The chemicals tested were the bleaching herbicides fluridone and topramazone, the ALS inhibitors penoxsulam and bensulfuron, and the protoporphyrinogen oxidase (protox) inhibitors carfentrazone and flumioxazin. Of these, topramazone and bensulfuron are not yet registered. The screenings were carried out in a controlled growth chamber (Percival® Low Temperature Incubator) under the same culturing conditions, and lasted for 14 days. Experimental units were 125ml flasks with added culture growing in log-growth phase and fresh media (1:10 ratio), and with added chemicals. Flasks were gently agitated and positions within the incubator rotated daily to ensure equal exposure to the 115 μmol m^{-2} s^{-1}fluorescent lights.

The experimental dosage gradient included a wide range of concentrations, with some exceeding the manufacturer's recommendations (in the case of fluridone and penoxsulam). For fluridone, penoxsulam, topramezone and bensulfuron, chemical concentrations were 0, 6.25, 12.5, 25, 50, 100 and 500 μg/L. For carfentrazone and flumioxazin, concentrations of 0, 25, 50 100, 200 and 400 μg/L were used. Stock solutions concentrations of 0.5 μg/L were used for fluridone, carfentrazone and flumioxazin and 1.0 μg/L for bensulfuron, penoxsulam and topramezone. To achieve the desired final concentration, 1ml of each stock was diluted into 1 L so that a manageable

aliquot could be pipetted into each flask and gently swirled to mix thoroughly. Triplicate flasks of *P. parvum* culture were used for each concentration and *in-vivo* fluorescence of each flask was measured with a 10-AU fluorometer (Turner Designs® 10 AU) every 2 days as a proxy for algal biomass. Flask positions within the incubator were also rotated daily to ensure equal light exposure. For the purposes of initial chemical screening, we assumed that variation in fluorescence per cell was not significantly different over the 14 days of the screening experiment. Statistical analysis of the *in-vivo* fluorescence was carried out using a Repeated Measures General Linear Model with Tukeys test for within subjects differences (SPSS 14).

2.2.2 Cove Monitoring

Lake Granbury is a sinuous, shallow lake in southcentral USA that experiences recurrent *P. parvum* blooms in late winter and early spring when low inflows have led to higher salinities (Roelke et al., 2010, 2011). We timed our field experiments to coincide with the period of blooms, with two in-lake experiments, one conducted in March and the other in April. To better understand the initial conditions for these in-lake experiments, we sampled several parameters (see below) weekly in the lake as a time-series prior to and during our experiments (February 17 to April 20, 2010). In addition, online data for inflows to Lake Granbury were collected (Brazos River Authority website, USGS Data, station 08090800 near Dennis, TX).

We estimated total phytoplankton biomass (fluorometry) and *P. parvum* population density (direct counts) in surface water. For *P. parvum*, we collected 100-mL water samples preserved in 3% v/v glutaraldehyde. Subsamples of 1 to 2 mL were

settled for 24 hours and cells were counted using an inverted phase-contrast light microscope (400x, Leica)(Uttermohl 1958). Fields of view were randomly selected and counted with a target total number of 200 *P. parvum* cells (Roelke et al., 2007). Additionally, we looked for incidences of other algal species and shifts in relative abundance. As a proxy for total algal biomass, chlorophyll *a* was measured. For each sampling, we collected triplicate 50-mL water samples, filtered through 47mm GF/F filters, and extracted with 90% acetone, centrifuged, then analyzed fluorometrically as μg L^{-1} Chl-*a*(Strickland and Parsons 1968). This technique does not enable taxonomic resolution of phytoplankton.

Direct counts of zooplankton were performed. We concentrated 12-L water sample through a 63-μm screened cod end net down to 50-mL samples preserved with 5 mL of 10% buffered formalin (Roelke et al., 2007). Subsamples of 10 to 15 mL were then settled for 24 hours and enumerated using phase contrast inverted light microscopy (40X, 200X) following the Utermohl technique. The shape and dimensions of each zooplankter in 8~12 ml subsamples were measured in order to estimate biovolume, a proxy for biomass (Wetzel and Likens 1991, Roelke et al., 2007), and observed individuals were classified into the following groups: total copepod adults, total copepod nauplii, total rotifers and total cladocera. We report both the biomass estimates (in units of $\mu m^3 L^{-1}$) and population density (in units of individuals L^{-1}) for each of the four groups of zooplankton. Both quantification measures are important, as biomass estimates are best related to foodweb mass budgets and population densities are important when estimating grazing pressure.

Water quality parameters were also measured weekly. Nutrient analyses were done using a nutrient auto-analyzer (O-I Analytical) following methodology from Armstrong and Sterns 1967 and Harwood and Kuhn, 1970; water samples were first filtered through pre-combusted Whatman 47mm GF/F filters (~0.7μm pore size) and then frozen prior to analysis. For our study, we identified soluble reactive phosphorus (SRP) and the sum of NO_2, NO_3 and NH_4, which we refer to as dissolved inorganic nitrogen (DIN). Other abiotic parameters measured included temperature, pH and salinity (Hydrolab multiprobe) and water transparency (Secchi disk).

2.2.3 In-lake Experiments

Our field experiments were conducted in 20-L polycarbonate carboys, each filled with 18 L of lake water collected from ~0.5m below the surface in the sample cove at Lake Granbury. An empty headspace of 2 L was left to allow for gas exchange and neutral buoyancy. Each carboy was attached to a free-moving, but anchored floatation device to ensure natural wave action and was covered with optically neutral density mesh to reduce sunlight exposure by approximately 50%, which for these waters is likely more representative of natural light conditions occurring over the mixing depth (see Roelke et al., 2010, Schwierzske et al. 2010).

Our first *in-lake* experiment was initiated on March 9, 2010 and ended on March 16, a period coinciding with the initiation of a fish-killing *P. parvum* bloom in the lake. For this experiment, we deployed 15 carboys, which comprised triplicate treatments of 0, 25, 50, 100 and 200 μg/L flumioxazin. Our second *in-lake* experiment was initiated on April 20, 2010 and ended on April 27, a period coinciding with the bloom decline in the

lake. For this experiment 18 carboys were deployed for triplicate treatments of 0, 25, 50, 100, 200 and 400 μg/L flumioxazin. The additional dosage level of 400 μg/L was added to elicit a stronger die off response as indicated by preliminary findings from our first *in-lake* experiment. At the start and end (seven days later) of both experiments, *P. parvum*, chlorophyll *a*, zooplankton, inorganic nutrients and other abiotic parameters were measured following methods previously described. As for the cove monitoring, other than the direct counts of *P. parvum*, florometry did not enable taxonomic resolution of phytoplankton composition.

2.2.4 Statistical Analyses

For the laboratory screening experiments, the effect of chemical dosage on *P. parvum* biomass was tested using repeated measures ANOVAs. Within-subjects differences were evaluated using Tukeys tests (SPSS 14). For the in-lake experiments, differences in *P. parvum* densities, zooplankton biovolumes, chl-*a* and nutrient concentrations between dosages were evaluated using one-way ANOVAs, where the level of significance was p=0.05.

2.3. Results

2.3.1 Laboratory Screening

Repeated measures ANOVA of *in-vivo* fluorescence from the *P. parvum* monocultures during the chemical screening laboratory experiments showed dose dependent growth responses that varied among chemicals (Figure 2.1). Exposure to fluridone at 500 μg/L killed off *P. parvum* within 8 days (significant p<0.05), while concentrations at 100 μg/L and less produced little effect, thus *P. parvum* biomass was

able to accumulate. Penoxsulam at all dosages was ineffective, at slowing accumulation of *P. parvum* biomass. The label-maximum use rate for fluridone and penoxsulam is 150 μg/L. Exposure to topramezone and bensulfuron produced similar results to penoxulam, with only higher dosages significantly slowing the accumulation of *P. parvum* biomass (for topramezone at 25 μg/L and greater; for bensulfuron at 500 μg/L only). A gradient of dose-response effects were observed for carfentrazone and flumioxazin; with *P. parvum* biomass accumulation significantly decreased with increased chemical concentration (for carfentrazone at 50 μg/L and greater; for flumioxazin at 25 μg/L and greater). Dosage for flumioxazin of 100 μg/L and greater resulted in the elimination of *P. parvum* within 14 days. Because the lowest effective dosage (i.e., producing significant reduction in biomass accumulation of *P. parvum*) was observed with flumioxazin, it was selected for further evaluation in our *in-lake* experiments.

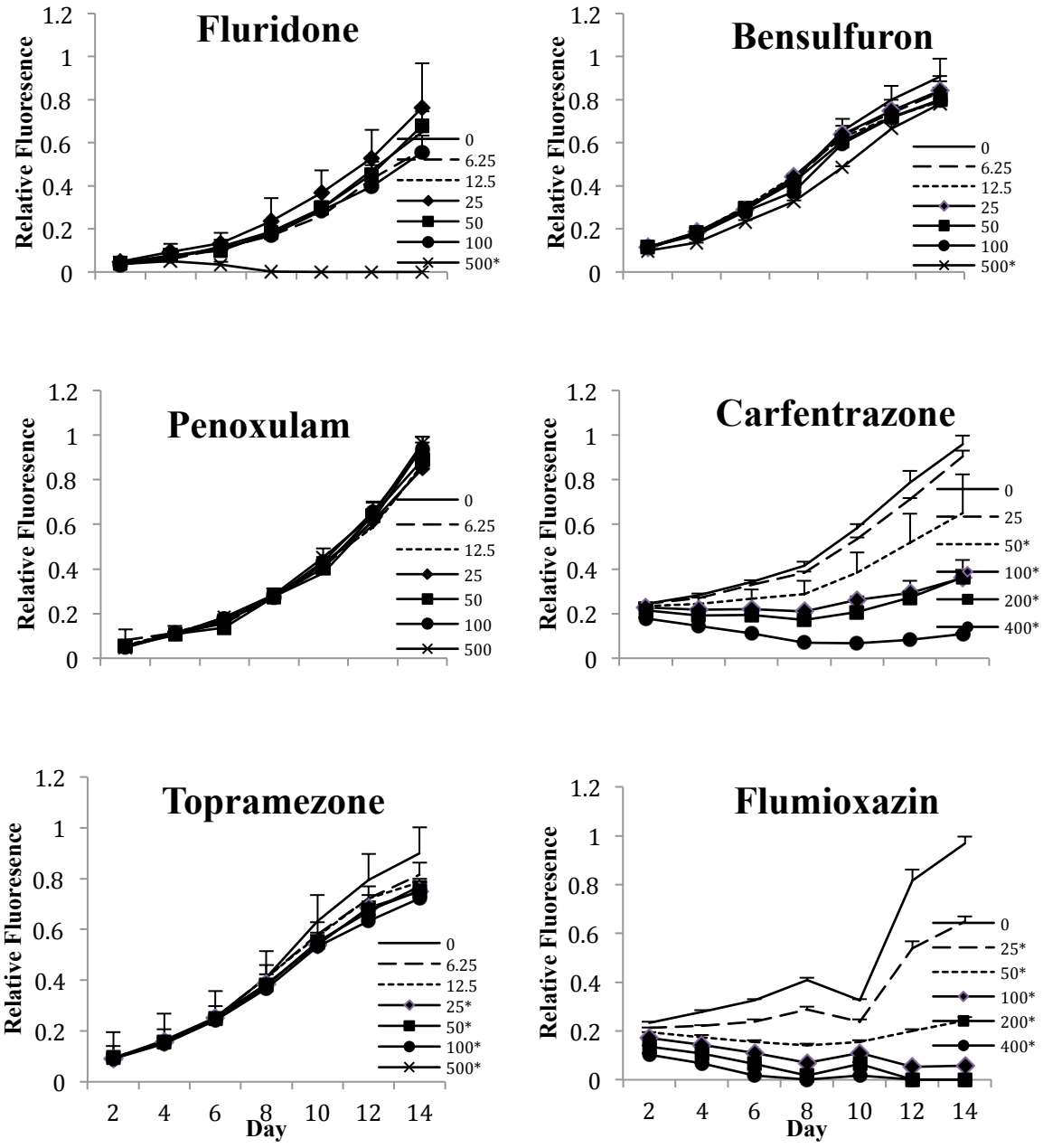

Figure 2.1: Relative fluorescence of *in-vivo* culture of *P. parvum* after a 14 day screening. Chemicals (A) fluridone, (B) bensulfuron, (C) penoxsulam, (D) carfentrazone, (E) topramezone, (F) flumioxazin at various recommended

concentrations. Data are averages of 3 replications and normalized. Error bars indicate + standard deviation.

2.3.2 Cove Monitoring

During the period preceding the start of our first experiment, *P. parvum* populations in the sample cove changed little, remaining at ~0.5 x10^6 cells liter^{-1}. Following our first in-lake experiment, *P. parvum* in the cove increased 5-fold, nearing typical bloom proportions and cove waters were toxic to fish (Figure 2.2). Prior to the start of our second experiment, cove waters were non-toxic and *P. parvum* populations were declining rapidly; then were nearly obliterated (0.1 x10^6 cells liter^{-1}) after the lake experienced a large inflow event that pushed a large amount of debris into our sample cove. Therefore, we refer to our experiments as pre-bloom (experiment 1) and post-bloom (experiment 2).

Total phytoplankton biomass (as chlorophyll *a*) exhibited a trend similar to that of *P. parvum*. Chlorophyll *a* samples taken weeks prior to the pre-bloom experiment increased to ~12 μg L^{-1} and then subsequently declined. After the pre-bloom experiment was over however, cove chlorophyll *a* levels increased to ~22 μg L^{-1} and then decreased to ~13 μg L^{-1} by the start of our post-bloom experiment. Thus, each experiment began with different cove chlorophyll a levels.

Zooplankton accumulated biomass until the *P. parvum* density peaked, after which zooplankton declined. Biovolume of Copepod adults (1.26x10^8 μm^3 L^{-1}, ~10.1 individuals L^{-1}), copepod nauplii (5.11x10^8 μm^3 L^{-1}, ~33.01 individuals L^{-1}), and rotifers (3.99 x10^7 μm^3 L^{-1}, ~67.15 individuals L^{-1}) all were increasing at the start of the pre-

bloom experiment. In contrast, at the start of the post-bloom experiment, biovolumes of Copepod adults (2.49 x10^7 $\mu m^3 L^{-1}$, ~7.31 individuals L^{-1}) and copepod nauplii (1.14 x10^8 $\mu m^3 L^{-1}$, ~123.19 individuals L^{-1}) and rotifer (6.17 x10^7 $\mu m^3 L^{-1}$, ~94.10 individuals L^{-1}) all were decreasing. Cladocera biomass peaked at 3.5 x 10^7 $\mu m^3 L^{-1}$, ~1.06 individuals L^{-1}, two weeks prior to the initiation of the pre-bloom experiment and continued this decreasing trend for the remainder of the sampling period. No cladocera were found in water samples from the sample cove at the start of the post-bloom experiment. Values for Most abiotic parameters followed predictable seasonal trends during the experimental period (Figure 2.3). Water temperature, for example, steadily increased from 7.8°C to 11°C at the start of the pre-bloom experiment (Feb.-Mar 2010) then increased further to 18.9°C at the start of the post-bloom experiment in April. Kwon et al. (2004) and Senseman (2007), reported that the half-life due to hydrolysis of flumioxazin was 16.4 h, 9.1h and 0.3h at pHs of 5, 7 and 9, respectively, indicating that very short flumioxazin exposures under high pH conditions may influence activity. The pH in our studies remained relatively stable at ~8 for both experiments. Prior to the pre-bloom experiment salinity decreased from 0.5 to 0.3 PSS in the cove. After that experiment, cove salinity increased to 0.7 PSS at the start of the post-bloom experiment. Similar to the trend described for temperature, light penetration steadily increased throughout the sampling period, from mid February to April 1. Inorganic nutrient levels in the cove were relatively stable prior to the pre-bloom experiment, but then both DIN (~45 μM) and SRP (~0.6 μM) began to decline to near zero, before increasing again to ~20 μM and ~0.3 μM. Thus, at the start of the post-bloom experiment, nutrient

concentrations were approximately ½ the levels measured at the start of the pre-bloom experiment.

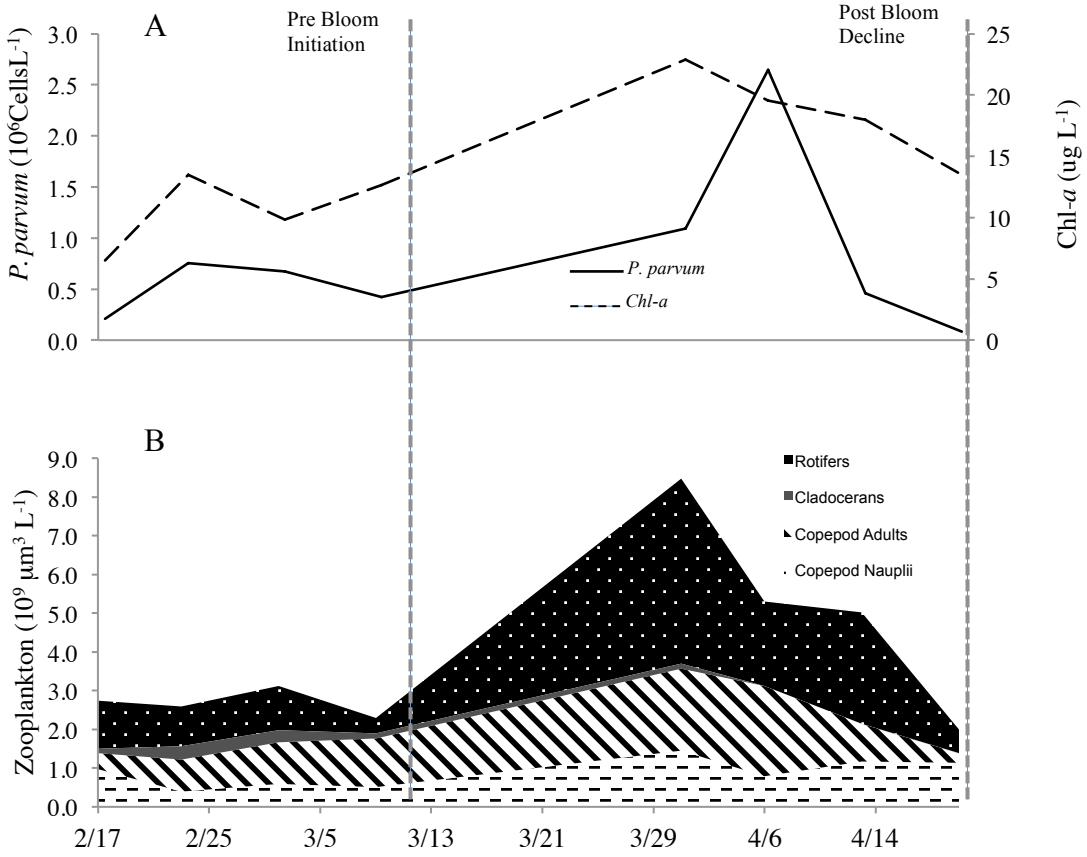

Figure 2.2: Weekly monitoring of biotic cove conditions. (A) *P. parvum* cell densities (solid line) and Chl-*a* concentration (broken line). (B) Zooplankton biovolume as represented by rotifers, cladocera, copepod adults, and copepod nauplii. Vertical broken grey lines represent start dates of the in-field pre-bloom initiation (3-9-2010) experiment and the post bloom decline experiment (4-20-2010)

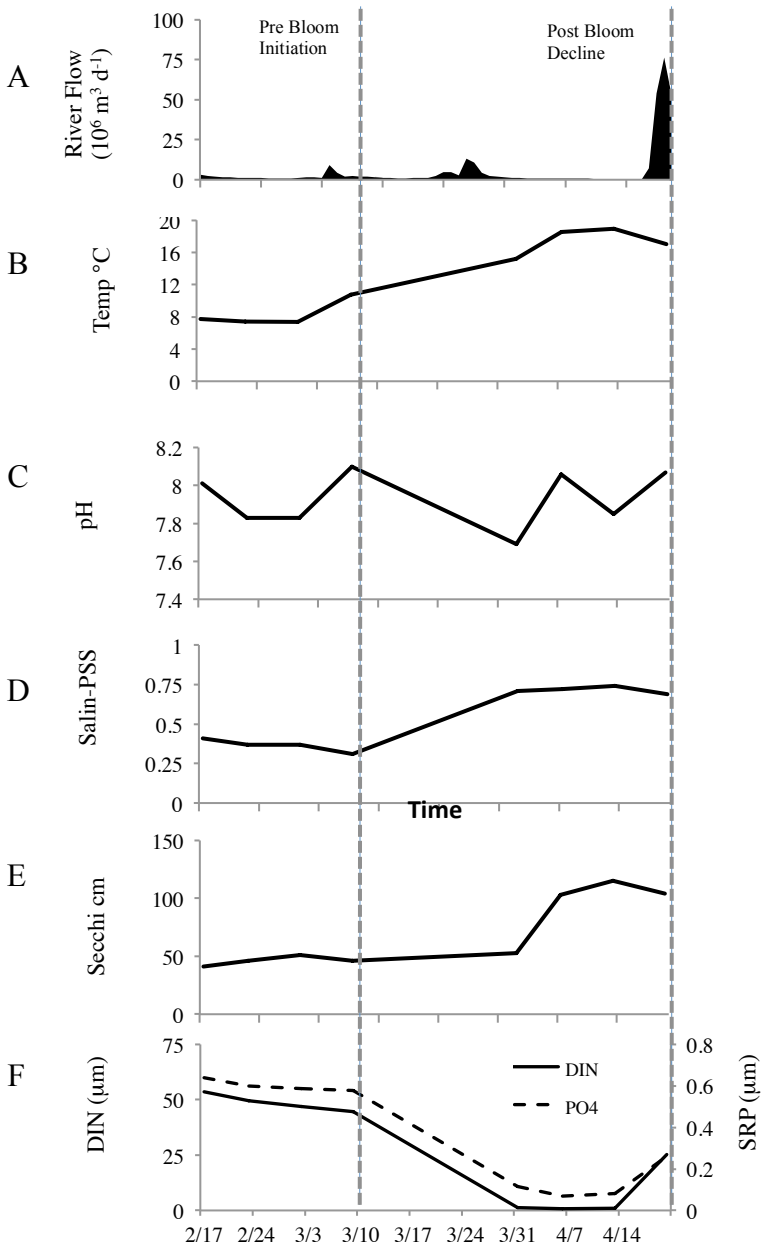

Figure 2.3: Weekly monitoring of abiotic cove conditions. (A) river flow, (B) temperature, (C) pH, (D) salinity, (E) light penetration, (F) nutrients

2.3.3 In-Lake Experiments

2.3.3.1 Pre-Bloom Initiation Experiment

Treatment effects were strong in the pre-bloom experiment. *P. parvum* population density declined after seven days in all treatments, with the decline being more pronounced in carboys with flumioxazin additions (Figure 2.4). At 200 μg/L, where the effect was strongest, *P. parvum* density was 96.4% lower than the control (p<0.01). On the other hand, total phytoplankton biomass increased in all carboys. The effects of flumioxazin additions were still negative, however, as significantly less chlorophyll *a* was measured in those treatments (ANOVA P<.0001). Inorganic nutrients showed opposite trends from phytoplankton, where declines in DIN and SRP from the initial condition were lessened with flumioxazin dosage.

A trend of decreasing adult copepod accumulation was observed with flumioxazin dosage, where in the extreme (200 μg/L) population decreased (Figure 2.5). Copepod nauplii and rotifers decreased in all carboys with flumioxazin, regardless of dosage. Cladocera abundance, on the other hand, increased in all carboys.

2.3.3.2 Post-Bloom Decline Experiment

Treatment effects were not as strong in the post-bloom experiment. After seven days, *P. parvum* density increased from initial cove conditions for all carboys with little distinguishable effect of increasing flumioxazin dosage (Figure 2.6). Similarly, total phytoplankton biomass showed little distinguishable trend with flumioxazin dosage.

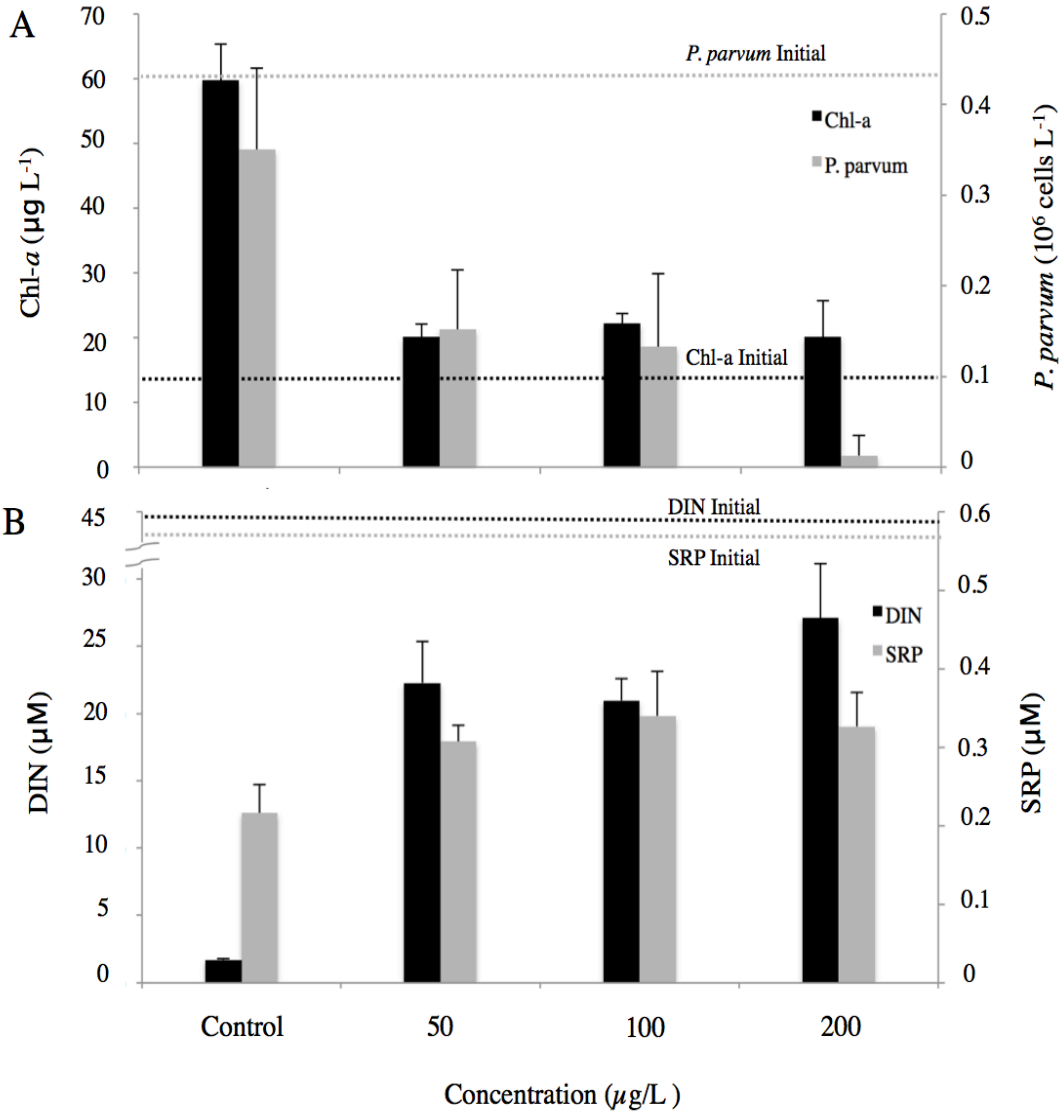

Figure 2.4: Pre-bloom initiation experimental effects on *P. parvum*, Chl-*a*, and nutrients. (A) *P. parvum,* and Chl-*a* after seven days, (B) nutrients: dissolved inorganic carbon and SRP for chemical concentrations of 0, 50, 100, and 200 µg/L. Data are averaged of 3 replications averaged + SD. Broken lines show initial concentrations for Chl-*a*, *P. parvum*, DIN and SRP.

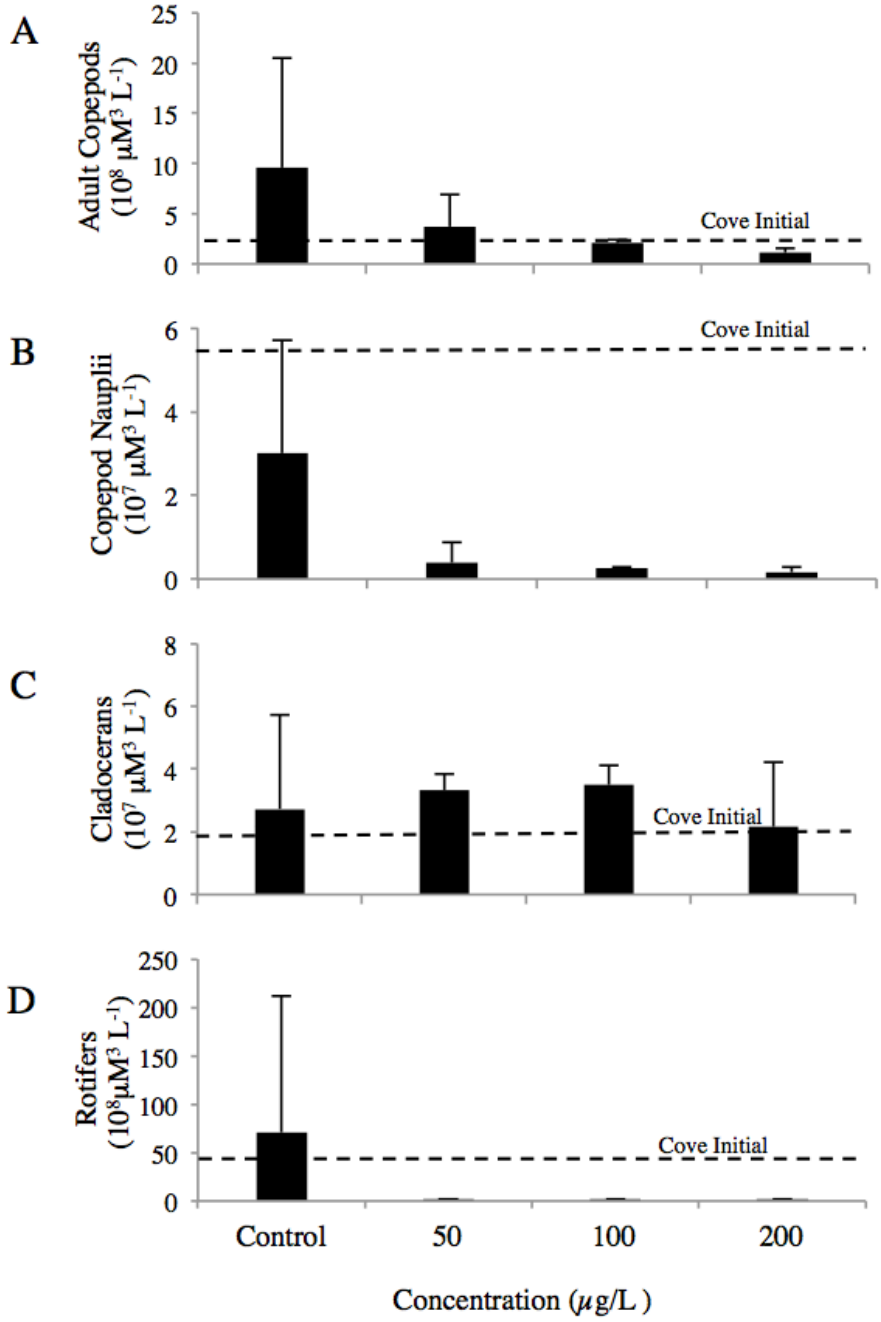

Figure 2.5: Pre-bloom initiation experimental effects on zooplankton taxa. Effects of flumioxazin on mean Biovolumes + SD for (A) adult copepod, (B) copepod nauplii, (C) cladoceran, (D) rotifer. Broken lines show initial biovolumes.

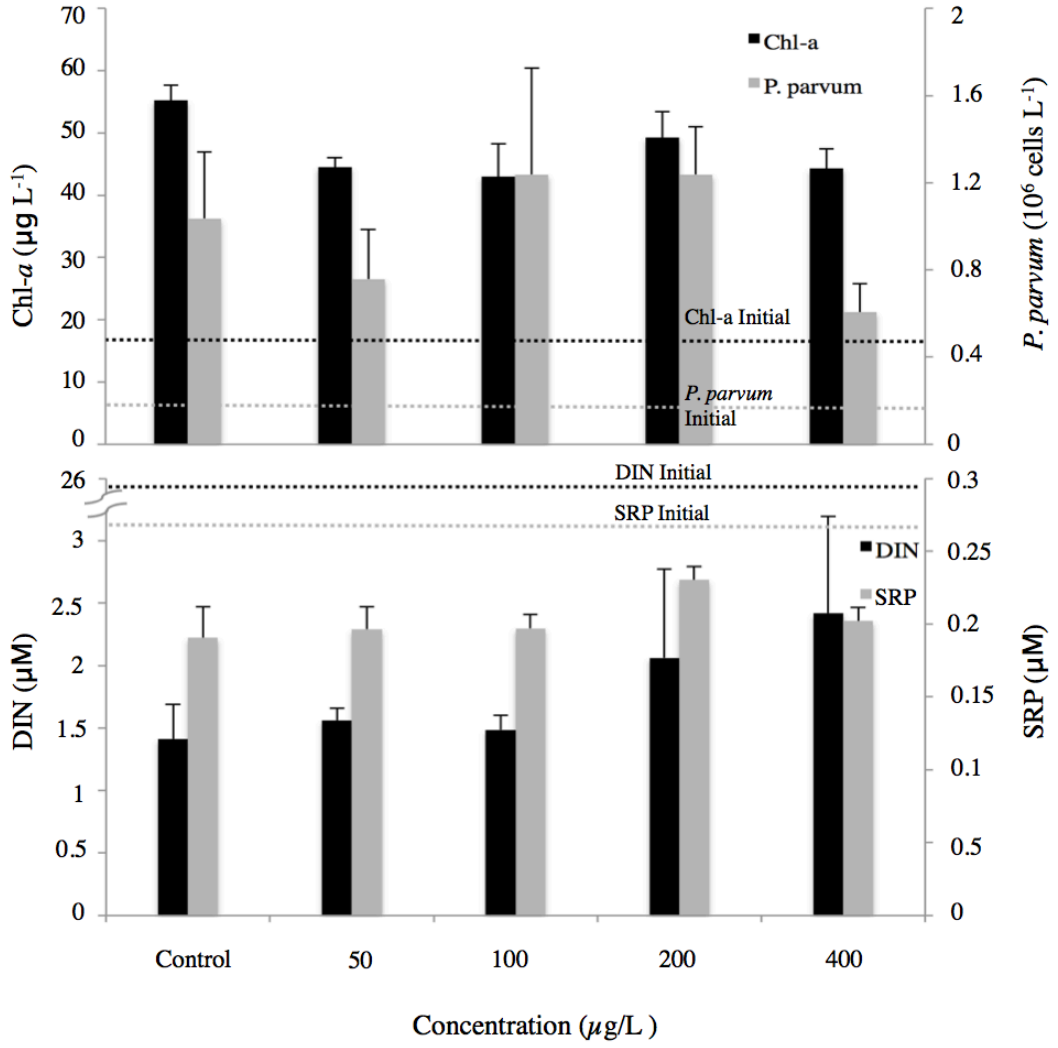

Figure 2.6: Post-bloom decline experimental effects on *P. parvum*, Chl-*a* and nutrients. Effects of flumioxazin on mean + SD for (A) *P. parvum,* and Chl-*a*, (B) nutrients: dissolved inorganic carbon and SRP for chemical concentrations of 0, 50, 100, 200, 400 µg/L. Broken lines show initial concentrations for Chl-*a*, *P. parvum*, DIN and SRP.

DIN and SRP decreased in all carboys from initial conditions during the seven-day experiment but showed a general increase in the nutrients with increasing concentration of flumioxazin.

Adult copepods again accumulated biomass from the initial conditions, but less so with increased flumioxazin dosage (p=0.006) (Figure 2.7). Copepod nauplii decreased in all carboys from the initial conditions, with the decrease being more pronounced at higher flumioxazin dosage (p=0.09). Cladocera and rotifers showed no trends with flumioxazin dosage.

2.4. Discussion

The herbicides screened in this research elicited different responses in *P. parvum* monocultures. The effects of the protoporphyrinogen oxidase (Protox) inhibitors carfentrazone and flumioxazin were most promising, with all showing dose responses. Of these, the rapid action of flumioxazin, the low dosage required to elicit a response, and rapid product degradation via pH-dependent hydrolysis suggest some appealing attributes as an algaecide of choice for *P. parvum* mitigation. The acetolactate synthase (ALS) inhibitors penoxsulam and bensulfuron were least effective in reducing *P. parvum* densities, and the response to topramezone was very weak. The phyotene desaturase (PDS) inhibitor fluridone was only effective at reducing *P. parvum* densities to zero at a dosage that is 10 to 20X greater than current rates used for invasive plant control.

The overall results, however suggest certain limitations to flumioxazin application. The mode of action for this herbicide involves protoporphyrinogen oxidase inhibition.

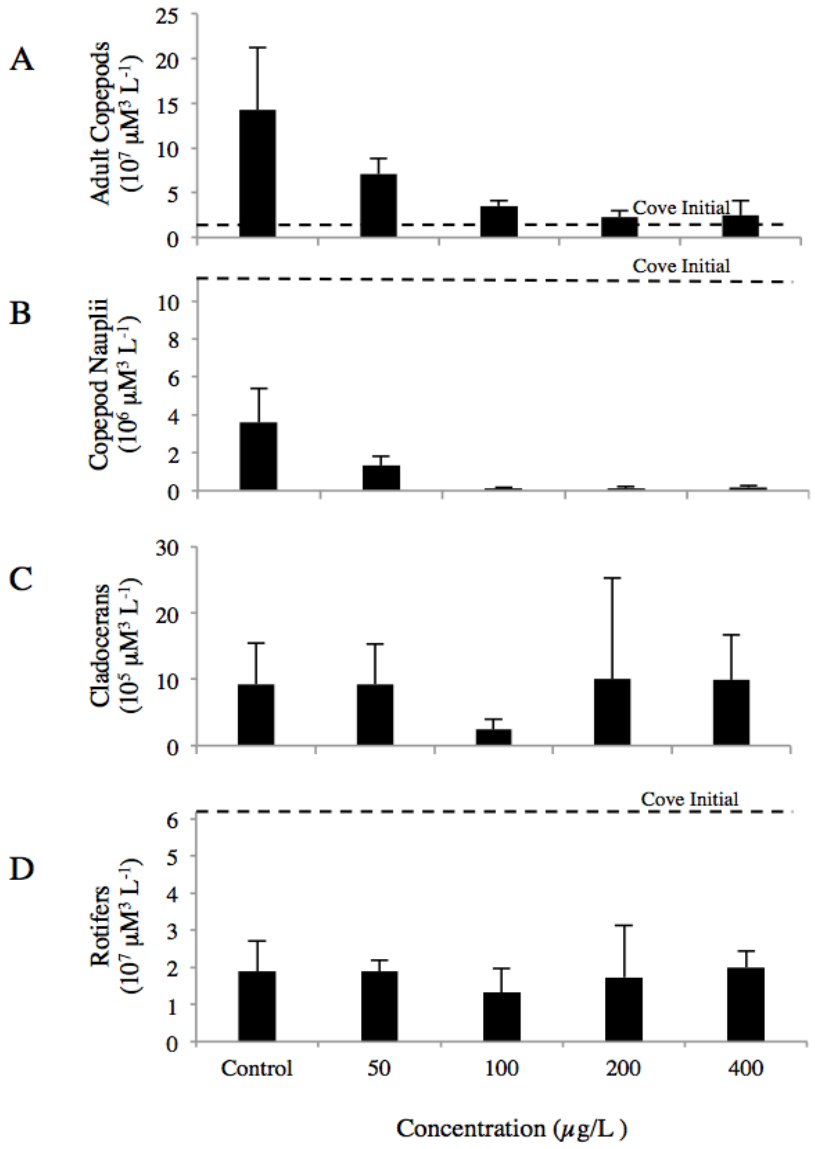

Figure 2.7: Post-bloom decline experimental effects on zooplankton taxa. Effects of flumioxazin on (A) adult copepod biovolume, (B) copepod nauplii biovolume, (C) cladoceran biovolume, (D) rotifer biovolume + SD. Broken lines show initial biovolumes.

The inhibition of this enzyme where light is present leads to lipid peroxidation of the cell's plasma membrane (Devine et al. 1993, Lee et al 1993, Hayes 2010). This dependency on light seems evident in our experiments. *P. parvum* population densities and chlorophyll *a* concentrations showed strong decreasing trends with increase in flumioxazin dosage in the pre-bloom experiment, but no effects in the post-bloom experiment. Recall, just prior to the post bloom experiment a large inflow event pushed excessive amounts of sediment and debris into our sampling area, thus the experimental carboys were filled with highly turbid waters. As a result, phytoplankton in the post-bloom experiment were exposed to a reduced amount of sunlight in comparison to the pre-bloom experiment and likely were growing at lower rates. This light dependent peroxidation was also observed by Mudge et al. (2012), where flumioxazin efficacy on *Hydrilla verticillata* increased with increasing light exposure. In addition, other studies have shown a relation between growth rates and enzyme turnover, and Protox inhibitors such as flumioxazin have been shown to be much more effective on small actively growing plants as opposed to large mature plants (Duke et al. 1991).

There are other considerations regarding flumioxazin addition, beyond efficacy for mitigating *P. parvum* blooms. For example, zooplankton herbivores can cause shifts in nutrient availability through stoichiometric processes such as digestion, metabolism and growth that alter nutrient recycling (Lehman, 1984, Sterner, 1989). Reductions of copepod:cladoceran ratios like those seen in our field experiments, will likely cause a species shift in dominant phytoplankton not only from grazer selection from remaining zooplankton, but changes in N:P recycling ratios as well. In the case of cladocera

dominance, a non-selective grazer with a fixed N:P stoichiometry of ~12 (Sterner 2002), application of flumioxazin may increase the evenness of phytoplankton species composition, and the ambient N:P may also increase, thereby creating a selective advantage for phytoplankton taxa , such as chlorophytes and diatoms optimized for that nutrient condition. On the other hand, cladocera are known to be sensitive to *P. parvum* toxins (Urena-Boeck 2008) and in a moderately toxic bloom where no treatment is applied, they may be succeeded by copepods, a selective grazer with a fixed N:P stoichiometry of ~35 (Sterner 2002). In this later hypothetical scenario, and assuming there is some degree of toxin resistance in plankton co-occurring with *P. parvum*, the evenness of phytoplankton species would likely decrease due to the selectivity of grazing along with the ambient N:P.

Another consideration involves the magnitude of inorganic nutrient concentrations. We can surmise that the differences observed in nutrients from the sample cove and carboys between the pre-bloom and post-bloom experiment were directly related to nutrient uptake by phytoplankton. During the pre-bloom initiation experiment, less *P. parvum* and total phytoplankton biomass allowed higher levels of DIN and SRP. During the post-bloom experiment, increased algal concentrations led to higher metabolic demand that decreased DIN and SRP. So in an event where flumioxazin proved efficacious, an increase in DIN and SRP also would result and be available to un-affected algae for growth. However, such increases in inorganic nutrients are cause for concern in downstream environments sensitive to eutrophication, especially marine systems, which tend to be N-limited.

From a management perspective, the application of flumioxazin may be an effective mitigation tool for *P. parvum* blooms, provided that light is not limiting or preventing its mode of action. The fast acting nature of flumioxazin is appealing from a manager's perspective as it may enable a rapid response to fish killing blooms. Additional research is needed, however, to better determine optimal application rates, timing, relations between cell density and efficacy, and influence of light and pH on efficacy of flumioxazin.

CHAPTER III

THE EFFECT OF FLUMIOXAZIN ON GROWTH, SURVIVAL AND FEED CONVERSION OF THE BLUEGILL SUNFISH, *Lepomis macrochirus*

3.1. Purpose

Bluegill sunfish (*Lepomis macrochirus*) is an important species in recreational fisheries. Originally native to the Mississippi River Drainage, *L macrochirus* now inhabits the entire United States and can be found worldwide (Welcomme, 1988). In the USA, *L. macrochirus* is considered a valuable primary forage fish for larger game species such as largemouth and smallmouth bass, and itself is also sought after for angling (Thomas et al. 2007). Therefore, *L. macrochirus* was a suitable candidate for a six-week experiment to evaluate the effect of the herbicide flumioxazin on growth, survival and feed conversion ratio.

3.2. Methods

3.2.1 Acclimation Period

This experiment was carried out at the Texas A&M Aquatic Research and Teaching Facility in College Station, TX. Prior to the initiation of the experiment, a fourteen-day period was allowed for disease free *L. macrochirus* (The Bait Barn Fisheries, Bryan TX) to acclimate to new 23-L tank conditions. During this period, water temperature remained ~22°C ± 1° with a pH of ~8.3 ± .1 while dissolved oxygen

remained ~8.0 mg L^{-1} ± 1 mg L^{-1}. Our ambient lighting conditions were set on a 12:12 day/night cycle. To allow adequate removal of wastes, water was recirculated at a rate of ~1 L min^{-1} with a residence time of 23 minutes and filtered through mechanical/biological media. Low-pressure electrical blowers provided adequate aeration though the use of air stones. To reach salinities typical of *P. parvum* bloom conditions, mixed Stock Salt (United Salt Corp) was added to reach a final salinity of four psu. Feeding was done once daily at 15:00 to apparent satiation with 1.6-mm floating pellets comprised of 44% protein (Rangen EXTR 450).

3.2.2 Screening Experiment

Twenty-one 23-L aquaria were utilized in our experiment to evaluate the effect of flumioxazin on the growth of *L. macrochirus*. Three tanks (triplicate) were randomly selected for each of five treatment concentrations of flumioxazin (25, 50, 100, 200 and 400 μg L^{-1}). Six tanks were randomly selected for our control treatments (no flumioxazin). Upon initiation, fish were graded by size, weighed in groups of 12 fish , all of which were similar size, and added to each tank.

The experimental design that was implemented had limitations. Operation of the single pump and filtration system fitted to 21 aquaria would have resulted in complete mixing of all treatment concentrations of flumioxazin. To prevent this, at the initiation of the experiment recirculating pumps were turned off and each aquarium became static except for oxygenation via air stones. After an additional 0.5-h acclimation period, packets of pre-weighed flumioxazin granules were added to achieve the designated concentrations. A subsequent 3-h period was allowed for the flumioxazin granules to

fully dissolve before fish were fed to apparent satiation. Feed ration was adjusted daily to minimize waste, and after each feeding the weight of feed in grams was recorded for each tank.

Previous monitoring of total ammonia nitrogen in densely stocked tanks with *L. macrochirus* indicated acute toxicity at levels of 1.3 mg L-1 (Umphres personal observation). Similar results were seen in Mayes et al. 1986 and Ruffier et al. 1981. To minimize stress caused by ammonia buildup and declining water quality, every seventh day a 100% water exchange was performed. To accomplish this, oxygen (~12 mg L^{-1}) was added to pre-mixed salt water (4 mg L^{-1}) in a secondary 950-L tank with pure liquid O2 (Airgas Inc.). Water from aquaria was then pumped out and replaced with new water. During this period of water exchange, fish from each tank were recounted and weighed as a group before being returned to their respective tank. Then, flumioxazin was again added following the methods previously described. Thus the treatment levels represent a static experimental design with weekly replacement of flumioxazin dosages.

Total nitrogen ammonia and dissolved oxygen were measured bi-weekly. Alkalinity, hardness, salinity and pH were measured weekly. Additionally, during our daily feeding period, fish were counted, and general swimming and feeding behavior (i.e. erratic fin movement, lethargy and gain or loss of appetite) was observed. Throughout the experiment, just prior to feeding, any dead fish were removed and weighed, and additional ammonia levels were measured and recorded for only those tanks in which fish died.

3.2.3 Statistical Analysis

Mean individual weights of fish across all three replicates of each treatment were analyzed using a general linear model (GLM) repeated measures ANOVA (JMP Pro 9) where treatment differences of the seven repeated measurements of weight were considered significant at $p<0.05$. Additionally, overall mean percent survival, percent total weight gain and feed conversion ratios of triplicate tanks for each treatment were analyzed using One-Way ANOVAs (JMP Pro 9).

3.3 Results

For the duration of this experiment, total ammonia nitrogen remained belowtoxic levels for *L. macrochirus* (mean=0.17 mg L^{-1} SD± 0.06). Moderately high levels of Alkalinity and Hardness were observed throughout the experiment (190.5 SD± 12.6, 691.5 SD± 23.3). Salinity remained at approximately 4.5 mg L^{-1} SD± 0.08. pH also remained stable at 8.31 SD± 0.04. During our experiment, dissolved oxygen, was highest at the start of each week due to the 100% water exchange with highly oxygenated water (12.0 ± 1 mg L^{-1}) but steadily decreased to a steady state at ~8.0 ± 1 mg L^{-1} by the second or third day.

L. macrochirus mean individual fish weights across all replicates per treatment increased throughout the experiment for all treatments (Figure 3.1). Repeated measures GLM showed no significant differences among treatments (p=0.11). In this study, fish growth and feeding were not affected by the flumioxazin dosage (Table 3.1) Total survival was relatively high (92%) and no significant difference was observed among overall treatment concentrations effects (p=0.83). Similarly, no statistically significant

differences in percentage of initial-weight gained (p=0.76) or feed conversion ratios (p=0.89) were observed among treatments. By visual observation, some *L. macrochirus* seemed more aggressive than others, with resulting mortality within some tanks.

Still, other factors must be considered. For example, problematic *P. parvum* blooms in Texas occur in winter and early spring (Baker et al. 2007). Typically, in nutrient rich lakes, algal species and planktonic invertebrates also undergo rapid growth during early spring (Sommer 1989), which is followed by predation on planktonic invertebrates by recently hatched juvenile herbivorous fish. The application of flumioxazin to waters to suppress or mitigate *P. parvum* blooms have indirect food-web effects that could influence growth of wild *L. macrochirus* and herbivorous fishes by decreasing the availability of planktonic prey (Umphres et al. 2012).

3.4. Discussion

Visual observations through the duration of the experiment revealed no apparent difference in behavior (i.e., erratic swimming, gill movement, aggression) of fish in tanks among control and treatment levels with flumioxazin. Additionally, the lack of statistical difference between treatments for percent survival, weight gain and feed conversion ratios suggest that weekly repeated exposure of the herbicide flumioxazin is not likely to cause detriment to these parameters for *L. macrochirus*, in short term applications in high pH waters (pH > 8.0). However, long-term effects on reproduction and recruitment were not carried out and no inferences can be made from the current study on their fate.

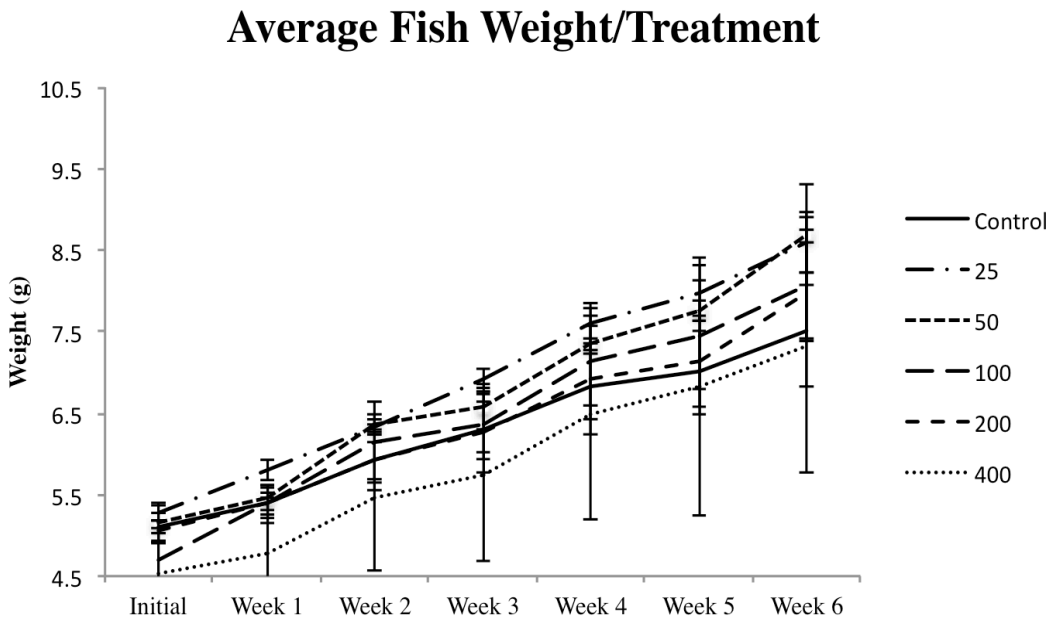

Figure 3.1. Average fish weight/treatment. Mean individual weight ± 1 SD across three replicates per treatment recorded weekly (25, 50, 100, 200 and 400 μg L^{-1}.) and control (no flumioxazin)

Table 3.1 Survival, Percent Weight Gain and Feed Conversion Ratio (FCR) of *L. macrochirus* across a gradient of concentrations of the herbicide flumioxazin

Treatment	Survival (%)	Weight Gain (%)	FCR (g fed/g gained)
Control	90.0	0.47	1.29
25 $\mu g/L^{-1}$	94.4	0.63	1.22
50 $\mu g/L^{-1}$	86.1	0.69	1.43
100 $\mu g/L^{-1}$	94.4	0.73	1.20
200 $\mu g/L^{-1}$	91.7	0.58	1.28
400 $\mu g/L^{-1}$	91.7	0.67	1.35
ANOVA			
P>F	0.83	0.76	0.89

Table 3.1. Survival, Mean Percent Weight Gain and Mean Feed Conversion Ratio (FCR) of *L. macrochirus* across a gradient of concentrations of flumioxazin. *p*-values calculated via One-Way ANOVA (JMP Pro 9). Standard Error calculated using a pooled estimate of error variance.

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In this experiment, observed mortalities appeared to be caused by agonistic territorial behavior among *L. macrochirus* within some aquaria. Not all aquaria experienced mortalities and fish in some aquaria appeared to coexist without contention regardless of experimental treatment. The establishment of social hierarchies by dominant *L. macrochirus* is well known (Medvick et al. 1987, Beitinger and Magnuson 1975) and the level of boldness and "personality" vary by individual (Wilson and Godin 2010). Similarly, the behavior of fish in the present experiment varied among individuals. Although no treatment-related declines in growth of *L. macrochirus* were observed, other factors that determine the health of an ecosystem also must be

The management of harmful invasive species is currently a popular subject and the options for mitigation of their effects on native species are not without potential trade-offs. The ability to target *P. parvum* prior to bloom formation and subsequent toxin production could provide a useful strategy in mitigating toxic impacts to gill breathing organisms. Nonetheless, careful consideration of ecological ramifications must be evaluated before the application of any chemical herbicide to the environment.

CHAPTER IV

CONCLUSIONS*

4.1 Summary

In the preceding experiments, the herbicide flumioxazin was proven to be most efficacious among the six herbicides screened on pure cultures of *P. parvum* in laboratory experiments. Flumioxazin also proved to be successful in reducing *P. parvum* abundance in the pre-bloom initiation experiments using in-lake mesocosms, although the chemical's toxic effects were observed for a variety of plankton taxa. In the post bloom decline experiment, flumioxazin showed little effect on all plankton except adult copepods. Results infer that the lack of significant effects in the post-bloom decline experiment was in part due to conditions of decreased light availability and slower growth rates of *P. parvum* , which would reduce the effect of protox inhibition by flumioxazin. Findings from these experiments suggest that flumioxazin could be used to inhibit toxic *P. parvum* bloom formation in small areas such as lake coves, which in turn may provide fish refuge during an ongoing bloom, as no adverse effects were observed on *L. macrochirus* in the pulsed experimental lab study.

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4.2 Management Recommendations

The findings in this research suggest that the control of *P. parvum* bloom formation may be feasible through the application of flumioxazin. However, due to the broad range of organisms affected by flumioxazin, managers should approach the bloom mitigation campaign with discretion. For example, seasonal succession of phytoplankton and zooplankton communities are influenced by factors such light, temperature, nutrient availability as well as many other physical, chemical and biotic factors (Reynolds1989). Herbicidal treatment with flumioxazin will likely affect many of these parameters as shown in this research causing concerns for bottom-up cascades and eutrophication downstream.

Additionally, the chemical products of flumioxazin hydrolysis, photolysis and metabolic breakdown include 7-fluoro-6 [(2-carboxyl-cyclohexenoyl)amino]-4-(2-popynyl)-1,4-benzoxazin-3(2H)-one (482-HA), 6-Amino-fluoro-4-(2-propynyl)-1,4-benzoxazin-3(2H)-one (APF),3,4,5,6-tetrahydrophthalic acid (THPA), and 3,4,5,6-tetrahydrophthalic acid anhydride (TPA). An EPA study indicates some potential for these degradates to accumulate in groundwater (Federoff et al. 2003). However, due of the lack of research on these particular chemicals, their toxicity and ultimate environmental fate is unknown, making risk assessment difficult at this time.

Nevertheless, flumioxazin appears to be a useful tool against the *P. parvum's* toxic effects on inland lakes and rivers. This research suggests that herbicidal application during pre-log or log growth phase of *P. parvum* would be most efficacious especially in waters with low turbidity and pH < 8.5.

4.3 Future Research Considerations

The next steps in this research should include experiments examining ecosystem level effects over extended durations. These would help identify more comprehensive approaches to fisheries management where *P. parvum* blooms are prevalent. For example, experiments in small impoundments or reservoirs where natural assemblages of flora and fauna are present would be useful for understanding the extent that energy transfer is affected by the reduction in plankton biomass. Furthermore, there is a need for knowledge of the fate of the final degradation products of flumioxazin and their toxicity.

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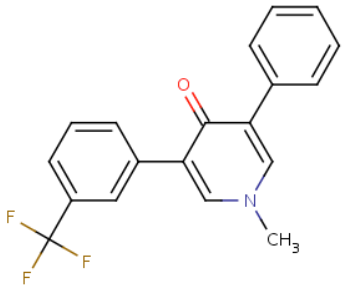
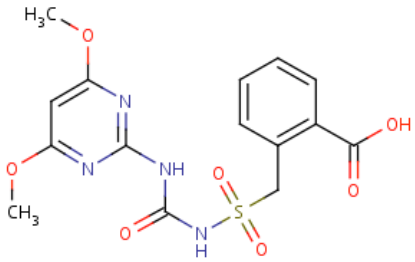
APPENDIX A

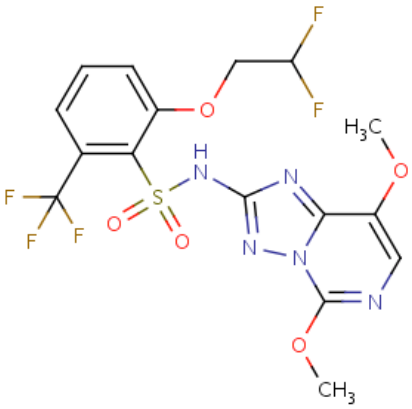
HERBICIDE CHEMICAL STRUCTURE



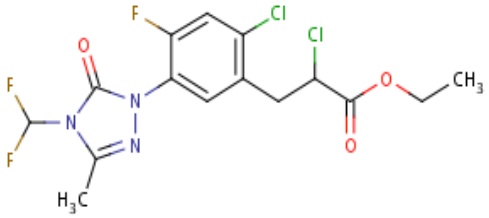
Fluridone



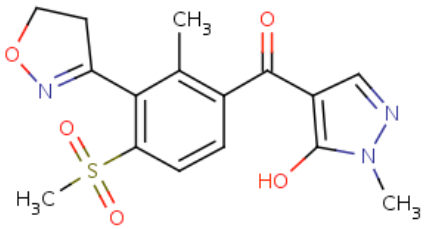
Bensulfuron



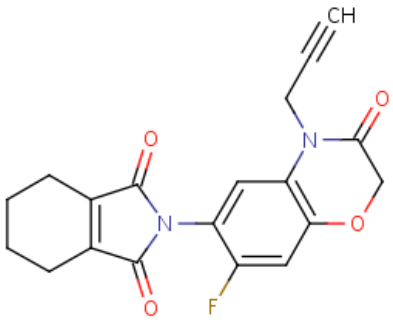
Penoxsulam



Carfentrazone-ethyl



Topramezone



Flumioxazin

(Judson et al. 2012)