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Responses of Target and Non-target Species to Algaecide Exposures

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RESPONSES OF TARGET AND NON-TARGET SPECIES TO ALGAECIDE EXPOSURES

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ABSTRACT

Laboratory experiments are often used to predict the responses of target and non-target species to chemical exposures in the field. In the first two experiments of this thesis, a rigorous evaluation of six algal viability measures was conducted. A definitive evaluation of the algal response measures was conducted using heat treatment to create known live: dead cell suspensions. Results from the response measures were compared to the known viability of the cell suspensions to determine their variance and accuracy. Copper-based algaecides were then used as a more realistic exposure to test the algal viability measures. When algal viability measures had a monotonic response curve, EC_{50s} and potency slopes were calculated to compare the relative sensitivities of *Planktothrix agadhii* and *Pseudokirchneriella subcapitata* to copper sulfate pentahydrate and Cutrine-Ultra. Lastly, experiments were conducted in the laboratory to predict the responses of target and non-target organisms in Lay Lake to an ongoing algaecide treatment and a potential alternative algaecide treatment for *Lyngbya wollei* (*L. wollei*). Lay Lake is a man-made reservoir in central Alabama that has experienced noxious *L. wollei* growths for the past 10 years. Results from the first two experiments highlight the advantages, limitations and utility of some algal viability measures. In the last experiment, no measureable copper residuals were present and no adverse effects to benthic invertebrates (*Hyalella azteca* and *Chironomus dilutus*) were discerned from sediments after 10 years of periodic algaecide applications. An effective

alternative treatment for *L. wollei* from Lay Lake was predicted that may enhance the margin of safety for non-target species in the field.

DEDICATION

I would like to dedicate this thesis to my family and friends. Thanks especially to my parents who have supported me throughout my education and taught me that happiness and education are paramount. A final thanks to my boyfriend, Michael Eck for always being available to talk about the ups and downs, his support and finding ways to make my experience at Clemson easier through last minute flights.

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CHAPTER ONE

Introduction

Laboratory toxicity testing is commonly used to predict the responses of organisms in the field to constituents of concern (Lewis 1995, Doig and Liber 2010, Ankley and Villeneuve 2006); however, issues arise regarding the reliability, accuracy and utility of results from standardized toxicity testing (Lewis and Hamm, 1986; Lewis, 1995; Chapman, 2000; Janssen and Heijerick, 2003). The experiments in this thesis provide a rigorous evaluation of algal viability measures and use target and non-target species responses in the laboratory to make predictions about potential risks associated with algaecide applications in the field.

Cell density and chlorophyll *a* concentration are commonly used algal response measures although they may be inadequate in certain situations (Nyholm, 1985; Chao and Chen, 2001). Algae have a number of responses to exposures of chemicals including loss in cell membrane integrity or decreased respiratory rates (Gibson 1972). Traditional response measures (e.g. cell density and chlorophyll *a* concentration) would be incapable of discerning these differences. The viability measures assessed in "Evaluation of six measures for algal (*Microcystis aeruginosa*, *Planktothrix agardhii* and *Pseudokirchneriella subcapitata*) viability" included cell density, vital staining (neutral red [NR]), mortal staining (erythrosin b [EB]), chlorophyll *a* concentration, pheophytin *a* concentration and respiration (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium formazan [INT formazan] production). These response measures assess different aspects of algal viability.

Cell density measures viable cells, staining can be used to assess cell membrane integrity, chlorophyll *a* and pheophytin *a* concentrations are a measure of photosynthesis and INT formazan absorbance can be used to estimate respiration. Algal viability measures were assessed using known live: dead cell suspensions following heat treatment.

Copper-based algaecides were then used to test these algal viability measures following a more realistic exposure in "Responses of *Planktothrix agardhii* and *Pseudokirchneriella subcapitata* to copper sulfate ($CuSO_4 \bullet 5H_2O$) and a chelated copper compound (Cutrine®-Ultra)". Copper based algaecides have been used for more than a century to reduce densities of noxious algal growths (Moore and Kellerman 1905). Currently, numerous copper-based compounds are available for use as algaecides with different chelators and adjuvant packages. Following applications of copper based algaecides, algae may manifest physiological changes, which may be dependent on the algaecide form or concentration (Gibson 1972; Stauber and Florence 1987; Perales-Vela 2007). Accurate and precise algal viability measures with the resolution to discern physiological changes in algae can be used to calculate EC_{50s} and potency slopes for measuring differences in the relative sensitivities of algae to copper based algaecides.

The ultimate goal of toxicity testing is to predict responses of organisms in the field. In "Responses of *Lyngbya wollei* to algaecide exposures and a risk characterization associated with their use," laboratory toxicity testing was used to identify an effective algaecide and aid management decisions for Lay Lake.

Lay Lake is a man made reservoir, in central Alabama. Since 2004, selected coves in the reservoir have required algaecide treatment because *L. wollei* growths interfered with the intended uses of the water body. A copper based algaecide in conjunction with a peroxide algaecide have been applied periodically for the past 10 years. Because copper has a lithic biogeochemical cycle, questions about the potential risks to non-target benthic invertebrates were addressed. Questions also arose concerning the continued efficacy of the ongoing algaecide treatment because of the potential for an algal assemblage shift from long-term algaecide use (e.g. 10 years). Laboratory experiments were used to identify potential alternative treatments for *L. wollei.* The responses of fish to aqueous algaecide exposures from two alternative treatments were then assessed to understand the relative sensitivity of *Pimephales promelas* to the two alternatives. Laboratory experimentation was conducted to make site-specific predictions and recommendations regarding the potential risk of the ongoing treatment and an alternative treatment identified in the study.

Objectives

"Evaluation of six measures for algal (*Microcystis aeruginosa*, *Planktothrix agardhii* and *Pseudokirchneriella subcapitata*) viability"

The overall objective of this experiment was to rigorously test the ability of cell density, a vital stain (neutral red), a mortal stain (erythrosin b), chlorophyll *a* concentration, pheophytin *a* concentration and a respiration measurement (INT formazan absorbance) to discern known viable and non-viable cell ratios. Specific objectives of this study were: 1) to measure properties of individual cells in known populations of viable and non-viable cells using cell density, a vital stain (neutral red) and a mortal stain (erythrosin b); 2) to measure chlorophyll *a* concentration, pheophytin *a* concentration and respiration activity (INT formazan absorbance) as aggregate measures of known populations; and 3) to evaluate the accuracy, precision and utility of these measurements.

"Responses of *Planktothrix agardhii* and *Pseudokirchneriella subcapitata* to copper sulfate ($CuSO_4 \bullet 5H_2O$) and a chelated copper compound (Cutrine®-Ultra)"

The overall objective of this research was to evaluate the utility of a stain and biochemical indicators of algal viability for measuring the relative health of two freshwater algae (*P.* agardhii and *P.* subcapitata) exposed to two copper based algaecides. The specific objectives of this research were to compare and contrast: 1) individual (cell densities, and erythrosin b stained cells) and aggregate (chlorophyll *a* and pheophytin *a* concentrations and INT formazan absorbance) algal viability measures for *P. agardhii* and *P. subcapitata* exposed to a copper salt ($CuSO_4 \bullet 5H_2O$) and a chelated copper compound (Cutrine®-Ultra) every 24h over the course of

7days, and 2) the relative sensitivities of *P. agardhii* and *P. subcapitata* to the two copper compounds in 96h static laboratory toxicity tests.

"Responses of *Lyngbya wollei* to algaecide exposures and a risk characterization associated with their use"

The overall objective of this research was to compare results from the current algaecide treatment versus alternative algaecide treatments. The specific objectives were to 1) assess responses of *Hyalella azteca* and *Chironomus dilutus* in terms of survival to potential copper-based algaecide residuals in sediments following 9 years of periodic algaecide treatments (Phycomycin®-SCP and Algimycin®- PWF); 2) measure the responses in terms of wet weight, chlorophyll *a* concentration and visual observations of *L. wollei* to candidate algaecides (Cutrine®-Ultra, Phycomycin®-SCP, Clearigate®, Algimycin®- PWF) and an adjuvant (Cide-Kick II) and determine an effective algaecide treatment for Lay Lake; and 3) measure and compare survival of *Pimephales promelas* to exposures of the effective algaecide treatment and the treatment of Phycomycin®-SCP and Algimycin®- PWF.

Organization of Thesis

This thesis is arranged in subsequent chapters intended for publication in peer-reviewed journals. Therefore, chapters two through four are written and formatted for a

specific journal, and some of the introductory information and materials and methods were repeated. Chapter two has been submitted for peer-review in *Ecotoxicology and Environmental Safety*; chapter three has been submitted for peer-review in *Water, Air, and Soil Pollution*; and chapter four is targeted for submission to the journal *Ecotoxicology and Environmental Safety*.

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CHAPTER TWO

Evaluation of six measures for algal (*Microcystis aeruginosa*, *Planktothrix agardhii* and *Pseudokirchneriella subcapitata*) viability

Abstract

Reliable viability measures are needed to discern responses of algae to a variety of exposures including pesticides, personal care products and complex mixtures such as runoff and effluents. To definitively evaluate six algal viability measures, algae were heat-treated to produce known live:dead cell ratios. Cultures of two prokaryotic algae (*Microcystis aeruginosa and Planktothrix agardhii*) and a eukaryotic alga (*Pseudokirchneriella subcapitata*) were boiled for five minutes and mixed after cooling with untreated cultures to produced suspensions of 0, 25, 50, 75 and 100% live algal cells. Optical microscopy was used to assess the viability of algae on a cell-by-cell basis by measuring cell density, uptake of a vital stain (neutral red) and exclusion of a mortal stain (erythrosin b). Aggregate measures of algal cell viability included chlorophyll *a* concentrations, pheophytin *a* concentrations and respiration (measured as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium formazan absorbance (INT)). Cell densities, erythrosin b stained cells and chlorophyll *a* concentrations correlated with viable *M. aeruginosa, P agardhii* and *P. subcapitata* cells (R^2 = 0.97-0.78, 0.98-0.85 and 0.99-0.93 respectively). Pheophytin *a* concentrations and neutral red stained cells did not correlate with viable algae (R^2 = 0.41-0.01 and 0.15-0.03 respectively). For INT formazan absorbance, 50, 75 and 100% viable algae had greater variances and did not strongly correlate (R^2 = 0.75-

0.54). This result was likely confounded by respiration associated with resident bacteria. Three of the six methods provided accurate and precise information regarding the viability of both prokaryotic and eukaryotic algae. These methods have a relatively low initial expense and can be used widely.

Introduction

Responses of algae to potentially phytotoxic exposures have been studied in the laboratory since the early 1900s for a variety of reasons (Lewis, 1995). Some laboratory experiments were initiated to evaluate potential effects of complex mixtures such as industrial effluents and agricultural runoff (Chapman, 2000). Other experiments measured effects of commercial chemicals that may enter aquatic systems such as pharmaceutical and personal care products (Cleuvers, 2003) as well as pesticides (algaecides and herbicides) (Lewis, 1990; Murray-Gulde et al., 2002). And other experiments were designed to assess the potential for site waters to support algal biomass (Miller et al., 1978; Shoaf, 1981).

Questions have arisen regarding the accuracy, precision and utility of results from standardized algal toxicity tests conducted in the laboratory (Lewis and Hamm, 1986; Lewis, 1995; Chapman, 2000; Janssen and Heijerick, 2003). Algal toxicity experiments should provide repeatable results that predict responses of algae in aquatic environments to potentially phytotoxic chemicals. There are concerns regarding the environmental relevance of these tests since results are impacted by the test species selected, culture medium, test conditions, and measures of effects used (Lewis, 1995). This study focused on measures of algal viability. Traditional algal viability measures (cell density and chlorophyll *a* concentration) are often inadequate response endpoints to accurately reflect responses to exposures (Nyholm, 1985; Chao and Chen, 2001). For example, there are uncertainties interpreting some morphological and physiological responses of

algae in cell density measurements as algae may be deformed, decrease in cell size, or cease reproduction in response to exposures to metals or phytotoxic chemicals (Nyholm, 1985; Mares et al., 1997; Chao and Chen, 2001). In addition, chlorophyll *a* concentrations may overestimate the viability of algae because of interferences by other pigments (e.g. pheophytins) (Rai, 1980; APHA, 2005).

The viability measures evaluated in the present study included cell density, vital staining (neutral red [NR]), mortal staining (erythrosin b [EB]), chlorophyll *a* concentration, pheophytin *a* concentration and respiration (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium formazan [INT formazan] production). A rigorous evaluation of the six algal viability measures was conducted by formulating known ratios of viable and non-viable algae in a controlled laboratory experiment. If these measures are precise and accurate in this controlled experiment, then they may be useful for algal toxicity testing.

The selection criteria for the six viability measures evaluated in this study included relative cost and ease of implementation. Each algal viability measure uses a light microscope or spectrophotometer, instruments that are commonly available in laboratories. Cell density, vital staining and mortal staining measure the viability of individual cells when coupled with light microscopy. For the individual measures, determinations of viable and non-viable algae are made on a cell-by-cell basis. The methods for measuring chlorophyll *a,* pheophytin *a,* and respiration aggregate cells with a concentration step prior to analysis and determinations of viable or non-viable algae are based on the aggregate measurement. Individual cell measures and

aggregate measures should lead to the same conclusions regarding the effects of exposures.

Cell density provides an estimate of viable algal cells in a sample or volume of water. Neutral red and erythrosin b are organic stains used to measure algal cell membrane integrity (Reynolds et al., 1973; Krause et al., 1984; Markelova, 2000; Zetsche and Meysman, 2012). Neutral red may stain cytoplasm or diffuse into the tonoplast of viable eukaryotic cells staining the vacuole (Reynolds et al., 1978; Dubrovsky et al., 2006). Erythrosin b will diffuse into cells that have lost the capacity to maintain membrane integrity (Krause et al., 1984). Algae contain chlorophyll *a*, an essential pigment for photosynthesis, and an inactive degradation product of chlorophyll *a*, pheophytin *a* is found in damaged and dying cells (Rai, 1980; Louda et al. 1998). Cells that contain more chlorophyll *a* than pheophytin *a* are assumed to be viable. Cells with more pheophytin *a* than chlorophyll *a* lack the ability to photosynthesize and eventually become non-viable. Algae must also respire to remain viable, and INT formazan can be used to estimate this. In viable algae, INT (pale yellow) is reduced to INT formazan (red) by the electron transport system for respiration. INT formazan can be quantified using a spectrophotometer (Packard, 1971).

Algae selected for the initial evaluation of these response endpoints included two prokaryotes (*Microcystis aeruginosa* (Kützing) and *Planktothrix agardhii* (Gomont)) and one eukaryote (*Pseudokirchneriella subcapitata* (Korshikov)). *M. aeruginosa* and *P. agardhii* are widely distributed and often produce microcystin (a

hepatotoxin) that may cause liver failure as well as tumor promotion (WHO, 2003). These two potential toxin producers can become problematic when total microcystin concentrations exceed 1ug/L (WHO, 2003). *M. aeruginosa* as well as *P. subcapitata* are commonly used in algal toxicity tests (Lewis, 1995).

Response measures were used to evaluate a series of viable and nonviable algal suspensions. The overall objective of this experiment was to rigorously test the ability of cell density, a vital stain (neutral red), a mortal stain (erythrosin b), chlorophyll *a* concentration, pheophytin *a* concentration and a respiration measurement (INT formazan absorbance) to discern known viable and non-viable cell ratios. Specific objectives of this study were: 1) to measure properties of individual cells in known populations of viable and non-viable cells using cell density, a vital stain (neutral red) and a mortal stain (erythrosin b); 2) to measure chlorophyll *a* concentration, pheophytin *a* concentration and respiration activity (INT formazan absorbance) as aggregate measures of known populations; and 3) to evaluate the accuracy, precision and utility of these measurements.

Materials and methods

2.1. Algal cultures

M. aeruginosa, *P. agardhii* and *P. subcapitata* were cultured in modified BG-11 medium (UTEX) at 21°C with a 18:6 h light: dark cycle. Illumination was provided by cool-white fluorescent bulbs (Residential Ecolux 40 W, GE) at 1980-3340 LUX. *P. agardhii* was collected from Grand Lake St. Marys in Lima, Ohio, and *P. subcapitata* (UTEX 1648) and *M. aeruginosa* (UTEX 2385) were obtained from University of Texas culture collection (UTEX) in Austin, Texas. All algae were sampled during the logarithmic growth phase (OD_{730} = 0.2 for *P. agardhii* and *M. aeruginosa* and OD_{730} = 1.0 for *P. subcapitata*) measured using a Spectra Max M_2 spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.2. Preparation of known viable algae ratios

Viable cells were boiled for 5 minutes to prepare non-viable cells, and this treatment was sufficient to eliminate regrowth for two weeks. Heat-treated algae and viable algae were mixed to produce algal suspensions containing 0, 25, 50, 75 and 100% viable cells (Sato et al., 2004).

2.3. Individual algal viability measures

Cell densities were determined using a Leitz Wetzlar Dialux 20 light microscope (Leitz USA Scopes, Paramount, California) and an Improved Neubauer Hemocytometer at 250x magnification (Rodgers et al., 2010). *P. agardhii* cell densities were estimated by counting the number of trichomes per 0.2 mm^2 gridded area on the hemocytometer and multiplied by the number of cells (12 cells) spanning this gridded area.

Neutral red (NR; 0.1% w/v) was prepared in NANOpure® water (Barnstead, Thermo Fisher Scientific Inc.) (Reynolds et al., 1978). NR solution (0.04 mL) was added to 1 mL of the algal treatments with a staining duration of 1 h. One mL of 0.25 % (w/v) EB dissolved in phosphate buffered saline was added to 1mL of algal suspension and stained for 15 min. Cells were enumerated to 100 total cells and calculated as a ratio of stained cells to total cells.

2.4. Aggregate algal viability measures

Chlorophyll *a* and pheophytin *a* concentrations were analyzed using a modified spectrophotometric method (APHA, 2005). Acidification of chlorophyll *a* was extended to 20 min to allow for complete acid hydrolysis of chlorophyll *a* to pheophytin *a.* Absorbance values were converted to chlorophyll *a* and pheophytin *a* concentrations using a linear regression calculated from chlorophyll *a* and pheophytin *a* standards (C6144 Sigma).

INT was analyzed using a modified method from Packard (1971). Nitrocellulose filters (0.45 μ m pore size) were substituted for glass-fiber filters, and the final dilution step was excluded. Absorbance was determined using a Spectra Max M_2 spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.5. Statistical analyses

Three subsamples were collected from each algal treatment (0, 25, 50, 75 and 100% live algal cells) for evaluation of the algal viability measures, and the results were averaged. Precision is a measurement of the variability of multiple results and was measured using ANOVA and Tukey's multiple comparison tests with the Statistical

Analysis System ($\alpha = 0.05$) (APHA 2005; SAS 9.2 2010). Tukey's test was used to discern significant differences among algal treatments (0, 25, 50, 75 and 100% live algal cells). Simple linear regression analyses were used to determine relationships between results from viability measures and 0, 25, 50, 75 and 100% viable cells for the three algae (SAS 9.2 2010). Coefficients of correlation were used to measure the accuracy of these viability parameters or the closeness of measured values to their true value (APHA 2005).

Results

3.1 Individual algal viability measures

Cell densities of *M. aeruginosa, P. agardhii* and *P. subcapitata* were positively correlated with percentages of live algal cells, while erythrosin b stained cells were negatively correlated (Figs. 2.1 and 2.2). The ratios of NR stained cells did not correlate with percent viable algae (Fig. 2.3). Results from statistical analyses yielded low R^2 values for neutral red stained cells and high R^2 values for cell densities and erythrosin b stained cells (Table. 2.1). For cell density, the coefficient of correlation was highest for *P. subcapitata* (R^2=0.97). For erythrosin b staining, *P. subcapitata* and *M. aeruginosa* both had high R^2 values (0.98 and 0.96 respectively). For *P. subcapitata*, significant differences could be determined between all algal treatments using cell density and erythrosin b stained cells. Significant differences among 0, 25, 50, 75 and 100% living *M. aeruginosa* cells was determined using erythrosin b staining.

3.2 Aggregate algal viability measures

Results from chlorophyll *a* concentrations demonstrated a positive linear relationship with percentages of viable algal cells (Fig. 2.4). No strong correlation was observed between pheophytin *a* concentration and viable algae ratios (Fig. 2.5). INT fomazan absorbance demonstrated a positive non-linear relationship with increasing algal viability (Fig. 2.6). Regression analyses yielded R^2 values for chlorophyll *a* concentrations, pheophytin *a* concentrations and INT absorbances (Table. 2.2). Using Tukey's test, chlorophyll *a* concentrations distinguished each algal treatment (0, 25, 50, 75 and 100% live algal cells) for the three algae studied. Pheophytin *a* concentrations

cannot be used to distinguish viable algae ratios. INT absorbances can distinguish between 0, 25 and higher viable algae percentages, but was not useful for discerning differences among 50, 75 and 100% viable algal cells.

Discussion

Chlorophyll *a* concentrations and cell densities are commonly used algal response measures for laboratory evaluations of potentially phytotoxic elements, compounds or mixtures (Vocke et al., 1980; Chao and Chen, 2001; Murray-Gulde, 2002; Boudreau et al., 2003). Other measures may be unavailable to laboratories because of upfront equipment expenses and are not often used. Flow cytometers ($15,000-50,000) and fluorescent microscopes ($7,500-60,000 (personal communication Martin Microscope Company)) are relatively costly and because of this, not available for use in many laboratories. The response measures evaluated in this study were selected for their simplicity and ease of use. The results obtained in this study contributed to understanding the accuracy, precision and utility of the six response measures. These accuracy and precision data can aid in selection of appropriate response measures for algal studies.

For algal viability measurements to be accurate, live:dead parameters must be capable of measuring continuous data (e.g. percent viable cells). Viability measures with sufficient resolution to discern 0, 25, 50, 75 and 100% viable algae can be used to obtain required information from the dataset (e.g. predict toxicities of compounds, measure responses of algae following a phytotoxic exposure and/or measure responses of algae to an exposure of less than EC100). Information such as the no observed effects concentration (NOEC), lowest observed effects concentration (LOEC) and effective concentration in which half or all of the organisms respond (EC50 and EC100 respectively) are valuable for making risk-based decisions concerning aquatic

environments (Dobbins et al. 2010). However, decisions based on information gained from algal viability measures must be tempered with an understanding of the strengths, weaknesses and limitations of each method.

Advantages and caveats of the six algal viability measures assessed in this study were evident in individual and aggregate measures. Individual measures (cell density and staining in this study) have the specificity to distinguish between algal genera or species. Light microscopy provided the magnification and resolution necessary to distinguish the alga of interest from other organisms visible in the sample and allowed for an assessment of viability on a cell-by-cell basis. Aggregate measures [i.e. chlorophyll *a* concentration, pheophytin *a* concentration and respiration (INT formazan absorbance), for this study] are generic because they yield a single value for photosynthetic or respiring organisms in a sample. Using aggregate measures, responses of a particular algal cell or taxon are not discernable.

Cell density as an individual measure obtained using a counting chamber and light microscopy can have advantages and disadvantages in some situations. Considerable training is initially required for investigators to define salient features to permit identification of organisms (Culverhouse, 1993). Cell densities can be difficult to enumerate with algae that are motile (Southard, 2005), colonial (Joung et al. 2006), or in filaments/trichomes (Ernst et al. 2006). In this experiment, precision of cell density measurements declined with *P. agardhii* at 25, 50 and 75 % viable algae due to the inherent difficulty enumerating algae in trichomes (Fig. 2.1). Differentiating viable from non-viable cells can be challenging. Following a phytotoxic exposure, physical changes

occur in cells, and interpreting these physical changes for cell density measurements is problematic (Chao and Chen, 2001). Cell stains (erythrosin b and neutral red) can assist in interpretation of these cellular changes following phytotoxic exposures.

The decline in precision for erythrosin b staining of *P. agardhii* at 25 and 50% was likely due to methodological imprecision counting cells in trichomes using a hemocytometer and light microscopy (Fig. 2.2). Cells stained bright red with erythrosin b were easily distinguished from cells that remained unstained. Using eight different stains and twenty-one algal taxa in newly inoculated cultures and "long-term cultures", Markelova et al. (2000) also found that erythrosin b stained cells were easily discerned from non-stained cells.

As a vital stain, neutral red was not useful for measuring the viability of individual cells because cells stained indiscriminately and became shrunken and deformed. The inability to discern stained from non-stained cells using neutral red resulted in large variability and a low R^2 value (0.02) (Fig. 2.3). Reynolds et al. (1978) reported that results using neutral red were unreliable after exposing eight species of algae to chlorine-produced oxidants. In the Reynolds et al. (1978) study, seven of the eight algal species took up the vital stain, however, the stain quickly leached from the cells. In this study, neutral red staining was not a reliable measure of algal viability because of arbitrary staining of cells.

Chlorophyll *a* concentration as an aggregate measure had sufficient resolution to discern differences among percentages of viable algae for *M. aeruginosa, P. agardhii* and *P. subcapitata.* For this study, algal cultures were dominated by one species (>95%).

Difficulties may arise measuring chlorophyll *a* concentrations for assemblages or mixed cultures. Weber (1973) mentioned that mass to chlorophyll *a* concentration ratio can vary greatly (by 1,042%) for assemblages of algae likely due to variations in species composition, yield of chlorophyll *a* on extraction as well as environmental conditions (i.e. light intensity, nutrients, and/or temperature; Collins and Weber, 1978). Given the variability in chlorophyll *a* concentrations between algal species as well as with environmental conditions, chlorophyll *a* measurements for algal viability will likely be reliable for unialgal laboratory cultures, cultures dominated by one alga, and experiments where the response of an algal assemblage is desired.

Chlorophyll degradation products include chlorophyllides, pheophorbides and pheophytins (Louda et al. 1998). The chlorophyll *a* degradation product, pheophytin *a,* is a commonly used pigment for algal viability studies because it is relatively stable (Rai, 1980; Louda et al. 1998). In this study, pheophytin *a* concentrations were unreliable as an algal viability measure because variances for the three algae studied were large and significant differences could not be discerned (Fig. 2.5). Calculations to convert acidified chlorophyll *a* to pheophytin *a* are based on the assumption that viable algae will have more chlorophyll *a* than pheophytin *a*, therefore the ratio (chlorophyll *a*: pheophytin *a*) will be approximately 1.7 (APHA, 2005). Based on this assumption, algal assemblages that are non-viable will have a ratio of 1 (APHA, 2005). However, this was not confirmed in the present study. This assumption must be valid for pheophytin *a* concentrations calculated from acidified chlorophyll *a* concentrations to be reliable. A factor affecting this ratio may be degradation of pheophytin *a* to non-fluorescent

degradation products (Rehnberg et al. 1982). Chlorophyll degradation products initially maintain sufficient structural integrity to either fluoresce or absorb light (Louda et al. 1998). Subsequently, the degradation products are structurally modified to the extent that they are colorless or do not fluoresce (Louda et al. 1998). If pheophytin *a* is decomposing to non-fluorescent products at the same rate that chlorophyll *a* is degrading, the ratio would remain the same regardless of algal viability.

INT formazan production declined in reliability at higher percentages of viable algae (50 and 75% viable algae). Variability in respiration measurements was likely due to decomposition of dead algae by resident bacteria (Goecke et al. 2013). In experimental situations where more than one species is present, an aggregate measure such as INT formazan absorbance is non-selective and cannot distinguish the species responsible for respiratory activity (Zimmermann et al. 1978).

Ultimately, selection of algal response measures is guided by the hypothesis at hand. If the response of an algal assemblage or an axenic culture is of interest, aggregate measures of cell viability will be sufficient. If differentiating the response of one alga among an assemblage of other organisms is of interest, individual measures (e.g. cell density or erythrosin b staining) will be useful. This study utilized heat-treatment to elicit a known algal response for definitive assessment of the viability measures. Each measure used in this study involved different measures of algal viability (cell density=presence of viable cells, staining=cell membrane integrity, chlorophyll *a* and pheophytin *a* = photosynthesis and INT formazan absorbance =respiration). In other studies, the

specificity of the stimulus (modes of action) should be considered prior to determination of appropriate algal response endpoints.

Conclusions

Using known ratios of viable to non-viable algae, this study provided a rigorous evaluation of six viability measures for algal assays. Cell density, erythrosin b stained cells and chlorophyll *a* concentration were useful response endpoints, while neutral red stained cells and pheophytin *a* concentration were not. Cell density and erythrosin b stained cells have the greatest precision when determining the viability of unicellular algae. These techniques have resolution to distinguish between algal taxa and would be useful for discerning responses of a genus from other algae in mixed cultures, natural assemblages as well as laboratory cultures. Chlorophyll *a* concentrations will give accurate results in unialgal cultures or cultures dominated by one algal species. Chlorophyll *a* concentrations can be used as a response endpoint along with microscopic observations of algal assemblages in the sample as algal dynamics may change following an exposure. Respiration measured as INT formazan absorbance may be useful in axenic cultures. Selection of appropriate response measures could also depend on the stimuli. If the mode of action of a stimulus is known to impact the photosynthetic apparatus in an algal cell, changes in chlorophyll *a* concentrations may be informative. If a stimulus disrupts cell membrane integrity, erythrosin b may be useful in staining the cells impacted by this stimulus. If impaired respiration is a mode of action, INT formazan absorbance may be able to discern a response. If there are ambiguities related to the mode of action of the stimulus or multiple mechanisms are involved, an approach involving multiple response measures of multiple algae will provide

valuable information regarding cell membrane integrity, respiration and photosynthesis of algae in response to an exposure.

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Table 2.1: Coefficients of correlation for *M. aerginosa, P. agardhii* and *P. subcapitata* in known viable cell ratios for cell densities, neutral red and erythrosin b stained cells.

Algae	Individual Algal Viability Measures		
	Cell Density	Percent Neutral Red Stained Cells	Percent Erythrosin b Stained Cells
M. aerginosa	0.91	0.04	0.98
P. agardhii	0.78	0.15	0.85
P. subcapitata	0.97	0.03	0.96

Table 2.2: Coefficients of correlation for *M. aerginosa, P. agardhii* and *P. subcapitata* in known viable cell ratios for chlorophyll *a* concentrations, pheophytin *a* concentrations and INT formazan absorbances.

Algae	Aggregate Algal Viability Measures		
	Chlorophyll *a* concentration	Pheophytin *a* concentration	INT formazan absorbance
M. aerginosa	0.99	0.22	0.54
P. agardhii	0.99	0.01	0.75
P. subcapitata	0.97	0.41	0.65

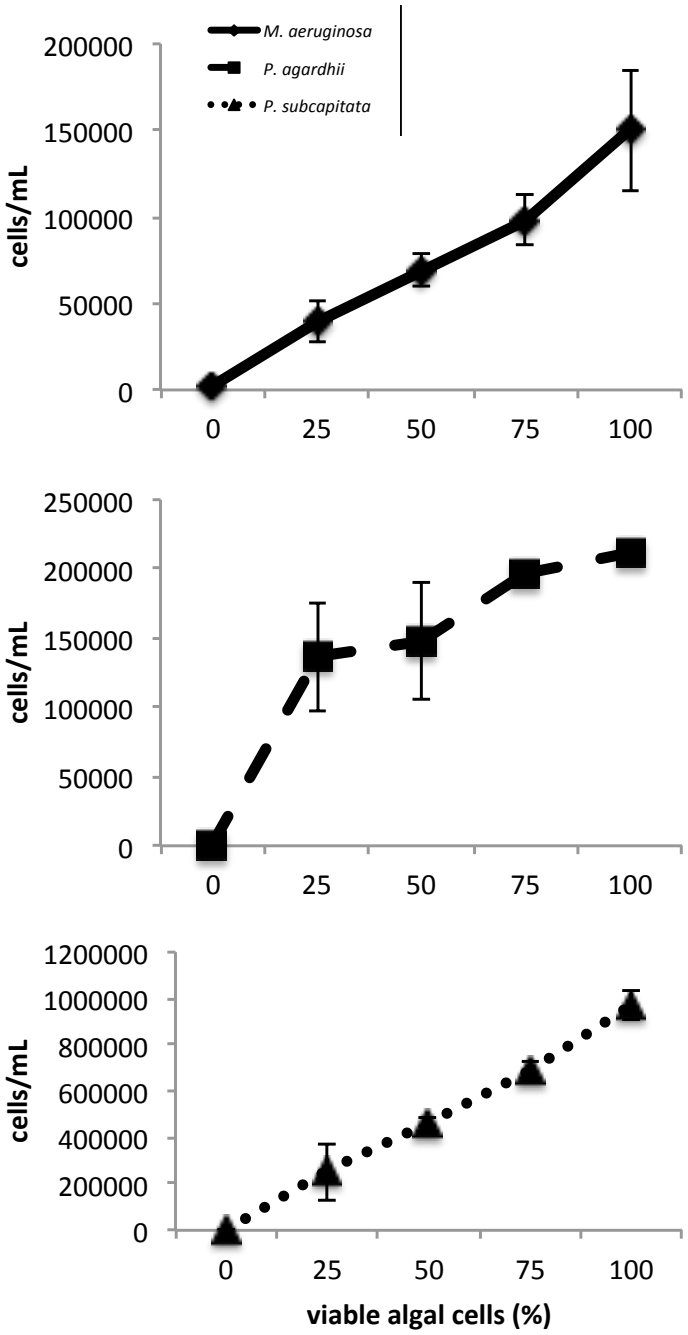

Figure 2.1: Mean cell densities of *M. aeruginosa*, *P. agardhii* and *P. subcapitata* in known viable cell ratios. Error bars represent +/- 1 standard deviation. (n=3)

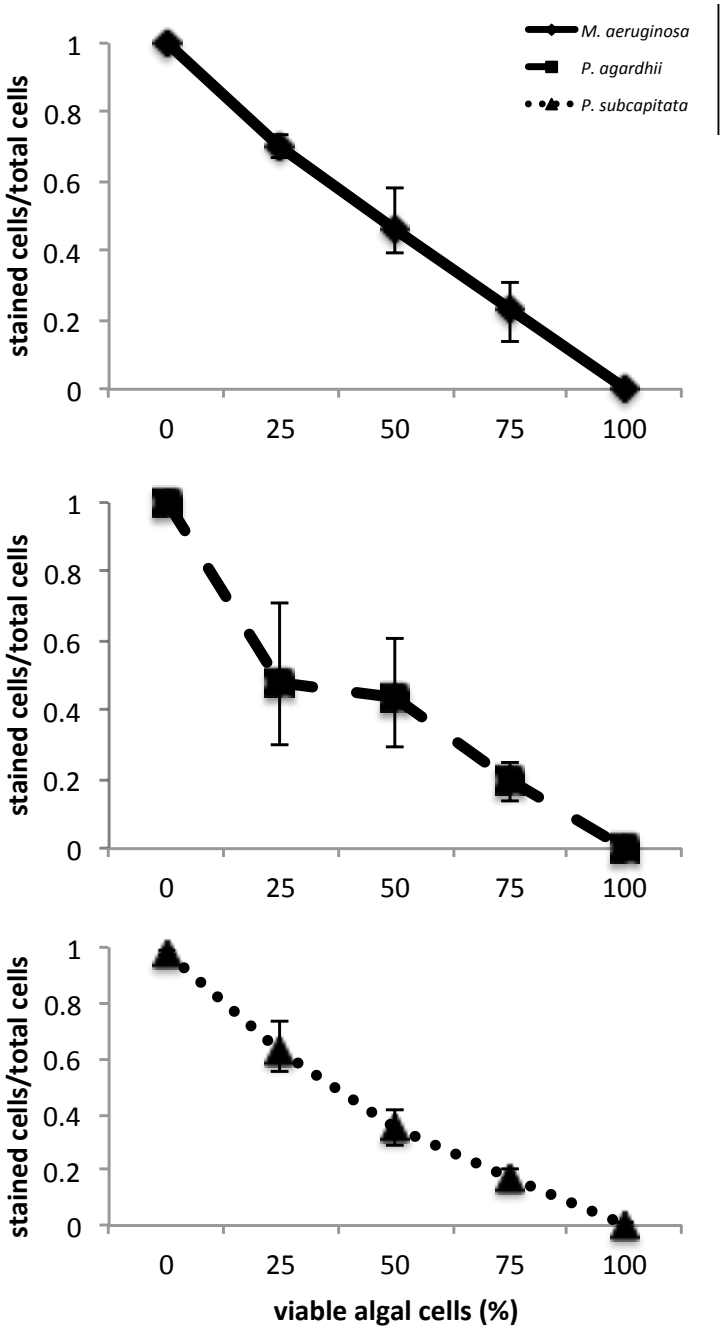

Figure 2.2: Mean erythrosin b stained *M. aeruginosa*, *P. agardhii* and *P. subcapitata* cells in known viable cell ratios. Error bars represent minimum and maximum values. (n=3)

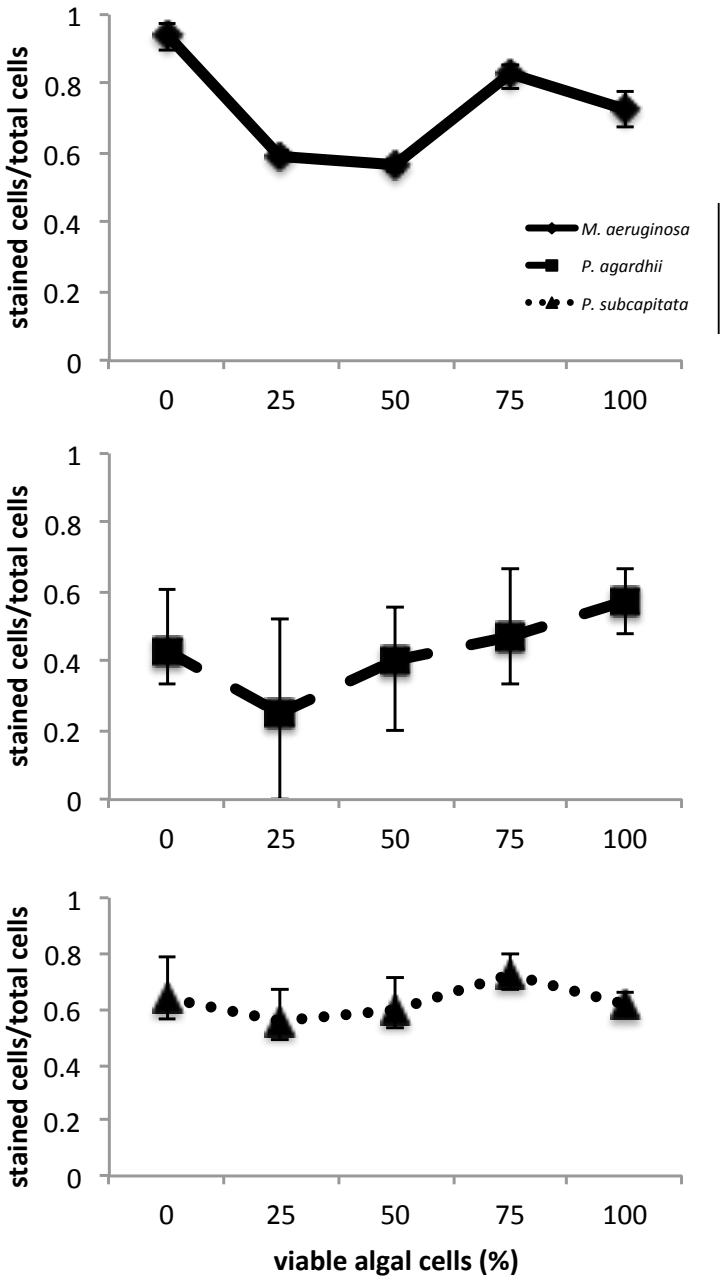

Figure 2.3: Mean neutral red stained *M. aeruginosa*, *P. agardhii* and *P. subcapitata* cells in known viable cell ratios. Error bars represent minimum and maximum values. (n=3)

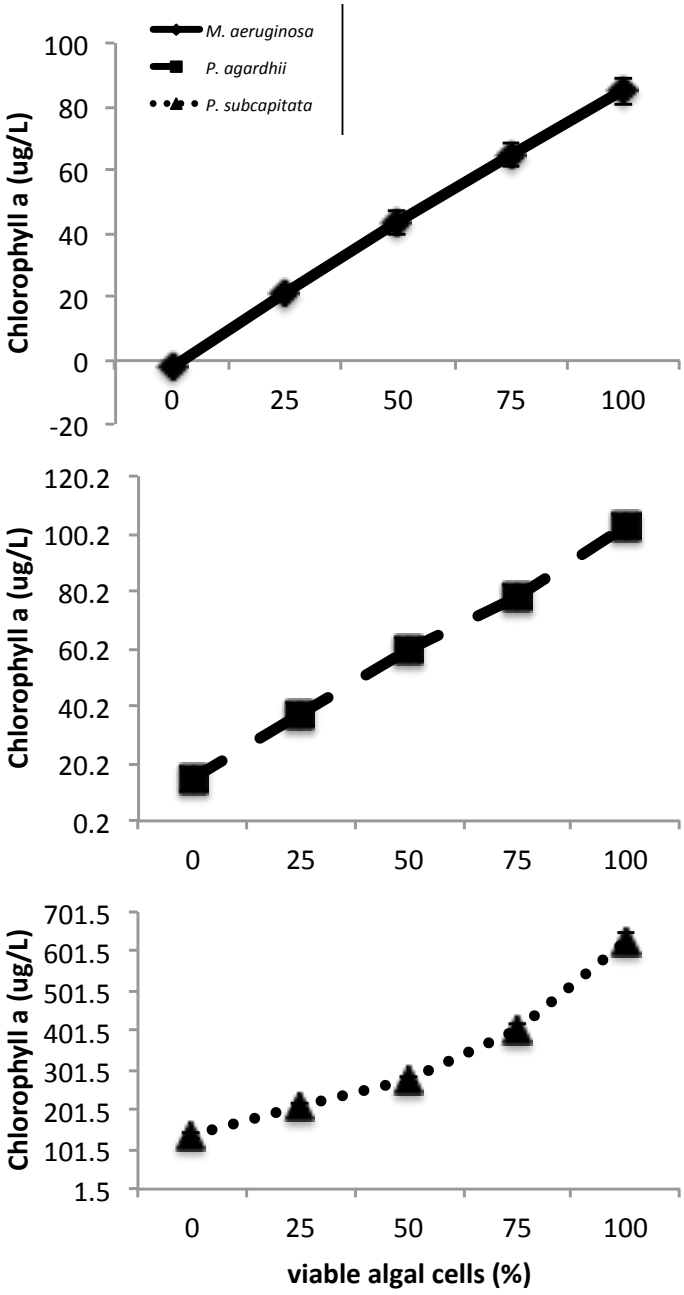

Figure 2.4: Mean chlorophyll *a* concentrations of *M. aeruginosa*, *P. agardhii* and *P. subcapitata* in known viable cell ratios. Error bars represent +/- 1 standard deviation. (n=3)

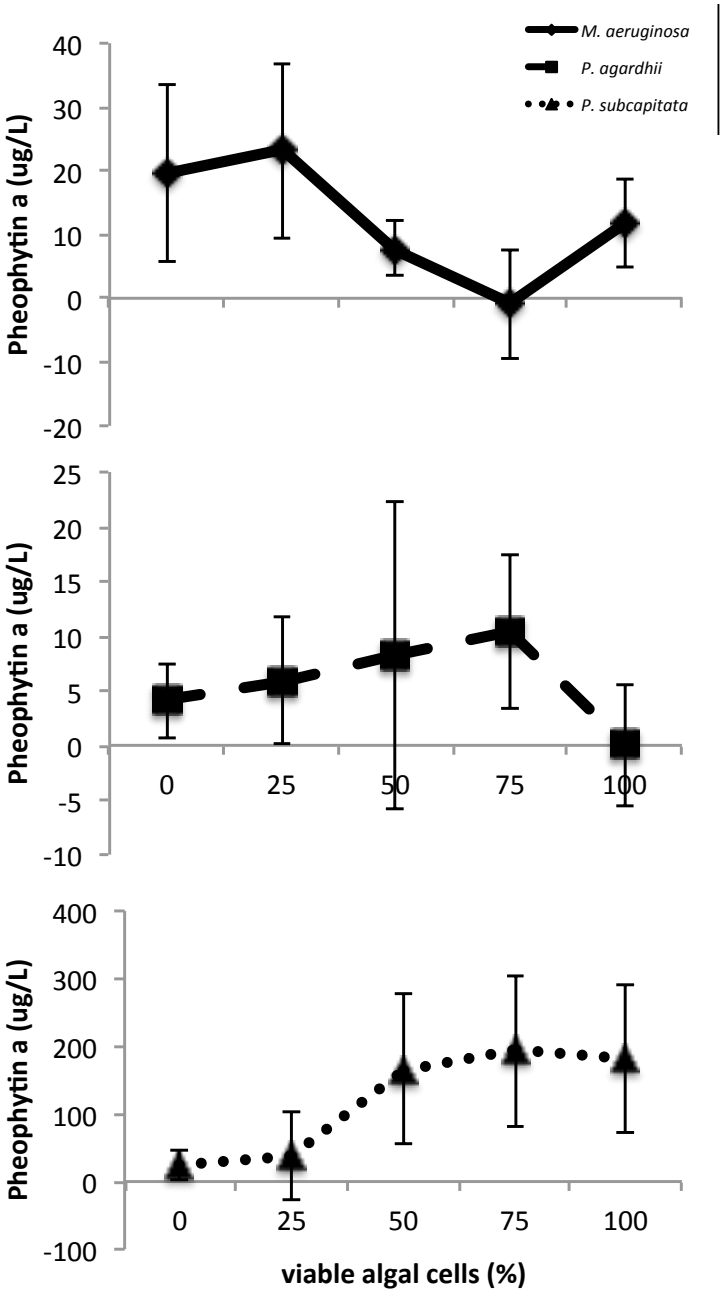

Figure 2.5: Mean pheophytin *a* concentrations of *M. aeruginosa*, *P. agardhii* and *P. subcapitata* in known viable cell ratios. Error bars represent +/- 1 standard deviation. (n=3)

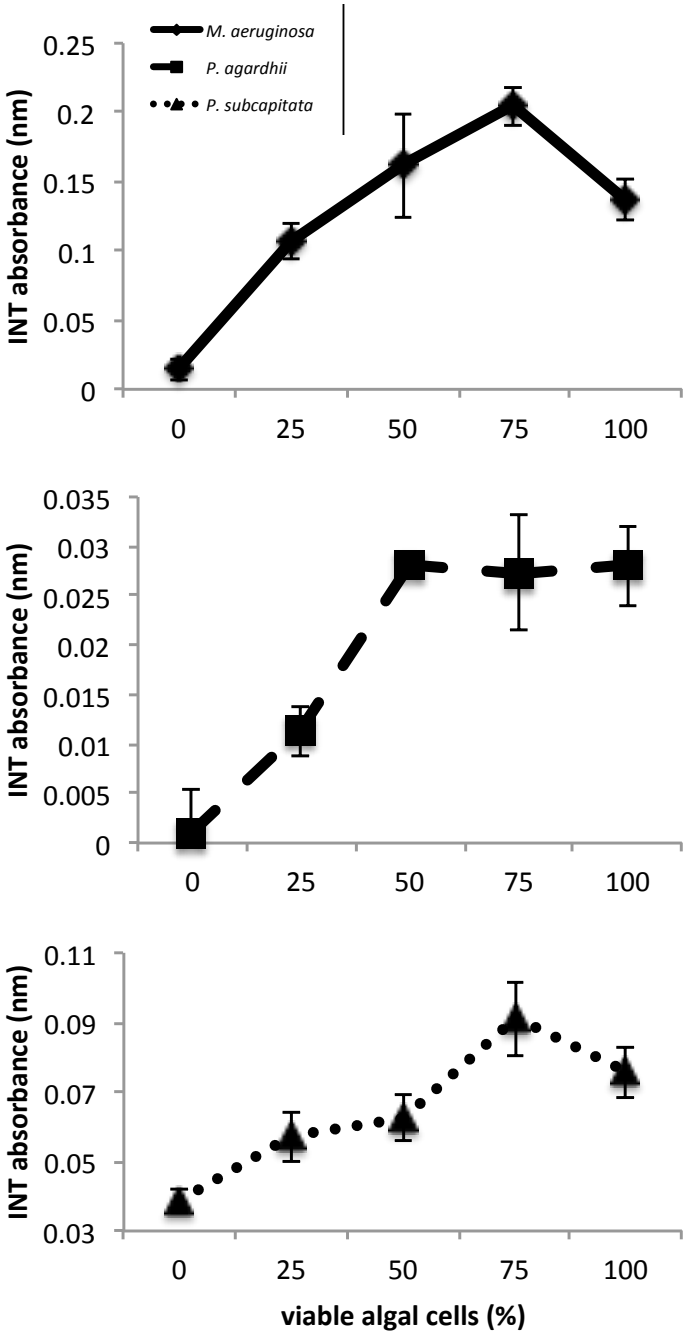

Figure 2.6: Mean INT formazan absorbances of *M. aeruginosa*, *P. agardhii* and *P. subcapitata* in known viable cell ratios. Error bars represent +/- 1 standard deviation. (n=3)

CHAPTER THREE

Responses of *Planktothrix agardhii* and *Pseudokirchneriella subcapitata* to copper sulfate ($CuSO_4 \bullet 5H_2O$) and a chelated copper compound (Cutrine®-Ultra)

Abstract

Reliable viability measures are needed to predict responses of algae to phytotoxic exposures. Non-axenic laboratory cultures of *Planktothrix agardhii* and *Pseudokirchneriella subcapitata* were exposed to a series of concentrations of $CuSO_4 \bullet 5H_2O$ (0.1-0.5 and 1.0-8.0 mg Cu/L) and a chelated copper compound with an adjuvant, Cutrine®-Ultra (0.05-0.25 and 1.0-8.0 mg Cu/L) in 7 d static laboratory experiments. Algal viability measures in terms of cell density, uptake of mortal stain (erythrosin b), chlorophyll *a* concentration, pheophytin *a* concentration and respiration (measured as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium formazan absorbance (INT)) were discerned daily. The time and concentration required to achieve algal control, 96h $EC_{50s,}$ potency slopes and doubling times were calculated from algal responses if algal viability measures were accurate and precise. Cell densities and erythrosin b stained cells had sufficient accuracy and precision to differentiate responses of *P. agardhii* and *P. subcapitata* from resident algae while chlorophyll *a* concentrations and INT formazan absorbances did not discriminate. In this study, pheophytin *a* concentrations lack precision and accuracy. *P. agardhii* was an order of magnitude more sensitive than *P. subcapitata,* and Cutrine®-Ultra was more than twice as potent as $CuSO_4 \bullet 5H_2O$. This laboratory study corroborates previous laboratory and field studies suggesting that chelated copper-based algaecides when applied in the field can selectively control specific target algae at a lower copper concentration than $CuSO_4 \bullet 5H_2O$. Individual algal

viability measures such as cell density and erythrosin b staining can measure the relevant change in algal assemblage as a result of exposure.

Introduction

Algal viability measures can be used to assess responses of algae to exposures of biocides and algaecides (Zetsche and Meysman 2012; Lewis 1990; Murray-Gulde et al. 2002; Calomeni and Rodgers in review). Responses of algae to algaecides depend on several factors such as the algaecide formulation (Fitzgerald and Jackson 1979; Murray-Gulde et al. 2002; Deaver and Rodgers 1996), characteristics of the site water or growth medium, and algal species (Fitzgerald and Jackson 1979). Copper-based algaecides have been used for more than a century to control growths of noxious algae (Moore and Kellerman 1905) and are currently available in a variety of formulations. Following exposures to copper, algae manifest morphological and physiological effects that may not be discerned using common algal viability measures such as chlorophyll *a* concentration and cell density. For example, copper exposures may decrease respiration rates and compromise cell membranes (Gibson 1972). Copper-based algaecides are used in aquatic environments to control noxious algal species with co-occurring prokaryotic and eukaryotic algae. Therefore, useful algal viability measures need to be specific and robust to discern responses of target species.

The present study focuses on responses of a prokaryotic alga, *Planktothrix agardhiii* (*P. agardhii* (Gomont)) and a eukaryotic alga, *Pseudokirchneriella subcapitata* (*P. subcapitata* (Korshikov)) to exposures of a copper salt ($CuSO_4 \bullet 5H_2O$) and an ethanolamine chelated copper compound formulated with d-limonene as an adjuvant (Cutrine®-Ultra) (Table 3.1). Since copper speciation greatly affects the responses of algae and other organisms, one would expect the responses of algae to these two copper

compounds to differ. Hypothetically, the two copper sources ($CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra) will dissociate into Cu^{2+} and ethanolamine chelated copper compounds, respectively. For algae, chelated copper compounds are often more potent than $CuSO_4 \bullet 5H_2O$ (Bishop and Rodgers 2011, Rodgers et al. 2010).

Prokaryotic and eukaryotic algae can interfere with the intended or designated uses of water resources and require mitigation to restore those uses (Bishop and Rodgers 2011; Rodgers et al. 2010). *P. agardhii* is a filamentous cyanobacterium that produces microcystin, a hepatotoxin that can become problematic when total microcystin concentrations exceed 1 ug/L (WHO 2003). *P. subcapitata,* a eukaryotic green alga, is commonly used by the United States Environmental Protection Agency (US. EPA) in algal toxicity experiments (Wehr and Sheath 2003; Miller 1978), is an example of a primary producer for aquatic food webs (Schrader et al. 1998) and does not produce any known toxins. Exposures to a series of concentrations of two copper-based algaecides can identify the relative sensitivities of the prokaryotic alga (*P. agardhii*) and the eukaryotic alga (*P. subcapitata*). Five algal viability measures were used to measure the effects of $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra on *P. agardhii* and *P. subcapitata*.

The algal viability measures included density of viable cells (cell density), cell membrane integrity (erythrosin b staining), measures for photosynthesis (chlorophyll *a* and pheophytin *a* concentration) and respiration (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium (INT) formazan absorbance). The algal responses in this study discern different aspects of algal viability, and may be used to measure specific physiological and morphological alterations following copper exposures. After an

exposure, algal responses in terms of photosynthesis, respiration and cell membrane integrity will occur over time, often depending on the algaecide concentration and form (Gibson 1972; Stauber and Florence 1987; Perales-Vela 2007). Therefore, viability measures in this study were assessed daily to capture the response when and if any occurred.

The overall objective of this research was to evaluate the utility of a stain and biochemical indicators of algal viability for measuring the relative health of two freshwater algae (*P. agardhii* and *P. subcapitata*) exposed to two copper-based algaecides. The specific objectives of this research were to compare and contrast: 1) individual (cell densities and erythrosin b stained cells) and aggregate (chlorophyll *a* and pheophytin *a* concentrations and INT formazan absorbance) algal viability measures for *P. agardhii* and *P. subcapitata* exposed to a copper salt ($CuSO_4 \bullet 5H_2O$) and a chelated copper compound (Cutrine®-Ultra) every 24 h over the course of 7 days, and 2) the relative sensitivities of *P. agardhii* and *P. subcapitata* to the two copper compounds in 96 h static laboratory toxicity tests.

Methods

Algal cultures

P. agardhii and P. subcapitata (UTEX 1648) cultures were grown and maintained separately in COMBO medium (Kilham et al. 1998) at 21°C with a 18:6 h light:dark cycle. Illumination was provided by cool-white fluorescent bulbs (Residential Ecolux 40 W, GE) at 2660 LUX. The culture of *P. subcapitata* was obtained from University of Texas culture collection (UTEX 1648, http://web.biosci.utexas.edu/utex/default.aspx) in Austin, Texas. The source of the non-axenic *P. agardhii* culture was Grand Lake in St. Marys, Ohio. This culture was dominated by *P. agardhii* (>90%) but also contained *Chlamydomonas sp.*, *Scenedesmus sp.* and *P. subcapitata*.

Algaecide exposures

Stock algaecide solutions of 1,000 mg Cu/L for *P. agardhii* and 10,000 mg Cu/L for *P. subcapitata* were prepared from $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra using NANOpure® water. *P. agardhii and P. subcapitata* in 200 mL of COMBO medium were placed into separate 250 mL acid-washed borosilicate beakers. Stock copper solutions were used to prepare nominal exposure concentrations from 0.1-0.5 mg Cu/L as $CuSO_4 \bullet 5H_2O$ and 0.05-0.25 mg Cu/L as Cutrine®-Ultra for *P. agardhii* (Table 3.2). *P. subcapitata* was exposed to concentrations ranging from 1.0-8.0 mg Cu/L as $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra (Table 3.2). Copper concentrations were arrayed to capture the 96h EC_{50} based on preliminary range finding experiments prior to initiation of definitive toxicity experiments. For each algaecide, five concentrations and an untreated control were tested. Acid soluble copper concentrations were measured at the initiation

of the experiment using flame atomic absorption spectroscopy and graphite furnace atomic absorption spectroscopy (Agilent PSD 120 Atomic absorption spectrometer) (APHA 2005).

Algal viability measures

Because responses of the algae to copper exposures were expected to occur over time, subsamples of algae from each exposure were collected daily over the course of 7 days for assessment of algal viability. Immediately before collection of subsamples, cells were re-suspended in the exposure chambers. Cell densities were determined using a Leitz Wetzlar Dialux 20 light microscope (Leitz USA Scopes, Paramount, California) and an Improved Neubauer hemacytometer at 250x magnification (Rodgers et al. 2010). *P. agardhii* cell densities were estimated by counting the number of trichomes per 0.2 mm^2 gridded area on the hemocytometer and multiplied by the number of cells (12 cells) in this gridded area. For erythrosin b staining, one mL of a 2.5mg erythrosin b /L solution was added to 1 mL of algal suspension, and cells were stained for 15 min. Cells were enumerated to 100 total cells and calculated as a ratio of stained cells to total cells. Individual algal viability measures (cell density and erythrosin b stained cells) employ light microscopy to discern live and dead cells on a cell-by-cell basis.

Chlorophyll *a* and pheophytin *a* concentrations were measured using a modified spectrophotometric method (APHA 2005). Acidification of chlorophyll *a* was extended to 20 min to allow for complete acid hydrolysis of chlorophyll *a* to pheophytin *a.* Absorbance values were converted to chlorophyll *a* and pheophytin *a* concentrations using linear regressions calculated from chlorophyll *a* and pheophytin *a* standards

(C6144 Sigma). To measure respiration, INT formazan was analyzed using a method modified from Packard (1971). Nitrocellulose filters (0.45 μm pore size) were substituted for glass-fiber filters, and the final dilution step was excluded. Absorbance was determined using a Spectra Max M_2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Aggregate algal viability measures (chlorophyll *a* concentration, pheophytin *a* concentration and INT formazan absorbance) concentrate cells in a water sample to discern the collective responses of cells in the sample. Both types of response measures (individual and aggregate) should result in the same conclusions concerning the viability of algal cells in axenic cultures. However, in mixed cultures, aggregate viability measures may be misleading or ambiguous due to differential sensitivities of algal species to exposures.

Analyses

Response parameters calculated using the individual and aggregate viability measures included 1) the time and concentration required to achieve algal control, 2) the concentration after 96h of exposure where control of 50% of the algal population was observed (i.e. 96h EC_{50}), 3) the potency slopes and 4) doubling times for the untreated. In this experiment, algal control is defined as an appropriate change in mortality (e.g. 90%) for the target alga indicated by the viability measures (i.e. EC_{90}; Murray-Gulde et al. 2002; Bishop 2011). Ninety-six hour EC_{50s} and potency slopes were used to estimate the relative sensitivities of the two algae to the two algaecides and, doubling times were used to estimate growth in the untreated controls. Ninety-six hour EC_{50s} and potency slopes were calculated using Probit and Regression analyses with the Statistical Analysis

System ($\alpha = 0.05$) (APHA 2005; SAS 9.2 2010). The linear portion of the potency curves (LOEC to EC100) was used to derive the regression equations estimating the potency slopes (Fuentes et al. 2011). Estimates of time needed to achieve control and doubling times were based on graphical interpolation using targeted copper concentrations.

Results and Discussion

Cell densities of P. agardhii and P. subcapitata in response to copper algaecides

Resolution of cell density measurements was sufficient to measure responses of *P. agardhii* and *P. subcapitata* in non-axenic cultures to a series of concentrations of $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra in 7 day toxicity experiments (Figs. 3.1 and 3.2). Cell density measurements are accurate, precise and discrete for discerning the responses of one algal species or genus in a mixed culture or a field-collected sample (Calomeni and Rodgers in review). Cell density measurements utilize light microscopy and the microscopist's discretion to identify viable and non-viable algae of the taxa of interest.

Separate experiments to determine responses of *P. agardhii* and *P. subcapitata* to $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra were conducted; therefore, there were two untreated controls for *P. agardhii* and two untreated controls for *P. subcapitata.* *P. agardhii* and *P. subcapitata* densities in untreated controls increased throughout the course of the experiment. Doubling times for *P. agardhii* in untreated controls were 2.8 and 3.1 days for experiments using $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra, respectively (Fig. 3.1). Doubling times for *P. subcapitata* in untreated controls were 8.3 and 4.4 days for $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra, respectively (Fig. 3.2).

For *P. agardhii* exposed to $CuSO_4 \bullet 5H_2O$, algal control (defined as a decrease in cell density by 90% or EC_{90}) was achieved at 0.2 mg Cu/L and 4.8 days after treatment (DAT) (Fig 3.1). For *P. agardhii* exposed to Cutrine®-Ultra, control was achieved at 0.2 mg Cu/L and 3.3 DAT. At 0.05 mg Cu/L, regrowth of the post-treatment algae occurred 7 DAT (Fig. 3.1). For *P. subcapitata* exposed to $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra,

control was achieved at 6 mg Cu/L on 5.1 DAT and 4 mg Cu/L on 4.1 DAT, respectively (Fig. 3.2).

Erythrosin b stained cells (P. agardhii and P. subcapitata) in response to exposures of copper algaecides

Erythrosin b staining was effective for detecting compromised cell membranes of *P. agardhii* and *P. subcapitata* following algaecide exposures; however, staining lacked precision (Figs. 3.3 and 3.4). For *P. agardhii* exposed to both copper-based algaecides, erythrosin b staining appears to be inversely correlated with live cell density (Figs. 3.1 and 3.3). For *P. subcapitata,* responses as indicated by erythrosin b staining were less evident than for *P. agardhii* (Figs. 3.3 and 3.4). At concentrations and times after exposures when algal control occurred (indicated by cell density), about half of the *P. subcapitata* cells were stained. For *P. subcapitata,* following exposures of Cutrine®-Ultra, cells became stained at a lower copper concentration (1-4 mg Cu/L) compared to 6 mg Cu/L for $CuSO_4 \bullet 5H_2O$ exposures (Fig. 3.4).

The chemical composition of prokaryotic (*P. agardhii*) and eukaryotic (*P. subcapitata*) cell walls may explain the difference in cell membrane permeability indicated by erythrosin b staining. The cell walls of prokaryotic and eukaryotic cells are located external to the cell membrane (Machie and Preston 1974). Prokaryotic algal cell walls are composed of peptidoglycan while cell walls for the eukaryotic algal cells are composed of cellulose (Baulina 2012; Machie and Preston 1974). It is likely that copper was more easily internalized in *P. agardhii* than *P. subcapitata* and was then capable of efficiently catalyzing Fenton and Haber-Weiss type reactions (Stevenson et al. 2013)

forming radicals that can cause lipid peroxidation (Gutteridge 1995). Internalized copper could also interact with redox sensitive reactions such as photosynthesis and respiration (Stauber and Florence 1987) since copper is a redox active element (Stevenson et al. 2013).

Chlorophyll a concentrations of P. agardhii and P. subcapitata in response to exposures of copper algaecides

The sensitivity of chlorophyll *a* concentrations as a response measure differed between algae. Chlorophyll *a* concentrations lacked the accuracy necessary to discern responses of *P. agardhii* to copper exposures (Fig. 3.5). It is likely that the eukaryotic algae in the non-axenic culture of *P. agardhii* were less sensitive to copper exposures than *P. agardhii* causing a shift in the algal assemblages in the sample. Post treatment cell densities supported this, as densities of eukaryotic algae (*Chlamydomonas sp.*, *Scenedesmus sp.* and *P. subcapitata*) 7 DAT were 10^6 cells/mL. Chlorophyll *a* concentrations from these cells could have confounded the results.

For *P. subcapitata* exposed to copper-based algaecides, chlorophyll *a* concentrations had sufficient precision and accuracy to discern differences in responses (Fig. 3.6). Copper concentrations for *P. subcapitata* were an order of magnitude greater than concentrations required to elicit a similar effect for *P. agardhii*. These copper concentrations (1-8mg Cu/L) likely controlled other resident algae. In the two untreated controls for *P. subcapitata*, chlorophyll *a* concentrations increased with DAT. Doubling times in the untreated controls were 5.9 and 2.7 days corresponding with the experiments using $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra (Fig. 3.6). Control of *P. subcapitata* (defined as a

90% decrease in chlorophyll *a* concentrations) occurred at 4 mg Cu/L as $CuSO_4 \bullet 5H_2O$ and 8 mg Cu/L as Cutrine®-Ultra on 3.4 and 6.8 DAT, respectively (Fig. 3.6).

Pheophytin a concentrations of P. agardhii and P. subcapitata in response to copper algaecides

Pheophytin *a* concentrations lacked the accuracy and precision to discern responses of either alga to copper-based algaecide exposures as indicated by large variances (Figs. 3.7 and 3.8). Calculations to convert acidified chlorophyll *a* to pheophytin *a* are based on the assumption that viable algae will have more chlorophyll *a* than pheophytin *a*; therefore, the ratio (chlorophyll *a*: pheophytin *a*) will be approximately 1.7 (APHA 2005). Based on this assumption, algal assemblages that are non-viable will have a ratio of 1 (APHA 2005). However, this was not confirmed in the present study, and is why some pheophytin *a* concentrations were negative (Figs. 3.7 and 3.8). For pheophytin *a* concentrations calculated from acidified chlorophyll *a* concentrations to be reliable, this assumption must be valid.

INT formazan absorbances of P. agardhii and P. subcapitata in response to copper algaecides

INT formazan absorbances lacked the accuracy needed to detect responses of the algae to algaecide exposures (Figs. 3.9 and 3.10). This is indicated by the lack of relationship between INT formazan absorbance and concentration for all of the treatments except the untreated controls for *P. agardhii* (Figs. 3.9 and 3.10). Doubling

times for *P. agardhii* indicated by INT formazan absorbances were 4.6 and 6.5 days for the experiments using $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra, respectively (Fig. 3.9).

Respiration is a non-specific or generic parameter and cannot discern the species responsible for the respiratory activity (Packard 1971). Once the algae were treated with copper-based algaecides, they began to degrade supporting bacterial respiration (Goecke et al. 2013). Resident bacterial respiration may have contributed to the lack of accuracy and precision needed to discern the responses of *P. agardhii* and *P. subcapitata* to copper-based algaecide exposures. INT formazan absorbance may be an accurate parameter when used in a laboratory setting with an axenic culture to reduce "noise" from resident bacterial respiration.

Relative sensitivities

Ninety-six hour EC_{50s} and potency slopes were calculated from cell densities because this parameter had a monotonic response curve for both algae. *P. agardhii* was an order of magnitude more sensitive than *P. subcapitata* to exposures of both copper-based algaecides (Table 3.3). Studies have suggested that generally cyanobacteria are more sensitive to copper than eukaryotic algae although there are limited published data to support this (Brand et al. 1986; Hadjoudja et al. 2009). Hypothetically, prokaryotic organisms should be more sensitive than eukaryotic organisms based on algal fine structure. The photosynthetic and respiratory apparatus in prokaryotic algae (*P. agardhii*) are located in the thylakoid membrane and inter membrane space (Baulina 2012) in proximity to the outer membrane. For eukaryotic algae, the structures necessary for photosynthesis and respiration are encased in membrane bound organelles

(chloroplasts and mitochondria, respectively). To maintain copper homeostasis inside cells, chelators such as glutathione are available in the cytoplasm to bind with copper preventing formation of oxidizing byproducts inside the cell (Stevenson et al. 2013). Therefore, copper may be less likely to contact redox sensitive pathways (photosynthesis and respiration) in eukaryotic cells than in prokaryotic cells.

Estimated EC_{50s} of $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra for *P. agardhii* were not significantly different (α=0.05), likely due to the high sensitivity of this alga to the algaecides and because the EC_{50} values were relatively small (0.18 ppm copper as $CuSO_4 \bullet 5H_2O$ and 0.10 ppm copper as Cutrine®-Ultra). Predicted EC_{50s} of $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra for *P. subcapitata* were significantly different (α=0.05). The 96 h EC_{50} for $CuSO_4 \bullet 5H_2O$ was approximately double that for Cutrine®-Ultra.

Ninety-six hour EC_{50s} are commonly used algal response estimates for evaluations of the relative sensitivity of algae to exposures. In the present study, this estimated value was not sufficient to detect a difference in the relative sensitivity of *P. agardhii* to $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra. However, the slope of the effect of an incremental increase in exposure on the response parameter (cell density) can discern a difference in the potency of compounds when the algae are sensitive. For *P. agardhii,* Cutrine®-Ultra was more than twice as potent as $CuSO_4 \bullet 5H_2O$ (Table 3.3). Potency slopes were not significantly different for *P. subcapitata* exposed to the two copper-based algaecides (Table 3.3).

Responses of algae to the same nominal copper concentration but in different forms (copper salt vs. chelated copper) range widely. This study supports the notion that

for algae, chelated copper-based algaecides are often more potent than non-chelated algaecides (Bishop and Rodgers 2011; Rodgers et al. 2010). Bishop and Rodgers (2011) determined that chelated copper algaecides (Algimycin®-PWF and Clearigate®) can achieve algal (*Lyngbya* wollei) control at lower concentrations of sorbed copper than a non-chelated algaecide (i.e. copper sulfate). Therefore, the toxicity of chelated copper-based algaecides may be better correlated with internalized copper reaching physiologically active sites in the algae.

Conclusions

The capacity to detect responses of algae to phytotoxic compounds depends on the sensitivity, accuracy and precision of the algal viability measures used. In this experiment, copper-based algaecides provided the stimulus to test the utility of five algal viability measurements. The individual algal response measures (cell density and uptake of erythrosin b) could clearly discern responses of *P. agardhii* and *P. subcapitata* in non-axenic cultures to $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra. Therefore, individual algal viability measures will likely be useful for measuring responses of mixed cultures or field-collected samples to exposures. Chlorophyll *a* concentrations and INT formazan absorbances did not discriminate responses in a non-axenic culture but may be useful for an axenic laboratory culture. If used with field samples and assemblages of algae, these parameters should be used with care (i.e. in conjunction with microscopy and an understanding of their limitations). Pheophytin *a* concentrations lacked both precision and accuracy.

Cell densities were used to estimate 96 h EC_{50s} and potency slopes for the two algae and algaecides. *P. agardhii* was an order of magnitude more sensitive than *P. subcapitata* to $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra. In freshwater resources, often the goal of an algaecide treatment is to reduce densities of a problematic alga (e.g. toxin producer) and maintain densities of non-target algae. This laboratory study corroborates previous laboratory and field studies suggesting that copper-based algaecides can be applied at concentrations that will selectively control noxious algae with minimal to no impacts on non-target algal species (e.g. *P. subcapitata*).

Ninety-six hour EC_{50s} and potency slopes indicated that Cutrine®-Ultra was roughly twice as potent as $CuSO_4 \bullet 5H_2O$ for *P. agardhii* and *P. subcapitata.* This means that less copper can be applied as a chelated algaecide to elicit the same effect as a copper salt and consequently decrease copper residuals from algaecide applications in the field while increasing margins of safety for non-target organisms.

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Table 3.1: Physical and chemical characteristics of copper sulfate pentahydrate and Cutrine®-Ultra.

Characteristics	$CuSO_4 \cdot 5H_20$	Cutrine®-Ultra
Active ingredient	Copper	Copper
% Active ingredient	23.7	9.0[a]
Maximum label concentration as copper	2 mg/L[b]	1 mg/L[a]
Formulation	Copper sulfate pentahydrate	Copper ethanolamine in an emulsified complex[a]
Appearance	Blue crystals	Viscous blue liquid
Water solubility	415,997 mg/L	Miscible[*]
Boiling point (°C)	106[**]	113
Specific gravity (g/cm^3)	1.21[**]	1.20
pH	3.22[**]	10.0-10.5[b]

Physical and chemical characteristics are of the original compound unless otherwise noted.
[*] Cutrine®-Ultra can mix with water in all proportions.
[**]Physical and chemical characteristics of saturated solution
[a]Applied Biochemists product label
[b] Applied Biochemists MSDS

Table 3.2: Experimental design of toxicity experiments for *P. agardhii* and *P. subcapitata* exposed to $CuSO_4 \cdot 5H_2O$ and Cutrine®-Ultra

Algae	Algaecide	Targeted Acid-Soluble Cu Concentration (mg/L)	Measured Acid-Soluble Cu Concentration (mg/L)	Number of replicates/ concentration	Initial Cell Density (cells/mL)
P. agardhii	$CuSO_4 \cdot 5H_2O$	0, 0.1, 0.2, 0.3, 0.4, 0.5	0.024, 0.114, 0.176, 0.290, 0.393, 0.578	3	1.27×10^6
	Cutrine®-Ultra	0, 0.05, 0.1, 0.15, 0.2, 0.25	0.024, 0.047, 0.091, 0.149, 0.195, 0.242	3	2.21×10^6
P. subcapitata	$CuSO_4 \cdot 5H_2O$	0, 1, 2, 4, 6, 8	0.024, 0.82, 1.97, 4.25, 5.20, 7.25	3	3.22×10^6
	Cutrine®-Ultra	0, 1, 2, 4, 6, 8	0.024, 0.66, 2.28, 4.43, 6.72, 8.50	3	3.02×10^6

Table 3.3: 96 h EC_{50} values and potency slopes for *P. subcapitata* and *P. agardhii* exposed to $CuSO_4 \bullet 5H_2O$ and Cutrine®-Ultra in static non-renewal laboratory toxicity tests with laboratory formulated moderately hard water.

Algae	Estimates	$CuSO_4 \bullet 5H_2O$ (mg Cu/L ± 1 fiducial limit)	Cutrine®-Ultra (mg Cu/L ± 1 fiducial limit)
P. agardhii	96 h EC_{50}	0.18 (0.14-0.22)	0.10 (0.05-0.14)
	Potency Slope	-1.50	-3.35
P. subcapitata	96 h EC_{50}	3.0 (2.2-3.8)	1.18 (0.16-1.8)
	Potency Slope	-0.073	-0.085

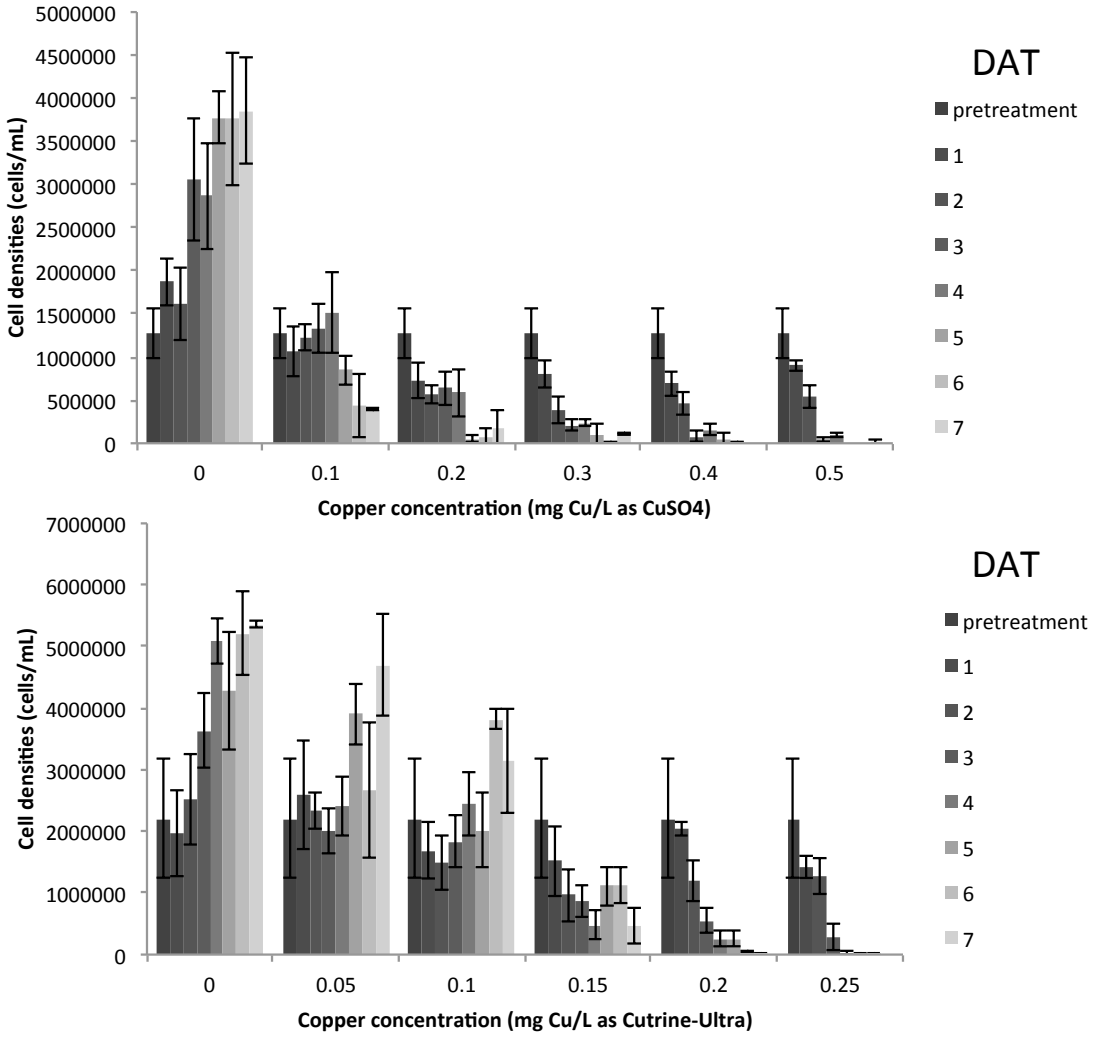

Figure 3.1: Mean responses of *P. agardhii* measured by cell density to copper exposures for 7 days (n=3). Error bars represent $\pm$ 1 standard deviation. DAT represents days after treatment.

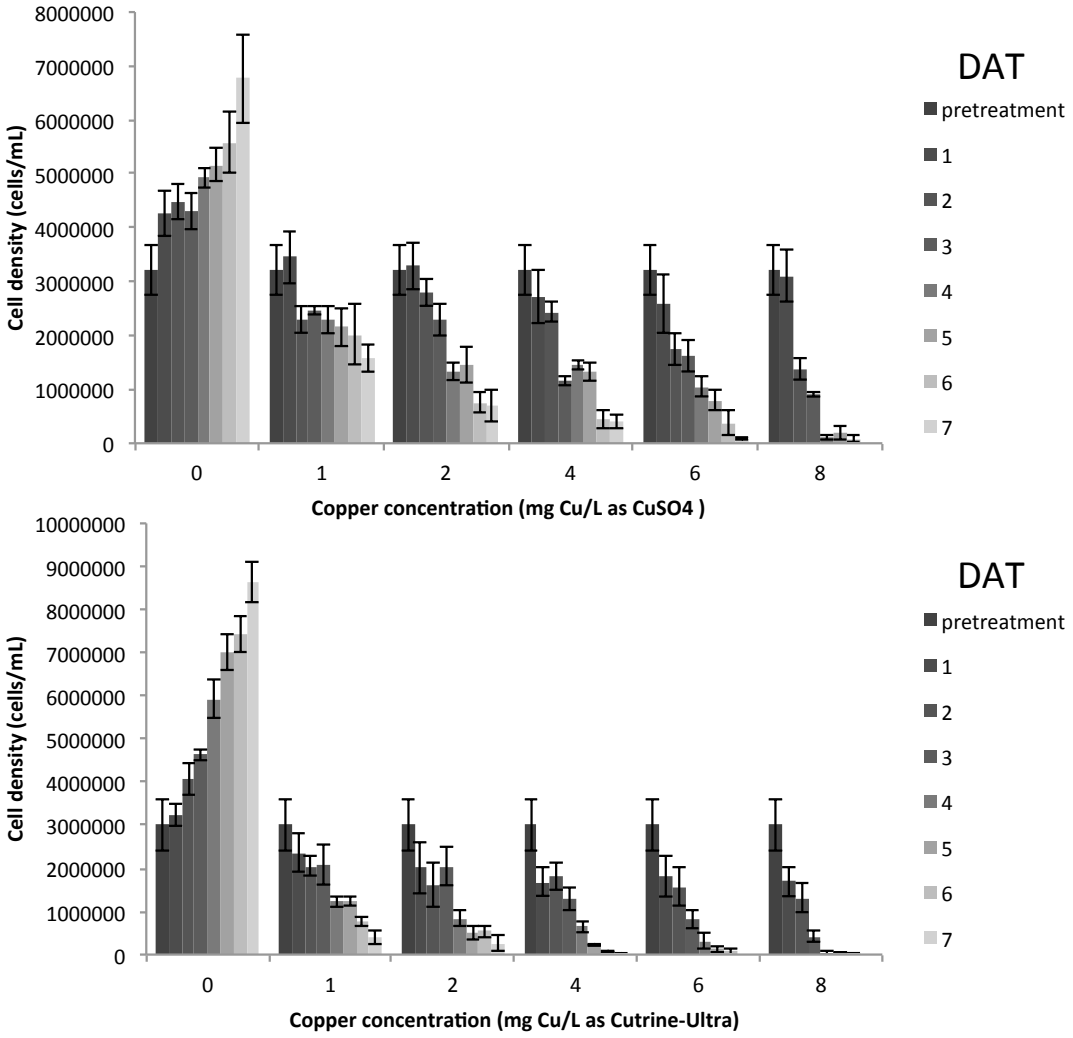

Figure 3.2: Mean responses of *P. subcapitata* measured by cell density to copper exposures for 7 days (n=3). Error bars represent $\pm$ 1 standard deviation. DAT represents days after treatment.

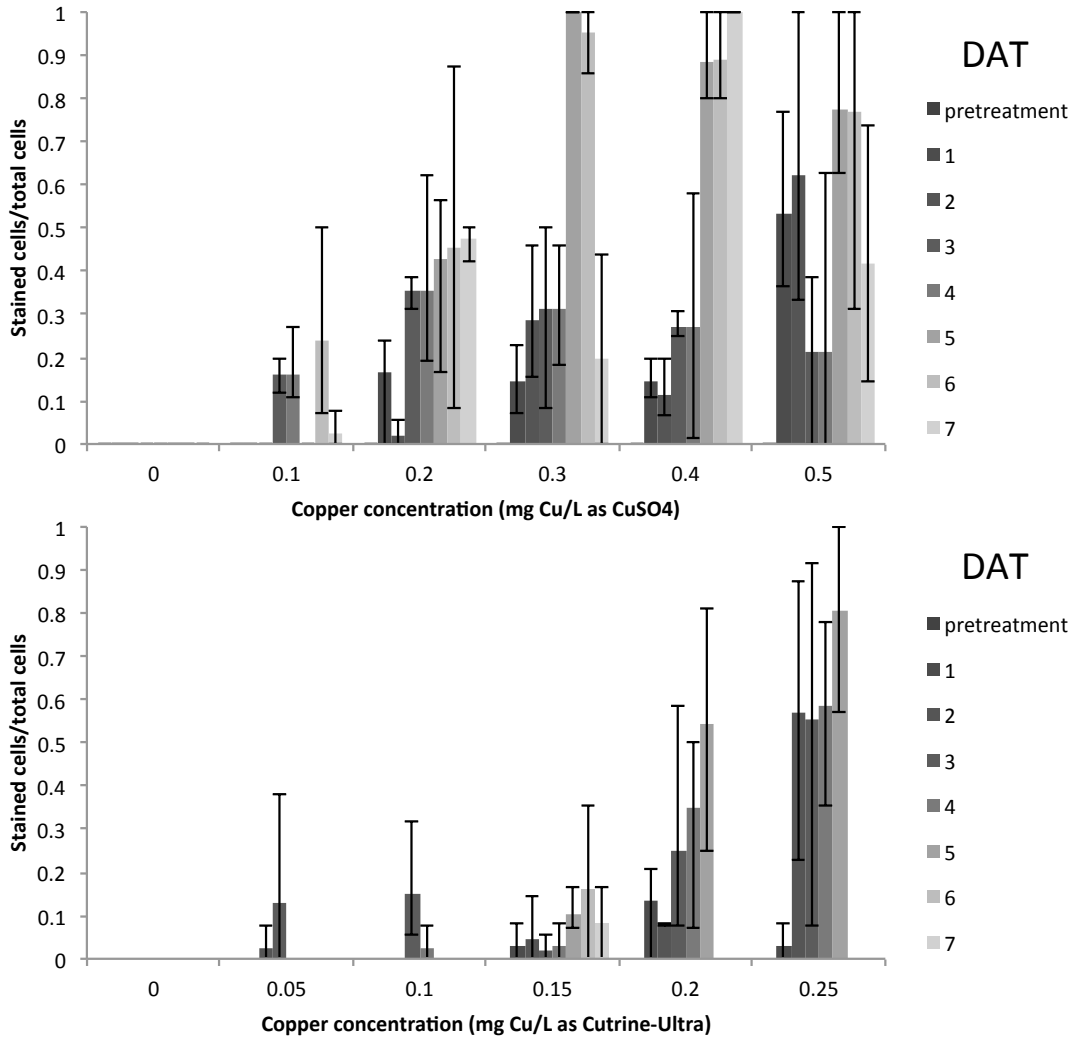

Figure 3.3: Mean responses of *P. agardhii* measured by erythrosin b stained cells to copper exposures for 7 days (n=3). Error bars represent the range. DAT represents days after treatment.

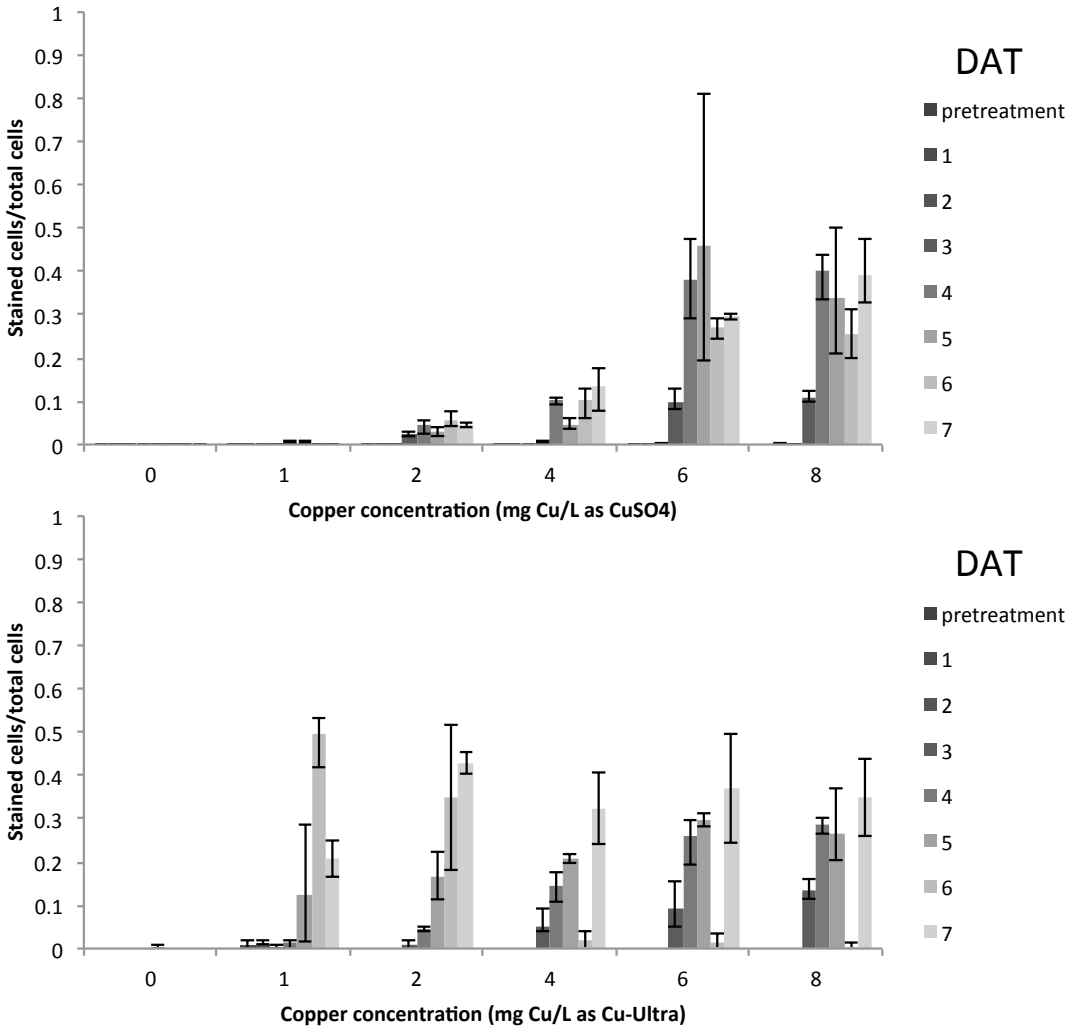

Figure 3.4: Mean responses of *P. subcapitata* measured by erythrosin b stained cells to copper exposures for 7 days (n=3). Error bars represent the range. DAT represents days after treatment.

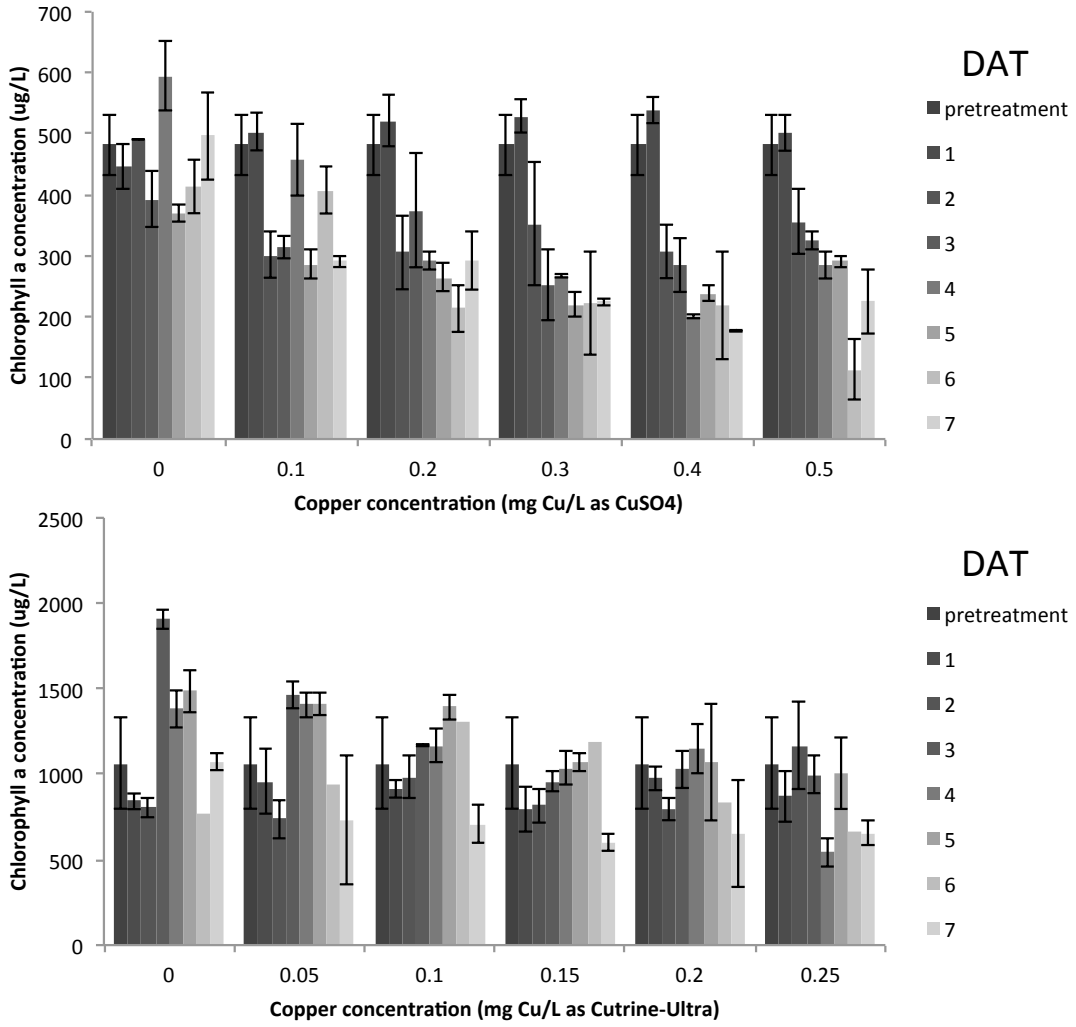

Figure 3.5: Mean responses of *P. agardhii* measured by chlorophyll *a* concentration to copper exposures for 7 days (n=3). Error bars represent ± 1 standard deviation. DAT represents days after treatment.

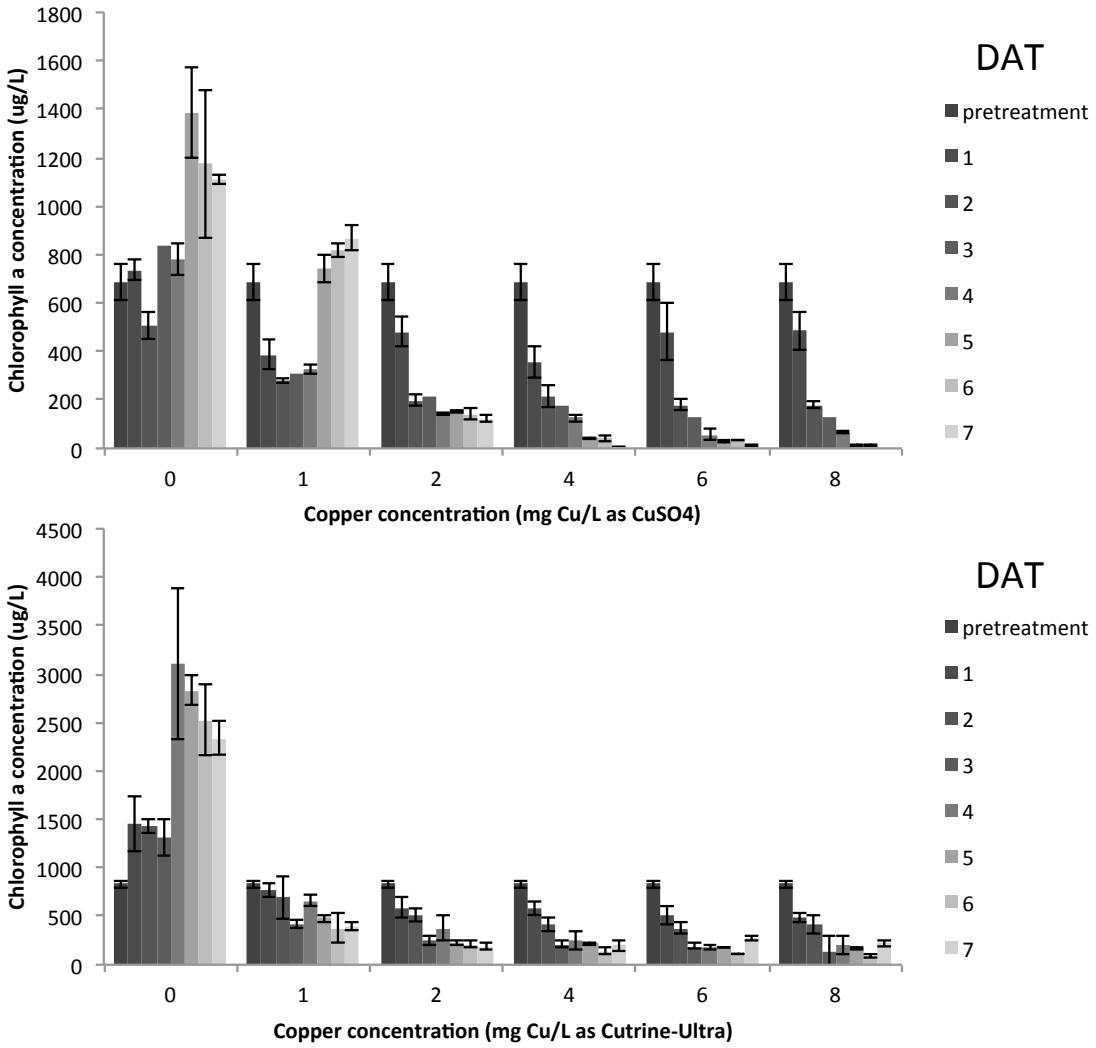

Figure 3.6: Mean responses of *P. subcapitata* measured by chlorophyll *a* concentration to copper exposures for 7 days (n=3). Error bars represent $\pm$ 1 standard deviation. DAT represents days after treatment.

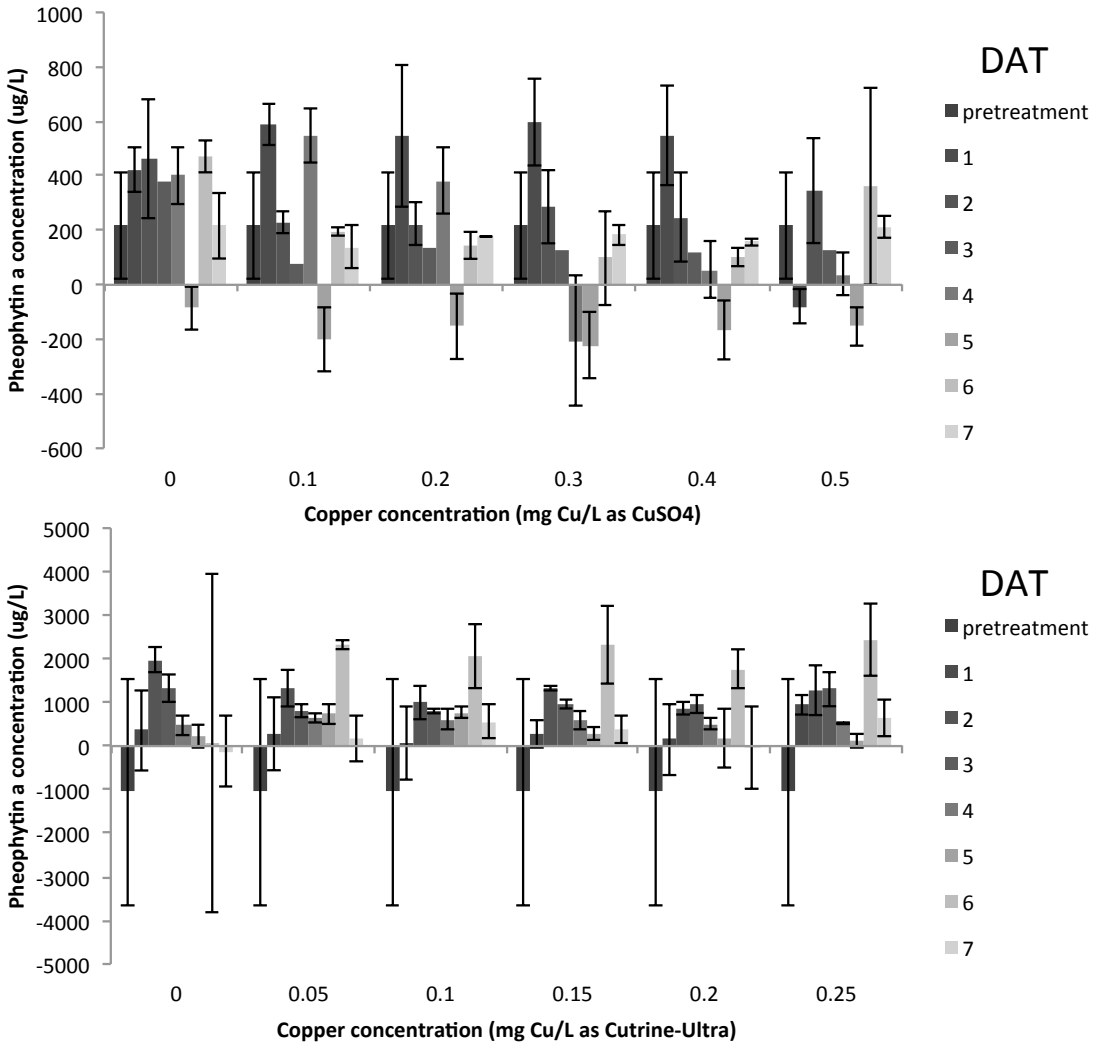

Figure 3.7: Mean responses of *P. agardhii* measured by pheophytin *a* concentration to copper exposures for 7 days (n=3). Error bars represent ± 1 standard deviation. DAT represents days after treatment.

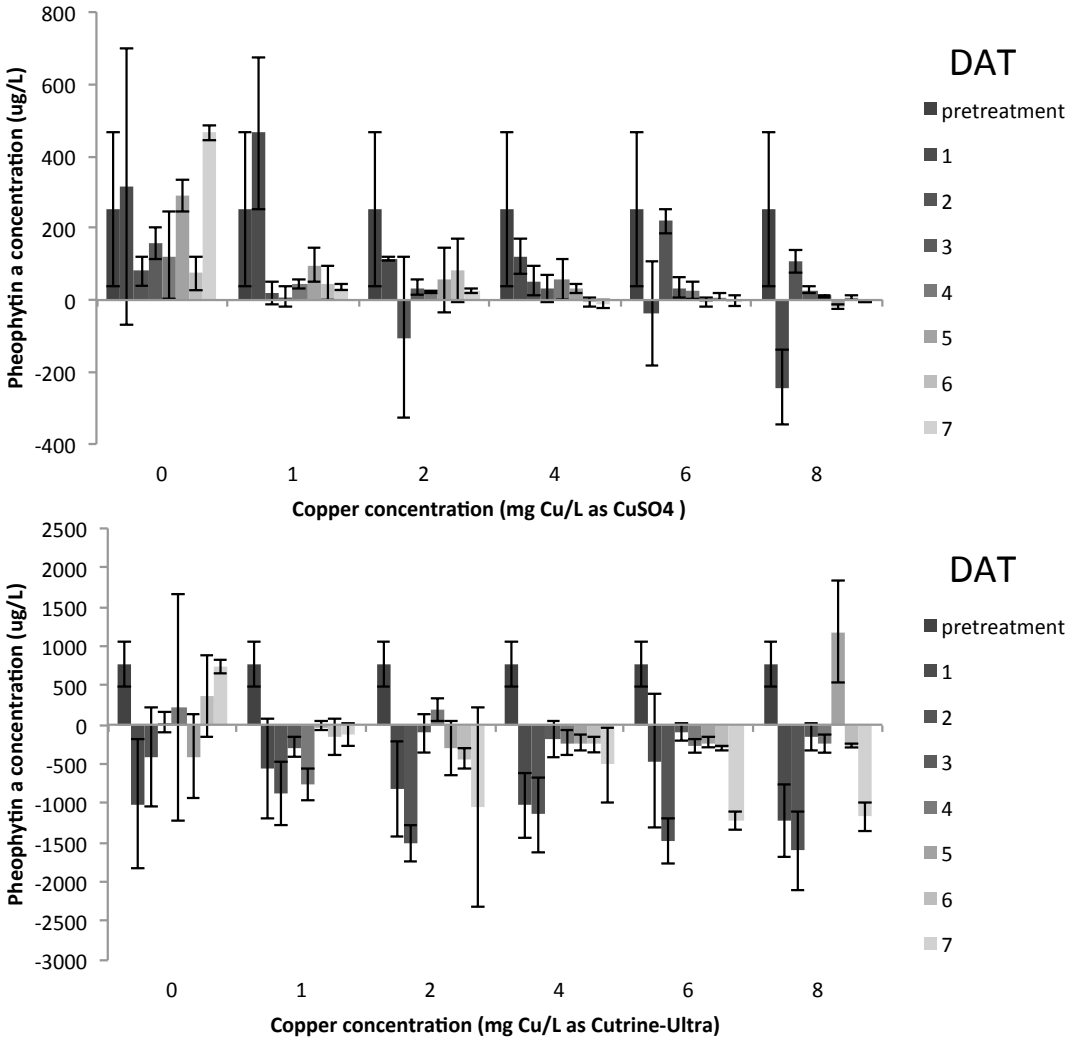

Figure 3.8: Mean responses of *P. subcapitata* measured by pheophytin *a* concentration to copper exposures for 7 days (n=3). Error bars represent ± 1 standard deviation. DAT represents days after treatment.

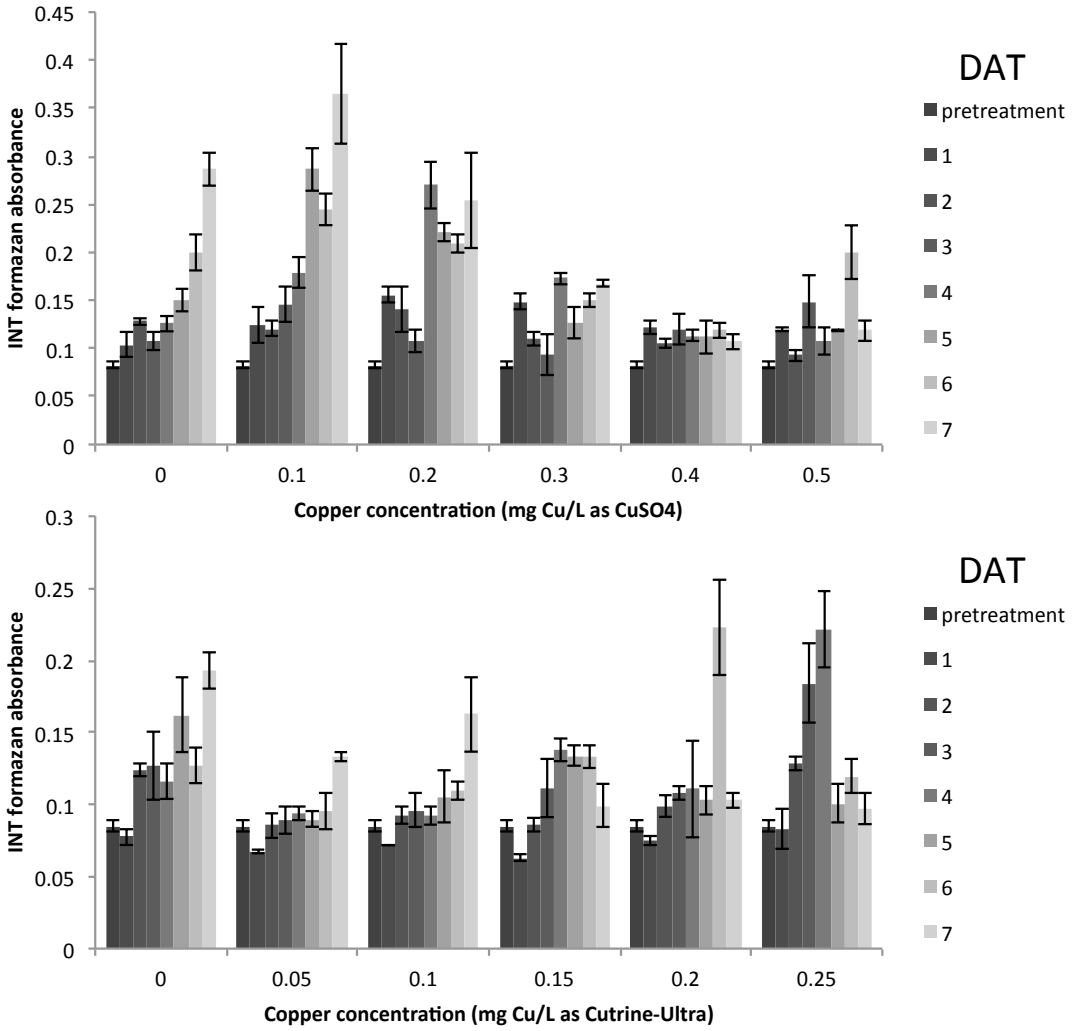

Figure 3.9: Mean responses of *P. agardhii* measured by INT formazan absorbances to copper exposures for 7 days (n=3). Error bars represent ± 1 standard deviation. DAT represents days after treatment.

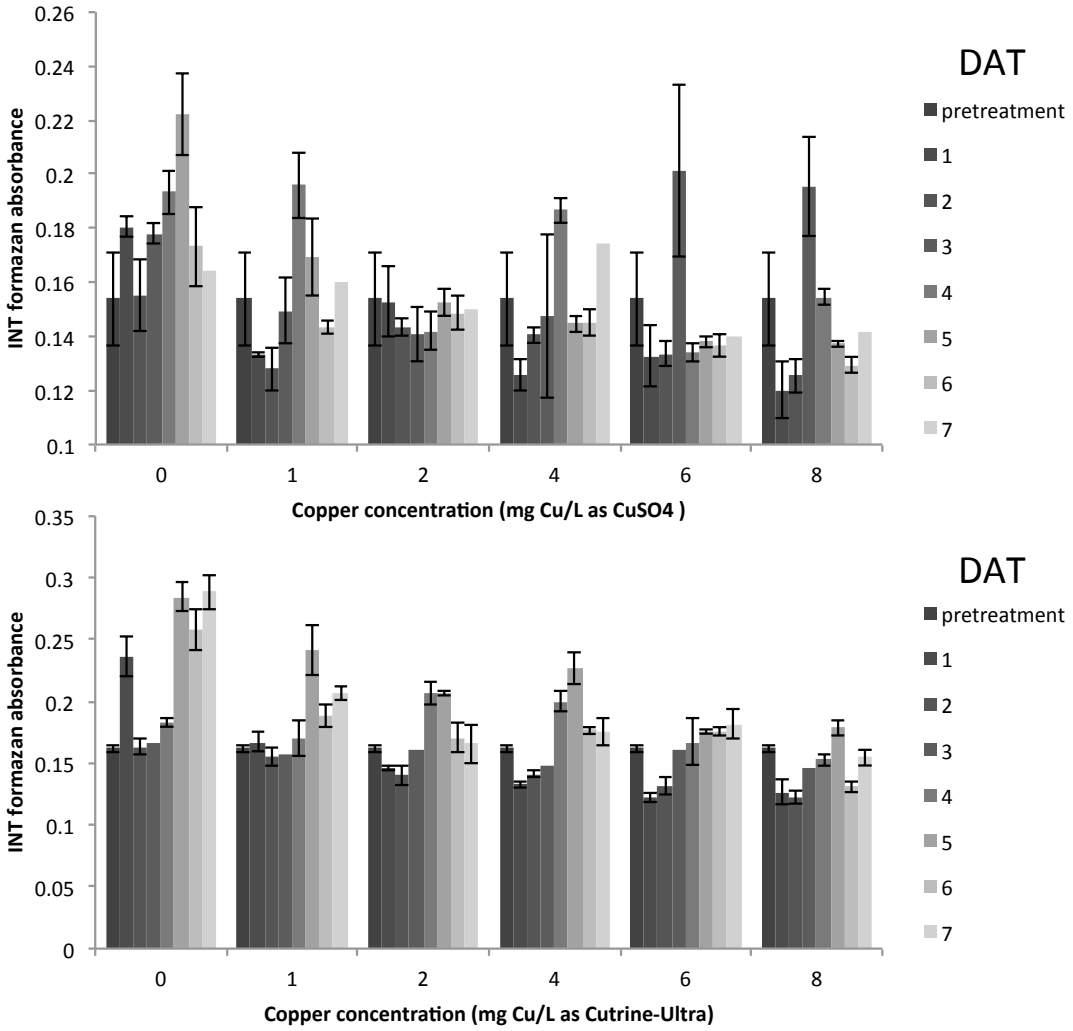

Figure 3.10: Mean responses of *P. agardhii* measured by INT formazan absorbances to copper exposures for 7 days (n=3). Error bars represent $\pm$ 1 standard deviation. DAT represents days after treatment.

CHAPTER FOUR

Responses of *Lyngbya wollei* to algaecide exposures and a risk characterization associated with their use

Abstract

To make informed decisions regarding management of noxious algal growths, water resource managers require information on responses of target and non-target species to algaecide exposures. Periodic treatments of Phycomycin®-SCP (sodium carbonate peroxyhydrate) followed by Algimycin®- PWF (gluconate and citrate chelated copper) to control *Lyngbya wollei* growths for ten years provided an opportunity for a risk evaluation of treated coves in Lay Lake, AL. Abiotic sediment characteristics (acid soluble copper concentrations, acid volatile sulfides, percent organic matter and cation exchange capacity) and survival of *Hyalella azteca* and *Chironomus dilutus* were measured in sediment samples from treated and untreated coves to assess the bioavailability of potential copper-residuals. In laboratory studies to seek a more effective approach for managing the growth of Lyngbya, six algaecide treatments consisting of combinations of copper-based algaecides (Cutrine®-Ultra, Clearigate® and Algimycin®- PWF), a hydrogen peroxide based algaecide (Phycomycin®-SCP) and an adjuvant (Cide-Kick II) were assessed for efficacy in controlling *Lyngbya wollei* sampled from Lay Lake. The most efficient algaecide treatment was determined based on post-treatment algal wet weight and visual observations of responses to exposures. To estimate the margin of safety for non-target organisms, *Pimephales promelas* was exposed to the most efficacious

treatment and a treatment of Phycomycin®-SCP followed by Algimycin®- PWF. Results from sediment experiments demonstrated that there were no measureable copper residuals and no adverse effects on *Hyalella azteca* and *Chironomus dilutus* from sediments following ten years of copper-based algaecide treatments. Based on the laboratory results, a treatment of Phycomycin®-SCP at 10.1 mg H_2O_2/L followed by Cide-Kick II at 0.2mg/L and Algimycin®- PWF at 0.26mg Cu/L could control the growth of *Lyngbya wollei* from Lay Lake, AL and enhance the margin of safety for non-target species (e.g. *Pimephales promelas*).

Introduction

Water resources serve multipurpose functions and often have competing uses. Noxious algae can interfere with valuable water resources and intended uses such as potable water supply, industrial needs and aesthetic value of shoreline properties. When noxious algae approach or exceed decision thresholds such as human health risks and economic pressures, control measures such as algaecides are often initiated in critical water resources to efficiently and effectively achieve control of algae and restore the uses of the water resources.

Lay Lake, a 4,900 hectare man-made reservoir, is located within St. Clair, Talladega, Shelby, Coosa and Chilton counties in Alabama and is managed for contact recreation, drinking water, food processing, fishing, industrial uses, agricultural uses and propagation of fish and wildlife. Lay Lake also has aesthetic value for shoreline and other nearby properties. Since 2004, coves in Lay Lake have experienced persistent *Lyngbya wollei* (*L. wollei*) growths. *L. wollei,* a prokaryotic cyanobacterium, can produce dense bi-phasic mats (Speziale and Dyck, 1992), taste-and-odor compounds (Brown and Boyd, 1982), dermatotoxins and neurotoxins (Carmichael et al., 1997). *L. wollei* may also decrease densities of beneficial zooplankton (e.g. *Daphnia magna*) and cause avoidance responses in fish (e.g. *Pimephales promelas*) (Mastin et al., 2002).

The need to maintain the designated uses of water in some coves in Lay Lake plagued by growths of *L. wollei* compelled intervention. Chemical (algaecides), physical (raking) and mechanical (harvesters) tactics were initially implemented to

decrease *L. wollei* densities in these coves with marginal success. In 2004, results from laboratory toxicity experiments discerned an effective algaecide combination for controlling *L. wollei* in coves (Duke 2007). A sequential treatment of Phycomycin®-SCP at 2.03 mg H_2O_2/L followed by 0.94 mg Cu /L as Algimycin®- PWF was used 5 times a year for the last 10 years to maintain sustained control of *L. wollei.* Phycomycin®-SCP is a hydrogen peroxide-based algaecide that oxidizes organic material and then decomposes into water and oxygen (Barroin and Feuillade, 1985). Algimycin®- PWF is a copper-based algaecide that is chelated with citrate and gluconate (Applied Biochemists Inc., 2010a).

With repeated copper-based algaecide exposures, questions arose about responses of non-target species in treated coves. Copper has a lithic biogeochemical cycle and will partition to sediments after an application. Sediment characteristics such as percent organic matter (%OM) (Besser et al., 2003; Milani et al., 2003), acid-volatile sulfides (AVS) (Allen et al., 1993), cation exchange capacity (CEC) (Chapman et al., 1998) and particle size distribution (Hoss et al., 1997) influence the bioavailability of copper sorbed to sediments. Therefore, analytically measured copper concentrations in sediment are not always directly correlated with bioavailability. Bioavailability can be assessed using organism responses to copper in sediments. The non-target organisms, *Hyalella azteca* Saussure and *Chironomus dilutus* Fabricius are commonly used for sediment toxicity experiments and can tolerate a wide range of organic matter concentrations and particle sizes (Deaver and Rodgers, 1996; Suedel et al., 1996).

After repeated uses of Phycomycin®-SCP and Algimycin®- PWF, questions also arose regarding the continued performance of this algaecide treatment. Treatments used to decrease densities of a specific alga at a site can lead to the availability of an ecological niche for other species. Following a long-term (10 year) treatment plan for Lay Lake, the algal assemblage in treated coves may have shifted. Laboratory studies in which algae are exposed to a series of concentrations of different algaecides in site water can assist in decision making for selection of an appropriate algaecide and concentration for a field application (Mastin et al., 2002; Murray-Gulde et al. 2002; Bishop and Rodgers, 2012). An appropriate algaecide will be one that is efficient and effective for controlling *L .wollei* in coves and will not cause unreasonable harm to non-target species (e.g. fish, benthic invertebrates).

If an alternative treatment is proposed based on the results of laboratory experiments, risks for non-target species must also be evaluated. Following an algaecide application, the initial exposure would originate from the aqueous phase. Since one of the designated uses for Lay Lake is fishing, questions arise about responses of fish to aqueous algaecide exposures. *Pimephales promelas* Rafinesque is a commonly used freshwater species for toxicity experiments because of its extensive distribution in the United States (Allan, 1952). Decisions to proceed with an algaecide treatment usually involve target and non-target species effects, economic considerations and social acceptance.

Algaecides applied 5 times/ year over a period of 10 years and questions regarding a more efficacious approach for Lay Lake, AL provided the opportunity to

gather site-specific data regarding responses of target and non-target species to copper exposures. The overall objective of this research was to compare results from the current algaecide treatment versus alternative algaecide treatments. The specific objectives were to 1) assess responses of *Hyalella azteca* and *Chironomus dilutus* in terms of survival to potential copper-based algaecide residuals in sediments following 10 years of periodic algaecide treatments (Phycomycin®-SCP and Algimycin®- PWF), 2) measure the responses in terms of wet weight, chlorophyll *a* concentration and visual observations of *L. wollei* to candidate algaecides (Cutrine®-Ultra, Phycomycin®-SCP, Clearigate®, Algimycin®- PWF) and an adjuvant (Cide-Kick II) and determine an effective algaecide treatment for Lay Lake, and 3) measure and compare survival of *Pimephales promelas* to exposures of an effective algaecide treatment and a treatment of Phycomycin®-SCP and Algimycin®- PWF.

Materials and Methods

2.1. Study site

Lay Lake is a man-made reservoir centrally located in Alabama (33.15°,-86.48°). *L. wollei* growths were present in coves of interest for this study with an average depth of 3.0 m. The treated cove selected for toxicity experiments has records of algaecide applications 5 times/year for the past 10 years. The untreated cove was used to determine pretreatment copper concentrations and serve as reference sediment for copper bioavailability experiments using *H. azteca* and *C. dilutus*.

2.2. Sediment characteristics and copper bioavailability from Lay Lake sediments

To assess the potential for adverse effects on benthic organisms from 10 years of copper based algaecide applications and to determine if an increase in copper concentration or bioavailability could be detected following one algaecide application, four sediment samples were collected (2 samples from a treated cove – one pre- and one post- treatment, and 2 from an untreated cove). Approximately 3,000 cm^3 of surficial sediment samples were collected on 23 September 2013 from treated and untreated coves before an algaecide treatment was applied to the treated cove. Two weeks (7 October 2013) following application of the algaecide, additional sediment samples were collected from treated and untreated coves. This sampling date was selected because in previous studies, copper partitioned to sediment within two weeks following an application (Murray-Gulde et al., 2002). Sediment samples were transported on ice to Clemson University and stored at 4°C

until analysis. Before analyses, sediment samples were gently homogenized with a spatula, and large detritus was removed with a sieve (2 cm mesh).

To analytically determine if copper concentrations increased in sediments following repeated algaecide treatments over 10 years and after one treatment in Lay Lake, four sediment samples were analyzed. Pre and post-treatment sediment samples from the treated cove and 2 samples from the untreated cove were acid digested and copper concentrations were measured using graphite furnace atomic absorption spectroscopy [Agilent PSD 120 Atomic absorption spectrometer (USEPA 3050b; APHA, 2005)]. Particle size distribution (Gee and Bauder, 1986), organic matter content (Nelson and Sommers, 1986), acid volatile sulfide concentration (Plumb, 1981) and cation exchange capacity (Mehlich, 1953) were measured as these sediment characteristics may impact the bioavailability of copper in sediments. The method for determining cation exchange capacity was modified to include Zn, Mn, Cu and B, and the cations were measured using Inductively Coupled Plasma Optical Emission Spectroscopy (Spectro Arcos, Kleve, Germany).

Method detection limits (MDLs) for copper concentrations in sediment samples depend on sediment characteristics, the extraction procedure and the analytical equipment used (Willis and Rodgers, 2013). Therefore MDLs were determined empirically for each sediment sample. Sediments were amended with a series of copper concentrations as copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$; Thermo Fisher Scientific, Inc.). A contact period of two weeks was allowed before acid digestion and copper measurement [(Agilent PSD 120 Atomic absorption

spectrometer (USEPA 3050b; APHA, 2005)]. MDLs or the lowest amendment with a statistically significant increase in copper concentration greater than the unamended sediments were discerned using ANOVA and Tukey's tests ($\alpha = 0.05$)

The potential bioavailability of copper from sediments was determined using *H. azteca* and *C. dilutus* (USEPA 1994). Experimental organisms were cultured at Clemson University following the methods of de March (1981) and Townsend et al. (1981) for *H. azteca* and *C. dilutus*, respectively. Two to three week old amphipods (Deaver and Rodgers, 1996) and second instar *C. dilutus* were collected for sediment toxicity experiments. Three replicate experiments were initiated by adding ten *H. azteca* to 250mL borosilicate glass beakers containing 40mL of wet sediment and 150mL of site water in 10-day toxicity experiments (Deaver and Rodgers, 1996). Three replicate experiments were also conducted with ten *C. dilutus.* Four sediments were used for toxicity tests, pre and post-treatment samples from the treated cove and 2 samples from the untreated cove. Three 7mm diameter leached maple leaf disks and 0.1mL TetraMin® (Tetra United Pet Group, Blacksburg, VA) slurry were added to each beaker at the start of the experiment as a food source for *H. azteca* and *C. dilutus,* respectively (Deaver and Rodgers, 1996). For *C. dilutus,* test chambers were aerated to maintain dissolved oxygen concentrations above 4mg O_2/L. Significant differences in organism responses to copper in the four sediments were determined using ANOVA and Tukey's tests [SAS (9.3)]. If significant differences in percent survival of benthic organisms were not discerned between treated and untreated coves, treated sediments were amended with a series of

concentrations of Algimycin®- PWF until a 10 day LC_{50} was discerned. The 10 day LC_{50} can be used as a reference value for understanding the concentration of copper where adverse effects to *H. azteca* could be anticipated for this specific sediment matrix. Post-treatment sediment from the treated cove was amended using a modified method outlined in Huggett et al. 1999. Specific volumes of 10,000mg Cu/L as Algimycin®- PWF were substituted for copper sulfate and added to 200mL site water prior to application to sediment. 10 *H. azteca* were then added and survival was measured after 10 days. Sediment copper concentrations were measured at the completion of the toxicity test using flame atomic absorption spectroscopy [Agilent PSD 120 Atomic absorption spectrometer (USEPA 3050b).

2.3. L. wollei responses to candidate algaecides

L. wollei and site water were collected from the treated cove in Lay Lake and transported to Clemson University. Algaecides selected for evaluation of algal responses in laboratory toxicity experiments included copper-based algaecides (Cutrine®-Ultra, Clearigate® and Algimycin®- PWF), a peroxide-based algaecide (Phycomycin®-SCP) and an adjuvant (Cide-Kick II) (Tables 4.1 and 4.2). As laboratory experiments are scaled versions of conditions in the field, algaecide and adjuvant concentrations were modified to accurately represent exposures that occur in the field. Algaecide and adjuvant concentrations applied for this experiment were based on the maximum label concentration and the assumption that the cove was 3.0 m deep (Table 4.2). Site water characteristics pre and post-treatment of 1) Phycomycin®-SCP and Algimycin®- PWF and 2) Phycomycin®-SCP,

Cide-Kick II and Algimycin®- PWF with algaecide concentrations adjusted for 3.0m water depth are displayed in Table 4.3.

For this screening level 14-day laboratory algal toxicity experiment, 0.5 g (± 0.09 g) of *L. wollei* was placed in 250 mL borosilicate beakers. Three replicates and untreated controls were tested for each algaecide treatment. Stock solutions of the copper based algaecides and Cide-Kick II were prepared within 4 hours of experiment initiation. To achieve nominal exposure concentrations, stock solutions were diluted in site water. Phycomycin®-SCP was weighed, added to the appropriate volume of site water and inverted until dissolution. Copper concentrations and H_2O_2 concentrations were confirmed with a flame atomic absorption spectrometer (Agilent PSD 120 Atomic absorption spectrometer (APHA, 2005) and methods outlined in Klassen et al. (1994), respectively.

Algal responses were measured on day 7 and 14 by wet weight (Bishop and Rodgers, 2012), chlorophyll *a* concentrations (APHA, 2005) and visual observations. The fluorescence method for chlorophyll *a* concentrations was modified from Standard Methods to account for the robust structure of *L. wollei* trichomes. *L. wollei* was frozen at -20°C for 24 hours, ground at room temperature and placed at -20°C again for 24 hours. Chlorophyll *a* concentration was measured using a SpectraMax M2 spectrofluorometer (Molecular Devices Corp, Sunnyvale, CA). Statistical differences in wet weight and chlorophyll *a* concentrations were identified using ANOVA and Tukey's test (SAS, 9.3). The algaecide treatment that

elicited a response significantly different from the untreated control was identified as a potential alternative treatment.

Using the lowest concentration of algaecide that elicits an algal response not significantly different from the maximum label concentration will decrease costs for treatment as well as decrease risks for non-target species. Following the screening level algal toxicity experiment the algaecide concentrations were discerned in the definitive algal toxicity test.

2.4. Pimephales promelas responses to algaecide treatments

Non-target species' responses are an important consideration for selection of an algaecide for a field site. A conservative experiment was designed to assess the difference in response of *P. promelas* to aqueous exposures from two algaecide treatments. The two treatment concentrations were calculated from 1) the concentrations applied in the ongoing algaecide treatment and 2) the concentrations used in the effective algaecide treatment identified in the present study. These algaecide concentrations were adjusted based on the assumption that all of the algaecide applied in a 3.0 m column of water was not mixed homogenously but was applied to 1L. *P. promelas* toxicity experiments were conducted with site water and 0.5g (± 0.09g) of *L. wollei.* Three untreated controls were tested along with the treatment of Phycomycin®-SCP and Algimycin®- PWF and the alternative treatment from the present study. Ten *P. promelas* fry (<24 hours old) cultured at Clemson University according to Lewis et al. (1994) were then added to each beaker containing *L. wollei,* site water and algaecide treatment. To ensure that the

dissolved oxygen concentration did not decrease to less than 4mg/L from the decomposing algae, aeration was maintained throughout the course of the experiment.

Results and Discussion

3.1. Physical and chemical sediment characteristics

In Lay Lake, sediments in treated coves were dominated by sand (>90%), had a low OM% (<0.05%), low AVS (<1μmol/g) and average CEC (2.05 and 5.00 meq/100g) in comparison to freshwater sediments in the United States (Suedel and Rodgers, 1991) (Table 4.4). Sediment samples from the untreated coves consisted of mostly silt (>50%) and had an average OM% (0.133 and 0.537%) (Suedel and Rodgers, 1991) (Table 4.4). The sediment sample collected on 23 September 2013 from the untreated cove had minimal AVS (0.077μmol/g) and an average CEC (4.15 meq/100g). The sediment sample collected on 7 October 2013 from the untreated cove had an order of magnitude greater AVS concentration and a high CEC (14.2 meq/100g) (Suedel and Rodgers, 1991) (Table 4.4). In general, sediment samples collected from the treated cove were dominated by sand had a low organic matter percentage and AVS concentration. Differences in sediment characteristics in terms of particle size, AVS and OM% for sediment collected from the treated cove on 23 September 2013 and 7 October 2013 are not environmentally significant (Table 4.4). Sediment characteristics within physiographic provinces are highly variable and can be as variable as characteristics among physiographic provinces across the United States (Suedel and Rodgers 1991). The similarity in sediment characteristics for sediment samples collected from the treated cove on two separate sampling dates suggests that these samples were representative. Generally, sediment samples collected from the untreated cove were dominated by silt.

Organic matter concentration (Besser et al. 2003), cation exchange capacity (Chapman et al. 1998), acid volatile sulfides (Allen et al. 1993), and % clay (Hoss et al. 1997) are inversely correlated with copper bioavailability. The bioavailability of copper is decreased in sediments with high organic matter, cation exchange capacity, sulfides and clays because of the strength of these ligands for binding copper. In the presence of sulfides, strong bonds are formed (K_{sp} for covelite=$10^{-22.19}$; Di Toro et al., 1992). The sediment characteristics in the sand-dominated treated coves, suggests that copper from these sediment samples may have a higher bioavailability because of the lack of available ligands for copper binding sites. To definitively determine the concentration of copper in which adverse effects to benthic invertebrates could be anticipated for this specific sediment matrix, sediment toxicity tests with benthic invertebrates were conducted.

MDLs for copper in sediment can be influenced by the sediment matrix, extraction procedure and analytical equipment (Willis et al., 2013). To confirm that the extraction procedure (EPA method 3050.b) and analytical equipment (GFAAS) had the sensitivity to discern an increase in copper from one or repeated applications, MDLs were estimated. MDLs for treated sediments were 1.5mg Cu/kg and MDLs for untreated sediments were 2 and 3mg Cu/kg for the samples collected on 23 September 2013 and 7 October 2013 respectively (Table 4.4).

A mass balance equation can be used to estimate the mass of copper that will partition to sediments from one algaecide application. The values used for the variables in this equation are scaled to a 3.0 m column with the same radius as a

standard 250 mL beaker for simplicity. Assuming that the copper from an application will partition to the top 3 cm of sediment (Liu et al. 2006), the application was applied 0.6 m above the sediment surface, and there is no bioturbation or accretion, one can conservatively predict the copper concentration in sediments from one application.

$$\frac{C\ (mg\ Cu) \times V\ (L)\ (1000g\ kg^{-1})}{V_1\ (L) \times S\ (g)} = \frac{MA\ (mg)}{MS\ (kg)} \qquad \text{(Eq. 1)}$$

Where: C = Mass of copper applied (0.94 mg Cu)

V= Volume of water in 3.0 m column with the diameter of a 250 mL beaker (11 L)

V_1 = Volume of water treated (2 L)

S = Mass of sediment in a 250 mL beaker at 3 cm depth (136.2 g)

MA = Mass of copper

MS = Mass of sediment

The estimated mass of copper that would partition to sediment from each application is 38.0mg Cu/kg. If the estimated mass of copper is greater than the MDLs for each sediment sample, then the incremental increase in copper concentration following an algaecide treatment should be discerned. As the measured MDLs were between 1.5 and 3mg Cu/kg, we had sufficient resolution to discern an increase in copper concentration from a single application and could also discern the additional copper from repeated applications (Table 4.5).

Measured acid soluble copper concentrations were significantly different among the four sediment samples ($\alpha = 0.05$, Table 4.5), although these differences

are likely not environmentally relevant. Reported 96 hour LC_{50}s for *H. azteca* and *C. dilutus* are 351 and 1,905mg Cu/kg, respectively, for a silt-dominated sediment containing roughly 5% organic matter (Suedel et al. 1996). As copper concentrations ranged from 3.078-9.043mg Cu/kg, it is unlikely that adverse effects on *H. azteca* and *C. dilutus* would be observed (Suedel et al., 1996; Willis and Rodgers in review).

3.2 Responses of H. azteca and C. dilutus to sediment samples

Laboratory toxicity experiments with *H. azteca* and *C. dilutus* were used to confirm the presence and degree of copper toxicity in sediments (Suedel et al., 1996). No significant differences in percent survival were measured from sediment samples collected from treated and untreated coves (Table 4.6). Percent survival ranged from 80-100%, which is an acceptable range in sediment toxicity experiments to conclude no significant effects (Table 4.6) (US EPA, 1996).

To provide a conservative estimate for a sediment copper concentration where adverse effects would be anticipated with sediments of similar characteristics (i.e. sand, low OM% and low AVS), sediment samples from the treated cove were amended with a series of concentrations of Algimycin®- PWF. Extracted and analytically determined copper concentrations from amended sediments ranged from 33.5mg Cu/kg to 413mg Cu/kg. Survival of *H. azteca* following exposures of copper from amended sediments did not follow a typical exposure response curve. Sediments amended with lower copper concentrations (i.e. 33.5-50.9mg Cu/kg) at the completion of the 10-day experiment had dense algal

mats covering the sediment surface. When the *H. azteca* were recovered at the termination of the experiment, *H. azteca* carapaces were found entrapped in the algal mats. The predicted 10-day LC_{50} value was calculated using the sigmoidal portion of the exposure response curve and measured sediment copper concentrations. The 10-day LC_{50} for *H. azteca* exposed to copper amended sediments was 168.43mg Cu/kg (95% fiducial limits, 144.49-193.27mg Cu/kg). The copper concentration where adverse effects to 50% of the benthic invertebrates (*H. azteca*) could be anticipated is two orders of magnitude greater than the copper concentration measured in treated sediments. For this site, the actual risk incurred would be inferred from the relationship between the rate and concentration of copper algaecide applications, the sedimentation rate and the rate and extent of formation of ligands (i.e. AVS and OM%) (Willis et al. 2013)..

3.3 Responses of L. wollei to candidate algaecides in a screening level experiment

In the screening experiment to determine an effective algaecide treatment to control *L. wollei*, targeted copper concentrations were 11mg Cu/0.5 g algae as Algimycin®-PWF and Cutrine®-Ultra and 6.3mg Cu/0.5 g algae as Clearigate® with a mean analytical Cu recovery between 102 and 105%. Targeted Phycomycin®-SCP concentrations were 118mg H_2O_2/0.5g algae with a mean analytical H_2O_2 recovery of 98% when Phycomycin®-SCP was applied as the first algaecide in the treatment and 72% when Phycomycin®-SCP was applied as the second algaecide in the treatment (Table 4.2). Analytically determined Phycomycin®-SCP concentrations

were consistently lower than targeted concentrations in this experiment. To measure a homogenous nominal H_2O_2 concentration, Phycomycin®-SCP was mixed until dissolution. It is likely that Phycomycin®-SCP would be more effective in a field application as the solid is applied directly to the algae (Bishop and Rodgers, 2011).

For the experiment comparing the responses of *L. wollei* to candidate algaecides, the treatment effectiveness was measured using wet weight, chlorophyll *a* concentration and visual observations. Based on these parameters, algaecides that were effective in the treatment of *L. wollei* included 1) Phycomycin®-SCP followed by Cutrine®-Ultra, 2) Clearigate®, 3) Phycomycin®-SCP followed by Clearigate®, 4) Phycomycin®-SCP followed by Algimycin®-PWF and 5) Phycomycin®-SCP followed by Cide-Kick II and Algimycin®-PWF (Fig. 4.1). Algal responses to the above algaecide treatments were approximately the same. For these algaecide treatments, wet weight decreased by 25% and, chlorophyll *a* concentration decreased by an order of magnitude 14 DAT (Figs. 4.1 and 4.2). A lower density, loss in buoyancy and a decrease in pigmentation for *L. wollei* following effective treatment were discerned based on visual observations. In this 14-day algal toxicity experiment, wet weight and visual observations were more closely correlated than chlorophyll *a* concentrations. Based on these results in terms of wet weight and visual observations as well as preliminary experiments, sequential treatments of Phycomycin®-SCP, Cide-Kick II and Algimycin®-PWF were

reliable for reducing the density of *L. wollei* and could be adapted to decrease risks to non-target species by adjusting Algimycin®-PWF concentrations.

3.4 Responses of L. wollei to Phycomycin®-SCP, Cide-Kick II and Algimycin®-PWF in a definitive experiment

To select an effective algaecide treatment for Lay Lake, it is important to consider risks to non-target species due to aqueous exposures of algaecides. Selecting the lowest concentration of algaecide that elicits control of *L. wollei* can decrease those risks as well as decrease cost of an algaecide treatment. Based on preliminary experiments, Phycomycin®-SCP and Cide-Kick II were applied at the maximum label concentration. In this definitive algal toxicity experiment a series of concentrations of Algimycin®-PWF were used to treat *L. wollei* to select an algaecide concentration that elicited a response not significantly different from the response achieved by the maximum label concentration. Targeted copper concentrations were 0.7, 3, 5, 8 and 11mg Cu/0.5 g algae, and analytically recovered copper concentrations were 120, 103, 99, 103 and 105%, respectively. Based on wet weight and visual observations, 3mg Cu/0.5 g algae as Algimycin®-PWF when applied in combination with Phycomycin®-SCP and Cide-Kick II was toxic to *L. wollei* (Fig. 4.3). This application would decrease the aqueous copper exposure from an algaecide treatment by 74% and also decrease the mass loading of copper to sediments from algaecide applications. The mass loadings of copper to sediments

were 38.0mg Cu/kg for the ongoing treatment and 10.9mg Cu/kg per application for the effective treatment identified in the current study.

3.5 Responses of P. promelas to candidate algaecides

Propagation of fish and wildlife is a designated use of Lay Lake, and responses of *P. promelas* to aqueous exposures of the effective algaecide treatment were of interest. Laboratory experiments to test the toxicity of a constituent to an organism often provide conservative estimates based on "worse case scenarios". This laboratory toxicity experiment was designed to be conservative in order to effectively assess differences in the responses of *P. promelas* to two alternative treatments. The treatments were 1) Phycomycin®-SCP (23.6mg H_2O_2/L) and Algimycin®-PWF (11mg Cu/L) applied sequentially and 2) Phycomycin®-SCP (118mg H_2O_2/L), Cide-Kick II (6.4mg Cide-Kick II/L) and Algimycin®-PWF (3mg Cu/L) applied sequentially. The algaecide concentrations used do not represent concentrations that would be applied in the field but were adjusted in order to identify differences in the response of *P. promelas* if a difference was evident. This experiment was conservative because the most sensitive life stage of naïve organisms opposed to resident organisms was confined to an area with the highest algaecide concentration. The avoidance response of fish to metals such as copper has been studied extensively (Sprague, 1964; Svecevicius, 2011). It is likely that fish in the field would swim away from an algaecide application and would decrease their exposure concentration and duration.

The responses of *P. promelas* to algaecide treatments were significantly different. In the untreated control, there was 100%±0% survival at the completion of the experiment. Percent survival of *P. promelas* exposed to Phycomycin®-SCP and Algimycin®- PWF was 0%±0% at the concentrations used in the present study. Percent survival of *P. promelas* exposed to Phycomycin®-SCP, Cide-Kick II and Algimycin®- PWF was 87±6% at the concentrations used in the present study. The algaecide treatment identified in the current study (Phycomycin®-SCP, Cide-Kick II and Algimycin®- PWF) could potentially enhance the margin of safety for fish from an algaecide application.

Again, the results displayed here serve as points of reference to compare the survival of *P. promelas* to different aqueous algaecide exposures in a conservative laboratory experiment. The conservatism of these experiments is confirmed by the presence of *Gammarus sp.* and *Hyalella sp.* in *L. wollei* samples collected 14 days after algaecide exposures. Reported LC_{50s} for copper as $CuSO_4$ for *P. promelas, Hyalella azteca* and *Gammarus pulex* are 0.66 mg Cu/L (96h), 0.43 mg Cu/L (48h) and 0.20 mg Cu/L (96h) respectively (Murray-Gulde 2002; Charles et al. 2014). Therefore, the presence of these organisms shortly after the algaecide treatment (14 days) suggests that no unreasonable adverse effects to organisms occurred after the ongoing algaecide treatment was applied.

Conclusions

This research provided a prospective and retrospective risk characterization for ongoing and alternative algaecide treatments in Lay Lake. The approach used was site specific although it could be applied to other sites and situations. The current research resulted in predictions and information that can be used to make informed decisions for water resource management for Lay Lake.

Following 10 years of copper-based algaecide treatment, no measurable copper residuals were present in sediments and no adverse effects to *H. azteca* and *C. dilutus* could be discerned in laboratory experiments from sediments collected from treated coves. The alternative algaecide treatment identified in this study may enhance the margin of safety for non-target species and decrease the calculated mass loading of copper to sediments by approximately 70% while maintaining the efficacy of the algaecide treatment for the target species (*L. wollei*).

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Table 4.1: Physical and chemical characteristics of candidate algaecides and the adjuvant Cide-Kick II. Characteristics are of original compound unless otherwise specified.

Characteristics	Algimycin®-PWF	Cutrine®-Ultra	Clearigate®	Phycomycin®-SCP	Cide-Kick II
Active ingredient	Copper [a]	Copper [a]	Copper [a]	Hydrogen peroxide [a]	d-limonene [a]
% Active ingredient	5.0 [a]	9.0[a]	3.8 [a]	85.0 [a]	100 [a]
Maximum label concentration as active ingredient	1mg /L [a]	1 mg /L [a]	0.6 mg /L [a]	10.2 mg /L [a]	0.5 mg /L [a*]
Formulation	Copper citrate and gluconate [a]	Copper ethanolamine in an emulsified complex[a]	Copper ethanolamines in an emulsified complex[a]	Sodium carbonate peroxyhydrate [a]	d-limonene and alcohol ethoxylated fatty acid [a]
Appearance	Blue liquid	Viscous blue liquid	Viscous blue liquid	Granular white solid	Clear yellow liquid
Water solubility	Miscible[**]	Miscible[**]	Miscible[**]	14,856 mg/L	Miscible[**]
Boiling point (°C)	100	113	108	100 for saturated solution	165
Specific gravity (g/cm^3)	1.25	1.20	1.06	1.10 for saturated solution	0.90
pH	1.7-1.9[b]	10.0-10.5 [b]	9.7-10.0 [b]	10.36 for saturated solution	4.36

[*] Calculated assuming 3.0m water depth
[**] Can mix with water in all proportions
[a]Applied Biochemists specimen labels
[b]Applied Biochemists MSDS

Table 4.2: Algaecide concentrations for *L. wollei* laboratory toxicity experiments

Algaecides/adjuvant	Experimental concentrations **	*In situ* concentrations
Phycomycin®-SCP	118 mg H_2O_2/0.5g algae	10.1 mg H_2O_2/L
Phycomycin®-SCP and Cutrine®-Ultra *	118 mg H_2O_2/0.5g algae and 11 mg Cu/0.5g algae	10.1 mg H_2O_2/L and 1 mg Cu/L
Cide-Kick II and Phycomycin®-SCP *	6.4 mg Cide-Kick II/0.5g algae and 118 mg H_2O_2/0.5g algae	0.2 mg Cide-Kick II/L and 10.1 mg H_2O_2/L
Clearigate®	6.3 mg Cu/0.5 g algae	0.6mg Cu/L
Phycomycin®-SCP and Clearigate® *	118 mg H_2O_2/0.5g algae and 6.3 mg Cu/0.5 g algae	10.1 mg H_2O_2/L and 0.6 mg Cu/L
Phycomycin®-SCP and Algimycin®-PWF *	118 mg H_2O_2/0.5g algae and 11mg Cu/0.5g algae	10.1 mg H_2O_2/L and 1.0 mg Cu/L
Phycomycin®-SCP, Cide-Kick II and Algimycin®-PWF *	118 mg H_2O_2/0.5g algae, 6.4 mg Cide-Kick II/0.5g algae and 11mg Cu/0.5g algae	10.1 mg H_2O_2/L, 0.2 mg Cide-Kick II/L and 1.0 mg Cu/L

* Subsequent algaecides were applied 24 hours later
** Calculated assuming 3.0 m water depth

Table 4.3: Site water characteristics for Lay Lake pretreatment and 4 days after treatment of ongoing and effective algaecide treatments from the current study.

	Pretreatment	Phycomycin®-SCP and Algimycin®-PWF	Phycomycin®-SCP, Cide-Kick II and Algimycin®-PWF
pH (S.U.)	8.16	7.69± 0.02	8.13± 0.07
Dissolved Oxygen (mg O_2 / L)	8.50	8.49± 0.04	8.51± 0.02
Alkalinity (mg $CaCO_3$ / L)	44	103± 15	323± 12
Hardness (mg $CaCO_3$ / L)	60	77± 15	80± 17
Conductivity (µS)	219.8	385.0± 7.0	766.6± 20.0
Temperature (°C)	22	22	22

Table 4.4: Physical and chemical sediment characteristics for treated and untreated coves from sampling events on 23 September 2013 and 7 October 2013. Timing of treatment (pre or post) was not applicable (NA) for the untreated cove because no algaecide was applied.

Physical/ chemical sediment characteristics	Pre or post-treatment	AVS (µmol/g)	% Sand	% Silt	% Clay	CEC (meq/100g)	OM(%)
Treated							
Sample collection date: 09/23/13	Pretreatment	0.120 ± 0.035	93.0	7.0	0.0	5.00	0.047
Sample collection date: 10/07/13	Post-treatment	0.387 ± 0.055	95.0	4.0	1.0	2.05	0.014 ± 0.008
Untreated							
Sample collection date: 09/23/13	NA	0.077 ± 0.033	37.3	58.7	4.0	4.15	0.133 ± 0.016
Sample collection date: 10/07/13	NA	13.02 ± 2.326	23.4	69.6	7.0	14.2	0.537 ± 0.027

Table 4.5: Method detection limits (MDL) and acid soluble copper concentrations for sediments from treated and untreated coves from sampling events on 23 September 2013 and 7 October 2013. Timing of treatment (pre or post) was not applicable (NA) for the untreated cove because no algaecide was applied.

Sediment Location	Pre or post-treatment	MDL (mg Cu/kg)	Acid soluble copper (mg Cu/kg)
Treated cove	pretreatment	1.5	5.486 $\pm$ 0.733
	post-treatment	1.5	3.078 $\pm$ 0.455
Untreated cove	NA	2	4.843 $\pm$ 0.303
	NA	3	9.043 $\pm$ 0.431

Table 4.6: Percent survival of *H. azteca* and *C. dilutus* exposed to sediments from treated and untreated coves from sampling events on 23 September 2013 and 7 October 2013 in 10 day laboratory toxicity experiments. Timing of treatment (pre or post) was not applicable (NA) for the untreated cove because no algaecide was applied.

	Sediment Location			
	Untreated Coves		**Treated Coves**	
Organism	Sample from 09/23/13 (% survival)	Sample from 10/07/13 (% survival)	Sample from 09/23/13 (% survival)	Sample from 10/07/13 (% survival)
Pre or post-treatment	NA	NA	pretreatment	post-treatment
Hyalella azteca	80	97	100	90
Chironomus dilutus	90	93	83	87

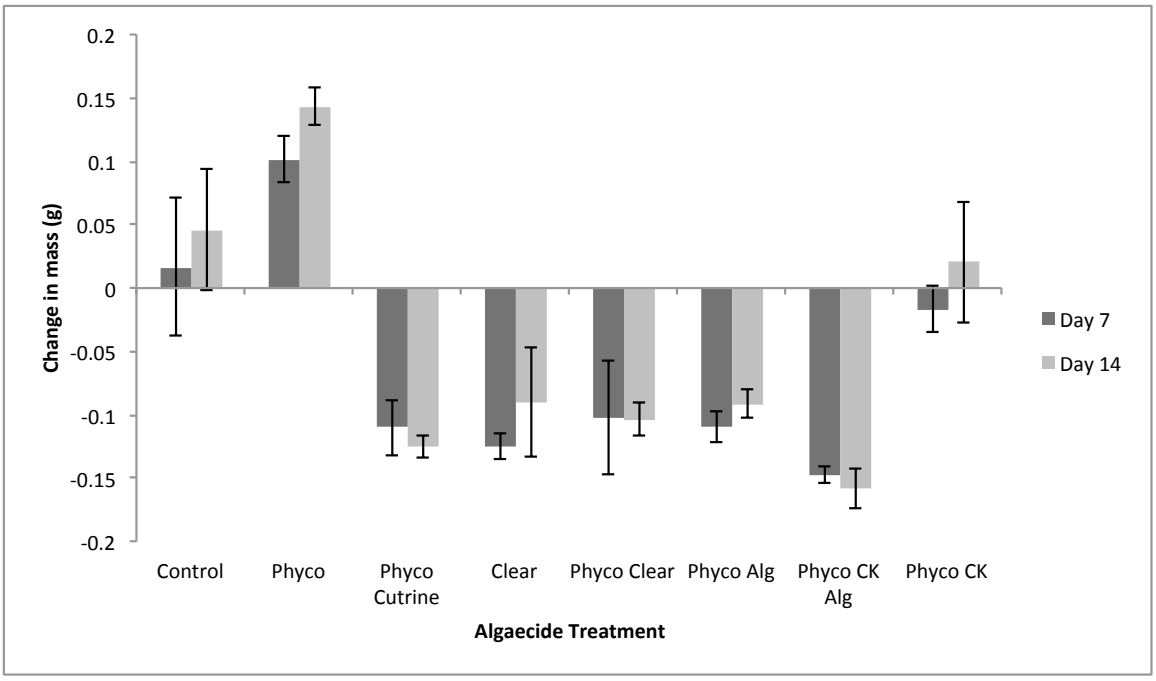

Figure 4.1: Responses of *L. wollei* measured by wet weights 7 and 14 days after treatment to candidate algaecide exposures.

Phyco= Phycomycin®-SCP

Cutrine= Cutrine®-Ultra

Clear= Clearigate®

Alg= Algimycin®-PWF

CK= Cide-Kick II

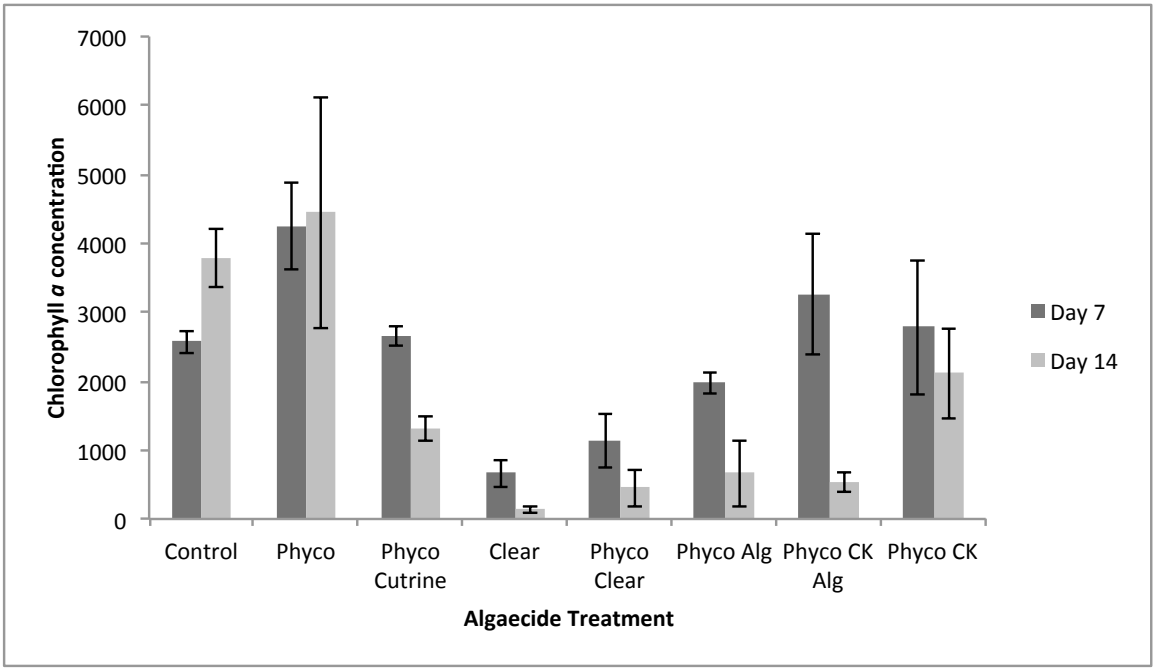

Figure 4.2: Responses of *L. wollei* measured by chlorophyll *a* concentrations 7 and 14 days after treatment to candidate algaecide exposures.

Phyco= Phycomycin®-SCP

Cutrine= Cutrine®-Ultra

Clear= Clearigate®

Alg= Algimycin®-PWF

CK= Cide-Kick II

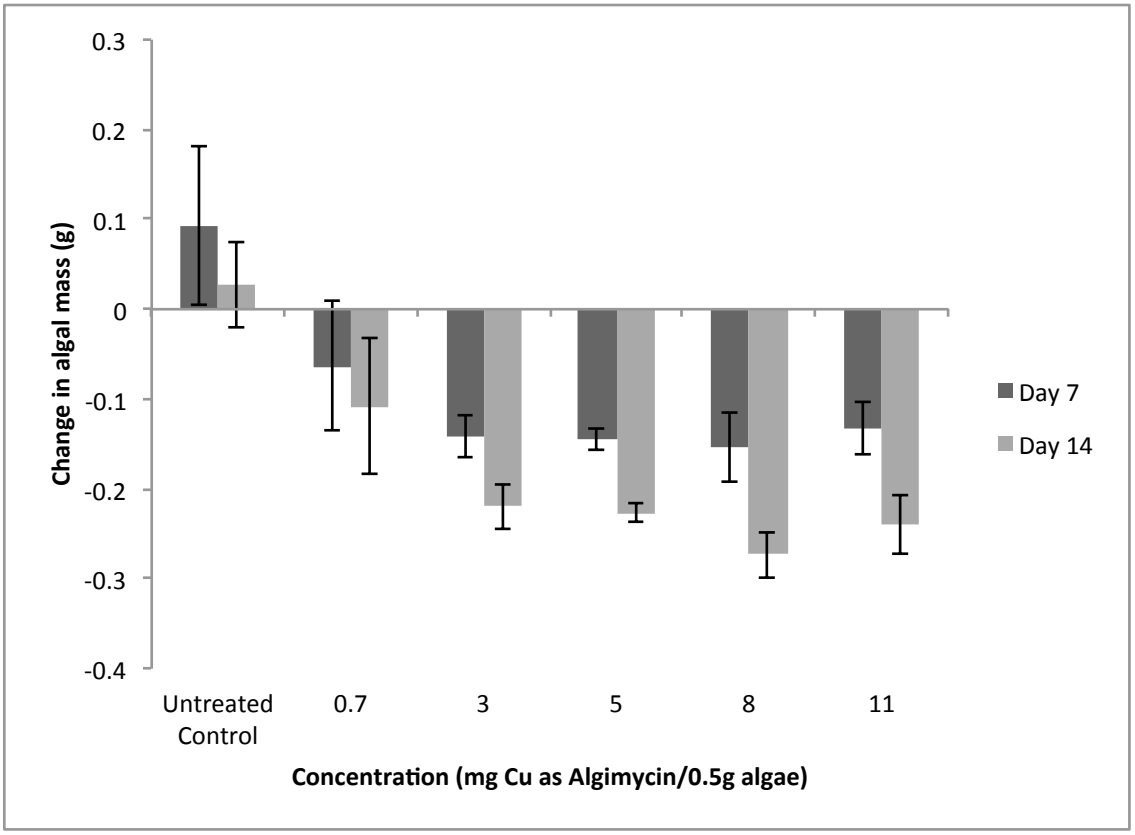

Figure 4.3: Responses of *L. wollei* measured by wet weights 7 and 14 days after treatment of Phycomycin®-SCP, Cide-Kick II and series concentrations of Algimycin®-PWF.

CHAPTER FIVE

Summary and Conclusions

Results from these studies aid in understanding responses of target and non-target species to exposures. Information from this research enhances the understanding of the advantages and limitations of algal viability measures for field-collected samples, mixed cultures and axenic cultures exposed to constituents. Results from this research have also provided insight for water resource management in Lay Lake.

The manuscript "Evaluation of six measures for algal (*Microcystis aeruginosa*, *Planktothrix agardhii* and *Pseudokirchneriella subcapitata*) viability" was a rigorous evaluation of six algal response parameters. This experiment utilized known live and dead cell ratios to discern the accuracy and precision of the response measures in a controlled laboratory environment. Cell density and erythrosin b staining have resolution to distinguish between algal taxa and would be useful for discerning responses of a genus from other algae in mixed cultures, natural assemblages as well as laboratory cultures. Neutral red and pheophytin *a* concentration lacked accuracy and precision. Chlorophyll *a* concentration was capable of discerning differences in algal viability in cultures dominated by one alga, but in a mixed culture or field-collected sample, light microscopy will aid in the utility of this parameter. INT formazan absorbance may be useful in an axenic culture, but lacked accuracy and precision in mixed cultures likely due to the respiratory activity of resident bacteria.

The manuscript "Responses of *Planktothrix agardhii* and *Pseudokirchneriella subcapitata* to copper sulfate ($CuSO_4 \bullet 5H_2O$) and a chelated copper compound (Cutrine®-Ultra)" utilized two copper-based algaecides to assess the accuracy and precision of algal response measures to a more realistic exposure. Cell density had a monotonic response curve and could therefore be used to calculate EC_{50s} and potency slopes for *Planktothrix agardhii* and *Pseudokirchneriella subcapitata* exposed to copper sulfate pentahydrate and Cutrine®-Ultra. *P. agardhii* was an order of magnitude more sensitive than *P. subcapitata* to the two copper based algaecides and Cutrine®-Ultra was approximately twice as potent as $CuSO_4 \bullet 5H_2O$ for both algae. Therefore, a lower concentration of copper applied as a chelated copper based algaecide as opposed to copper sulfate pentahydrate may be used in the field to reduce densities of toxin producing algae (i.e. *P. agardhii*) and maintain densities of green algae (i.e. *P. subcapitata*).

"Responses of *Lyngbya wollei* to algaecide exposures and a risk characterization associated with their use" provided laboratory data to address the potential for risk of two alternative treatments in Lay Lake. Following 10 years of copper-based algaecide applications at 0.94mg Cu/L as Algimycin®- PWF, no adverse effects to benthic invertebrates were discerned using sediment toxicity testing. An alternative algaecide treatment was identified with a similar efficacy for *L. wollei* as the ongoing treatment which remained effective after 10 years of periodic application. The alternative algaecide treatment included the use of Phycomycin®-SCP at 10.1 mg H_2O_2/L followed by Cide-Kick II at 0.2mg/L and

Algimycin®- PWF at 0.26mg Cu/L. Because this treatment had a lower concentration of copper (0.26mg Cu/L compared to 0.94mg Cu/L), the mass loading of copper to sediments would be decreased. In addition, the margin of safety for fish from aqueous algaecide exposures may be enhanced with the alternative algaecide treatment.

Ultimately, this research highlights important considerations to make in the selection of appropriate algal viability measures for experiments utilizing algae as indicators of exposures. The results from the third experiment were used to support algaecide application decisions for Lay Lake.