MANAGEMENT OF *PRYMNESIUM PARVUM* AT TEXAS STATE FISH HATCHERIES

Edited by

Aaron Barkoh and Loraine T. Fries

MANAGEMENT DATA SERIES
No. 236
2005

Texas Parks and Wildlife Department
Inland Fisheries Division
4200 Smith School Road
Austin, Texas 78744

INLAND FISHERIES DIVISION *PRYMNESIUM PARVUM* TASK FORCE

2001-2003 Members

Aaron Barkoh (Chair), *Heart of the Hills Fisheries Science Center*
Tom Dorzab, *Dundee State Fish Hatchery*
Loraine T. Fries, *Fish Health and Genetics Lab., A. E. Wood Fish Hatchery*
Jake Isaac, *Possum Kingdom State Fish Hatchery*
Gerald Kurten, *A. E. Wood Fish Hatchery*
John Paret, *Possum Kingdom State Fish Hatchery*
Dennis G. Smith, *Dundee State Fish Hatchery*
Gregory M. Southard, *Fish Health and Genetics Lab., A. E. Wood Fish Hatchery*
Jason Vajnar, *Dundee State Fish Hatchery*
H. Joe Warren, *Dundee State Fish Hatchery*

2004-2005 Members

Aaron Barkoh (Chair), *Heart of the Hills Fisheries Science Center*
Drew Begley, *Dundee State Fish Hatchery*
Loraine T. Fries, *Fish Health and Genetics Lab., A. E. Wood Fish Hatchery*
Pamela Hamlett, *Environmental Chemistry Lab*
David Klein, *Environmental Chemistry Lab*
Gerald Kurten, *Texas Freshwater Fisheries Center*
Dale Lyon, *Possum Kingdom State Fish Hatchery*
John Paret, *Possum Kingdom State Fish Hatchery*
Dennis G. Smith, *Dundee State Fish Hatchery*
Gregory M. Southard, *Fish Health and Genetics Lab., A. E. Wood Fish Hatchery*
Thomas A. Wyatt, *Dundee State Fish Hatchery*

CONTRIBUTING AUTHORS

Aaron Barkoh
Heart of the Hills Fisheries Science Center
5103 Junction Hwy
Ingram, TX 78025

Tom Dorzab
Dundee State Fish Hatchery
Route 1, Box 123A
Electra, TX 76360

Loraine T. Fries
A. E. Wood Fish Hatchery
507 Staples Road
San Marcos, TX 78666

Steven Hamby
A. E. Wood Fish Hatchery
507 Staples Road
San Marcos, TX 78666

Jake Isaac
A. E. Wood Fish Hatchery
507 Staples Road
San Marcos, TX 78666

David Klein
Inland Fisheries ECL
506 Staples Road
San Marcos, TX 7866

Gerald Kurten
Texas Freshwater Fisheries Center
5550 Flat Creek Rd
Athens, TX 75751

Dale Lyon
Possum Kingdom State Fish Hatchery
600 S Highway 16
Graford, TX 76449

John Paret
Possum Kingdom State Fish Hatchery
600 S Highway 16
Graford, TX 76449

Dennis G. Smith
Dundee State Fish Hatchery
Route 1, Box 123A
Electra, TX 76360

Gregory M. Southard
A. E. Wood Fish Hatchery
507 Staples Road
San Marcos, TX 78666

CONTENTS

PREFACE v

ACKNOWLEDGMENTS vi

CHAPTER 1: Management Strategies for *Prymnesium parvum* Ichthyotoxicity in Aquaculture 1

CHAPTER 2: Control of *Prymnesium parvum* using Ammonium Sulfate or Copper Sulfate in Plastic-Lined Ponds for Koi Carp Production 9

CHAPTER 3: Efficacy of Potassium Permanganate to Reduce *Prymnesium parvum* Ichthyotoxicity 17

CHAPTER 4: Toxicity of Copper Sulfate and Potassium Permanganate to Rainbow Trout and Golden Alga *Prymnesium parvum* 20

CHAPTER 5: Toxicity of Copper Sulfate Pentahydrate to Rainbow Trout *Oncorhynchus mykiss* 25

CHAPTER 6: Effects of pH on *Prymnesium parvum* Cell Viability and Toxicity 29

CHAPTER 7: Use of Hydrogen Peroxide as an Algaecide for *Prymnesium parvum* 35

CHAPTER 8: Efficacy of Nitrogen:Phosphorus Ratios for Controlling *Prymnesium parvum* in Fish Culture Ponds: Summary of 2002 Experiments 39

CHAPTER 9: Efficacy of Ultraviolet Radiation to Control *Prymnesium parvum* Cells and Toxicity 66

CHAPTER 10: Evaluation of an Ultrasonic Device to Control Golden Alga *Prymnesium parvum* in Fish Hatchery Ponds 71

CHAPTER 11: Microscopy and *Prymnesium parvum*: Observations and Challenges 74

CHAPTER 12: Dundee State Fish Hatchery *Prymnesium parvum* Management Plan 80

CHAPTER 13: Possum Kingdom State Fish Hatchery *Prymnesium parvum* Management Plan 85

REFERENCES 89

APPENDIX A: Identification and Enumeration of *Prymnesium parvum* cells, Version AEW-IDE 1.1 97

APPENDIX B: Standard Bioassay of *Prymnesium parvum* Toxin, Version AEW-ITU 1.2 99

APPENDIX C: Recommended Treatments for P. *parvum* Blooms using Liquid Mmmonia, Ammonium Sulfate, or Copper Sulfate as Related to Temperature and pH 102

APPENDIX D: Copper – Bathocuproine Method, Version AEW-COP 1.1 103

PREFACE

Although golden alga *Prymnesium parvum* appears to have been in Texas for more that 20 years, it only became a public issue in 2001 when it caused massive fish kills at Dundee State Fish Hatchery and in major economically important reservoirs in north and central Texas. Following the devastating effects of *P. parvum* on fish at the Dundee State Fish Hatchery, a group of Inland Hatcheries biologists formed the *P. parvum* Task Force to develop strategies to control the alga and maintain the viability of the fish hatcheries – Dundee and Possum Kingdom State Fish Hatcheries – located in the affected region.

After months of synthesizing available information, group discussions and conducting various research studies, the task force developed strategies for controlling the alga to allow successful culture of fish at the affected hatcheries. This document contains some of the written reports prepared by members of the task force on research findings, review summaries and management plans; a few reports published in mainstream journals are not included here. The purpose of this document is to consolidate the reports for easy access and to share what strategies work at Texas hatcheries and how to implement them. Because of design shortfall (e.g., small sample sizes or inadequate replications) associated with some of the research studies, we caution readers to consider some of the results as preliminary. Efforts continue to revisit some of these studies, as resources become available, to confirm findings as well as to conduct new research to find more effective and efficient ways of dealing with the alga. As new strategies are developed, the management plans in this document will be updated. We invite interested persons to call the Dundee or Possum Kingdom State Fish Hatchery from time to time for any future updates.

Aaron Barkoh
Loraine T. Fries

ACKNOWLEDGMENTS

This document is the culmination of cooperation, assistance and efforts provided by several individuals willing to serve in whatever capacity possible to contribute to finding a solution to the *Prymnesium parvum* problem in Texas. We thank Gary Saul and Dick Luebke for approving the formation of the Inland Hatcheries *P. parvum* Task Force, members of the task force for their dedication to duty, Roger McCabe and Joan Glass for participating in some of the task force meetings, and Annette Sudyka for help with literature search.

We appreciate the zeal and commitment of the contributing authors for making publication of this document possible as well as all those who helped them with their research efforts particularly the Inland Fisheries staffs at Dundee State Fish Hatchery, Possum Kingdom Fish Hatchery, Fish Health and Genetic Lab, and Management District 1C. These individuals spent long hours collecting data or water for research, or provided analytical assistance.

Jerry Brand and Alexandra Holland of the University of Texas – UTEX Culture Collection of Algae in Austin, Texas donated a pure *P. parvum* culture for research, and Isaac Bejerano and Elizabeth Maor of the Central Fish Health Laboratory in Israel offered expertise and protocols for monitoring *P. parvum* ichthyotoxin and treating toxic blooms in fish culture ponds. We are indebted to them for their generosity and professionalism.

Funding for the studies published in this document was provided in part by Federal Aid in Sport Fish Restoration, Grants FFD95-PARVM and FFD96-PARVM to the Texas Parks and Wildlife Department.

CHAPTER 1

Management Strategies for *Prymnesium parvum* Ichthyotoxicity in Aquaculture

GREGORY M. SOUTHARD AND LORAINE T. FRIES

Abstract

This report contains general information on the toxin-producing haptophyte, *Prymnesium parvum*, which began to threaten the Texas Parks and Wildlife Department (TPWD) Inland Fisheries Division hatchery program in 2001. Included are methods to identify and enumerate the organism, a bioassay to determine the ichthyotoxin level, and treatment or control methods. Additionally, this report documents efforts to modify the bioassay protocol for easier or efficient implementation at TPWD fish hatcheries.

Introduction

"Golden alga" is the name commonly applied to *Prymnesium parvum* - a small, halophilic organism of considerable economic importance due to its ability to produce ichthyotoxin. The organism has a cosmopolitan distribution and has been implicated in extensive fish kills in brackish waters across an extensive geographic range (e.g., Otterstrom & Steelmann-Nielson 1940; Holdway et al. 1978; Kaartvedt et al. 1991; Guo et al.1996; Lindholm et al. 1999), including Texas (James and de la Cruz 1989). Although the cells contain chlorophyll, phagotrophy has been documented in cultures of *P. parvum*, and the organism is believed to be a mixotroph capable of feeding on bacteria and protists of various sizes (Skovgaard et al. 2003). The organism also has a vitamin requirement in culture media (Droop 1954). The taxonomy of *P. parvum* has undergone a revision in recent years. Once considered a chrysomonad alga (e.g., Bold and Wynne 1983), the organism now is considered a haptophyte protist (Green and Leadbeater 1994).

P. parvum produces at least three toxins collectively known as prymnesins: they include an ichthyotoxin (Ulitzer and Shilo 1966), a cytotoxin (Ulitzer and Shilo 1970a) and a hemolysin (Ulitzer 1973). The toxic effect for all prymnesins appears to be a change in the permeability of cell membranes (Shilo 1971). Prymnesin is difficult to isolate and has been variously characterized as saponin, proteolipid, or carbohydrate (Igarashi et al. 1996). Igarashi et al. (1996, 1999) elucidated the structure and stereochemistry of prymnesin-1 and prymnesin-2 as glycosides. Prymnesins have been described and evaluated by various methods and detail (Yariv and Hestrin 1961; Ulitzer 1973; Kim and Padilla 1977; Shilo 1971, 1981; Kozakai et al. 1982; Meldahl et al. 1994, 1995; Igarashi et al. 1996, 1999), but the ichthyotoxin has received the most attention.

The ichthyotoxin produced by *P. parvum* affects gill-breathing aquatic animals including fish, mollusks, and brachiated tadpoles (Shilo 1967). In fish, the primary effect of

the ichthyotoxin is loss of selective permeability of the gill epithelial cells followed by mortality resulting from sensitization of the fish to any number of toxicants in the surrounding medium (Ulitzer and Shilo 1966; Shilo 1967). In affected ponds, fish have been observed to concentrate in the shallows and appear to attempt to leap from the water to escape (Sarig 1971). Gill repair occurs within hours if fish are removed during the early stages of intoxication into freshwater (Shilo 1967). Interestingly, there is no direct correlation between the *P. parvum* cell density and toxicity (Shilo 1981) probably because toxicity is enhanced by various environmental factors including temperature < 30°C (Shilo and Aschner 1953), pH > 7.0, and phosphate limitation (Shilo 1971).

In Texas, *P. parvum* has been implicated in fish kills in the Pecos River since at least 1985 (James and de la Cruz 1989). In 1989, a major fish kill occurred in the Colorado River near E. V. Spence Reservoir (TPWD, unpublished data). However, *P. parvum* had not been known at TPWD fish hatcheries until spring 2000 when Possum Kingdom Reservoir, the source of water supply for the Possum Kingdom State Fish Hatchery (PKSFH), and Lake Diversion, the source of water supply for the Dundee State Fish Hatchery (DSFH), experienced toxic blooms of *P. parvum*. The PKSFH was undergoing renovation at the time and was thus not in production, but many ponds at DSFH became toxic and the entire 2000 crop of striped bass *Morone saxatilis* and palmetto bass (*M. saxatilis* ♀ × *M. chrysops* ♂), in addition to many other species, was lost. Hatchery staff had to implement methods for identification and control of *P. parvum* toxicity. Although new to TPWD fish hatcheries, many references were available for *P. parvum*, including its control in aquaculture ponds and methods to assess toxicity (Sarig 1971). This report is a compilation of methods for identification and enumeration of *P. parvum*, a bioassay protocol to evaluate toxicity levels in water samples and *P. parvum* control methods. Also included are findings from investigations into bioassay protocol modifications to improve efficiency.

Identification of P. parvum

Examination of unpreserved, discrete water samples is crucial since *P. parvum* cells will pass through most plankton nets and may be distorted by fixatives. Cells can be detected even at low concentrations using fluorescent microscopy on live samples.

Green et al. (1982) described *P. parvum* N. Carter as small, subspherical, swimming cells, 8-10 μm long (maximum to 15 μm), with two equal or subequal heterodynamic flagella of 12-15 μm (up to 20 μm) long and a short (3-5 μm), flexible, non-coiling haptonema. Green et al. (1982) included the swimming motion in the species description as smoothly forward movement while the cell spins on the longitudinal axis and flagellar pole. Cells have two chloroplasts, yellow-green to olive in color, which may appear "c-shaped". Additional descriptors of *P. parvum* are found in Green (1982). Figure 1 has images of *P. parvum* using different types of light microscopy.

P. parvum cells have calcareous scales, which can be observed by electron microscopy and are used to distinguish congeneric species (Green et al. 1982; Chang and Ryan 1985; Green and Leadbeater 1994; Nicholls 2003). Classification of the haptophytes has undergone scrutiny and the distinction between *Chrysochromulina* and *Prymnesium* has

been considered 'arbitrary' and in need of review (Nicholls 2003): sequences of the 18S ribosomal DNA confirms the closeness of these two genera (Edvardsen et al. 2000). Indeed, Nicholls (2003) stated that there are no confirmed reports of *P. parvum* in fresh water in North America. However, the present consensus of workers in Texas is that the organism implicated in fish kills is *P. parvum.* Scientists at the University of Texas reported that DNA from *P. parvum* blooms in Texas and North Carolina are very similar, if not identical, and both also are very similar to the *P. parvum* isolate archived from England (e.g., UTEX LB995).

Enumeration of P. parvum

A light microscope with 400X magnification and a standard hemacytometer are used to enumerate *P. parvum* cells. Cells normally swim actively and it may be necessary to slow their movement for identification or enumeration, which can be achieved by refrigeration or the addition of a few drops of Lugol's solution to the water sample.

A hemacytometer consists of microscope slide with two counting chambers and a cover slip. Each chamber has a grid etched into the slide consisting of nine large squares. Each large square is 1 mm × 1 mm (i.e., 1 mm^2) and the depth under the cover slip is 0.1 mm; hence, the volume underneath the slide for each chamber is 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equal to 1 mL, cells/mL is calculated by multiplying the average count per large square by 10^4, unless samples are diluted in which case a correction factor must be introduced.

Errors that can lead to inaccuracies in hemacytometer counts include inadequate dispersion of cells (e.g., cells clumping in samples), inaccurate dilution of cell suspension, incomplete mixing of cell suspension, improper filling of chambers, and improperly cleaned slide or cover slip, which can result in the presence of air bubbles or dirt particles (Burleson et. al 1992). To avoid errors, the chamber is filled via pipette using capillary action, without under-filling or overfilling. Between counts, the hemacytometer and its cover slip should be thoroughly cleaned using 70% isopropanol solution and wiped dry with lens paper. Appendix A includes a standard operating protocol (SOP) for enumerating *P. parvum* using a hemacytometer.

A Bioassay for Toxicity

Israeli aquaculture has been affected by *P. parvum* for many years and the organism is now considered endemic in most ponds (Shilo 1967). Israeli scientists have developed methods for evaluating and controlling *P. parvum* ichthyotoxin, including a bioassay to measure ichthyotoxin units (ITU) (Ulitzer and Shilo 1964). An ITU is defined as the minimal amount of prymnesin required to kill all of the test organisms during a two-hour period at 28°C in test water to which a cofactor has been added. The bioassay described herein and in Appendix B is adapted from the one developed in Israel (Dr. Isaac Bejerano, Central Fish Health Laboratory, personal comm.), which is based on the work of Ulitzer and Shilo (1964).

A bioassay should be used whenever *P. parvum* cells are observed in pond water. However, not all water containing *P. parvum* becomes toxic to fish. A three-year survey of 900 ponds in Israel showed that only 2-6% of the *P. parvum*-infested ponds had enough ITU to kill fish (Sarig 1971). At Texas hatcheries, however, it has been observed that a higher percentage of *P. parvum*-infested ponds become toxic to fish if left untreated.

The bioassay is designed to detect prymnesin toxins at sublethal levels. Briefly, affected water is treated with a cationic activator (cofactor) such as 3'3-diaminodipropylamine (DADPA) and tris buffer to pH 9. The test is run for 2 hours at 28°C using fish suitable for the test vessels (i.e., sized to allow use of 4 fish without depletion of dissolved oxygen during the 2-hour test). The Israeli protocol suggests *Gambusia* as a test organism, but fathead minnows *Pimephales promelas* or guppies *Poecillia reticulata* are suitable substitutes.

Ideally, the test consists of side-by-side comparisons of control water (e.g., conditioned tap water or other water known to be free of *P. parvum*), control water with cofactor, undiluted pond water, undiluted pond water with cofactor, and a 1:5 dilution of pond water with cofactor. Death of test organisms in the undiluted pond water without cofactor is the most toxic condition, followed by death in the diluted pond water with cofactor added. Ambiguous results occur when death occurs in the control water or if there is partial mortality in one or more of the test vessels (e.g., if 2 of 4 test organisms die): if test water is toxic, all test organisms should die. The bioassay protocol is found in Appendix B.

Control of P. parvum Blooms

In addition to developing the bioassay procedure to detect the *P. parvum* ichthyotoxin, Israeli aquaculturists also developed strategies to control the alga. Copper sulfate at about 2 mg/L is recommended for *P. parvum* control at temperatures below 20°C (see Appendix C for more details). Ammonium sulfate is used at concentrations based on temperature and pH; however, it is not recommended for low temperatures (<15° C). Ammonium sulfate causes swelling and lysing of *P. parvum* cells (Shilo and Shilo 1953). Observations at DSFH and the literature suggest that un-ionized ammonia concentrations ≥ 0.20 mg/L are effective at controlling *P. parvum* (e.g., Barkoh et al. 2003).

Bioassay Test Manipulations

In an attempt to simplify and improve the efficiency of the Israeli bioassay procedure, several limited laboratory investigations were made including comparisons between fathead minnow fry and adult as test organism and 28°C vs. room temperature (i.e., 24°C) as test temperature. Brine shrimp *Artemia* spp. also was evaluated as a test organism. The methods and results of these trials are described below.

Fathead minnow (FHM) fry verses FHM adults.—Fathead minnows are a commonly cultured baitfish and test organism and are readily available as adults and sub-adults. However, the fry which are preferred as test animals since they take up less space and require smaller beaker are not always readily available. To ascertain that bioassays conducted with

larger FHM would be valid, the relative sensitivity of FHM fry and adults to the ichthyotoxin produced by *P. parvum* was evaluated.

Water from E. V. Spence Reservoir was collected in fall 2001 during a toxic *P. parvum* bloom event for this study. Cell density was estimated to be 3 million cells/mL using the protocol in Appendix A. Fry were tested in 50-mL volumes while 500-mL volumes of the test water were used for adults. The bioassay protocol (Appendix B) was followed for the side-by side comparison of FHM fry and adults as bioassay organism.

Fathead minnow fry appeared to be more susceptible to the *P. parvum* toxin than the adults. In the undiluted water sample, 50% of the FHM fry died compared to none of the adults (Table 1). Additionally, in the diluted water sample + cofactor there was complete mortality of all FHM fry after 2 hours compared to 50% of the adults. Although these results are somewhat unclear, we recommend using FHM fry for the evaluation of *P. parvum* ichthyotoxin. Fry should provide better detection of sublethal toxin concentrations than adults and thus promote a more conservative approach to therapeutic treatments of ponds.

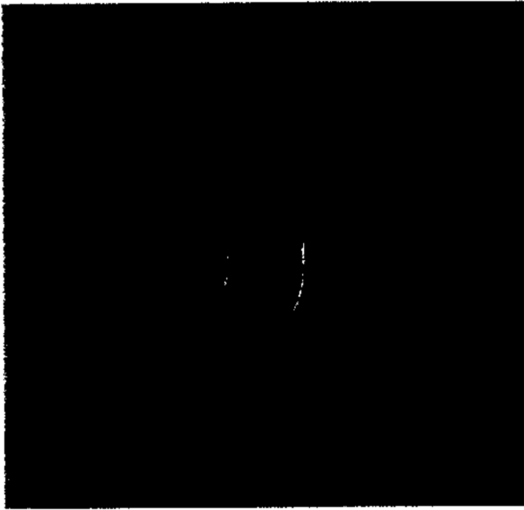
Toxin Activity at Ambient Temperature (e.g., room temperature, 24°C) versus 28°C.—It takes a considerable amount of time to bring the temperatures of water samples from hatchery ponds to the recommended temperature (28°C) for the bioassay test, in addition to tempering of the test fish. If the tempering step could be eliminated, the time required to conduct bioassays would be reduced allowing more time for hatchery personnel to focus on other activities. Thus, bioassay test results were compared at 28°C and ambient room temperature (i.e., 24°C). The water used in this test was the same as described above and the test organisms were FHM adults. Except for the temperature manipulation, test procedures followed Appendix B.

Fish mortality in the undiluted water + cofactor was less (75%) at 24°C than at 28°C, which had complete mortality (Table 1). However, there were no differences in mortality between temperatures in the diluted sample + cofactor or the undiluted sample without cofactor. Although this temperature comparison was conducted on a small scale, the results suggested that the bioassay should not be done at 24°C, as the toxicity of *P. parvum* ichthyotoxin was greater at 28°C than at 24°C. Since the purpose of the bioassay is to provide sensitive detection of *P. parvum* ichthyotoxin at sub-lethal levels in order to allow time to treat ponds or move fish, this test should be conducted at 28°C.

Evaluation of Artemia *spp. as Alternative Test Organism.*—Brine shrimp *Artemia* spp. commonly are used as aquatic test organisms. They are easily cultured on demand from commercially available cysts using established protocols. Furthermore, brine shrimp have been used to evaluate toxicity of laboratory-cultured *P. parvum* (Meldahl et al. 1994; Larsen and Bryant 1998). Those tests involved dose titrations of toxins extracted from algal cultures without use of a cofactor, and it is not clear how comparable those laboratory experiment results are to those of the standard bioassay. Brine shrimp have gill-like processes and may be susceptible to the ichthyotoxic prymnesin. If a correlation between ichthyotoxin and brine shrimp mortality can be established, brine shrimp could become a preferred test organism. Besides being available on demand, Artemia are very small and bioassays could be

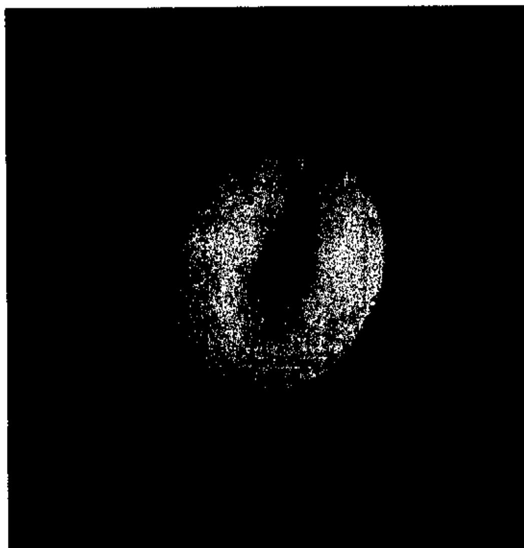
conducted in very small vessels, minimizing both space and tempering time required to conduct the test.

Our trials were conducted using water collected in February 2002 from E. V. Spence Reservoir with a cell density of 4,000 cells/mL. The standard bioassay (Appendix B) was used to compare the toxicity of the water to FHM fry and 24-hour-old brine shrimp. With the exception of the control water, the test was conducted using 4 replicates of 50 mL each per water type (undiluted, undiluted + cofactor, and diluted + cofactor). The water appeared very toxic to the FHM, as all fish died within 45 minutes except those in the control vessels. The brine shrimp appeared unaffected by the toxin and thus are unsuitable as a test organism for the bioassay.

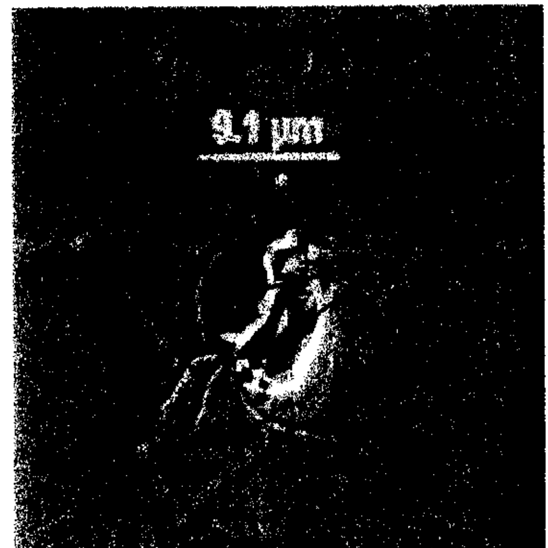


A.—Photo by Carmelo Tomas, University, North Carolina,Wilmington.

B.—Photo by John LaClaire, University of Texas, Austin



C.—Photo by Gregory M. Southard, TPWD, San Marcos.



D.—Photo by Gregory M. Southard, TPWD San Marcos.

FIGURE 1.—*Prymnesium parvum* under (**A**) bright field, (**B**) phase contrast, and (**C**) epifluorescent miscroscopy. (**D**) diagram of key morphological characteristics.

TABLE 1.—Susceptibility of fathead minnow fry and adult to *Prymnesium parvum* ichthyotoxin, and activity of ichthyotoxin at 28°C verses ambient room temperature (24°C) for standard toxin bioassay. Details of the bioassay procedure are found in Appendix B.

Water Sample	Percent Mortality*			
	Fry	Adult	28°C	24°C
Undiluted	50	0	0	0
Undiluted + cofactor**	100	100	100	75
1/5 Dilution + cofactor**	100	50	50	50
Control	0	0	0	NA
Control + cofactor**	0	0	0	NA

* Separate bioassays were conducted for fathead minnow fry vs. adults and for 28°C vs. ambient temperature (24°C).
** Cofactor = 0.003 M 3,3'-iminobispropylamine (DADPA)/0.02 M Tris, pH = 9.0
N/A = not available

CHAPTER 2

Control of *Prymnesium parvum* using Ammonium Sulfate or Copper Sulfate in Plastic-Lined Ponds for Koi Carp Production

DENNIS G. SMITH

Abstract

Prymnesium parvum is a toxic alga that has been responsible for numerous fish kills in reservoirs, rivers, and hatcheries in Texas and many other locations around the world. We compared the effectiveness of ammonium sulfate and copper sulfate treatments in controlling *P. parvum* and its associated toxicity in plastic-lined ponds for rearing koi carp, a strain of common carp *Cyprinus carpio,* fingerlings. Treatments were 9.5-mg/L ammonium sulfate, 2-mg/L copper sulfate, and control (no chemical treatment). After the initial treatments, treatments were reapplied to all ponds if weekly bioassay results revealed ichthyotoxin in any pond. All ponds were harvested after 75 or 76 days of koi carp rearing. Bioassay and cell density results revealed that ammonium sulfate and copper sulfate were effective in reducing *P. parvum* density and toxicity. Fish production was highest in ammonium sulfate ponds, followed by the copper sulfate ponds and was zero in the control ponds. The copper sulfate treatment ponds had a mean net loss of 0.9 kg in fish biomass while the ammonium sulfate treatment ponds had a net gain in mean fish biomass of 266.8 kg. Both chemical treatments were effective in controlling *P. parvum*; however, ammonium sulfate is recommended because fish production was significantly ($P \leq 0.05$) better in ponds treated with that chemical.

Introduction

The presence of the halophilic phytoflagellate *Prymnesium parvum* and its associated toxins in Dundee State Fish Hatchery (DSFH) ponds during spring 2001 resulted in the combined loss of over 5 million fry and fingerling striped bass *Morone saxatilis* and palmetto bass (female *M. saxatilis* × male *M. chrysops*). In addition, significant numbers of smallmouth bass *Micropterus dolomieu* and largemouth bass *M. salmoides* brood stock, adult rainbow trout *Oncorhynchus mykiss*, and fingerling koi carp and channel catfish *Ictalurus punctatus* also perished.

Various control methods, including applications of ammonium sulfate, copper sulfate and mud, nutrient manipulation, and reduction in salinity, have been employed to control *P. parvum* throughout the world with mixed results (Guo et al. 1996). Of these, ammonium sulfate and copper sulfate generally are most frequently recommended (Sarig 1971). Ammonium sulfate, when added to a pond, raises the total ammonia level. It has been suggested that the un-ionized portion of the total ammonia is responsible for causing the *P. parvum* cells to swell and lyse (Shilo and Shilo 1953, 1962). This is supported by the fact

that ammonium sulfate treatments are more effective in controlling *P. parvum* at higher temperatures and pH levels (Shilo and Shilo 1953). At higher temperature and pH levels, a greater proportion of the total ammonia exists in the un-ionized form (Emerson et al. 1975), which helps explain why ammonium sulfate treatments are not always effective (Guo et al. 1996). Copper sulfate is usually effective at eliminating *P. parvum* from ponds; however, it is an indiscriminate algaecide and its use may result in anoxic conditions due to decomposition of dead algae or reduction of oxygen production from photosynthetic activity. Copper sulfate also is toxic to a variety of fish species (Irwin 1997) and its toxicity to phytoplankton and fish is influenced by alkalinity (Boyd 1990).

Because the DSFH water supply has high levels of alkalinity (88-90 mg/L as $CaCO_3$) and none of the published literature on control of *P. parvum* was conducted in plastic-lined ponds, we evaluated the effectiveness of ammonium sulfate and copper sulfate at the recommended doses for controlling *P. parvum* in plastic-lined ponds. The specific objectives were to determine the effects of ammonium sulfate (10 mg/L) and copper sulfate (2 mg/L) on *P. parvum* density and toxicity and koi carp production in plastic-lined ponds.

Materials and Methods

This study was conducted at the DSFH near Wichita Falls, Texas from August through mid-October 2001. The experimental design was three replicates of each of ammonium sulfate (9.5 mg/L), copper sulfate (2 mg/L), and untreated control. Treatment concentrations were selected from the recommendations in published literature (Sarig 1971). The recommended minimum dose of 10 mg/L ammonium sulfate was not used due to an investigator error that led to an initial treatment rate of 9.5 mg/L which also was used in follow-up treatments for consistency. All treatments were based upon full pond volumes.

Nine 0.4-ha plastic-lined ponds were utilized for this study. The catch basin of each pond was cleaned of sediment, and containment screens were installed prior to filling ponds. Fill water was filtered through 500-µm socks to prevent contamination by wild fish. Pond filling was started on the same day and all ponds reached full volume at least two days prior to stocking of fish. Once full, no additional water was added to the ponds in an attempt to prevent reintroduction of *P. parvum* from Lake Diversion, the water source for the hatchery. The water characteristics were alkalinity 88 mg/L as $CaCO_3$, hardness 900 mg/L as $CaCO_3$, pH 8, and 4 ppt salinity. Ponds were randomly assigned to the treatment and control groups. Initial treatments were applied to the ponds two days prior to stocking of fish. Water samples were collected from each pond prior to treatment and again the day after treatment for bioassay and *P. parvum* enumeration.

Temperature, dissolved oxygen, and pH were measured and recorded twice daily with a Yellow Springs Instrument® (YSI) model 600XL sonde attached to an YSI model 610DM data logger. Once every seven days, water was collected from each pond to perform cell enumeration, conduct a bioassay, and measure nitrogen and phosphorous concentrations using established protocols (Appendix A; Appendix B; APHA 1995). Total ammonia was measured with an ion specific Accumet® brand electrode connected to a Denver Instruments® model 250 meter, and phosphorous concentrations were determined using the

stannous chloride method (APHA 1995). Test fish for the bioassay were wild-caught red shiner *Cyprinella lutrensis* that were maintained in a laboratory culture. A positive bioassay result (half or more of the test fish dying in any one bioassay container using water from a treated pond) triggered follow-up treatments of all ponds. If follow-up treatments were required, bioassays and cell enumerations were performed again the day after the treatments to monitor treatment effects. Copper concentrations in ponds were not measured because materials required for the testing were not available to investigators during the study.

Koi carp fingerlings (≈ 27 mm TL) were obtained from the A. E. Wood Fish Hatchery in San Marcos, Texas on 2 August 2001 and transported to DSFH. These fish were stocked into ponds at 250,000 fish/ha. Feeding of fish commenced the day after stocking and fish were fed a daily ration of 6% body weight based on the initial stocking weight. The fish were fed equal rations twice per day using a mechanical feed blower. Eleven days after stocking and every seven days thereafter, fish in each pond were sampled by seining. At least one hundred fish were collected from each pond, counted, and weighed in aggregate to determine number fish per kg. The number fish per kg and the initial number of fish stocked were used to estimate the biomass of fish in each pond. Feed amounts were adjusted according to biomass estimates each week. Thirty randomly selected fish from each sample also were measured for total length.

The fish were harvested by completely draining the ponds, allowing the fish to concentrate in the pond catch basins, and removing them with a plastic basket attached to a crane. At harvest, the total biomass, mean total length, and number of fish per kg were determined for each pond. The total biomass and number per kg were used to estimate the total number fish harvested. Six ponds (two each from the treatments and control) were harvested on 16 October 2001 and the remaining three were harvested the following day. These production data were compared using a t-test.

Results and Discussion

Follow-up treatments were triggered on two occasions. On 27August 2001, a bioassay indicated that three ponds contained ichthyotoxin: two ponds were ammonium sulfate treatment and one was a copper sulfate treatment. Mean cell density in the ammonium sulfate treatment ponds was 3,333 cells/mL at that time but decreased to 667 cells/mL one day after treatment. Cells were not detected in the copper treatment pond containing the ichthyotoxin either on August 27 or 28. Bioassays were performed again on August 28 and no test fish died in water from any of the treated ponds. Bioassays and cell enumerations conducted on 17 September 2001 indicated that one of the ammonium sulfate treated ponds had ichthyotoxin and a cell density of 48,000 cells/mL. All ponds were treated again, and cell counts and bioassays conducted the following day revealed neither cells nor toxicity in any of the treated ponds.

In one of the control ponds, *P. parvum* cells were first detected on 6 August 2001 and again on 27 August 2001. Thereafter, *P. parvum* persisted in the control ponds and steadily increased in density, reaching a peak mean of 66,000 cells/mL on 18 September 2001, and then gradually declined. Cells remained in the control ponds up to the final cell counts

performed one week prior to fish harvest. There were no positive bioassays in water from the control ponds until August 27. Beginning on that date, all but five of the 27 bioassays conducted with water from the control ponds indicated the ichthyotoxin was present.

On 28 August 2001, fish in one of the untreated control ponds appeared stressed. Mortalities were noticed the following day and continued through 31 August 2001. No live fish were collected from this pond during a weekly seine sampling conducted on 4 September 2001. Signs of stress and mortality were noticed in the other two control ponds on 4 September 2001 and continued to worsen throughout the week. No live fish were collected from any of the control ponds during weekly seine sampling conducted on 10 September 2001 or thereafter, and no fish were harvested from any of the control ponds.

When follow-up treatments were applied, the same amount of chemical as the initial treatment was used. Pond water levels declined from evaporation, and actual pond volumes were not determined, which resulted in higher application rates for the follow-up treatments. Doses higher than the target are evidenced in Figure 1, which illustrates progressively higher total ammonia levels on the days after treatments as the pond volumes were reduced by evaporation over time.

Table 1 summarizes cell enumeration, bioassays, nutrient analysis, and water quality measurements. Mean cell density was four-fold higher in the ammonium sulfate treatment ponds than in the copper sulfate treatment ponds, however the difference was not statistically significant. Conversely, cell densities in the treated ponds were significantly lower than that of control ponds, indicating that both treatments were effective at reducing *P. parvum* populations. The results of bioassays showed a similar trend, implying that reducing cell densities also diminished toxin levels.

As expected, total ammonia was significantly higher in the ammonium sulfate treatment ponds than in the copper sulfate treatment or control ponds. Other studies have shown that the total ammonia concentrations in earthen ponds following applications of ammonium sulfate were reduced by half in 24 hours (Shilo and Shilo 1953, Sarig 1971). During this study, total ammonia concentrations declined by approximately 50% per week in the plastic-lined ponds (Figure 1). Although more frequent sampling would better show the rate of decline, ammonia was more persistent in our plastic-lined ponds than has been reported for earthen ponds. Phosphorous levels were low and similar among treatment and control groups. Afternoon temperatures were similar among treatments whereas morning temperature was significantly lower in control ponds. However, the small temperature difference was likely not biologically significant. Morning dissolved oxygen levels did not significantly differ among treatment and control groups; while afternoon dissolved oxygen level was highest in ammonium sulfate treatment ponds, followed by the control, then copper sulfate ponds. Similarly, the greatest diel shift in mean dissolved oxygen level was found in the ammonium sulfate ponds while the least was found in the copper sulfate treatment ponds, probably a reflection of the differences in phytoplankton standing crops among treatment and control groups.

Mean morning and afternoon pH readings were significantly different among treatments and control ponds (Table 1). Both morning and afternoon mean pH levels were significantly lower in copper sulfate ponds that in the ammonium sulfate treatment or the control ponds. Ammonium sulfate treated ponds also had the greatest diel shift in pH values, followed by the control, then copper sulfate ponds. The diel shifts in dissolved oxygen and pH levels are indicators of primary productivity with larger shifts indicating greater primary productivity. Thus, primary productivity was highest in the ammonium sulfate treatment ponds that were richer in nitrogen. The copper sulfate treatment ponds had the least amounts of primary productivity and probably had the lowest mean phytoplankton standing crop (Boyd 1990).

Initial numbers, weights, and mean lengths of fish stocked were not significantly different among treatments and control (Table 2). Mean production days (75.3) were identical for all treatments. Harvest information varied significantly among treatment and control groups. Ammonium sulfate treatment ponds had significantly higher fish biomass, survival, and mean length than copper sulfate treatment ponds. No fish survived in the control ponds. Fish in one of the ammonium sulfate treated ponds suffered some mortality during harvest due to high sediment loading and low dissolved oxygen levels. The loss was estimated at 25 kg of fish and excluded from data analysis. If these fish had not been lost, harvest differences between treatments would have been even more pronounced. Ponds treated with copper sulfate had a net loss of biomass from the time of stocking to harvest, even though the mean length at harvest was almost 20 mm greater than the length at stocking, due to the low mean survival rate in these ponds.

During weekly seine sampling, fish collected from the ammonium sulfate treated ponds appeared to have a prevalence of gill hyperplasia, with the gill filaments actually extending beyond the operculum. This observation was confirmed by laboratory examination and although no direct cause could be determined, the hyperplasia could have been the result of exposure to high ammonia concentrations (Thurston et al. 1984). Another possible explanation is that the fish could have mistakenly ingested ammonium sulfate granules as food particles since both feed and ammonium sulfate were distributed with the same machinery. However, despite the hyperplasia, survival from these ponds was comparatively high (58.3%) and biomass production was well above the historic average of 53% survival and for DSFH koi fingerlings reared in plastic-lined ponds.

Summary

Complete fish mortality in the control ponds dictate that some form of control must be employed to prevent fish losses when *P. parvum* is present in ponds. Treatment with ammonium sulfate or copper sulfate was effective in reducing *P. parvum* density and toxicity. Both appeared to work within 24 hours after treatment. Although *P. parvum* cells persisted in some ponds the day after treatment, none of the treated ponds were toxic. Due to large differences in harvested biomass, survival, and mean total length of fish, ammonium sulfate appears to be much better than copper sulfate for controlling *P. parvum* in plastic-lined ponds for rearing koi carp when temperature and pH are high enough to make such

treatments feasible. The ammonia concentration applied to plastic-lined ponds was reduced to about 50% in approximately one week.

TABLE 1.—Mean values of water quality variables, nutrient levels, cell density, and toxicity in plastic-lined ponds for rearing koi carp fingerlings treated to control *Prymnesium parvum* with 9.5 mg/L ammonium sulfate or 2 mg/L copper sulfate, or untreated control. Values in a row bearing the same letter are not significantly different ($P > 0.05$). Standard deviations are in parentheses.

Variable	Treatment		
	$(NH_4)_2SO_4$	$CuSO_4 \cdot 5H_2O$	Control
Cell density (no./mL)	1,867 z (7,266)	400 z (1,175)	16,089 y (25,023)
Toxicity (bioassay % mortality)			
Whole water	6.1 z (22.2)	2.9 z (11.7)	50.0 y (49.0)
1:5 dilution	5.6 z (22.0)	2.4 z (11.4)	35.3 y (47.2)
Total ammonia (mg/L)	1.06 z (1.05)	0.05 y (0.10)	0.07 y (0.09)
Phosphorous (mg/L)	0.004 z (0.004)	0.003 z (0.004)	0.004 z (0.006)
Temperature (°C)			
Morning	24.0 z (3.9)	23.8 z (3.9)	24.3 y (3.7)
Afternoon	26.0 z (3.4)	25.9 z (3.8)	26.4 z (3.6)
Dissolved oxygen (mg/L)			
Morning	6.7 z (0.9)	7.1 z (0.8)	7.0 z (1.0)
Afternoon	9.3 z (1.0)	8.4 y (1.0)	8.9 x (0.9)
pH			
Morning	8.69 z (0.37)	8.28 y (0.24)	8.75 x (0.31)
Afternoon	8.92 z (0.36)	8.34 y (0.23)	8.88 x (0.32)

TABLE 2.—Mean ± SD values of harvest variables for koi carp fingerlings reared in plastic-lined ponds and treated to control *Prymnesium parvum* with 9.5 mg/L ammonium sulfate or 2 mg/L copper sulfate, or untreated control. Values in a row bearing the same letter are not significantly different ($P > 0.05$). Standard deviations are in parentheses.

	Treatment		
Harvest variable	$(NH_4)_2SO_4$	$CuSO_4 \cdot 5H_2O$	Control
Number	58,958 z (15,255)	23,800 y (4,804)	0 x
Weight (kg)	299.8 z (52.2)	30.7 y (4.0)	0 x
Length (mm)	71.3 z (3.6)	46.7 y (2.0)	0 x
Survival (%)	58.3 z (15.0)	23.3 y (4.5)	0 x

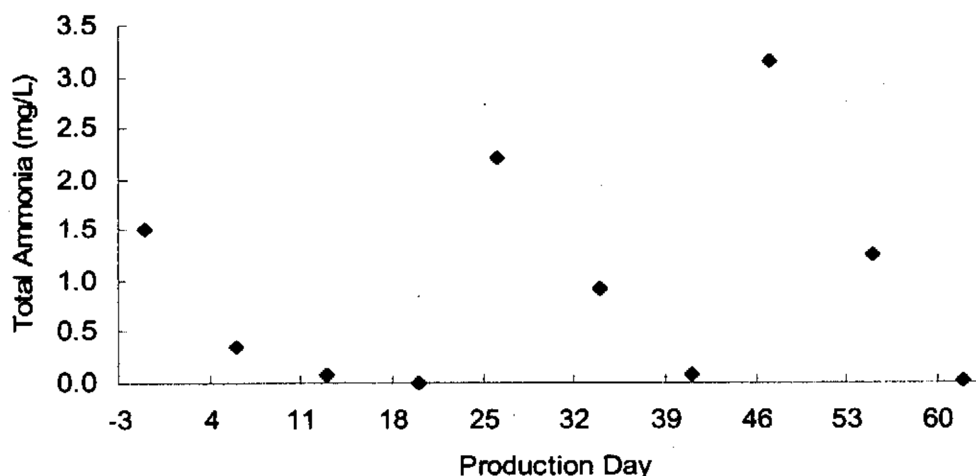


FIGURE 1.—Mean total ammonia levels in ammonium sulfate treatment ponds. Initial treatments were applied two days prior to fish stocking (production day = 0). Follow-up treatments were applied on days 25 and 46 after stocking.

CHAPTER 3

Efficacy of Potassium Permanganate to Reduce *Prymnesium parvum* Ichthyotoxicity

DENNIS G. SMITH

Abstract

Highly toxic water containing *Prymnesium parvum* and associated ichthyotoxin was subjected to potassium permanganate treatments to determine the minimum effective concentration required to detoxify the toxin and allow fish survival. Potassium permanganate concentrations of 0-6 mg/L were tested at 2-mg/L intervals. Bioassays using fathead minnows were performed shortly after the toxic water was treated and test fish were observed for mortality at 15-minute intervals for 2 hours. All fish died in the control (0 mg/L $KMnO_4$) and 2-mg/L $KMnO_4$ treatments within 30 min and 90 min, respectively, while no fish died in the 4- or 6-mg/L potassium permanganate treatments. Because the potassium permanganate demand of the toxic water was 2 mg/L, it was concluded that potassium permanganate mitigated the ichthyotoxin at a minimum residual concentration of 2 mg/L (i.e., concentration above the potassium permanganate demand). Further research involving better resolution of potassium permanganate treatment concentrations would be needed to define the true minimum effective concentration of potassium permanganate that would detoxify the ichthyotoxin.

Introduction

The presence of *Prymnesium parvum* and the production of toxins (collectively called prymnesins) by this alga in fish rearing ponds at the Dundee State Fish Hatchery (DSFH) have resulted in significant fish mortalities. Effective control strategies must be implemented to make fish production possible. Control methods may target the alga or the toxin (Shilo and Shilo 1953) although preventing toxin accumulation by controlling *P. parvum* blooms appears to be the most popular strategy. This study targeted the ichthyotoxin to determine if and at what concentration potassium permanganate could neutralize the ichthyotoxin and consequently prevent fish mortality. Potassium permanganate has the potential to detoxify the ichthyotoxin due to its oxidative properties which are reported to detoxify fish toxins such as rotenone and antimycin (Lawrence 1956; Marking and Bills 1975).

Potassium permanganate is a widely used chemical. It oxidizes organic matter, reduces inorganic substances, and has been used as an antimicrobial to treat several fish diseases. However, potassium permanganate is toxic to phytoplankton, fish, and bacteria at relatively low concentrations (Boyd 1990). The amount of permanganate that quickly is reduced to manganese dioxide in water is called the potassium permanganate demand of the water (Boyd 1990) making the effectiveness of treatments strongly influenced by water quality characteristics and efficient use of potassium permanganate requires determination of

the potassium permanganate demand. The specific objective of this study was to determine the minimum effective concentration of potassium permanganate above the ambient demand required to detoxify *P. parvum* ichthyotoxin and allow fish survival for at least 2 h.

Materials and Methods

In order to calculate effective treatment rates, potassium permanganate demand was determined according to Boyd (1979). Dose titration for ichthyotoxin detoxification was then conducted using 12 1.9-L glass jars. Each jar was filled with 1 L of water obtained from a pond known to contain *P. parvum* density of 86,000 cells/mL and be highly toxic to fish (i.e., a bioassay resulted in complete mortality of all test fish in whole pond water without the co-factor, Appendix B). Appropriate volumes of a 1000-mg/L potassium permanganate stock solution were added to the test containers to achieve 0, 2, 4, or 6 mg/L concentrations. These concentrations were selected based partly on the potassium permanganate demand of the water and the recommendation of Tucker (1989) and each treatment concentration was in triplicate. Four or five test fish were placed into each container shortly after the treatments were applied to the test water. The fish were observed every 15 minutes for 2 hours and the total number of dead in each container was recorded. The water temperature was 27.8°C and both the temperature and duration of the test were based on the toxicity bioassay protocol in Appendix B

Results and Discussion

The potassium permanganate demand of the water was determined to be 2 mg/L. In the detoxification test, fish mortality was 100% for the 2-mg/L potassium permanganate treatment and 0% for the 4-mg/L or 6-mg/L potassium permanganate treatment (Table 1). Because the potassium permanganate demand of the test water was 2 mg/L, the minimum residual concentration of potassium permanganate that detoxified the water and allowed test fish survival was 2 mg/L. These results suggest that a minimum potassium permanganate concentration of 2 mg/L above the permanganate demand may be used for at least a short-term relief of ichthyotoxicity to allow culturists time to implement measures that eradicate *P. parvum* and thus prevent further production of ichthyotoxin. Future research should address the long-term effects of potassium permanganate on the ichthyotoxin and *P. parvum* populations. In this study, a residual concentration of 2 mg/L of potassium permanganate was the lowest concentration tested. Residual concentrations < 2 mg/L should be tested to define the true minimum effective concentration required to detoxify the ichthyotoxin.

TABLE 1.—Bioassay results for toxic pond water treated with various concentrations of potassium permanganate or not treated (control) to neutralize the toxin produced by *Prymnesium parvum*.

Treatment	Replicate	Fish per jar	Percent mortality	Comments
Control (0 mg/L)	1	4	100	All dead within 30 minutes
	2	4	100	All dead within 30 minutes
	3	4	100	All dead within 30 minutes
2 mg/L	1	4	100	All dead within 90 minutes
	2	4	100	All dead within 90 minutes
	3	4	100	All dead within 90 minutes
4 mg/L	1	4	0	
	2	4	0	
	3	4	0	
6 mg/L	1	4	0	
	2	4	0	
	3	4	0	

CHAPTER 4

Toxicity of Copper Sulfate and Potassium Permanganate to Rainbow Trout and Golden Alga *Prymnesium parvum*

TOM DORZAB AND AARON BARKOH

Abstract

The effects of copper sulfate and potassium permanganate on *Prymnesium parvum* cell density and ichthyotoxicity and on rainbow trout *Oncorhynchus mykiss* survival were investigated in 0.1-ha plastic-lined hatchery ponds. Treatments were 4 mg/L $KMnO_4$, 1 mg Cu/L and 0.5 mg Cu/L as $CuSO_4$, and untreated ponds which received no chemical addition. Treatments were applied once and rainbow trout were stocked at 5 fish/pond 4 days thereafter. The $KMnO_4$ and 1 mg Cu/L treatments appeared to eradicate *P. parvum* and eliminate ichthyotoxin within 3 days after treatment application. The 0.5mg Cu/L did not appear effective to control cells or toxicity. *P. parvum* persisted in the control ponds throughout the study and sublethal levels of the toxin also existed in these ponds. The potassium permanganate demand of the toxic water was 2 mg/L. Rainbow trout survival did not significantly differ among treatment and control groups. The mortalities that occurred were caused by factors not measured in this study.

Introduction

In 2001, 7,000 rainbow trout *Oncorhynchus mykiss* died in ponds at the Dundee State Fish Hatchery (DSFH). These deaths were attributed to *Prymnesium parvum* ichthyotoxicity. Ammonium sulfate, which is used successfully to control *P. parvum* during warmer months, is ineffective in winter because of lower temperatures and pH levels (Shilo and Shilo 1953). Consequently, Sarig (1971) recommended copper sulfate as the most suitable chemical for controlling *P. parvum* in winter.

Copper sulfate is an effective algaecide (Boyd 1990), but it is toxic to many species of fish at or near the concentration necessary for algal control (Irwin 1997). Hipkins (2002) advised against using copper sulfate in trout ponds, probably because of the adverse effects of copper on growth and survival (Hansen et al. 2002a, b). However, the toxicity of copper is related to water quality characteristics such as hardness, alkalinity, pH, and dissolved organic carbon. Increases in these water quality variables result in decreased copper toxicity and subsequent increased in tolerance by fishes (USACE 1985; Straus and Tucker 1993) and higher concentrations required to control algae (Boyd 1990).

The water quality characteristics of DSFH include: total hardness, 959 mg/L as $CaCO_3$; calcium hardness, 255 mg/L as $CaCO_3$; total alkalinity, 75 mg/L as $CaCO_3$; total chloride, 1,304 mg/L and salinity, 4 ppt. During the period rainbow trout are cultured at DSFH (i.e., late November to early March), pH values of 7-8 are common. We hypothesized

that use of relatively lower concentrations of copper than the recommended 2 mg Cu/L for most algal control may not harm trout but may kill the planktonic *P. parvum* in DSFH ponds. Besides copper sulfate, potassium permanganate has the potential to kill *P. parvum* or detoxify the ichthyotoxin. Potassium permanganate is reported to be toxic to bacteria and phytoplankton (Fitzgerald 1964; Kemp et al. 1966; Tucker and Boyd 1977) and to detoxify fish toxins such as rotenone and antimycin (Lawrence 1956; Marking and Bill 1975). We compared the effects of copper sulfate and potassium permanganate on *P. parvum* survival and ichthyotoxicity and survival of 229- to 250-mm rainbow trout.

Materials and Methods

Filling was begun 10 January 2002 for eight 0.1-ha study ponds One day later, the ponds were full and *P. parvum* ichthyotoxicity bioassay and cell count were performed using established protocols (Appendices A and B). The bioassay was performed on incoming lake water while cell count was performed on incoming lake water as well as on water from all study ponds. The study design was 4 X 2 (treatments X replicates) and ponds were randomly assigned to treatments of 4 mg/L potassium permanganate, 0.5 or 1 mg/L copper sulfate pentahydrate, or untreated (e.g., controls). Before chemical treatments were applied, a potassium permanganate demand test was performed (Boyd 1990) on water from the ponds designated for the potassium permanganate treatment. On 14 January 2002, *P. parvum* cell counts and ichthyotoxicity bioassays were performed on water samples from each study pond. Water samples were collected from 25-30 cm depths. One day later, copper analysis (APHA 1995) was performed on water samples from all ponds and five rainbow trout were stocked into each pond. Morning dissolved oxygen concentration, pH, and temperature were recorded daily for each pond using the Yellow Springs Instrument (YSI) Model 650 MDS data logger equipped with a YSI Model 600XL probe. On 21 January 2002, cell counts were performed and all ponds were drained to harvest the fish.

Results and Discussion

Due to inadequate pond availability, the study design was limited to two replicates per treatment or control so these results should be considered preliminary. The initial *P. parvum* cell counts from the study ponds ranged from 1,000 to 6,000 cells/mL, whereas the source water was 3,000 cells/mL. These cell counts indicate relatively light densities of *P. parvum* in both the water source and the study ponds.

Cell count data suggest that *P. parvum* was eradicated in the 1.0-mg/L copper sulfate and 4.0-mg/L potassium permanganate treatment ponds (Table 1). The potassium permanganate demand of the pond water was between 0.0 and 1.0 mg/L, thus the effective concentration of potassium permanganate was <4 mg/L. Although the ponds treated with 0.5-mg/L copper sulfate were initially free of cells following treatment, cells were subsequently seen in one of these ponds. Thus, it appears the 0.5-mg/L copper sulfate is not as effective as 1 mg/L for controlling *P. parvum*. Cell counts indicate that *P. parvum* persisted in the control ponds throughout the study.

The incoming water from the lake was toxic at the time of pond filling and remained so through at least three days when the bioassay performed on 14 January 2001 indicated high fish mortality in water from the control ponds (Table 2). Potassium permanganate at 4.0 mg/L appeared most effective in reducing ichthyotoxicity in this 3-day period whereas some toxicity persisted in the copper sulfate treated ponds.

The copper analysis revealed residual concentrations that increased with the increasing concentration applied to ponds (Table 1). The ambient copper concentration measured in the control ponds averaged 0.057 mg/L and was higher that the average concentration of 0.007 mg/L in ponds treated with potassium permanganate. The lower copper concentrations in the potassium permanganate treated ponds may have resulted from reduction of the Cu^{2+} by the potassium permanganate and subsequent precipitation of free copper.

Survival of rainbow trout was high in all study ponds, averaging 80% for potassium permanganate, 90% for 0.5 mg/L copper sulfate, and 100% for both the1 mg/L copper sulfate and control ponds (Table 1). Because of the small number of trout stocked into each pond, it is difficult to discern with confidence the treatment effects on trout survival. However, because no fish died in the ponds treated with 1 mg/L copper sulfate ponds, the mortality in the ponds treated with 0.5 mg/L copper sulfate probably was not due to copper poisoning. Similarly, the mortalities in the potassium permanganate and 0.5 mg/L copper sulfate treated ponds could not be attributed to ichthyotoxicity. The toxicity was relatively highest in the control ponds and lowest in the permanganate ponds (Table 2) and yet trout mortality was highest in the potassium permanganate pond whereas there was no mortality in the control pond. Obviously, factors not measured in this study caused the trout mortalities.

TABLE 1.—Residual copper concentrations (mg/L), mean rainbow trout survival (%), and *Prymnesium parvum* density (cells/mL) in water samples from ponds treated with potassium permanganate, copper sulfate, or untreated (control).

Date	Treatment			
	$KMnO_4$ 4 mg/L	$CuSO_4 \cdot 5H_2O$ 0.5 mg/L	$CuSO_4 \cdot 5H_2O$ 1.0 mg/L	Control
		Residual copper		
15 Jan 2002	0.005	0.220	0.209	0.036
	0.008	0.139	0.228	0.078
Mean	0.007	0.180	0.219	0.057
		Survival		
21 Jan 2002	80	90	100	100
		***P. parvum* density**		
11 Jan 2002	3,000	1,000	2,000	6,000
	2,000	2,000	4,000	5,000
Mean	2,500	1,500	3,000	5,500
14 Jan 2002	0	0	0	2,000
	0	0	0	1,000
Mean	0	0	0	1,500
21 Jan 2002	0	0	0	3,000
	0	1,000	0	2,500
Mean	0	500	0	2,750

TABLE 2.—Mean fish mortality (percent) observed in bioassays conducted on incoming lake water (11 January 2002) and on pond water (14 January 2002). Ponds were full and treated with potassium permanganate, copper sulfate, or left untreated (control) on 11 January 2002.

		Treatment			
			$CuSO_4 \cdot 5H_2O$		
Bioassay water	Incoming water	$KMnO_4$4 mg/L	0.5 mg/L	1.0 mg/L	Control
Undiluted + cofactor	100	0	12.5	25	75
Diluted (1:5) + cofactor	25	0	12.5	0	0
Control	0	0	0	0	0

CHAPTER 5

Toxicity of Copper Sulfate Pentahydrate to Rainbow Trout *Oncorhynchus mykiss*

TOM DORZAB AND AARON BARKOH

Abstract

The effect of copper sulfate on survival of 230- to 250-mm rainbow trout *Oncorhynchus mykiss* was investigated in indoor tanks for seven days. Rainbow trout were exposed to three copper concentrations (0.5, 1.0 and 2.0 mg Cu/L) and a control (0 mg Cu/L). In a 3 X 4 study design (replicates X treatments), 12 2,271-L circular tanks were stocked at five fish per tank. Mean survival of rainbow trout in copper sulfate treated tanks was 74% for 2 mg/L, 86% for 1 mg/L, 94% for 0.5mg /L treatments, and 100% for the untreated controls. The differences in survival were not significant among treatment and control groups. Survival declined progressively with increasing copper concentration, and the onset of fish mortality was sooner for the highest copper concentration treatment and later for the lowest (0.5 mg Cu/L) treatment. No mortality occurred in the control tanks. Although the differences in survival were not statistically significant, the observed differences could be biologically or economically important. Because of the small sample size, the effect of the copper concentrations on survival of rainbow trout was unclear.

Introduction

In February 2001, the presence of the toxic alga *Prymnesium parvum* was confirmed in ponds at the Dundee State Fish Hatchery (DSFH), located in Archer County, Texas. Among the species adversely affected was rainbow trout *Oncorhynchus mykiss*, with approximately 7,000 (mean length = 229 mm) lost to the toxic alga. Methods used to control *P. parvum* in Israeli fish farm ponds involve the use of ammonium sulfate (Shilo and Shilo 1953) or copper sulfate (Sarig 1971). Both chemicals were used successfully to control *P. parvum* at the DSFH during summer 2001. Cold water temperatures (8-10 °C) typically encountered when holding trout at the DSFH limit treatment options to copper sulfate(Sarig 1971), but rainbow trout is known to be sensitive to copper toxicity (Anderson and Mayer 1993).

Because the toxicity of copper is influenced by water quality, especially alkalinity (Boyd 1990), we suspected that the high alkalinity of the DSFH water might reduce copper toxicity to rainbow trout. The characteristics of the DSFH water are: total hardness, 959 mg/L as $CaCO_3$; calcium hardness, 255 mg/L as $CaCO_3$; and total alkalinity, 88 mg/L as $CaCO_3$. The objective of this study was to compare the effects of three concentrations of copper (0.5, 1.0, and 2.0 mg Cu/L) on the survival of rainbow trout in the DSFH water.

Materials and Methods

This study was conducted indoors using 12 2,271-L circular fiberglass tanks. Each tank was filled with 1,279 L water from Lake Diversion, the hatchery water source, which was passed through both a rapid sand filter and an ultraviolet (UV) filter. The treated water was examined for the presence of *P. parvum* using a hemacytometer, then tested for ichthyotoxicity using established protocols (Appendices A and B). Calcium carbonate alkalinity was determined with a HACH Model AL-DT Alkalinity Test Kit. Aeration in each tank was provided by compressed air and tanks were covered with netting to prevent fish escapement. On 15 January 2002, the day following filling, tanks were randomly assigned to treatments. Treatments were 0.5 mg Cu/L, 1.0 mg Cu/L and 2.0 mg Cu/L as copper sulfate pentahydrate (100 % active ingredient) or untreated (controls). Five rainbow trout were placed into each tank. Temperature, dissolved oxygen, and pH were measured daily in each tank. Copper analysis from water samples taken from the tanks was performed using the bathocuproine method (Appendix D) at 4 and 24 hours post-treatment. The 4-hour samples were preserved with HCl (APHA 1995) and analyzed at the same time the unpreserved 24-hour samples were analyzed. Dead fish were removed from each tank and the numbers recorded daily. The study lasted 7 days. The water quality and fish mortality (or survival) data were analyzed by analysis of variance with differences among treatment and control groups considered significant at $P \leq 0.05$.

Results and Discussion

The water used for the study was determined to be free of *P. parvum* and ichthyotoxin. No cells were found when the water was examined microscopically, and toxin was not detected with the bioassay. Furthermore, water quality did not differ significantly among treatment and control groups (Table 1).

The concentrations of free copper in the 24-h water samples were slightly higher than those in the 4-h samples for all treatments (Table 2), although one would expect to find less free copper in the 24-hour samples. Apparently, it is important to analyze for copper in water samples as soon as possible after collection since copper can be adsorbed onto sample containers (APHA 1995) or form complexes (Boyd 1990). The preservation of the 4-hour water samples with hydrochloric acid was intended to prevent copper adsorption; however, some adsorption appears to have occurred even with the addition of the acid. Whether the added acid was insufficient to stabilize all of the free copper was unclear. The concentrations of copper in the control ponds were 0.043-0.053 mg Cu/L, reflecting the ambient concentrations of copper in the lake water used for the study. This observation suggests that the ambient copper concentration of water should be considered in the calculation of copper treatments to better determine actual concentrations of copper exposed to fish.

The concentrations of copper added to the water declined very rapidly to mean cumulative percent declines of 69.5-74.3 after 4 h and 64.9-71.2 after 24 h. Essentially, the lowest concentrations were achieved after the first 4 h. This agrees with other reports (e.g., McIntosh 1975; Button et al. 1977) that most of the soluble copper applied to pond water disappears quickly.

The survival of rainbow trout ranged from 74 to 100% and did not significantly differ among treatments and the control, perhaps due to small sample size. Whereas none of the fish died in the control tanks, there was progressively higher mortality among the treatment groups as copper concentration increased. Additionally, the onset of mortality was sooner at higher copper concentrations. Although the differences in survival were not statistically significant, these differences could be biologically or economically important. A 74% survival over a 7-day period could be unacceptable, whereas a 94% survival might be acceptable. Further research should be done with higher sample sizes before conclusions can be drawn about the effect of copper on the survival of trout in the DSFH water.

TABLE 1.—Mean (±SD) values of dissolved oxygen, temperature, pH, and copper concentrations measured at 4- and 24-hr post-treatment (percent declines in parentheses) in study tanks and survival of rainbow trout exposed to copper sulfate treatments for seven days.

Variable	Copper sulfate treatment (mg Cu/L)			
	0	0.5	1.0	2.0
Dissolved oxygen (mg/L)	10.14 ± 0.29	10.19 ± 0.35	10.20 ± 0.33	10.14 ± 0.32
Temperature (°C)	11.82 ± 0.45	11.60 ± 0.44	11.68 ± 0.45	11.77 ± 0.47
pH	7.99 ± 0.04	7.99 ± 0.05	8.00 ± 0.04	8.01 ± 0.04
24-h copper (mg/L)	0.043 ± 0.014	0.152 ± 0.006 (69.5 ± 1.2)	0.274 ± 0.033 (72.6 ± 3.3)	0.514 ± 0.029 (74.3 ± 1.4)
24-h copper (mg/L)	0.053 ± 0.016	0.175 ± 0.021 (64.9 ± 4.1)	0.327 ± 0.037 (67.3 ± 3.7)	0.576 ± 0.046 (71.2 ± 2.3)
Survival	100	93.3 ± 11.5	86.7 ± 11.5	73.3 ± 11.5

CHAPTER 6

Effects of pH on *Prymnesium parvum* Cell Viability and Toxicity

GREGORY M. SOUTHARD AND DAVID KLEIN

Abstract

Toxic water obtained from E. V. Spence Reservoir during an ongoing *Prymnesium parvum*-related fish kill was used to evaluate the effect of lower pH levels on *P. parvum* cell integrity and toxicity. Hydrochloric and sulfuric acids were used separately to lower pH in toxic water to see if the *P. parvum* cells and ichthyotoxin would be destroyed or deactivated. The treatments ranged from pH 5.5 to pH 7.0 in 0.5-SU increments. Untreated water (control) had a pH of 8.3. The acidic pH levels were effective in reducing the density of viable *P. parvum* cells, and the percent reduction in density increased as the pH decreased. Reductions in density were 23.6% and 87.6 % for pH 6 and 5.5, respectively, 3 hours after treatment and 41.6% and 94%, respectively, 28 hours after treatment with hydrochloric acid. Sulfuric acid treatments reduced cell density by 38.3% and 61.7% for pH 6.5 and 6, respectively 1 hour after treatment and 35.8% and 82.3% 18 hours after treatment. A bioassay demonstrated that at pH 6 and 6.5, toxicity was reduced but not completely eliminated.

Introduction

The toxin-producing haptophyte *Prymnesium parvum* has been known to occur in Texas since at least 1985 when it was implicated in a fish kill in the Pecos River (James and de la Cruz 1989). In 2001, *P. parvum* was responsible for the loss of the entire crop of fingerling striped bass *Morone saxatilis* and palmetto bass (female striped bass ×male *M. chrysops*) at the Dundee State Fish Hatchery (DSFH). Additionally, the alga has been resident in Possum Kingdom Reservoir, the water supply for the Possum Kingdom State Fish Hatchery (PKSFH). The alga is reported to become endemic once established in hatchery ponds (Shilo 1967), thus successful fish culture at affected hatcheries may be dependent upon successful management of the organism or its toxins. One way to deactivate toxins may be to manipulate pH to acidic or alkaline levels, depending on the type of toxin. The activity of the *P. parvum* ichthyotoxin has been demonstrated to increase between pH 6 and 8 (Ulitzer and Shilo 1970b), and from pH 7 to 9 where it complexes better with cationic cofactors (Shilo and Aschner 1953). However, McLaughlin (1958) reported that the toxic properties of *P. parvum* culture fluids were lost at pH 6.0-6.5. The pH requirement for growth of *P. parvum* in media has been variably reported as pH 8.2-8.4 (Padan et al. 1967), pH 8.0 (Ulitzer and Shilo 1970b), and pH 9.0 (Padilla and Martin 1973). It appears slightly alkaline pH is suitable for *P. parvum* growth and ichthyotoxin activity, whereas acidic pH levels may be detrimental for toxicity.

Hatchery ponds typically experience diurnal fluctuations in pH with the lowest values occurring by just before sunrise and highest values by mid- to late afternoon (e.g. 6 p.m.; Boyd 1990). The recommended pH values for aquaculture are 6.7-8.6 (US EPA 1980). Boyd (1990) stated that optimum growth of fish occurs at pH 6-9 whereas slow growth occurs at pH 5-6, reproduction fails at pH 4-5, and acid death occurs at pH 4. At the DSFH pond pH levels fluctuated from a low of 7.2 to a high of 10.1 during the 2001 9-inch channel catfish *Ictalurus punctatus* production period in ponds treated with ammonium sulfate to control *P. parvum*. The best time to manipulate the pH to kill *P. parvum* likely would be during the mid-morning when pH values are lowest. This experiment was undertaken to evaluate the effects of pH manipulation, using hydrochloric acid or sulfuric acid, on *P. parvum* viability and toxicity.

Materials and Methods

Toxic water for the study was collected from E. V. Spence Reservoir at the Wildcat Creek Marina during an active *P. parvum*-fish kill in October 2001. Brightfield microscopy and a hemacytometer were used to determine *P. parvum* density prior to pH manipulation. Aliquots (50 mL each) of toxic water were transferred into 100-mL beakers, and either hydrochloric or sulfuric acid was added to each to achieve the final pH values of 7.0, 6.5, 6.0, and 5.5. The control (pH 8.2) received no acid. Six replicate cell counts were performed for each study beaker at 3 h and 28 h after the addition of hydrochloric acid and at 1 h and 18 h after adding sulfuric acid. Density was expressed as cells/mL. Using the mean cell density of the control 3 h post-treatment as reference point, the percent decline in cell density was calculated for each hydrochloric acid-adjusted pH treatment. Similarly, the mean cell density of the control 1 h post-treatment was used as reference point to calculate cell density for each sulfuric acid-adjusted pH treatment. The standard bioassay for toxicity testing (Appendix B) also was used 1 h after pH adjustments to determine the effect of pH on toxicity.

Results and Discussion

Acidic pH was effective in reducing the density of viable *P. parvum*, and percent reduction in viable cells increased as the pH decreased. Percent reductions in density for pH 6 and 5.5 were 23.6% and 87.6 %, respectively, 3 h after treatment and 41.6% and 94%, respectively, 28 h after treatment with the hydrochloric acid (Table 1). A similar pattern was observed for the sulfuric acid treatments. One hour after treatment the cell density was reduced by 38.3% and 61.7% for pH 6.5 and 6, respectively, and 35.8% and 82.3%, respectively, after 18 h of treatment (Table 2). These results suggest that decreasing pH to 6 or less would destroy *P. parvum* and cause considerable reduction in cell density in 1-28 h.

The pH among the treated beakers returned to basic conditions when they were tested at 18 h and 28 h post-treatment for the sulfuric acid and hydrochloric acid treatments, respectively (Tables 1 and 2). The temporary nature of the acid treatments may enhance the feasibility of using pH to control *P. parvum* in fish culture systems. Since fish can tolerate pH 5-6 but suffer slow growth at these conditions (Boyd 1990), the temporal nature of the acidic treatments would allow *P. parvum* to be treated without significant loss of fish growth.

Also, infested culture systems could be treated with pH 6 or less to reduce *P. parvum* densities and then pH allowed to rise to neutral or basic conditions before fish are stocked or effluents are discharged. The minimum pH for aquaculture effluents is pH 6, thus some minimum post-treatment holding time following acid treatments may be required before effluents are discharged.

The bioassays revealed that pH 6-6.5 reduced the toxicity but did not eliminate it completely. Fish mortality in the undiluted water without cofactor was 33.3% for the untreated control (pH 8.3) and 0% for pH 6.5 or 6 (Table 3). However, when cofactor was added, fish died (33-100% mortality) at all pH levels. The pattern of mortalities suggest that toxicity was reduced by the lower pH, which support the findings of McLaughlin (1958) who reported that the toxicities of *P. parvum* culture fluids were lost at pH 6.0-6.5.

This was a pilot study intended to provide a cursory view of the potential effects of acidic conditions on *P. parvum* and its toxicity. The study design was limited with no replicates within treatments. Although the results of this study appear promising, it is unknown if the toxin returns with the increasing pH. Also, it was calculated that approximately 875 L of concentrated hydrochloric acid would be required to effectively treat a typical 1-acre pond, which is a major impediment to the efficacy of such treatments. If further studies are undertaken, they should determine at what rate pH returns to basic conditions and evaluate longer-term effects on cell density and toxicity, as well as how pH deactivates the toxin. Further studies also should use water from affected hatcheries, which may have different buffering capacities and require different volumes of acid for efficacious treatment.

TABLE 1.—Mean *Prymnesium parvum* densities (percent reduction in parenthesis) in toxic water from E. V. Spence Reservoir, Texas following additions of concentrated hydrochloric acid to reduce pH.

		Mean cell density (cells/mL)*	
Treatment pH	Final pH	3 h post-treatment	28 h post-treatment
8.2	8.6	22,250 (0)	26,000 (-16.8)
7.0	8.3	22,000 (1.1)	22,000 (1.1)
6.5	8.2	20,750 (6.8)	18,000 (19.1)
6.0	8.1	17,000 (23.6)	13,000 (41.6)
5.5	8.1	2,750 (87.6)	1,333 (94)

* $N = 6$ replicates

** Untreated control

TABLE 2.—Mean *Prymnesium parvum* densities (percent reduction in parenthesis) in toxic water from E. V. Spence Reservoir, Texas following addition of sulfuric acid to reduce pH.

		Mean cell density (cells/mL)*	
Treatment pH	Final pH	1 h post-treatment	18 h post-treatment
8.3 (control)	8.3	24,300 (0.0)	19,300 (20.5)
7.0	8.1	17,300 (28.8)	17,000 (30.0)
6.5	8.0	15,000 (38.3)	15,600 (35.8)
6.0	7.6	9,300 (61.7)	4,300 (82.3)

* $N = 6$ replicates

TABLE 3.—Bioassay results for toxic water (due to *Prymnesium parvum*) from E. V. Spence Reservoir, Texas 1 h following pH adjustment using sulfuric acid.

	Dead fish/total fish		
pH	Undiluted water	Undiluted water + cofactor	1:5 dilution + cofactor
8.3 (control)	1/3	3/3	3/3
6.5	0/3	3/3	3/3
6.0	0/3	3/3	1/3

CHAPTER 7

Use of Hydrogen Peroxide as an Algaecide for *Prymnesium parvum*

GREGORY M. SOUTHARD

Abstract

Hydrogen peroxide was investigated as a potential algaecide for the toxic alga *Prymnesium parvum*. The goal was to determine if hydrogen peroxide could be used to eliminate *P. parvum* in fish transportation water to prevent the incidental spread during fish stocking operations. Hydrogen peroxide concentrations of 62.5-12,500 mg/L were tested for their ability to lyse *P. parvum* cells or inhibit cell motility at 15 min, 1 h, or 24 h post-exposure. Only the highest concentration (12,500 mg/L) caused lysis of *P. parvum* cells within 15 min while concentrations ≥ 3,125 mg/L lysed all the algal cells after 1 h. At 24 h, complete lysis was observed for all concentrations. Since most cultured fish species cannot tolerate hydrogen peroxide concentrations > 500 mg/L for prolonged periods and the concentrations needed to lyse all of the algal cells within 1 h exceeded the U.S. Food and Drug Administration low regulatory treatment rates (250-500 mg/L), this chemical is not recommended as an algaecide in fish hauling water.

Introduction

Prymnesium parvum, a halophilic chrysomonad, is a small (up to 15 µm long) biflagellate phytoplankton responsible for extensive fish mortalities in brackish waters in northern Europe (Kaartvedt et al. 1991; Lindolm et al. 1999), Israel and China (Guo et al. 1996), and North America (Holdway et al. 1978a). The alga is a free-living phytoflagellate having two long flagella, a shorter haptonema, a C-shaped or saddle-shaped chloroplast, and scaly surface. *P. parvum* produces several toxins (Shilo 1981) which affect many gilled aquatic species, including fish, bivalves, and brachiated tadpoles.

In mid-April through early May 2001, the entire crop of striped bass (*Morone saxatilis*) and palmetto bass (female *M. saxatilis* × male *M. chryopsis*) was lost at the Texas Parks and Wildlife Department's (TPWD) Dundee State Fish Hatchery (DSFH). High densities of *P. parvum* were identified in Lake Diversion, the source of water for the hatchery, during active fish kills in January and February 2001. Subsequently, biologists determined the alga was responsible for the widespread mortality of striped bass and hybrid striped bass fry at DSFH. The Possum Kingdom Reservoir was similarly affected during the same period, although the Possum Kingdom State Fish Hatchery (PKSFH) was not in production at the time and no hatchery fish were lost. As of 2004, the alga continued to be present at least sporadically at both Diversion and Possum Kingdom reservoirs.

In 2002, after extensive study into chemical control methods for this alga, striped bass, palmetto bass, channel catfish, and other fish species were successfully produced at

both affected hatcheries and transported to water bodies throughout Texas. Although *P. parvum*-free well water is used in hauling tanks at PKSFH to transport fish, it is virtually impossible to avoid incidental contamination of the transport water with *P. parvum*. Therefore, there is concern regarding the possible spread of this pathogen from affected hatcheries to uninfected Texas waters and, as a matter of internal policy, TPWD Inland Fisheries does not want to disperse the organism via fish deliveries.

The U. S. Food and Drug Administration (FDA) considers hydrogen peroxide a low regulatory priority drug when used as a fungicide at rates up to 500 mg/L (based on active ingredient) on all species and life stages of fish. Some species of fish are more sensitive to hydrogen peroxide than others. Rach et al. (1997) reported that several fish species such as fathead minnow *Pimephales promelas*, brown trout *Salmo trutta*, bluegill *Lepomis machrochirus*, and channel catfish *Ictalurus punctatus* tolerate hydrogen peroxide concentrations up to 1000 mg/L for 45 min. However, walleye *Stizostedion vitreum* were intolerant to exposures as low as 100 mg/L for 15 min. Rainbow trout *Oncorhynchus mykiss* fry tolerate hydrogen peroxide concentrations up to 1000 mg/L, whereas fingerlings and adults were more sensitive at concentrations ≤ 500 mg/L for the same exposure time. Rach et al. (1997) also reported that the toxicity of hydrogen peroxide increased as temperature increased. These results indicate that considerations must be made for species, life stage, and water temperature when using hydrogen peroxide in aquaculture.

The use of peroxides as algaecides is somewhat controversial. Although there are many claims regarding the efficacy of peroxides as algaecides, there are few to no formal, scientific studies that prove or disprove these claims. The mechanism by which peroxides work is corrosive oxidation which damages unicellular structures. This process is easily observed as gas formation and bubble production when hydrogen peroxide is applied to a wound infected with bacteria. Arguments against the use of hydrogen peroxide as an algaecide include that oxidation may also kill beneficial alga and bacteria, have detrimental effects on fish, and the compound is unapproved for such purposes. Despite the controversy, the lack of an effective method to remove *P. parvum* from fish hauling tanks warrants investigation into whether it can be a useful algaecide for aquaculturists.

This study was conducted to determine the effectiveness of hydrogen peroxide as an algaecide for *P. parvum*. The specific objectives were to investigate if selected concentrations of hydrogen peroxide would kill or lyse *P. parvum* cells in 15-60 min and to determine the effects that 15-min to 24-h exposures of concentrations of hydrogen peroxide considered tolerable by fish may have on *P. parvum*.

Materials and Methods

An axenic culture of *P. parvum* was obtained from the University of Texas – UTEX Culture Collection of Algae in Austin, Texas. The concentration of the hydrogen peroxide solution was determined by a standard test using potassium permanganate (Jeffery et al. 1989). Aliquots of this solution were diluted with sterile, glass-filtered deionized water to 10-mL volumes to produce the target concentrations of hydrogen peroxide. The test containers were 15-mL disposable tubes.

Three concentrations (781.25, 3,125, and 12,500 µg/mL) of hydrogen peroxide were used to determine the acute (immediate) effects of high concentrations to *P. parvum* (Table 1). A 1-mL aliquot of the algal culture was added to each of these concentrations, as well as a control consisting of sterile, glass-filtered deionized water. The density of the *P. parvum* cells was determined to be 8,000 cells/mL using a hemacytometer. The experiment consisted of two replicates per treatment (hydrogen peroxide or control) and was repeated three times for each of two dates at room temperature (25°C). The cells were observed at 15 min and 60 min post-treatment for cell integrity (i.e., lysed or intact) and movement (i.e., motile, slow motility, and flagella motion) using an Olympus CH-2 brightfield microscope.

The second part of the experiment used hydrogen peroxide concentrations reported to tolerated by fish (Rach et. al 1997). The experimental design and conditions were similar to the one above except that the treatment concentrations were 0 (control), 62.5, 125, 250, and 500 µg/mL and the *P. parvum* cells were observed at 15 min, 1 h, and 24 h post-treatment for cell integrity and movement as described above.

Results and Discussion

Among the hydrogen peroxide concentrations tested, only 12,500 µg/mL lysed all *P. parvum* cells within 15 min while 250-3,125 µg/mL inhibited cell motility (Tables 1 and 2). For 1-h exposures, hydrogen peroxide concentrations ≥ 3,125 µg/mL lysed all cells whereas no lysis was observed for concentrations ≤ 781.25 µg/mL. Motility was inhibited at 1-h exposure and concentrations ≥ 500 µg/mL (Tables 1 and 2). Complete lysis of cells was observed in all 24-h hydrogen peroxide treatments while no lytic effect or changes in motility were observed in the control (Table 2).

These results suggest that hydrogen peroxide exposures from 62.5 to 500 µg/mL for 24 h would lyse *P. parvum*, but this exposure time is greater than typical fish transport times in Texas. Treatment regimes demonstrated safe for fish vary by species. Largemouth bass *Micropterus salmoides* tolerate hydrogen peroxide at <150 µg/mL for 60 min; walleye, bluegill and channel catfish tolerate <100 µg/mL for 60 min; and fathead minnow fry tolerate <50 µg/mL for 60 min (Gaikowski 1999). Lumsden et al. (1998) demonstrated hydrogen peroxide effective against bacterial gill disease in rainbow trout when used at concentrations between 100-250 µg/mL for 1 h, although significant gill damage was observed at treatment rates greater than 175 µg/mL. Although Rach et al. (1997) showed that some fish species could tolerate hydrogen peroxide at concentrations up to 1000 µg/mL for 15-45 min for four consecutive days, the present study indicates that hydrogen peroxide is ineffective as an algaecide against *P. parvum* at concentrations ≤ 3,125 µg/mL for 15-min treatments and at concentrations ≤ 781.25 µg/mL for 60-min treatments. Since hydrogen peroxide exposures tolerated by fish are much less than those required to lyse *P. parvum* cells, the chemical is not recommended for this purpose.

TABLE 1.—Motility and integrity of *Prymnesium parvum* cells at 15-min and 1-h exposures to various concentrations of hydrogen peroxide.

Exposure time	12,500 μg/mL	3,125 μg/mL	781.25 μg/mL	0 μg/L
15 min	lysis	no lysis no motility	no lysis no motility	motility motility
1 h	lysis	lysis	no lysis no motility	motility motility

TABLE 2.—Motility and integrity of *Prymnesium parvum* cells at 15-min, 1-h, and 24-h exposures to various concentrations of hydrogen peroxide (μg/L).

		H_2O_2 (μg/L)				
Date	Exposure	500	250	125	62.5	0
13 Aug 2002	15 min	no lysis no motility	no lysis FM	no lysis slow motility	no lysis motility	motility
	1 h	no lysis no motility	no lysis FM	no lysis FM	no lysis slow motility	motility
	24 h	lysis	lysis	lysis	lysis	motility
14 Aug 2002	15 min	no lysis no motility	no lysis FM	no lysis slow motility	no lysis motility	motility
	1 h	no lysis no motility	no lysis FM	no lysis FM	no lysis slow motility	motility
	24 h	lysis	lysis	lysis	lysis	motility

FM = flagella motion, no motility observed

CHAPTER 8

Efficacy of Nitrogen:Phosphorus Ratios for Controlling *Prymnesium parvum* in Fish Culture Ponds: Summary of 2002 Experiments

GERALD KURTEN AND DENNIS G. SMITH

Abstract

The goal of this project was to determine if two specific concentrations and ratios of nitrogen and phosphorus would deter dominance and toxin production by *Prymnesium parvum* in warmwater fish culture ponds at the Dundee State Fish Hatchery. The initial objective was to establish phosphorus fertilization rates that would sustain 60 µg P/L in hatchery ponds and simultaneously determine if phosphorus fertilization alone would reduce *P. parvum* density and toxicity. An average phosphorus addition of 99 µg/L (82 – 137 µg/L) was required to achieve a target concentration of 60 µg/L. Pond temperatures averaged 13°C and neither pond productivity nor *P. parvum* cell densities appeared to be affected by phosphorus fertilization alone. The second objective was to determine if nitrogen concentration of 300 µg/L and phosphorus concentration of 30 µg/L (N:P = 10:1; low-P) or nitrogen concentration of 300 µg/L and phosphorus concentration of 60 µg/L (N:P = 5:1; high-P) would reduce the incidence and toxicity of *P. parvum* and produce water quality conditions and food sources suitable for zooplanktivorous fish. The number of fish produced was significantly different between low-P and control ponds as well as between high-P and control ponds. Numbers of fish produced in high-P and low-P ponds were statistically similar but relatively more fish were produced in the low-P ponds. At temperatures typical of striped bass *Morone saxatilis* culture (22°C), *P. parvum* cells appeared to be eliminated in ponds fertilized with high concentrations of phosphorus (92 µg/L).

Introduction

Recently, *Prymnesium parvum* has caused massive mortalities of brood fish, fingerlings, and fry at the Dundee and Possum Kingdom State Fish Hatcheries (DSFH and PKSFH, respectively). Control practices at these facilities have focused on killing the alga with either copper sulfate or ammonium sulfate. Unfortunately, the use of these compounds provides short-term relief and each has undesirable consequences in pond systems dependent upon zooplankton as food for small fish. Ammonium sulfate concentrations high enough to control algal blooms may yield un-ionized ammonia concentrations approaching levels toxic to fry and fingerlings. Copper sulfate has the side effect of killing desirable algae and can negatively impact zooplankton food resources.

Laboratory, pond, and reservoir research suggest that toxin production and dominance by *P. parvum* may be related to nutrient concentrations within the system. High N:P ratios combined with phosphorus limitation is suspected to be responsible for toxic blooms of *P.*

parvum in western Norway (Kaartvedt et al. 1991; Aure and Rey 1992), England (Holdway et al. 1978), and Finland (Lindholm et al. 1999). Apparently, *P. parvum* is a good scavenger of phosphorus at low concentrations and is able to acquire phosphorus from a wide array of organic compounds (McLaughlin 1958). Therefore, fish ponds with low concentrations of phosphorus may provide *P. parvum* the opportunity to dominate the phytoplankton community. However, once available phosphorus supplies are exhausted, phosphorus-limited growth appears to result in toxin production via over-synthesis of membrane intermediates (Dafni et al. 1972; Holdway et al. 1978; Shilo 1981; Johansson 2000). Toxicity coincided with phosphorus declines in control ponds at the DSFH in 2001 during studies designed to control *P. parvum* with ammonium sulfate and copper sulfate (TPWD, unpublished data). Conversely, nitrogen limitation does not appear to be a factor in toxicity of *P. parvum*. Holdway et al. (1978) reported that limiting levels of nitrogen, thiamine, or vitamin B_{12} do not result in increased toxin production. Apparently, *P. parvum* has the ability to use a wide variety of nitrogen-containing compounds including nitrates, ammonia, and amino acids (Paster 1973).

At DSFH and PKSFH, recently used pond management practices include limiting pond fertilization to applications solely of organic materials (e.g., cottonseed meal), which may supply the thiamine and B_{12} required for *P. parvum* growth (Shilo 1972). However, the N:P ratio of cottonseed meal is very high (22:1), whereas the available phosphorus is very low (Anderson 1993). Chinese aquaculturists have achieved good control of *P. parvum* in ponds containing planktivorous fish species by regular fertilization with livestock and poultry manure (Gou et al. 1996). This strategy is based upon the observation that *P. parvum* is relatively slow growing and does not compete well with other algal species when nutrients are replete, but it can tolerate extremely deplete nutrient conditions. Ponds were kept free of *P. parvum* toxicity for up to three months by fertilizing with 50-70 kg/ha/d of manure (dry weight), whereas all fish died in the control (no manure) ponds. Based upon published composition of cattle manure (Boyd 1990) and an average pond depth of one meter, this application rate would supply approximately 420 µg N/L and 98 µg P/L per day (N:P = 4.3:1) to ponds. These fertilization rates are much higher than those customarily used at TPWD hatcheries and likely would result in low dissolved oxygen in ponds.

Recent data suggest that DSFH fish culture ponds are consistently phosphorus limited. When phosphorus has been measured in hatchery ponds, concentrations generally have been at or below detectable limits (< 7 µg/L). Additionally, characteristics of Lake Diversion, the water supply for DSFH, apparently result in low solubility and high precipitation rates of phosphorus (Wetzel 1983). Boyd and Daniels (1993) reported that in brackish, high-alkalinity ponds, phosphorus must be applied frequently due to its low solubility in such waters. Phosphorus concentrations of 30-35 µg/L have been suggested as the lower limit for phytoplankton growth (Sommer 1985; Culver et al. 1993). Phosphorus concentrations in Lake Diversion have been below detectable limits in recent years (TNRCC, unpublished data), suggesting that phosphorus concentrations have been lower than that required for phytoplankton growth. However, Barkoh (1996) compared two N:P ratios with alfalfa meal against alfalfa meal fertilization alone at DSFH and demonstrated that high phosphorus concentrations were not difficult to achieve.

The practice of using organic fertilizers alone in production ponds resulted from concerns that high pH and un-ionized ammonia can adversely affect survival of moronids (Anderson 1993, Bergerhouse 1993, Barkoh 1996). High phosphorus levels could increase phytoplankton growth, if nitrogen is not limiting, resulting in high afternoon pH. Therefore, fertilization strategies aimed at controlling *P. parvum* density and toxicity should not result in phytoplankton densities that affect pond pH in an adverse manner. Inland hatcheries rearing largemouth bass *Micropterus salmoides* fingerlings typically fertilize ponds to achieve approximately 250 µg P/L and 500 µg N/L. These concentrations of inorganic nutrients typically result in good phytoplankton and zooplankton populations although resultant pH levels generally exceed those considered acceptable for striped bass *Morone saxatilis* production. Culver (1993) reported good production of percids with 30 µg P/L and 600 µg N/L. However, pH in some ponds reached 9.7 and may have influenced fish survival. Anderson (1993) used similar target nutrient concentrations in striped bass ponds. Although concentrations of about 300 µg N/L were achieved, phosphorus never exceeded 5 µg/L and ponds appeared to be phosphorus limited. Anderson (1993) thus suggested that a target concentration of 30 µg P/L might be too low in hatchery ponds with hard water supply.

The N:P ratio used by Culver (1993) was 20:1 and is similar to the ratio suggested for algal growth media (APHA 1995). However, others have stated that N:P ratios higher than 10:1 may result in phosphorus limitation (Groeger et al. 1997), although the theoretical N:P ratio for balanced phytoplankton growth is 7:1 (Cromar and Fallowfield 1997; Welch 1980). Barkoh (1996) targeted nutrient ratios of 7:1 and 15:1 to stimulate predominance of nanoplankton in ponds for striped bass production at DSFH. However, the measured N:P ratios were 11:1 and 3:1, respectively, and phosphorus concentrations were highly variable during the culture period.

The goal of this project was to determine if two specific concentrations and ratios of nitrogen and phosphorus would deter dominance and toxin production by *P. parvum* in warmwater fish culture ponds at the DSFH. The initial objective was to determine phosphorus fertilization rates that would sustain phosphorus concentrations of 60 µg/L in hatchery ponds and simultaneously determine if phosphorus fertilization alone would reduce *P. parvum* density and toxicity. The second objective was to determine if nitrogen level of 300 µg/L and phosphorus level of 30 µg/L (N:P = 10:1; high ratio, low-P) or nitrogen level of 300 µg/L and phosphorus level of 60 µg/L (N:P = 5:1; low ratio, high-P) would reduce the incidence and toxicity of *P. parvum* and produce suitable water quality conditions and food base for zooplanktivorous fish. The standard DSFH fertilization regimen using only organic fertilizers served as the control treatment. Both high-P and low-P ponds also received the same organic fertilizers as control ponds.

Materials and Methods

Objective 1.—Determine phosphorus demand and loss rates at known chlorophyll a concentrations and the effects of phosphorus fertilization on P. parvum density and toxicity, if present, and simultaneously assess phosphorus fertilization effects on water quality and zooplankton densities.

Determining appropriate phosphorus application rates requires understanding phosphorus loss rates under various scenarios. Both Anderson (1993) and Barkoh (1996) reported difficulties in achieving target phosphorus concentrations in striped bass ponds, either exceeding or never reaching target concentrations. This experiment was designed to examine phosphorus loss rates at low algal densities typical of the early fish culture period (April-May) and at high algal densities typical of late fish culture period (June-July). These data were used for fertilizer application calculations for the second objective which was to determine the affect of fertilization on *P. parvum* dominance and toxicity.

Three ponds of equal volume were fertilized with phosphoric acid at 60 µg P/L. Three control ponds were filled at the same time and remained unfertilized. Ponds were at full volume before fertilizer applications and, once full, received no additional water. Soluble reactive phosphorus (SRP) was measured for each experimental pond at the pond fill valve and also the day before fertilization using the stannous chloride method (APHA 1995). Chlorophyll *a* (µg/L) was measured at the same time using the filters required for the SRP determination. Chlorophyll *a* was extracted with 95% acetone and measured using a spectrophotometer (APHA 1995).

Phosphorus and chlorophyll *a* were determined at 24-h intervals following the initial fertilization until SRP concentrations fell below detectable levels on two successive intervals or after 4 days. If SRP was undetectable or lower than the target of 60 µg P/L at the first sampling interval following fertilization, an additional 60 µg P/L was applied until a minimum residual of 60 µg P/L was achieved. The amount of phosphorus required to achieve the minimum residual of 60 µg P/L was used to determine the phosphorus demand as follows:

(Total P applied – initial pond P) + (final pond P – 60) = phosphorus demand.

Phosphorus loss rates were calculated as µg P/L/h at ambient chlorophyll *a* concentrations. Regression analysis was used to develop a relationship between chlorophyll *a* and phosphorus loss.

To determine the effects phosphorus on *P. parvum*, cell counts (Appendix A) and bioassays (Appendix B) were conducted for each pond immediately prior to fertilization and twice weekly thereafter, ending one week after the last phosphorus and chlorophyll *a* sampling. All ponds also were monitored twice daily (morning and afternoon) for pH, temperature, and dissolved oxygen.

Zooplankton was sampled from each experimental pond on Monday and Thursday each week between 0600 and 0700 hours by an oblique 4-m tow with a 5.75-cm diameter 80-µm Wisconsin plankton net. Each sample was dewatered to 90 mL and densities of major zooplankton groups (i.e., cladocerans, copepod nauplii, adult copepod, and rotifers) were determined on two separate 1-mL subsamples on a plankton counting wheel under a variable-magnification dissecting microscope.

This experiment was to be repeated on the same treatment and control ponds, with minimum chlorophyll *a* concentrations near 40 µg/L (Anderson 1993) to determine P demand and loss rates at higher algal biomass. To achieve high chlorophyll *a* concentrations for this test, both control and treatment ponds were to be fertilized with P at 60 µg/L following the previous experiment if chlorophyll *a* concentrations were below 40 µg/L.

Objective 2.—Determine phosphorus and nitrogen fertilization effects on P. parvum density and toxicity, water quality, zooplankton, and striped bass production.

Nine ponds were used for this experiment, which consisted of two treatments and a control. Three control ponds were fertilized, stocked with fish, and received feed according to striped bass production guidelines (Warren 2001). Briefly, ponds were fertilized at pond filling with 280 kg/ha cottonseed meal and fertilized again at 3 days and 12 days post-stocking with 56 kg/ha cottonseed meal. Fry were stocked at 3-5 days after hatching (7 days after pond filling) and supplemental feeding was begun 14 days after stocking fry. No steps were taken to control *P. parvum* densities and toxicity in these ponds. Three ponds each served as the "high-ratio" (N:P = 10:1; 300 µg N/L, 30 µg P/L) treatment and "low-ratio" (N:P = 5:1; 300 µg N/L, 60 µg P/L) treatment, respectively. These ponds were filled, fertilized, and stocked as the control ponds. No additional water was added to ponds once they were filled.

Initial inorganic fertilization.—Ponds in high- and low-ratio treatments were fertilized immediately at pond filling to achieve 300 µg N/L with liquid urea nitrogen (URAN). Ambient nitrogen concentrations (ammonia + nitrate) were determined one day before fertilization. If nitrogen concentrations exceeded 300 µg/L, no additional nitrogen was applied; otherwise, the amount applied was equal to 300 µg/L minus ambient concentration. Likewise, on the same day, high- and low-ratio ponds were fertilized with liquid phosphoric acid. Phosphoric acid was added to achieve either the high rate (60 µg P/L; low N:P ratio ponds) or the low rate (30 µg P/L; high N:P ratio ponds). Application rates were calculated as follows:

(P demand – initial P concentration) + anticipated P loss for three days.

This calculation required that chlorophyll *a* also be determined prior to pond fertilization.

Follow-up inorganic fertilization.—Every third day following initial fertilization, both nitrogen and phosphorus were reapplied. Phosphorus was reapplied as for initial fertilization using the phosphorus demand calculation described above. Nitrogen was applied at a rate equivalent to the desired concentration (300 µg/L) minus the concentration in the pond.

Soluble reactive phosphorous, nitrate nitrogen, ammonia nitrogen, and chlorophyll *a* were measured every third day after initial pond fertilization. Morning and afternoon dissolved oxygen, temperature, and pH were measured daily. *P. parvum* cell counts and toxicity bioassays were conducted twice weekly on each pond for the duration of the culture period. Control ponds were sampled in a manner identical to fertilized ponds. Zooplankton

was sampled as described for objective one. Pond harvest and termination of the experiment occurred approximately 40 days after initial pond filling.

Analysis

Objective 1.—Phosphorus demand was calculated from the initial fertilization of each pond using the equation presented above, and then averaged to determine the application concentration required to achieve a minimum phosphorus residual of 60 µg/L. Daily phosphorus decline rate, following achievement of the 60-µg P/L target, was computed as follows:

(Initial P concentration – Final P concentration)/24 h = P loss/h.

Regression analysis was used to resolve the relationship between phosphorus loss and initial phosphorus and chlorophyll *a* concentrations. Phosphorus demand and loss rates were used to calculate the appropriate fertilization rates for objective 2. To retain target concentrations at the end of the three-day fertilization interval, the phosphorus fertilization rate was calculated as follows:

P target + P demand + P loss for 72-h interval

Because phosphorus fertilization alone could reduce *P. parvum* density and toxicity and likely has effects on water quality and pond productivity, data were examined graphically to discern trends in water quality, zooplankton, nutrient concentrations, algal biomass (chlorophyll *a*), *P. parvum* cell density, and daily net primary productivity (afternoon dissolved oxygen - morning dissolved oxygen). When differences appeared significant between treatment and control ponds, daily paired means were compared. Differences in measured variables between treatment and control were compared with repeated measures analysis of variance.

Objective 2.—The most successful fertilization strategy was judged as that treatment with the lowest incidence of toxicity and *P. parvum* cell density, which were evaluated as for objective 1. Additionally, treatment means or daily means were evaluated based on production of appropriate zooplankton for striped bass feeding and pH, un-ionized ammonia and dissolved oxygen concentrations for suitable striped bass survival (Barkoh 1996; Warren 2001). Zooplankton and water quality also were evaluated as in objective 1. Survival, growth, production rate (kg/ha/d), and net fish biomass (harvest biomass-stocking biomass) were compared among the two treatments and the control by analysis of variance. Survival was log (X+1) transformed prior to analysis. Significance for all tests was set at $P \leq 0.05$.

Results and Discussion

Objective 1

Phosphorus demand and decline.—Two applications of 60-µg/L phosphorus were required to achieve target concentrations of at least 60 µg P/L. Phosphorus concentrations

averaged 86 µg/L the morning after the second addition. The calculated application rate to achieve 60 µg/L was 88 µg P/L. No additional phosphorus was applied for the next five days. Ninety-six hours after target concentrations of phosphorus were measured, concentrations declined to an average of 60 µg P/L (Figure 1). Average phosphorus loss rate was 0.03 µg/L/h (0.65 µg/L/d).

Chlorophyll *a* concentrations averaged 19.1 µg/L during the phosphorus application period and increased to 23.0 µg/L in fertilized ponds 5 days later when the experiment ended. Changes in chlorophyll *a* concentrations were not significantly different between beginning and end of sampling or between treated (fertilized) and control (untreated) ponds. Similarly, there were no statistically significant differences among water quality variables between treated and control ponds. Morning and afternoon pond temperatures averaged 13 and 16°C, respectively. Morning and afternoon dissolved oxygen averaged 10.7 and 12.0 mg/L, respectively, and morning and afternoon pH both averaged 8.2. Apparently, productivity was not measurably stimulated during the 5-day period at these ambient temperatures because the indicators of pond productivity (algal biomass, pH, and afternoon dissolved oxygen) did not differ between fertilized and unfertilized ponds.

A second series of phosphorus applications was made to the three unfertilized ponds to determine phosphorus demand. Chlorophyll *a* concentrations averaged 22.0 µg/L. Calculated phosphorus required to achieve 60 µg P/L averaged 110 µg P/L. The study was not continued as planned to examine phosphorus decline rates at higher algal biomass due to labor constraints. The 12 applications of phosphorus during the first and second application series and the ratio of applied phosphorus to measured phosphorus was used to calculate the amount of phosphorus required to achieve 60 µg P/L. The mean additional phosphorus required was 99 µg /L (range: 82 – 137 µg/L).

P effects on P. parvum toxicity and densities.—*P. parvum* cell densities on the morning of the first phosphorus application averaged 13,000 cells/mL (10,000-15,000 cells/mL) and bioassays indicated that both fertilized and unfertilized ponds were toxic to fathead minnow fry. On the morning after the second application of phosphorus when target concentrations had been achieved, cell densities were significantly different ($P = 0.029$) between treatment and control ponds, averaging 12,333 cells/mL for P-fertilized ponds and 6,667 cells/mL for unfertilized ponds (Figure 2). Bioassays at the same time were inconclusive because more fish died in diluted pond water than in undiluted pond water. At the termination of the experiment, all fathead minnow fry died in both diluted and undiluted pond water for both P-fertilized and unfertilized ponds. *P. parvum* cell densities were statistically similar. These results seem to indicate that phosphorus fertilization alone had no short-term effect on toxicity or cell density. The difference in cell densities detected the morning after the second phosphorus application may have been a result of an error associated with the sampling technique rather than actual differences, since cell densities were similar between treatments on dates before and after that sampling date.

Objective 2

Ponds were started filling on 17 April 2002, first fertilized on 18 April 2002, and stocked with striped bass fry on 25 April 2002. All ponds were stocked at a density of 500,000 fish/ha. All ponds were fertilized with cottonseed meal following the standard regimen used at the DSFH for striped bass production. Inorganic fertilizers were added twice weekly to high-P and low-P ponds. Pond temperatures did not differ between treatment and control ponds and averaged 22°C in the morning and 24°C in the afternoon.

P effects on P. parvum toxicity and densities.—Bioassays were complicated by factors that rendered the data difficult to interpret. On one date striped bass were used rather than fathead minnows. On three sampling dates controls were completed without co-factor additions and in 15 samples on various sampling dates more fish died in diluted pond water samples than in undiluted samples. Therefore, the toxicity of pond waters could not be reliably determined. However, among undiluted pond water, a significant difference in test animal mortalities occurred between control ponds and P-fertilized ponds. Overall, more fish died in bioassays using control pond water than P-fertilized pond water (Figure 3). No fish died in bioassays with undiluted pond water after 7 May 2002 in either P-fertilization treatment, and in none of the bioassays did all test animals die. In control ponds, test animals died in bioassays through 13 May 2002. Unlike P-fertilized ponds, numerous bioassays of control pond water resulted in mortality of all test animals. On 22 April 2002 (3 days before fish stocking) all control ponds had complete mortality among all test animals, whereas on the same date in both high-P and low-P ponds no test animals died in bioassays using undiluted pond water.

There were no significant differences in overall mean *P. parvum* densities among treatments and the control. However, in high-P ponds no *P. parvum* cells were found from 20 days after initial fertilization through pond harvest (Figure 4). The trend in cell density in low-P and control ponds was a gradual decline through the study period with a moderate peak in cell density during the third week after fish stocking.

Water quality and nutrients.—Analysis of nitrate-nitrogen samples was unreliable and as a result total nitrogen concentrations were not determined. Ammonia-nitrogen concentrations averaged 24 μg/L in the low-P ponds and 32 μg/L in the high-P ponds (Figure 5). The difference in ammonia-nitrogen concentration between high- and low-P treatments was not statistically significant. Although no inorganic nitrogen was added to control ponds, ammonia concentrations averaged 63 μg/L and were significantly higher in the control than in both high-P and low-P ponds.

Because total nitrogen concentrations were not determined, the objective of maintaining specific target N:P ratios was not achieved. However, elevated phosphorus concentrations were maintained in P-fertilized ponds and low levels of phosphorus were observed in unfertilized ponds allowing the examination of phosphorus fertilization effects on water quality and cell density. Due to an error in calculating fertilization rates, phosphorus concentrations were almost double the target levels (Figure 6). Phosphorus concentrations averaged 67.7 μg/L in the low-P ponds, 91.9 μg/L in the high-P ponds, and

2.9 µg/L in the control ponds. These concentrations were significantly different between treatments and among treatments and the control.

Trends in algal growth were similar between high-P and low-P ponds. Chlorophyll *a* concentrations increased after pond fertilization and peaked six to seven weeks later (Figure 7). High-P and low-P ponds reached chlorophyll *a* maxima of around 100 µg/L by 30 days after first fertilization. Control ponds achieved chlorophyll *a* maxima of 70 µg/L. High-P and low-P ponds did not differ significantly in chlorophyll *a* however, both had significantly higher chlorophyll *a* concentrations than the control ponds. Chlorophyll *a* averaged 54.9 µg/L, 52 µg/L, and 32 µg/L in high-P, low-P, and control ponds, respectively.

Average morning and afternoon pH values differed significantly among treatments and control. Both morning and afternoon pH were highest for high-P ponds (Figure 8 and Figure 9), intermediate for low-P ponds and lowest for control ponds. Morning and afternoon pH appeared to be a function of phosphorus concentration. In both high-P and low-P ponds, pH values exceeded those recommended by Warren (2001) for the production of fingerling striped bass and their hybrids.

No differences were detected in mean morning dissolved oxygen among treatments and control ponds (Figure 10 and 12). However, near time of harvest, low morning dissolved oxygen concentrations were observed in all ponds. From 24 May 2002 through harvest, most ponds experienced 3-5-day episodes of repeated morning dissolved oxygen concentrations between 4 and 5 mg/L. One high-P pond had five mornings with dissolved oxygen concentrations of less than 4 mg/L (range 0.6-3.4 mg/L). Afternoon dissolved oxygen was significantly different between low-P ponds and control ponds (Figure 11), but no differences were found between high-P and low-P ponds or between high-P ponds and control ponds. Pond temperatures were similar among treatments and the control (Figure 12).

Zooplankton.—Densities were similar among treatments and control ponds for most zooplankton groups. Rotifers, copepod nauplii, and total zooplankton densities were statistically similar among treatments and control (Figure 13). For cladocerans, densities in high-P and control ponds were statistically different. High-P ponds averaged 209 cladocerans/L while control ponds averaged 43 cladocerans/L (Figures 13 and 14). For adult copepod, densities were statistically different among treatments and control ponds (Figure 15). Low-P ponds averaged 492 adult copepod /L while high-P ponds averaged 289 organisms/L and control ponds averaged 171 organism/L. For all treatments, zooplankton densities appeared to be adequate to support fish growth, and food limitation did not appear to be likely despite differences in densities of some zooplankton groups.

Fish production.—The most fish was produced in low-P ponds (Table 1). The number of fish produced was significantly different between low-P and control ponds ($P < 0.05$). Numbers of fish produced in high-P and low-P ponds were not statistically different. Near the end of the study two control ponds (pond 12 and 14) had observable mortality on 31 May 2002 and 1 June 2002. These mortalities were not due to low dissolved oxygen since morning concentrations were near 6 mg/L in both ponds. Such mortality is typical of previous mortality episodes observed in Dundee striped bass culture ponds, which has been

attributed to a wide variety of causes, but most recently *P. parvum*. No such mortality events were noted in P-fertilized ponds. It is likely, however, that low morning dissolved oxygen of 0.63 mg/L observed in one high-P pond may have resulted in fish mortality.

Conclusions

Phosphorus decline at cool pond temperatures was estimated to be 0.03 µg/L/h. Phosphorus decline rates were not determined at varied chlorophyll *a* concentrations nor at different temperatures. Under the conditions of this study an average phosphorus addition of 99 µg/L was required to achieve a target concentration of 60 µg/L.

Bioassays were not very helpful in determining phosphorus effects on *P. parvum* because of inconsistent results and variable bioassay methods. Bioassay methods should be standardized to avoid this problem in the future.

Cell densities did not appear to be affected by phosphorus fertilization in the short term at low temperatures (13°C). However, at temperatures typical of striped bass culture (22°C) *P. parvum* cells appeared to be eliminated in ponds fertilized with high levels of phosphorus (92 µg/L).

Under the culture conditions during objective 2, target phosphorus rates were grossly exceeded and total nitrogen concentrations were not determined so N:P ratios were unknown. No differences were found in indicators of productivity (dissolved oxygen, pH and chlorophyll *a*) between fertilization rates. However, productivity of control ponds differed significantly from that of P-fertilized ponds. It is likely that phosphorus was in excess of algal needs in both treatments and it is also possible that nitrogen limitation affected algal dynamics since nitrogen concentrations were lower in P-fertilized ponds than control ponds. Both phosphorus fertilization rates may be unsafe for striped bass production due to resulting elevated pH.

The most important indicator of efficacy of any fish culture pond treatment is fish production. The standard DSFH fertilization regime (control ponds) resulted in complete mortality of fish in two ponds near the end of the culture period. However, bioassays did not confirm that this mortality was due to *P. parvum* toxicity. If mortality was due to *P. parvum* it occurred at low cell densities. One control pond had cell densities of 1,000/mL and cells were not found in the other two control ponds. Algal cell counts may be inadequate to accurately warn of impending fish kills if mortality was due to *P. parvum*. Low dissolved oxygen in P-fertilized ponds likely resulted in fish mortality, which may have obscured differences in fish production between treatments. It is possible that fertilization rates more in line with those targeted in the original proposed rates may have less affect on pH and dissolved oxygen while providing a reduction in *P. parvum* toxicity. However, this can only be adequately determined by repeating the experiment.

Overall, this study provided some compelling evidence that phosphorus fertilization was beneficial in reducing *P. parvum* densities and toxicity in striped bass production ponds; however, more work is required to better validate these findings and to refine inorganic

fertilization strategies. The following problems, modifications and recommendations need to be addressed before the study is repeated:

1. The ability to accurately measure nitrate and ammonia concentrations needs to be insured through appropriate training and quality control measures.
2. Bioassays should be standardized with respect to test organisms, use of cofactor, and criteria used to determine if the test should be repeated.
3. Fertilization rates should be accurately calculated and verified via entry into the FHS system.
4. Phase 1 should be completed between now and initiation of phase 2.
5. Nutrient ratios and concentrations may need to be reconsidered or the number of treatments expanded based on results from the high-P treatments.
6. Methods for assessing *P. parvum* cell densities may need to be refined.