

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

Lorraine 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 <i>Prymnesium parvum</i> Ichthyotoxicity in Aquaculture.....	1
CHAPTER 2: Control of <i>Prymnesium parvum</i> using Ammonium Sulfate or Copper Sulfate in Plastic-Lined Ponds for Koi Carp Production	9
CHAPTER 3: Efficacy of Potassium Permanganate to Reduce <i>Prymnesium parvum</i> Ichthyotoxicity	17
CHAPTER 4: Toxicity of Copper Sulfate and Potassium Permanganate to Rainbow Trout and Golden Alga <i>Prymnesium parvum</i>	20
CHAPTER 5: Toxicity of Copper Sulfate Pentahydrate to Rainbow Trout <i>Oncorhynchus mykiss</i>	25
CHAPTER 6: Effects of pH on <i>Prymnesium parvum</i> Cell Viability and Toxicity	29
CHAPTER 7: Use of Hydrogen Peroxide as an Algaecide for <i>Prymnesium parvum</i>	35
CHAPTER 8: Efficacy of Nitrogen:Phosphorus Ratios for Controlling <i>Prymnesium parvum</i> in Fish Culture Ponds: Summary of 2002 Experiments.....	39
CHAPTER 9: Efficacy of Ultraviolet Radiation to Control <i>Prymnesium parvum</i> Cells and Toxicity.....	66
CHAPTER 10: Evaluation of an Ultrasonic Device to Control Golden Alga <i>Prymnesium parvum</i> in Fish Hatchery Ponds.....	71
CHAPTER 11: Microscopy and <i>Prymnesium parvum</i> : Observations and Challenges	74
CHAPTER 12: Dundee State Fish Hatchery <i>Prymnesium parvum</i> Management Plan	80
CHAPTER 13: Possum Kingdom State Fish Hatchery <i>Prymnesium parvum</i> Management Plan	85
REFERENCES	89
APPENDIX A: Identification and Enumeration of <i>Prymnesium parvum</i> 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 Ammonia,
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 than 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 LORAIN 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 chryomonad 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 mm²) and the depth under the cover slip is 0.1 mm; hence, the volume underneath the slide for each chamber is 0.1 mm³ or 10⁻⁴ cm³. Since 1 cm³ is equal to 1 mL, cells/mL is calculated by multiplying the average count per large square by 10⁴, 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 \geq 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.



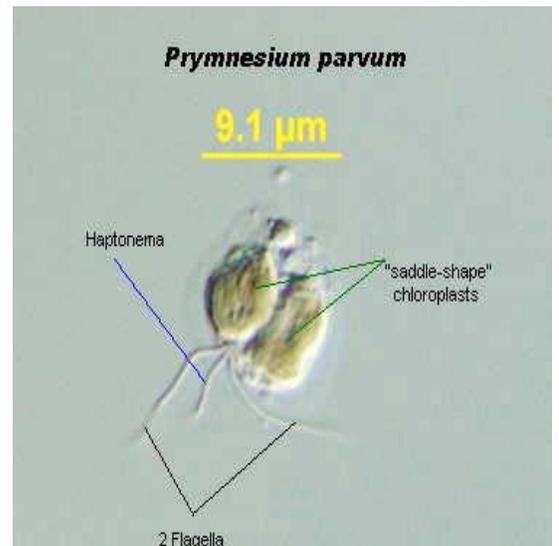
A.—Photo by Carmelo Tomas, University, North Carolina, Wilmington.



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 microscopy. (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 algicide 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 CaCO₃) 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- μ m 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 CaCO₃, hardness 900 mg/L as CaCO₃, 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 27 August 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 than 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) ₂ SO ₄	CuSO ₄ ·5H ₂ O	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 \pm 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.

Harvest variable	Treatment		
	(NH ₄) ₂ SO ₄	CuSO ₄ ·5H ₂ O	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

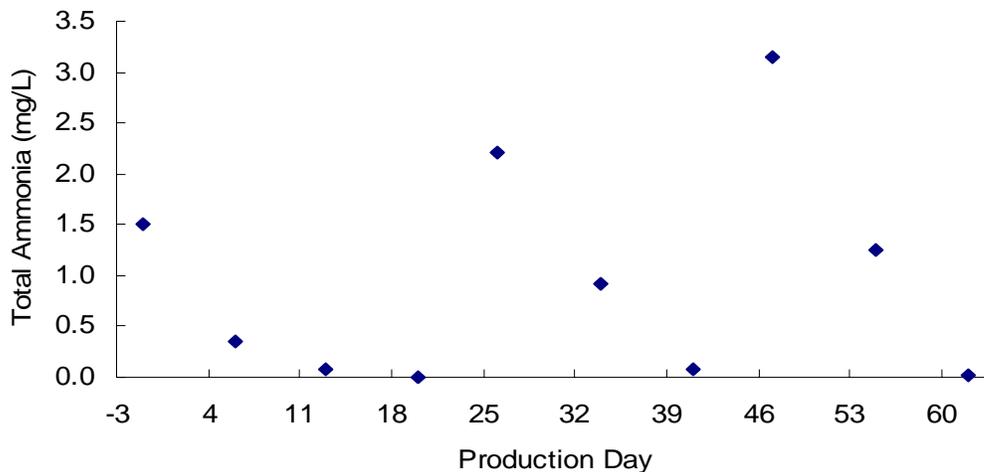


FIGURE 1.—Mean total ammonia levels in ammonium sulfate treatment ponds. Initial treatments were applied two days prior to fish stocking (production day = 0). Follow-up treatments were applied on days 25 and 46 after stocking.

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 KMnO₄, 1 mg Cu/L and 0.5 mg Cu/L as CuSO₄, 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 KMnO₄ 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 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 CaCO₃; calcium hardness, 255 mg/L as CaCO₃; total alkalinity, 75 mg/L as CaCO₃; 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 than 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 the 1 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 ·5H ₂ O		Control
		0.5 mg/L	1.0 mg/L	
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
<i>P. parvum</i> 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.

Bioassay water	Incoming water	Treatment			
		KMnO ₄ mg/L	CuSO ₄ ·5H ₂ O		Control
			0.5 mg/L	1.0 mg/L	
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 CaCO₃; calcium hardness, 255 mg/L as CaCO₃; and total alkalinity, 88 mg/L as CaCO₃. 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 (\pm 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 \pm 0.29	10.19 \pm 0.35	10.20 \pm 0.33	10.14 \pm 0.32
Temperature ($^{\circ}$ C)	11.82 \pm 0.45	11.60 \pm 0.44	11.68 \pm 0.45	11.77 \pm 0.47
pH	7.99 \pm 0.04	7.99 \pm 0.05	8.00 \pm 0.04	8.01 \pm 0.04
24-h copper (mg/L)	0.043 \pm 0.014	0.152 \pm 0.006 (69.5 \pm 1.2)	0.274 \pm 0.033 (72.6 \pm 3.3)	0.514 \pm 0.029 (74.3 \pm 1.4)
24-h copper (mg/L)	0.053 \pm 0.016	0.175 \pm 0.021 (64.9 \pm 4.1)	0.327 \pm 0.037 (67.3 \pm 3.7)	0.576 \pm 0.046 (71.2 \pm 2.3)
Survival	100	93.3 \pm 11.5	86.7 \pm 11.5	73.3 \pm 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.

Treatment pH	Final pH	Mean cell density (cells/mL)*	
		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.

Treatment pH	Final pH	Mean cell density (cells/mL)*	
		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.

pH	Dead fish/total fish		
	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 $\geq 3,125$ 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 chrysoomonad, 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 brachiopods.

In mid-April through early May 2001, the entire crop of striped bass (*Morone saxatilis*) and palmetto bass (female *M. saxatilis* \times 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 $\mu\text{g}/\text{mL}$) 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 be 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 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ lysed all *P. parvum* cells within 15 min while 250-3,125 $\mu\text{g}/\text{mL}$ inhibited cell motility (Tables 1 and 2). For 1-h exposures, hydrogen peroxide concentrations $\geq 3,125$ $\mu\text{g}/\text{mL}$ lysed all cells whereas no lysis was observed for concentrations ≤ 781.25 $\mu\text{g}/\text{mL}$. Motility was inhibited at 1-h exposure and concentrations ≥ 500 $\mu\text{g}/\text{mL}$ (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 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ for 60 min; walleye, bluegill and channel catfish tolerate <100 $\mu\text{g}/\text{mL}$ for 60 min; and fathead minnow fry tolerate <50 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ for 1 h, although significant gill damage was observed at treatment rates greater than 175 $\mu\text{g}/\text{mL}$. Although Rach et al. (1997) showed that some fish species could tolerate hydrogen peroxide at concentrations up to 1000 $\mu\text{g}/\text{mL}$ for 15-45 min for four consecutive days, the present study indicates that hydrogen peroxide is ineffective as an algacide against *P. parvum* at concentrations $\leq 3,125$ $\mu\text{g}/\text{mL}$ for 15-min treatments and at concentrations ≤ 781.25 $\mu\text{g}/\text{mL}$ 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).

Date	Exposure	H ₂ O ₂ (µg/L)				
		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 B₁₂ 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 B₁₂ 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:

$$(\text{Total P applied} - \text{initial pond P}) + (\text{final pond P} - 60) = \text{phosphorus demand.}$$

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:

$$(\text{P demand} - \text{initial P concentration}) + \text{anticipated P loss for three days.}$$

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:

$$(\text{Initial P concentration} - \text{Final P concentration})/24 \text{ h} = \text{P loss/h.}$$

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:

$$\text{P target} + \text{P demand} + \text{P loss for 72-h interval}$$

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.

TABLE 1.—Striped bass fingerling production in ponds fertilized with high or low levels of phosphorus or no phosphorus (control) at the Dundee State Fish Hatchery in Spring 2002.

Treatment (N:P ratio)	N:P rates ($\mu\text{g/L}$)	Pond	Fish stocked	Fish harvested	Weight harvested (kg)	Harvest length (mm)	Survival (%)
Low P (10:1)	300 : 30	7	50,156	6,792	6.85	44.3	0.14
Low P (10:1)	300 : 30	15	50,184	1,392	0.68	36.0	0.03
Low P (10:1)	300 : 30	16	50,184	10,270	7.17	41.6	0.20
High P (5:1)	300 : 60	9	50,000	4,317	5.22	47.2	0.09
High P (5:1)	300 : 60	13	50,184	0	0.00	0.00	0.00
High P (5:1)	300 : 60	67	52,693	0	0.00	0.00	0.00
Control	0	10	50,184	0	0.00	0.00	0.00
Control	0	12	50,184	0	0.00	0.00	0.00
Control	0	14	50,184	167	0.00	39.2	0.00

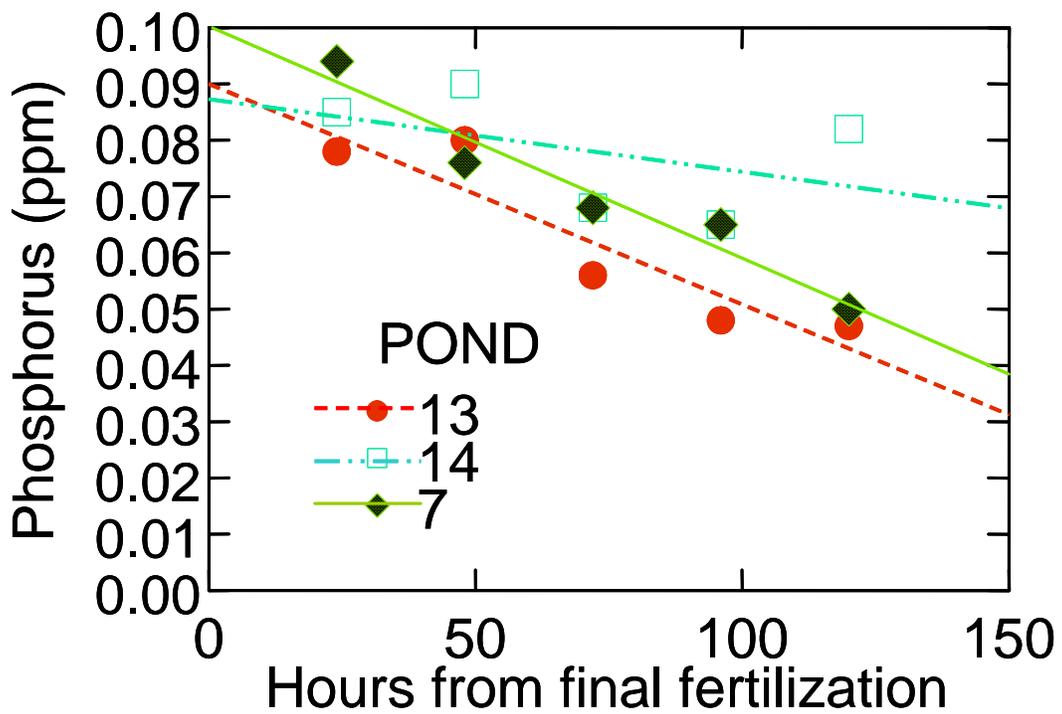


FIGURE 1.—Phosphorus decline rates in ponds fertilized with two 60- μ g/L applications of phosphorus at the Dundee State Fish Hatchery in Spring 2002.

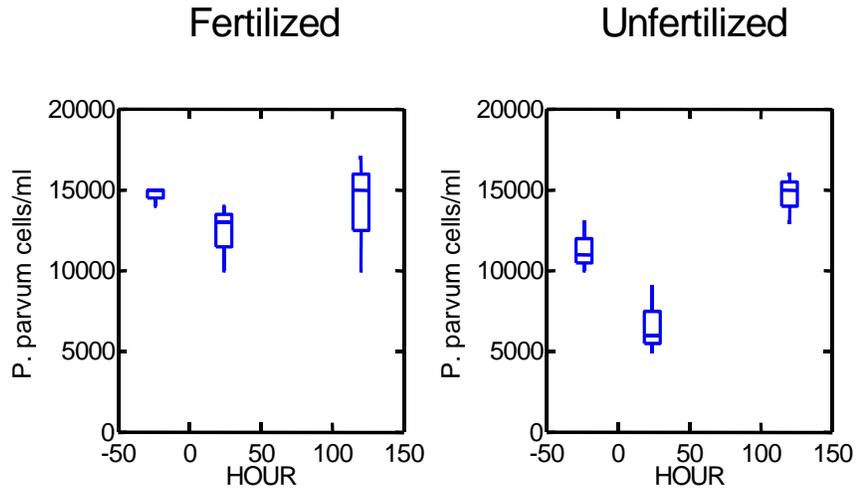


FIGURE 2.—*Prymnesium parvum* cell densities in ponds fertilized with 60 μg P/L or unfertilized with phosphorus at the Dundee State Fish Hatchery in Spring 2002.

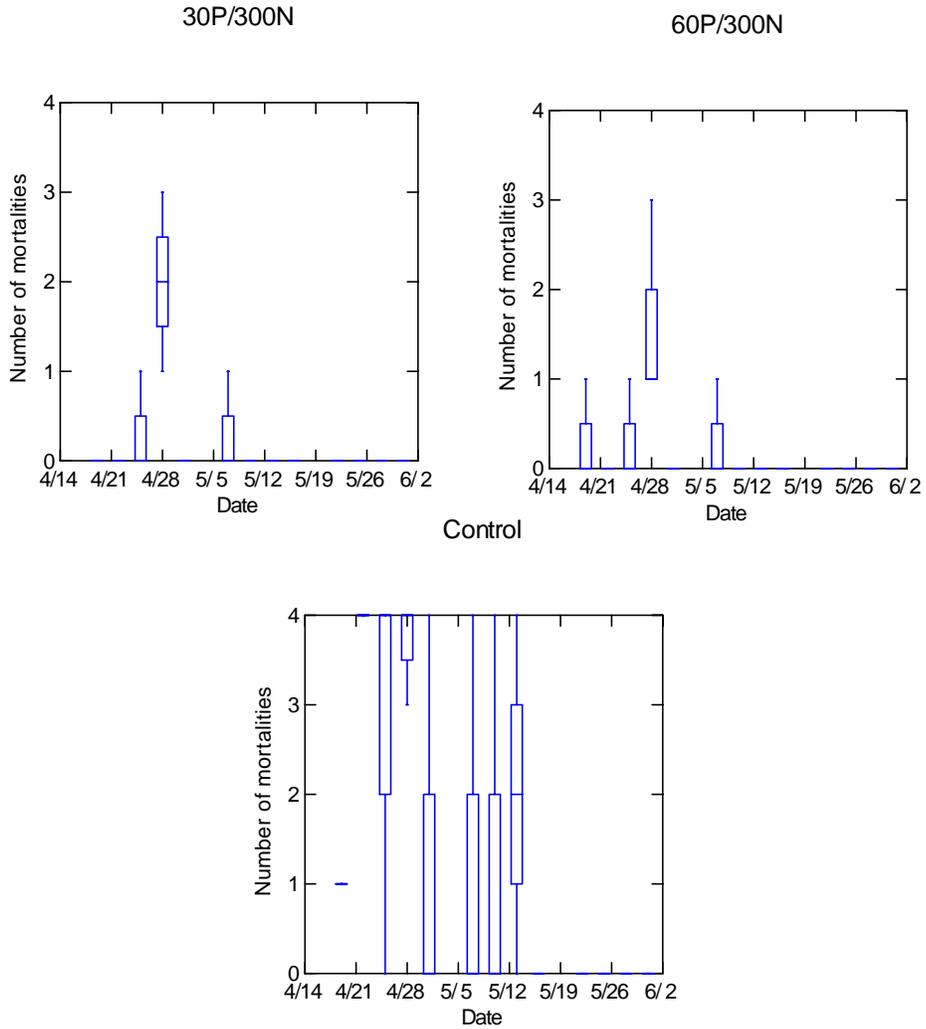


FIGURE 3.—Mortality among test fish in bioassays with undiluted water from ponds fertilized with high or low levels of phosphorus or no phosphorus (control) at the Dundee State Fish Hatchery in Spring 2002.

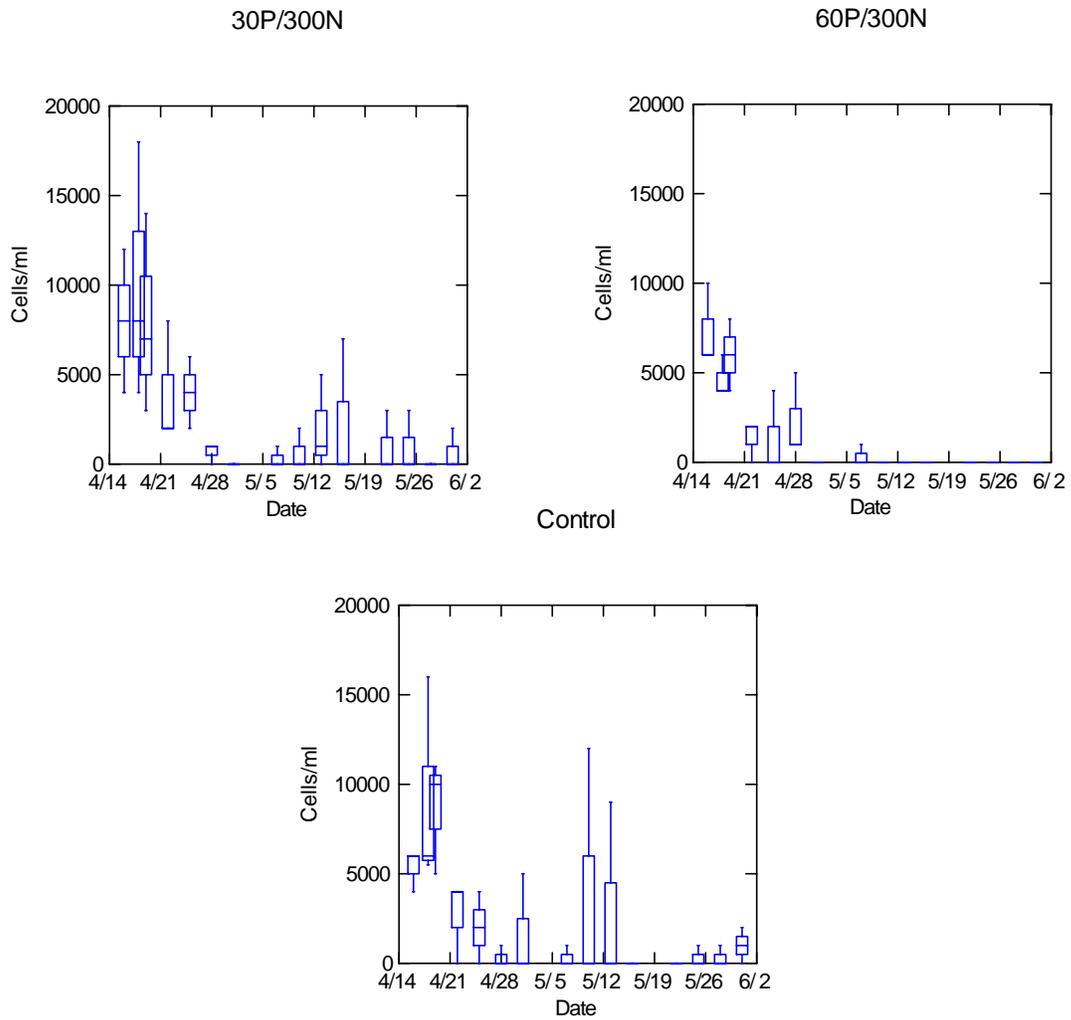


FIGURE 4.—*Prynnesium parvum* cell densities in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

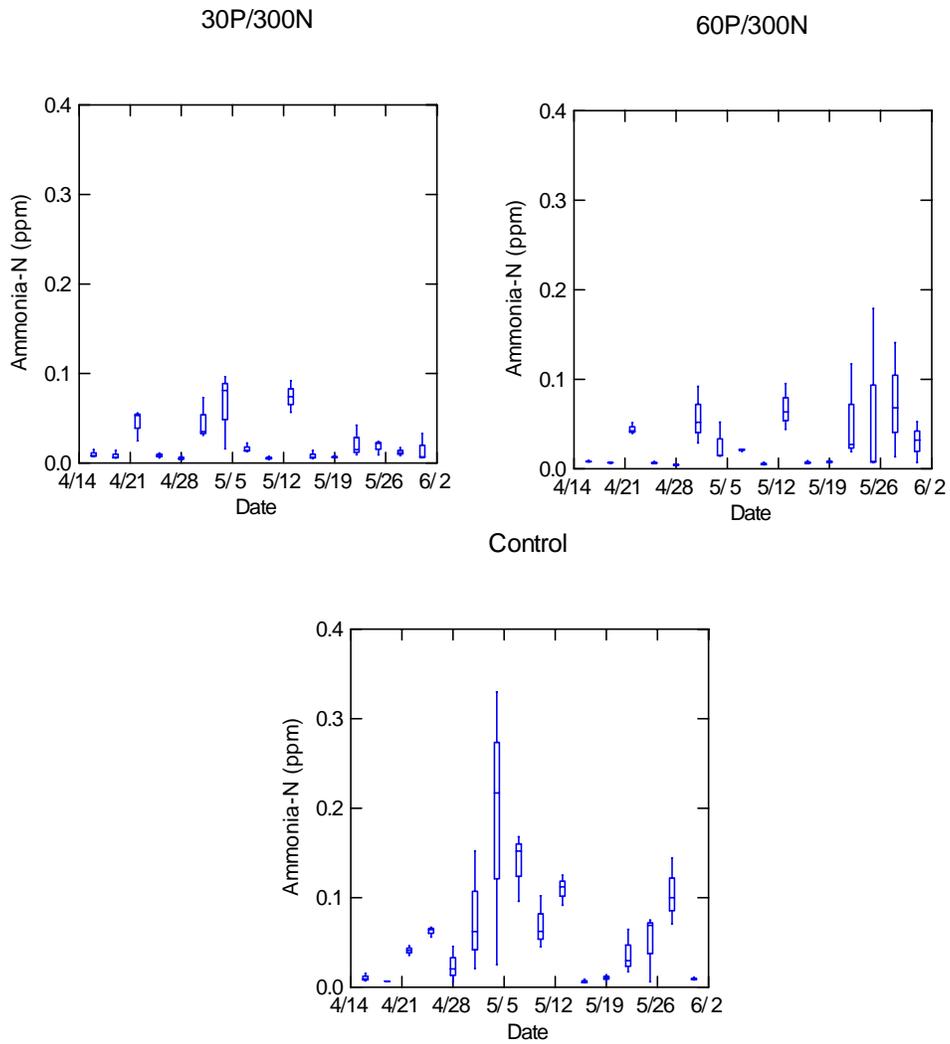


FIGURE 5.—Ammonia nitrogen concentrations in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

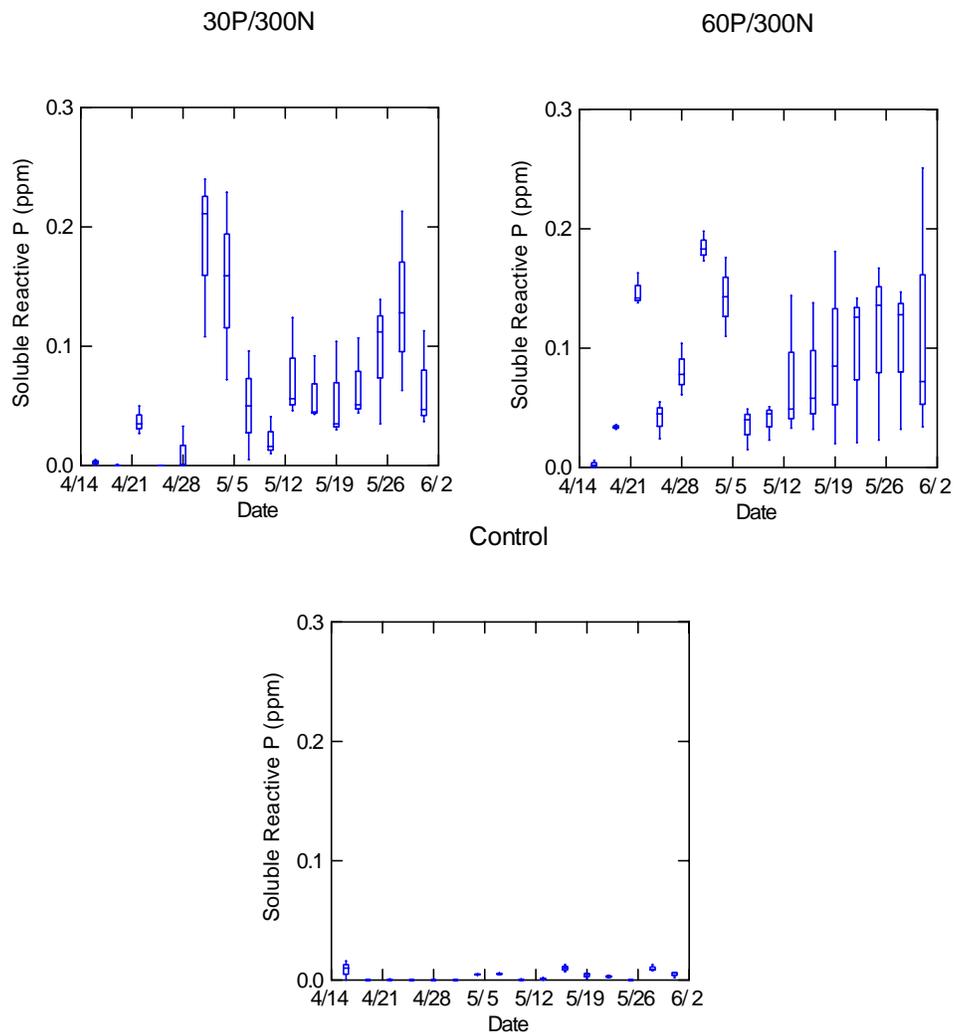


FIGURE 6.—Phosphorus concentrations in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

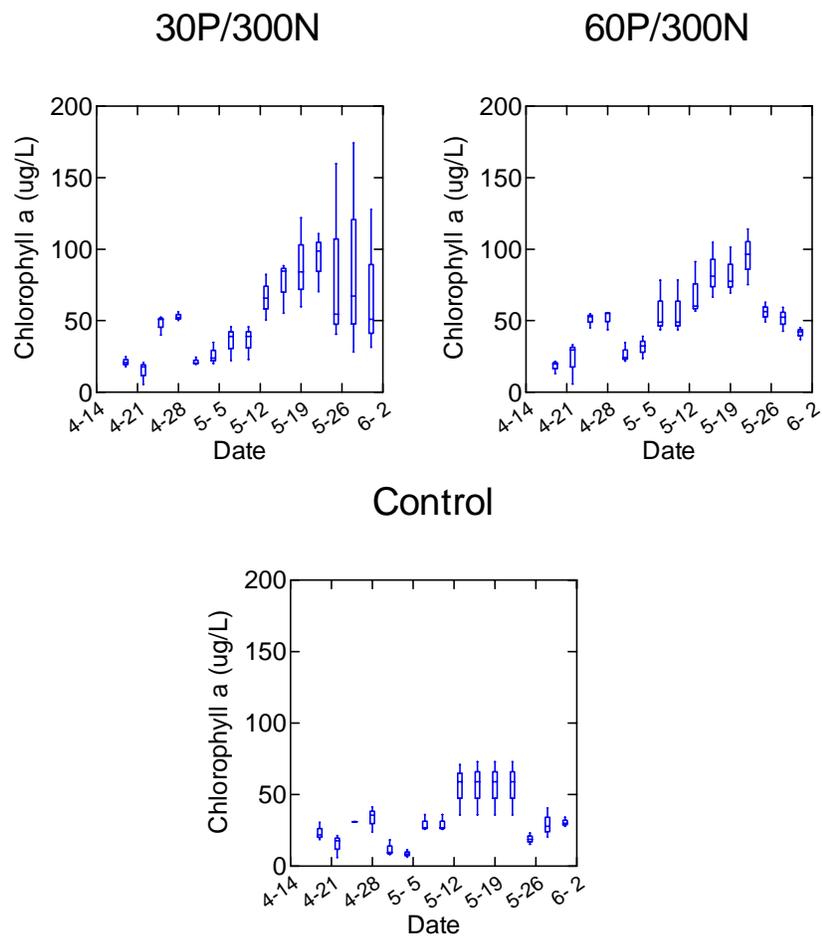


FIGURE 7.—Chlorophyll *a* concentrations in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

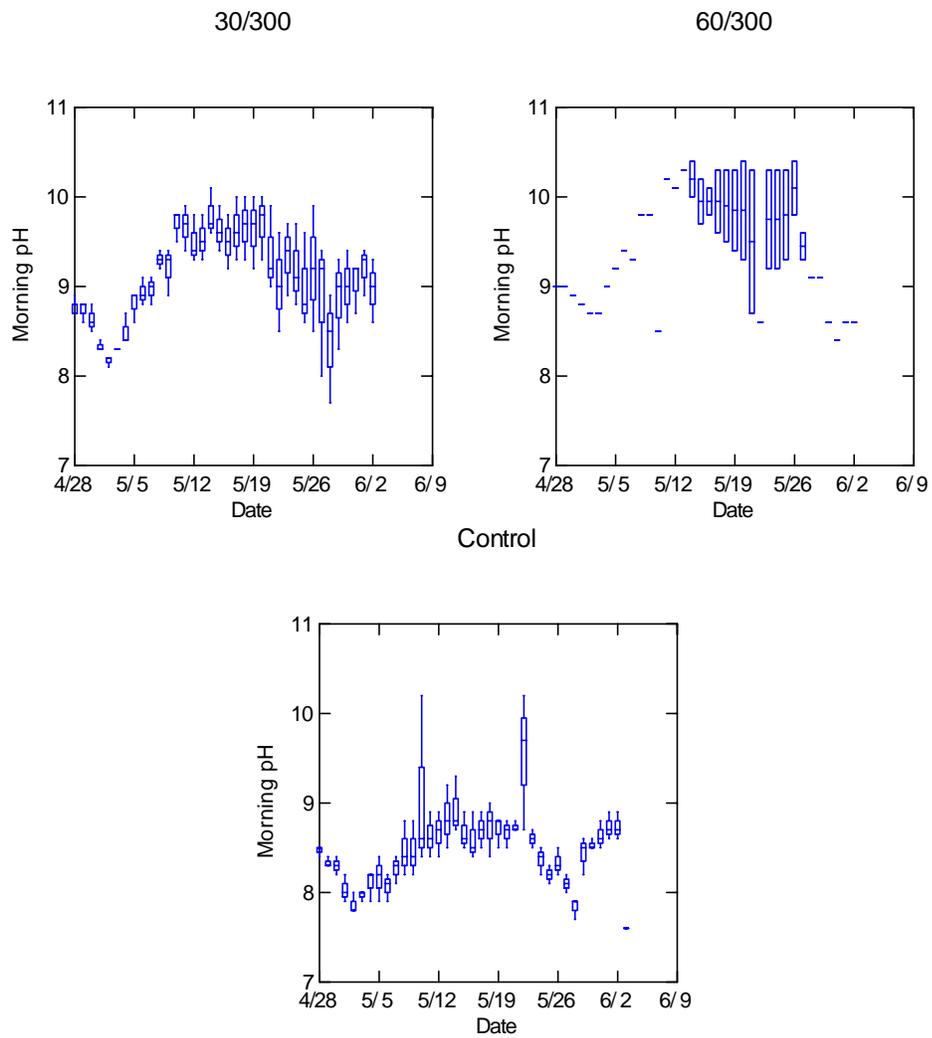


FIGURE 8.—Morning pH values in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

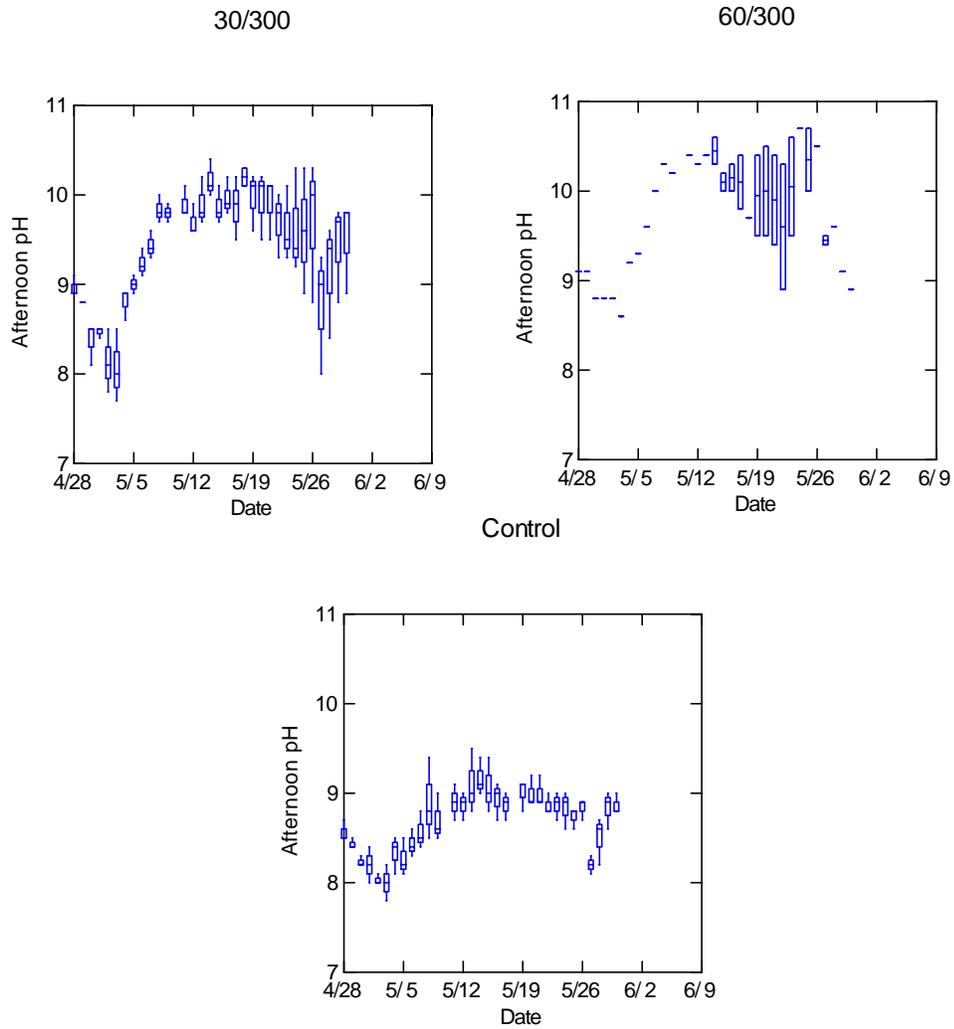


FIGURE 9.—Afternoon pH values in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

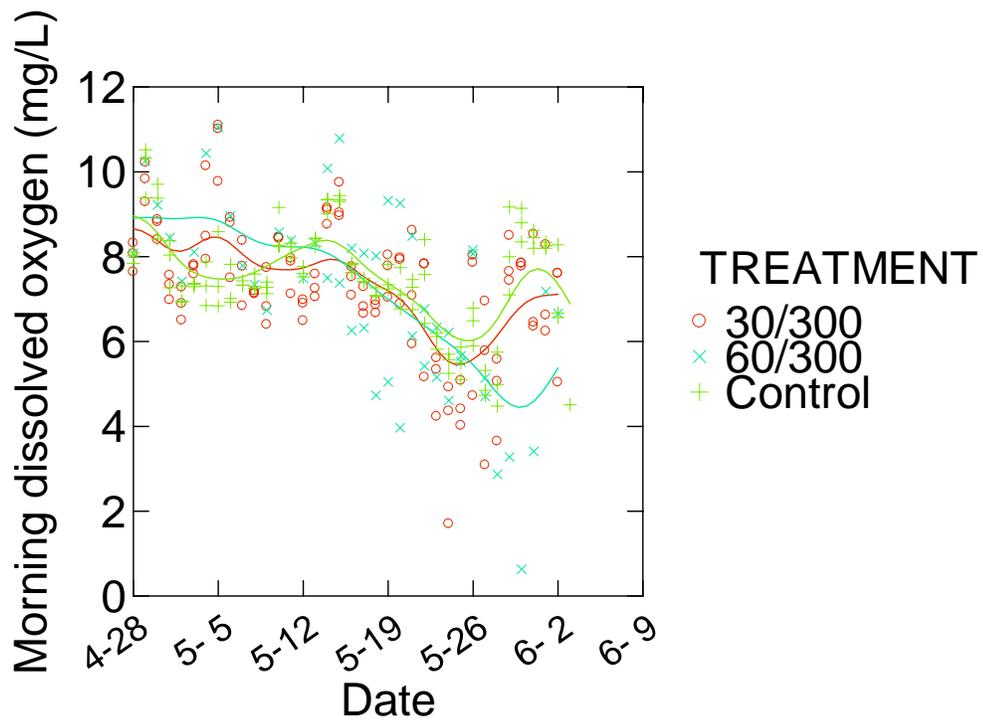


FIGURE 10.—Morning dissolved oxygen concentrations in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

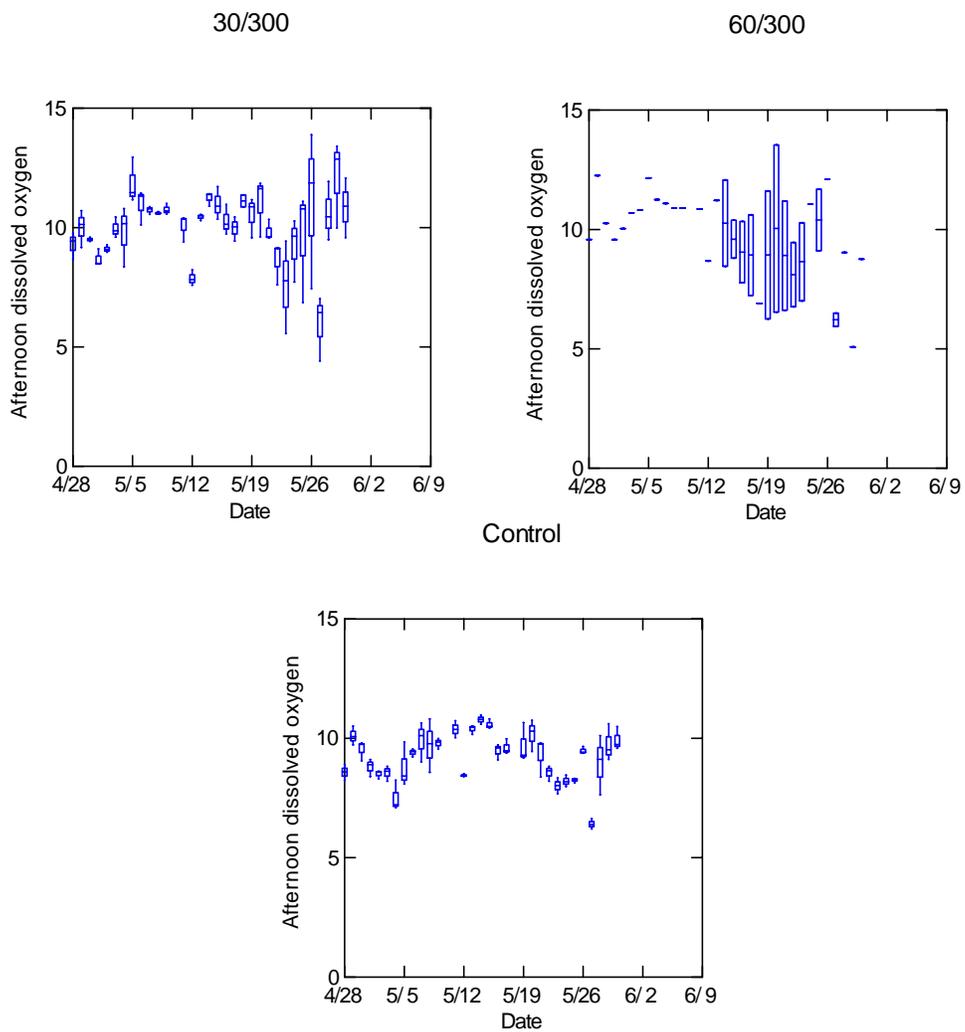


FIGURE 11.—Afternoon dissolved oxygen concentrations in ponds fertilized with high or low levels of phosphorus or no phosphorus at the Dundee State Fish Hatchery in Spring 2002.

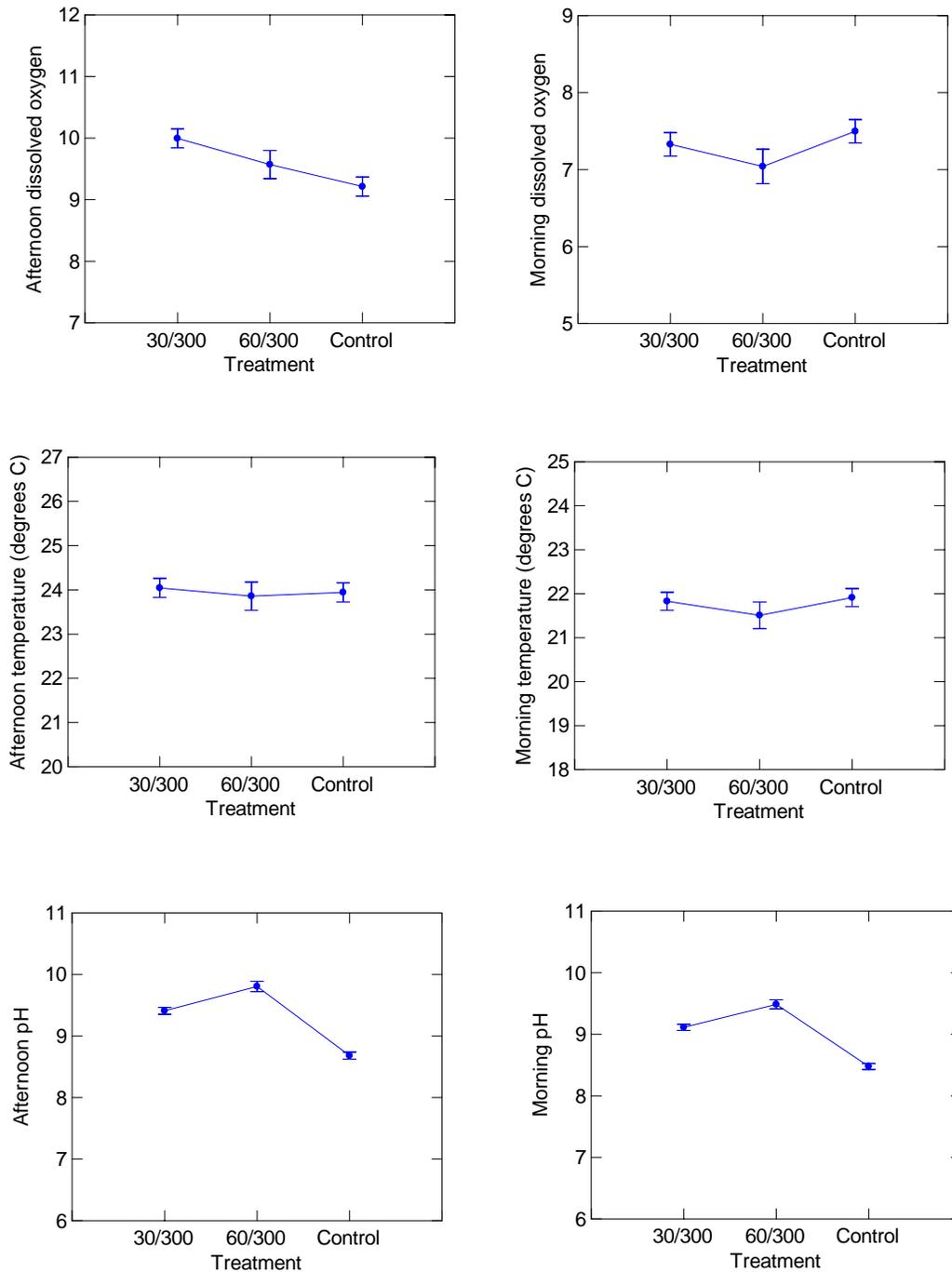


FIGURE 12.—Least squared means for water quality parameters in ponds fertilized with two concentrations of phosphorus or no phosphorus (control) at the Dundee State Fish Hatchery in Spring 2002.

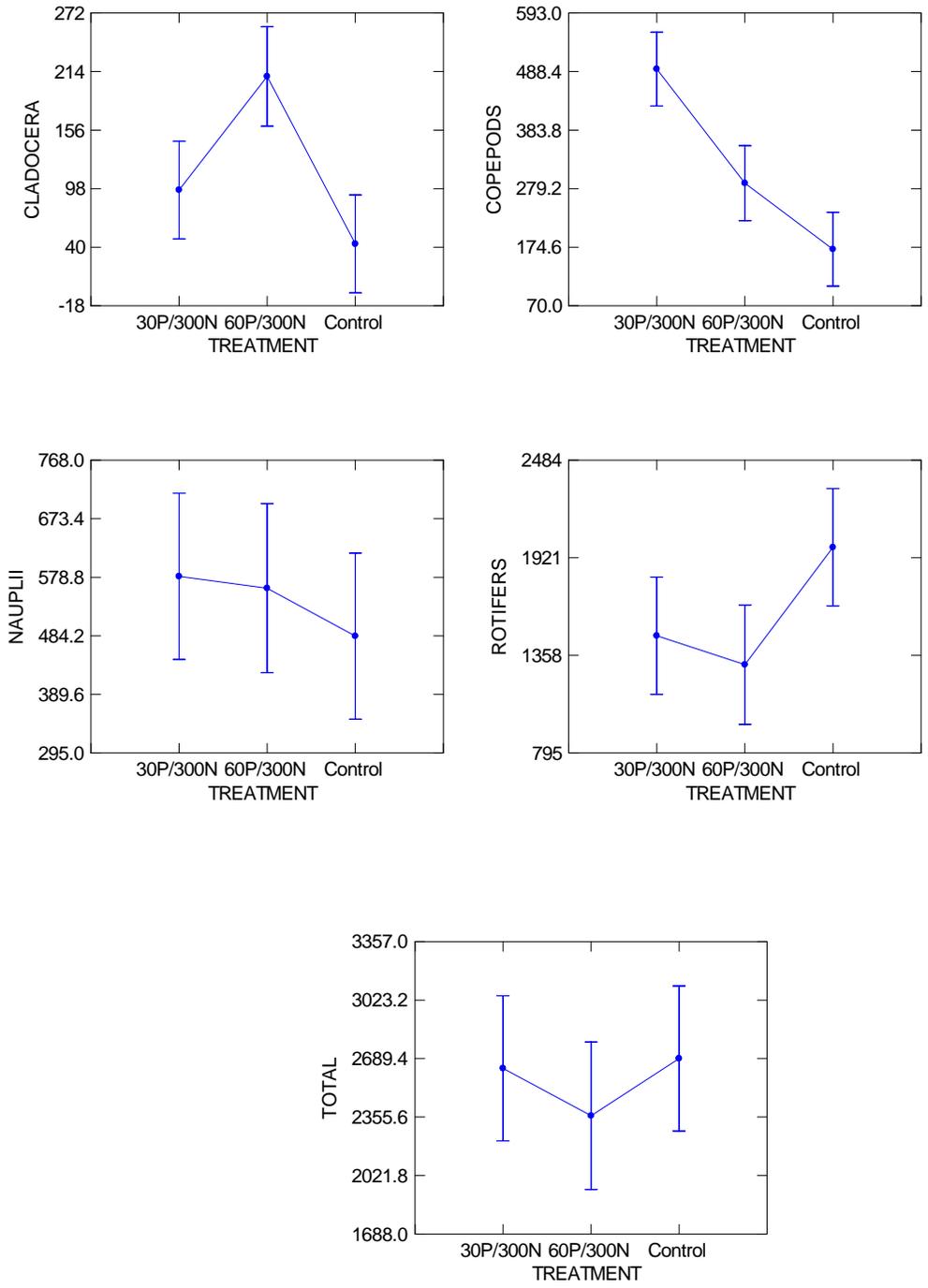


FIGURE 13.—Least squared means of zooplankton densities in ponds fertilized with two concentrations of phosphorus or no phosphorus (control) at the Dundee State Fish Hatchery in Spring 2002.

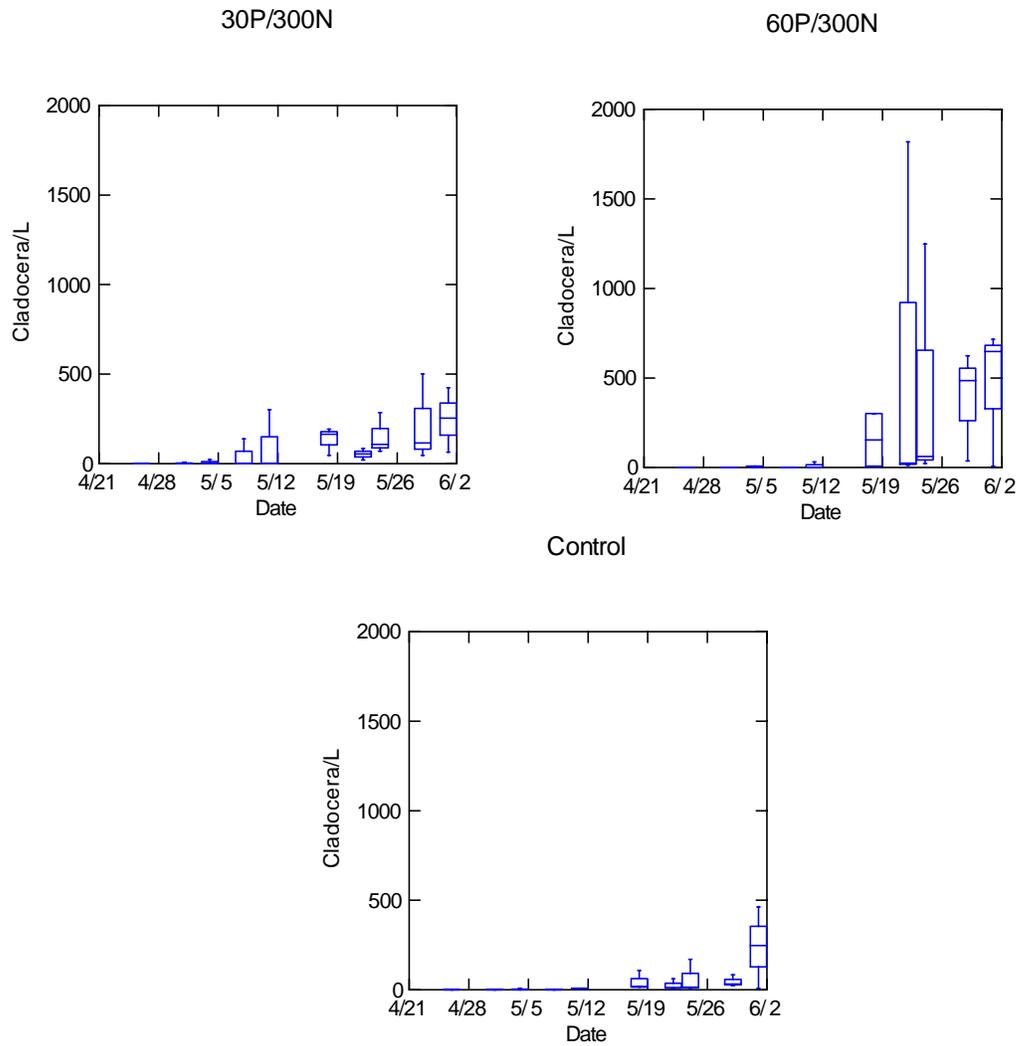


FIGURE 14.—Cladoceran densities in ponds fertilized with two concentrations of phosphorus or no phosphorus (control) at the Dundee State Fish Hatchery in Spring 2002.

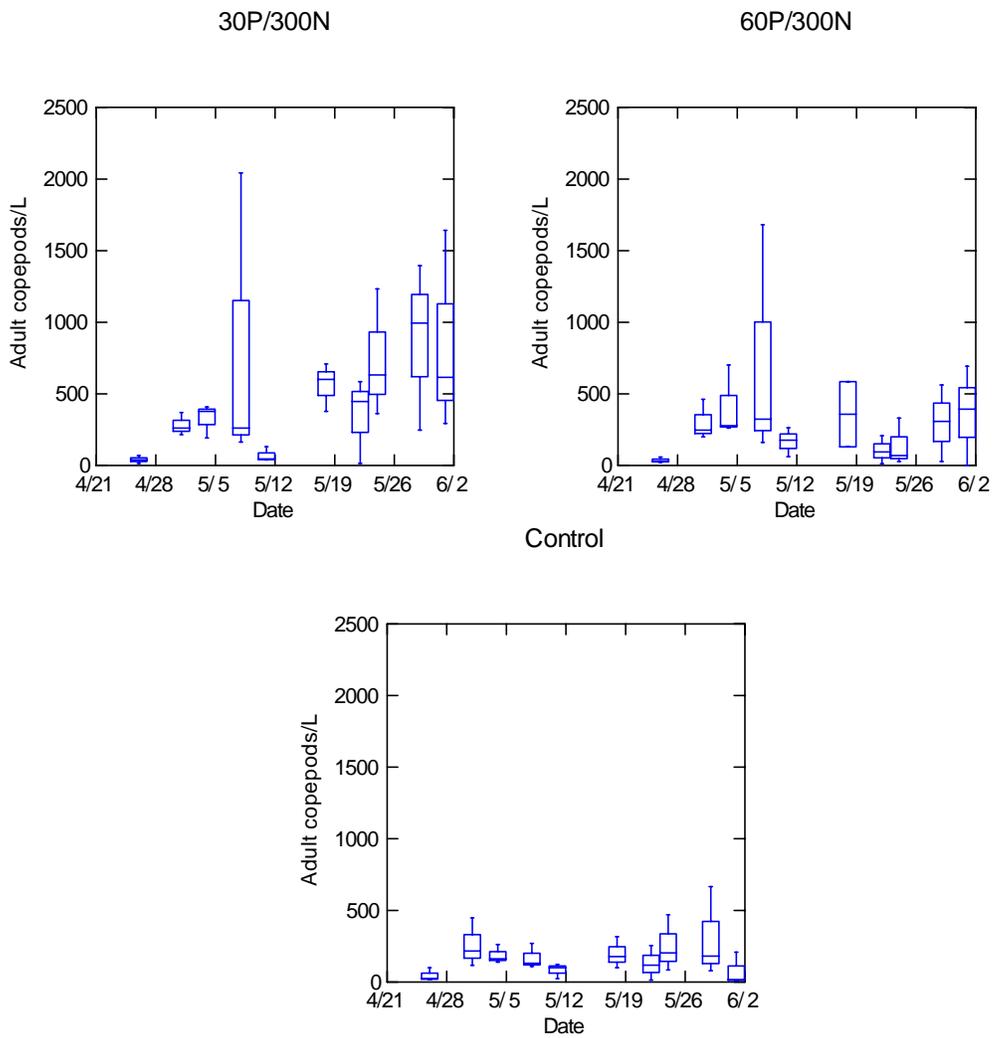


FIGURE 15.—Adult copepod densities in ponds fertilized with two concentrations of phosphorus or no phosphorus (control) at the Dundee State Fish Hatchery in spring 2002.

CHAPTER 9

Efficacy of Ultraviolet Radiation to Control *Prymnesium parvum* Cells and Toxicity

DENNIS G. SMITH

Abstract

The effects of ultraviolet (UV) radiation on *Prymnesium parvum* cells and ichthyotoxicity were investigated using water from Lake Diversion, Texas. Reservoir water was toxic to fish on three of four testing days and sublethal on the remaining day. *P. parvum* cells were present in the water throughout the study. The water flowed through the UV unit at 11.5-11.6 CFM (i.e., cubic feet per min). The radiation was emitted at a mean dose of 210 (range = 193- 220) mJ/cm² and mean intensity of 91.5 (range = 84-96) mW/cm². The radiation completely destroyed all *P. parvum* cells and reduced toxicity from lethal to sublethal levels or from a sublethal to undetectable level.

Introduction

Prymnesium parvum, which produces toxins collectively known as prymnesins (Igarashi et al. 1996), has caused extensive fish mortality in rearing ponds at the Dundee State Fish Hatchery (DSFH) which necessitated a need for effective control of *P. parvum* cells and toxicity. This study was designed to determine whether ultraviolet (UV) radiation was a viable method to destroy *P. parvum* cells and ichthyotoxin. If UV radiation was determined to be effective, a treatment unit could be installed to sterilize incoming water to the hatchery. Water to the hatchery flows under gravity through a pipeline from Lake Diversion which is known to contain *P. parvum* and has experienced periodic fish kills attributable to the alga.

Materials and Methods

A UV-treatment unit was rented from Aquionics® and installed on a levee of a 0.1-ha rearing pond. Water flowed from the fill line of the pond through the UV unit and a flow meter into the rearing pond. A polyvinyl polycarbonate (PVC) pipe on either side of the treatment unit was fitted with a drain valve as close as possible to the unit. These drain valves allowed acquisition of untreated (inlet side of UV unit) and treated (outlet side of UV unit) water samples. The control and display panel of the UV unit was mounted on the safety railing of the pond. Water flow was adjusted with the pond fill valve to maintain a rate of approximately 327 L/min. The UV unit was in continuous operation for 11 days. The radiation dose and intensity and related data as well as water flow rates were recorded once or twice daily (Table 1). Water samples for cell counts were collected twice daily from the drain valves by flushing water through them for a few minutes and then simultaneously collecting some water from each drain valve in 19-L buckets. Three separate aliquots were

taken from each bucket for cell counts using a hemacytometer (Appendix A). Toxicity bioassays (Appendix B) also were performed on water collected in the same manner on the second, fourth, sixth, and eleventh day of operation. Bioassays were performed in duplicate for each sample.

Results and Discussion

Water was treated with a mean radiation dose of 210 (range = 193-220) mJ/cm² and mean intensity of 91.5 (range = 84-96) mW/cm² (Table 1). Mean cell density of the untreated water ranged from 0 to 14,000 cells/mL (Table 2), which is reflective of a patchy distribution of *P. parvum* in Lake Diversion. There were no viable cells in any of the treated water samples examined, indicating that UV radiation at the dose and intensity tested was effective at destroying *P. parvum* cells. This suggests that UV sterilization could prevent contamination of rearing ponds with *P. parvum* during pond filling. Furthermore, UV irradiation could be used to reduce or eliminate the number of viable *P. parvum* cells in water for fish transport and thereby lower the risk of unintentional introduction of *P. parvum* into lakes where the fish are stocked.

The bioassay results revealed that UV radiation did not always eliminate the toxin, but effectively reduced toxicity from lethal to sublethal levels or from sublethal to undetectable levels. The mortality data showed that toxicity was present in Lake Diversion water throughout the study occurring at lethal concentrations during the first three sampling days and at a sublethal concentration during the last sampling day. In the undiluted water with no co-factor (i.e., water that fish in rearing ponds would be exposed to), there were no mortalities in UV-treated water for the first three test dates (Table 3), although on those same days mortalities did occur in the other bioassay treatments (those with the cofactor). This observation indicates that toxicity was present at sublethal levels in the UV-treated water. On 8 October 2001, mortality occurred only in the untreated undiluted water without co-factor suggesting that the sublethal concentration of the toxin was further reduced to an undetectable level. These results suggest that, for a given radiation and water flow rate, detoxification is dependent upon toxin level such that complete removal is only accomplished at lower toxicity levels and at higher levels, toxicity is reduced but not eliminated.

TABLE 1.—Ultraviolet radiation characteristics and flow rate (cubic feet per min) of water subjected to radiation to destroy *Prymnesium parvum* and its ichthyotoxin.

Date	Time	Intensity (%)	Intensity (mW/cm ²)	Low UV Set point	Cumulative Run time (h)	Temperature (°C)	Dose (mJ/cm ²)	Wiper delay	Wiper cycles	Flow (CFM)
27 Sep 01	1430	98	84	70	47	20	193	2	90	11.5
28 Sep 01	1300	101	86	70	69	19	197	2	101	11.6
29 Sep 01	0901	105	90	70	89	19	207	2	111	11.5
29 Sep 01	1342	105	90	70	94	20	204	2	113	11.6
30 Sep 01	0853	108	92	70	113	19	211	2	122	11.6
30 Sep 01	1310	107	91	70	117	20	209	2	124	11.5
1 Oct 01	1100	108	92	70	140	19	211	2	135	11.5
1 Oct 01	1500	108	92	70	143	19	211	2	137	11.5
2 Oct 01	0900	110	94	70	161	19	216	2	145	11.6
2 Oct 01	1430	108	92	70	167	19	211	2	148	11.6
3 Oct 01	1030	108	92	70	187	19	211	2	158	11.5
3 Oct 01	1500	105	90	70	191	19	207	2	160	11.5
4 Oct 01	0900	109	93	70	209	19	213	2	168	11.5
4 Oct 01	1500	108	92	70	215	19	211	2	171	11.5
5 Oct 01	0800	109	93	70	232	18	213	2	180	11.5
5 Oct 01	1300	110	90	70	237	18	216	2	182	11.5
7 Oct 01	0900	112	96	70	256	18	220	2	203	11.6
7 Oct 01	1255	110	94	70	260	18	216	2	205	11.5
8 Oct 01	1100	111	95	70	282	18	218	2	216	11.5
Mean		107.4	91.5	70		18.9	210.3	2	151	11.5

TABLE 2.—Mean densities (range) of *Prymnesium parvum* in UV-treated and untreated water from Lake Diversion. Treated water passed through a UV unit at 11.5 CFM and subjected to radiation of 210.3 mJ/cm² at 91.5-mW/cm² intensity. Untreated water received no UV-radiation.

Date	N	<i>Prymnesium parvum</i> density (cells/mL)	
		Untreated water	Treated water
27 Sep 01	6	9,000 (2,000 – 30,000)	0
28 Sep 01	3	4,000 (2,000 – 6,000)	0
28 Sep 01	6	8,333 (4,000 – 16,000)	0
30 Sep 01	6	6,000 (2,000 – 10,000)	0
1 Oct 01	6	6,667 (2,000 – 16,000)	0
2 Oct 01	6	6,333 (2,000 – 10,000)	0
3 Oct 01	6	4,667 (0 – 10,000)	0
4 Oct 01	6	1,667 (0 – 4,000)	0
5 Oct 01	6	2,000 (0 – 4,000)	0
7 Oct 01	6	333 (0 – 2,000)	0
8 Oct 01	6	666 (0 – 2,000)	0

TABLE 3.—Bioassay (toxicity) results for untreated and treated water. Treated water passed through a UV unit at 11.5 ft³/min and subjected to radiation of 210.3 mJ/cm² at 91.5-mW/cm² intensity. Untreated water received no UV radiation.

Date	Water type	Whole water with cofactor		1:5 Dilution with cofactor		Whole water with no cofactor	
		Test fish	Dead fish	Test fish	Dead fish	Test fish	Dead fish
28 Sep 01	Untreated	3	3	3	3	3	3
	Treated	3	3	3	2.5	3	0
1 Oct 01	Untreated	4	4	4	4	4	0.5
	Treated	4	4	4	0	4	0
3 Oct 01	Untreated	4	4	4	4	4	4
	Treated	4	4	4	0	4	0
8 Oct 01	Untreated	4	4	4	0	4	0
	Treated	4	0	4	0	4	0

CHAPTER 10

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

TOM DORZAB

Abstract

An ultrasonic device (i.e., Aquasonic Algae Controller) was evaluated to determine its efficacy at controlling *Prymnesium parvum* in hatchery ponds. This pilot study consisted of one pond with one Aquasonic Algae Controller (treatment) and two untreated ponds (control). Each 0.1-ha pond was stocked with five adult rainbow trout *Oncorhynchus mykiss*. Cell density *P. parvum* cell density was monitored in each pond for 21 days. The ultrasonic device appeared to be ineffective in reducing *P. parvum* cell density and had no discernable effect on survival of rainbow trout.

Introduction

The toxin producing, brackish-water phytoflagellate *Prymnesium parvum* was first identified at the Dundee State Fish Hatchery in March 2001. During the preceding month, about 7,000 rainbow trout *Oncorhynchus mykiss* died from exposure to the alga and its toxin. Since rainbow trout are known to be sensitive to copper sulfate (Hansen et al. 2002c) and ammonium sulfate is ineffective at controlling *P. parvum* at temperatures below 18°C (Sarig 1971), an alternative control method was needed for cold water conditions. This study was initiated to determine the effectiveness of the Aquasonic Algae Controller to control *P. parvum* in water with temperatures up to 18°C. This device is advertised for control of aquatic vegetation and reported to have successful application and wide acceptance (VoR Environmental, <http://www.vor-env.com>). The device works by emitting sound waves that cause cell death by breaking the cell vacuole (VoR Environmental)

Materials and Methods

Three 0.1-ha ponds were filled on 16 December 2001. Two of the ponds were used as control and received no treatment and the ultrasonic device was installed in the remaining pond on December 17. Each pond was stocked with five rainbow trout on December 17. *P. parvum* cell counts were conducted on December 17 and continued periodically through the end of the trial on January 7, 2002 using an established protocol (Appendix A). Morning temperatures, dissolved oxygen, and pH readings were recorded daily in each pond. Ponds were harvested on January 7, 2002 and the number of fish recovered from each pond was recorded.

Results and Discussion

At the beginning of the trial, cell counts showed *P. parvum* cells were present in one of the two control ponds as well as in the Aquasonic-treated pond (Table 1). Three days later, the same pattern was observed. Ten days after the start of the trial, *P. parvum* cells were present in the control pond that had previously had none while the two ponds that previously had cells now had none. Sixteen days after the trial began, cells were not observed in any of the ponds and after 21 days, *P. parvum* cells were found only in the pond with the ultrasonic unit. These observations suggest that the Aquasonic device was not effective in eliminating *P. parvum* from the pond after 21 days.

One fish (20%) died in the Aquasonic-treated pond while no fish died in either control pond, though the cause of the mortality is unclear. Although this study is not robust due to a lack of replication, the results presented herein suggest that further study of ultrasonic devices is unwarranted.

TABLE 1.—*Prymnesium parvum* cell densities and rainbow trout *Oncorhynchus mykiss* mortality in ponds with or without the Aquasonic Algae Controller.

Treatment	Pond	Fish mortality (%)	Cell density (number/mL)				
			17 Dec 01	20 Dec 01	27 Dec 01	2 Jan 02	7 Jan 02
Aquasonic	13	20	2,000	2,000	0	0	4,000
Control	9	0	0	0	2,000	0	0
Control	12	0	2,000	8,000	0	0	0

TABLE 2.—Water quality variables of trout ponds with or without the Aquasonic device for controlling *Prymnesium parvum*.

Treatment	Pond	Dissolved oxygen (mg/L)	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Temperature (°C)	pH
Control	9	12.0 10.8 - 12.8	5.1 1.7 - 9.5	7.91 7.80 - 8.10	12.0 11.4 - 12.9	6.4 3.8 - 10.3	7.98 7.80 - 8.10
Control	12	11.8 10.6 - 13.0	5.3 1.9 - 9.3	7.91 7.80 - 8.00	12.1 11.0 - 13.0	5.8 3.9 - 9.9	7.96 7.90 - 8.00

CHAPTER 11

Microscopy and *Prymnesium parvum*: Observations and Challenges

GREGORY M. SOUTHARD

Abstract

Prymnesium parvum is an algal species responsible for toxic fish kills in Texas reservoirs and two freshwater fish hatcheries. The current Texas Parks and Wildlife Department (TPWD) method for identifying and estimating densities of *P. parvum* in water samples uses a compound light microscope, hemacytometer, and trained personnel adept at identifying this particular species among mixed algal communities. Repeated observations of *P. parvum*-infested water samples using epifluorescence microscopy suggest that *P. parvum* may yield distinct fluorescence emission pattern(s) compared to the other types of algae in mixed samples. This observation led to an investigative effort regarding the feasibility of specialized microscopic techniques, flow cytometry, and other fluorescence-based applications that might facilitate *P. parvum* cell enumeration. The following report is an overview of that investigation.

Introduction

Toxin-producing algae have been an area of health and economic concerns and study for marine biologists for decades and recently have become an issue for freshwater systems in the United States. *Prymnesium parvum*, also called “golden alga”, is a chryomonad haptophyte, which was first implicated in minor fish kills along the Pecos River in Texas during the 1980s (Linam et al. 1991). In recent years, this species has caused catastrophic fish mortalities in several Texas river systems and reservoirs as well as being problematic at two Texas Parks and Wildlife Department (TPWD) freshwater fish hatcheries.

An important aspect of toxic algal management plans at TPWD fish hatcheries involves monitoring cell densities to assess the need to implement control methods. Currently, trained personnel monitor *P. parvum* in reservoirs and hatchery ponds using light microscopy at magnification up to 1000X to identify the organism and a hemacytometer to calculate cell concentration.

Description of P. parvum

Microscopically, *P. parvum* has often been described as having variations in size and shape depending upon the phase of the life cycle as well as nutrient availability. Examination is facilitated by using unpreserved and unfiltered water samples since *P. parvum* can pass through or be damaged by most plankton nets and the cells are distorted by fixatives. Additionally, living cells have a characteristic swimming motion that aids in their identification. When samples must be preserved for long-term storage, buffered Lugol's

solution at 4°C is recommended although there may be some deleterious effects upon the cells, including loss of the haptonema.

The following description of *P. parvum* is adapted from Green et al. (1982). Cells glide smoothly in a forward direction while spinning on their longitudinal axis. Cells are 8-15 µm long and 4-10 µm wide, sub-spherical to elongate in shape with a rounded posterior end and tapered anterior end that bears two equal or sub-equal flagella of 12-20 µm long or approximately 1.5 times the length of the cell. A short haptonema (3-5 µm) is directed forward and arises from the same groove as the two flagella. There are two large yellow-green chloroplasts situated laterally and parietally, often deeply lobed and each containing an immersed pyrenoid. The nucleus lies centrally between the two chloroplasts. A golgi body is parabaasal and a contractile vacuole is sometimes present. The flagellar action is heterodynamic in swimming cells with the anterior flagellum beating with a rapid, almost “flicking” movement (Green et al. 1982) as the posterior flagellum undulates freely. In resting cells, the flagellar action is slower and more regular with the amplitude of waves increasing towards the distal end of the flagellum. Cells are covered by organic scales of two types and in two layers: scales of the outer layer have narrow inflexed rims, a pattern of radiating fibrils on the proximal face and concentrically arranged fibrils, sometimes observed only in the central area, on the distal face; scales of the inner layer have surface patterning similar to the outer scales but with a rim strongly inflexed over the distal face. These scales require electron microscopy to be observed.

Light Microscopy

The oldest and simplest method for identifying algal species is by visual observation using a light microscope and a glass slide. While this approach is commonplace, it has many limitations. It requires trained personnel with an aptitude to distinguish subtle differences among taxa or variations associated with life history or nutrient availability (Culverhouse 1995). Cell counts can be very time-consuming, especially when phytoplankters are numerous. Additionally, density estimates using the hemacytometer likely are unreliable at low cell concentrations. In cases where the alga is present in low numbers or as a part of a complex mixture, it may be undetectable leading to the conclusion that the alga is absent when it is actually present. Despite these drawbacks, TPWD Inland Fisheries staff currently use light microscopy and hemacytometer counting chambers to identify and quantify *P. parvum* cells because faster, more accurate and inexpensive methods are unavailable.

Epifluorescent Microscopy

Epifluorescent microscopy was explored as an option to improve *P. parvum* cell identification and enumeration methodology. When illuminated using epifluorescence, *P. parvum* cells fluoresce a gold color that contrasts with the green or red color of many other algal species (Figure 1). This observation prompted further investigation into use of fluorescent microscopy to identify and quantify algae samples. Also of note, in some water samples we observed an organism that appeared to preferentially graze on *P. parvum* (Figures 2A, B, C, and D). The organism closely resembles the ciliate *Coleps* sp. (Fig. 2E and 2F), but it has not been positively identified. This protist could be useful as a biological

control agent if it is a significant predator of *P. parvum*, but more research is needed to demonstrate feasibility of using this organism as a control method. *Coleps* spp. have occasionally been associated with morbidity and mortality among fish (Szekely and Berezky 1992; Wooster and Bowser 1994)

Pigment analysis via fluorescence excitation spectra has been studied and widely used for *in vivo* characterization of marine and freshwater phytoplankton communities. *In vivo* fluorometry (IVF) uses direct measurement of chlorophyll fluorescence in living cells and has been used by oceanographers and limnologists for locating and measuring algae for over 20 years. The IVF method is useful for gross estimates of algal growth patterns, but not very helpful to identify algal species.

Various attempts have been made to achieve taxonomic identification of phytoplankton based on *in vivo* bio-optical characteristics such as fluorescent excitation and absorption spectra (Hoepffner and Sathyendranath 1991). Oldham et al. (1985) were the first researchers to report identification of marine phytoplankton using total fluorescence “fingerprinting”. Neveux and Panouse (1987) reported fluorescence characteristics of the chlorophylls and phaeophytins and the patterns associated with different algal taxa. Currently, fluorescence “signatures” of four taxonomic groups can be readily distinguished: chromophytes (golden-brown algae), chlorophytes (green algae), cryptophytes and rhodophytes (red algae), and cyanobacteria (blue-green algae). Specifically, the chlorophyll accessory pigment (CAP) ratio is used to differentiate these taxa in marine waters.

Other techniques for monitoring algal communities include estimation of chlorophyll *a* concentration, algal biomass, and primary productivity (USEPA 446.0, 1997; Yentsch and Yentsch, 1979). Recently developed methods measure fluorescence based on fixed wavelength fluorometry using a filter system for excitation and detection, scanning spectrofluorometry based upon the excitation or emission spectra of fluorescence, fast repetition rate fluorometry, and a pump and probe technique (Kolber and Falkowski 1993).

Although the new techniques are promising, there are some drawbacks of using fluorescent patterns to characterize algal species. A common feature among these techniques is pigment concentration is estimated through the correlation of *in vivo* fluorescence with the isolated photosynthetic pigment values. Attempts to quantify phytoplankton pigments *in situ* using fluorescence characteristics counter the fact that photosynthetic pigments are bound to protein in living cells (Cowles et al. 1993; Kolber and Falkowski 1993). Because of that, *in vivo* fluorescence of phytoplankton should also take into consideration these pigment-protein complexes, which have a variety of structures and different roles in photosynthesis. *In vivo* fluorescence excitation spectra of phytoplankton depend not only on the taxonomic position of algae, but also on the photoadaptation state. The cellular pigment content, the ratio of total chlorophyll *a* to accessory pigments, and the efficacy of energy transfer to chlorophyll *a* are sensitive to the light conditions of culture growth (Wilhelm and Manns 1991). Vertical profiles of a species will vary in photoadaptive state due to the gradient of ambient light. The bulk of chlorophyll and carotenoid-protein complexes consists of various light harvesting pigment proteins that absorb light and then convert light energy into chemical energy. The characteristics of phytoplankton *in-vivo* fluorescence, including its fluorescence

efficiency, depend upon various factors other than only taxonomy. For *P. parvum*, quantitative pigmentation has been found to be nearly independent of growth phase (Wilhelm and Manns 1991) and the pigment pattern is sensitive to nutrient limitations. Also of note, for *P. parvum*, the predominant fraction of the light harvesting protein gave absorption spectra rich in chlorophyll *c* (c_1 , c_2 , and c_3) as well as xanthophylls (Wilhelm and Wiedemann 1991).

Flow Cytometry

Fluorescence becomes a powerful tool in cell identification when the cells can be separated and analyzed individually, as with flow cytometry (Børsheim et al. 1989). With the aid of laser-based flow cytometry and cell sorting, pigment autofluorescence, stain-induced fluorescence, and light scatter are used as probes to quantify and sort subpopulations of phytoplankton cultures and natural populations (Yentsch et al. 1983). Once fluorescence signature coordinates are established, flow cytometry is a convenient way to count individual species of phytoplankton in mixed samples. Chlorophyll *a* fluorescence and forward light scatter are two parameters measured using flow cytometry and that produce discriminatory signature ranges, but they are highly variable depending upon life history, distribution, concentration, and other factors (Yentsch et al. 1983). Thus, as with the visual method, there also are disadvantages with using flow cytometry to identify and quantify algal cells. Flow rates typically make selection of individual cells nearly impossible or investigation of organisms in low-concentrations time-consuming or impossible. Also, there is natural variation in the form or biochemistry of the algae themselves, ranging from morphological features to amount of chlorophyll or associated pigments produced (Culverhouse 1995).

Challenges

The TPWD Inland Fisheries Division has the need for a quicker and more reliable method to identify and quantify *P. parvum* in water samples, whether it occurs in the hatchery setting or as part of management plan for affected water bodies. Fluorescence characteristics may be useful to distinguish this alga from other species, but techniques in the field of microscopy or cytometry need to be developed and current TPWD personnel lack the expertise to develop these methods. Additionally, the specialized equipments are expensive and bulky. Thus, cost-effective alternatives to these technologies that are suitable for hatchery or field operations are needed

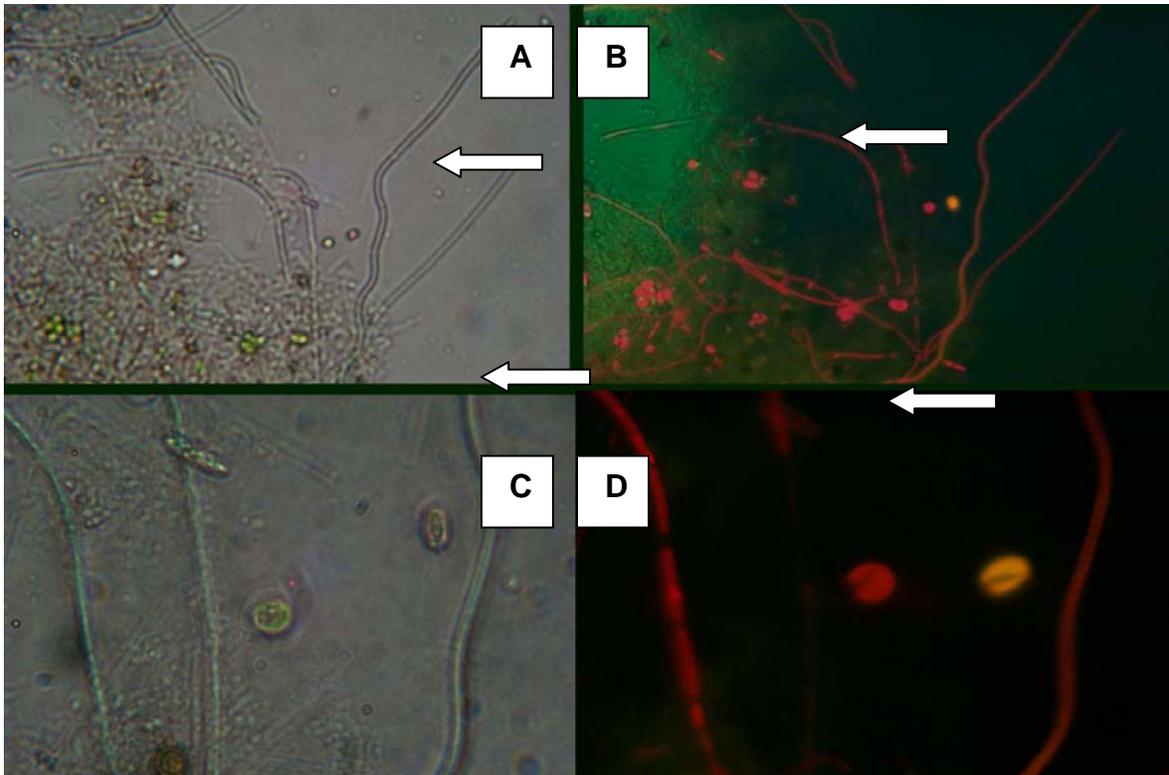


FIGURE 1.—Brightfield and epifluorescence microscopy images of mixed phytoplankton community containing *Prymnesium parvum* (arrows) cells: (A) brightfield (100X); (B) epifluorescence (100X); (C) brightfield (400X); (D) epifluorescence (400X)

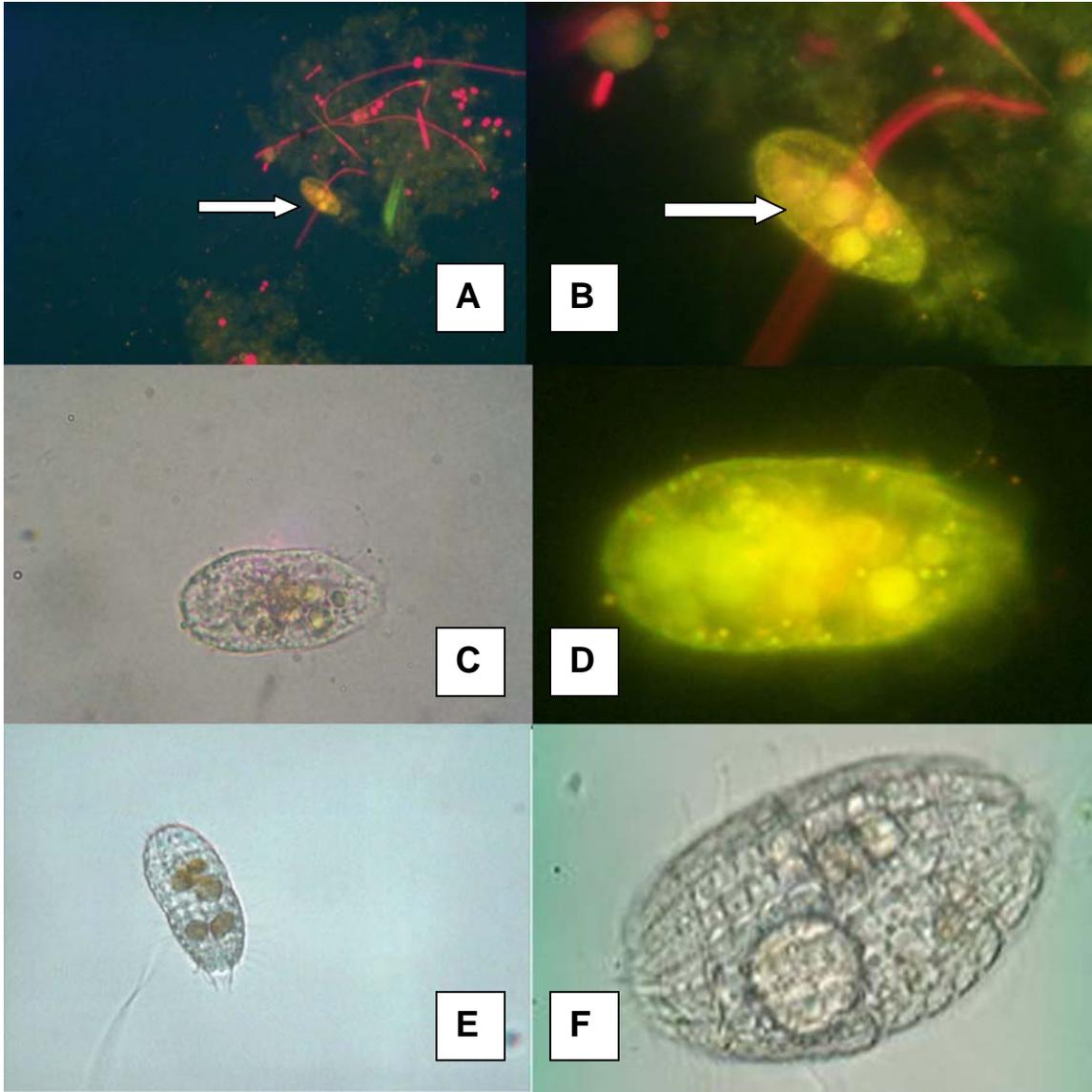


FIGURE 2.—Brightfield and epifluorescent images of mixed algal community, suspect *Prymnesium parvum* grazer (arrows), and the protist *Coleps* sp. (A) Mixed algal species (epifluorescence 100X); (B) Mixed algal species (epifluorescence 400X); (C) Suspect *P. parvum* grazer (brightfield 400X); (D) Suspect *P. parvum* grazer (epifluorescence 1000X); (E) *Coleps* species (brightfield 400X) [http://www.funsci.com/fun3_en/protists/prot_14.jpg]; (F) *Coleps* species (brightfield 1000X) [<http://www.ph-karlsruhe.de/NATUR/GARTEN/2001/kraeuterspirale/index.html>].

CHAPTER 12

Dundee State Fish Hatchery *Prymnesium parvum* Management Plan

DENNIS G. SMITH

Abstract

This management plan was prepared as a guide to control the toxic alga *Prymnesium parvum* and its ichthyotoxin and eliminate, or at least minimize, its adverse impact on fish production. The plan includes monitoring presence and abundance of *P. parvum* and concentration of un-ionized ammonia nitrogen, and application of effective chemical treatments. Ammonium sulfate is applied at concentrations to raise the un-ionized ammonia nitrogen concentrations to 0.2-0.4 mg/L when water temperatures are 15°C or higher, and copper sulfate (or Cutrine-Plus) is applied at 0.2-0.4 mg Cu²⁺/L when water temperatures are up to 15°C. The selected target concentrations of un-ionized ammonia nitrogen and copper depend on the tolerance of the fish that would be exposed to the treatments.

Introduction

The Dundee State Fish Hatchery is located in Archer County, Texas below Lake Diversion which supplies water to the hatchery. The hatchery has 97 ponds: 73 are plastic-lined totaling 24 ha (59.5 acres) and 24 are earthen ponds totaling 9.3 ha (23 acres) of surface water. Other culture units include four outdoor raceways and indoor 12, 1.8-m fiberglass round tanks, 90-jar egg incubation system and 4-trough (970-L) rearing system. All indoor culture systems can be operated as flow-through or closed systems. The spawning and rearing building which houses the indoor culture units also is equipped with an ozone generator and UV system for treating lake water containing *Prymnesium parvum* cells or toxins.

Fish species cultured at this facility include striped bass *Morone saxatilis*, palmetto bass (striped bass ♀ × *M. chrysops* ♂), channel catfish *Ictalurus punctatus*, black basses *Micropterus* spp., koi carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mykiss*, walleye *Stizostedion vitreum* and saugeye (female walleye × male *S. canadense*).

In 2001 fishes on the hatchery suffered substantial mortality from *P. parvum* ichthyotoxicity. Losses included 5.1 million striped bass and palmetto bass, 1,500 black basses, and thousands of channel catfish, rainbow trout and koi carp. Through the efforts of hatchery staff and the Hatcheries Golden Alga Task Force, strategies have been developed to control *P. parvum*. These strategies form the basis of the *P. parvum* management plan described herein. This plan continues to evolve and modifications are made to it as more effective or efficient solutions to the *P. parvum* toxicity problem are discovered.

Prymnesium parvum Management Plan

Pond Management

- Fill ponds well in advance of fish stockings to allow water temperatures to rise so treatment with ammonium sulfate, if needed, can be effective.
- Avoid flushing ponds too rapidly and decreasing temperature if ponds must be flushed. If possible avoid pond flushing.
- Treat ponds at least two days prior to anticipated stockings to allow treatments to work and toxins to decompose.
- Perform bioassays and check for cells any time *P. parvum* toxicity is suspected and on the days before fish stockings.
- Maintain a minimum of 0.18 mg/L un-ionized ammonia nitrogen (UIA-N) or 2 mg Cu²⁺/L in ponds depending on treatment option.

Prophylactic Treatments of P. parvum in Ponds

- Measure pond water temperature and pH
- If pond water temperatures are consistently above 28°C
 - *P. parvum* may be absent or present in very low numbers and ichthyotoxicity is unlikely. Treatment should be unnecessary.
 - Monitor ponds for presence of the alga and signs of toxicity at least once per week.
- If pond water temperature is 28°C
 - Check for presence of *P. parvum* cells twice per week.
 - If cells are present measure ammonia, temperature, and pH.
 - Calculate concentration of UIA-N.
 - Apply ammonium sulfate to raise UIA-N to 0.3 mg/L if UIA-N is less than 0.18 mg/L.
- If pond temperatures are below 28°C, consult an ammonia ionization table (Piper et al. 1992) or hatchery ammonia spreadsheet to determine proportion of total ammonia in the un-ionized form.
 - If the proportion of total ammonia in the un-ionized form is less than 5%
 - Apply Cutrine-Plus® or copper sulfate to raise copper concentration to 0.25 mg/L.
 - Measure copper concentration once per week.
 - Maintain copper concentration above 0.2 mg/L.
 - Check for presence of *P. parvum* cells once per week for monitoring purposes.
 - If the proportion of total ammonia in the un-ionized form exceeds 5%
 - Measure ammonia, temperature, and pH once per week (twice per week for sensitive species such as striped or palmetto bass).
 - Calculate concentration of UIA-N.
 - Apply ammonium sulfate to raise UIA-N to 0.3 mg/L if UIA-N is less than 0.18 mg/L.
 - Check for presence of *P. parvum* cells once per week for monitoring purposes.

- If the proportion of total ammonia in the un-ionized form is low (5-15%) and pH is expected to increase above 8.5
 - Reduce target ammonium sulfate treatment to achieve UIA-N of 0.25 mg/L. This treatment level is high enough to control *P. parvum* but requires less ammonium sulfate and lower total ammonia. Thus, should pH rise the UIA-N generated may not be toxic to the fish. Treatments at this lower UIA-N rate may require more frequent applications.

Indoor Culture Units

- Use UV- and ozone-treated lake water (treated water) for all culture activities in the spawning and rearing building if lake water contains *P. parvum* or its toxin. High dosage UV (180 to 200 mJ/cm²) and ozone treatment is required to eliminate *P. parvum* toxicity if toxins are present in the supply water.
 - Check treated water for presence of *P. parvum* or toxin to be sure the system is working.

Treatment of Ichthyotoxicity

- Treat ponds or other culture units with potassium permanganate at the demand rate or up to 2 mg/L above the demand rate for temporary relief if fish show signs of ichthyotoxicity.

Fish Harvest

- Check incoming lake water for toxicity and presence of *P. parvum* one day before fish harvest.
- If *P. parvum* or toxin is absent in lake water
 - Harvest fish using routine hatchery procedures.
- If *P. parvum* or toxin is present in lake water but water not toxic.
 - Do partial pond draining the day before harvest.
 - Harvest fish as scheduled within 2 hours using lake water.
 - Treat pond water with potassium permanganate if fish exhibit signs of ichthyotoxicity.
- If lake water is toxic
 - Suspend fish harvest until the condition improves.
 - If fish must be harvested, use non-toxic water from adjacent ponds or treated water and potassium permanganate treatment if fish show signs of ichthyotoxicity.

Fish Hauling Units

- Fill fish hauling units with treated water.
- Rinse fish to be transported off the hatchery with treated water before loading to avoid introducing *P. parvum* into hauling tanks and ultimately into stocked lakes.
- After fish loading check hauling unit water for *P. parvum*.
 - If *P. parvum* is absent deliver fish according to hatchery guidelines.
 - If *P. parvum* is present drain out some water, refill with treated water, and recheck for *P. parvum*. Repeat until no *P. parvum* is found.
- Upon return to the hatchery, disinfect hauling units with 10% chlorine bleach.

- Use lake water free of *P. parvum* cells or toxins, or treated water to transfer fish between hatchery culture units.

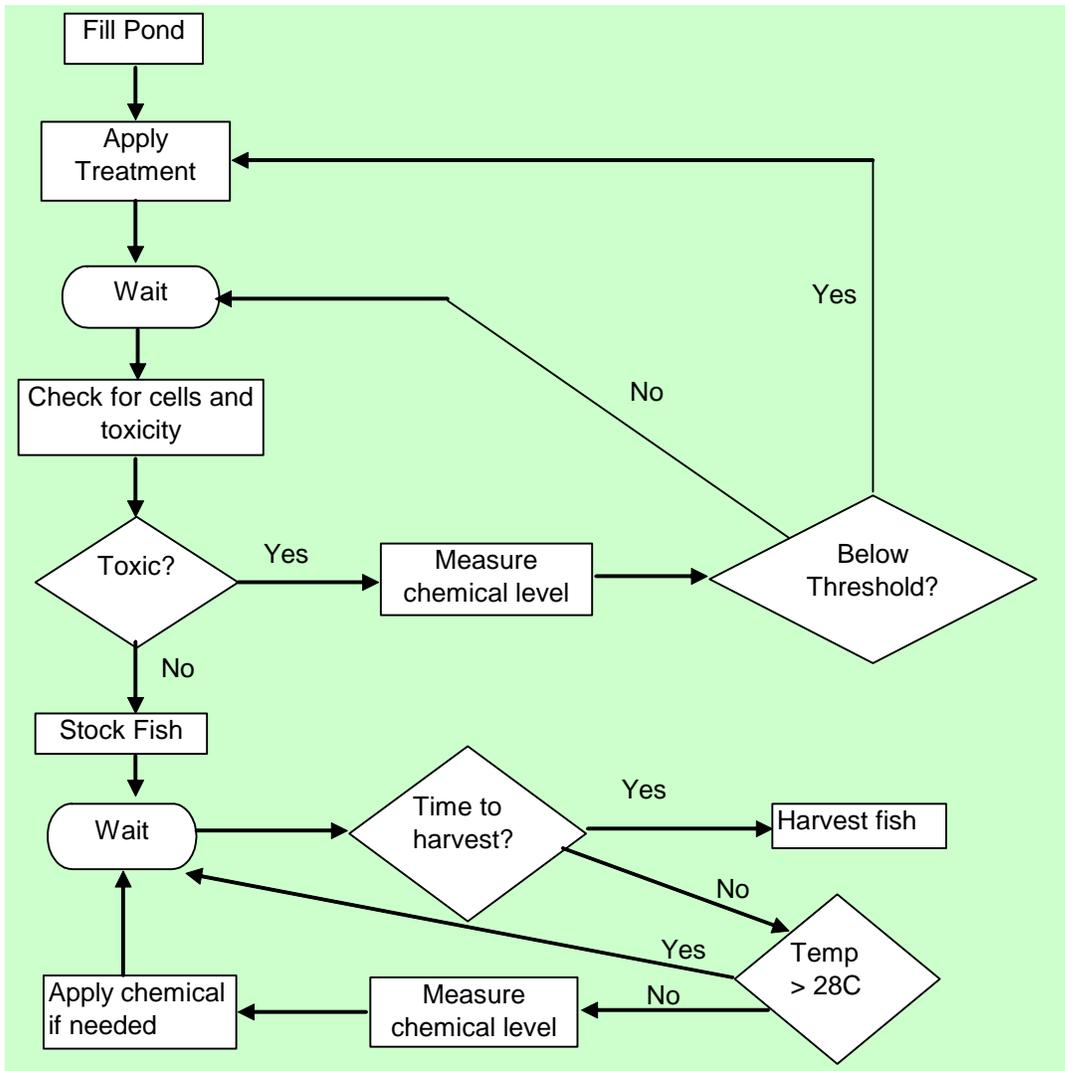


FIGURE 1.—A schematic diagram of the Dundee State Fish Hatchery pond management plan.

CHAPTER 13

Possum Kingdom State Fish Hatchery *Prymnesium parvum* Management Plan

DALE D. LYON, JAKE ISAAC, AND JOHN PARET

Abstract

This *Prymnesium parvum* management plan was prepared to provide a systematic approach to controlling this toxin-producing alga to make fish production possible at the Possum Kingdom State Fish Hatchery. The essential facets of the plan are monitoring presence and density of *P. parvum* and un-ionized ammonia levels, and application of chemical treatments. Ammonium sulfate is applied at 10 mg/L or concentrations to raise the un-ionized ammonia concentration to 0.2-0.4 mg/L when water temperatures are 15°C or higher and copper sulfate or Cutrine-Plus is applied at 0.75-1.0 mg Cu/L when water temperatures are up to 15°C. The target concentration of un-ionized ammonia or copper depends on the fish species being cultured.

Introduction

Possum Kingdom State Fish Hatchery is located in Palo Pinto County, Texas below Possum Kingdom Lake, the main source of water for the hatchery. The lake water comes to the hatchery through a 4.5-m (14.8 ft) deep intake valve (shallow water) or an 18-m (59-ft) deep intake valve (deep water). Additional water for the hatchery is provided by a well. Effluent water from ponds and indoor culture units can be reused in ponds after filtration by a re-circulation system. Culture units include 38 plastic-lined ponds (9.4 ha or 23.2 acres) and indoor raceway, 48-McDonald jar egg incubation system and six holding troughs. The incubation system can be operated as flow-through or closed system with filtration, heating and cooling capabilities. All holding troughs have flow-through capabilities but only four have re-circulation capabilities. The indoor re-circulation systems are equipped with an ultraviolet sterilizer for treating water infected with *P. parvum*.

Fish species cultured at this hatchery include striped bass *Morone saxatilis*, palmetto bass (female striped bass × male *M. chrysops*), channel catfish *Ictalurus punctatus*, smallmouth bass *Micropterus dolomieu*, koi carp *Cyprinus carpio*, bluegill *Lepomis macrochirus*, crappie *Pomoxis* spp., rainbow trout *Oncorhynchus mykiss*, and walleye *Stizostedion vitreum*.

P. parvum was first confirmed in Possum Kingdom Lake in 2001 following extensive toxin-related fish kills in the reservoir. This alga was found in our hatchery ponds in 2002 when ponds were filled with lake water following a renovation in 2001. This alga consistently appears to bloom during colder months (January-March), and blooms are usually associated with fish kills. During summer months, when temperatures exceed 28° C, the alga

usually disappears or occurs in very low density and toxin-related fish kills are rare. Spring and fall appear to be transitional periods when *P. parvum* densities fluctuate and fish kills are sporadic.

Since 2001 staffs at Possum Kingdom and Dundee hatcheries in cooperation with the Hatcheries Golden Alga Task Force have been developing strategies for controlling the alga. The strategies that seem to work best for this facility are formulated into the management plan described below. As more effective or efficient strategies are developed this management plan will be updated.

***P. parvum* Management Plan**

This facility has adopted a prophylactic approach to managing *P. parvum* with the goal of elimination the alga from culture systems or keeping densities as low as possible. Therefore, if a single cell is detected in a water sample (i.e., 2,000 cells/mL), the infected pond is treated to control the alga. Before treatment, the un-ionized ammonia nitrogen (UIA-N) or Cu^{2+} concentration in the pond is determined and the difference needed to achieve the target treatment level is provided by applying ammonium sulfate or copper sulfate.

Brood fish Holding (striped bass or white bass)

- Fill indoor holding troughs with well water and operate as closed system
 - Check for the presence of *P. parvum* to be sure the system is free of the alga.
 - If no cells are present there should be no need for further monitoring.

Jar Rack Egg Incubation

- Fill egg incubation system with well water and operate as a closed system.
- Check system water for *P. parvum* cells.
 - If cells are present treat with UV radiation.
 - If no cells are present there should be no need for further monitoring.

Spring Fry Rearing (striped bass, smallmouth bass, koi carp, etc)

- Clean all pond bottom sediments 12-14 days before fry stocking.
- Begin filling ponds 11 days before fry stocking with deep lake water.
- Treat ponds with ammonium sulfate to achieve UIA-N level of 0.3 mg/L 6 days before stocking.
- Check ponds for presence of *P. parvum* 4 days and 1 day before fry stocking; treat if cells are present.
- For striped bass conduct 24-hour survival tests on all ponds before stocking.
- Check all ponds with fish for *P. parvum* once per week.
 - If *P. parvum* is present check affected ponds twice per week
 - Treat ponds containing *P. parvum* with ammonium sulfate to achieve UIA-N level of 0.3 mg/L if UIA-N is low and temperature is 15°C or higher.
 - Treat ponds containing *P. parvum* with copper sulfate (or Cutrine-Plus) to achieve 0.75 mg Cu^{2+} /L if temperature is less than 15 °C.

Spawning Ponds (smallmouth bass)

- Fill ponds with deep lake water
 - Check ponds for *P. parvum* once per week; when *P. parvum* is present check twice per week.
 - If *P. parvum* is present treat with ammonium sulfate to achieve 0.4 mg/L UIA-N.

Summer-Fall Fingerling Rearing (channel catfish and koi carp)

- Begin to fill ponds with lake water 7 days before stocking.
- Check ponds for *P. parvum* 2 days before stocking
 - If *P. parvum* is absent continue to fill ponds according to culture guidelines.
 - If *P. parvum* is present treat to raise UIA-N to 0.4 mg/L if temperature is 15 °C or higher, or treat to raise Cu²⁺ to 0.75 mg/L if temperature is below 15 °C.
- Check ponds for toxin 1 day before stocking and select ponds with no toxin for stocking with fish.
- After stocking fish monitor pond temperature and pH daily and *P. parvum* once per week.
 - If pond temperatures are consistently above 28°C.
 - No treatment should be necessary but monitor *P. parvum* twice per week.
 - If pond temperatures are 15-28°C.
 - Monitor UIA-N and treat to raise UIA-N to 0.4 mg/L if *P. parvum* present.
 - If pond temperatures are below 15°C.
 - Monitor Cu²⁺ and toxin, and treat with Cutrine-Plus to raise Cu²⁺ to 0.75 mg/L if toxicity is present.

Winter Holding Ponds

- Monitor ponds for *P. parvum* once per week or twice per week if *P. parvum* present.
 - If water temperatures are up to 15°C treat to raise Cu²⁺ to 0.75 mg/L if *P. parvum* is present.

Raceway or Trough Culture (rainbow trout and channel catfish)

- 8 days before fish stocking fill with lake water and check for *P. parvum* cells.
 - If *P. parvum* is absent stock fish and operate raceway/trough as flow-through.
 - If *P. parvum* is present perform bioassay to test toxicity.
 - If lake water is not toxic stock fish and operate raceway/trough as flow-through.
 - If lake water is toxic do not use raceway/trough (Go to Trout Pond Production).

Trout Pond Production

Use ponds for trout production or holding, instead of indoor raceway or troughs, when lake water is toxic.

- 8 days before stocking fill ponds with lake water.
 - Treat with Cutrine-Plus to raise Cu²⁺ level to 1.0 mg/L if temperatures are less than 15°C.
 - Treat with ammonium sulfate to raise UIA-N to 1.0 mg/L if temperatures are 15°C and higher.

- 3 days before stocking check for *P. parvum* cells
 - If *P. parvum* is present treat as above.
- 1 day before stocking check for *P. parvum*.
 - If *P. parvum* is present test for toxicity.
- Stocking day
 - Stock only ponds with no toxicity.
- After stocking
 - Check for *P. parvum* twice per week and if present treat as described above.
- If lake conditions improve harvest fish (e.g., trout) and move to indoor raceway.

Fish Harvest

- At harvest check incoming lake water for *P. parvum*
 - If *P. parvum* is absent harvest fish using lake water
 - If *P. parvum* is present perform bioassay: if negative harvest fish using lake water; if positive use well water.
 - Fish leaving the hatchery must be rinsed in well water before loading into hauling unit.
 - Fish to be transferred between hatchery culture units need not be rinsed with well water.

Fish Transportation

- Fill hauling unit with well water and check all compartments for *P. parvum* after loading fish (Note: all fish leaving the hatchery must be rinsed in well water before loading).
 - If *P. parvum* is absent deliver fish according to hatchery guidelines.
 - If *P. parvum* is present drain out some water, refill and re-check for *P. parvum*. Repeat until no *P. parvum* is found.
 - Upon return to the hatchery, disinfect hauling unit with 10% chlorine bleach.
- Use *P. parvum*-free lake water or well water to transfer fish between culture units on the hatchery.

Monitoring Sites

- Monitor *P. parvum* in lake water at the dam, hatchery intake water, and ponds and indoor culture units in use.

REFERENCES

- Anderson, R. O. 1993. Apparent problems and potential solutions for production of fingerling striped bass. *Journal of Applied Aquaculture* 2(3/4):101-118.
- Anderson, D. P., and F. L. Mayer. 1993. Salmonid pharmacology and toxicology. Pages 432-439 in M. K. Stoskopf, editor, *Fish Medicine*. W. B. Saunders Company, Philadelphia, Pennsylvania.
- APHA (American Public Health Association), American Water Works Association, and Water Pollution Control Federation. 1995. Standard methods for the examination of water and wastewater, 19th edition. APHA, Washington, D.C.
- Aure, J., and F. Rey. 1992. Oceanographic conditions in the Sandfjord system, western Norway, after a bloom of the toxic prymnesiophyte *Prymnesium parvum* Carter in August 1990. *Sarsia* 76:247-254.
- Barkoh, A. 1996. Effects of three fertilization treatments on water quality, zooplankton, and striped bass fingerling production in plastic-lined ponds. *Progressive Fish-Culturist* 58:237-247.
- Barkoh, A., D. G. Smith, and J. W. Schlechte. 2003. An effective minimum concentration of un-ionized ammonia nitrogen for controlling *Prymnesium parvum*. *North American Journal of Aquaculture* 65:220-225.
- Bergerhouse, D. L. 1993. Lethal effects of elevated pH and ammonia on early life stages of hybrid striped bass. *Journal of Applied Aquaculture* 2(3/4):81-100.
- Bold, H. C., and M. J. Wynne. 1983. Introduction to the algae, 2nd edition. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.
- Børsheim, K.Y., Harboe, T., Johnson, T., Norland, S., and K. Nygaard. 1989. Flow cytometric characterization and enumeration of *Chrysochromulina polylepis* during a bloom along the Norwegian coast. *Marine Ecology Progress Series* 54:307-309.
- Boyd, C. E. 1979. Aluminum sulfate (Alum) for precipitating clay turbidity from fish ponds. *Transactions of the American Fisheries Society* 108:307-313.
- Boyd, C. E. 1990. Water quality in ponds for aquaculture. Alabama Agricultural Experiment Station, Auburn University, Alabama.
- Boyd, C. E., and H. V. Daniels. 1993. Liming and fertilization of brackish water shrimp ponds. *Journal of Applied Aquaculture* 2:221-234.
- Burleson, F. G., Chambers, T. M., and D. L. Wiedbrauk. 1992. *Virology: A laboratory manual*. Academic Press, Inc. San Diego, California.

- Button, K. S., H. P. Hostetter, and D. M. Mair. 1977. Copper dispersal in a water supply reservoir. *Water Resources* 11:539-544.
- Chang, F. H., and K. G. Ryan. 1985. *Prymnesium calathiferum* sp. Nov. (Prymnesiophyceae), a new species isolated from Northland, New Zealand. *Phycologia* 24:191-198.
- Cowles, T. J., R. A. Desiderio, and S. Neuer. 1993. *In situ* characterization of phytoplankton from vertical profiles of fluorescence emission spectra. *Marine Biology* 115:217-222.
- Cromar, N. J., and H. J. Fallowfield. 1997. Effect of nutrient loading and retention time on performance of high rate algal ponds. *Journal of Applied Phycology* 9:301-309.
- Culver, D. A., S. P. Maden, and J. Qin. 1993. Percid pond production techniques: timing enrichment and stocking density manipulation. *Journal of Applied Aquaculture* 2(3/4):9-31.
- Culverhouse, P. F. 1995. Automatic methods for the detection of harmful algae. Pages 695-706 in P. Lassus, E. Erard, P. Gentine, and C. Marcaillou, editors. *Harmful Marine Algal Blooms*. Lavoisier, Paris, France.
- Dafni, Z, S. Ulitzur and M. Shilo. 1972. Influence of light and phosphate on toxin production and growth of *Prymnesium parvum*. *Journal of General Microbiology*. 70:199-207.
- Droop, M. R. 1954. A note on the isolation of small marine algae and flagellates for pure cultures. *Journal of the Marine Biology Association of the U. K.* 33:511-514.
- Edwardsen, B., W. Eikrem, J. C. Green, R. A. Andersen, S. Y. Moon-van der Staay, and L. K. Medlin. 2000. Phylogenetic reconstructions of the Haptophyta inferred from 18S ribosomal DNA sequences and available morphological data. *Phycologia* 39:19-35.
- Emerson, K., R. C. Russo, R. E. Lung, and R. V. Thurston. 1975. Aqueous ammonia equilibrium calculations: effects of pH and temperature. *Journal of the Fisheries Research Board of Canada* 32:2379-2383.
- Fitzgerald, G. P. 1964. Laboratory evaluation of potassium permanganate as an algacide for water reservoirs. *Southwest Water Works Journal* 45:16-17.
- Gaikowski, M. P., J. J. Rach, and R. T. Ramsay. 1999. Acute toxicity of hydrogen peroxide treatments to selected life stages of cold-, cool-, and warmwater fish. *Aquaculture* 178:191-207.
- Green, J. C., D. J. Hibberd, and R. N. Pienaar. 1982. The taxonomy of *Prymnesium* (Prymnesiophyceae) including a description of a new cosmopolitan species, *P.*

- patellifera* sp. Nov., and further observations on *P. parvum* N. Carter. British Phycological Journal 17:363-382.
- Green, J. C., and B. S. C. Leadbeater, editors. 1994. The haptophyte algae. Systematics Association Special Volume No. 51. Clarendon, Oxford, England.
- Groeger, A. W., P. F. Brown, T. E. Tietjen, and T. C. Kelsey. 1997. Water quality of the San Marcos River. Texas Journal of Science 49:279-294.
- Guo, M., P. J. Harrison, and F. J. R. Taylor. 1996. Fish kills related to *Prymnesium parvum* N. Carter (Haptophyta) in the People's Republic of China. Journal of Applied Phycology 8:111-117.
- Hansen, J. A., P. G. Welsh, J. Lipton, and D. Cacela. 2002a. Effects of copper exposure on growth and survival of juvenile bull trout. Transactions of the American Fisheries Society 131:690-697.
- Hansen, J. A., P. G. Welsh, and J. Lipton. 2002b. Relative sensitivity of bull trout *Salvelinus confluentus* and rainbow trout *Oncorhynchus mykiss* to acute copper toxicity. Environmental Toxicology and Chemistry 21:633-639.
- Hansen, J. A., J. Lipton, P. G. Welsh, J. Morris, D. Cacela, and M. J. Suedkamp. 2002c. Relationship between exposure duration, tissue residues, growth, and mortality in rainbow trout (*Oncorhynchus mykiss*) juveniles sub-chronically exposed to copper. Aquatic Toxicology 58:175-188.
- Hipkins, P. L. 2002. Weed Control in Ponds and Lakes (Aquatic Weeds). Horticultural & Forest Crops. Virginia Department of Agriculture and Consumer Services Publication.
- Hoepffner, N., and S. Sathyendranath. 1991. Effect of pigment composition on absorption properties of phytoplankton. Marine Ecology Progress Series 73:11-23.
- Holdway, P. A., R. A. Watson, and B. Moss. 1978. Aspects of the ecology of *Prymnesium parvum* (Haptophyta) and water chemistry in the Norfolk Broads, England. Freshwater Biology 8:295-311.
- Igarashi, T., M. Satake, and T. Yasumoto. 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from red tide alga *Prymnesium parvum*. Journal of the American Chemical Society 118:479-480.
- Igarashi, T., M. Satake, and T. Yasumoto. 1999. Structures and partial stereochemical assignments for prymnesin-1 and prymnesin-2: potent hemolytic and ichthyotoxic glycosides isolated from the red tide alga *Prymnesium parvum*. Journal of the American Chemical Society 121:8499-8511.

- Irwin, R. J. 1997. Environmental Contaminants Encyclopedia, 1-7. Copper Entry. National Park Service, Water Resources Divisions, Water Operations Branch. Fort Collins, Colorado.
- James, T. L., and A. De La Cruz. 1989. *Prymnesium parvum* Carter (Chrysophyceae) as a suspect of mass mortalities of fish and shellfish communities in western Texas. The Texas Journal of Science 41:429-430.
- Jeffery, G. H., J. Bassett, J. Mendham, and R. C. Denney. 1989. Titrimetric analysis. Pages 372-373 in Vogel's Textbook of Qualitative Chemical Analysis, 5th edition, Longman Scientific and Technical, Essex, England.
- Johansson., N. 2000. Ecological implications of the production of toxic substances by fish killing phytoplankton species grown under variable N:P ratios. Ph. D. Dissertation, University of Lund, Lund, Sweden.
- Kaartvedt, S., T. M. Johnsen, D. L. Aksnes, U. Lie, and H. Svedsen. 1991. Occurrence of the toxic phytoflagellate *Prymnesium parvum* and associated fish mortality in a Norwegian Fjord system. Canadian Journal of Fisheries and Aquatic Science 48: 2316-2323.
- Kemp, H. T., R. G. Fuller, and R. S. Davidson. 1966. Potassium permanganate as an algicide. Journal of the American Water Works Association 58:255-263.
- Kim, Y. S., and G. M. Padilla. 1977. Hemolytically active components from *P. parvum* and *G. breve* toxins. Life Sciences 21:1287-1292.
- Kolber, Z., and P. G. Falkowski. 1993. Use of active fluorescence to estimate phytoplankton photosynthesis *in situ*. Limnology and Oceanography 38(8):1646-1665.
- Kozakai, H., Y. Oshima, and T. Yasumoto. 1982. Isolation and structural elucidation of hemolysin from the phytoflagellate *Prymnesium parvum*. Agricultural and Biological Chemistry 46:233-236.
- Larsen, A., and S. Bryant. 1998. Growth rate and toxicity of *Prymnesium parvum* and *Prymnesium patelliferum* (Haptophyta) in response to changes in salinity, light, and temperature. Sarsia 83:409-418.
- Lawrence, J. M. 1956. Preliminary results on the use of potassium permanganate to counteract the effects of rotenone on fish. Progressive Fish-Culturist 18:15-21.
- Linam, G., J. Ralph, and J. Glass. 1991. Toxic blooms, an unusual algae threatens aquatic resources. Chihuahuan Desert Discovery. No. 28(winter):6-7.

- Lindholm, T., P. Öhman, K. Kurki-Helasmo, B. Kincaid, and J. Meriluoto. 1999. Toxic algae and fish mortality in a brackish-water lake in Åland, SW Finland. *Hydrobiologia* 397:109-120.
- Lumsden, J. S., V. E. Ostland, and H. W. Ferguson. 1998. Use of hydrogen peroxide to treat experimentally induced bacterial gill disease in rainbow trout. *Journal of Aquatic Animal Health* 10:230-240.
- Marking, L. L., and T. D. Bills. 1975. Toxicity of potassium permanganate to fish and its effectiveness for detoxifying antimycin. *Transactions of the American Fisheries Society* 104:579-583.
- Meldahl, A. S., B. Edvardsen, and F. Fonnum. 1994. Toxicity of four potentially ichthyotoxic marine phytoflagellates determined by four different test methods. *Journal of Toxicity and Environmental Health* 42:289-301.
- Meldahl, A. S., J. Kvernstuen, G. J. Grاسبakken, B. Edvardsen, and F. Fonnum. 1995. Toxic activity of *Prymnesium* spp. and *Chrysochromulina* spp. tested by different test methods. Pages 315-320 in P. Lassus, E. Erard, P. Gentein, and C. Marcaillou, editors. *Harmful Marine Algal Blooms*. Lavoisier, Paris, France.
- McIntosh, A. W. 1975. Fate of copper in ponds. *Pesticide Monitoring Journal* 8:225-231.
- McLaughlin, J. L. A. 1958. Euryhaline chrysoomonads; nutrition and toxigenesis of *Prymnesium parvum* with notes on *Isochrysis galbana* and *Monochrysis luteri*. *Journal of Protozoology* 5(1):75-81.
- Neveux, J., and M. Panouse. 1987. Spectrofluorometric determination of chlorophylls and phaeophytins. *Archives of Hydrobiology: Advances in Limnology* 4:567-581
- Nicholls, K. H. 2003. Haptophyte Algae. Pages 511-521 in J. D. Wehr and R. G. Smith, editors. *Freshwater Algae of North America*. Academic Press, Boston, Massachusetts.
- Oldham, P., E. Zillioux, and I. Warner. 1985. Spectral "finger printing" of phytoplankton population by two dimensional fluorescence and Fourier-transform-based pattern recognition. *Journal of Marine Research* 43: 893-906.
- Otterstrom, C. V., and E. Steelmann-Nielson. 1940. Two cases of extensive mortality in fishes caused by flagellate *Prymnesium parvum* Carter. *Reports of the Danish Biological Station* 44:4-24.
- Padan, E., Ginzburg, D., and M. Shilo. 1967. Growth and colony formation of the phytoflagellate *Prymnesium parvum* Carter on solid media. *Journal of Protozoology* 14(3):477-480.

- Padilla, G. M., and D. F. Martin. 1973. Interactions of prymnesin with erythrocyte membranes. Pages 265-295 in D. F. Martin and G. M. Padilla, editors. *Marine Pharmacognosy: action of marine biotoxins at the cellular level*. Academic Press, New York.
- Paster, Z. 1973. Pharmacology and mode of action of *Prymnesium*. Pages 241-263 in G. M. Padilla and D. F. Martin, editors. *Marine pharmacognosy: action of marine biotoxins at the cellular level*. Academic Press, New York.
- Piper, R. G., I. B. McElwain, L. E. Orme, J. P. McCraren, L. G. Fowler, and J. R. Leonard. 1982. *Fish hatchery management*. U. S. Fish and Wildlife Service, Washington, D.C.
- Rach, J. J., T. M. Schreier, G. E. Howe, and S. D. Redman. 1997. Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *Progressive Fish-Culturist* 59:41-46.
- Sarig, S. 1971. Toxin-producing algae: *Prymnesium parvum* Carter. Pages 17-43 in S.F. Snieszko and H.R. Axelrod, editors. *Disease of fishes – Book 3: The prevention and treatment of disease of warmwater fishes under subtropical conditions, with special emphasis on intensive fish farming*. T. F. H. Publications, Inc. Ltd., Neptune, New Jersey.
- Shilo, M. 1967. Formation and mode of action of algal toxins. *Bacteriological Reviews* 31: 180-193.
- Shilo, M. 1971. Toxins of Chrsophyceae. Pages 67-103 in S. Kadis, A. Ciegler, and S. J. Ajl, editors. *Microbial toxins, volume 7*. Academic Press, New York.
- Shilo, M. 1972. Toxigenic algae. Pages 233-265 in O. J. D. Hockenhill II, editor. *Progress in industrial microbiology, volume 2*. Churchill Livingstone Press, Edinburgh, England.
- Shilo, M. 1981. The toxic principles of *Prymnesium parvum*. Pages 37-47 in W. W. Carmichael, editor. *The water environment: algal toxins and health, Volume 20*. Plenum Press, New York.
- Shilo, M., and M. Aschner. 1953. Factors governing the toxicity of cultures containing the phytoflagellate *Prymnesium parvum* Carter. *Journal of General Microbiology* 8:333-343.
- Shilo, M., and M. Shilo. 1953. Conditions which determine the efficiency of ammonium sulfate in the control of *Prymnesium parvum* in fish breeding ponds. *Applied Microbiology* 1:330-333.

- Shilo, M. and M. Shilo. 1962. The mechanism of lysis of *Prymnesium parvum* by weak electrolytes. *Journal of General Microbiology* 29:645-658.
- Skovgaard, A., C. Legrand, P.J. Hansen, and E. Graneli. 2003. Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*. *Aquatic Microbial Ecology* 31:259-265.
- Sommer, U. 1985. Comparison between steady state and non-steady state competition experiments with natural phytoplankton. *Limnology and Oceanography* 30:346-353.
- Straus, D. L., and C. S. Tucker. 1993. Acute toxicity of copper sulfate and chelated copper to channel catfish *Ictalurus punctatus*. *Journal of the World Aquaculture Society* 24:390-395.
- Szekely, C., and M. C. Berezky. 1992. An unusual case of disease in pet fish stocks caused by *Coleps* sp. *Diseases of Aquatic Organisms* 13(2):143-145.
- Thurston, R. V., and seven authors. 1984. Chronic toxicity of ammonia to rainbow trout. *Transactions of the American Fisheries Society* 113:56-73.
- Tucker, C. S. 1989. Method for estimating potassium permanganate disease treatment rates for channel catfish in ponds. *Progressive Fish-Culturist* 51:24-26.
- Tucker, C. S., and C. E. Boyd. 1977. Relationship between potassium permanganate treatment and water quality. *Transactions of the American Fisheries Society* 106:481-488.
- Ulitzer, S. 1973. The amphiphatic nature of *Prymnesium parvum* hemolysin. *Biochemica et Biophysica Acta* 298:673-679.
- Ulitzer, S., and M. Shilo. 1964. A sensitive assay system for determination of the ichthyotoxicity of *Prymnesium parvum*. *General Microbiology* 36:161-169.
- Ulitzer, S., and M. Shilo. 1966. Mode of action of *Prymnesium parvum* ichthyotoxin. *Journal of Protozoology* 13:332-336.
- Ulitzer, S., and M. Shilo. 1970a. Effect of *Prymnesium parvum* toxin, cetyltrimethylammonium bromide and sodium dodecyl sulphate on bacteria. *Journal of General Microbiology* 62:363-370.
- Ulitzer, S., and M. Shilo. 1970b. Procedure for purification and separation of *Prymnesium parvum* toxins. *Biochemica et Biophysica Acta* 201:350-363.
- USACE (US Army Corps of Engineers). 1985. Monoecious *Hydrilla* in the Potomac River. Miscellaneous paper A-85-5. Department of the Army, US Corps of Engineers, Washington, D.C.

- USEPA. 1997. *In vitro* determination of chlorophylls a, b, c1 + c2 and pheopigments in marine and freshwater algae by visible spectrophotometry. National Exposure Research Laboratory, EPA/446.0/R 2/September 1997.
- Warren, H. J. 2001. Striped bass production plan 2001. Management Data Series No. 185. Texas Parks and Wildlife Department. Austin, Texas.
- Welch, E. B. 1980. Ecological effects of wastewater. Cambridge University Press, New York.
- Wetzel, R.G. 1983. Limnology. CBS College publishing, Philadelphia, Pennsylvania.
- Wilhelm, C., and L. Manns. 1991. Changes in pigmentation of phytoplankton species during growth and stationary phase-consequences for reliability of pigment-based methods of biomass determination. *Journal of Applied Phycology* 3:305-310.
- Wilhelm, C., and I. Wiedemann. 1991. Evidence of protein-bound chlorophyll c₃ in a light-harvesting protein isolated from the flagellate alga *Prymnesium parvum* (*Prymnesiophyceae*)*. *Photosynthetica* 25(2):249-255.
- Wooster, G. A., and P. R. Bowser. 1994. Unusual infection of rainbow trout *Oncorhynchus mykiss* by the protozoan *Coleps* sp. (Ciliophora: Prostomatea) in a closed recirculation aquaculture system. *Journal of the World Aquaculture Society* 25(4):566-570.
- Yariv, J., and S. Hestrin. 1961. Toxicity of the extracellular phase of *Prymnesium parvum* cultures. *Journal of General Microbiology* 24:165-175.
- Yentsch, C. M., and 13 coauthors. 1983. Flow cytometry and cell sorting: a technique for analysis and sorting of aquatic particles. *Limnology and Oceanography* 28(6):1275-1280.
- Yentsch, C. S., and C. M. Yentsch. 1979. Fluorescence spectral signatures: the characterization of phytoplankton populations by the use of excitation and emission spectra. *Journal of Marine Science* 37:471-483.

APPENDIX A

Identification and Enumeration of *Prymnesium parvum* cells, Version AEW-IDE 1.1

GREGORY M. SOUTHARD

Purpose and Scope:

This protocol outlines the accepted procedure for identification and enumeration of *P. parvum* in water samples.

Materials required:

Standard hemacytometer
Cover slip
Light microscope with scanning and
high dry objectives
Pasteur pipette

Reagents/consumables:

Immersion oil
Lugol's solution
- 200 mL DI water
- 20 mL glacial acetic acid
- 20 g KI
- 10 g I

Procedure:

1. Carefully load the hemacytometer chamber using a Pasteur pipette at the loading groove, allowing capillary action to pull the water sample under the cover slip until chamber is full.
2. Using the scanning objective, observe for small, fast moving organisms. This is a tentative indication that *P. parvum* is present.
3. Switch to the 40X objective and look for *P. parvum* at 400X magnification. *P. parvum* will be identified by its small size (9-12 μm), two large chloroplasts (which may appear C-shape or saddle-shape), two long flagella, and one haptonema. All of the structures can be seen using 400X magnification, except the haptonema that can be visualized using the oil immersion lens (i.e., 1000X magnification). However, the oil immersion lens cannot be used with the hemacytometer.
4. Count the number of *P. parvum* cells in each of the five large squares of the hemacytometer grid, in each of the chambers (a total of 10 counts). The average number of cells per chamber (5 large squares) is the total cell count. For statistical accuracy, the total cell counts for each chamber should be within 10% of each other. If there is greater than 50 cells per large square, dilute the water sample to minimize time.
5. Total cells per mL is determined as follows:

$$\text{Total cells/mL} = (\text{total cell count}/5) \times (1/\text{dilution}) \times 10^4$$

Counting cell graphic (from Burleson, F. G., T. M. Chambers, and D. L. Wiedbrauk. 1992).
Virology – a laboratory manual. Academic Press, Inc. New York. 250 pp.

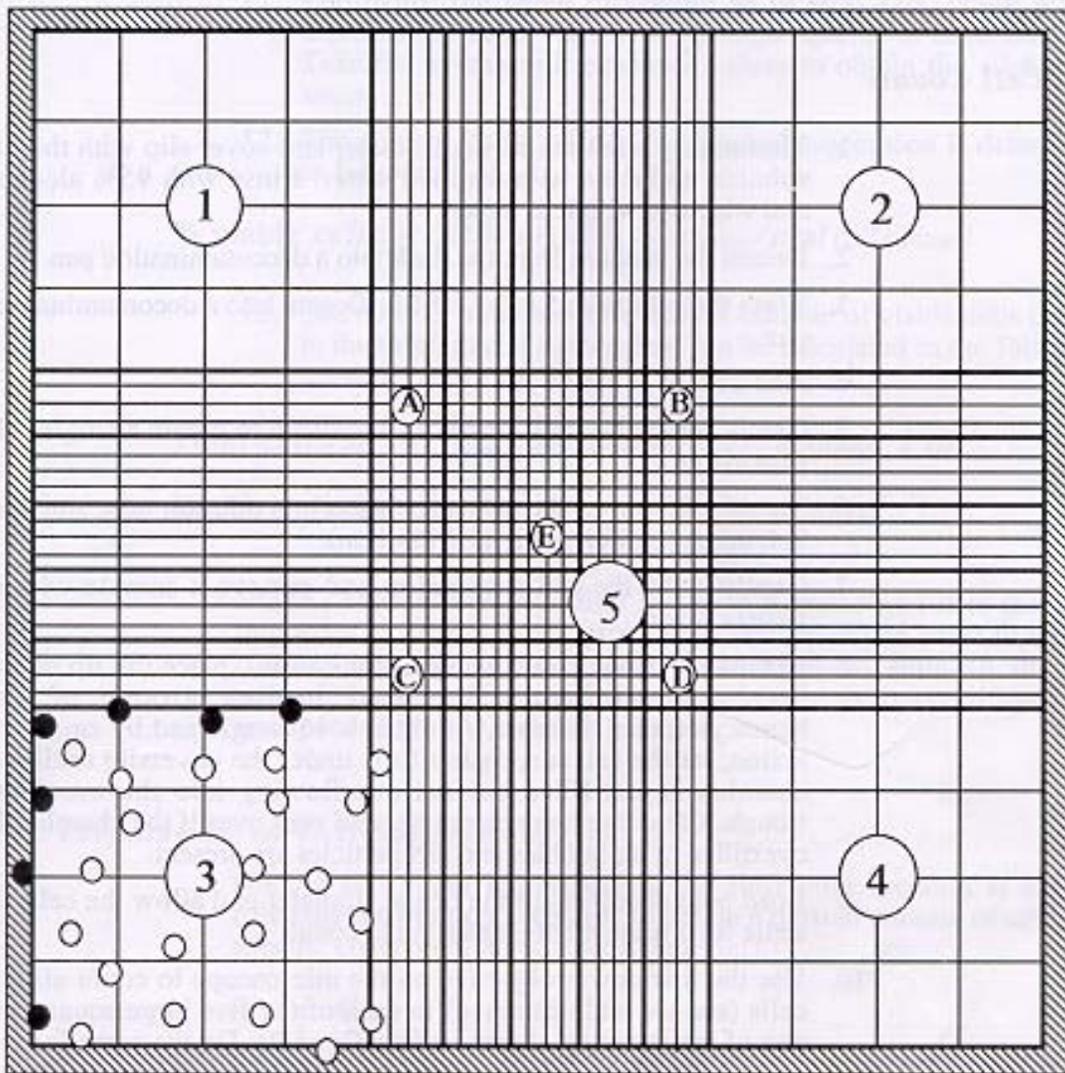


Figure 5.2 Enlarged view of the hemacytometer counting chamber, as it would appear through the low power of the microscope. The cells in the five large squares (1-5) are counted and this number is divided by 5 to obtain the average number of cells per large square. This value is multiplied by 10^4 and by the reciprocal of the dilution factor to obtain the number of cells/ml of the original cell suspension. Square #3 has a sample cell count. The open circles would be counted, whereas the dark circles indicate cells that would not be counted since they touch the top and left lines of that square.

Red blood cell counts are performed using the high dry objective of the microscope and counting the cells in 5 of the small squares (A,B,C,D, and E) of square #5. The cells/ml in the original suspension is calculated by multiplying the total number of cells in the 5 small squares by 5×10^4 , and the reciprocal of the dilution factor.

APPENDIX B

Standard Bioassay of *Prymnesium parvum* Toxin, Version AEW-ITU 1.2*¹

GREGORY M. SOUTHARD

Purpose and Scope: This test should be used when *Prymnesium parvum* is detected in hatchery water. The purpose is to evaluate the concentration of prymnesin toxin in ichthyotoxic units (ITU*) in hatchery water to determine the need for algaecide treatment.

*ITU (ichthyotoxic unit) = 1/25th the lethal dose of prymnesin ichthyotoxin/mL for fish.

Equipment required:

Glass/metal aquarium (ca. 10 L)
with aquarium heater (a water bath
may be substituted if available)
Thermometer
5 beakers (100 mL-1L)
pH meter
Graduated cylinder
Pipettes (mL and µL)

Reagents/consumables:

Distilled water
Pond water (test water)
Cofactor solution (see below)
12 test fish

Cofactor Solution Preparation:

1. Cofactor: 0.003 M DADPA /0.02 M tris buffer in distilled water, pH = 9.0.
To 491.15 mL of distilled water in a dark storage container, add 9.85 mL of DADPA (= 0.15 M) and 60.5 g tris (= 1.0 M)
2. Store in the dark at 4°C.

(Note: 1 mL cofactor solution per 50 mL water is needed for each of four test beakers.
DADPA = 3,3'-iminobispropylamine, Sigma cat # I 7006, supplied as 1.772-M solution.)

Procedure:

- 1) Fill the aquarium or water bath with enough water to incubate but not float the beakers.
- 2) Maintain temperature at 28°C ± 1.
- 3) Five beakers are used for each test. Size beakers according to size of test fish.
(Note: Fish lengths and beaker sizes are guidelines and may be adjusted as needed.)
- 4) Add 1 mL of prepared cofactor per 50-mL total volume in each of the 4 beakers. 'Clean' water used for dilutions and controls is from *P. parvum*-free source (See Table 1 below).
- 5) Add 4 test fish to each beaker.
- 6) Observe for mortality and maintain constant temperature in aquarium.
- 7) Results are noted after 2 hours.

¹ Adapted from the Central Fish Health Lab, Nir David, Israel.

Interpretation:

- 1) Record mortality in each beaker (see Table 1) after 2 hours.
 - a) Mortality in beaker #2 (undiluted pond water + cofactor) indicates the presence of at least 1 ITU/mL, which is 1/25th the lethal dose to fish in ponds. Toxicity is considered to be low. Treatment is not required, but continued monitoring is advised.
 - b) Mortality in beakers #2 and #3 (1/5 dilution + cofactor) indicates 5 ITU/mL, one-fifth the dangerous level in the ponds, which indicates the need for immediate treatment of the ponds. Toxicity is considered to be moderate.
 - c) Mortality in beakers # 2, 3 and #1 (undiluted pond water) indicates a high level of toxicity (25 ITU/mL or greater). The pond is toxic and fish should be displaying signs of prymnesin toxicity.
 - d) Partial mortality can occur in a beaker, which would indicate a level of ichthyotoxicity between those parameters confined by the dilutions and cofactor used in the bioassay. For example, there can be complete mortality in beaker #2, but only partial (e.g. 1 of 3 test fish) in beaker #3, which would indicate an ITU level between 1 and 5 ITU/mL. The same phenomenon can occur at higher toxin levels, with complete mortality occurring in beakers #2 and #3, or with partial mortality in beaker #1, indicating an ITU level between 5 and 25 ITU/mL. Partial mortality cannot occur in two of the beakers and if does, some other lethal mechanism is likely the cause.
 - e) Mortality in either control beaker suggests some other lethal mechanism. If fish die in one of the control beakers, the test should be repeated.

TABLE 1.—Suggested beaker size and experimental conditions to test for the presence of ichthyotoxin (from *Prymnesium parvum*) in hatchery water. If more than one pond is tested at a time, beakers 1 and 2 must be repeated for each pond, but only one set of controls (beakers #4 and 5) is required.

Fish length (mm)	Beaker size (mL)	Pond Water (mL)	Dilution water (mL)*	Cofactor (mL)	Dilution
≤ 50	100	50	0	0	1:1
		49	0	1	1:1
		10	39	1	1:5
		control	50	0	
		control + cofactor	49	1	
50 < length ≤ 75	400	200	0	0	1:1
		196	0	4	1:1
		40	156	4	1:5
		control	400	0	
		control + cofactor	396	4	
> 75	800	600	0	0	1:1
		588	0	12	1:1
		120	468	12	1:5
		control	600	0	
		control + cofactor	588	12	

* Dilution water must be free of *Prymnesium parvum* cells and toxin (e.g., conditioned tap water).

APPENDIX C

Recommended treatments for *Prymnesium parvum* blooms using liquid ammonia, ammonium sulfate, or copper sulfate as related to temperature and pH (after the Central Fish Health Lab (Israel) recommendations).

Temperature (°C)	pH	Liquid ammonia (mg/L)	Ammonium sulfate (mg/L)	Copper sulfate (mg/L)
> 20	> 9.0		10-12	
	8.6-9.0	10-12	15	
	< 8.6	12-13	15-17	
18-20	> 9.0	10-12	15	2
	8.6-9.0	12-13	20	2
	< 8.6	13	25	
12-18	> 9.0	13	25	2-3
	8.6-9.0	13		2-3
	< 8.6			2-3

Note: The toxicity of copper sulfate increases as water alkalinity decreases and a more appropriate treatment concentration may be determined as follows:

$$\text{Treatment rate (mg/L)} = \text{alkalinity as CaCO}_3 \div 100.$$

APPENDIX D

Copper – Bathocuproine Method, Version AEW-COP 1.1

STEVEN HAMBY

Purpose and Scope: This protocol describes methods used for measuring copper at concentrations of 0.02 to 0.5 mg/L. Color and turbidity of the sample can affect the accuracy of the method.

Materials Required:

Equipment

Class A 100-mL or 50-mL glassware
(volumetric or graduated cylinder)
125-mL flask
Spectrophotometer, 1-cm path length
Hot plate

1 + 1 Hydrochloric acid (Conc. HCl diluted 1:1
v:v with water)

Hydroxylamine hydrochloride solution.

Sodium citrate solution

Disodium bathocuproine disulfonate solution

Stock copper solution (20 mg/L) for Cu
standards:

10 mg/L intermediate

0.05 mg/L

0.1 mg/L

0.5 mg/L

0.2 mg/L

Reagents/consumables:

Deionized (D.I.) water that has been distilled
in a glass still

Procedure:

- Prior to use, wash all glassware with concentrated HCl then rinse with copper-free water.
Note: Many domestic waters have copper in detectable concentrations due to leaching from copper pipes.
- Turn spectrophotometer on, set absorbance to 484 nm, and allow the unit to warm up.
- Pour 50 mL of the blank (reagent water), standard, or sample into a separate 125-mL flask.
- To each flask add, with mixing after each addition, the following: 1 mL (1 + 1) HCl, 5 mL $\text{NH}_2\text{OH} \cdot \text{HCL}$ solution, 5 mL sodium citrate solution, 5 mL disodium bathocuproine disulfonate solution.
- After all flasks have been mixed, pour an aliquot into a cuvet, place it in the spectrophotometer, and record the absorbance. The following order is suggested: blank, 0.05 mg/L, 0.1 mg/L, 1 mg/L, samples, and the 0.5-mg/L continuing calibration verification standard.

Calculations:

Use the absorbencies from each standard to plot readings on a calibration curve to determine the amount of copper present.

Chemical recipes:

- Hydroxylamine hydrochloride solution: 50g $\text{NH}_2\text{OH} \cdot \text{HCL}$ in 450 mL water.
- Sodium citrate solution: dissolve 300 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and make up to 1 L with water.
- Disodium bathocuproine disulfonate solution: dissolve 1 g $\text{C}_{12}\text{H}_4\text{N}_2(\text{CH}_3)_2(\text{C}_6\text{H}_4)_2(\text{SO}_3\text{Na})_2$ in water and make up 1 L.
- Stock copper solution (20 mg/L): into a 250 mL flask, add 10 mL reagent water, 20 mg copper wire, and 5 mL conc. HNO_3 . As reaction slows, warm to completely dissolve the copper, then

boil to expel nitrogen oxides. Cool and add 50 mL reagent water, carefully transfer to a 1-L volumetric flask, and fill to 1 L.

E. Cu standards:

10 mg/L intermediate (50 mL of 20 mg/L to 100 mL) or (25 mL of 20 mg/L to 50 mL)

0.5 mg/L (5 mL of 10 mg/L to 100 mL)

0.1 mg/L (1 mL of 10 mg/L to 100 mL)

0.05 mg/L (5 mL of 1 mg/L to 100 mL)

0.2-mg/L continuing calibration verification standard (1 mL of 20 mg/L to 100 mL)

Quality Control:

A. A laboratory duplicate sample should be tested along with the original sample. An analytical precision control chart should be kept to monitor these results.

B. Percent recovery of the spike standard can be calculate as follows:

$$\% \text{ Recovery} = \frac{(C_s - C_{sx}) \times 100}{C_a}$$

Where C_s = result of spiked sample

C_{sx} = average of the original and duplicate sample

C_a = value of the spike added

Reference:

Standard Methods for the Examination of Water and Wastewater 3500-Cu E, 18th edition.

NOTE: This method measures the free copper. To measure the chelated form perform the preliminary digestion described in Standard Methods 3500-Cu Neocuproine Method 4b then adjust pH to 4-5.