CHAPTER 1

Management Strategies for *Prymnesium parvum* Ichthyotoxicity in Aquaculture

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Abstract

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

Introduction

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

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

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

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

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

Identification of P. parvum

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

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

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

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

Enumeration of P. parvum

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

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

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

A Bioassay for Toxicity

Israeli aquaculture has been affected by *P. parvum* for many years and the organism is now considered endemic in most ponds (Shilo 1967). Israeli scientists have developed methods for evaluating and controlling *P. parvum* ichthyotoxin, including a bioassay to measure ichthyotoxin units (ITU) (Ulitzer and Shilo 1964). An ITU is defined as the minimal amount of prymnesin required to kill all of the test organisms during a two-hour period at 28ºC 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 28ºC 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 20ºC (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 (<15º 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 28ºC vs. room temperature (i.e., 24ºC) 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, 24ºC) versus 28ºC.—It takes a considerable amount of time to bring the temperatures of water samples from hatchery ponds to the recommended temperature (28ºC) 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 28ºC and ambient room temperature (i.e., 24ºC). 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 24ºC 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 24ºC, as the toxicity of *P. parvum* ichthyotoxin was greater at 28°C than at 24ºC. Since the purpose of the bioassay is to provide sensitive detection of *P. parvum* ichthyotoxin at sub-lethal levels in order to allow time to treat ponds or move fish, this test should be conducted at 28°C.

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

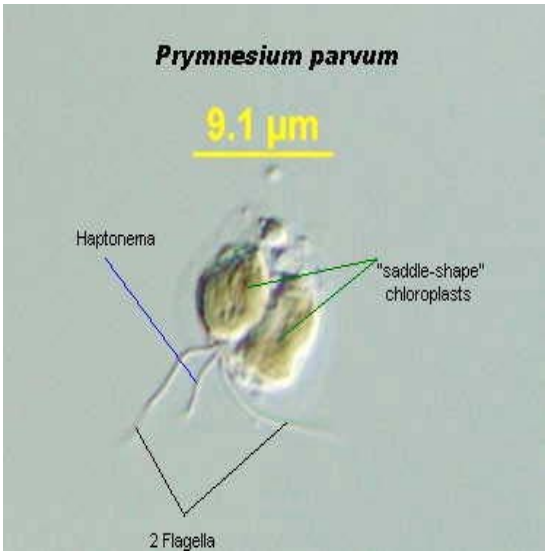
conducted in very small vessels, minimizing both space and tempering time required to conduct the test.

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

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

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

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



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

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

TABLE 1.—Susceptibility of fathead minnow fry and adult to *Prymnesium parvum* ichthyotoxin, and activity of ichthyotoxin at 28°C verses ambient room temperature (24ºC) 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