

Identification, Fractionation and Quantification of Prymnesin Toxins Collected from the Salt River Reservoirs, Arizona.

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Introduction and Background

Since the winter of 2004, several large-scale fish kills have occurred sporadically in Apache, Canyon, and Saguaro reservoirs along the Salt River, Arizona. All of these are multi-use reservoirs for drinking water, recreation, and angling.

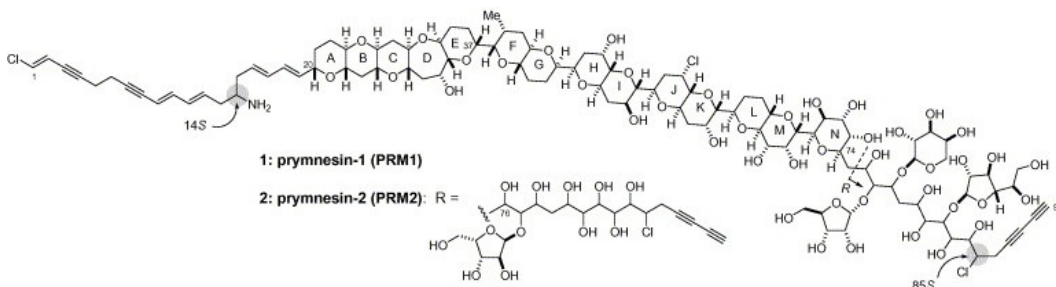
The primary cause of these fish kills is believed to be the powerful exotoxin prymnesin (actually two toxins; see below) produced by *Prymnesium parvum*, a haptophyte identified from the affected reservoirs in 2004 by Arizona Game and Fish Department personnel and confirmed by both the University of Arizona and Aquatic Consulting and Testing. Since 2004, *P. parvum*-related fish kills have been sporadic, occurring in any of the three reservoirs seemingly at random. During 2006, fish kills occurred in Canyon at the earliest date thus far, whereas large kills previously occurred in Saguaro during early June. Apache, while not having sustained fish kills of the magnitude observed in Saguaro, should no longer be considered a viable fishery for smallmouth bass (*Micropterus dolomieu*). The bivalve mollusc *Corbicula fluminea* (*Asian clam*), once prevalent in the riverine portion of Saguaro Reservoir, is also now almost completely absent (Marc Dahlberg, AzG&FD pers. comm.). Such elimination of species suggests the strong potential for a trophic cascade with bottom-up control by algal toxin production. Long-term effects of chronic toxicity, often of much greater ecological significance than acutely toxic events, are poorly understood for *P. parvum*.

Determining environmental triggers for toxin production is our best hope of determining possible management strategies to prevent or reduce fish kills within these reservoirs. The extremely sporadic nature of the kills and toxin production, and the unstable nature of the toxins, makes documentation of fish kill causality and toxin quantification difficult. Prymnesin consists of two hemolytic toxins referred to as prymnesin-1 and prymnesin-2 (Igarishi et al. 1996, Murata & Yasamoto 2000). For each, the toxic fraction likely is relatively small when compared to the size of the individual molecules. Moreover, it is possible that *P. parvum* produces several other toxins, any one of which might exhibit ichthyotoxic, neurotoxic, hemolytic, and/or cytotoxic properties (Shilo 1981). Due to the difficulty in quantifying the toxic fraction(s) of prymnesin, toxicity is usually estimated using various bioassays (Larsen et al. 1993, Larsen and Bryant 1998) or *in vitro* hemolytic assays (Johansson and Granéli 1999, Barriero et al. 2005). Such assays are most relevant, however, when interpreted against the quantity of toxin(s) present (Burkholder et al. 2005). Methodologies to quantify prymnesins are not yet available, so bioassay-guided studies of the role of different environmental factors in prymnesin production have met with limited success (Baker et al. 2007, Larsen and Bryant 1998).

Prymnesin-1 and prymnesin-2, the *P. parvum* toxins that have been characterized thus far, are similar in structure to brevetoxins. Both forms of prymnesin are large organic molecules with mass greater than 1400 AMU (Fig. 1). Despite the apparent structural similarity of brevetoxins and prymnesins, the two types of toxin have very different modes of action. Brevetoxins are potent neurotoxins, whereas prymnesins cause massive haemolysis of red blood cells. This similarity in structure but distinct modes of action suggests that elucidation of the toxic fractions within *P. parvum*, in addition to prymnesin, will be highly ecologically relevant considering these known compounds to be the principal etiological agent. Another point that supports this premise is that research thus far has not shown a significant correlation between water-column concentrations of prymnesin and fish toxicosis.

Prymnesin toxins possess several unique structural features such as an unbranched single chain of 90 carbons with just a single methyl group, a fused polycyclic ether ring system, four distinct 1,6-dioxadecalin units, conjugated double and triple bonds, chlorine atoms, an amino group, and glycosidic residues including an uncommon L-xylose (Sasaki et al. 2006). The structure and large size of the two characterized prymnesins would make cell entry difficult, in turn making fish toxicosis difficult to explain and supporting the premise that other, smaller toxins made by *P. parvum* may be important in affecting fish health.

Figure 1. Structure of prymnesin-1 and prymnesin-2 (Sasaki et al. 2006).



Although allelopathy and grazing defense have been proposed as major factors controlling *P. parvum* toxicity, the evolutionary factors that control toxin production by any species of alga are poorly known. Granéli and Johansson (2003) examined the release of allelopathic substances by *P. parvum* when grown in N- or P-deficient conditions. They found that cell-free filtrate from *P. parvum* cultures grown under either N or P limitation significantly inhibited growth of the diatom *Thalassiosira weissflogii*, the potentially toxic dinoflagellate *Prorocentrum minimum*, and the cryptophyte *Rhodomonas cf. baltica*. In contrast, these species were not inhibited when exposed to nutrient-replete *P. parvum*. Nutrients apparently also interact with temperature, photoperiod, light intensity, salinity and likely other factors to affect prymnesin production, but the various roles of these interactions in controlling toxicity are poorly understood.

The reservoirs along the Salt River provide an ideal model system for advancing scientific understanding about prymnesin toxicity. Roosevelt Lake, the reservoir upstream from the affected reservoirs, receives incoming water via either the Salt River or Tonto Creek and has not sustained a *P. parvum*-related fish kill. The earliest fish kills in 2004 occurred in the riverine section of Apache reservoir; thus, kills occurred on one side of Roosevelt Dam but not the other. Roosevelt may act as a trap of allochthonous nutrients; it has higher levels of N, P, and C than the affected downstream reservoirs, which are generally considered to be N+P co-limited. Most of the urban lakes in the Metropolitan Phoenix area that have sustained *P. parvum*-related fish kills are connected to the Salt River Project canal system which receives flows from the three affected reservoirs.

Scope of Services

This project team has the expertise in algal strain isolation, mass culture, and toxin analysis needed to strengthen understanding about environmental triggers for toxin production by *P. parvum*. Uni-algal cultures of *P. parvum* will be grown under a range of environmental factors to identify the conditions that support maximum toxicity. Using

those conditions, we will mass-culture toxic *P. parvum* to develop improved techniques for biochemical fractionation and identification of the toxins produced.

The first step will be to assess nutrient/light conditions that support maximal toxicity. Two bioassay systems will be used to assess toxicity, including the sheepshead minnow assay, as well as the Israeli prymnesin activity assay. We will focus on altered nitrogen, phosphorus, and trace metal concentrations in controlling toxin production. These culture conditions for maximal toxin production will be used for mass-culture by CoPIs Burkholder and Zimba. By using two facilities for grow-out, we will produce the necessary biomass for chemical fractionation more quickly. Cultures will be grown in increasingly larger containers, up to 50 L, to maintain conditions optimized for toxin production and cell biomass. Culture conditions will be 28°C, 90 µmol light intensity (photosynthetically active radiation), and photoperiod 16-hr light: 8-hr darkness, with illumination under optimal nutrient limitation conditions to maximize toxicity.

The algal cells and culture filtrate will be processed for toxin analysis as follows: Cells will be harvested by filtration (> 10 µm retained biomass). Cell pellets will be sent to Dr. Moeller for serial fractionation. Cell pellets will be serially extracted to determine toxin classes present. Filtrate will be passed through C18 solid phase cartridges by Dr. Zimba to collect dissolved toxic fractions. These cartridges are routinely useful for concentrating various types of toxins belonging to proteins, lipids, and sugar complexes (e.g. Hallegraef et al. 2003, Moeller et al. 2007). This material will be serially eluted from the solid phase cartridges using an elution sequence of water, methanol, chloroform, and ethyl acetate. Each fraction will be concentrated by rotary evaporation for toxin purification and identification. Drs. Burkholder and Zimba will provide cell pelleted material from > 500 L of toxic *P. parvum* culture for this effort during the first 1.5 years of the project; Zimba will provide >100L of solid-phase extract (SPE) retained filtrate to assess dissolved toxins during the first 15 months of this project.

Dr. Moeller will use bioassay-guided fractionation to facilitate identification of toxic components from *P. parvum*. Bioassays will use known cell lines known to respond to hemolytic agents. Activity is identified by living cells producing carbon dioxide – this alters the pH and changes color from the initial yellow. Active fraction(s) will be separated using HPLC methodology. This will include use of selective normal and reverse phase mobile phases and column conditions to isolate the toxin(s). All steps of isolation will use bioassay methods to confirm activity of that fraction.

We have assembled a strong multidisciplinary team to attempt this scope of research within the proposed two-year timeframe.

Timeline

Year 1:

Walker: Project management/data synthesis/reporting.

Burkholder: Isolate and grow *P. parvum* unialgal cultures to assess environmental conditions for maximal toxicity.

Zimba / Burkholder: Using these conditions, mass-culture *P. parvum* (from 100 L minimum culture volume), concentrate cell biomass and dissolved organics, and send cell pellets and SPE material to CoPI Moeller for toxin extraction and assessment (estimated cell pellet, >50 grams).

Moeller: Biochemical fractionation, toxin class identification, development of toxin bioassay(s)

Year 2:

Walker: Project management/data synthesis/reporting.

Burkholder/Zimba: Continued mass culture of *P. parvum*; cell pellets from 500 L of culture sent to CoPI Moeller (estimated cell pellet >250 g).

Moeller: Continued toxin identification and refinement of improved methods for toxin assessment.

The University of Arizona will serve as the lead institution and will be in charge of disbursing funds to other investigators.

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