ABSTRACT

Title of Dissertation: TOXINS AND TOXICITY FROM THE COSMOPOLITAN,
BLOOM-FORMIMG DINOFLAGELLATE *Karlodinium micrum*.

Jonathan R. Deeds, Doctor of Philosophy, 2003

Dissertation directed by: Professor Allen R. Place
University of Maryland Biotechnology Institute
Center of Marine Biotechnology
Marine, Estuarine, and Environmental Science Program

Karlodinium micrum (Leadbeater and Dodge) Taylor was first described in the United States during an investigation into the cause of repeated fish kills at an estuarine aquaculture facility located on a tributary of the Chesapeake Bay, USA. As part of that investigation we described toxins in this species for the first time. Named karlotoxins (KmTx), these compounds possess hemolytic, cytotoxic, and anti-fungal properties. However, the primary harmful effect associated with blooms of this organism is ichthyotoxicity. Karlotoxins are lethal to fish through damage to gill epithelia. This research focused on two of these toxins, KmTx 1 (1338 Da.) and KmTx 2 (1344 Da.) These two toxins have been the main toxins, in terms of amount and potency, in all US isolates tested to date. Using a range of mammalian cell types, the mode of KmTx 2 cytotoxicity was shown to be non-selective permeabilization of cell membranes to a range of small ions and molecules resulting in cell death through osmotic lysis. Membrane sterol composition appears to play a role in the sensitivity of different species to KmTx's membrane disrupting effects. This sterol specificity also appears to be responsible for the apparent immunity of *K. micrum* from its own toxins. We have described various karlotoxins in *K. micrum* isolates and bloom samples from US east coast estuaries from Maryland to Florida. Among US east coast isolates, a geographic strain variation appears to exist in that KmTx 1 has only been found in Maryland isolates while KmTx 2 has been found in all other isolates tested from North Carolina, South Carolina, and Florida. Recently, a KmTx 2-like compound (1342 Da.) has been isolated from bloom samples from Western Australia, being the first confirmation of karlotoxin production outside of the United States. This work confirms the association between blooms of *K. micrum* and fish kills that has been observed in temperate estuaries around the world for over half a century. It also lays the foundation for future studies to determine the ecological function of toxin production in this species and the consequences of this production both on *K. micrum*'s environment and ours.

TOXINS AND TOXICITY FROM THE COSMOPOLITAN, BLOOM-FORMING DINOFLAGELLATE *Karlodinium micrum*

By

Jonathan R. Deeds

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2003

Advisory Committee:

Allen R. Place, Ph.D., Chair
Renate Reimschuessel, Ph.D., D.V.M.
Diane K. Stoecker, Ph.D.
Daniel E. Terlizzi, Ph.D.
John M. Trant, Ph.D.

DEDICATION

To my parents, Ron and Marie, who sacrificed so much for my education. You may never have fully understood what I was doing with my life but you never questioned why I was doing it. And to my wife Bethany whose support and understanding made this accomplishment possible. I thank her for agreeing to postpone our lives for a little while.

ACKNOWLEDGEMENTS

First and foremost I must acknowledge my advisor and friend Allen Place. I know he took a chance on me five years ago and I will always be grateful to him for that. We may not have always agreed on our interpretation of the data, but trying to prove my theories to him made me a better scientist.

I also wish to acknowledge my research committee members, Renate Reimschuessel, Diane Stoecker, Danial Terlizzi, and John Trant. Each contributed significantly to the quality of the research contained in this text.

Lastly, I must acknowledge Tony Mazaccarro, owner and proprietor of HyRock Fish Farm. The observations of Tony and Dan back in 1996 were the inspiration for this entire line of research. Your questioning of the party line did not go in vain.

Additional acknowledgements are contained at the end of each chapter.

TABLE OF CONTENTS

List of Tables -- vi

List of Figures-- vii

Chapter 1 – Introduction -- 1

Tables/Figures 7

Chapter 2 – Toxic Activity from Cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) – A Dinoflagellate Associated with Fish Mortalities in an Estuarine Aquaculture Facility. ------------------------- 9

Abstract 10
Introduction 12
Materials and Methods 14
Results 26
Discussion 33
Tables/Figures 42

Chapter 3 - Treatment Options for the Control of the Ichthyotoxic Dinoflagellate *Karlodinium micrum* in an Estuarine Aquaculture Facility. ------------- 62

Abstract 63
Introduction 64
Materials and Methods 66
Results 69
Discussion 70
Tables/Figures 75

Chapter 4 - Mode of Cytotoxicity of KmTx 2, A New Fish-Killing Toxin from the Dinoflagellate *Karlodinium micrum.* -------------------------------------- 81

Abstract 82
Introduction 84
Materials and Methods 85
Results 99
Discussion 103
Tables/Figures 112

Chapter 5 - Mode of Ichthyotoxicity of the Toxins from the Dinoflagellate *Karlodinium micrum.* --- 138

Abstract 139

Introduction 140
Materials and Methods 141
Results 144
Discussion 146
Tables/Figures 151

Chapter 6 - Geographic Strain Variation in Toxin Production in *Karlodinium micrum* (Dinophyceae) from Southeastern Estuaries of the United States. --- 163

Abstract 164
Introduction 165
Materials and Methods 166
Results 168
Discussion 169
Tables/Figures 172

Addendum – Investigation of the 2003 Swan and Canning River Fish Kills (Perth, Western Australia) Associated with Blooms of *Karlodinium micrum*. --- 182

Abstract 183
Introduction 184
Materials and Methods 185
Results 185
Discussion 186
Tables/Figures 190

Chapter 7 – Summary and Future Research Directions. -------------------------------- 198

Tables/Figures 211

Literature Cited-- 221

Chapter 1 221
Chapter 2 223
Chapter 3 230
Chapter 4 232
Chapter 5 239
Chapter 6 243
Chapter 7 245

LIST OF TABLES

Table 2.1. Copper chelation experiments measuring free copper in solution after the addition of EDTA (2 mM). p. 43

Table 2.2. Summary of toxic activity from cultures of *Karlodinium micrum*. p. 45

Table 4.1. Comparison of anti-fungal and hemolytic activities of KmTx 2 and amphotericin B. p. 113

Table 6.1 *Karlodinium micrum* samples screened for the presence of KmTx 1 and KmTx 2. p. 173

Table 7.1 Summary of current data for toxins from U.S. populations of *Karlodinium micrum.* p. 212

LIST OF FIGURES

Figure 1.1. SEM image of *Karlodinium micrum*. p. 8

Figure 2.1. Map of the Chesapeake Bay showing location of HyRock Fish Farm. p. 47

Figure 2.2. Dose dependence for the lysis of rainbow trout erythrocytes, compared to cells lysed with 10 µg saponin. p. 49

Figure 2.3. Percent hemolysis, measured as release of hemoglobin compared to cells lysed with 10 µg saponin, in aliquots of separated lipid classes from *Karlodinium micrum* (CCMP 1974). p. 51

Figure 2.4. Hemolytic activity in 0.5 min. HPLC fractions (flow rate 1 ml/min) of 80% MeOH C_{18} elutions from *Karlodinium micrum* (CCMP 1974) cells and culture filtrate. p. 53

Figure 2.5. UV absorbance spectra (mAU) of reversed phase HPLC fractions Tox A and Tox B. p. 55

Figure 2.6. *In-vitro* cytotoxicity assay, based on the release of lactate dehydrogenase, testing 0.5 min. reversed phase HPLC fractions of a 50 µl injection of a concentrated 80% MeOH C_{18} elution of *Karlodinium micrum* (CCMP 1974) culture filtrate. p. 57

Figure 2.7. Cell lysis in *Karlodinium micrum* (CCMP 1974) and *Prorocentrum minimum* (strain PM-1) cultures exposed to either $CuSO_4$ or $KMnO_4$. p. 59

Figure 2.8. Time course of hemolytic material release, measured as release of hemoglobin from rainbow trout erythrocytes, compared to cells lysed with 10 µg saponin, in *Karlodinium micrum* (CCMP 1974) cultures exposed to either $CuSO_4$ or $KMnO_4$. p. 61

Figure 3.1. Schematic diagram of HyRock Fish Farm. p. 76

Figure 3.2. Effect of algicidal treatments on cell lysis of *Karlodinium micrum*. p. 78

Figure 3.3. Effect of algicidal treatments on hemolytic activity from both cells and isolated toxins from *Karlodinium micrum*. p. 80

Figure 4.1. Purification and molecular weight determination of KmTx 2. p. 115

Figure 4.2. Purification and identification of gymnodinosterol isolated from *Karlodinium micrum* (CCMP 2282). p. 117

Figure 4.3. Fura 2 pre-loaded rat cardiac myocyte exposed to 0.25 μg/ml KmTx 2. p. 119

Figure 4.4. Measurement of Ca^{2+} flux into rat embryonic fibroblast (REF 52) cells using the intracellular fluorescent indicator fura-2. p. 121

Figure 4.5. Additional intracellular fluorescent indicator experiments to test the specificity of cation permeability in REF-52 cells upon exposure to KmTx 2. p. 123

Figure 4.6. KmTx 2 induced current in whole cell patch-clamped rabbit vagal sensory nodose ganglion neuron (NGN). p. 125

Figure 4.7. Voltage ramp constructed-current voltage (I-V) relationship for KmTx 2 induced current in whole cell patch clamped rabbit vagal sensory nodose ganglion neuron (NGN). p. 127

Figure 4.8. Inhibition of hemolysis of rainbow trout erythrocytes due to exposure to 0.25, 0.5 or 1 μg/ml KmTx 2 after co-incubation with 30 mM of various molecular weight osmolytes (300-10,000 Da.). p. 129

Figure 4.9. Effect of KmTx 2 on *Oxyrrhis marina* and *Karlodinium micrum* (CCMP 2282). p. 131

Figure 4.10. Hemolysis of rainbow trout erythrocytes due to exposure to 0, 0.1, 0.5, or 1 μg/ml KmTx 2 after co-incubation with various membrane sterols. p. 133

Figure 4.11. Hemolysis of rainbow trout erythrocytes due to exposure to 0, 0.1, 0.5, or 1 μg/ml KmTx 2 after co-incubation with membrane lipids. p 135

Figure 4.12. H&E stained sections of whole 60-day old zebrafish (*Danio rerio*) exposed to an increasing concentration of KmTx 2. p. 137

Figure 5.1. Dose response relationship for zebrafish (*Danio rerio*) mortality upon exposure to *Karlodinium micrum* toxins. p. 152

Figure 5.2. 15-minute exposure of zebrafish (*Danio rerio*) larvae to 4 μg/ml KmTx 2. p. 154

Figure 5.3. Larval Sheepshead minnows (*Cyprinodon variegates*) (24 hours old post-hatch) exposed to a sonicated suspension of *Karlodinium micrum.* p. 156

Figure 5.4. H&E stained control (MeOH exposed) zebrafish (*Danio rerio*) gills in KmTx 2 exposure experiments. P. 158

Figure 5.5. H&E stained gill arches of 0.1 µg/ml KmTx 2 exposed zebrafish (*Danio rerio*). p. 160

Figure 5.6. H&E stained gill arches of 0.5 µg/ml KmTx 2 exposed zebrafish (*Danio rerio*). p. 162

Figure 6.1. Comparison of four *K. micrum* isolates grown both autotrophically and mixotrophically. p. 175

Figure 6.2. Map of the Southeastern coast of the United States. Numbers represent sampling locations and correspond to sample information in (Table 1). p. 177

Figure 6.3. Dose response curves for KmTx 1 and KmTx 2 based on lysis of rainbow trout erythrocytes. p. 179

Figure 6.4. HPLC co-injection of purified KmTx 1 isolated from a Maryland isolate of *Karlodinium micrum*, and KmTx 2 isolated from a South Carolina isolate of *Karlodinium micrum*. p. 181

Figure A6.1. Map of Western Australia showing location of Swan and Canning River estuaries. p. 191

Figure A6.2. HPLC traces of Swan River water samples. p. 193

Figure A6.3. Comparison of Swan River KmTx 2-like compound to KmTx 2 from US isolates. p. 195

Figure A6.4. LC/MS analysis of Swan River KmTx 2-like compound. p. 197

Figure 7.1. Map of the Chesapeake Bay, Maryland, USA showing location of collection sites for isolates and water samples for the analysis of the distribution of the toxins from *Karlodinium micrum*. p. 214

Figure 7.2. Chemical structures of sterols tested in this study for their ability to inhibit fish red blood cell hemolysis upon exposure to KmTx 2. p. 216

Figure 7.3. Comparison of dose response relationships for the lysis of fish red blood cells for several of the toxins from *Karlodinium micrum*. p. 218

Figure 7.4. H&E stained section of mouse pancreas after 24-hour exposure to an IP injection of 10 µg KmTx 2. p. 220

CHAPTER 1

Introduction

Karlodinium micrum, then known as *Gymnodinium galatheanum*, was first described in 1950 in Walvis Bay, South Africa during a large bloom-related fish mortality (Braarud, 1957; Pieterse and Van Der Post, 1967). *Gyrodinium galatheanum* (Braarud) Taylor *sensu* Taylor is synonymous with *Gymnodinium galatheanum* Braarud *sensu* Kite and Dodge and *Gymnodinium micrum* (Leadbeater and Dodge) Loedlich III (Daugbjerg et al., 2000). Based on ultrastructure and molecular data, Daugbjerg et al. (2000) recently redescribed this species as *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen *comb. nov*. This species is identified based on the presence of a displaced cingulum, a straight apical groove (see Figure 1), a normal nuclear envelope, fucoxanthin or fucoxanthin derivatives as accessory pigments, and lack of a nuclear connective (Daugbjerg et al., 2000). To this point, this description is identical to the well-known and closely related fish-killing dinoflagellates *Karenia brevis* and *Karenia mikimotoi*. Additional characters which distinguish *Karlodinium* sp. from *Karenia* sp. include an amphiesma with an array of plug-like structures arranged in a hexagonal configuration and the presence of a ventral pore (see Figure 1) (Daugbjerg et al., 2000).

Since its original description in 1950, few associations have been made in the scientific literature linking blooms of this organism to negative impacts on aquatic fauna, although, laboratory experiments have shown this organism causes mortality in juvenile cod and reduced growth in mussel (Nielsen and Stromgren, 1991; Nielsen, 1993).

On July 30, 1996 a large mortality of ca. 15,000 hybrid striped bass occurred following an algicidal copper sulfate treatment to arrest a dense dinoflagellate bloom at

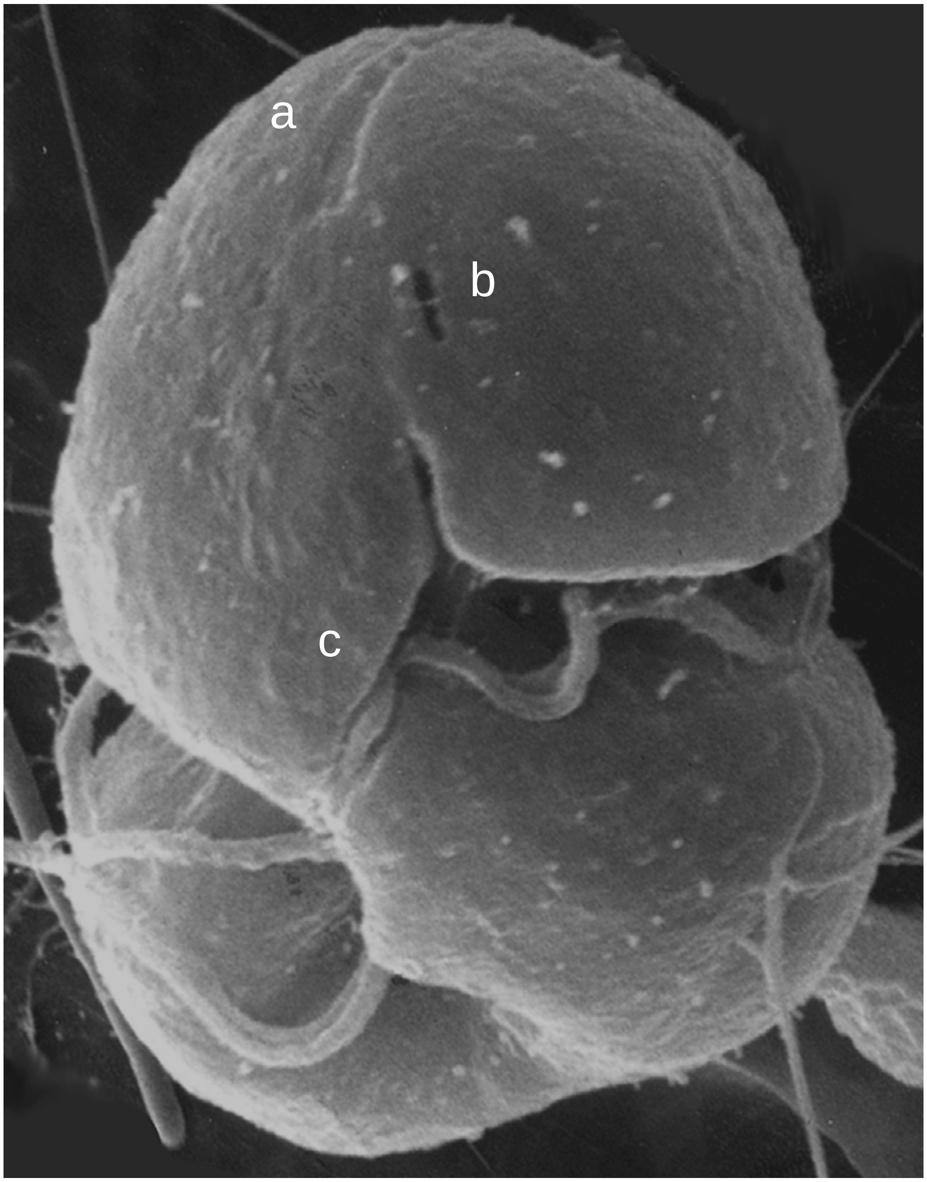
HyRock Fish Farm (Latitude 38.1733N, Longitude 75.7374W), located on Maryland's lower Eastern Shore. HyRock Fish Farm is an estuarine aquaculture facility supplied with water from the Manokin River, a tributary of the Chesapeake Bay located in Princess Anne, MD, USA. Potassium permanganate treatment of a neighboring pond with similar dinoflagellate populations appeared to arrest the bloom without fish mortality. The bloom was eventually determined to be dominated by the 10-15 μm, non-thecate, mixotrophic dinoflagellate *Karlodinium micrum*, originally identified as *Gyrodinium estuariale*, (ca. 6×10^4 cells ml^{-1}), with < 1,000 cells ml^{-1} of an unidentified dinoflagellate (*Gymnodinium* sp.) and several additional < 10 μm unidentified species (Wayne Coats, Smithsonian Environmental Research Center, Edgewater MD, personal communication). Some of the additional < 10 μm dinoflagellates present resembled life history stages of *Pfiesteria piscicida*, as described in Burkholder et al. (1992) and Steidinger et al. (1996), so samples were forwarded to the laboratory of Dr. Karen Steidinger (Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg FL) who confirmed through SEM and light microscopy that both *K. micrum* and *P. piscicida* were present in the sample (ca. 300 cells ml^{-1} *P. piscicida*). This was the first confirmation of *K. micrum* in U.S. waters. In contrast, *G. estuariale* was first described in the U.S. in the late 1950's and its presence had been recorded in Atlantic Coast estuaries for decades (Hulburt, 1957). In the Chesapeake Bay *G. estuariale* had been observed throughout the year, often dominating the 10-20 μm phytoplankton assemblage during the summer months (Marshall, 1980). Although it has been observed to bloom in very high numbers, blooms of *G. estuariale* had never been associated with toxic effects in the scientific literature. In subsequent years, two additional fish mortality events co-occurred with blooms of *K. micrum* at HyRock Fish Farm, 8000 adults on August 13, 1997, and 5000 fingerlings on May 15, 1999. In both circumstances, mortality was stopped following a potassium permanganate treatment, with fish returning to feed in 3-5 days.

Our initial goal in this investigation was to show that *Karlodinium micrum* was potentially responsible for the mortalities of hybrid striped bass that had co-occurred with blooms of this organism at HyRock Fish Farm since 1996. As part of this research thus far, we have shown that *K. micrum* produces a suite of compounds with ichthyotoxic, cytotoxic, and hemolytic properties (Deeds et al., 2002; Deeds et al., *submitted* a). We have shown that exposure to environmentally relevant concentrations of these toxins results in extensive damage to fish respiratory epithelia. We have isolated these compounds, in sufficient amounts found to be toxic, directly from water samples collected during fish kills both in the United States and abroad (Kempton et al., 2002; Deeds et al., *submitted* a). In confirmation of observations made at HyRock Fish Farm, we have shown algicidal copper treatment to result in *K. micrum* cell disruption, which in turn results in the release of toxic compounds leading to fish death (Deeds et al., 2002). Further, we have shown that strong oxidizing agents, such as potassium permanganate and ozone, cause dinoflagellate cell destruction without subsequent toxic effects (Deeds et al., 2002; Deeds et al., *submitted* b).

As part of the investigation into the cause of the HyRock Fish Farm dinoflagellate bloom-related mortalities, we described toxic fractions (initially named Tox A and Tox B) from Chesapeake Bay cultures of *K. micrum* (Deeds et al., 2002). These same compounds were later isolated directly from water collected during a non-aquaculture related fish kill in Maryland in 2002 in which high *K. micrum* densities were present (Goshorn et al., *submitted*). We isolated a similar compound (Tox A-like) both directly from water samples collected during a non-aquaculture related estuarine fish kill near Charleston, South Carolina and later from a *K. micrum* strain isolated from that same kill [strain JW020205-B4, provided by Jennifer Wolney, Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg FL] (Kempton et al., 2002). To date, we have confirmed the presence of these and similer compounds in nine isolates and ten natural water samples, both with and without fish kills, some grown both mixotrophically and autotrophically, from Maryland, North Carolina, South Carolina, Florida, and most recently from an estuarine river experiencing fish kills associated with a persistent bloom of *K. micrum* in Western Australia (Deeds et al., *submitted* a). In the Deeds et al. (*Submitted* a) study, we confirmed an observation that an apparent geographic strain variation in toxin type exists between Chesapeake Bay, MD isolates and isolates collected from estuaries located South of the Chesapeake Bay. We have since named the main toxin from Chesapeake Bay populations KmTx 1 (formerly the active principle of Tox A) and the main toxin from the other, more southern, populations KmTx 2. Several additional toxic compounds have been isolated from the above-described isolates and water samples. They too appear to follow a geographic pattern in their distribution. Initial analyses indicate that they are related both in activity and chemical class to KmTx 1 and 2, but confirmation of these observations must await a more detailed investigation of both their nature and purpose. We are putatively naming this newly described group of toxins **karlotoxins**, after Karl Tangen, after whom this species is named.

In this work we report the following: The first description of toxic compounds from *Karlodinium micrum* with properties consistent with effects observed during fish kills associated with high densities of this organism (Chapter 2). An account of potential management strategies for the control of *K. micrum* blooms including a description of an ozone generation system designed to limit the introduction of harmful algal species into estuarine aquaculture facilities (Chapter 3). A detailed description of the cytotoxic mode of action of KmTx 2, the main toxin from *K. micrum* population from Southeastern United States estuaries located South of the Chesapeake Bay, including hypotheses concerning potential ecological functions of these toxins as well as how *K. micrum* protects itself from these toxins harmful effects (Chapter 4). An account of the ichthyotoxic effects of KmTx 2, including a detailed description of its effects on fish gill epithelia (Chapter 5). Finally, we also report here on an apparent geographic strain variation in toxin type for *K. micrum* populations from estuaries of the Southeastern United States (Chapter 6), including a description of a KmTx 2-like compound recently isolated from a Western Australia *K. micrum* related fish kill (Chapter 6 addendum).

Figure 1.1. SEM image of *Karlodinium micrum* identified by Karen Steidinger (Florida Marine Research Institute, Florida Fish and Wildlife Commission). Image provided by Beverly Roberts (FMRI). Original image designation FMRI ID# 1161-4846. Note: (a) straight apical grove, (b) ventral pore, and (c) displaced cingulum.


a
b
c

CHAPTER 2

Toxic Activity from Cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - A Dinoflagellate Associated with Fish Mortalities in an Estuarine Aquaculture Facility[I].

[I] Deeds, J.R., Terlizzi, D.E., Adolf, J.E., Stoecker, D.K., and Place, A.R. 2002. Toxic activity from cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - A dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. Harmful Algae 1, 169-189.

ABSTRACT

The goal of this study was to test for, and partially characterize, toxic activity associated with the dinoflagellate *Karlodinium micrum*. Since 1996, three fish kill events associated with blooms of *K. micrum* have occurred at HyRock Fish Farm, an estuarine pond aquaculture facility raising hybrid striped bass on the Chesapeake Bay, MD, USA. Using an assay based on the lysis of rainbow trout erythrocytes, cultures of a Chesapeake Bay isolate of *K. micrum* have been shown to produce toxic substances which are released upon cell disturbance or damage. The LC_{50} for hemolysis of a sonicated cell suspension was 2.4×10^4 cells ml^{-1}, well within the range of cell concentrations observed associated with fish kills. The toxic activity from *K. micrum* cells and culture filtrates was traced to two distinct fractions that co-elute with polar lipids. The LC_{50} for hemolysis of the larger of these two fractions, KmTx 1 (formerly Tox A) was 284 ng ml^{-1} while the LC_{50} of the second, smaller, fraction *p*KmTx 3 (formerly Tox B) was 600 ng ml^{-1}. For comparison, the LC_{50} for the standard hemolysin saponin was 3203 ng ml^{-1}. At concentrations of 800 and 2000 ng ml^{-1}, respectively, KmTx 1 was further shown to be ichthyotoxic to zebrafish (*Danio rerio*) larvae (80% mortality), and cytotoxic to a mammalian GH(4)C(1) cell line (100% LDH release). At a concentration of 600 ng ml^{-1} *p*KmTx 3 was shown to be cytotoxic to a mammalian GH(4)C(1) cell line (>30% LDH release), but not ichthyotoxic to zebrafish (*Danio rerio*) larvae up to a concentration of 250 ng ml^{-1}. Although treatment with either algicidal copper or potassium permanganate

caused significant lysis of *K. micrum* cells (> 70%), toxic activity was released after treatment with copper and eliminated following treatment with potassium permanganate. This observation in cultures is consistent with observations made at HyRock Fish Farm where significantly higher mortality was observed following treatment of a *K. micrum* bloom with copper sulfate compared to treatment with potassium permanagante. This study represents the first direct evidence of the toxicity of *K. micrum* isolated from the Chesapeake Bay.

INTRODUCTION

Opened in 1993, HyRock Fish Farm consists of 37 acres of impoundments supplied with water from the Manokin River, a tributary of the Chesapeake Bay located in Princess Anne, MD, USA (Figure 2.1). Average salinity of the incoming Manokin river water is 10 psu (range 4.5 – 18 psu). On July 30, 1996 a large mortality of ca. 15,000, 1-1.25 lb. (2.20-2.75 kg) reciprocal cross hybrid striped bass (*Morone saxatilis* male × *Morone chrysops* female) occurred following a copper sulfate treatment (< 2 mg L^{-1}) to arrest a dense dinoflagellate bloom. The bloom had been developing for over one week prior to the events of July 30, but no mitigating actions were taken due to the misdiagnosis of the deep mahogany water coloration as tannins. Limited fish mortalities had occurred in the days preceding July 30. To avoid low dissolved oxygen problems, paddle wheel aeration was initiated maintaining dissolved oxygen levels > 5 ppm. Alkalinity was 75 ppm and other water quality conditions immediately prior to the main kill were within normal mid-summer ranges for HyRock Fish Farm (see Glibert and Terlizzi, 1999). Foaming and a “petroleum-like” odor were present in the days preceding the large kill. Treatment using potassium permanganate (< 4 mg L^{-1}), a strong oxidizing agent, of a neighboring pond with similar mixed dinoflagellate populations, appeared to arrest the bloom without fish mortality.

The bloom was subsequently determined to be dominated by the 10-15 μm, non-thecate, mixotrophic dinoflagellate *Karlodinium micrum*, originally identified as *Gyrodinium estuariale*, (ca. 6×10^4 cells ml^{-1}), with < 1,000 cells ml^{-1} of an unidentified dinoflagellate (*Gymnodinium* sp.) and several additional < 10 μm unidentified species

(Wayne Coats, Smithsonian Environmental Research Center, Edgewater MD, personal communication). Some of the < 10 µm dinoflagellates present resembled life history stages of *Pfiesteria piscicida*, as described in Burkholder et al. (1992) and Steidinger et al. (1996), so samples were forwarded to the laboratory of Dr. Karen Steidinger (Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg FL) who confirmed through SEM and light microscopy that *P. piscicida* was present in the sample (ca. 300 cells ml^{-1}). Pathological examination of both live and preserved specimens at the time of the kill suggested that suffocation due to gill inflammation was the cause of mortality, although brain tissue also showed some abnormalities (Eric May, University of Maryland Eastern Shore, Salisbury MD, personal communication). Since that time, two additional fish mortality events have co-occurred with blooms of this organism; 8000 adults on August 13, 1997, and 5000 fingerlings on May 15, 1999. In both circumstances, mortality was stopped following a < 4 mg L^{-1} potassium permanganate treatment, with fish returning to feed in 3-5 days.

We hypothesize that *Karlodinium micrum* was in part responsible for the mortalities of hybrid striped bass that have co-occurred with blooms of this organism at HyRock Fish Farm since 1996. We do not believe that copper alone was responsible for the fish mortality of July 30, 1996, considering it had been used previously at HyRock to control phytoplankton densities at the same concentration and alkalinity without problems. Rather, we hypothesize that copper treatment lead to dinoflagellate cell disruption which in turn led to the release of a toxic and/or irritating substance(s). This, possibly in combination with residual copper, moderately high ammonia, and moderately low dissolved oxygen, led to the complete mortality observed in the pond. Rapid cell disruption was suggested due to a substantial increase in foaming observed upon copper sulfate addition. An anecdotal observation was that workers removing dead fish reported that the water felt "hot" (actual water temperature was 28-30 °C), further suggesting that a dermal irritant was present in the water. To our knowledge, no toxic substance has yet been identified in *K. micrum*.

MATERIALS AND METHODS

The following dinoflagellate isolates were chosen for initial toxicity screening: *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen *comb. nov*. (CCMP 1974; Chesapeake Bay isolate) and (CCMP 1975; HyRock Fish Farm isolate). For comparison purposes, a Maryland isolate of *Prorocentrum minimum* (strain PM-1; provided by the University of Maryland Center for Environmental and Estuarine Science Horn Point Laboratory, Cambridge MD), a North Carolina *P. minimum* isolate (provided by Patricia A. Tester, NOAA Center for Coastal Fisheries and Habitat Research, Beaufort NC), *Cryptoperidiniopsis* sp. (CCMP 1828; Chesapeake Bay isolate), and *Pfiesteria piscicida* (CCMP 1921; Chesapeake Bay isolate), were also screened for hemolytic activity. The *P. piscicida* isolate used in this study, original designation MMRCC #981020BR01C5, was a gift from Karen Steidinger, Florida Marine Research Institute, and has been maintained on algae since its arrival on 12/17/1998. Additional species tested were the cryptophytes *Rhodomonas* sp. (CCMP 767) and *Storeatula major* (strain g; Chesapeake

Bay isolate) (used in lipid class separation experiments only), two commonly used food sources for heterotrophic and mixotrophic dinoflagellates in this size class (10-20 μm).

Culturing

Karlodinium micrum (CCMP 1974 and 1975) and *Prorocentrum minimum* (strain PM-1 and the North Carolina isolate) all clonal but not axenic, were cultured autotrophically in 12 psu, filtered (0.22 μm), artificial sea water (ASW) (Instant Ocean Brand), with added f/2–Si nutrient mixture (Guillard 1975), and 1.5% soil extract and 0.3% chicken manure extract. Soil and chicken manure extracts were produced by autoclaving (121 °C and 15 psi) either 400 g of air dried soil, not previously exposed to pesticides or fertilizers, or 50 g of air dried chicken manure, in 1L of distilled-deionized (ddi) water for 1 hour. Mixtures were allowed to sediment for 24 hours, decanted, and the supernatant was further centrifuged to remove remaining particulates. The pH of the extract was adjusted to 6.8-7.0, then filter sterilized (0.22 μm) and stored at 4°C. The additional extracts were added to simulate the nutrient rich, i.e. high DON, environment typical of fish farms. In later experiments, aimed at the isolation of toxic compounds, the addition of extracts to culture media was reduced to 1% soil extract only. This reduction had no apparent effect on culture growth but its effect on the overall production of hemolytic substances was not tested. Cultures were maintained at 20 °C, with an alternating 12 hour light / dark cycle with 100-120 μmol m^{-2} s^{-1} of illumination, measured using a Li-COR model LI-250 light meter with a LI-190S Quantum sensor (Li-

COR, inc., Lincoln NE). Several researchers have noted that maximal growth rates of *K. micrum* were achieved at irradiances ca. 120 μmol m^{-2} s^{-1} (Nielsen, 1996; Li et al., 1999).

Pfiesteria piscicida (CCMP 1921) and *Cryptoperidiniopsis* sp. (CCMP 1828) were grown in 15 psu ASW (Instant Ocean Brand), with added f/2-Si nutrient mixture (Guillard 1975), at 20 °C, and 170 μmol m^{-2} s^{-1} illumination, with an alternating 12 hour light / 12 hour dark cycle, using *Rhodomonas* sp. (CCMP 767) as a food source. *P. piscicida* (CCMP 1921) and *Cryptoperidiniopsis* sp. (CCMP 1828) were starved for 48 hours prior to all experiments to reduce the number of food organisms. *Rhodomonas* sp. (CCMP 767) was grown at 32 psu under the same conditions as described for *P. piscicida* and *Cryptoperidinopsis* sp.

Hemolytic Assay

A hemolytic assay based on the lysis of fish erythrocytes was utilized to screen for bioactive materials. Cultures and culture fractions that were positive in the hemolytic assay were tested further using assays for ichthyotoxicity and cytotoxicity.

Erythrocyte suspensions were prepared as described in Edvardsen et al. (1990). Blood was extracted from the caudal vein of rainbow trout (*Oncorhynchus mykis*) provided by the Center of Marine Biotechnology's Aquaculture Research Center. Needles were heparin (Sigma Chemical Co., St. Louis MO) treated and 10 units ml^{-1} of additional heparin was added to whole blood samples to prevent clotting. Erythrocyte suspensions were prepared by washing three times (2500 g for 5 min.) with ice cold buffer [150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4$, and 12.2 mM Tris base]. Buffer pH was adjusted to 7.4 at 10 °C with 1N HCl, then filter sterilized (0.22 μm). After the third wash, cells were stored in the Tris buffer with 3.75 mM $CaCl_2$ at 50% of their original concentration. Suspensions were stored at 4 °C for no longer than 10 days.

Hemolytic assays were performed by diluting test material in Tris buffer + $CaCl_2$ (100 μl total) and adding this to a 5% erythrocyte suspension (100 μl). Assays were run in 96 well, V-bottom, non-treated, polystyrene plates (Corning Inc., Corning NY) sealed with Falcon 3073 pressure sensitive film (Becton Dickinson Labware, Lincoln Park NJ). Assays were incubated on an orbital shaker (80-100 rpm) at 20 °C for 1 hour. Plates were then centrifuged at 2500 g for 5 min. and the supernatant (100 μl) was transferred to another flat bottom 96 well polystyrene plate, where the absorbance of released hemoglobin was read at 540 nm. Saponin (10 μg) (from *Quillaja* bark; Sigma Chemical Co., St. Louis MO) was used as a positive hemolysin control. All treatments were run in quadruplicate.

Ichthyotoxicity Assay

Ichthyotoxicity was evaluated using a static, acute (24 hour), small volume (2 ml), larval fish bioassay. Exposures were performed at 20°C in 24-well non-tissue culture treated polystyrene plates (Becton Dickinson Labware, Franklin Lakes NJ). Two species were utilized for ichthyotoxicity testing, zebrafish (*Danio rerio*) and sheepshead minnows (*Cyprinodon variegatus*), depending on their availability and salinity tolerance, with sheepshead minnows being tolerant to a wider range of salinities, but zebrafish being more readily obtainable in large numbers. Therefore, zebrafish, 5 - < 48 hrs old post-hatch, were used for toxic fraction testing, while sheepshead minnows, 3 - < 24 hours old post-hatch, were used to test whole dinoflagellate cultures. Larvae were not fed prior to or during testing. Previous experiments had shown that, at the biomass / water ratios used for each species, oxygen saturation remained > 60% during the 48 hour exposure, as recommended in the Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians (ASTM, 1992). All treatments were run in triplicate.

Cytotoxicity Assay

Cytotoxicity was assessed using an *in-vitro* toxicology assay kit based on the release of lactate dehydrogenase (LDH) (TOX-7, Sigma Chemical Co., St. Louis, MO). A GH(4)C(1) rat pituitary tumor cell line (ATCC, CCL-82.2) was utilized for the assay. The GH(4)C(1) cell line has previously been shown to be sensitive to several marine algal toxins (Young et al., 1995; Xi et al., 1996; Fairey et al., 1999). Saponin (10 μg) was used as a positive control. The 1 hour assay was run in duplicate, according to the manufacturers instructions.

Toxic Activity of Dinoflagellate Cultures

Hemolytic and ichthyotoxic activity were assayed in both lysed and non-lysed cultures of *Karlodinium micrum* (CCMP 1974) and *Prorocentrum minimum* (strain PM-1). Hemolytic activity alone was assayed in lysed and non-lysed cultures of *K. micrum*

(CCMP 1975), *Pfiesteria piscicida* (CCMP 1921), *Cryptoperidiniopsis* sp. (CCMP 1828), *Rhodomonas* sp. (CCMP 767), and *P. minimum* (North Carolina isolate). All cultures were between 1.5 - 2.5 × 10^5 cells ml^{-1}, with the exception of *K. micrum* (CCMP 1975) (5 × 10^4 cells ml^{-1}) which was assayed immediately upon arrival to test for culturing artifacts. *Rhodomonas* sp. (CCMP 767) cultures were diluted to 15 psu with ddi H_2O. Cultures were lysed through a pulsed sonication (30 sec. on / 30 sec. off), on ice, for 5 minutes using a microtip sonicator (50 Watt, 3 mm tip, 60 amplitude). Cultures were confirmed to be > 70% lysed by using a Coulter Multisizer II particle counter with enumeration of the 7 – 20 µm size fraction using a Coulter Accucomp software package (Coulter Electronics Limited, Miami FL).

Isolation of Toxic Fractions from Karlodinium micrum Cultures

Lipid Class Separation

Total lipids and separated lipid classes were obtained following procedures described in Parrish et al. (1998) and Yongmanitchi and Ward (1992). All solvents used were HPLC grade or equivalent. To obtain the total lipid fraction, cultures of *K. micrum* (CCMP 1974) and *P. minimum* (North Carolina isolate) (ca. 1 × 10^8 total cells ea^{-1}) were filtered onto pre-combusted type GF/F filters (Whatman International Ltd., Maidstone England) and total lipids were extracted through washes (3 × 4 ml ea^{-1}) with 2:1, 1:1, and 1:2 CH_2Cl_2 / MeOH. Each wash consisted of a 30-minute incubation in a Branson 1200 sonicated water bath (Branson Ultrasonics Corp., Danbury CT). Next, to the combined washes (ca. 12 ml total) was added 25% of the volume (ca. 3 ml) of a 0.88% KCl solution and vortexed to mix. The mixture was centrifuged at low speed to separate, and the upper phase was removed and discarded. The lower phase was dried under nitrogen, resuspended in $CHCl_3$ (1 ml), and stored at –20 °C until further separation.

Lipid class separations were performed using disposable silica cartridges (Sep-Pak Plus Silica, Waters Corp., Milford MA). The silica cartridge was attached to a vacuum manifold and equilibrated with MeOH (20 ml) followed by CH_3Cl_2 (2×15 ml). The lipid extract (< 3 mg lipid), in $CHCl_3$, was then loaded onto the column. The elution procedure and expected products, based on the results of Yongmanitchi and Ward (1992) for *Phaeodactylum tricornutum*, were as follows: (i.) 7.5 ml $CHCl_3$ eluting neutral lipids, pigments, chlorophylls, and carotenoids; (ii.) 9 ml $CHCl_3$ / acetone (11:9) eluting monogalactosly-diacylglycerol (MGDG); (iii.) 9 ml $CHCl_3$ / acetone (7:13) eluting digalactosyl-diacylglycerol (DGDG); (iv.) 15 ml $CHCl_3$ / MeOH (7:1) eluting sulfoquinovo-diacylglycerol; (v.) 9 ml $CHCl_3$ / MeOH (7:3) eluting an unknown acyl-lipid; (vi.) 9 ml $CHCl_3$ / MeOH (1:1) eluting phosphatidylcholine (PC), and finally (vii.) 6 ml MeOH eluting lysophospholipid (LC). After elution, each fraction was evaporated to dryness in a Savant SpeedVac AES1010 concentrator (Savant Instrument Inc., Farmingdale NY) at 40 °C, resuspended in MeOH (1 ml), and stored at –80 °C until tested for toxicity.

Hemolytic and ichthyotoxic activity was assayed for in aliquots of the seven lipid classes obtained from *K. micrum* (CCMP 1974) (= 4.78×10^7 cells ml^{-1}) and *P. minimum* (North Carolina isolate) (= 6.70×10^7 cells ml^{-1}). Zebrafish were used for ichthyotoxicity testing of lipid fractions, as previously described, using aerated, reconstituted fresh water (soft) pH 7.3-7.5, hardness 40-48 mg L^{-1} $CaCO_3$, alkalinity 30-35 mg L^{-1} $CaCO_3$ as the diluent (ASTM, 1992). In some cases, lipid samples were concentrated to reduce MeOH additions in the assay to < 1%. In a separate experiment, hemolytic and ichthyotoxic activity was assayed for in aliquots of lipid classes, separated using the above mentioned procedure, from *K. micrum* (CCMP 1974), grown both autotrophically (= 1.55×10^7 cells ml^{-1}) and mixotrophically (= 1.0×10^7 cells ml^{-1}), using the cryptophyte *Storeatula major* (strain g) as the food source. Hemolytic and ichthyotoxic activity was also assayed for in aliquots of separated lipid classes from *S. major* (= 3.44×10^7 cells ml^{-1}).

Isolations from Cells and Culture Filtrates

An exponentially growing culture of *K. micrum* (CCMP 1974) (400 ml; 5.5×10^4 cells ml^{-1}) was filtered (at 15 in.Hg) onto a pre-combusted type GF/F filter (Whatman International Ltd., Maidstone England), and cells were extracted through a 30 second sonication on ice using a microtip sonicator (50 Watt , 3mm tip , amplitude 60) in 70% MeOH (3 × 4 ml). The culture filtrate was saved and stored at −80°C for later analysis. The supernatants from the three sonication steps were combined (12 ml total) and placed in a glass separatory funnel. The sonicated extract was then washed with both hexane (C_6H_{14}) and methylene chloride (CH_2Cl_2) (3 × 12 ml ea^{-1}). Hexane partitioned to the top phase, while methylene chloride partitioned to the bottom. Appropriate washes were combined, evaporated to dryness at 50 °C in a rotavapor (Buchi model R110, Switzerland), and resuspended in methanol (12 ml). The hemolytic activity was measured in the original aq-methanol extract, the hexane extract, the methylene chloride extract, and in the aq-MeOH fraction remaining after the hexane and methylene chloride washes.

The saved culture filtrate (thawed and at room temp.) was passed through a Sep-Pak Plus tC_{18} disposable cartridge (Waters Corp., Milford MA), attached to a vacuum manifold. The column was pre-equilibrated with methanol (20 ml) followed H_2O (20 ml). The cartridge was subsequently eluted with increasing concentrations of MeOH / H_2O as follows: 100% H_2O, 5%, 10%, 20%, 40%, and 100% MeOH (12 ml ea^{-1}). In a second experiment, another *K. micrum* (CCMP 1974) culture (2 L; 3.0×10^4 cells ml^{-1}) was processed, as previously described, and eluted with 40%, 60%, 80%, and 100% MeOH solutions (15 ml ea^{-1}). An aliquot of each elution from the two experiments was diluted back to its original culture concentration (=5.5×10^4 or 3.0×10^4 cells ml^{-1} for the first and second experiments, respectively) and tested for hemolytic activity as previously described.

The hemolytic extracts isolated from both the *K. micrum* cells and from the culture filtrate were dried under N_2 gas, weighed, and the material resuspended in MeOH (500 μl). An aliquot (50 μl) of each suspension was injected onto a LiChroDART 125-4 / RP-8 (5 μm) reversed phase HPLC column (Waters Corp., Milford MA) and eluted with a 95% H_2O / 5% MeOH to 5% H_2O / 95% MeOH linear gradient, over 20 min., at a flow rate of 1 ml $min.^{-1}$ (Hewlett Packard Series 1100 HPLC System, Hewlett Packard Corp., Wilmington DE). Fractions were collected every 0.5 min. and assayed for hemolytic activity. Cytotoxic activity was evaluated, as previously described, for all 80 fractions at a cellular equivalent of 6.0×10^5 cells ml^{-1}. As a negative control, MeOH (50 μl) was injected onto the column and fractions were collected and tested for both hemolytic and cytotoxic activity.

Based on hemolytic and cytotoxic screening, three HPLC fractions, fractions 36, 46, and 47, corresponding to elution times of 18, 23, and 23.5 min, respectively, were further tested for ichthyotoxicity. Ichthyotoxic testing was performed using zebrafish larvae, as previously described, at a cell equivalent of 2.4×10^5 *K. micrum* cells ml^{-1}.

Based on spectral analyses, fractions 46 and 47 were combined, and along with fraction 36, were evaporated to dryness under N_2 gas and weighed. From here on, combined fractions 46/47 will be referred to as KmTx 1, and fraction 36 will be referred to as *p*KmTx 3. In earlier work these two compounds were referred to as toxic fractions Tox A and Tox B, respectively.

LC_{50}'s were calculated from a dilution series of KmTx 1, *p*KmTx 3, saponin, and a sonicated suspension of *K. micrum* (CCMP 1974) (5 ml; 1.2×10^5 cells ml^{-1}). LC_{50} values and ranges were determined by Probit analysis (SPSS Base 10.0, SPSS Inc., Chicago IL).

Dinoflagellate Lysis due to Copper Sulfate and Potassium Permanganate

Isolates of both *Karlodinium micrum* (CCMP 1974) and *Prorocentrum minimum* (strain PM-1) were exposed to either $CuSO_4 \cdot 5\ H_2O$ (0.5, 2, or 8 mg L^{-1} $CuSO_4$) [= 0.2, 0.8, 3.18 mg L^{-1} Cu], or $KMnO_4$ (2, 4, or 16 mg L^{-1}). Mid-range values of $CuSO_4$ and $KMnO_4$ (2 mg L^{-1} and 4 mg L^{-1}, respectively) approximated dosages typically applied at HyRock Fish Farm, while high range values (8 mg L^{-1} $CuSO_4$ and 16 mg L^{-1} $KMnO_4$)

approximated published LC_{50} values for aquaculture species (Tucker, 1987; Reardon and Harrell, 1990). Cell lysis was evaluated using the methods previously described.

Toxic Activity of Copper Sulfate and Potassium Permanganate Treated Dinoflagellate Cultures and Culture Fractions

Karlodinium micrum (CCMP 1974), *Prorocentrum minimum* (strain PM-1), and *Pfiesteria piscicida* (CCMP 1921) cultures ($1.5 - 2.5 \times 10^5$ cells ml^{-1}) were exposed in 6 well polystyrene non-tissue culture treated plates (Becton Dickinson Labware, Franklin Lakes NJ) to either $CuSO_4 \cdot 5\ H_2O$ (2 mg L^{-1} $CuSO_4$) [= 0.8 mg L^{-1} Cu] or $KMnO_4$ (4 mg L^{-1}) and assayed for hemolytic activity. Controls consisted of untreated cultures of the same density. Analysis of variance with Scheffe's F post-hoc test was used to test for statistically significant ($p<0.05$) differences among treatments (StatView 4.5, Abacus Concepts Inc., Berkley CA).

To validate that hemolytic activity observed in Cu treated *K. micrum* cultures was not due to exogenous free Cu, two additional experiments were performed. The first experiment involved the addition of EDTA (2 mM) [as EDTA + 4 Na $\cdot$ 2 H_2O] to 0.9% NaCl solutions containing $CuSO_4 \cdot 5\ H_20$ (0.1, 0.5, 2.5, or 10 mg L^{-1} Cu). Free Cu was measured using the porphyrin method (range 0-210 µg L^{-1}) (HACH, Loveland CO). An accuracy check, according to the manufacturer's recommendations, was within acceptable limits. Each solution, with and without EDTA, was assayed for hemolytic activity. The second experiment involved exposing cultures of *K. micrum* (CCMP 1974) and *P. minimum* (Maryland and North Carolina isolates), all 1.5×10^5 cells ml^{-1}, to

$CuSO_4 \cdot 5\ H_2O$ (2 mg L^{-1} $CuSO_4$) then testing for hemolytic activity with and without the subsequent addition of EDTA (2 mM).

Ichthyotoxic activity was assayed by adding 3 – sheepshead minnow larvae (< 24 hour old post-hatch) to *K. micrum* (CCMP 1974) culture (2 ml; 1.5×10^5 cells ml^{-1}) exposed to the same Cu and $KMnO_4$ treatments described above. This experiment was run in triplicate. For these experiments, 12 psu ASW (Instant Ocean Brand) with added f/2-Si nutrient mixture (Guillard, 1975) and 1.5% soil extract and 0.3% chicken manure extract (alkalinity 75 mg L^{-1} $CaCO_3$) was used as the diluent. Controls were run exposing sheepshead minnow larvae to $CuSO_4$ or $KMnO_4$ alone.

An additional set of experiments was run in which aliquots of both the hemolytic methanolic extracts, isolated from both the *K. micrum* cells and from the culture filtrate, and aliquots of KmTx 1 and *p*KmTx 3, were diluted back to a cellular equivalent of 5.5×10^4 cells ml^{-1} for the methanolic cellular extracts, and 3.0×10^5 cells ml^{-1} for the HPLC fractions, using filter sterilized (0.22 μm) culture media as the diluent, and mixed with either $CuSO_4 \cdot 5\ H_2O$ (2 mg L^{-1} $CuSO_4$), $CuSO_4 \cdot 5\ H_2O$ (2 mg L^{-1} $CuSO_4$) with the subsequent addition of EDTA (2 mM), or $KMnO_4$ (4 mg L^{-1}). Each was then tested again for hemolytic activity as previously described.

RESULTS

Toxic Activity from Dinoflagellate Cultures

Throughout the course of these experiments, hemolytic activity was consistently observed in sonicated cultures of *Karlodinium micrum* (CCMP 1974 and 1975) (0.1-2.5 × 10^5 cells ml^{-1}) [1]. No hemolytic activity was observed in either whole or sonicated cultures of *Prorocentrum minimum* (Maryland and North Carolina isolates), *Cryptoperidiniopsis* sp. (CCMP 1828), *Pfiesteria piscicida* (CCMP 1921), or *Rhodomonas* sp. (CCMP 767), all 1.5 × 10^5 cells ml^{-1}. Hemolytic activity in whole *K. micrum* cultures (non-sonicated) was highly variable, ranging from 0% to >80% lysis of rainbow trout erythrocytes, and did not appear to be correlated exclusively with *K. micrum* cell number (data not shown). Hemolytic activity in sonicated cultures was detectable (>10%) in dilutions equivalent to ca. 5000 cells ml^{-1} (Figure 2.2B).

Ichthyotoxicity (100%, 9 of 9 larvae) was observed in sonicated but not whole cultures of *K. micrum* (CCMP 1974) (1.5 × 10^5 cells ml^{-1}) using the static, acute, 24 hour bioassay with sheepshead minnow (*Cyprinodon variegatus*) larvae. At 5 min. post exposure, effects at the pectoral and caudal fins in sonicated *K. micrum* exposures were noticeable. At 1 hour post-exposure, severe epithelial damage / sloughing was observed, including complete destruction of the pectoral and caudal fins, but heartbeat and peripheral circulation were still present.

---

[1] Filtering small volumes (> 5 ml) of either whole or sonicated *Karlodinium micrum* suspensions through filters composed of nylon (0.22 µm), hydrophobic PTFE (0.22 µm), or glass fibers (0.7 µm) was found to remove or greatly reduce hemolytic activity. Hemolytic activity was still present in supernatants after a 20 min. centrifugation at 16,000 g at 4 °C. Filtering either larger volumes of culture through glass fiber filters (0.7 µm) or any volume of the aq-MeOH C_{18} elutions through either nylon or hydrophobic PTFE membranes (0.22 µm) had little or no affect on hemolytic activity.

Isolation of Toxic Fractions from Karlodinium micrum Cultures

In the lipid class separation from *K. micrum* (CCMP 1974) cells, hemolytic activity (>90%) was observed in aliquots of fraction (vi.) (= 4.78×10^7 cells ml^{-1}) (eluted with 1:1 chloroform / methanol), which co-eluted with phosphatidylcholine (PC) according to Yongmanitchi and Ward (1992). Additional hemolytic activity (ca. 30%) was detected in fraction (vii.) (eluted with 100% methanol), co-eluting with lysophospholipid (LC) (Figure 2.3A). No hemolytic activity was observed in aliquots of separated lipid classes from *P. minimum* (North Carolina isolate) (= 6.7×10^7 cells ml^{-1}).

Ichthyotoxicity testing using zebrafish (*Danio rerio*) larvae showed 100% mortality (15 of 15 larvae) in fraction (vi.) (PC) after 18 hours. Epithelial damage to pectoral and caudal fin tissue was evident at 2 hours post-exposure. No effects were observed in separated lipid class exposures from *P. minimum* (North Carolina isolate).

In aliquots of autotrophic and mixotrophically grown *K. micrum* (CCMP 1974) separated lipid classes (= 1.55×10^7 and 1.0×10^7 cells ml^{-1}, respectively) hemolytic activity was again observed mainly in fraction (vi.) (PC). Some hemolytic activity was also observed in fraction (vii.) (LC) for both autotrophic culture lipids (ca. 60%) and mixotrophic culture lipids (ca. 30%) (Figure 2.3B,C). No hemolytic activity was observed in aliquots of separated lipid classes from *Storeatula major* (strain g) (= 3.44×10^7 cells ml^{-1}) (Figure 2.3D).

No mortality of zebrafish larvae occurred in 48 hours due to exposure to separated lipid classes from *K. micrum* (CCMP 1974) or *S. major* (strain g), in the autotrophic and mixotrophic culture experiments. However, pronounced epithelial damage was observed in 100% (15 larvae) of zebrafish exposed to aliquots of autotrophic and mixotrophic culture-derived fraction (vi.) (PC) (= 1.55×10^7 and 1.0×10^7 cells ml^{-1}, respectively). Similar epithelial effects, although to a lesser degree, were observed in ca. 50% (7 of 15 larvae) of zebrafish exposed to aliquots of fraction (vii.) (LC) from *K. micrum* grown autotrophically only (= 1.55×10^7 cells ml^{-1}). Zebrafish larvae exposed to aliquots of separated lipid classes from *S. major* (= 3.55×10^7 cells ml^{-1}) showed no gross damage to epithelial tissues. All fish possessed a strong heartbeat and visible peripheral circulation during these exposures.

Hemolytic activity was present in the 70% methanol extract of filtered *K. micrum* (CCMP 1974) cells (= 5.5×10^4 cells ml^{-1}). This activity was retained in the aq-MeOH extract, even following hexane or CH_2Cl_2 washes. This activity was also present in the *K. micrum* (CCMP 1974) culture filtrate after passage through the type GF/F filter, but not after passage through the Sep-Pak Plus tC_{18} cartridge. In the first culture filtrate extraction experiment (400 ml ; 5.5×10^4 *K. micrum* cells ml^{-1}) elution from the Sep-Pak Plus tC_{18} cartridge resulted in hemolytic activity only in the final 100% methanol elution, while in the second experiment (2 L; 3.0×10^4 *K. micrum* cells ml^{-1}) hemolytic activity was found only in the 80% methanol elution.

After washing with hexane and CH_2Cl_2, the aq-MeOH extract of cells from a *K. micrum* culture (400 ml; 5.5×10^4 cells ml^{-1}) yielded 6.1 mg of organic material, whereas only 0.7 mg of material was obtained from the culture filtrate. On the other hand, five times more hemolytic activity was present in the extract from the culture filtrate.

Reversed phase HPLC separation and subsequent hemolytic testing of the concentrated extracts from both the cellular and culture filtrate portions of the *K. micrum* (CCMP 1974) cultures yielded hemolytic activity associated with a prominent peak eluting at ca. 23 min. (KmTx 1, formerly Tox A) (Figure 2.4). A second peak in hemolytic activity (*p*KmTx 3, formerly Tox B), eluting at ca. 17.5 min., was found only in the extracts derived from culture filtrates (Figure 2.4). Based on both retention time and UV spectrum data these two fractions appear to contain distinct compounds (Figure 2.5). Additional reversed phase HPLC analysis of the *p*KmTx 3 fraction using a C_{18} column (Altex Ultrasphere - ODS, 5 µm, 4.6 mm ID × 250 mm L; Beckman, Inc.), under the previously described conditions except with a 60 min linear MeOH / H_20 gradient, revealed that this fraction contains as many as four discrete compounds.

Reversed phase HPLC analysis of fractions (vi.) and (vii.) from the lipid class separation experiments from autotrophic and mixotrophically grown *K. micrum* (CCMP 1974) revealed that for both fractions the hemolytic activity was associated with a peak eluting at ca. 23 min. UV spectral analysis of this peak matched that of KmTx 1. According to peak area, the material in this fraction was ca. 100 fold less than the peak obtained from the culture filtrate fractions [290 mAU for fraction (vi.) and 140 mAU for fraction (vii.) compared to 27,000 mAU from the C_{18} extraction of culture filtrates].

Dilution series of sonicated *K. micrum* (CCMP 1974) cultures, as well as hemolytic HPLC fractions, responded in the hemolysis assay in a typical dose dependant manner (Figure 2.2C,D). Probit analysis from these dilution series revealed that the compound eluting at 23 min. (KmTx 1) had an LC_{50} for the lysis of rainbow trout erythrocytes of 284 ng ml^{-1} (range 128 – 744 ng ml^{-1}). Likewise, the compound eluting at 17.5 min. (*p*KmTx 3) had an LC_{50} of 600 ng ml^{-1} (range 287 – 2864 ng ml^{-1}). The LC_{50} for the standard hemolysin saponin was 3203 ng ml^{-1} (range 1836 – 4693 ng ml^{-1}) (Figure 2.2A). Finally, the LC_{50} for sonicated *K. micrum* (CCMP 1974) cell concentration (original culture = 1.2×10^5 cells ml^{-1}) was 2.4×10^4 cells ml^{-1} (range 8.4×10^3 - 7.9×10^4 cells ml^{-1}).

In-vitro cytotoxicity testing of aliquots of the 80 HPLC fractions (= 6.0×10^5 *K. micrum* cells ml^{-1} ea^{-1}) showed toxic activity in fractions 47 (KmTx 1) (equivalent to 2000 ng ml^{-1}) and 36 (*p*KmTx 3) (equivalent to 600 ng ml^{-1}) (Figure 2.6). Ichthyotoxic testing of aliquots of KmTx 1 and *p*KmTx 3 (= 2.4×10^5 cells ml^{-1} ea^{-1}) showed 80% mortality (12 of 15 larvae) of zebrafish after 24 hours due to exposure to KmTx 1 only (800 ng ml^{-1}). *p*KmTx 3 (250 ng ml^{-1}) showed no ichthyotoxic activity. Ichthyotoxic effects did not change after 48 hours of exposure.

Dinoflagellate Lysis Due to Copper Sulfate and Potassium Permanganate

Cell Lysis (range 15% - 90%) occurred in both *Karlodinium micrum* (CCMP 1974) and *Prorocentrum minimum* (strain PM-1) cultures upon exposure to $KMnO_4$ at all concentrations and times tested (Figure 2.7). Cell lysis due to $CuSO_4$ exposure occurred in *K. micrum*, but not in *P. minimum,* cultures at exposures ≥ 2 mg L^{-1} and only after 2 hours (Figure 2.7). Microscopic observation confirmed that copper exposed *K. micrum* cells were swelling and lysing. In both algicidal exposures, swimming dinoflagellates were typically observed after 24 hours in low treatments only (0.5 mg L^{-1} $CuSO_4$ and 2 mg L^{-1} $KMnO_4$).

The Effects of Copper Sulfate and Potassium Permanganate Treatment on the Toxicity of Dinoflagellate Cultures and Culture Fractions

Hemolytic activity was significantly greater in *Karlodinium micrum* (CCMP 1974) cultures (2.5×10^5 cells ml^{-1}) exposed to $CuSO_4$ (2 mg L^{-1}) compared to controls and to cultures exposed to $KMnO_4$ (4 mg L^{-1}) ($p<0.0001$) at 30 min. and 2 hours, but not at 5 min. and 24 hours, using ANOVA with Scheffe's F post-hoc test (Figure 2.8). No hemolytic activity was observed in cultures of *Prorocentrum minimum* (North Carolina and Maryland isolates) or in cultures of *Pfiesteria piscicida* (CCMP 1921), all 1.5×10^5 cells ml^{-1}, exposed to either $CuSO_4$ or $KMnO_4$.

In EDTA / Cu chelation experiments, EDTA (2 mM) was shown to chelate >90% of free Cu (range 90.4% - 96.3%) at all concentrations tested (Table 2.1). The addition of EDTA was shown to completely remove hemolytic activity due to Cu alone up to the highest concentration tested (10 mg L^{-1}). Finally, EDTA did not significantly ($p>0.05$) reduce hemolytic activity in *K. micrum* (CCMP 1974) cultures (1.5×10^5 cells ml^{-1}) exposed to $CuSO_4$ (2 mg L^{-1}).

Ichthyotoxicity to sheepshead minnow larvae was observed at 24 hours post-exposure in $CuSO_4$ treated, but not in $KMnO_4$ treated *K. micrum* (CCMP 1974) cultures (1.5×10^5 cells ml^{-1}) at all concentrations tested (80% mortality, 7 of 9 larvae, in 0.5 mg L^{-1} $CuSO_4$ treatment and 100% mortality, 9 of 9 larvae, in 2 and 8 mg L^{-1} treatments). No mortality or epithelial damage was observed in $CuSO_4$ treated control larvae, but 100% mortality did occur at 24 hours at the highest level of $KMnO_4$ tested (16 mg L^{-1}). Prior to mortality in copper treated *K. micrum* exposures, several sub-lethal effects were observed. As early as 1 hour post-exposure, severe epithelial damage to the sheepshead minnow larvae occurred in *K. micrum* cultures at all levels of $CuSO_4$ tested. These effects included epithelial sloughing, and the complete deterioration of both pectoral and caudal fin tissue. Heartbeat and peripheral circulation were still present at this time. In all $KMnO_4$ control exposures, an orange flocculent was present which increased in abundance with increasing $KMnO_4$ concentration. Except for the 16 mg L^{-1} treatment, this did not result in any mortality. Sheepshead minnow larvae exposed to control (untreated) *K. micrum* cultures showed no mortality or epithelial effects. These results did not change after 96 hours of exposure.

The addition of either $CuSO_4$ (2 mg L^{-1}), $CuSO_4$ (2 mg L^{-1}) with the subsequent addition of EDTA (2 mM), or $KMnO_4$ (4 mg L^{-1}) to hemolytic extracts from both the *K. micrum* (CCMP 1974) cells and culture filtrates, as well as to hemolytic HPLC fractions KmTx 1 and *p*KmTx 3, resulted in the complete disappearance of hemolytic activity in extracts exposed to $KMnO_4$ only. The addition of $CuSO_4$, both with and without the subsequent addition of EDTA, resulted in no significant, ($p>0.05$) change in hemolytic activity compared to untreated hemolytic extracts.

DISCUSSION

Gymnodinium galatheanum Braarud was first described in 1950 in Walvis Bay, South Africa during a large bloom-related fish mortality (Braarud, 1957; Pieterse and Van Der Post, 1967). *Gyrodinium galatheanum* (Braarud) Taylor *sensu* Taylor is synonymous with *Gymnodinium galatheanum* Braarud *sensu* Kite and Dodge and *Gymnodinium micrum* (Leadbeater and Dodge) Loedlich III (Daugbjerg et al. 2000). Based on ultrastructure and molecular data, Daugbjerg et al. (2000) recently redescribed the species as *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen *comb. nov*. Since its original description in 1950, few associations have been made in the literature linking blooms of this organism to negative impacts on aquatic fauna, although, laboratory experiments have shown this organism causes mortality in juvenile cod and reduced growth in mussels (Nielsen and Stromgren, 1991; Nielsen, 1993).

We hypothesize that blooms of the unarmored dinoflagellate *Karlodinium micrum* have in part been responsible for several kills of hybrid striped bass at HyRock Fish Farm since 1996. We have shown that *K. micrum* produces at least one substance which is hemolytic, ichthyotoxic, and cytotoxic. This compound is released in varying amounts into the culture medium but can be released in greater amounts through cell disturbance or disruption. Finally, dinoflagellate cell disruption, leading to release of the compound(s), followed by toxic activity, is promoted through copper sulfate ($CuSO_4$) treatment, while potassium permanganate ($KMnO_4$) treatment causes cell disruption with no subsequent toxic effects (Table 2.2).

Hallegraff (1993) stated, “Aquaculture operations act as sensitive ‘bioassay systems’ for harmful algal species and can bring to light the presence in water bodies of problem organisms not known to exist there before”. Such has proven to be the case with *Karlodinium micrum* in the Chesapeake Bay. Since the first fish kill at HyRock in 1996, several additional reports of aquaculture related fish kills associated with blooms of this organism have surfaced. In 1998, the death of thousands of southern king whiting (*Menticirrhus americanus*) co-occurred with a bloom of *K. micrum* at a farm in South Carolina (A. Lewitus, Belle W. Baruch Institute for Marine Biology and Coastal Research, Charleston SC, personal communication). In 1997, a 30% loss of red drum (*Sciaenops ocellatus*) was associated with a mixed dinoflagellate bloom, including *K. micrum,* at a Texas fish farm (K. Steidinger, Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg FL, personal communication). Before death, fish were observed to be listless and at the water’s surface, with sporadic bursts of agitated activity. Fish showed heavy mucus production from the gills but were found to contain no gill or body parasites. Water quality was reported as normal. The blooms and associated mortality in the pond were also reported as re-occurring.

To our knowledge, only one other report exists in the literature evidencing the direct toxic effects of this dinoflagellate in a laboratory setting. Nielsen (1993) showed that juvenile cod exposed to 1×10^5 cells ml^{-1} of *K. micrum* resulted in death within two days. Further, compared to controls, affected fish were lethargic, appeared to stay at the surface, often with heads out of the water, possessed increased blood osmolarity, and showed an extensive separation of the respiratory epithelium from the underlying pillar cells. Based on nuclear SSU rDNA sequences, the strains of *K. micrum* used in this study (CCMP 1974 and 1975), isolated from the Chesapeake Bay and from HyRock Fish Farm, respectively, were shown to be genetically identical to each other and to differ by only two base pairs from the available European strains (CCMP 415 and 416) as well as from the Braarud strain (Tengs et al., 2001).

Karlodinium micrum has been shown to be an important component of the phytoplankton community in both the Maryland and Virginia portions of the Chesapeake Bay (Marshall, 1999; Li et al., 2000). In the Chesapeake Bay, Li et al. (2000) found that *K. micrum* reached maximum densities ca. 4×10^3 cells ml^{-1} in the main-stem of the mid to upper Bay during late spring and early summer, often dominating the 2-20 μm photosynthetic nanoflagellate community. These densities are below those typically associated with fish mortalities, but surface densities ranging from 6×10^4 to 2×10^5 cells ml^{-1} have been observed at HyRock Fish Farm during blooms both in ponds and in a channel of the Manokin River from which the farm draws water.

As mentioned previously, *Pfiesteria piscicida* was shown to be present at HyRock Fish Farm during the first kill in 1996 (ca. 300 cells ml^{-1}). Because of its common co-occurrence in nature and its similarity in appearance to *P. piscicida* under light microscopic examination, *K. micrum* has been grouped, along with *Cryptoperidiniopsis* sp., into the category of "*Pfiesteria*–like organisms" (PLOs) (Marshall, 1999). *P. piscicida* has been implicated as the causative agent in numerous fish kills in Mid-Atlantic and southeastern U.S. estuaries (see Burkholder and Glasgow, 1997), therefore its involvement in the kills at HyRock cannot be ruled out. In the current study, both *P. piscicida* (CCMP 1921) and *Cryptoperidiniopsis* sp., both grown on algae, were not found to produce any hemolytic substances up to densities of 1.5 × 10^5 cells ml^{-1}. Glasgow et al. (1998) observed that *P. piscicida* does feed on fish erythrocytes, but during the one hour incubation in this study measurable amounts of hemoglobin were not released. These findings still do not rule out the possible involvement of *P. piscicida* in the fish kills at HyRock considering "toxic" and "non-toxic" varieties have been reported (Burkholder et al., 2000; Burkholder et al. 2001). But, considering the repeated associations between high *K. micrum* cell numbers and fish mortality and now the laboratory confirmation of toxic material production with properties consistent with observed effects during kills, *K. micrum* appears to have been a contributing factor in the fish mortalities at HyRock Fish Farm making it a new management concern for the estuarine aquaculture industry.

Several phytoplankton species, responsible for substantial aquacultural related losses, have been shown to kill fish through the production of chemical substances grouped loosely into the category of "hemolysins". Some examples of these include the raphidophyte *Heterosigma akashiwo*, the prymnesiophytes *Chrysochromulina polylepis* and *Prymnesium parvum*, and the dinoflagellates *Gyrodinium auroelum* and *Gyrodinium mikimotoi* (syn. *G. nagasakiense*) (Roberts et al., 1983; Guo et al., 1996; Khan et al., 1997; Johansson and Graneli, 1999). Exposure to high densities (10^4 – 10^5 cells ml^{-1}) of several of these species have been shown to elicit a stereotypical response in fish gills including increased ion permeability, edema, hyperplasia, and epithelial necrosis (Ulitzer and Shilo, 1966; Jones et al., 1982; Roberts et al., 1983; Edvardsen and Paasche, 1998). In the current study, all ichthyotoxic fractions were found to have a generalized necrotic effect on the epidermis of larval zebrafish (*Danio rerio*) and sheepshead minnows

(*Cyprinodon variegatus*), typically starting with the pectoral and caudal fins, but not initially affecting heartbeat or peripheral circulation. More detailed studies on the effects of *K. micrum* and its toxin(s) on fish gills are currently being performed.

In most circumstances, the extraction procedures utilized to assay for hemolytic materials from the aforementioned group of organisms were those that targeted polar-lipids (Ulitzer and Shilo, 1970; Yasumoto et al., 1990; Arzul et al., 1994). For several of the above mentioned species, the hemolytic activity has been attributed to a combination of glycoglycerolipids, lyso-glycoglycerolipids, and free fatty acids, mainly the poly-unsaturated fatty acid octadecapentaenoic acid (18:5n3) (Yasumoto et al., 1990; Parrish et al., 1993; Arzul et al., 1995; Bodennec et al., 1995; Parrish et al., 1998; Fossat et al., 1999; Sola et al., 1999). We have previously shown *K. micrum* contains from 5-15% of its total fatty acids as 18:5n3, depending on culture conditions (Deeds et al., 2000). Therefore, our initial attempt to isolate the material(s) responsible for the observed hemolytic activity in sonicated and copper treated *K. micrum* cultures followed those described for the isolation of hemolytic glycoglycerolipids (Parrish et al., 1998). Our results differed from those of Parrish et al. in that the hemolytic and ichthyotoxic material(s) co-eluted from the silica column with phosphatidylcholine and lysophospholipids and not the 18:5n3 containing glycoglycerolipids monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG) (Figure 2.3). Further, we had previously shown that *Prorocentrum minimum* contained from 6-19% of its total fatty acids as 18:5n3 (Deeds et al., 2000). In the current study, two strains of *P. minimum* were found to contain no hemolytic activity. These facts led us to the conclusion that

18:5n3 containing glycoglycerolipids are likely not responsible for the observed toxic activity in this strain of *K. micrum*.

It was observed that the cellular equivalent of material required to cause lysis of rainbow trout erythrocytes was ca. 100 fold higher using hemolytic materials obtained from the lipid extraction procedure compared to sonicated cell suspensions alone (10^7 compared to mid 10^4-10^5 cells ml^{-1}, respectively). It was later confirmed through reversed phase HPLC analysis that the more prominent of the two toxic fractions, Tox A, was in fact found in ca. 100 fold lower amounts in equivalent injections of extracts obtained through the lipid class separation compared to the C_{18} culture filtrate procedure (based on peak area, 290 mAU from fraction (vi.) (PC) and 140 mAU from fraction (vii.) (LC) compared to 27,000 mAU from the C_{18} extraction of culture filtrates). Therefore, it was determined that extraction procedures targeting cellular lipids, using silica columns, are not the most efficient means of toxin isolation. In the C_{18} column extraction of culture filtrates, five times more hemolytic activity was present in nine times less total material, suggesting that the isolation of toxic materials from the culture filtrate using C_{18} columns is the most efficient method among those tested in this study.

The hemolytic activity in the aq-MeOH extract of *K. micrum* cells did not partition into either hexane or methylene chloride indicating that the toxins behave like polar-lipids. Reversed phase HPLC separation of extracts from both the *K. micrum* cellular fraction and culture filtrates, as well from the hemolytic lipid fractions (vi.) (PC) and (vii.) (LC) from the initial lipid class separation, showed that the main peak in toxic activity corresponded to an elution time of ca. 23 min. (see Figure 2.4). This suggests that the same compound (KmTx 1) was isolated in all three procedures. Using material extracted from the culture filtrate, this peak was further shown to be both ichthyotoxic and cytotoxic. On a per weight basis this compound is 10 times more potent than the standard hemolysin saponin.

A second peak in hemolytic activity (*p*KmTx 3), eluting at ca. 17.5 min., was found in the materials isolated from the culture filtrate only. This peak, containing several discrete compounds, was further shown to be cytotoxic, but not ichthyotoxic, and was 5 times more potent than saponin. This 17.5 min. peak was not associated with any toxic activity in materials isolated from the aq-methanol extraction of *K. micrum* cells or from the separated lipid classes. It is possible that these are merely issues of concentration, but further characterization of both of these described fractions will be necessary to answer this and many other pertinent questions regarding the nature of these toxic compounds. Purification and structural elucidation of these compounds is currently in progress.

Management

Few algicidal treatments are available to the aquaculture industry, due mainly to concerns over both expense and the regulatory issues involved with the use of harmful chemical substances with food fish. In this study, two of the more common treatments for the control of phytoplankton abundance in aquaculture ponds, copper sulfate and potassium permanganate, were examined. Copper sulfate was shown to lyse *K. micrum* cells, within the range of dosages used at HyRock, and in doing so promoted the release of toxic substance(s). Potassium permanganate was shown also to lyse *K. micrum* cells within the range of dosages applied at HyRock, but at the same time, was also shown to remove the toxic activity from whole cultures, from cell and filtrate extracts, as well as from *K. micrum* toxins of KmTx 1 and *p*KmTx 3. Despite the vast difference in treatment cost [Treatment of one-5 acre pond (avg. depth 5 ft.) costs ca. $25.00 vs. $500.00 for copper sulfate and potassium permanganate, respectively (A. Mazzaccaro, HyRock Fish Farm, personal communication)]. based on this data, potassium permanganate is recommended over copper sulfate for the treatment of *K. micrum* blooms in aquaculture facilities (see Chapter 3 for more details).

Conclusions

Aquaculture currently represents about 20% of world fisheries production, and this number is only projected to increase in the future (Botsford et al., 1997; Boyd, 1999). The results of this study have shown that *Karlodinium micrum* produces at least one compound which is hemolytic, ichthyotoxic and cytotoxic. Historically, this organism has not been associated with aquatic faunal mortalities in the main stem of the Chesapeake Bay, though it has been observed in sufficient numbers in mid-salinity tributaries to be a new management concern. The effects of these newly described toxins on mammals and their potential for bioaccumulation in aquatic organisms has yet to be assessed. A better understanding of the ecophysiology of this noxious “weed” species, as well as the harmful compounds it produces, will be essential for the development of better management practices for the mitigation and even prevention of its toxic effects.

Such research will be necessary for estuarine aquaculture to continue to develop in this and similar regions.

ACKNOWLEDGEMENTS

We thank Dr. Eric Lund and Robin Way for performing much of the initial fatty acid analyses on *Karlodinium micrum* and *Prorocentrum minimum*, Dr. Danara Krupatkina for technical assistance in dinoflagellate culturing, and finally, we thank Dr. Anthony Mazzaccaro for both allowing access to HyRock Fish Farm and for his invaluable observations during fish kills over the years. We also thank Dr. Tomas Drgon and Dr. Renate Reimschuessel for critical review of this manuscript. This research project was funded by grants from NIEHS (PO1-ES9563), NOAA ECOHAB (NA860P0492), and NSF (OCE-9819670).

Table 2.1. Copper chelation experiments measuring free copper in solution after the addition of EDTA (2 mM). Measured using porphyrin method, n=2.

CALCULATED AMOUNT (mg L^{-1})	MEASURED AMOUNT (mg L^{-1})				% DECREASE
	Copper		Copper + EDTA (2 mM)		
	AVG	Range	AVG	Range	
0.2	0.197	0.196-0.197	0.0073	0.0071-0.0075	96.3
1	1.034	1.011-1.057	0.042	0.032-0.053	95.9
5	4.957*	4.940-4.975	0.477	0.440-0.515	90.4
20	21.110*	20.760-21.460	1.570	1.560-1.580	92.6

* Indicates presence of hemolytic activity when mixed with an equal volume of a 5% rainbow trout erythrocyte suspension.

Table 2.2. Summary of toxic activity from cultures of *Karlodinium micrum*.

TOXICITY ASSAY	WHOLE CULTURES			HPLC FRACTIONS	
	Sonicated	Cu Treated	$KMnO_4$ Treated	KmTx 1	*p*KmTx 3
Ichthyotoxicity	+	+	-	+	-
Cytotoxicity*	n.a.	n.a.	n.a.	+	+
Hemolytic Activity	+	+	-	+	+

*Cytotoxicity assay was not available for testing whole dinoflagellate cultures due to incompatibilities in culture media requirements between mammalian cells and dinoflagellates.

Figure 2.1. Map of the Chesapeake Bay showing location of HyRock Fish Farm (Latitude 38.1733N, Longitude 75.7374W), positioned on the Manokin River in Princess Ann County, MD, USA.

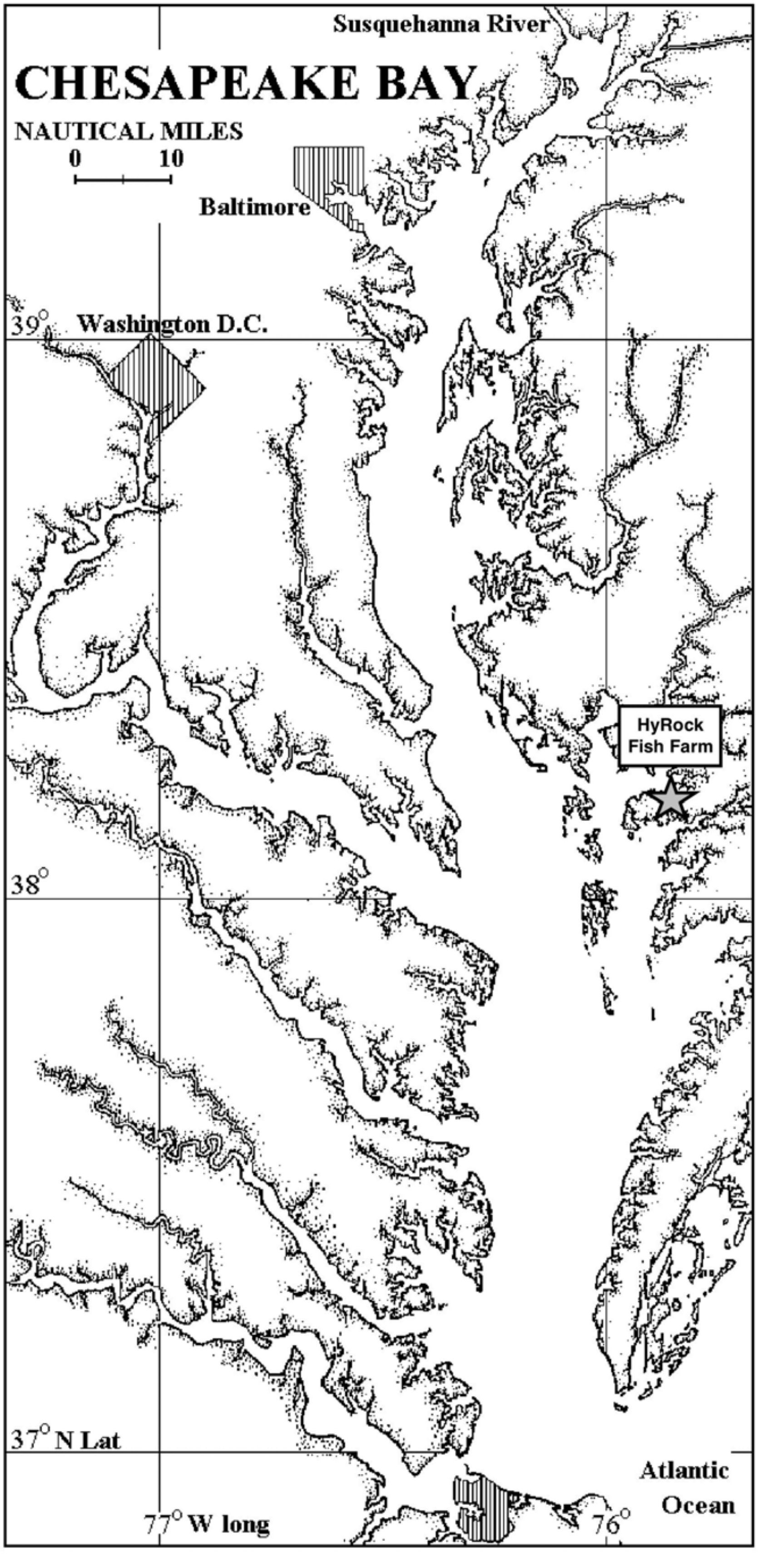

Susquehanna River
CHESAPEAKE BAY
NAUTICAL MILES
0
10
Baltimore
39°
Washington D.C.
HyRock
Fish Farm
38°
37° N Lat
Atlantic
Ocean
77° W long
76°

Figure 2.2. Dose dependence for the lysis of rainbow trout erythrocytes, compared to cells lysed with 10 μg saponin, for [A] The standard hemolysin saponin, [B] A diluted suspension of sonicated *Karlodinium micrum* (CCMP 1974; 1.2×10^5 cells ml^{-1}), [C] Reversed-phase HPLC fraction 46/47 (KmTx 1, formerly Tox A), elution time 23 min., [D] Reversed-phase HPLC fraction 36 (*p*KmTx 3, formerly Tox B), elution time 17.5 min. n=4.

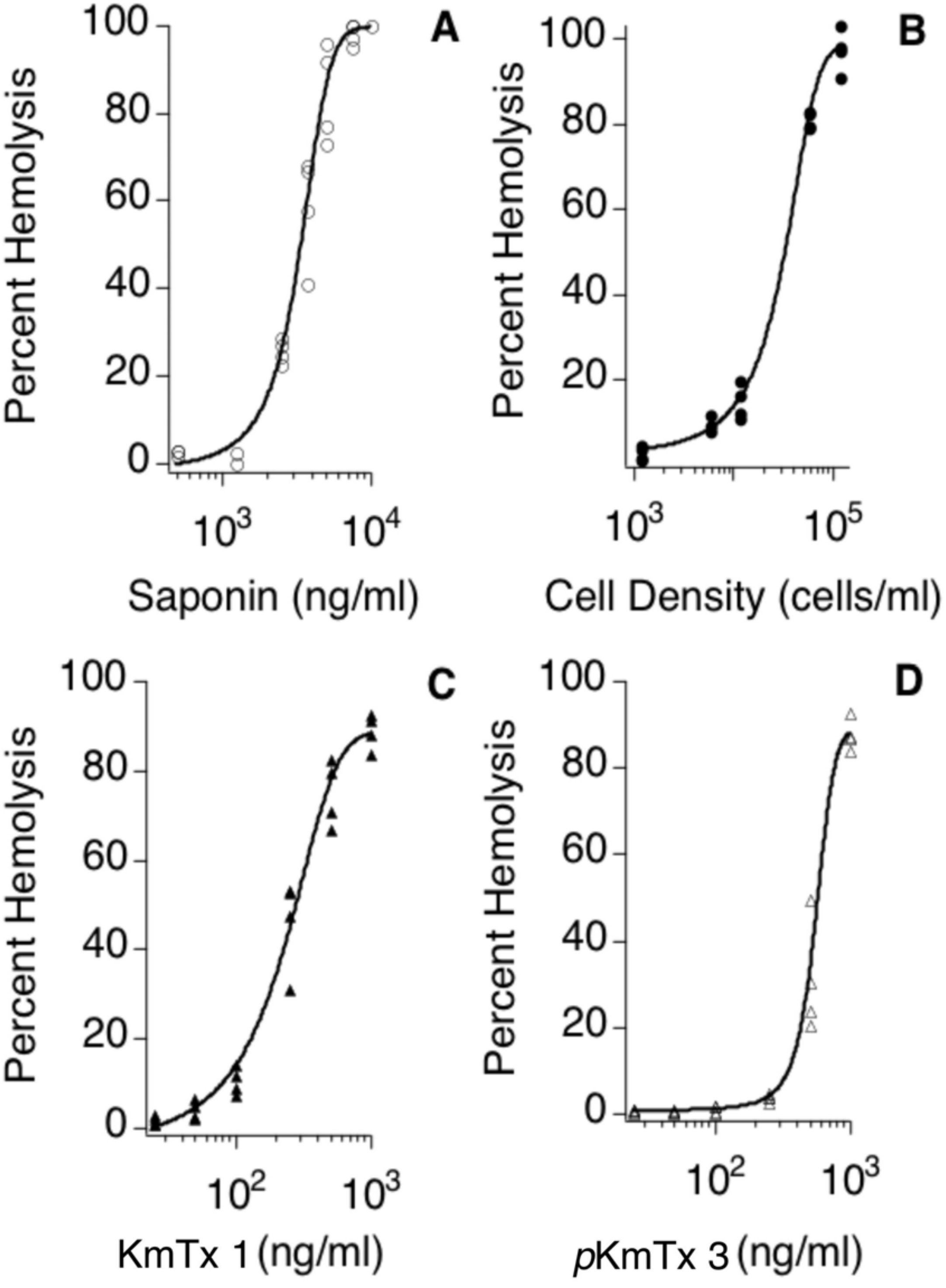

A
B
C
D
Percent Hemolysis
0
20
40
60
80
100
10^3
10^4
Saponin (ng/ml)
10^5
Cell Density (cells/ml)
10^2
KmTx 1 (ng/ml)
*p*KmTx 3 (ng/ml)

Figure 2.3. Percent hemolysis, measured as release of hemoglobin compared to cells lysed with 10 μg saponin, in aliquots of separated lipid classes from *Karlodinium micrum* (CCMP 1974). [A] Grown autotrophically (= 4.78 × 10^7 cells ml^{-1}). [B] Grown autotrophically (= 1.55 × 10^7 cells ml^{-1}). [C] Grown mixotrophically (= 1.0 × 10^7 cells ml^{-1}). [D] Cryptophyte food source *Storeatula major* (strain g) (= 3.44 × 10^7 cells ml^{-1}). According to Yongmanitchi and Ward (1992) fractions included: (i.) neutral lipids, (ii.) monogalactosyl-diacylglycerol (MGDG), (iii.) digalactosyl-diacylglycerol (DGDG), (iv.) sulfoquinovo-diacylglycerol (SQDG), (v.) unknown acyl-lipid, (vi.) phosphatidylcholine (PC), (vii.) lysophospholipid (LC). Control consisted of an equivalent amount of MeOH. Bars represent one standard deviation; n=4 (x) Signifies 100% mortality (15 of 15 larvae) occurred in 48 hour bioassay using zebrafish (*Danio rerio*). (y) Signifies sub-lethal epithelial effects in 100 % (15 of 15 larvae) to zebrafish (*Danio rerio*) in 48 hour bioassay. (z) Signifies sub-lethal epithelial effects in ca. 50% of zebrafish (*Danio rerio*) (7 of 15 larvae) in 48 hour bioassay.

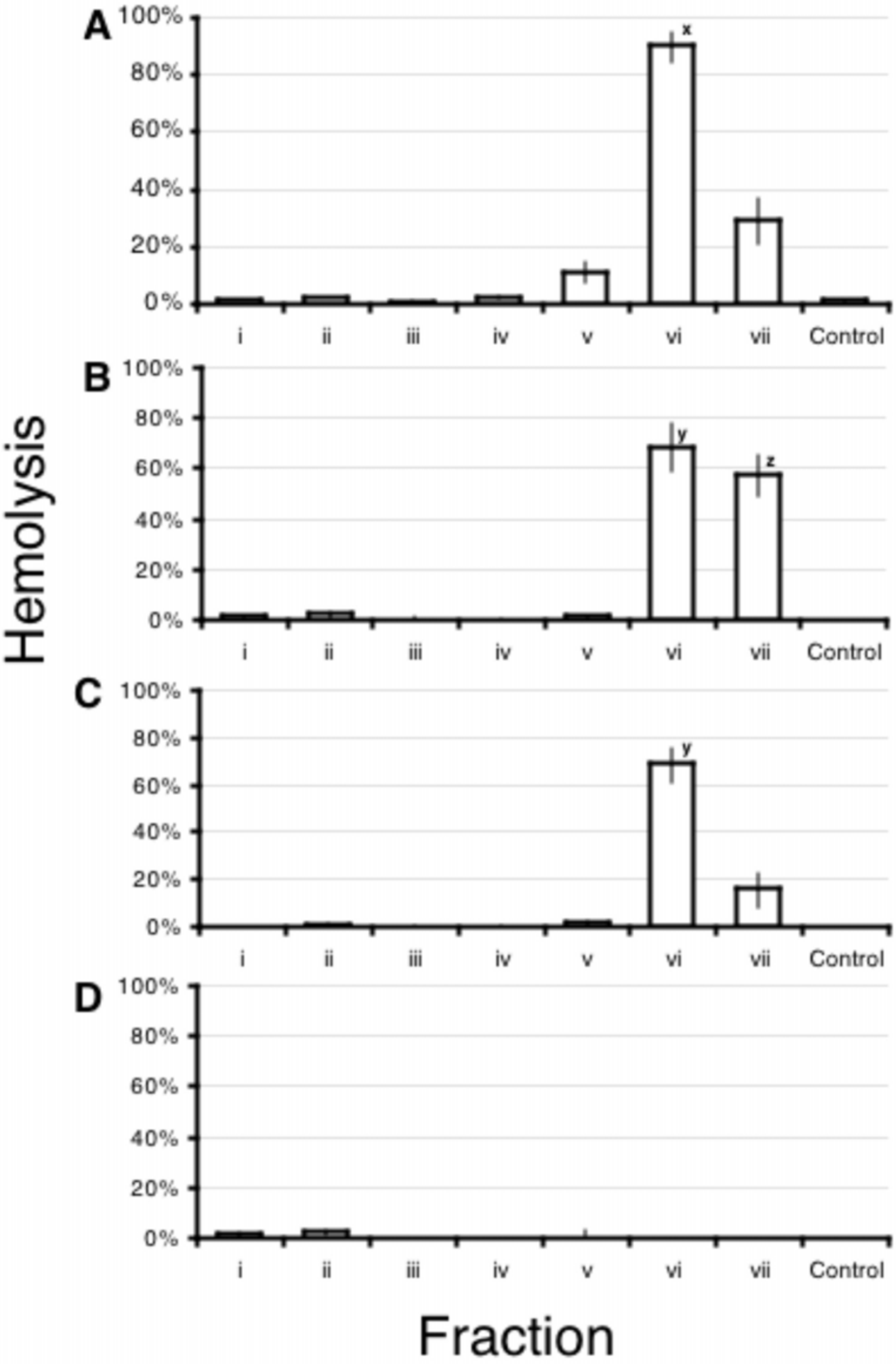

Hemolysis
A
100%
80%
60%
40%
20%
0%
x
i
ii
iii
iv
v
vi
vii
Control
B
100%
80%
60%
40%
20%
0%
y
z
i
ii
iii
iv
v
vi
vii
Control
C
100%
80%
60%
40%
20%
0%
y
i
ii
iii
iv
v
vi
vii
Control
D
100%
80%
60%
40%
20%
0%
i
ii
iii
iv
v
vi
vii
Control
Fraction

Figure 2.4. [A] Reversed phase HPLC elution profile at 230 nm (dotted line) and 254 nm (solid line) for a 50 µl injection of a concentrated 70% MeOH extraction of filtered *Karlodinium micrum* (CCMP 1974) cells (400 ml; 5.5 × 10^4 cells ml^{-1}). [B] Reversed phase HPLC elution profile at 230 nm (dotted line) and 254 nm (solid line) for a 50 µl injection of a concentrated 100% MeOH tC_{18} elution of the culture filtrate from a *Karlodinium micrum* (CCMP 1974) culture (400 ml; 5.5 × 10^4 cells ml^{-1}). For Both: Flow rate of separation was 1 ml min^{-1}. Overlaid histogram (gray bars) is the hemolytic activity, compared to cells lysed with 10 µg saponin, in 0.5 min. collected fractions.

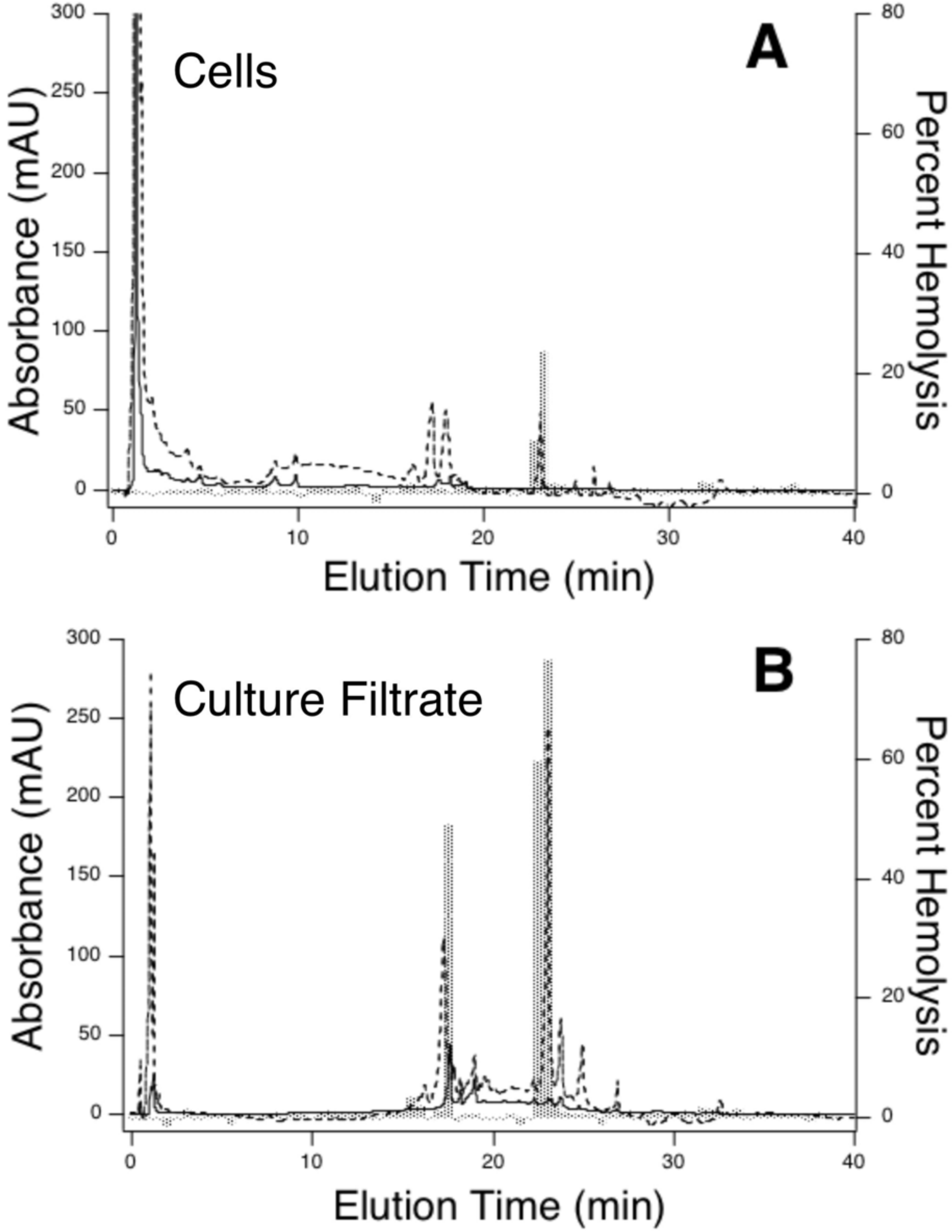
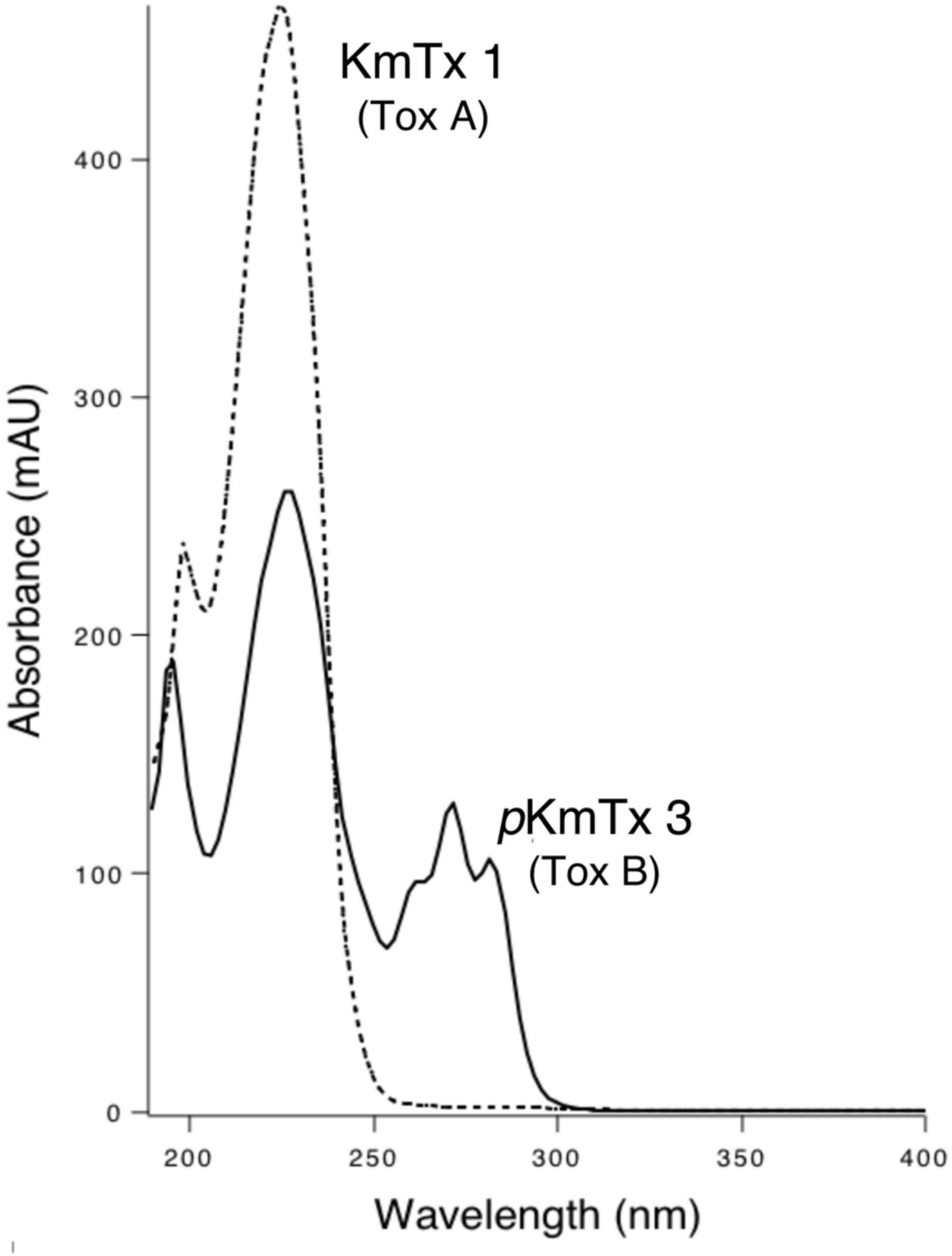

A
Cells
Absorbance (mAU)
300
250
200
150
100
50
0
Percent Hemolysis
80
60
40
20
0
0
10
20
30
40
Elution Time (min)
B
Culture Filtrate
Absorbance (mAU)
300
250
200
150
100
50
0
Percent Hemolysis
80
60
40
20
0
0
10
20
30
40
Elution Time (min)

Figure 2.5. UV absorbance spectra (mAU) of reversed phase HPLC fractions KmTx 1 (formerly Tox A), from elution time 22.5–23.5 min. [dotted line] and *p*KmTx 3 (formerly Tox B), from elution time 17-18 min. [solid line].



KmTx 1
(Tox A)
*p*KmTx 3
(Tox B)
Absorbance (mAU)
0
100
200
300
400
Wavelength (nm)
200
250
300
350
400

Figure 2.6. [A.] Reversed phase HPLC elution profile at 230 nm (dotted line), and 254 nm (solid line) for a 50 µl injection of a concentrated 80% MeOH tC_{18} elution of *Karlodinium micrum* (CCMP 1974) culture filtrate (2 L; 3.0×10^4 cells ml^{-1}). Flow rate of separation was 1 ml min^{-1}. [B.] *In-vitro* cytotoxicity assay, based on the release of lactate dehydrogenase, testing 0.5 min. reversed phase HPLC fractions of a 50 µl injection of a concentrated 80% MeOH tC_{18} elution of *Karlodinium micrum* (CCMP 1974) culture filtrate (2 L; 3.0×10^4 cells ml^{-1}). Cells tested were a GH(4)C(1) rat pituitary tumor cell line (ATCC CCL-82.2). LDH release (histogram) is represented as the percentage of release compared to cells lysed with 10 µg saponin. Bars represent range; n=2.

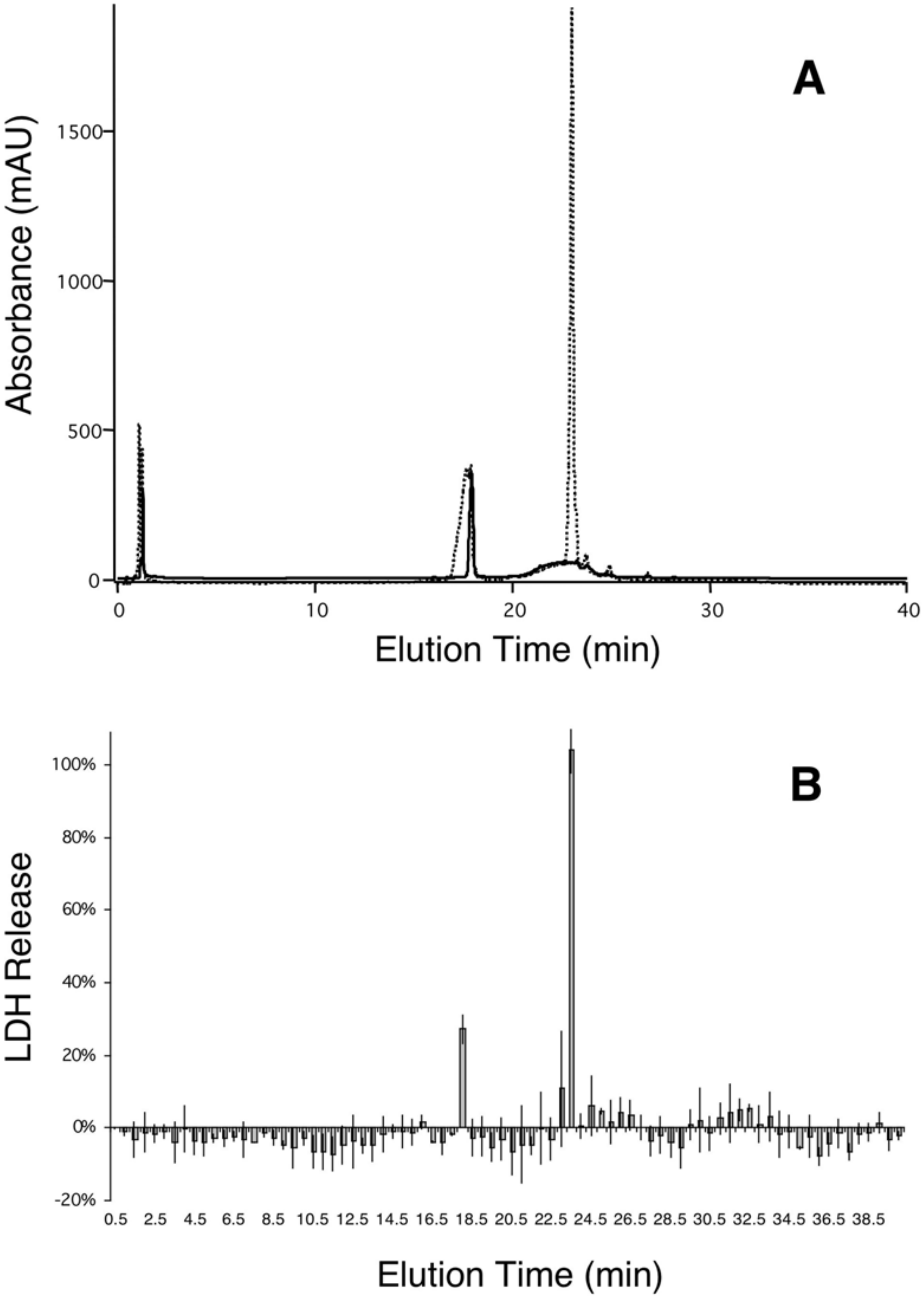

A
Absorbance (mAU)
1500
1000
500
0
0
10
20
30
40
Elution Time (min)
B
LDH Release
100%
80%
60%
40%
20%
0%
-20%
0.5
2.5
4.5
6.5
8.5
10.5
12.5
14.5
16.5
18.5
20.5
22.5
24.5
26.5
28.5
30.5
32.5
34.5
36.5
38.5
Elution Time (min)

Figure 2.7. Cell lysis in *Karlodinium micrum* (CCMP 1974) and *Prorocentrum minimum* (strain PM-1) cultures (3.5-4 × 10^4 cells ml^{-1}) exposed to either $CuSO_4$ (0.5, 2, or 8 mg L^{-1}) [= 0.2, 0.8, or 3.18 mg L^{-1} Cu] or $KMnO_4$ (2, 4, or 16 mg L^{-1}). Mid-range values of $CuSO_4$ and $KMnO_4$ (2 mg L^{-1} and 4 mg L^{-1}, respectively) approximated dosages applied at HyRock fish farm, while upper-range values of $CuSO_4$ and $KMnO_4$ (8 mg L^{-1} and 16 mg L^{-1}, respectively) were similar to published LC_{50} values for aquaculture species under comparable water quality conditions. Cell lysis was determined by enumerating the 7-20 µm size fraction using a Coulter particle counter. Bars represent one standard error; n=3.

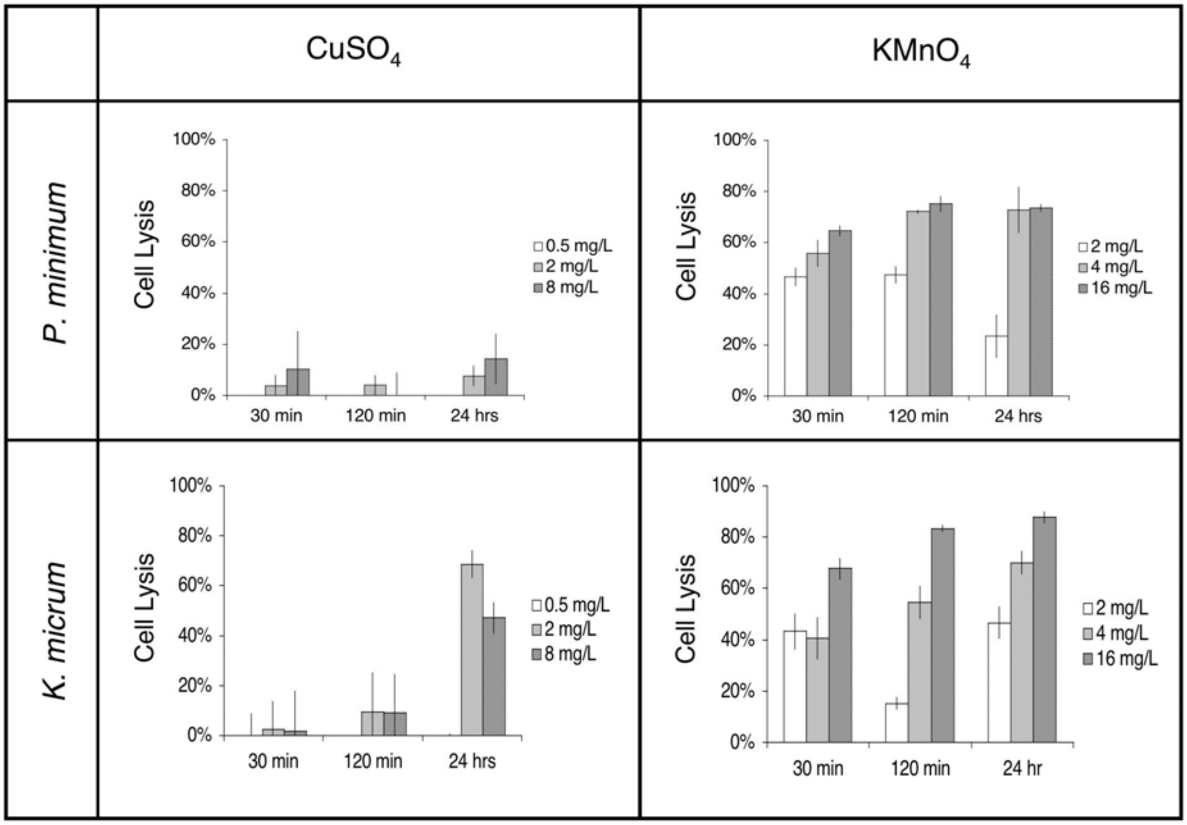
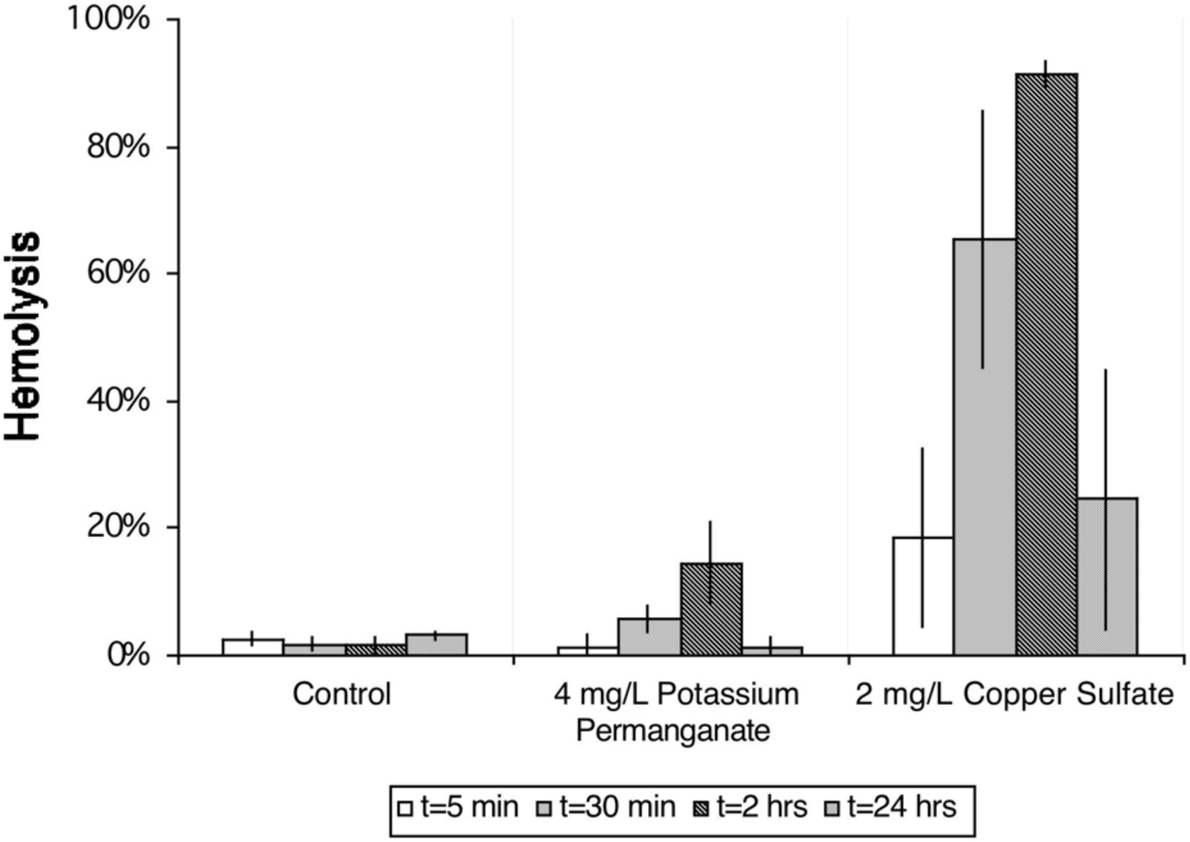

CuSO$_4$
KMnO$_4$
P. minimum
K. micrum
Cell Lysis
100%
80%
60%
40%
20%
0%
30 min
120 min
24 hrs
24 hr
□ 0.5 mg/L
▨ 2 mg/L
▨ 8 mg/L
□ 2 mg/L
▨ 4 mg/L
▨ 16 mg/L

Figure 2.8. Time course of hemolytic material release, measured as release of hemoglobin from rainbow trout erythrocytes, compared to cells lysed with 10 µg saponin, in *Karlodinium micrum* (CCMP 1974) cultures exposed to either $CuSO_4$ (2 mg L^{-1}) [= 0.8 mg L^{-1} Cu] or $KMnO_4$ (4 mg L^{-1}). Cu exposures received EDTA (2 mM) prior to hemolysis testing to remove any hemolytic effects due to free Cu remaining in solution. Final cell concentrations in assay were equivalent to 1.5×10^5 cells ml^{-1}. Bars represent one standard deviation; n=4


100%
80%
60%
40%
20%
0%
Hemolysis
Control
4 mg/L Potassium Permanganate
2 mg/L Copper Sulfate
t=5 min
t=30 min
t=2 hrs
t=24 hrs

CHAPTER 3

Treatment Options for the Control of the Ichthyotoxic Dinoflagellate *Karlodinium micrum* in an Estuarine Aquaculture Facility.[II]

[II] Deeds, J.R., Mazzaccaro, A.P., Terlizzi, D.E., Place, A.R. *Submitted*. Treatment Options for the Control of the Ichthyotoxic Dinoflagellate *Karlodinium micrum* in an Estuarine Aquaculture Facility: A Case Study. Proceedings of the Second International Conference on Harmful Algae Management and Mitigation, Qingdao, China, November 12-16, 2001.

ABSTRACT

Between 1996-1999, three fish kill events have been associated with blooms of the toxic dinoflagellate *Karlodinium micrum* at HyRock Fish Farm, an estuarine pond production facility raising hybrid striped bass along a tributary of the Chesapeake Bay, MD, USA. At HyRock, it was observed that ponds containing blooms of *K. micrum* treated with copper sulfate resulted in greater fish mortality than ponds treated with potassium permanganate. We were able to show that a Chesapeake Bay isolate of *K. micrum* produces several toxic fractions which are released upon cell destruction due to copper sulfate [2 mg L^{-1} $CuSO_4$ caused > 70% cell lysis with a significant ($p<0.0001$) increase in hemolytic activity compared to controls and Cu alone]. Treatment with 4 mg L^{-1} $KMnO_4$ also resulted in > 70% cell lysis but hemolytic activity was significantly reduced [($p<0.0001$) compared to the 2 mg L^{-1} $CuSO_4$ treatment]. The same results were obtained with purified toxic fractions. Copper had no effect on toxicity while potassium permanganate removed it. At HyRock, the addition of an ozone generation system inline with the main estuarine water supply appeared to reduce the occurrence of fish kills associated with *K. micrum* blooms. We showed that a 0.4 mg L^{-1} ozone treatment also caused cell lysis (> 80%) along with the removal of hemolytic activity. It appears that several algicidal treatments will kill *K. micrum*, but only strong oxidizing agents such as potassium permanganate and ozone will also reduce mortality due to its associated toxins.

INTRODUCTION

Opened in 1993, HyRock Fish Farm consists of 37 acres of impoundments supplied with water from the Manokin River, a tidally influenced tributary of the Chesapeake Bay located in Princess Anne, MD, USA. Average salinity of the incoming Manokin river water is 10 psu (range 4.5 – 18 psu). On July 30, 1996 a large mortality of ca. 15,000, 1-1.25 lb. (2.20-2.75 kg) reciprocal cross hybrid striped bass (*Morone saxatilis* male × *Morone chrysops* female) occurred following a copper sulfate treatment (< 2 mg L^{-1}) used to arrest a dense bloom containing 6×10^4 cells ml^{-1} of the toxic dinoflagellate *Karlodinium micrum*. The bloom had been developing for over one week prior to the events of July 30, but no mitigating actions were taken due to the misdiagnosis of the deep mahogany water coloration as tannins. Limited fish mortalities had occurred in the days preceding July 30. To avoid low dissolved oxygen problems, paddle wheel aeration was initiated maintaining dissolved oxygen levels > 5 ppm. Alkalinity was 75 ppm and other water quality conditions immediately prior to the main kill were within normal mid-summer ranges for HyRock Fish Farm (Glibert and Terlizzi, 1999). Foaming and a “petroleum-like” odor were present in the days preceding the large kill. Treatment of a neighboring pond using potassium permanganate (< 4 mg L^{-1}), a strong oxidizing agent, appeared to arrest the bloom without fish mortality. Since that time, two additional fish mortality events have co-occurred with blooms of this organism, 8000 adults on August 13, 1997, and 5000 fingerlings on May 15, 1999. In both circumstances, mortality was stopped following a < 4 mg L^{-1} potassium permanganate treatment, with fish returning to feed in 3-5 days. In an attempt to minimize the continued introduction of bloom-forming dinoflagellate species, common in the Manokin River, an ozone generation system was added in-line with the main water supply in the summer of 1998.

As part of the investigation into the causes of these repeated kills at HyRock fish farm, we eventually discovered that cultures of a Chesapeake Bay isolate of *K. micrum* produce an easily released suite of toxins with hemolytic, cytotoxic, and ichthyotoxic properties (Chapter 1). Similar compounds were later isolated directly from water samples, containing $> 4 \times 10^4$ cells ml^{-1} of *K. micrum*, collected from a South Carolina brackish water retention pond in which a mixed fish kill had recently occurred (Kempton et al., 2002)

The purpose of this report is to chronicle the methods employed at HyRock fish farm to control and/or prevent toxic dinoflagellate blooms, and also to describe a commercial scale ozonation system employed for these same purposes. The events described in this report took place between the years 1993-2000 (the last year fish were commercially raised at the facility). To our knowledge, this is the first report of a commercial scale ozone generation system used to prevent the introduction of bloom-forming dinoflagellates into an estuarine aquaculture facility.

MATERIALS AND METHODS

Facilities

HyRock Fish Farm consists of 5-2.2 acre ea^{-1} fingerling ponds, and 5-5.1 acre ea^{-1} grow-out ponds (avg. depth for all was 4 -5 ft.) (Figure 3.1). Water was supplied from the Manokin River by way of a 25-ft. wide × 8-ft. deep (tidal avg.) × 1600-ft. long channel. Water was delivered to the ponds by a Babcock and Wilcox pump (10-inch line in - 8-inch line out) at a max. rate of 1800 gallons min^{-1}. Individual ponds were filled via a 12-inch main line with 8-inch spurs to each pond (Figure 3.1). Ponds were constructed above ambient water levels so that ponds could be drained by gravity through 10-inch lines into a ditch which flowed into a man-made salt marsh before draining back into the Manokin River. Approximate time to completely replace the water in a pond was 5 days.

To control green algal densities, copper sulfate was occasionally used (Blue Stone, large crystals). Copper was applied by hanging a sufficient amount to achieve 1-2 mg/L in porous bags in front of paddle wheel aerators. After the first kill on July 30, 1996, associated with a dinoflagellate bloom and copper treatment, potassium permanganate was used to control dinoflagellate blooms (CAIROX ZM free-flowing grade, Carus Chemical Company, Peru, IL). A solution of potassium permanganate was hand distributed to achieve a concentration ca. 4 mg/L.

In the summer of 1998 an ozone generation system was installed in-line with the main water supply pump. The system was a PCI-WEDECO model GS-40, water-cooled, coronal discharge ozone generator supplied with pure O_2 that was generated on-site.

Calculated and measured maximum system capacities were 40 lbs of O_3 day^{-1} and 31 lbs. of O_3 day^{-1}, respectively. The system was typically run at 2/3 of capacity giving an ozone output of ca. 20 lbs. of O_3 day^{-1} or an initial aqueous concentration of 1 mg L^{-1} O_3. The 12-inch main line to the ponds acted as the ozone contact chamber. Distance from the point of ozone generation to the first and last ponds was ca. 330 ft. and 1900 ft., respectively.

Algicidal Treatments.

Effect on Dinoflagellate Cell Lysis and Release of Toxic Activity.

To assess the effects of algicidal treatments used at HyRock Fish Farm on cell lysis and the subsequent release of toxic activity, a Chesapeake Bay isolate of *Karlodinium micrum* (CCMP 1974) (4×10^4 cells ml^{-1}) was exposed to either 2 mg L^{-1} $CuSO_4$ (as $CuSO_4 \cdot 5\ H_2O$) [= 0.8 mg L^{-1} Cu], 4 mg L^{-1} $KMnO_4$, or ozone (approx. 0.2, 0.3, or 0.4 mg L^{-1}). Values of $CuSO_4$ and $KMnO_4$ (2 mg L^{-1} and 4 mg L^{-1}, respectively) approximated dosages typically applied at HyRock Fish Farm. Dissolved ozone concentrations were achieved by directly adding O_3 to 100 ml dinoflagellate cultures using a Pacific Ozone Technologies ozone generator (SGA Series). Ozone was delivered using Teflon lined tubing with a course glass diffuser for either 0.5, 1, or 2 min. at a flow rate of 1 SCFH. Ozone dosages (mg L^{-1}) were determined by immediately testing concentrations in culture media controls using a colorimetric ozone test kit (AccuVac, HACH, Loveland CO). Cell lysis was evaluated using a Coulter Multisizer II particle counter with enumeration of the 7 – 20 μm size fraction using a Coulter Accucomp software package (Coulter Electronics Limited, Miami FL). Hemolytic activity remaining in solution was assayed as described previously (see Chapter 1).

Effect on Purified Toxic Fractions.

As part of a previous study (Chapter 1), aliquots of two toxic HPLC fractions (KmTx 1 and *p*KmTx 3), isolated from *K. micrum* (CCMP 1974), were diluted back to a cellular equivalent of 3.0×10^4 cells ml^{-1} and mixed with either 2 mg L^{-1} $CuSO_4$ (as $CuSO_4 \cdot 5\ H_2O$), or 4 mg L^{-1} $KMnO_4$. After a one-hour incubation, each was tested for hemolytic activity as described previously (Chapter 1). EDTA (2 mM) was added after the one hour Cu exposure but prior to hemolytic testing to ensure any hemolytic activity was due to released toxins and not due to exogenous copper. Previous studies had shown that the 2 mM EDTA treatment was sufficient to bind > 90% of free Cu (see Chapter 1). For all of these experiments, dinoflagellate culture media (12 psu artificial sea water, Instant Ocean Brand, alkalinity 75 mg/L $CaCO_3$, with f/2 nutrient mixture and 1% soil extract, preparation described previously (see Chapter 1)) was used for controls and toxin dilutions.

Statistics.

Analysis of variance with Scheffe's F post-hoc test was used to test for statistically significant (p<0.05) differences among treatments (StatView 4.5, Abacus Concepts Inc., Berkley CA).

RESULTS

Treatment with either copper, potassium permanganate, or ozone caused significant lysis of *K. micrum* cells (> 70%) (Figure 3.2A-C). Toxic activity was enhanced only after treatment with copper and eliminated following treatment with potassium permanganate or ozone (minimum residual ozone concentration 0.40 mg L^{-1}) (Figure 3.3A,C). This observation in cultures is consistent with observations made at HyRock Fish Farm where significantly higher mortality was observed following treatment of a *K. micrum* bloom with copper sulfate compared to treatment with potassium permanganate. In laboratory trials, 2 mg L^{-1} $CuSO_4$ had no effect on the hemolytic activity of *K. micrum* toxic HPLC fractions KmTx 1 and *p*KmTx 3, while 4 mg L^{-1} $KMnO_4$ caused the complete elimination of hemolytic activity in both fractions (Figure 3.3B).

DISCUSSION

Few algicidal treatments are available to the aquaculture industry, due mainly to concerns over both expense and the regulatory issues involved with the use of harmful chemical substances with food fish. Two of the more commonly utilized treatments for the control of phytoplankton abundance in aquaculture ponds are copper sulfate ($CuSO_4$) and potassium permanganate ($KMnO_4$).

Costing ca. $25.00 USD to treat one 5-acre pond (avg. depth 5 ft.) (1999-2000 pricing), copper sulfate is one of the most commonly used chemicals for the control of both noxious weed species and infectious diseases in fishponds and hatcheries (Boyd, 1990; Masser, 2000). Application rates for copper sulfate at HyRock typically ranged from 1-2 mg L^{-1}, well below the experimentally determined 96h LC_{50} of ca. 8 mg L^{-1}, determined for striped bass fingerlings at comparable salinities (although the Rearden and Harrell study was performed at higher alkalinities than those typically found at HyRock) (Reardon and Harrell, 1990). Regardless, prior to the events of July 30, 1996, this application dosage had been used at HyRock, at the same alkalinities present during the 1996 fish kill, to control green algal blooms without difficulty.

Potassium permanganate has been used in aquaculture facilities for various reasons ranging from disease and external parasite treatment to the oxidation of organic and inorganic substances to reduce both biological and chemical oxygen demands (Tucker and Boyd, 1977; Tucker, 1987; Tucker, 1989; Boyd, 1990; Noga, 2000). An added benefit of algicidal $KMnO_4$ treatment is that during the reduction of potassium permanganate to manganese dioxide (MnO_2), the manganese dioxide forms a colloid with an outer layer of exposed OH groups. These groups are capable of adsorbing both charged and neutral particles from the water column, thereby further promoting the precipitation of microorganisms (US EPA, 1999). The 96h LC_{50} for potassium permanganate was shown to range from 4.5 to 17.6 mg L^{-1} for channel catfish fingerlings depending on the amount of dissolved organics in the system (Tucker, 1987). Any confined animal feeding operation, HyRock being no exception (see Glibert and Terlizzi, 1999), tends to possess very high organic loads. One would expect this to push the lethal concentration for potassium permanganate towards the upper end of this range. A simple method for determining the required dosage in such an environment is the 15-min. $KMnO_4$ demand in which the concentration of $KMnO_4$ required to color the water for 15 min. is multiplied by 2.5 to determine the application rate (Tucker, 1989). At HyRock, the typical application was < 4 mg L^{-1}, actually below recommended, due mainly to cost constraints. Treatment of one 5-acre pond (avg. depth 5 ft.) costs ca. $500.00 USD (1999-2000 pricing).

Ozone has been used for years as an alternative treatment method in the wastewater and drinking water industries for such varied purposes as bacterial and viral disinfection, oxidation of taste and odor compounds, increased organic biodegradation, and coagulation and filter improvement (for review see United States Environmental Protection Agency Alternative Disinfectants and Oxidants Manual at www.epa.gov/safewater/mdbp/pdf/alter/chapt_3.pdf). More recently, these properties of ozone have begun to be exploited by the aquaculture industry (for review see Timmons et al., 2001). Additional benefits to the use of ozone in aquaculture include the production of oxygen as a byproduct and the oxidation of nitrogenous waste products, particularly nitrite, to the less toxic form nitrate (Colberg and Lingg, 1978). Ozone is a powerful oxidant second only to the hydroxyl free radical among chemicals used in water treatment. Because ozone is very unstable, it must be generated on-site. Aqueous molecular ozone will either react directly with organic or inorganic substrates or will decompose spontaneously generating hydroxyl free radicals which will further react with available substrates. Under certain conditions more stable reactive byproducts can be created. For example, in seawater ozone reacts with bromide ions to produce reactive compounds such as hypobromous acid (HOBr) and hypobromite ions (OBr^-) which are more persistent in solution than the original ozone molecules (Timmons et al, 2001). More research needs to be done to determine the harmful effects of these byproducts and to find reliable methods for the removal of these compounds under varying environmental conditions. At HyRock, adverse effects were never observed following addition of ozonated water. On the contrary, on several occasions reduced parasite loads were observed in ponds that received ozone treated waters, although these observations were never quantified.

The use of ozone has been suggested for use in both the removal of toxin producing dinoflagellates and for the direct destruction of the toxins they produce (Schneider and Rodrick, 1992, Flecher et al. 1998). In this study, exposure to an increasing oxidative residual due to ozone resulted in both the destruction of *K. micrum* cells and the removal of released hemolytic activity due to toxic compounds. However, it should be noted that a minimum residual ozone concentration of 0.4 mg L^{-1} was required to remove hemolytic activity while lower amounts were required for cell lysis and release of hemolytic activity. Therefore, there is a definite danger in under-dosing. This observation will apply to any oxidative compound that is utilized as it reacts with dissolved and particulate organic matter.

Despite the vast difference in treatment cost, based on the data from this and previous studies (see Chapter 1), potassium permanganate is recommended over copper sulfate for the treatment of *K. micrum* blooms in aquaculture facilities. Although the ozone generation system at HyRock was only in operation for a few seasons, ozonation of incoming water did appear to improve water quality and reduce the occurrences of fish mortality due to toxic dinoflagellate blooms. The system as described was only useful in treating incoming "replacement" water and could not be used to treat blooms that developed within the ponds *in-situ*. With minimal engineering, it would have been possible to rout the drainage outlets from the individual ponds back through the pumping station, thereby creating isolated loops to each pond. By doing this it would have been possible to treat individual ponds with ozone when needed.

HyRock Fish Farm stands as an example of the successful use of ozone on a commercial scale to reduce the introduction of harmful algal species from source waters into an estuarine aquaculture facility. Still, at present, ozonation systems are expensive to install and maintain, and are relatively complex. But with increasing limitations on the use of chemicals for food fish, both from a food safety and environmental impact perspective, these technologies appear to be well suited for the aquaculture industry. With an ever-increasing demand for quality seafood coupled with the continuing depletion of natural stocks, hopefully, the demand will also increase for the production of lower-cost and more reliable ozone generation and distribution technologies that will make these treatment options more accessible and economically feasible to the aquaculture industry worldwide.

ACKNOWLEDGEMENTS

This research was funded by grants from NOAA ECOHAB (NA860P0492), the University of Maryland Rural Development Center, and the Maryland Industrial Partnerships Program.

Figure 3.1. Top: Aerial view of HyRock Fish Farm during construction circa. 1993. Bottom: Schematic diagram of HyRock Fish Farm. Ponds 1-5 are 2.1 acres each. Ponds 6-10 are 5.2 acres each. Average depth for both is 4-5 ft. Shaded ponds indicate sites of fish kills that co-occurred with blooms of *Karlodinium micrum*. Pond 6: July 1996, 15,000 adults. Pond 8: August 1997, 8,000 adults. Pond 1 - May 1999, 5,000 fingerlings.

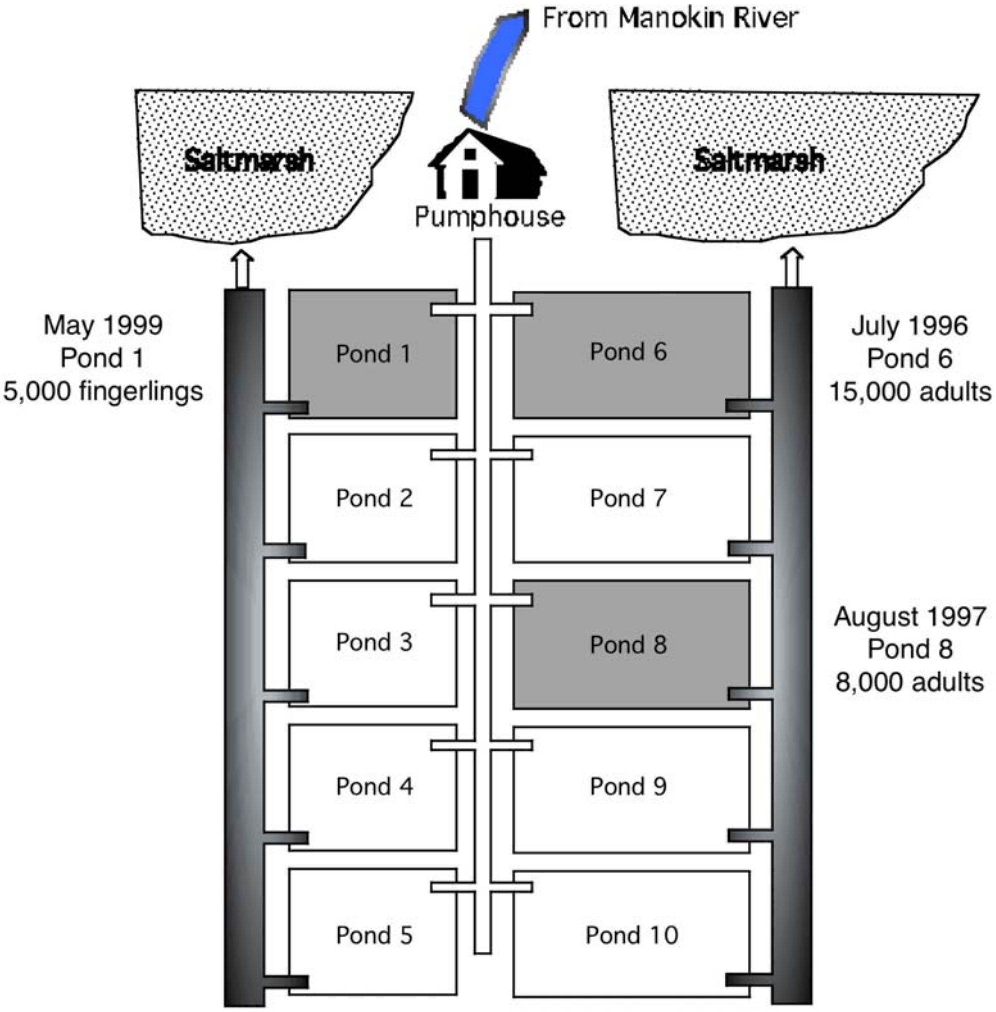

From Manokin River
Saltmarsh
Pumphouse
Saltmarsh
May 1999
Pond 1
5,000 fingerlings
Pond 1
Pond 6
July 1996
Pond 6
15,000 adults
Pond 2
Pond 7
Pond 3
Pond 8
August 1997
Pond 8
8,000 adults
Pond 4
Pond 9
Pond 5
Pond 10

Figure 3.2. Effect of algicidal treatments on cell lysis of *Karlodinium micrum*. [A.] 2 mg L^{-1} copper sulfate. [B.] 4 mg L^{-1} potassium permanganate. [C.] An increasing concentration of ozone delivered by sparging cultures for 0.5, 1, or 2 min at 1 SCFH ozone. Data for A and B are adapted from Chapter 2. Bars represent one standard deviation, n=3.

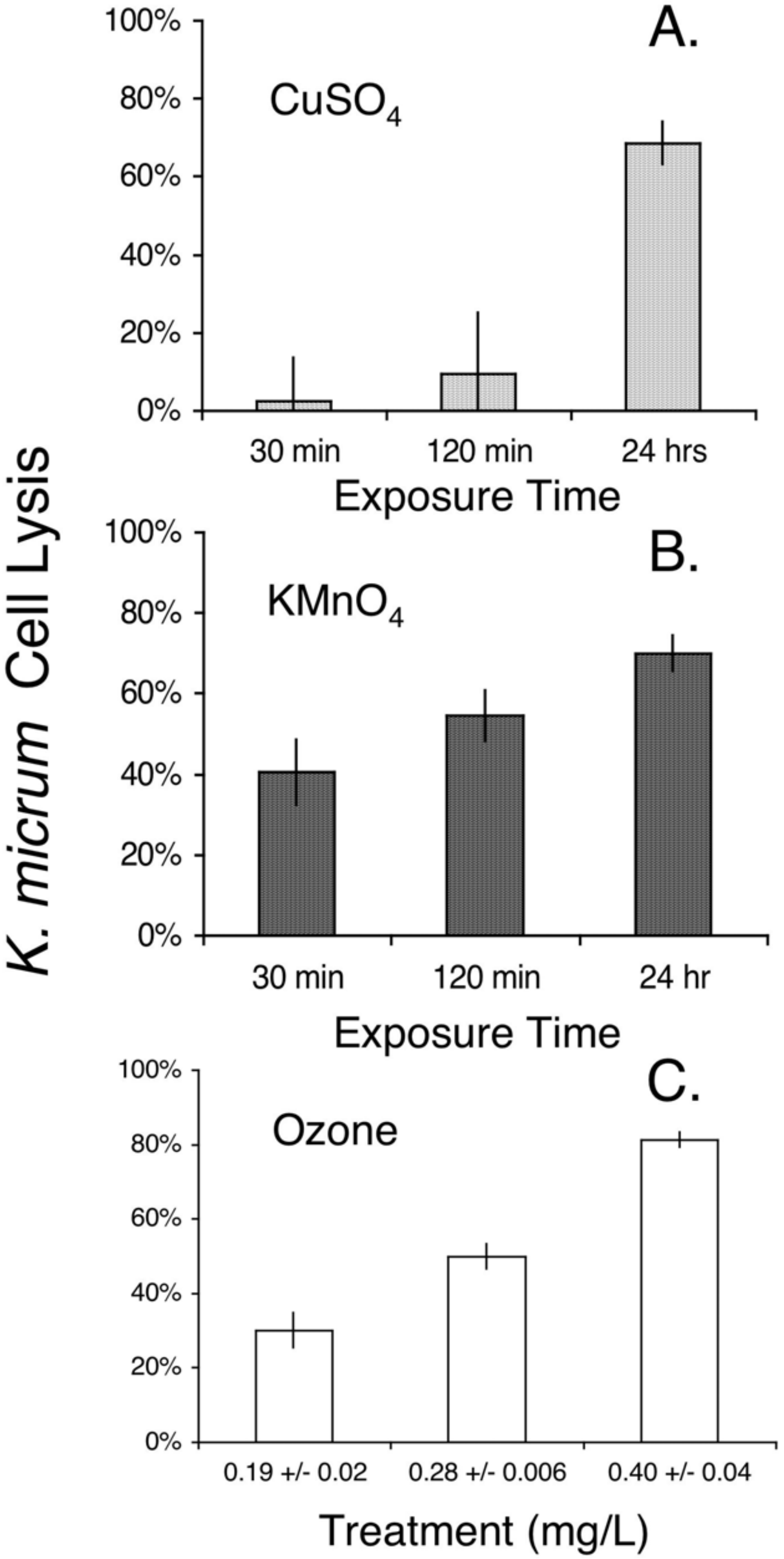
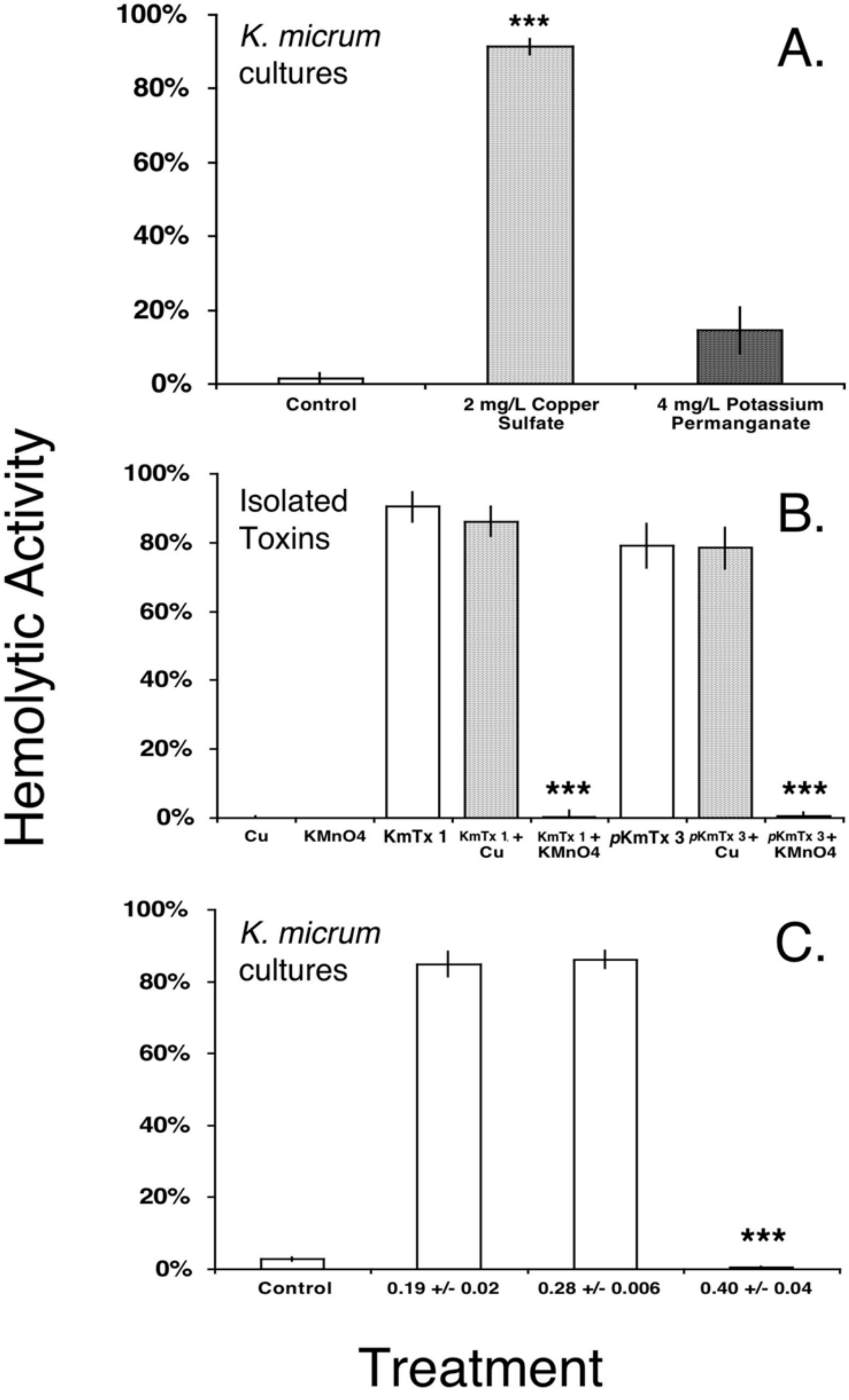

K. micrum Cell Lysis
A.
CuSO$_4$
100%
80%
60%
40%
20%
0%
30 min
120 min
24 hrs
Exposure Time
B.
KMnO$_4$
100%
80%
60%
40%
20%
0%
30 min
120 min
24 hr
Exposure Time
C.
Ozone
100%
80%
60%
40%
20%
0%
0.19 +/- 0.02
0.28 +/- 0.006
0.40 +/- 0.04
Treatment (mg/L)

Figure 3.3. Effect of algicidal treatments on hemolytic activity from both cells and isolated toxins from *Karlodinium micrum*. [A.] Effect of copper sulfate and potassium permanganate on release of hemolytic compounds from *Karlodinium micrum* cells (Controls = no treatment). [B.] Effect of copper sulfate and potassium permanganate on toxic fractions KmTx 1 and *p*KmTx 3 from *Karlodinium micrum*. [C.] Effect of an increasing concentration of ozone on the release of hemolytic compounds from *Karlodinium micrum* cells. Control cultures were sparged with air for 2 min. Data for A and B adapted from Chapter 1. Bars represent one standard deviation, n=4, *** signifies (p<0.0001).


Hemolytic Activity
100%
80%
60%
40%
20%
0%
K. micrum cultures
A.
***
Control
2 mg/L Copper Sulfate
4 mg/L Potassium Permanganate
100%
80%
60%
40%
20%
0%
Isolated Toxins
B.
***
***
Cu
KMnO4
KmTx 1
KmTx 1 + Cu
KmTx 1 + KMnO4
pKmTx 3
pKmTx 3 + Cu
pKmTx 3 + KMnO4
100%
80%
60%
40%
20%
0%
K. micrum cultures
C.
***
Control
0.19 +/- 0.02
0.28 +/- 0.006
0.40 +/- 0.04
Treatment

CHAPTER 4

Mode of Cytotoxicity of KmTx 2, A New Fish Killing Toxin from the Dinoflagellate *Karlodinium micrum*[III]

[III] Deeds, J.R., Hoesch, R.E., Kao, J.P.Y., and Place, A.R. *Submitted.* Cellular mode of action of a new fish-killing toxin from the common estuarine dinoflagellate *Karlodinium micrum*. Nature.

ABSTRACT

The common estuarine dinoflagellate *Karlodinium micrum*, previously reported as non-toxic in the United States, is now shown to be just the opposite. Here we provide a detailed description of the cytotoxic mode of action of KmTx 2, a newly described fish-killing toxin from *K. micrum*. KmTx 2 was first isolated during a fish kill in South Carolina, USA, and has subsequently been identified in US Atlantic coast isolates from North Carolina to Florida. KmTx 2 is toxic to all mammalian cell types tested, including epithelial cells, neurons, fibroblasts, cardiac myocytes, and lymphocytes. Whole-cell voltage-clamp and single-cell microfluorimetry studies revealed that cytotoxicity occurs through permeabilization of the plasma membrane to cation and water fluxes, which results in osmotic cell lysis. This study also reveals mechanisms that underlie the historical association between *K. micrum* blooms and fish kills in the marine environment: KmTx 2 is lethal to zebrafish (*Danio rerio*) at environmentally relevant concentrations (0.1-0.8 μg/ml), while sub-lethal doses severely damage gill epithelia. In addition, KmTx 2 is toxic to representative fungal and dinoflagellate species, but is not toxic to *K. micrum* itself. Membrane sterol composition appears to be critical in determining both cellular susceptibility to KmTx 2 toxicity, and immunity of *K. micrum* from the membrane disrupting properties of its own toxins. In US Atlantic coastal states, *K. micrum* co-occurs with the ichthyocidal dinoflagellate *Pfiesteria piscicida*, but has been reported as non-toxic under putatively ecologically realistic conditions. This study,

in conjunction with recent work from our laboratory, proves that *K. micrum* is far from being benign, and is in fact highly toxic. This study confirms the association between high densities of *K. micrum* and fish kills that has been observed in temperate estuarties around the world for over half a century.

INTRODUCTION

As part of an investigation into the cause of repeated fish kills associated with blooms of *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen (=*Gyrodinium/ Gymnodinium galatheanum* (Braarud) Taylor) (Daugbjerg et al., 2000) that occurred at a Maryland, USA aquaculture facility from 1996-1999, we recently described toxic compounds KmTx 1 (formerly Tox A) and *p*KmTx 3 (formerly Tox B) from Chesapeake Bay cultures of *K. micrum* (Deeds et al., 2002) (Chapter 2). These same compounds were later isolated directly from water collected during a non-aquaculture related fish kill in Maryland in 2002 in which high *K. micrum* densities were present (Goshorn et al., *submitted*). We isolated a similar compound, KmTx 2, both directly from water samples collected during a non-aquaculture related estuarine fish kill near Charleston, South Carolina and later from a *K. micrum* strain (JW020205-B4) isolated from that same kill (Kempton et al., 2002; Qian et al., *submitted*). Subsequently, we confirmed the presence of these compounds in eleven isolates and six natural water samples, both with and without fish kills, some grown both mixotrophically and autotrophically, from Maryland, North Carolina, South Carolina, and Florida. Just recently, we have also isolated a KmTx 2-like compound from an estuarine river experiencing fish kills associated with a persistent bloom of *K. micrum* in Western Australia (Deeds et al., *submitted*; Chapter 6; Chapter 6 addendum). In the Deeds et al. (*submitted*) study, we confirmed an earlier observation that an apparent geographic strain variation in toxin type exists between Chesapeake Bay, MD isolates and isolates collected from estuaries located South of the Chesapeake Bay. KmTx 1 appears to be produced only by Maryland *K. micrum* populations while populations from estuaries located South of the Chesapeake Bay appear to produce only KmTx 2.

This study was undertaken for several reasons: 1. To determine the cellular mode of bioactivity of the newly described toxins from *Karlodinium micrum* , 2. To confirm the role of *K. micrum* in fish kills that has been reported as associations in temperate estuaries around the world since the 1950's (Braarud, 1957; Nielsen, 1996; Landsberg, 2002), and 3. To show that *Karlodinium micrum*, which had previously been reported as non-toxic in the United States, actually is highly toxic. This study focused on the biological activities of KmTx 2, previously isolated from North Carolina, South Carolina, and Florida isolates of *K. micrum*.

MATERIALS AND METHODS

KmTx 2 Isolation and Characterization

KmTx 2 used in this study was isolated directly from water collected during a fish kill in a South Carolina brackish pond described in Kempton et al. (2002). Previously frozen and thawed water samples (1.6L total) were first passed through type GF/F filters (Whatman International Ltd., Maidstone, England), then lipophilic materials were isolated by passing the filtrates through several small (3 ml) disposable C_{18} cartridges (Sep-Pak Plus tC_{18}, Waters Corporation, Milford, MA). C_{18} cartridges were first pre-equilibrated with methanol (MeOH) then water (20 ml ea^{-1}). After 40% and 60% MeOH washing steps (15 ml ea^{-1}), hemolytic materials eluted from the cartridges with 80% MeOH (15 ml). The hemolytic 80% MeOH fraction was dried under N_2, re-suspended in a small volume of MeOH and fractionated further using HPLC. Aliquots were injected onto a LiChroDART 125-4/RP8 (5 μm) reversed-phase column (Waters Corporation, Milford, MA) and eluted at 30 °C with a MeOH/H_2O (30:70) to (95:5) linear gradient, over 20 min, at a flow rate of 1 ml/min (Hewlett Packard Series 1100 HPLC System, Agilent Technologies, Inc. Wilmington, DE). Fractions were collected every 0.5 min and assayed for hemolytic activity as previously described (see Chapter 2). This particular sample contained only one hemolytic peak. Molecular weight of this peak was determined through liquid chromatography / mass spectroscopy (LC/MS) analysis. To first confirm sample purity a 0.5 μg sample was separated on a Zorbax 2.1×50mm SB-C8 column using an Agilent 1100 series HPLC system (Agilent Technologies, Wilmington, DE), eluted at 30 °C with a MeOH/H_2O (60:80) to (80:60) linear gradient, over 6 min, at a flow rate of 0.7 ml/min (Figure 4.1A). Both MeOH and H_2O contained 0.1% formic acid to facilitate sample ionization. Samples were then analyzed both in the positive and negative modes using an Agilent G1946D single quad mass spectrometer detector (Agilent Technologies, Inc., Wilmington DE). Positive ionization yielded several prominent peaks in the 1300-1400 Da range. In the positive ionization mode, the most abundant peak was at 1345.8 Da $(M+H)^+$, followed in abundance by peaks at 1361.8 $[(M+H)^+ +16]$, 1367.8 $(M+Na)^+$, and 1383.8 $[(M+Na)^+ +16]$. Previous attempts at mass determination, all in the positive ionization mode, yielded a mass ion of 1367.8 Da $(M+Na)^+$ (data not shown). Negative ionization yielded only prominent peaks at 1343.8 $(M-H)^+$ and 1359.8 Da $[(M-H)^+ +16]$ (Figure 4.1B). These data led us to the conclusion that the molecular weight of KmTx 2 was 1344.8 Da. (+/- 0.13 - published sensitivity of mass detector), and the molecule had a tendency to form Na adducts in the positive ionization mode. Several, less abundant, peaks were also in the samples, differing from the molecular ion by multiples of 16 Da, suggesting a difference of one or more oxygen atoms, possibly additional hydroxyl groups. Similar methods utilizing LC/MS analysis were used to determine the molecular weight of KmTx 1 at 1338.8 Da. (Jeffrey L.C. Wright, University of North Carolina at Wilmington, personal communication). KmTx 1 was also found to form Na adducts in the positive ionization mode and also contained similar additional peaks differing from the mass ion by multiples of 16 Da.

Hemolysis Assays.

Hemolytic activity was assessed through the use of a microtiter assay utilizing rainbow trout erythrocytes that is proportional to the amount of toxin present. The details of this assay have been described previously (see Chapter 2).

Ion Permeation Measurements

The following cell types were tested for toxicity and increased membrane permeability due to exposure to KmTx 2; rat embryonic fibroblasts (REF 52), rat intestinal epithelial cells (IEC-6), acutely dissociated rabbit vagal sensory neurons, human T-lymphocytes (Jurkat), and acutely dissociated rat ventricular cardiac myocytes. At the start of an experiment, trypsinized cells were typically inoculated (to ca. 30%

cover) on 25-mm round No. 1 glass coverslips (Fisher Scientific, Newark, DE) coated with poly-D-lysine (0.1 mg/mL; M.W. 30 – 70 kD, Sigma, St. Louis, MO). Coverslips were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum in humidified 5% CO_2/95% air at 37°C. After 24 hours, or until cell confluence, cells were loaded with the intracellular fluorescent Ca^{2+} indicator Fura-2 (Molecular Probes Inc., Eugene OR) by incubation with the acetoxymethyl (AM) ester of the indicator for > 60 min. Cells were examined on an inverted epifluorescence microscope (model Diaphot; 40X CF Fluor objective, N.A. 1.30; Nikon Corp.) coupled to a spectrofluorometer (model CM1T10I, SPEX Industries) operating in the microfluorometry mode. Cells were bathed in 4 ml of DMEM buffered with HEPES (pH 7.4). KmTx 2 (stock solution in DMSO) was directly bath-applied with gentle convective mixing. Fura-2 was alternately excited at 340 and 380 nm. Fluorescence emission was passed through a 510-nm bandpass filter before photometric quantitation. DATAMAX software (SPEX Industries) was used for data acquisition and instrument control. Origin software (OriginLab Corp.) was used for data reduction and analysis. Data reduction and analysis were performed as previously described (Kao, 1994). For measurement of Na^+ fluxes the Na^+ specific fluorescent indicator SBFI (AM) (Molecular Probes, Eugene OR) was used. Methods were as described for fura-2.

Electrophysiological Measurements

Cell Isolation

Male New Zealand White rabbits, weighing 3 – 4 kg, were purchased from RSI Biotechnology (Clemmons, NC) and killed by sodium pentobarbital overdose (100 mg/kg), as approved by the Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute. Dissociated nodose ganglion neurons (NGNs) were prepared as described in Leal-Cardoso et al. (1993) with the exception that sterile technique was used and the final neuronal pellet was resuspended in Leibovitz L-15 medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS), and 0.1% by volume penicillin-streptomycin (10,000 units penicillin G and 10,000 μg/ml streptomycin sulfate; Gibco-BRL, Grand Island, NY). The resulting cell suspension was then plated in 0.2-mL aliquots onto 25-mm round No. 1 glass coverslips (Fisher Scientific, Newark, DE) coated with poly-D-lysine (0.1 mg/mL; M.W. 30 – 70 kD, Sigma, St. Louis, MO). NGNs were incubated at 37°C for 12 h, then maintained at room temperature to prevent neurite outgrowth, and used for experiments for up to 48 h.

Extracellular Solution and Superfusion

Neurons were superfused with physiological saline solution (20-24°C) that contained (mM): 120 NaCl, 3.0 KCl, 1.0 NaH_2PO_4, 25.0 $NaHCO_3$, 1.5 $MgCl_2$, 2.2 $CaCl_2$, and 10.0 dextrose, equilibrated with 95%O_2-5%CO_2; pH adjusted to 7.4.

A recording chamber with a narrow rectangular flow path allowed superfusion of NGNs on a glass coverslip at 7 ml/min via a gravity flow system. The chamber was mounted on an inverted microscope (Diaphot, Nikon, Melville, NY) equipped with a X40 phase-contrast oil-immersion objective (Fluor, N.A. 1.3, Nikon) to allow fluorescence measurements and direct visualization of NGNs for positioning patch pipettes. Solution changes were complete in 14 sec, as determined with fluorescent tracers. For toxin exposures, KmTx 2 (1 μg/ml) was applied by superfusion to NGN's. Through the use of fluorescent tracers, it was determined that toxin exposure lasted ca. 60 sec.

Intracellular Solution

The patch pipettes were filled with intracellular solutions containing (mM): 152 KCH_3SO_3, 10.0 HEPES, 2.0 $MgCl_2$, 1.0 Na_3ATP, 1.0 Na_3GTP, and 1.0 KCl; pH adjusted with KOH to 7.2. KCH_3SO_3 was used to avoid excess intracellular Cl^-, which is known to inhibit G proteins (Lenz et al., 1997). Aliquots of stock pipette solution were stored frozen at 0^oC. Each aliquot of pipette solution was thawed, stored on ice, and used for only one day. K_5Fura-2 (Molecular Probes, Inc., Eugene OR) was added to the pipette solution to a final concentration of 50 μM; sufficient $CaCl_2$ was added to set free $[Ca^{2+}]$ = 100 nM (taking the Ca^{2+} dissociation constant of fura-2 under physiological conditions to be 224 nM (Grynkiewicz et al., 1985).

Patch-Clamp Recording

The whole-cell configuration of the patch clamp technique (Hamill et al., 1981) was used to measure membrane currents. Patch pipettes (2 – 3 MΩ resistance), fabricated from borosilicate glass stock (O.D. 1.5 mm, I.D. 1.12 mM, World Precision Instruments, Sarasota, FL) on a Flaming-Brown P97 micropipette puller (Sutter Instruments, Novato, CA) were connected to an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Data acquisition through the Digidata 1200 interface was controlled with pClamp 8 software (Axon Instruments). NGNs were first loaded with fura-2/AM to allow fluorimetric measurement of $[Ca^{2+}]_i$ in parallel with electrophysiological recording. After a gigaohm seal (>1.0 GΩ) was formed, the whole-cell configuration was established, with neurons voltage-clamped to -50 mV. Membrane input resistance and capacitance were determined from current transients elicited by 5-mV depolarizing voltage steps from the holding potential. NGNs were considered suitable for study if membrane input resistance was >150 MΩ and holding current was <200 pA.

Determination of I-V Relationship

To determine I-V relations, the following three-phase voltage command protocol was applied to voltage-clamped NGNs: 1) From a holding potential of –50 mV, the membrane potential was stepped to +50 mV for 100 msec to inactivate voltage-gated Na^+ channels; 2) An I-V relation was then generated by a voltage ramp that decreased from +70 mV to –110 mV at 1.125 mV/msec; 3) At the end of the ramp, the cell was returned to a holding potential of –50 mV. This protocol was applied twice to each NGN tested: first immediately before toxin application, and again 90 sec after a 60-sec toxin application by superfusion, when the toxin-induced current had developed substantially (as determined by the previous patch-clamp experiments). Taking the difference between these two I-V relations yielded the I-V relation for the toxin-induced current.

Osmotic Protection Assays.

The following osmolytes (all purchased from Sigma-Aldrich Co., St Louis, MO) were prepared as 30 mM solutions using the Tris buffer + $CaCl_2$ (described in Chapter 2): sucrose (MW 342.3), polyethylene glycol (PEG) (MW 400), polyethylene glycol (MW 600), maltohexaose (MW 990.0), polyethylene glycol (MW 8,000), dextran (MW 10,000). Osmolarity of each solution was measured using a Vapro 5520 vapor pressure osmometer (Wescor Inc., Logan Utah). Osmolarity of all solutions, with the exception of PEG 8,000 and 10,000 MW dextran, did not differ significantly from the Tris buffer (300-320 mOsm). The osmolarities of PEG 8,000 and 10,000 MW dextran were approximately double this amount (ca. 640 mOsm).

Assays were performed by preparing trout erythrocyte suspensions and toxin dilutions (0, 0.25 0.5, and 1 μg/ml, in triplicate) in the appropriate osmolyte solution and performing the hemolysis assay as previously described (see Chapter 2).

Effect of KmTx 2 on Additional Eukaryotic Cell Types

To assess the effects of KmTx 2 on additional eukaryotic cell types, growth inhibition and cytotoxicity assays were performed, respectively, on model yeast and dinoflagellate species. *Aspergillis niger* was chosen as a representative species of filamentous fungi, while *Candida albicans* was chosen as a representative species of yeast. To test the hypothesis that KmTx 2 functions as an anti-grazing compound, *Oxyrrhis marina*, a co-occurring, similarly sized, potential grazer, as well as a KmTx 2 producing South Carolina *K. micrum* isolate were exposed to a range of KmTx 2 concentrations.

Anti-Fungal Assays

The following fungal strains were purchased from the American Type Culture Collection (Manassas, Virginia), *Aspergillus niger* (ATCC 1004) as a representative filamentous fungi, and *Candida albicans* (ATCC 14053) as a representative yeast. Assays were performed according to the following National Committee for Clinical Laboratory Standards (NCCLS) documents; M27-A2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard – Second Edition (NCCLS, 2002a), and M38-A Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard (NCCLS, 2002b). Procedures for the broth microdilution modification were followed for both species with the following exceptions; *A. niger* stocks were maintained on Potato Dextrose Agar at 25

°C, while *C. albicans* was maintained on Yeast Malt Agar at 28 °C, both according to ATCC recommendations. Amphotericin B was used as a positive control. Microdilution assays were performed in HyQ-RPMI-1640 media (HyClone, Logan, UT), with 2.05 mM L-glutamine, without sodium bicarbonate, pH 7.2 using dilutions of 1 mg/ml DMSO stocks of KmTx 2 and amphotericin B. The following concentrations were tested for both KmTx2 and amphotericin B: 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 μg/ml. A maximum of 1.6% DMSO was added to any given well. Plates were incubated at 25 and 28 °C, respectively, for *A. niger* and *C. albicans*. Plates were checked daily for growth and minimal inhibitory concentrations (MIC) were defined as the lowest dose at which no growth was observed after 96 hours. Amphotericin B and all culture media were purchased from Sigma-Aldrich (St. Louis, MO).

Hemolytic LC_{50} values were calculated from a dilution series of KmTx 2 and amphotericin B. LC_{50} values and ranges were determined by Probit analysis using a SPSS Base 10.0 statistical software package (SPSS Inc., Chicago, IL).

Effect of KmTx 2 on a Potential Grazer

Oxyrrhis marina (1.7×10^4 cells/ml) and *K. micrum* (CCMP 2282) (3.5×10^4 cells/ml) were exposed, in triplicate, to 0, 0.1, 0.5, and 1 μg/ml KmTx 2 in six well non-tissue culture treated polystyrene plates (Becton Dickinson Labware, Lincoln Park NJ). The *O. marina* culture was obtained through single cell isolation by Dr. Danara Krupatkina (Center of Marine Biotechnology, Baltimore MD) from a Chesapeake Bay water sample and was maintained in 15 psu artificial sea water (Instant Ocean Brand) using *Rhodomonas* sp. (CCMP 767) as a food source. *O. marina* was starved for 24 hours prior to exposures to reduce the number of food organisms. *K. micrum* (CCMP 2282) was maintained in 12 psu artificial sea water (Instant Ocean Brand) with f/2 nutrient mixture plus 1% soil extract as described in Deeds et al. (2002) (Chapter 2). Cell densities were measured at 1 and 24 hours using a Coulter Multisizer II particle counter by enumerating the 7-20 μm and the 15-30 μm size fractions, respectively, for *K. micrum* and *O. marina* using a Coulter Accucomp software package (Coulter Electronics Limited, Miami FL). Significant differences ($p<0.05$) among mean cell numbers for different treatments were tested for using one-way analysis of variance with Scheffe's *post hoc* test using SPSS Base 10.0 statistical software (SPSS Inc., Chicago, IL).

Hemolytic activity remaining in solution was measured at 1 hour by mixing 100 μl of each treatment with an equal volume of diluted rainbow trout RBC suspension as described previously (see Chapter 2).

Effect of Membrane Lipids on KmTx 2 Bioactivity

Gymnodinosterol Purification.

Gymnodinosterol, the primary sterol from *K. micrum*, was isolated from *K. micrum* (CCMP 2282) cells, grown as described in Deeds et al (2002) (Chapter 2). Cells were filtered onto pre-combusted type GF/F filters (Whatman International Ltd., Maidstone, England) and extracted twice with chloroform/methanol (2:1). Extracts were concentrated under vacuum at 50 °C using a rotavapor (Buchi model R110, Switzerland) then placed in a large glass column containing 100 ml (dry volume) of Bio-Sil A (100-200 mesh) activated silica (Bio-Rad Laboratories, Richmond, CA) that had been pre-equilibrated with 250 ml methanol followed by 300 ml chloroform. Neutral lipids were eluted first using 250 ml chloroform, according to Yongmanitchi and Ward (1992). Neutral lipids were dried under vacuum at 50 °C, then re-suspended in a small volume of chloroform. This material was then applied to a 20×20-tapered layer TLC plate (Uniplate Silica Gel G, Analtech, Newark DE) that had been pre-developed for 2 hours with 1:1 chloroform/methanol. Next, the plates were pre-focused with 1:1 chloroform/methanol, then developed for 2.5 hrs using 250 ml of n-hexane/diethyl ether/acetic acid (80:20:1.5). After drying, several pigment bands were visible on the bottom half of the TLC plate. Additional lipid bands on the top half of the TLC plate were visualized by adding several iodine crystals to the emptied developing chamber. Four pale yellow bands became visible upon iodine vapor exposure. These 4 bands were scraped into 6 ml glass reaction chambers that contained Teflon frits (Supelco, Bellefonte PA). Each was eluted with 25 ml of n-hexane/diethyl ether (80:20). Fractions was then dried under vacuum at 50 °C, weighted, and re-suspended in chloroform to a concentration of 5 mg/ml. To confirm which bands contained sterols, 5 μg of each sample was spotted onto activated S-III Chromarods and analyzed by thin layer chromatography/flame ionization detection (TLC/FID) on an Iatroscan TH-10 TLC/FID Analyzer (Iatro Laboratories, Tokyo, Japan). Spotted rods were pre-focused with chloroform/methanol (1:1) then developed in n-hexane/diethyl ether/formic acid (85:15:0.1) for 45 min. Bands were tentatively identified by comparison to known lipid standards. The band corresponding to sterols was dried under nitrogen, and re-suspended in a small volume of acetonitrile/methanol

(1:1). The sterol-containing fraction was separated further using an Agilent 1100 series HPLC system (Hewlett Packard Corporation, Wilmington, DE). The fraction was injected onto a STERI-5 220×4.6 mm RP-18 (1μm) column (Applied Biosystems, Foster City, CA) and eluted at 51°C with an isocratic mixture of acetonitrile/methanol/water (48.5:48.5:3). Presumptive sterol fractions were collected using cholesterol and ergosterol as standards. Collected sterol fractions were positively identified by gas chromatography mass spectroscopy (GC/MS) analysis using an Agilent 6890 Series GC with a 60 m DB5ms, 0.32 ID, 0.25-μm film column (JW Scientific, Folsom, CA). Sterols were identified as trimethylsilyl ether derivatives (TMS). TMS derivatives were created by drying the samples under nitrogen then re-suspending in 1 ml of BSTFA 25% pyridine with 50 μl of TMS. This mixture was heated at 50 °C for 15 min, then re-dried under nitrogen and re-suspended in a small volume of methylene chloride. 10 μg of each sample was injected onto the GC/MS. (24S)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol (gymnodinosterol) was positively identified according to LeBlond and Chapman (2002) (approx. 80% pure) (Figure 4.2).

Membrane Lipid Inhibition Assays

To assess the inhibitory effects of exogenous membrane lipids on KmTx 2 hemolytic activity, the following sterols and lipids were tested: cholesterol, ergosterol, gymnodinosterol, and both natural (unsaturated) and synthetic (saturated) phosphatidylcholine. Cholesterol (5-cholesten-3β-ol) and L-α-phosphatidylcholine (brain, porcine) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL); ergosterol

(5,7,22-cholestatrien-24β-methyl-3β-ol) was purchased from Steraloids, Inc. (Newport, RI). Synthetic phosphatidylcholine (L-α-lecithin (β-γ-dipalmitoyl) was purchased from Calbiochem (San Diego, CA). Gymnodinosterol was isolated from filtered *K. micrum* (CCMP 2282) cells (isolation described previously). KmTx 2: 0, 0.1, 0.5, and 1 μg/ml was added to solutions of Tris buffer + $CaCl_2$ (described in Chapter 2) with either: 0, 0.001, 0.01, 0.1, 1 or 10 μM selected sterol or membrane lipid. Stock solutions of toxin and lipid were made in methanol and no more than 1% of each (2% total) was added to any given well. After brief mixing, these solutions were added 1:1 with a diluted suspension of rainbow trout erythrocytes and hemolytic activity was assessed as described previously (Chapter 2).

Effect of KmTx 2 on Fish

Zebrafish (*Danio rerio* - 60 days old) were exposed to 0.1, 0.5, 1, or 2 μg/ml KmTx 2 in 50 ml of aerated reconstituted fresh water (soft) (ASTM, 1992). Toxin dilutions were made in MeOH (200 μl max. addition per treatment) and controls were exposed to 200 μl MeOH only. Three fish were exposed per replicate, three replicates per treatment. Fish were observed for mortality hourly. Upon death, fish were preserved in neutral buffered formalin and prepared for histological examination as described in Noga (2000). At six hours post exposure, controls and any fish that did not die were euthanised by rapidly lowering the water temperature and prepared for histological examination as previously described.

RESULTS

Ion Permeation Experiments

(Figure 4.3A-D) shows the effect of KmTx 2 on a rat cardiac myocyte loaded with fura-2 indicator. At rest, the myocyte displayed normal relaxed morphology (bright-field micrograph, Figure 4.4A) and low resting $[Ca^{2+}]_i$ (pseudo-color image, Figure 4.3B). Seconds after application of 0.25 µg/ml KmTx 2, $[Ca^{2+}]_i$ was dramatically elevated (Figure 4.3C), and the myocyte was irreversibly contorted by hyper-contraction (Figure 4.3D). When applied to rat embryo fibroblasts at 0.25 µg/ml, KmTx 2 caused a marked increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which then declined slowly (Figure 4.4A). In contrast, at 1 µg/ml, KmTx 2 caused a sharp and irreversible rise in $[Ca^{2+}]_i$ (Figure 4.4B), and eventual cell lysis. There was a marked difference in the rate of Ca^{2+} increase between 0.25 and 1 µg/ml KmTx 2 treatments with the 0.25 µg/ml treatment taking ca. 100 × longer to reach an intracellular Ca^{2+} concentration of 1 µM (Figure 4.4C). Essentially similar results were obtained when these experiments were repeated on additional cell types, including rat intestinal epithelial cells, vagal sensory neurons from adult rabbit, as well as lymphocytes and rat ventricular cardiac myocytes. The increased membrane permeability induced by KmTx 2 was not selective for Ca^{2+}. Through methods that have been used previously to characterize microbial toxins (Raimondi et al. 2000), we determined that KmTx 2 promoted the permeation of cations as different as Na^+ and Mn^{2+}, in addition to Ca^{2+} (Figure 4.5).

Patch Clamp Recording

Isolated New Zealand White rabbit nodose ganglion neurons (NGN's), loaded with fura-2 intracellular Ca^{2+} indicator to allow fluorimetric measurement of $[Ca^{2+}]_i$ in parallel with electrophysiological recording, showed that upon a 60 sec. transient exposure to 1 μg/ml KmTx 2 there was a sharp rise in intracellular Ca^{2+} levels concomitant with a increase in membrane current (Figure 4.6). However, the timecourse of the rise in intracellular Ca^{2+} was delayed relative to the corresponding change in current. This may imply that ions other than Ca^{2+} are the principal carriers of the toxin-invoked current.

Determination of I-V Relationship

Based on the voltage-ramp experiments, an I-V (current-voltage) relationship was established for the isolated rabbit NGN KmTx 2 induced current (Figure 4.7). The reversal potential was established to be E_{Rev} = +3.6 +/- 5.4 mV (n=7). This is consistent with a non-selective cation current. The relative permeabilities of Na^+ and K^+ were estimated through the Goldman-Hodgkin-Katz equation to be P_K/P_{Na} = 0.79. These results indicate that KmTx 2 acts principally to increase membrane permeability to Na^+ and K^+, while causing a more modest, but still significant, increase in Ca^{2+} permeability.

Osmotic Protection Assays

Co-incubation of rainbow trout erythrocytes with a range (342.3-10,000 MW) of inert osmolytes, including sugars, polyethylene glycols, and dextrans, causes a progressive inhibition of lysis with complete prevention at molecular weights > 8,000 Da (Figure 4.8). This effect was graded based on the amount of toxin used with lower inhibition at higher toxin concentrations.

Effect of KmTx 2 on Additional Eukaryotic Cell Types

Anti-Fungal Assays

Minimal inhibitory concentrations (MIC) of 8 and 16 μg/ml, respectively, were found for *A. niger* and *C. albicans* for exposure to KmTx 2. Results did not change between 24 and 96 hours. Amphotericin B, a common anti-fungal antibiotic was used as a positive control in the fungal growth inhibition assays. For amphotericin B, no growth was observed in either species at any concentration after 24 hours, but trailing growth resulted in MIC values of 0.5 and 0.125 μg/ml, respectively, for *A. niger* and *C. albicans* after 96 hours. Using probit analysis, hemolytic LC_{50} values for KmTx 2 and amphotericin B were calculated to be 0.368 μg/ml (range: 0.190-0.605) and 3.759 μg/ml (range: 2.067-7.858), respectively (Table 1).

Effect of KmTx 2 on a Potential Grazer

Incubation of KmTx 2 with a Chesapeake Bay isolate of *O. marina* and a South Carolina isolate of *K. micrum* (CCMP 2282) resulted in significant (p<0.05) cell lysis to *O. marina* in 0.5 and 1 µg/ml treatments at 1 hour (Figure 4.9). Membrane blebing and cellular swelling were observed in periodic microscopic observations of *O. marina* cells during the exposures. Hemolytic activity remaining in solution after 1 hour was reduced at all KmTx 2 concentrations in *O. marina* exposed cultures compared to *K. micrum* exposed cultures (Figure 4.8). In the 1 µg/ml KmTx 2 treatment, *O. marina* cell densities were reduced by > 90% after 24 hours, while *K. micrum* cultures appeared unaffected (data not shown).

Membrane Lipid Inhibition Assays

Both cholesterol and ergosterol began to cause inhibition of hemolytic activity in the range of 10-100 nM, but for ergosterol this inhibition plateaued ca. 1 µM, whereas with cholesterol, inhibition continued until complete inhibition was reached at levels ca. 10 µM (Figure 4.10B,C). Co-incubation of fish erythrocytes with gymnodinosterol, isolated from *K. micrum*, had no effect on hemolytic activity due to KmTx 2 (Figure 4.10A). Likewise, both synthetic (dipalmitoyl) and natural brain phosphotidylcholine had no effect on KmTx 2 induced hemolysis of fish erythrocytes (Figure 4.11).

Effect of KmTx 2 on Fish

To confirm the ichthyotoxicity of KmTx 2, 60 day old zebrafish (*Danio rerio*) were exposed to an increasing concentration of toxin. Fish exposed to ≥ 0.5 μg/ml KmTx 2 all died in < 1 hour. Fish exposed to 0.1 μg/ml survived the full 6 hr exposure but respiration rates were noticeably elevated, and significant mucus production was observed coming from the opercular cavity. Histological examination of sectioned whole fish showed loss of secondary structure of gill lamellae, including edema, lamellar bridging, and sloughing of gill epithelial tissue in all 0.1 μg/ml KmTx 2 treated fish (Figure 4.12). Severe necrosis and degeneration of gill tissue was observed in all ≥ 0.5 μg/ml treated fish. For all 0.1 μg/ml treated fish, gross histological changes were not observed in any other organs including skin, liver, kidney, heart, brain, muscle, pseudobranch, thymus, or gonads. Similar results (gill damage with death due to apparent asphyxiation) were described previously for juvenile cod exposed to a high concentration (>1.5×10^5 cells/ml) of a North Atlantic isolate of *K. micrum* (Nielsen, 1993).

DISCUSSION

The use of cytolytic, pore-forming agents, is a widely used and highly successful means of cellular attack and defense (Thelestam and Mollby, 1979). Various cytolysins are produced by organisms ranging from prokaryotes, to protozoans, to higher vertebrates. Examples of this group of compounds include glycoside saponins (Baumann et al., 2000) produced by plants, polyene-macrolide antibiotics (Bolard, 1986) and cytolytic proteins (Fivaz et al., 2001) produced by bacteria, venoms produced by aquatic invertebrates, such as cnidarian jellyfish (Edwards et al., 2002), and even the complement proteins of the human immune system (Peitsch and Tschopp, 1991). The presumed function of these compounds varies as well, from defense as with the saponins (Wassler et al., 1990) and complement proteins (Peitsch and Tschopp, 1991), to aids for infection and proliferation as with bacterial cytolytic proteins (Fivaz et al., 2001), to prey capture as with the jellyfish venoms (Edwards and Hessinger, 2000). But the ultimate activity of this group of compounds is fairly consistent; permeabilization of target plasma membranes to a range of small ions and molecules resulting in an inward osmotic flux of water with subsequent cellular swelling. This process can lead to either direct osmotic lysis or a series of more complicated pathways such as endoplasmic reticulum vacuolation or apoptosis (Fivaz, 2001, and references therein).

In previous studies, we showed that the addition of a high dose (2 μg/ml) of KmTx 1 to a rat pituitary tumor cell line (GH_4C_1) resulted in 100% release of the cytosolic marker lactate dehydrogenase (LDH), suggesting cell lysis (Deeds et al. 2002; Chapter 2). Wassler et al. (1990) showed that the release of LDH from saponin exposed mammalian hepatocytes could be inhibited through the addition of osmotically active substances to the culture media. Theoretically, these osmolytes balanced the inward osmotic flux of water into permeabilized cells driven by the high concentration of cytosolic proteins. To confirm the hypothesis that KmTx 2 kills vertebrate cells by colloid osmotic lysis, we show here that co-incubation of rainbow trout erythrocytes with a range (342.3-10,000

MW) of inert osmolytes, including sugars, polyethylene glycols, and dextrans, cause a progressive inhibition of lysis with complete prevention, even at 1 μg/ml KmTx 2, at molecular weights > 8,000 Da (Figure 4.8). This effect was graded based on the amount of toxin used with lower inhibition at higher toxin concentrations. This data confirms the action of colloid osmotic lysis in cells exposed to KmTx 2.

To further assess the nature of the cytotoxic activity of KmTx 2, we exposed a variety of model mammalian cell types and measured the inward flux of various cations using both intracellular fluorescent indicators and direct electrophysiological measurements. Using intracellular fluorescent indicators, we showed that KmTx 2 promoted the inward flux of cations such as Ca^{2+}, Na^{+}, and Mn^{2+}. Through the use of direct whole cell patch clamping we showed that KmTx 2 exposure induced an increase in membrane permeability to Ca^{2+} that corresponded to a change in membrane current (Figure 4.5), although further analysis showed that Ca^{2+} flux was in fact a minor component of the toxin induced current. A voltage ramp constructed current-voltage (I-V) relationship showed that the reversal potential was close to zero (E_{Rev} = +3.6 mV) (Figure 4.7) indicating that the KmTx 2 induced pore is non-selective. In comparison, the theoretical reversal potential for Na^{+} would be E_{Na} = +55 mV, while E_K = -75 mV. Through the use of the Goldman-Hodgkin-Katz equation (1):

$$V_m = \frac{RT}{F} \ln \frac{P_K[K^+]_{out} + P_{Na}[Na^+]_{out} + P_{Cl}[Cl^-]_{in}}{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{out}} \qquad (1)$$

it was estimated that $P_K/P_{Na} = 0.79$. A value of 1 would have indicated that the KmTx 2 induced pore was equally permeable to Na^{+} and K^{+}. Therefore, the resultant current appears to be due mainly to Na^+ and K^+, with Ca^{2+} being a smaller, yet still significant, contributor.

For several cytolytic pore-forming compounds, such as the polyene antibiotics and certain bacterial protein toxins, membrane sterols play a critical role in toxicity (Gary-Bobo, 1989; de Kruijff, 1990). For the polyene antibiotic amphotericin B, binding to target membranes occurs whether sterols are present or not, but permeability leading to cell lysis is only induced when membrane sterols are present (Gary-Bobo, 1989). Additional examples of the importance of membrane sterols in cytolysin activity include the amphidinols, potent hemolytic and anti-fungal polyhydroxy-polyenes produced by the dinoflagellate *Amphidinium klebsii*, whose activity is enhanced in liposomes containing cholesterol (Paul et al., 1997), and prymnesins, potent ichthyotoxic and hemolytic polyketides produced by the prymnesiophyte *Prymnesium parvum*, whose activity is inhibited through co-incubation with cholesterol, ergosterol, and phosphotidylcholine (Igarashi et al., 1998). In this study, the hemolytic activity of KmTx 2 was inhibited by co-incubation with both cholesterol and ergosterol but was not inhibited when incubated with its own major sterol, gymnodinosterol (Figure 4.10). In addition, no inhibition was observed upon incubation with either synthetic dipalmitoyl (16:0), or natural brain phosphotidylcholine (Figure 4.11). This data suggests that KmTx 2 will competitively associate with free cholesterol or ergosterol over fish erythrocyte membranes, thereby inhibiting hemolysis, while it will not competitively associate with other common membrane lipoproteins or its own major sterol.

Amphotericin B is reported to have a higher affinity for ergosterol compared to cholesterol (Bolard, 1986), which is the entire basis for its use as a treatment for human systemic fugal infections. Through molecular modeling, Baginski et al. (2002) predicted that amphotericin B would form larger, more stable channels in membranes containing ergosterol compared to cholesterol, resulting in higher ion conductances. In this study, anti-fungal activity of KmTx 2 was 10-100 fold lower than for amphotericin B, depending on species tested (Table 1). In addition, hemolytic activity towards rainbow trout erythrocytes was ten fold higher for KmTx 2 compared to amphotericin B. All of our data suggest that KmTx 2 has higher activity towards cholesterol containing membranes than ergosterol containing membranes.

Leblond and Chapman (2002) recently found that *K. micrum* has a unique sterol profile, shared only by the closely related dinoflagellates *Karenia brevis* and *Karenia mikimotoi*, dominated by (24S)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol. This sterol has recently been named gymnodinosterol (Giner et al., 2003). We confirmed that the *K. micrum* cultures used in this study possess gymnodinosterol as their major sterol. In this study, KmTx 2 was toxic towards the co-occurring heterotrophic dinoflagellate, and potential grazer, *Oxyrrhis marina* while it had no effect on cultures of *K. micrum* (Figure 4.9). Furthermore, hemolytic activity remaining in solution after the one-hour incubation period was significantly reduced in *O. marina* cultures compared to *K. micrum* cultures suggesting that KmTx 2 will partition into *O. marina* membranes but not *K. micrum* membranes. *O. marina* has been reported to both synthesize cholesterol and acquire it from its prey (Klein Breteler et al., 1999). Giner et al. (2003) suggested that the unique sterol composition of *K. brevis*, which *K. micrum* shares, helps to reduce predation due to its poor nutritional qualities. Here we propose that the unusual structural features of *K. micrum* sterols play a role in the protection of this organism from the membrane disrupting properties of its own toxins by not allowing toxin penetration and subsequent permeabilization. Additional testing will be required to confirm this intriguing hypothesis.

Implications for K. micrum and fish kills

K. micrum was first identified in the United States as part of investigations into fish kills associated with the ichthyocidal dinoflagellate *Pfiesteria piscicida* (Li et al., 2000; Terlizzi et al., 2000). Due to their common co-occurrence, and prior to the advent of molecular diagnostic techniques that could easily distinguish these species (Oldach et al., 2000), *K. micrum*, along with several additional small gymnodinioid dinoflagellates, were placed in the category of *Pfiesteria*-like organisms (Steidinger et al., 1996; Marshall, 1999). In the Maryland aquaculture related fish kill, that originally inspired this line of research, *P. piscicida* was present (ca. 300 cells/ml *P. piscicida* vs. > 60,000 cells/ml *K. micrum*) (Deeds et al., 2002) and initially was suggested as the causative agent (Burkholder and Glasgow, 1997). Before this event, Maryland monitoring authorities were misidentifying *K. micrum* as *Gyrodinium estuariale* Hulburt (Li et al., 2000; Terlizzi et al., 2000), which had never been linked with fish kills in the scientific literature. *K. micrum*, formerly *Gyrodinium/Gymnodinium galatheanum* (Daugbjerg et al., 2000), has been reported to be associated with fish kills since its first description in Southwestern Africa in the 1950's (Braarud, 1957; Pieterse and Van Der Post, 1967). Several reports have stated that in the standardized fish bioassay used to confirm ichthyotoxicity of *P. piscicida* isolates, which is reported to simulate environmentally relevant conditions, *K. micrum* is not toxic (Seaborn et al., 2000; Burkholder et al., 2001b; Glasgow et al., 2001). Based on these reports, it was further suggested that management, policy, and research considerations should be discouraged on such species of unknown toxicity status until their ichthyotoxicity could be confirmed (Burkholder et al., 2001a; and references therein). Quesenberry et al. (2002) clarified these earlier observations by showing that that *K. micrum* in fact does not proliferate, therefore would not cause toxicity, in assays similar to the standardized fish bioassay used to confirm toxicity of *P. piscicida*. The standardized fish bioassay is a semi-closed system, starts with low cell numbers (ca. 500 cells/ml), has aeration (i.e. rapid mixing of the water column), and a high biomass of fish present. Fish kills associated with *K. micrum* to date have occurred typically in shallow, stratified systems with high concentrations of *K. micrum* (> 10,000 cells/ml) and occurred in situations with both low and high fish biomass (Deeds et al., 2002; Kempton et al., 2002; Goshorn et al., *submitted*). Many dinoflagellates, *K. micrum* included, do not proliferate well under conditions of rapid mixing (unpublished data). In this study, KmTx 2 concentrations ≥ 0.5 μg/ml were acutely toxic (< 1 hr) to zebrafish and exposures of 0.1 μg/ml caused severe damage to gill epithelia. Toxin concentrations of 0.4-0.8 μg/ml have been isolated from water collected during fish kills associated with blooms of *K. micrum* (Deeds et al., *submitted*, unpublished data). Therefore, based on the data presented here and in similar recent studies (Deeds et al., 2002; Kempton et al., 2002; Deeds et al., *submitted*; Goshorn et al., *submitted*), *K. micrum* should be considered potentially ichthyotoxic in the United States. Research is in progress to screen *K. micrum* isolates from outside the United States for their ability to produce Karlotoxin-like compounds.

Summary/Conclusions

In summary, KmTx 2, the main toxin from *K. micrum* populations from North Carolina, South Carolina, and Florida, appears to function by permeabilizing plasma membranes to a range of ions resulting in cell destruction through colloid osmotic lysis. Through the use of intracellular fluorescent indicators and whole cell voltage clamping we have shown that exposure to KmTx 2 induces a non-selective increase in ionic permeability concomitant with a change in membrane current due mainly to Na^+ and K^+ flux. Cell lysis can be inhibited through co-incubation with the membrane sterols cholesterol and ergosterol, but hemolytic and anti-fungal assays suggest that activity is higher in membranes containing cholesterol. KmTx 2 appears to partition into the membrane of a cholesterol-containing potential grazer, resulting in cell lysis, while it will not partition into it own membrane. This suggests that the natural role of these compounds may be to function as anti-grazing agents. The unusual sterol composition of K. micrum's own membranes appear to play a role in the protection of this organism from its own toxins. Finally, KmTx 2 was toxic towards zebrafish within the range of toxin concentrations found present during fish kills. Sublethal exposure to KmTx 2 resulted in extensive damage to gill epithelia. This work further solidifies the potential ichthyotoxicity of *K. micrum*, in contrast to previous US reports, and confirms the associations between high densities of this organism and fish kills that have been observed in temperate estuaries around the world for decades.

ACKNOWLEDGEMENTS

The authors would like to thank Scott LaPatra (Clear Springs Foods, Inc., Buhl Idaho, USA) and the staff of the Center of Marine Biotechnology Aquaculture Research Center for providing and maintaining trout that were used extensively in this study. They would also like to thank Roger Harvey and Rachael Dyda (Chesapeake Biological Laboratory, Solomans, Maryland, USA) for GC-MS analysis of *K. micrum* sterols, Jennifer Wolny (Florida Marine Research Institute, St. Petersburg, Florida, USA) for providing *K. micrum* isolates CCMP 2282 and JW020205-B4, Peter Mrozinski, William Barrett, and Jim Lau (Agilent Technologies Inc., Wilmington Delaware, USA) for LC-MS analyses of KmTx 2, Renate Reimschuessel (US Food and Drug Administration Center for Veterinary Medicine, Laurel Maryland, USA) for pathological examination of fish, and Danara Krupatkina (Center of Marine Biotechnology, Baltimore, Maryland, USA) for providing the Chesapeake Bay *O. marina* culture. This research was funded by a grant from NOAA ECOHAB (NA860P0492).

Table 4.1. Comparison of anti-fungal and hemolytic activities of KmTx 2 and amphotericin B. *Aspergillus niger* (ATCC 1004) was chosen as a representative filamentous fungi, and *Candida albicans* (ATCC 14053) was chosen as a representative yeast. Minimal inhibitory concentration (MIC) defined as concentration of anti-fungal agent required to completely inhibit visible growth for 96 hours. Hemolytic LD_{50} with ranges determined by probit analysis using SPSS Base 10.0 statistical software package (SPSS Inc., Chicago, IL).

	KmTx 2	Amphotericin B
A. niger (MIC)*	8 μg/ml	0.5 μg/ml
C. albicans (MIC)*	16 μg/ml	0.125 μg/ml
Hemolytic LC_{50}	0.368 μg/ml (0.190-0.605)	3.759 μg/ml (2.067-7.858)

*MIC - Minimal Inhibitory Concentration

Figure 4.1. Purification and molecular weight determination of KmTx 2. [A.] LC/MS trace of purified KmTx 2. Solid line [left axis] 230 nm absorbance. Dashed line [right axis] mass intensity. [B.] Negative ion mass spectra of KmTx 2.

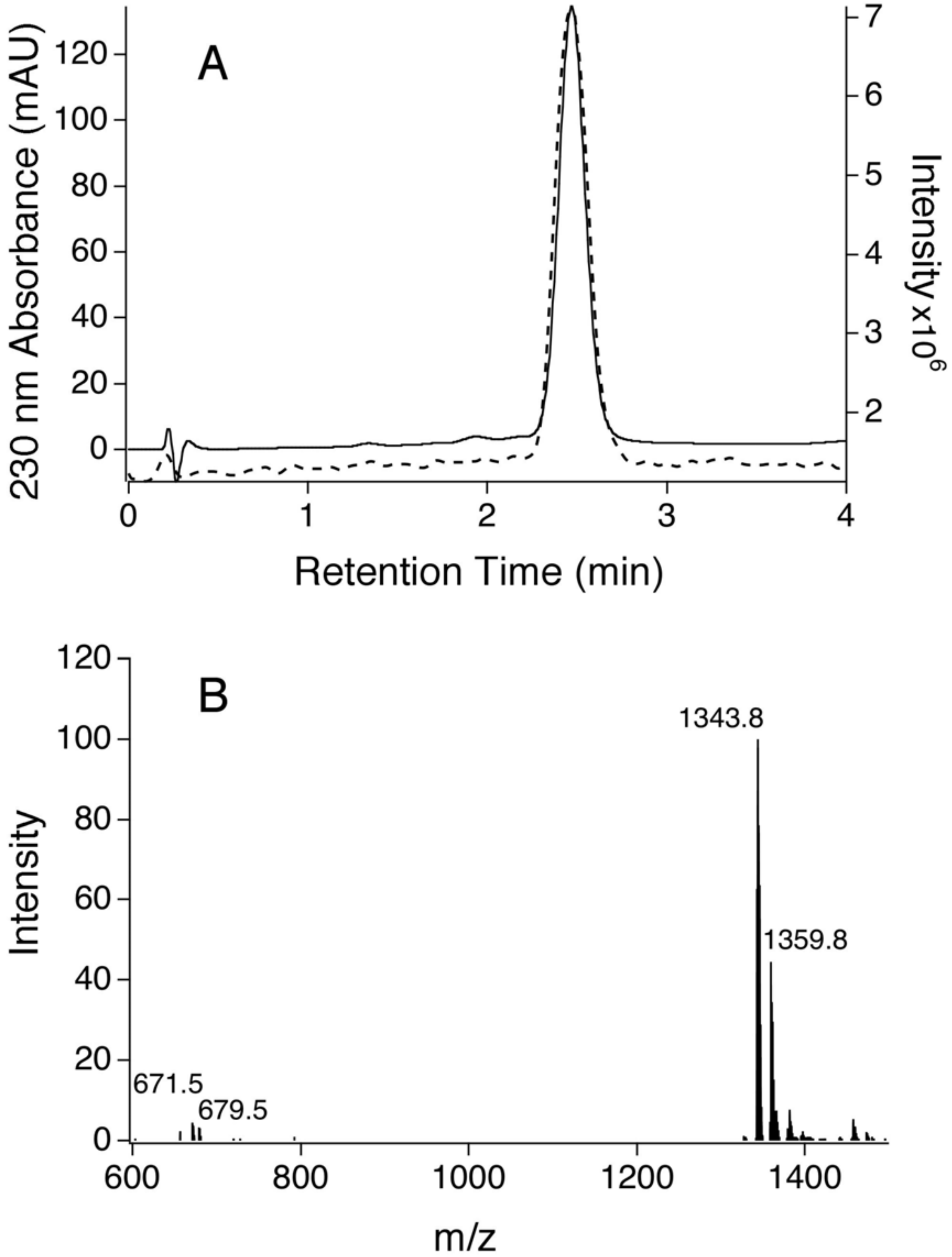

A
230 nm Absorbance (mAU)
0
20
40
60
80
100
120
Intensity x10^6
2
3
4
5
6
7
Retention Time (min)
0
1
2
3
4
B
Intensity
0
20
40
60
80
100
120
1343.8
1359.8
671.5
679.5
m/z
600
800
1000
1200
1400

Figure 4.2. Purification and identification of gymnodinosterol isolated from *Karlodinium micrum* (CCMP 2282). [A.] HPLC trace of gymnodinosterol (ca. 80% pure). [B.] GC/MS spectra of gymnodinosterol. *Molecular weight of gymnodinosterol is reported to be 484 Da. According to Leblond and Chapman 2002)

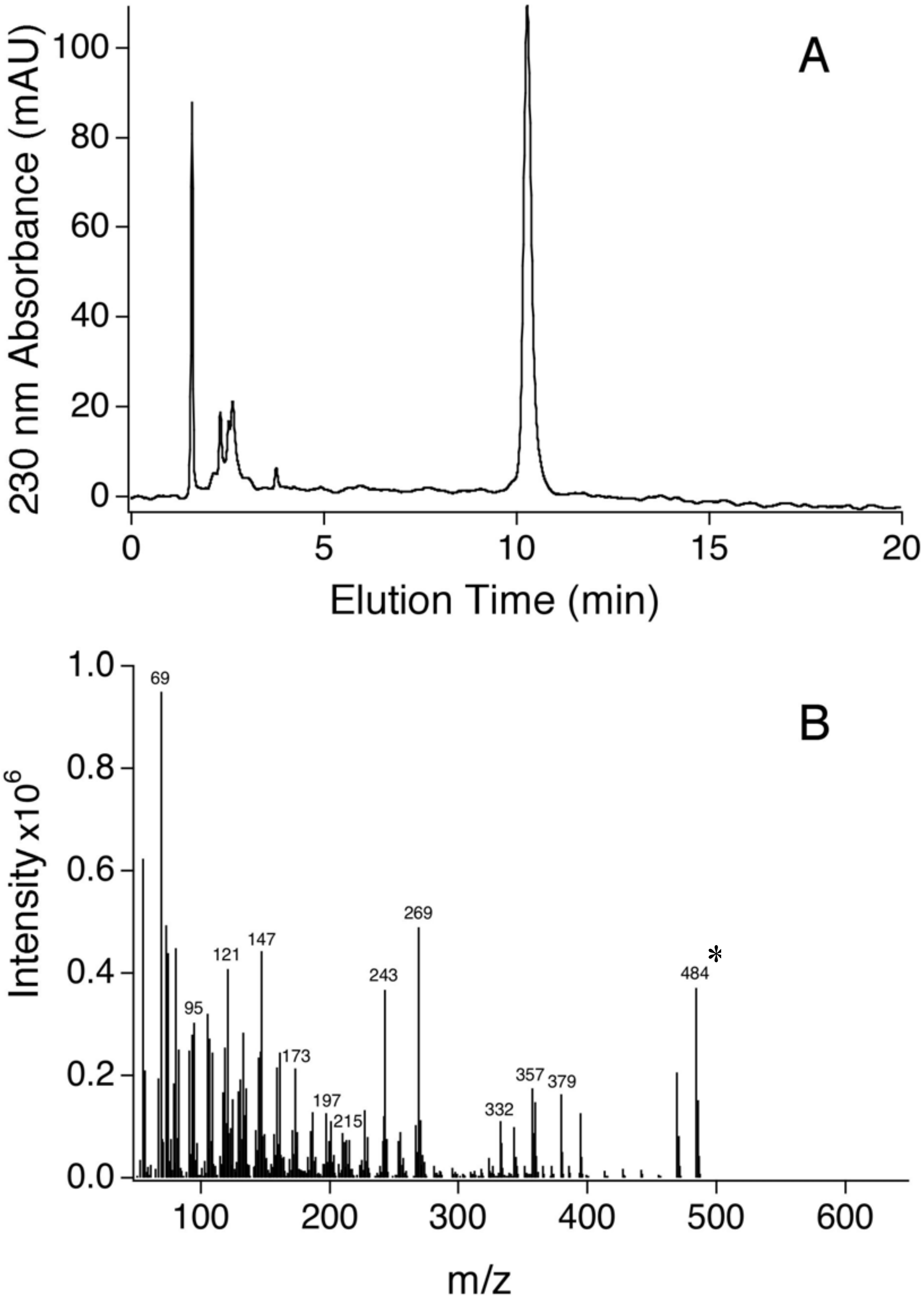

A
230 nm Absorbance (mAU)
0
20
40
60
80
100
Elution Time (min)
0
5
10
15
20
B
Intensity x10^6
0.0
0.2
0.4
0.6
0.8
1.0
m/z
100
200
300
400
500
600
69
95
121
147
173
197
215
243
269
332
357
379
484
*

Figure 4.3. Fura 2 pre-loaded rat cardiac myocyte exposed to 0.25 μg/ml KmTx 2. [A.] Normal relaxed cell morphology of rat cardiac myocyte viewed under bright field microscopy. [B.] Pseudo-colored image of fura 2 pre-loaded rat cardiac myocyte showing low resting cytoplasmic Ca^{2+}. [C.] Pseudo-colored image of fura 2 pre-loaded rat cardiac myocyte after exposure to 0.25 μg/ml KmTx 2. Note elevated cytoplasmic Ca^{2+} levels. [D.] Rat cardiac myocyte after exposure to 0.25 μg/ml KmTx 2 viewed under bright field microscopy. Note severe cell contraction and membrane blebs (white arrows). Bar represents 20 μm. Experiments performed with Dr. Joseph P.Y. Kao, University of Maryland Biotechnology Institute, Center for Biomedical Research, Baltimore, MD.

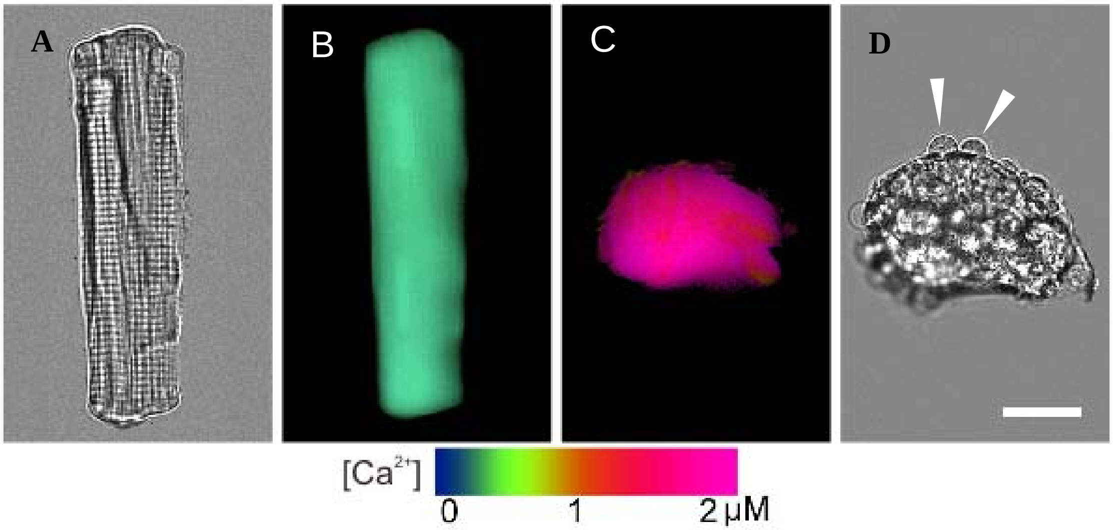

A
B
C
D
[Ca2+]
0
1
2 µM

Figure 4.4. Measurement of Ca^{2+} flux into rat embryonic fibroblast (REF 52) cells using the intracellular fluorescent indicator fura-2. [A.] Addition of DMSO control followed by the addition of 0.25 μg/ml KmTx 2, note rapid increase in cytosolic Ca^{2+} levels followed by slight recovery. [B.] Addition of 0.2 μM vasopressin followed by the addition of 1 μg/ml KmTx 2. For vasopressin, a carrier type Ca^{2+} ionophore, note spike in cytosolic Ca^{2+} levels followed by rapid decline to level above baseline. For KmTx 2 addition note rapid rise in cytosolic Ca^{2+} levels with no recovery. [C.] Comparison of time course of Ca^{2+} influx for 0.25 and 1 μg/ml KmTx 2 additions. Experiments performed with Dr. Joseph P.Y. Kao, University of Maryland Biotechnology Institute, Center for Biomedical Research, Baltimore, MD.

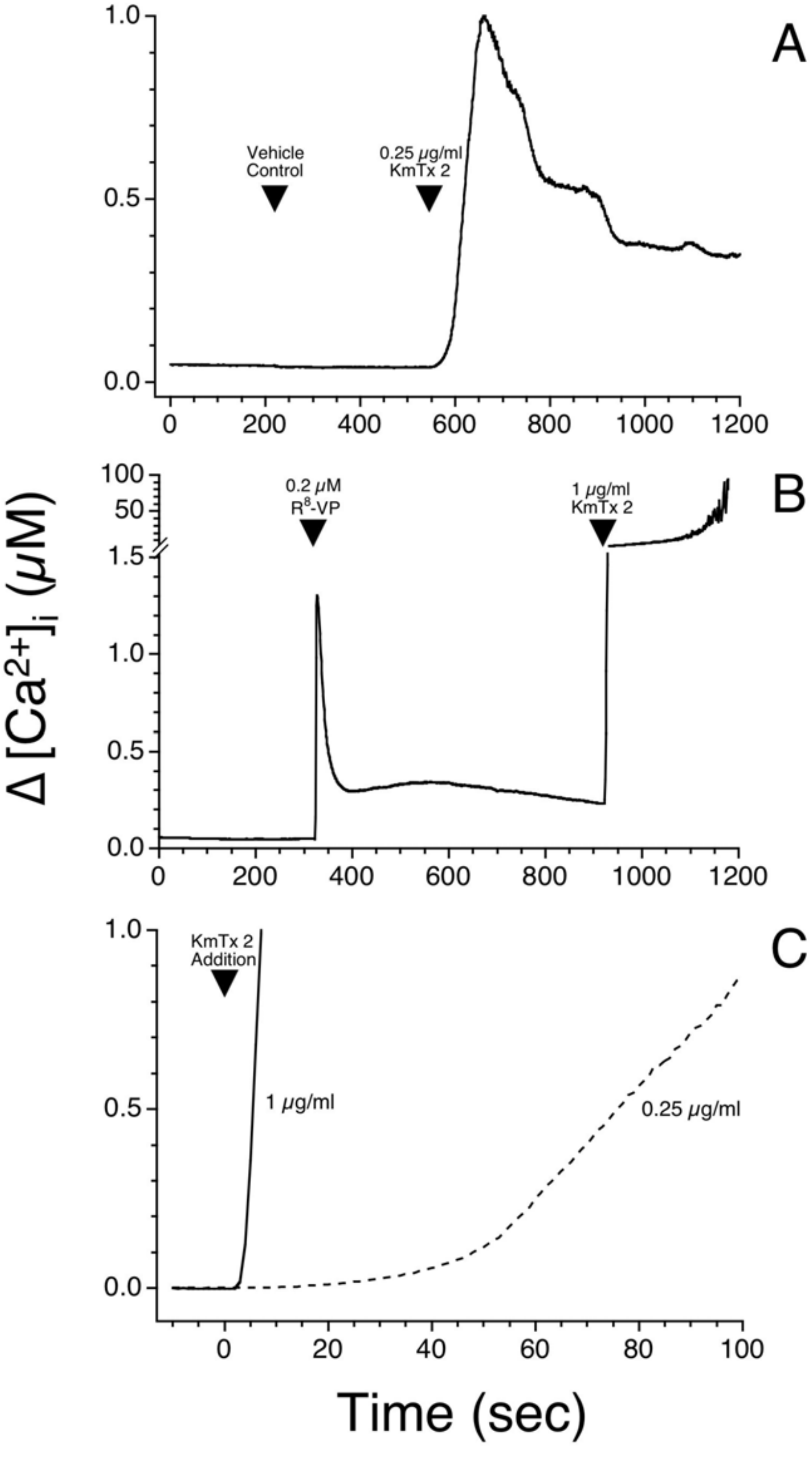
A
Vehicle Control
0.25 μg/ml KmTx 2
1.0
0.5
0.0
0
200
400
600
800
1000
1200
B
0.2 μM R8-VP
1 μg/ml KmTx 2
100
50
1.5
1.0
0.5
0.0
Δ [Ca2+]i (μM)
C
KmTx 2 Addition
1 μg/ml
0.25 μg/ml
0
20
40
60
80
100
Time (sec)

Figure 4.5. Additional intracellular fluorescent indicator experiments to test the specificity of cation permeability in REF-52 cells upon exposure to KmTx 2. [A.] REF-52 cells loaded with the Na^+ specific intracellular fluorescent indicator SBFI (AM ester) exposed to 300 ng/ml KmTx 2. Fluorescence normalized to Na^+ levels. [B.] REF-52 cells loaded with fura-2 indicator exposed to 500 ng/ml KmTx 2. First arrow represents addition of 50 μM Mn^{2+} to external media. Second arrow represents addition of 500 ng/ml KmTx 2. Drop in fluorescence represents displacement of Ca^{2+} on fura-2 indicator by Mn^{2+}. Experiments performed with Dr. Joseph P.Y. Kao, University of Maryland Biotechnology Institute, Center for Biomedical Research, Baltimore, MD.

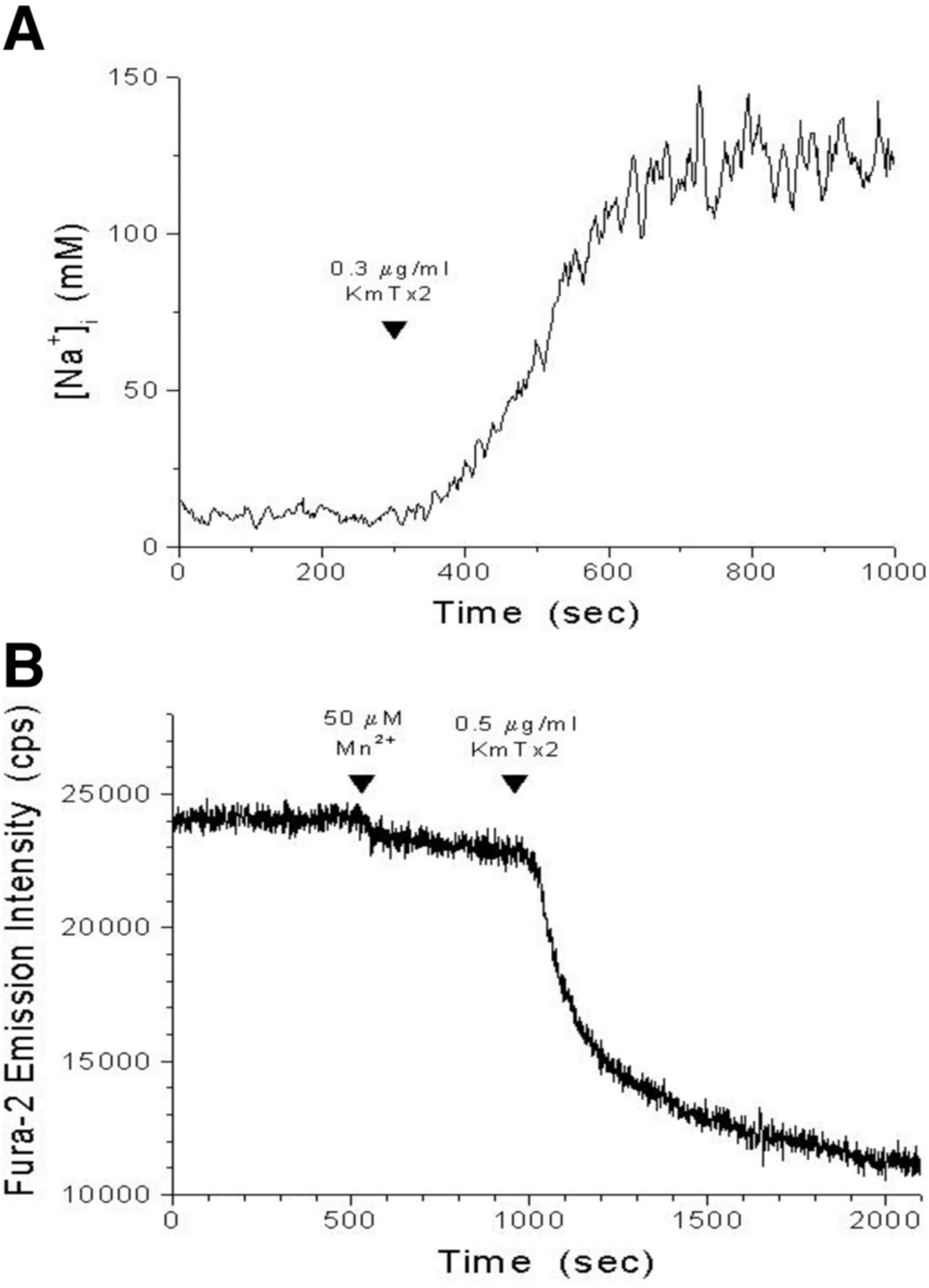

A
[Na$^+$]$_i$ (mM)
150
100
50
0
0.3 µg/ml
KmTx2
0
200
400
600
800
1000
Time (sec)
B
Fura-2 Emission Intensity (cps)
25000
20000
15000
10000
50 µM
Mn^{2+}
0.5 µg/ml
KmTx2
0
500
1000
1500
2000
Time (sec)

Figure 4.6. KmTx 2 induced current in whole cell patch-clamped rabbit vagal sensory nodose ganglion neuron (NGN). Upper line indicates change in intracellular Ca^{2+} level (μM) upon 60 sec. transient exposure to 1 μg/ml KmTx 2. (Based on fluorescence increase due to pre-loaded fluorescent Ca^{2+} indicator fura-2). Lower line indicates change in current (nA) upon 60 sec. transient exposure to 1 μg/ml KmTx 2. Inset line represents KmTx 2 transient exposure estimated through the use of a fluorescent tracer. Note delay in onset of change in intracellular Ca^{2+} compared to change in current. Experiments performed with Dr. Joseph P.Y. Kao, University of Maryland Biotechnology Institute, Center for Biomedical Research, Baltimore, MD.

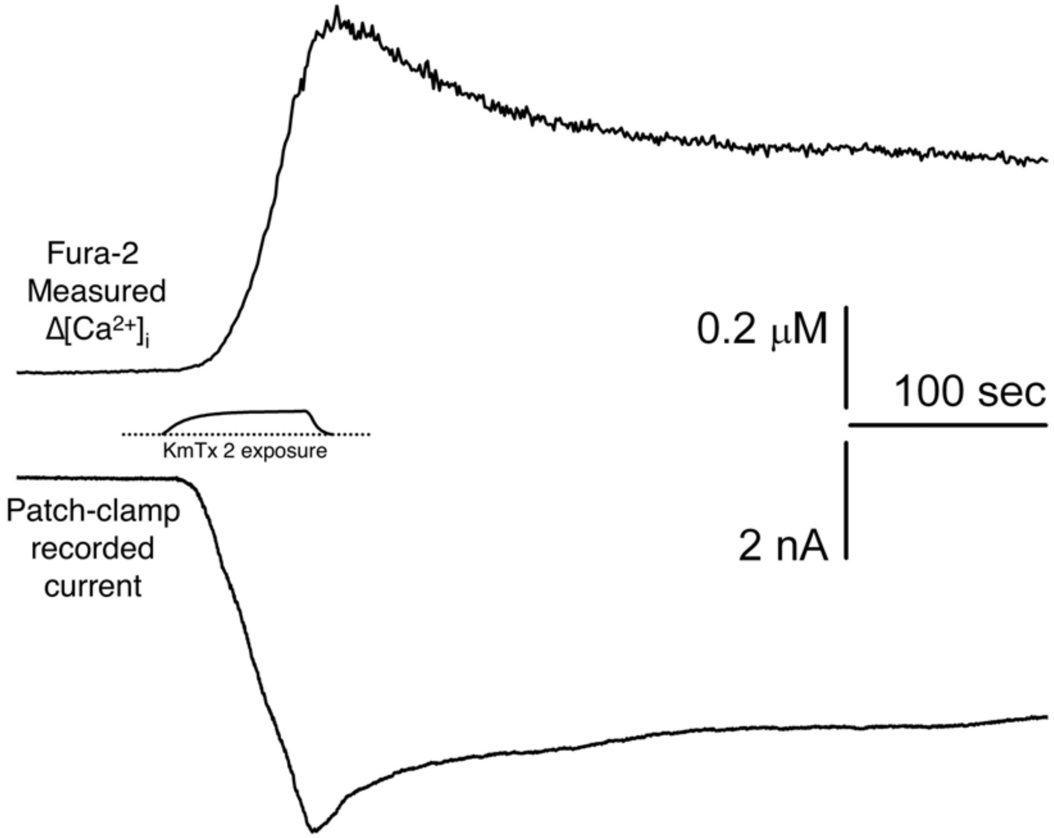

Fura-2
Measured
Δ[Ca2+]i
0.2 µM
100 sec
KmTx 2 exposure
Patch-clamp
recorded
current
2 nA

Figure 4.7. Voltage ramp constructed-current voltage (I-V) relationship for KmTx 2 induced current in whole cell patch clamped rabbit vagal sensory nodose ganglion neuron (NGN). Reversal potential (E_{Rev}) is estimated to be +3.6 +/- 5.4 mV (n=7). Experiments performed with Dr. Joseph P.Y. Kao, University of Maryland Biotechnology Institute, Center for Biomedical Research, Baltimore, MD.

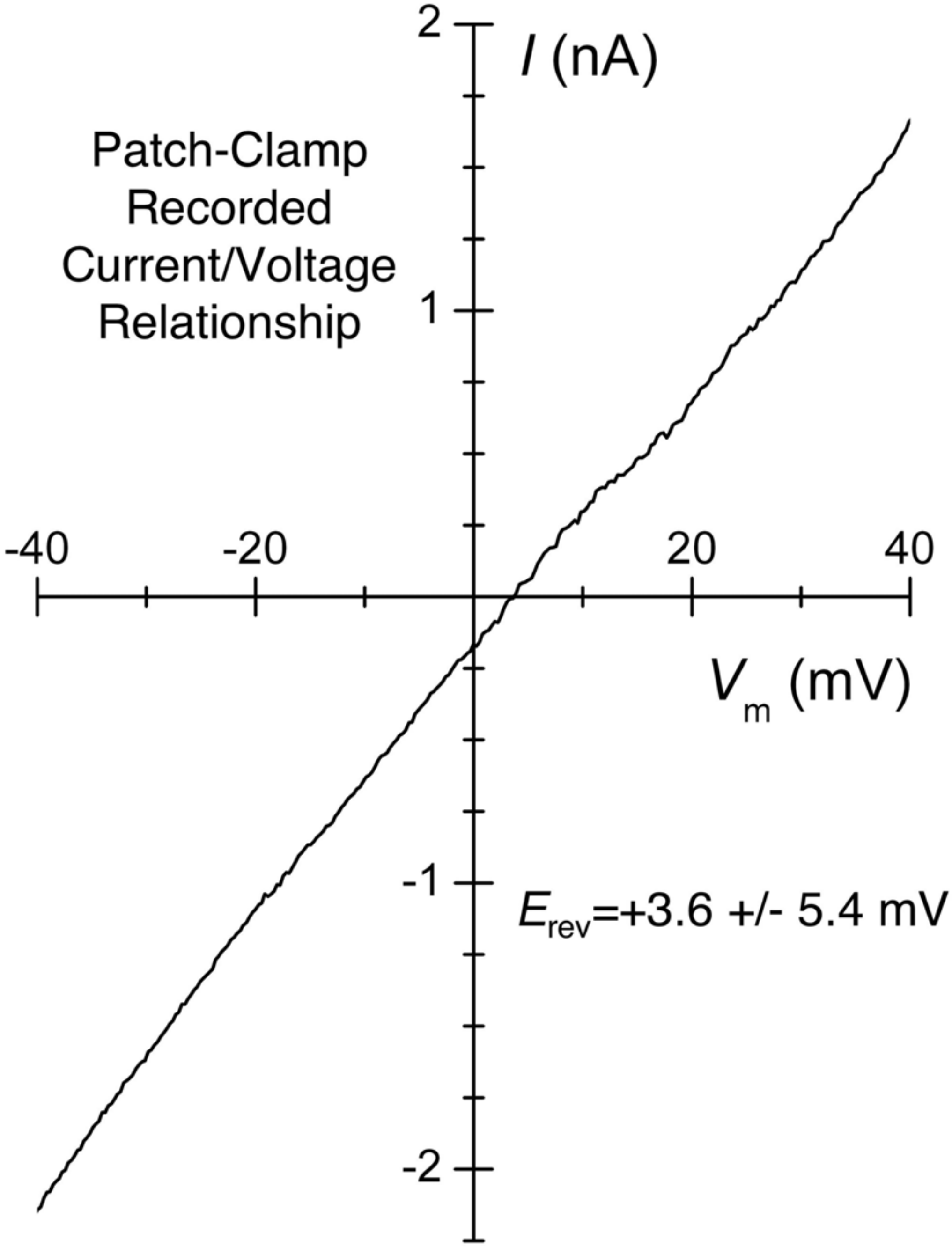

2
I (nA)
Patch-Clamp
Recorded
Current/Voltage
Relationship
1
-40
-20
20
40
V_m (mV)
-1
E_{rev}=+3.6 +/- 5.4 mV
-2

Figure 4.8. Inhibition of hemolysis of rainbow trout erythrocytes due to exposure to 0.25, 0.5 or 1 μg/ml KmTx 2 after co-incubation with 30 mM of either sucrose (MW 342.3), polyethylene glycol (MW 400), polyethylene glycol (MW 600), maltohexaose (MW 990.0), polyethylene glycol (MW 8,000), dextran (MW 10,000).

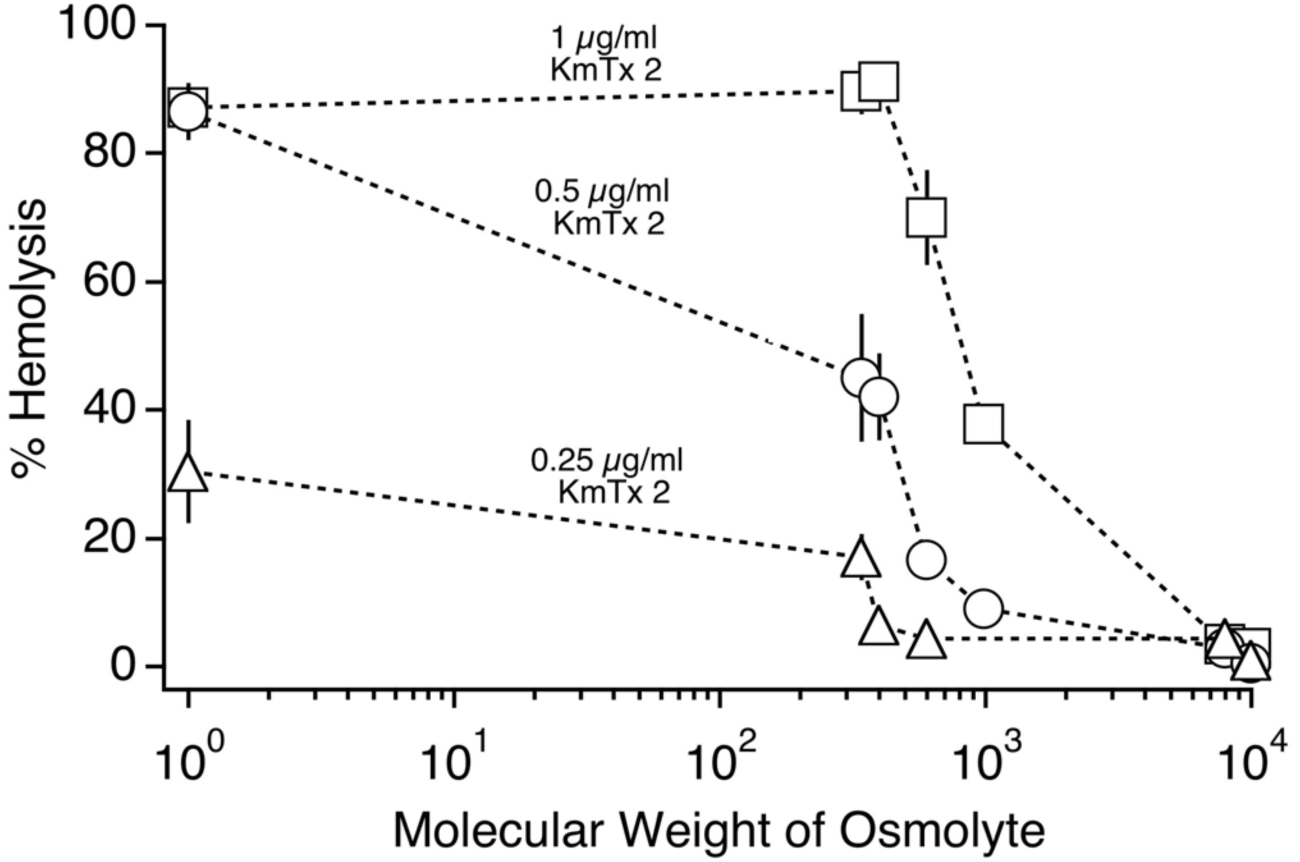

1 μg/ml
KmTx 2
0.5 μg/ml
KmTx 2
0.25 μg/ml
KmTx 2
% Hemolysis
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
Molecular Weight of Osmolyte

Figure 4.9. [A.] One-hour exposure of *Karlodinium micrum* (CCMP 2282) to an increasing concentration of KmTx 2. Histogram [left axis] represents number of *K. micrum* counted using a Coutler Multisizer II particle counter enumerating the 7-20 µm size fraction. Filled diamonds [right axis] represents hemolysis of rainbow trout erythrocytes due to KmTx 2 remaining in solution after one-hour exposure. Lines represent one standard deviation (n=3) for both. [B.] One-hour exposure of a Chesapeake Bay isolate of *Oxyhhris marina* exposed to an increasing concentration of KmTx 2. Histogram [left axis] represents number of *O. marina* counted using a Coutler Multisizer II particle counter enumerating the 15-30 µm size fraction. Filled diamonds [right axis] represents hemolysis of rainbow trout erythrocytes due to KmTx 2 remaining in solution after one-hour exposure. Lines represent one standard deviation (n=3) for both.

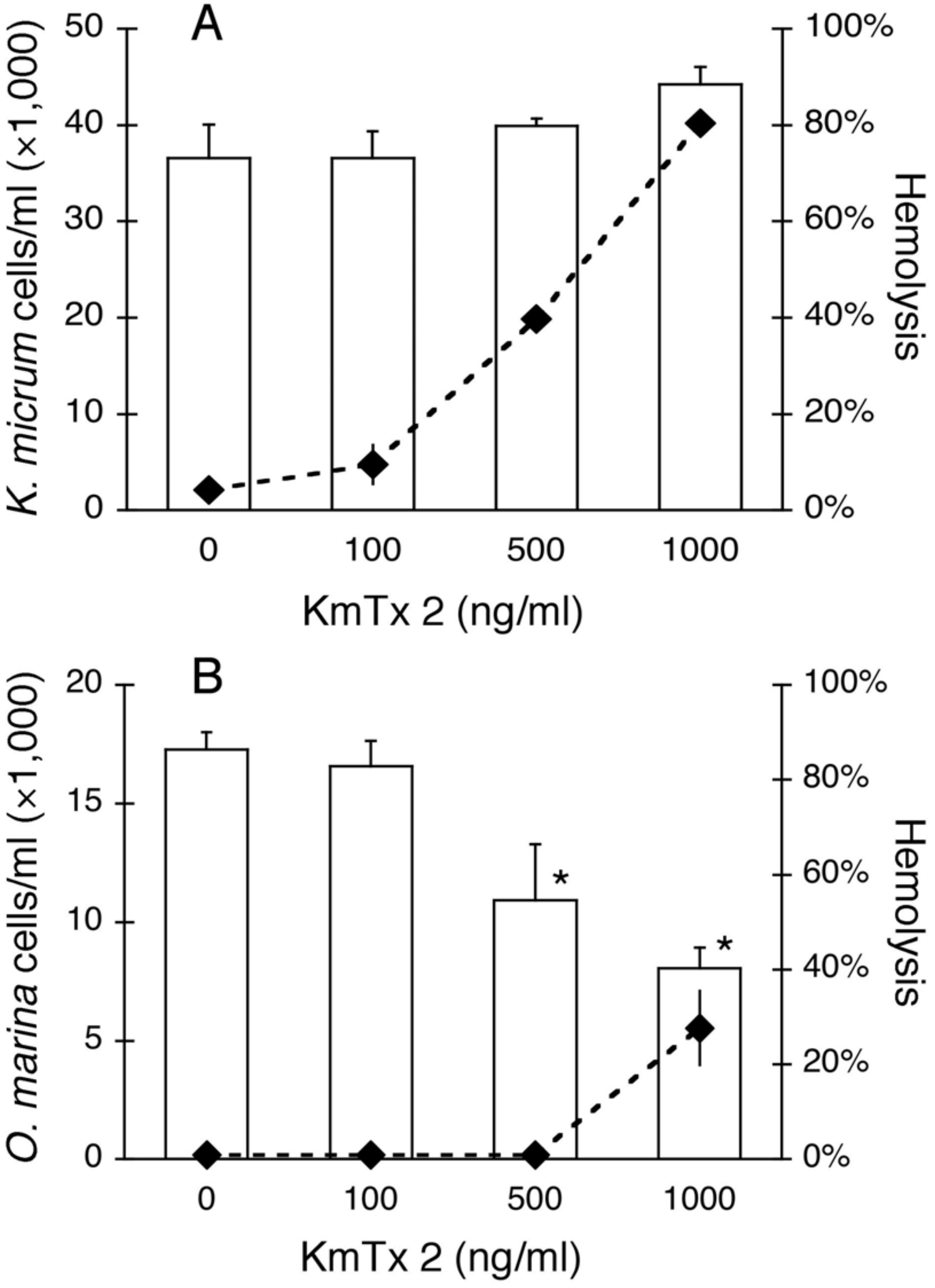

A
K. micrum cells/ml (×1,000)
50
40
30
20
10
0
100%
80%
60%
40%
20%
0%
Hemolysis
0
100
500
1000
KmTx 2 (ng/ml)
B
O. marina cells/ml (×1,000)
20
15
10
5
0
100%
80%
60%
40%
20%
0%
Hemolysis
*
*
0
100
500
1000
KmTx 2 (ng/ml)

Figure 4.10. Hemolysis of rainbow trout erythrocytes due to exposure to 0, 0.1, 0.5, or 1 μg/ml KmTx 2 after co-incubation with [A.] gymnodinosterol isolated from *Karlodinium micrum* (CCMP 2282), [B.] ergosterol, and [C.] cholesterol. For all: [□] 0, [○] 0.001, [Δ] 0.01, [∇] 0.1, [◊] 1, [⊗] 10 μM sterol.

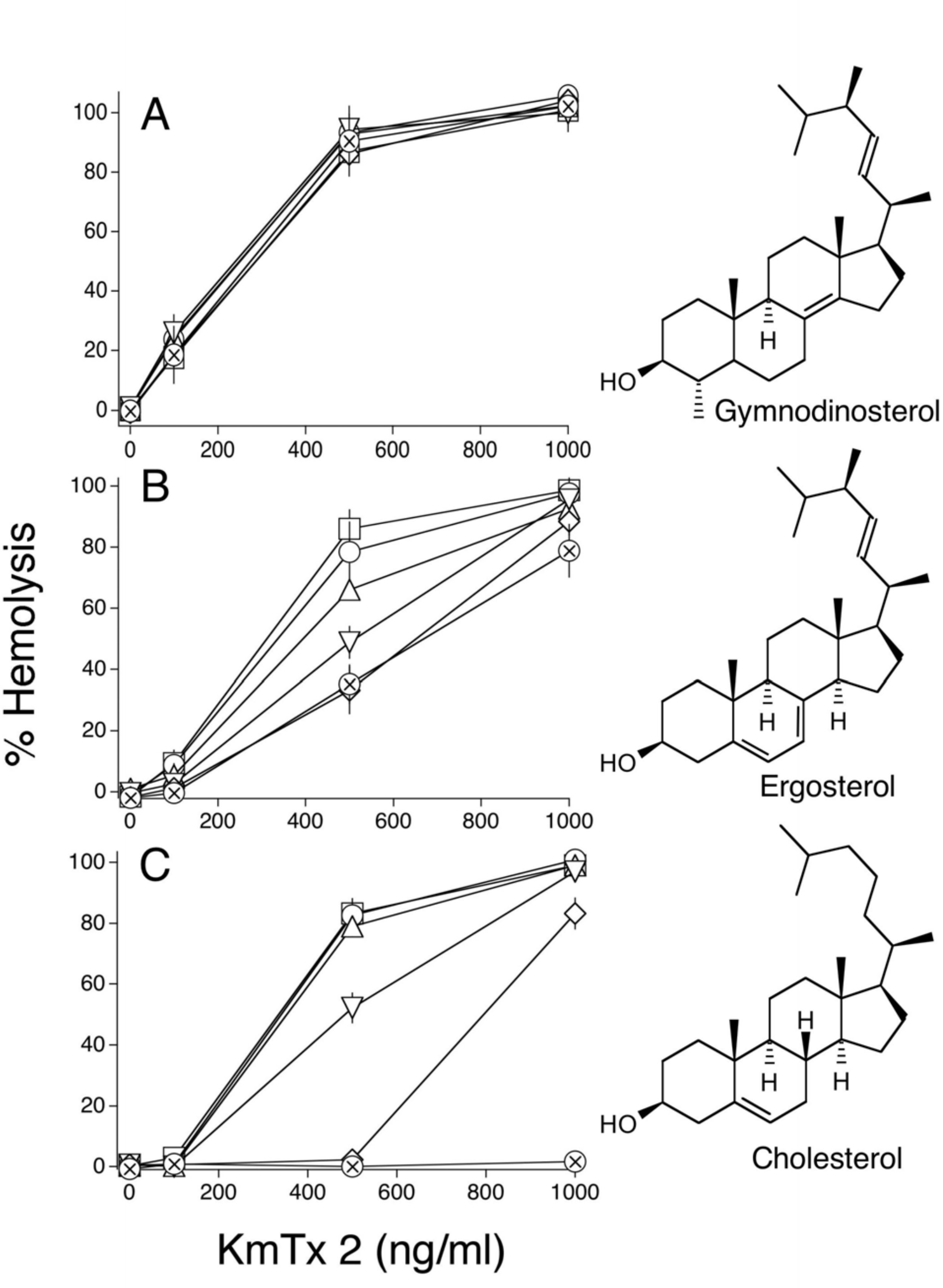

A
B
C
% Hemolysis
KmTx 2 (ng/ml)
0
20
40
60
80
100
0
200
400
600
800
1000
HO
H
Gymnodinosterol
Ergosterol
Cholesterol

Figure 4.11. Hemolysis of rainbow trout erythrocytes due to exposure to 0, 0.1, 0.5, or 1 μg/ml KmTx 2 after co-incubation with [A.] synthetic dipalmitoyl (16:0) phosphotidylcholine [□] 0, [○] 5, [Δ] 10, [∇] 20 μM sterol. [B.] Natural brain (porcine) phosphotidylcholine. [□] 0, [○] 0.001, [Δ] 0.01, [∇] 0.1, [◊] 1, [⊗] 10 μM sterol.

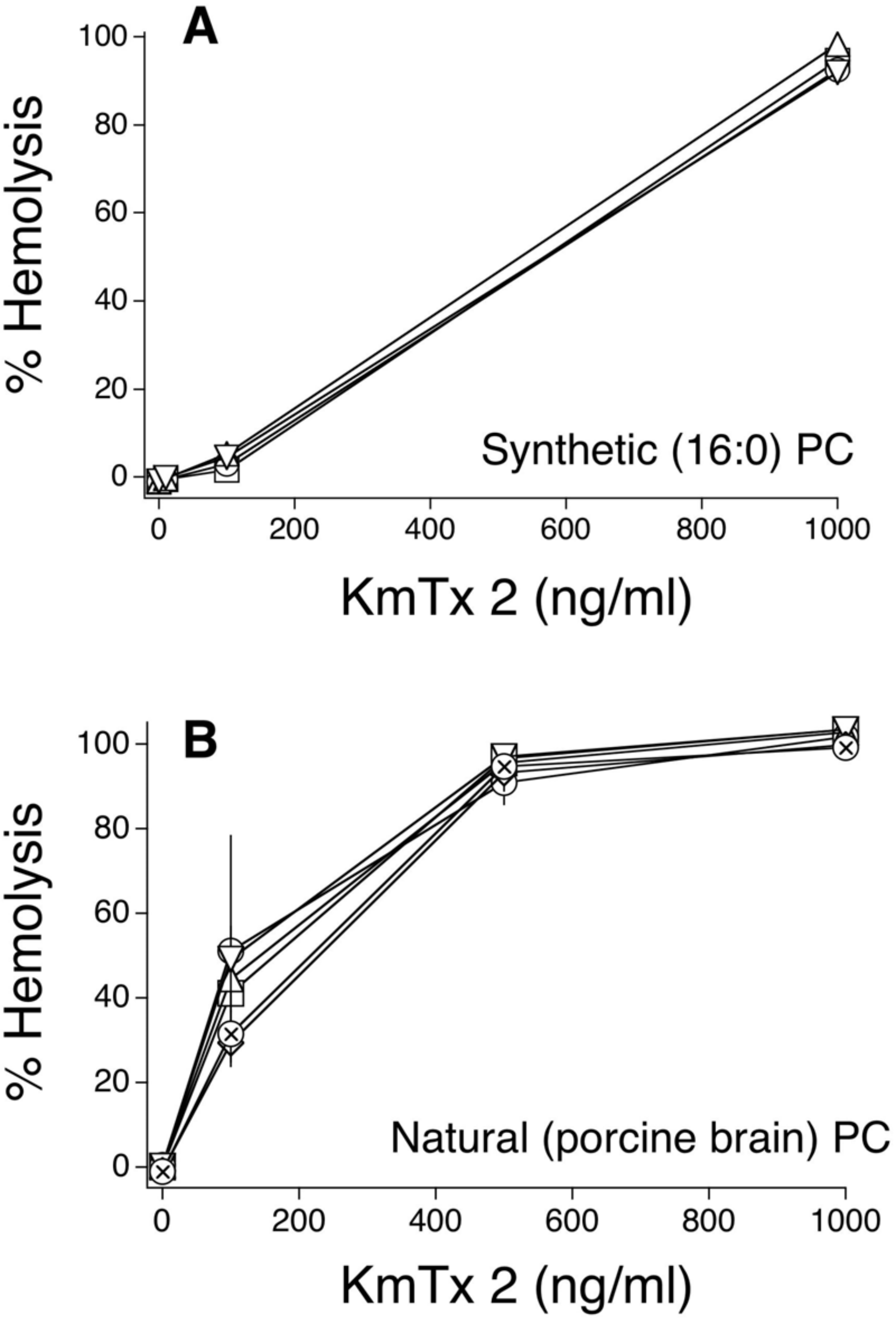
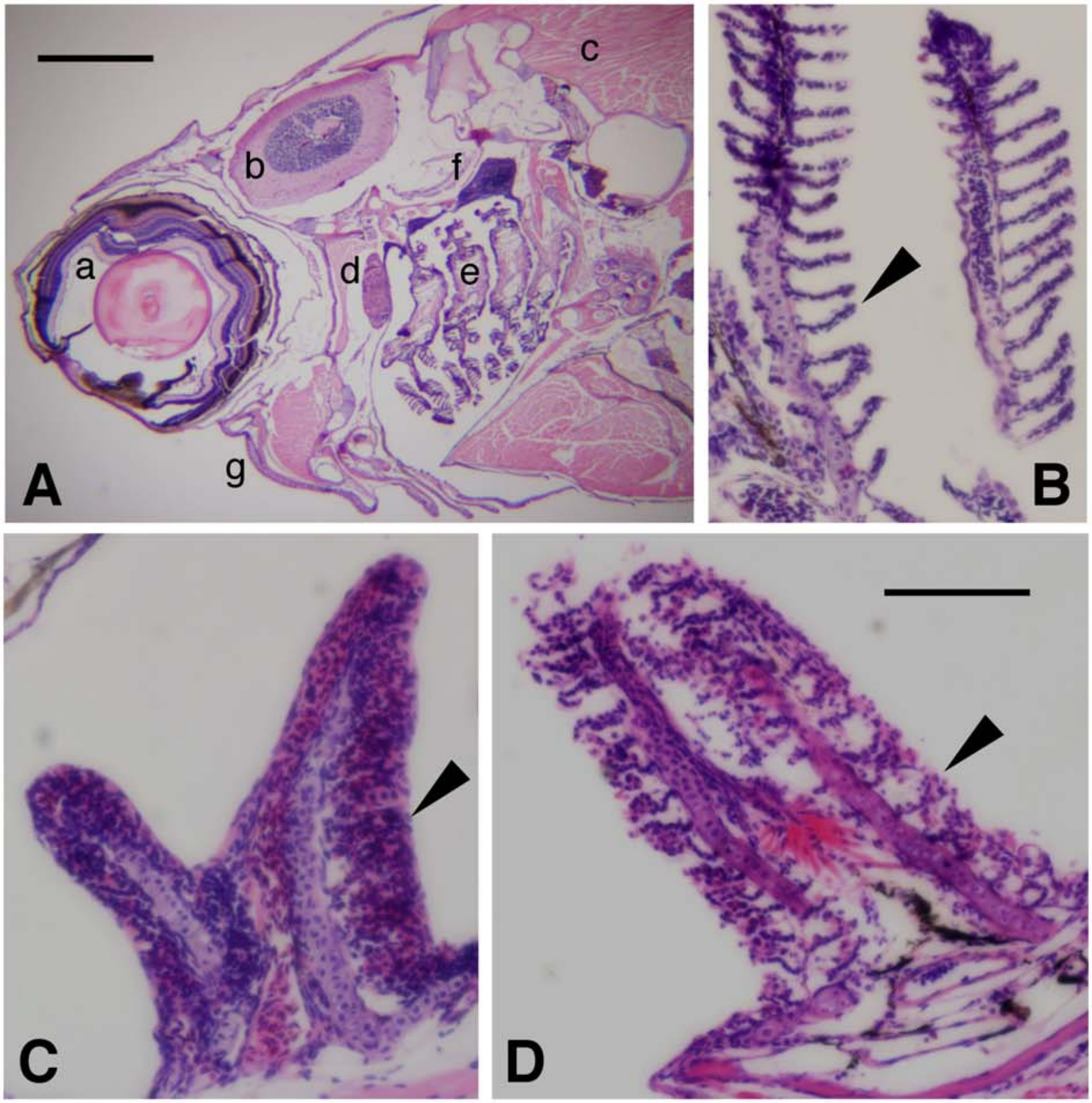

A
% Hemolysis
100
80
60
40
20
0
Synthetic (16:0) PC
0
200
400
600
800
1000
KmTx 2 (ng/ml)
B
% Hemolysis
100
80
60
40
20
0
Natural (porcine brain) PC
0
200
400
600
800
1000
KmTx 2 (ng/ml)

Figure 4.10. H&E stained sections of whole 60-day old zebrafish (*Danio rerio*) exposed to an increasing concentration of KmTx 2. [A.] Control at 6 hrs. [a. eye, b. brain, c. skeletal muscle, d. pseudobranch, e. gills, f. thymus, g. skin] [B.] Control gills at 6 hrs. [C.] 0.1 μg/ml KmTx 2 exposure at 6 hrs. showing loss of interlamellar spacing and edema. [D.] 0.5 μg/ml KmTx 2 exposure at 1 hr. showing extensive necrosis of gill epithelia. For [B.], [C.], and [D.] arrows indicate secondary gill lamellae. Bars equal 350 μm for [A.], and 50 μm for [B.], [C.], and [D.]



a
b
c
d
e
f
g
A
B
C
D

CHAPTER 5

Mode of Ichthyotoxicity of the Toxins from *Karlodinium micrum*

ABSTRACT

High densities of the estuarine dinoflagellate *Karlodinium micrum* have been associated with fish kills both in natural systems and aquaculture facilities for decades. As part of the current research effort, we have shown that the toxins from *K. micrum* are acutely toxic to both larval and adult fish (Chapters 2 and 4). This study was undertaken to further explore the nature of the ichthyotoxic mode of action of the toxins from *K. micrum*. Larval zebrafish (*Danio rerio*) were exposed to both KmTx 1, from Chesapeake Bay, MD *K. micrum* cultures, and KmTx 2, from South Carolina *K. micrum* populations. Dose response relationships for exposure to both KmTx 1 and 2 were similar for both toxins. Mortality (100%) was observed at concentrations ≥ 1 μg/ml toxin with the entire epithelial surface being affected. For adult zebrafish exposed to KmTx 2 mortality began to occur at concentrations ≥ 0.1 μg/ml toxin with gills being the primary site of histopathologic damage. Hematoxylin and eosin stained sections of isolated gill arches showed fusion of secondary lamellae and mucous hypersecretion in all 0.1 μg/ml treated fish. Fish exposed to 0.5 μg/ml KmTx 2 died in < 1 hour. H&E stained sections of isolated gill arches showed extensive necrosis to the entire gill respiratory epithelium. These results confirm the ichthyotoxicity of the toxins from *K. micrum* and begin to provide regulatory data on toxin levels and fish exposure for environmental managers concerned with *K. micrum* blooms and fish health.

INTRODUCTION

Since its original description in Southwestern Africa in the 1950's, blooms of the dinoflagellate *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen have been associated with fish kills worldwide (Braarud, 1957; Hallegraeff, 1993; Landsberg, 2002). Nielsen (1993) showed for the first time that high densities of a North Atlantic strain of *K. micrum* were directly toxic to juvenile cod in laboratory exposures. *K. micrum* was not described in the United States until 1996, when it was associated with several fish kills, originally attributed to *Pfiesteria piscicida,* at a Maryland estuarine aquaculture facility (Burkholder and Glasgow, 1997; Li et al., 2000; Terlizzi et al., 2000); Chapters 2 and 4). It is believed that *K. micrum* was present in US East coast estuaries prior to this event but was misidentified as *Gyrodinium estuariale*, a common estuarine dinoflagellate that has not been linked to toxic events in the scientific literature (Marshall, 1980). As part of the investigation into fish kills at the Maryland aquaculture facility, we described several toxic fractions from a Chesapeake Bay culture of *K. micrum* (Deeds et al., 2002; Chapter 2). Since then we have isolated similar compounds from additional *K. micrum* isolates and from natural bloom samples, both with and without fish kills, from Maryland, North Carolina, South Carolina, Florida, and recently from a fish kill in Western Australia (Kempton et al., 2002; Goshorn et al., *Submitted*; Chapter 6). It has been established that *K. micrum* commonly co-occurs with the ichthyocidal dinoflagellates of the genus *Pfiesteria* (Marshall, 1999; unpublished data). However, several publications on the toxicity of *Pfiesteria* spp. have stated that *K. micrum* is non-toxic under putatively environmentally relevant conditions (for more details see Chapter

4). We have previously established that KmTx 1, the main toxin from *K. micrum* populations from the Chesapeake Bay is toxic to larval zebrafish (*Danio rerio*) and sheepshead minnows (*Cyprinodon variegatus*) (Deeds et al., 2002; Chapter 2). Furthermore, we have shown that KmTx 2, the main toxin from *K. micrum* populations originating from estuaries located South of the Chesapeake Bay, is toxic to adult zebrafish within the range of toxin concentrations found during fish kills associated with blooms of this organism (see Chapter 4). In that same study, it was shown that the primary site of histopathological damage in fish exposed to KmTx 2 is the gills.

This study used a combination of fish bioassays and standard histological techniques to further explore the nature of the ichthyotoxicity of the toxins from *K. micrum,* thereby establishing the potential role of this organism in estuarine fish kills.

MATERIALS AND METHODS

Isolation of KmTx 1 and KmTx 2

The isolation and characterization of KmTx 1, from Chesapeake Bay populations of *K. micrum*, and KmTx 2, from South Carolina *K. micrum* populations, were as previously described (see Chapters 2 and 4).

Exposures to Fish

Larvae

Exposures of both KmTx 1 and KmTx 2 (0-2 μg/ml) to larval zebrafish (*Danio rerio*) were as previously described (see Chapter 2). For one high dose experiment (4 μg/ml), larval zebrafish were exposed to KmTx 2 and gross morphological changes were observed and recorded using a digital video camera (Canon Elura 2, Canon, USA, Inc.) linked to an inverted microscope (Olympus IX70, Olympus America, Inc.). Still images were captured through the use of digital video editing software (iMovie, v. 2.1.2, Apple Computer, Inc.)

Adults

Zebrafish (*Danio rerio* - 90 days old) were exposed to either 0.1 or 0.5 μg/ml KmTx 2 in 50 ml of aerated reconstituted fresh water (soft) (ASTM, 1992). These concentrations were chosen as representative "low" and "high" doses based on previous experiments (see Chapter 4). Toxin dilutions were made in MeOH (200 μl max. per treatment) and controls were exposed to 200 μl MeOH only. Two fish were exposed per replicate, three replicates per treatment. For the high toxin dose (0.5 μg/ml) three replicates were sacrificed (6 fish) at 15 minutes post exposure. Three additional replicates (6 fish) were sacrificed at 1-hour post exposure. Three control replicates (6 fish) (MeOH exposed) were also sacrificed at 1 hour. For the low toxin dose treatments, (0.1 μg/ml) three replicates (6 fish each) were sacrificed at 1, 2, and 4 hours post exposure (18 fish total). An additional 3 control replicates (6 fish) were sacrificed at 4 hours. For both treatments, any fish that appeared moribund (i.e. swimming upside down either at the surface or at the vessels bottom, or unresponsive to gentle probing) were sacrificed and preserved immediately. Beakers with fish to be sacrificed were killed by rapidly lowering the water temperature by placing beakers in ice. Immediately upon fish death, 4F/1G preservative (4% formalin, 1% gluteraldehyde in phosphate buffered saline) was applied via syringe to the oral cavity and gills, then whole gills were dissected and placed in fresh 4F/1G. Out of 42 total fish (12 - 0.5 μg/ml treated, 6 - 1 hour controls, 18 – 0.1 μg/ml treated, and 6 – 4 hour controls), 10 fish (4 - 0.5 μg/ml treated, 2 - 1 hour controls, and 4 – 0.1 μg/ml treated) were preserved as whole decapitated heads. Tissues were preserved for at least 24 hours, then either whole heads or single dissected gill arches were rinsed in distilled-deionized H_2O, dehydrated in an ethanol series, then embedded in paraffin. Paraffin blocks were cut into 5-6 μm sections and stained with hematoxylin and eosin (H&E) according to Clark (1981).

RESULTS

Larval Exposures to KmTx 1 and KmTx 2

The 24-hour dose response relationship for mortality of 24-hour old (post-hatch) zebrafish (*Danio rerio*) was similar for KmTx 1 and KmTx 2 (Figure 5.1A). For both toxins, a sharp concentration dependence was observed (0% mortality for 0.5 µg/ml treated larvae and 80-100% mortality for 1 µg/ml treated larvae). It was an observation from both this and previous experiments (see Chapter 2) that larvae that survived the initial 24-hour exposure, even if some epithelial damage was evident, would typically survive for at least 96 additional hours (the limit that was tested). Based on data from previous experiments (see Chapter 4), adult zebrafish exposed to the same concentrations of KmTx 2 were found to be more sensitive to the toxins effects (Figure 5.1B). For adults exposed to KmTx 2, 100 % mortality occurred in 0.5 µg/ml treated fish, while all 0.1 µg/ml treated fish were moribund and showed severe damage to gill epithelia.

In order to observe gross histological changes to zebrafish larvae, larvae were exposed to a high dose (4 µg/ml) of KmTx 2 and the first 30 min. post-exposure was videotaped using a digital video camera linked to an inverted microscope. At this dose, fish died within 5-10 minutes. All epithelial surfaces appeared to be affected with severe cellular swelling and epithelial sloughing. These effects were easily observed at the corneal epithelium and the trunk region distal to the pectoral fins (Figure 5.2). In previous experiments (see Chapter 2) similar results were observed for sheepshead minnow (*Cyprinodon variegates*) larvae exposed to a sonicated *K. micrum* culture (Figure 5.3).

Adult exposures to KmTx 2

For adult zebrafish exposed to a high dose of KmTx 2 (0.5 µg/ml), 5 of 6 fish died within the 1 hour-exposure period (83%). Average time to death was 18 min. (+/- 4 min.)

with the remaining fish being sacrificed at 15 min. For the low dose exposure (0.1 μg/ml) 8 of 18 fish died within the 4 hour-exposure period (44%). Of those that died, the average time to death was 81 min. (+/- 35 min.). No controls (MeOH exposed) died or showed signs of stress during the experiment. All toxin treated fish showed excessive mucous production during the experiment. Strings of mucus were observed trailing from the opercular cavity, and clusters of mucus strings were often observed on the exposure vessel bottoms.

Sectioned and H&E stained control gill arches (both 1 and 4 hour) showed no obvious pathologies (Figure 5.4). For sectioned and H&E stained isolated gill arches of 0.5 μg/ml KmTx 2 treated fish, observed pathologies included: extensive necrosis and edema of the respiratory epithelium, with curling of secondary lamellae and some thrombosis in both primary and secondary lamellae (Figure 5.5). For 0.1 μg/ml KmTx 2 treated fish, observed pathologies of sectioned and H&E stained isolated gill arches included: extensive fusion of secondary lamellae and congestion of both primary and secondary lamellar vessels, with some cellular hyperplasia and shortening of secondary lamellae and some mild necrosis of respiratory epithelia (Figure 5.6). In H&E stained sections of zebrafish heads, cellular damage was only observed at the gills. This is in accord with previous experiments where whole zebrafish were sectioned after exposure to a range of KmTx 2 concentrations (0.1-2 μg/ml) (see Chapter 4). Histological damage was similar to that observed for dissected gill arches.

DISCUSSION

In his 1993 review of the global effects of harmful algal blooms, Hallegraeff divided HAB's into three main types: 1. Species whose harmful effects are related mainly to biomass and result in indiscriminate aquatic faunal kills due to low dissolved oxygen, 2. Species which produce potent toxins that bioaccumulate through the food chain and can effect humans, and 3. Species which are non-toxic to humans but have harmful effects on fish and aquatic invertebrates due mainly to either physical or chemical damage to delicate gill structures. Based on all of our data collected thus far, the toxins produced by *Karlodinium micrum* fall into category three, species which produce toxins that have their most pronounced harmful effects on fish and aquatic invertebrates.

Several phytoplankton species, responsible for substantial aquacultural related losses, have been shown to kill fish through the production of chemical substances grouped loosely into the category of "hemolysins". Some examples of these include the raphidophyte *Heterosigma akashiwo*, the prymnesiophytes *Chrysochromulina polylepis* and *Prymnesium parvum*, and the dinoflagellate *Gyrodinium auroelum* (now *Gyrodinium mikimotoi* - syn. *G. nagasakiense*) (Roberts et al., 1983; Guo et al., 1996; Khan et al., 1997; Johansson and Graneli, 1999). Exposure to high densities ($10^4 – 10^5$ cells ml^{-1}) of several of these species have been shown to elicit a stereotypical response in fish gills including increased ion permeability, edema, hyperplasia, and epithelial necrosis (Ulitzer and Shilo, 1966; Jones et al., 1982; Roberts et al., 1983; Edvardsen and Paasche, 1998).

In the current study, relatively high doses (> 1 μg/ml) of both KmTx 1 and KmTx 2 were found to have a generalized necrotic effect on the entire epithelial surface of larval zebrafish (*Danio rerio*). Previous studies found this same effect on larval sheepshead minnows (*Cyprinodon variegatus*) exposed to both KmTx 1 and a sonicated *K. micrum* culture (see Chapter 2). For both, effects typically started with the pectoral and caudal fins and did not initially affect heartbeat or peripheral circulation. Gross anatomical observations included non-discriminate cellular swelling, followed by sloughing, of all epithelial surfaces (Figure 5.2). These observations led us to an initial hypothesis that the cellular mode of action of the toxins from *K. micrum* was to increase membrane permeability resulting in cell death through osmotic cell lysis. This hypothesis was later substantiated (see Chapter 4). On a cellular level, KmTx 2 was found to be equally toxic to a range of mammalian cell types including epithelial, nervous, connective, and muscular (see Chapter 4). But in adult fish exposed to KmTx 2, at lethal concentrations (> 0.1 μg/ml), gill tissue appeared to be the primary site of histopathological damage. This study was undertaken to both confirm the observation that gills were the primary site of toxicity in adult fish exposed to *K. micrum* toxins and to provide a more detailed histological record of these toxic effects.

In the majority of fish, the gill epithelium has been shown to be the primary site of gas exchange, ionic-regulation, acid-base balance, and nitrogenous waste excretion (Laurent and Dunel, 1980; Evans, 1987; 1998). In comparison, for a given amount of damage to a fish's gill epithelium, to cause the same organism level effects in a mammalian system a toxin would have to damage three major organs, the lungs, the kidneys, and the gut. The fish gill is sensitive to a range of environmental pollutants and has been used a model for toxic effects in aquatic systems (Baker, 1969; Abel, 1976; Toyoshima et al., 1985; Evans, 1987; Karan et al., 1998). However, a large statistical review of the scientific literature by Mallatt (1985) found that upon exposure to a range to aquatic pollutants and toxicants, as well as extremes in temperature and pH, branchial alterations and lesions were largely non-specific in nature. The most frequently observed alterations and lesions were: epithelial lifting, necrosis, hyperplasia, hypertrophy, rupture, bulbing and/or fusion of lamellae, hypersecretion and proliferation of mucocytes, and changes in chloride cells and gill vasculature (Mallatt, 1985). It was Mallatt's conclusion that the most frequently recorded types of gill epithelial alterations and lesions were stereotyped physiological reactions to stress and many should be considered defensive responses. In this study, many of the gill epithelial alterations recorded in Mallot's literature review were observed in adult zebrafish exposed to KmTx 2. These included, for the low dose (0.1 µg/ml which caused 44% mortality in 4-hours), secondary lamellar fusion, mucus hypersecretion, vascular congestion, and some necrosis. For the high dose (0.5 µg/ml which caused 83% mortality in < 1-hour), histopathological effects to gill epithelium included extensive necrosis and epithelial lifting (edema), extensive congestion with some thrombosis and hemorrhage, mucous hypersecretion and curling of secondary lamellae. Mucous hypersecretion and loss of mucous goblet cells was observed in yellowtail (*Seriola quinqueradiata*) upon exposure to ichthyotoxic blooms of several HAB species (Shimada et al., 1982; Shimada et al., 1983). It was Shimada's (1982; 1983) conclusion that loss of the gills protective mucous coating, due to exposure to these sea blooms, resulted in fish death due to osmoregulatory impairment which caused the observed histologic gill epithelial damage. Abel (1976) showed that for brown trout (*Salmo trutta* L.) exposed to several concentrations of an anionic detergent, the histopathological effects on gill tissues were graded based on exposure concentration.

Abel found that at low concentrations (median survival times > 1 hour), epithelial cell death was due mainly to autolysis and was associated with acute inflammation of gill tissues and detachment of the respiratory epithelium. At higher exposure concentrations (median survival times < 1 hour), rapid cellular lysis occurred with complete disruption of cellular and tissue structures. Similar results were found in this study for fish exposed to either 0.1 or 0.5 μg/ml KmTx 2, with cellular damage being more extensive and rapid in nature at higher toxin concentrations. These observations would seem to fit with the established mode of cellular toxicity being increased membrane permeability due to non-specific ionic pore formation (see Chapter 4). Considering the non-specific nature of the pore formation due to exposure to KmTx 2, the exact reason why fish death appears to primarily result from gill damage prior to additional tissues being affected is not yet known. It should be noted that the effects on fish gills in this and previous studies (see Chapter 4) are occurring at concentrations below (100 ng/ml) the previously established cellular LD_{50}'s for KmTx 1 and 2 (284 ng/ml and 368 ng/ml, respectively). The mucous hypersecretion observed in this study, both extruding from the opercular cavity and on the exposure vessel bottoms, could possibly represent a loss of protection from the gill epithelial surface, in accord with Shimada et al.'s (1982; 1983) findings, which led to a loss of osmoregulatory function, which led to the observed histopathological damage. This could help to explain the lack of damage to less-exposed (internal organs) and more highly protected (external epithelia) at these low toxin concentrations. The extensive epithelial damage to larvae in this and previous experiments (see Chapter 2) occurred at higher toxin exposures (0.5-4 μg/ml). Regardless of the exact cause, the gill epithelia appears to be the primary site of histopathological damage leading to fish death upon exposure to toxic blooms of *K. micrum*. In addition, the sub-acute histological gill effects observed in this study would be expected to hyper-sensitize fish to environmental stressors (i.e. low dissolved oxygen or osmotic changes, both typical of estuarine environments) that they would typically survive with non-impaired gill function. The toxin levels required to cause extensive cellular damage to gill epithelia (0.1 μg/ml) are well within those found during some blooms of *K. micrum* (0.1-0.8 μg/ml) (Deeds et al., *Submitted*; Goshorn et al., *Submitted*; Chapter 6). This data further solidifies the role of *K. micrum* in estuarine fish kills and begins to provide regulatory data for toxin levels at which environmental managers should be concerned with exposure and fish health.

ACKNOWLEDGEMENTS

The author would like to acknowledge the University at Maryland at Baltimore's EM and Histology Laboratory for aid in the processing of tissue samples for histological examination and Dr. Renate Reimschuessel of the FDA Center for Veterinary Medicine, Laurel, MD, for aid in the histological evaluation of tissue samples.

Figure 5.1. Dose response relationship for zebrafish (*Danio rerio*) mortality upon exposure to *Karlodinium micrum* toxins. [A.] 24-hour exposure of zebrafish larvae to either KmTx 1 or KmTx 2. [B.] 6-hour exposure of 60-day old zebrafish to KmTx 2 (data from Chapter 4).

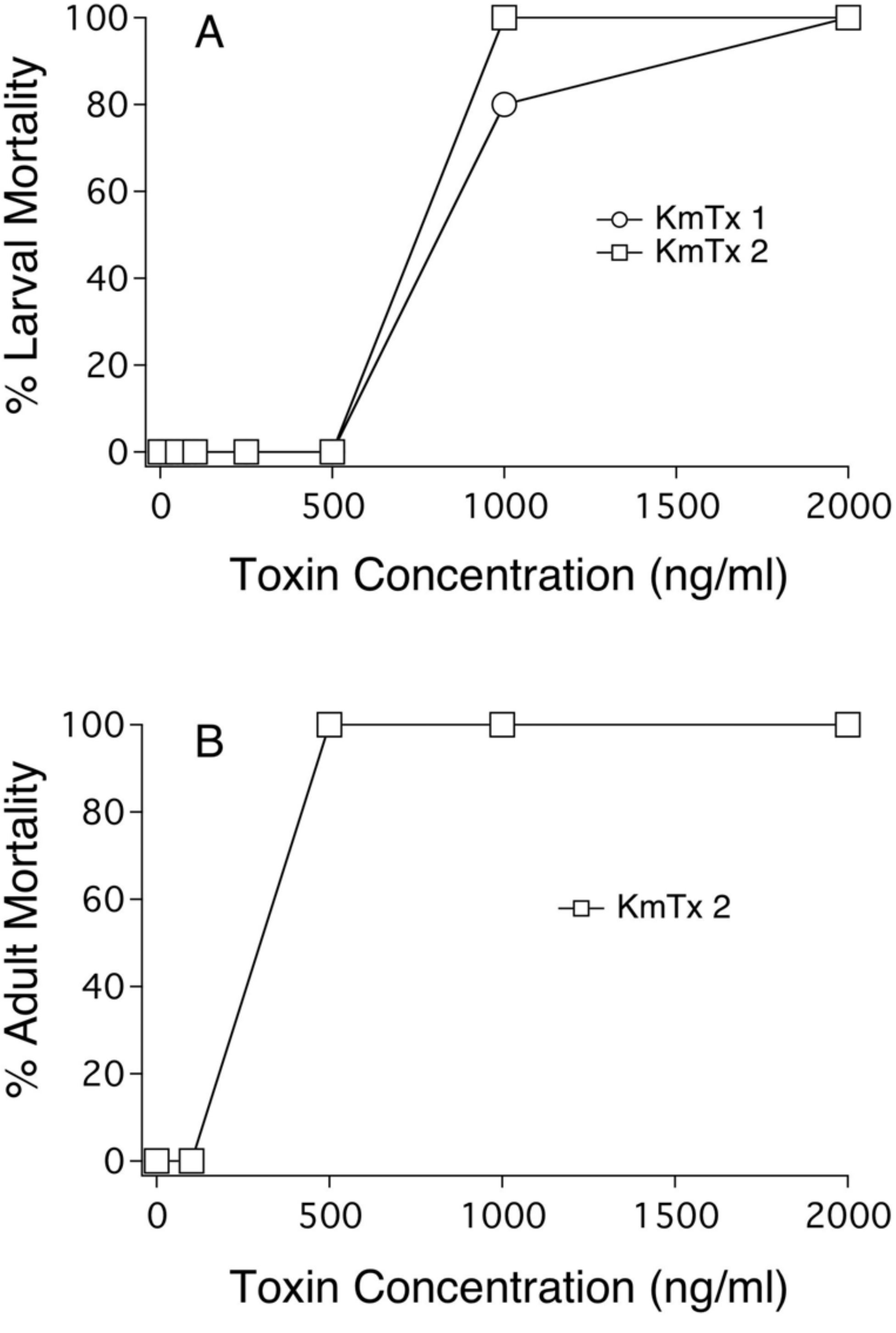

A
% Larval Mortality
0
20
40
60
80
100
0
500
1000
1500
2000
Toxin Concentration (ng/ml)
KmTx 1
KmTx 2
B
% Adult Mortality
0
20
40
60
80
100
0
500
1000
1500
2000
Toxin Concentration (ng/ml)
KmTx 2

Figure 5.2. 15-minute exposure of zebrafish (*Danio rerio*) larvae to 4 μg/ml KmTx 2. [A.] Control cornea. [B.] Control trunk. [C.] 4 μg/ml KmTx 2 exposed cornea. Note difference in corneal epithelium compared to control. [D.] 4 μg/ml KmTx 2 exposed trunk. Note epithelial swelling.

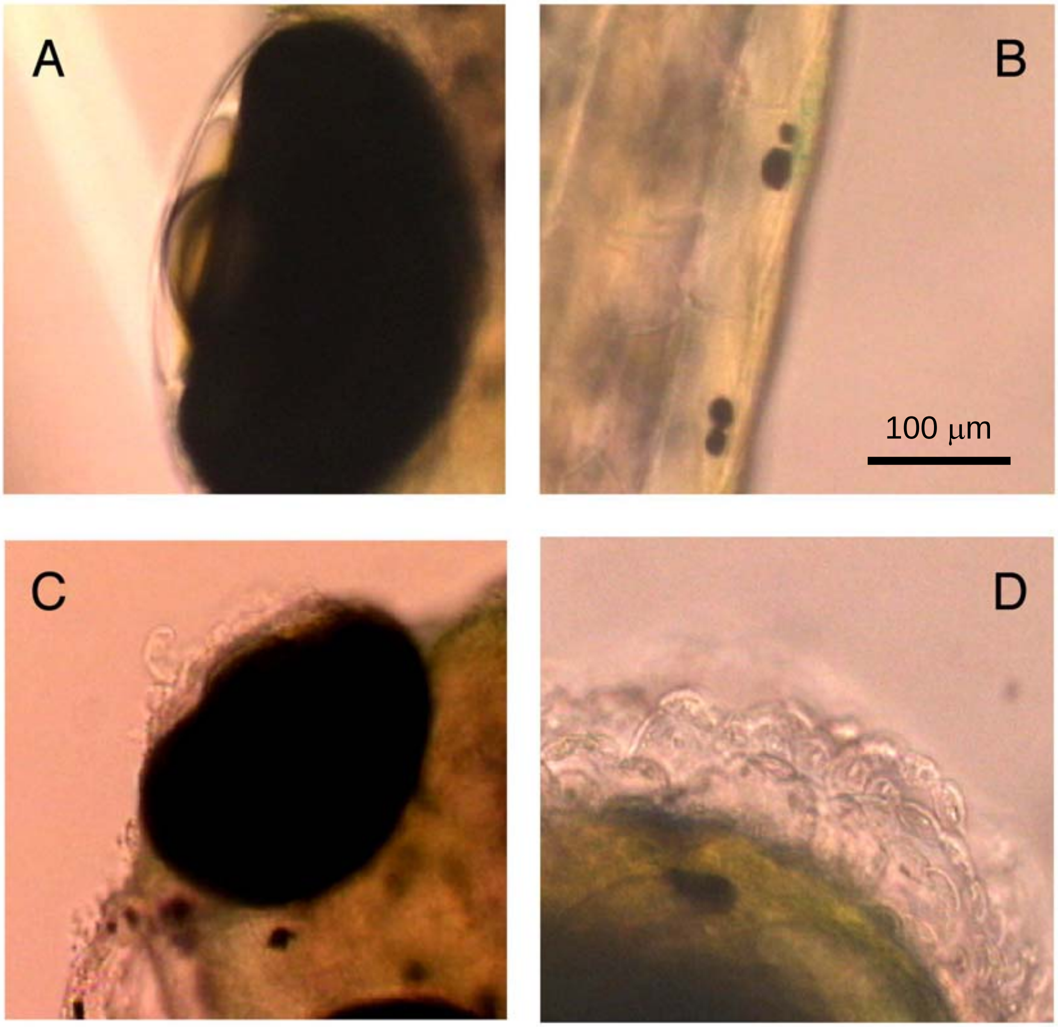

A
B
100 μm
C
D

Figure 5.3. Larval Sheepshead minnows (*Cyprinodon variegates*) (24 hours old post-hatch) exposed to a sonicated suspension of *Karlodinium micrum* (5.0×10^5 cells/ml). [A.] Control larvae. [B.] Larvae exposed to a non-sonicated culture of *K. micrum* (5.0×10^5 cells/ml). [C.] Larvae exposed to a sonicated culture of *K. micrum* (5.0×10^5 cells/ml) Note extensive cellular sloughing. (Described further in Chapter 2).

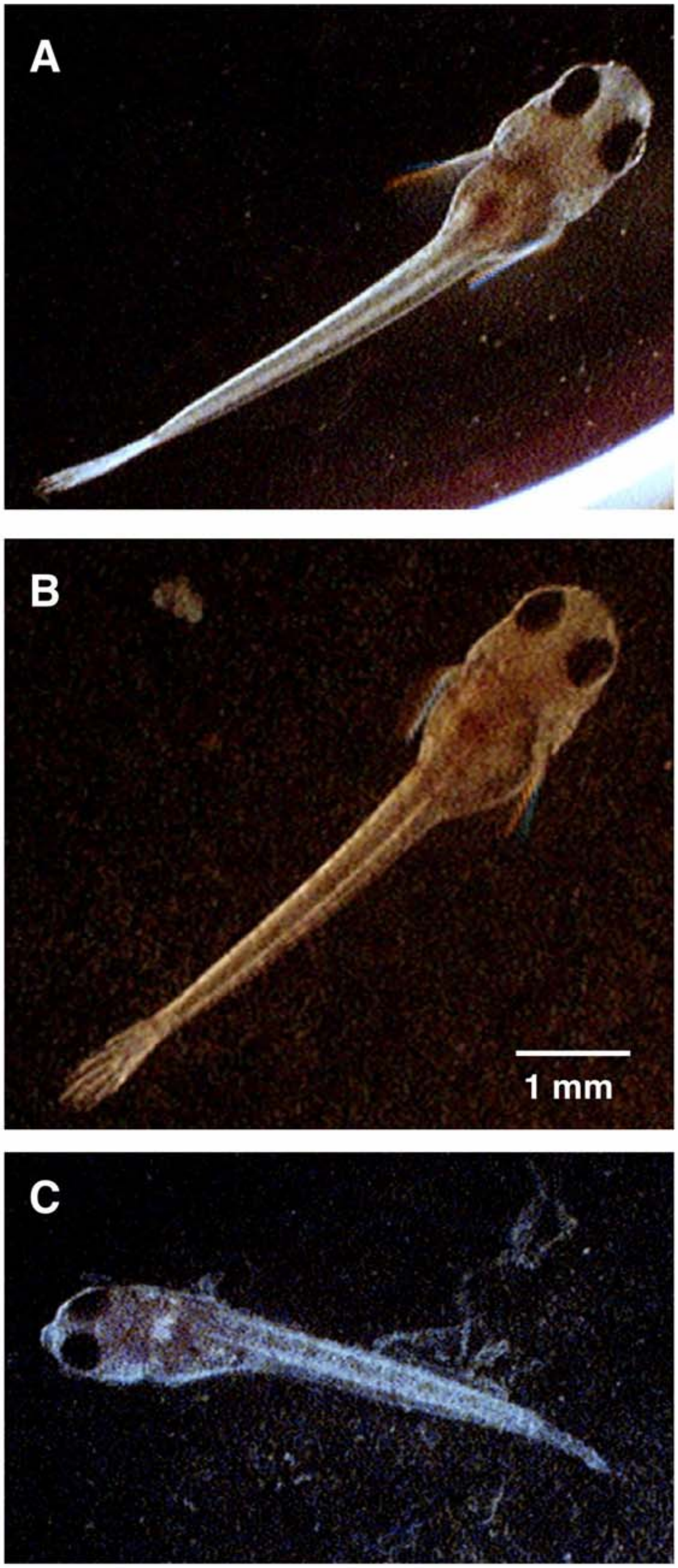

A
B
1 mm
C

Figure 5.4. H&E stained control (MeOH exposed) zebrafish (*Danio rerio*) gills in KmTx 2 exposure experiments. [A.] Control gill arch. [B.] Higher magnification of individual control gill filaments.

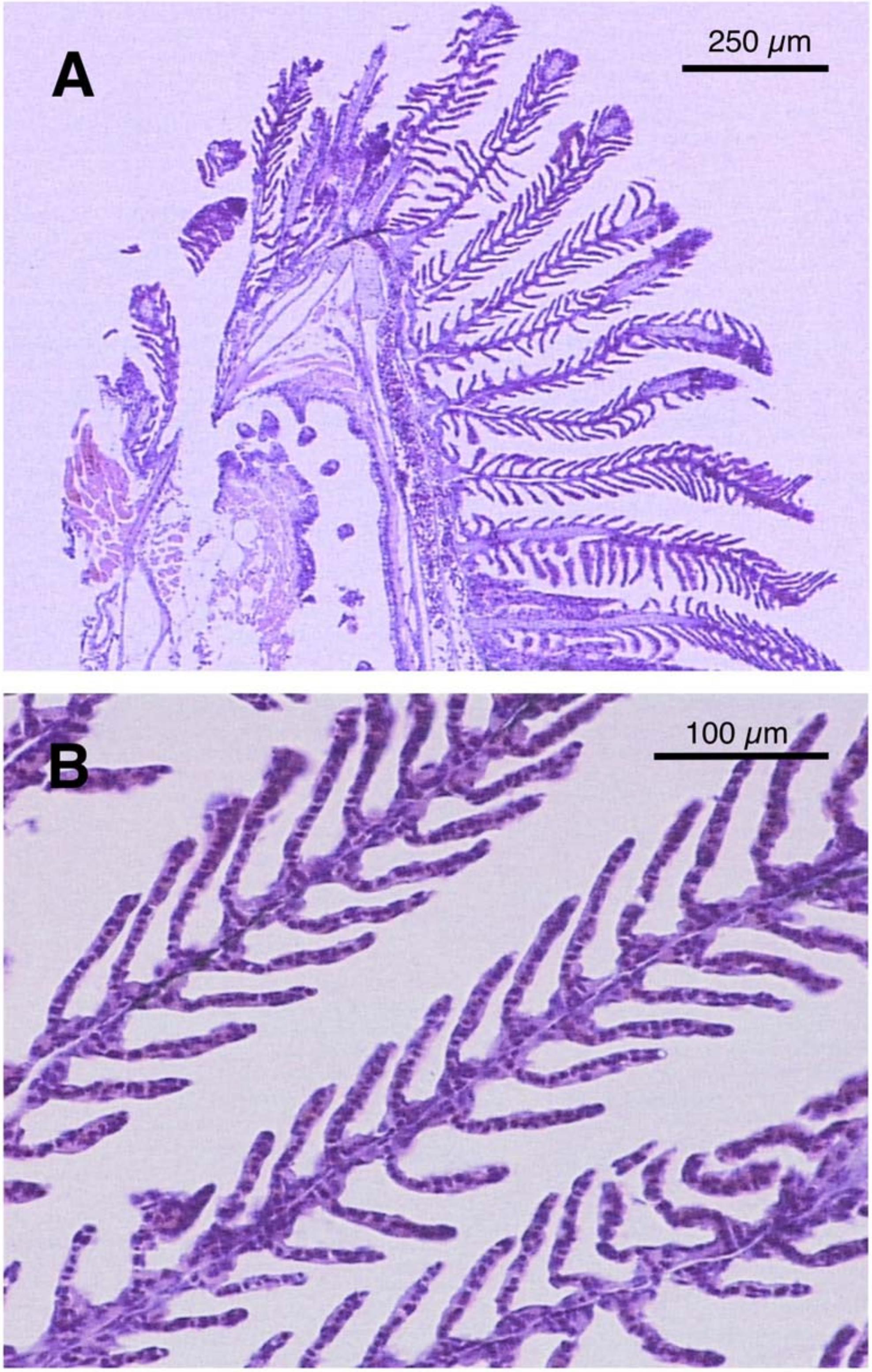

A
250 μm
B
100 μm

Figure 5.5. H&E stained gill arches of 0.1 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). [A.] H&E stained gill arch of 0.1 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). [B.] Higher magnification of primary gill filament of H&E stained gill arch of 0.1 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). Note loss of intra-lamellar spacing due to secondary lamellar collapse. [C.] Higher magnification of primary gill filament of H&E stained gill arch of 0.1 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). Arrows denote area of mild necrosis of respiratory epithelium. B and C are the same magnification.

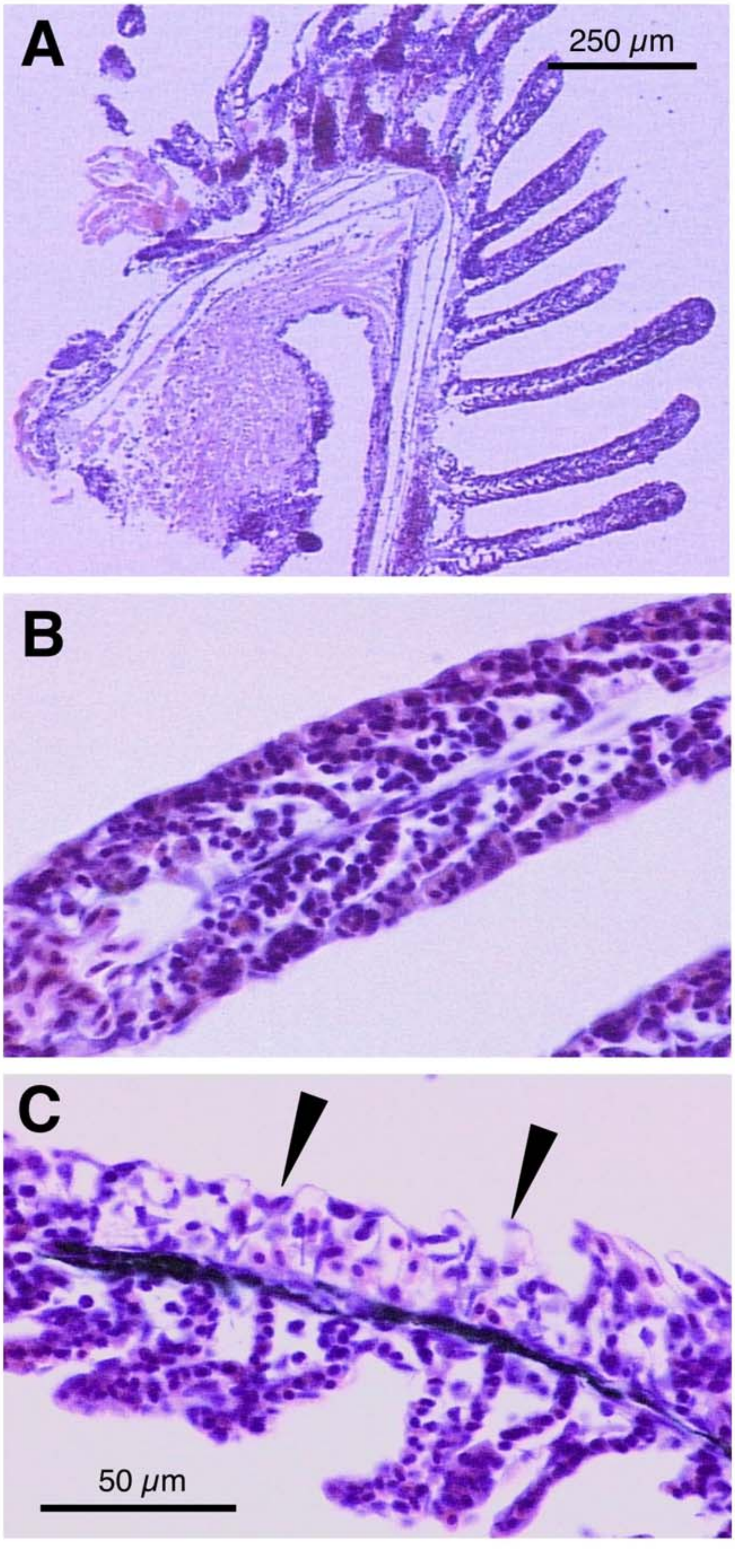
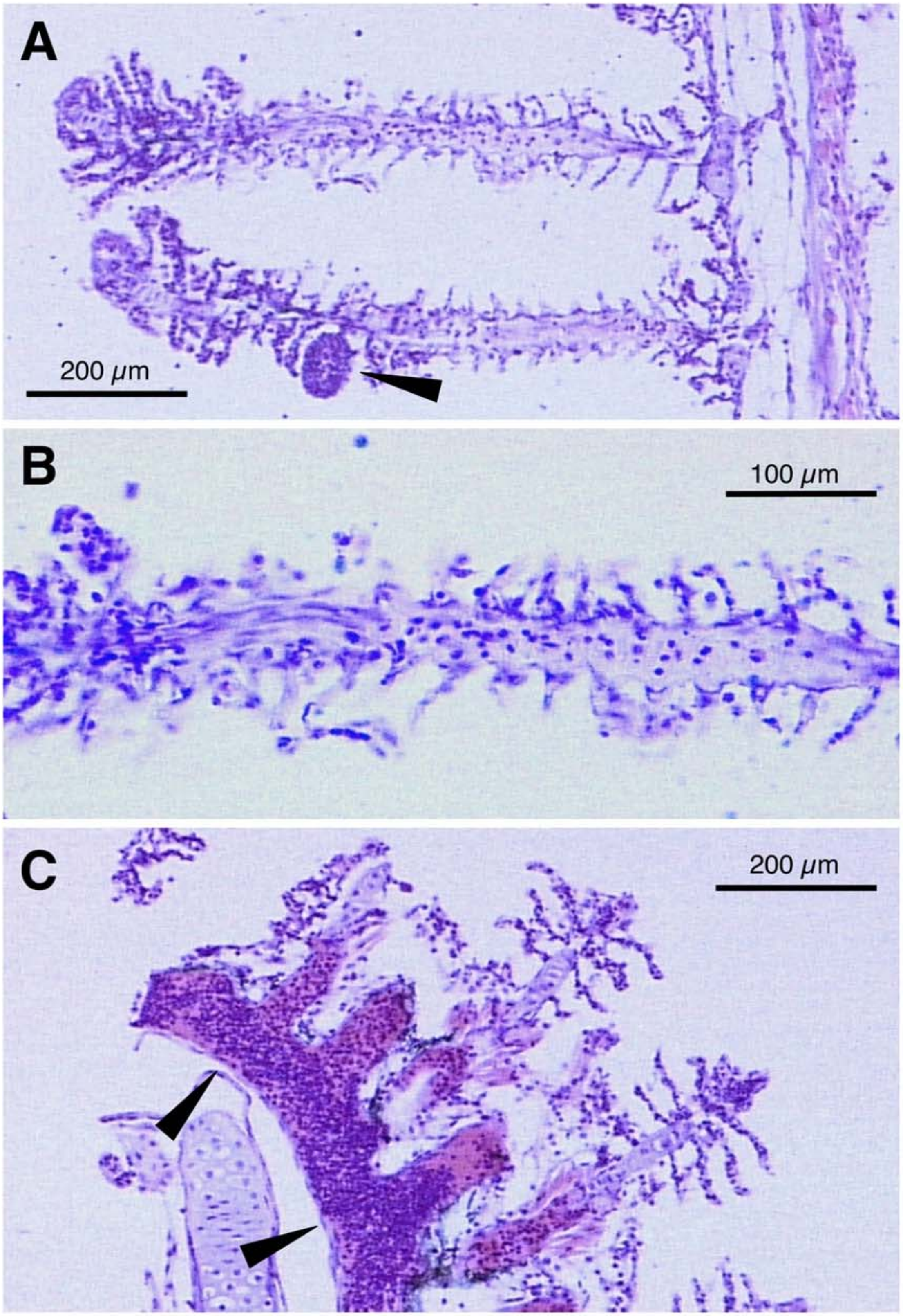

A
250 μm
B
C
50 μm

Figure 5.6. H&E stained gill arches of 0.5 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). [A.] H&E stained gill arch of 0.5 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). For A: arrow denotes thrombosis following rupture of capillary in secondary gill filament. [B.] Higher magnification of primary gill filament of H&E stained gill arch of 0.5 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). Note extensive necrosis of respiratory epithelium. [C.] H&E stained gill arch of 0.5 μg/ml KmTx 2 exposed zebrafish (*Danio rerio*). For C: arrows denote area of extensive congestion in central gill sinus.


A
200 µm
B
100 µm
C
200 µm

CHAPTER 6

Geographic Strain Variation in Toxin Production in *Karlodinium micrum* (Dinophyceae) from Southeastern Estuaries of the United States.[IV]

[IV] Deeds, J.R., Kibler, S.R., Tester, P.A., and Place, A.R. *Submitted*. Geographic strain variation in toxin production in *Karlodinium micrum* (Dinophyceae) from Southeastern estuaries of the United States. Proceedings of the Xth International Conference on Harmful Algae, St. Pete Beach, FL, USA, October 21-25, 2002.

ABSTRACT

Cultured isolates and natural water samples containing the ichthyotoxic dinoflagellate *Karlodinium micrum* were screened for the presence of recently discovered toxic compounds to test the hypothesis that the main toxin from Chesapeake Bay, Maryland, USA, isolates and water samples differed from isolates and water samples collected from other Southeastern estuaries of the United States. For the Chesapeake Bay, it was found that in four cultured isolates and four water samples, two with a fish kills and two without, KmTx 1 was the main toxin both in terms of amount and potency. For samples collected from estuarine waters from North Carolina, South Carolina, and Florida, in seven isolates and two water samples, both collected during fish kills, KmTx 2 was the main toxin. KmTx 2 was not detected in any KmTx 1 producing strains and KmTx 1 was not detected in any KmTx 2 producing strains. Based on this data, there does appear to be a geographic strain variation in toxin production among *K. micrum* populations from Southeastern estuaries of the United States.

INTRODUCTION

For decades, *Karlodinium micrum* has been associated with fish mortalities in temperate latitudes worldwide (Braarud 1957; Nielsen 1993; Landsberg, 2002). Recently in the United States, high densities (> 30,000 cells ml^{-1}) of this organism have been observed to co-occur with fish mortalities, both aquaculture and non-aquaculture related, typically in shallow, poorly flushed, estuarine tributaries (Deeds et al. 2002; Kempton et al. 2002).

In an attempt to determine the cause of repeated fish kills in an estuarine aquaculture facility in Maryland, USA, we have recently shown that *K. micrum* produces a unique suite of compounds with hemolytic, cytotoxic, and ichthyotoxic properties (Deeds et al., 2002) (Chapter 2). The discovery of these toxins may help to explain the adverse effects associated with high densities of this organism. Thus far, we have been able to detect these compounds in cultured isolates collected from estuarine waters both from Maryland and South Carolina, USA. In addition, we were able isolate these same toxins directly from water samples collected during a fish kill in a South Carolina brackish water pond in which high densities of *K. micrum* were present (Kempton et al., 2002).

Kempton et al. (2002), found that the main toxin isolated from both cultured cells and directly from a water sample collected during a fish kill (KmTx 2), was similar but not identical to the main toxin isolated previously from several Maryland, USA isolates (KmTx 1). This study was undertaken to test the hypothesis that there is a geographic strain variation in toxin production (KmTx 1 vs. KmTx 2) among *K. micrum* isolates from estuaries of the Southeastern United States. To accomplish this, eleven *K. micrum* isolates were acquired from estuarine waters ranging from Maryland to Florida. In addition, we were fortunate to acquire and test six water samples, four of which were collected during fish kills in which high *K. micrum* densities were present. In addition, we recently acquired two water samples that were collected from an estuarine river system in Western Australia that had been experiencing intermittent fish kills associated with a persistent bloom of *K. micrum* (See Addendum).

MATERIALS AND METHODS

The following *K. micrum* isolates were cloned by single cell isolation: GE (syn. CCMP 1974); GE 2-1 (syn. CCMP 1975), both acquired from D. Stoecker, Horn Point Environmental Laboratory, Cambridge MD; MD5CR0599; MD6CR0599, both acquired from M. Johnson, Horn Point Environmental Laboratory, Cambridge MD; 010410-B1 (CCMP 2282), 010410-C6 (CCMP 2283), and JW020205-B4 all acquired from J. Wolny, South Carolina Department of Natural Resources. All were grown at 20 °C, under 100 $\mu E\ m^{-2}s^{-1}$ illumination, in f/2-Si enriched artificial sea water (Instant Ocean Brand), either 12 or 32 PSU depending on original isolation, plus 1% soil extract, prepared according to Deeds et al. (2002) (Chapter 2). All were harvested for toxin extraction / identification, at cell concentrations ca. 1×10^5 cells ml^{-1}, according to procedures described in Deeds et al. (2002) (Chapter 2). Additional *K. micrum* isolates: Cell J; HR1NovC4; PIM05JulC4; and F205AprD2 were all grown at 23 °C, under 50 $\mu E\ m^{-2}s^{-1}$ illumination, in Gulf

Stream, USA, water diluted to 15 psu. Each of these four isolates were grown in parallel either autotrophically, by adding f/2-Si nutrient mixture, or mixotrophically, by periodically adding *Rhodomonas* (CCMP 767) as the only food source. All were harvested, at concentrations of 3-5 × 10^4 cells ml^{-1}, by gentle filtration using Whatman GF/F filters. Toxin extraction / identification was performed on frozen and thawed 500 ml filtrate samples, according to procedures described in Deeds et al. (2002) (Chapter 2). Four water samples, collected by the Maryland Department of Natural Resources and Department of the Environment, were also included in this study. Each sample contained cells, visually identified by representatives from the same organizations, as *K. micrum*. Two were nearly unialgal samples of *K. micrum* (4-5 × 10^4 cells ml^{-1}) collected during mixed fish kills. The others were: 1. a mixed algal sample collected during routine sampling, containing ca. 50% *K. micrum* (7 × 10^3 cells ml^{-1}), and 2. a sample containing 1.6 × 10^5 cells ml^{-1} *K. micrum* collected in an area where two small fish kills had occurred in the preceding weeks, both associated with low DO events. Another water sample associated with a fish kill was sent to our laboratory (frozen) for analysis by researchers at the North Carolina State University Pamlico Aquaculture Field Laboratory. The sample had been collected five days after a large unexplained kill of hybrid striped bass in a pond receiving water from South Creek, a tributary of the Pamlico river, North Carolina, USA. On the day of the kill *K. micrum* densities were reported to be ca. 3.5 × 10^4 cells ml^{-1}. The researchers had not previously observed kills in these ponds. The final water sample was collected during a mixed fish kill in a South Carolina brackish retention pond and contained 7 × 10^4 cells ml^{-1} *K. micrum*. Identification and toxin processing of this sample are described in Kempton et al. (2002). Prior to processing in our lab, all isolates and water samples were positively identified as containing *K. micrum* using a modified Taqman assay with PCR specific probes as described in Tengs et al. (2001).

RESULTS

For all of the isolates and water samples collected from the Chesapeake Bay, Maryland, USA, KmTx 1 was found to be the main hemolytic toxin both in terms of amount and potency. For all other isolates and water samples collected from North Carolina, South Carolina, and Florida, KmTx 2 was found to be the main toxin (Table 6.1). KmTx 1 was not detected in any KmTx 2 producing strains and KmTx 2 was not detected in any KmTx 1 producing strains. The estimated amount of toxin per cell for all of the isolates tested varied greatly (ca. 0.1 - 1 pg cell^{-1}). No trends in amount of toxin per cell were found between isolates containing KmTx 1 vs. KmTx 2 as the main toxin, nor were any trends observed for the amount of toxin per cell compared to length of time in culture. The estimated amount of toxin per cell for natural water samples tested in this study, with the exception of the North Carolina fish kill sample, was substantially greater (5-12 pg cell^{-1}) than for the cultured isolates. The sample sent from the North Carolina State University Aquaculture Field Laboratory, collected five days after the fish kill, contained trace amounts of KmTx 2 only. The NCSU Aquaculture Field Laboratory had not previously reported fish kills associated with *K. micrum*. The isolates; Cell J, F205AprD2, PIM05JulC4, and HR1NovC4, were each found to contain KmTx 2 as the primary toxin regardless of trophic state. Furthermore, although the amount of toxin per cell varied among individual isolates, within the range previously reported for cultures, no major differences were observed for the amount of toxin per cell based on their mode of nutrition (Figure 6.1).

DISCUSSION

In this study, *K. micrum* clonal isolates and water samples, from sites both with and without fish kills, were screened from the Chesapeake Bay, Maryland; The Neuse and Pamlico River estuaries, North Carolina; Charleston and Hilton Head, South Carolina; and the St. John's and St. Lucie River systems, Florida (Figure 6.2). After testing a total of eleven isolates and six water samples, we found that KmTx 1 was the main toxin in cells from the Chesapeake Bay and KmTx 2 was prevalent in all other Southeastern USA samples.

KmTx 1 (formerly Tox A) along with another hemolytic fraction *p*KmTx 3 (formerly Tox B), which has not yet been fully purified, was first discovered as part of an investigation into the cause of repeated fish kills associated with blooms of *K. micrum*. These kills occurred at an estuarine aquaculture facility in Maryland, USA described in Deeds et al (2002) (Chapter 2). KmTx 2 was first discovered as part of an investigation into the cause of a mixed fish kill along a tributary of Charleston Harbor, South Carolina, USA (Kempton et al., 2002). Using model vertebrate systems, the biological activities of KmTx 1 and KmTx 2 appear to be similar (Figure 6.3, Chapter 5) yet these two compounds have distinct HPLC retention times (Figure 6.4A), differing spectral characteristics (UV max: 224 nm for KmTx 1 and UV max: 235 nm for KmTx 2) (Figure 6.4B), and distinct molecular weights (1338.8 Da +/- 0.13 for KmTx 1 and 1344.8 Da +/- 0.13 for KmTx 2 (See Chapter 4)). It now appears that KmTx 1 and 2 also have distinct geographic distributions.

An interesting observation from this research has been that water samples containing high concentrations of *K. micrum* collected directly from waters in which a fish kill had recently occurred contained 5-100 times the amount of toxin, on a per cell equivalent, than all of the cultured isolates we have tested thus far. Due to the fact that both KmTx 1 and KmTx 2 are easily released from cells, it has been difficult thus far to assess the exact amount of toxin contained within a cell as opposed to being present in the surrounding water. By simply measuring the amount of hemolytic activity present in undisturbed cultures it has been estimated that >90% of the toxins are typically stored within the cells (Deeds et al., 2002; unpublished data). However, due to these technical difficulties it cannot be discriminated whether the high amounts of toxin present during fish kills are due to higher toxin producing populations of *K. micrum* or prolonged toxin build up in these waters due to cell disruption. Once released from cells and into the culture media, containing cellular debris and all associated bacteria, hemolytic activity is lost (at room temperature) over a period of 24-48 hours (Deeds et al., 2002; Chapter 2). Therefore, it is unlikely that strains with low toxin production could generate sufficient toxin to reach levels observed to be associated with fish kills, particularly at the warm temperatures (> 20 °C) at which this organism typically blooms (Li et al., 2000).

Additional research into the factors regulating *K. micrum* blooms and toxin production, such as nutrient limitation and selective grazing, may yield insights into why this observed variability in both toxin quality (type) and quantity occurs.

ACKNOWLEDGEMENTS

The author wishes to thank the members of the Maryland Departments of Natural Resources and the Environment, in particular Dave Goshorn, Peter Tango, Chris Lucket, and Walt Butler for providing *K. micrum* bloom samples and fish kill data. He would also like to thank Amber Garber and Charlene Couch of North Carolina State University for providing samples and observations from the North Carolina aquaculture fish kill. And finally, we would like to thank Wayne Litaker for critical review of this manuscript. This research was funded by grants from NOAA ECOHAB (NA860PO492), and NIEHS (PO1-ES9563).

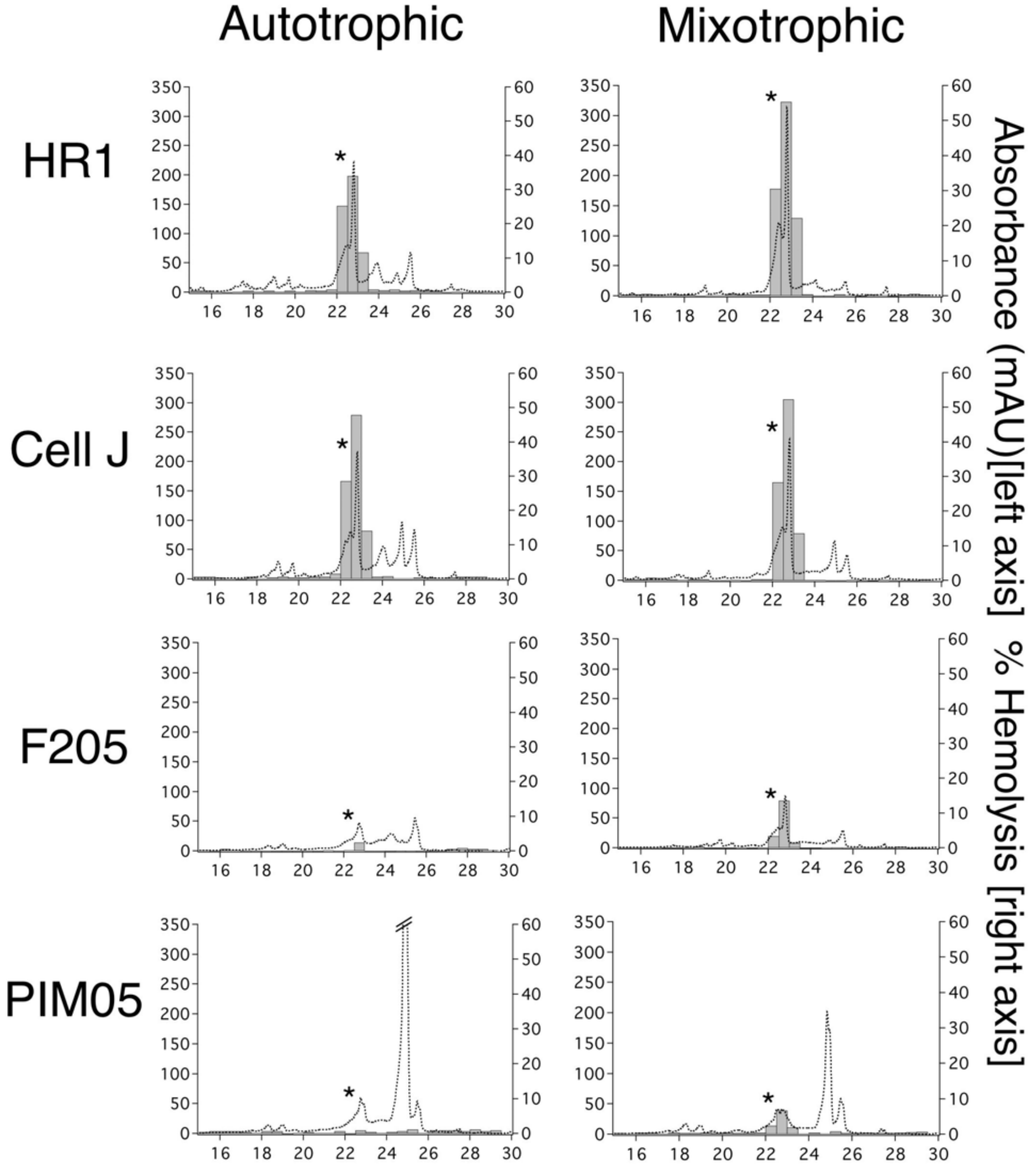
Table 6.1. *Karlodinium micrum* samples screened for the presence of KmTx 1 and KmTx 2.

Map Code	Isolate / Sample	Collection Location	Isolation Date	Salinity (PSU)	Main* Toxin
1	CCMP 1974	Chesapeake Bay, MD	5/95	12	KmTx1
2	CCMP 1975	HyRock Farm, MD	7/96	12	KmTx1
3	MD5CR0599	Choptank River, MD	5/99	12	KmTx1
4	MD6CR0599	Choptank River, MD	5/99	12	KmTx1
5	Water Sample[a]	Fishing Creek, MD	6/02	9	KmTx1
6	Water Sample[b]	Sharp's Island, MD	4/02	15	KmTx1
7	Water Sample[C]	Oak Creek, MD	6/03	6	KmTx1
8	Water Sample[D]	Goose Creek, MD	7/03	8	KmTx1
9	Cell J[e,f]	Neuse River, NC	7/99	15	KmTx2
10	Water Sample[g]	NCSU Field Lab	6/02	6	KmTx2
11	010410-B1	Hilton Head, SC	3/01	32	KmTx2
12	010410-C6	Hilton Head, SC	3/01	32	KmTx2
13	Water Sample[h]	Charleston, SC	2/02	11	KmTx2
14	JW020205-B4	Charleston, SC	2/02	15	KmTx2
15	F205AprD2[e]	St. John's River, FL	4/01	15	KmTx2
16	PIM05JulC4[e]	St. John's River, FL	7/00	15	KmTx2
17	HR1NovC4[e]	St. Lucie River, FL	11/00	15	KmTx2

[a] Mixed fish kill; 4×10^{4} cells ml^{-1} *K. micrum*.
[b] Routine sampling; 7×10^{3} cells ml^{-1} *K. micrum*.
[c] Suspect area, no fish kill, 1.6×10^{5} cells ml^{-1} *K. micrum.*
[D] Fish kill (3600 silversides), 5.7×10^{4} cells ml^{-1} *K. micrum.*
[e] Two samples each, one autotrophic, one mixotrophic.
[f] Genbank numbers AF352365, AF352367 (Litaker et al., 2003)
[g] Sampled five days after kill, 3.5×10^{4} cells ml^{-1} *K. micrum* during kill.
[h] Mixed fish kill; 7×10^{4} cells ml^{-1} *K. micrum*.

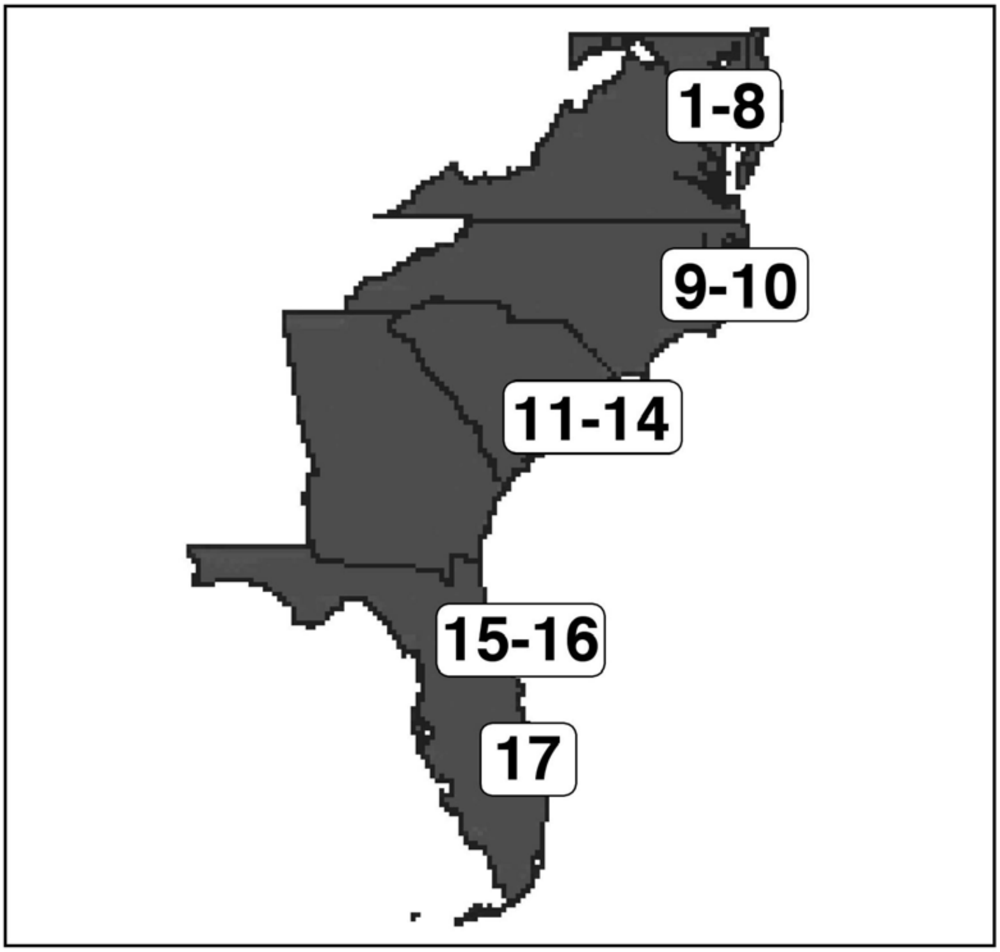
* In terms of amount and potency.

Figure 6.1. Comparison of four *K. micrum* isolates grown both autotrophically and mixotrophically. X axis: Elution Time (min). Left Y axis: (dashed line) Absorbance (mAU). Right Y axis: (histogram) % Hemolysis. Autotrophic samples avg. 3.4 × 10^4 cells ml^{-1}. Mixotrophic samples avg. 4.6 × 10^4 cells ml^{-1}. All graphs scaled to equivalent cell numbers. * indicates KmTx 2.



Autotrophic
Mixotrophic
HR1
Cell J
F205
PIM05
*
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
16
18
20
22
24
26
28
30
Absorbance (mAU)[left axis] % Hemolysis [right axis]
Elution Time (min)

Figure 6.2. Map of the Southeastern coast of the United States. Numbers represent sampling locations and correspond to sample information in (Table 1). Samples [1-8] were found to contain KmTx 1 as their main hemolytic toxin and samples[9-17] were found to contain KmTx 2 as their main hemolytic toxin.


1-8
9-10
11-14
15-16
17

[1-8] KmTx 1
[9-17] KmTx 2

Figure 6.3. Dose response curves for KmTx 1 and KmTx 2 based on lysis of rainbow trout erythrocytes.

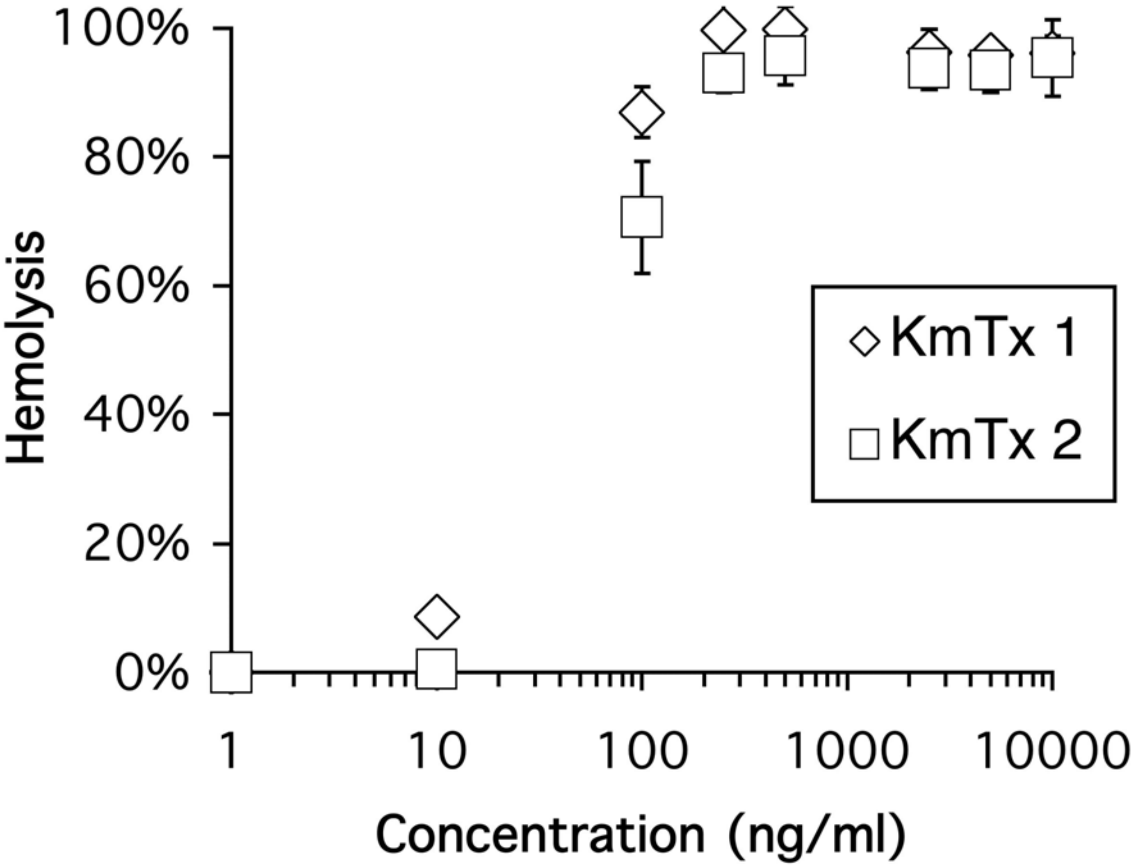
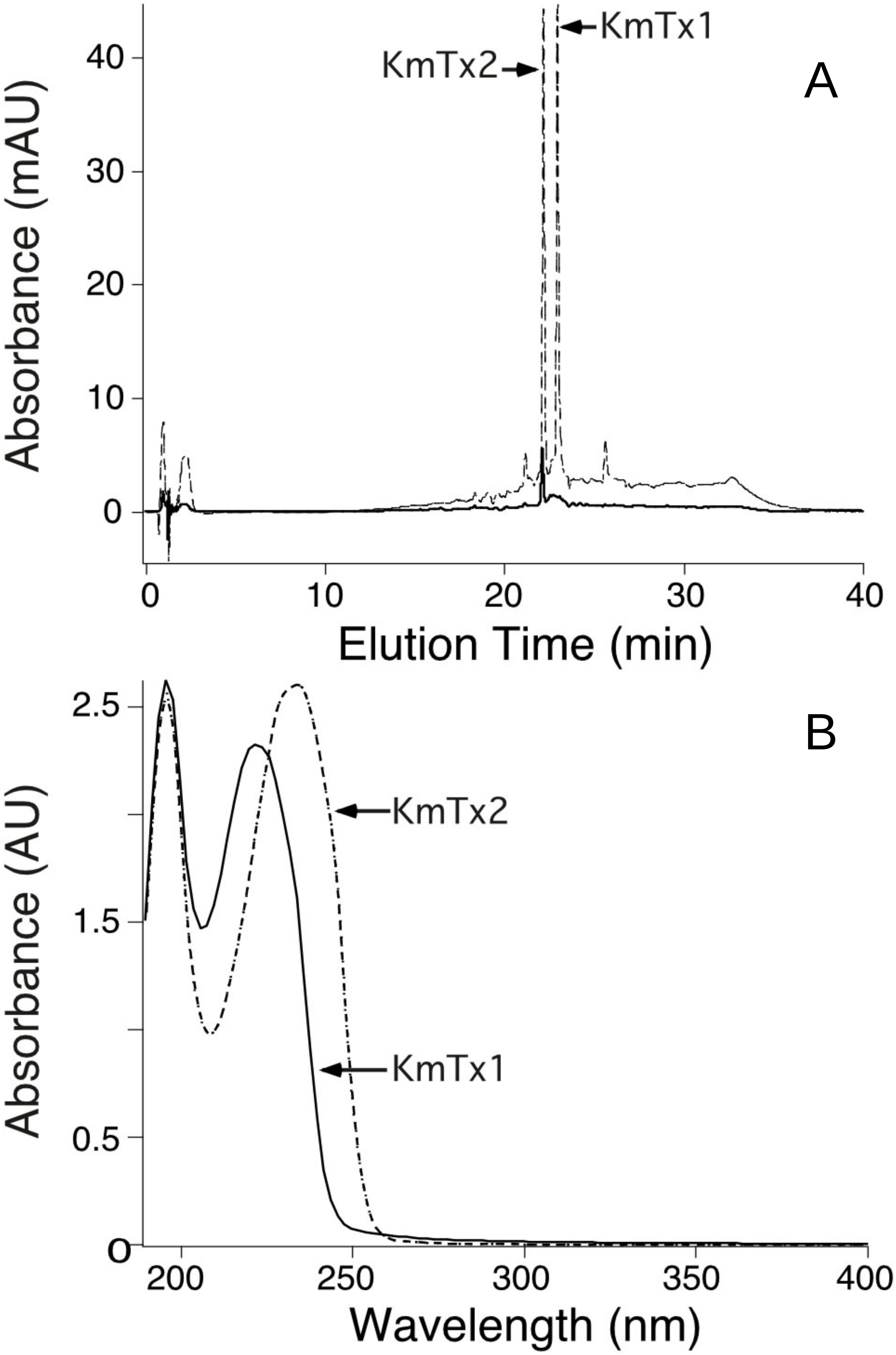

100%
80%
60%
40%
20%
0%
Hemolysis
1
10
100
1000
10000
Concentration (ng/ml)
◇ KmTx 1
□ KmTx 2

Figure 6.4. HPLC co-injection of purified KmTx 1 isolated from a Maryland isolate of *Karlodinium micrum*, and KmTx 2 isolated from a South Carolina isolate of *Karlodinium micrum*. [A] HPLC trace of co-injected KmTx 1 and KmTx 2. Dashed line represents 230 nm absorbance. Solid line represents 254 nm absorbance. [B] Overlaid UV spectra of KmTx 1 and KmTx 2. HPLC conditions described in Chapter 2.

A
Absorbance (mAU)
40
30
20
10
0
KmTx2
KmTx1
0
10
20
30
40
Elution Time (min)
B
Absorbance (AU)
2.5
1.5
0.5
0
KmTx2
KmTx1
200
250
300
350
400
Wavelength (nm)

CHAPTER 6

ADDENDUM

Investigation of the 2003 Swan and Canning River Fish Kills (Perth, Western Australia) Associated with Blooms of *Karlodinium micrum*.

ABSTRACT

In 2003, several fish kills co-occurred with blooms of *Karlodinium micrum* in the Swan and Canning River estuaries in Perth Australia. Two water samples collected from the Swan River were analyzed for the presence of toxins similar to those produced by US isolates of *K. micrum*. In both samples a KmTx 2-like compound was found in sufficient quantities to cause the observed effects on fish. Results of histopathological examination of fish kill samples by the Australian authorities were similar to the effects noted for zebrafish exposed to KmTx 2. This was the first confirmation of *K. micrum* populations outside of the United States producing karlotoxin-like compounds.

INTRODUCTION

From April-July 2003, numerous fish kills occurred in both the Swan and Canning River estuaries located in Perth, Western Australia (Figure A6.1). These kills coincided with a persistent bloom of *Karlodinium micrum* which started in the Swan River in early April and spread to the Canning River in Mid-June. The bloom officially ended in early July following a large rainfall event. The bloom was believed to be fueled by unusually high rainfall in the area during early April followed by a prolonged period of calm, sunny weather. *K. micrum* densities fluctuated during this period, often reaching densities of 1.0×10^5 cells ml^{-1}. Numerous species were affected, including black bream (*Chrysophrys australis*) a popular local sport fish, Perth herring (*Nematalosa ulaminghi*), and Swan River gobies (*Pseudogobius olorum*). Most kills contained < 1,000 fish, but two kills were estimated to contain in excess of 100,000 fish each (Peter Musk, Swan River Trust, Perth Australia, personal communication).

In early June, 2003, we were contacted by Andrew McTaggert of the Swan River Trust, and asked to analyze water samples containing *K. micrum* for the presence of toxins that potentially could have been responsible for the repeated fish kills in the Swan and Canning River systems.

MATERIALS AND METHODS

Two - 1 L water samples were collected from the Swan River, one on June 12, 2003 and the other on June 23, 2003. Both were immediately filtered through glass fiber filters and shipped to our laboratory frozen. Upon arrival, both samples were thawed and processed for toxin analysis as described previously (Chapter 2), with the exception of the elution gradient during the HPLC analysis being shortened from a 5% MeOH to 95% MeOH linear gradient over 25 min. to a 30% MeOH to 95% MeOH linear gradient over 20 min. Salinity of both samples was determined to be approx. 30 ppt. Presence of toxic activity in water samples and lipid fractions was determined through the use of a hemolytic assay described previously (Chapter 2). Molecular weight determination of toxic fractions was performed using LC/MS analysis on an Agilent G1946D single quad mass spectrometer detector (Agilent Technologies, Inc., Wilmington DE). Methods were as previously described (Chapter 4) with the exception of the addition of 0.01% TFA to facilitate ionization in the negative scan mode.

RESULTS

Initial analysis of the two thawed water samples showed hemolytic activity in both with activity being higher in the 6/23 sample (Figure A6.2). Initial C_{18} column extraction of raw water samples showed all of the hemolytic activity eluting in the 80% MeOH fraction. This data was in accord with previous results for the analysis of KmTx 1 and KmTx 2 from US *K. micrum* isolates and water samples (see Chapter 2). HPLC separation of the 80% MeOH - C_{18} eluates showed a single prominent hemolytic peak eluting at ca. 17 min. in both samples (Figure A6.2). Toxin concentration was estimated from the 230 nm UV peak area using a known amount of KmTx 2 for comparison (e_{230} = 1828 mAU*s/µg). The 6/23 sample contained 170 µg/L of the toxic fraction while the 6/12 sample contained 92 µg/L. Retention time and UV spectral characteristics of the toxic peaks for both samples were very similar to KmTx 2 isolated from US East coast *K. micrum* isolates from estuaries located South of the Chesapeake Bay (Figure A6.3).

Mass determination in the positive ionization mode yielded a $(M+Na)^+$ ion at 1365.4 Da and a $(M+H)^+$ ion at 1343.6 (Figure A6.4A). In addition, positive ionization yielded several smaller doubly charged ions ranging from 654.4-695.2. The identity of these ions is not yet known. Negative ionization yielded a prominent peak at 1455.6 Da, believed to be a $(M-H)^+$+TFA adduct [TFA=114 Da] (Figure A6.4B). These results led us to the conclusion that the molecular weight of the KmTx 2-like toxin from the Swan River samples was 1342.6 Da. +/- 0.13. Previous studies (see Chapter 4) have found that KmTx 2 has a molecular weight of 1344.8 Da. +/- 0.13.

DISCUSSION

All of our analyses suggest that the Swan and Canning River fish kills, that occurred between April and July, 2003 in Perth, Western Australia, could have potentially been caused by the observed elevated densities of *K. micrum.* Initial characterization of toxic fractions from two water samples collected on the Swan River on 6/12 and 6/23, 2003 suggest it is similar but not identical to KmTx 2, previously isolated from both *K. micrum* isolates and natural water samples from US East coast estuaries located South of the Chesapeake Bay, MD.

Observations made by fisheries managers during the Swan River fish kills included: several species appearing to be affected, affected fish showed lethargy with advanced cases showing dullness to the point of stupor, and all affected fish showed increased respiration rates. Necroscopies of black bream collected from the Swan River on April 10, 2003 and May 22, 2003 both suggested death caused by asphyxiation due to acute branchial toxicity. Fish samples from April 10, showed brown-green fibrous material in the secondary gill lamellae, granulocyte infiltration, and extensive hyperplasia of the gill epithelial tissue. It should be noted that post mortem autolysis was also observed in external tissues of the liver and spleen, with underlying tissue appearing normal. In the May 22 bream samples, no abnormalities were observed in brain, kidney, heart, liver, pyloris, or swim bladder tissues. However, gills appeared very congested with increased amounts of mucus covering the gill lamellae and internal opercular epithelium. Wet preparations revealed no parasites or bacteria. Interlamellar spaces contained large amounts of clear mucus and an amorphous brown-green material. Histopathological examination of gill tissue showed severe branchial degeneration. Diffuse epithelial hyperplasia with lamellar fusion and bridging was observed. No bacteria or parasites were observed in histopathological examination (John Creeper, fish pathologist report c/o Jane Latchford, Swan River Trust, Perth Australia, personal communication). All of these observations are consistent with effects observed during exposure of zebrafish to purified KmTx 2 (described in Chapter 5).

The molecular weight of the toxin purified from the Swan River fish kill samples was very similar to what we have previously shown for KmTx 2 (1342 vs. 1344 Da for KmTx 2). This difference suggests one additional unsaturation in the Swan River toxin. In addition, based on retention time and UV spectral characteristics, it was concluded that the Swan River toxin was more similar to KmTx 2 than KmTx 1 and therefore, at present, is being referred to as a KmTx 2-like compound.

The amount of toxin isolated from the Swan River fish kill samples (170 μg/L for the 6/23 sample and 92 μg/L for the 6/12 sample) are lower than those observed for fish kills in the United States (400-800 μg/L). However, 100 μg/L KmTx 2 was sufficient to cause acute mortality and similar pathologies in gill epithelial tissue in laboratory-exposed zebrafish (see Chapter 5).

All of this data suggests that the prolonged blooms of *K. micrum* that occurred in the Swan and Canning River estuaries in 2003 could have potentially been responsible for the observed fish mortalities. The similarities in the Swan River toxin and KmTx 2 are interesting but complete structural determination of both of these compounds will be required to establish their true relationship. The distinctness of both of these compounds from KmTx 1 is also interesting in that it further solidifies the uniqueness of Chesapeake Bay *K. micrum* populations and their toxins.

ACKNOWLEDGEMENTS

The author wishes to thank Andrew McTaggert, Peter Musk, and Jane Latchford (Swan River Trust, Perth Australia) for providing the water samples from the Swan River fish kills used in this study. The author also wishes to thank Peter Mrozinski, William Barrett, and Jim Lau (Agilent Technologies Inc., Wilmington Delaware, USA) for assistance in LC/MS analyses of the KmTx 2-like Swan River toxin.

Figure A6.1. Map of Western Australia showing location of Swan and Canning River estuaries. Map acquired from http://www.wrc.wa.gov.au/srt/aboutriver/index.html

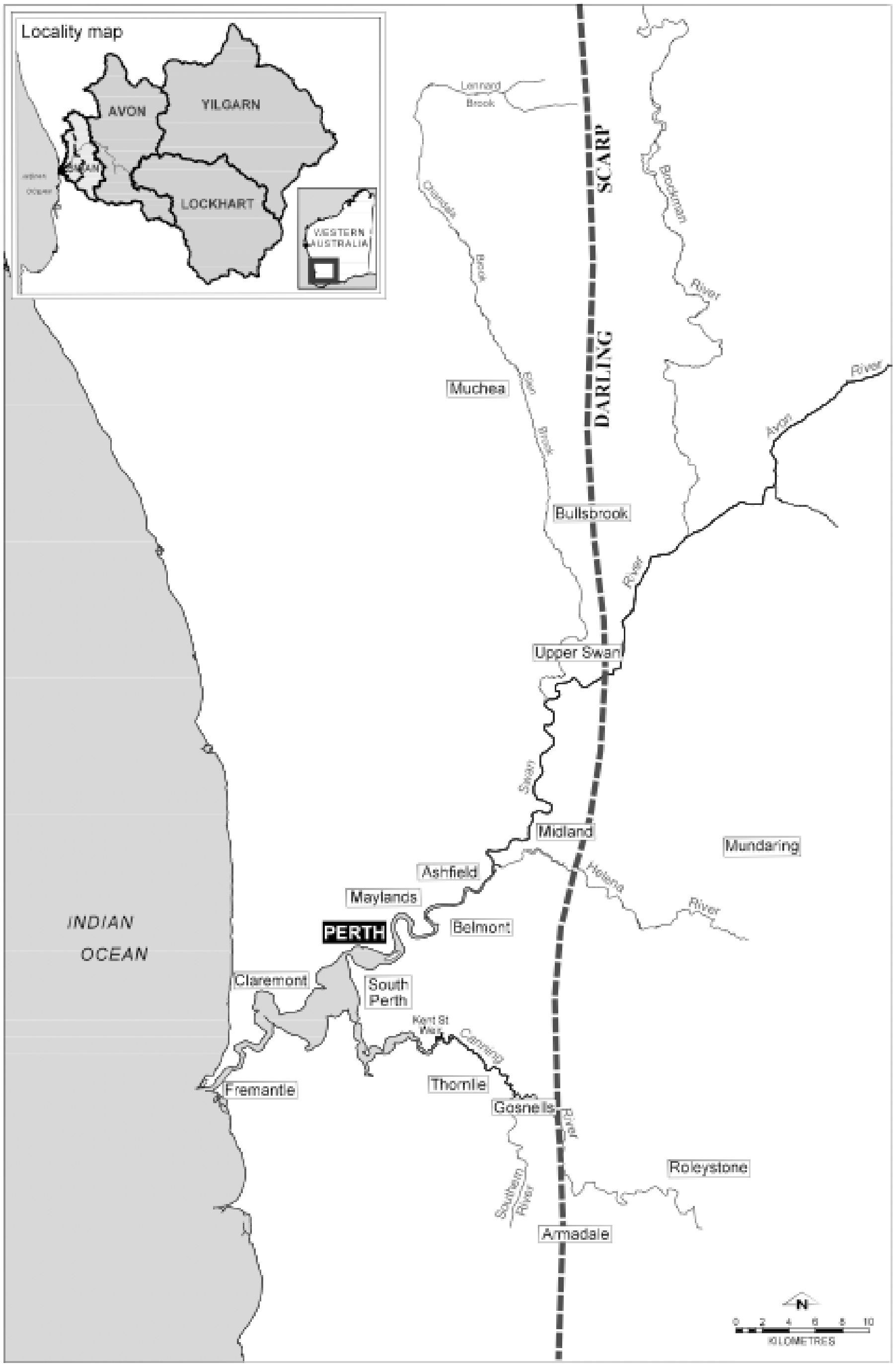
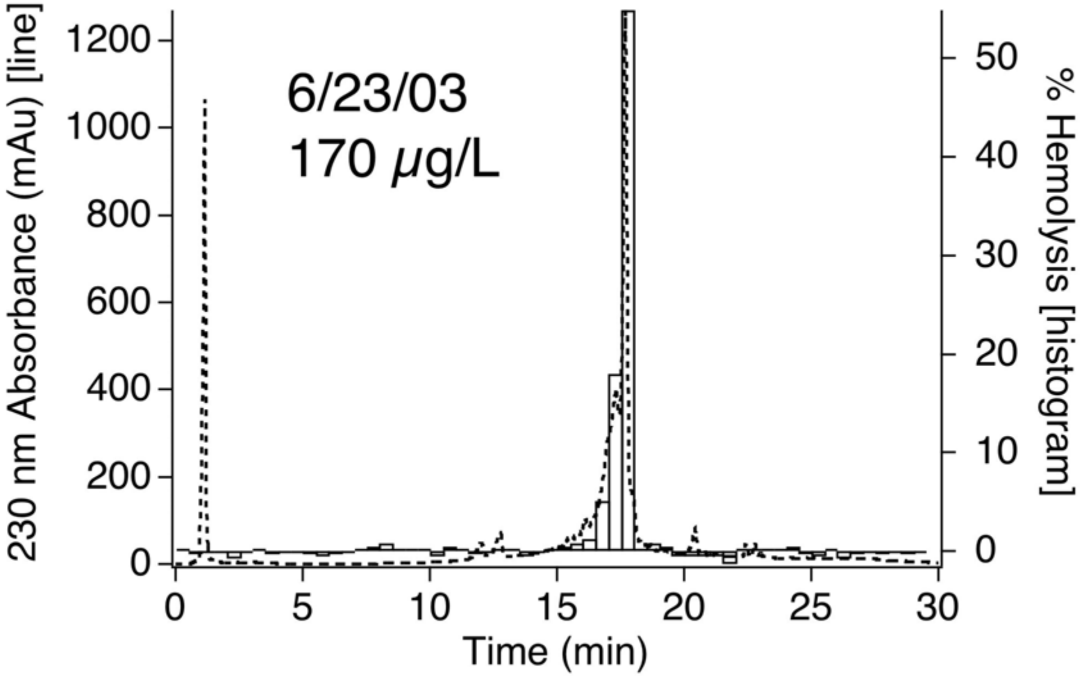

Locality map
AVON
YILGARN
SWAN
LOCKHART
Indian
Ocean
WESTERN
AUSTRALIA
Lennard
Brook
Chandala
Brook
Brockman
River
SCARP
DARLING
Muchea
Ellen
Brook
Avon
River
Bullsbrook
River
Upper Swan
Swan
Midland
Mundaring
Ashfield
Helena
River
Maylands
PERTH
Belmont
INDIAN
OCEAN
Claremont
South
Perth
Kent St
Weir
Canning
Fremantle
Thornlie
Gosnells
River
Roleystone
Southern
River
Armadale
N
0 2 4 6 8 10
KILOMETRES

Figure A6.2. HPLC traces of Swan River water samples. [A] HPLC trace of sample from 6/23/2003 (dashed line) overlaid with hemolytic activity in 0.5 min collected fractions (histogram). [B] HPLC trace of Swan River water sample from 6/12/2003 (solid line) overlaid with HPLC trace of purified sample of KmTx 2 isolated from US isolate of *Karlodinium micrum* (CCMP 2282). Inset histogram is hemolytic activity in raw water tested immediately upon thawing of samples.


6/23/03
170 μg/L
230 nm Absorbance (mAu) [line]
% Hemolysis [histogram]
Time (min)
0
200
400
600
800
1000
1200
0
10
20
30
40
50
0
5
10
15
20
25
30

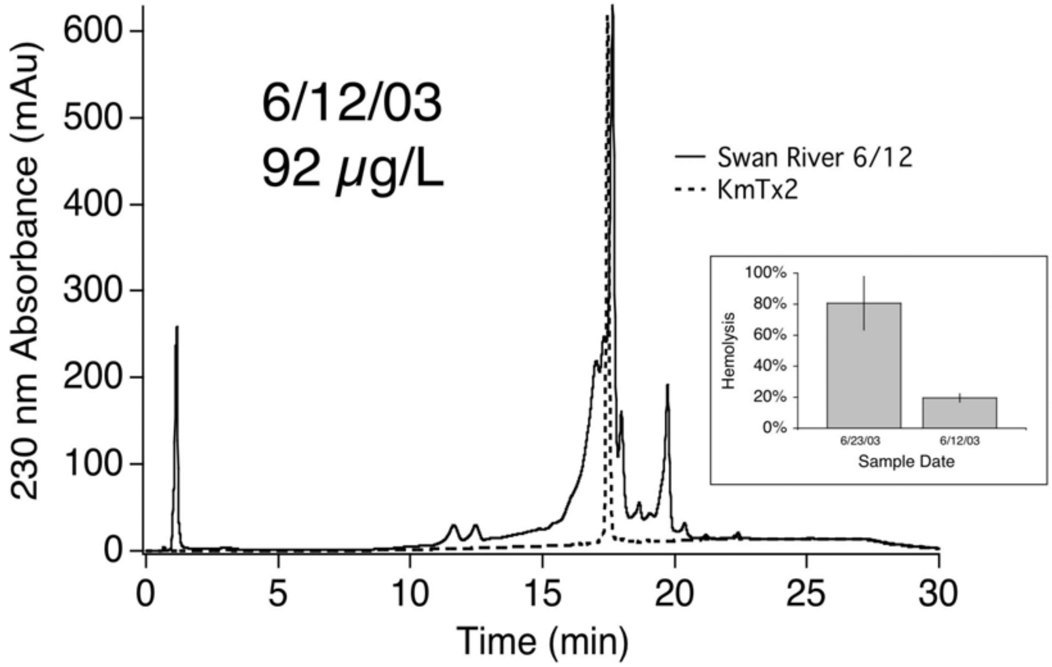

6/12/03
92 μg/L
230 nm Absorbance (mAu)
Time (min)
Swan River 6/12
KmTx2
0
100
200
300
400
500
600
0
5
10
15
20
25
30
Hemolysis
100%
80%
60%
40%
20%
0%
6/23/03
6/12/03
Sample Date

Figure A6.3. Comparison of Swan River KmTx 2-like compound to KmTx 2 from US isolates. [A] HPLC trace of isolated Swan River KmTx 2-like compound overlaid with HPLC trace of purified KmTx 2 from US isolates of *Karlodinium micrum*. [B] UV spectra of isolated Swan River KmTx 2-like compound overlaid with UV spectra of purified KmTx 2 from US isolates of *Karlodinium micrum*.

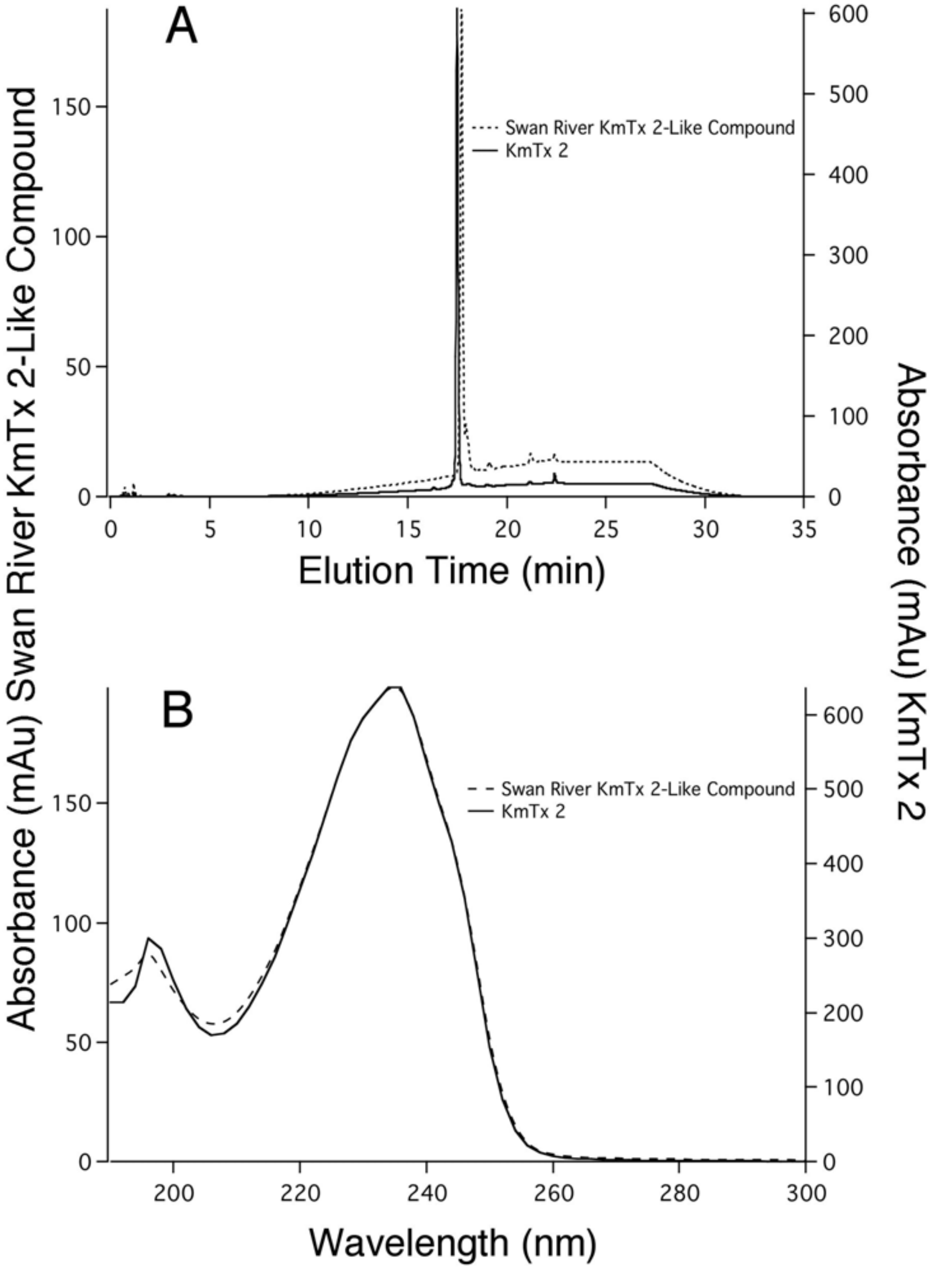
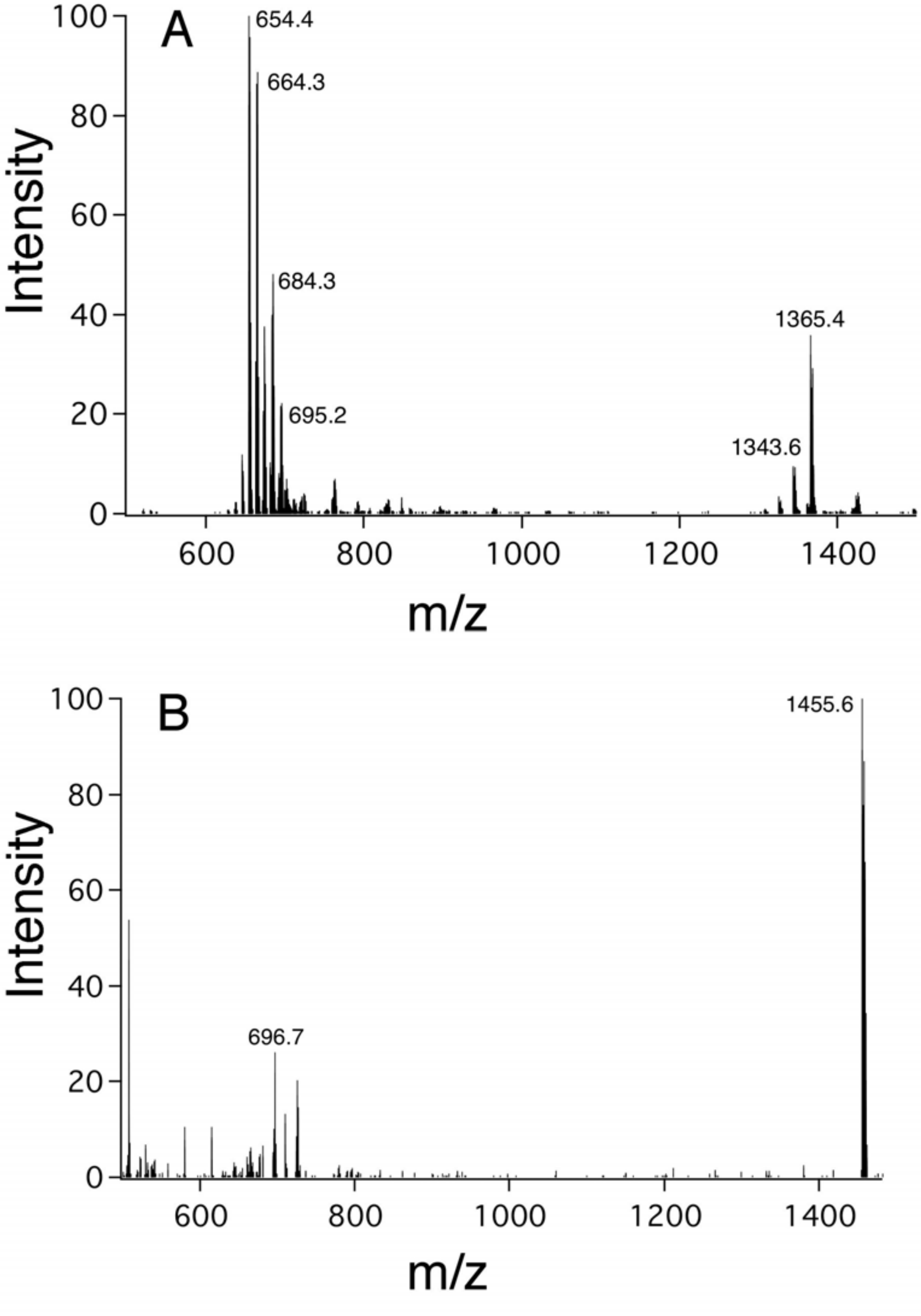

A
B
Absorbance (mAu) Swan River KmTx 2-Like Compound
Absorbance (mAu) KmTx 2
Swan River KmTx 2-Like Compound
KmTx 2
Elution Time (min)
0
5
10
15
20
25
30
35
0
50
100
150
0
100
200
300
400
500
600
Swan River KmTx 2-Like Compound
KmTx 2
Wavelength (nm)
200
220
240
260
280
300
0
50
100
150
0
100
200
300
400
500
600

Figure A6.4. LC/MS analysis of Swan River KmTx 2-like compound. [A] LC/MS in positive ionization mode for Swan River KmTx 2-like compound. 1365.4 Da ion believed to be $(M+Na)^+$ ion. 1343.6 Da ion believed to be $(M+H)^+$ ion. [B] LC/MS in negative ionization mode with 0.01% TFA added to facilitate ionization. 1455.6 Da ion believed to be the $(M-H)^+$ + TFA adduct.


A
Intensity
100
80
60
40
20
0
654.4
664.3
684.3
695.2
1343.6
1365.4
600
800
1000
1200
1400
m/z
B
Intensity
100
80
60
40
20
0
1455.6
696.7
600
800
1000
1200
1400
m/z

CHAPTER 7

Summary and Future Research Directions

In 1996, when the first fish kill occurred at HyRock fish farm, the original diagnosis of the cause of the kill was *Pfiesteria piscicida*, even though it was a minor component of the bloom. This was due mainly to the fact that the dominant organism in the bloom was misidentified, at first, as *Gyrodinium estuariale*. *G. estuariale* had been known for years to occur in the Chesapeake Bay and had never been associated with fish kills in the scientific literature, whereas *P. piscicida*, at the time, had recently been associated with several high profile fish kill events in North Carolina estuaries. It was not until later that the composition of the bloom was properly identified as *Gyrodinium galatheanum*, later renamed as *Karlodinium micrum*. This was the first record of *K. micrum* occurring in U.S. waters. *K. micrum* was first identified (then as *Gyrodinium galatheanum*) in 1950 in Southwestern Africa. Since then it only occurred sparingly in the scientific literature. Most notably in the early 1990's where several Norwegian studies showed that high densities of the organism caused fish death and reduced bivalve growth in laboratory experiments (Nielsen and Stromgren, 1991; Nielsen, 1993). Despite these studies, it was not until several years later, after two additional fish kills occurred at HyRock fish farm, both associated with large blooms of *K. micrum*, that *K. micrum* was considered as the causative agent in fish kills in Maryland waters (Deeds et al., 2002). In 1999, when this line of research began, no toxic substance had yet been identified from *K. micrum*. Initially, several fractions were identified from *K. micrum* cultures with hemolytic, cytotoxic, and ichthyotoxic properties (Deeds et al. 2002; Chapter 2). These initial toxic fractions were shown to possess properties that were consistent with effects observed to be associated with blooms of *K. micrum* at Hyrock fish farm, namely toxic activity was shown to be increased through copper sulfate algicidal treatments, while toxic activity was shown to be diminished in blooms treated with an algicidal potassium permanganate solution. Again in accord with observations from HyRock fish farm, ozonation of water containing *K. micrum* was shown to remove both cells and toxic activity (see Chapter 3). Several toxins, putatively named karlotoxins, were subsequently isolated from *K. micrum* cultures and fish kills. Karlotoxins have now been identified in U.S. east coast *K. micrum* isolates from Maryland to Florida (see Chapter 6). These toxins have also been identified directly from fish killing waters collected during blooms of *K. micrum* in Maryland, South Carolina, and most recently from an estuarine river experiencing repeated fish deaths associated with a large persistent bloom of *K. micrum* in western Australia (Chapter 6; Chapter 6 addendum). One of these toxins, KmTx 2, was shown here to function by non-specifically permeabilizing plasma membranes to a range of ions and small molecules resulting in cell death through colloid osmotic lysis (see Chapter 4). KmTx 2 was also shown here to be more active against cholesterol containing membranes compared to ergosterol containing (fungal) membranes. Exposure to KmTx 2 resulted in the lysis of a co-occurring, potential grazer while it had no effect on a KmTx 2 producing strain of *K. micrum*. We believe this to be due to the fact that the grazer used in these studies (*Oxyrrhis marina*) is reported to contain cholesterol in its membranes (Klein Breteler et al., 1999) while *K. micrum* was confirmed to possess the unusual sterol gymnodinosterol as its primary membrane sterol (see Chapter 4). It was shown here that incubation of KmTx 2 with gymnodinosterol, isolated from *K. micrum*, had no effect on hemolytic activity while incubation with both cholesterol and ergosterol reduced this activity, with effects being more pronounced with cholesterol. Finally this report showed that the site of ichthyotoxic action in KmTx 2 appears to be the gills, particularly the respiratory epithelium where concentrations approaching the LD_{50} (0.1 ug/ml) resulted in extensive changes to secondary lamellaer gill architecture and mucous hypersecretion, while concentrations above this amount ($\geq$ 0.5 ug/ml) resulted in rapid fish death due to severe necroses of the gill epithelium (see Chapter 5). These concentrations are well within those found during fish kills associated with blooms of *K. micrum* (0.1-0.8 ug/ml KmTx 1 or 2) (see Chapter 6).

Despite the numerous advances in our knowledge of the toxicity, pharmacology, ecological function, and geographic distribution of the toxins from *Karlodinium micrum*, numerous gaps still remain. First and foremost, the structural determination of karlotoxins KmTx 1 and KmTx 2 needs to be completed. Thus far we know that the molecular weight of KmTx 1 is 1338 Da. while the molecular weight of KmTx 2 is 1344 Da. They are both amphipathic lipid-like molecules with a high UV absorbance ca. 230 nm. (KmTx 1 - UV max. 224 nm, KmTx 2 – UV max. 235 nm). KmTx 1 and KmTx 2 have distinct HPLC retention times (see Chapter 6). Furthermore, KmTx 1 has no absorbance above 250 nm, while the spectra of KmTx 2 is shifted slightly to the right thereby giving this compound a small but consistent shoulder in its UV absorbance at 250 nm. These features make KmTx 1 and KmTx 2 easily distinguishable in standard HPLC analyses without the requirement of mass detection. In collaboration with Dr. Jeffrey L.C. Wright, University of North Carolina at Wilmington, thus far, the majority of the structural elucidation work has focused on the first toxin discovered, KmTx 1. High resolution mass spectrographic data indicates that KmTx 1 is a compound with a molecular mass of 1338.8589 Da. High-resolution ^{1}H and ^{13}C NMR data, acquired in collaboration with Bruker BioSpin scientists using an instrument equipped with a Bruker Cryoprobe, has enabled us to gain a preliminary understanding of the nature of the compound. KmTx 1 contains approximately 96 carbons, consistent with the molecular weight of the molecule. Of these, some 34 are directly bonded to oxygen, typical of a polyether or polyhydroxy structure, and the molecule also contains as many as 20 olefinic carbons, and three carbonyl groups. A particularly striking feature of the NMR spectra is the occurrence of a protonated carbon resonating at *ca.* 168 ppm. This unusual, and we believe characteristic signal, is tentatively assigned to an imine carbon, a function that has been found in a number of dinoflagellate metabolites (Hu *et al.,* 1996). Although the structure of KmTx 1 has still not been fully elucidated, some important features can be reported. Like other toxic dinoflagellate metabolites, KmTx 1 has a high percentage of carbons bearing oxygen (*ca* 33%), in line with the percentage found in linear polyethers such as amphidinols (*ca* 31%) and pectenotoxins (*ca* 33%). Importantly, this is lower than observed in fused polyethers (*ca* 40-44%) such as PbTx-2 (Baden and Adam, 2000), gymnosin-A (Satake *et al.,* 2002) and gambieric acid (Morohashi *et al*., 2000) and amphidinol (Paul *et al.,* 1995). This indicates less frequency of fused polyether ring systems, though it doesn’t discount their existence within the toxin molecule. Another distinctive feature of KmTx 1 is the large number of olefinic carbons (20) present in the molecule representing over 20% of the total carbon count. Only the amphidinols with an average of about 12% contain such a large fraction of olefinic carbons. This is also a considerably higher than the typical percentage (4-5%) of unsaturated carbons found in fused polyether structures. A lower frequency of olefinic carbons in ladder-frame polyethers is consistent with their proposed biosynthesis (Lee *et al.,* 1989; Moore, 1999), in which the double bonds present in the nascent polyketide chain are consumed in the process of cyclization to form the fused polyether structure. Finally, KmTx 1 contains three carbonyls, a remarkably high number compared with all other polyethers regardless of their general structure. All of this information points to a novel polyketide structure for KmTx 1, in which the molecule contains some features associated with known polyethers, particularly the linear polyethers, while at the same time possessing other unique features. In the immediate future, work must focus on the production of larger amounts of more highly purified toxins so the work on the structural elucidation of the toxins from *K. micrum* may be completed.

The biological activities of KmTx 1 and 2 are nearly identical, yet they appear to have distinct geographic distributions (see Chapter 6). KmTx 1 appears to be produced by *K. micrum* populations from the Chesapeake Bay, while KmTx 2 appears to be produced by *K. micrum* populations from estuaries located south of the Chesapeake Bay. Due to its UV spectral characteristics, the compound isolated from the Swan River, Australia, fish kills is currently considered to be a KmTx 2-like compound (Chapter 6 addendum). To truly determine if KmTx 1 is produced (in the U.S.) exclusively by Chesapeake Bay *K. micrum* populations, additional isolates will need to be acquired and tested. Particularly, isolates from Delaware’s inland bays, Maryland’s Atlantic coastal bays, Virginia’s estuarine rivers and North Carolina estuarine rivers located outside of the mouth of the Chesapeake Bay. All of these locations have been reported to contain *K. micrum*. This will determine not only if KmTx 1 is produced exclusively inside of the confines of the Chesapeake Bay, but also where the demarcation lies for its geographic distribution. In an initial attempt to answer this question a *K.micrum* isolate was cloned by single cell isolation from a water sample collected form a Maryland Department of Natural Resources permanent monitoring site located on the Upper St. Martin's River (38.4056° -75.1461°) on Maryland's Atlantic coast (isolate XDN4486-0603B3) (Figure 7.1). This isolate was found to contain KmTx 1 as its primary toxin. This would suggest that KmTx 1 can be produced by Maryland *K. micrum* isolates from outside of the Chesapeake Bay watershed. Additional isolates will need to be acquired and tested to answer this interesting question.

As mentioned previously (see Chapter 4) membrane specificity for the toxins from *K. micrum* appear to be determined by the sterol composition of the target membrane. This same feature also appears to render *K. micrum* immune from the membrane disrupting properties of its own toxins. Once the chemical structures of the toxins from *K. micrum*, particularly KmTx 1 and 2, are elucidated, the nature of this specificity for particular sterols may be better understood. For the pore-forming cytolysin amphotericin B, one of the most common treatments for human systemic fungal infections, the nature of the specificity for fungal cell membranes compared to vertebrate cell membranes is based on a greater specificity for ergosterol compared to cholesterol (Bolard, 1986). Even though KmTx 2 was shown to have a greater specificity for cholesterol compared to ergosterol, making it non-useful, in its present form, as a potential anti-fungal drug, a better understanding of the toxins structural components which make it specific to such structurally similar sterols could greatly benefit the design of new drugs for the treatment of systemic fungal infections. Giner (2003) recently proposed that the unusual side chain of the sterols from *K. brevis*, which *K. micrum* shares, make them undigestible by potential grazers and therefore aid in anti-grazing defense. Considering the fact that ergosterol shares the same side chain with gymnodinosterol (Figure 7.2), we propose that other, less common, structural features such as the (8,14) double bond of the sterols ring system are more signifigant as the reason why this group of dinoflagellates possess this ususual sterol as an integral membrane component. More work will need to be done to answer this interesting question as well.

In Chapter 2, it was found that the Chesapeake Bay isolate used in that study contained two distinct toxic fractions, initially called Tox A and Tox B. Once purified further, the active principle from Tox A was renamed KmTx 1. Since that time, we have found that in all U.S. isolates and water samples tested, either KmTx 1 or KmTx 2 have been found to be the major toxin, both in terms of amount and potency. Therefore the majority of this research effort has focused on the toxicity, pharmacology, and ecology of these two compounds in various *K. micrum* isolates. But as we have also found, several additional toxins appear to be present in *K. micrum* isolates and water samples from estuaries of the U.S. east coast (Table 7.1). At the present time, we are referring to these compounds as putative KmTx-like (*pKmTx*) toxins. Several of these toxins have not yet been purified to the point where they can be compared in detail to KmTx 1 and 2 where significantly more information had been gained on toxicity and distribution. In the few that have been sufficiently purified, they appear to be ca. 10 fold less potent (hemolytic activity on a per weight basis) compared to KmTx 1 and 2 (Figure 7.3). These compounds may represent metabolic precursors, or possibly bacterial breakdown products of the parent compounds KmTx 1 and 2, but sufficient structural information has not been gained at this point to reasonably speculate as to the relationship of these compounds to KmTx 1 or 2.

A major question that remains to be determined is how *Karlodinium micrum* stores its toxins and, in turn, how they are released. This information may aid in the ultimate determination of the ecological function of toxin production in this species. Our data suggests that the toxins are routinely sequestered and/or only released in small amounts without major cell disturbance (see Chapter 2). An interesting possibility is that toxin release is somehow related to ejectosomes, such as trichocysts, which many dinoflagellates have been shown to possess (Lee, 1992). Li (1998) observed fine, thread-like, structures, possibly trichocysts, associated with the mixotrophic feeding behavior and prey capture of Chesapeake Bay *K. micrum*. But in this study (see Chapter 6), it was found that four isolates of KmTx 2 producing *K. micrum*, all grown in parallel both autotrophically, using f/2 growth media as the sole nutrient source, and mixotrophically, using cryptophyte prey as the sole nutrient source, did not differ substantially in toxin production/cell. Therefore, an offensive function of *K. micrum* toxins, i.e. prey capture, is still in question. In other protozoa, such as paramecium, trichocysts have been shown to have a defensive function (Sugibayashi and Harumoto, 2000). In this study (see Chapter 4), it was shown that KmTx 2 caused the direct cell lysis of a co-occurring heterotrophic dinoflagellate, and potential grazer, *Oxyhhris marina*, while having no effect on itself. Further, in another preliminary study, grazing rates by *O. marina* were found to be significantly different over time on two *K. micrum* isolates shown to produce differing amounts of toxin (Matt Johnson, Horn Point Laboratory, Cambridge MD, personal communication). Paramecium trichocyst exocytosis has been shown to be stimulated by Ca^{2+} influx upon exposure to veratridine (Blanchard et al., 1999). Tsim et al. (Tsim et al., 1997) showed that the inducement of encystment could be stimulated in the dinoflagellates *Alexandrium catenella* and *Crypthecodinium cohnii,* also due to the elevation of intracellular Ca^{2+} levels, upon exposure to indoleamines, such as melatinon, and Ca^{2+} ionophores such as A23187. In an attempt to stimulate trichocyst exocytosis in *K. micrum*, we exposed cells to veratridine (1 mM), melatonin (2 mM), and 5-methoxytryptamine (0.5 mM) and both observed cells under light microscopy for trichocyst exocytosis and measured hemolytic activity in solution as an indicator of toxin release. In all treatments, cells were observed to lose flagella, develop membrane blebs and settle to the container bottom. After 24 hours, cells at the bottom of wells died and dissolved. No trichocysts were observed to discharge as has been observed for paramecium under identical conditions. It is possible that *K. micrum* trichocysts are simply not observable under normal light microscopic examination. Hemolytic activity did increase in cultures exposed to the above compounds (ca. 20-30% increase in hemolytic activity) but this could potentially be explained through the observed cell death. All that could be concluded from these experiments was that *K. micrum* possesses membrane receptors that are responsive to veratridine, melatonin, and 5-methoxytryptamine. Trichocysts have been observed in the closely related dinoflagellate *Gyrodinium estuariale* (Gardiner et al., 1989), but TEM analysis of *K. micrum* cells will be required to confirm the presence of trichocysts in this species. Once this is established, further experimentation will be required to determine the proper stimulus for their expulsion and subsequent determination of any relation with *K. micrum* toxin release. Once the toxins structures are determined, antibodies could be developed and, in conjunction with TEM analyses, sub-cellular localization of the toxins may be established. All of this information will be critical to the determination of the ecological role of toxin production in *K. micrum*.

Perhaps one of the most important questions, from a regulatory standpoint, that remains to be answered is the potential for bioaccumulation of the toxins from *K. micrum* and what effect this may have in mammalian systems due to the consumption of contaminated seafood. Bioaccumulation studies for the toxins from *K. micrum* have not yet been performed and will be required in the future. On a cellular level, all mammalian cell types tested, including epithelial, nervous, muscular, and connective were sensitive to the toxins membrane disrupting effects (see Chapter 4). But as was seen with the whole fish exposures, not all tissues are equally affected when whole organisms are exposed (see Chapters 4 and 5). To assess the effects of KmTx 2 on mammals we chose to employ the gold standard for algal toxin regulation and contaminated seafood consumption, the mouse bioassay (AOAC, 1965; Cummins and Hill, 1969). The standard mouse bioassay employs an intraperitoneal toxin injection of 20g mice, typically in a saline solution containing a small amount of surfactant such as Tween. In an initial study, 20 g, female, balb c, mice were IP injected with 1, 2, or 10 μg of KmTx 2 [50, 100, and 500 μg/kg respectively]. Ten mice were injected per treatment. Injections consisted of the appropriate amount of 1 mg/ml KmTx 2 MeOH stock added to 1 ml of sterile PBS containing 1% Tween 60. The KmTx2 used in this experiment had been isolated directly from water collected during a fish kill described in Kempton et al. (2002). Although no mice died in 24 hours, the 10 μg dose mice showed a definite behavioral effect. High dose animals displayed a classic “door mouse” posture with eyes closed, limited movements, and rapid breathing. All animals appeared to recover, with behavior similar to controls, after several hours. After 24 hours, 2 mice from each treatment were sacrificed by cervical dislocation and all of the major abdominal organs were fixed and sectioned for standard H&E histological examination. Differences compared to controls were only observed in the 10 μg dose. Inflammation of the peritoneum was observed in both animals, but this tissue was not specifically sampled for, therefore this effect was only observed in the pieces of peritoneum still attached to the removed organs. In one of the two high dose mice that were sacrificed, pancreatic necrosis was observed (Figure 7.4). The proliferate nature of pancreatic damage would fit with the non-selective cellular damage previously observed for KmTx2 exposures in assays using mammalian cell lines (see Chapter 4). But, due to the low sample size (n=2 with only one animal showing this effect) this result will have to be confirmed in subsequent experiments. Based on this initial mouse bioassay, 10 μg KmTx 2 per mouse is considered the low observed effects concentration (LOEC). Future experiments should be conducted to continue assessing the effects of KmTx in model mammalian systems using mouse bioassays if for no other reason then to make comparisons to the more well studied algal toxins with known effects in mammals which also have established regulatory limits for contaminated seafood consumption. In addition, experiments should also be conducted comparing toxicity via oral exposure vs. IP injections considering others have found differences in toxicity for certain toxins based on the route of exposure (Aune et al., 2002).

Much research is still required before we can fully understand the reasons why *K. micrum* produces the toxic compounds described in this report, and what effect this has on both its immediate environment and ultimately ours. The work described in this research report is but the first step. Hopefully it will provide a blueprint both in terms of its methods and results for future investigators to continue this much needed research on the ecology and ecophysiology of *Karlodinium micrum* and its toxins.

Table 7.1. Summary of current data for toxins from U.S. populations of *Karlodinium micrum.*

Name	**Retention Time* (min.)**	**UV Maximum**	**280 nm Absorbance**	**Source**
KmTx 1 (a.k.a. Tox A)	23.350	224 nm	N	CCMP 1974,1975 (MD isolates)
KmTx 2**	22.613	235 nm	N	CCMP 2282, 2283 (SC isolates)
pKmTx 3 (a.k.a. Tox B)	17.527	224 nm	Y	CCMP 1974, 1975 (MD isolates)
pKmTx 4	24.690	224 nm	Y	CCMP 1974, 1975 (MD isolates)
pKmTx 5	21.623	235 nm	N	CCMP 2282, 2283 (SC isolates)
pKmTx 6	22.909	NA	NA	Maryland water samples

*Based on HPLC elution protocol described in Chapter 2.
**e_{230nm} = 1828 mAU*s/µg

Figure 7.1. Map of the Chesapeake Bay, Maryland, USA showing location of collection sites for isolates and water samples for the analysis of the distribution of the toxins from *Karlodinium micrum*. [1] Oak Creek, water sample from 6/03, suspect area – no fish kill, 1.6×10^5 cells/ml *K. micrum*. [2] Fishing Creek, fish kill water sample from 6/02, 4×10^4 cells/ml *K. micrum*. [3] Goose Creek, fish kill water sample from 7/03, 5.7×10^4 cells/ml *K. micrum*. [4] Sharps Island, routine sampling on 4/02, no fish kill, 7×10^3 cells/ml. [5] Isolate MD5CR0599 from Matt Johnson, Horn Point Environmental Laboratory, Cambridge, MD, Isolated from Choptank River on 5/99. [6] Isolate MD6CR0599 from Matt Johnson, Horn Point Environmental Laboratory, Cambridge, MD, Isolated from Choptank River on 5/99. [7] Isolate CCMP 1974 from Diane Stoecker, Horn Point Environmental Laboratory, Cambridge, MD, isolated from approx. 37.8500N, 76.1000W on 5/95. [8] Isolate CCMP 1975, from Diane Stoecker, Horn Point Environmental Laboratory, Cambridge, MD, isolated from HyRock Fish Farm (38.1733N, 75.7374W) on 7/96. [9] Isolate XDN4486-0603B3, isolated from upper St. Martin's River (38.4056°N, 75.1461°W) on 6/03.

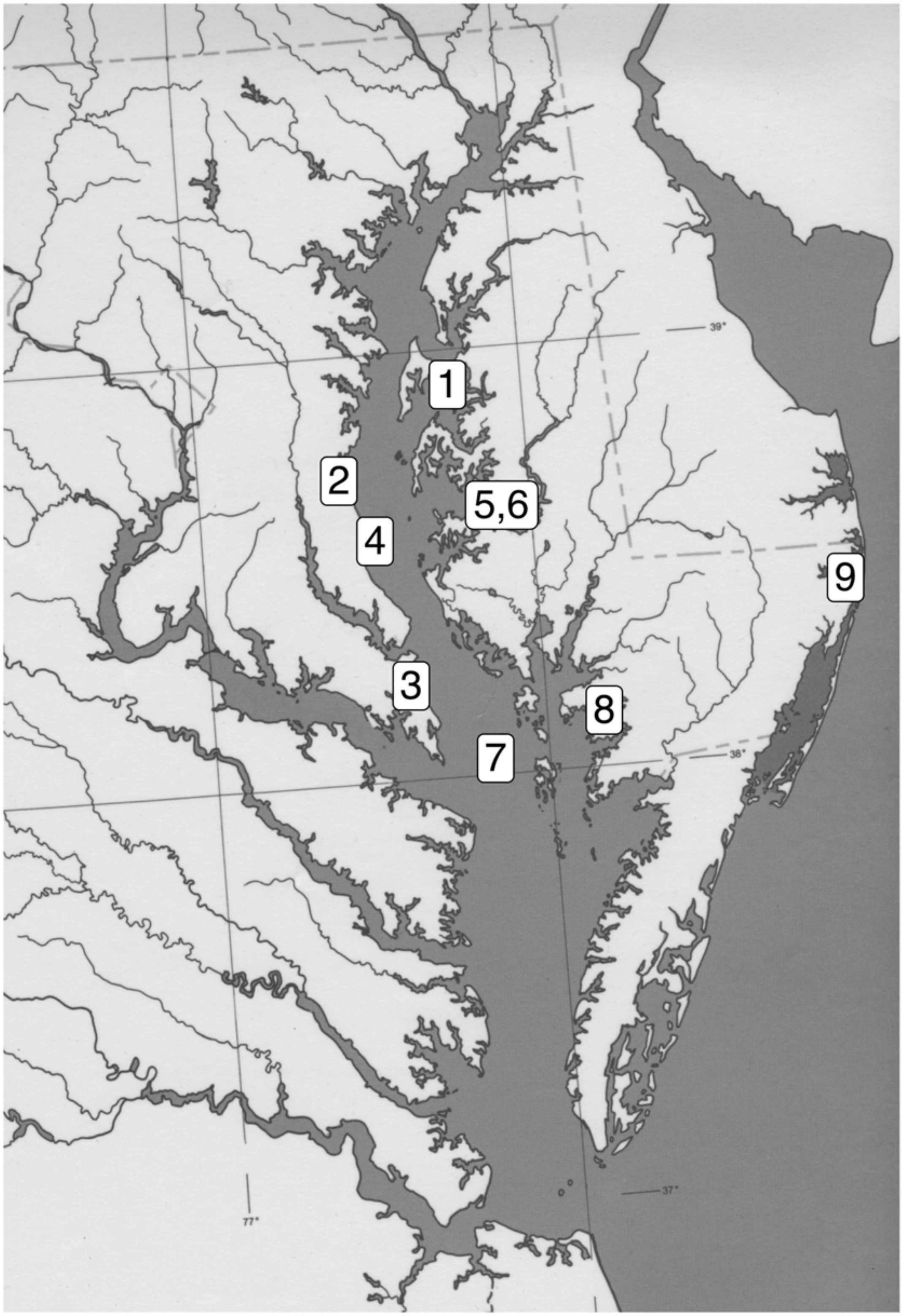
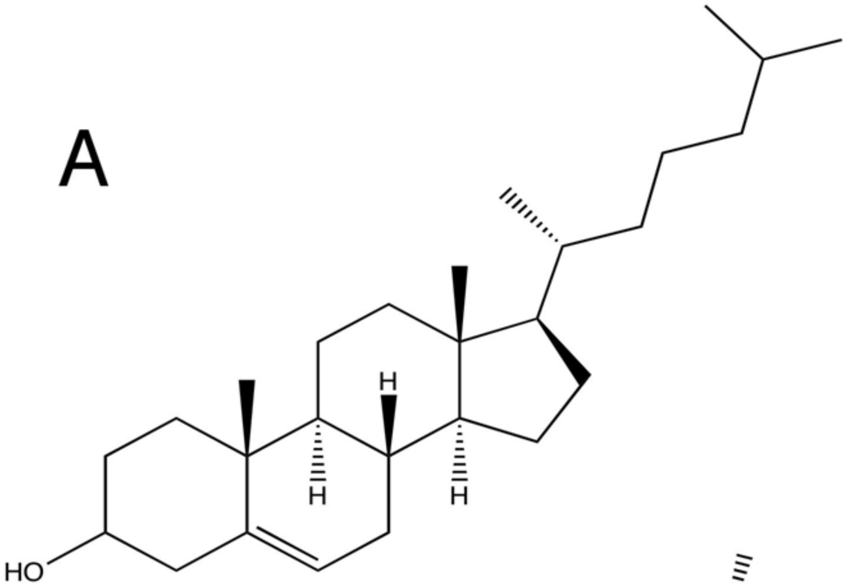
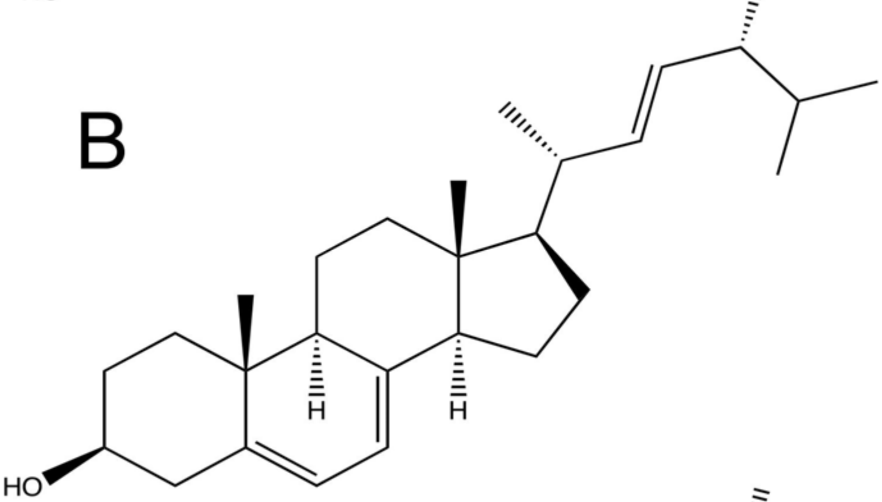

39°
1
2
5,6
4
9
3
8
7
38°
37°
77°

Figure 7.2. Chemical structures of sterols tested in this study for their ability to inhibit fish red blood cell hemolysis upon exposure to KmTx 2. [A.] Cholesterol. [B.] Ergosterol. [C.] Gymnodinosterol from *Karlodinium micrum*. (*) denotes location of (8,14) double bond.

A



B



C

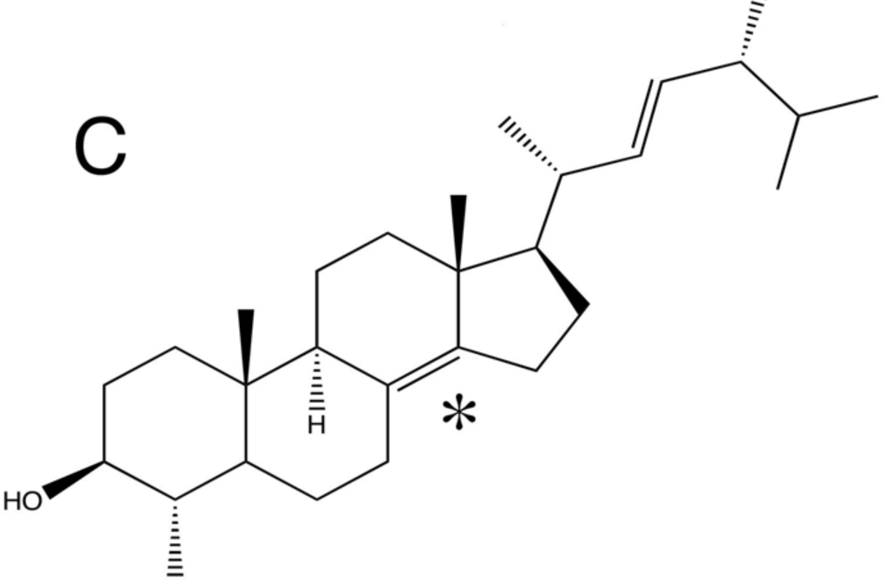


Figure 7.3. Comparison of dose response relationships for the lysis of fish red blood cells for several of the toxins from *Karlodinium micrum*. *pKmTx* denotes “putative *Karlodinium micrum* toxin”. Dashed line represents LD_{50}.

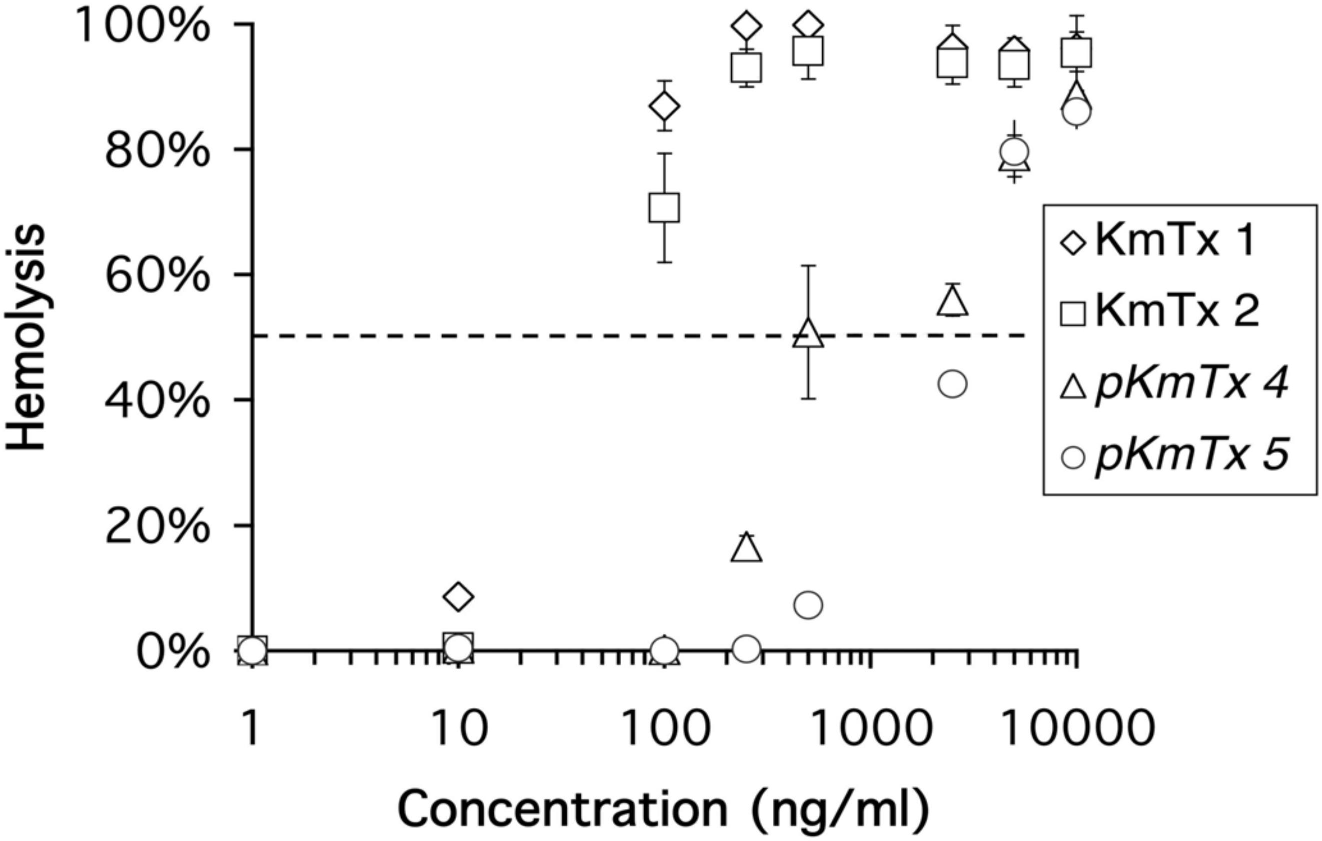
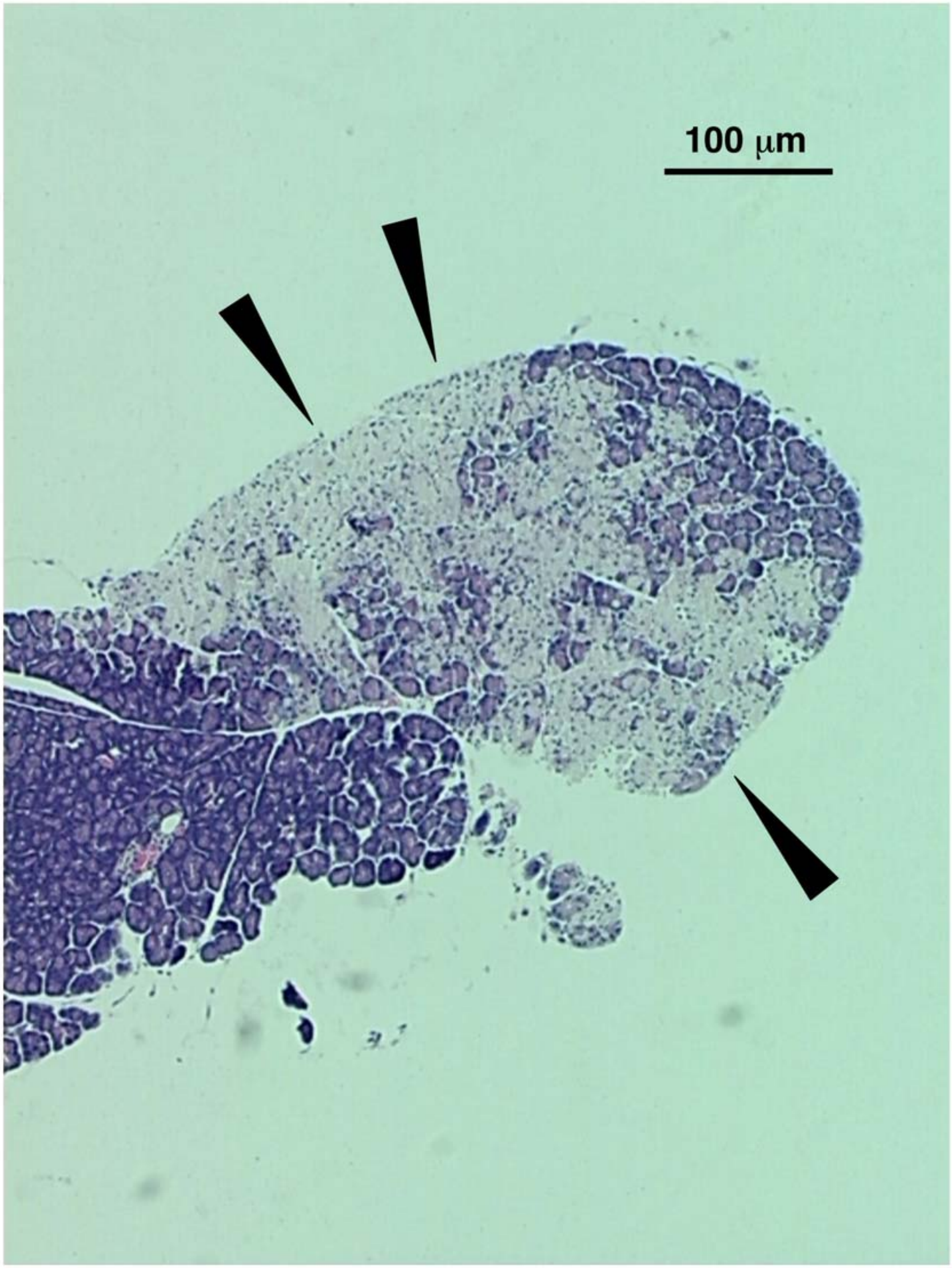

100%
80%
60%
40%
20%
0%
Hemolysis
1
10
100
1000
10000
Concentration (ng/ml)
◇ KmTx 1
□ KmTx 2
△ *pKmTx 4*
○ *pKmTx 5*

Figure 7.4. H&E stained section of mouse pancreas after 24-hour exposure to an IP injection of 10 μg KmTx 2. Arrows indicate areas of necrosis.



100 μm

CHAPTER 1 - LITERATURE CITED

Braarud, T. 1957. A red tide organism from Walvis Bay. Galathea Report 1, 137-138.

Burkholder, J. M., Noga, E. J., Hobbs, C. W., Glasgow, H. B. J., and Smith, S. A. 1992. New "phantom" dinoflagellate is the causitive agent of major estuarine fish kills. Nature 358, 407-410.

Daugbjerg, N., Hansen, G., Larsen, J., and Moestrup, O. 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39, 302-317.

Deeds, J. R., Kibler, S. R., Tester, P. A., and Place, A. R. *Submitted* a. Geographic strain variation in toxin production in *Karlodinium micrum* (Dinophyceae) from Southeastern estuaries of the United States. Proceedings of the Xth International Conference on Harmful Algae, 2002.

Deeds, J. R., Mazzaccaro, A. A., Terlizzi, D. E., and Place, A. R. *Submitted* b. Treatment options for the control of an ichthyotoxic dinoflagellate in an estuarine aquaculture facility: A case study. Proceedings of the Second International Conference of Harmful Algae Management and Mitigation, 2002.

Deeds, J. R., Terlizzi, D. E., Adolf, J. E., Stoecker, D. K., and Place, A. R. 2002. Toxic activity from cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - A dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. Harmful Algae 1, 169-189.

Goshorn, D., Deeds, J., Tango, P., Poukish, C., Place, A. R., McGinty, M., Butler, W., Lucket, C., and Magnien, R. *Submitted*. Occurrence of *Karlodinium micrum* and its association with fish kills in Maryland estuaries. Proceedings of the Xth International Conference on Harmful Algae.

Hulburt, E. M. 1957. The taxonomy of unarmoured Dinophyceae of shallow embayments on Cape Cod, Massachusetts. Biol. Bull. 112, 196-219.

Kempton, J. W., Lewitus, A. J., Deeds, J. R., Law, J. M., and Place, A. R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. Harmful Algae 1, 233-241.

Marshall, H. G. 1980. Seasonal phytoplankton composition in the lower Chesapeake Bay and Old Plantation Creek, Cape Charles, Virginia. Estuaries 3, 207-216.

Nielsen, M. V. 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar. Ecol.-Prog. Ser. 95, 273-277.

Nielsen, M. V., and Stromgren, T. 1991. Shell growth response of mussels (*Mytilus edulis*) exposed to toxic microalgae. Mar. Biol. 108, 263-267.

Pieterse, F., and Van Der Post, D. C. 1967. The Pilchard of South West Africa (*Sardinops ocellata*) Oceanographic conditions associated with red-tides and fish mortalities in the Walvis Bay region. Investigational Report No. 14, 8-27.

Steidinger, K. A., Burkholder, J. M., Glasgow, H. B. J., Hobbs, C. W., Garrett, J. K., Truby, E. W., Noga, E. J., and Smith, S. A. 1996. *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), A new toxic dinoflagellate with a complex life cycle and behavior. J. Phycol. 32, 157-164.

CHAPTER 2 - LITERATURE CITED

Arzul, G., Gentien, P., Bodennec, G., Toularastel, F., Youenou, A., and Crassous, M. P., 1995. Comparison of toxic effects in *Gymnodinium* cf. *nagasakiense* polyunsaturated fatty acids. In: Lassus, P., Arzul, G., Erard, E., Gentain, P. and Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd., pp. 395-400.

Arzul, G., Gentien, P., and Crassous, M., 1994. A haemolytic test to assay toxins excreted by the marine dinoflagellate *Gyrodinium* cf. *aureolum*. Water Res. 28(4), 961-965.

ASTM, 1992. Standard guide for conducting toxicity tests with fishes, macroinvertebrates, and amphibians - E 729-88a. American Society for Testing and Materials, Philadelphia, pp. 403-422.

Bodennec, G., Gentien, P., Parrish, C. C., Arzul, G., Youenou, A., and Crassous, M. P., 1995. Production of suspected lipid phycotoxins by *Gymnodinium* cf. *nagasakiense* in batch cultures. In: Lassus, P., Arzul, G., Erard, E., Gentain, P. and Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd., pp. 407-412.

Botsford, L. W., Castilla, J. C., and Peterson, C. H., 1997. The management of fisheries and ecosystems. Science 227(5325), 509-515.

Boyd, C.E. 1999. Aquaculture sustainability and environmental issues. World Aquaculture 30(2), 10-13.

Braarud, T., 1957. A red tide organism from Walvis Bay. Galathea Report 1, 137-138.

Burkholder, J. M., and Glasgow, H. B., Jr., 1997. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: Behavior, impacts, and environmental controls. Limnol. Oceanogr. 42(5), 1052-1075.

Burkholder, J.M., Glasgow, H.B., Deamer-Melia, N.J., Springer, J, Parrow, M.W., Zhang, C., Cancellieri, P.J., 2001. Species of the toxic *Pfiesteria* complex, and the importance of functional type in data interpretation. Environ. Health Perspec. (Supp.) 109(5), 667-679

Burkholder, J. M., Glasgow, H. B., Jr, Lewitus, A., Parrow, M., Zheng, C., Cancellieri, P., and Deamer-Melia, N., 2000. Functional type (toxicity status) controls *Pfiesteria* response to nutrients and algal prey versus fish prey. Symposium on Harmful Marine Algae in the U.S., Woods Hole, Massachusetts, pp. 24.

Burkholder, J. M., Noga, E. J., Hobbs, C. W., Glasgow, H. B., Jr., and Smith, S. A., 1992. New "phantom" dinoflagellate is the causitive agent of major estuarine fish kills. Nature 358, 407-410.

Daugbjerg, N., Hansen, G., Larsen, J., Moestrup, O., 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39(4), 302-317.

Deeds, J. R., Terlizzi, D. E., Stoecker, D. K., Way, R., and Place, A. R., 2000. Relationship between octadecapentaenoic acid, algicidal treatments, and toxicity in two bloom forming dinoflagellates, *Karlodinium micrum* and *Prorocentrum minimum*, found in the Chesapeake Bay, MD. Symposium on Harmful Marine Algae in the U.S., Woods Hole, Massachusetts, pp. 106.

Edvardsen, B., Moy, F., and Paasche, E., 1990. Hemolytic activity in extracts of *Chrysochromulina polylepis* grown at different levels of selenite and phosphate. In: Graneli, E. (Ed.), Toxic Marine Phytoplankton. Elsevier Science Publishing Co., Inc., Amsterdam, pp. 284-289.

Edvardsen, B., and Paasche, E., 1998. Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. In: Anderson, D.M., Cembella, A. D. and Hallegraeff, G. M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin Heidelberg, pp. 193-208.

Fairey, E.R., Edmunds, J.S., Deamer-Melia, N.J., Glasgow, H., Jr., Johnson, F.M., Moeller, P.R., Burkholder, J.M., and Ramsdell, J.S., 1999. Reporter gene assay for fish-killing activity produced by Pfiesteria piscicida. Environ. Health Perspect. 170(9), 711-714.

Fossat, B., Porthe-Nibelle, J., Sola, F., Masoni, A., Gentien, P., and Bodennec, G., 1999. Toxicity of fatty acid 18:5n3 from *Gymnodinium* cf. *mikimotoi*: II. Intracellular pH and K^+ uptake in isolated trout hepatocytes. J. Appl. Toxicol. 19, 275-278.

Glasgow, H.B., Jr., Lewitus, A.J., and Burkholder, J.M., 1998. Feeding behavior of the ichthyotoxic dinoflagellate, *Pfiesteria piscicida*, on amino acids, algal prey, and fish vs. mammalian erythrocytes. In. Reguera, B., Blanco, J., Fernandez, M.L., and Wyatt, T. (Eds.), Proceedings, VIIIth International Conference on Harmful Algal Blooms. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Paris.

Glibert, P. M., and Terlizzi, D. E., 1999. Cooccurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. Appl. Environ. Microb. 65(12), 5594-5596.

Guillard, R. R. L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., and Chanley, M. H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 29-60.

Guo, M., Harrison, P. J., and Taylor, F. J. R., 1996. Fish kills related to *Prymnesium parvum* N. Carter (Haptophyta) in the People's Republic of China. J. Appl. Phycol. 8, 111-117.

Hallegraeff, G. M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32(2), 79-99.

Johansson, N., and Graneli, E., 1999. Cell density, chemical composition and toxicity of *Chrysochromulina polylepis* (Haptophyta) in relation to different N:P supply ratios. Mar. Biol. 135, 209-217.

Jones, K. J., Ayres, P., Bullock, A. M., Roberts, R. J., and Tett, P., 1982. A red tide of *Gyrodinium auroelum* in sea lochs of the Firth of Clyde and associated mortality of pond-reared salmon. J. Mar. Biol. Assoc. U.K. 62, 771-782.

Khan, S., Arakawa, O., and Onoue, Y., 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. Aquac. Res. 28, 9-14.

Li, A., Stoecker, D. K., and Adolf, J. E., 1999. Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. Aquat. Microb. Ecol. 19, 163-176.

Li, A., Stoecker, D. K., and Coats, D. W., 2000. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. J. Plankton. Res. 22(11), 2105-2124.

Marshall, H. G., 1999. *Pfiesteria piscicida* and dinoflagellates similar to *Pfiesteria*. VA. J. Science 50(4), 281-285.

Nielsen, M. V., 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar. Ecol.-Prog. Ser. 95, 273-277.

— 1996. Growth and chemical composition of the toxic dinoflagellate *Gymnodinium galatheanum* in relation to irradiance, temperature and salinity. Mar. Ecol.-Prog. Ser. 136, 205-211.

Nielsen, M. V., and Stromgren, T., 1991. Shell growth response of mussels (*Mytilus edulis*) exposed to toxic microalgae. Mar. Biol. 108, 263-267.

Parrish, C. C., Bodennec, G., and Gentien, P., 1998. Haemolytic glycolipids from *Gymnodinium* species. Phytochemistry 47(5), 783-787.

Parrish, C. C., Bodennec, G., Sebedio, J.-L., and Gentien, P., 1993. Intra- and extracellular lipids in cultures of the toxic dinoflagellate, *Gyrodinium aureolum*. Phytochemistry 32(2), 291-295.

Pieterse, F. and Van Der Post, D. C., 1967. The Pilchard of South West Africa (*Sardinops ocellata*) Oceanographic conditions associated with red-tides and fish mortalities in the Walvis Bay region. Investigational Report No. 14, 8-27.

Roberts, R. J., Bullock, A. M., Turner, M., Jones, K., and Tett, P., 1983. Mortalities of *Salmo gairdneri* exposed to cultures of *Gyrodinium aureolum*. J. Mar. Biol. Assoc. U.K. 63, 741-743.

Sola, F., Masoni, A., Fossat, B., Porthe-Nibelle, J., Gentien, P., and Bodennec, G., 1999. Toxicity of fatty acid 18:5n3 from *Gymnodinium* cf. *mikimotoi*: I. Morphological and biochemical aspects on *Dicentrarchus labrax* gills and intestines. J. Appl. Toxicol. 19, 279-284.

Steidinger, K. A., Burkholder, J. M., Glasgow, H. B. J., Hobbs, C. W., Garrett, J. K., Truby, E. W., Noga, E. J., and Smith, S. A., 1996. *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), A new toxic dinoflagellate with a complex life cycle and behavior. J. Phycol. 32, 157-164.

Tengs, T., Bowers, H. A., Ziman, A. P., Stoecker, D. K., and Oldach, D. W., 2001. Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. Mol. Ecol. 10(2), 515-23.

Ulitzer, S., and Shilo, M., 1966. Mode of action of *Prymnesium parvum* ichthyotoxin. J. Protozool. 13(2), 332-336.

— 1970. Procedure for purification and separation of *Prymnesium parvum* toxins. Biochim. Biophys. Acta 201, 350-363.

Xi, D., Kurtz, D.T., and Ramsdell, J.S., 1996. Maitotoxin-elevated cytosolic free calcium in GH4C1 rat pituitary cells nimodiphine-sensitive and -insensitive mechanisms. Biochem. Pharmacol. 51(6) 759-769.

Yasumoto, T., Underdal, B., Aune, T., Hormazabal, V., Skulberg, O. M., and Oshima, Y., 1990. Screening for hemolytic and ichthyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In: Granell, E., (Ed.), Toxic Marine Phytoplankton. Elsevier Science Publishing Co., Inc., Amsterdam, pp. 436-440.

Young, R.C., McLaren, M., and Ramsdell, J.S., 1995. Maitotoxin increases voltage independent chloride and sodium currents in GH4C1 rat pituitary cells. Nat. Toxins 3(6), 419-427.

Yongmanitchai, W., and Ward, O. P., 1992. Separation of lipid classes from *Phaeodactylum tricornutum* using silica cartridges. Phytochemistry 31(10), 3405-3408.

CHAPTER 3 - LITERATURE CITED

Boyd, C.E. 1990. Water Quality in Ponds for Aquaculture. Birmingham Publishing Co., Birmingham, Alabama.

Colberg, P.J., Lingg, A.J. 1978. Effect of ozonation on microbial fish pathogens, ammonia, nitrate, nitrite, and BOD in simulated reuse hatchery water. J. Fish. Res. Board Can. 35, 1290-1296.

Fletcher, G.C., Hay, B.E., Scott, M.F. 1998. Detoxifying Pacific oysters (*Crassostrea gigas*) of the neurotoxic shellfish poison (NSP) produced by *Gymnodinium breve*. J. Shellfish Res. 17(5), 1637-1641.

Glibert, P.M., Terlizzi, D.E. 1999. Co-occurrence of elevated urea levels and dinoflagellate blooms in temperate estuarine aquaculture ponds. Appl. Environ. Microb. 65 (12), 5594-5596.

Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J.M., and Place, A.R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. Harmful Algae 1, 233-241.

Masser, M.P. 2000. Aquatic vegetation control. In: Encyclopedia of Aquaculture. R. R. Stickney, (Ed.), John Wiley and Sons Inc., New York, pp. 51-61.

Noga, E.J. 2000. Fish Disease: Diagnosis and Treatment. Iowa State University Press, Ames, Iowa.

Reardon, I.S., Harrell, R.M. 1990. Acute toxicity of formalin and copper sulfate to striped bass fingerlings held in varying salinities. Aquaculture 87, 255-270.

Schneider, K.R., Rodrick, G.E. 1995. The use of ozone to degrade *Gymnodinium breve* toxins, R. Poggi, J.-Y. Le Gall, (Eds)., Shellfish Depuration. Second International Conference on Shellfish Depuration, Rennes 6-8 April, 1992 (IFREMER, Plouzane).

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J. 2001. Recirculating Aquaculture Systems. Cayuga Aqua Ventures.

Tucker, C.S. 1987. Acute toxicity of potassium permanganate to channel catfish fingerlings. Aquaculture 60, 93-98.

Tucker, C.S. 1989. Method of estimating potassium permanganate disease treatment rates in channel catfish in ponds. Prog. Fish. Cult. 51, 24-26.

Tucker, C.S., Boyd, C.E. 1977. Relationships between potassium permanganate and water quality. T. Am. Fish. Soc. 106, 481-488.

U. S. Environmental Protection Agency. 1999. "Alternative Disinfectants and Oxidants Guidance Manual - EPA 815-R-99-014” *EPA 815-R-99-014* . United States Environmental Protection Agency Office of Water.

CHAPTER 4 - LITERATURE CITED

ASTM. 1992. Standard guide for conducting toxicity tests with fishes, macroinvertebrates, and amphibians - E 729-88a. Philadelphia: American Society for Testing and Materials. Report nr E 729-88a. 403-422.

Baginski M, Resat H, Borowski E. 2002. Comparative molecular dynamics simulations of amphotericin B-cholesterol/ergosterol membrane channels. Biochim. Biophys. Acta 1567:63-78.

Baumann E, Stoya G, Volkner A, Richter W, Lemke C, Linss W. 2000. Hemolysis of human erythrocytes with saponin affects the membrane structure. Acta Histochem. 102:21-35.

Bolard J. 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta 864:257-304.

Braarud T. 1957. A red tide organism from Walvis Bay. Galathea Report 1:137-138.

Burkholder JM, Glasgow HBJ. 1997. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: Behavior, impacts, and environmental controls. Limnol. Oceanogr. 42(5):1052-1075.

Burkholder JM, Glasgow HBJ, Deamer-Melia N. 2001a. Overview and present status of the *Pfiesteria* complex (Dinophyceae). Phycologia 40(3):186-214.

Burkholder JM, Glasgow HBJ, Deamer-Melia NJ, Springer J, Parrow MW, Zhang C, Cancellieri PJ. 2001b. Species of the toxic *Pfiesteria* complex, and the importance of functional type in data interpretation. Environ. Health Perspec. 109(5):667-679.

Daugbjerg N, Hansen G, Larsen J, Moestrup O. 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39(4):302-317.

de Kruijff B. 1990. Cholesterol as a target for toxins. Bioscience Rep. 10(2):127-130.

Deeds JR, Kibler SR, Tester PA, Place AR. *Submitted*. Geographic strain variation in toxin production in *Karlodinium micrum* (Dinophyceae) from Southeastern estuaries of the United States; October 21-25, 2002; St. Pete's Beach, FL.

Deeds JR, Terlizzi DE, Adolf JE, Stoecker DK, Place AR. 2002. Toxic activity from cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - A dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. Harmful Algae 1:169-189.

Edwards L, Hessinger DA. 2000. Portuguese Man-of-war (*Physalia physalis*) venom induces calcium influx into cells by permeabilizing plasma membranes. Toxicon 38:1015-1028.

Edwards L, Whitter E, Hessinger DA. 2002. Apparent membrane pore-formation by Portuguese Man-of-war (*Physalia physalis*) venom in intact cultured cells. Toxicon 40(9):1299-1305.

Fivaz M, Abrami L, Tsitrin Y, van der Goot G. 2001. Not as simple as just punching a hole. Toxicon 39:1637-1645.

Gary-Bobo CM. 1989. Polyene-sterol interaction and selective toxicity. Biochimie 71:37-47.

Giner J-J, Faraldos JA, Boyer GL. 2003. Novel sterols of the toxic dinoflagellate *Karenia brevis* (Dinophyceae): a defensive function for unusual marine sterols. J. Phycol. 39:315-319.

Glasgow HBJ, Burkholder JM, Mallin MA, Deamer-Melia N, Reed RE. 2001. Field ecology of toxic *Pfiesteria* complex species and a conservative analysis of their role in estuarine fish kills. Environ. Health Perspec. 109 (supplement 5):715-730.

Goshorn D, Deeds J, Tango P, Poukish C, Place AR, McGinty M, Butler W, Lucket C, Magnien R. *Submitted*. Occurrence of *Karlodinium micrum* and its association with fish kills in Maryland estuaries; October 21-25, 2002; St. Pete's Beach, FL.

Grynkiewicz G, Poenie M, R.Y. T. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3340-3350.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. 1981. Improved parch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391:85-100.

Igarashi T, Aritake S, Yasumoto T. 1998. Biological activities of prymnesin-2 isolated from a red tide alga *Prymnesium parvum*. Nat. Toxins 6:35-41.

Kao JYP. 1994. Practical aspects of measuring [Ca^{2+}] with fluorescent indicators. Methods in Cell Biology: Academic Press.

Kempton JW, Lewitus AJ, Deeds JR, Law JM, Place AR. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. Harmful Algae 1:233-241.

Klein Breteler WCM, Schogt N, Baas M, Schouten S, Kraay GW. 1999. Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. Mar. Biol. 135:191-198.

Landsberg JH. 2002. The effect of harmful algal blooms on Aquatic organisms. Rev. Fish. Sci. 10(2):113-390.

Leal-Cardoso H, Koschorke GM, Taylor G, Weinreich D. 1993. Electrophysiological properties and chemosensitivity of acutely isolated nodose ganglion neurons of the rabbit. J. Auto. Nerv. Sys. 45:29-39.

Leblond JD, Chapman PJ. 2002. A survey of the sterol composition of the marine dinoflagellates *Karenia brevis*, *Karenia mikimotoi*, and *Karlodinium micrum*: distribution of sterols within other members of the class dinophyceae. J. Phycol. 38:670-682.

Lenz RA, Pilter TA, Alger BE. 1997. High intracellular Cl- concentrations depress G-protein-modulated ionic conductances. J. Neurosci.:6133-6141.

Li A, Stoecker DK, Coats DW. 2000. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. J. Plankton Res. 22(11):2105-2124.

Marshall HG. 1999. *Pfiesteria piscicida* and dinoflagellates similar to *Pfiesteria*. VA J Science 50(4):281-285.

NCCLS. 2002a. NCCLS Document M27-A2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Second Edition. Wayne, PA, USA: NCCLS.

NCCLS. 2002b. NCCLS Document M38-A Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. Wayne, PA, USA: NCCLS.

Nielsen MV. 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar. Ecol.-Prog. Ser. 95:273-277.

Nielsen MV. 1996. Growth and chemical composition of the toxic dinoflagellate *Gymnodinium galatheanum* in relation to irradiance, temperature and salinity. Mar. Ecol.-Prog. Ser. 136:205-211.

Noga EJ. 2000. Fish Disease: Diagnosis and Treatment. Ames, Iowa: Iowa State University Press. 367 p.

Oldach DW, Delwiche CF, Jakobsen KS, Tengs T, Brown EG, Kempton JW, Schaefer EF, Bowers HA, Glasgow HBJ, Burkholder JM, Steidinger KA, Rublee PA. 2000. Heteroduplex mobility assay-guided sequence discovery: Elucidation of the small subunit (18S) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal culture and environmental sample DNA pools. Pro. Nat. Acad. Sci. 97(8):4303-4308.

Paul GK, Matsumori N, Murata M, Tachibana K. 1997. Chemical structures of amphidinols 5 and 6 isolated from marine dinoflagellate *Amphidinium klebsii* and their cholesterol-dependent membrane disruption. J. Mar. Biotech. 5:124-128.

Peitsch MC, Tschopp J. 1991. Assembly of macromolecular pores by immune defense systems. Curr. Opin. Cell Biol. 3:710-716.

Pieterse F, Van Der Post DC. 1967. The Pilchard of South West Africa (*Sardinops ocellata*) Oceanographic conditions associated with red-tides and fish mortalities in the Walvis Bay region. Investigational Report No 14:8-27.

Qian, W., Deeds, J.R., Place, A.R., and Belas, R. 2003. Dinoflagellate community analysis of a fish kill using denaturing gradient gel electrophoresis. Harmful Algae, *Submitted*.

Quesenberry MS, Saito S, Krupatkina DN, Robledo JAF, Drgon T, Pecher WT, O'Leary N, Alavi M, Miller T, Schneider RE, Belas R, Deeds JR, Place AR, Zohar Y, Vasta GR. 2002. Bioassay for ichthyocidal activity of *Pfiesteria piscicida*: characterization of a culture flask assay format. J. Appl. Phycol. 14(4):241-254.

Raimondi, F., Kao, J.P.Y., Fiorentini, C., Fabbri, A., Donelli, G., Gasparini, N., Rubino, A. & Fasano, A. 2000. Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in *in vitro* systems. Infect. Immun. 68, 3180-3185.

Seaborn DW, Dunstan WM, Marshall HG, Gordon AS, Seaborn AM. 2000. Growth and toxicity studies of the dinoflagellates *Cryptoperidiniopsis* sp., *Gyrodinium galatheanum*, and *Pfiesteria piscicida*. In: Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ, (Eds.) Harmful Algal Blooms: Intergovernmental Oceanographic Commission of UNESCO.

Steidinger KA, Burkholder JM, Glasgow HBJ, Hobbs CW, Garrett JK, Truby EW, Noga EJ, Smith SA. 1996. *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), A new toxic dinoflagellate with a complex life cycle and behavior. J. Phycol. 32:157-164.

Terlizzi DE, Stoecker D, Glibert PM. *Gyrodinium galatheanum*: a threat to estuarine aquaculture. In: Flos R, Cressell L, (Eds.); 2000; Nice, France. European Aquaculture Society.

Thelestam M, Mollby R. 1979. Classification of microbial, plant, and animal cytolysins based on their membrane-damaging effects on human fibroblasts. Biochim. Biophys. Acta. 557:156-169.

Wassler M, Westman J, Fries E. 1990. Permeabilization of hepatocytes by a saponin and the effects of dextran. Eur. J. Cell Biol. 51:252-258.

Yongmanitchai, W., and Ward, O. P., 1992. Separation of lipid classes from *Phaeodactylum tricornutum* using silica cartridges. Phytochemistry 31(10), 3405-3408.

CHAPTER 5- LITERATURE CITED

Abel, P.D. 1976. Toxic action of several lethal concentrations of an anionic detergent on the gills of the brown trout (*Salmo trutta* L.). J. Fish Biol. 9:441-446.

ASTM. 1992. Standard guide for conducting toxicity tests with fishes, macroinvertebrates, and amphibians - E 729-88a. Philadelphia: American Society for Testing and Materials. Report nr E 729-88a. 403-422.

Baker, J.T.P. 1969. Histological and electron microscopical observations on copper poisoning in the winter flounder (*Pseudopleuronectes americanus*). J. Fish Res. Board Can. 26(11):2785-2793.

Braarud, T. 1957. A red tide organism from Walvis Bay. Galathea Report 1:137-138.

Burkholder, J.M, and Glasgow H.B.J. 1997. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: Behavior, impacts, and environmental controls. Limnol. Oceanogr. 42(5):1052-1075.

Clark, G. 1981. Staining Procedures. Williams and Wilkins, Baltimore, MD, USA.

Deeds, J.R., Kibler, S.R., Tester, P.A., and Place, A.R. *Submitted*. Geographic strain variation in toxin production in *Karlodinium micrum* (Dinophyceae) from Southeastern estuaries of the United States; October 21-25, 2002; St. Pete's Beach, FL.

Deeds, J.R., Terlizzi, D.E., Adolf, J.E., Stoecker, D.K., and Place, A.R. 2002. Toxic activity from cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - A dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. Harmful Algae 1:169-189.

Edvardsen, B., and Paasche, E. 1998. Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. *In*: Anderson DM, Cembella AD, Hallegraeff GM, (Eds.) Physiological Ecology of Harmful Algal Blooms. Berlin Heidelberg: Springer-Verlag. 193-208.

Evans, D.H. 1987. The fish gill: site of action and model for toxic effects of environmental pollutants. Environ. Health Perspec. 71:47-58.

Evans, D.H. (Ed.) 1998. The Physiology of Fishes - Second Edition. New York: CRC Press.

Goshorn, D., Deeds, J., Tango, P., Poukish, C., Place, A.R., McGinty, M., Butler, W., Lucket, C., and Magnien, R. *Submitted.* Occurrence of *Karlodinium micrum* and its association with fish kills in Maryland estuaries; October 21-25, 2002; St. Pete's Beach, FL.

Guo, M., Harrison, P.J., and Taylor, F.J.R. 1996. Fish kills related to *Prymnesium parvum* N. Carter (Haptophyta) in the People's Republic of China. J Appl. Phycol. 8:111-117.

Hallegraeff, G.M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32(2):79-99.

Johansson, N., and Graneli, E. 1999. Cell density, chemical composition and toxicity of *Chrysochromulina polylepis* (Haptophyta) in relation to different N:P supply ratios. Mar. Biol. 135:209-217.

Jones, K.J., Ayres, P., Bullock, A.M., Roberts, R.J., and Tett, P. 1982. A red tide of *Gyrodinium auroelum* in sea lochs of the Firth of Clyde and associated mortality of pond-reared salmon. J. Mar. Biol. Assoc. UK 62:771-782.

Karan, V., Vitorovic, S., Tutundzic, V., and Poleksic, V. 1998. Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. Ecotox. Environ. Safety 40:49-55.

Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J.M., and Place, A.R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. Harmful Algae 1:233-241.

Khan, S., Arakawa, O., Onoue, Y. 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. Aquac. Res. 28:9-14.

Landsberg, J.H. 2002. The effect of harmful algal blooms on Aquatic organisms. Rev. Fish. Sci. 10(2):113-390.

Laurent, P., Dunel, S. 1980. Morphology of gill epithelia in fish. Am. J. Physiol. 238:R147-R159.

Li, A., Stoecker, D.K., and Coats, D.W. 2000. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. J Plankton. Res. 22(11):2105-2124.

Mallatt, J. 1985. Fish gill structural changes induced by toxicants and other irritants: A statistical review. Can. J. Fish. Aquat. Sci. 42:630-648.

Marshall, H.G. 1980. Seasonal phytoplankton composition in the lower Chesapeake Bay and Old Plantation Creek, Cape Charles, Virginia. Estuaries 3(3):207-216.

Marshall, H.G. 1999. *Pfiesteria piscicida* and dinoflagellates similar to *Pfiesteria*. VA J Sci. 50(4):281-285.

Nielsen, M.V. 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar. Ecol.-Prog. Ser. 95:273-277.

Roberts, R.J., Bullock, A.M., Turner, M., Jones, K., and Tett, P. 1983. Mortalities of *Salmo gairdneri* exposed to cultures of *Gyrodinium aureolum*. J. Mar. Biol. Assoc. UK 63:741-743.

Shimada, M., Murakami, T.H., Doi, A., Abe, S., Okaichi, T., Watanabe, M. 1982. A morphological and histochemical study on gill primary lamellae of the teleost, *Seriola quinqueradiata*, exposed to sea bloom. Acta Histochem. Cytochem. 15(4):497-507.

Shimada, M., Murakami, T.H., Mahayashi, T., Ozaki, H.S., Toyoshima, T., and Okaichi, T. 1983. Effects of sea bloom, *Chattonella antiqua*, on gill primary lamellae of the young yellowtail, *Seriola quinqueradiata*. Acta Histochem. Cytochem. 16(3):232-244.

Terlizzi, D.E., Stoecker, D., and Glibert, P.M. *Gyrodinium galatheanum*: a threat to estuarine aquaculture. *In*: Flos, R., Cressell, L., (Eds.) AQUA 2000 Proceedings; Nice, France. European Aquaculture Society.

Toyoshima, T., Ozaki, H.S., Shimada, M., Okaichi, T., and Murakami, T.H. 1985. Ultrastructural alterations on chloride cells of the yellowtail *Seriola quinqueradiata*, following exposure to the red tide species *Chattonella antiqua*. Mar. Biol. 88:101-108.

Ulitzer, S., and Shilo, M. 1966. Mode of action of *Prymnesium parvum* ichthyotoxin. J Protozool. 13(2):332-336.

CHAPTER 6- LITERATURE CITED

Braarud, T. 1957. Galathea Rep. 1, 137-138.

Deeds, J.R., Terlizzi, D.E., Adolf, J.A., Stoecker, D.K., and Place, A.R. 2002. Toxic activity from cultures of *Karlodinium micrum* (=*Gryodinium galatheanum*) (Dinophyceae)-a dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. Harmful Algae 1, 169-189.

Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J.M., and Place, A.R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish retention pond. Harmful Algae 1, 233-241.

Landsberg, J.H. 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10(2), 113-390.

Li, A., Stoecker, D.K., and Coats, D.W. 2000. Spatial and temporal aspects *Gyrodinium galatheanum* in the Chesapeake Bay: distribution and mixotrophy. J. Plankton Res. 22(11), 2105-2104.

Litaker, R.W., Vandersea, M.W., Kibler, S.R., Reece, K.R., Stokes, N.A., Steidinger, K.A., Millie, D.A., Bendis, B.J., Pigg, R.M. and Tester, P.A. 2003. Identification of *Pfiesteria piscicida* (Dinophyceae) and *Pfiesteria*-Like organisms using ITS-specific PCR assays. J. Phycol. 39: 754-761.

Nielsen, M.V. 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar.Ecol,-Prog.Ser. 95, 273-277.

Tengs, T., Bowers, H.A., Ziman, A.P., Stoecker, D.K., Oldach, D.W. 2001. Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. Mol. Ecol. 10 (2), 515-523.

CHAPTER 7 - LITERATURE CITED

Association of Official Agricultural Chemists. 1965. Paralytic Shellfish Poison, Biological Method (18) *In*: Official Methods of Analysis of the Association of Official Agricultural Chemists (10th ed.) Washington D.C., 282-284.

Aune, T., Sorby, R., Yasumoto, T., Ramstad, H., and Landsverk, T. 2002. Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. Toxicon 40, 77-82.

Baden, D.G., and Adam, D., 2000. Brevetoxins: Chemistry, mechanism of action and methods of detection. *In* Seafood and freshwater toxins: Pharmacology, physiology, and detection. Luis M. Botana (ed.) Marcel Dekker Inc., NY. pp 505-532.

Blanchard, M.-P., Klauke, N., Zitzmann, S., Plattner, H. 1999. Veratridine-mediated Ca^{2+} dynamics and exocytosis in Paramecium cells. J. Membrane Biol. 169:155-165.

Bolard J. 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta 864:257-304.

Cummins, J.M., and Hill, W.F. 1969. Special Report: Method for the Bioassay of *Gymnodinium breve* Toxin(s) *In*: Shellfish. Gulf Coast Marine Health Sciences Laboratory, Dauphin Island, Alabama 36528.

Deeds, J.R., Terlizzi, D.E., Adolf, J.E., Stoecker, D.K., and Place, A.R. 2002. Toxic activity from cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - A dinoflagellate associated with fish mortalities in an estuarine aquaculture facility. Harmful Algae 1**,** 169-189.

Gardiner, W.E., Rushing, A.E., Dawes, C.J. 1989. Ultrastructural observations of *Gyrodinium estuariale* (dinophyceae). J. Phycol. 25:178-183.

Giner, J.-J., Faraldos, J.A., Boyer, G.L. 2003. Novel sterols of the toxic dinoflagellate *Karenia brevis* (Dinophyceae): a defensive function for unusual marine sterols. J. Phycol. 39:315-319.

Hu, T., deFreitas, A.S.W., Curtis, J.M., Oshima, Y., Walter, J.A. and Wright, J.L.C. 1996. Isolation and structure of prorocentrolide B, a fast-acting toxin from *Prorocentrum maculosum*. J. Nat. Prod. 59: 1010-1014.

Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J.M., and Place, A.R. 2002. Toxicity of *Karlodinium micrum* (Dinophyceae) associated with a fish kill in a South Carolina brackish pond. Harmful Algae 1, 233-241.

Klein Breteler WCM, Schogt N, Baas M, Schouten S, Kraay GW. 1999. Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. Mar. Biol. 135:191-198.

Lee, R.E. 1992. Phycology, Second Edition. New York: Cambridge University Press.

Lee, M.S., Qin, G., Nakanishi, K. and Zagorski, M.G. 1989. Biosynthetic studies of brevetoxins, potent neurotoxins produced by the dinoflagellate *Gymnodinium breve*. J. Am. Chem. Soc., 111: 6234-6241.

Li, A. 1998. The Feeding Physiology and Ecology of the Mixotrophic Dinoflagellate

Gyrodinium galatheanum. Ph.D. Dissertation. University of Maryland at College Park.

Moore, B. 1999. Biosynthesis of marine natural products: microorganisms and macroalgae. Nat. Prod. Reps., 16: 653-674.

Morohashi, A., Satake, M., Nagai, H., Oshima, Y. and Yasumoto, T. 2000. The absolute configuration of gambieric acids A-D, potent antifungal polyethers, isolated from the marine dinoflagellate *Gambierdiscus toxicus*. Tetrahedron, 56: 8995-9001.

Nielsen, M.V. 1993. Toxic effect of the marine dinoflagellate *Gymnodinium galatheanum* on juvenile cod *Gadus morhua*. Mar. Ecol. Prog. Ser. 95, 273-277.

Nielsen, M.V. and Stromgren, T. 1991. Shell growth response of mussels (*Mytilus edulis*) exposed to toxic microalgae. Mar. Biol. 108, 263-267.

Paul, G. K., Matsumori, N., Murata, M., and Tachibana, T. 1995. Isolation and structure of amphidinol 2 a potent hemolytic compound from the marine dinoflagellate *Amphidinium klebsii*. Tetrahedron Lett., 36: 6279-6282.

Satake, M., Shoji, S., Oshima, Y., Naoki, H., Fujita, T. and Yasumoto, Y. 2002. Gymnocine-A, a cytotoxic polyether from the notorious red tide dinoflagellate *Gymnodinium mikimotoi*. Tetrahedron Letts., 43: 5829-5832.

Sugibayashi R, Harumoto T. 2000. Defensive function of trichocysts in Paramecium tetraurelia against heterotrich ciliate *Climacostomum virens*. Eur. J. Protozool. 36(4):415-422.

Tsim, S-T, Wong, J.T., Wong, Y.H. 1997. Calcium ion dependancy and the role of inositol phosphates in melatonin-induced encystment of dinoflagellates. J. Cell Sci. 110:1387-1393.