ABSTRACT

Influences of Nutrients and Salinity on *Prymnesium parvum* Elicited Sublethal Toxicity in Two Common Fish Models

Bridgett N. Hill, M.S.

Mentor: Bryan W. Brooks, Ph.D.

The magnitude, frequency, and duration of harmful algal blooms (HABs) are increasing worldwide primarily due to climate change and anthropogenic activities. *Prymnesium parvum* is a euryhaline and eurythermal HAB forming species that has expanded throughout North America resulting in massive fish kills. Previous ecotoxicological work supported an understanding of conditions resulting in HABs and fish kills; however, the primary endpoint selected for these studies was acute mortality. Whether adverse sublethal responses to *P. parvum* occur in fish are largely unknown. To begin to address this question, fish molecular and biochemical oxidative stress (OS) responses and behavioral alterations in two common fish models were investigated. Varying nutrient and salinity conditions influenced *P. parvum* related OS and fish behavioral responses of two common fish models, and these responses were heightened by conditions nonoptimal for *P. parvum* growth. Such sublethal observations present important considerations for future assessment and management of *P. parvum*.

Influences of Nutrients and Salinity on *Prymnesium parvum* Elicited Sublethal Toxicity in Two Common Fish Models

by

Bridgett N. Hill, B.S.

A Thesis

Approved by the Department of Environmental Science

_____

George P. Cobb, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Master of Science

Approved by the Thesis Committee

_____

Bryan W. Brooks, Ph.D., Chairperson

_____

Cole W. Matson, Ph.D.

_____

J. Thad Scott, Ph.D.

Accepted by the Graduate School

August 2018

_____

J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

A special thank you to my advisor, Dr. Bryan Brooks, for support, mentorship, and offering me endless opportunities to learn and engage with other scientists/collaborators. All of which helped me become the scientist I am today. A big thank you to my committee members, Dr. Thad Scott and Dr. Cole Matson for their guidance and input towards refining this thesis. I would like to thank Dr. Jone Corrales for her constant mentorship and training for all the oxidative stress related work presented in this thesis and Dr. Jeffery Back of the Center for Reservoir and Aquatic Science Research for performing nutrient analyses. Thank you to my fellow lab mates Sam Haddad, Bekah Burket, Lea Lovin, Rachel Molé and especially Gavin Saari, Baylor Steele, Casan Scott, and Dr. Lauren Kristofco for advice and assistance during the exposures, behavioral analysis, and data collection. I would also like to thank all the undergraduate workers including Brian Burbidge and Zach Rundell in addition to Ben Castellon who helped during the exposure and tissue collection process. My family and friends for all their support as I traveled halfway across the U.S. to pursue my dreams. Support for my conference travel was provided by the Department of Environmental Science and Baylor University Graduate School. Additional thanks to the Baylor Environmental Science Department and the Center for Reservoir and Aquatic Science Research for their support and assistance throughout my time at Baylor.

DEDICATION

To Kenny, my favorite fisherman and my brother, Barton

CHAPTER ONE

Influence of Nutrients on Sublethal Toxicity of the Harmful Haptophyte, *Prymnesium parvum*, to Two Common Fish Models

Introduction

The magnitude, frequency, and duration of harmful algal blooms (HABs) are increasing globally, primarily due to anthropogenic activities including altered hydrology, nutrient enrichment, salinization, and storm water and agriculture runoff in addition to climate change (Granéli et al., 2008; Hallegraff, 1993; Paerl and Huisman, 2009). Many HAB forming species are opportunistic and take advantage of altered habitat conditions leading to their proliferation, which is primarily facilitated by the production of secondary metabolites. Altered community structures, production of taste and odor compounds, fish kills and human health HAB-related illnesses are common results (Paerl et al., 2001). Monitoring and managing HABs are challenging due to the wide array of site-specific factors that can lead to these scenarios.

Prymnesium parvum, commonly referred to as golden algae or the "Texas Tide", is an invasive mixotrophic haptophyte capable of forming HABs oftentimes resulting in large economic losses (estimated in millions of dollars) due to the extensive fish and other gill-breathing organism mortality events (Roelke et al., 2015). *P. parvum* HABs are most often found in marine and estuarine waters, although blooms in brackish and inland water can also occur. *P. parvum* was first discovered in North America after a toxic bloom resulted in a massive fish kill in the Pecos River, Texas, U.S.A. in 1985. Since its first discovery *P. parvum* has invaded inland bodies of water throughout the southern

United States and has expanded northward where a devastating bloom occurred in Dunkard Creek along the Pennsylvania/West Virginia border as a result of introduced produced water (Brooks et al., 2011; Roelke et al., 2015). However, in the U.S.A., Texas has often experienced severe, frequent, and extensive *P. parvum* HABs including where threatened and endangered organisms appear susceptible (Southard et al., 2010). The expansion of *P. parvum* and recurrent fish kills raises aquatic ecosystem disruption and potential public health concerns (Brooks et al., 2011b; Brooks et al., 2016).

P. parvum related acute toxicity, and thus apparently toxin production, has been found to occur under conditions that deviate from optimal growth conditions, leading to potential associations with allelopathy in order to gain a competitive advantage over other algal species and deter predation. Various environmental factors, such as salinity, temperature, pH, and irradiance, have been shown to influence *P. parvum* growth and toxicity (Baker et al., 2007; Roelke et al., 2010; Valenti et al., 2010). However, a consensus over which specific factor(s) drives toxin production has not been determined due to the large geographical distribution of *P. parvum*, a number of site-specific factors influencing toxicity, and the inability to measure analytically associated toxins. Baker et al. (2007) proposed 22 ppt salinity, 27 ◦C and 275 μmol photons m^{-2} s^{-1} as the optimal growth conditions for *P. parvum*. Inland Texas *P. parvum* HABs typically occur during winter months, with temperatures ranging from 10-15 ◦C and salinities less than 4 ppt, clearly deviating from optimal conditions (Baker et al., 2007). *P. parvum* blooms can grow at suboptimal conditions as long as population losses are lower than the reproductive growth rate, which appears facilitated by toxins production (Brooks et al., 2011).

Nutrient imbalances, involving varying concentrations or stoichiometry, also influence *P. parvum* toxicity. Laboratory cultures grown under nutrient deficiency resulted in lower cell densities and higher toxicity when compared to cultures grown under nutrient sufficient conditions (Baker et al., 2009). Nutrient enrichment to field enclosures led to a decrease or elimination in toxicity when grown along different seasons (Roelke et al., 2007). Stoichiometric imbalances have also been shown to influence toxicity, with higher toxicity occurring in cultures grown under N and P limitation (Graneli, 2003). Eutrophication allows for the proliferation of *P. parvum*, often resulting in imbalanced N:P ratios leading to stressful conditions which result in increased toxicity (Manning and LaClaire, 2010).

Toxicity studies involving *in vitro* and *in vivo* models are commonly employed to determine the potential behavior of *P. parvum* toxins in the environment and resulting effects on organisms. Building from previous efforts (Brooks et al., 2010), I performed a literature review and identified a total of 155 toxicity studies of *P. parvum* in aquatic systems (Table 1.1). *In vivo* acute mortality was the most commonly studied endpoint accounting for 59 studies, followed by *in vivo* sublethal (56), *in vitro* acute mortality (34), and *in vitro* sublethal toxicity endpoints (5). Hemolysis and acute fish mortality were the most employed and sensitive *in vitro* and *in vivo* assays, respectively. Previous sublethal responses in *in vivo* fish models include equilibrium losses, changes in oxygen consumption and respiration and immunological impairment determined by the contraction of a virus (Table 1.1). Knowledge of sublethal responses in fish is imperative to understanding population level effects when devastating *P. parvum* blooms occur. Fish communities in Texas, including the Colorado River Basin, have exhibited declines or

altered community structures after *P. parvum* HABs (VanLandeghem et al., 2013). Unfortunately, an understanding of biochemical, molecular and behavioral responses *P. parvum* are lacking.

Oxidative stress (OS) is a physiological response resulting from an imbalance between reactive oxygen species (ROS) and antioxidant capabilities to detoxify these molecules. OS has been implicated in damage to tissues, inflammation, many disease states including neurodegenerative diseases and carcinogenesis in fish and mammals (Kohen and Nyska, 2002; Scandalios, 2005). The primary targets of ROS include proteins, lipids, and nucleic acids; therefore, OS can occur in virtually all intracellular organelles and across the entire organism (Scandalios, 2005). An increase in the use of biochemical responses in aquatic organisms have occurred in response to poorly understood cause and effect relationships among toxins/toxicants in conjunction with the need for early warning signals of ensuing pathology (Di Giulio et al., 1989). Biomarkers of OS have been studied for environmentally induced OS from exposure to xenobiotics, radiation, and metals (Limon-Pacheco and Consebatt, 2009; Lushchak, 2011; Valavanidis et al., 2006). The development of commercially available biochemical assays provide for rapid OS determination. The application of behavioral and OS biomarkers and thus contribution of OS toxicity by algae has been demonstrated (Baganz et al., 1998b; Lasley-Rasher et al., 2016b; Lefebvre et al., 2004; Rao and Bhattacharya, 1996; Wiegand et al., 1999).

Behavior responses, which represent an organism's response to changes in internal (physiological) and external (social) environments, have been shown to be rapid and sensitive indicators of environmental exposure (Gerhardt, 2007). Behavioral

syndromes have been established to determine signs of stress that are classified based on chemical structure and mode of toxicity (Drummond and Russom, 1990). Behavioral ecotoxicology utilizes these syndromes for various biomonitoring efforts to determine the extent of environmental exposure (Hellou, 2011). Further, behavioral endpoints are utilized in biomedical and toxicological studies to determine phycotrophic effects of pharmaceuticals, thus establishing behavioral fingerprints to easily predict and screen pharmaceuticals that have received less attention (Rihel et al., 2010). Recent research by my laboratory has extended these efforts to identify behavioral response profiles for various contaminants eliciting toxicity through nonpolar narcosis, polar narcosis, electrophilic and specifically acting modes and mechanisms of action (Steele et al 2018, Steele et al accepted). Therefore, behavior represents a biomonitoring tool for environmental exposures, and also may provide an early tier estimate of the mechanisms behind *P. parvum* sublethal toxicity.

Table 1.1. Studies of aquatic toxicity associated with *Prymnesium parvum* Carter following experiments performed under various environmental conditions for samples collected in the laboratory or field. Table represents a continuation of previously published work (Brooks et al. 2010).

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml^{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature (°C)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Aquatic Vertebrates									
Schug *et al.* 2010	*Pimpephales promelas*	NS; Fractionated Samples	Mortality	Lab	6	20	NS	f/2 medium	150-200 /12:12
Valenti *et al.* 2010	*Pimephales promelas*	1.3-2.1 x 10^5	Mortality	Lab	5.8	20	NS	f/2 and f/8 media	140 /12:12
Valenti *et al.* 2010	*Pimpephales promelas*	29-61.5 x 10^3	Mortality	Field	NS	NS	8.2-8.4	NS	Ambient spring light conditions
James *et al.* 2011	*Pimephales promelas*	NS; cell-free filtrate	Mortality	Lab	2.4	20	NS	f/2 medium	12:12
Prosser *et al.* 2012	*Pimephales promelas*	0.21-1.1 x 10^6	Mortality	Field	<1	8-17.5	7.5-8.5	NS	Ambient winter and spring conditions
Roelke *et al.* 2012	*Pimephales promelas*	0-35 x 10^5	Mortality	Field	NS	NS	7.7-8.8	Varied	Ambient conditions
Bertin *et al.*2012	red drum larvae	NS; fractionated samples	Mortality	Lab	6	NS	NS	BG-11 media with N/4 and P/4	NS
Anderson *et al.* 2016	Rainbow trout fingerlings	0.8-1 x 10^6	Susceptibility to virus	Lab	15	10	NS	f/2 medium	120 /12:12
VanLandegham *et al.* 2015	*Pimephales promelas*	1-100 x 10^3	Mortaltiy	Field	1.1-3.2	17.7-21.1	8-8.7	Varied	Ambient winter conditions

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml_{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature ($_oC$)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m_{-2} s_{-1})/ Light: Dark Cycle (h)
Zamor *et al.* 2014	*Pimephales promelas; Morone saxatilis; Menidia beryllina; Dorosoma cepedianum*	20-40 x 10_4	Mortality	Lab	15	NS	NS	NS	NS
Zamor *et al.* 2014	*Pimephales promelas*	25-233 x 10_4	Mortality	Field	NS	NS	NS	NS	NS
Barkoh *et al.* 2011	*Pimephales promelas*	<6,000	Mortality	Field	NS	12.5	8.18 - 8.24	Varied	Ambient fall and winter light conditions
Schwierzke-Wade *et al.* 2011	*Pimephales promelas*	18-37.8 x 10_5	Mortality	Field	1.3-1.7	9-17	8.1-8.6	Varied	Ambient light conditions
Schwierzke *et al.* 2010	*Pimephales promelas*	15-54 x 10_5	Mortality	Field	NS	9-17	8.1-8.6	Vaired	Ambient conditions
Hambright *et al.* 2014	*Pimephales promelas*	2-250 x 10_4	Mortality	Lab	0-30	25	NS	COMBO media with added salts	12:12
Kurten *et al.* 2011	*Pimephales promelas*	~5-200 x 10_2	Mortality	Field	3	19.3-21.6	8.2	300:30, 117:15	Ambient April and May light conditions
Hayden *et al.* 2012	*Pimephales promelas*	Natural; starting density 0.75 x 10_6	Mortality	Field	0.3-0.7	7.7-18.9	7.8-8.1	Varied	Winter and spring ambient conditions

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml_{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature ($_oC$)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m_{-2} s_{-1})/ Light: Dark Cycle (h)
Blossom *et al.* 2014b	*Oncorhynchus mykiss*	NS; late exponential phase	Moribund (loss of equilibrium)	Lab	15	15	NS	f/2 medium	250 /14:10
VanLandegham *et al.* 2012	*Danio rerio*	NS	Mortality	Field	0.5-12	7.2-26	6.28 - 8.35	Varied	Annual ambient light conditions
Henrikson *et al.*2010	*Pimpephales promelas*	2 x 10_6 extract	Mortality	Lab	NS	NS	7.8	COMBO media with added salts	12:12
Vasas *et al.* 2012	*Lebistes reticulatus*	0.3-40 x 10_3	Mortaltiy	Field	NS	22-28	8.2-9.2	NS	Ambient spring light conditions
Remmel & Hambright 2012	*Pimephales promelas*	0-20 x 10_4	Mortality	Lab	5.5 & 13.7	25	NS	COMBO media with varying N and P concnetrations	30 /12:12
Van Landegham *et al.* 2014	*Pimephales promelas*	0-10 x 10_5	Mortaltiy	Field	0.5-2.9	18.6-20.3	8.1-8.5	NS	Annual ambient conditions
Roelke *et al.* 2010	*Pimephales promelas*	0.31-0.86 x 10_5	Mortality	Field	~1	NS	NS	Natural lake water with f/2 enrichment	Ambient conditions
Lundgren *et al.* 2015	*Menidia beryllina*	5-65 x 10_6	Mortality	Field	NS	NS	NS	Varied	Ambient fall and winter light conditions
Zooplankton									
Valenti *et al.* 2010	*Daphnia magna*	29-61.5 x 10_3	Reproduction	Field	NS	NS	8.2, 8.4	NS	Ambient spring light conditions
Vasas *et al.* 2012	*Daphnia magna*	300-40,000	Mortality	Field	NS	22-28	8.2-9.2	NS	Ambient spring light conditions

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml_{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature ($_oC$)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m_{-2} s_{-1})/ Light: Dark Cycle (h)
Yu *et al.* 2016	*Harpacticus sp.*	3.6 x 10_4	Feeding rates and Reproduction	Lab	NS	20	NS	f/2 medium	60 /12:12
Prosser *et al.* 2012	*Daphnia magna*	0.21-1.1 x 10_6	Reproduction	Field	<1	8-17.5	7-8.5	NS	Ambient winter and spring light conditions
Remmel *et al.* 2011	*Daphnia pulicaria, Daphnia magna, Daphnia pulex*	31,000	Reproduction, Feeding rate, Life History	Lab	6-15	25	NS	COMBO media; N:P 16:1	12:12
Schwierzke *et al.* 2010	*Daphnia magna*	15-45 x 10_5	Reproduction	Field	NS	9-17	8.1-8.6	Varied	Ambient light coniditions
Aylagas *et al.* 2014	*Artemia fransiscana, Corophium multisetosum*	5-250 x 10_3	Mortality	Lab	35	20	NS	f/2 medium with added silica	100/14:10
Schwierzke-Wade *et al.* 2011	*Daphnia magna*	18-37.8 x 10_5	Reproduction	Field	1.3-1.7	9-17	8.1-8.6	Varied	Ambient light conditions
Remmel & Hambright 2012	*Daphnia pulex, Daphnia pulicaria*	0-20 x 10_4	*P. parvum* attachment rate	Lab	5.5 & 13.7	25	NS	COMBO media with varied nutrients	30/12:12
Roelke *et al.* 2010	*Daphnia magna*	0.31-0.86 x 10_5	Reproduction	Field	~1	NS	NS	Natural lake water with f/2 enrichment	Ambient light conditions

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml^{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature (°C)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m^{-2} s^{-1})/ Light: Dark Cycle (h)
Phytoplankton									
Lundgren *et al.* 2016	*Rhodomonas salina*	1.87-8 x 10^5	Cell density	Lab	7	20	8.2	10:1, 1:1, 100:1	100/16:8
Carvalho & Granéli 2010	*Rhodomonas salina*	5 x 10^4	Cell density	Lab	26	16	NS	f/20 medium	100/16:8
Driscoll *et al.* 2013	*Dunaliella tertiolecta*	.1-10 x 10^4	Cell density	Lab	6	22	NS	f/2 medium with silica	12:12
Blossom *et al.* 2014a	*Teleaulax acuta*	65-150 x 10^3 supernatant	Mortality	Lab	30	15	NS	f/2 medium	250/14:10
Blossom *et al.* 2015b	*Teleaulax acuta*	NS; late exponential phase	Mortality	Lab	12	12	NS	f/2 medium	250/14:10
Davis *et al.* 2015	Natural plankton community	200-4,000	Cell Density	Field	1	13	NS	Varied	Ambient lighting
Vidyarathna *et al.* 2014	*Rhodomonas salina*	6.38 x 10^5	Cell Density	Lab	7	20	8.2-9.4	f/10 medium, N:P 20:1	100/16:8
Freitag *et al.* 2011	*Rhodomonas baltica*	4.69-67.5 x 10^3	Cell Density	Lab	16-26	5-25	NS	NS	0-77/14:10
Weissbach & Legrand 2012	*Rhodomonas salina*	15-800 x 10^3	Cell Density	Lab	7 & 26	15	NS	f/10 medium	90/14:10
Miscellaneous *in vitro* cells									
Bertin *et al* 2012a	Rat pituitary and mouse neuroblastoma cells	Fractionated samples	Cell viability	Lab	6	NS	NS	BG-11 media with N/4 and P/4	NS

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml_{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature ($_oC$)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m_{-2} s_{-1})/ Light: Dark Cycle (h)
Bertin *et al* 2012b	Mouse neuroblastoma cells, rainbow trout gill cells	NS, cell-free filtrate; fatty acid amides isolated	Cell viability	Lab	NS	NS	6.5-8.5	NS	NS
Bertin *et al* 2014	Rainbow trout gill cells	NS, Oleamide, oleic acid, linoleamide, linoleic acid	Cell viability	Lab	NS	NS	6.5,8.5	NS	NS
Henrikson *et al.* 2010	Human cancer cell line	2 x 10_6 extract	Cell viability	Lab	NS	NS	7.8	COMBO media with added salts	12:12
Dorantes-Aranda *et al.* 2015	Rainbow trout gills cells	19-57 x 10_4	Antioxidant Enzyme Activity	Lab	35	20	NS	GSe Medium	150/12:12
Vidyarathna *et al.* 2014	Unidentified cell line	6.38 x 10_5 supernatant	Cell Viability	Lab	7	20	8.2-9.2	f/10 medium, N:P 20:1	100/16:8
Rasmussen *et al.* 2016	Rainbow trout gill cells	1-15 x 10_5	Cell viability	Lab	35	15	NS	f/2 medium	250
Erythrocytes									
Bertin *et al.* 2012	Sheep erythrocytes	NS, cell-free filtrate; fatty acid amides isolated	Hemolysis	Lab	NS	NS	6.5-8.5	NS	NS
Schug *et al.* 2010	Sheep erythrocytes	Fractionated samples	Hemolysis	Lab	6	20	NS	f/2 medium	150-200/12:12

Author(s) and Date	Model System	*P. parvum* Cell Density Used for the Toxicity Test (cells ml_{-1})	Endpoint(s)	Lab/ Field Study	Environmental Conditions for *P. parvum* Growth				
					Salinity (psu)	Temperature ($_oC$)	pH	N:P NO_3: PO_4 (µM)	Light Range (µmol m_{-2} s_{-1})/ Light: Dark Cycle (h)
Lundgren *et al.* 2016	Horse erythrocytes	1.87-8 x 10_5	Hemolysis	Lab	7	20	8.2	10:1, 1:1, 100;1	100/16:8
Carvalho & Granéli 2010	Horse erythrocytes	Cell-free filtrate	Hemolysis	Lab	26	16	NS	f/20 medium	100/16:8
Lindehoff *et al.* 2010	Horse erythrocytes	3-16 x 10_4	Hemolysis	Lab	7	15	NS	f/2 medium, N:P 4:1	100/16:8
Driscoll *et al.* 2013	Sheep erythrocytes	2 x 10_5	Hemolysis	Lab	6	22	NS	f/2 medium with silica	12:12
Vasas *et al.* 2012	Sheep erythrocytes	300-40,000	Hemolysis	Field	NS	22-28	8.2-9.2	NS	Ambient spring light conditions
Skingel *et al.* 2010	Sheep erythrocytes	0.2-10 x 10_5 supernatant	Hemolysis	Lab	6	20	7-9.6	f/2 medium	150-200/12:12
Freitag *et al.* 2011	Erythrocytes	1 x 10_7 (resuspended pellet)	Hemolysis	Lab	16-26	5-25	NS	NS	0-700/14:10
Weissbach & Legrand 2012	Erythrocytes	15-800 x 10_3	Hemolysis	Lab	7 & 26	15	NS	f/10 medium	90/14:10
Miscellaneous invertebrates									
Aylagas *et al.* 2014	*Paracentrotus lividus*	1-300 x 10_3	Embryo Development	Lab	35	20	NS	f/2 medium with silica	100/14:10

$_1$NS=not specified

The primary objective of the present study was to examine sublethal effects of *P. parvum* by evaluating common biochemical biomarkers of OS and photomotor behavioral response patterns. Sublethal responses were determined by these endpoints in order to assess the sensitivity at two different levels of biological organization (cellular and organismal). Sublethal responses were examined with two common fish models, the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*). To determine the toxicological effects of varying nutrient concentrations, *P. parvum* cultures were grown under nutrient sufficient and deficient conditions. Exposure to sublethal cell densities of *P. parvum* would result in OS and altered behavioral responses was hypothesized due to the influence of OS in other HAB toxicity. In addition, these responses were expected to be heightened under nutrient limited conditions due to the previously reported increases in acute mortality in response to stressful growth conditions. Lastly, differential toxicity to fathead minnow and zebrafish was expected due to previously reported comparative toxicological investigations by my laboratory (Corrales et al., 2018).

Methods

Laboratory Cultures and Experimental Design

A Texas strain of *P. parvum* was obtained from the University of Texas at Austin Culture Collection of Algae (UTEX LB 2797, Austin, TX, USA). Stock cultures were grown at Baylor University in a temperature controlled incubator at 25 ◦C under a 12:12 light:dark cycle at 25 ppt salinity and f/2 nutrients. Experimental cultures were inoculated when the stock culture reached late exponential growth phase. Approximately 100 cells/mL of the above stock culture was introduced to 19 L of media. Artificial seawater

(ASW) was prepared by dissolving Instant Ocean salt in Nanopure water (18.2 megohm ionic purity; Barnstead, ThermoFisher, Wilmington, DE, USA,). A super stock ASW solution of 35 ppt was first prepared, and then diluted to the target salinity of 2.4 ppt using Nanopure water according to previously published methods by my laboratory (Brooks et al., 2010). Water was enriched with either f/2 or f/8 nutrients, with full trace metals and vitamins for all cultures (Table A1, Appendix A; Guillard, 1975). ASW and all media stocks were autoclaved before use.

An experiment was performed at 15 ◦C, on a 12:12 light:dark cycle. Two replicate experimental units with either sufficient (f/2) or deficient (f/8) nutrient treatment levels were swirled and rotated daily. Every second day, 40 mL subsamples from each culture was preserved using 40 μL glutaraldehyde for cell counts. Cell counts were determined using a haemocytometer following a previously reported protocol (Southard, 2005). Additionally, cell growth was examined using chlorophyll a fluorescence using a hand held fluorometer (Turner Designs, San Jose, CA, USA) in order to rapidly assess condition of experimental units. Acute fish mortality was assessed on each culture during exponential and stationary growth phases on day 27 and 42, which were confirmed by cell counts and chlorophyll a fluorescence. At study initiation, dissolved nitrogen (nitrate/nitrite) and phosphorus (phosphate) concentrations were analyzed for each nutrient condition according to standard methods using a flow-injection auto-analyzer (Lachat QuikChem 8500 and Series 520 XYZ Autosampler; APHA (Clesceri et al. 1998). Media N and P concentrations (μg/L) for each treatment level (f/2 or f/8) are reported in Table A2. (Appendix A).

Fish Cultures

Fathead minnow and zebrafish used in toxicity studies were cultured at Baylor University. Fathead minnow (*P. promelas*) cultures were maintained at Baylor University using a flow through system that introduced aged dechlorinated tap water to individual aquaria. Cultures were maintained at 25±1 ◦C under a 16:8 light:dark cycle. Fathead minnow were fed brine shrimp (Artemia sp. nauplii; Pentair AES, Apopka, FL, USA) twice daily. Embryos were collected from sexually mature adults aged to at least 120 d before breeding (1:4-5 male to female ratio). Larvae within 24-48 h post hatch were used for toxicity studies. Tropical 5D wild type zebrafish (*D. rerio*) were cultured using a z-mod recirculating system (Marine Biotech Systems, Beverly, MA, USA). Zebrafish were maintained at a density of < 4 fish per liter in 260 ppm instant ocean with a pH of 7.0, temperature of 27±1 ◦C under a 16:8 light:dark cycle. Zebrafish were fed artemia (Artemia sp. nauplii; Pentair AES, Apopka, FL, USA) with flake food (Pentair AES, Apopka, FL, USA) twice daily. Embryos used for this experiment were collected from sexually mature adults, and were used for toxicity experiments at 48 h post fertilization. All experimental procedures and fish culture protocols followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

Acute Bioassays

Due to the lack of analytical standards, acute bioassays using fathead minnow were employed to determine toxicity of cultures throughout growth stages similar to previous published methods in our laboratory (Valenti et al., 2010). Bioassays were initiated when cell densities reached 10,000 cell/mL, the minimum cell density that is considered to be toxic (Roelke et al., 2007). Acute mortality of fathead minnow was

determined following U.S. Environmental Protection Agency (EPA) Toxicity Identification Evaluation (TIE) (US EPA 1991). Serial dilutions following a 0.5 dilution scheme were prepared for a total of six dilutions (e.g. 100, 50, 25, 12.5, 6.25, 3.125% of *P. parvum* culture). Media (2.4 ppt with f/2 or f8) served as the dilution water and treatment control. Five < 24 hour post hatch (hph) fathead minnow larvae were loaded into 80 mL of each culture dilution in duplicates. The pH of each solution was manipulated to 8.5 using 1 N hydrochloric acid or 1 N sodium hydroxide, due to the positive relationship between pH and toxicity (Valenti et al., 2010). Bioassays were performed in a temperature controlled incubator at 25 ◦C in the dark to prevent photo-degradation (James, 2011). Acute mortality was assessed at 24 and 48 hours to estimate the dilution to cause 50% organism lethality (LC_{50}) which was then normalized to *P. parvum* cell density. Identical acute mortality studies were performed during the *P. parvum* culture stationary growth phase with fathead minnow and zebrafish larvae (48 hph fathead minnow, 48 hour post fertilization (hpf) zebrafish) to determine LC_{50} values and subsequent sublethal study doses (cell densities). For larval zebrafish, the bioassays were performed in a temperature controlled incubator at 27 ◦C in the dark.

Sublethal Bioassays

Experimental design followed that of previous biochemical and molecular work conducted by my laboratory (Corrales et al, 2017). Sublethal culture studies with fathead minnow and zebrafish were performed according to standardized toxicity methods from the US EPA Whole Effluent Toxicity (WET) testing and Organization for Economic Cooperation and Development (OECD) Fish Embryo Toxicity Test (FET), respectively (EPA 2002, OECD no. 236). From each experimental carboy, dilution levels (10, 50,

100% *P. parvum* whole culture) were determined after a 48 h acute fish mortality bioassays from each carboy resulted in no mortalities. Media (2.4 ppt, f/2 or f/8) served as the diluent and control to derive these dilution levels. Fish were acclimated to experimental media for approximately 2 hours in a 50:50 ratio of culture water: *P. parvum* media or reconstituted hard water (RHW),which served as a negative control. The pH of each solution was manipulated to 8.5 using 1 N hydrochloric acid or 1 N sodium hydroxide, due to the influence of increasing pH on toxicity (Valenti et al., 2010). Bioassays were conducted in temperature controlled chambers set to 25 or 27 ◦C for fathead minnow and zebrafish, respectively, and in the dark to prevent toxin photodegration (James et al., 2011). Briefly, 48 hph fathead minnow larvae were placed in 200 mL of each dilution solutions. Each dilution level consisted of eight replicates of ten fathead minnow. Fifteen zebrafish at 48 hpf were exposed to 30 mL of each dilutions. Each dilution level consisted of twelve replicates with fifteen zebrafish each. These volumes were chosen to ensure that the loading density did not exceed acceptable levels for standardized guidelines. At 24 and 48 h mortalities and abnormalities were observed.

At 48 h, tissue samples were collected, frozen at -80 ◦C, and saved for biochemical OS determination and fathead minnow and zebrafish behaviors were observed. Collection of tissue and behavioral analysis were randomized per experimental unit using random.org for each dilution of experimental replicate 20 L carboy. Five fathead minnow were pooled per replicate for a total of three units for OS biochemical endpoints (total glutathione, lipid peroxidation, and DNA damage). Four fathead minnow were analyzed individually for behavior from one experimental unit for a total of three units. Fifteen zebrafish were pooled per experimental unit for a total of three units for OS

biochemical analyses. Six zebrafish were analyzed individually for behavior from one experimental unit for a total of four units. If no mortalities occurred, remaining fathead minnow and zebrafish were collected for additional DNA extractions. Each collection of organisms per experimental unit represented a biological replicate. Therefore, a total number of three biological replicates were statistically analyzed for all endpoints (except for zebrafish behavior) per each dilution of experimental replicate 20 L carboy. Four biological replicates were analyzed for zebrafish behavior from each dilution of experimental replicate 20 L carboys (following protocol developed by Steele et al, In press).

Biochemical Oxidative Stress Assays

Total glutathione concentration, lipid peroxidation, and oxidative DNA damage followed previously described methods (Corrales et al., 2017). All biochemical marker analyses consisted of three biological replicates and two technical replicates per dilutions of experimental 15 L carboys. Each biological replicate contained five pooled individual fathead minnow or fifteen zebrafish larvae. Briefly, total glutathione (GSH) concentration was determined using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA). Prior to conducting the assay, samples were deproteinated with 1.25 M metaphosphoric acid and 0.2 M triethanolamine. DTNB (5,5,-dithio-bis-2-nitrobenzoic acid) was added to deproteinated tissue supernatant initiating a reaction between the GSH present in tissue samples and DTNB yielding TNB (5-thio-2-nitrobenzoic acid). The rate of TNB production is directly proportional to the GSH concentration due to the recycling of GSH by glutathione reductase present. Total glutathione concentrations were normalized to sample protein content. Protein content was determined following the

Bradford protein assay by which a Bio-Rad protein dye was reacted with tissue supernatant (Sigma-Aldrich, St. Louis, MO, USA Cat. No. A7906 and 5000006).

Lipid peroxidation was determined by the concentration of malondialdehyde (MDA) present in tissue samples. MDA is a reactive carbonyl compound that is a natural product of lipid peroxidation. MDA concentration was quantified using a Thiobarbituric Acid Reactive Substances assay (TBARS) (Cayman Chemical Company, Ann Arbor, MI, USA). Thiobarbuturic acid (TBA) was added to each tissue sample, producing a MDA-TBA adduct which was fluorometrically detected. Elevated MDA concentrations are proportional to the MDA-TBA adducts formed. MDA concentration was also normalized to sample protein content. Protein content was determined following the Pierce BSA assay by which a working dye reagent was reacted with tissue supernatant (Thermo Scientific Wilmington, DE, USA, Cat No. 23225).

DNA oxidative damage was determined by presence of the oxidatively damaged guanine species, 8-hydroxy-2'-deoxyguanosine (8-OH-dG), measured using a commercially available enzyme immunoassay (EIA) (Cayman Chemical Company, Ann Arbor, MI, USA). Prior to development of EIA, DNA was extracted using DNAzol (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. Extracted DNA samples were cleaned and purified using Zymo Genomic DNA Clean and Concentrator (Zymo Research, Irvine, CA, USA) prior to DNA quantification. DNA concentrations were quantified using a Nanodrop2000 (Thermo Scientific, Wilmington, DE, USA), and 5 µg DNA per sample was prepared for the EIA by diluting DNA with Cayman Ultrapure water (Cayman Chemical Company, Ann Arbor, MI, USA) to yield a 50 µg/mL sample. The amount of 8-OH-dG present in the

sample competed with an added 8-OH-dG-acetylcholinesterase conjugate for binding to an oxidative damage monoclonal antibody. This antibody was bound to seeded goat polyclonal antimouse IgG cells. After an 18 hour incubation, each plate was washed five times and Ellman's reagent was added to develop the plate. The intensity of the signal is inversely proportional to the amount of free 8-OH-dG or oxidatively damaged DNA.

Behavioral Analysis

The behavioral responses of fathead minnow and zebrafish were observed after 48 h sublethal exposures to *P. parvum* following previously described methods (Kristofco et al., 2016; Steele et al., In press; Steele et al., Accepted). Twelve fathead minnows of each dilution from each experimental carboy replicate were loaded into 24 well plates, with each well containing two mL of treatment level solution. Twenty-four zebrafish of each dilutions from each experimental carboy replicate were loaded into 48 well plates with each well containing one mL of dilution level solution. All well plates were preloaded and maintained in exposure conditions until analysis. Fish were acclimated to the well plate for at least 30 minutes before behavioral platform loading. To minimize time of day behavioral effects, plates were analyzed from approximately 9:00 am to 2:00 pm for fathead minnow and 2:00-7:00 pm for zebrafish with each plate analyzed immediately after the conclusion of previous plate (Kristofco et al., 2016).

Larval swimming patterns were observed and recorded using automated tracking software (ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon France). This system was set in tracking mode and behavioral recordings took place over 50 minutes with a ten minute dark acclimation period followed by two altering ten minute light/dark cycles. Observations were recorded for total distance swam and total

number of movements. Additionally, distance swam, number of movements, and duration of movements were recorded for activity across three different speed thresholds. These speeds are categorized as bursting (>20mm/s), cruising (5-20 mm/s), and freezing (<5 mm/s) to characterize stimulatory and refractory behaviors. To measure larvae swimming responses to a sudden change in photoperiod condition, a photomotor response (PMR) was observed following methods previously used (Beker van Woudenberg et al., 2013) with slight modifications. PMR for each photoperiod transition (2 light and 2 dark responses) was calculated as the change in mean distance traveled (in mm) between the last minute of an initial photoperiod and the first minute of the following period.

Statistical Analysis

Specific growth rates of *P. parvum* cultures across nutrient conditions were calculated using the equation:

$$r = (\ln Nd - \ln N0)/ t$$

where r is the growth rate (divisions/day), Nd is the number of organisms at the beginning of the steady growth state, N0 is the number of organisms at study initiation, and t is the time (days) to reach steady state growth. The beginning of steady state was determined as the time at which the maximum *P. parvum* density was reached and followed by a decline in cell density.

The lethal concentration to cause 50% morality (LC_{50}) values from acute studies were calculated for each culture using the Toxicity Relationship Analysis Program version 1.30a (EPA). Sigma Plot (Systat Software Inc., San Jose, CA, USA) software was used for statistical analysis of *P. parvum* growth, biochemical and behavioral data. Prior to analysis, data were normalized to cell density (cells/mL) after which normality and

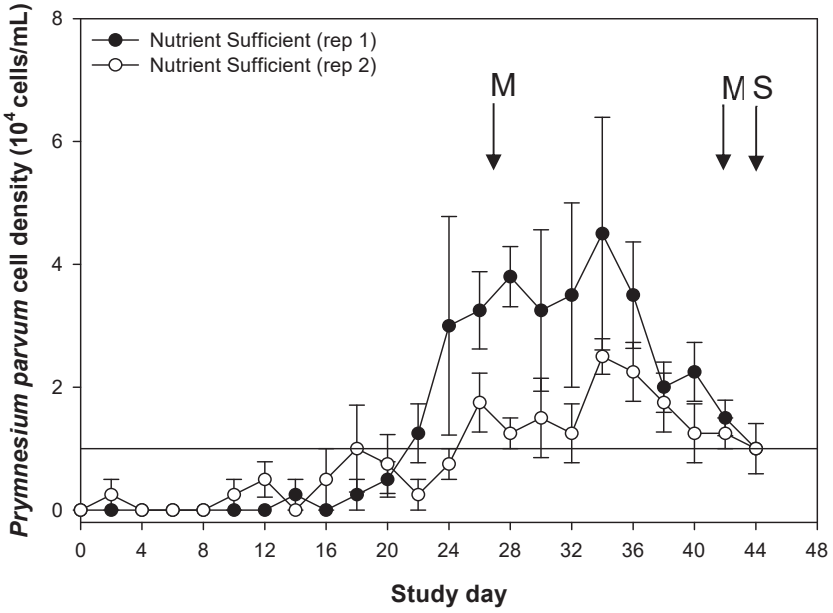
equivalence of variance were analyzed. Significant differences (α=0.05) of maximal cell densities and growth rates were identified among culture conditions using an analysis of variance (ANOVA) if normality and equivalence of variance assumptions were met followed by a Tukey post hoc test. Significant differences (α=0.05) in biochemical endpoints and movement patterns (per minute) were identified among treatments using ANOVA if normality and equivalence of variance assumptions were met. Dunnett's post hoc test was performed to identify dilution level differences from media controls. For data not meeting ANOVA assumptions, an ANOVA on ranks was performed.

Results

Cell Densities and Specific Growth Rates

Throughout the duration of the study, *P. parvum* cell densities exceeded or met the toxicity bloom threshold of 10,000 cell/mL after exponential growth phase began approximately on day 20 for all experimental units (Figure 1.1). Nutrient sufficient conditions resulted in variations among the replicate cultures. Maximum cell densities for these cultures were of 4.5×10^4 and 2.2×10^4 cells/mL. Nutrient deficient cultures resulted in similar maximum cell densities among the replicates, 3.0 and 3.25×10^4 cells/mL respectively. No significant difference was determined for maximal cell densities observed. Growth under nutrient deficiency resulted in higher variation in cell densities observed throughout the study (Figure 1.1). The specific growth rates ranged from 0.1618-0.1852 divisions per day and were not significantly difference among nutrient conditions (Table 1.2). No significant differences were observed between nutrient conditions or culture replicates.

A.



B.

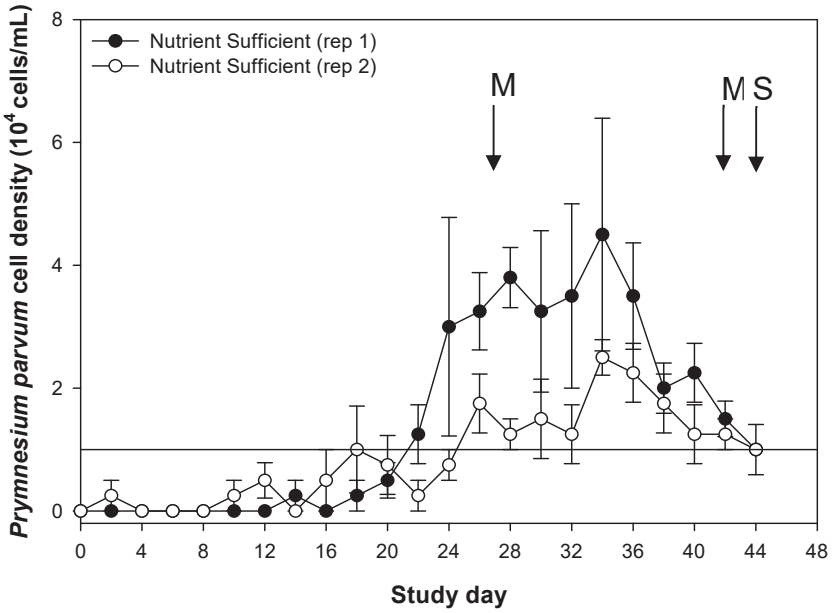


Figure 1.1. *Prymnesium parvum* represented as mean ± S.D. (n=5) cell density per study day. Two replicate carboys were grown under the following conditions: 2.4 ppt, 15 ◦C, 3,325 Lux, 12:12 light dark cycle with sufficient (A) or deficient (B) nutrient concentrations. Nutrient sufficient (f/2) and deficient (f/8) conditions were prepared following Guillard 1975. 100 cells/mL were introduced to each carboy on day 0. Cell densities were counted using a hemocytometer. Horizontal line represents the toxic bloom threshold of 10,000 cells/mL. M=acute mortality studies, S=sublethal study

Table 1.2. *P. parvum* growth rates (divisions/day) represented as mean ± S.D grown at 2.4 ppt, 15 °C with a 12:12 light:dark cycle, under nutrient sufficient (f/2) and deficient (f/8) conditions. Growth rates were determined when stationary growth phase was reached between study days 30-34.

Nutrient sufficient (rep 1)	Nutrient sufficient (rep 2)	Nutrient deficient (rep 1)	Nutrient deficient (rep 2)
0.1728 ± 0.0224	0.1618 ± 0.0069	0.1852 ± 0.0226	0.1705 ± 0.0212

Acute Mortality

Acute mortality was assessed on day 27 after exponential growth phase was observed. The 48 h LC_{50} value for cultures grown under nutrient sufficient conditions were 71.9% and 86.7% of *P. parvum* culture, corresponding to 25,156 and 13,010 cells/mL, and 68.2% and 58.2% of *P. parvum* culture, corresponding to 16,801 and 17,376 cells/mL grown under nutrient deficiency (Table 1.3). No significant differences were observed between the 24 and 48 h LC_{50} values (Table 1.3). The nutrient sufficient treatment level (f/2) resulted in significantly different acute mortality between replicate carboys indicated by 95% confidence intervals. This nutrient treatment level elicited the lowest and highest acute mortality responses and were not significantly different when compared to nutrient limited culture toxicity (Table 1.3).

Table 1.3. *P. parvum* 24 and 48 h LC_{50} values (±95% CI, cells/mL) for larval fathead minnow (*P. promelas*). Exposures occurred on study day 27 during exponential growth phase to determine culture toxicity. NA=not available due to lack of partial mortalities.

Culture Condition	24 h LC_{50}	48 h LC_{50}
Nutrient Sufficient (rep 1)	28,820 (21,264-36,376)	25,156 (16541-33,902)
Nutrient Sufficient (rep 2)	13,010 (NA)	13,010 (NA)
Nutrient Deficient (rep 1)	21,409 (16,332-26,488)	16,801 (NA)
Nutrient Deficient (rep 2)	21,258 (14,510-28,005)	17,376 (13,079-21,674)

No mortalities were observed for media controls. On day 42, acute mortality was reassessed during stationary growth phase. No acute mortalities were observed for any nutrient treatment level or replicate carboy for fathead minnow and zebrafish larvae. Therefore, sublethal bioassays were initiated on study days 44 and 45 with the following dilution levels: 10, 50 and 100% with cell densities ranging between 1,000-15,000 cells/mL (Table 1.4). No mortalities occurred in dilution levels or controls during the sublethal exposure for both fish species.

Table 1.4. *P. parvum* whole culture dilutions and corresponding nominal cell densities (cells/mL) used in the sublethal studies. Cell densities were counted using a haemocytometer. Dilutions were prepared using either nutrient sufficient (f/2) or deficient (f/8) culture media, which also served as control for toxicology studies.

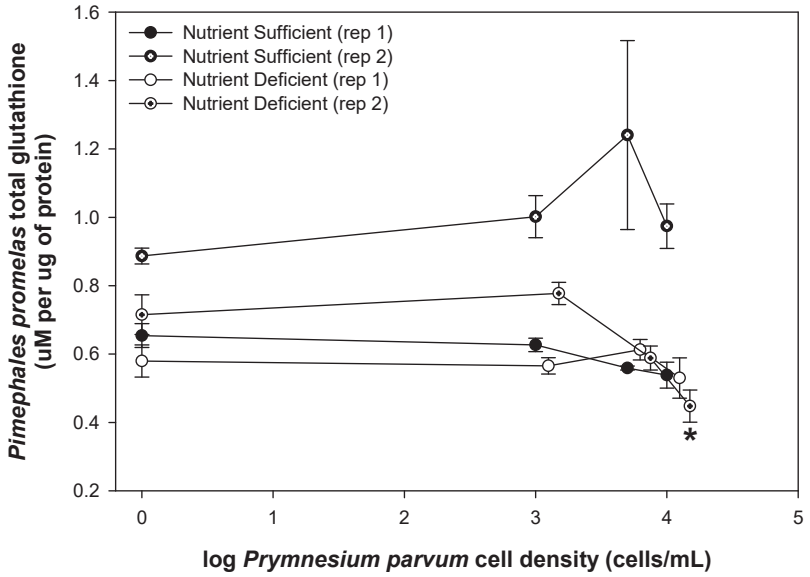
Treatment Level	Nutrient Sufficient (rep 1)	Nutrient Sufficient (rep 2)	Nutrient Deficient (rep 1)	Nutrient Deficient (rep 2)
10%	1,000	1,000	1,250	1,500
50%	5,000	5,000	6,250	7,500
100%	10,000	10,000	12,500	15,000

Biochemical Oxidative Stress Endpoints

Differential responses were observed among fish species and *P. parvum* dilutions of nutrient treatment levels. A significant ($p < 0.05$) decrease in total glutathione concentration was observed in fathead minnow after exposure to nutrient deficient conditions. Although not significant ($p > 0.05$), a nutrient sufficient culture resulted in overall higher glutathione concentrations in fathead minnow with an induction at 50% *P. parvum* culture when compared with culture one. Zebrafish significantly ($p < 0.05$) elicited total glutathione depletion under nutrient sufficient conditions for all dilution levels of experimental carboy replicate 2 with maximal depletion at 50% *P. parvum* culture (Figure 1.2).

Fathead minnow larvae experienced an increase, though not significant, in MDA concentration with increasing cell density of a nutrient deficient culture (Figure 1.3). Unfortunately, samples were lost for fathead minnow larvae exposed to nutrient sufficient and deficient replicate one carboys, therefore MDA could not be determined for these cultures. A significant ($p < 0.05$) increase in MDA concentration in zebrafish larvae was observed for 10% of a nutrient sufficient culture (Figure 1.3). In fathead minnow, a significant ($p < 0.05$) increase in oxidative DNA damage occurred after exposure to 10% of a nutrient sufficient culture (Figure 1.4). This 8-OH-dG concentration was almost double that of the remaining cultures with an overall higher response across dilution levels. A significant ($p < 0.05$) increase in zebrafish oxidative DNA damage resulted from exposure to 100% of nutrient deficient culture.

A.



B.

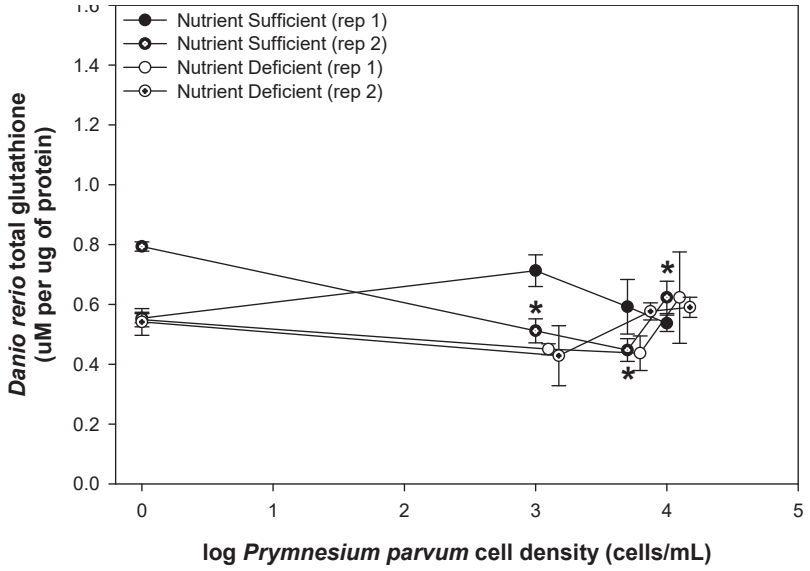


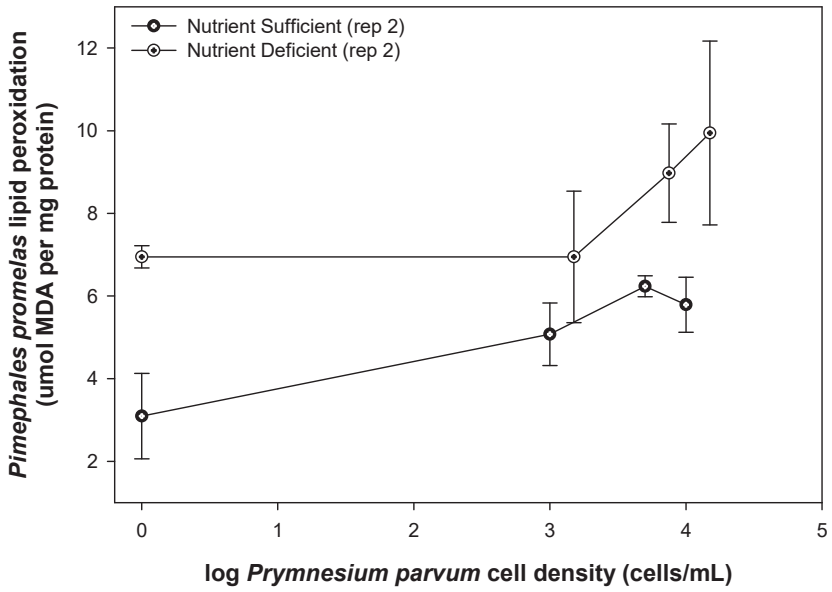
Figure 1.2. Total glutathione concentration (μM) in fathead minnow (*P. promelas*, A) and zebrafish (*D. rerio*, B) following a 48 h exposure to *P. parvum* grown under two nutrient conditions (f/2, nutrient sufficient and f/8, nutrient deficient) in duplicate (represented as rep 1 and 2). Controls were f/2 or f/8 media without *P. parvum* cells. Results analyzed using Sigma Plot and represented as mean ± S.E. One-way ANOVA followed by Dunnett's post hoc test determined statistical significance (N=3,* $p \leq 0.05$) from control.

Behavior Analysis

Behavioral responses varied among species and nutrient condition (Figures 1.5-8). The photomotor responses (PMR) was originally used in the biomedical sciences as a tool to identify therapeutic targets for novel compounds, but has since been adopted to toxicological studies (Kokel et al., 2010; Kokel and Peterson, 2011; Noyes et al., 2015). PMR observed for fathead minnow exposed to media control indicate a preference for light conditions due to the negative or smaller changes in distance traveled from light to dark. By contrast, zebrafish PMR indicated a preference for dark conditions by the negative dark to light and positive light to dark PMR after exposure to media controls and *P. parvum* treatment levels. Significant ($p < 0.05$) increases in fathead minnow PMR were observed after exposure to nutrient deficient cultures (Figure 1.6). A larger zebrafish PMR was observed for sudden changes from dark to light conditions regardless of culture condition (Figure 1.5 & 1.6). Fathead minnow PMR responses were more variable than zebrafish PMR (Figures 1.5 & 1.6).

Exposure to nutrient sufficient cultures resulted in no significant ($p > 0.05$) behavioral alterations for fathead minnow (Figure 1.7). By contrast, zebrafish behavior increased in both light and dark conditions. Fathead minnow and zebrafish behavior exhibited similar behavioral effects after exposure to nutrient deficient cultures. Stimulatory responses for both species are indicated by an increase in bursting activity in light and dark conditions (Figure 1.8). Fathead minnow behavior decreased significantly ($p < 0.05$) for the majority of swimming behavior end points selected, however, a stimulatory response across bursting speeds was observed in the light and dark conditions.

A.



B.

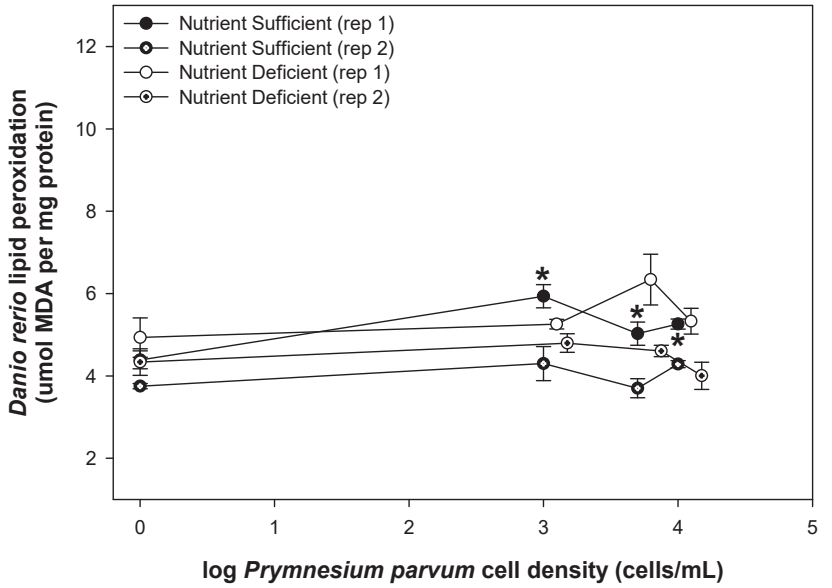
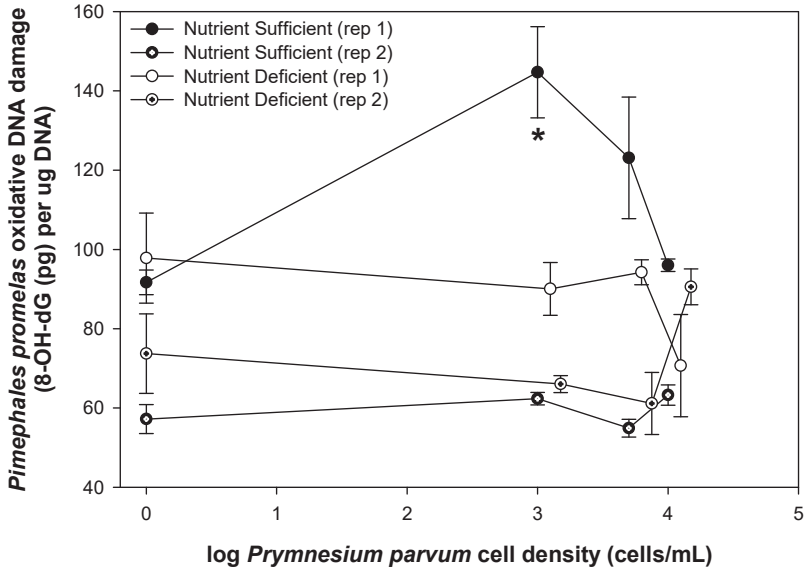


Figure 1.3. Lipid peroxidation (malondialdehyde (MDA) concentration (µM per mg protein)) in fathead minnow (*P. promelas*; A) and zebrafish (*D. rerio*; B) following a 48 h exposure to *P. parvum* grown under two nutrient conditions (f/2, nutrient sufficient and f/8, nutrient deficient) in duplicate (represented as rep 1 or 2). Controls were f/2 or f/8 media without P. parvum cells. Results analyzed using Sigma Plot and represented as mean ± S.E. One-way ANOVA followed by Dunnett's post hoc test determined statistical significance (N=3,* $p \leq 0.05$) from control.

A.



B.

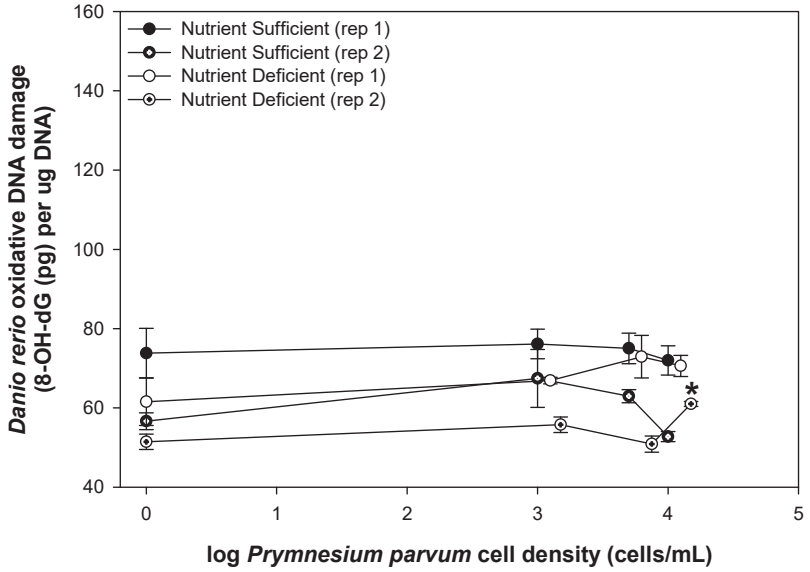


Figure 1.4. DNA damage (8-OH-dG (pg) per mg of DNA) in fathead minnow (*P. promelas*; A) and zebrafish (*D. rerio*; B) following a 48 h exposure to *P. parvum* grown under two nutrient conditions (f/2, nutrient sufficient and f/8, nutrient deficient) in duplicate (represented as rep 1 or 2). Controls were f/2 or f/8 media without *P. parvum* cells. Results analyzed using Sigma Plot and represented as mean □ S.E. One-way ANOVA followed by Dunnett's post hoc test determined statistical significance (N=3,* $p \leq 0.05$) from control.

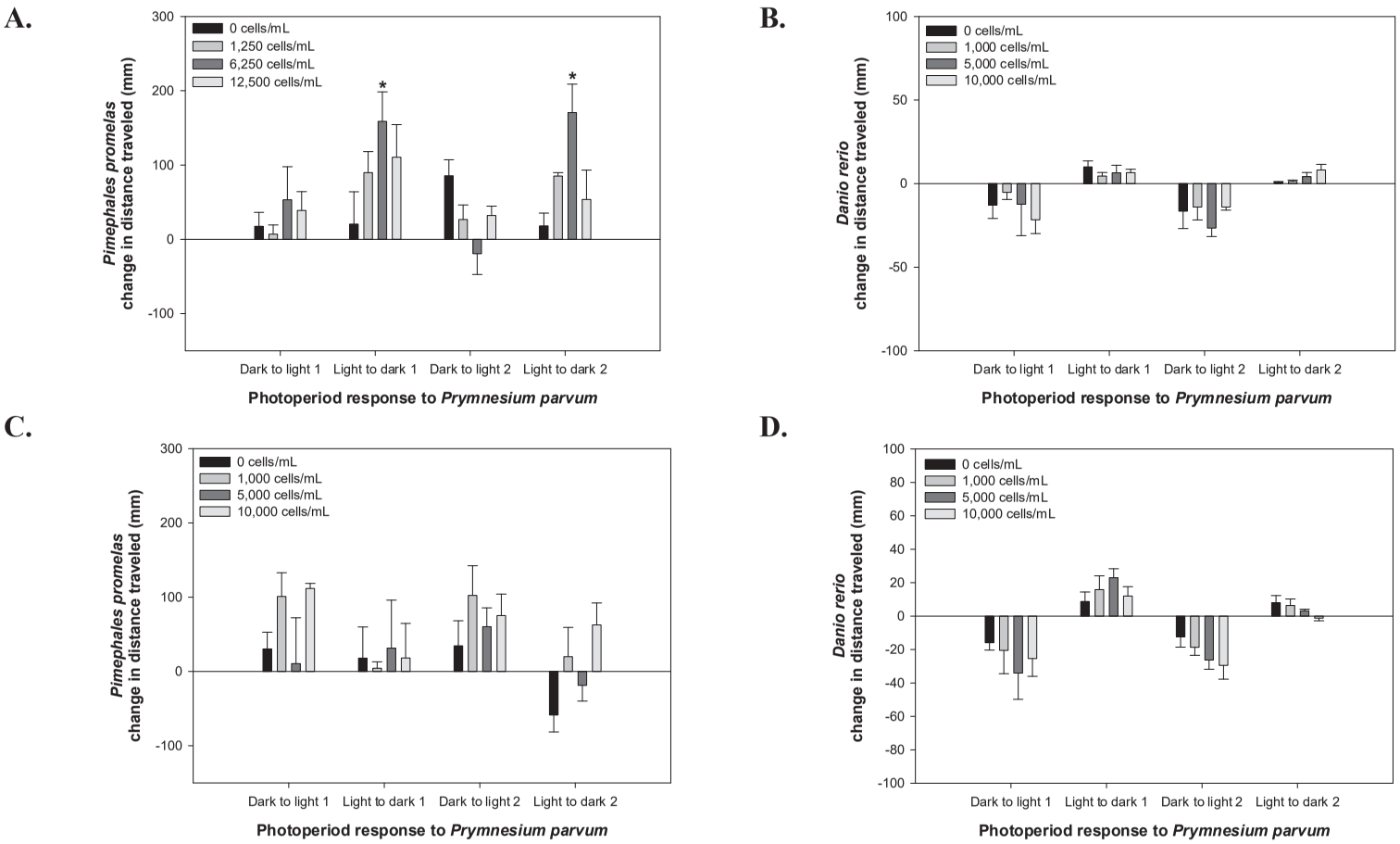


Figure 1.5. Fathead minnow (*P. promelas*; A, and C) and zebrafish (*D. rerio*; B and D) photomotor swimming responses (PMR) following a sudden change in light condition (Light to Dark and Dark to Light) after 48 h exposure to *P. parvum* grown in duplicate (top: replicate 1, bottom: replicate 2) under nutrient sufficient (f/2) conditions. Bars represent the mean ± S.E. PMR per minute. * N=3 fathead minnow, N=4 zebrafish, $p \leq 0.05$, one-way ANOVA followed by Dunnett's post hoc test.

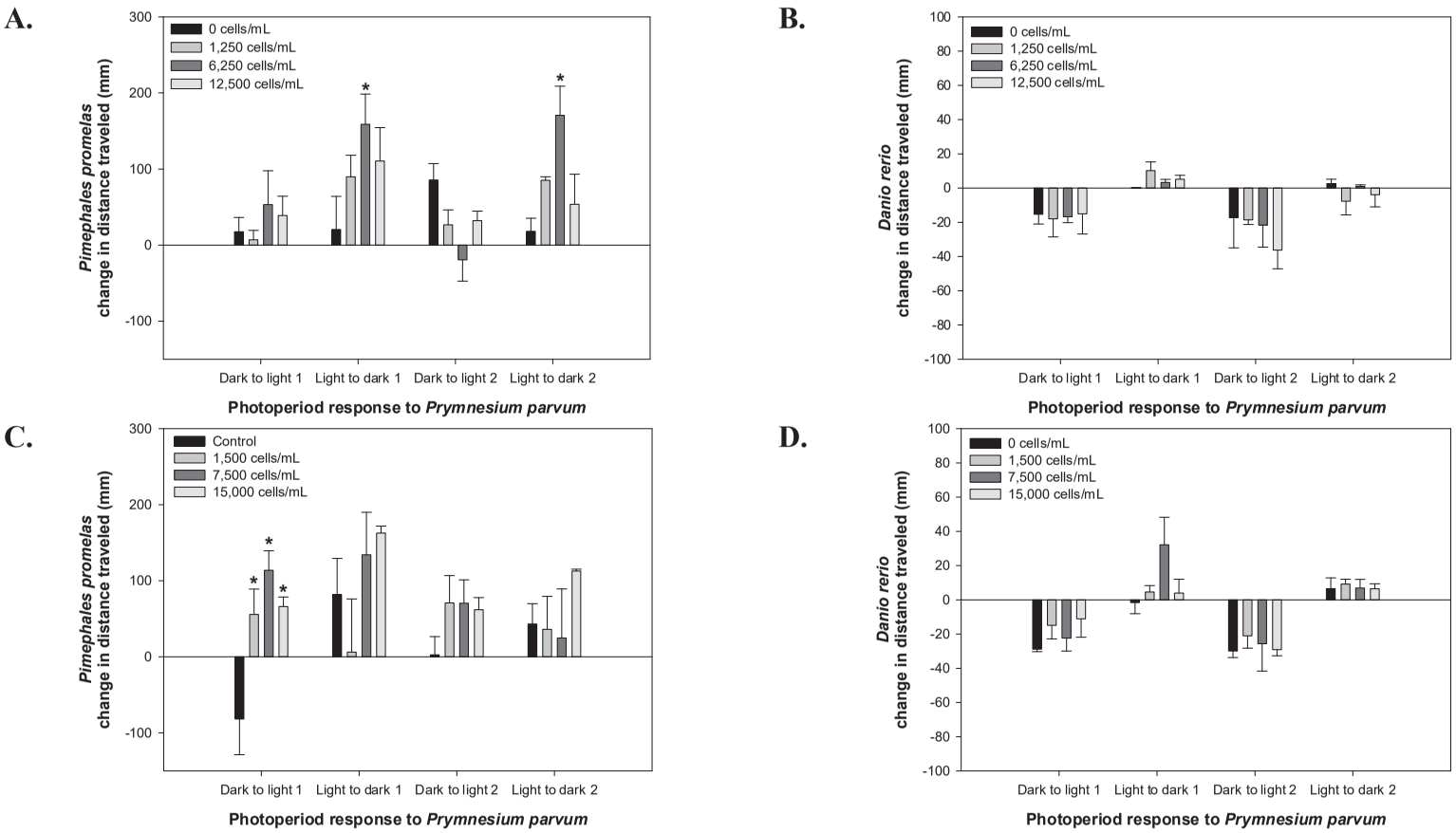


Figure 1.6. Fathead minnow (*P. promelas*; A, and C) and zebrafish (*D. rerio*; B and D) photomotor swimming responses (PMR) following a sudden change in light condition (Light to Dark and Dark to Light) after 48 h exposure to *P. parvum* grown in duplicate (top: replicate 1, bottom: replicate 2) under nutrient deficient (f/8) conditions. Bars represent the mean ± S.E. PMR per minute. * N=3 fathead minnow, N=4 zebrafish, $p \leq 0.05$, one-way ANOVA followed by Dunnett's post hoc test.

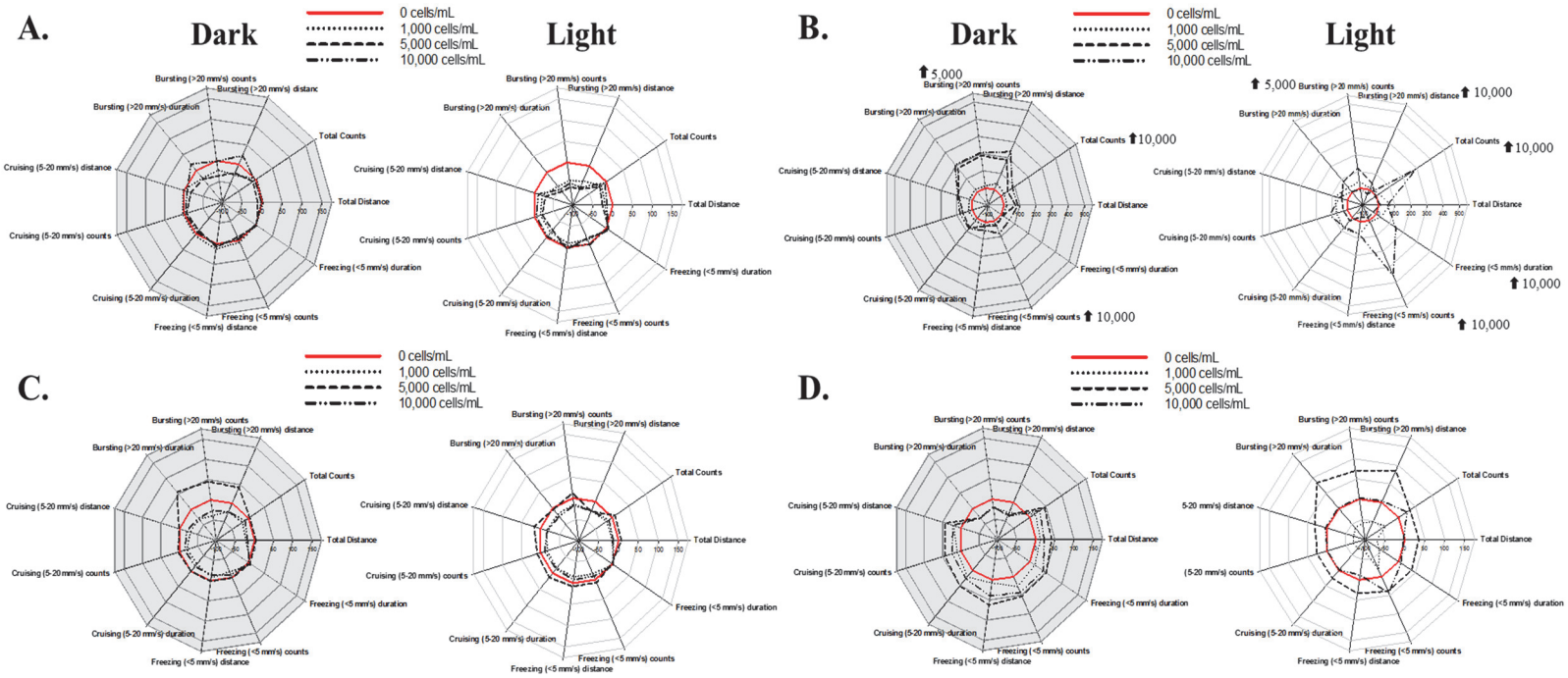


Figure 1.7. Mean fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B, D) swimming activity during light and dark photoperiods after a 48 h exposure to *P. parvum* cultures grown in duplicate (top: replicate 1, bottom: replicate 2) under nutrient sufficient conditions (f/2). Data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to media control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test determined statistical significance from control. Arrows = statistically significant (N=3, fathead minnow, N=4, zebrafish; $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

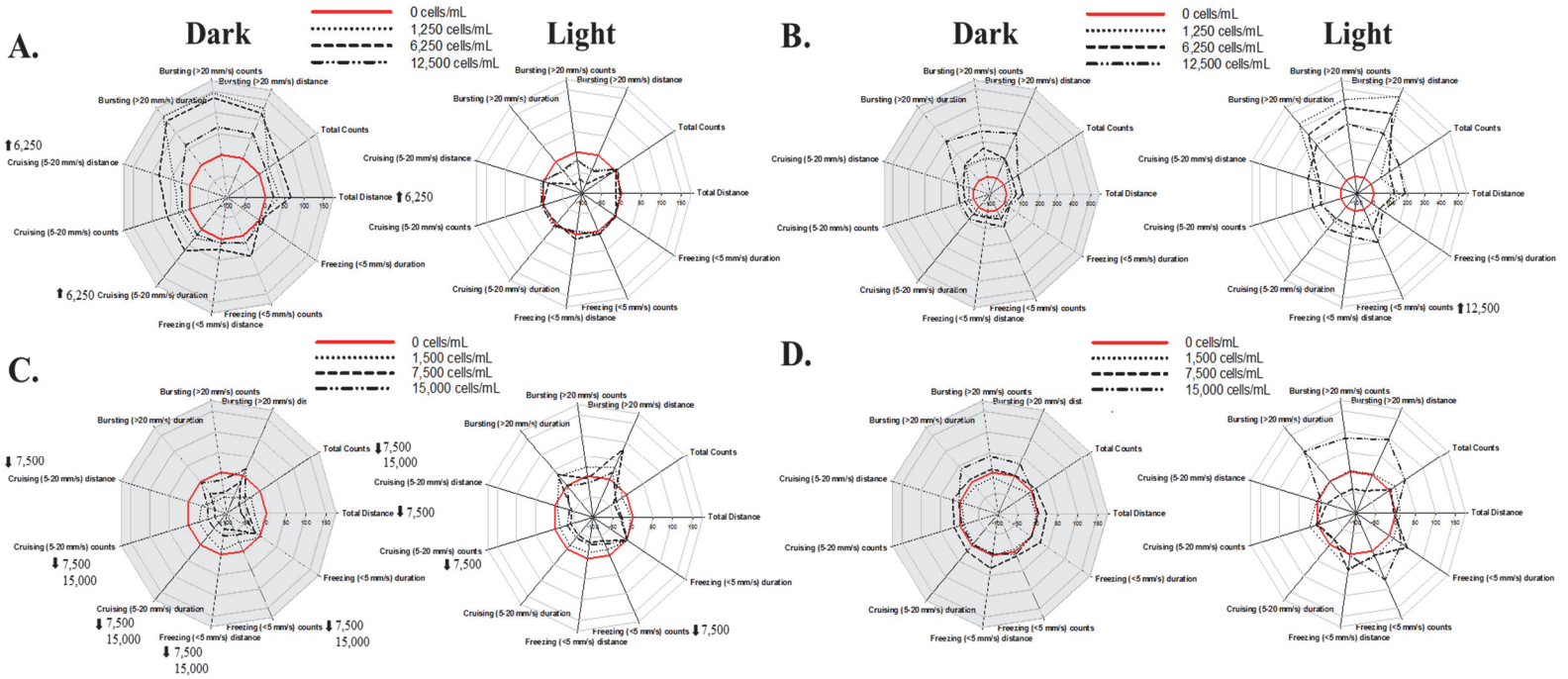


Figure 1.8. Mean fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B, D) swimming activity during light and dark photoperiods after a 48 h exposure to *P. parvum* cultures grown in duplicate (top: replicate 1, bottom: replicate 2) under nutrients deficient conditions (f/8). Data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to media control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test determined statistical significance from control. Arrows = statistically significant (N=3,fathead minnow; N=4,zebrafish, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

Discussion

P. parvum is a eury- and thermohaline species that continues to invaded inland bodies of water along the southern regions of North America and has begun expansion to northern U.S.A. States, primarily due to climate change and anthropogenic activities (hydrologic alterations, storm water runoff, and unconventional natural gas extraction) (Roelke et al., 2015). Understanding the effects of *P. parvum* to fish is extremely important due to the mass mortality and community alterations that occur after *P. parvum* HAB events that often result in large economic losses. As previously noted, current literature of *P. parvum* sublethal toxicity is lacking and comparative *in vivo* toxicological studies between vertebrates are nonexistent (Table 1.1). Sublethal responses are important to understand potential impacts on an organism/population and may provide biomonitoring strategies for blooms. This study sought to examine sublethal effects of *P. parvum* cultured under nutrient sufficient or insufficient conditions by evaluating photomotor behavioral response patterns and common biochemical biomarkers of OS to answer the simple question: what happens to fish when they don't die following a HAB event? The results of the current study demonstrate, for the first time, that exposure to sublethal cell densities of *P. parvum* alter behavioral responses and elicit OS.

P. parvum is a relatively slow growing species with optimal growth rates between 0.8-1.8 d^{-1} (Baker et al., 2007). Under stressful conditions representative of inland Texas HABs (including nutrient limitation, lower salinity, and winter temperatures), reproductive growth rates have been shown to decrease to around 0.1-0.3 d^{-1} (Baker et al., 2009a). The specific growth rates of the current study represent cultures below optimal reproductive growth rates and fall within the range of growth rates observed for inland

Texas HABs. Maximal cell densities of the present study were smaller than previously reported laboratory *P. parvum* cultures at the end of exponential growth phase (Baker et al., 2009a; Brooks et al., 2010; Lindehoff et al., 2010; Lundgren et al., 2016; Skingel et al., 2010; Valenti, 2010). However, these studies were inoculated with higher cell densities of *P. parvum* with cultures grown under higher temperatures or salinities, which may explain the differences observed in maximum cell densities.

Relationships between algal respiration, growth and temperature have been established through the Q_{10} principle (Davison, 1991). The stock culture was maintained at a higher salinity and temperature, close to the optimal growth conditions mentioned previously for this algal species. Inoculation of this culture to the experimental conditions with lack of acclimation to these conditions may have resulted in decreased ability to maintain homeostasis and thus cell lysis. Physiological shock treatments, including lower temperature and salinity, were studied for effects on *P. parvum* toxicity. Lower toxicity and down regulation of polyketide synthase gene expression was noted for the low temperature shock treatment, which was within the temperature inhibition range for *P. parvum* growth (Baker et al., 2007; Freitag et al., 2011).

Acute mortality bioassays were employed to monitor and confirm acute toxicity of all cultures throughout the study because analytical methods verifying toxins production are not available. Roelke et al (2007) determined an approximate cell density of 10,000 cells/mL is associated for acute toxicity to be observed in the field. The results of the current study are similar to LC_{50} values reported for laboratory grown cultures and confirm that all cultures were toxic during exponential growth phase (Blossom et al., 2014; Brooks et al., 2010; Valenti, 2010). Acute mortality was greatest when *P. parvum*

cultures were grown under nutrient deficiency, which is consistent with reported laboratory and field studies (Baker et al., 2009a; Errera et al., 2008; Graneli, 2003; Roelke et al., 2007). Similarly, the majority of the sublethal responses observed in the present study occurred after exposure to nutrient limited conditions in fathead minnow suggesting that sublethal endpoints would be an adequate marker of *P. parvum* toxicity. Further investigation of nutrient limitation in conjunction with other environmentally stressful *P. parvum* growth parameters will lead to a better understanding of sublethal responses and resulting toxicity to enhance monitoring strategies.

Two toxins have been identified as prymnesins 1 and 2, while other metabolites contributing to the deleterious effects of *P. parvum* exposure are relatively unknown (Bertin, 2012, 2014; Blossom. H.E., 2014; Henrikson, 2012; Igarashi et al., 1996, 1999b). Toxicity tests are routinely employed to determine bioavailable toxins due to lack of analytical standards and an inability to detect toxins in the water. Consistent with toxin elucidation, no mechanisms of actions have been determined for these metabolites. The gills have been suggested as the primary target tissue of toxicity in fish by disrupting ion permeability and regulation (Shilo, 1967). Other studies have reported cell lysis (e.g., plankton, erythrocytes, human cancer and gill cells) occurring following *P. parvum* exposure further recognizing that different toxins are responsible for the multiple effects observed.

The degree of acute toxicity has been associated with other environmental abiotic factors including temperature, pH, irradiance, and salinity similar to other algal toxins (Baker et al., 2007; Baker et al., 2009b; James et al., 2011; Valenti, 2010). For example, previous research done by my laboratory linked pH increases with increased toxicity and

concluded that prymnesins are ionizable weak bases and uptake of toxins is greatest under basic pH conditions (Valenti, 2010). The structure of prymnesins is similar to another algal toxin, maitotoxin, that is frequently involved in hemolysis, fish mortality, and the human illness ciguatera fish poisoning (Murata, 2000). A comparable pH dependency has also been demonstrated for this toxin (Igarashi et al., 1999a). In addition, maitotoxin is a calcium channel activator inducing mortality through calcium influxes to the cell (Igarashi et al., 1999b). Similar calcium signaling has been disrupted following *P. parvum* exposure, however these perturbations were observed more rapidly than maitotoxin. In addition, *P. parvum* toxicity to a freshwater fish was enhanced after addition of Ca^{2+} contributing to conformational changes of toxins allowing for interactions with specific components on cell membranes (Igarashi et al., 1998; Manning and La Claire, 2010). Oxidative stress (OS) causes Ca^{2+} influxes to the cytoplasm from the external environment via cell membrane or endoplasmic reticulum (Ermak and Davies, 2002). This influx of Ca^{2+} disrupts normal cell function leading to cell death. The influence of OS has not been studied for maitotoxin or *P. parvum*, which could be a contributing mechanism behind the Ca^{2+} toxicity and gill impairment observed for both algal toxins.

Glutathione concentration, lipid peroxidation, and DNA damage have been well-studied biochemical markers of OS and previously used to assess adverse effects of chemicals in commerce and other algal toxins (Evans et al., 2004; Kohen and Nyska, 2002). The role of OS has been previously studied for *P. parvum* using an *in vitro* model. No significant differences were observed for superoxide dismutase, catalase, and lactate dehydrogenase activities in rainbow trout gills cells after exposure to *P. parvum* cell

densities reaching 5.725 x 10^5 cells/mL (Dorantes-Aranda et al., 2015). The production of superoxide radical was also quantified for lyzed *P. parvum* cells, which revealed very low concentrations (0.15 pmol $cell^{-1}$ hr^{-1}) (Dorantes-Aranda et al., 2015). These results suggest that OS is minimal at the site of the gill. The biochemical markers of the present study can only elucidate the contribution of OS toxicity on the entire organism, and not a specific cell or tissue.

GSH is a tripeptide that serves as a cosubstrate during phase II metabolism for xenobiotic detoxification and is an essential electron donor for the reduction of hydroperoxides, by serving as an electron donor to glutathione peroxidases. GSH is located largely in the cytosol primarily in its reduced form and is oxidized into glutathione disulfide (GSSG) upon contact with electrophilic compounds. An increase in GSSG leads to a depletion of cellular GSH, suggesting oxidative stress and other pathological conditions (Wu et al., 2004). Therefore, the GSH depletion observed for larval zebrafish and fathead minnow is indicative of oxidative stress and suggests that GSH is actively involved in detoxification or decreased synthesis in response to exposure to *P. parvum* toxins. Compared to the other biochemical markers chosen, GSH was the most sensitive endpoint selected which highlights the utility of this endpoint as a biomarker of *P. parvum* exposure. Previous comparative toxicological work with the fathead minnow and zebrafish showed GSH more significantly affected among the same biochemical markers chosen in the present study (Corrales et al., 2017).

Lipid peroxidation is the oxidative damage to lipids, often leading to the impairment of cell membranes. MDA has been a commonly studied end product of lipid peroxidation that can react with biomolecules forming adducts. These adducts can then

undergo secondary reactions with DNA and proteins, altering properties of biomolecules and accumulate during aging and chronic diseases (Ayala et al., 2014). Consequently, an increase in lipid peroxidation is suggestive of OS as observed in the present study by the increase in MDA concentration for both species. Fathead minnow MDA concentration increased in a dose dependent manner under the same *P. parvum* condition that depleted GSH concentration (nutrient limitation). Zebrafish MDA concentration did not exhibit an increase in concentration with dilution level. However, similar to the fathead minnow, the same culture condition elicited both an increase in MDA and GSH depletion (nutrient sufficient). These observations suggest a relationship between GSH and MDA. Mice exposed to a cyanotoxin exhibited an increase in lipid peroxidation at 16 h post exposure and a decrease at 24 and 32 h in conjunction with a large decrease in unconjugated glutathione (Gehringer et al., 2004). Similar results were observed in zebrafish exposed to low and high concentrations of the cyanotoxin alphantoxin in which MDA content increased within the first 12 h and then decreased to levels similar to the control (Zhang et al., 2013). The OS biochemical markers were only analyzed at the conclusion of 48 h, therefore the results do not provide a representation of lipid peroxidation effects through time.

8-hydroxy-2'-deoxuguanosine (8-OH-dG) is the most common oxidative DNA damage product that has been studied as an indicator of oxidative stress and carcinogenesis. Oftentimes, 8-OH-dG will be analyzed in conjunction with other biological markers to confirm that an increase in 8-OH-dG is accurately representative of oxidative stress (Kasai, 1997). The results of this study reveal that exposure to *P. parvum* cell densities increases 8-OH-dG concentrations in larval fathead minnow and zebrafish,

indicating oxidative stress. These responses occurred under opposite culture conditions that elicited GSH and lipid peroxidation further supporting that different toxins may be produced in response to varying levels of stress. Similarly, accumulation of oxidized purines were observed in human hepatoma HepG2 cells after exposure to microcystin-LR (Zegura et al., 2004). Other studies have also shown the role of pretreatment protection (including GSH) against microcystin-LR DNA damage, however DNA damage still occurred in pretreated individuals but to a lesser extent (Lakshmana Rao and Bhattacharya, 1996). In the present study, cultures that exhibited highest DNA damage did not have significantly different GSH content compared to controls. However, the cultures with the highest GSH content, exhibited the lowest DNA oxidative damage when comparing across cultures, suggesting similar GSH protection.

The biochemical endpoints chosen have differential sensitivities and occur at various points throughout a cell. Glutathione is one of the most prevalent antioxidants that is utilized rapidly upon exposure to xenobiotics. Whereas MDA and oxidative DNA damage are products of OS. Although repair mechanisms exist, sustained perturbations lead to life history constraints ultimately leading to population level consequences (Birnie-Gauvin et al., 2016; Metcalfe and Alonso-Alvarez, 2010). An understanding of mechanisms responsible for the observed toxicity is important for development of adverse outcome pathways to better understand effects at different biological classifications that will aid in conducting risk assessments (Ankley et al., 2010). Fish behavior is an invasive tool linking biochemical and molecular responses wo whole organism responses. Behavior includes sensitive endpoints at which effects are observed at concentrations magnitudes below those that induce acute mortality (Gerhardt, 2007).

Fish behavioral responses are utilized in both ecotoxicology and biomedical applications, with a particular emphasis on zebrafish behavior for pharmacological applications. Some observation behaviors of adult fish after exposure to *P. parvum* has been documented, but these behavioral changes were not quantified by standardized behavior methods (tracking system or scoring) and occurred during an active bloom site. Effected fish were lethargic, swam with coordination problems, slow movement, and remained at the surface of the water gasping for air (Vasas et al., 2012). To my knowledge, this is the first quantitative examination of fish behavioral responses to *P. parvum*.

High throughput, behavioral profiling (rest/wake and anxiety-related phenotypes) tools using early life stage zebrafish have been utilized in pharmacology to screen behavioral changes associated with mechanisms of action (Egan et al., 2009; Rihel et al., 2010a). Behavioral syndromes were established for juvenile fathead minnow and are defined as hypoactivity, hyperactivity and physical deformity syndrome (Drummond and Russom, 1990c). The larval behavioral responses to *P. parvum* indicate hyperactivity responses due to the observed stimulatory responses for both species (Figures 7 & 8). Chemical classes that also produced a hyperactivity syndrome in 30 d old fathead minnows include primary aliphatic amines, phenols and halogenated phenols (Drummond and Russom, 1990c). These compounds elicit toxicity through disruption of metabolic activity and function, which is consistent with the proposed fish mode of action for *P. parvum* (Shilo, 1967). Stimulatory responses have been observed in copepods after exposure to the red tide, *Alexandrium fundyense* (Lasley-Rasher et al., 2016a). However for fathead minnow, exposure to one nutrient deficient culture, resulted in hypoactivity

suggestive of nervous system disruption (Figure 8; Drummond and Russom, 1990b). Neurotransmitter disturbances in rat synaptosomes and decreased muscle contractions in guinea pig intestine after neurotransmitter inhibition have been observed following exposure to isolated prymnesin toxins (Bergmann et al., 1964; Mariussen et al., 2005; Meldahl and Fonnum, 1995).

Different behavioral responses were observed for chemicals within the same class due to variations in mode of actions inferring that nutrient stress could cause variations in *P. parvum* secondary metabolite production (Drummond and Russom, 1990c). Similar differences between doses were observed for microcystin-LR. Lower concentrations of microcystin-LR (0.5 and 5 μg/L) increased daytime and decreased nighttime motility, whereas higher concentrations (15 and 50 μg/L) decreased daytime and increased nighttime motility as well as shifted the maximum behavioral peak by 11 hours (Baganz et al., 1998a). As mentioned previously, species sensitivity to *P. parvum* suggests that various secondary metabolites are produced under varying conditions and/or elicit varying effects through multiple modes of action. Although investing these mechanisms were outside the scope of this study, the different behavioral responses observed for the various growth conditions, may indicate differential toxin production.

Consistent with acute mortality, fathead minnows were more susceptible to *P. parvum* grown under nutrient limitation, with the exception of DNA oxidative damage. *P. parvum* acute mortality data are lacking for zebrafish; therefore, comparisons among previous work are difficult (Table 1.1). As stressed in Brooks et al. (2010), absence of standardized culture conditions presents a challenge when comparing previous toxicological work as growth conditions in laboratory and field studies have been shown

to influence growth and toxicity. The observed differences among the behavioral responses and three biochemical OS markers could be explained by the model organisms. Although acute and sublethal responses were examined for the Texas strain, *P. parvum* HABs occur globally. To maximize comparisons of study findings for fathead minnow and zebrafish, standardized methods were chosen for each species that are employed in regulatory and international settings. The initiation of these methods occurred at early life stages at which susceptibility to contaminants is believed to be greatest and involve dosing each species at different development stages. The influence of development on toxicity was outside the scope of this study, although influences in uptake and metabolism along development have been demonstrated for multiple fish species exposed to environmental contaminants including algal toxins (Kristofco et al., 2018; Otte et al., 2010; Wiegand et al., 1999). Whether the observed effects between the fathead minnow and zebrafish in the present study are species, age, or both species and age related is unclear and requires future attention.

Oxidative stress responses to other HAB toxins are dose and time dependent. The exposures of the current study only focused on one growth phase of *P. parvum*, which has been shown to influence toxicity. The behavior and OS responses varied among culture dilution of *P. parvum* cultures, suggesting that there may be a dose/cell density dependency. However, *P. parvum* cell density does not always determine the degree of acute toxicity, which may be consistent with sublethal responses (Baker et al., 2007). Future work is needed to understand the influence of different *P. parvum* growth phases on behavioral and OS effects. In addition, comparisons among multiple model organisms

and toxicity across developmental stages and exposure duration time are essential to furthering understanding of toxicity.

In conclusion, exposure to sublethal cell densities of *P. parvum* resulted in behavioral alterations and OS. Future research needed to determine the extent to which the observed effects lead to population level impacts, and whether such effects would occur in natural fish populations. OS has been implicated in redox cycling disruptions leading to inflammation and many disease states while behavioral alterations increase susceptibility to predation, alter feeding and social (courting and schoaling/schooling) behaviors. Responses were observed at cell densities at or below the current toxicity threshold, therefore a reevaluation of what is deemed toxic especially for environmental assessment and management purposes is required. Behavioral activity across slower speed thresholds was the most sensitive behavioral endpoint for both species. Whether such responses would translate to increased predation risks is unknown. In addition, total glutathione concentration was the most sensitive biochemical endpoint selected across treatment level and fish species, showing the potential utility of these endpoint as a biomarker for *P. parvum* exposure. Fathead minnow and zebrafish assays, which followed standardized methods and were thus initiated at different ages, displayed different sensitivities to *P. parvum* grown under varying nutrient conditions. Selection of other sublethal endpoints and the examination of selected endpoints across multiple *P. parvum* growth conditions along with identification of secondary metabolites across these conditions will further the understanding of underlying mechanisms of *P. parvum* toxicity, which will ultimately advance bloom monitoring, assessment and management.

CHAPTER TWO

Salinity and Nutrients Influence *Prymnesium parvum* Related Oxidative Stress and Behavioral Responses in Two Common Fish Models

Introduction

The frequency, duration, and intensity of harmful algal blooms (HABs) are increasing at a global scale primarily due to eutrophication, urbanization, climate change, altered hydrology, and storm water and agricultural runoff (Granéli et al., 2008; Hallegraeff, 1993; Paerl and Huisman, 2009; Roelke et al., 2012a). The successful invasion of HABs are due to multiple interlinking factors (both anthropogenic and natural) typically specific to each algal species providing challenges when assessing and managing HABs. Environmental impacts include lower dissolved oxygen concentrations, altered community structure, impairments to recreational uses, and nuisance taste and odor compounds (Paerl et al., 2001). Adverse effects resulting from HABs include human health illnesses and extensive fish kills leading to potential substantial threats to coastal and inland waters (Brooks et al., 2016; Grattan et al., 2016).

Prymnesium parvum is a mixotrophic (autotrophic and heterotrophic modes of nutrition) invasive species capable of forming HABs leading to massive fish kills resulting in large economic losses (estimated damages in millions of U.S.A. dollars) (Roelke et al., 2015; Southard et al., 2010). *P. parvum* typically blooms in marine and brackish waters, although blooms have occurred in inland bodies with characteristic lower salinity concentrations. The presence of *P. parvum* in the United States was first observed in the Pecos River, Texas, U.S.A. Since its discovery, recurrent blooms of *P.*

parvum have occurred in Texas and expansion into other areas of the U.S. across latitudes and various climates has occurred (Roelke et al., 2015). The successful invasion of *P. parvum* can be attributed to toxin production which functions as an allelochemical to outcompete other algal species, an antipredatory mechanism to deter grazers and/or as an aid for mixotrophic nutrition (Carvalho and Granéli, 2010; Driscoll et al., 2013; Granéli and Johansson, 2003; Tillmann, 1998; Tillmann, 2003). In addition, *P. parvum* is tolerable to various temperatures and salinities (e.g., 10 ◦C below and salinities 10 times lower than optimal conditions) that have been shown to influence toxin production (Baker et al., 2007).

Two toxins have been identified as prymnesins I and II, however, the full suite of toxins have not been identified but remains an active area of research (Bertin et al., 2012a, 2012b; Blossom et al., 2014a; Blossom et al., 2014b; Henrikson et al., 2010; Manning and La Claire, 2010), and quantification of these toxins remains elusive. Monitoring of *P. parvum* HABs has thus been challenged due to lack of sufficient characterization of the toxins produced and their corresponding analytical standards for sufficient verification. To supplement these challenges, aquatic toxicity studies are employed to determine the occurrence of bioavailable toxins. Adding to the challenge of understanding *P. parvum* HAB potency is a lack in standard published culture procedures leading to inconsistencies with culture conditions and the use of different toxicological study methods, making the comparison between *P. parvum* toxicity studies difficult (Brooks et al., 2010). The importance of establishing standardized culturing conditions for toxicological experiments is due to the influence of environmental stressors that have been linked to *P. parvum* bloom formation and toxicity, in which the greatest adverse

acute toxicity effects to aquatic life have been observed under suboptimal growth conditions (Baker et al., 2007; Roelke et al., 2015).

Global climate change and increasing urbanization create stress on aquatic ecosystems resulting in changes to inland water chemistry metrics (e.g., temperature, salinity, pH). In addition, water transportation practices will increase to meet water demands, potentially facilitating the introduction of *P. parvum* to new areas. In combination with these concerns, increased water sequestration could be exacerbated by climate change, which is predicted to decrease instream flow by 60% in the South Central U.S. alone and has been shown to influence *P. parvum* HABs (Brooks et al., 2011). Practices including natural resource extraction operations have the potential of discharging produced waters containing high total dissolved solids (TDS) levels into aquatic ecosystems. This scenario has already occurred along Dunkard Creek located along the West Virginia/Pennsylvania border triggering a *P. parvum* HAB that resulted in devastating ecological losses (Brooks et al., 2011). Therefore, salinization of inland bodies of water, as a result of evaporation and anthropogenic sources, have the potential to increase the development of *P. parvum* HABs especially in water bodies prone to eutrophication.

Eutrophication resulting from storm water/agriculture runoff and effluent discharges lead to imbalanced nitrogen to phosphorus ratios that select for opportunistic primary producers which can produce toxic secondary metabolites when stressed. Similar to salinity, nutrient limitation and stoichiometric imbalances (primarily nitrogen and phosphorus) are a contributing factor in *P. parvum* bloom dynamics and differential toxicity (Granéli and Johansson, 2003; Lindehoff et al., 2010; Lundgren et al., 2016;

Roelke et al., 2007). Historical data of three Texas reservoirs revealed a relationship between salinity and in flow events that have the potential to dilute blooms/secondary metabolites but also increase nutrient loading, which has been shown to deter *P. parvum* HABs (Roelke et al., 2011). However, in stream flows are predicted to decrease as a result of climate change and urbanization (Roelke et al., 2012). A predicted increase in HABs, including *P. parvum*, as a result anthropogenic activities and global climate change create additional challenges and a need to develop appropriate assessment, monitoring and management strategies to prevent/mitigate blooms before ecosystem disruption occurs.

In addition to acute mortality responses, sublethal endpoints associated with adverse outcomes in aquatic life are relevant to ecological risk assessment and management (Ankley et al., 2010; Bradbury et al. 2004). For example, my preliminary results revealed sublethal responses following exposure to *P. parvum* cell densities below those inducing acute mortality, which is currently the main endpoint determinate for toxicity (see CH 1). Traditional sublethal endpoints (growth and reproduction) can have severe chronic impacts on species survival; however, these assays are relatively nonspecific regarding molecular and cellular pathways by which toxicity is elicited. In addition, these assays can be time and resource intensive. Adverse outcome pathways (AOP) provide a conceptual framework linking molecular initiating events cascading across multiple levels of biological organization leading to adverse outcomes at the individual and population level. AOPs can be used to develop acute and chronic HAB toxicity pathways (Ankley et al., 2010). An AOP was recently developed for *P. parvum* after oxygen consumption and respiration were affected in rainbow trout. However, the

molecular initiating event and osmotic cost of gill damage up to the population level remains unknown (Svendsen et al., 2018). Endpoints including fish behavioral alterations and biochemical and molecular markers have been examined for multiple environmental stressors including algal toxins (Amado and Monserrat, 2010; Drummond and Russom, 1990; Lushchak, 2011; Valavanidis et al., 2006); however, these endpoints have not been investigated following *P. parvum* exposure. Further, an understanding of sublethal toxicity associated with *P. parvum* HABs are largely unstudied, particularly across gradients of factors known to alter acute toxicity.

Fish are the most sensitive in vivo model organism to exposure; however, the most common endpoint selected for these exposures have been acute mortality (Brooks et al., 2010). Because fish kills are routinely caused by *P. parvum*, fish responses provide important information for various toxins/toxicants and share conserved toxicity pathways relevant to human health. Fathead minnows (*Pimephales promelas*) and zebrafish (*Danio rerio*) are commonly employed fish models used in toxicological research. Early life stage studies have been demonstrated to routinely be sensitive developmental stage following contaminant exposures (McKim, 1977). Application and comparisons of larval bioassays evaluating multiple sublethal endpoints are lacking following *P. parvum* exposure and would provide insight to more effective biomonitoring strategies and environmental assessments.

The current study objectives were to determine the influence of various growth conditions, including higher and lower salinity and nutrients, to the acute and sublethal toxicity of *P. parvum* using two common model fish species, fathead minnow and zebrafish. In addition to current acute mortality endpoints, antioxidant gene expression,

common biochemical oxidative stress (OS) markers and fish behavior were examined to determine sublethal toxicity effects in fish. Fathead minnow and zebrafish endpoints were compared to determine whether species specific differences exist between the two model fishes following *P. parvum* exposure to potentially identify HAB biomarkers of exposure and/or toxicity. I hypothesized that exposure to sublethal cell densities of *P. parvum* would result in OS and altered behavioral responses due to preliminary investigations (CH 1). In addition, these responses were hypothesized to be heightened under conditions representative of the most suboptimal to growth (low salinity, low nutrients) due to the previously reported increases in acute mortality and sublethal toxicity (CH 1) in response to stressful growth conditions. Lastly, differential toxicity to fathead minnow and zebrafish was expected due to previously reported comparative toxicological investigations by this laboratory (CH 1).

Methods

Laboratory Cultures

A Texas strain was obtained from the University of Texas at Austin Culture Collection of Algae (UTEX LB 2797, Austin, TX, USA). Stock cultures were grown at 4 ppt, f/2 medium, 20 ◦C on a 12:12 light:dark cycle at Baylor University. Prior to study initiation, stock culture were gradually acclimated to 15 ◦C and evaluated for acute mortality to fish to confirm that the stock culture was acutely toxic prior to performing this experiment. A super stock of artificial seawater (ASW) was prepared by dissolving Instant Ocean salt (Spectrum Brands, Blacksburg, VA, USA) in Nanopure water (18.2 megohm ionic purity; Barnstead, ThermoFisher, Wilmington, DE, USA; Brooks et al.,

2010). Media for salinity treatment levels were prepared by diluting the ASW super stock using Nanopure water to 2.4 and 5 ppt. Water was then enriched with either f/2 or f/8 nutrients, with full trace metals and vitamins for both nutrient conditions (Table A1, Appendix A; Guillard, 1975), for nutrient treatment levels. Two replicates of each experimental unit were included in a 2 x 2 experimental design for 8 total experimental units. ASW and all media stocks were autoclaved before use and acclimated to 15 ◦C. Approximately 100 cells/mL were introduced to 15 L for experimental unit, which were 20 L glass carboys. Experimental units were incubated at 15 ◦C, on a 12:12 light:dark cycle, swirled and rotated daily. Every second day, chlorophyll *a* fluorescence was determined using a handheld fluorometer (Turner Designs, San Jose, CA) to identify growth status of each culture. These subsamples collected for chlorophyll *a* fluorescence were preserved with 200 µL of 25% aqueous glutaraldehyde for cell counts. Cell counts were determined using a haemocytometer following previously published methods (Southard, 2005). At study initiation, during exponential and stationary growth, dissolved nitrogen (nitrate/nitrite) and phosphorus (phosphate) concentrations were analyzed for each treatment level (salinity and nutrient conditions) according to standard methods using a flow-injection auto-analyzer (Lachat QuikChem 8500 and Series 520 XYZ Autosampler; APHA (Clesceri et al. 1998). N and P concentrations (µg/L) for each treatment level and experimental carboy are reported in Table B1. (Appendix B).

Fish Cultures

Fathead minnow (*P. promelas*) and zebrafish (*D. rerio*) used in toxicity studies were cultured at Baylor University. Fathead minnow cultures were maintained using a flow through system that introduced aged dechlorinated tap water to individual aquaria.

Cultures were maintained at 25±1 ◦C under a 16:8 light:dark cycle. Fish were fed brine shrimp (*Artemia* sp. naupli; Pentair AES, Apopka, FL, USA) twice daily. Embryos were collected from sexually mature adults aged to at least 120 d before breeding (1:4-5 male to female ratio). Larvae within 24-48 h post hatch were used for toxicity studies. Tropical 5D wild type zebrafish were cultured using a z-mod recirculating system (Marine Biotech Systems, Beverly, MA, USA). Fish were maintained at a density of < 4 fish per liter in 260 ppm instant ocean with a pH of 7.0, temperature of 27±1 ◦C under a 16:8 light:dark cycle. Fish were fed *Artemia* sp. nauplii (Pentair AES, Apopka, FL, USA) with flake food (Pentair AES, Apopka, FL, USA) twice daily. Embryos used for this experiment were collected from sexually mature adults, and were used for toxicity experiments at 48 h post fertilization. All experimental procedures and fish culture protocols followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

Acute Bioassays

Due to the lack of *P. parvum* toxin analytical standards, acute mortality bioassays using fathead minnow were employed to determine acute toxicity of cultures throughout growth stages similar to previous published methods in our laboratory (Brooks et al., 2010; Valenti et al., 2010). Bioassays were conducted weekly and initiated when cell densities reached 10,000 cell/mL, the minimum cell density that is considered to be associated with HAB events in the field (Roelke et al., 2007). Acute mortality of fathead minnow was determined following U.S. Environmental Protection Agency (EPA) Toxicity Identification Evaluation (TIE) (US EPA 1991). Serial dilutions following a 0.5 dilution scheme were prepared for a total of six dilutions (e.g. 100, 50, 25, 12.5, 6.25, 3.125). Media matched to experimental treatments (2.4 and 5 ppt salinity, f/2 and f/8

nutrients) served as the dilution water and treatment control. Five < 24 hour post hatch (hph) fathead minnow larvae were loaded into 80 mL of each culture dilution in duplicates. The pH of each solution was titrated to 8.5 using 1 N hydrochloric acid or 1 N sodium hydroxide, due to the relationship between increasing pH and acute toxicity (Valenti et al., 2010). Bioassays were performed in temperature controlled incubators at 25 ◦C in the dark to prevent photo-degradation (James et al., 2011). Acute mortality was assessed 48 hours to estimate the dilution to cause 50% organism lethality (LC_{50}), which was then normalized to *P. parvum* cell density. Identical acute mortality studies were performed during *P. parvum* culture stationary growth phase with fathead minnow and zebrafish larvae (48 hph fathead minnow, 48 hour post fertilization (hpf) zebrafish) to determine LC_{50} values and subsequent sublethal study cell densities. Zebrafish bioassays were conducted in a temperature controlled chamber set to 27 ◦C in the dark.

Sublethal Bioassays

The experimental design for toxicity studies followed previously published methods by this laboratory (Corrales et al., 2017; Valenti et al., 2010). Sublethal studies with fathead minnow and zebrafish were performed according to standardized toxicity methods from the US EPA Whole Effluent Toxicity (WET) methods (EPA 2002) and Organization for Economic Cooperation and Development (OECD) Fish Embryo Toxicity Test (FET), respectively (OECD no. 236). Three dilution levels (Table 2.1) per experimental replicate carboy were selected below the 48 h LC_{50} value and cell densities were targeted at which responses were observed in preliminary studies (CH 1). This approach was taken to aid in comparisons between treatment and dilution levels. Here again, media (2.4 or 5 ppt, f/2 or f/8) served as the diluent and control to derive dilution

levels. Fish were acclimated to experimental media for approximately 2 hours in a 50:50 ratio of culture water: *P. parvum* media or reconstituted hard water (RHW) served as a negative control. The pH of each solution was titrated to 8.5 using 1 N hydrochloric acid or 1 N sodium hydroxide, due to the positive relationship between pH and toxicity (Valenti et al., 2010). Bioassays were conducted in temperature controlled chambers set to 25 or 27 ◦C for fathead minnow and zebrafish respectively, and in the dark to prevent toxin photodegradation (James et al., 2011). Briefly, 48 hph fathead minnow larvae were placed in 200 mL of experimental solutions. Each experimental carboy replicate (8 total) dilution level consisted of eight experimental units of ten fathead minnow. Zebrafish at 48 hpf were exposed to 30 mL of each experimental dilution. Each experimental carboy dilution consisted of twelve experimental units with fifteen zebrafish each. These volumes were chosen to ensure that the loading density did not exceed acceptable levels for standardized guidelines. At 24 and 48 h mortalities and abnormalities were observed.

At 48 h, tissue samples were collected, frozen at -80 ◦C, and saved for antioxidant related gene expression and biochemical OS determination and fathead minnow and zebrafish behaviors were observed. Collection of tissue and behavioral analysis were randomized per experimental unit using random.org per dilution of each experimental 15 L carboy. Four fathead minnow were analyzed individually for behavior from one experimental unit. Five and ten fathead minnow were pooled per experimental unit for qPCR gene expression and OS biochemical determination respectively. A total of three experimental units were collected for both endpoints. A total of three experimental units were randomly selected for behavioral analysis. Fifteen and ten zebrafish were pooled per beaker for OS biochemical and qPCR gene expression respectively. Similar to fathead

minnow, a total of three experimental units were collected per endpoint. If no mortalities occurred, remaining tissue were collected for additional DNA and RNA extractions. Six zebrafish were analyzed individually for behavior from one experimental unit. A total of four experimental units were randomly selected for behavioral analysis. Each collection of organisms per experimental unit represented a biological replicate. Therefore, a total number of three biological replicates were statistically analyzed for all endpoints for each dilution of experimental 15 L carboys. Zebrafish behavior consisted of four biological replicates that were statistically analyzed following previously published methods by this laboratory (Corrales et al, 2018; Steele et al., Accepted).

Table 2.1. *P. parvum* whole culture dilutions (low, medium and high doses with nominal cell densities (cells/mL)) selected for sublethal exposure to larval fathead minnow and zebrafish. Cultures were grown in duplicate and represented as rep 1 and 2. Media (2.4 or 5 ppt and f/2 or f/8) served as diluent and control. Significant mortalities (represented by *, N=2, $p \leq 0.05$) of each dilution level from media control were determined by a Fisher Exact test. Dilution levels that were significant from media control were not measured for sublethal endpoints.

	Fathead minnow			Zebrafish		
Culture Condition	Low	Medium	High	Low	Medium	High
2.4 ppt f/2 medium (rep 1)	1,540	15,400	38,500	1,540	15,400	38,500
2.4 ppt f/2 medium (rep 2)	2,120	21,200	53,000*	2,120	21,200	53,000
2.4 ppt f/8 medium (rep 1)	1,240	12,400	31,000*	1,240	12,400	31,000
2.4 ppt f/8 medium (rep 2)	1,080	10,800*	27,000*	1,080	10,800	27,000
5 ppt f/2 medium (rep 1)	2,320	4,640	11,600	11,600	23,200	232,000*
5 ppt f/2 medium (rep 2)	2,336	4,672	11,680*	11,680	23,360	233,600*
5 ppt f/8 medium (rep 1)	1,496	2,992	7,480	7,480	14,960	149,600*
5 ppt f/8 medium (rep 2)	1,488	2,976	7,440*	7,440	14,880	148,800*

Antioxidant Gene Expression; qPCR

Changes in mRNA abundance were measured for glutamate cysteine ligase catalytic subunit (*gclc*), glutathione-*s*-transferase (*gst*), superoxide dismutase (*sod*), and nuclear factor erythroid-2 like 2 (*nrf2*) in order to determine changes in genomic activity associated with OS. Specific isoforms measured in zebrafish were *gstp1*, *nrf 2a*, and *sod1*, though due to the poor annotation of this gene family in fathead minnows we were not able to identify the specific isoforms in this species. Gene expression was determined following previously described methods (Corrales et al., 2017). RNA was extracted from whole larval fish using RNAzol (Molecular Research Center, Cincinnati, OH, USA) and cleaned and purified using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was measured using a Nanodrop20000 and 500 ng of RNA was reversed transcribed. RNA was reversed transcribed to cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) to yield 25 ng/µL reaction.

Relative abundance of target genes was determined by real time reverse transcription polymerase chain reaction (qPCR). This reaction consists of 1 µL cDNA, 300 nM of each forward and reverse primer, and 1X Power SYBR Green PCR Master Mix. Gene amplification reaction conditions were 95 ◦C for 10 min, followed by 40 cycles of 95 ◦C for 10 s, and 60 ◦C for 1 min using a StepOnePlus Real-Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Reaction of each sample was performed with two technical replicates per biological replicate (triplicate). Prior to performing assays, amplification efficiencies of all primer pairs were determined at ≥90%. Beta-actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase

(*gapdh*), and hypoxanthine phosphoribosyltransferase 1 (*hprt1*) were selected as reference genes. The geometric mean of *actb1* and *gapdh* for zebrafish and that of *gapdh* and *hprt1* for fathead minnow were used as controls to normalize the starting quantity of mRNA in target genes.

Biochemical Oxidative Stress Assays

Total glutathione concentration, lipid peroxidation, and oxidative DNA damage also followed previously described methods by my lab (Corrales et al., 2017). All biochemical marker analyses consisted of three biological replicates and two technical replicates per dilutions of experimental 15 L carboys. Each biological replicate contained pooled organisms, including five fathead minnow or fifteen zebrafish. Briefly, total glutathione (GSH) concentration was determined using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA). Prior to conducting the assay, samples were deproteinated with 1.25 M metaphosphoric acid and 0.2 M triethanolamine. DTNB (5,5,-dithio-bis-2-nitrobenzoic acid) was added to deproteinated tissue supernatant initiating a reaction between the GSH present in tissue samples and DTNB yielding TNB (5-thio-2-nitrobenzoic acid). The rate of TNB production is directly proportional to the GSH concentration due to the recycling of GSH by glutathione reductase present. Total glutathione concentrations were normalized to sample protein content. Protein content was determined following the Bradford protein assay by which a Bio-Rad protein dye was reacted with tissue supernatant (Sigma-Aldrich, St. Louis, MO, USA Cat. No. A7906 and 5000006).

Lipid peroxidation was determined by the concentration of malondialdehyde (MDA) present in tissue samples. MDA is a reactive carbonyl compound that is a natural

product of lipid peroxidation. MDA concentration was quantified using a Thiobarbituric Acid Reactive Substances assay (TBARS) (Cayman Chemical Company, Ann Arbor, MI, USA). Thiobarbuturic acid (TBA) was added to each tissue sample, producing a MDA-TBA adduct which was fluorometrically detected. Elevated MDA concentrations are proportional to the MDA-TBA adducts formed. MDA concentration was also normalized to sample protein content. Protein content was determined following the Pierce BSA assay by which a working dye reagent was reacted with tissue supernatant (Thermo Scientific Wilmington, DE, USA, Cat No. 23225).

DNA oxidative damage was determined by presence of the oxidatively damaged guanine species, 8-hydroxy-2'-deoxyguanosine (8-OH-dG), measured using a commercially available enzyme immunoassay (EIA) (Cayman Chemical Company, Ann Arbor, MI, USA). Prior to development of EIA, DNA was extracted using DNAzol (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. Extracted DNA samples were cleaned and purified using Zymo Genomic DNA Clean and Concentrator (Zymo Research, Irvine, CA, USA) prior to DNA quantification. DNA concentrations were quantified using a Nanodrop2000 (Thermo Scientific, Wilmington, DE, USA), and 5 µg DNA per sample was prepared for the EIA by diluting DNA with Cayman Ultrapure water to yield a 50 µg/mL sample. The amount of 8-OH-dG present in the sample competed with an added 8-OH-dG-acetylcholinesterase conjugate for binding to an oxidative damage monoclonal antibody. This antibody was bound to seeded goat polyclonal antimouse IgG cells. After an 18 hour incubation, each plate was washed five times and Ellman's reagent was added to develop

the plate. The intensity of the signal is inversely proportional to the amount of free 8-OH-dG or oxidatively damaged DNA.

Behavioral Analysis

Behavioral responses of fathead minnow and zebrafish were observed after 48 h sublethal exposures to *P. parvum* following previously described methods (Kristofco et al., 2016; Steele et al., In press; Steele et al., Accepted). Twelve fathead minnows of each dilution level were loaded into 24 well plates, each well containing two mL of dilution level solution. Twenty-four zebrafish of each dilution level were loaded into 48 well plates with each well containing one mL of dilution level solution. All well plates were preloaded and maintained in exposure conditions until analysis. Fish were acclimated to the well plate for at least 30 minutes before behavioral platform loading. To minimize time of day behavioral effects, plates were analyzed from approximately 9:00 am to 2:00 pm for fathead minnow and 2:00-7:00 pm for zebrafish with each plate analyzed immediately after the conclusion of previous plate (Kristofco et al., 2016).

Larval swimming patterns were observed and recorded using automated tracking software (ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon France). This system was set in tracking mode and behavioral recordings took place over 50 minutes with a ten minute dark acclimation period followed by two altering ten minute light/dark cycles. Observations were recorded for total distance swam and total number of movements. Additionally, distance swam, number of movements, and duration of movements were recorded for activity across three different speed thresholds. These speeds are categorized as bursting (>20mm/s), cruising (5-20 mm/s), and freezing (<5 mm/s) to characterize stimulatory and refractory behaviors. To measure larvae swimming

responses to a sudden change in photoperiod condition, a photomotor response (PMR) was observed following methods previously used (Beker van Woudenberg et al., 2013) with slight modifications. PMR for each photoperiod transition (two light and two dark responses) was calculated as the change in mean distance traveled (mm) between the last minute of an initial photoperiod and the first minute of the following period.

Statistical Analysis

P. parvum specific growth rates were calculated using the equation:

$$r = (\ln N_d - \ln N_0)/ t$$

where r is the growth rate (divisions/day), N_d is the number of organisms at the beginning of the steady growth state, N_0 is the number of organisms at study initiation, and t is the time (days) to reach steady state growth. Steady state growth was determined as the time at which the maximum *P. parvum* density was reached and followed by a general decline.

The lethal concentration to cause 50% morality (LC_{50}) values from acute studies with each of the cultures were calculated using the Toxicity Relationship Analysis Program version 1.30a (EPA). Sigma Plot (Systat Software Inc., San Jose, CA, USA) software was used for statistical analyses of *P. parvum* growth and antioxidant gene expression, biochemical (glutathione concentration, lipid peroxidation, and DNA damage), and fish behavioral data. Significant mortalities (α=0.05) from control at 48 h were determined for all sublethal dilutions using a Fisher Exact test. All sublethal responses were not measured for treatment dilutions with significant mortalities from media control. Prior to analysis antioxidant gene expression data, data were normalized to the geometric mean of reference/endogenous genes and then normalized to media controls to determine linearized $2^{-\Delta Ct}$ values, prior to normality and equivalence of

variance analyses. For biochemical and behavioral data were normalized to cell density (cells/mL) after which normality and equivalence of variance were analyzed.

Significant differences (α=0.05) in mRNA fold changes, biochemical OS endpoints, and behavior movement patterns were identified among dilution levels from each experimental replicate carboy using a One-Way ANOVA if normality and variance assumptions were met. Dunnett's post hoc test was performed to identify dilution level differences from media controls and used to derive No Observable Effect Concentration (NOEC) and Lowest Observable Effect Concentration (LOEC). For data not meeting ANOVA assumptions, an ANOVA on ranks was performed. NOEC values were log transformed prior to statistical analysis to meet normality assumptions. NOEC values less than the lowest dilution level selected were excluded in statistical analysis. Significant differences (α=0.05) of maximal cell densities and growth rates were identified for main and interacting treatment factors (salinity, nutrients, salinity x nutrients) for NOEC values determined for each experimental carboy replicate using General Linear Models (GLM) with SPSS software (IBM Corp., Armonk, NY, USA). Main and interacting treatment factors (salinity, nutrients, salinity x nutrients) effects were also determined with GLMs for NOEC values for OS related endpoints and behavior.

Results

P. parvum Growth

The specific growth rate of each culture ranged from .1456-.1606 divisions per day (Table 2.2). Cultures grown under the same growth conditions were not significantly different ($p > 0.05$) and were influenced by salinity and nutrient factors (Table 2.3). Cultures grown under nutrient deficient conditions had a slower growth rate and lower

maximal cell densities when compared to nutrient sufficient conditions regardless of salinity (Figure 2.1). The low salinity, nutrient sufficient cultures were comparable to those grown under high (5 ppt) salinity with deficient nutrients. Maximal cell densities also varied with salinity and nutrient condition and were significantly ($p < 0.05$) influenced by main and interacting growth factor conditions (Table 2.3). High salinity and sufficient nutrients resulted in the highest cell densities observed, followed by high salinity with deficient nutrients, low salinity with sufficient nutrients and low salinity with deficient nutrients. Exponential growth began on day 26 of the study for all cultures. Stationary growth was reached at day 48 for 2.4 ppt cultures and day 54 for 5 ppt cultures.

Acute Bioassays and Sublethal Survival

Larval fathead minnow LC_{50} values indicate that all cultures were highly toxic throughout the duration of *P. parvum* growth (Table 2.4). Fathead minnow and zebrafish survival was not affected by both salinity and nutrient condition (control survival >90%). Acute mortality was observed at the start of exponential growth and continued to stationary growth phase (Table 2.4). Acute mortality of fathead minnow decreased per study day (Figure 2.2 and Table 2.4) for all cultures with the highest mortality occurring during early exponential growth phase. Acute toxicity was highest under nutrient limitation across both salinities. However, acute mortality was greatest on a per cell basis under higher salinity and deficient nutrient conditions (Figure 2.2). Zebrafish exhibited biphasic toxicity after exposure to the high salinity cultures, with greatest mortality between 37,200-292,000 cells/mL (Table 2.5). Similarly, survival was greatest for nutrient sufficient conditions compared to nutrient deficient conditions during the

sublethal exposures for both species (Figure 2.3). Significant ($p < 0.05$) differences in mortality were observed for the highest dilution levels selected for experimental carboy replicates that were acutely toxic prior to sublethal exposures; these dilution levels were omitted in sublethal response evaluations (Figure 2.3).

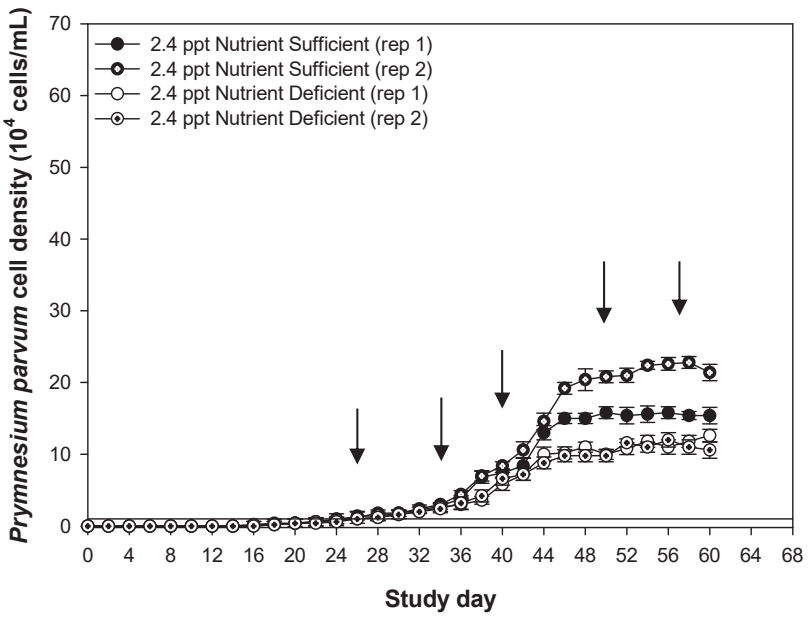
Table 2.2. *P. parvum* growth rates (divisions/day) represented as mean ± S.D (n=5) grown at 2.4 and 5 ppt, 15 °C with a 12:12 light:dark cycle, under nutrient sufficient (f/2) and deficient (f/8) conditions in duplicate (represented as rep 1 and 2). Growth rates were determined when stationary growth phase was reached at study day 48 for 2.4 ppt cultures and study day 54 for 5 ppt cultures.

2.4 ppt f/2 medium (rep 1)	2.4 ppt f/2 medium (rep 2)	2.4 ppt f/8 medium (rep 1)	2.4 ppt f/8 medium (rep 2)	5 ppt f/2 medium (rep 1)	5 ppt f/2 medium (rep 2)	5 ppt f/8 medium (rep 1)	5 ppt f/8 medium (rep 2)
0.15236 ±0.00099	0.15874 ±0.00055	0.14586 ±0.00134	0.14586 ±0.00134	0.1605 ±0.00021	0.1606 ±0.00043	0.1522 ±0.00042	0.1529 ±0.00057

Table 2.3. Influence of *P. parvum* growth treatment factors (salinity, nutrients, salinity x nutrients) on *P. parvum* mean growth rate (divisions/day, n=5) and maximal cell densities (cells/mL, n=5). Growth conditions included 2.4 or 5 ppt with sufficient (f/2) or deficient (f/8) nutrient conditions at 15 ◦C with a 12:12 light:dark cycle in duplicate. Statistical differences (N=2, $p \leq 0.05$, in bold) determined using General Linear Models.

	Interactive effect	Salinity	Nutrients
Mean growth rate (divisions/day)	0.626	**0.022**	**0.05**
Mean maximal cell density (cells/mL)	**0.001**	**<0.001**	**<0.001**

A.



B.

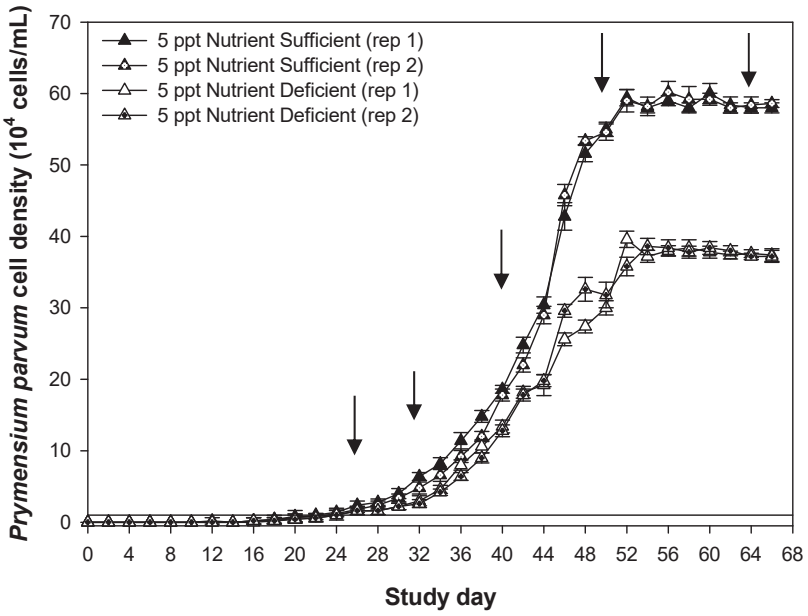
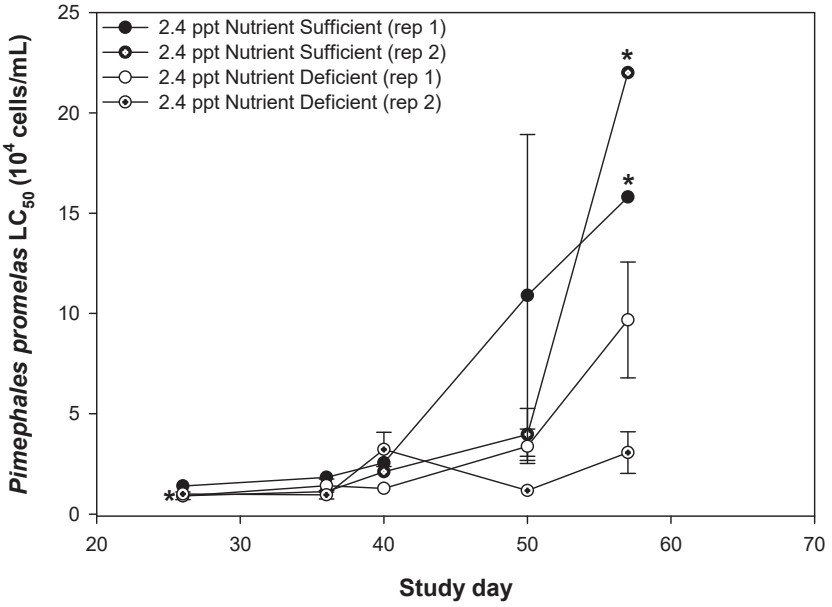


Figure 2.1. Growth of *P. parvum* represented as mean cell density (cells/mL, n=5) ± S.D. per study day. Cultures were grown in duplicate at A) 2.4 and B) 5 ppt 15 °C with a 12:12 light:dark cycle, under nutrient sufficient (f/2) and deficient (f/8) conditions. Cell densities were determined using a haemocytometer. Acute toxicity tests were initiated every 7 days after exponential growth phase began as indicated by arrows. Line indicates bloom toxicity threshold (10,000 cells/mL).

A.



B.

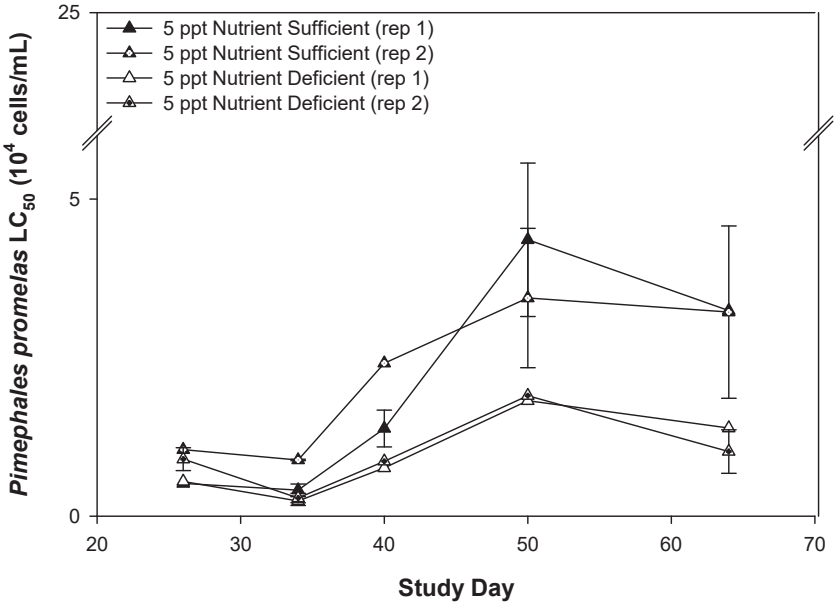


Figure 2.2. *P. promelas* lethal concentration to cause 50% mortality (LC_{50} ±95% C.I.; *P. parvum* cells/mL) per study day. Cultures were grown in duplicate at A) 2.4 and B) 5 ppt under nutrient sufficient (f/2) and deficient (f/8) conditions. Acute mortality bioassays employing larval fathead minnow (*P. promelas* <24 hph) were performed weekly after exponential growth phase began through stationary growth phase. Cultures that resulted in no mortality are represented by observed cell density at the time of initiation (denoted by *).

Table 2.4. *P. parvum* 48 h LC_{50} values (±95% CI, cells/mL) for larval fathead minnow (*P. promelas*). Exposures occurred weekly to determine acute toxicity which began after the initiation of exponential growth phase and continued throughout stationary growth phase. Cultures were grown in duplicate (represented as rep 1 and 2). NA=not available due to lack of partial mortalities.

	Study Day					
Culture Condition	26	34/36	40	50	57	64
2.4 ppt f/2 medium (rep 1)	Not Toxic	18,349.8 (NA)	25,641 (NA)	109,056 (28,851-189,268)	Not Toxic	
2.4 ppt f/2 medium (rep 2)	9,120 (7,227-11,016)	11,2479 (NA)	21,114 (NA)	39,726 (9,727-69,726)	Not Toxic	
2.4 ppt f/8 medium (rep 1)	9,100 (NA)	14,220 (10,497-17,974)	12,857 (NA)	37,378 (24,342-50,413)	96,816 (67,899-125,735)	
2.4 ppt f/8 medium (rep 2)	Not Toxic	9,604 (7,483-11,740)	32,255 (23,707-40,803)	21,725 (NA)	30,661 (20,240-41,083)	
5 ppt f/2 medium (rep 1)	5,168 (NA)	4,106 (3,170-5,041)	13,829 (10,931-16,727)	43,580 (31,479-55,682)		32,438 (NA)
5 ppt f/2 medium (rep 2)	10,500 (8,838-12,617)	8,856 (NA)	24,119 (NA)	34,440 (23,469-45,410)		32,174 (18,582-45,767)
5 ppt f/8 medium (rep 1)	5,472 (NA)	2,384 (1,697-3,100)	7,607 (NA)	18,213 (NA)		13,029 (NA)
5 ppt f/8 medium (rep 2)	9,000 (7,186-10,814)	2,867 (2,127-3,608)	8,658 (NA)	19,018 (NA)		10,213 (6,785-27,211)

Table 2.5. Percent (%) survival of larval zebrafish (*D. rerio*) per dilution level after 48 h exposure to *P. parvum* grown under varying salinity (2.4 or 5ppt) and nutrient (sufficient; f/2 or deficient/8) conditions in duplicate (represented as rep 1 and 2). Media (2.4 or 5 ppt, f/2 or f/8) served as the diluent and control. Survival (100%) of zebrafish were not affected by media.

	P. parvum whole culture dilution (%)					
Culture condition	3.125%	6.25%	12.5%	25%	50%	100%
2.4 ppt f/2 medium (rep 1)	100	100	100	100	100	100
2.4 ppt f/2 medium (rep 2)	100	100	100	100	100	100
2.4 ppt f/8 medium (rep 1)	100	100	100	100	100	100
2.4 ppt f/8 medium (rep 2)	100	100	100	100	100	100
5 ppt f/2 medium (rep 1)	100	90	60	40	80	100
5 ppt f/2 medium (rep 2)	100	100	70	70	20	70
5 ppt f/8 medium (rep 1)	100	70	20	50	70	100
5 ppt f/8 medium (rep 2)	90	60	10	30	0	60

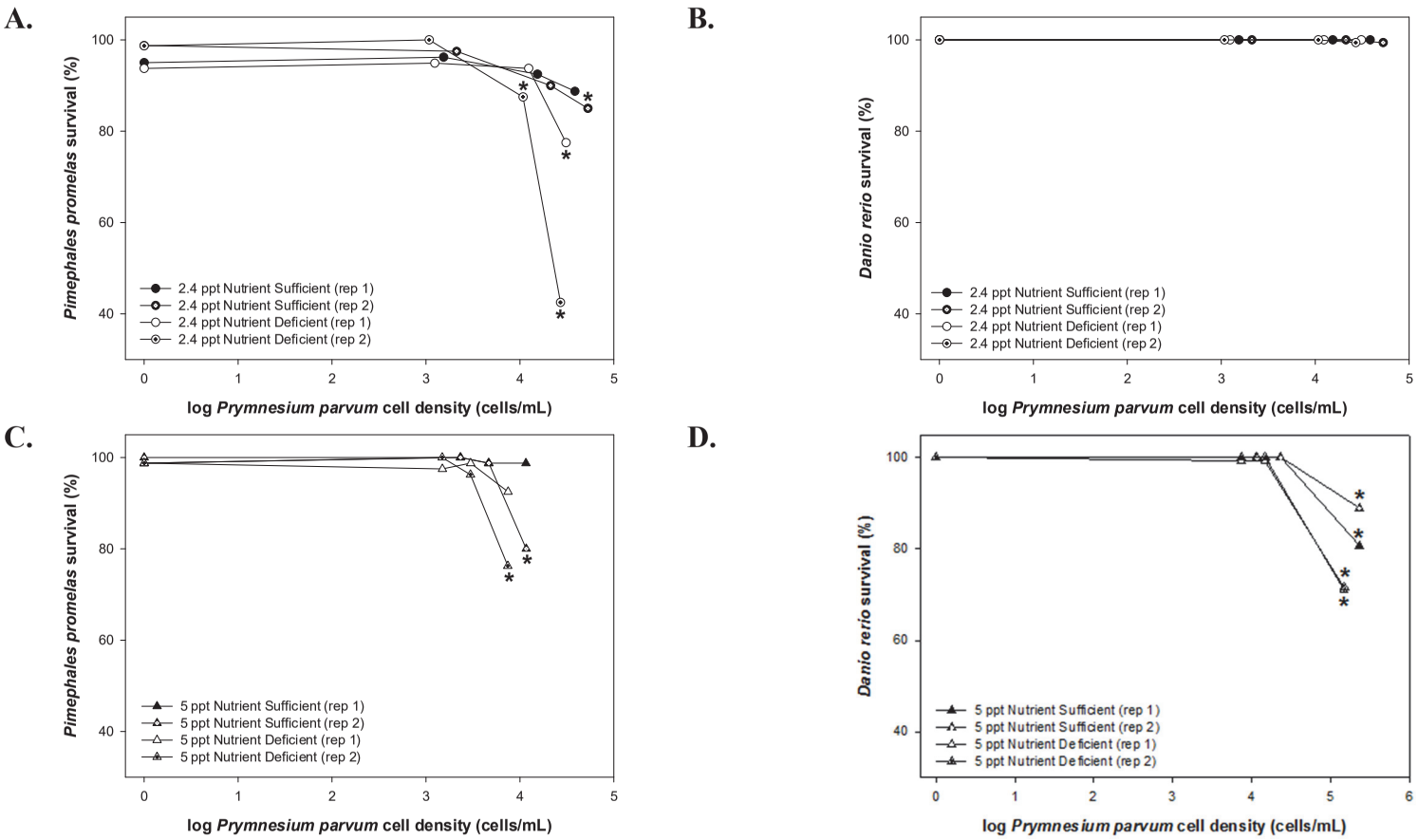


Figure 2.3. Percent survival of larval fathead minnow (*P. promelas*) (A,C) and zebrafish (*D. rerio*) (B,D) after 48 h sublethal exposure to *P. parvum* cultures grown in duplicate under 2.4 (A,B) and 5 (C,D) ppt and nutrient sufficient (f/2) or deficient (f/8) conditions. Significant mortalities from the media control of each treatment level (represented as *, N=2, $p \leq 0.05$) were determined by a Fisher Exact test and omitted in sublethal analyses.

Antioxidant Gene Expression

Relative gene expression (mRNA fold change) were significantly ($p < 0.05$), unless otherwise stated, induced in both species but varied among *P. parvum* culture condition. Fathead minnow *gclc* and *nrf2* induction ($p<0.05$) occurred after exposure to high salinity treatments (Figures 2.4 & 2.6). A low salinity deficient nutrient culture also significantly ($p<0.05$) induced *nrf2* expression (Figure 2.6). Fathead *nrf2* NOEC values were the lowest cell densities observed and comparable among cultures that elicited acute mortality prior to sublethal study (Tables 2.4& 2.7). High salinity sufficient nutrients cultures induced *gclc* expression in zebrafish which was significantly ($p<0.05$) influenced by salinity (Figures 2.4, Table 2.6, 2.9). Significant ($p<0.05$) inductions of *nrf2a* were also observed after exposure to this same culture (Figure 2.6). Comparable to GSH and lipid peroxidation, *nrf2* expression in fathead minnow was significantly ($p<0.05$) influenced by nutrient condition and interactive effects (Table 2.9), whereas *nrf2a* expression was only influenced by nutrients (Table 2.9). Although not significant ($p > 0.05$), large inductions were observed for fathead minnow while zebrafish elicited depletions of *gclc* and *gst* when exposed to nutrient deficient cultures (Figures 2.4-5). Similar to fathead minnow, *nrf2a* expression was induced after exposure to the same low salinity deficient nutrient culture (Figure 2.6). High salinity with deficient nutrients resulted in a significant ($p<0.05$) depletion of *gstp1* in zebrafish (Figure 2.5). No significant differences were observed for *sod/sod1* expression for either species however, significant influences of salinity and nutrients were observed for zebrafish NOEC values (Figure 2.7, Table 2.9).

Table 2.6. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) (*P. parvum* cells/mL) values to elicit antioxidant gene expression in larval fathead minnow and zebrafish following a 48 h exposure to *P. parvum* grown under varying salinity (2.4 or 5 ppt) and nutrient conditions (nutrient sufficient; f/2 or deficient; f/8) in duplicate (represented as rep 1 and 2). Values determined by One-Way ANOVA followed by a Dunnett's post hoc test (N=3, $p \leq 0.05$; induction or depletion represented by ↑ ↓)

	Fathead minnow				Zebrafish			
	gclc		*gst*		*gclc*		*gst*	
Culture condition:	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
2.4 ppt f/2 medium (rep 1)	38,500	>38,500	38,500	>38,500	38,500	>38,500	38,500	>38,500
2.4 ppt f/2 medium (rep 2)	21,200	>21,200	21,200	>21,200	53,000	>53,000	53,000	>53,000
2.4 ppt f/8 medium (rep 1)	12,400	>12,400	12,400	>12,400	31,000	>31,000	31,000	>31,000
2.4 ppt f/8 medium (rep 2)	1,080	>1,080	1,080	>1,080	27,000	>27,000	1,080	10,800 (↓)
5 ppt f/2 medium (rep 1)	4,640	11,600 (↑)	11,600	>11,600	23,200	>23,200	23,200	>23,200
5 ppt f/2 medium (rep 2)	4,672	>4,672	4,672	>4,672	11,600	23,200 (↑)	23,360	>23,360
5 ppt f/8 medium (rep 1)	2,992	7,480 (↑)	7,480	>7,480	14,960	>14,960	14,960	>14,960
5 ppt f/8 medium (rep 2)	2,976	>2,976	2,976	>2,976	14,880	>14,880	14,880	>14,880

Abbreviation: *gclc*: glutamate-cysteine ligase catalytic subunit, *gst*: glutathione-*s*-transferase

Table 2.7. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) (*P. parvum* cells/mL) values to elicit antioxidant gene expression in larval fathead minnow and zebrafish following a 48 h exposure to *P. parvum* grown under varying salinity (2.4 or 5 ppt) and nutrient conditions (nutrient sufficient; f/2 or deficient; f/8) in duplicate (represented as rep 1 and 2). Values determined by One-Way ANOVA followed by a Dunnett's post hoc test (N=3, $p \leq 0.05$; induction or depletion represented by ↑ ↓).

	Fathead minnow				Zebrafish			
	nrf2		*sod*		*nrf2*		*sod*	
Culture condition:	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
2.4 ppt f/2 medium (rep 1)	38,500	>38,500	38,500	>38,500	38,500	>38,500	38,500	>38,500
2.4 ppt f/2 medium (rep 2)	21,200	>21,200	21,200	>21,200	53,000	>53,000	53,000	>53,000
2.4 ppt f/8 medium (rep 1)	1,240	12,400 (↑)	12,40	>12,400	31,000	>31,000	31,000	>31,000
2.4 ppt f/8 medium (rep 2)	1,080	>1,080	1,080	>1,080	10,800	27,000 (↑)	27,000	>27,000
5 ppt f/2 medium (rep 1)	2,320	4,640 (↑)	11,600	>11,600	23,200	>23,200	23,200	>23,200
5 ppt f/2 medium (rep 2)	4,672	>4,672	4,672	>4,672	23,360	>23,360	23,360	>23,360
5 ppt f/8 medium (rep 1)	2,992	7,480 (↑)	7,480	>7,480	14,960	>14,960	14,960	>14,960
5 ppt f/8 medium (rep 2)	1,488	2,976 (↑)	2,976	>2,976	14,880	>14,880	14,880	>14,880

Abbreviation: *nrf2*: nuclear factor erythroid 2-like 2, *sod:* superoxide dismutase

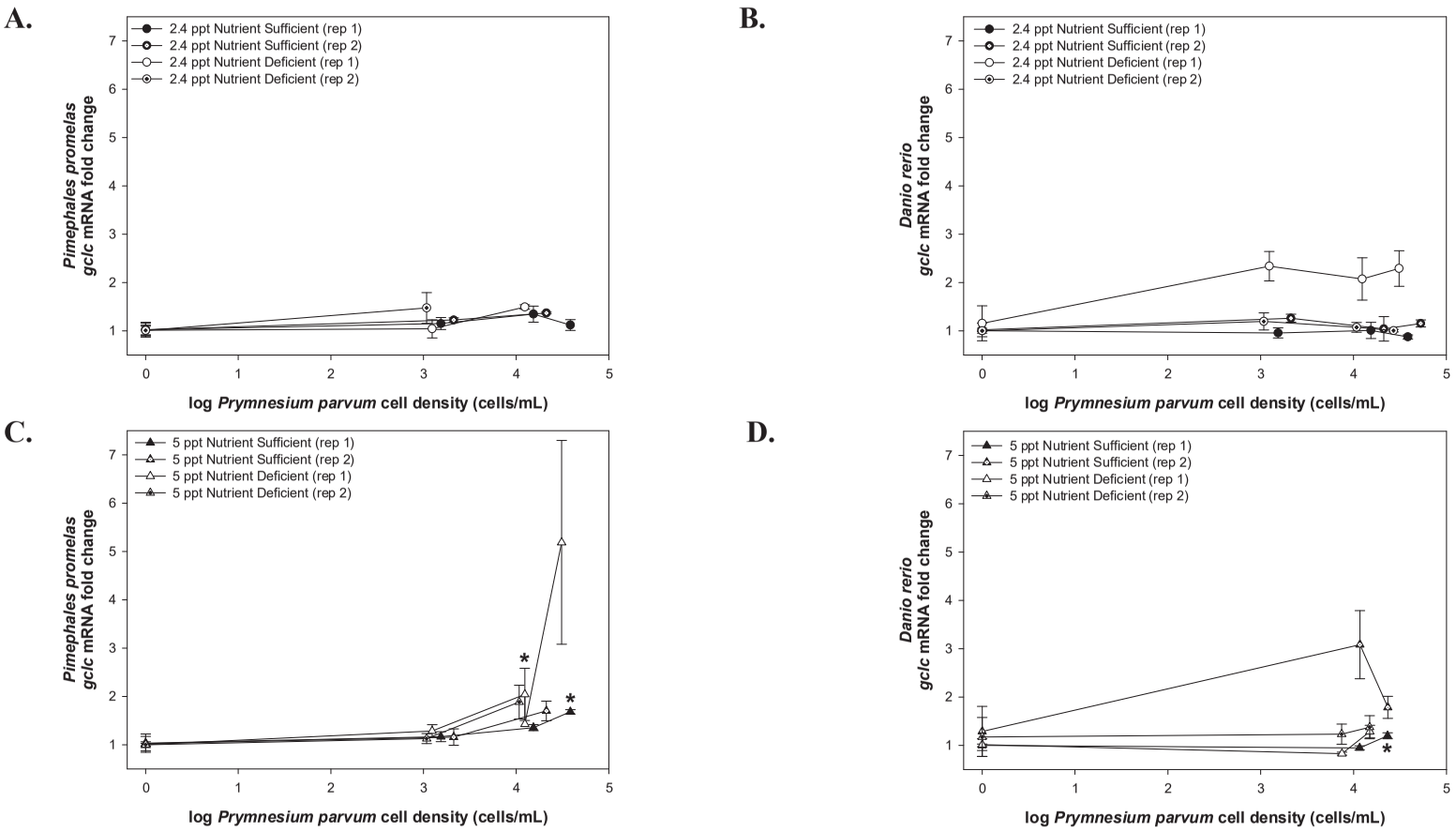


Figure 2.4. Relative *gclc* gene expression (mRNA fold induction change) determined by qPCR in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were first normalized to reference genes and then compared to control. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance (N=3, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

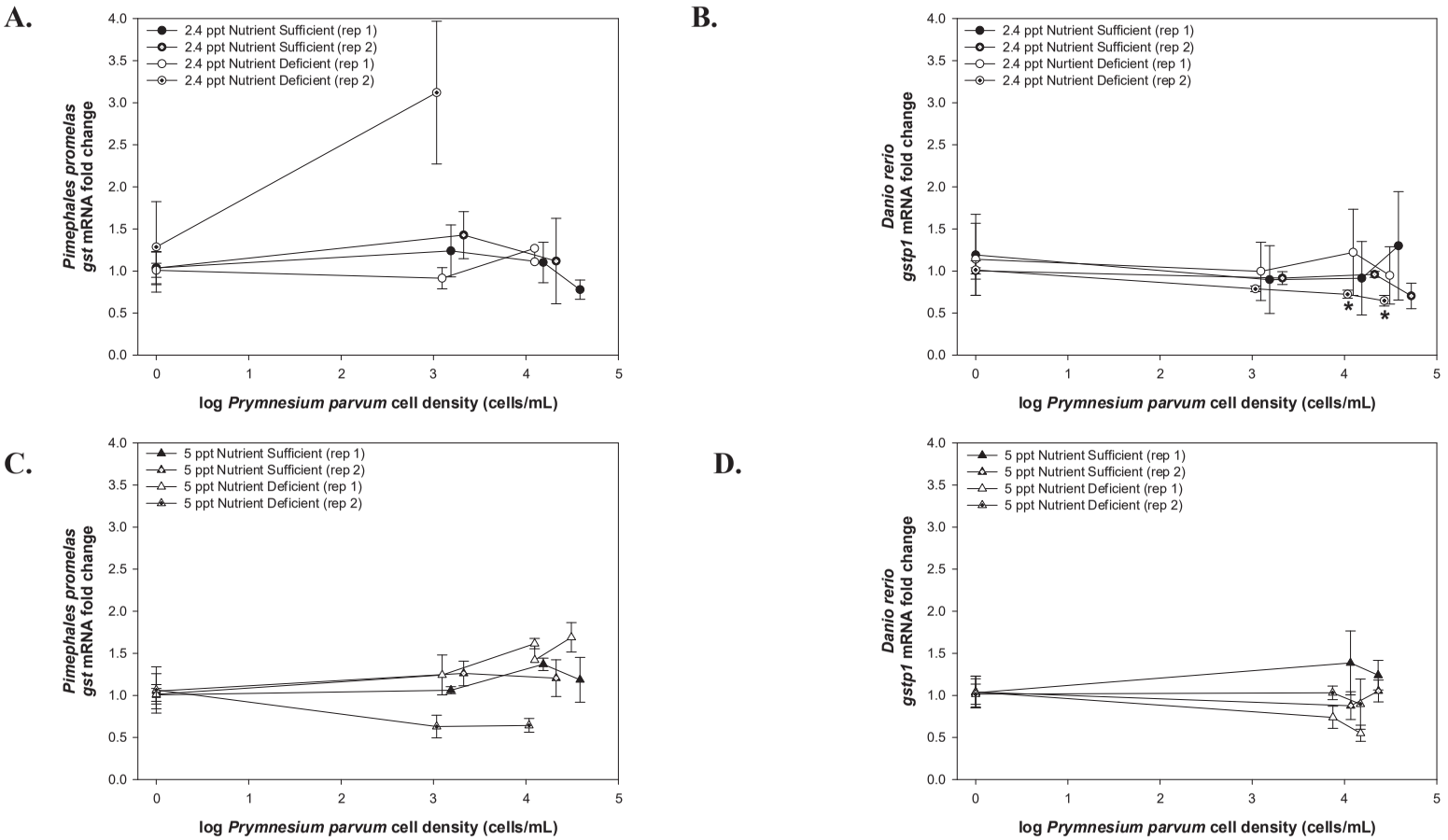


Figure 2.5. Relative gst gene expression (mRNA fold induction change) determined by qPCR in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were first normalized to reference genes and then compared to control. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance (N=3, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

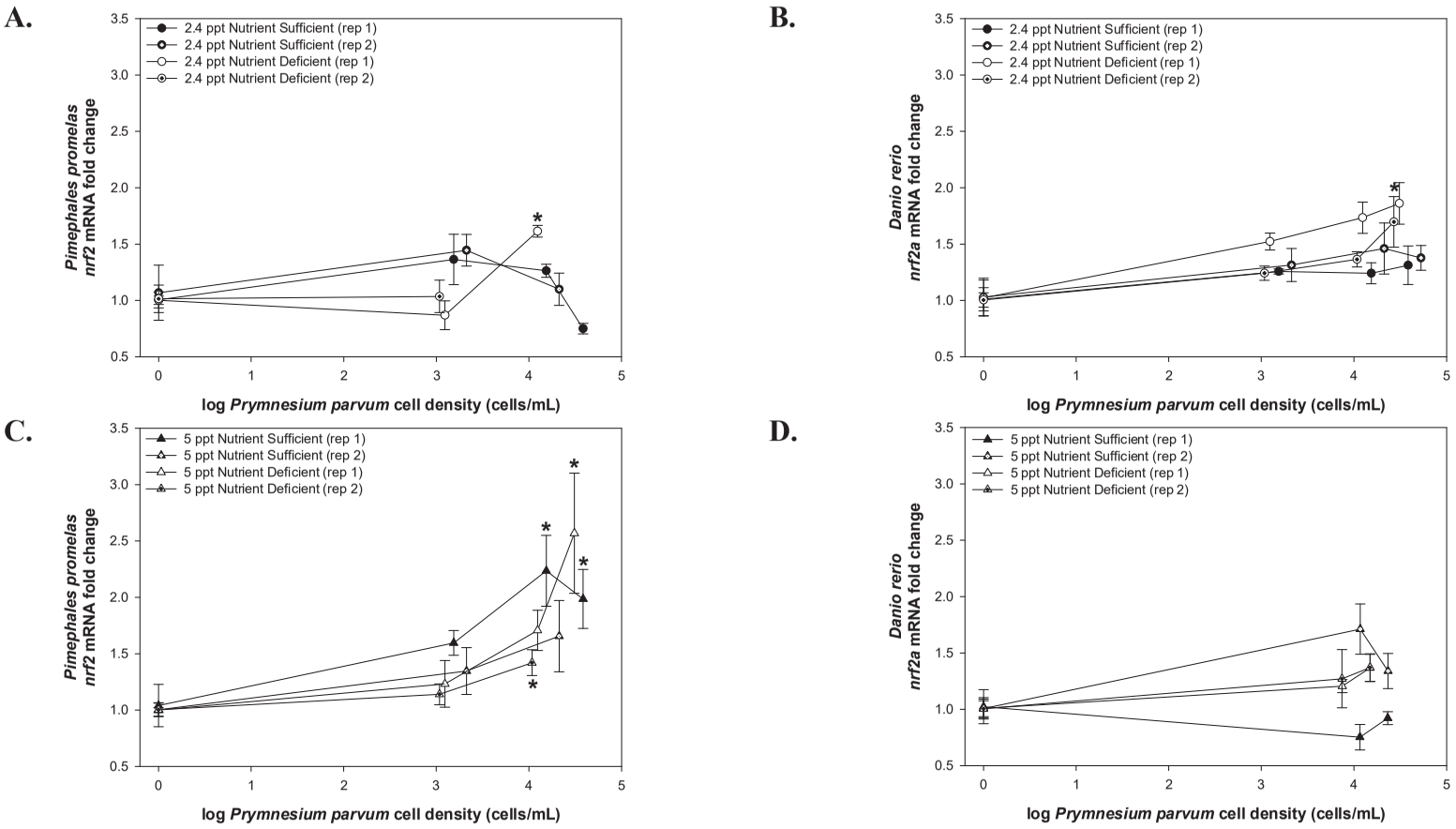


Figure 2.6. Relative *nrf2* gene expression (mRNA fold induction change) determined by qPCR in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were first normalized to reference genes and then compared to control. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance (N=3, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

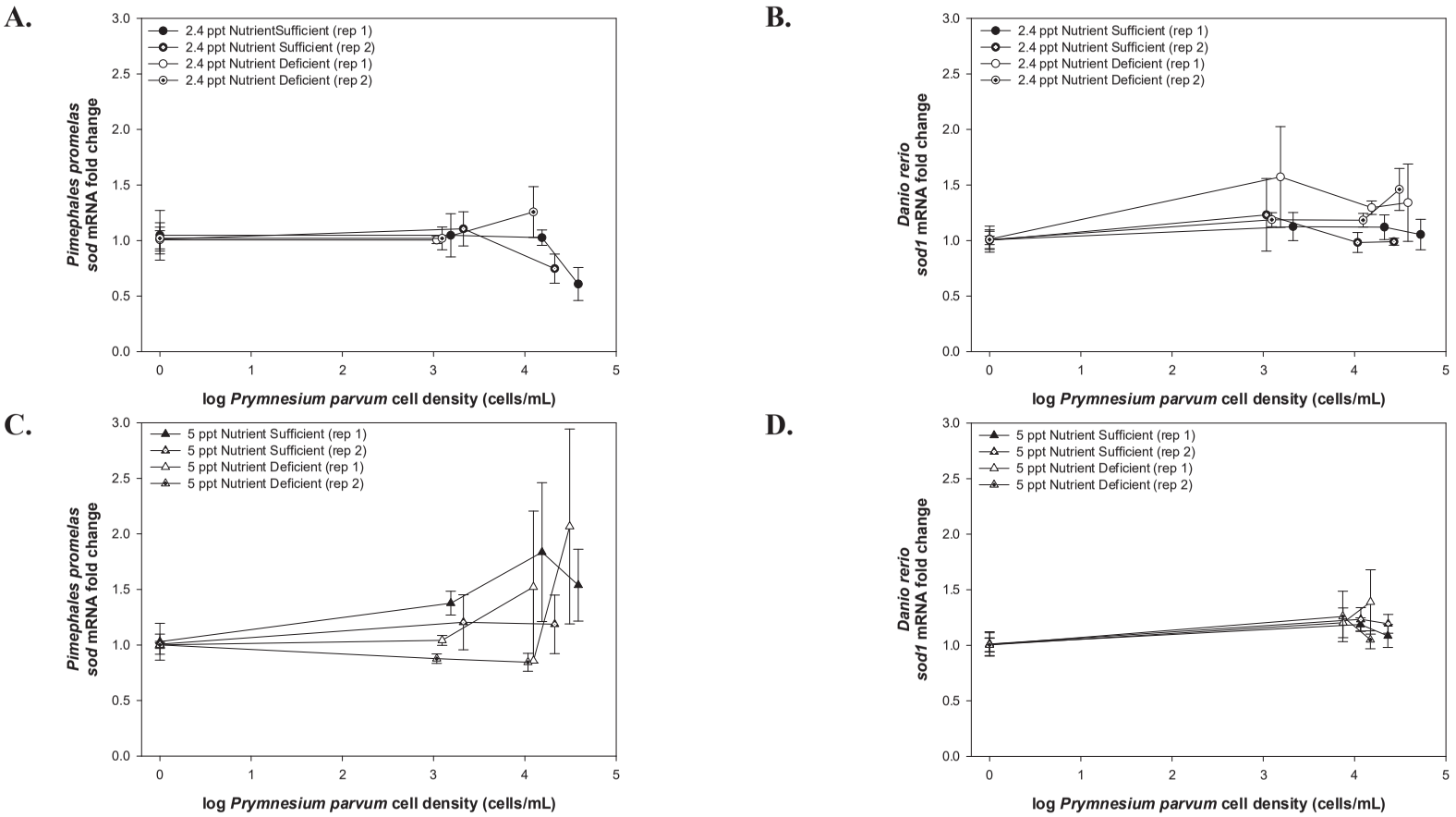


Figure 2.7. Relative sod gene expression (mRNA fold induction change) determined by qPCR in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were first normalized to reference genes and then compared to control. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance (N=3, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

Biochemical Oxidative Stress Endpoints

Fathead minnow and zebrafish exhibited differential biochemical OS responses across culture conditions. Statistically significant ($p < 0.05$) depletion of total glutathione and increases in lipid peroxidation were observed in fathead minnow exposed to low salinity, deficient nutrient conditions with similar trends after exposure to remaining culture conditions (Figures 2.8-9). Both of these responses were significantly ($p < 0.05$) influenced by nutrient condition and the interaction between salinity and nutrients (Table 2.9). NOECs were comparable among cultures that elicited acute fathead minnow mortality with the most significant responses observed after exposure to low salinity, low nutrient conditions (Table 2.8). Increases in oxidative DNA damage were observed after exposure to high salinity cultures, however these were not significant (Figure 2.10). By contrast, zebrafish elicited a decrease in lipid peroxidation after exposure to high salinity, high nutrient conditions (Figure 2.9). High salinity, low nutrient conditions resulted in a significant increase in oxidative DNA damage in zebrafish (Figure 2.10). Although not significant ($p > 0.05$), high salinity low nutrients induced total glutathione concentration in zebrafish (Figure 2.8). GSH, lipid peroxidation and DNA damage in zebrafish exhibited a significant influence of salinity and nutrient conditions (Table 2.9). No significant glutathione depletion was observed as indicated by NOEC values, therefore this significant influence could be controlled by the dose of *P. parvum* cells (Table 2.8). Lipid peroxidation and DNA damage were observed at the lowest dose thus an accurate NOEC value could not be determined (Table 2.8).

Table 2.8. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) (*P. parvum* cells/mL) values to elicit total glutathione (GSH), lipid peroxidation (LPO) and DNA damage responses in larval fathead minnow and zebrafish following a 48 h exposure to *P. parvum* grown under varying salinity (2.4 or 5 ppt) and nutrient conditions (nutrient sufficient; f/2 or deficient; f/8) in duplicate (represented as rep 1 and 2). Values determined by One-Way ANOVA followed by a Dunnett's post hoc test (N=3, $p \leq 0.05$; induction or depletion represented by ↑ ↓).

	Fathead minnow						Zebrafish					
	GSH		LPO		DNA damage		GSH		LPO		DNA damage	
Culture condition:	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
2.4 ppt f/2 medium (rep 1)	38,500	>38,500	38,500	>38,500	38,500	>38,500	38,500	>38,500	38,500	>38,500	38,500	>38,500
2.4 ppt f/2 medium (rep 2)	21,200	>21,200	21,200	>21,200	21,200	>21,200	53,000	>53,000	53,000	>53,000	53,000	>53,000
2.4 ppt f/8 medium (rep 1)	1,240	12,400 (↑)	1,240	12,400 (↑)	12,400	>12,400	31,000	>31,000	31,000	>31,000	31,000	>31,000
2.4 ppt f/8 medium (rep 2)	1,080	>1,080	1,080	>1,080	1,080	>1,080	27,000	>27,000	27,000	>27,000	27,000	>27,000
5 ppt f/2 medium (rep 1)	11,600	>11,600	11,600	>11,600	11,600	>11,600	23,200	>23,200	23,200	>23,200	23,200	>23,200
5 ppt f/2 medium (rep 2)	4,672	>4,672	4,672	>4,672	4,672	>4,672	23,360	>23,360	<11,680	11,680 (↓)	23,360	>23,360
5 ppt f/8 medium (rep 1)	7,480	>7,480	7,480	>7,480	7,480	>7,480	14,960	>14,960	14,960	>14,960	<7,480	7,480 (↓)
5 ppt f/8 medium (rep 2)	2,976	>2,976	2,976	>2,976	2,976	>2,976	14,880	>14,880	14,880	>14,880	14,880	>14,880

Table 2.9. Influence of *P. parvum* growth treatment factors (salinity, nutrients, salinity x nutrients) on oxidative stress (OS) biochemical responses in larval fathead minnow (FHM) and zebrafish (ZF). No Observable Effect Concentrations (NOEC) were calculated after 48 h exposure to *P. parvum* grown at 2.4 or 5 ppt with sufficient (f/2) or deficient (f/8) nutrient conditions in duplicate. Statistical differences (N=2, $p \leq 0.05$, in bold) determined using General Linear Models.

		Growth Treatment Factor					Growth Treatment Factor		
Biochemical OS endpoint	Species	Interactive effect	Salinity	Nutrient	Relative gene expression	Species	Interactive effect	Salinity	Nutrients
GSH	FHM	**0.018**	0.948	**0.007**	*gclc*	FHM	0.269	0.183	0.117
	ZF	0.997	**0.002**	**0.007**		ZF	0.425	**0.013**	0.237
LPO	FHM	**0.018**	0.948	**0.007**	*gst*	FHM	0.318	0.479	0.152
	ZF	0.987	**0.010**	**0.029**		ZF	0.394	0.874	0.212
DNA damage	FHM	0.318	0.479	0.152	*nrf2*	FHM	**0.009**	0.055	**0.003**
	ZF	0.993	**0.010**	**0.029**		ZF	0.452	0.191	**0.071**
					sod	FHM	0.318	0.479	0.152
						ZF	0.997	**0.002**	**0.007**

Abbreviations: GSH:total glutathione concentration, LPO:lipid peroxidation, *gclc*: glutamate-cysteine ligase catalytic subunit, *gst*: glutathione-*s*-transferase, *nrf2*: nuclear factor erythroid 2-like 2, *sod:* superoxide dismutase

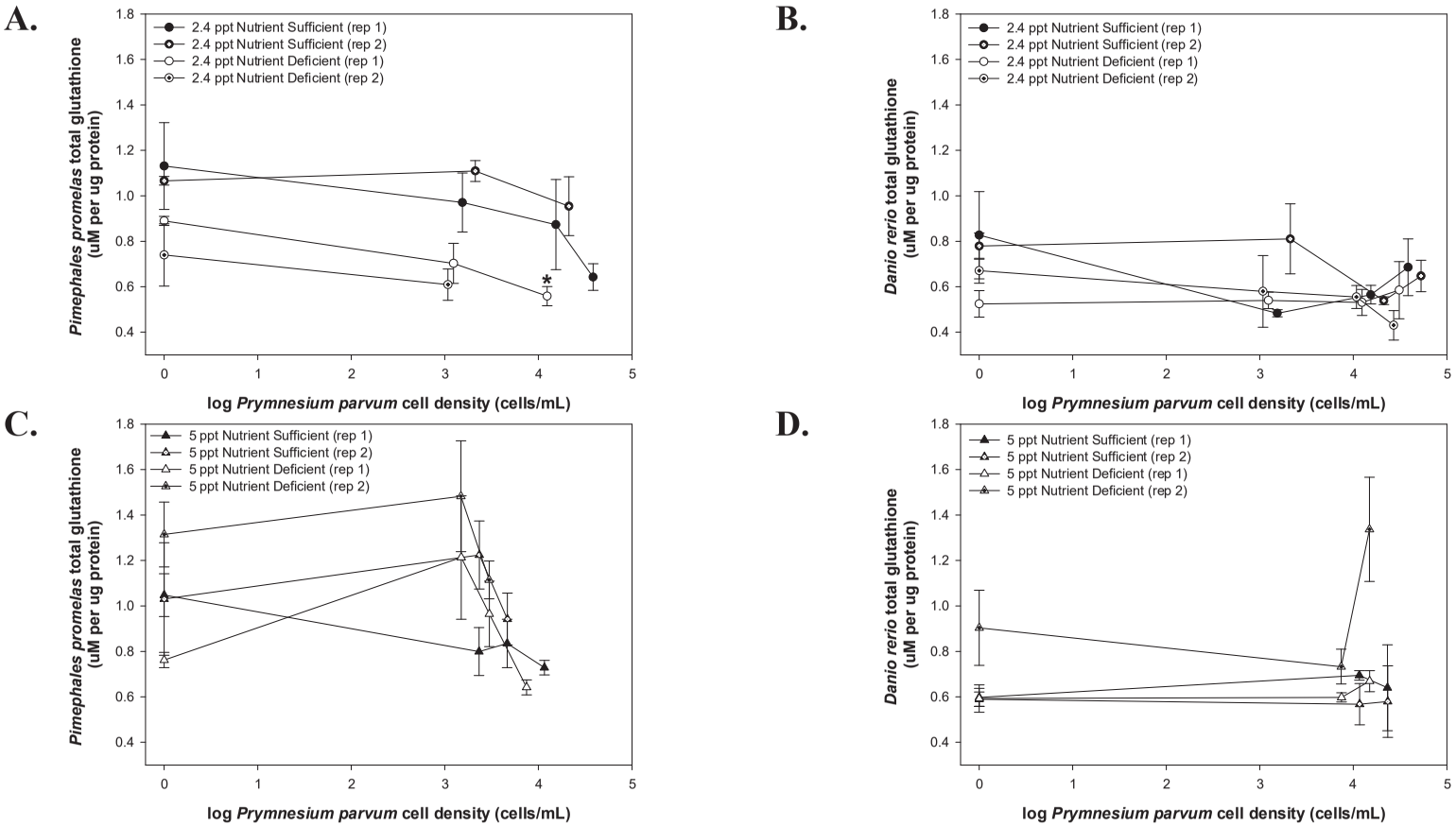


Figure 2.8. Total glutathione concentrations (μM per μg of protein) in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance ($N= 3$, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

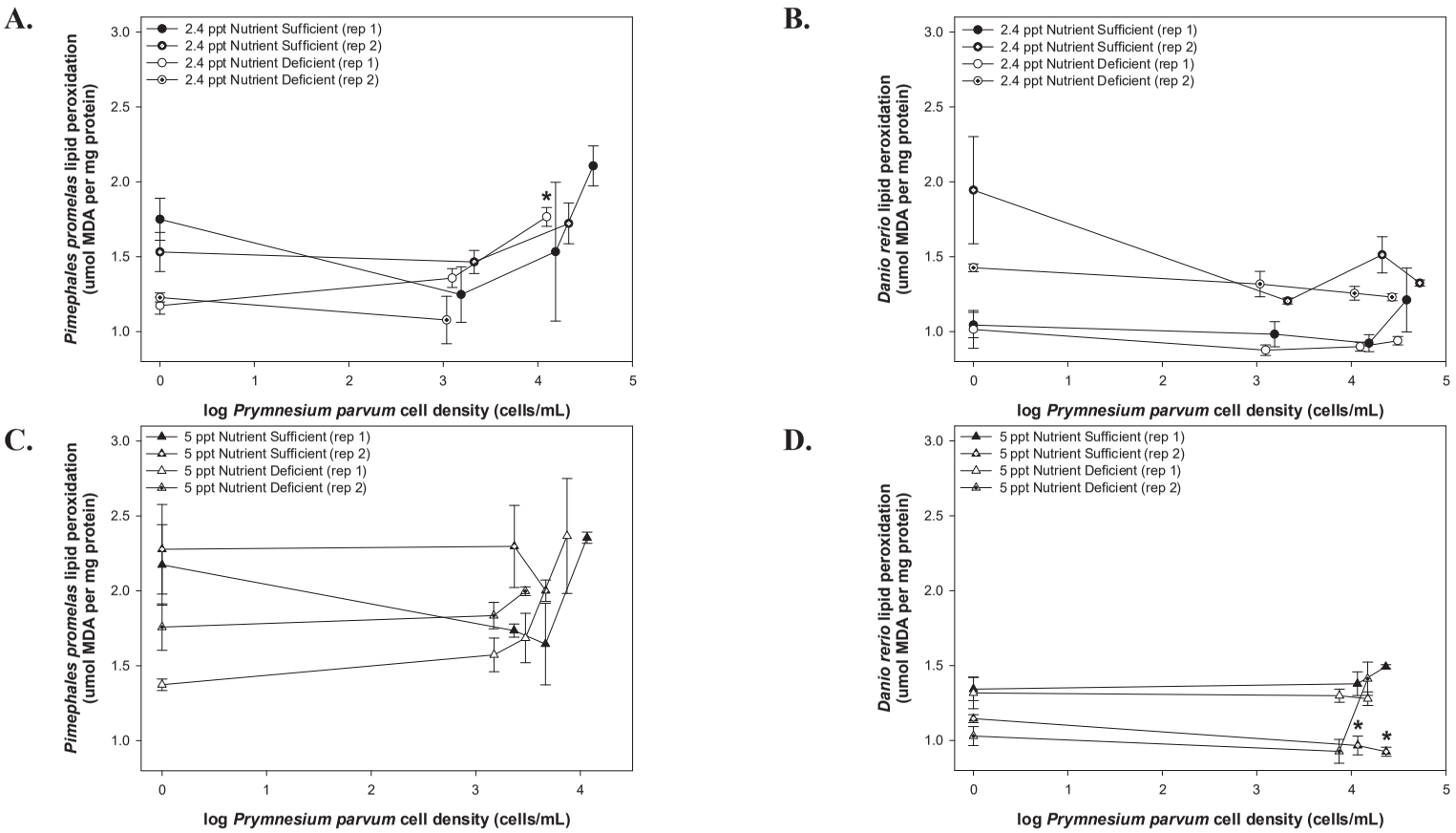


Figure 2.9. Lipid peroxidation (malondialdehyde (MDA) concentration, µM per µg of protein) in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance (N=3, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

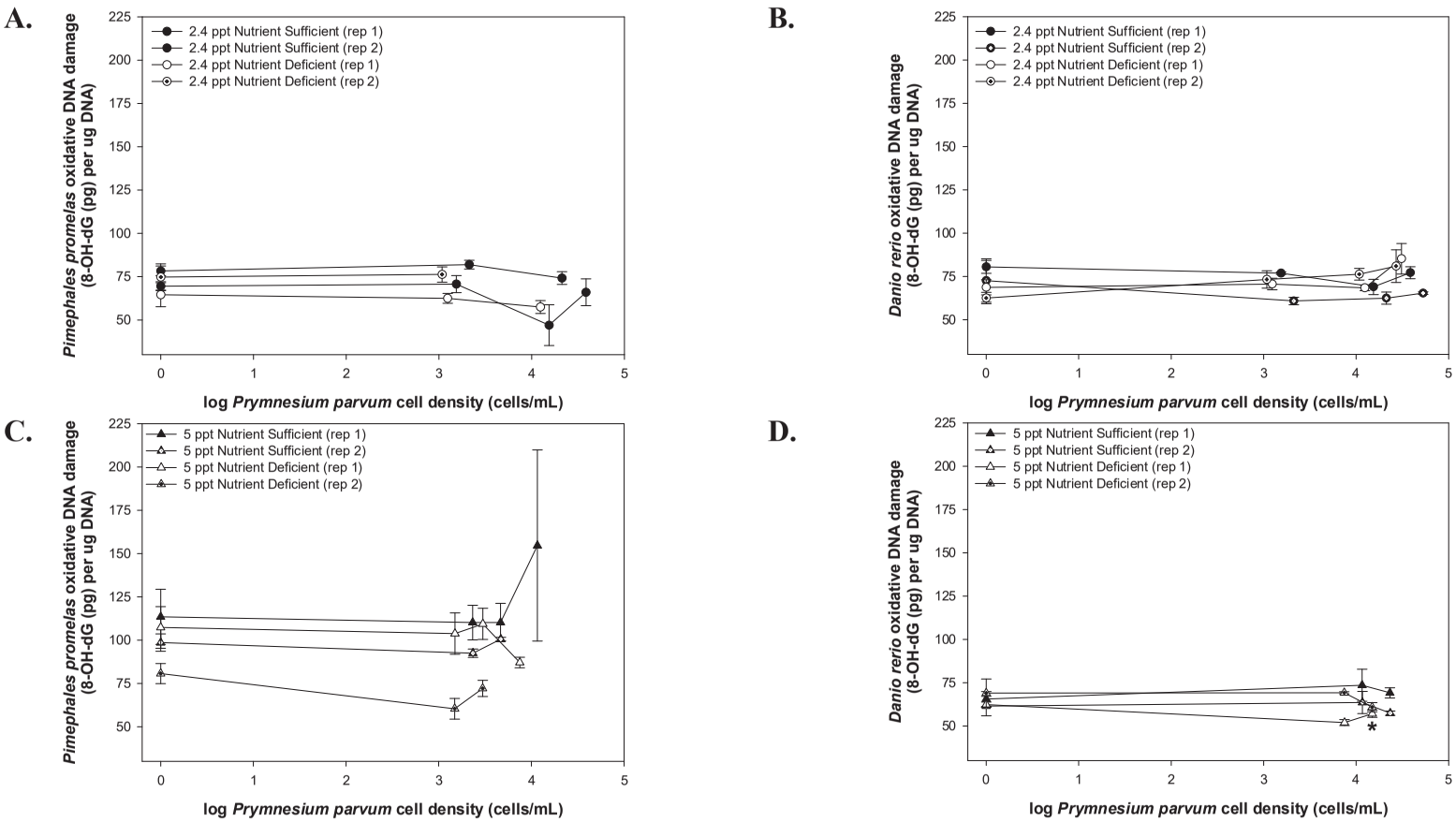


Figure 2.10. Oxidative DNA damage (8-OH-dG (pg) per µg of DNA) in larval fathead minnow (*P. promelas*; A, C) and zebrafish (*D. rerio*; B,D) after 48 h exposure to *P. parvum* cultures grown under 2.4 (A-B) or 5 (C-D) ppt and nutrient sufficient (high, f/2) or deficient (low, f/8) conditions. Data were analyzed using Sigma Plot and represented as mean ± S.E. Statistical significance was determined using a one-way ANOVA followed Dunnett's post hoc test (* indicates statistical significance (N=3, $p \leq 0.05$) Media control (0 cells/mL) contained no *P. parvum* cells.

Fish Behavior

Behavior varied across species and culture conditions. Fathead minnow activity was greatest in light conditions indicated by the positive dark to light photomotor responses (PMRs) and reduced light to dark PMRs (Figures 2.11-14). Statistically significant ($p < 0.05$) decreases in swimming activity occurred in the light and dark conditions for low salinity with sufficient nutrients and high salinity with deficient nutrients, with responses observed at lower cell densities when grown under higher salinity conditions (Figures 2.11 & 2.14). Although decreased activity was significantly ($p < 0.05$) affected across freezing and cruising speed thresholds, stimulatory response trends were observed for bursting speed thresholds (Figure 2.11-14). An increase in activity was observed for fathead minnow exposed to high salinity with sufficient nutrients (Figure 2.13). Stimulatory response trends across bursting thresholds were observed for fathead minnow exposed to all culture conditions. Salinity and nutrient conditions in addition to interaction effects did not significantly ($p > 0.05$) influence swimming behavior or PMR responses (Tables 2.10-11).

Table 2.10. Influence of *P. parvum* growth treatment factors (salinity, nutrients, salinity x nutrients) on behavior photomotor response (PMR) and total swimming behavior in larval fathead minnow (FHM) and zebrafish (ZF). No Observable Effect Concentrations (NOEC) were calculated after 48 h exposure to *P. parvum* grown at 2.4 or 5 ppt with sufficient (f/2) or deficient (f/8) nutrient conditions in duplicate. Statistical differences (N=2, $p \leq 0.05$, in bold) determined using General Linear Models.

		Growth Treatment Factor					Growth Treatment Factor		
Behavior Endpoint (Light)	Species	Interactive effect	Salinity	Nutrient	Behavior Endpoint (Dark)	Species	Interactive effect	Salinity	Nutrients
PMR light to dark cycle 1	FHM	0.318	0.479	0.152	PMR dark to light cycle 1	FHM	0.318	0.479	0.152
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
PMR light to dark cycle 2	FHM	0.318	0.479	0.152	PMR dark to light cycle 2	FHM	0.318	0.479	0.152
	ZF	0.987	**0.010**	**0.029**		ZF	0.997	**0.002**	**0.007**
Total counts	FHM	0.811	0.324	0.104	Total counts	FHM	1.00	0.803	0.664
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Total distance	FHM	0.629	0.555	0.196	Total distance	FHM	0.454	0.216	0.667
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**

Table 2.11. Influence of *P. parvum* growth treatment factors (salinity, nutrients, salinity x nutrients) on swimming behavior responses across three speed thresholds in larval fathead minnow (FHM) and zebrafish (ZF). No Observable Effect Concentrations (NOEC) were calculated after 48 h exposure to *P. parvum* grown at 2.4 or 5 ppt with sufficient (f/2) or deficient (f/8) nutrient conditions in duplicate. Statistical differences (N=2, $p \leq 0.05$, in bold) determined using General Linear Models.

Behavior Endpoint (Light)	Species	Growth Treatment Factor: Interactive effect	Salinity	Nutrient	Behavior Endpoint (Dark)	Species	Growth Treatment Factor: Interactive effect	Salinity	Nutrients
Freezing (<5mm/sec) counts	FHM	0.811	0.346	0.104	Freezing (<5mm/sec) counts	FHM	0.515	0.683	0.305
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Freezing distance	FHM	0.437	0.308	0.091	Freezing distance	FHM	0.848	0.452	0.112
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Freezing duration	FHM	0.318	0.479	0.152	Freezing duration	FHM	0.318	0.479	0.152
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Cruising (5-20mm/sec) counts	FHM	0.811	0.346	0.104	Cruising (5-20mm/sec) counts	FHM	1.00	0.803	0.664
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Cruising distance	FHM	0.629	0.555	0.196	Cruising distance	FHM	1.00	0.803	0.664
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Cruising duration	FHM	0.625	0.448	0.129	Cruising duration	FHM	1.00	0.803	0.664
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Bursting (>20 mm/sec) counts	FHM	0.318	0.479	0.152	Bursting (>20 mm/sec) counts	FHM	0.318	0.479	0.152
	ZF	0.997	**0.002**	**0.007**		ZF	0.997	**0.002**	**0.007**
Bursting distance	FHM	0.318	0.479	0.152	Bursting distance	FHM	0.318	0.479	0.152
	ZF	0.505	0.236	0.525		ZF	0.997	**0.002**	**0.007**
Bursting duration	FHM	0.318	0.479	0.152	Bursting duration	FHM	0.318	0.479	0.152
	ZF	0.505	0.236	0.525		ZF	0.997	**0.002**	**0.007**

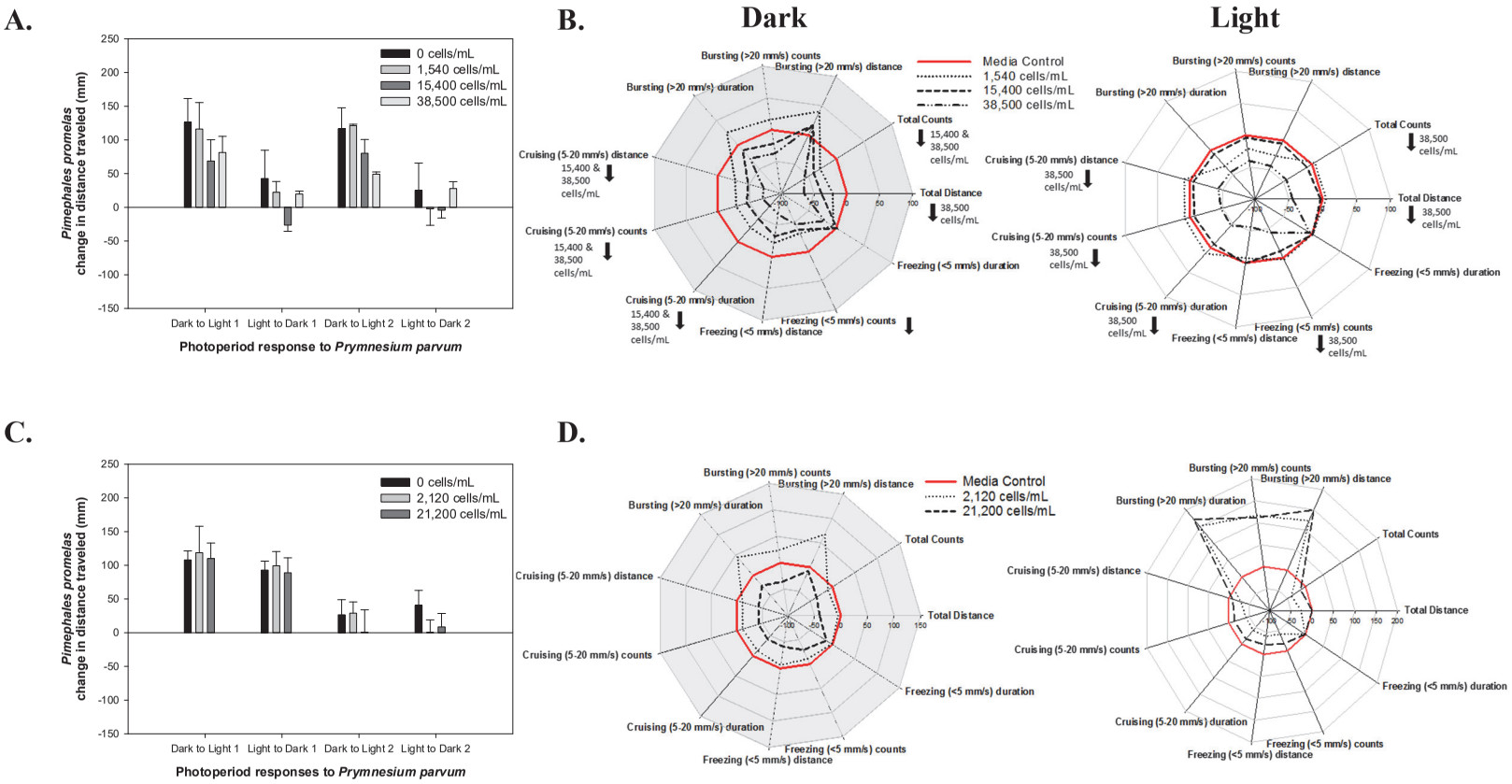


Figure 2.11. Fathead minnow (*P. promelas*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 2.4 ppt under nutrient sufficient (f/2) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=3, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

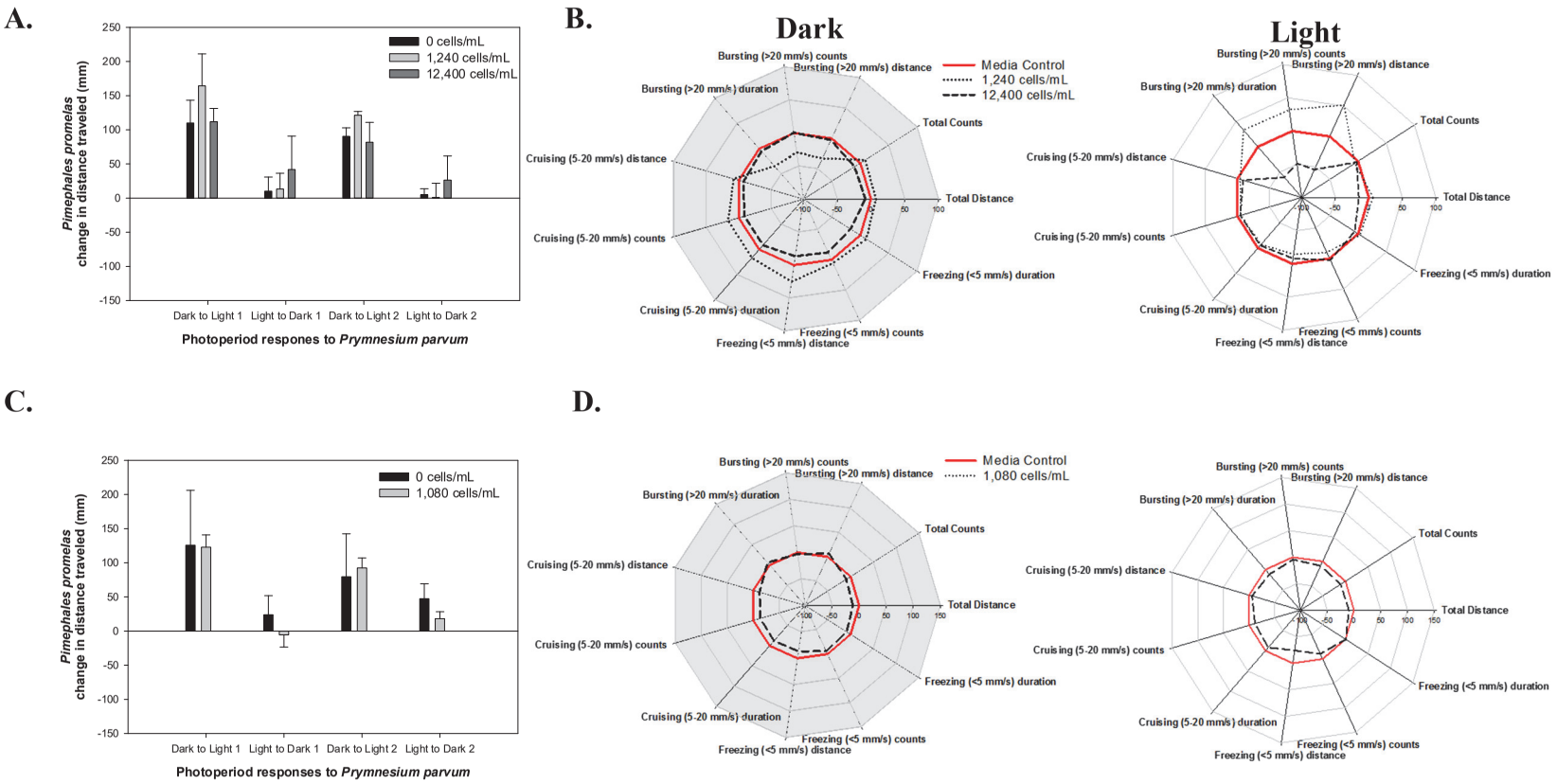


Figure 2.12. Fathead minnow (*P. promelas*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 2.4 ppt under nutrient deficient (f/8) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=3, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

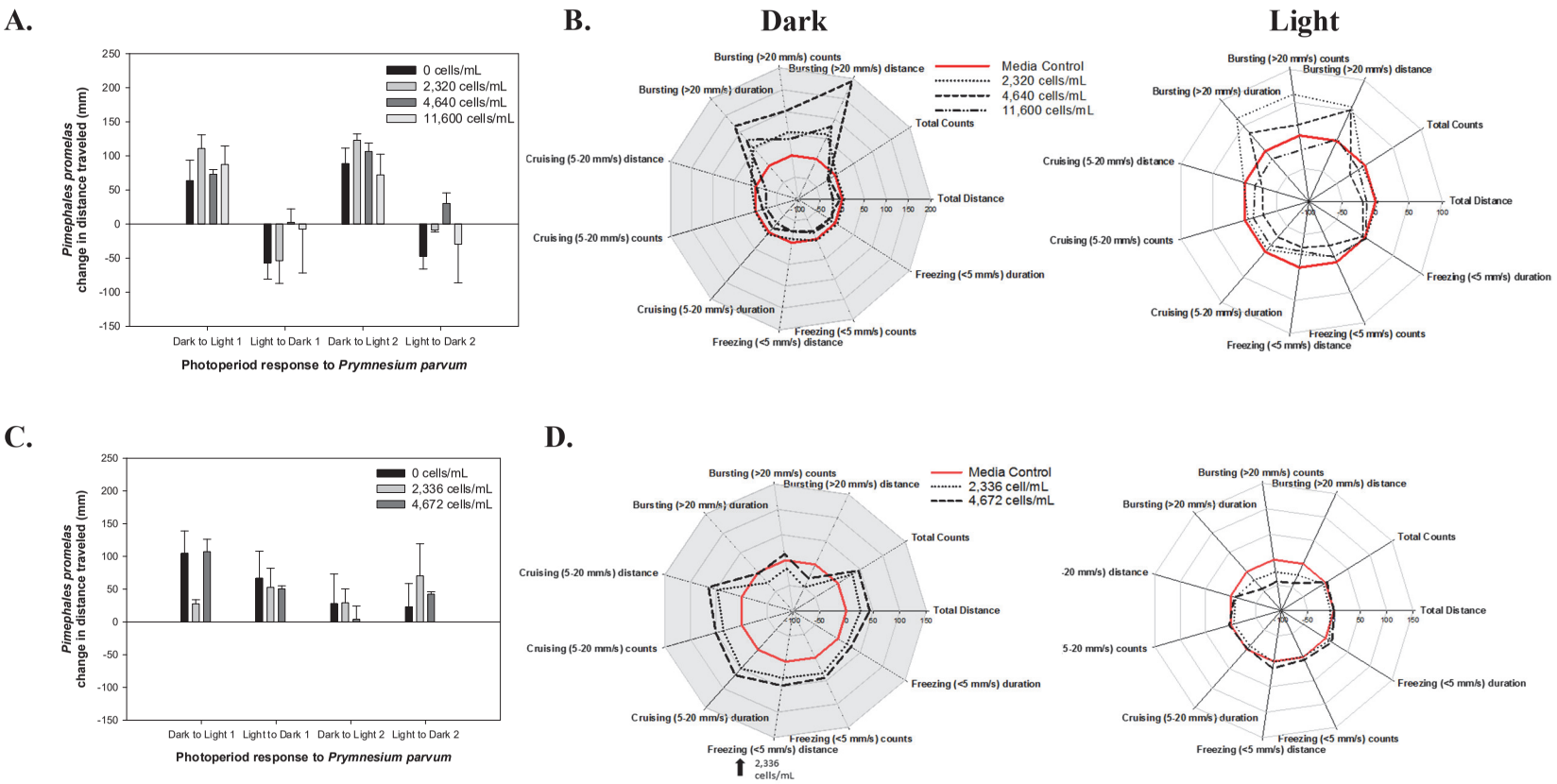


Figure 2.13. Fathead minnow (*P. promelas*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 5 ppt under nutrient sufficient (f/2) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=3, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

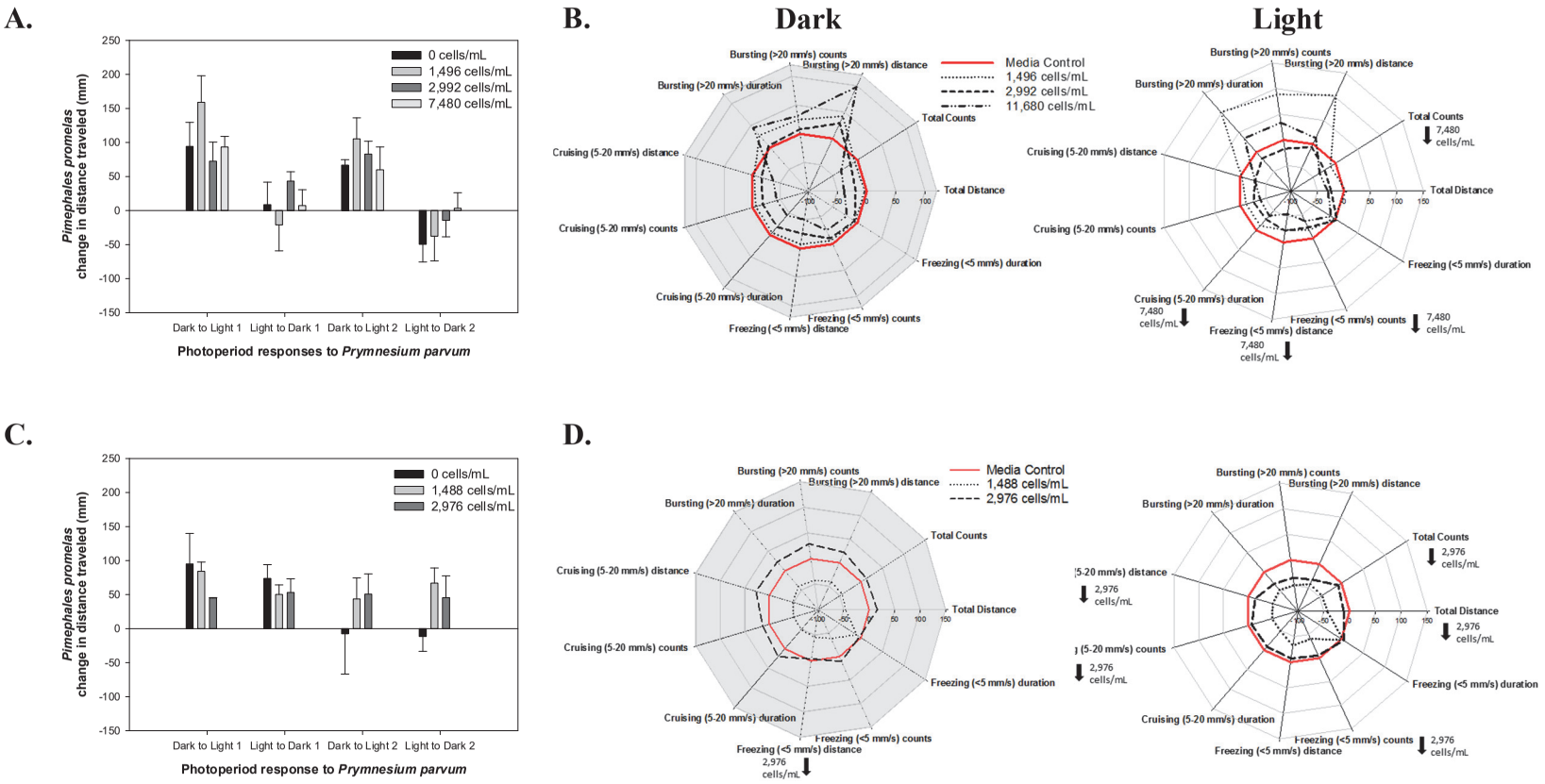


Figure 2.14. Fathead minnow (*P. promelas*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 5 ppt under nutrient deficient (f/8) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=3, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

Zebrafish activity was greatest in dark conditions indicated by the negative dark to light and positive light to dark PMRs. A statistically significant ($p < 0.05$) increase in PMR was observed under high salinity low nutrient conditions (Figures 2.17). Low salinity with sufficient nutrients elicited significant ($p < 0.05$) stimulatory responses in zebrafish swimming behavior in the light (Figure 2.15). Similar to the fathead minnow, stimulatory trends were observed for bursting speed threshold with heightened responses in light conditions, although some decreases in activity were observed (Figures 2.15-18). Contrary to fathead minnow, salinity and nutrients did influence swimming behavior and PMR zebrafish responses (Tables 2.10-11). Although there were a significant ($p < 0.05$) influence of both these experimental factors, it should be noted that these NOEC values were the highest dilution level exposed to zebrafish, except for light to dark PMR.

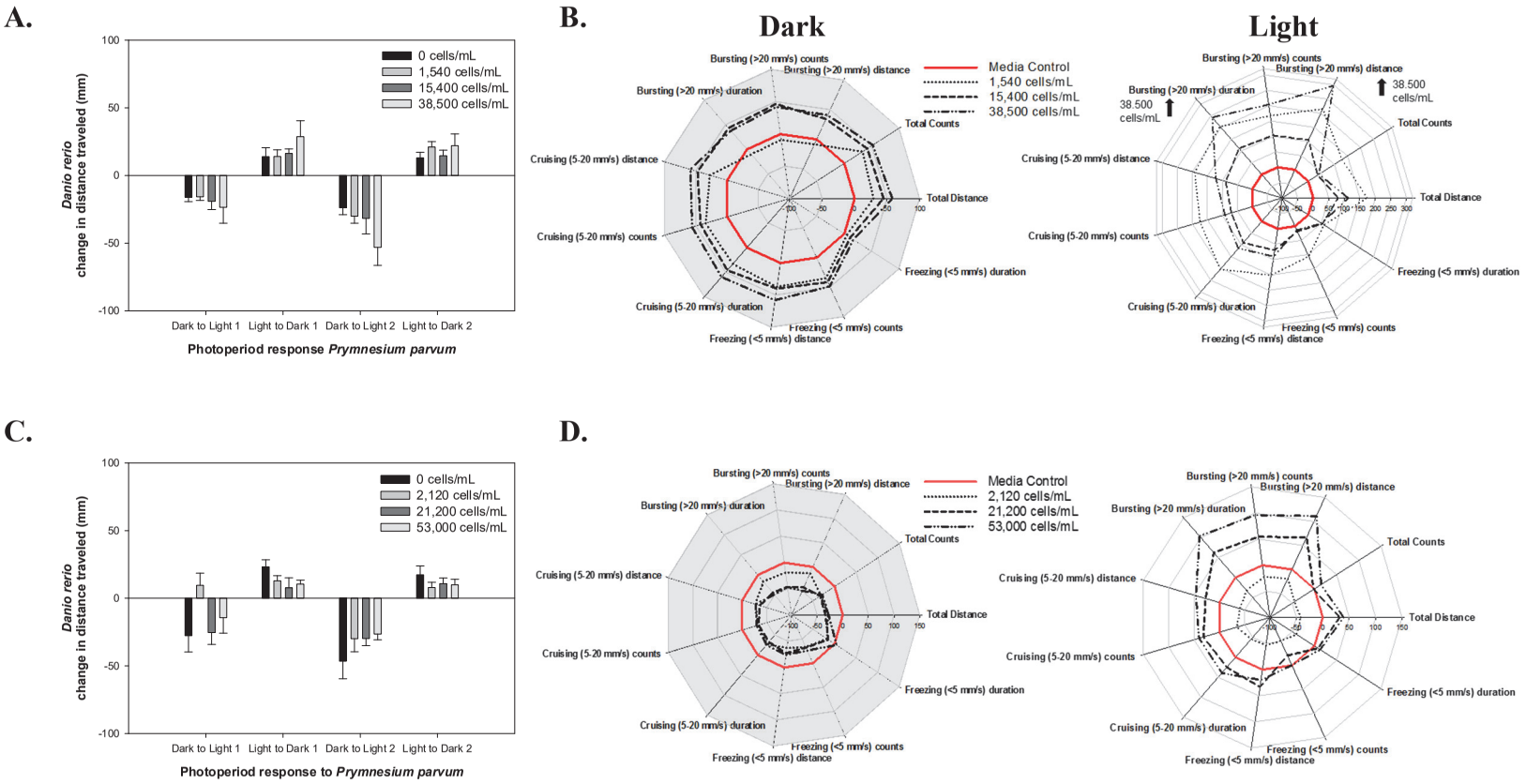


Figure 2.15. Zebrafish (*D. rerio*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 2.4 ppt under nutrient sufficient (f/2) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=4, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

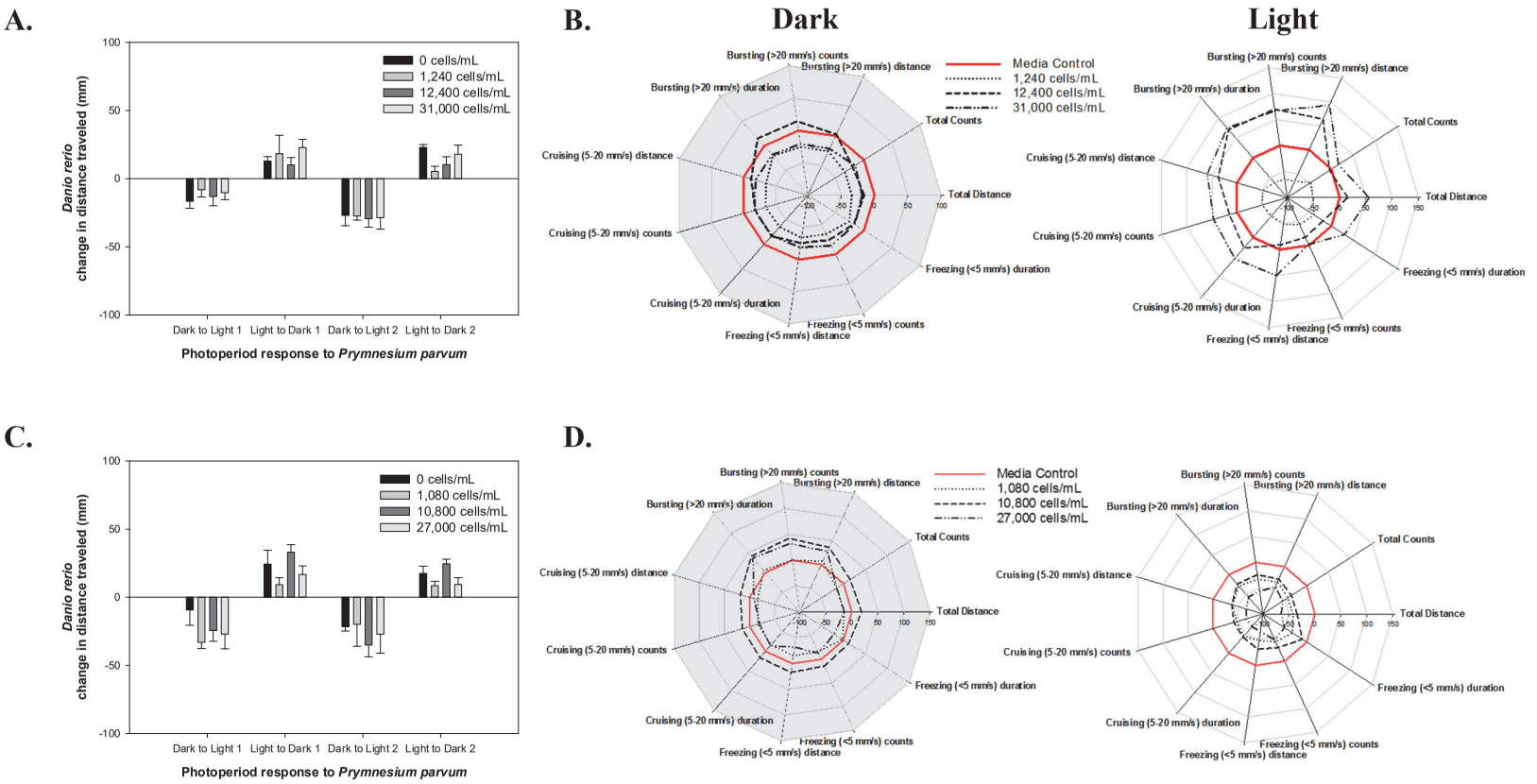


Figure 2.16. Zebrafish (*D. rerio*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 2.4 ppt under nutrient deficient (f/8) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=4, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

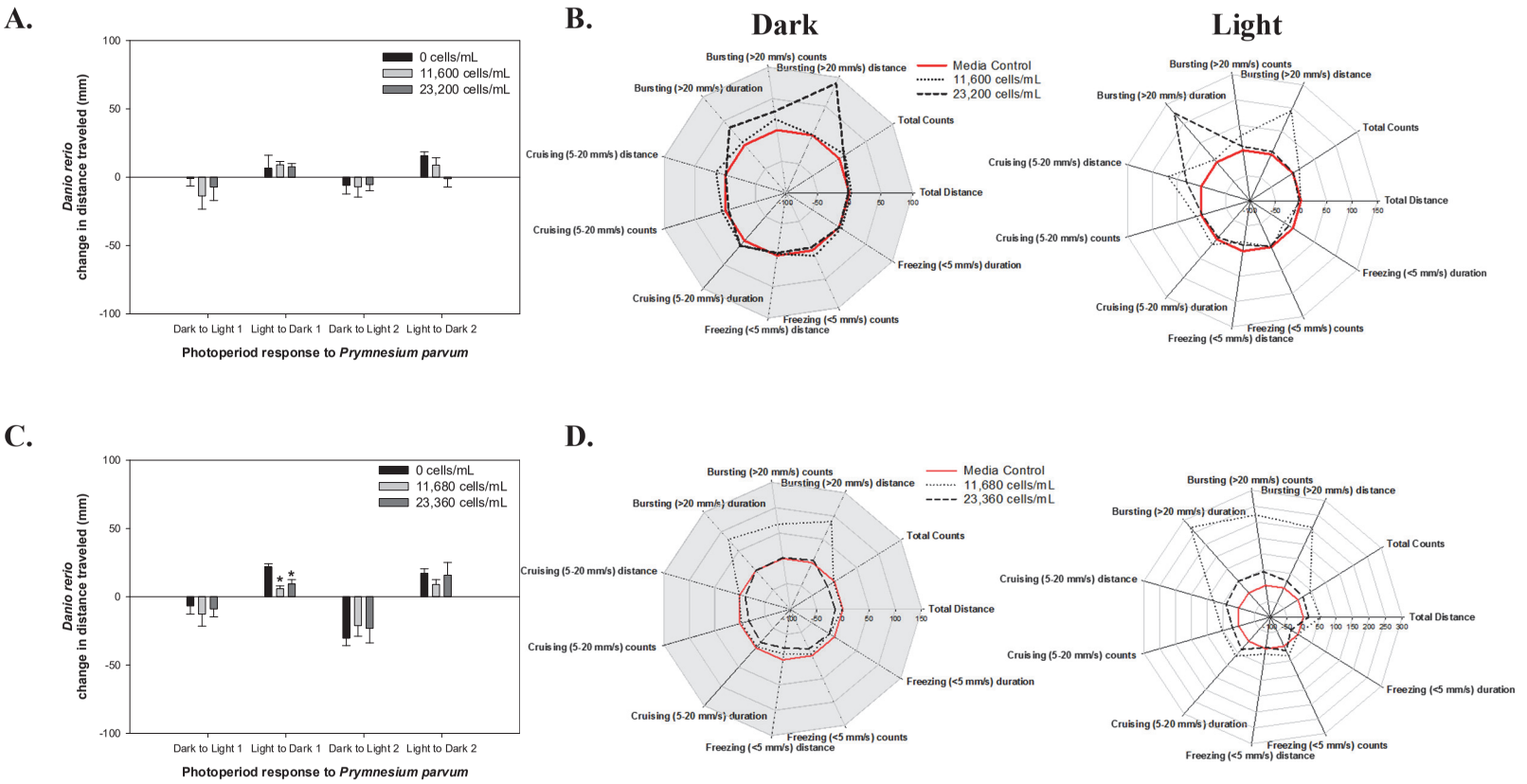


Figure 2.17. Zebrafish (*D. rerio*) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to *P. parvum* grown at 5 ppt under nutrient sufficient (f/2) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=4, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

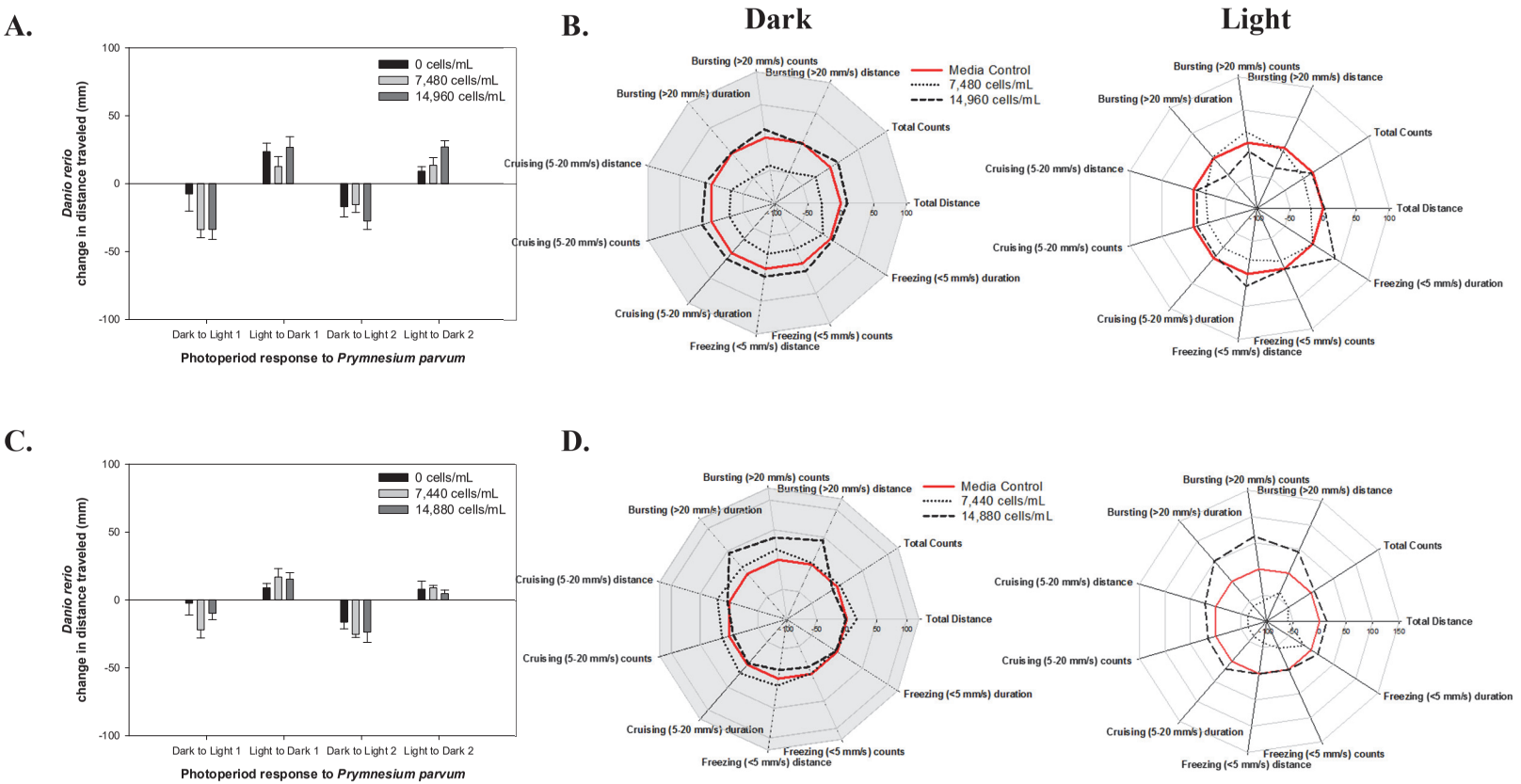


Figure 2.18. Zebrafish (D. rerio) photomotor swimming responses (A, C) following a sudden change in light condition (Light to Dark and Dark to Light) and swimming activity (B,D) during light and dark conditions after 48 h exposure to P. parvum grown at 5 ppt under nutrient deficient (f/8) conditions. Swimming data depicts distance swam, total number of movements (counts), and duration of each movement across 3 speed levels: freezing (<5 mm/sec), cruising (5-20 mm/sec), and bursting (>20 mm/sec). Data were normalized to control, representing 0 on the axis. One-way ANOVA followed by Dunnett's post hoc test. * and arrows = statistically significant (N=4, $p \leq 0.05$) increased (↑) or decreased (↓) behavioral response.

Discussion

P. parvum is a mixotrophic and euryhaline alga capable of forming harmful algal blooms (HABs) enabling for the invasion of Texas inland waters and other regions of the U.S. *P. parvum* HABs are characterized by massive mortality events that primarily target gill breathing organisms and are predicted to increase due to climate change, watershed modifications and urbanization. Nation and international scale monitoring networks and databases are relatively nonexistent for *P. parvum* identifying concern due to the projected increase and expansion in HABs. Establishment of standardized monitoring methods is necessary to properly assess and predict *P. parvum* blooms, especially since this species is expanding. In the present study, a novel approach was taken to understand sublethal *P. parvum* toxicity by examining multiple molecular, biochemical and behavioral responses. The influence of nutrients and salinity were investigated to understand these effects at conditions representative of Texas inland HABs. To our knowledge, this is the first study to report induced oxidative stress and behavioral alterations in two common fish models after exposure to sublethal cell densities of *P. parvum*. I found that these responses were heightened under suboptimal growth conditions, which is consistent with previously reported acute mortality.

The growth rates observed in the present study indicate that *P. parvum* growth was not optimal for any of the experimental conditions and are comparable with those of wintertime, low salinity Texas HABs (Baker et al., 2007; Baker et al., 2009). Suboptimal growth conditions such as low salinity, temperature, and nutrients have been studied and suggested as indicators of *P. parvum* bloom formation and acute toxicity (Brooks et al., 2011; Roelke et al., 2015). However, previous laboratory studies have seldom compared

multiple salinity and nutrient conditions concurrently (Brooks et al., 2010). A double salinity threshold has been established for *P. parvum* in which growth is able to occur between 1-25 ppt that causes enough stress to produce toxins supporting *P. parvum* bloom development (Roelke et al., 2015). A relationship between temperature and low salinity conditions exist in which the optimal temperature for growth decreases with decreasing salinity (Baker et al., 2009). In the current study, *P. parvum* growth (maximal cell density and growth rate) was greatest under the high salinity condition chosen. Slight increases in salinity have been shown to increase *P. parvum* blooms in South Central U.S.A. (Hambright et al., 2014; Roelke et al., 2011), which are consistent with the present study. Conversely, sublethal toxicity in the present study was greatest under lower salinity conditions consistent with conditions observed for documented Texas HABs. In addition, I observed lower maximal cell densities and growth rates and an increase in acute fish mortality and sublethal responses in cultures grown under nutrient limitation regardless of salinity. In fact, the growth treatment factor that significantly influenced responses observed most frequently was nutrient limitation (Table 2.9-11). Nutrient availability is a major environmental parameter frequently observed to influence harmful plankton succession (Heisler et al., 2008; Paerl and Scott, 2010).

Associations among water quality parameters have been established for *P. parvum* in Texas (Israël et al., 2014; Patiño et al., 2014; VanLandeghem et al., 2014). However, with anticipated human population growth and climate change alterations in precipitation and evaporation will occur in arid to semi-arid regions and ultimately influence in flow events, which have been shown to alleviate *P. parvum* HABs (Roelke et al, 2012; Roelke et al., 2015). In addition, nutrient loadings from point (effluent) and

nonpoint (urban storm water and agriculture runoff) sources may increase with watershed modification (Brooks et al., 2011). In the present study, significant main and interactive effects between salinity and nutrients were observed for OS biochemical responses for both species (Table 2.9). *P. parvum* is mixotrophic and can therefore assimilate nutrients from multiple sources. For example, additions of tertiary sewage effluent stimulated growth and substantially increased cellular nitrogen content of laboratory grown *P. parvum* cultures confirming that this species can assimilate nutrients from multiple inorganic and organic sources (Lindehoff et al., 2009). Autotrophy and heterotrophy appear to be permanent nutritional adaptations; however, the degree of contribution likely varies among growth nutrient condition and presence/absence of prey. Laboratory grown cultures resulted in higher organic nitrogen content than those grown under nutrient sufficiency suggesting higher phagotrophic activity that is believed to be enabled by toxin production (Carvalho and Granéli, 2010).

Highest concentrations of prymnesins have been reported throughout late exponential and stationary growth for cultures grown under similar conditions as the present study (La Claire et al., 2015). As mentioned previously, no analytical standards exist for prymnesin I and II. Therefore, acute bioassays were employed to determine the presence of toxins. Low salinity and nutrient sufficient conditions became nontoxic during late stationary phase suggesting that prymnesins were not the primary toxins responsible for the acute fish mortality observed (Figure 2.2, Table 2.4). In fact, fathead minnow mortality decreased per study day with the highest toxicity per cell observed during early exponential phase for all cultures (Figure 2.2). Whether prymnesins

contributed to the observed mortality and sublethal toxicity is unknown and requires further investigation.

Multiple toxins, in addition to prymnesins have been suggested including fatty acids, fatty acid amides, and other golden algae toxins (GATs) with much discrepancy among these results (Schug et al., 2010; Henrikson et al.,2010; Bertin et al., 2012a; Bertin et al., 2012b; Blossom et al, 2014). Different *P. parvum* culturing conditions were chosen and different toxicological bioassays were utilized to confirm toxicity/presence of toxins. Growth and toxicological endpoints have been shown to be influenced by *P. parvum* growth condition. In addition, comparative toxicity work has confirmed differential responses of routine model organisms selected for these assays (Brooks et al., 2010). A plausible explanation for the discrepancy among proposed toxins could be influenced by different toxins and/or the magnitude of these toxins produced under different growth conditions, during different growth phases, observed toxicity, and/or a combination of these factors. Future investigations are required to better understand mechanisms contributing to toxin production and resulting toxicity.

Fathead minnow and zebrafish were found to exhibit differential acute toxicity. Fathead minnow mortality was greatest under nutrient limited conditions for both salinities which is consistent with previously reported results (Baker et al., 2009a; Errera et al., 2008; Graneli, 2003; Valenti et al, 2010). *P. parvum* grown at 2.4 ppt under the same nutrient limited conditions (f/8) resulted in a mean LC_{50} value of 21,800 cells/mL for larval fathead minnow during stationary growth phase (Brooks et al., 2010) similar to one carboy grown under 2.4 ppt and nutrient deficient conditions in the present study. Fish mortality in response to *P. parvum* grown under 5 ppt conditions have not been

previously reported (CH 1, Table 1.1). Mortality of 10-14 day old fathead minnow following exposure to *P. parvum* grown at 6 ppt resulted in a mean LC_{50} value of 51,560 cells/mL during stationary growth (Remmel and Hambright, 2012). Although this suggests that increasing salinity conditions decreases fish morality responses, increased larval fathead minnow mortality in response to increased *P. parvum* salinity growth conditions have been observed previously by this laboratory (Prosser et al., unpublished). As stressed in Brooks et al. (2010) the development and application of standardized growth and toxicity procedures are required in order to adequately compare reported toxicity. Embryonic zebrafish assays were employed to determine *P. parvum* toxicity across urban and rural landscapes (VanLandeghem et al. 2012). The authors noted similar mortality responses between zebrafish and fathead minnow, contrary to the observed mortality responses. In the present study, fathead minnow mortality was more sensitive to *P. parvum* cultures than zebrafish (Table 2.4, 2.5).

Fathead minnow were more sensitive to *P. parvum* exposure than zebrafish (consistent with CH 1), which exhibited biphasic toxicity. Valenti et al (2010) predicted various physiochemical properties of prymnesin toxins, which indicate that bioavailability influences of pH to these toxins are similar to bases. Although study solutions were manipulated to pH 8.5, physiochemical properties of toxin metabolites not related to pH such as salinity may explain the biphasic toxicity observed for zebrafish. Additions of co-factors, especially mono- and divalent cations led to increased toxicity of *P. parvum*, which led to an earlier conclusion that an activation of these toxins by cofactors is required (Ulitzer and Shilo, 1964). Acute mortality was greatest under high salinity conditions which could be explained by more available cations (Na^+) in the

media. Clearly this area deserves additional study. Model organism sensitivity also varied and followed similar trends for the sublethal responses chosen.

Although the Texas strain was the focus of the present study, *P. parvum* HABs occur globally. Regulatory and international standardized methods for fathead minnow and zebrafish were chosen to maximize comparisons of study findings. The initiation of these standardized methods occurred at early life stages at which susceptibility to contaminants is believed to be particularly sensitive and involved initiating studies with each species at different development stages. Although the influence of development on toxicity was outside the scope of this study, increased uptake and metabolism along development have been demonstrated for multiple fish species exposed to environmental contaminants including algal toxins (Kristofco et al., 2018; Otte et al., 2010; Wiegand et al., 1999). Zebrafish have been used as a model organism to determine adverse effects of algal toxins; however, to my knowledge, this is the first study to employ larval zebrafish in an acute or sublethal *P. parvum* toxicological experiment. Zebrafish are a global toxicological fish model that contains similar copies of human genes facilitating read across extrapolations (Gunnarson et al., 2008; Howe et al., 2013; Rand-Weaver et al., 2013).

Oxidative stress (OS) is a common component in any substantial stress that results in an imbalance between reactive oxygen species (ROS) and antioxidant capabilities to detoxify these molecules. Since exposure to increased ROS may fluctuate, organisms are able to respond to this stress by inducing antioxidant related enzymes. The Keap1-Nrf2 signaling pathway is important in regulating antioxidant enzymes in response to xenobiotics that is conserved in vertebrate systems including the fathead minnow and

zebrafish (Kaspar et al., 2009; Lushchak, 2011; Nguyen et al., 2009). Nrf2 is a leucine zipper transcription factor that under normal physiological conditions is complexed with the repressor protein Keap1 (Kelch-like ECH associating protein 1) in the cytosol. Cell exposure to oxidants changes the confirmation of Keap1, leading to the release of Nrf2, which migrates to the nucleus and binds to the antioxidant response element (ARE) activating gene transcription (Lushchak, 2011). The present study evaluated the expression changes of four genes after exposure to *P. parvum*: nuclear factor erythroid 2–like 2 (*nrf2*), glutamate cysteine ligase catalytic subunit (*gclc*), glutathione-s-transferase (*gst*) and superoxide dismutase (*sod*). The specific gene isoforms for zebrafish were *nrf2a*, *gstp1*, and sod1. To my knowledge, this is the first study to investigate relative gene expression changes to *gclc*, *gst*, and *nrf2* after exposure to *P. parvum* toxins. *Gclc*, *gst*, *nrf2* and *sod* expression changes have been reported for mammalian (mice) and fish following other algal exposures, primarily cyanobacteria (Gonçalves-Soares et al., 2012; Jos et al., 2005; Qiu et al., 2007; Wang et al., 2006).

Relative gene expression was widely induced across both species, highlighting the sensitivity of these antioxidants in response to OS elicited by algal toxins. For fathead minnow, *nrf2* expression was the most sensitive antioxidant gene selected. This is not surprising due to the major role that the Nrf2-Keap1 pathway plays in OS defense as mentioned previously. The second most sensitive antioxidant gene was *gclc* for both species. Glutamate cysteine ligase (*gcl*) consists of a catalytic (*gclc*) and a light or modifier subunit (*gclm*) that is involved in the first step of GSH synthesis. *Gclc* is the rate limiting step for GSH synthesis thus expression is upregulated when increased cellular defenses are needed but if insults persist may become dysregulated (Lu, 2013). Another

GSH related enzyme, glutahtione-*s*-transferase (*gst*), specifically the cytosolic family, was also selected for investigation and exhibited induction in zebrafish. *Gst* facilitates the conjugation between GSH and a reactive molecule increasing hydrophilicity for excretion. Induction of *gst* expression has occurred in response to OS induced by environmental contaminants and algal toxins (Limon-Pacheco and Consebatt, 2009). Superoxide dismutase (*sod*) is responsible for the partitioning of superoxide radicals into hydrogen peroxide and molecular oxygen that is often induced in aquatic organisms as a result of OS (Di Giulio et al., 1989). No significant responses were observed for *sod* in both species, which is supported by *in vitro* investigations of *P. parvum* exposure to gill cells (Dorantes-Aranda et al., 2015). Changes in gene expression were more statistically significant for zebrafish when compared to fathead minnow responses, especially under conditions at high salinity. These responses suggest a successful antioxidant protection due to lack of altered biochemical markers.

The implications of OS include damage to tissues, inflammation, carcinogenesis and neurodegenerative diseases in humans and wildlife (Kensler et al., 2007; Scandalios, 2005). OS is somewhat paradoxical due to the requirement of oxygen for life; however, organisms have adapted adequate antioxidant defenses to combat the generation of ROS. The main targets of ROS include proteins, lipids, and nucleic acids therefore the alterations of glutathione concentrations, lipid peroxidation, and DNA damage have been well studied biomarkers of OS. Environmentally induced OS and the utility of biomarkers in aquatic organisms have been reviewed (Di Giulio et al., 1989; Limon-Pacheco and Consebatt, 2009; Lushchak, 2011; Valavanidis et al., 2006).

In the present study, all fish OS biochemical markers responded similarly, with neither more sensitive than the others. Glutathione depletion, increased lipid peroxidation and DNA damage have all been associated with OS and were observed in both species indicating *P. parvum* induced OS. Similar to previous sublethal toxicity studies (CH. 1), DNA damage was highest under different growth conditions that elicited glutathione and lipid peroxidation responses, further suggesting that different secondary toxin metabolites are produced under varying growth conditions (refer to CH 1). Interestingly, lipid peroxidation was decreased in zebrafish, indicating an antioxidant response rather than an OS elicited effect. A possible explanation for this decline is metabolism of MDA and detoxification as supported by an induction of *nrf2a* observed after exposure to the same culture (Ayala et al., 2014). Lipid peroxidation was induced within 12 h of exposure to microcystin-LR but decreased to control levels after 24 h in zebrafish brain (Zhang et al., 2013). This recovery, although incomplete due to induction of antioxidant related genes at 24 h, suggests that these responses occur rapidly. A similar increase and decrease in lipid peroxidation corresponding with an induction of an antioxidant enzyme was observed in mice (Gehringer et al., 2004). *P. parvum* toxins elicit high lethality to fish due to the rapid mortality observed in a short amount of time and exposures to an invertebrate resulted in decreased toxicity overtime indicating potential loss of bioavailable toxins (Tillmann, 2003). The sublethal endpoints were only evaluated at 48 h post exposure, which may only partially represent the antioxidant response. Investigation of the selected biochemical endpoints at smaller durations of exposure are needed to understand the full scope of biochemical mechanisms following *P. parvum* exposure. In addition, linking these responses to higher levels of biological organization will

contribute to the development of AOPs and facilitate risk assessment of *P. parvum* HABs.

Behavior represents an organism's adaptable response to internal (physiological) and external (social) factors and is essential for survival (Gerhardt, 2007). Therefore, alterations to behavior after exposure to algal toxins may result in negative impacts on individual survival. Behavioral responses to other environmental contaminants including algal toxins have been observed at concentrations magnitudes below which would elicit effects routinely examined for acute toxicity studies (Lasley-Rasher et al., 2016; Lefebvre et al., 2004; Steele et al., In press: Steele et al., Accepted; Valenti et al., 2012; Zhang et al., 2013). Behavioral alterations have been revealed for algal toxins including stimulatory and refractory behavior in response to photoperiod and stimuli. Zebrafish exposed to a low dose of microcystin-LR exhibited stimulatory responses while behavior activity decreased in response to a high dose in light conditions (Baganz et al., 1998). By contrast, zebrafish locomotor activity and acetylcholinesterase activity decreased as a result of low and high doses of aphantoxins (Zhang et. al, 2013). In the present study, behavioral alterations were observed at cell densities below those inducing acute mortality and were one of the most sensitive endpoints selected (Figures 2.5-12). A current limitation of behavioral ecotoxicology is the application of laboratory observations to field applications. However, behavioral observations including predator avoidance, food capture, and social behavior (courting and shoaling/schooling) have been used as indicators of exposure and monitoring strategies (Chew et al., 2009; Kuklina et al., 2013). Recent advances in computational and tracking technologies allow for high throughput screening (HTS) of fish models, primarily during early life stages. Fish

behavioral syndromes and phenotypes of a wide array of chemicals have been studied in order to associate specific behavioral alterations with chemical mode/mechanism of action (Drummond and Russom, 1990; Rihel et al., 2010). As mentioned previously, behavioral observations have been demonstrated for algal toxins, however specific behavioral phenotypes have not been associated with mechanisms of action, and comparisons among classes of algal toxins are lacking.

Swimming activity was significantly reduced in fathead minnow across the freezing and cruising speed thresholds indicating refractory responses after exposure to *P. parvum* grown under all experimental conditions. These behavioral responses observed in the present study differ from those observed in our previous studies (refer to CH 1). Algal growth was reduced and acute mortality was nonexistent in the previous study, suggesting that different toxins may have been produced at varying growth phases. Fish behavioral responses may not only represent *P. parvum* toxin exposure but also indicate differences in the amounts or types of toxins produced at specific growth phases. Zebrafish behavior exhibited differential responses to varying doses of microcystin with decreased behavior and spawning at higher toxin concentrations and some stimulatory responses at lower doses (Baganz et al., 2004; Baganz et al., 1998). The consistent behavioral patterns observed across both species demonstrate the utility of using behavioral assays to indicate exposure to *P. parvum* HABs. Further investigation is needed to understand if these responses may result in population level impacts, and whether such effects would occur in natural fish populations.

Some consistencies were present among the sublethal endpoints. The same *P. parvum* culture (low salinity x deficient nutrient) elicited total glutathione depletion and

an increase in lipid peroxidation in conjunction with an increase in *nrf2a* expression in fathead minnow. Decreased swimming behavior was observed for fathead minnows exposed to the same culture conditions (low salinity x deficient nutrient) suggesting that sublethal toxicity increases with increasing suboptimal growth conditions. A similar relationship has been established with acute fish mortality further proposing that sublethal endpoints are an adequate endpoint to determine *P. parvum* exposure and resulting toxicity. Fathead minnow morality was observed throughout the growth of low salinity nutrient deficient conditions, although this toxicity was reduced on a per cell basis compared to cultures grown under higher salinity. Higher salinity induced gene expression of *gclc* and *nrf2* in fathead minnow and resulted in behavioral alterations. In the present study, relative gene expression was a more sensitive endpoint in relation to the selected biochemical endpoints. For example, induction of *nrf2* was observed after exposure to *P. parvum* grown at multiple salinities and nutrient conditions whereas significant glutathione depletion and increased lipid peroxidation were only observed following exposure to low salinity and low nutrient treatment in fathead minnow (Figures 2.4-6 & 2.8-9). Similarly, more significant relative gene expression (*nrf2a, gclc*) was observed in zebrafish compared to biochemical endpoints (glutathione and lipid peroxidation). The greater gene induction and behavioral responses may suggest different toxin production from that inducing fish morality or produced to a different degree.

As mentioned previously, an induction of *nrf2a* occurred in zebrafish in conjunction with a decrease in MDA concentration. Similar to the fathead minnow, stimulatory response trends occurred in conjunction with molecular and biochemical markers. These observations occurred after exposure to growth conditions that were more

optimal (higher salinity, higher nutrients) thus *P. parvum* toxins may have been produced to a lesser extent. Zebrafish mortality was only observed at higher salinities with greater mortality under nutrient deficient conditions (Table 2.5). Although significant molecular and biochemical responses were primarily observed for fish exposed to nutrient sufficient conditions, toxins produced in response to salinity changes may have contributed to both the mortality and sublethal response observed. More research is therefore warranted to understand the influence of OS in *P. parvum* toxicity in addition to the mechanisms behind stress tolerance and toxin production.

Variations in sublethal responses occurred between culture replicates warranting future investigations of sublethal and/or chronic longer-term responses during exposure to *P. parvum* HAB events. Focusing on influences across environmental gradients including temperature, salinity, and varying N:P will further aid in this understanding. Isolation and elucidation of toxin profile will facilitate not only linking specific toxicological responses to specific toxins but how concentrations of these vary with growth phase. Other algal toxins have shown varying time and dose-response OS relationships. Whether a similar response pattern exists for *P. parvum* toxins is currently unknown and may explain the variations between culture replicates. The difference in mortality and sublethal responses suggest that the identified prymnesins may not be the most important toxins contributing to fish mortality during late stationary growth phase. The rapid expansion of *P. parvum* throughout the U.S. indicates a strong need for well-established monitoring networks and a proactive approach to bloom management due to the sublethal responses observed in the present study occurring below induced mortality.

Conclusions

Historical HABs of *P. parvum* elicit profound fish kills, and thus fish mortality has been most commonly studied. My novel observations indicate sensitive sublethal responses to *P. parvun* under conditions associated with HABs, and further suggest that these sublethal endpoints represent important considerations for the assessment and management of *P. parvum*, especially due to the predicted increase and expansion of these HABs. To my knowledge this is the first study to combine gene expression, biochemical and behavioral changes associated with OS following *P. parvum* exposure with two common toxicological fish models. Traditional biochemical markers indicated a contribution of OS to *P. parvum* toxicity. Induction of antioxidant gene expression confirmed this contribution and indicated that organisms can potentially combat the algal toxin induced OS. Behavior alterations were a sensitive indicator of *P. parvum* exposure and were associated with cellular and molecular responses. Two experimental factors (salinity and nutrients) associated with HAB events influenced both *P. parvum* growth rates and sublethal fish responses; however, future investigations of such relationships are needed across environmental gradients. More research is also needed to understand the mechanistic underpinning of these responses, and the identification and production of *P. parvum* toxins. In addition, read across approaches from fish to mammals is unknown for *P. parvum* and deserves attention due to heightened antioxidant gene expression elicited in relation to the conserved antioxidant pathway in all vertebrate systems.

APPENDICES

APPENDIX A

Media Recipe and Measured Nutrients for Chapter One

Table A1. Media stock recipes for culturing *P. parvum* experiments. Stocks were added at a concentration of 1 mL per 1L of artificial seawater. All stocks were autoclaved and cooled prior to use. Vitamin stock was filtered sterilized using a 45 μm pore filter. Recipes were modified from standardized culture methods (Guillard, 1975).

Stock Solution	Nutrient Condition	Reagent	Amount	Nanopure Volume (mL)
Nutrients	f/2	$NaNO_3$	7.50 g	100
		NaH_2PO_4 H_2O	0.50 g	100
	f/8	$NaNO_3$	1.88 g	100
		NaH_2PO_4 H_2O	0.13 g	100
Trace Metals	f/2, f/8	Na_2 EDTA	2.18 g	500
		$FeCl_3$ $6H_2O$	1.58 g	
		$CuSO_4$ $7H_2O$	3.4 mg	
		$ZnSO_4$ $7H_2O$	11.5 mg	
		$CoCl_2$ $4H_2O$	7.1 mg	
		$MnCl_2$ $2H_2O$	76.0 mg	
		Na_2MoO_4 $2H_2O$	3.70 mg	
Vitamins	f/2, f/8	Thiamin HCl	100 mg	500
		Biotin	0.53 mg	
		B_{12}	0.28 mg	

Table A2. Analytically measured dissolved nitrogen (nitrate/nitrite) and phosphorus (phosphate) concentrations of nutrient stock solutions for Chapter 1 experiment. Samples were collected at time of inculcation.

Culture Condition	Dissolved Nitrogen (µg/L)	Dissolved Phosphorus (µg/L)
2.4 ppt f/2 medium	1450	119
2.4 ppt f/8 medium	332	27.7

APPENDIX B

Measured Nutrients for Chapter Two

Table B1. Analytically measured dissolved nitrogen (nitrate/nitrite) and phosphorus (phosphate) concentrations of experimental carboys for Chapter Two experiment. Samples were collected at time of inculcation (day 0), during exponential growth (day 34), and stationary growth phase (day 60 for 2.4 ppt or 67 for 5 ppt cultures).

		Dissolved Nitrogen (µg/L)			Dissolved Phosphorus (µg/L)		
Culture condition	Replicate	Study Day 0	34	60/67	0	34	60/67
2.4 ppt, high (f/2) nutrients	1	8550	2060	5920	292	268	523
	2	7710	1480	4680	1640	154	564
2.4 ppt, low (f/8) nutrients	1	2590	1190	1290	82.5	96.1	108
	2	1670	3880	960	189	370	63.5
5 ppt, high (f/2) nutrients	1	3590	1630	9260	390	147	308
	2	1530	6970	9350	165	132	322
5 ppt, low (f/8) nutrients	1	6170	3090	2565	584	502	18.1
	2	6430	4480	5270	534	181	75.5

BIBLIOGRAPHY

Amado, L.L., Monserrat, J.M., 2010. Oxidative stress generation by microcystins in aquatic animals: Why and how. Environment International 36(2), 226-235.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environmental Toxicology and Chemistry 29(3), 730-741.

Ayala, A., Munoz, M., Arguelles, S., 2014b. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. Oxidative Medicine and Cellular Longevity 2014, 1-31.

Baganz, D., Staaks, G., Pflugmacher, S., Steinberg, C.E.W., 2004. Comparative study of microcystin-LR-induced behavioral changes of two fish species, *Danio rerio* and *Leucaspius delineatus*. Environmental Toxicology 19(6), 564-570.

Baganz, D., Staaks, G., Steinberg, C., 1998. Impact of the cyanobacteria toxin, microcytin-LR on behaviour of zebrafish, *Danio rerio*. Water Research 32(3), 948-952.

Baker, J.W., Grover, J.P., Brooks, B.W., Urena-Boeck, F., Roelke, D.L., Errera, R., Kiesling, R.L., 2007. Growth and toxicity of *Prymnesium parvum* (haptophyta) as a function of salinity, light, and temperature. Journal of Phycology 43.

Baker, J.W., Grover, J.P., Ramachandrannair, R., Black, C., Valenti, T.W., Brooks, B.W., Roelkec, D.L., 2009. Growth at the edge of the niche: An experimental study of the harmful alga *Prymnesium parvum*. Limnology and Oceanography 54(5), 1679-1687.

Beker van Woudenberg, A., Wolterbeek, A., Te Brake, L., Snel, C., Menke, A., Rubingh, C., de Groot, D., Kroese, D., 2013. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: the value of the zebrafish (*Danio rerio*) embryotoxicity test (ZET). Reprod Toxicol 41, 35-44.

Bergmann, F., Parnas, I., Reich, K., 1964. The action of the toxin *Prymnesium parvum* Carter on the guinea-pig ileum. British Journal of Pharmacology 22, 47-55.

Bertin, M., Voronca, D., Chapman, R., Moeller, P., 2014. The effect of pH on the toxicity of fatty acids and faty acid amides to rainbow trout gill cells. Aquatic Toxicology 146, 1-11.

Bertin, M., Zimba, P., Beauchesne, K., Huncik, K., Moeller, P., 2012a. The contribution of fatty acid amindes to *Prymnesium parvum* Carter toxicity. Harmful Algae 20, 117-125.

Bertin, M.J., Zimba, P.V., Beauchesne, K.R., Huncik, K.M., Moeller, P.D.R., 2012b. Identification of toxic fatty acid amides isolated from the harmful alga *Prymnesium parvum* carter. Harmful Algae 20, 111-116.

Birnie-Gauvin, K., Costantini, D., Cooke, S.J., Willmore, W.G., 2017. A comparative and evolutionary approach to oxidative stress in fish: A review. Fish and Fisheries 18, 928-942.

Blossom, H.E., Anderson, N.G., Rasmussen, S.A., Hansen, P.J., 2014a. Stability of the intra- and extracellular toxins of *Prymnesium parvum* using a microalgal bioassay. Harmful Algae 32, 11-21.

Blossom, H.E., Rasmussen, S.A., Anderson, N.G., Larson, T.O., Nielsen, K.F., Hansen, P.J., 2014b. *Prymnesium parvum* revisited: Relationship between allelopathy, ichthyotoxicity, and chemical profiles in 5 strains. Aquatic Toxicology 157, 159-166.

Bradbury, S.P., Feijtel, T.C.J., Van Leeuwen, C., J., Meeting the Scientific Needs of Ecological Risk Assessment in a Regulatory Context. Environmental science & technology 38(23), 463A-470A.

Brooks, B.W., Grover, J.P., Roelke, D.L., 2011. *Prymnesium parvum*: An emerging threat to inland waters. Environmental Toxicology and Chemistry 30(9), 1955-1964.

Brooks, B.W., James, S.V., Valenti Jr., T.W., Urena-Boeck, F., Serrano, C., Berninger, J.P., Schwierzke, L., Mydlarz, L.D., Grover, J.P., Roelke, D.L., 2010. Comparative Toxicity of *Prymnesium parvum* in Inland Waters. Journal of the American Water Resources Association 46(1), 45-62

Brooks, B.W., Lazorchak, J.M., Howard, M.D.A., Johnson, M.V.V., Morton, S.L., Perkins, D.A.K., Reavie, E.D., Scott, G.I., Smith, S.A., Steevens, J.A., 2016. Are Harmful Algal Blooms Becoming the Greatest Inland Water Quality Threat to Public Health and Aquatic Ecosystems? Environmental Toxicology and Chemistry 35(1), 6-13.

Carvalho, W., Granéli, E., 2010. Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency.

Chew, F., Eng, H.-L., Thida, M., 2009. Vision-based Real-time Monitoring on the Behavior of Fish School.

Corrales, J., Kristofco, L.A., Steele, W.B., Saari, G.N., Kostal, J., Williams, E.S., Mills, M., Gallagher, E.P., Kavanagh, T.J., Simcox, N., Shen, L.Q., Melnikov, F., Zimmerman, J.B., Voutchkova-Kostal, A.M., Anastas, P.T., Brooks, B.W., 2018. Toward the design of less hazardous chemicals: exploring comparative oxidative stress in two common animal models. Chemical Research in Toxicology 30, 893-904.

Davison, I.R., 1991. Environmental effects on algal photosynthesis: temperature. Journal of Phycology 27, 2-8.

Dorantes-Aranda, J., Seger, A., Mardones, J., Nichols, P., Hallegraeff, G., 2015. Progress in understanding algal bloom-mediated fish kills: the role of superoxide radicals, phycotoxins, and fatty acids. PLoS One 10(7).

Driscoll, W.W., Espinosa, N.J., Eldakar, O.T., Hackett, J.D., 2013. Allelopathy as an emergent, exploitable public good in the bloom-forming microalga *Prymnesium parvum* Evolution 67(6), 1582-1590.

Drummond, R.A., Russom, C.L., 1990. Behavioral toxicity syndromes: A promising tool for assessing toxicity mechanisms in juvenile fathead minnows. Environmental Toxicology and Chemistry 9(1), 37-46.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behavioral Brain Research 205, 38-44.

Ermak, G., Davies, K.J.A., 2002. Calcium and oxidative stress: from cell signaling to cell death. Molecular Immunology 38(10), 713-721.

Errera, R.M., Roelke, D.L., Kiesling, R.L., Brooks, B.W., Grover, J.P., Schwierzke, L., Urena-Boeck, F., Baker, J.W., Pinckney, J.L., 2008. Effect of imbalanced nutrients and immigration on *Prymnesium parvum* community dominance and toxicity: results from in-lake micrososm experiments. Aquatic Microbial Ecology 52.

Evans, M.D., Dizdaroglu, M., Cooke, M.S., 2004. Oxidative DNA damage and disease: induction, repair and significance. Mutation Research 567, 1-61.

Freitag, M., Beszteri, S., Vogel, H., John, U., 2011. Effects of physiological shock treatments on toxicity and polyketide synthase gene expression in *Prymnesium parvum* (Prymnesiophyceae). European Journal of Phycology 46(3), 193-201.

Gehringer, M., Shepard, E., Downing, T., Wiegand, C., Neilan, B., 2004. An investigation into the detoxification of microcysin-LR by the glutathione pathway in Balb/c mice. The International Journal of Biochemistry & Cell Biology 36, 931-941.

Gerhardt, A., 2007. Aquatic Behavioral Ecotoxicology—Prospects and Limitations. Human and Ecological Risk Assessment: An International Journal 13(3), 481-491.

Giulio, R.T.D., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: A review of determinants of oxidative stress. Environmental Toxicology and Chemistry 8(12), 1103-1123.

Guillard, R.L., 1975. Culture of Phytoplankton for Feeding Marine Invertebrates. In: Culture of Marine Invertebrate Animals. W.L.Smith and M.H. Charley (Editors). Plenum Press, New York.pp. 29-60.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. Environmental Science & Technology 42, 5807-5813.

Gonçalves-Soares, D., Zanette, J., Yunes, J.S., Yepiz-Plascencia, G.M., Bainy, A.C.D., 2012. Expression and activity of glutathione S-transferases and catalase in the shrimp Litopenaeus vannamei inoculated with a toxic Microcystis aeruginosa strain. Marine Environmental Research 75, 54-61.

Granéli, E., Johansson, N., 2003. Effects of the toxic haptophyte *Prymnesium parvum* on the survival and feding of a ciliate: the influence of different nutrient conditions. Marine Ecology Progress Series 254, 49-56.

Granéli, E., Johansson, N., 2003. Effects of the Toxic Haptophyte *Prymnesium parvum* on the Survival and Feeding of a Ciliate: The Influence of Different Nutrient Conditions.

Granéli, E., Weberg, M., Salomon, P.S., 2008. Harmful algal blooms of allelopathic microalgal species: The role of eutrophication. Harmful Algae 8(1), 94-102.

Grattan, L.M., Holobaugh, S., Morris, J.G., 2016. Harmful algal blooms and public health. Harmful Algae 57, 2-8.

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

Hambright, K.D., Easton, J.D., Zamor, R.M., Beyer, J., Easton, A.C., Allison, B., 2014. Regulation of growth and toxicity of a mixotrophic microbe: implications for understanding range expansion in *Prymnesium parvum*. Freshwater Science 33(3).

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8(1), 3-13.

Hellou, J., 2011. Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. Environmental Science and Pollution Research 8, 1-11.

Henrikson, J.C., Gharfeh, M.S., Easton, A.C., Easton, J.D., Glenn, K.L., Shadfan, M., Mooberry, S.L., Hambright, K.D., Cichewicz, R.H., 2010. Reassessing the ichthyotoxin profile of cultured *Prymnesium parvum* (golden algae) and comparing it to samples collected from recent freshwater bloom and fish kill events in North America. Toxicon 55, 1396-1404.

Howe, K., et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496(7446), 498-503.

Igarashi, T., Satake, M., Yasumoto, T., 1996. A Potent Ichthyotoxic and Hemolytic Glycoside Isolated from the Red Tide Alga *Prymnesium parvum*. Journal of the American Chemical Society 118, 479-480.

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

Igarashi, T., Aritake, S., Yasumoto, T., 1999a. Mechanisms underlying the hemolytic and ichthyotoxic activities of maitotoxin. Natural Toxins 7(2), 71-79.

Igarashi, T., Satake, M., Yasumoto, T., 1999b. Structures and Partial Stereochemical Assignments for Prymnesin-1 and Prymnesin-2: Potent Hemolytic and Ichthyotoxic Glycosides Isolated from the Red Tide Alga *Prymnesium parvum*. Journal of the American Chemical Society 121, 8499-8511.

Israël, N.M.D., VanLandeghem, M.M., Denny, S., Ingle, J., Patiño, R., 2014. Golden alga presence and abundance are inversely related to salinity in a high-salinity river ecosystem, Pecos River, USA. Harmful Algae 39, 81-91.

James, S.V., Valenti, T.W., Prosser, K.N., Grover, J.P., Roelke, D.L., Brooks, B.W., 2011. Sunlight amelioration of *Prymnesium parvum* acute toxicity to fish. Journal of Plankton Research 33(2), 265-272.

Jos, Á., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis sp*.) under laboratory conditions. Aquatic Toxicology 72(3), 261-271.

Kasai, H., 1997. Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. Mutation Research 387, 147-163.

Kaspar, J.W., Niture, S.K., Jaiswal, A.K., 2009. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radical Biology and Medicine 47(9), 1304-1309.

Kensler, T.W., Wakabayashi, N., Biswal, S., 2007. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. Annual Review of Pharmacology and Toxicology 47(1), 89-116.

Kohen, R., Nyska, A., 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicological pathology 30(6), 620-650.

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C.Y.J., Mateus, R., Healey, D., Kim, S., Werdich, A.A., Haggarty, S.J., MacRae, C.A., Shoichet, B., Peterson, R.T., 2010. Rapid behavior—based identification of neuroactive small molecules in the zebrafish. Nature chemical biology 6(3), 231-237.

Kokel, D., Peterson, R.T., 2011. Using the Zebrafish Photomotor Response for Psychotropic Drug Screening. Methods in cell biology 105, 517-524.

Kristofco, L.A., Cruz, L.C., Haddad, S.P., Behra, M.L., Chambliss, C.K., Brooks, B.W., 2016. Age matters: Developmental stage of Danio rerio larvae influences photomotor response thresholds to diazinion or diphenhydramine. Aquatic toxicology (Amsterdam, Netherlands) 170, 344-354.

Kristofco, L.A., Haddad, S.P., Chambliss, C.K., Brooks, B.W., 2018. Differential uptake of and sensitivity to diphenhydramine in embryonic and larval zebrafish. Environmental Toxicology and Chemistry 0(0).

Kuklina, I., Kouba, A., Kozák, P., 2013. Real-time monitoring of water quality using fish and crayfish as bio-indicators: a review. Environmental Monitoring and Assessment 185(6), 5043-5053.

La Claire, J.W., Manning, S.R., Talarski, A.E., 2015. Semi-quantitative assay for polyketide prymnesins isolated from *Prymnesium parvum* (Haptophyta) cultures. Toxicon 102, 74-80.

Lakshmana Rao, P., Bhattacharya, R., 1996. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver *in vivo*. Toxicology 114, 29-36.

Lasley-Rasher, R.S., Nagel, K., Angra, A., Yen, J., 2016. Intoxicated copepods: ingesting toxic phytoplankton leads to risky behaviour. Proceedings of the Royal Society B: Biological Sciences 283(1829).

Lefebvre, K.A., Trainer, V.L., Scholz, N.L., 2004. Morphological abnormalities and sensorimotor deficits in larval fish exposed to dissolved saxitoxin. Aquatic Toxicology 66(2), 159-170.

Limon-Pacheco, J., Consebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutation Research 674, 137-147.

Lindehoff, E., Graneli, E., Gilbert, P.M., 2010. Influence of prey and nutritional status on the rate of nitrogen uptake by *Prymnesium parvum* (Haptophyte). Journal of the American Water Resources Association 46(1), 121-132.

Lindehoff, E., Granéli, E., Granéli, W., 2009. Effect of tertiary sewage effluent additions on *Prymnesium parvum* cell toxicity and stable isotope ratios. Harmful Algae 8(2), 247-253.

Lu, S.C., 2013. Glutathione synthesis. Biochimica et biophysica acta 1830(5), 3143-3153.

Lundgren, V.M., Gilbert, P.M., Graneli, E., Vidyarathna, N.K., Fiori, E., Ou, L., Flynn, K.J., Mitra, A., Stoecker, D.K., Hansen, P.J., 2016. Metabolic and physiological changes in *Prymnesium parvum* when grown under, and grazing on prey of, variable nitrogen:phosphorus stoichiometry. Harmful Algae 55, 1-12.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101(1), 13-30.

Manning, S.R., La Claire, J.W., 2010. Prymnesins: Toxic Metabolites of the Golden Alga, *Prymnesium parvum* Carter (Haptophyta). Marine Drugs 8(3), 678.

Mariussen, E., Nelson, G.N., Fonnum, F., 2005. A toxic extract of the marine phytoflagellate *Prymnesium parvum* induces calcium-dependent release of glutamate from rat brain synaptosomes. Journal of Toxicology and Environmental Health Part A 68, 697-679.

McKim, J.M., 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. Journal of the Fisheries Research Board of Canada 34, 1148-1154.

Meldahl, A.S., Fonnum, F., 1995. Th effects of a purified toxic extract of *Prymnesium patelliferum* on transport of ions through the plasma membrane of synaptosomes. Toxicon 33(8), 1071-1086.

Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Functional Ecology 24, 984-996.

Murata, M., 2000. The structure elucidation and biological activities of high molecular weight algal toxins: maitotoxin, prymnesins and zooxanthellatoxins (1993 to 1999). Natural product reports 17(3), 293-314.

Nguyen, T., Nioi, P., Pickett, C.B., 2009. The Nrf2-Antioxidant Response Element Signaling Pathway and Its Activation by Oxidative Stress. The Journal of Biological Chemistry 284(20), 13291-13295.

Noyes, P.D., Haggard, D.E., Gonnerman, G.D., Tanguay, R.L., 2015. Advanced Morphological — Behavioral Test Platform Reveals Neurodevelopmental Defects in Embryonic Zebrafish Exposed to Comprehensive Suite of Halogenated and Organophosphate Flame Retardants. Toxicological Sciences 145(1), 177-195.

OECD, 2013. 'Test No. 236: Fish Embryo Acute Toxicity (FET) Test', OECD Guidelines for Testing of Chemicals Section 2:Effects on Biotic Systems, OECD Publishing, Paris, France

Otte, J.C., Schmidt, A.D., Hollert, H., Braunbeck, T., 2010. Spatio-temporal development of CYP1 activity in early life-stages of zebrafish (*Danio rerio*). Aquatic Toxicology 100(1), 38-50.

Paerl, H.W., Fulton, R.S., Moisander, P.H., Dyble, J., 2001. Harmful Freshwater Algal Blooms, With an Emphasis on Cyanobacteria. The Scientific World Journal 1.

Paerl, H.W., Huisman, J., 2009. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environmental Microbiology Reports 1(1), 27-37.

Paerl, H.W., Scott, J.T., 2010. Throwing Fuel on the Fire: Synergistic Effects of Excessive Nitrogen Inputs and Global Warming on Harmful Algal Blooms. Environmental science & technology 44(20), 7756-7758.

Patiño, R., Dawson, D., VanLandeghem, M.M., 2014. Retrospective analysis of associations between water quality and toxic blooms of golden alga (*Prymnesium parvum*) in Texas reservoirs: Implications for understanding dispersal mechanisms and impacts of climate change. Harmful Algae 33, 1-11.

Qiu, T., Xie, P., Ke, Z., Li, L., Guo, L., 2007. In situ studies on physiological and biochemical responses of four fishes with different trophic levels to toxic cyanobacterial blooms in a large Chinese lake. Toxicon 50(3), 365-376.

Rand-Weaver, M., Margiotta-Casaluci, L., Patel, A., Panter, G.H., Owen, S.F., Sumpter, J.P., 2013. The Read-Across Hypothesis and Environmental Risk Assessment of Pharmaceuticals. Environmental Science & Technology 47(20), 11384-11395.

Rao, P.V.L., Bhattacharya, R., 1996. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver *in vivo*. Toxicology 114(1), 29-36.

Remmel, E.J., Hambright, K.D., 2012. Toxin-assisted micropredation: experimental evidence shows that contract micropredation rather than exotoxicity is the role of Prymnesium toxins. Ecology Letters 15, 126-132.

Rihel, J., Prober, D., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S., Kokel, D., Rubin, L., Peterson, R., Schier, A., 2010a. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327.

Roelke, D.L., Barkoh, A., Brooks, B.W., Grover, J.P., Hambright, K.D., LaClaire, J.W., Moeller, P.D.R., Patino, R., 2015. A chronicle of a killer algal in the west: ecology, assessment, and management of *Prymnesium parvum* blooms. Hydrobiologia 746, 29-50.

Roelke, D.L., Brooks, B.W., Grover, J.P., Gable, G.M., Schwierzke-Wade, L., Hewitt, N.C., 2012. Anticipated human population and climate change effects on algal blooms of a toxic haptophyte in the south-central USA. Canadian Journal of Fisheries and Aquatic Sciences 69, 1389-1404.

Roelke, D.L., Errera, R.M., Kiesling, R., Brooks, B.W., Grover, J.P., Schwierzke, L.S., Urena-Boeck, F., Baker, J., Pinckney, J.L., 2007. Effects of nutrient enrichment on *Prymnesium parvum* population dynamics and toxicity: results from field experiments, Lake Possum Kingdom, USA. Aquatic Microbial Ecology 46, 125-140.

Roelke, D.L., Grover, J.P., Brooks, B.W., Glass, J., Buzan, D., Southard, G.M., Fries, L., Gable, G.M., Schwierzke-Wade, L., Byrd, M., Nelson, J., 2011. A decade of fish-killing *Prymnesium parvum* blooms in Texas: roles of inflow and salinity. Journal of Plankton Research 33(2), 243-253.

Scandalios, J.G., 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Brazilian Journal of Medical and Biological Research 38, 995-1014.

Schug, K.A., Skingel, T.R., Spencer, S.E., Serrano, C.A., Le, C.Q., Schug, C.A., Valenti, T.W., Brooks, B.W., Mydlarz, L.D., Grover, J.P., 2010. Hemolysis, fish mortality, and LC-ESI-MS of cultured crude and fractionated golden alga (*Prymnesium parvum*). Journal of the American Water Resources Association 46, 33-44.

Shilo, M., 1967. Formation and Mode of Action of Algal Toxins. Bacteriological Reviews 31(3), 180-193.

Skingel, T.R., Spencer, S.E., Le, C.Q., Serrano, C.A., Mydlarz, L.D., Scarbrough, B.J., Schug, K.A., Brooks, B.W., Grover, J.P., 2010. Hemolytic toxicity and nutritional status of *Prymnesium parvum* during population growth. Aquatic Microbial Ecology 61, 141-148.

Southard, G.M., 2005. Appendix A: Identification and Enumeration of *Prymnesium parvum* Cells, Version AEW-IDE 1.1. In: Management of *Prymnesium parvum* at Texas State Fish Hatcheries, A. Barkoh and L.T. Fries (Editors). Texas Parks and Wildlife Department, Management Data Series 236, PWD RP T3200-1138 (9/05), Austin, Texas, pp. 97-98

Southard, G.M., Fries, L.T., Barkoh, A., 2010. *Prymnesium parvum*: The Texas Experience. JAWRA Journal of the American Water Resources Association 46(1), 14-23.

Steele WB, Kristofco LA, Corrales J, Saari GN, Haddad SP, Gallagher EP, Kavanagh TJ, Kostal J, , Zimmerman JB, Voutchkova-Kostal A, Anastas PT, and Brooks BW. Accepted with revision. Comparative behavioral toxicology of two common larval fish models: exploring relationships between modes of action and locomotor responses. Science of the Total Environment

Steele WB, Mole R, Brooks BW. 2018, In press. Experimental protocol for examining behavioral response profiles in larval fish: Application to the neurostimulant caffeine. Journal of Visualized Experiments

Svendsen M, Andersen N, Hansen P, Steffensen J. 2018. Effects of Harmful Algal Blooms on Fish: Insights from Prymnesium parvum. Fishes 3:11.

Tillmann, U., 1998. Phagotrophy by a plastidic haptophyte, *Prymnesium patelliferum*. Aquatic Microbial Ecology 14, 155-160.

Tillmann, U., 2003. Kill and Eat Your Predator: A Winning Strategy of the Planktonic Flagellate *Prymnesium parvum*.

Ulitzur, S., Shilo, M., 1964. A Sensitive Assay System for Determination of the Ichthyotoxicity of *Prymnesium parvum*. Microbiology 36, 161-169.

USEPA. 1991. Methods for aquatic toxicity identification evaluations: Phase i toxicity characterization procedures. Washington, D.C.: Office of Research and Development.

USEPA. 2002. Methods for measuring the acute toxicity of effluents receiving waters to freshwater and marine organisms. Washington, D.C.: Office of Water.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64(2), 178-189.

Valenti, J., T.W., James, S.V., Lahousse, M.J., Schug, K.A., Roelke, D.L., Grover, J.P., Brooks, B.W., 2010. A mechanistic explanation for pH-dependent ambient aquatic toxicity of *Prymnesium parvum* carter. Toxicon 55, 990-998.

Valenti, T.W., Gould, G.G., Berninger, J.P., Connors, K.A., Keele, N.B., Prosser, K.N., Brooks, B.W., 2012. Human Therapeutic Plasma Levels of the Selective Serotonin Reuptake Inhibitor (SSRI) Sertraline Decrease Serotonin Reuptake Transporter Binding and Shelter-Seeking Behavior in Adult Male Fathead Minnows. Environmental Science & Technology 46(4), 2427-2435.

Valenti, T.W., James, S.V., Lahousse, M.J., Schug, K.A., Roelke, D.L., Grover, J.P., Brooks, B.W., 2010. A mechanistic explanation for pH-dependent ambient aquatic toxicity of *Prymnesium parvum* carter. Toxicon 55, 990-998.

VanLandeghem, M.M., Meyer, M.D., Cox, S.B., Sharma, B., Patino, R., 2012. Spatial and temporal patterns of surface water quality and ichthyotoxicity in urban and rural river basins in Texas. Water Research 46, 6638-6651.

VanLandeghem, M.M., Farooqi, M., B., F., Patino, R., 2013. Impacts of golden alga *Prymnesium parvum* on fish population in reservoirs of the upper Colorado River and Brazos River Basins, Texas. Transactions of the American Fisheries Society 142(3), 581-595.

VanLandeghem, M.M., Farooqi, M., Southard, G.M., Patino, R., 2014. Associations between water physiochemistry and *Prymnesium parvum* prescence, abundance, and toxicity in west Texas reservoirs. Journal of the American Water Resources Association 51(2), 1-16.

Wang, L., Liang, X.-F., Liao, W.-Q., Lei, L.-M., Han, B.-P., 2006. Structural and functional characterization of microcystin detoxification-related liver genes in a phytoplanktivorous fish, Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 144(3), 216-227.

Wiegand, C., Pflugmacher, S., Oberemm, A., Meems, N., Beattie, K., Steinberg, C., A. Codd, G., 1999. Uptake and Effects of Microcystin-LR on Detoxication Enzymes of Early Life Stages of the Zebra Fish (Danio Rerio).

Wu, G., Fang, Y., Yang, S., Lupton, J., Turner, N., 2004. Glutathione metabolism and its implications for health. Nutritional Sciences, 489-492.

Zegura, B., Lah, T., Filipic, M., 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology 200, 59-68.

Zhang, D., Hu, C., Li, D., Liu, Y., 2013. Lipid peroxidation and antioxidant responses in zebrafish brain induced by *Aphanizomenon flos-aquae* DC-1 aphatoxins. Aquatic Toxicology 144-145, 250-256.