UNDERSTANDING THE ROLE DIRECT CONTACT TOXICITY AND MIXOTROPHY PLAY IN THE POPULATION DYNAMICS OF *PRYMNESIUM PARVUM*

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ABSTRACT

UNDERSTANDING THE ROLE DIRECT CONTACT TOXICITY AND MIXOTROPHY PLAY IN THE POPULATION DYNAMICS OF *PRYMNESIUM PARVUM*

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The University of Texas at Arlington, 2019

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Harmful algae disrupt other organisms and ecosystems by multiple mechanisms. Many taxa of harmful algae produce toxic compounds that likely contribute to such problems. Though there is little evidence that the harmful golden algal species such as *Prymnesium parvum* are harmful to human health, this species has been linked to many toxic blooms leading to fish kills. A clear relationship between the concentrations of *P. parvum* cells or *P. parvum* toxins and fish kills is not well established in natural settings. Filtration techniques to generate cell-free filtrates of *P. parvum* may create toxigenic artifacts that are a result of high-pressure during filtration. Toxic activity in cultures of *P. parvum* is not strong unless cultures are already at a high density, suggesting that *P. parvum* may use other traits to become competitive initially, such as mixotrophy. Mixotrophy has been demonstrated in many protists that take up nutrients via photosynthetic processes in addition to phago- and/or osmotrophy. One goal of this study was to examine whether toxicity in *P. parvum* targeting a potential competitor could be attributed to

diffusion of dissolved substances, or if direct contact was required. Using an experimental apparatus that could separate cells of the two species, or allow direct contact, only arrangements allowing direct contact resulted in a rapid decline of cell density for competitor *Rhodomonas salina*. An experiment was conducted with cultures in which *P. parvum* was supplied with limiting phosphorus, either as orthophosphate in sterile medium, or as an equivalent amount of total phosphorus in the form of a culture of the cryptophyte *R. salina*. Rapid lysis of the latter occurred, along with consumption of phosphorus by *P. parvum*, and net population growth.

Together these observations suggest *P. parvum* individuals produce toxins that kill potential competitors, and then benefit nutritionally by consuming the victim. Thus, the relationship between *P. parvum* and *R. salina*, and perhaps with other potential competitors of the former species, is one of intraguild predation.

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GENERAL INTRODUCTION

Harmful algae have been known to cause ecological and economic problems worldwide (Anderson et al. 2002). Such algae often produce toxins that act against a variety of other aquatic organisms. The functions of such toxins have been much discussed. Among the functions thought to be important is allelopathy, a common competitive mechanism, and a way harmful alga can dominate in an otherwise diverse aquatic community. Allelopathy in harmful algae is often attributed to dissolved substances that promote the growth of the producer species by interfering with metabolic process or structural cell integrity of competing species in proximal spatial areas. Such dissolved allelopathic substances may provide a way for organisms with low nutrient affinity to be more competitive (Smayda 1997). Additionally, dissolved substances used for allelopathy may be toxic to other organisms such as potential predators and prey.

Prymnesium parvum, the focus of this dissertation, is a harmful alga whose toxins affect a variety of aquatic species (Brooks et al., 2010), and many functions including allelopathy and protection against predators have been assigned to its broad-spectrum toxins. It is has also been difficult to isolate and quantify the dissolved substances (if indeed there are any, see Remmel and Hambright, 2012) that harm predators, prey, or competitors. The argument that dissolved substances used for allelopathy contribute to the formation of harmful bloom presents some ambiguity, because a sufficiently dense producing population must already exist for such toxins to reach effective concentrations (Jonsson et al. 2009). Furthermore, the supposed allelopathic dissolved substances may not be the same substances that deter predators and kill prey. More issues arise with the evolutionary aspects of allelopathy in aquatic settings. There are apparent costs incurred to the producing species, such as a reduction in growth rate within the genus *Alexandrium (*Blossom et al. 2019*)*. Complicating matters further is the potential existence of

cheaters, non-producers not sensitive to dissolved substances, but which benefit from the reduction competition brought about by producers. Cheaters are especially likely to arise in well mixed aquatic systems, and would undermine the evolutionary advantage conferred by dissolved allelopathic substances.

The aims of the dissertation were to attain a better understanding of the toxic nature of the harmful algal *P. parvum*. *Prymnesium parvum* has been known to change the biodiversity and population dynamics of natural aquatic ecosystems (Michaloudi et al. 2009). Laboratory studies have demonstrated the toxic nature of *P. parvum* on competitors (Barreiro et al. 2005), potential predators (Carvalho and Granéli 2010; Tillmann 2003) and prey (Carvalho and Granéli 2010). It is unclear, however, how toxicity arises or how it is regulated within *P. parvum*. Mere filtration artifacts may erroneously suggest that dissolved substances contribute to the toxicity of *P. parvum* (Remmel and Hambright 2012).

Thus one aim of this study was to understand whether dissolved substances truly contribute to the toxic effects that *P. parvum* has on a potential competitor, the flagellated alga *Rhodomonas salina*. Toxicity in *P. parvum* may contribute to the mixotrophic nature of *P. parvum*, by killing potential prey. Thus another aim of this study was to understand how mixotrophy contributed to the growth of *P. parvum*. Though laboratory studies have shown mixotrophic potential within *P. parvum* (Skovgaard et al. 2003; Tillmann 2003), the benefits conferred to population growth of *P. parvum*, though implied, have not been quantified. To gain a better understanding of toxicity and the role of mixtrophy in the population dynamics of *P. parvum*, laboratory studies involving monocultures of *P. parvum* and mixed cultures with other species (primarily *R. salina*) were set used.

The first experimental chapter elucidates the population dynamics between *P. parvum* and well-known potential competitor species (*Rhodomonas salina* and *Scenedesmus obliquus*). It demonstrates that within hours to days of being in mixed cultures with *P. parvum*, *R. salina* disappears. Notably different, *S. obliquus* persists and grows in the presence of *P. parvum*. Though consistent with other studies regarding population dynamics between *P. parvum* and species similar to *R. salina* and *S. obliquus*, the population dynamics do not reveal whether toxicity, mixotrophy, or both is the cause of the disappearance of *R. salina*. Nor did these experiments adequately demonstrate that dissolved toxins were involved.

The second experimental chapter establishes the role of direct cell-to-cell contact in interspecific interactions involving *P. parvum*. Two different cell populations (*R. salina* and *P. parvum*) were separated with a permeable barrier allowing the diffusion of dissolved substances. Lysis and eventual disappearance of *R. salina* was observed only in treatments in which *P. parvum* was in direct contact with *R. salina*. In other interspecific setups that did not involve direct cell-to cell contact, *R. salina* survived.

The third experimental chapter examined the benefit of mixotrophy to population growth of *P. parvum* by using two different forms of phosphorus as the limiting nutrient, at the same supply concentrations. Cultures of *P. parvum* were given either inorganic phosphate or organic phosphorus in potential the competitor/prey species *R. salina*. Organic phosphorus in this target species supported population growth, but led to lower growth rates of *P. parvum* than did inorganic phosphate.

Rapid lysis and disappearance of *R. salina* in a matter of hours only occurred in mixed cultures having direct contact with *P. parvum* strongly suggesting that direct cell-to-cell contact mechanisms contribute largely to the competitive edge of *P. parvum*. Any role of dissolved

toxins is weaker, and acts on a much slower time scale (days). Moreover, nutritional benefits of this direct contact toxicity were found, because the limiting nutrient phosphorus was consumed from victim cells and supported population growth. As such, there now evidence that toxicity is mediated by direct cell-to-cell mechanisms in conjunction with mixotrophy.

This form of toxicity coupled with mixotrophy allows *P. parvum* to grow when provided with a limiting nutrient not easily attainable. If the role of toxicity mediated by direct cell-to-cell contact mechanism rather than diffusion of dissolved substances, such a phenomenon may resolve ecological and evolutionary questions about *P. parvum* in ways that might apply to other harmful algae. Ecologically, the killing and eating of a potential competitor has come to be called intraguild predation, in distinction to simpler competitive relationships. Moreover, the capability to poison and eat competitors can contribute to the formation of large populations from sparse ones, and contribute to the formation of harmful blooms. Evolutionarily, the individual level of the action of direct contact toxicity, and of the nutritional benefits obtained presents a resolution to the public goods problem that is posed for the evolution of allelopathy by dissolved substances in aquatic ecosystems.

REFERENCES

Anderson DM, Glibert PM, Burkholder JM. 2002. Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. Estuaries 25(4, Part B: Dedicated Issue: Nutrient Over-Enrichment in Coastal Waters: Global Patterns of Cause and Effect):704-26.

Barreiro A, Guisande C, Maneiro I, Lien TP, Legrand C, Tamminen T, Lehtinen S, Uronen P, Granéli E. 2005. Relative importance of the different negative effects of the toxic haptophyte *Prymnesium parvum* on *Rhodomonas salina* and *Brachionus plicatilis*. Aquat Microb Ecol 38(3):259-67.

Blossom HE, Markussen B, Daugbjerg N, Krock B, Norlin A, Hansen PJ. 2019. The cost of toxicity in microalgae: Direct evidence from the dinoflagellate *Alexandrium*. Frontiers in Microbiology 10.

Brooks BW, et al. 2010. Comparative toxicity of *Prymnesium parvum* in inland waters. J Am Water Resour Assoc 46(1):45-62.

Carvalho WF and Granéli E. 2010. Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency. Harmful Algae 9(1):105-15.

Jonsson PR, Pavia H, Toth G. 2009. Formation of harmful algal blooms cannot be explained by allelopathic interactions. Proc Natl Acad Sci USA 106(27):11177-82.

Michaloudi E, Moustaka-Gouni M, Gkelis S, Pantelidakis K. 2009. Plankton community structure during an ecosystem disruptive algal bloom of *Prymnesium parvum*. Journal of Plankton Research 31(3):301-9.

Remmel EJ and Hambright KD. 2012. Toxin-assisted micropredation: Experimental evidence shows that contact micropredation rather than exotoxicity is the role of *Prymnesium* toxins. Ecol Lett 15(2):126-32.

Skovgaard A, Legrand C, Hansen PJ, Graneli E. 2003. Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*. Aquat Microb Ecol 31:259-65.

Smayda TJ. 2008. Complexity in the eutrophication–harmful algal bloom relationship, with comment on the importance of grazing. Harmful Algae 8(1):140-51.

Tillmann U. 2003. Kill and eat your predator: A winning strategy of the planktonic flagellate *Prymnesium parvum*. Aquat Microb Ecol 32(1):73-84.

CHAPTER 2

THE VARIABLE INTERSPECIFIC COMPETITIVE EFFECTS OF CELL-FREE AND WHOLE CELL *PRYMNESIUM PARVUM* PREPARATIONS ON *RHODAMONAS SALINA*[1]

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ABSTRACT

Prymnesium parvum is one amongst many species of algae thought to produce compounds harmful to other algae and aquatic organisms, including fish. The mechanism of interspecific competition between *P. parvum* and two other algal species was explored using cell-free and whole cell preparations of *P. parvum* and exposing it to two different algal species. In 24-hour cytotoxic assays, *P. parvum* caused lysis of one algal species, *Rhodomonas salina*, with more than 90% of *R. salina* being lysed. Such high lysis occurred when cellular ratios of *R. salina* to *P. parvum* were approximately 1:1. No such lysis due to *P. parvum* was observed for a second algal species, *Scenedesmus obliquus*. In growth experiments with mixed batch cultures of *P. parvum* and *R. salina*, the final cell density of *P. parvum* was more than double that of *P. parvum* in monoculture, suggesting *P. parvum* may benefit nutritionally from subsequent lysis *R. salina*. In mixed semicontinuous cultures, elevated abundance of *P. parvum* also lasted many days after the disappearance of *R. salina* due to lysis.

INTRODUCTION

Harmful algae are a diverse group of organisms that cause water quality problems, economic losses, and public health problems worldwide (Glibert et al. 2005, Brooks et al. 2016). Harmful algae disrupt other organisms and ecosystems by multiple mechanisms. Dead and decaying mats of algae stimulate oxygen consumption by detritivores and stimulate the production of sulfide gaseous compounds, impairing cellular respiration (Zinder et al. 1977, Marshall et al, 2005). Other groups of algae may cause harm through mechanical means. Diatoms and spine-bearing algae can impair the respiration of fish by clogging their gills (Zingone and Enevoldsen 2000). Many groups of algae produce toxic compounds (Anderson et al, 2002). Certain species of dinophytes and cyanobacteria are thought to release compounds that interfere with hepatic and nervous organs (Anderson et al, 2002).

Prymnesium parvum is one amongst many species of algae thought to produce toxic compounds that interfere with respiration in gill-bearing organisms (Ulitzer and Shilo 1964, Roelke et al, 2010, 2016). Mass finfish mortalities worldwide have been blamed on the presence of *P. parvum* (Kaartvedt et al, 1991, Guo et al, 1996). However, a clear relationship between the concentration of *P. parvum* cells and fish kills is not well established in natural settings (Shilo and Aschner 1953; Johansson and Graneli 1999). Though the nature of these compounds is not well characterized or understood (Ulitzer and Shilo 1964, Blossom et al 2014), there is evidence that these compounds may negatively affect many other organisms including ciliates, grazers, and other phytoplankton species (Rosetta and McManus 2003, Graneli and Johansson 2003). In addition, some zooplankton grazers such as *Eurytemora affinis* and *Acartia bifilosa* avoid feeding on *P. parvum* when other palatable prey is present (Sopanen et al, 2006). Otherwise, *P. parvum* or the toxins released from *P. parvu*m negatively affect the growth and survival of many

zooplankton species (Nejstgaard and Solberg 1996, Graneli and Johansson 2003, Brooks et al, 2010).

Ecologically important phytoplankton are affected by the presence of *P. parvum*. Populations of cryptomonads and diatoms declined during a natural *P. parvum* bloom (Michaloudi et al, 2009). Cell-free preparations extracted from *P. parvum* have demonstrated lytic activity or immobilization activity (Skovgaard and Hansen 2003). Because *P. parvum* and other phytoplankton demand similar inorganic resources such as nitrogen (N) and phosphorus (P), toxic compounds may be produced by *P. parvum* to reduce competition from other phytoplankton (Graneli and Johansson 2003). Such a phenomenon, allelopathy, is thought to cause shifts in ecological communities and changes in phytoplankton abundances (Michaloudi et al, 2009). Many species, including *P. parvum*, have demonstrated high levels of toxic activity (including potential allelopathy) when grown in artificial medium lacking adequate concentrations of N or P (Fistarol et al, 2003). Though cell-free preparations have exhibited a strong allelopathic effect on other phytoplankton communities, studies using diffusion chambers and gentle filtration techniques raise questions about whether toxins produced by *P. parvum* are truly released to the dissolved phase (Remmel and Hambright 2012). This study uses whole cell and filtered preparations to address the following questions about competitive interactions of *P. parvum* and other algae. Are observations consistent with allelopathy mediated by toxins produced by *P. parvum*? Are such allelopathic effects related to cell concentrations of *P. parvum* or competitors? Are observations of allelopathic effects consistent with the action of dissolved substances released by *P. parvum*? Though it has been asserted that *P. parvum* is a strong competitor due to production of toxic compounds, the mechanism of its competitive nature needs further exploration in these directions.

METHODS

Maintenance of Stock Algal Cultures

Prymnesium parvum (obtained from UTEX culture collection, Austin, Texas, **UTEX** 2763) was maintained in batch culture flasks in f/2 growth medium (Guillard and Ryther 1962) modified to N = 882 µM and P = 36 µM, to give an N:P ratio ca. 24.5:1. Salinity was maintained at 5.8 practical salinity units, using artificial seawater (Baker et al. 2007). Culture flasks were maintained under a light:dark regime of 12:12 h at 20°C. *Rhodomonas salina* (obtained from UTEX culture collection, Austin, Texas) and *Scenedesmus obliquus* (provided by the Walsh Ecology Lab, University of Texas – Arlington) were grown under the same conditions as *P. parvum*.

Interspecific and Intraspecific Interactions in Batch Culture

The effect of interspecific and intraspecific interactions among *P. parvum*, *R. salina* and *S. obliquus* on their population dynamics were tested by setting up unialgal and mixed species batch cultures. One experiment was done with simultaneous cultures of *P. parvum* and *R. salina*, and another with simultaneous cultures of *P. parvum* and *S. obliquus*. Each experiment had 3 replicates for each treatment combination. Mixed batch cultures were prepared by adding *P. parvum* and *R. salina* or *S. obliquus* to triplicate 0.5 L Erlenmeyer flasks containing 200 ml of f/2 growth medium at a total concentration of 10^4 cells ml^{-1} per species. Unialgal cultures were prepared by adding a single species to 0.2 L of f/2 growth medium at a total concentration of 10^4 cells ml^{-1}. All culture flasks (mixed and unialgal) were incubated at 20°C, under a light:dark regime of 12:12 h. Samples of 5 ml were taken from all cultures at different intervals and preserved with 2% Lugol's iodine. Phytoplankton abundance was estimated microscopically

using Sedgwick-Rafter chambers, counting between 300 – 500 cells in a sample. Growth rates were calculated by regression of the natural log of abundance versus time, excluding the lag phase. Differences in endpoint abundances and growth rates were compared using analysis of variance (ANOVA) with the program R.

Interspecific and Intraspecific Interactions in Semicontinuous Cultures

The effects of interspecific and intraspecific interactions among *P. parvum* and *R. salina* on their population dynamics were tested by setting up unialgal and mixed algal semicontinuous cultures, with 3 replicates for each treatment combination. Mixed cultures were made by adding *P. parvum* and *R. salina* to 0.5 L Erlenmeyer flasks containing 200 ml of f/2 growth medium to give an initial concentration of 10^4 cells ml^{-1} per species. Unialgal cultures were prepared by adding a single species to 0.2 L of f/2 growth medium to give a total concentration of 10^4 cells ml^{-1}. Every other day for 18 days, 10% of the volume in all cultures was replaced with fresh f/2 medium. All culture flasks (mixed and unialgal) were incubated at 20°C, under a light:dark regime of 12:12 h. Every other day for the first ten days, then every four days for eight additional days, cultures were sampled and preserved with 2% Lugol's iodine. Phytoplankton abundance was estimated microscopically using Sedgwick –Rafter chambers. Differences in endpoint abundances were compared using an ANOVA with the program R.

Acute Cytotoxicity Assay using R. salina as the Target and Whole Cells and Filtrates of a P. parvum Monoculture

To test the 24-hour acute effect of *P. parvum* whole cells on *R. salina*, three different ratios of *R. salina* to *P. parvum* were prepared from stationary stock batch cultures. Samples were set up in polypropylene scintillation vials to achieve the following ratios: 1:1 (5×10^4 cells

ml^{-1}: 5×10^4 cells ml^{-1}), 5:1 (5×10^4 cells ml^{-1}: 10^4 cells ml^{-1}), 10:1 (5×10^4 cells ml^{-1}: 5×10^3 cells ml^{-1}), along with controls containing only *R. salina* cells (5×10^4 cells ml^{-1}). Vial volumes were brought to 10 ml total volume with f/2 medium. There were three replicates for each treatment combination. Samples were incubated for 24 hours at 20°C under a light:dark cycle of 12:12 h. A similar experiment was set up to examine the acute effects of *P. parvum* monoculture filtrate on whole cells of *R. salina*. The experiment was set up in a similar fashion to whole cell treatments. Cultures of *P. parvum* were vacuum filtered (7 kPa). Different cell concentrations of *R. salina* were exposed to different amounts of this filtrate to obtain the same ratios to *P. parvum* cells in the source culture for the whole cell treatment. After 24 hours, all samples were preserved with 2% Lugol's iodine. The concentration of intact *R. salina* cells was estimated microscopically in a Sedgwick-Rafter chamber. Differences in lysis among treatments were compared using two-factor ANOVA, followed by Tukey's Honestly Significantly Differences (HSD) Test using the program R.

Acute Cytotoxicity Assay using R. salina as the Target and Filtrates of a Mixed Culture

To understand the effect of mixed cultures containing *P. parvum* as a source of potential toxins, an experiment was set up similarly to the whole cell and monoculture filtrate treatment just described above, using *R. salina* as a target. To make the mixed cultures, stock cultures of each species were mixed together in a separate 1L Erlenmeyer flask to obtain 1:1 ratio of *R. salina* and *P. parvum.* The mixed culture was incubated in f/2 medium for 24 hours. This mixed culture was then vacuum filtered (7 kPa). *R. salina* was exposed to different amounts of the resulting mixed culture filtrate to yield the following approximate ratios of *R. salina*: *P. parvum,* considering the concentration of *P. parvum* in the source mixed culture: 1:1, 5:1, and 10:1, along with controls containing only *R. salina* whole cells with no treatment added. There were three

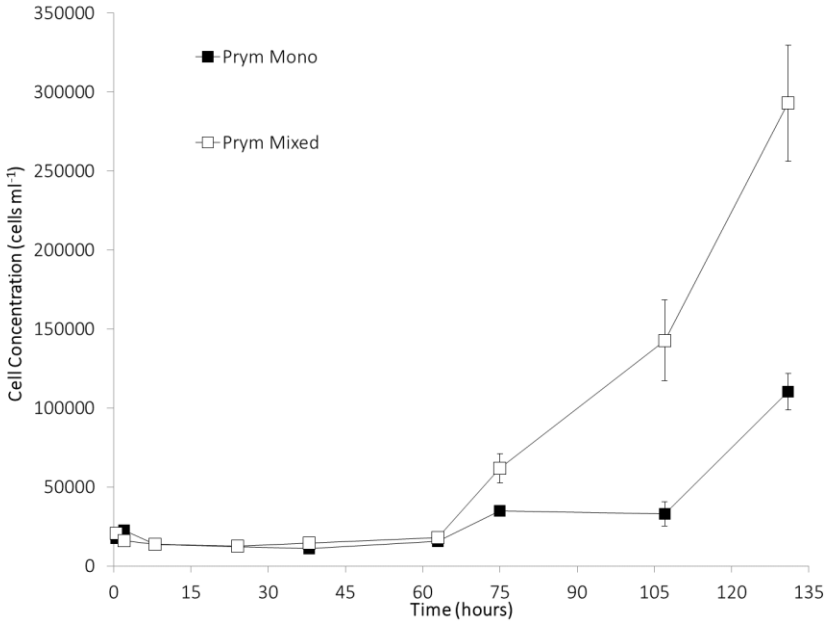
replicates for each treatment combination. The filtrate treatments were set up in polypropylene scintillation vials brought to 10 ml total volume with f/2 medium. Samples were incubated for 24 hours at 20°C under a light:dark cycle of 12:12 h. After 24 hours, samples were preserved with 2% Lugol's iodine. The concentration of intact *R. salina* cells was estimated microscopically in a Sedgwick-Rafter chamber. Differences in lysis among treatments were compared using a single-factor analysis of variance (ANOVA), followed by a Tukey's Honestly Significantly Differences Test using the program R.

RESULTS

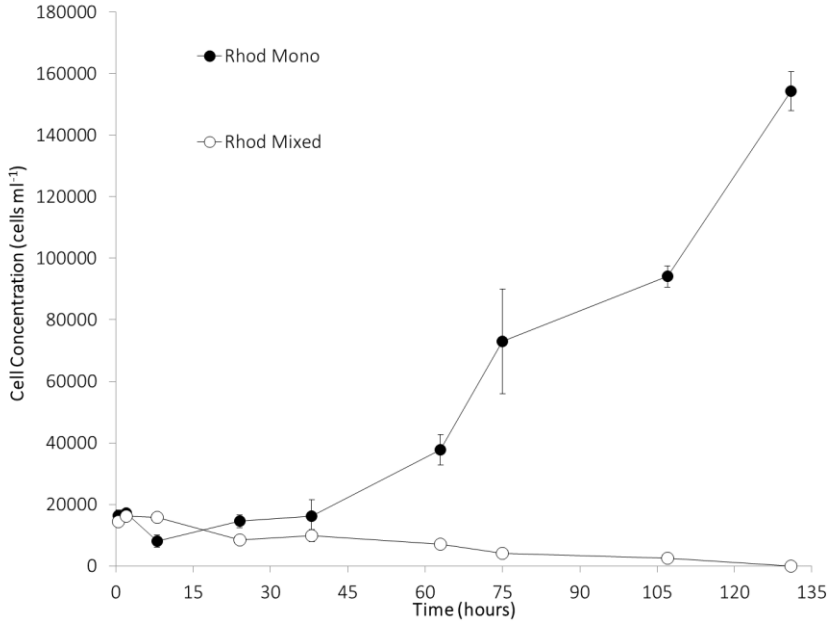
Interspecific and Intraspecific Interactions in Batch Culture: *P. parvum* and *R. salina*

An initial lag phase for unialgal cultures of *P. parvum* and *R. salina* was observed within the first 2-4 days of culture growth (Figure 1), after which both unialgal populations grew throughout the duration of the batch culture experiment. After a similar lag, *P. parvum* grew in mixed cultures with *R. salina*, but the latter species did not. At the end of the experiment, the cell concentration of *P. parvum* was more than twice as high in mixed culture (average of 2.9×10^5 cells ml^{-1}) than in unialgal cultures (average 1.1×10^5 cells ml^{-1}) (Figure 1a). Final cell abundances in monocultures and mixed cultures of *P. parvum* were significantly different from each other (ANOVA $F_{1,4} = 49.0$, $p < .01$). The final cell concentration of *R. salina* averaged ca. 1.5×10^5 cells ml^{-1} in monocultures, but cells of *R. salina* could not be detected in mixed culture flasks after five days (Figure 1b), and microscopic observations suggested lysis and consumption by *P. parvum* had occurred. Final cell abundances in mixed and unialgal cultures of *R. salina* were significantly different from each other (ANOVA $F_{1,4} = 401.3$, $p < 0.001$). The mean growth rates in mixed and monoculture conditions for *P. parvum* differed significantly (ANOVA $F_{1,4} = 30.4$, $p < .01$). The mean growth was higher for *P. parvum* in mixed culture (0.0314 h^{-1})

compared to *P. parvum* growth rate in monoculture (0.020 h^{-1}) Table 1. The growth rates of *R. salina* differed significantly as well (ANOVA $F_{1,4} = 2057.2$, $p < .001$). *Rhodomonas* in monoculture maintained a comparable growth rate to *P. parvum* at 0.024 h^{-1}. In mixed cultures, growth rate of *R. salina* was negative, averaging at -0.069 h^{-1} (Table 1).



1a



1b

Figure 1. Population dynamics in batch monocultures (filled symbols) and mixed cultures (open symbols) of *P. parvum* (a) and *R. salina* (b). Values are mean ± SD (n = 3).

Interspecific and Intraspecific Interactions in Batch Culture: P. parvum and S. obliquus

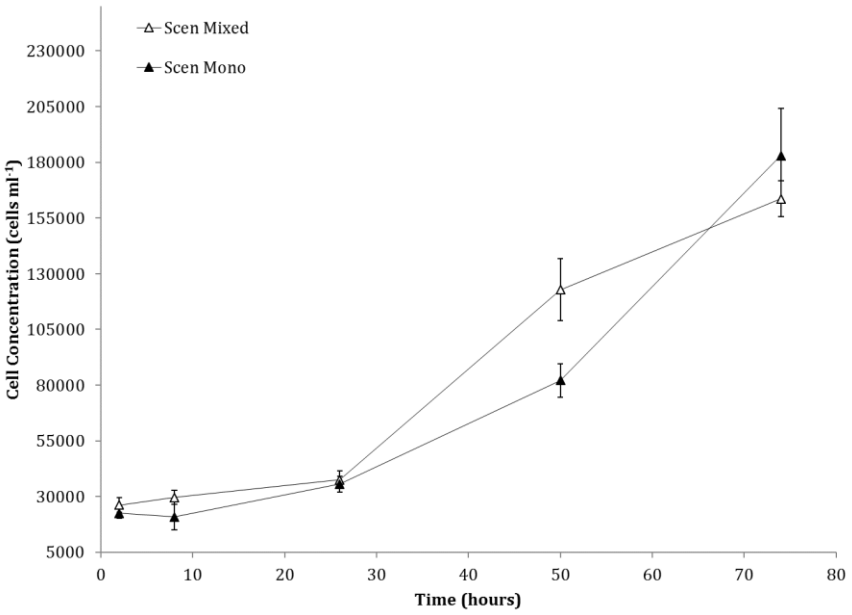
An initial lag phase for unialgal and mixed cultures of *P. parvum* and *S. obliquus* was observed within the first day of growth (Figure 2a and 2b). Both unialgal populations then grew throughout the duration of the batch culture experiment. At the end of the experiment, the final cell concentration of *P. parvum* averaged 2.3×10^4 cell ml^{-1} in monocultures (Figure 2b), and the cell concentration *S. obliquus* averaged 1.8×10^5 cell ml^{-1} (Figure 2a). The abundance of both species also increased over the duration of the batch culture experiments in mixed culture conditions, and there were no microscopic observations of cell lysis or abnormalities in *S. obliquus*. The average final cell concentration of *S. obliquus* was similar in mixed and unialgal culture conditions. Final cell abundances of *S. obliquus* in mixed and unialgal culture conditions were not significantly different from each other (ANOVA $F_{1,4} = 0.76$, $p = 0.43$). Average final cell concentration of *P. parvum* in mixed culture was 5.2×10^4 cell ml^{-1}, while that in unialgal cultures was 2.3×10^4 cell ml^{-1} (Figure 2b). Final cell abundance of *P. parvum* cultures were significantly different from each other in monoculture and mixed cultures (ANOVA $F_{1,4} = 16.9$, $p < .05$). The mean growth rates in mixed and monoculture conditions for *P. parvum* (Table 2) were not statistically different from each other (ANOVA $F_{1,4} = 2.93$, $p > .05$). The growth rates of *S. obliquus* in mixed and monoculture conditions also were not statistically different (ANOVA $F_{1,4} = 1.84$, $p > .05$).

Table 1: Mean growth rates (n=3) of *P. parvum* and *R. salina* in different culture conditions with the standard deviation (SD).

Growth Rate	**± SD**	**Species**	**Condition**
0.0202	0.0028	*P. parvum*	Mono
0.0314	0.0021	*P. parvum*	Mixed
0.0239	0.0035	*R. salina*	Mono
-0.0690	0.0002	*R. salina*	Mixed

Table 2: Mean growth rates (n=3) of *P. parvum* and *S. obliquus* in different culture conditions with the standard deviation (SD).

Growth Rate	**± SD**	**Species**	**Condition**
0.017	0.010	*P. parvum*	Mono
0.023	0.005	*P. parvum*	Mixed
0.045	0.019	*S. obliquus*	Mono
0.031	0.002	*S. obliquus*	Mixed



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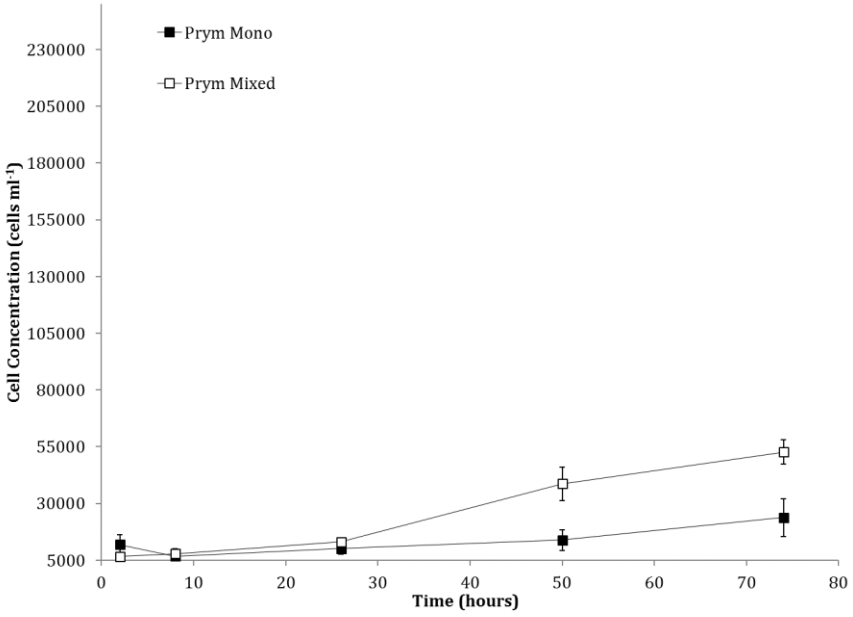
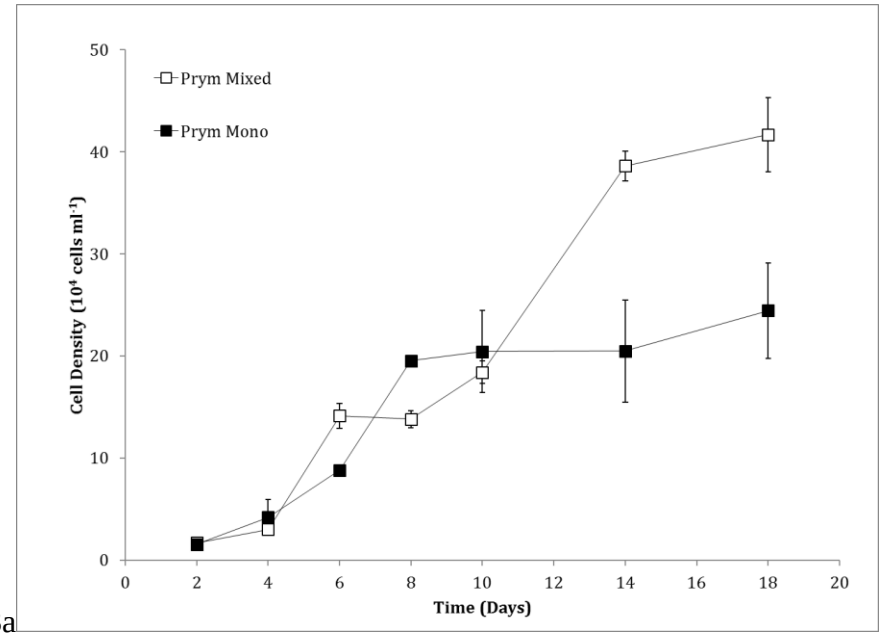


2b

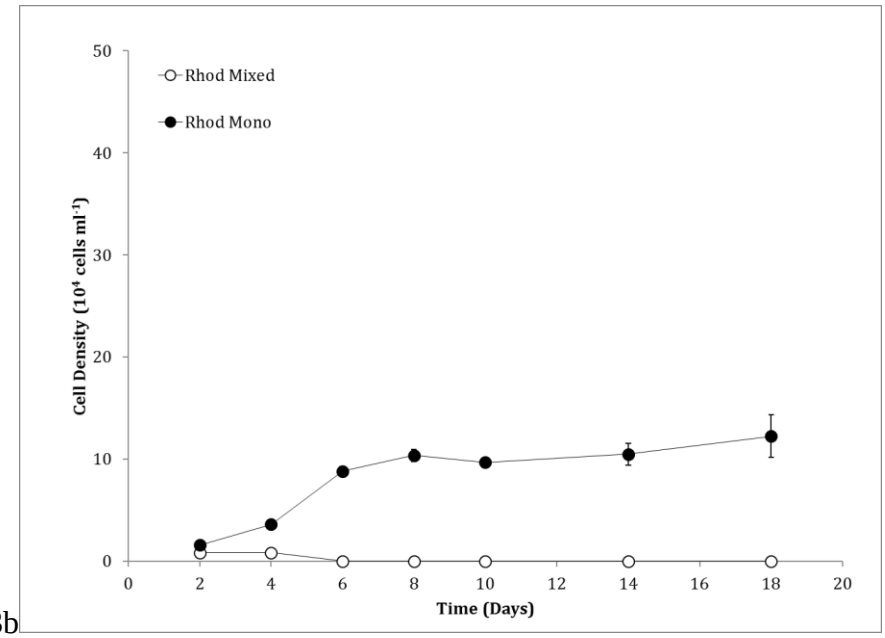
Figure 2. Population dynamics in batch monocultures (closed symbols) and mixed cultures (open symbols) of *S. obliquus* (a) and *P. parvum* (b). Values are mean ± SD (n = 3).

Interspecific and Intraspecific Interactions in Semicontinuous Cultures

Similar dynamics were observed in the semi-continuous culture experiment with *P. parvum* and *R. salina* as in batch cultures. Though each species grew in their respective unialgal cultures, *P. parvum* reached a higher maximum abundance (average of 2.0×10^5 cell ml^{-1}) compared to *R. salina* (average of 1.0×10^5 cell ml^{-1}) (Figure 3a and 3b). In mixed cultures, the abundance of *R. salina* declined within four days of the start of the experiment. After six days in mixed culture conditions, cells of *R. salina* could not be detected (Figure 3b). *Prymnesium parvum* reached an average maximum cell concentration of 4.2×10^5 cell ml^{-1} at the end of the experiment (18 days), more than double the cell concentration of *P. parvum* in unialgal culture conditions (Figure 3a). The final cell densities of *P. parvum* in monoculture were significantly different from the final densities of *P. parvum* in mixed cultures (ANOVA $F_{1,4} = 16.9$, $p < .05$). Final cell abundances of *R. salina* were significantly different in mixed culture and unialgal culture conditions (ANOVA $F_{1,4} = 68.8$, $p < .05$).



3a



3b

Figure 3. Population dynamics in semicontinuous monocultures (filled symbols) and mixed cultures (open symbols) of *P. parvum* (a) and *R. salina* (b). Values are mean ± SD (n = 3).

Acute Cytotoxicity Assay using R. salina as the Target: Whole Cell Treatment

Whole cell preparations of *P. parvum* from a monoculture demonstrated a dose-response curve for lysis of *R. salina* heavily dependent upon the ratio of *Rhodomonas: Prymnesium* (Figure 4a). When *R. salina* cells outnumbered *P. parvum* cells 10:1, approximately 15% of the *R. salina* cells were lysed in 24 hours. Approximately 27% of *R. salina* cells were lysed after 24 hours when ratios were 5:1 and nearly 100% when ratios were 1:1. Controls (*R. salina* cells with no *P. parvum* cells), had the lowest incidence of lysed *R. salina* (ca. 5%). Means at different ratios were significantly different from each other (ANOVA $F_{3,16} = 814.8$, $p < .001$).

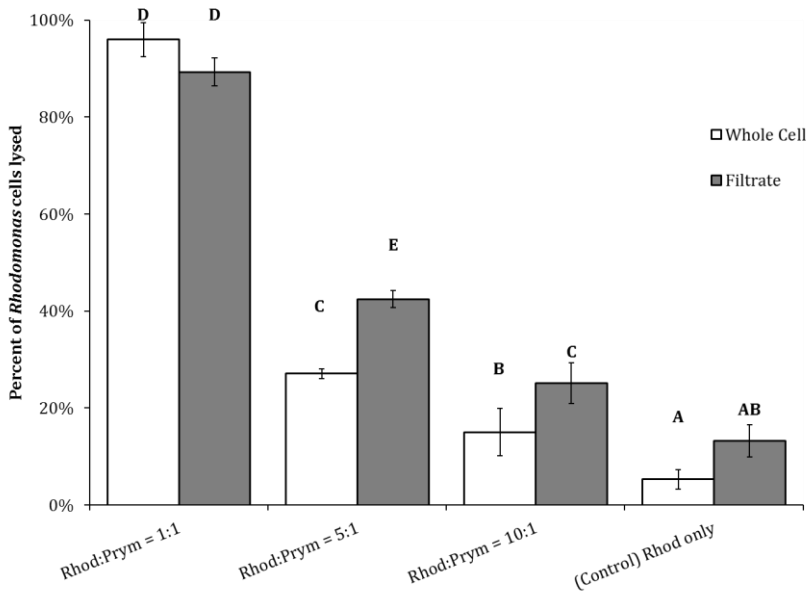
Acute Cytotoxicity Assay using R. salina as the Target: Monoculture Filtrate Treatment

Filtrate from monocultures of *P. parvum* generally demonstrated a very similar a dose-response curve heavily dependent upon the ratio of *Rhodomonas: Prymnesium* (Figure 4a). At the highest ratio (10:1) 25% of the *R. salina* cells lysed in 24 hours. As the ratios decreased, the effects of the filtrate were more pronounced. Approximately 42% of *R. salina* cells were lysed after 24 hours at ratios of ca. 5:1, and nearly 95% at a ratio of 1:1. Controls (*R. salina* with no *P. parvum* filtrate), had the lowest incidence of lysed *R. salina* at 13%. Means at different ratios were all significantly different from each other (ANOVA $F_{3,16} = 814.8$, $p < .001$). However, when comparing lysis between whole cells and filtrate, means of some of the ratios differed significantly but inconsistently (ANOVA $F_{1,16} = 26.0$, $p < .001$). There was no significant difference between the controls for both treatments (TukeyHSD, $p = .11$). There was a significant difference between whole-cell and filtrate means of the 10:1 ratio (TukeyHSD, $p = .0005$), and the 5:1 ratio (TukeyHSD, $p = .02$). There was no significant difference at the 1:1 ratio (TukeyHSD, $p = .19$). There was not a statistical difference between the 5:1 whole cell

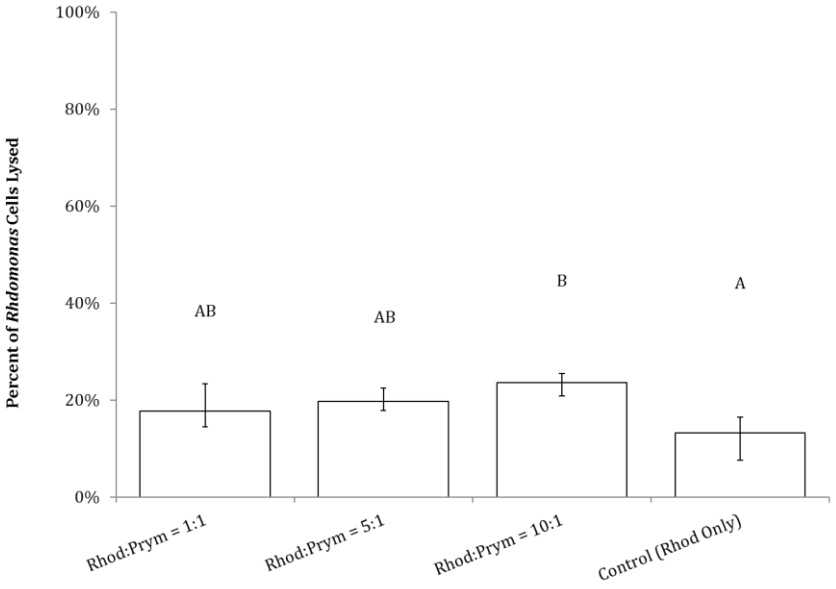
mean and the 10:1 filtrate mean (TukeyHSD, p = .98), nor between the filtrate" control mean and the 10:1 whole cell mean (TukeyHSD, p =.99).

Acute Cytotoxicity Assay using R. salina as the Target: Mixed Culture Filtrate Treatment

A mixed culture of *P. parvum* and *R. salina* was filtered, and *R. salina* was exposed to differing amounts of filtrate constructed to produce different *Rhodomonas: Prymnesium* ratios, based on the concentration of *P. parvum* in the mixed culture. Results differed considerably from those obtained from whole-cell or filtrate preparations from a *P. parvum* monoculture, just described in the previous section. Average percent lysis hovered between 17% to 25% at different *Rhodomonas: Prymnesium* ratios. Differences between means were only marginally significant ($F_{3,8} = 4.2$, $p > 0.047$). The control mean, *Rhodomonas* cells with no added filtrate, was significantly different from the *R. salina* cells treated with filtrate from a culture with a ratio of 10:1 (*R. salina: P.parvum*). Otherwise, the control mean was not different from the other treatment means (Figure 4b). Likewise, the mean of the treatment ratio 10:1 was not significantly different from the other treatment means.



4a



4b

Figure 4. Acute cytotoxicity (lysis) of *R. salina* by exposure to whole cell suspensions or filtrates of a *P. parvum* monoculture (a), and filtrates of a mixed culture of *P. parvum* and *R. salina* (b). Means are expressed ± SD (n = 3). Letters above columns show groups of means that are not significantly different (Tukey HSD, $P > 0.05$).

DISCUSSION

The results of all experiments reported show that toxic activity likely contributes to making *P. parvum* a better competitor than *R. salina*. During both the batch and semicontinuous culture experiments involving mixed cultures of *P. parvum* and *R. salina* there was evidence of lysis and eventual disappearance of the latter species. At the end of both experiments, there were no traces of intact or lysed *R. salina* cells in mixed culture conditions, and *P. parvum* reached higher population densities when grown with *R. salina* than when grown alone. In stark contrast, at a comparable point in time in batch cultures, *P. parvum* grown with *S. obliquus* was at lower population densities than when grown alone. These observations suggest that the lytic effect of *P. parvum* on *R. salina* is crucial to its competitive success. This result is not surprising; there is abundant documentation that *P. parvum* induces lysis in a wide range of aquatic microorganisms (Brooks et al. 2010), including *R. salina* (Barreiro et al. 2005, Uronen et al. 2005). The extent of lysis varies between target organisms, and some taxa are apparently invulnerable, as was found for the chlorophyte *S. obliquus* here. Interestingly, a bloom of *P. parvum* was shown to have minimal effect upon the chlorophytes in a natural community of phytoplankton (Michaloudi et al. 2009). Many chlorophytes possess cell walls with sporopollenin or related compounds (Atkinson et al. 1972), which can make cells resistant to enzyme degradation and chemical detergents (Burczyk and Dworzanski 1988, Xiong et al. 1997). *Rhodomonas salina* does not possess a rigid cell wall with sporopollenin or related compounds (Burczyk and Dworzanski 1988), perhaps making susceptibility to toxic compounds likely.

Prymnesium species have demonstrated capabilities of mixotrophy (Legrand et al. 2001, Tillman 2003, Granéli et al. 2012), and prey species may be immobilized by toxins, making it easier to feed on prey (Skovgaard et al. 2003). Observations from the batch cultures presented

here (Figure 1) suggest that *P. parvum* in mixed cultures obtained a nutritional benefit from exposure to *R. salina*. After a lag of about 3 days, during which time the initial population of *R. salina* experienced substantial lysis, *P. parvum* grew more rapidly and achieved a higher density than it did in corresponding monocultures. Toxin production by *P. parvum* may be used for several purposes, including competition, predation on other microorganisms, and defense against its own predators. For example, *P. parvum* can effectively become a predator, killing and ingesting a predatory heterotrophic dinoflagellate, thus reversing the relationship between a grazing dinoflagellate and its prey (Tillman 2003).

The concentration of *P. parvum* relative to *R. salina* strongly affects the degree of lysis that *R. salina* suffers. At equal cell concentrations of *P. parvum* and *R. salina*, more than 80% of cells of *R. salina* were destroyed within a short time (≈ 24 hours). As the ratio between of *R. salina* cells to *P. parvum* increased, the rate of lysis amongst *R. salina* cells decreased. Similar extents of lysis were seen when filtrates of a *P. parvum* monoculture were dosed to *R. salina* at concentrations equivalent to whole cell suspensions. Filtrates were prepared with a pressure differential (7 kPa) lower than that found to produce filtration artifacts (17 kPa), in which apparently cell-bound toxins were mobilized to the dissolved phase (Remmel and Hambright 2012). Nevertheless, such artifacts are still possible, and the role of dissolved versus cell-bound toxins remains uncertain.

Some studies suggest there is no clear relationship between population size and toxicity in *P. parvum* (Shilo and Aschner 1953). However, in this study *P. parvum* populations were prepared under standardized conditions and a quantitative relationship was found between toxic activity and the cell concentration of *P. parvum* relative to its target (Figure 4a). The well-known variation of the toxic activity of *P. parvum* with a variety of environmental factors is likely to

complicate the relationship between cell concentration and toxic activity (Ulitzur and Shilo 1964). Monitoring and mitigating *P. parvum* blooms in natural settings is often complex. In a dynamic setting, resources and other factors fluctuate daily, and continued uncertainty about the nature and roles of the associated toxins poses additional challenges.

REFERENCES

Anderson, D. M., Glibert, P. M., & Burkholder, J. M. (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries, 25(4), 704-726.

Atkinson, J. A., Gunning, B. E. S., & John, P. C. L. (1972). Sporopollenin in the cell wall of *Chlorella* and other algae: ultrastructure, chemistry, and incorporation of 14 C-acetate, studied in synchronous cultures. Planta, 107(1), 1-32.

Barreiro, A., Guisande, C., Maneiro, I., Lien, T.P., Legrand, C., Tamminen, T., Lehtinen, S., Uronen, P., & Granéli, E. (2005). Relative importance of different negative effects of the toxic haptophyte *Prymnesium parvum* on *Rhodomonas salina* and *Brachionus plicatilis*. Aquatic Microbial Ecology, 38, 259-267.

Blossom, H.E., Rasmussen, S.A., Andersen, N.G., Larsen, T.O., Nielsen, K.F., & Hansen, P.J. (2014). Prymnesium parvum revisited: relationship between allelopathy, ichthyotoxicity, and chemical profiles in 5 strains. Aquatic Toxicology, 157, 159-166.

Brooks, B.W., James, S.V., Valenti, T.W., Jr., Urena-Boeck, F., Serrano, C., Berninger, J.P., Schweirzke, 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.

Burczyk, J., & Dworzanski, J. (1988). Comparison of sporopollenin-like algal resistant polymer from cell wall of *Botryococcus*, *Scenedesmus* and *Lycopodium clavatum* by GC-pyrolysis. Phytochemistry, 27(7), 2151-2153.

Fistarol, G. O., Legrand, C., & Granéli, E. (2003). Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Marine Ecology Progress Series, 255, 115-125.

Glibert, P. M., Anderson, D. M., Gentien, P., Granéli, E., & Sellner, K. G. (2005). The global, complex phenomena of harmful algal blooms.

Granéli, E., Edvardsen, B., Roelke, D.L., & Hagström, J.A. (2012). The ecophysiology and bloom dynamics of *Prymnesium* spp. Harmful Algae, 14, 260-270.

Granéli, E., & Johansson, N. (2003). Increase in the production of allelopathic substances by *Prymnesium parvum* cells grown under N-or P-deficient conditions. Harmful Algae, 2(2), 135-145.

Guillard, R. R., & Ryther, J. H. (1962). Studies of marine planktonic diatoms: I. Cyclotella Nana Hustedt, and Detonula Confervacea (CLEVE) Gran. Canadian journal of microbiology, 8(2), 229-239.

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.

Guo, M., Harrison, P. J., & Taylor, F. J. R. (1996). Fish kills related to *Prymnesium parvum* N. Carter (Haptophyta) in the People's Republic of China. Journal of Applied Phycology, 8(2), 111-117.

Igarashi, T., Satake, M., & Yasumoto, T. (1996). Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. Journal of the American Chemical Society, 118, 479-480.

Johansson, N., & Granéli, E. (1999). Influence of different nutrient conditions on cell density, chemical composition and toxicity of *Prymnesium parvum* (Haptophyta) in semi-continuous cultures. Journal of Experimental Marine Biology and Ecology, 239(2), 243-258.

Kaartvedt, S., Johnsen, T. M., Aksnes, D. L., Lie, U., & Svendsen, H. (1991). Occurrence of the toxic phytoflagellate *Prymnesium parvum* and associated fish mortality in a Norwegian fjord system. Canadian Journal of Fisheries and Aquatic Sciences, 48(12), 2316-2323.

Larsen, A. (1999). Prymnesium parvum and P. patelliferum (Haptophyta)—one species. Phycologia, 38(6), 541-543.

Legrand, C., Johansson, N., Johnsen, G., Borsheim, K.Y., & Granéli, E. (2001). Phagotrophy and toxicity variation in the mixotrophic *Prymnesium patelliferum* (Haptophyceae). Limnology and Oceanography, 46(5), 1208-1214.

Manning, S.R., & LaClaire, J.W., II. (2013). Isolation of polyketides from *Prymnesium parvum* (Haptophyta) and their detection by liquid chromatography / mass spectrometry metabolic fingerprint analysis. Analytical Biochemistry 442, 189-195.

Marshall, J. A., Ross, T., Pyecroft, S., & Hallegraeff, G. (2005). Superoxide production by marine microalgae. Marine biology, 147(2), 541-549.

Michaloudi, E., Moustaka-Gouni, M., Gkelis, S., & Pantelidakis, K. (2009). Plankton community structure during an ecosystem disruptive algal bloom of *Prymnesium parvum*. Journal of Plankton Research, 31(3), 301-309.

Nejstgaard, J. C., & Solberg, P. T. (1996). Repression of copepod feeding and fecundity by the toxic haptophyte Prymnesium patelliferum. Sarsia, 81(4), 339-344.

Roelke, D. L., Grover, J. P., Brooks, B. W., Glass, J., Buzan, D., Southard, G. M., ... & Nelson, J. (2010). A decade of fish-killing *Prymnesium parvum* blooms in Texas: roles of inflow and salinity. Journal of Plankton Research, 33(2), 243-253

Roelke, D.L., Barkoh, A., Brooks, B.W., Grover, J.P., Hambright, K.D., LaClaire, J.W., II, Moeller, P.D.R., & Patino, R. (2016). A chronicle of a killer alga in the west: ecology, assessment, and management of *Prymnesium parvum* blooms. Hydrobiologia, 764, 29-50.

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

Rosetta, C. H., & McManus, G. B. (2003). Feeding by ciliates on two harmful algal bloom species, *Prymnesium parvum* and Prorocentrum minimum. Harmful Algae, 2(2), 109-126.

Shilo, M., & Aschner, M. (1953). Factors governing the toxicity of cultures containing the phytoflagellate *Prymnesium parvum* Carter. Microbiology, 8(3), 333-343.

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.

Skovgaard, A., & Hansen, P. J. (2003). Food uptake in the harmful alga *Prymnesium parvum* mediated by excreted toxins. Limnology and Oceanography, 48(3), 1161-1166.

Skovgaard, A., Legrand, C., Hansen, P. J., & Granéli, E. (2003). Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*. Aquatic Microbial Ecology, 31(3), 259-265.

Sopanen, S., Koski, M., Kuuppo, P., Uronen, P., Legrand, C., & Tamminen, T. (2006). Toxic haptophyte *Prymnesium parvum* affects grazing, survival, egestion and egg production of the calanoid copepods *Eurytemora affinis* and *Acartia bifilosa*. Marine Ecology Progress Series, 327, 223-232.

Tillmann, U. (2003). Kill and eat your predator: a winning strategy of the planktonic flagellate *Prymnesium parvum*. Aquatic Microbial Ecology, 32(1), 73-84.

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

Ulitzur, S., & Shilo, M. (1966). Mode of action of *Prymnesium parvum* ichthyotoxin. Journal of Eukaryotic Microbiology, 13(2), 332-336.

Uronen, P., Lehtinen, S., Legrand, C., Kuuppo, P., & Tamminen, T. (2005). Haemolytic activity and allelopathy of the haptophyte *Prymnesium parvum* in nutrient-limited and balanced growth conditions. Marine Ecology Progress Series, 299, 137-148.

Xiong, F., Komenda, J., Kopecký, J., & Nedbal, L. (1997). Strategies of ultraviolet‐B protection in microscopic algae. Physiologia Plantarum, 100(2), 378-388.

Zinder, S. H., Doemel, W. N., & Brock, T. D. (1977). Production of volatile sulfur compounds during the decomposition of algal mats. Applied and environmental microbiology, 34(6), 859-860

Zingone, A., & Enevoldsen, H. O. (2000). The diversity of harmful algal blooms: a challenge for science and management. Ocean & Coastal Management, 43(8), 725-748.

CHAPTER 3

DIRECT CONTACT MECHANISMS MEDIATE TOXICITY OF *PRYMNESIUM PARVUM* WHEN INTERACTING WITH TARGET SPECIES *RHODOMONAS SALINA*

ABSTRACT

A common working assumption is that such toxic effects of *Prymnesium parvum* are mediated by dissolved substances released into surrounding water and dispersed over distances much larger than cell length. It has recently been suggested, however, that the toxic activity of *P. parvum* targeting certain species such as fish larva requires direct cellular contact. To resolve these incongruencies, an experiment was conducted at two phosphorus levels using different configurations of mixed populations and those separated by permeable barriers, using *Rhodomonas salina* as the target species for *P. parvum*. Cell abundances of *R. salina* in direct contact treatments with *P. parvum* declined and were eliminated completed during the early phase of the experiment, while cell abundances of *R. salina* in no-contact treatments were sustained over time. Only those experimental arrangements allowing direct contact, in which *P. parvum* and *R. salina* were together on the same side of a permeable barrier resulted in a rapid decline of cell density for *R. salina*, suggesting that direct contact cell-to-cell mechanisms are important for rapid lysis of the target species. In a second experiment, additional configurations of mixed populations and those separated by permeable barriers ruled out a hypothesis that signals released during direct contact of the two species induce production of dissolved toxins by *P. parvum*.

INTRODUCTION

For several decades, large-scale algal blooms have been globally widespread causing major ecological disruption in aquatic habitats. Such disruptions have included massive fish kills attributed to algal species that can produce toxins (Anderson et al. 2002). Many species that form blooms have been shown to have a variety of toxic effects. For example, for the prymnesiosphyte *Chrysochromulina polylepis,* the toxins it produces can impede the motility of competing dinoflagellates (Hansen and Schmidt 2001). In other cases, toxins not only hindered motility in motile species, but also led to rapid lysis under certain external conditions, as was the case between the dinoflagellate *Alexandrium tamarense* and target species *Rhodomonas salina* (Zhu and Tillmann 2012). Similar observations have been reported for this target species, when exposed to the prymnesiophyte *P. parvum*. A common working assumption is that such toxic effects are mediated by dissolved substances released into surrounding water and dispersed over distances much larger than cell length.

It has recently been suggested, however, that the toxic activity of *P. parvum* targeting fish larvae requires direct cellular contact, and that separation procedures such as filtration commonly produce artifacts falsely indicating mediation by dissolved toxins (Remmel and Hambright 2012). Suggesting that toxicity by direct contact could be used against many targets, from microbes to metazoan, to lyse cells and obtain nutrition, Remmel and Hambright named this phenomenon “micropredation”. Many harmful algae express both toxic activity and mixotrophic nutrition, and many observations suggest that lack of nutrients necessary for growth such as phosphorus (P), nitrogen (N), and carbon (C), trigger toxin production. For example, P limitation has frequently been observed to increase toxic activity amongst harmful algae (Xu et al. 2012).

In much of the literature addressing the ecological and evolutionary implications of algal toxicity, it is implicitly or explicitly assumed that dissolved toxins are produced. Many studies have presented evidence that harmful algae do secrete exotoxins ((RW.ERROR - Unable to find reference:174; Emura, Matsuyama, Oda 2004; Zhu and Tillmann 2012), although possible artifacts of separating cells from surrounding water are often not critically examined. Additionally, the functions of toxins vary between species. Although it may be that *P. parvum* toxicity is useful for predation, toxicity may also play a role in defense against predators such as ciliates (Rosetta and McManus 2003). Toxic activity might even enable *P. parvum* to obtain nutrition from the predators that it kills (Tillman 2003). Dissolved substances isolated from *P. parvum* have also been shown to be harmful to zooplankton, and to have varying effects on the different phytoplankton (Michaloudi et al. 2009). There is evidence that toxicity may play a role in allelopathy (Graneli and Hansen 2006), here defined as toxic activity mediated by dissolved substances and targeting potential competitors. Because many algal species are competing for similar resources such as N and P, allelopathy with the use of toxins may provide a feasible solution to acquire nutrients that are limited. The severity of hemolytic activity of *P. parvum* has been linked low phosphorus or low nitrogen concentrations (Johansson and Graneli 1999).

Regarding other competing species of algae, *P. parvum* has been shown to negatively impact populations of species of cryptophyta in laboratory settings, with the assumption that dissolved toxins produced by *P. parvum* serve as the major factor in making *P. parvum* a superior competitor (Barreiro et al. 2005). By extension, it is often suggested that production of dissolved toxins might enable harmful algae to eliminate their competitors and become abundant to start a bloom. However, if toxins must be released into the water and dispersed to have their effect on competitors, the producing species must first obtain a sufficient abundance to produce

an effective toxin concentration (Jonsson et al. 2009). Thus it has been questioned whether the toxicity of harmful algae is an important ecological mechanism enabling them to form blooms (Jonsson et al. 2009). An evolutionary problem also arises for toxins released into the aquatic environment: the potential for "cheaters". Nearby individuals that are not vulnerable to these same dissolved substances could also reap the benefits from toxin production and release, without themselves incurring the costs of production, thus undermining the selective advantage of toxin production (Jonsson et al. 2009; Driscoll et al. 2013). One way to mitigate cheating, is for the expression of toxin production to be regulated in relation to signals received from other producers. For example, quorum sensing can signal the presence of a dense population of producers, capable of collectively producing an effective toxin concentration. In addition, signaling could be linked to presence of potential competitors or prey, to further limit toxin production to the environmental conditions when it best enhances producer fitness.

Though harmful algae may present toxicity in different ways, whether toxicity is due to dissolved substances has rarely been critically evaluated. Filtrates derived from *P. parvum* also demonstrated varying toxicity effects dependent upon preparation, leading Remmel and Hambright (2012) to emphasize potential artifacts in much of the published literature. Such artifacts would all into question whether cell-free filtrates or other preparations accurately represent the toxicity of harmful algae such as *P. parvum*, and whether ecological and evolutionary dynamics of such species should be interpreted in a context of dissolved toxic substances.

The goal of this study was to examine whether toxicity in *P. parvum* targeting a potential competitor (*Rhodomonas salina*) could be attributed to diffusion of dissolved substances, or if alternatively, direct contact was required. The methodology of previously used to assess the role

of dissolved substances in toxicity to fish by *P. parvum* (Remmel and Hambright (2012) and *Pfisteria piscicida* (Vogelbein et al. 2002) was adapted to use *R. salina* as a target. This methodology uses 6-well tissue cultures plates with inserts that place a permeable barrier between the putative toxin producer and the target, preventing direct cellular contact but allowing diffusion of dissolved substances. This technique was used to ask the following questions: Does the lysis of *R. salina* cells by *P. parvum* require direct cellular contact, or can it be attributed to the diffusion of dissolved substances? Does the toxic activity of *P. parvum*, however mediated, become more severe under nutrient limitation? To answer these questions, an experiment was conducted at two P supplies using different configurations of mixed and separated populations.

METHODS

Prymnesium parvum and *Rhodomonas salina* culture

Stock cultures of *Prymnesium parvum* (obtained from UTEX culture collection, Austin, Texas, UTEX LL 2797) and *Rhodomonas salina* (obtained from UTEX culture collection, Austin, Texas, LB 2763) were maintained in separate batch culture 1L Erlenmeyer flasks in f/2 growth medium (Guillard and Ryther 1962) modified to N = 882 µM and P = 36 µM, to give an N:P ratio ca. 24.5:1. Salinity was maintained at 5.8 practical salinity units, using artificial seawater (Baker et al. 2007). Culture flasks were maintained under a light:dark regime of 12:12 h at 20°C. Cultures were grown to stationary phase.

Membrane Insert Study

Experiments were conducted in 6-well tissue culture plates with 1.0 µm hanging inserts that divided each well into 5 ml of culture outside of insert and 5 ml of culture within the hanging insert chambers (Figure 1). The porous walls of the insert prevented cells within the insert from making physical contact with those outside, and yet allowed dissolved substances (including exotoxins) to diffuse across the membrane. Individual experimental units were one well together with its insert, creating a diffusion culture. For all experimental treatments, 1 ml of culture was removed from within the insert and from area surrounding the insert and replenished with fresh f/2 media every day for 19 days.

Intraspecific treatments were set up in such a way that both the inside and the outside of the hanging insert both had the same species, with one type of control for *P. parvum* and the other for *R. salina* (Figure 1A and 1B). Both inner and outer chambers were inoculated with

approximately 2.0 x 10^5 cells/ml in 5 milliliters of f/2 medium. Controls were quadruplicated in different tissue plates.

Units in the "Interspecific No Contact" treatments contained one cell type on one side of the insert, and another cell type on the other side of the insert in the same well (Figure 1C and 1D). Units with no direct contact were set up by coupling *P. parvum* and *R.salina* in the same well on different sides of the porous barrier. Any dissolved substances produced and excreted by *P. parvum* could diffuse across the membrane to the target species *R. salina*, though direct contact was prevented. Each type of interspecific no contact treatment (i.e. with *P. parvum* inside or outside) was quadruplicated across different tissue plates. Both inner and outer chambers were inoculated with 2.0 x 10^5 cells/ml of a cell type.

Interspecific direct contact treatments were set up by inoculating both the inner and surrounding boundaries of the porous barrier with mixed cultures of *P. parvum* and *R. salina* (Figure 1E). Both inner and outer chambers were inoculated with 2.0×10^5 cell/ml of both cell types in five milliliters of f/2 medium, and the unit was quadruplicated in both experiments across different tissue plates. In order to see the effect of phosphorus concentration, all wells were subjected to either high phosphorus concentration (N:P = 24.5:1) or low phosphorus concentration (N:P = 245:1).

Estimating Cell Abundance

Samples of culture were taken at ca. 1, 2, 4, 6, 8, 11, 14, 17, 20 days after the initiation of the experiment. All culture samples were preserved with 2% Lugol's iodine. The concentration of intact *R. salina* and *P. parvum* cells were quantified microscopically in a Sedgwick-Rafter chamber.

Statistics

To examine trends over time for repeated sampling of abundance in the same well, a repeated measures univariate ANOVA (RM-ANOVA) by reducing each set of measurements (cell abundances) to a weighted sum by employing orthogonal polynomial coefficients. This RM-ANOVA was employed throughout two phases of the experiment: the first five sampling times and the last five sampling times.

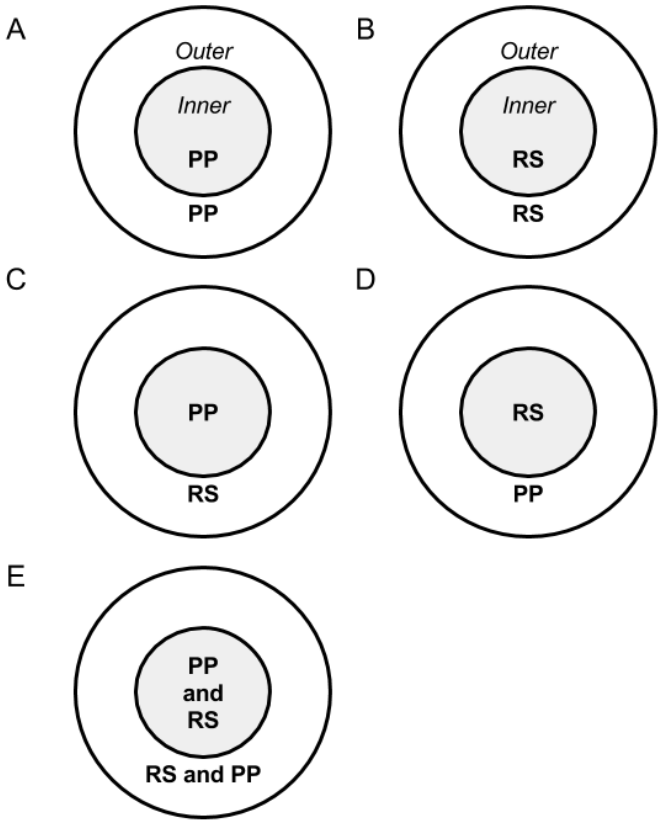


Figure 1: Set-up of millicell experiment. PP = *P. parvum* and RS = *R. salina*. Both A and B represent intraspecific treatments, C and D represent interspecific, no contact treatments, and E represents interspecific direct contact treatments.

RESULTS: EFFECT OF HIGH PHOSPHORUS CONCENTRATION

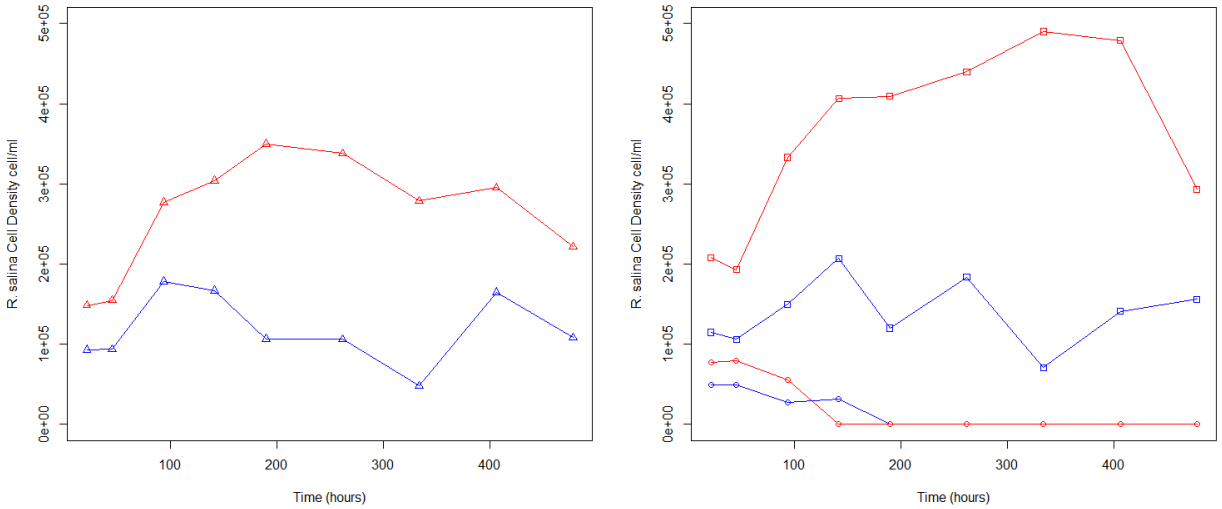


Figure 2: High Phosphorus concentration. Left panel – *R. salina* in inner (red) and outer (blue) chambers for the intraspecific treatment (△). Right panel – *R. salina* in inner (red) and outer (blue) for the interspecific treatments, contact (○), and no-contact (□). All treatments and chambers were inoculated at a calculated cell concentration of 200,000 cells ml^{-1}, for both species. Means are expressed (n = 4).

R. salina cell abundances in intraspecific and interspecific no-contact treatments sustained overall over time. *R. salina* cell abundances in interspecific direct contact treatments declined before being eliminated completed during the early phase of the experiment (Fig. 2). The details of the dynamics were related to both chamber and experimental treatment. For the intraspecific treatment, abundance in the inner chamber was higher for all sampling points. The same was true for *R. salina* abundances in the interspecific no contact treatments. For the contact interspecific treatment, however, *R. salina* in both chambers was undetectable after 120 h and remained so for the duration of the experiment.

Table 1: *R. salina* early phase under high P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	1.47×10^{13}	**<0.0001**	1.74×10^{10}	0.44	1.65×10^{12}	**<0.0001**
Block	1	5.42×10^{10}	**0.042**	4.90×10^{10}	0.20	3.74×10^{10}	0.30
Chamber	3	6.24×10^{11}	**<0.0001**	3.69×10^{11}	**0.0025**	3.45×10^{10}	0.29
Treatment	2	1.00×10^{12}	**<0.0001**	1.07×10^{12}	**<0.0001**	2.53×10^{10}	0.42
Cham×Trtmt	2	1.31×10^{11}	**0.0034**	1.59×10^{11}	**0.015**	1.59×10^{10}	0.58
Error	15	1.55×10^{10}		2.81×10^{10}		2.83×10^{10}	

EARLY PHASE: *R. salina*

In the RM-ANOVA for the early phase, significance of the grand mean for the constant contrast indicated that the average abundance was positive throughout the early phase (Table 1). Both the treatment and chamber were significant factors for the constant contrast, as was their interaction. The contact treatment produced low abundance of *R. salina* in both chambers, but for the other treatments where *R. salina* persisted, it attained higher abundance in the inner chamber than the outer chamber. The grand mean term was not significant for an overall linear trend. However, both the chamber and treatment factors were significant for the linear contrast, as was their interaction. The contact treatment produced a decline of *R. salina* in both chambers. For the other treatments, there were increases during the early phase for the inner chamber, but no consistent trend for the outer chamber. The grand mean for the quadratic contrast was significant, suggesting curvature in *R. salina* dynamics, however, neither chamber nor treatment contributed significantly to variation in this curvature.

Table 2: *R. salina* late phase under high P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	1.67×10^{13}	**<0.0001**	9.54×10^{10}	0.15	8.33×10^{10}	0.26
Block	1	1.77×10^{11}	**0.020**	2.46×10^{10}	0.62	3.43×10^{10}	0.64
Chamber	3	3.84×10^{12}	**<0.0001**	2.10×10^{11}	**0.039**	2.83×10^{11}	**0.046**
Treatment	2	1.16×10^{12}	**<0.0001**	2.98×10^{10}	0.50	1.03×10^{11}	0.21
Cham×Trtmt	2	1.09×10^{12}	**<0.0001**	6.73×10^{10}	0.23	1.73×10^{11}	0.086
Error	15	4.26×10^{10}		4.11×10^{10}		5.95×10^{10}	

LATE PHASE: *R. salina*

In the RM-ANOVA for the late phase, significance of the grand mean for the constant contrast shows that the average abundance maintained a positive value (Table 2). Both the treatment and chamber were significant factors for the constant contrast, as was their interaction. The contact treatment had zero abundance of *R. salina* during the late phase, while the other treatments had positive average abundance, which was higher in the inner chamber than the outer chamber. The linear and quadratic contrasts were not significant indicating there was no overall linear trend nor curvature. The chamber term for both contrasts was marginally significant, indicating possible weak differences in trends between inner and outer chambers when *R. salina* cells persisted.

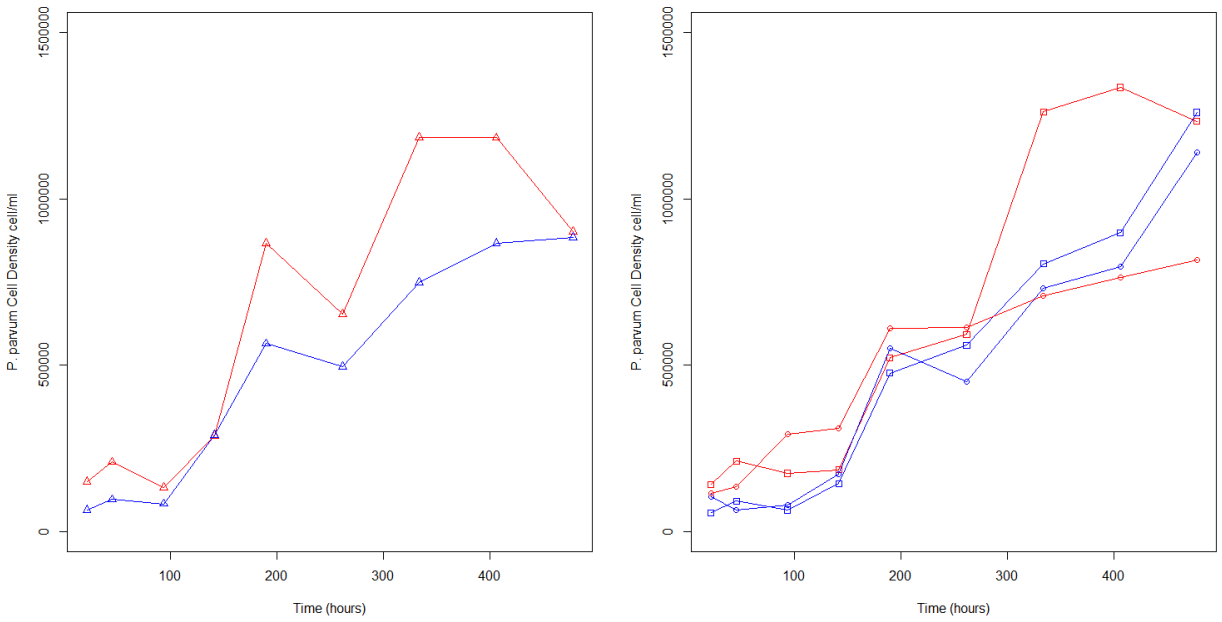


Figure 3: High phosphorus concentration. Left panel – *P. parvum* in inner (red) and outer (blue) chambers for the intraspecific treatment (△). Right panel – *P. parvum* in inner (red) and outer (blue) for the interspecific treatments, contact (○), and no-contact (□). All treatments and chambers were inoculated at a calculated cell concentration of 200,000 cells ml^{-1}, for both species. Means are expressed (n = 4).

In the early phase of the experiment, abundances of *P. parvum* generally increased over time across all treatments (intraspecific, interspecific contact, and interspecific no contact) regardless of chamber position (inner and outer) (Figure 3). In intraspecific treatments, *P. parvum* abundances in inner chambers were generally higher than those in outer chambers overall over time. The same was true for abundances in interspecific no contact treatments. Although abundances of P. parvum increased in interspecific direct contact treatments, the cell abundances were higher in the outer chambers during the last three sampling times.

Table 3: *P. parvum* early phase under high P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	1.71×10^{13}	**<0.0001**	2.92×10^{15}	**0.010**	2.99×10^{12}	**<0.0001**
Block	1	2.59×10^{10}	0.70	3.32×10^{10}	0.30	3.23×10^{10}	0.22
Chamber	3	1.07×10^{12}	**0.00041**	3.32×10^{11}	**0.012**	4.28×10^{11}	**0.00029**
Treatment	2	1.61×10^{11}	0.14	3.32×10^{12}	**0.020**	1.33×10^{11}	**0.0076**
Cham×Trtmt	2	7.56×10^{9}	0.87	3.32×10^{13}	0.014	5.05×10^{10}	0.11
Error	15	5.25×10^{10}		3.32×10^{14}		1.95×10^{10}	

EARLY PHASE*: Prymnesium parvum*

Average abundances of *P. parvum* was positive, as indicated by the significant grand mean for the constant contrasts (Table 3) Though the RM-ANOVA identified an artifact of chamber position in the constant contrast, the treatment term and the interaction between chamber and treatment were not significant, indicating that the treatments had no effect on the cell abundance. There was also significant positive slope and significant curvature, as indicated by the significant grand means of the linear and quadratic contrasts (Table 3). For the linear contrast, both the chamber position and treatment were significant. There was also significant interaction between chamber and treatment. Both chamber and treatment factors were significant for the quadratic contrasts. Overall, the ANOVA results indicate that the experimental treatment and chamber position does play a role in the abundances of *P. parvum*, and the experimental treatment effects are not as strong in the early phase as those for the abundances of *R. salina.*

Table 4: *P. parvum* early phase under high P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	4.00×10^{14}	**<0.0001**	3.99×10^{13}	**<0.0001**	1.44×10^{11}	0.41
Block	1	2.25×10^{11}	0.486	2.37×10^{11}	0.381	2.56×10^{12}	**0.0002**
Chamber	3	2.79×10^{12}	**0.005**	8.70×10^{11}	0.063	4.82×10^{12}	**0.0001**
Treatment	2	1.56×10^{12}	**0.013**	3.44×10^{12}	**<0.0001**	1.11×10^{12}	**0.0157**
Cham×Trtmt	2	1.02×10^{12}	**0.044**	7.83×10^{11}	0.052	3.04×10^{11}	0.2499
Error	15	2.63×10^{11}		2.16×10^{11}		2.00×10^{11}	

LATE PHASE: *Prymnesium parvum*

P. parvum in high phosphorus conditions generally increased throughout the late phase across all treatments regardless of chamber position. Average abundances of *P. parvum* was positive as indicated by the significant grand mean for the constant contrast. Within the constant contrast, both the chamber and treatment factors were also significant, indicating a possible effect of resource competition, and an artifact of chamber position. The interaction between treatment and chamber was also significant. The linear contrast also had a significant grand mean, indicating an overall positive slope in the linear trends. However, the treatment effect was the only factor that was significant within the linear contrasts. There was no overall curvature, as indicated by the non-significant grand mean of the quadratic term, however, both the chamber and treatment effect were significant, indicating a strong effect of chamber position, and a mild to moderate effect of experimental treatment. Overall, the treatment effect in the late phase of *P. parvum* growth was stronger compared to the early phase, which may indicate a resource competition effect that may be exaggerated by position.

RESULTS: EFFECT OF LOW PHOSPHORUS CONCENTRATION

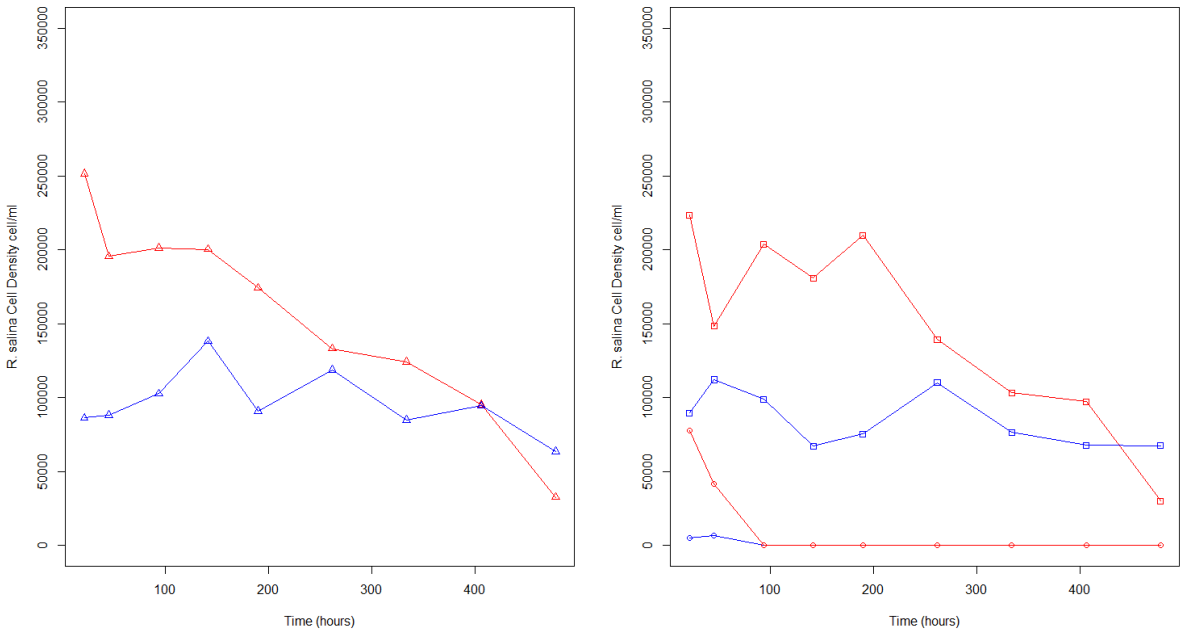


Figure 4: Low phosphorus concentration. Left panel – *R. salina* in inner (red) and outer (blue) chambers for the intraspecific treatment (△). Right panel – *R. salina* in inner (red) and outer (blue) for the interspecific treatments, contact (○), and no-contact (□). All treatments and chambers were inoculated at a calculated cell concentration of 200,000 cells ml^{1}, for both species. Means are expressed ($n = 4$).

R. salina cell abundances declined overall over time (Fig. 4) in high phosphorus conditions, and the details of the dynamics were related to chamber and experimental treatment. For the intraspecific treatment, abundance in the outer chamber was lower for all but the last two sampling times and declined faster than abundance in outer chamber. A similar pattern was seen for the no-contact interspecific treatment, where cells of *R. salina* were separated physically from those of *P. parvum*. For the contact interspecific treatment, however, *R. salina* in both chambers was undetectable after 100 h and remained so for the duration of the experiment.

Before the disappearance of *R. salina* in direct contact with *P. parvum*, its abundance was lower in the outer chamber than in the inner chamber.

Table 5: *R. salina* early phase under low P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	9.56×10^{12}	**<0.0001**	9.83×10^{11}	**<0.0001**	6.10×10^{11}	**0.0008**
Block	1	3.93×10^{10}	**0.0050**	7.42×10^{9}	0.88	8.56×10^{10}	0.10
Chamber	3	4.96×10^{11}	**<0.0001**	1.29×10^{9}	0.84	1.601×10^{11}	**0.048**
Treatment	2	6.69×10^{11}	**<0.0001**	1.84×10^{11}	**0.017**	7.20×10^{10}	0.17
Cham×Trtmt	2	9.32×10^{10}	**0.0002**	8.70×10^{10}	0.11	5.55×10^{10}	0.23
Error	15	6.08×10^{9}		3.39×10^{10}		3.44×10^{10}	

For the early phase prior to hour 264, the RM-ANOVA identified significant differences due to chamber and treatment in both the average abundances of *R. salina* over the entire early phase (constant contrast), and in the linear trends (Table 5). Significance of the grand mean for the constant contrast indicates that the average abundance of *R. salina* was positive during the early phase. The low average abundance *R. salina* for the contact interspecific treatment, where it did not persist during the latter part of the early phase, led to a strongly significant treatment effect for the constant contrast ($p < 0.0001$). The interaction of chamber and treatment was also significant for the constant contrast, because abundances in the outer chamber were consistently lower than the inner chamber for those treatments where *R. salina* persisted. Significance of the grand mean for the linear contrast indicates that the overall trend of *R. salina* abundance during the early phase was negative. The treatment effect for the linear contrast was marginally significant ($0.01 < p < 0.05$), indicating weakly differing rates of decline for the different treatments. Significance of the grand mean for the quadratic contrast indicates detectable curvature during the early phase, but no individual effect was significant, except for the very weak ($p \approx 0.05$) effect of chamber. The results for all contrasts indicate a detectable artifact of chamber, with reduced abundance in the outer chamber. In addition, there was a strong effect of experimental treatments, where direct interspecific contact with *P. parvum* led to the extirpation of *R. salina*. The other treatments, in which *R. salina* cells were either not exposed to *P. parvum*

at all, or were exposed only to its dissolved substances, allowed persistence over the early phase, albeit with an ongoing decline.

Table 6: *R. salina* late phase under low P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	df	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	2.51×10^{12}	**<0.0001**	1.18×10^{12}	**<0.0001**	8.10×10^{9}	0.344
Block	1	3.68×10^{9}	0.60	3.29×10^{9}	0.78	3.93×10^{10}	0.71
Chamber	3	4.34×10^{10}	**0.014**	6.09×10^{10}	**0.020**	1.53×10^{10}	0.20
Treatment	2	6.31×10^{11}	**<0.0001**	2.96×10^{11}	**<0.0001**	1.676×10^{10}	0.17
Cham×Trtmt	2	1.18×10^{10}	0.16	2.31×10^{10}	0.11	1.29×10^{10}	0.25
Total	15	5.62×10^{9}		8.98×10^{9}		8.52×10^{9}	

For the late phase after hour 264, the RM-ANOVA again identified significant differences due to chamber and treatment in the average abundances of *R. salina* over the early phase (constant contrast), and in the linear slopes of decline (Table 6). *R. salina* dynamics were again significantly related to treatment and chamber position. Significance of the grand mean for the constant contrast indicates that the average abundance of *R. salina* was positive during the late phase, even though its abundance was zero in the contact interspecific treatment throughout this phase, and positive only in the other treatments. Significance of the treatment term for the constant contrast identifies this difference as a strong effect ($p < 0.0001$). Significance of the grand mean for the linear contrast indicates that the overall trend of *R. salina* abundance during the late phase was negative. The chamber effect was marginally significant ($0.01 < P < 0.05$) for the constant and linear contrasts, because where *R. salina* persisted, it started higher but declined faster in the inner chamber than in the outer chamber. The late phase analysis shows a strong treatment effect due to the elimination of *R. salina* when in direct contact with cells of *P. parvum*, with declining abundance but persistence otherwise. There was also evidence of a weaker chamber artifact.

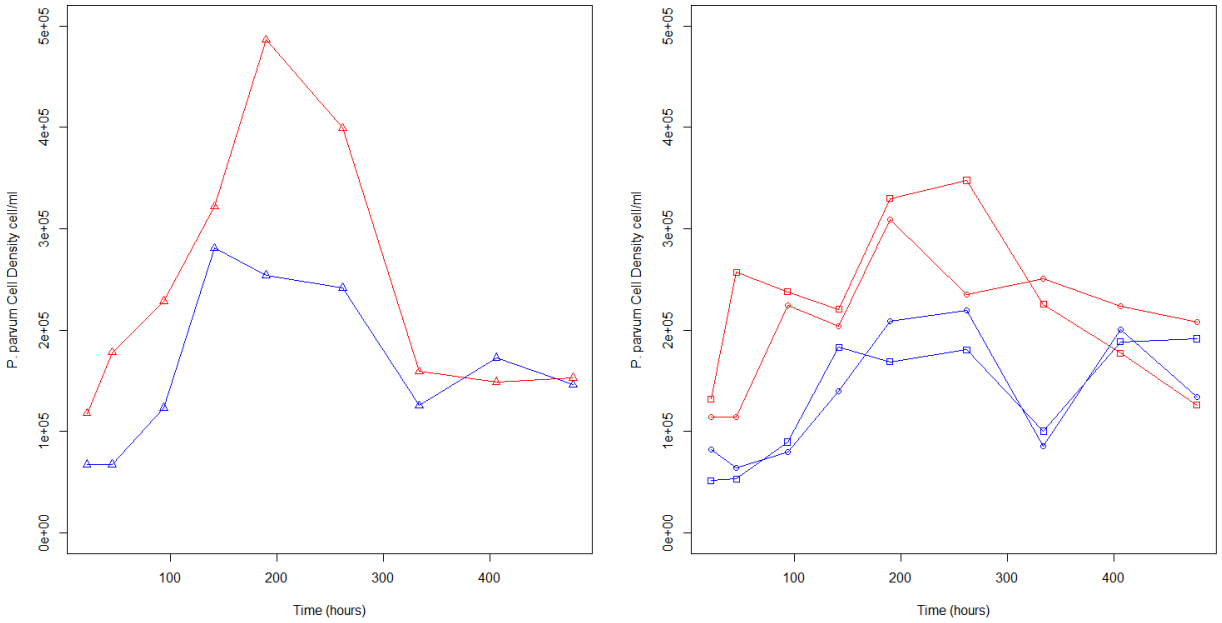


Figure 5: Low phosphorus concentration. Left panel – *P. parvum* in inner (red) and outer (blue) chambers for the intraspecific treatment (△). Right panel – *P. parvum* in inner (red) and outer (blue) for the interspecific treatments, contact (○), and no-contact (□). All treatments and chambers were inoculated at a calculated cell concentration of 200,000 cells ml^{1}, for both species. Means are expressed (n = 4).

Prymnesium parvum cell abundances increased in the early phase of the experiment before declining towards the end in some instances (Fig. 5). For the intraspecific treatment, abundances in the inner chamber were higher for all but the last two sampling times and declined faster from the initial peak than in the outer chamber. A similar pattern was seen for the no-contact interspecific treatment, where cells of *P. parvum* were separated physically from those of *R. salina*. For the contact interspecific treatment, *P. parvum* abundances were consistently higher in inner chambers as opposed to outer chambers.

Table 7: *P. parvum* early phase under low P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	1.49×10^{13}	**<0.0001**	.0189	0.892	1.36×10^{12}	**<0.0001**
Block	1	6.50×10^{10}	**0.022**	4.4×10^{10}	0.084	1.7×10^{11}	**0.02**
Chamber	3	8.80×10^{11}	**<0.0001**	1.1×10^{11}	**0.019**	7.7×10^{11}	**.00040**
Treatment	2	2.70×10^{10}	0.202	4.8×10^{10}	0.085	8.6×10^{10}	0.14
Cham×Trtmt	2	2.80×10^{10}	0.191	1.2×10^{10}	0.511	8.1×10^{10}	0.16
Total	15	1.50×10^{10}		1.6×10^{10}		3.9×10^{10}	

For the early phase prior to hour 264, the RM-ANOVA identified significant differences due to chamber in both the average abundances of *P. parvum* over the entire early phase (constant contrast), in the linear trends, and in the curvature of these trends (Table 7). Significance of the grand mean for the constant contrast indicates that the average abundance of *P. parvum* was positive during the early phase. Significance of chamber effect for the constant contrast corresponds to consistently higher average abundances in the inner chamber during the early phase. Non-significance of grand mean for the linear contrast indicates a lack of consistent linear trends, but the marginal significance of the chamber term ($0.01 < P < 0.05$) indicates more rapid growth in the inner chamber. Significance of the grand mean for the quadratic contrast indicates curvature in the growth patterns for *P. parvum* during the early phase, which was accompanied by a significant chamber term. These results for all contrasts indicate a detectable artifact of chamber during the early phase, where *P. parvum* was more abundant and appeared to grow faster in the inner chamber. Effects of the experimental treatments on *P. parvum* dynamics in the early phase were weaker than the chamber artifact and were not strong enough to rise to statistical significance.

Table 8: *P. parvum* late phase under low P: summary ANOVA table

		Constant		Linear		Quadratic	
Source	*df*	MS	*p*	MS	*p*	MS	*p*
Grand mean	1	1.41×10^{13}	**<0.0001**	1.14×10^{12}	**<0.0001**	4.14×10^{11}	**0.013**
Block	1	2.40×10^{10}	0.44	3.1×10^{10}	0.35	3.10×10^{10}	0.61
Chamber	3	6.60×10^{11}	**0.0001**	1.1×10^{10}	0.52	2.61×10^{9}	0.83
Treatment	2	4.10×10^{11}	**0.0001**	5.6×10^{11}	**<0.0001**	1.24×10^{11}	0.13
Cham×Trtmt	2	1.60×10^{11}	**0.0091**	1.6×10^{11}	**0.011**	1.22×10^{11}	0.13
Total	15	2.50×10^{10}		2.6×10^{10}		5.23×10^{10}	

LATE PHASE: *P. parvum*

For the late phase after hour 264, the RM-ANOVA identified significant differences due to both chamber and experimental treatments in both the average abundances of *P. parvum* over the entire late phase (constant contrast), and in the linear trends (Table 8). Significance of the grand means for the constant, linear, and quadratic contrasts, indicates overall positive abundance, a declining trend, and curvature respectively. Significance of chamber by treatment interactions and some of the associated main effects, for both constant and linear contrasts, is associated with differences in average abundances and trends. Inner chambers had higher abundances than outer chambers early in the late phase, but later abundances were about equal in both chambers for the intraspecific treatment, higher in the inner chamber for the interspecific contact treatment, but higher in the outer chamber for the interspecific no-contact treatment. Additionally, there was a rapid decline from an early peak for the inner chamber-intraspecific treatment combination, slower declines for inner chambers with other treatments, and slow or inconsistent trends for outer chambers. Though the grand mean for the quadratic contrast was marginally significant ($0.01 < P < 0.05$), indicating curvature to these trends, neither chamber nor treatment effects on curvature were significant during the late phase. Overall, the late phase analysis indicates declining *P. parvum* abundances that may have been converging to a common

long-term value with dynamics that varied with both experimental treatment and chamber position.

RESULTS: SPLIT PLOT ANALYSIS

The previous analyses addressed experimental factors applied at the level of subplots (wells on culture plates). Phosphorus supply at high and low levels was applied as a main plot factor, to entire plates. Accordingly, a split plot analysis was done to analyze responses to this factor. Presentation of the split plot analysis focuses on the main effect of phosphorus supply and its interactions with the subplot factors of chamber position and experimental treatment. The effects of the subplot factors already presented are not reiterated.

The main effect of phosphorus on *R. salina* abundance during the early phase was significant only for the linear contrast (Table 9), but interactions were significant for both constant and linear contrasts. For the constant contrast, the significant phosphorus × treatment interaction in combination with a non-significant main effect of phosphorus arose because the average abundance of *R. salina* during the early phase was more strongly enhanced by high phosphorus supply for the no-contact interspecific treatment than it was for other treatments, and especially so in the inner chamber (Fig. 6). For the linear contrast during the early phase, the main effect of phosphorus was marginally significant ($0.01 < p < 0.05$), and two interactions were significant: phosphorus × treatment and chamber × phosphorus. Under low phosphorus conditions, the linear trend of *R. salina* was negative for all treatments (Fig. 7), and most negative for the contact interspecific treatment, where it was extirpated during the early phase. With high phosphorus supply, the linear trend of *R. salina* was negative for the contact interspecific treatment, where it was again extirpated, while the trend was positive for the other treatments in the inner chamber, and near zero in the outer chamber. Thus it appeared that high

phosphorus supply enabled growth of *R. salina* during the early phase provided it was not in direct contact with *P. parvum*, an effect that was strongest for the inner chamber.

The main effect of phosphorus on *R. salina* during the late phase of the experiment was again significant only for the linear contrast, and interactions with all subplot factors were also significant only for this contrast (Table 10). With high phosphorus supply, the linear trend of *R. salina* was positive in the no contact interspecific and intraspecific treatment conditions, but was zero in the contact treatment, where it had already been eliminated (Fig. 8). Furthermore, the inner chambers had a more positive trend than outer chambers. With low phosphorus supply, *R. salina* had a negative linear trend, except for the contact interspecific treatment where its trend was zero, again due to its absence during the late phase. Inner chambers had stronger negative trends than outer chambers. Similar to the early phase, high phosphorus supply appeared to enable *R. salina* to grow, provided it was not in direct contact with *P. parvum*.

Table 9: *R. salina* early phase split plot ANOVA summary

		Constant		*Linear*		*Quadratic*	
Source	df	MS	*p*	MS	*p*	MS	*p*
Phos	1	2.78×10^{11}	0.11	6.32×10^{11}	**0.02**	1.27×10^{11}	0.32
Error	3	5.53×10^{10}		2.83×10^{10}		9.16×10^{10}	
Chamber	1	1.11×10^{12}	**<0.0001**	2.07×10^{11}	**0.015**	1.71×10^{11}	0.26
Treatment	2	1.61×10^{12}	**<0.0001**	1.03×10^{12}	**<0.0001**	6.00×10^{9}	0.83
Cham×Trtmt	1	2.08×10^{11}	**<0.0001**	2.41×10^{11}	**0.002**	1.23×10^{10}	0.68
Cham×Phos	2	3.68×10^{9}	0.56	1.63×10^{11}	**0.029**	2.30×10^{10}	0.4
Trtmt×Phos	1	6.30×10^{10}	**0.0072**	2.23×10^{11}	**0.0028**	9.13×10^{10}	0.07
Trtmt×Phos×Cham	2	1.67×10^{10}	0.23	5.51×10^{9}	0.84	5.91×10^{10}	0.17
Error	30	1.08×10^{10}		3.10×10^{10}		3.14×10^{10}	

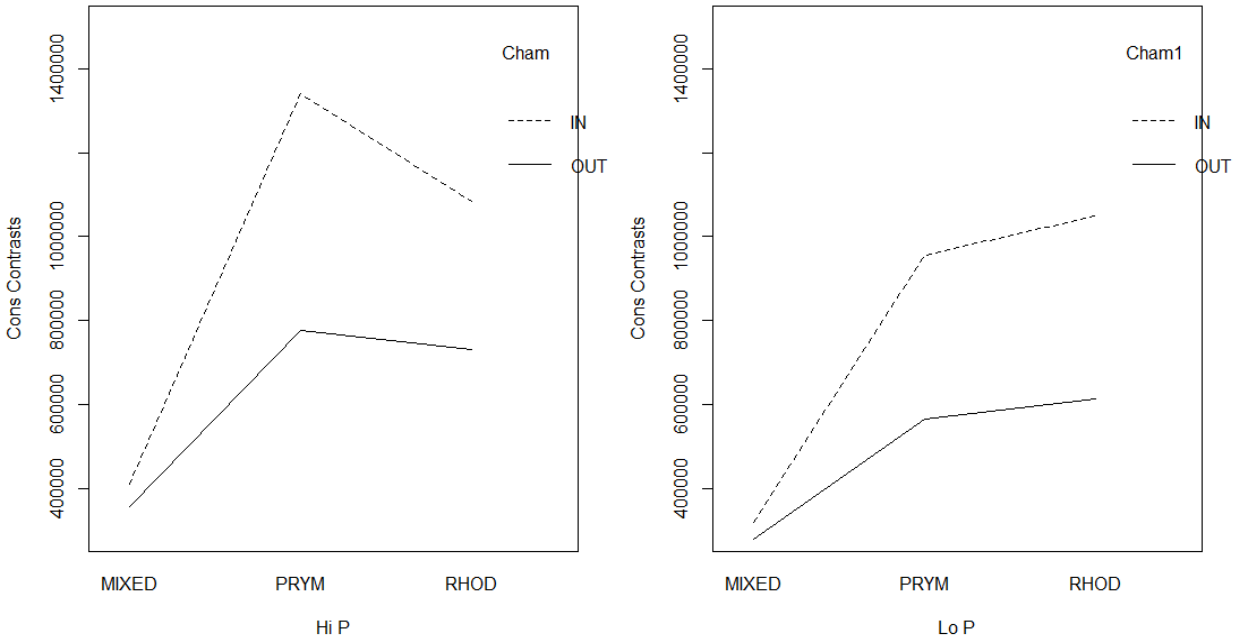


Figure 6: Interaction plot of constant contrast between experimental treatment (X-axis), average of response variable for the early phase of *R. salina* (Y-axis), and chamber position.

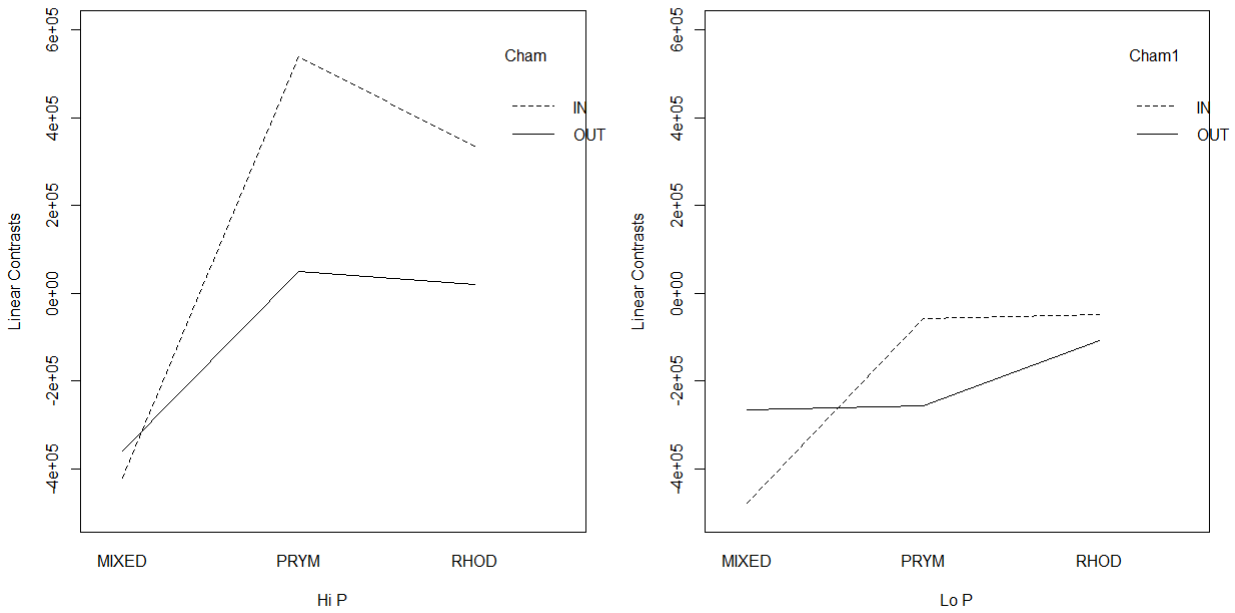


Figure 7: Interaction plot of linear contrasts between experimental treatment (X-axis), average of response variable for the early phase *of R. salina* (Y-axis), and chamber position.

Table 10: *R. salina* late phase split plot ANOVA summary

		Constant		*Linear*		*Quadratic*	
Source	df	MS	*p*	MS	*p*	MS	*p*
Phos	1	7.04×10^{7}	0.66	1.52×10^{12}	**0.0015**	1.81×10^{8}	0.43
Error	3	2.93×10^{8}		1.18×10^{10}		2.24×10^{8}	
Chamber	1	7.97×10^{10}	**0.0006**	1.61×10^{10}	0.22	2.36×10^{10}	0.11
Treatment	2	1.25×10^{12}	**<0.0001**	2.40×10^{10}	0.11	4.69×10^{10}	**0.0093**
Cham:Trtmt	1	2.27×10^{10}	**0.026**	4.07×10^{9}	0.67	3.20×10^{10}	**0.035**
Cham:Phos	2	1.53×10^{8}	0.87	2.26×10^{11}	**<0.0001**	1.15×10^{9}	0.82
Treat:Phos	1	1.41×10^{8}	0.97	3.83×10^{11}	**<0.0001**	4.60×10^{8}	0.88
Trtmt:Phos:Cham	2	9.73×10^{7}	0.98	7.07×10^{10}	**0.0031**	8.05×10^{8}	0.91
Error	30	1.65×10^{11}		1.00×10^{10}		8.54×10^{9}	

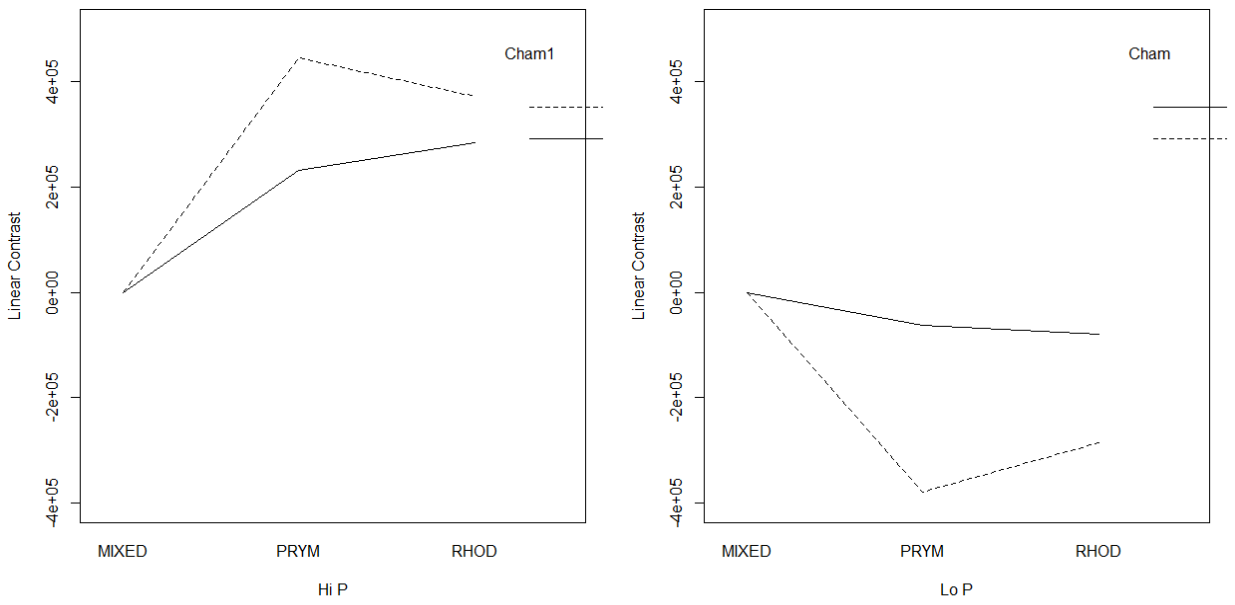


Figure 8: Interaction plot of linear contrasts between experimental treatment (X-axis), average of response variable for the late phase for *R. salina* (Y-axis), and chamber position.

The main effect of phosphorus supply on *P. parvum* abundance during the early phase was significant only for the linear contrast, along with the treatment × phosphorus interaction (Table 11). The linear trend was positive for all treatments and chambers, but *P. parvum* grew much more rapidly during the early phase with high phosphorus supply than with low supply (Fig. 9), an effect that was more pronounced than the weaker interaction with treatment.

The main effect of phosphorus supply on *P. parvum* abundance during the late phase was significant for both the constant and linear contrasts (Table 12), and at least some interactions with phosphorus supply were significant for all contrasts. For all three contrasts, the treatment × phosphorus interaction was significant, and the three-way interaction (treatment × phosphorus × chamber position) was significant in the constant and linear contrasts. For the constant contrast, the average abundance of *P. parvum* was highest in the inner chambers with high phosphorus

supply when not in direct contact with *R. salina* cells (Fig. 10). The chamber and treatment effects were not as apparent with low phosphorus suply, however (Figure 4). For the linear constrast, an interaction plot (Fig. 11) shows that high phosphorus supply resulted in a strong positive trend for *P. parvum* during the late phase when in direct contact with *R. salina*, and a mix of weaker trends under other conditions. With low phosphorus supply, trends were again mixed, but generally more positive for *P. parvum* in inner chambers. For the quadratic contrast, high phosphorus supply resulted in negative curvature in the inner chambers and positive curvature in the outer chambers (Fig. 12), while both the magnitude of curvature and the differences between inner and outer chambers were smaller with low phosphorus supply.

Table 11: *P. parvum* early phase split plot ANOVA summary

		Constant		*Linear*		*Quadratic*	
Source	df	MS	*p*	MS	*p*	MS	*p*
Phos	1	3.92×10^{10}	0.52	1.00×10^{11}	**0.038**	1.59×10^{11}	0.27
Error	3	7.45×10^{10}		7.92×10^{9}		8.76×10^{10}	
Chamber	1	1.95×10^{12}	**<0.0001**	3.04×10^{11}	**0.00058**	1.17×10^{12}	**<0.0001**
Treatment	2	8.67×10^{10}	0.094	2.13×10^{10}	0.37	8.31×10^{10}	0.074
Cham×Trtmt	1	1.42×10^{10}	0.66	1.15×10^{11}	**0.0086**	1.20×10^{11}	**0.027**
Cham×Phos	2	4.53×10^{9}	0.71	6.27×10^{9}	0.58	2.47×10^{10}	0.36
Trtmt×Phos	1	5.65×10^{10}	0.21	1.55×10^{11}	**0.0022**	1.36×10^{11}	0.17
Trtmt×Phos×Cham	2	2.16×10^{10}	0.54	3.85×10^{10}	0.172	1.19×10^{10}	0.67
Error	30	3.39×10^{10}		2.06×10^{10}		2.91×10^{10}	

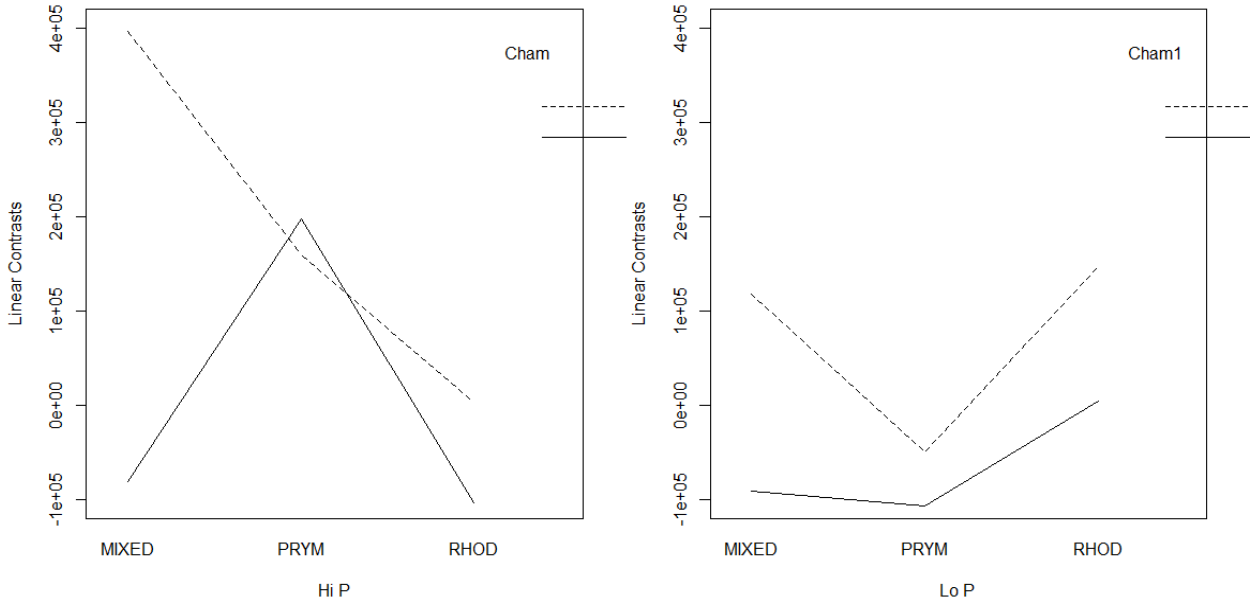


Figure 9: Interaction plot of linear contrasts between experimental treatment (X-axis), average of response variable for the early phase for *P. parvum* (Y-axis), and chamber position.

Table 12: *P. parvum* late phase split plot ANOVA summary

		Constant		*Linear*		*Quadratic*	
Source	df	MS	*p*	MS	*p*	MS	*p*
Phos	1	1.32×10^{14}	**<0.0001**	2.73×10^{13}	**0.0005**	9.17×10^{7}	0.99
Error	3	1.53×10^{11}		1.00×10^{11}		9.76×10^{11}	
Chamber	1	3.08×10^{12}	**<0.0001**	5.41×10^{11}	**0.043**	3.29×10^{12}	**<0.0001**
Treatment	2	7.77×10^{11}	**0.01**	8.83×10^{11}	**0.0027**	3.65×10^{11}	0.53
Cham×Trtmt	1	6.36×10^{11}	**0.021**	1.96×10^{11}	0.22	1.61×10^{11}	0.26
Cham×Phos	2	3.69×10^{11}	0.12	3.41×10^{11}	0.1	1.67×10^{12}	**0.0006**
Trtmt×Phos	1	1.198×10^{12}	**0.0014**	3.11×10^{12}	**<0.0001**	7.99×10^{11}	**0.003**
Trtmt×Phos×Cham	2	5.54×10^{11}	**0.033**	7.50×10^{11}	**0.006**	2.00×10^{11}	0.19
Error	30	1.44×10^{11}		1.21×10^{11}		1.13×10^{11}	

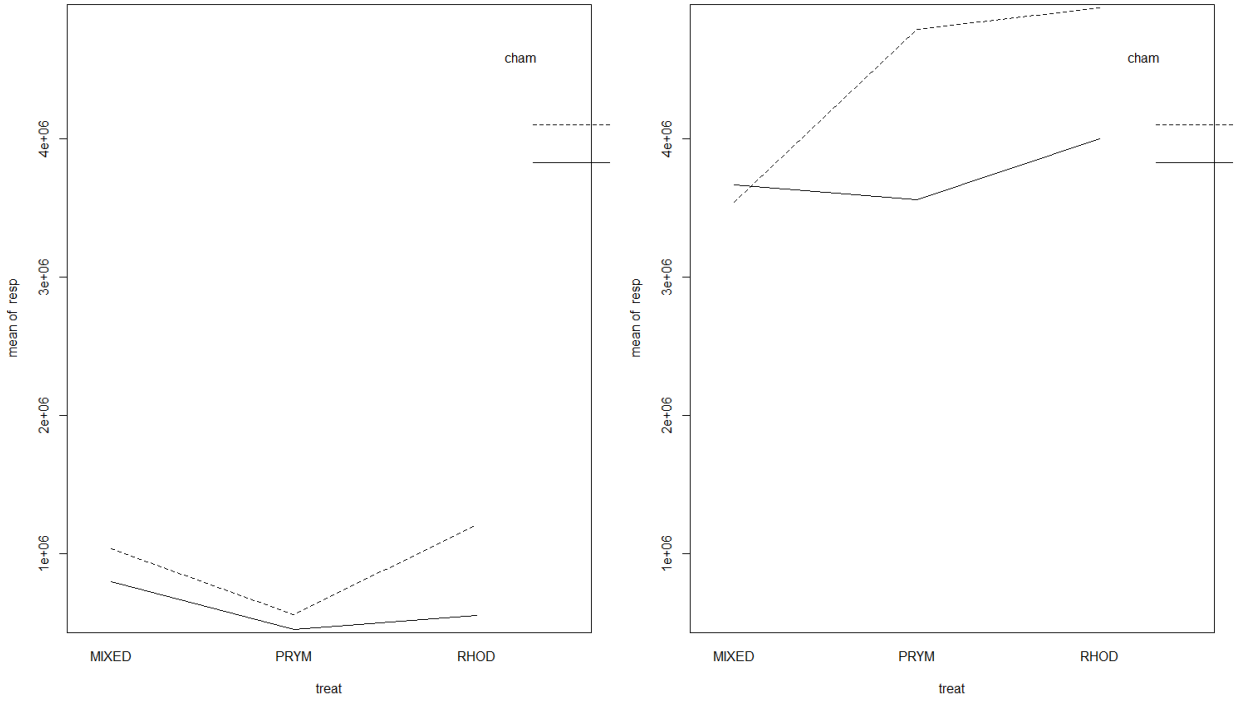


Figure 10: Interaction plot of constant contrasts between experimental treatment (X-axis), average of response variable for the late phase for *P. parvum* (Y-axis), and chamber position.

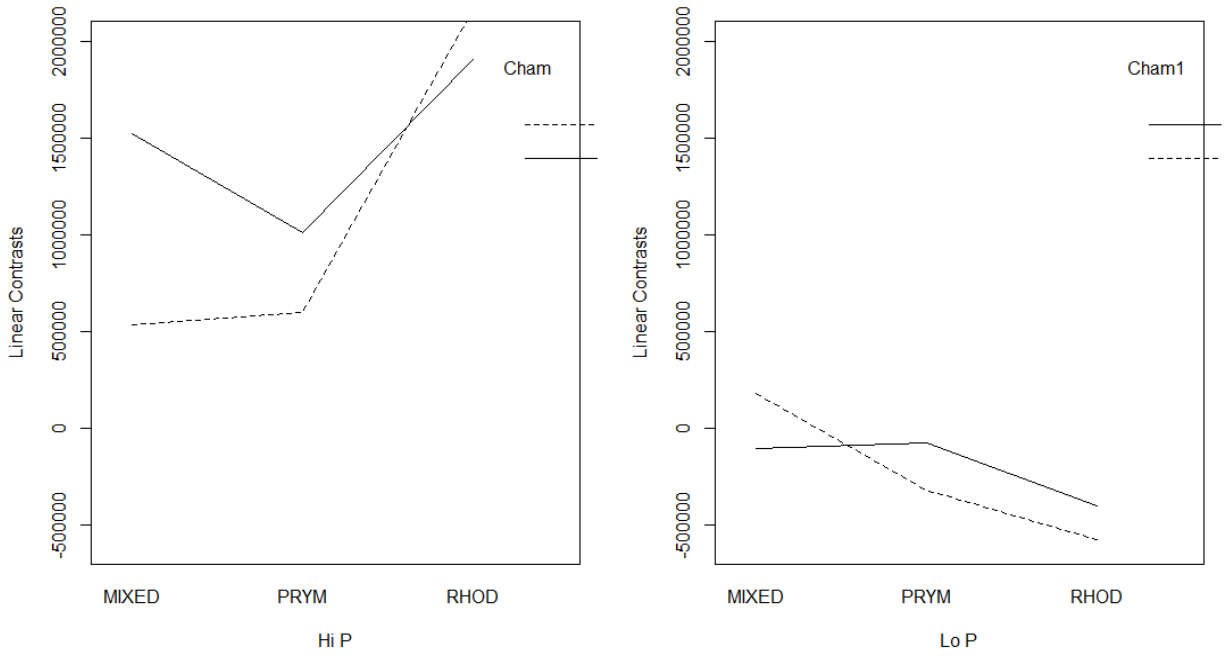


Figure 11: Interaction plot of linear contrasts between experimental treatment (X-axis), average of response variable (Y-axis), and chamber position.

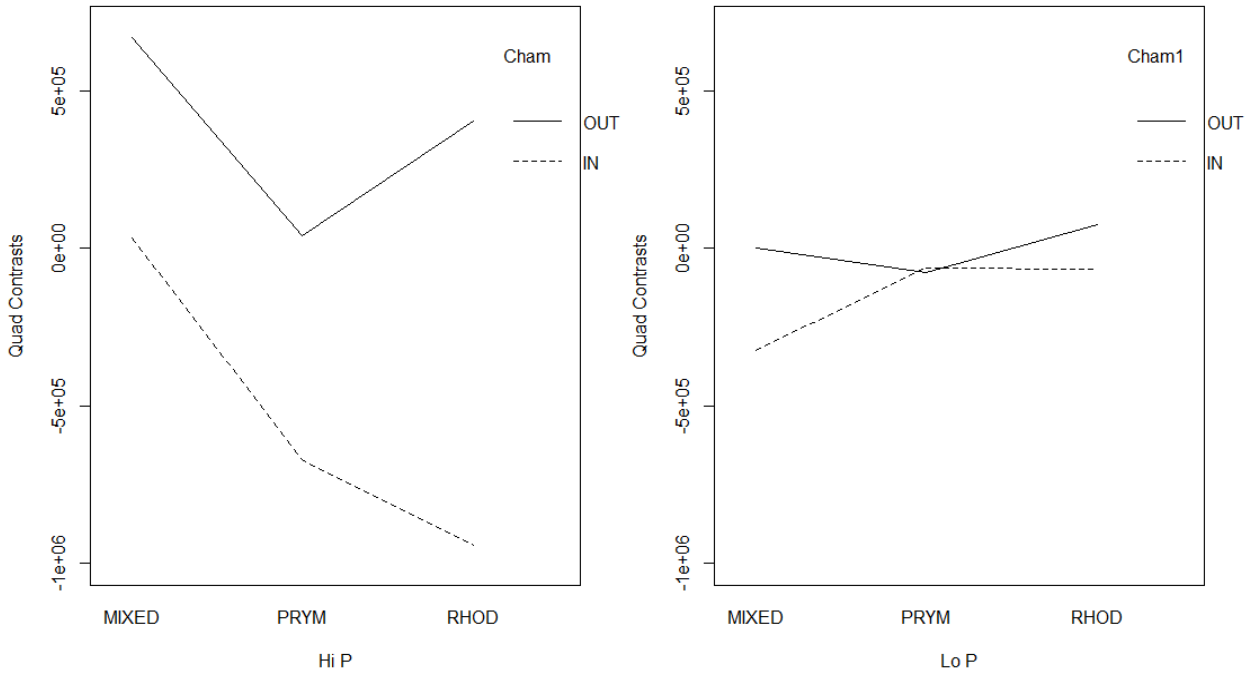


Figure 12: Interaction plot of quadratic contrasts between experimental treatment (X-axis), average of response variable for the late phase for *P. parvum* (Y-axis), and chamber position.

DISCUSSION

This study strongly suggests that direct contact between *P. parvum* and *R. salina* is required for rapid cellular death of the latter to occur. Only those experimental arrangements allowing direct contact, in which *P. parvum* and *R. salina* were together on the same side of the chamber resulted in a rapid decline of cell density for *R. salina*, Experimental arrangements that involved no contact between the two species resulted in a gradual rather than rapid decline of *R. salina* cell density. Phenomena that may be responsible for this gradual decline of cell density could include: a) dissolved toxic substances produced by *P. parvum* (e.g. exotoxins) diffusing slowly through permeable barrier separating the two species in some experimental treatments; b) competition for resources such as dissolved phosphorus diffusing through the permeable barrier; c) a combination of these two mechanisms; or d) a culture dilution rate was slightly higher than the maximal growth rate of *R. salina*. The hypothesis that *P. parvum* constitutively produces dissolved toxins that induce lysis of *R. salina* was rejected. In addition, a more subtle hypothesis was also rejected that intraspecific signaling resulting from direct contact induced production of dissolved toxins that then produce lysis. To ascertain whether nutrient supply would affect the interspecific interactions studied here, experimental treatments were conducted with high and low P supplies. Although *R. salina* cell densities generally differed detectably in relation to P supply, the conclusion that direct contact was responsible for the rapid decline of this target species did not differ with nutrient supply.

Though this study did not describe the exact mechanics of the direct contact effect of *P. parvum*, there are some plausible explanations as to why such contact resulted in a rapid cell death of *R. salina*. Several studies show the negative effects of *P. parvum* on predators, competitors, and potential prey (Fistarol 2003; Tillmann 2003; Barreiro et al. 2005; Remmel and

Hambright 2012). Filtrates and cells extracted from *P. parvum* cultures have both exhibited harmful effects on other planktonic species. However, Remmel and Hambright (2012) showed that high pressure filtration technique creates an artifact of toxicity in cell free extracts. Additionally, they showed that direct contact with fathead minnow larva resulted in 100% mortality for the target species. Other studies have also shown a varied effect between *P. parvum* whole cells and filtrates extracted from *P. parvum* cultures. Lytic effect of *P. parvum* has been shown to be more pronounced with whole cells as opposed to filtrates with target cell *Oxyrrhis marina* (Tillmann 2003). It could be postulated that phagotrophy in *P. parvum* is facilitated by prey cell lysis and cell immobilization via toxic agents deployed on its cell surface (Skovgaard and Hansen 2003).

Phagotrophy, in the case of eukaryotic protists, is a nutritional mode that cells employ to gain access to nutrients (Raven 1997). Evidence has shown that phagotrophy in *P. parvum* is an important nutritional mode whether dissolved inorganic nutrients are high or low in concentration (Carvalho and Graneli 2010). Indeed, *P. parvum* although largely photoautotrophic, may use phagotrophy to obtain access to nutrients, suggesting phagotrophy complements photoautrophy (Jones 2000). *Prymnesium parvum* has employed phagotrophy to neutralize a potential predator, *Oxyrrhis marina*, before consuming it (Tillman 2003). Similar interactions have been shown with *P. parvum* and *R. salina* (Barreiro et al. 2005). The lysis and subsequent uptake particulates may supplement *P. parvum* with additional nutrients. Thus, it is possible that *P. parvum* immobilized and/or consumed *R. salina* cells using direct contact mechanisms (explaining the eventual disappearance of *R. salina*) to obtain to additional nutrients.

If so, the direct nutritional benefits to individuals that induce lysis in adjacent prey cells could resolve some ecological and evolutionary questions concerning the functions of toxicity in *P. parvum*, and perhaps in other harmful algae. Although *R. salina* is a potential competitor of *P. parvum*, the ability to poison this competitor (allelopathy) via dissolved toxins would not have an important effect on the competitor unless *P. parvum* was at a sufficient population density to produce an effective toxin concentration. Thus, production of dissolve toxins would not contribute to growth of *P. parvum* from a sparse to a dominant population, nor would it contribute to formation of harmful blooms (Jonsson et al.). Other events or processes would be needed to permit *P. parvum* to become abundant, though dissolved toxins could perhaps contribute to the maintenance of dominance, once obtained. The evolution of dissolved toxin production can also be undermined by cheater subpopulations, which obtain the benefits of reduced competition without themselves producing the toxins involved. This evolutionary paradox has been raised for *P. parvum* (Driscoll et al. 2013), and is a classical puzzle concerning the world's many toxic microbes (Boenigk and Stadler 2004; Fistarol et al. 2004). Toxins that require direct contact can resolve this puzzle for *P. parvum*, if the nutritional benefit of consuming lysed prey cells accrues primarily to the cell that produced the toxins. If the interpretation offered here is correct, then the relationship between *P. parvum* and *R. salina* is neither competition nor predation, it is instead intraguild predation (Polis et al. 1989), in which both species compete for a resource (e.g. dissolved phosphorus), but one (e.g. *P. parvum*) also preys on the other (*R. salina*).

There was an artifact of the experimental apparatus that was revealed by this study. A chamber (position) artifact was a strong factor affecting both *P. parvum* and *R. salina* abundance. Cells residing in the inner chambers usually had higher population density than the

comparable cells residing in the outer chamber. This difference in abundance may be due to the geometry of the well and insert apparatus used. The outer chambers had a higher surface area to volume ratio than the inner chambers. Cell adherence to the well material may have been exaggerated in outer chambers as opposed to inner chambers, accounting for the artifact. Because of the strong effect of chamber position, there were often interactions between chamber position and the main plot (P supply) or subplot (cell contact) treatments. The artifact of the chamber position, though strong enough to detect, did not negate the consistent results concerning direct contact and lytic toxicity to *R. salina*. Disappearance of this target species always occurred rapidly (< 48 h) then there was direct contact with *P. parvum*, and never occurred otherwise. In addition to this chamber artifact, which was controlled by the experimental design, and accounted for in statistical analysis, other artifacts are possible. Pore size within the membrane separating the target cell from *P. parvum* could have affected the efficacy of any dissolved substances produced by *P. parvum*. If dissolved substances bound to the membrane rather than passing through the membrane, the toxin in question would not have drastic effects on target cells. The dissolved toxin produced by *P. parvum* may aggregate in aqueous solutions, creating filterability issues if pore sizes were smaller than 0.6 µm (Ulitzur 1973). However, since the pore size of the inserts used in the present study was larger than the suggested sizes of the proposed hemolytic and toxic aggregates (Ulitzur 1973), the issue of diffusion through the membrane should not have been problematic. It is also possible that the slow declines of *R. salina* observed late in the experiments in some treatments arose from a culture dilution rate that only slightly exceeded the maximal growth rate of this species in the culture apparatus used. In other culture apparatus, however, *R. salina* has been observed to have a higher maximal growth rate than the dilution rate imposed here.

This study provided strong evidence that fast elimination of the target species, *R. salina*, in the presence of *P. parvum* is heavily dependent upon a direct cell-to-cell contact mechanism of toxic activity. Direct contact between *P. parvum* and victim species might help to resolve ecological and evolutionary questions about the toxic activity displayed by this widespread harmful species. Killing competitors directly, rather than via dissolved toxic substances, could be effective at low population densities of *P. parvum*, thus contributing to the formation of harmful blooms. Additionally, if the killing of competitors by direct contact is associated with a subsequent nutritional benefit to the individual cell responsible, then the evolution of cheaters would be less likely to undermine the production of toxins involved, than it would be if the toxins were dissolved rather than cell-bound. Thus, demonstrating a nutritional benefit of direct contact toxicity is an important next step.

REFERENCES

Barreiro A, Guisande C, Maneiro I, Lien TP, Legrand C, Tamminen T, Lehtinen S, Uronen P, Granéli E. 2005. Relative importance of the different negative effects of the toxic haptophyte *Prymnesium parvum* on *Rhodomonas salina* and *Brachionus plicatilis*. Aquat Microb Ecol 38(3):259-67.

Boenigk J. Stadler P. 2004. Potential toxicity of chrysophytes affiliated with Poteriochromonas and related 'Spumella-like' flagellates. J Plankton Res 26(12):1507-1514

Carvalho WF and Granéli E. 2010. Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency. Harmful Algae 9(1):105-15.

Driscoll WW, Espinosa NJ, Eldakar OT, Hackett JD. 2013. Allelopathy as an emergent, exploitable public good in the bloom-forming microalga *Prymnesium parvum*. Evolution 67(6):1582-90.

Emura A, Matsuyama Y, Oda T. 2004. Evidence for the production of a novel proteinaceous hemolytic exotoxin by dinoflagellate *Alexandrium taylori*. Harmful Algae 3(1):29-37.

Fistarol GO, Legrand C, Granéli E. 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Mar Ecol Prog Ser 255:115-25.

Fistarol GO, Legrand C, Selander E, Hummert C, Stolte W, Granéli E. 2004. Allelopathy in *Alexandrium* spp.: Effect on a natural plankton community and on algal monocultures. Aquat Microb Ecol 35(1):45-56.

Granéli E and Hansen PJ. 2006. Allelopathy in harmful algae: A mechanism to compete for resources? In: Ecology of harmful algae. Springer. 189 p.

Johansson N and Granéli E. 1999. Influence of different nutrient conditions on cell density, chemical composition and toxicity of *Prymnesium parvum* (haptophyta) in semi-continuous cultures. J Exp Mar Biol Ecol 239(2):243-58.

Jones RI. 2000. Mixotrophy in planktonic protists: An overview. Freshwat Biol 45(2):219-26.

Jonsson PR, Pavia H, Toth G. 2009. Formation of harmful algal blooms cannot be explained by allelopathic interactions. Proc Natl Acad Sci USA 106(27):11177-82.

Michaloudi E, Moustaka-Gouni M, Gkelis S, Pantelidakis K. 2009. Plankton community structure during an ecosystem disruptive algal bloom of *Prymnesium parvum*. Journal of Plankton Research 31(3):301-9.

Polis GA, Myers CA, Holt RD. 1989. Intraguild predation: the dynamics of complex trophic interactions. Annu Rev Ecol Syst 20(1):297-330.

Raven J. 1997. Phagotrophy in phototrophs. Limnol Oceanogr 42(1):198-205.

Remmel EJ and Hambright KD. 2012. Toxin-assisted micropredation: Experimental evidence shows that contact micropredation rather than exotoxicity is the role of *Prymnesium* toxins. Ecol Lett 15(2):126-32.

Rosetta CH and McManus GB. 2003. Feeding by ciliates on two harmful algal bloom species, *Prymnesium parvum* and *Prorocentrum minimum*. Harmful Algae 2(2):109-26.

Schmidt LE and Hansen PJ. 2001. Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: Effect of cell concentration, growth phase and pH. Mar Ecol Prog Ser 216:67-81.

Skovgaard A, Legrand C, Hansen PJ, Graneli E. 2003. Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*. Aquat Microb Ecol 31:259-65.

Tillmann U. 2003. Kill and eat your predator: A winning strategy of the planktonic flagellate *Prymnesium parvum*. Aquat Microb Ecol 32(1):73-84.

Ulitzur S. 1973. The amphiphatic nature of *Prymnesium parvum* hemolysin. Biochimica Et Biophysica Acta (BBA) - Biomembranes 298(3):673-9.

Vogelbein WK, Lovko VJ, Shields JD, Reece KS, Mason PL, Haas LW, Walker CC. 2002. *Pfiesteria shumwayae* kills fish by micropredation not exotoxin secretion. Nature 418(6901):967-70.

Xu J, et al. 2012. Effects of inorganic and organic nitrogen and phosphorus on the growth and toxicity of two *Alexandrium* species from Hong Kong. Harmful Algae 16:89-97.

Zhu M and Tillmann U. 2012. Nutrient starvation effects on the allelochemical potency of *Alexandrium tamarense* (dinophyceae). Mar Biol 159(7):1449-59.

CHAPTER 4

PRYMNESIUM PARVUM OBTAINS PHOSPHORUS TO SUPPORT POPULATION GROWTH BY LYSING CELLS OF *RHODOMONAS SALINA*

ABSTRACT

A major factor that is thought to play a role in the competitive edge of *Prymnesium parvum* is the allelopathy demonstrated during blooms. Other traits that *P. parvum* has demonstrated to support its competitive edge include mixotrophy. The aim of this study was to quantify the benefits of mixotrophy for population growth in *P. parvum*. An experiment was conducted with semicontinuous cultures in which *P. parvum* was supplied with limiting phosphorus, either as orthophosphate in sterile medium, or as an equivalent amount of total phosphorus in the form of a culture of the cryptophyte *Rhodomonas salina*. Cells of the latter species were rapidly lysed after transfer to *P. parvum* cultures. Dissolved reactive P was much lower than the supplied concentration, regardless of the source provided, and P quota was higher in the *P. parvum* cells fed *R. salina*, demonstrating consumption. Even though the supply of total P fed to *P. parvum* in the form of *R. salina* was comparable to the concentration of inorganic P in cultures fed sterile medium, the population growth rates from the different P sources differed. Growth was positive but slower for *P. parvum* when it was fed *R. salina* as the major source of P that was obtained through mixotrophy.

INTRODUCTION

Golden algal blooms have had significant impacts on the economy by affecting recreational activities in major reservoirs (Oh and Ditton 2005). Though there is little evidence that golden algal species such as *Prymneisum parvum* are harmful to human health, they have been financially costly by affecting large construction projects (Linkov et al. 2009). Many toxic blooming algal species have been linked to fish kills (Bourdelais et al. 2002; Robineau et al. 1991). Golden alga has demonstrated many negative effects in planktonic communities. Cell free filtrates of *P. parvum* cause declines of abundance in cyanobacteria, dinoflagellates, diatoms, and nanoflagellates (Fistarol et al. 2003). Dinoflagellates such as *Alexandrium* spp. have also demonstrated a similar impact in diverse planktonic communities (Fistarol et al. 2004). Reduction in top-level control of the food chain along with a decrease in biodiversity can cause major shifts in natural ecosystems in addition to economic impacts (Hoagland and Scatasta 2006). *Prymnesium parvum* is the focus of this paper, as it shows the combination of toxicity, allelopathy, and mixotrophy.

A major factor that is thought to play a role in the competitive edge of *P. parvum* is the allelopathy demonstrated during blooms. Nutrient-limited conditions (nitrogen and phosphorus) are major factors in the toxigenic and hemolytic activity of *P. parvum* (Granéli and Johansson 2003). Indeed, *P. parvum* may lyse competing species with toxic substances releasing dissolved nutrients when nutrients are limited (Uronen et al. 2007). Though the evidence is overwhelming in showing the allelopathic nature of *P. parvum*, there is also evidence that suggests allelopathy may be a mere "side-effect" of *P. parvum* blooms. Filtration techniques to generate cell-free filtrates of *P. parvum* may create toxigenic artifacts that are a result of high pressure during filtration (Remmel and Hambright 2012). Furthermore, hemolytic activity in cultures of *P.*

parvum is not strong unless cultures are already at a high density, suggesting that *P. parvum* may use other traits to become competitive initially (Jonsson et al. 2009).

Other traits that *P. parvum* has demonstrated to support its competitive edge include mixotrophy. Mixotrophy has been demonstrated in many protists that take up nutrients via photosynthetic processes in addition to phago- and osmotrophy (Sanders and Porter 1988). Cells of *P. parvum* can rapidly kill and ingest potential grazers, effectively evading predatory species (Tillmann 2003). Phagotrophy and osmotrophy may also be important modes of obtaining nutrients when dissolved inorganic nutrient concentration is low (Carvalho and Granéli 2010). Understanding the modes of nutrition that prevail is key in understanding the bloom dynamics during disruptive events that affect ecosystems and the economy.

Though much evidence has shown that *P. parvum* exhibits mixotrophy in nutrient limited conditions, few studies directly quantify the benefits to population growth that result (Legrand 2001; Tillmann 2003). The aim of this study was to do so. An experiment was conducted with semicontinuous cultures in which *P. parvum* was supplied with limiting phosphorus, either as orthophosphate in sterile medium, or as an equivalent amount of total phosphorus in the form of a culture of the cryptophyte *R. salina*. This prey species is susceptible to rapid lysis in the presence of *P. parvum* (Laws and Grover 2019), and it is likely that complex phosphorus substrates become available for consumption. The population dynamics of *P. parvum* were then determined under these two nutritional conditions. Because concentrations of particulate P and soluble reactive P (SRP) were measured at different time points during the growth of *P. parvum*, it was also possible to calculate cell quotas of cultures that received the two different forms of nutrition. In this manner, it is possible to understand the contribution (if any) of mixotrophy plays to population growth and bloom activity of *P. parvum*.

METHODS

Preparation of Batch Cultures

Stock cultures of *Prymnesium parvum* (obtained from UTEX culture collection, Austin, Texas, UTEX LL 2797) and *Rhodomonas salina* (obtained from UTEX culture collection, Austin, Texas, LB 2763) were maintained in separate batch culture 1L Erlenmeyer flasks in f/2 growth medium (Guillard and Ryther 1962) modified to N = 882 µM and P = 1.5 µM, to give an N:P ratio ca. 588:1. Salinity was maintained at 5.0 practical salinity units, using artificial seawater (Baker et al. 2007). Culture flasks were maintained at 20°C under a light:dark regime of 12:12 h, with an irradiance of 150 µmol photons m^{-2} s^{-1}. Cultures were grown to stationary phase.

Semicontinuous Cultures of *R. salina*

Two 720 ml cultures of *R. salina* were set up in one-liter Erlenmeyer flasks using the same f/2 growth medium as described for batch cultures. External conditions (temperature, light:dark regime) were the also the same as the conditions for batch cultures. Semicontinuous cultures were inoculated with an estimated 20,00 cell ml^{-1}. The cultures were diluted daily for 17 days, with 240 ml withdrawn each day resulting in a 0.405 d^{-1} dilution rate. The volume removed was used to estimate dissolved phosphorus (50 ml), cell density (10 ml), and for feeding to *P. parvum cultures* (180 ml).

Semicontinuous Cultures of *P. parvum*

Two different semicontinuous *P. parvum* cultures were set up: one stage cultures and two stage cultures. For both types of *P. parvum* cultures, external conditions (e.g. temperature, light: dark regime) were the same as those used for batch cultures. Medium composition for the one

stage cultures was also the same as that used for batch cultures. However, medium for the two stage cultures had no phosphorus added.

Duplicate one-stage 180 ml *P. parvum* semicontinuous were cultivated in 0.5L Erlenmeyer flasks. Cultures were inoculated with an estimated 20,000 cell ml^{-1}. One stage cultures were started when *R. salina* semicontinuous cultures were 3 days old. The cultures were diluted daily for 14 days, with 60 ml withdrawn each day. Triplicate two stage 180 ml *P. parvum* semicontinuous were also cultivated in 0.5 Erlenmeyer flasks. Cultures were inoculated with an estimated 20,000 cell ml^{-1}. Two stage cultures were started when *R. salina* semicontinuous cultures were 7 days old. The two-stage cultures were diluted daily for 10 days, with 60 ml withdrawn each day, and replaced with 60 ml of *R. salina* semicontinuous culture. Only one replicate of *R. salina* semicontinous culture was used to feed the *P. parvum* two-stage cultures. The volume removed from both one and two stage semicontinuous cultures was used to estimate dissolved phosphorus (50 ml), and cell density (10 ml).

Estimation of Cell Density

Cell densities were estimated by counting > 450 cells in Lugol's fixed samples in a Sedgewick-Rafter counting cell. Cell densities of *R. salina* were estimated when cultures were 1, 2, 4, 8, 9, 11, 14, 16 and 17 days old. Cell densities of *P. parvum* one stage cultures were estimated when cultures were 1, 3, 5, 6, 9, 11, 13, and 14 days old. Cell densities of two stage *P. parvum* cultures were estimated when cultures were 1, 2, 4, 7, 9, 10 days old.

Estimation of Dissolved Phosphorus

Periodically, 50 ml portions of the volume that was withdrawn from semicontinuous cultures were filtered (0.2um) for soluble reactive phosphorus (SRP) analysis (Strickland and

Parsons 1972). On the last day of the experiment, 50 ml samples extracted from all semicontinous cultures were filtered onto GF/F filters for particulate phosphorus analysis using persulfate digestion (Menzel and Corwin 1965).

RESULTS

Cell Density and Growth Rate of *P. parvum* and *R. salina*

In semicontinuous culture setups, cell densities generally increased throughout the duration of the experiment. One stage *P. parvum* cultures were initiated when *R. salina* cultures were three days old, and two stage *P. parvum* cultures were initiated when *R. salina* cultures were seven days old (Figure 1, top). All cell cultures were inoculated with an initial estimated cell density of 20,000 cell ml^{-1}. Cultures of *R. salina*. reached a cell density of approximately 59,000 cell ml^{-1} (SE ± 353, n = 2) 17 days after inoculation. One stage cultures of *P. parvum* reached a cell density of approximately 184,000 cell ml^{-1} (SE ± 22980, n = 2) 14 days after inoculation. Two stage cultures of *P. parvum* cultures reached a cell density of 58,200 cell ml^{-1} (SE ±10356, n =3) 10 days after inoculation. Cell densities of *R. salina* were not enumerated in two stage cultures because *P. parvum* cells lysed all cells within a few hours of being in contact with *P. parvum*, therefore cell density estimates of *R. salina* within two stage *P. parvum* cultures are not reported. Population growth rates were calculated for all cultures (Table 1), by regressing ln of abundance versus time, and adding the culture dilution rate. The growth rates calculated from the one and two stage were significantly different from each other (Welch's t-test, $p \approx 0.0088$).

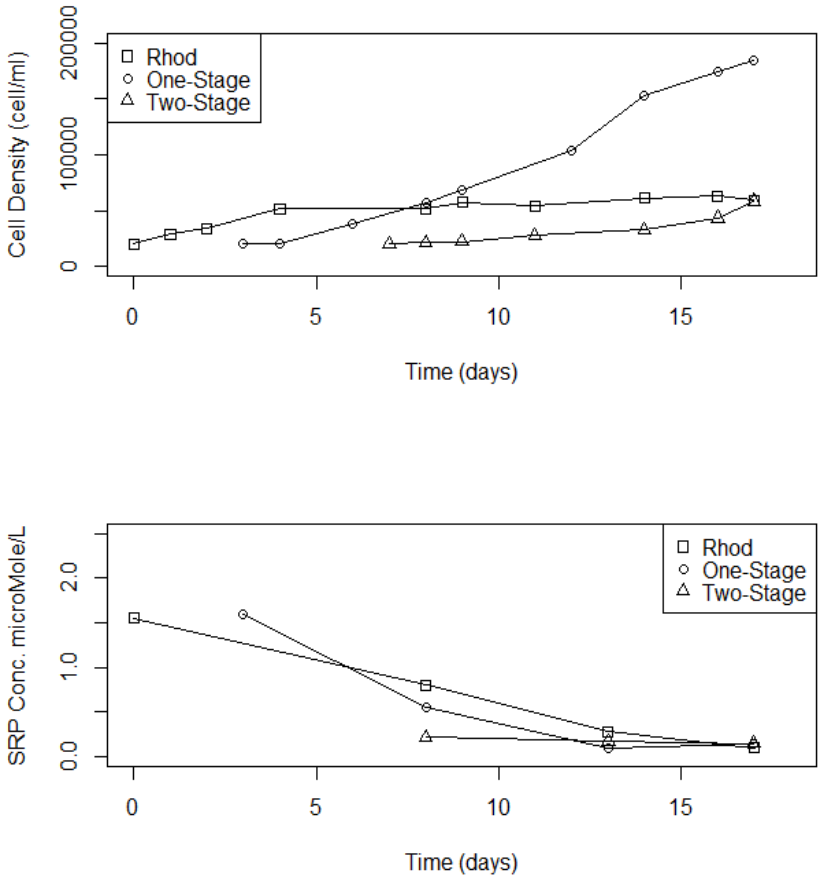


Figure 1: Plots of cell density against time (top) and SRP against time (bottom). Results are expressed as means (Rhod, *n* = 2; Prym one-stage, *n* = 2; Prym two-stage, *n* = 3).

Table 1: Calculated growth rates of individual replicates of *R. salina, P. parvum (one stage), and P. parvum (two-stage)* in each culture. All values are reported with units day^{-1}.

	R. salina	*P. parvum* (one stage)	*P. parvum* (two stage)
Replicate 1	0.46	0.57	0.51
Replicate 2	0.46	0.56	0.49
Replicate 3			0.49
Mean ± SE	**0.46 ± 0.0015**	**0.57 ± 0.0069**	**0.50 ± 0.0066**

Phosphorus Concentration

Generally, as cell densities increased, concentration of dissolved inorganic phosphorus (SRP) decreased (Figure 1, bottom). In *R. salina* cultures, the SRP concentration available at inoculation was estimated to be 1.55 µmol/L (analysis of uninoculated f/2 medium) and decreased to 0.102 µmol/L (SE ±0.0087, $n = 2$) over the course of 17 days. In one stage cultures of *P. parvum*, the available estimated SRP concentration was 1.6 µmol/L (analysis of uninoculated f/2 medium) that decreased to 0.14 µmol/L (SE ±0.026, $n = 2$) over the course of 14 days. Two stage cultures were inoculated in medium containing no dissolved phosphorus; however, the inoculum was suspected to contain a small concentration of SRP, thus the phosphorus concentration was measured one day after inoculation. The concentration of SRP of two stage culture was measured to be 0.22 µmol/L (SE ±0.018, $n = 3$) one day after inoculation and 0.15 µmol/L (SE ±0.041, $n = 3$) 10 days after inoculation. Particulate phosphorus concentrations were measured at the end of the experiment for all cultures. Measurements of soluble reactive phosphorus and particulate phosphorus on the last day of the experiment were

added together to estimate the total phosphorus in each culture at the end of the experiment (Fig 2). Cell quotas were calculated using the particulate phosphate measurements (Table 2). The differences in cell quotas between one and two stage cultures was marginally significant (Welch's t-test, $p \approx 0.025$).

Table 2: Cell quotas of individual replicates. All values are reported with units µmol cell^{-1}.

	R. salina	*P. parvum* (one stage)	*P. parvum* (two stage)
Replicate 1	2.84×10^{-8}	8.54×10^{-9}	3.13×10^{-8}
Replicate 2	2.29×10^{-8}	8.58×10^{-9}	2.24×10^{-8}
Replicate 3			3.30×10^{-8}
Mean ± SE	$\mathbf{2.57 \times 10^{-8} \pm 2.76 \times 10^{-9}}$	$\mathbf{8.56 \times 10^{-9} \pm 1.49 \times 10^{-11}}$	$\mathbf{2.89 \times 10^{-8} \pm 3.29 \times 10^{-9}}$

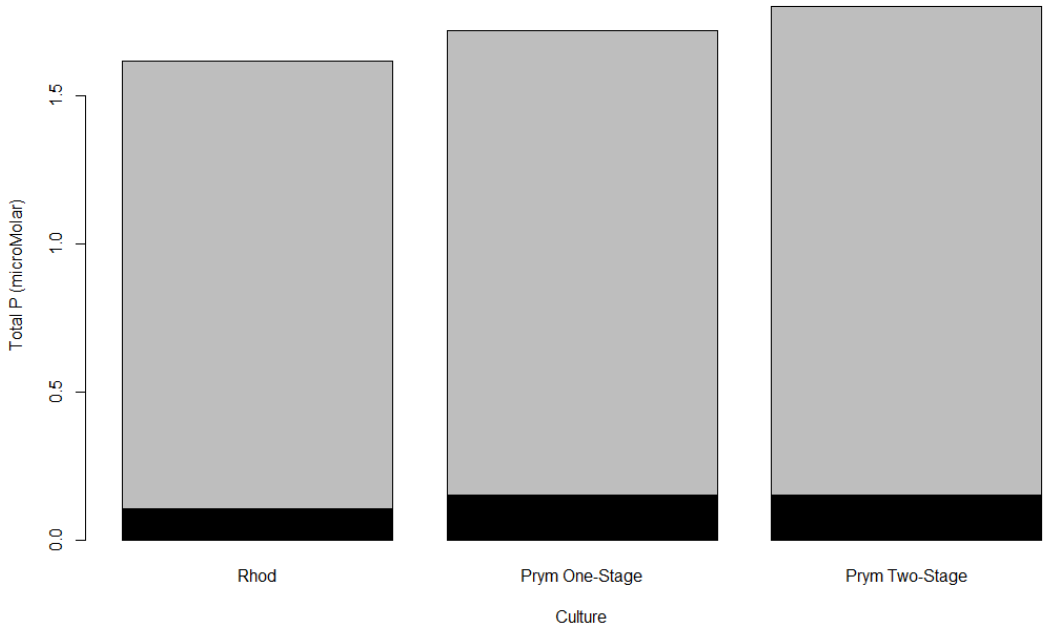


Figure 2: Stacked bar plot of SRP (black) and PP (grey). Values of SRP and PP are measured from the last day of each culture. Results are expressed as means (Rhod, n = 2; Prym one-stage, n = 2; Prym two-stage, n = 3)

DISCUSSION

This study demonstrated that *P. parvum* can use *R. salina* cells as the major source phosphorus (in the form of particulate P) to support population growth. When *R. salina* cells were fed to *P. parvum*, they were rapidly lysed, and converted to particulate P in *P. parvum* cells. However, even though the supply of P fed to *P. parvum* in the form of *R. salina* was comparable to the concentration of inorganic P in cultures fed sterile medium, the population growth rates from the different P treatments differed. Growth was slower for *P. parvum* when it was fed *R. salina* as the major source of P. This study along with several others, thus supports the current concept of *P. parvum* as a mixotroph (Brutemark and Granéli 2011; Skovgaard et al. 2003; Tillmann 2003). In quantifying the rates of growth from mixotrophic and autotrophic nutrition, it suggests further that mixotrophy is costly compared to autotrophy.

Though it is evident that *P. parvum* can assimilate inorganic P efficiently, there is also evidence that *P. parvum* and many other plankton species can utilize phosphate extracted from organic sources (Huang and Hong 1999). However, the study cited dealt with sources of organic phosphorus that were dissolved rather than insoluble. Because the source of organic phosphorus in this study was already incorporated in the target species, *R. salina*, it is assumed that cells of *P. parvum* would have to a) lyse *R. salina* cells to get access to P or b) ingest *R. salina* cells partially or wholly.

Lines of evidence suggest that *P. parvum* can lyse and ingest target cells. Diatoms and some heterotrophic dinoflagellates are lysed by *P. parvum*; particulates of the lysates were then ingested either partially or wholly by *P. parvum* cell aggregates (Martin-Cereceda, Novarino, Young 2003; Tillmann 2003). To accommodate for the presence of prey cells, *P. parvum* may shift certain patterns of gene expression to take advantage of nutrition present (Liu et al. 2015).

Though gene expression is not a direct correlate to protein expression and activity, it may explain why certain enzymes, such as alkaline phosphatase are more expressed in the presence of potential prey species. Though the expression and presence of the enzyme alkaline phosphatase was not measured in this study or the former studies, there is evidence alkaline phosphatase located in or near cell membranes is used to allow algae to absorb sources organic P (Bentzen, Taylor, Millard 1992; Huang and Hong 1999).

Though *P. parvum* has been linked to mixotrophy by the lysis and ingestion of potential prey species, it has not been clear what benefit, if any, is provided by the presence of prey in mixed cultures. Cultures of *P. parvum* showed no apparent increase in growth rate when in mixed cultures with the ciliate *Uronema marina* (Liu et al. 2015). Though these ciliates induced a change in gene expression, there was no evidence of the ciliate cell abundance after incubation with *P. parvum*, thus it is not known whether *P. parvum* lysed or consumed the ciliates for nutritional gain. Additionally, nutrient conditions (e.g. high or low concentrations of P) do not appear to cause a difference in feeding rate of *P. parvum* on *Rhodomonas baltica* (Skovgaard et al. 2003). Contrary to the previous studies, the present study revealed that there is a nutritional benefit to *P. parvum* from heterotrophic feeding. As such, direct nutritional benefits to individuals producing toxins may provide a resolution to the evolutionary public goods problem with dissolved allelochemicals (Lewis 1986; Driscoll et al 2013). Feeding on organic/particulate live matter may be mediated by toxic substances, though these substances are not necessarily dissolved. If toxic substances remain on or near the cell surface of *P. parvum*, target cells would have to come in close contact to result in lysis. This lysis results in gang feeding and particulate matter uptake, allowing *P. parvum* to have access to internal nutrient stores of *R. salina*. Interestingly, cultures of *P. parvum* fed *R. salina* obtained higher calculated cell quotas as

opposed to *P. parvum* cultures diluted with inorganic medium. According to Raven (1997), mixotrophs may have higher cell quotas due to the regulation of metabolic processes necessary for both photoautotrophy and a heterotrophic mode of nutrition. Larger cell quotas should result in higher population growth rates (Droop 1973). Because the results of the present study did not reveal such higher growth, it is possible that the calculation for the cell quotas are inaccurate. For example, possibly *P. parvum* did not incorporate all the particulate phosphorus into the cell, leaving detritus that was measured as particulate P and attributed to *P. parvum* cells when calculating their quotas.

In conclusion, mixotrophy is a mode of nutrition *P. parvum* employs against susceptible competitor species such as *R. salina*. Organic sources of phosphorus found in such victims do provide a source of nutrients that would be otherwise limiting. It is possible that the presence of susceptible prey items, such as *R. salina*, induced a series of gene expression changes allowing *P. parvum* to lyse and then feed on target species to gain access to limiting nutrients, though not without costs. Cell abundances increased slower in mixed cultures as compared to monocultures, though the concentration of phosphorus was comparable in both types of *P. parvum* cultures. The induction of gene expression and protein synthesis may be energetically costly. Changes may be necessary not only to lyse and eat *R. salina* cells, but to incorporate a different supply of phosphorus into *P. parvum* metabolism, also resulting in longer doubling times. Nonetheless, being a toxic mixotroph gives *P. parvum* the competitive edge to survive and thrive in complex planktonic communities, thus making it difficult to eliminate from food webs harmed by its presence.

REFERENCES

Baker JW, Grover JP, Brooks BW, Ureña-Boeck F, Roelke DL, Errera R, Kiesling RL. 2007. Growth and toxicity of *Prymnesium parvum* (haptophyta) as a function of salinity, light, and temperature 1. J Phycol 43(2):219-27.

Bentzen E, Taylor WD, Millard ES. 1992. The importance of dissolved organic phosphorus to phosphorus uptake by limnetic plankton. Limnol Oceanogr 37(2):217-31.

Bourdelais AJ, Tomas CR, Naar J, Kubanek J, Baden DG. 2002. New fish-killing alga in coastal delaware produces neurotoxins. Environ Health Perspect 110(5):465-70.

Brutemark A and Granéli E. 2011. Role of mixotrophy and light for growth and survival of the toxic haptophyte *Prymnesium parvum*. Harmful Algae 10(4):388-94.

Driscoll WW, Espinosa NJ, Eldakar OT, Hackett JD. 2013. Allelopathy as an emergent, exploitable public good in the bloom-forming microalga *Prymnesium parvum*. Evolution 67(6):1582-90.

Droop MR. 1974. The nutrient status of algal cells in continuous culture. J Mar Biol Assoc U K 54(04):825.

Fistarol GO, Legrand C, Selander E, Hummert C, Stolte W, Granéli E. 2004. Allelopathy in *Alexandrium* spp.: Effect on a natural plankton community and on algal monocultures. Aquat Microb Ecol 35(1):45-56.

Granéli E and Johansson N. 2003. Effects of the toxic haptophyte *Prymnesium parvum* on the survival and feeding of a ciliate: The influence of different nutrient conditions. Mar Ecol Prog Ser 254(4).

Guillard RR and Ryther JH. 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* hustedt, and *Detonula confervacea* (cleve) gran. Can J Microbiol 8(2):229-39.

Hoagland P and Scatasta S. 2006. The economic effects of harmful algal blooms. In: Ecology of harmful algae. Springer. 391 p.

Huang B and Hong H. 1999. Alkaline phosphatase activity and utilization of dissolved organic phosphorus by algae in subtropical coastal waters. Mar Pollut Bull 39(1-12):205-11.

Legrand C. 2001. Phagotrophy and toxicity variation in the mixotrophic *Prymnesium patelliferum* (haptophyceae). Limnol Oceanogr 46(5):1208-14.

Lewis Jr WM. 1986. Evolutionary interpretations of allelochemical interactions in phytoplankton algae. Am Nat 127(2):184-94.

Linkov I, Satterstrom F, Loney D, Steevens J. 2009. The Impact of Harmful Algal Blooms on USACE Operations.

Liu Z, Jones AC, Campbell V, Hambright KD, Heidelberg KB, Caron DA. 2015. Gene expression in the mixotrophic prymnesiophyte, *Prymnesium parvum*, responds to prey availability. Frontiers in Microbiology 6:319.

Martin-Cereceda M, Novarino G, Young JR. 2003. Grazing by *Prymnesium parvum* on small planktonic diatoms. Aquat Microb Ecol 33(2):191-9.

Menzel DW and Corwin N. 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation 1. Limnol Oceanogr 10(2):280-2.

Remmel EJ and Hambright KD. 2012. Toxin-assisted micropredation: Experimental evidence shows that contact micropredation rather than exotoxicity is the role of *Prymnesium* toxins. Ecol Lett 15(2):126-32.

Robineau B, Gagné J, Fortier L, Cembella A. 1991. Potential impact of a toxic dinoflagellate (*Alexandrium excavatum*) bloom on survival of fish and crustacean larvae. Mar Biol 108(2):293-301.

Sanders RW and Porter KG. 1988. Phagotrophic phytoflagellates. In: Advances in microbial ecology. Springer. 167 p.

Skovgaard A, Legrand C, Hansen PJ, Graneli E. 2003. Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*. Aquat Microb Ecol 31:259-65.

Strickland JD and Parsons TR. 1972. A practical handbook of seawater analysis.

Tillmann U. 2003. Kill and eat your predator: A winning strategy of the planktonic flagellate *Prymnesium parvum*. Aquat Microb Ecol 32(1):73-84.

Uronen P, Kuuppo P, Legrand C, Tamminen T. 2007. Allelopathic effects of toxic haptophyte *Prymnesium parvum* lead to release of dissolved organic carbon and increase in bacterial biomass. Microb Ecol 54(1):183-93.

CHAPTER 5

GENERAL CONCLUSION

To understand the purpose of the toxigenic nature of *Prymnesium parvum*, two questions were asked: how is toxicity mediated within *P. parvum*, and does toxicity promote a nutritional benefit through mixotrophy? Though several species of harmful algae do display evidence of toxicity, clarification is needed to understand if the toxicity is a result of dissolved substances as seen in allelopathy, or direct cell-to-cell contact mechanisms. Though evidence has shown dissolved substances negatively affect potential competitors through the use of cell-free filtrates extracted from *P. parvum* (Fistarolet al. 2003), high pressure filtration has been shown to create toxic artifacts (Remmel and Hambright 2012). This was also possible in the first experimental chapter. Furthermore, allelopathy may be difficult to discern from resource competition (Del Moral 1997). Indeed, resource competition and allelopathy may occur concomitantly in natural settings making its effects difficult to parse out from resource competition. Additionally, the physical constraint of a well-mixed aquatic environment makes it difficult for a producer of dissolved toxins to achieve a concentration that is effective against competitors (Lewis 1986). Regarding mixotrophy, *P. parvum* has demonstrated evidence of phagotropy and heterotrophy in addition to photoautotrophy (Carvalho and Granéli 2010; Skovgaard et al. 2003). Until now however, it was unknown if *P. parvum* populations could be sustained with the limiting nutrient phosphorus available in largely in organic form.

This dissertation showed that direct cell-to-cell contact is required for the lysis of the potential competitor *Rhodomonas salina* in the presence of *P. parvum*. Only *R. salina* cells in direct interspecific contact conditions showed rapid cell lysis and eventual disappearance prior to the end of laboratory experimentation. Cells of *R. salina* in interspecific no-contact configurations did not show the same level of lysis or the same level of cell disappearance, suggesting that allelopathic interactions via dissolved substances are weak if present and indiscernible from resource competition.

Another potential competitor, *Scenedesmus obliquus*, was insensitive to the presence of *P. parvum* whole cells. *Scenedemsus obliquus* cells, are structurally different from *R. salina*, as cells belonging to the *Scenedesmus* genus possess rigid exterior cellular integrity (Burczyk and Dworzanski 1988). Compounds produced by *P. parvum* may be ineffective against cells with such cellular structure. Likewise, the exterior cellular integrity of *S. obliquus* may also prove difficult prey for *P. parvum* to consume. This was not the case with *R. salina*. Disappearance of *R. salina* within mixed cultures highly suggested that *R. salina* was prey for *P. parvum*. When *P. parvum* was provided *R. salina* as the major source of phosphorus in mixed cultures, *P. parvum* growth was sustained, albeit at a lower growth rate compared cultures of *P. parvum* grown with sources of inorganic phosphorus dissolved in the aqueous medium. Costs associated with mixotrophy often include lower initial growth rates, possibly due to induction of genetic changes in response to prey availability to allow mixotrophy within *P. parvum* (Liu et al. 2015).

Mechanisms associated with direct cellular contact facilitate predation by *P. parvum* on *R. salina.* Though both species occupy similar niches, the complexity of their competitor-prey relationship suggests the presence of asymmetric intraguild predation (Polis et al. 1989). That is,

between *P. parvum* and *R.salina*, *P. parvum* is always the predator and *R. salina* is always the prey. In addition to sustaining population growth, intraguild predation within *P. parvum* may allow for a local limiting resource, such as phosphorus or nitrogen, to be freed by reducing the potential competition from exploiting the limiting resource, though it is not clear whether this is the adaptive function of intraguild predation or merely a beneficial byproduct. Either way, intraguild predation via direct contact provides a solution to public goods problem that is found with allelopathy in aquatic environments, in which cheaters may benefit from toxicity, without the cost of production. Intraguild predation directly increases fitness of the individual cell rather than the fitness of whole population or cheaters that may exist within a local population, since direct cell-to-cell interaction is required for toxicity and facilitating a predatory response. This type of predation would also explain how small populations of toxigenic *P. parvum* are able to bloom without invoking allelopathy mediated via dissolved substances (Jonsson et al. 2009).

Toxicity is a key factor in the growth dynamics of *P. parvum*, however the assumptions behind the understanding of toxicity may need additional revision. Allelopathy, although an important phenomenon demonstrated in terrestrial natural settings, is mechanistically and theoretically inefficient in aquatic settings, especially when abiotic factors such as turbidity and irradiance are taken into account (Jonsson et al. 2009). As demonstrated in Chapter 3, cultures of *R. salina* experienced a rapid loss in cell density when in direct contact with *P. parvum*. Cultures of *R. salina* separate from *P. parvum* but in the same culture well, persisted with a minute decline near the end of experimentation, indicating either *P. parvum* cell densities needed to increase to an effective size before becoming allelopathic or allelopathy was a slow-acting process from the beginning of experimentation. The accompanying disappearance of *R. salina* strongly suggests that cell-to-cell contact not only enables the toxigenic nature of P. *parvum*, but

also enables mixotrophy that in turn facilitates asymmetric intraguild predation. Though mixotrophy is not without costs, growth within *P. parvum* is sustained when phosphorus is primarily available in organic particulate form indicating mixotrophy may be an important means of obtaining nutrition. Mixotrophy may also be an initiating factor in bloom activity. In chapter 2, *P. parvum* reaches higher cell densities in mixed cultures as compared to *P. parvum* monocultures, strongly suggesting that *R. salina* (or easy prey similar to *R. salina*), would provide a source of nutrition, in addition to the inorganic forms of nutrition already provided by the culture medium.

Because direct cellular contact and mixotrophy were such important roles in the dominance of *P. parvum* as a competitor, these processes may need to be more closely examined in other harmful alga bloom species. Many of the species that form harmful blooms in coastal, marine systems are eukaryotes with known capabilities for mixotrophy. Toxicity to other microbes, including potential competitors in the phytoplankton, might often involve direct cellular contact followed by consumption for mixotrophic nutrition. If so, intraguild predation would be an important factor structuring plankton communities where harmful algal blooms arise.

REFERENCES

Burczyk J and Dworzanski J. 1988. Comparison of sporopollenin-like algal resistant polymer from cell wall of *Botryococcus, Scenedesmus* and *Lycopodium clavatum* by GC-pyrolysis. Phytochemistry 27(7):2151-3.

Carvalho WF and Granéli E. 2010. Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency. Harmful Algae 9(1):105-15.

Del Moral R. 1997. Is separating resource competition from allelopathy realistic? The Botanical Review 63(3):221-30.

Fistarol GO, Legrand C, Granéli E. 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Mar Ecol Prog Ser 255:115-25.

Jonsson PR, Pavia H, Toth G. 2009. Formation of harmful algal blooms cannot be explained by allelopathic interactions. Proc Natl Acad Sci USA 106(27):11177-82.

Lewis Jr WM. 1986. Evolutionary interpretations of allelochemical interactions in phytoplankton algae. Am Nat 127(2):184-94.

Liu Z, Jones AC, Campbell V, Hambright KD, Heidelberg KB, Caron DA. 2015. Gene expression in the mixotrophic prymnesiophyte, *Prymnesium parvum*, responds to prey availability. Frontiers in Microbiology 6:319.

Polis GA, Myers CA, Holt RD. 1989. The ecology and evolution of intraguild predation: Potential competitors that eat each other. Annu Rev Ecol Syst 20(1):297-330.

Remmel EJ and Hambright KD. 2012. Toxin-assisted micropredation: Experimental evidence shows that contact micropredation rather than exotoxicity is the role of *Prymnesium* toxins. Ecol Lett 15(2):126-32.

Skovgaard A, Legrand C, Hansen PJ, Graneli E. 2003. Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*. Aquat Microb Ecol 31:259-65.