Regulation of growth and toxicity of a mixotrophic microbe: implications for understanding range expansion in *Prymnesium parvum*

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Abstract: The rapid range expansion of the toxigenic marine haptophytic alga *Prymnesium parvum* in inland aquatic systems across the southern USA and beyond has prompted great interest in the ecology and evolutionary biology of this invasive bloom-forming species. Researchers have speculated that increased toxicity and heterotrophy in suboptimal environments allow blooms to develop in these new inland habitats that seem to represent extremes relative to *P. parvum*'s perceived optimal niche. We used a laboratory-based study to elucidate the roles of salinity and nutrient availabilities in *P. parvum* growth and toxicity under environmental conditions representative of hypereutrophic reservoirs of the southwestern USA in which *P. parvum* blooms are now common. We found evidence that nutrient conditions favoring toxigenesis in *P. parvum* are suboptimal for growth and bloom formation, whereas conditions conducive to high growth rates are less favorable for toxigenesis. In contrast, both growth and toxicity scaled positively with salinity. Taken in the context of the documented biogeography of *P. parvum* and that toxigenesis in *P. parvum* probably evolved in support of heterotrophic nutrient acquisition, our results suggest that *P. parvum*'s apparent range expansion into and across the USA is driven by increasing availabilities of salty and nutrient-rich systems, rather than by its toxigenic abilities.
Key words: harmful algal bloom (HAB), invasive species, toxigenic protist, N:P ratio, salinity

Biological invasions and range expansions have intrigued ecologists and biogeographers for nearly a century. Invasive species typically are defined as species that have been transported beyond their natural habitat into a new habitat through natural dispersal or by anthropogenic means and that cause disruptions to native biodiversity or ecosystem function, often with economic costs (Elton 1958, Lockwood et al. 2007, Sax et al. 2007). Invasive species are thought to thrive in the new system because they lack natural predators or diseases or they possess superior competitive abilities compared with the native taxa.

This view of invasive species has been adopted in discussions of *Prymnesium parvum*, a marine microbial mixotrophic species that is now commonly observed in inland aquatic systems across the southern USA (Baker et al. 2009, Hambright et al. 2010, Remmel et al. 2011, Roelke et al. 2011). The current inland distribution of *P. parvum* represents an extensive range expansion that has occurred over the past 3 decades and is thought to be a consequence, in part, of the alga's tolerance and success across a broad range of environmental conditions (Edvardsen and Paasche 1998) and its ability to dominate and bloom in suboptimal environmental conditions (Baker et al. 2009, Roelke et al. 2011). Many investigators have speculated that *P. parvum*'s toxigenic abilities provide an allelopathy-based competitive edge over native algae that allows blooms to develop during periods of stress, such as those created by low salinities, temperatures, and nutrient availabilities (reviewed by Granéli et al. 2012). Others have argued that toxicity may play important roles in predator avoidance and heterotrophy, but that toxicity is unlikely to provide a competitive advantage that could lead to bloom formation (Jonsson et al. 2009, Remmel and Hambright 2012). Examination of the distribution of *P. parvum* in Lake Texoma, (Oklahoma–Texas) reveals that it is dispersed throughout the lake, but blooms and fish kills are common only in areas and periods in which environmental conditions are conducive for growth (Hambright et al. 2010, Zamor et al. 2012, Zamor 2013). Thus, the recent expansion of *P. parvum* into new habitats suggests that habitats with suitable

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environmental conditions (e.g., elevated nutrients and salinities, see below) may be becoming more abundant, particularly in the southwestern USA, as growing human populations and development place increasing pressures on the quantity and quality of surface-water resources (Roelke et al. 2011).

A great deal of scientific effort has been directed toward characterizing *P. parvum*'s niche and the environmental conditions thought to be advantageous to *P. parvum* with respect to competition, predation, and toxicity. This focus is a result, in part, of its move into inland waters with devastating effects on fish and other aquatic animals. Laboratory and field studies have yielded an enigma with respect to *P. parvum*'s optimal niche. In laboratory studies, maximal growth rates of various strains of *P. parvum* are reached at relatively warm temperatures (15–30°C) and high salinities (8–30 practical salinity units [psu]). However, environmental conditions prevalent in the inland systems in which *P. parvum* has become predominant tend to be characterized by lower temperatures (10–20°C) and salinities (<4 psu), particularly during *P. parvum* blooms (Baker et al. 2007, Grover et al. 2010, Hambright et al. 2010). These lower field temperatures and salinities appear to enhance toxicity of *P. parvum* (Baker et al. 2007). In the laboratory, *P. parvum* tends to have higher toxicity when grown in N- or P-deficient conditions, i.e., unbalanced N:P (Granéli et al. 2012). Ambient environmental conditions during blooms of *P. parvum* generally corroborate these findings of stress-induced toxicity, i.e., blooms tend to occur under low N:P availability (Aure and Rey 1992, Bales et al. 1993, Hambright et al. 2010), and researchers have hypothesized that the evolutionary benefit of toxigenesis is likely to be related to nutrient acquisition (Lewis 1986, Beszteri et al. 2012, Remmel and Hambright 2012, Driscoll et al. 2013). The need for nutrient acquisition would generally signal unfavorable, hence stressful, conditions for growth.

The role of nutrient limitation in *P. parvum* toxicity has received considerable attention (Granéli et al. 2012). To date, most laboratory studies of the nutrient-toxicity relationship in *P. parvum* have been done in low-nutrient conditions rather than in the higher-nutrient conditions characteristic of the meso- and eutrophic systems in which *P. parvum* now thrives and blooms (e.g., Holdway et al. 1978, Guo et al. 1996, Hambright et al. 2010, Roelke et al. 2011). The results of studies showing dramatic N:P effects on growth or toxicity in *P. parvum* often are confounded by simultaneous manipulations of relative and absolute nutrient concentrations (e.g., Johansson and Granéli 1999, Granéli and Johansson 2003b). These manipulations serve as important foundational research, but such practice creates uncertainty with respect to how nutrient limitation ultimately dictates toxicity, toxigenesis, and competitive abilities—a situation reminiscent of the debate surrounding the roles and predictive capabilities of nutrient (N, P) concentrations and N:P ratios in cyanobacterial dominance of many freshwater systems (Smith 1983, Downing et al. 2001, Reynolds 2006).

We designed a laboratory-based study to elucidate the roles of salinity and relative and absolute nutrient availabilities in *P. parvum* growth and toxicity under environmental conditions representative of hypereutrophic reservoirs of the southwestern USA in which *P. parvum* blooms are common. We considered heterotrophy in an evolutionary context, and hypothesized generally that environmental conditions conducive to growth in *P. parvum* would be less favorable for toxigenesis, whereas conditions favoring toxigenesis would be suboptimal for growth. We hypothesized specifically that growth rates and maximum population sizes would scale positively with salinity and total available N and P and unimodally with N:P with growth reaching a maximum at intermediate N:P. In contrast, we hypothesized that toxicity would scale negatively with salinity and N and P concentrations and unimodally with N:P and that toxicity would be minimal at intermediate N:P (sensu Granéli and Johansson 2003b).

METHODS

Prymnesium stock batch cultures

We conducted all growth and toxicity experiments with 2 clonal isolates of *P. parvum*: UTEX-LB2797, isolated from the Colorado River, Texas basin (University of Texas algal collection) and UOBS-LP0109, isolated from Lebanon Pool, a 61-ha backwater basin of Lake Texoma with recurrent blooms of *P. parvum* (Hambright et al. 2010). The UTEX-LB2797 culture was shipped from the University of Texas in Erdschreiber's medium with salinity ~30 psu, but upon receipt in the laboratory, we initiated stock batch cultures in COMBO medium (Kilham et al. 1998) at a 16:1 N:P (800 μM N and 50 μM P). We manipulated salinity with either 6 or 15 g/L of Instant Ocean® (IO). Salinity of IO COMBO was related to NaCl salinity at room temperature as g IO/L = 1.085 × g NaCl/L + 0.086 (R^2 = 0.9996). We also maintained the UOBS-LP0109 culture in 6 and 15 g IO/L, 16:1 N:P COMBO. We maintained all stock batch cultures at room temperature under continuous lighting supplied by fluorescent aquarium tubes, mixed ≥1× weekly, and reinitiated into fresh medium upon visual signs of density decline.

Salinity effects on *Prymnesium* growth

We measured effects of salinity on *P. parvum* growth in batch culture as daily rate of change in cell density as a function of salinity using IO-supplemented COMBO (0–30 g IO/L). We initiated cultures from stock batch cultures of 6 and 15 g IO/L UTEX-LB2797 and UOBS-LP0109 separately. We established 3 replicate cultures of each strain and each initial culture salinity in 250-mL Erlenmeyer flasks containing 16:1 N:P COMBO at 1 of 6

test salinities (0, 2, 4, 6, 15, or 30 g IO/L). Flasks were swirled daily. Initially, and every 2nd d, we analyzed a 500-μL sample on a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, San Jose, California) to determine golden algal cell densities. We measured salinity for each sample initially and at the end of each experiment (Hach HQ40d meter, calibrated with NaCl; Hach, Loveland, Colorado).

N:P effects on *Prymnesium* toxicity

Based on the hypothesis that stoichiometric considerations (i.e., N:P) were important in understanding toxigenesis and toxicity in *P. parvum* (sensu Johansson and Granéli 1999), we established *P. parvum* chemostat cultures in UTEX-LB2797 with different N:P availabilities in modified COMBO medium. In the first series of chemostats, we held P constant at 50 μM and varied N from 200–1600 μM (hereafter fixed-P, variable-N chemostats), and in the 2nd series, we held N at either 200, 400, 800, or 1600 μM while varying P between 1.56 and 50 μM (hereafter variable-P, fixed-N chemostats). We verified nutrient treatments analytically. To examine the role of salinity in *P. parvum* toxicity, we established fixed-P, variable-N chemostats in salinities of 2, 4, 6, or 15 g IO/L, whereas we established all variable-P, fixed-N chemostats in 15 g IO/L salinity (see Table S1 for a complete list of nutrient and salinity treatments for chemostats).

We grew all cultures a 12:12 h light:dark daily cycle at 25°C under constant stirring and aeration. Cultures for use in bioassays were initially set up as batch and converted to chemostat once they were near steady-state densities (usually within 1–3 wk from initiation at densities of 20,000 to >3 million cells/mL, depending on nutrient concentrations and ratios). We monitored culture densities using microscopy (6–12 replicate hemocytometer counts at 200× magnification with an Olympus BX51 stereomicroscope; Center Valley, Pennsylvania), fluorometry (Turner Designs TD 700; Sunnyvale, California), or flow cytometry initially and at 1- to 3-d intervals, depending on culture and treatment. Cultures (~1.2 L) were maintained in chemostats supplied continuously with fresh medium at 7 to 9% daily replacement to yield ~100 mL of overflow from each culture each day for use in bioassays. All chemostats were replicated independently ≥2×. We conducted bioassays over the course of several years, so we replicated some chemostats (N:P = 16:1, 6 and 15 g IO/L) over the entire experimental period as internal checks against long-term culture-related artifacts.

Fathead-minnow bioassays

Many *P. parvum* toxins have been characterized (Shilo 1971, Igarashi et al. 1999, Henrikson et al. 2010, Bertin et al. 2012a, b), but no robust analytical chemical method is available for quantifying *P. parvum* toxicity. Therefore, we quantified culture toxicities to fish (our primary concern) with a modified 48-h lethal concentration to 50% of test animals (LC_{50}) approach with 10- to 14-d-old fathead minnows (*Pimephales promelas*) (Remmel and Hambright 2012) in 100-mL glass jars. Each of the 3 replicates/cell density consisted of 3 fish in a jar. Fish were acclimated for 1 h in 40 mL of aged, dechlorinated tap water following addition of 10 mL of *P. parvum* culture diluted to achieve final bioassay cell densities of 0 to 400,000 cells/mL. We harvested chemostat overflows daily, and subsampled and counted them as above. We used COMBO with nutrients and IO to compensate for nutrient and salinity differences in each of the jars during dilution to ensure that nutrient concentrations and salinity were equal across all treatments of the experiments and that fish were not affected by salt concentrations beyond their tolerance (all final salinities 0.2–3 g IO/L; Table S2). In preliminary trials, minnow fry could tolerate up to 6 g IO/L for 48 h. We ran all bioassays in a 12-h-dark:12-h-subdued light regime at 24°C. We recorded fish survival at 24 and 48 h. We used survival after 48 h to estimate LC_{50} concentrations of *P. parvum* with a nonlinear, 4-variable, dose-response regression with a variable slope parameter (Remmel and Hambright 2012).

Salinity effects on *Prymnesium* cell density and cell size

Addition of 2- to 15-g IO/L salinity cultures to aged tap water yielded final bioassay salinities between 0.2 and 3 g IO/L (Table S2). Preliminary microscopic observations indicated that extreme shifts in salinity (e.g., from 30 to near 0 g IO/L) resulted in lysis of *P. parvum* cells. However, *P. parvum* subjected to smaller, but substantial, salinity shifts, as experienced in our bioassays (e.g., 15–3 g IO/L), underwent changes in cell morphology (swelling), but showed little indication of cell lysis. Stress-induced lysis could affect release of intercellular or membrane-bound toxins and confound measures of toxicity in bioassays with living cells (Remmel and Hambright 2012). Therefore, we quantified these potentially confounding effects with time-series measurements of cell sizes and densities of *P. parvum* subjected to abrupt changes in salinities spanning the conditions created during our bioassays. An increase in mean cell size would indicate swelling, whereas a reduction in density would indicate lysis. We grew cultures in 16:1 N:P COMBO modified with 6 or 15 g IO/L at a constant 25°C on a 12-h-light:12-h-dark cycle. Initial densities of cultures were $\sim 0.6 \times 10^5$ cells/mL. We diluted *P. parvum* culture with 16:1 N:P COMBO without IO in 25-mL glass vials to create a gradient of decreasing salinities from 6 to 2.4, 1.2, 0.6, or 0.3 g IO/L and from 15 to 12, 9, 6, 3, or 0.8 g IO/L (n = 3 for each dilution). Following dilution and gentle mixing by inversion, we took 1-mL subsamples at 0.3, 1, and 24 h for analysis of cell density and size by flow cytometry. Densities presented were corrected for dilution.

Statistical analyses

All statistical analyses, with exception of estimation of LC_{50} concentrations (see above), were carried out in R (version 3.0.1; R Project for Statistical Computing, Vienna, Austria). To test for effects of salinity on *P. parvum* growth rates, we fit a general linear model with *P. parvum* growth rates in batch culture as the response variable, and initial culture salinity, experimental salinity, and strain as predictor variables. We $\log_{10}(x)$-transformed *P. parvum* growth rates to meet assumptions of normality. We excluded 2 outlying values in this analysis to satisfy assumptions of normality, but repeating the analysis with the outliers produced similar results.

We used 2-way analysis of variance (ANOVA) to test for the effects of salinity and N:P on LC_{50} values. The interaction term in this model represents how the slope of the relationship between N:P and toxicity varied between the salinity treatments.

We used linear mixed-effects models to test the effects of salinity on *P. parvum* cell density (corrected for dilution) and cell size. For each initial salinity (6 and 15 g IO/L), we used the *nlme* package (version 3.1-100; Pinheiro et al. 2013) to fit linear mixed-effects models with either cell density or cell size as response variables, time and dilution specified as fixed effects, and replicate ID specified as a random effect. We excluded 2 outliers from 15 g IO/L density analysis (1 each of 0.8 and 0.2 dilutions at 0.3 h).

RESULTS

Maximum growth rates of both *P. parvum* isolates (UTEX-LB2797 and UOBS-LP0109) in batch culture increased with increasing salinity in a log–linear fashion (Fig. 1, Table S3). Growth rates of the UOBS-LP0109 strain were consistently higher than growth rates of UTEX-LB2797 strain (likelihood ratio test, *LRT* statistic = 6.333, $p = 0.0119$). Chemostat cultures of UTEX-LB2797 also revealed a similar log–linear relationship of growth rates with salinity during the first 7 d after inoculation (but before initiation of chemostat), with mean (±1 SD) growth rates at 6 and 15 g IO/L uniformly high (0.333 ± 0.155/d) across all nutrient conditions, compared with growth rates at 2 g IO/L (0.056 ± 0.021/d) (Fig. 1 inset, Table S4). Once the chemostat was initiated at 7–9% daily volume replacement (equivalent to mortality), steady-state cell densities varied approximately unimodally with N:P regardless of salinity, N, or P concentration (Fig. 2).

All UTEX-LB2797 cultures, regardless of N:P or salinity revealed some level of toxicity, but toxicity was unquantifiable in ~7% (21/308) of all bioassays because of lack of complete mortality in the highest concentration used in a given bioassay. In fixed-P, variable-N cultures, toxicity of *P. parvum* decreased with increasing N:P (Fig. 3, Table S5). Toxicity was highest in 15-g IO/L cultures and

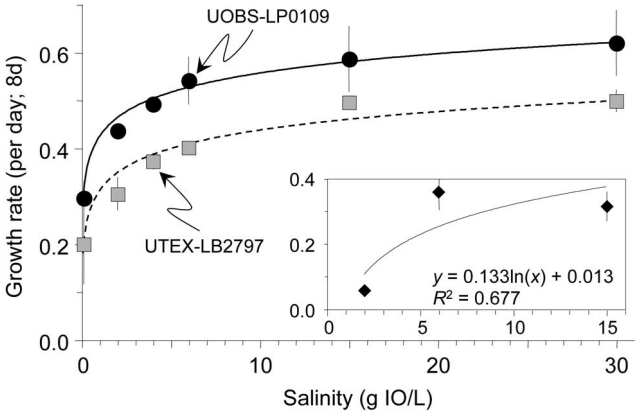

Figure 1. Mean (±1 SE, $n = 6$) growth rates (batch culture, 0–8 d) of 2 strains of *Prymnesium parvum* (UOBS-LP0109, UTEX-LB2797), cultured initially at either 6 or 15 g Instant Ocean (IO)/L salinity. Regression equations: UOBS-LP0109 growth rate = 0.0584ln(salinity) + 0.4207, $R^2 = 0.547$, $F_{1,34} = 41.08$, $p < 0.0001$; UTEX-LB2797 growth rate = 0.0576ln(salinity) + 0.3037, $R^2 = 0.683$, $F_{1,32} = 68.94$, $p < 0.0001$. Inset.—Mean growth rates (± 95% CI) of fixed-P, variable-N *P. parvum* (UTEX-LB2797) chemostat cultures, as functions of salinity (2 g IO/L, $n = 8$; 6 g IO/L, $n = 33$; 15 g IO/L, $n = 42$), prior to the initiation of 7–9% daily volume replacement (i.e., initial batch culture growth rates). Regression equation: growth rate = 0.0726ln(salinity) + 0.1498, $R^2 = 0.0775$, $F_{1,81} = 6.80$, $p < 0.011$.

declined markedly with decreasing culture salinity at all but the lowest N:P (salinity × N:P interaction, $t = -3.110$, $p = 0.002$; Table S5). The strength of the N:P effect (i.e., the slope of the log–linear regression between LC_{50} and N:P) decreased with increasing N:P (Fig. S1). Cultures from the Lake Texoma isolate (UOBS-LP0109) grown at 4 g IO/L and N:P = 8, 16, and 32 (data not shown) showed a similar decrease in toxicity with increasing N:P, but were generally less toxic than 2-g IO/L UTEX-LB2797 cultures ($LC_{50} \times 10^3 = 212\ln[N:P] - 432$, $R^2 = 0.644$, $F = 25.32$, $p = 0.0002$, $n = 16$). Compared with the fixed-P, variable-N cultures shown in Fig. 3, *P. parvum* toxicity increased dramatically with decreasing P above N:P = 32 in variable-P, fixed-N cultures (UTEX-LB2797) at 15 g IO/L salinity (i.e., with increasing N:P; Fig. 4A, B, Table S5). Toxicity also tended to increase with decreasing N, but the highest toxicities observed were in the 400-μM N cultures, rather than in the 200-μM N cultures. Regardless of absolute N and P concentrations, the *P. parvum* toxicity–N:P relationship was unimodal, with lowest toxicities at N:P between 16 and 32, and highest toxicities <16 and >32. This unimodal toxicity–N:P relationship, in conjunction with a similar unimodal relationship between steady-state chemostat culture density and N:P (see Fig. 2), yielded an exponential relationship between LC_{50} densities and steady-state culture densities (Fig. 5), suggesting an inverse relationship between nutrient-dependent growth and toxicity.

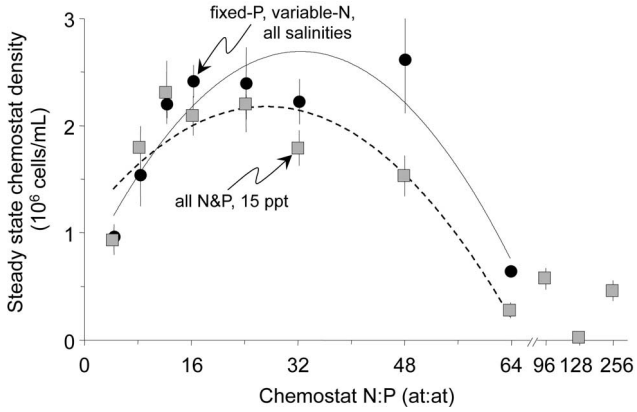

Figure 2. Mean (±1 SE) steady-state densities of *Prymnesium parvum* in chemostats used for fixed-P, variable-N toxicity bioassays shown in Fig. 3 (density = $-1733.7(\text{N:P})^2 + 10{,}783(\text{N:P}) + 936{,}359$, $R^2 = 0.196$, $F_{2,154} = 18.78$, $p < 0.0001$) and for all N:P, 15 g Instant Ocean (IO)/L salinity toxicity bioassays shown in Fig. 4 (density = $-1300(\text{N:P})^2 + 69{,}328(\text{N:P}) + 1{,}143{,}998$, $R^2 = 0.236$, $F_{2,136} = 21.03$, $p < 0.0001$).

The magnitude of salinity reduction, such as occurred during our bioassays, had no effect on cell densities in either 6- or 15-g IO/L cultures Fig. 6A, B). The time effect on density was significant in the 6-g IO/L experiments, with a slight decline in all dilutions at 1 h, whereas control densities continually increased with time. In the 15-g IO/L experiment, the time × dilution interaction effect on cell density was significant. Abrupt changes in salinity caused dramatic increases in cell diameter within the first 0.3 h (Fig. 6C, D, Table 1). In both 6- and 15-g IO/L experiments, salinity reduction significantly affected cell size, and the largest change in cell size corresponded to the largest change in salinity. The effect of time on cell size was significant in both experiments. Cell size decreased notably after 1 and 24 h. The magnitude of the effect of salinity reduction on cell size declined with time in the 6-g IO/L culture experiment (significant time × salinity-reduction interaction). Some dilutions showed declines in densities, whereas others increased slightly. However, overall, the abrupt changes in salinities did not result in substantial levels of cell lysis.

DISCUSSION

Prymnesium parvum blooms are paradoxical in that they occur in lakes when conditions are not optimal for reproductive growth. In other words, the fundamental and realized niches for *P. parvum* appear to be nonsynonymous (Baker et al. 2009, Granéli et al. 2012). However, optimal growth conditions generally have been defined in laboratory studies in which only a few selected factors (e.g., temperature, nutrients [N and P], and salinity) were manipulated, while other potentially important factors were held constant or were absent (Baker et al. 2009). Thus, the *n*-dimensional hypervolume (sensu Hutchinson 1957) for *P. parvum* has not been defined adequately. Measurement of growth performance under more natural conditions, including in the presence of grazers, competitors, parasites, and diseases, could potentially solve the apparent bloom paradox. Nevertheless, the bloom paradox, considered in light of *Prymnesium*'s ability to produce toxins, has led researchers to speculate that the suboptimal environmental conditions prevalent during *P. parvum* blooms are conducive to increased toxin production, which is thought to impart a competitive advantage to *P. parvum* through allelopathy and subsequent consumption of competitors or their cellular constituents (Granéli et al. 2012). Clearly, mixotrophic abilities can impart a competitive advantage over a purely photosynthetic species on an individual level (Legrand et al. 2003), but it does not seem reasonable that such behavior could foster development of a bloom (Jonsson et al. 2009, Remmel and Hambright 2012), particularly considering that rapid growth and blooms require substantial levels of nutrients.

The current dogma concerning *P. parvum* toxicity is based on the assumption that a stress-related mechanism underlies toxin production in this species, with some range of temperature, salinity, light, and nutrients that leads to high toxicity. From considerable study of these environmental factors on growth and toxicity in *P. parvum*, researchers generally assumed that poor growth conditions translated to increased heterotrophy, toxigenesis, and toxicity (Granéli et al. 2012). Given the mixotrophic nature of *P. parvum* and the assumed role of toxins in facilitating heterotrophic interactions (Skovgaard and Hansen 2003,

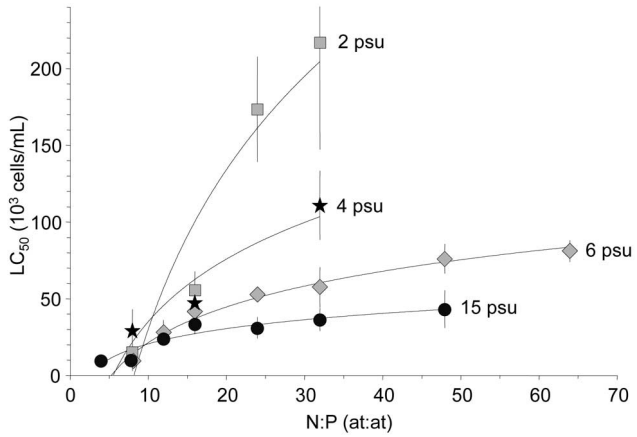

Figure 3. Mean (±1 SE) toxicity (lethal concentration to 50% of organisms [LC_{50}]) of *Prymnesium parvum* to 10- to 14-d-old fathead minnows in 48-h bioassays as functions of N:P and salinity. N:P (4, 8, 12, 16, 24, 32, 48, 64) was altered by holding P constant at 50 µM and varying N. 15 g IO/L: $LC_{50} \times 10^3 = 14.42\ln(\text{N:P}) - 12.77$, $R^2 = 0.243$, $F = 20.55$, $p < 0.0001$, $n = 66$; 6 g IO/L: $LC_{50} \times 10^3 = 33.49\ln(\text{N:P}) - 55.14$, $R^2 = 0.504$, $F = 52.90$, $p < 0.0001$, $n = 54$; 4 g IO/L: $LC_{50} \times 10^3 = 61.22\ln(\text{N:P}) - 114.03$, $R^2 = 0.391$, $F = 14.11$, $p = 0.001$, $n = 24$; 2 g IO/L: $LC_{50} \times 10^3 = 149.16\ln(\text{N:P}) - 328.22$, $R^2 = 0.615$, $F = 28.77$, $p < 0.0001$, $n = 20$.

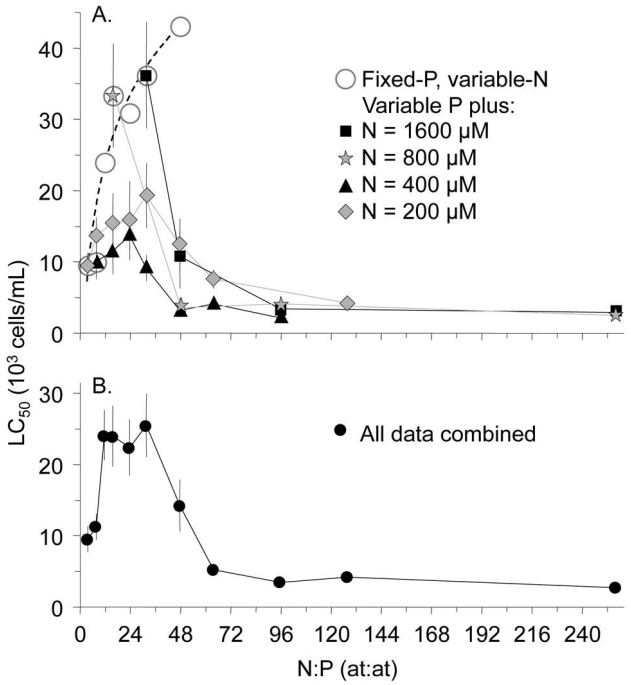

Figure 4. A.—Mean (±1 SE) toxicity of *Prymnesium parvum* to 10- to 14-d-old fathead minnows in 48-h bioassays using *P. parvum* cultured in different ratios of N:P at 15 g Instant Ocean (IO)/L. N:P was altered by holding N constant at 200 (n = 41), 400 (n = 53), 800 (n = 40), or 1600 (n = 35) µM and varying P accordingly. Fixed-P, variable-N curve from Fig. 3 (error bars omitted for clarity; note different scale from Fig. 3) are shown for comparison. B.—Same as A, but all data combined.

Remmel and Hambright 2012), a role for nutrient stress in toxicity regulation is logical because mixotrophy provides a means of ameliorating the effects of a nutrient-deficient environment. In contrast, it is less obvious how depressed growth because of unfavorable temperature, light, or salinity might contribute to enhanced toxigenesis or toxicity because mixotrophy cannot be used to make metabolic adjustments for temperature, to acquire more photons under low-light conditions, or to adjust osmoregulatory ability under variable salinity environments. Larsen and Bryant (1998) detected no relationships between salinity, temperature, or light with toxicity in 6 different strains of *P. parvum* isolated from Norwegian, Danish, English, and Australian coastal waters. We cannot draw inference with respect to light or temperature, but our results do not support the hypothesized role of salinity stress in toxicity regulation because toxicity appeared to scale positively, rather than negatively, with salinity (Fig. 3). However, our whole-cell toxicity bioassays with a freshwater fish species potentially could have confounded our results via stress to the *P. parvum* because of the abrupt salinity dilution by 81 to 84%. We found little indication that such stress was long lasting because the only salinity-dilution effect detected was a temporary increase in mean cell size. Moreover, Baker et al. (2009), who reported results similar to ours, and Baker et al. (2007), who report negative (at high temperatures) and unimodal (at low temperatures) relationships between salinity and toxicity, both used a salinity-dilution approach to conducting fish bioassays. In these cases, artificial seawater or lake-water cultures with salinities ranging from 0.5 to 35 psu were serially diluted in 50% increments with reconstituted hard water. Thus, higher-salinity *P. parvum* cultures were exposed to drastic salinity changes during the bioassays (final salinities were not reported, but the same freshwater *P. promelas* was used). Given the recent demonstration that *Prymnesium* toxins are not exotoxins, but instead probably are membrane-bound compounds that aid in contact-cell heterotrophy (Remmel and Hambright 2012), the potential dilutional-stress created by our bioassay method probably did not cause a release of toxins. However, no suitable analytical methods exist for toxin quantification, so we cannot verify this supposition at this time.

Data on growth and toxicity in *P. parvum* provide additional evidence of a weak link between toxicity and nonnutrient-related environmental stressors. Presumably, nonnutrient-related environmental stressors that result in poor growth also should be conducive to toxigenesis (Granéli et al. 2012). In some studies, including ours (Fig. 5), growth and toxicity were negatively related (*P. parvum* is most toxic under poor growth conditions; McLaughlin 1958, Uronen et al. 2005, Baker et al. 2007, Roelke et al. 2007), but in others, little relationship existed between growth and toxicity (Dafni et al. 1972, Larsen and Bryant 1998). We did not measure toxicities in the salinity–growth-rate experiments (Fig. 1), but comparison of the patterns in Figs. 1 and 3 suggest the possibility of a positive growth–toxicity relationship. Reich and Aschner (1947) found a positive relationship between population density and de-

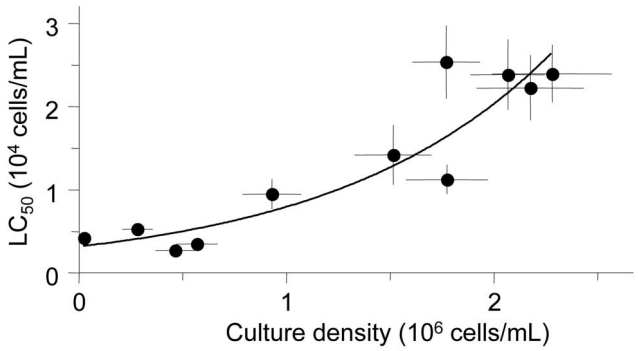

Figure 5. Relationship between mean (±1 SE) steady-state culture density and toxicity for all N:P, 15 g Instant Ocean (IO)/L salinity toxicity bioassays shown in Fig. 4. Best fit regression: $\ln(LC_{50}) = 0.954(\text{density} \times 10^6) + 8.00$, $R^2 = 0.849$, $F = 50.71$, $p < 0.0001$. LC_{50} = lethal concentration to 50% of organisms.

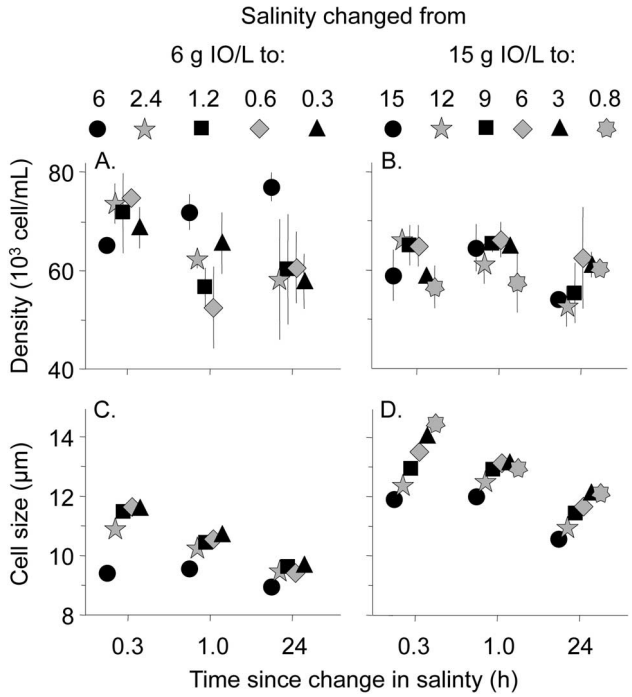

Figure 6. Effects of abrupt salinity change (as occurred during lethal concentration to 50% of organisms [LC_{50}] bioassays) on mean (±1 SE) *Prymnesium parvum* density (A, B) and mean cell diameter as measured over 24 h using flow cytometry (C, D) in 6-g Instant Ocean (IO)/L cultures diluted to 2.4, 1.2, 0.6, and 0.3 g IO/L using nutrient-spiked COMBO (A, C) and in 15-g IO/L cultures diluted to 12, 9, 6, 3, and 0.8 g IO/L using COMBO without added N and P (B, D).

gree of toxicity, and Padilla (1970) showed that both growth and toxicity in *P. parvum* were highest at high salinities (25 and 22.8‰, respectively). A positive relationship between growth and toxicity was further supported in a long-term field study on Lake Texoma (Oklahoma–Texas), which revealed that *P. parvum* net population growth rates were highest during periods of suboptimal (low) salinities and (cool) temperatures (Hambright et al. 2014), similar to conditions reported for Texas *P. parvum* blooms (Baker et al. 2009). The Lake Texoma blooms were large (up to ~200,000 cells/mL) and toxic throughout the bloom period as indicated by bioassays and large fish kills (Zamor et al. 2014).

Regardless of specific culture conditions, both strains of *P. parvum* used in our study were always toxic, but our results confirm the general hypothesis that nutrient stress in the form of unbalanced N:P yields lower growth rates and higher levels of toxicity. Under high and constant P availability, toxicity increased with decreasing N availability with no evidence of a unimodal relationship with N:P between 8 and 64. However, under lower P availabilities (i.e., fixed-N, variable-P experiments), we saw the more familiar (sensu Johansson and Granéli 1999) unimodal response of toxicity to N:P. Strong P limitation yielded dramatically higher toxicities than did strong N limitation. In addition to relative N:P effects, our results also showed a role for absolute nutrient concentrations because lower nutrients in general led to lower steady-state densities in culture and higher toxicities. Coupled with observational and behavioral study of *P. parvum* toxicity (Remmel et al. 2011, Remmel and Hambright 2012), these results corroborate the hypothesis that toxigenesis and toxicity in *P. parvum* are related to a biochemical demand for nutrients (e.g., auxotrophy) rather than physiological stress. Previous investigators have examined N:P effects on *P. parvum* grown in batch or semicontinuous cultures and reached the same conclusions (e.g., Johansson and Granéli 1999, Granéli and Johansson 2003b). Comparisons have been made across log-phase and steady-state-growth pe-

Table 1. Results of linear mixed-effects model (6- and 15-g Instant Ocean [IO]/L analyses run separately), with replicate specified as a random effect, and time (T) and dilution (D) specified as fixed effects. Two outliers excluded from 15 g IO/L density analysis (1 each of 0.8 and 0.2 dilutions at 0.3 h).

	Cell density					Cell size				
Source	Value	SE	df	*t*	*p*	Value	SE	df	*t*	*p*
6-g IO/L cultures										
Intercept	65224.90	2028.47	27	32.15	0.0000	11.31	0.0988	27	114.50	0.0000
T	−383.90	147.52	27	−2.60	0.0148	−0.07	0.0072	27	−9.82	0.0000
D	3233.67	4119.16	13	0.79	0.4465	−1.83	0.2005	13	−9.10	0.0000
T × D	685.61	297.16	27	2.31	0.0289	0.05	0.0145	27	3.37	0.0023
15-g IO/L cultures										
Intercept	60469.10	1764.44	32	34.27	0.0000	13.96	0.1050	34	132.95	0.0000
T	82.67	99.65	32	0.83	0.4129	−0.07	0.0076	34	−9.15	0.0000
D	3830.51	2907.35	16	1.32	0.2062	−1.90	0.1733	16	−10.95	0.0000
T × D	−561.40	164.31	32	−3.42	0.0017	0.01	0.0125	34	0.68	0.5038

riods, but identification of the exact limiting factor (i.e., the factor most strongly related to toxicity) has been difficult, particularly because N:P was manipulated in these studies by varying concentrations of N and P simultaneously. In contrast, our results were based on toxicities of *P. parvum* grown in continuous chemostat with N and P manipulated separately, which facilitated identification of the limiting factor that most strongly affected toxicity. Cells in our cultures always had a constant supply of N, P, vitamins, and trace metals. Only N or P concentration differed among cultures. This strategy revealed that level of toxicity was directly related to an imbalance between N and P, and to the absolute availability of both nutrients, as inferred from Figs. 4A, B, and 5. Effects of absolute and relative N and P concentrations are complex and are affected by temperature, salinity, and possibly multiple other conditions (our study and others already cited). However, nutrient limitation and N and P stoichiometry have general and important roles, and increased toxicity (i.e., the need for heterotrophy) is associated with limitation by one or both of these important nutrients.

Our results corroborate some previous findings, but important differences exist between our results and those of others. The most notable differences are the positive salinity–toxicity relationship and the negative N:P–toxicity relationship under fixed-P conditions (Fig. 3). Such differences could be caused by a number of factors, including genetic variation among strains, differing environmental conditions and culture media, and differences in the method of toxicity assessment. Considerable variation among strains has been documented (Larsen et al. 1993, Larsen and Bryant 1998), but the general patterns seem to be robust across strains. For example, we found similar toxicity and growth relationships as functions of salinity and N:P with both UTEX-LB2797 and UOBS-LP0109. Differences in culture medium, trace metals, and vitamins could generate the type of variation seen across studies, but how such differences would translate to growth–toxicity relationships is not clear. In contrast, differences across studies in the method of toxicity assessment could produce important differences in the growth–toxicity relationship. Some investigators have used fish bioassays to quantify toxicity, as we did (Baker et al. 2007). However, other investigators used hemolytic assays with horse blood (Johansson and Granéli 1999, Legrand et al. 2001), or bioassays with other protists (Granéli and Johansson 2003a, b) or brine shrimp (*Artemia*) nauplii (Larsen et al. 1993, Larsen and Bryant 1998). Numerous toxins have been isolated and characterized (Shilo 1971, Igarashi et al. 1999, Henrikson et al. 2010, Bertin et al. 2012a, b), but they do not all possess the same ichthyotoxic, hemotoxic, or cytotoxic properties. In the bioassay-guided extraction and isolation approach used by Henrikson et al. (2010), brine-shrimp toxicity revealed some toxin fractions that were toxic only to brine shrimp, some that were toxic to brine shrimp and juvenile fathead minnows, and some that were toxic only to juvenile fathead minnows (KDH, unpublished data). Bertin et al. (2012a, b) found similar variability in identity and toxicity of compounds isolated from *P. parvum*. We suspect that the variety of toxins characterized thus far, and the variability in toxin action across assay types, probably is the key factor underlying differences in growth–toxicity relationships among studies.

Our results indicate that environmental nutrient conditions that favor toxigenesis in *P. parvum* are suboptimal for growth and bloom formation, whereas those conditions that are conducive to high growth rates are less favorable for toxigenesis. Growth and toxicity in *P. parvum* scaled positively with salinity. These results suggest that *P. parvum*'s apparent range expansion into and across North America is driven by increasing availability of salty and nutrient-rich systems, rather than by its toxigenic abilities. *Prymnesium parvum* population density can be predicted on the basis of several environmental factors within Lake Texoma and across the Red and Canadian River basins of Texas and Oklahoma (Hambright et al. 2010, Zamor et al. 2012, Zamor 2013). Based on the typically immense population sizes of microbes, especially during blooms (e.g., during a bloom of 10^5 cells/mL in Lebanon Pool, which contains $\sim 3 \times 10^6$ m^3 of water, the total *P. parvum* population would be $>3 \times 10^{11}$ individuals), and the fact that hydraulic flushing and advective loses downstream are critical components of life in reservoirs (Thornton et al. 1990), the *P. parvum* propagule pressure on lower reaches of Lake Texoma probably are very high. We routinely detect *P. parvum* downstream using quantitative polymerase chain reaction (PCR; detection limit = 11 cells/mL for 0.5-L samples), and these cells probably are quite capable of heterotrophy and toxin production, but *P. parvum* has yet to bloom in any area of the lake in which salinities are lower than in upstream western bloom sites, even though nutrient concentrations can be quite high throughout the lake (Zamor et al. 2012, Hambright et al. 2014). Similar patterns have been observed across the Red and Canadian River basins (Zamor 2013). Such observations are consistent with the microbiologist's credo "everything is everywhere, but, the environment selects" (Baas-Becking 1934, de Wit and Bouvier 2006). In other words, dispersal probably is not the limiting factor that determines *P. parvum* biogeographic patterns. Rather, population establishment and blooms occur only when environmental conditions are favorable. Toxicity is highest under conditions of nutrient limitation, so little evidence exists that toxicity could lead to enhanced growth and bloom formation.

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