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Chattonella subsalsa (Raphidophyceae) growth and hemolytic activity in response to agriculturally-derived estuarine contaminants

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ABSTRACT

The potential for toxic contaminants and nutrient pollution to alter natural cycles of estuarine phytoplankton blooms is well known, yet few studies have examined how these combined stressors affect harmful algal species. Here, a robust testing protocol was developed to enable an ecotoxicological assessment of responses to commonly co-occurring estuarine contaminants by harmful algal bloom species. The population growth and toxicity (as cell density and hemolytic activity, respectively) of a cultured strain of the toxigenic raphidiophycean, *Chattonella subsalsa*, were assessed in two experiments (duration 10 days and 28 days) across a gradient of atrazine concentrations and N:P ratios simulating nutrient-rich versus nutrient-depleted regimes. The response of this large-celled, slowly growing alga to atrazine × nutrients depended on growth phase; atrazine was most inhibitory during early exponential population growth (day 10), whereas nutrient regime was a more important influence during later phases of growth (day 28). Without atrazine, toxicity toward fish was highest in low-P cultures. At atrazine levels > 25 μg L^{-1}, hemolytic activity was highest in low-N cultures, and increased with increasing atrazine concentration in all nutrient-limited cultures. Hemolytic activity varied inversely with atrazine concentration in N,P-replete conditions. Overall, atrazine inhibitory effects on population growth of this *C. subsalsa* strain depended on the growth phase and the nutrient regime; hemolytic activity was higher and further enhanced by atrazine in low N-P regimes; and atrazine inhibited hemolytic activity in nutrient-replete conditions. The data suggest that, depending on the growth phase and nutrient regime, atrazine can help promote toxic *C. subsalsa* blooms.

1. Introduction

Coastal zone estuaries and wetland habitats are among the most highly stressed natural systems in the world due to rapid urbanization and development of coastlands, and concomitant pollution from land-based runoff (Scott et al., 2006; U.S. EPA, 2016a). About two-thirds of the nation's coastal areas and more than one-third of the nation's estuaries have shown impairment from nutrient (nitrogen, N, and phosphorus, P) pollution (U.S. EPA, 2016b). As anthropogenic nutrient pollution increases, many estuarine and marine coastal waters are sustaining more frequent and extensive harmful algal blooms (HABs) (Hallegraeff, 1993; Glibert et al., 2005a,b; Heisler et al., 2008). Eutrophication is now recognized as one of the most important factors contributing to the global expansion of HAB species (Burkholder, 1998; Glibert et al., 2005a,b; Heisler et al., 2008). High levels of phosphate loading relative to dissolved inorganic nitrogen have been related to the increased occurrence of harmful algal species such as toxigenic raphidophyceans (Heterokontophyta, Raphidophyceae; Lewitus and Holland, 2003; Lewitus et al., 2003). Such inorganic N:P stoichiometric imbalances, coupled with high N and P supplies, are prevalent in many coastal waters worldwide (Pelley, 1998; Bricker et al., 2008; Burkholder and Glibert, 2013), along with many other chemical pollutants such as herbicides (Hapeman et al., 2002; Peters et al., 2005).

Among these, atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) is the second most commonly used herbicide in the U.S., and the most common surface water contaminant (Gilliom et al., 2006; Ryberg et al., 2010). This herbicide is a photosynthetic inhibitor which works by blocking electron transport in photosystem II (DeLorenzo et al., 2001; Vencill, 2002), and can elicit effects similar to nutrient dificiency in exposed algal populations (Weiner et al., 2007). It has been found at environmental concentrations as high as 1000 μg L^{-1} in surface waters adjacent to treated fields (deNoyelles et al., 1982; Pennington et al., 2001). Stormwater runoff in the Chesapeake Bay region, for example, has contained concentrations as high as 480 μg L^{-1} (Eisler, 1989; Lehotay et al., 1998), and in other estuaries concentrations above 10 μg L^{-1} are commonly reported (e.g. Starr et al., 2017).

Little is known about herbicide effects on harmful algal species, which can be difficult to culture and are rarely used in toxicity testing

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(Thursby et al., 1993; Lytle and Lytle, 2001). Still less is known about the response of harmful algal species to co-occurring contaminants such as atrazine and nutrients (Flood et al., 2018). Autecological and ecotoxicological studies of harmful algae are needed to gain insights about the mechanisms which lead to toxic blooms, and which regulate toxicity in coastal waters that are increasingly contaminated by chemical substances from human-related activities.

This study is the first to assess interactive effects of nutrients and atrazine exposure on the growth and toxicity of the harmful species, *Chattonella subsalsa* B. Biecheler. Species of *Chattonella* are widely distributed in temperate and subtropical/tropical estuarine and marine waters worldwide, and frequently have been linked to fish kills in some regions (e.g., Shimada et al., 1983; Hallegraeff et al., 1998; Imai et al., 1998; Cortés-Altamirano et al., 2006; Zhang et al., 2006; Imai and Yamaguchi, 2012; Lewitus et al., 2012). Recently, *C. subsalsa* blooms were linked to fish kills in eutrophic waters of coastal South Carolina in the southeastern U.S. as well (Lewitus et al., 2003, 2008). The autecology of *C. subsalsa* remains largely under-investigated, but this species appears to be especially well-adapted to thrive in shallow, eutrophic, turbid habitats (Zhang et al., 2006; Band-Schmidt et al., 2012). Regarding toxicity, *Chattonella* spp. can produce ROSs (e.g., hydrogen peroxide, superoxide, and hydroxyl radicals) at levels about 100-fold higher than other microalgal species (Marshall et al., 2005a). These ROSs can greatly enhance the toxicity of hemolytic free fatty acids such as eicosapentaenoic acid, which occurs at high levels in *C. subsalsa* and other raphidophyceans (Marshall et al., 2002). Here, the population growth and toxicity (as hemolytic activity) of a cultured strain of *C. subsalsa* were assessed across a concentration gradient of atrazine in nutrient-poor versus nutrient-rich regimes, using longer experimental durations (10 and 28 days) than in standard ecotoxicology assays to account for the slow growth of this large-celled organism. It was hypothesized that *C. subsalsa* response to atrazine would vary depending on its growth phase and the nutrient regime. It was also expected that exposure to low atrazine concentrations would act as a stressor that enhanced toxicity in this *C. subsalsa* strain, as has been shown for various harmful algae in response to other stressors (Varkitzi et al., 2010; Glibert et al., 2005a).

2. Materials and methods

2.1. Experimental organism

Strain CCMP2191 of *Chattonella subsalsa* was obtained in unialgal, non-axenic culture from the National Center for Marine Algae and Microbiota (Bigelow Laboratory for Ocean Sciences, East Boothbay, ME, USA) on 11 February 2009. It was originally isolated on 13 August 2001 from the Indian River Bay, Delaware, USA. This organism is a relatively large flagellate (length and width up to 50 µm and 25 µm, respectively; Hallegraeff and Hara, 2003), with highly variable shape due to the lack of a rigid cell wall (Band-Schmidt et al., 2012; Graham et al., 2016). The fragile cells are morphologically plastic and the cell shape is frequently lost with fixation, making identification difficult if based solely on morphological characteristics of preserved samples (Band-Schmidt et al., 2012). This species has broad environmental tolerances; it grows well at temperatures ranging from 10° to 30 °C, and salinities of 5–30, with optima (although strain-dependent) reported at 20–30 °C and salinities of 15–25 (Zhang et al., 2006). Like many other microalgae under environmentally unfavorable conditions, *C. subsalsa* forms cysts (benthic stage), which can remain dormant for months to years. This ability to remain a member of the "hidden flora" (Smayda, 2002) means that previous blooms can leave "seed" populations as cysts for years until conditions become favorable for germination. Thus, *C. subsalsa* may remain undetected and then suddenly become dominant in the plankton and potentially lethal to aquatic life (Imai et al., 1991, 1998; Imai and Yamaguchi, 2012).

Other characteristics of *C. subsalsa* (based, however, on assessment of few strains) that were important considerations for this work are its slow growth, its mixotrophic capabilities (Jeong et al., 2010), and its toxin production (Bourdelais et al., 2002). The maximum nutrient-saturated growth rate of this species is ~0.2–1.26 divisions day^{-1} (e.g., Zhang et al., 2006; Band-Schmidt et al., 2012; Imai and Yamaguchi, 2012), and it is generally considered to be a large, slowly growing flagellate. Half-saturation constants (K_s) for *C. subsalsa* have been reported at 0.84 µM for PO_4^3, 8.98 µM for NO_3^- and 1.46 µM for NH_4^+, indicating that this raphidophycean can use low concentrations of NH_4^+ more efficiently than NO_3^- (Zhang et al., 2006). Moreover, it appears to attain higher biomass (~double) when NH_4^+ is the major inorganic N source (Zhang et al., 2006). Organic forms of N (e.g., glutamic acid) and phosphorus (P, e.g. ATP/ADP, adenosine triphosphate and adenosine diphosphate, respectively) can be used by *C. subsalsa*, as well as inorganic N and P forms (Zhang et al., 2006; Yamaguchi et al., 2008). In addition to photosynthetic carbon assimilation, *C. subsalsa* can consume coccoid unicellular cyanobacteria, and possibly other small (< 2 µm) organisms, but phagotrophy is apparently limited to the size of the mucocyst openings on the cell surface which are believed to be the sites of ingestion (Jeong et al., 2010, Jeong, 2011).

2.2. Culture conditions

Batch cultures of *Chattonella subsalsa* strain CCMP2191 were maintained in 500-mL Erlenmeyer flasks containing 250 mL of salinity 20-modified L1–Si medium (Guillard and Hargraves, 1993) at 24 °C (ambient air temperature) and 106.31 (± 26.16) µmol photons of photosynthetically active radiation (PAR) $m^{-2}s^{-1}$ (cool white fluorescent tubes) under a 16:8 h light:dark cycle. Cultures were sterile-transferred under a laminar flow hood every 10–15 days as needed to maintain log growth phase. Bacterial densities remained low (below detection) between transfers based on subsamples examined under light microscopy. All media (culture and test) were prepared by adjusting the salinity of ultrapure Milli-Q water (18 MΩ cm^{-1} at 25 °C) with Instant Ocean® artificial sea salts (Aquarium Systems, Blacksburg, VA, USA) to the desired level, adjusting the pH as necessary to 8.1 (± 0.2) using HCl or NaOH, and adding trace mineral and vitamin solutions following autoclaving. Light was measured using a Biospherical QSL 101 quantum lab sensor (BSI, San Diego, CA, USA); salinity was measured using a YSI model 3200 conductivity/salinity bridge and cell (YSI [Yellow Springs Instruments], Yellow Springs, OH, USA); and pH was measured using an Orion Versa Star Pro® multi-parameter meter equipped with an Orion ROSS® Sure-Flow pH electrode (Thermo Scientific, Waltham, MA, USA). Trace mineral and vitamin solutions were sterile filtered (Whatman® Puradisc cellulose acetate syringe filter, nominal pore size 0.2 µm, Sigma-Aldrich, St. Louis, MO, USA), added to the autoclaved media aseptically, and the final media was vacuum-filtered (Corning® cellulose acetate filter, 0.22 µm nominal pore size, Sigma-Aldrich) before storage at 4 °C for no more than 30 days. All equipment was initially cleaned by scrubbing in hot, soapy tap water. All glassware and non-plastic equipment used in culturing and experiments was rinsed with pesticide-grade acetone before use, and all glassware and any non-metallic equipment used was cleaned in 10% HCl (v/v). All equipment was sterilized by autoclaving before use. Testing and culture transfers and media prep were conducted using aseptic techniques, and algal observation and testing methods were initially developed and optimized using *Dunaliella tertiolecta* as described in Flood et al. (2018). Stock cultures were maintained at 106 (± 26) µmol photons $m^{-2}s^{-1}$ and a 16:8-h light:dark period throughout the experiments, and were gently swirled once daily. Bioassays (controls and test cultures) were maintained on a culture rack under a fluorescent light bank. All cultures in bioassays were gently swirled by hand and randomly repositioned daily using computer derived culture positioning, to avoid any effects from possible differential light exposure.

2.3. Optical density versus direct cell counts

Samples (1 mL) were randomly withdrawn from each parent culture and thoroughly vortexed before absorbance was read on a Thermo Scientific Spectronic GENESYS 2 spectrophotometer (Thermo Scientific) using a freshly wiped Hellma® quartz cuvette (Sigma-Aldrich) with a 10-mm pathlength. All absorbance measurements for OD were performed in duplicate and adjusted using a media blank (made using fresh media) as a measurement baseline. Preceding all testing procedures, the absorbance of the parent culture of *C. subsalsa* was scanned, and the three highest peaks were selected as the most appropriate wavelengths for analysis of a series of 8-point dilution curves, using progressive dilutions of stock culture in growth media. The curve that produced the ΔOD/ΔPD [the change in optical density divided by the change in population density; (Sorokin, 1973)] closest to unity (1) was used to determine the growth rates from each stock culture for each strain tested. Observations of *C. subsalsa* under light microscopy indicated that the cells were similar in appearance across nutrient and atrazine gradients during assays, so that OD-to-cell density relationships were consistent. It should be noted that bacterial growth was comparable and below detection in the cultures, so that bacterial contamination did not affect the OD data.

Because *C. subsalsa* cells are fragile and become deformed or lyse easily upon exposure to standard preservatives (Band-Schmidt et al., 2012), a "fast-kill" preservative was used (1% HEPES-buffered paraformaldehyde with 1% glutaraldehyde; Katano et al., 2009). Preservation was adequate to preserve cells for ~1 week without appreciable cell distortion or loss while preparing dilution curves immediately before each experiment. Counts to determine ΔPD were obtained by collecting random 1-mL samples from the parent culture. Cells were quantified from subsamples under light microscopy using a Reichert Bright-Line phase contrast hemocytometer with improved Neubauer rulings (Hausser Scientific No. 1475, Horsham, Pennsylvania, USA) (Guillard, 1973). Two well mixed 100-µL samples were assessed by counting 18 grids or a minimum of 400 cells. The wavelength that produced the highest correlation between absorbance and cell numbers of the stock culture was used to monitor population density and calculate growth rates for the duration of the experiment. All modeled OD:cell number relationships used had a minimum observed r^2 value > 0.95 (e.g., Fig. 1).

2.4. Population growth rate

Net cell-specific growth rates (day^{-1}) were modeled using OD following Sorokin (1973). Samples were collected in duplicate daily from gently hand-swirled culture flasks and measured in a clean, wiped quartz cuvette as described above. A media blank served as a baseline measurement. The OD was converted to population density (PD), data were log-transformed ($\log_{10}$ + 1), and the linear portion of the growth curve (slope = a_k) was used to calculate growth rate k (Sorokin, 1973; Pennington and Scott, 2001):

$$k = a_k * 3.322 \quad (1)$$

2.5. Bioassays

Concentration-response bioassays were conducted to describe the effects of atrazine exposures and imbalanced versus balanced nutrient levels on the population growth rate of the tested *Chattonella subsalsa* strain, using the Redfield ratio (TN:TP – ratio by weight 7:1, atomic ratio 16:1; Redfield, 1934, 1958; Harris, 1986) as balanced. Three nutrient regimes were used including low N, low P, and N- plus P-replete media (below). During many direct observations of *C. subsalsa* in bioassay subsamples, bacterivory on the sparse bacterial populations was not observed, although *C. subsalsa* has been reported to consume bacteria (Jeong, 2011).

Analytical-grade atrazine standards (CASRN 1912-12-9, > 98% purity) were obtained from ChemService (West Chester, PA, USA), and stock solutions were prepared in 100% pesticide-grade acetone (Fisher Scientific, Fair Lawn, NJ, USA). Chemical stocks were diluted in pesticide-grade acetone to desired final concentrations and were introduced into fresh, sterile culture media aseptically under a laminar flow hood. Testing doses were administered to obtain a final concentration of 0.1% (v/v) acetone in each replicate, with exception of a solvent-free media control series which was included for all treatments (n = 3) in all assays. Controls and treatments were incubated in 50-mL Erlenmeyer flasks, filled to 25 mL and capped with foil. Five concentrations of atrazine were used to develop response curves (12.5, 25, 50, 100 and 200 µg atrazine L^{-1}, in addition to the media and solvent controls). Exposure levels were selected based on range-finding test estimates in preliminary work, and were designed to expose *C. subsalsa* to concentrations with 0% and 100% growth inhibition effects.

The three nutrient regimes were as follows: To assess low-nutrient effects, three inorganic N: inorganic P supply ratios (molar basis; selected based on deviations from the Redfield Ratio as explained above) were prepared, including 1:1, N-limited (Low N = 2.0 µM NO_3^- N, 2.0 µM PO_4^{-3} P); 160:1, P-limited (Low P = 32 µM NO_3^- N, 0.2 µM PO_4^{-3} P); and 16:1, nutrient-replete (32 µM NO_3^- N, 2.0 µM PO_4^{-3} P) media. Each low-nutrient treatment series contained corresponding solvent and media controls. These ratios and concentrations encompass conditions reported in estuarine and coastal marine waters (e.g., Burkholder et al., 2007a,b, 2008 and references therein). Nitrate was used in media rather than ammonium because this approach provided for continuity of media preparation procedures without alteration to the base L1 media recipe across multiple assay platforms (Flood et al., 2018).

Nutrient × herbicide effects were assessed using the above assay procedures in a simultaneously run, three-way bioassay format. Two separate experiments were conducted. The first was for a 10-day duration which corresponded to early exponential growth, but it was noted that low nutrient conditions had just been achieved by the end of that experiment (Fig. 2). Therefore, a second experiment was conducted for 28 days, at which point cultures were in late-to-post exponential growth, to enable improved assessment of *C. subsalsa* response to the low-nutrient regime.

Each assay included 63 50-mL flasks in identical set-up, inoculation and chemical preparation approaches as described above, but varied in culture media nutrient additions. Testing procedures were derivations on the standard 96-h static algal toxicity bioassay protocols (American Society for Testing and Materials (ASTM), 1996), and were extended to assess 10- and 28-day endpoints. The 10-day endpoint was used to evaluate growth and hemolytic responses (below) of *C. subsalsa* to treatments during early exponential growth phase and a 28-day endpoint was used to enable assessment of treatment effects during late phase growth under nutrient depletion, which can take 14 days or longer to observe in *Chattonella* spp. (Kim et al., 2004; Yamaguchi et al., 2008). Growth of *C. subsalsa* was slow in comparison to bioassays conducted with the small green flagellate, *Dunaliella tertiolecta* (Chlorophyta) (replete media K_{max} = 0.95 div day^{-1} versus 2.04, respectively), in work to develop the general test platform for these assays (see Flood et al., 2018). In assays with *C. subsalsa*, the lag phase continued for 48 to 72-h post-inoculation, and exponential growth did not begin to occur until about 96-h post-inoculation. Therefore, 96-h bioassay protocols commonly used in standard toxicity testing were not considered appropriate to assess effects of atrazine × nutrient stress, and were modified to accommodate the slow growth of this test species. The 96-h half-maximal inhibition concentrations (IC_{50}) were calculated in this work to facilitate comparisons of atrazine tolerance and sensitivity reported in the literature for other organisms, but these values are presented with the caveat that the 96-h endpoint may not effectively capture the most ecologically relevant response of *C. subsalsa* to the

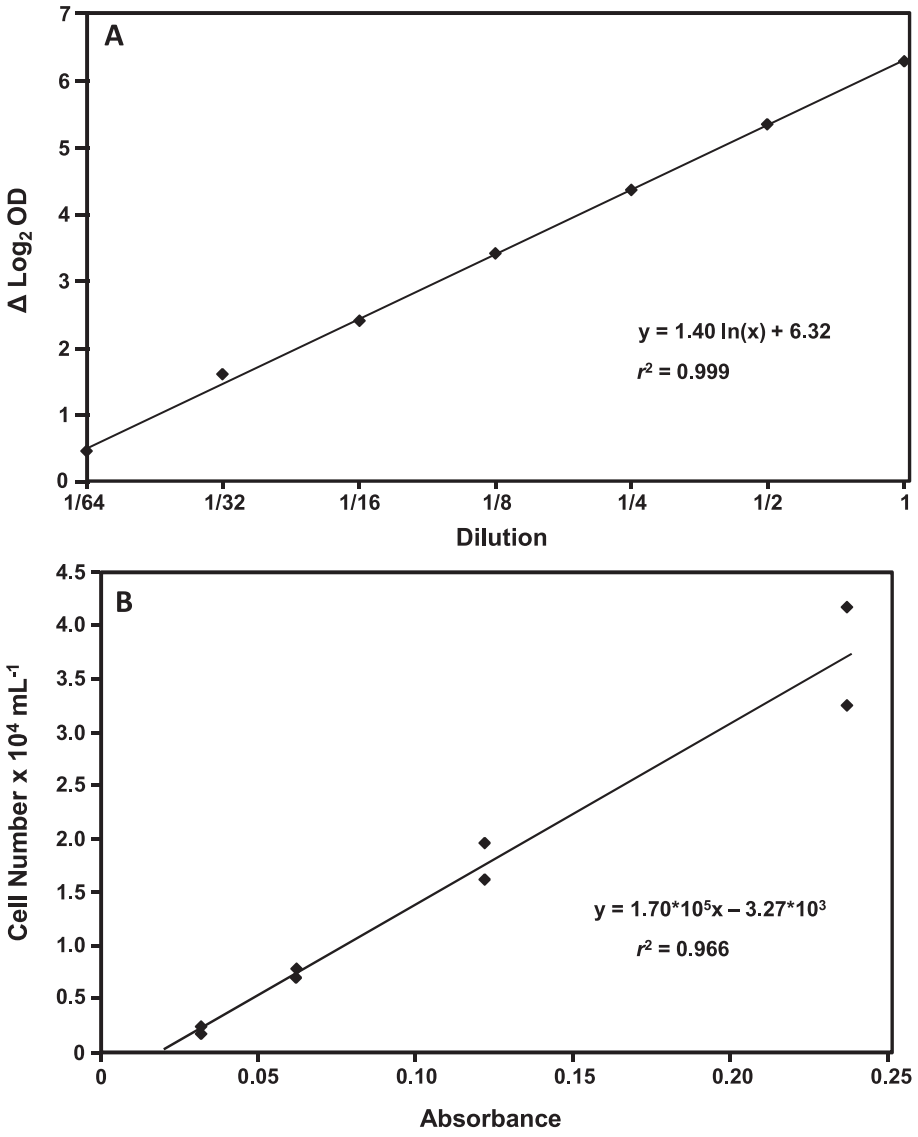

Fig. 1. Example of the modeled relationships between (A) optical density (OD) versus dilution at 415 nm, and (B) *C. subsalsa* cell number versus absorbance at 415 nm, used to monitor population growth in atrazine inhibition assays.

treatments. These analyses instead emphasized responses to treatments at endpoints that were 10 days and 28 days post-exposure.

For all experimental assays, when stock culture of *Chattonella subsalsa* was in exponential growth phase (assessed by periodic direct cell counts), test and control cultures were inoculated into foil-capped Erlenmeyer flasks under aseptic conditions to attain initial cell densities of ~1.5×10^3 cells mL^{-1} (10-day assay) or 1.0×10^3 cells mL^{-1} (28-day assay) in all experimental cultures and controls. Initially in experiments, the cultures were not nutrient-limited (as they had been taken from nutrient-replete stock culture), the cells were in exponential growth phase, and all cells had the same nutritional history. The inoculum was less than 5% of the total test culture volume (that is, less than 1.25 mL) to minimize the influence from the high nutrient levels in the stock culture on controls and treatments. Time-series testing began when *C. subsalsa* was added to testing media, and samples were collected immediately following inoculation (0 h) to assess the initial OD. Samples (n = 2) for OD measurements were taken during the assays at equally spaced daily intervals for the first 4 days following inoculation (0, 24, 48, 72, and 96 h), and then every third day for the remainder of the test duration. Immediately before each sample was taken, flasks were gently swirled by hand for 30–60 s, and then 1 mL was gently, randomly siphoned using a sterile transfer pipette and aseptically removed for OD measurement.

For each sample taken from control and test replicates, growth of *C. subsalsa* was determined by spectrophotometric determination of OD. The OD was then converted to cell density using equations derived from direct cell count: optical density relationships that had been established using the parent culture within one week prior to the test initiation, as described above. The percent inhibition (%I) of algal growth at each atrazine concentration was calculated by comparing mean growth rates for each treatment to the respective solvent controls for the treatment, as follows:

$$\%I = \left[1 - \left(\frac{k}{\overline{x}_{k_{solvent\ control}}}\right)\right] \cdot 100 \quad (2)$$

where k is the growth rate of the pertinent sample, calculated as described previously. Nominal concentrations of pesticide were quantified with enzyme-linked immunosorbent assay (ELISA) at the initiation and conclusion of each bioassay with enzyme-labeled paramagnetic particles of atrazine (method detection limit for atrazine = 0.046 µg/L; SDI, Delaware, MD, USA; see Flood, 2017 Appendix for raw ELISA data and QA/QC determination). The accuracy of this method has been shown to be comparable to gas chromatographic quantification of atrazine

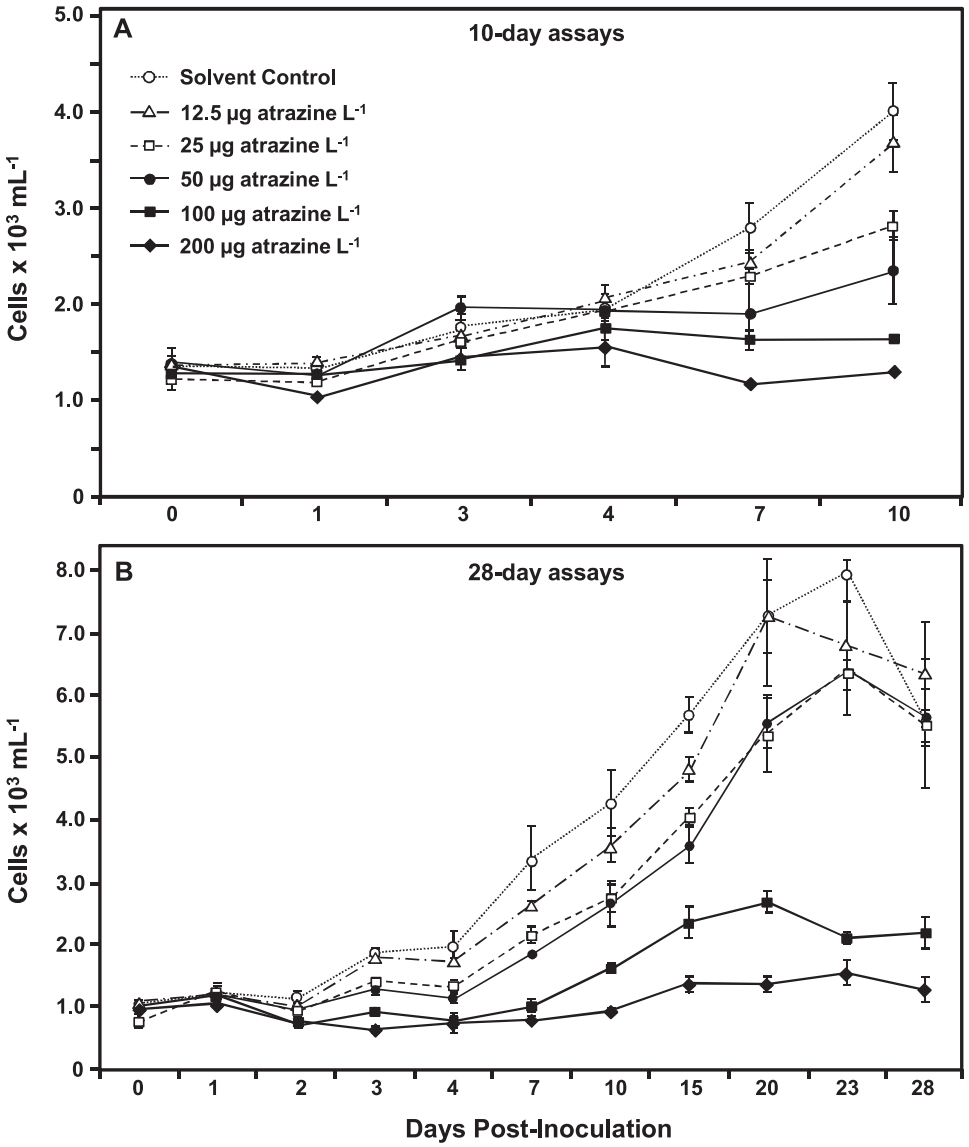

Fig. 2. Growth curves for *Chattonella subsalsa* (A) 10-day and (B) 28-day bioassays with atrazine under nutrient-replete conditions. Data are given as means + 1 SE; some SEs were too small to be evident.

concentrations in surface water samples (Gascón et al., 1997).

Salinity and pH were checked at test initiation and conclusion to ensure that changes were within acceptable limits (salinity 20 ± 1, pH 8.1 ± 0.2) based on the test platform developed by Flood et al. (2018). At assay initiation, all cultures were inoculated from the same parent culture. Solvent controls showed similar growth as the media-only controls for all tests (Fig. 2), so the solvent controls were used as the baseline activity for subsequent analytical comparisons.

2.6. Protocols for assays of hemolytic activity

Several mechanisms have been suggested for *Chattonella* toxicity to fish, including production of neurotoxic brevetoxins or brevetoxin-like substances (Ahmad, 1995; Khan et al., 1995, 1996; Bourdelais et al., 2002; Band-Schmidt et al., 2012); a synergistic interaction between reactive oxygen species (ROSs) and free fatty acids (FFAs) (Marshall et al., 2003, 2005a; also see Oda et al., 1992, 1997; Ishimatsu et al., 1996a,b; Kim et al., 2005, 2006, 2007); and gill damage by unidentified hemolytic substances (Shimada et al., 1983) or polyunsaturated fatty acids (Marshall et al., 2002) which can have hemolytic effects (Fu et al., 2004); and physical clogging of gills (Matsusato and Kobayashi, 1974). Hemolytic effects are common (Shimada et al., 1983; Ahmed et al., 1995; Kuroda et al., 2005; Pistocchi et al., 2012). Bioactive substances released by *Chattonella* spp. have also adversely affected diatoms (Matsuyama et al., 2000) and dinoflagellates (Fernández-Herrera et al., 2016), allowing these relatively large and slowly growing flagellates to become dominant under conducive conditions.

The lack of chemical structure analysis for toxins from *C. subsalsa* prevented analysis of toxicity by toxin quantification. As a proxy for the toxic potential of the control and test cultures, erythrocyte lysis assays (ELAs, developed for 96-well microtiter plates with well volume 120 µL; Eschbach et al., 2001; Kuroda et al., 2005) were modified for use with a larger sample volume (1 mL microcentrifuge tubes), and then ELAs were performed using freshly prepared fish erythrocytes to characterize the hemolytic activity (HA) of *C. subsalsa*. From this test, it was inferred that high HA indicated relatively higher toxicity, and low HA indicated relatively lower toxicity.

2.6.1. Blood collection and erythrocyte preparation

The blood from randomly selected, healthy adult tilapia (*Oreochromis niloticus*) was examined under light microscopy prior to experiments to ensure that it contained healthy erythrocytes and

showed no evidence of disease. The fish blood was used in tests of hemolytic activity in this strain of *C. subsalsa* strictly from a comparative standpoint (control conditions without the alga versus treatment with the alga). Fish were anesthetized with a pH-adjusted 0.02% (w/v) solution of aminobenzoic acid ethyl ester [Tricaine™ (MS-222) (Sigma-Aldrich, St. Louis, MO, USA)] in transport water. Blood was collected by gill puncture as follows:

Anaesthetized fish were placed on wet towels and blood was collected using a Pravaz No. 1 needle. Approximately 5 mL of blood per fish was collected into a 10-mL syringe prefilled with 5 mL of pH-adjusted (7.2) RPMI 1640 media (without phenol red), diluted to 10% (v/v) with Milli-Q water (to adjust for fish serum osmolarity), and supplemented with 50 IU sodium heparin salt (Sigma-Aldrich) added as an anticoagulant. Blood samples were transferred to 15-mL centrifuge tubes and gently hand-agitated by inverting two or three times. Red bloods cells were then separated from serum by gentle centrifugation (2100 rpm at 4 °C for 5 min) and washed by replacing supernatant with fresh media until the supernatant appeared clear after gentle mixing. Whole, washed erythrocytes were diluted 1:10 with RPMI 1640 culture medium containing 22.5 IU sodium heparin anticoagulant mL^{-1}. Erythrocytes ($\sim 10^7$ cells mL^{-1}) were stored at 4 °C for no more than 10 days, based on preliminary work wherein microscopic observations and spectrophotometric measurements (λ = 414 nm) indicated no noticeable lytic activity over that duration. The erythrocytes were resuspended daily until use.

2.6.2. Preparation of algal extracts

Because the hemolytic component of *Chattonella* is likely light-activated (Kuroda et al., 2005), all hemolytic (ELA) preparation and procedures were conducted at 4 °C (Eschbach et al., 2001) under continuous fluorescent lighting (30 µmol photons PAR $m^{-2} s^{-1}$). Following 10-day or 28-day assay endpoint OD measurements, *Chattonella subsalsa* cells were immediately centrifuged (IEC Centra-CL2, Needham Heights, MA, USA) for hemolytic analysis. Because of the low cell densities in high atrazine treatments, treatment replicates were pooled prior to extraction procedures as described below. Before replicates were pooled, they were checked to ensure that the variation in cell density and hemolytic activity between individual replicates was less than the variation between treatments (*F*-test, $p < 0.05$), following counsel from Dr. C. Arellano, Department of Statistics, NCSU. The low variation supported the validity of pooling the data. All remaining culture from two of the three replicates was transferred to a 50-mL centrifuge tube (which could fit the volume of only two replicates at a time) and spun at 2000 rpm for 10 min, during which time a cell pellet accumulated. The supernatant was carefully removed by pipetting and the volume of third test replicate was added and centrifuged, thus consolidating all cells from all three replicates of a control or a treatment into one pellet (n = 21 different pellets for assessment of treatment effects, including 5 concentrations and solvent and media controls for 3 different nutrient regimes).

Once the three replicates had been combined into a single visible cell mass, the pellet was gently aspirated and rinsed using cold, pH-adjusted, sterile ELA buffer (150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4$, 3.75 mM $CaCl_2$, and 12.2 mM Trisma base [TRIS], pH-adjusted to 7.4 with 1N HCl), and re-consolidated by centrifugation as above. The supernatant was again carefully removed, and the pellet was resuspended and rinsed in cold assay buffer (for a total of three rinses) to remove any residual test medium. The cell pellet was then resuspended in 5 mL of assay buffer, and the tube contents were transferred to a sterile, acid-stripped, 20-mL glass beaker. An additional 10 mL of assay buffer was added to the centrifuge tube to rinse any remaining cells into the beaker. The beaker contents were sonicated for 30 continuous seconds at amplitude 45 on a 20 kHz Sonic Dismembrator (Fisher Scientific Model 550, Fisher Scientific, Pittsburgh, PA, USA). The sonicated pellet was used immediately in ELAs.

2.6.3. Hemolytic assays

The modified ELAs used for analysis of hemolytic activity by *Chattonella subsalsa* were conducted in 1-mL conical microcentrifuge tubes. Algal cellular contents were extracted as described above, and equivolume amounts of erythrocytes and algal sample (300 µL) were added to a microcentrifuge tube (to enhance pellet formation; n = 3) and incubated for 24 h at 4 °C and 30 µmol photons PAR $m^{-2} s^{-1}$. Microcentrifuge tubes were then spun at 1250 rpm for 10 min in a refrigerated microcentrifuge (4 °C; Micromax RF, IEC, Needham Heights, MA, USA). Erythrocytes were also incubated in an equal volume of assay buffer alone as a negative control, and in an equal volume of 20 µg mL^{-1} saponin solution (Sigma-Aldrich preparation from the soapbark tree *Quillaja saponaria*, a known hemolytic compound) which was microscopically verified to cause 100% lysis of red blood cells and served as a positive control. The microcentrifuge tube supernatant was transferred to a quartz cuvette for measurement of sample absorbance (wavelength 414 nm, based on preliminary scans of initial preparations of lysed erythrocytes which showed the highest hemoglobin absorbance peaks, following Eschbach et al., 2001). The change in absorption at 414 nm following exposure to sonicated cells was used to measure hemoglobin released by lysed erythrocytes as a result of toxic activity of *C. subsalsa*, using the following normalization equations:

$$[(cells/mL)\cdot(V_{centrifuged})\cdot(V_{well})] = N \quad (3)$$

$$\left(\frac{sample - \bar{x}_{negative\ control}}{\bar{x}_{positive\ control}}\right)\cdot 100 = \%\ hemolysis \quad (4)$$

$$\left(\frac{\%\ hemolysis}{N}\right)\cdot 10^5 = Normalized\ \ Lytic\ \ Value\ (\mathrm{NLV}) \quad (5)$$

wherein ($V_{centrifuged}$) was the total volume of culture centrifuged to form assay pellets, and (V_{well}) (volume added to well) was 0.300 mL (300 µL).

2.7. Statistical analyses

All statistical analyses were performed using JMP software (version 12.2.0, SAS Institute, Cary, NC, USA). Normality of the data was checked with the Shapiro-Wilks test, and Bartlett's test for unequal variance was used to check variation. The only sample which failed normality checks was the low P series on day 4 of the 10-day assays. Analysis of variance (ANOVA) comparing nutrient and herbicide effects on growth considered the data from day 10 from that assay series, and day 28 from the 28-d assay series. The 10-day and 28-day bioassays were independently analyzed.

Atrazine effects on growth were determined using a one-way analysis of variance (ANOVA). No-observed effect concentrations (NOECs) and lowest-observed effect concentrations (LOECs) were determined ($p < 0.05$) using Dunnett's procedure for multiple comparisons to determine which specific treatments differed significantly from controls (Zar, 1999). Atrazine × nutrient comparisons were analyzed by two-way ANOVAs using nutrient levels and herbicide concentration as the fixed effects, and growth rate or normalized hemolytic activity as the dependent variable. The *F* statistic and associated *P* value for the independent variables and the interactions between them were also reported. Inhibition concentration point estimates of sublethal toxicity (IC_{50}s) were based on growth rates, and were calculated using the Inhibition Concentration linear interpolation (IC*p*) method of Norberg-King (1993):

$$ICp = C_J + [M_1(1 - p/100) - M_J]\frac{(C_{J+1} - C_J)}{(M_{J+1} - M_J)} \quad (6)$$

wherein:

C_J ≡tested concentration whose observed mean response is greater than M_1 (1-p/100);

C_{J+1} ≡ tested concentration whose observed mean response is less than $M_1(1\text{-}p/100)$;
M_1 ≡ smoothed mean response for the control;
M_J ≡ smoothed mean response for concentration J;
M_{J+1} ≡ smoothed response for concentration $J + 1$;
p ≡ percent reduction in response relative to the control response; and
ICp ≡ estimated concentration at which there is a percent reduction from the smoothed mean control response. The ICp is reported for the test together with the 95% confidence interval calculated by the ICPIN.EXE program.

Results were generated using bootstrap methods to derive point estimates and confidence intervals from no fewer than 80 resamplings (Efron, 1982).

3. Results

3.1. Growth

Media controls in the shorter-term (10-day) assay more than doubled in cell density relative to initial measured values by 96-h ($1.3 \pm 0.2 \times 10^3$ cells mL^{-1} at hour 0 versus $2.8 + 0.3 \times 10^3$ cells mL^{-1}) and had an almost 200% increase in density by day 10 ($4.0 \pm 0.3 \times 10^3$ cells mL^{-1}) (Fig. 2). Controls in the longer-duration bioassays (28 days) began to decrease in cell density after day 23 (replete media $\bar{x} = 7.4\ ^*10^4 \pm 3.0 \times 10^3$ on day 23 and replete media $\bar{x} = 5.1\ ^*10^4 \pm 2.5 \times 10^3$ on day 28). Some differences in the two experiments (10 days versus 28 days) were expected, considering that the inoculation cell densities were higher in the 10-day experiment ($\sim 1.5 \times 10^3$ cells mL^{-1}, vs. 1.0×10^3 cells mL^{-1} in the 28-day experiment), which also would have resulted in substantially more algal biomass initially in the 10-day experiment. Controls in nutrient-replete (Redfield ratio) media remained in lag phase for the first 48–72 h, and then increased in cell density until day 23 (Fig. 2). Exponential growth occurred approximately during days 4 through 20, and slowed post-day 20. Growth in nutrient-replete cultures was similar to that in "nutrient-limited" cultures for the first seven days, reflecting the extended influence of the nutrient supplies added with the initial parent stock solution of *Chattonella subsalsa*. Thereafter, effects of nutrient depletion were observable in controls (Fig. 3).

3.2. Atrazine effects

Atrazine concentration significantly affected the growth of *Chattonella subsalsa* in nutrient-replete media at all time points after 96-h (Dunnett's test for multiple comparisons, $p \leq 0.05$) as shown by IC_{50}s (Tables 1 and 2). In the 28-day assays, overall sensitivity to atrazine decreased until day 4 (96-h IC_{50} 36 μg L^{-1}), and increased post-day 15 (28-d IC_{50} 130 μg L^{-1}) – although there was no observed herbicide degradation based on ELISA checks of test samples on day 28. Effective dose estimates for *C. subsalsa* in nutrient-replete media initially increased rapidly before leveling off, and growth responses to atrazine were similar on days 15–28 (IC_{50} range = 105.3–130.8 μg L^{-1}). Atrazine effects on cell growth in nutrient-replete media followed typical dose-response curves after days 7–10. In the 10-day assay, nutrient-replete *C. subsalsa* cultures were most resistant to atrazine at 96-h (IC_{50} = 187 μg L^{-1}), and sensitivity to atrazine increased continuously until day 10 (IC_{50} = 46.9 μg L^{-1}; Table 1). In the 28-day assays with nutrient-replete media, higher atrazine (100–200 μg L^{-1}) had stronger growth-limiting effects on *C. subsalsa* over time, whereas responses in mid-range atrazine concentrations (25–50 μg L^{-1}) were similar to responses of control cultures without atrazine by day 28, and the lowest dose (12.5 μg atrazine L^{-1}) had cell numbers comparable to controls by day 28 (Figs. 2 and 3). Nutrient-limited treatments had no atrazine-induced growth inhibition after 10 days of exposure, and at 28 days multiple cultures at higher atrazine concentrations had significantly higher growth than controls (Fig. 3) (ANOVA, $p \leq 0.05$).

Inhibition effect concentration estimations based on growth rate were most precise (smaller CIs) on day 10 of the 10-day assay (Table 1) and from days 4 through 10 in the 28-day assay (Table 2). Dose-response curves for the 10-day assay illustrated the increasing potency of atrazine from days 4 to 10 (day 10 IC_{50} = 46.9 μg L^{-1} versus 187 μg L^{-1} on day 4); however, growth in the 28-day assay was most sensitive to herbicide exposures between days 1–4 (Table 2), followed by increasing tolerance from days 7–20 (day 10 IC_{50} = 69.1 μg L^{-1} versus 130 μg L^{-1} on day 28). In the ANOVA model, atrazine concentration had the strongest effect on cell growth at day 10, but all model variables were significant at that endpoint including the nutrient × atrazine interaction term (which did not outrank either treatment effect) (Table 3). In contrast, growth at the 28-day endpoint also had significant effects from all tested variables, but nutrient limitation had much stronger influence on modeled growth rates than herbicide concentration (two-way ANOVA, $p \leq 0.05$) (Table 3). By day 28, herbicide concentration had less influence on growth responses (LogWorth of 2.52 versus 22.37 from 10-day ANOVA model) (Table 3).

Nitrogen and phosphorus limitation had no observable effects on growth rates until after day 10, when growth under nutrient limitation began to noticeably decrease relative to growth in replete-media cultures (Fig. 3). For example, at day 7 the mean growth rate of *C. subsalsa* in nutrient-replete media controls was 0.16 div day^{-1}, not significantly different from growth rates (0.14 div day^{-1}) in both low-N and low-P media control treatments. In contrast, at day 10 the mean growth rate of *C. subsalsa* in nutrient-replete media controls (0.10 div day^{-1}) was significantly higher than growth in the low-N and low-P media controls (0.05 and 0.07 div day^{-1}, respectively; ANOVA, $p < 0.05$). The actual point of initial growth inhibition effects attributable to media nutrient depletion appeared to have occurred between days 7 and 10, but observations were restricted to the measured endpoints (Fig. 3). In the 10-day assay, both N- and P-limited *C. subsalsa* exhibited atrazine sensitivity, which was highest at 24-h (43.2 μg atrazine L^{-1} in low N; 11.5 μg atrazine L^{-1} in low P). Following a period of wide-ranging point estimates, IC_{50} values became more consistent by day 10 (Table 1). By contrast, in the 28-day assay series, there was significant growth stimulation by atrazine in both N- and P-limited cultures, relative to growth in the low-nutrient controls after day 10 (Dunnett's test for multiple comparisons, $p < 0.05$) (Fig. 3). Thus, IC_{50} values could not be determined for *C. subsalsa* in the low-nutrient treatments during late exponential growth, because observed concentration-related responses were stimulatory rather than inhibitory.

3.3. Toxicity as hemolytic activity

Both N and P limitation increased hemolytic activity (HA) relative to HA in nutrient-replete media in the *Chattonella subsalsa* atrazine-free controls at 28 days (without evidence of effects attributable to the presence of solvent in media), (Fig. 4; Dunnett's test for multiple comparisons, $p \leq 0.05$) supporting previous reports of increased toxicity under nutrient depletion in other harmful algal species (Johansson and Granéli, 1999a,b; Granéli and Johansson, 2003; Granéli et al., 2012). In the 10-day HA assay series, by contrast, hemolytic activity [reported as Normalized Lytic Value, NLV (Eq. (5))] was significantly higher in the nutrient-replete control cultures (9.6 NLV ± 0.8) relative to the low-N and low-P treatments (3.3 ± 0.2 and 4.2 ± 0.1 NLV, respectively) (Dunnett's test for multiple comparisons; $p \leq 0.05$; Fig. 4). These findings contrast with reports that ROS production (and, by proxy, toxicity) is not related to nutrient availability in other *Chattonella* (e.g., Liu et al., 2007), and potentially offers (growth phase-related) context for reports that hemolytic activity is not increased by nutrient limitation in other harmful raphidophyceans (de Boer et al., 2004). As nutrient depletion (both N and P) progressed over time, *C. subsalsa* exhibited significantly higher HA in cultures without atrazine,

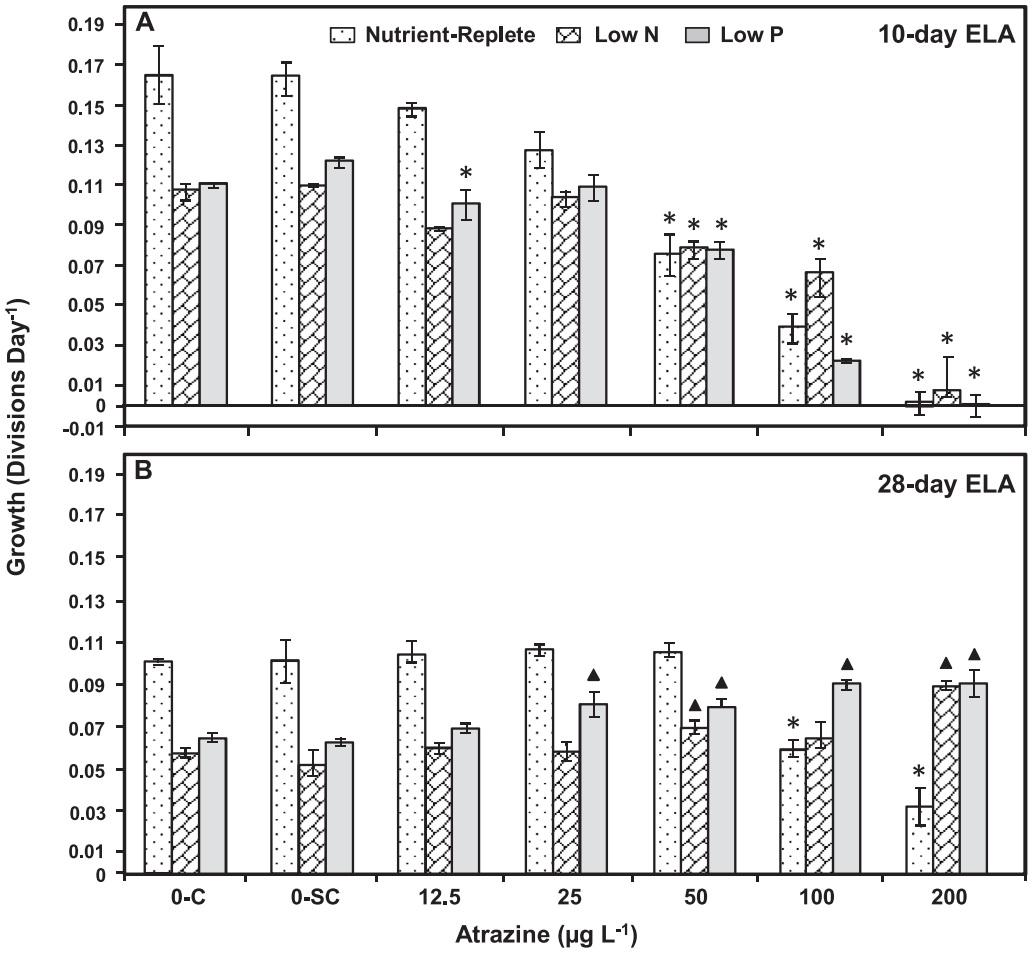

Fig. 3. Growth rates (divisions day^{-1}) in all tested atrazine concentrations, nutrient treatments and controls (C, control; SC, solvent control) for erythrocyte lysis assays. Hemolytic activity was examined in two different assays, first (A) after a 10-day growth period, and second (B) following 28-days of growth. Data are given as means ± 1 SE. Asterisks (*) indicate significant decreases in growth compared to carrier controls; triangles (▲) indicate increased growth relative to carrier controls (Dunnett's multiple comparison tests; $p \leq 0.05$).

Table 1
Growth inhibition (IC values (in bold) and 95% confidence intervals; µg atrazine L^{-1}) over time in 10-day bioassays with *Chattonella subsalsa* strain CCMP2191. BD = beyond detection.

Treatment	Day	IC_{50}	(95% CI)	IC_{10}	(95% CI)
Nutrient-Replete	1	**18.8**	BD	**13.8**	BD
	3	**88.6**	BD	**57.7**	(2.9–65.5)
	4	**186.5**	(166.7–208.6)	**62.8**	(5.6–65.8)
	7	**74.4**	(44.0–114.2)	**29.5**	(9.9–35.2)
	10	**46.9**	(40.4–56.9)	**12.9**	(7.5–21.3)
Low N	1	**43.2**	(6.3–104.5)	**28.4**	(1.3–55)
	3	**147.8**	BD	**40.2**	(4.1–72.5)
	4	**99.5**	BD	**8.3**	(3.6–54.4)
	7	**108.0**	(84.5–151.7)	**7.1**	(4.5–26.7)
	10	**119.7**	(105.7–140.2)	**9.9**	(8.4–25.6)
Low P	1	**11.5**	(7.8–82.1)	**2.3**	(1.5–30.5)
	3	**102.5**	BD	**21.8**	(17.8–62.4)
	4	**88.2**	(48.2–126)	**18.0**	(16.5–20.9)
	7	**50.0**	(39.3–57.1)	**7.3**	(5.2–12.6)
	10	**64.7**	(58.5–68.0)	**8.5**	(6.0–25.7)

Table 2
Growth inhibition (IC values and 95% confidence intervals; µg atrazine L^{-1}) over time in 28-day bioassays with *Chattonella subsalsa* strain CCMP2191 in nutrient-replete media. BD = beyond detection; ND = not determined.

Day	IC_{50} (CI)	IC_{10} (CI)
1	146.2 (BD)	61.5 (5.1–122.9)
2	ND	ND
3	42.8 (32.8–8.7)	10.6 (3.8–10.4)
4	36.1 (27.7–43.2)	7.5 (3.5–28.3)
7	46.5 (36.9–55.3)	6.3 (3.9–25.3)
10	69.1 (56.5–81.0)	8.8 (5.4–18.5)
15	105.3 (89.0–128.2)	11.69 (8.9–21.2)
20	130.8 (100.2–147.4)	36.7 (12.4–58.2)
23	124.3 (97.8–142)	55.6 (10.1–62.3)
28	129.7 (96.4–154.7)	61.9 (51.2–64.6)

Table 3
Results of two-way ANOVAs examining atrazine Concentration (Atra) and nutrient regime (Nut) effects on growth rates of *Chattonella subsalsa* strain CCMP2191 in bioassays at 10-day (R^2 adjusted 0.94) and 28-day (R^2 adjusted 0.85) endpoints.

Endpoint; Bioassay	Source	DF	F Ratio	LogWorth	Prob > F
Day 10; 10-day bioassay	Atra	5	151	22.4	< 0.0001
	Nut	2	15.6	4.89	< 0.0001
	Atra•Nut	10	7.19	5.37	< 0.0001
Day 28; 28-day bioassay	Atra	5	4.44	2.52	0.0030
	Nut	2	25.6	6.92	< 0.0001
	Atra•Nut	10	25.3	12.6	< 0.0001

relative to nutrient-replete controls (Fig. 4, 28 days), (7.4 ± 1.3 and 10.1 ± 0.4 NLV, respectively, compared to 0.5 ± 0.3 NLV) in the late-growth phase assay. When atrazine is also considered as a factor in production of hemolytic compounds, the highest HA (13.8 ± 0.2 NLV) was observed in both early-phase growth (10-d) with intermediate (25 µg atrazine L^{-1}) concentrations in nutrient-replete media and high concentration (100 µg L^{-1}) in late-growth phase, N-limited cultures (28-d; 12.8 ± 1.5 NLV; Fig. 5). Hemolytic activity in the atrazine + nutrient treatments also had differing 10-day versus 28-day

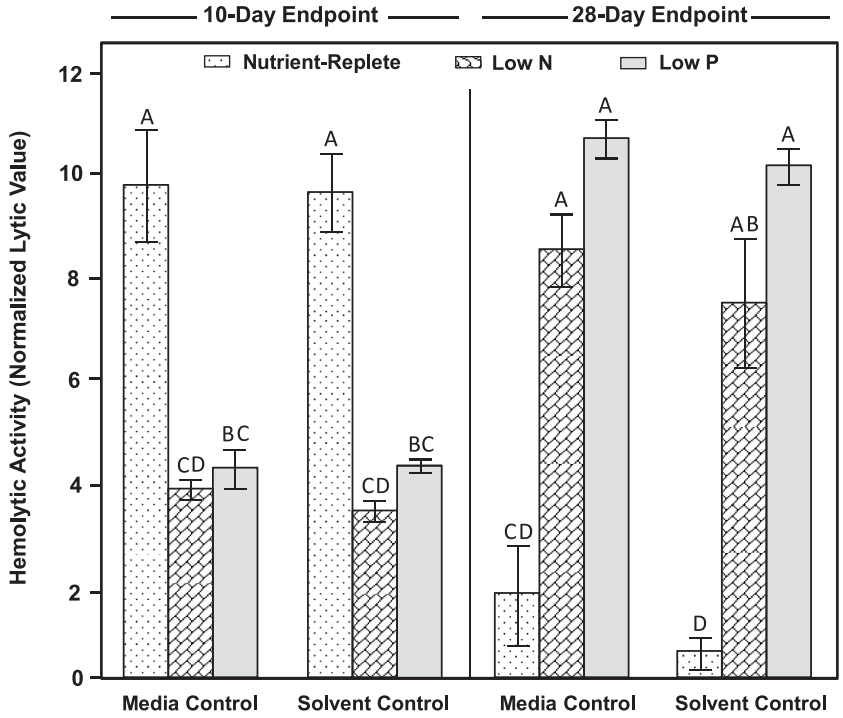

Fig. 4. Normalized Lytic Value (NLV) for hemolytic activity in control cultures, measured at the end of 10- and 28-day assays. Data are given as means ± 1 SE. Levels with the same letters are not significantly different ($p \leq 0.05$).

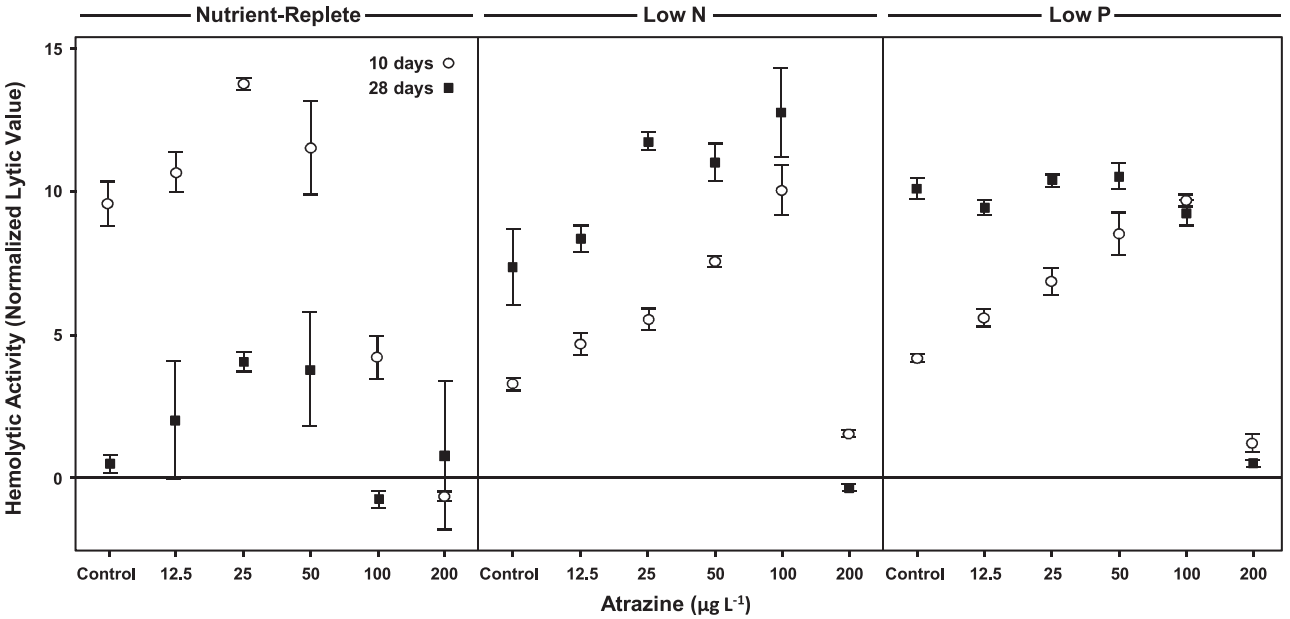

Fig. 5. Normalized Lytic Value (NLV) for hemolytic activity at different test endpoints, normalized to cell density and sample volume, in various nutrient regimes under a range of atrazine concentrations. Data are given as means ± 1 SE. Note that more hemolysis occurred in nutrient-replete cultures at 10 days, whereas there was more hemolysis at 28 days in N/P-limited cultures except at the highest atrazine concentration.

response dynamics (Fig. 5). The 10-day nutrient-limited assays indicated increasing HA with increasing atrazine concentrations up to 100 µg/L, with no observable growth or HA in any nutrient regime at 200 µg/L. HA in nutrient-replete media at lower atrazine concentrations (12.5 µg L^{-1}) in early exponential growth was similar to that of controls, but the highest HA observed in the 10-day assay occurred in nutrient-replete cultures with 25 µg atrazine L^{-1}. In contrast to nutrient-limited atrazine treatments, HA in nutrient-replete cultures varied non-monotonically with increasing atrazine concentrations (increased from 0 to 25 µg L^{-1} and decreased from 50 to 200 µg L^{-1}). As a result, highest levels of HA were observed (i) in early exponential growth phase under nutrient-replete conditions at an intermediate atrazine concentration (25 µg L^{-1}) (13.8 ± 0.2 NLV), and (ii) in late exponential growth phase under N limitation at a high atrazine concentration (100 µg L^{-1}) (12.8 ± 1. NLV) (Fig. 5). Hemolytic activity in atrazine + nutrient treatments also had differing 10-day versus 28-day response dynamics, as follows:

The 10-day nutrient-limited assays indicated *increasing* HA with increasing atrazine concentrations up to 100 µg/L, with no observable growth or HA in any nutrient regime at 200 µg atrazine L^{-1}. HA in

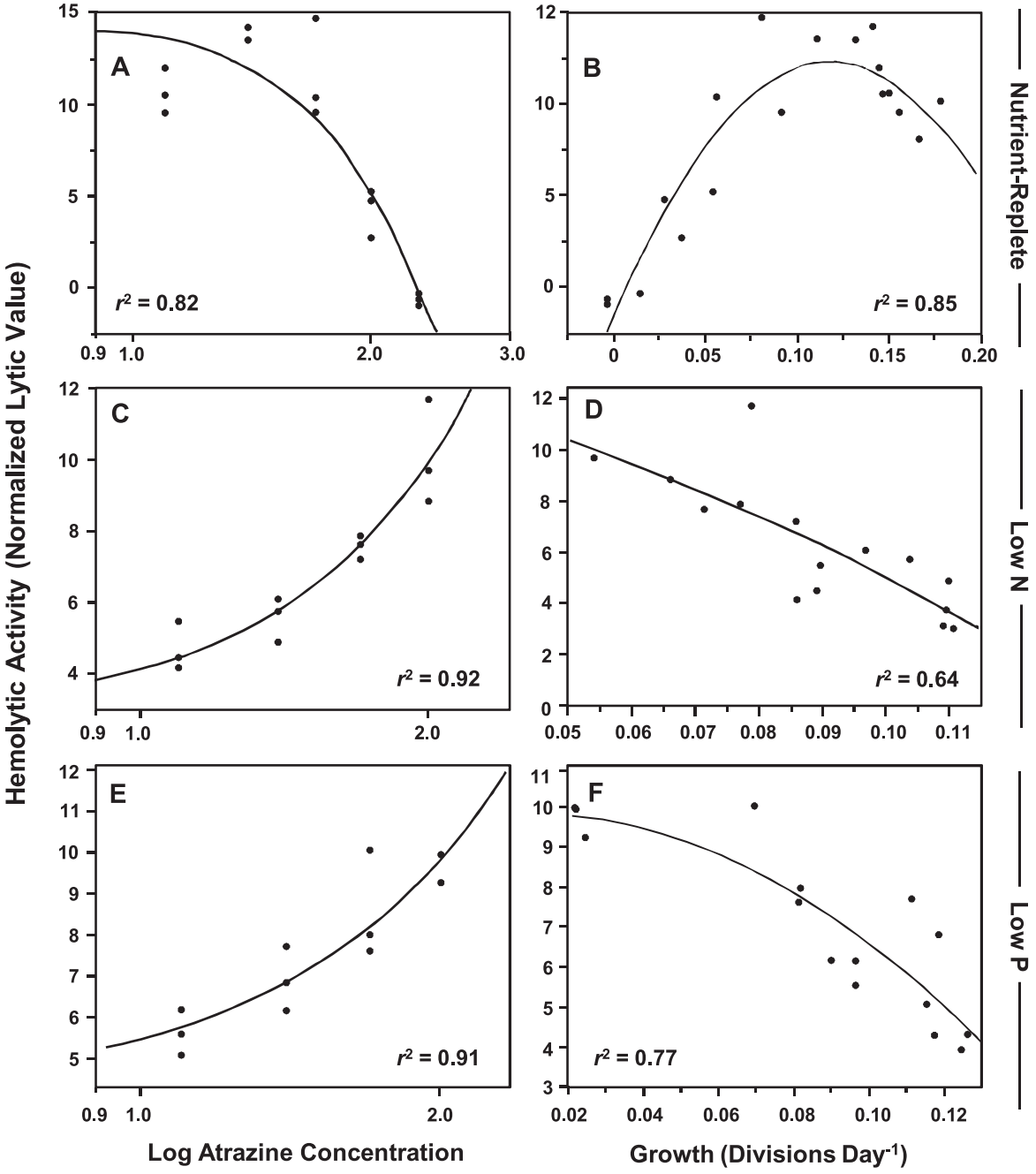

Fig. 6. Log-normal relationships between hemolytic activity (normalized to cell density and sample volume) and atrazine concentration (A, C, E), and between hemolytic activity (normalized as above) and growth rates (B, D, F) at the 10-day endpoint. Panels A and B ≡ nutrient-replete media; panels C and D = low N; and panels E and F = low P. Note that panels C–F (low-nutrient treatments) exclude 200 µg atrazine L^{-1} cultures due to nearly complete cell loss (see text).

nutrient-replete media at lower atrazine concentrations (12.5 µg L^{-1}) in early exponential growth was similar to that of controls, but the highest HA observed in the 10-day assay occurred in nutrient-replete cultures with 25 µg atrazine L^{-1}. In contrast to nutrient-limited atrazine treatments, HA in nutrient-replete cultures varied non-monotonically with increasing atrazine concentrations (increased from 0 to 25 µg L^{-}1 and decreased from 50 to 200 µg L^{-1}). As a result, in the early-exponential growth assay, HA in nutrient-replete controls was significantly higher (13.8 ± 0.2 NLV) at intermediate concentrations than in the highest atrazine concentrations [100 µg L^{-1} and 200 µg L^{-1}; 4.3 (± 0.8) to −0.6 (± 0.2), respectively]. The low-N and low-P cultures responses to atrazine in terms of HA were generally consistent with each other while distinct from the nutrient-replete treatments (Figs. 5 and 6). The nutrient-limited control series (both N and P) were lower in HA than the nutrient-replete cultures at the end of the 10-day assay (Fig. 4) but had higher HA across all atrazine concentrations at the end of the 28-day assay than nutrient-replete cultures (Fig. 5).

In the 28-day assay series, there was again no apparent concentration-related effect of atrazine on hemolytic activity in nutrient-replete cultures, which had low production of hemolytic compounds in all concentrations (−0.7 to 4.1 NLV) in comparison to late-phase nutrient-limited cultures at intermediate to high atrazine concentrations (25–100 µg L^{-1} had a range of 9.3–12.8 NLV). At day 28, with the exception of the 200 µg L^{-1} atrazine treatment, cultures in the low-P treatment were elevated in HA across the atrazine concentration gradient, whereas in the low-N treatment, HA of *C. subsalsa* was significantly higher at 100 µg atrazine L^{-1} (12.8 ± 1.5 NLV) than at low atrazine concentrations (0–12.5 µg L^{-1}; range 7.4 ± 0.5 to 8.5 ± 0.5

Table 4
Results of two-way ANOVAs examining atrazine concentration (Atra) and nutrient regime (Nut) effects on hemolytic activity of *Chattonella subsalsa* strain CCMP2191 in bioassays at the 10-day (R^2 Adjusted 0.93) and 28-day (R^2 Adjusted 0.86) endpoints.

Bioassay	Source	DF	F Ratio	LogWorth	Prob > F
10-day	Atra	5	119	18.0	< 0.0001
	Nut	2	191	8.54	< 0.0001
	Atra•Nut	10	55.8	12.7	< 0.0001
28-day	Atra	5	71.0	9.94	< 0.0001
	Nut	2	298	13.1	< 0.0001
	Atra•Nut	10	13.7	4.46	< 0.0001

NLV) (ANOVA; $p \leq 0.05$; Fig. 5). Both N- and P-limited *C. subsalsa* in late phase growth had significantly higher HA at atrazine concentrations above 25 μg L^{-1} (again, with exception of the cytotoxic level of 200 μg L^{-1}) than in any replete-media culture (0–4.0 NLV) (ANOVA; $p \leq 0.05$; Fig. 5).

Overall, HA in *C. subsalsa* was more influenced by atrazine concentration in the 10-day assay than in the 28-day assay, whereas nutrient regime was the best predictor of HA in the 28-day assay (two-way ANOVA variable ranking using LogWorth; Table 4). Log-normal relationships between HA (normalized to cell density and sample volume, and omitting the 200 μg atrazine L^{-1} treatment in low-nutrient regimes) and atrazine concentration had high r^2 values (0.82 to 0.92) across nutrient treatments in the 10-day assay (Fig. 6), but the strength of these relationships declined over time (see Flood, 2017, Appendix for modeled relationships at the 28-day endpoint). HA was inversely related to log-atrazine concentration in nutrient-replete cultures, but positively related to the log-atrazine concentration in low-nutrient treatments. The r^2 values were somewhat lower for HA versus growth rate in low-nutrient cultures (0.64–0.77) in comparison to r^2 values in nutrient-replete cultures (0.85), but the relationships still were clear: In nutrient-replete cultures, HA *decreased* with increasing atrazine concentration, whereas under nutrient-limited conditions, HA *increased* with increasing atrazine. In nutrient-replete cultures, HA also increased up to intermediate growth rates and then declined (parabolic response curve), whereas in nutrient-limited cultures, HA was inversely related to growth rate (Fig. 6).

4. Discussion

This study is the first to test atrazine sensitivity in a toxigenic raphidophycean species, wherein the toxicity (as HA) of a strain of *Chattonella subsalsa* was exposed to a range of photosystem II-inhibiting atrazine concentrations under different nutrient regimes. Inhibition by atrazine was the dominant predictor of growth rates for this strain in early exponential growth phase (10-day assay series), whereas nutrient regime was a more important predictor of *C. subsalsa* population growth (as cell density) during late exponential growth (28-day assay series). In the extended duration 28-day assay, *Chattonella subsalsa* appeared to exhibit some "stress-stimulation" effect, whereby populations in low-nutrient treatments with intermediate levels of atrazine had increased growth rates relative to nutrient-replete controls. A possible explanation for the increased OD in low-level atrazine exposures is that the presence of atrazine has been reported to mimic nutrient-limited conditions (Weiner et al., 2007). These stimulatory effects could be considered as a potential function of increased predatory activity by *C. subsalsa* on bacteria. Bacterial consumption was not tracked, but the studies were performed on unialgal batch cultures containing bacteria. This species is known to consume bacteria (Jeong 2010), and if bacterivory in *Chattonella subsalsa* is increased by reduced nutrient availability or reduced photosynthetic efficiency, this might explain the increased growth in multi-stress conditions such as was described here. Another explanation of increased OD in low-level atrazine exposures may be due to the presence of the three N atoms in the triazine ring of the atrazine structure, which can become available for uptake by the algal cells. This phenomenon has been observed with atrazine and other *s*-triazines in bacterial growth studies (Cook and Hutter, 1981; Strong et al., 2002). Alternatively, the increased OD in low-level atrazine exposures may have reflected the fact that inhibition by this herbicide primarily affects cell division rather than photosynthesis at these concentrations (Chao and Chen, 2001).

HA in the absence of atrazine was highest in low-P cultures among all controls and treatments. Hemolytic activity by *C. subsalsa* in the presence of > 25 μg atrazine L^{-1} was highest in low-N cultures, and increased with increasing atrazine concentration in all nutrient (N- or P-) limited cultures.

Research with other harmful algae, such as the haptophyte *Prymnesium parvum* and the dinoflagellate *Karenia brevis*, have shown increasing toxin production under nutrient depletion or nutrient limitation (Johansson and Granéli, 1999a,b; Granéli and Johansson, 2003; Hardison et al., 2013). Our previous work has also shown that other harmful algal populations (e.g., geographically-distinct strains of *Prymnesium parvum*) can have elevated toxicity (as HA) under combined atrazine + low nutrient stress (Flood and Burkholder, 2018).

These findings are consistent with the "chemical ecology hypothesis," which predicts enhanced toxicity of nutrient-limited HAB species (Ianora et al., 2011), if considered together with the underlying, fundamental point that these blooms develop in nutrient over-enriched waters (above references; also see Burkholder et al., 2008); the algae deplete required nutrient resources as the blooms continue, inevitably leading to either N or P limitation in eutrophic systems. The "chemical ecology hypothesis" was derived, in turn, from a "carbon: nutrient balance hypothesis" (Bryant et al., 1983; Lambers et al., 2008), which predicted increased production of toxins and other defenses in terrestrial plants under low resource conditions wherein grazing is reduced. The latter hypothesis may not hold up well in the terrestrial systems for which it was designed (Hamilton et al., 2001), but it has been used successfully to explain increased toxicity with nutrient limitation in unicellular algae (e.g., Ianora et al., 2006, 2011; Hardison et al., 2012). Production of toxic compounds by phytoplankton frequently has been associated with allelopathic interactions (Legrand, 2001; Fistarol et al., 2005; Uronen et al., 2005; Granéli et al., 2008; Driscoll et al., 2013; Fernández-Herrera et al., 2016), which may help reduce grazing and competition for scarce resources and promote survival in competitive (nutrient-imbalanced) systems.

Here, increasing toxicity as HA was also observed at low to moderate growth rates in nutrient-replete *C. subsalsa* cultures, and at low growth rates in nutrient-limited cultures. Others have reported that the closely related species, *Chattonella marina*, is most toxic during late exponential growth phase and toxicity decreases over stationary phase (measured as putative brevetoxins affecting fish; Khan et al., 1995; Marshall et al., 2003). The toxicity of *C. marina* has been highly correlated with growth phase, but not with cell density (Shen et al., 2010); or, lower cell densities of *C. marina* ($< 10^4$ cells mL^{-1}) produced more superoxide on a per-cell basis than more dense cultures (Marshall et al., 2005b). These differences may relate to the tested strain or other factors. Other studies on individual phytoplankton species responses to chronic atrazine exposures have yielded mixed results as well. Some have reported that algal populations with a history of exposure became more sensitive to atrazine upon repeated exposure (Pennington and Scott, 2001), whereas others have reported that the tested algae became more tolerant over time (Baxter et al., 2013).

These discrepancies may be attributed to the wide range of phylogenic diversity which is often mistakenly "combined" under the umbrella of "phytoplankton responses." The ecological diversity of the huge, extremely diverse organisms referred to as algae cannot be considered as one functional group (although they frequently are so considered), or even as one trophic level (e.g., phototrophs versus predatory mixotrophs; e.g., see Burkholder et al., 2008; Flynn et al., 2012;

Mitra et al., 2016). Phytoplankton assemblages have been investigated for their response to repeated atrazine exposure – also with conflicting results, including increased sensitivity over time (Hamala and Kollig, 1985; Guasch et al., 2007), no effect (Nyström et al., 2000; Pinckney et al., 2002), and exposure-induced tolerance (deNoyelles et al., 1982; Hamilton et al., 1987). These findings are not surprising, as phytoplankton assemblage recovery potential following exposure to photosystem II-inhibiting herbicides depends on the species composition of the original assemblage, their physiological status, the herbicide concentration (Gustavson and Wängberg, 1995; Kasai, 1999), the season (Guasch et al., 1997; Lorente et al., 2015), and other environmental factors.

Several consistent findings, nevertheless, are that (i) phytoplankton species and strains vary greatly in response to the same level of atrazine exposure (Blanck et al., 1984; Choi et al., 2012); (ii) intraspecific variation in responses can be very high (Kasai et al., 1993); and (iii) atrazine exposure can alter phytoplankton assemblage composition as sensitive species are eliminated and tolerant species gain a selective advantage (Hamala and Kollig, 1985; Hamilton et al., 1987; Graymore et al., 2001). Thus, it is important to strengthen understanding about how harmful, potentially ecosystem-disruptive phytoplankton species (those highlighted in Sunda et al., 2006, and others, sensu Burkholder et al., 2008) respond to common environmental contaminants, despite the challenges involved in attempting to apply standard ecotoxicological methods to organisms that can be much more difficult to culture than standard model organisms. Autecological and ecotoxicological studies of potentially toxic species are needed to understand the mechanisms that promote HABs, and the influence of common contaminants such as atrazine on toxin production. Regarding *Chattonella subsalsa*, this research offers insights about the complexities of nutrient interactions with atrazine in influencing growth and toxicity. Yet, as only one strain was examined, this work is only a first step toward understanding *C. subsalsa* response to atrazine × nutrients.

In the past few decades there has been a dramatic increase in the frequency, magnitude, and duration of HABs, with devastating impacts on global fisheries stocks, coastal economies and freshwater and marine ecosystems (Hallegraeff, 1993; Burkholder, 1998; Van Dolah, 2000; Glibert et al., 2005a,b; Heisler et al., 2008; Lewitus et al., 2012). It has also been more than two decades since Cloern (1996) discussed the potential for toxic contaminants and nutrient pollution to alter natural cycles of estuarine phytoplankton blooms, yet few studies have examined how these combined stressors affect HAB species. This research indicates that imbalanced nutrient regimes can act in conjunction with herbicide exposures to promote toxicity in *C. subsalsa*, a toxigenic species that is expected to continue to thrive in increasingly urbanized coastal zones (Lewitus and Holland, 2003; Lewitus et al., 2003).

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References

Ahmad, S., 1995. Antioxidant mechanisms of enzymes and proteins. In: Ahmad, S. (Ed.), Oxidative Stress and Antioxidant Defenses in Biology. Springer, New York, NY, pp. 238–272.

Ahmed, M.S., Khan, S., Arakawa, O., Onoue, Y., 1995. Properties of hemagglutinins newly separated from toxic phytoplankton. Biochim. Biophys. Acta 1243, 509–512.

American Society for Testing and Materials (ASTM), 1996. Standard Guide for Conducting Static 96-hour Toxicity Tests with Microalgae. ASTM, West Conshohocken, PA.

Band-Schmidt, C.J., Martinez-Lopez, A., Bustillos-Guzmán, J.J., Carréon-Palau, L., Morquecho, L., Olguín-Monroy, N., Zenteno-Savín, T., Mendoza-Flores, A., González-Acosta, B., Hernández-Sandoval, F., 2012. Morphology, biochemistry, and growth of raphidophyte strains from the Gulf of California. Hydrobiologia 693, 81–97.

Baxter, L., Brain, R., Prosser, R., Solomon, K., Hanson, M., 2013. Sensitivity of a green alga to atrazine is not enhanced by previous acute exposure. Environ. Pollut. 181, 325–328.

Blanck, H., Wallin, G., Wangberg, S.A., 1984. Species-dependent variation in algal sensitivity to chemical compounds. Ecotoxicol. Environ. Saf. 8, 339–351.

Bourdelais, A., Tomas, C., Naar, J., Kubanek, J., Baden, D., 2002. New fish-killing alga in coastal Delaware produces neurotoxins. Environ. Health Perspect. 110, 465–470.

Bricker, S.B., Longstaff, B., Dennison, W., Jones, A., Boicourt, K., Wicks, C., Woerner, J., 2008. Effects of nutrient enrichment in the nation's estuaries: a decade of change. Harmful Algae 8, 21–32.

Bryant, J.P., Chapin III, F.S., Klein, D.R., 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40, 357–368.

Burkholder, J.M., Glibert, P.M., 2013. Eutrophication and oligotrophication. In: 2nd edition. In: Levin, S. (Ed.), Encyclopedia of Biodiversity, vol. 3. Academic Press, Waltham, MA, pp. 347–371.

Burkholder, J., Libra, B., Weyer, P., Heathcote, S., Kolpin, D., Thome, P.S., Wichman, M., 2007a. Impacts of waste from concentrated animal feeding operations on water quality. Environ. Health Perspect. 308–312.

Burkholder, J.M., Tomasko, D., Touchette, B.W., 2007b. Seagrasses and eutrophication. J. Exp. Mar. Biol. Ecol. 350, 46–72.

Burkholder, J.M., Glibert, P.M., Skelton, H.M., 2008. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae 8, 77–93.

Burkholder, J.M., 1998. Implications of harmful marine microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. Ecol. Appl. 8, S37–S62.

Chao, M.R., Chen, C.Y., 2001. Discrepancies between different response parameters in batch and continuous algal toxicity tests. J. Hazard. Mater. 82, 129–136.

Choi, C.J., Berges, J.A., Young, E.B., 2012. Rapid effects of diverse toxic water pollutants on chlorophyll *a* fluorescence: variable responses among freshwater microalgae. Water Res. 46, 2615–2626.

Cloern, J.E., 1996. Phytoplankton bloom dynamics in coastal ecosystems: a review with some general lessons from sustained investigation of San Francisco Bay, California. Rev. Geophys. 34, 127–168.

Cook, A.M., Hutter, R., 1981. *s*-Triazines as nitrogen sources for bacteria. J. Agric. Food Chem. 29, 1135–1143.

Cortés-Altamirano, R., Alonso-Rodríguez, R., Sierra Beltrán, A., 2006. Fish mortality associated with *Chattonella marina* and *C.* cf. *ovata* (Raphidophyceae) blooms in Sinaloa (Mexico). Harmful Algae News 31, 7–8.

de Boer, M.K., Tyl, M.R., Vrieling, E.G., van Rijssel, M., 2004. Effects of salinity and nutrient conditions on growth and haemolytic activity of *Fibrocapsa japonica* (Raphidophyceae). Aquat. Microb. Ecol. 37, 171–181.

DeLorenzo, M.E., Scott, G.I., Ross, P.E., 2001. Toxicity of pesticides to aquatic microorganisms: a review. Environ. Toxicol. Chem. 20, 84–98.

deNoyelles, F., Kettle, W.D., Sinn, D.E., 1982. The responses of plankton communities in experimental ponds to atrazine, the most heavily used pesticide in the United States. Ecology 63, 1285–1293.

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

Efron, B., 1982. The Jackknife, The Bootstrap and Other Resampling Plans Vol. 38 Society for Industrial and Applied Mathematics (SIAM).

Eisler, R., 1989. Atrazine hazards to fish, wildlife, and invertebrates: a synoptic review. Biological Report 85(1.8), Contaminant Hazard Reviews Report No. 18. U.S. Fish and Wildlife Service, Laurel, MD.

Eschbach, E., Scharsack, J.P., John, U., Medlin, L.K., 2001. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. J. Appl. Toxicol. 21, 513–551.

Fernández-Herrera, L.J., Band-Schmidt, C.J., López-Cortés, D.J., Hernández-Guerrero, C.J., Bustillos-Guzmán, J.J., Núñez-Vázquez, E., 2016. Allelopathic effect of *Chattonella marina* var. *marina* (Raphidophyceae) on *Gymnodinium catenatum* (Dinophyceae). Harmful Algae 51, 1–9.

Fistarol, G.O., Legrand, C., Granéli, E., 2005. Allelopathic effect on a nutrient-limited phytoplankton species. Aquat. Microb. Ecol. 41, 153–161.

Flood, S.L., Burkholder, J.M., 2018. Imbalanced nutrient regimes increase *Prymnesium parvum* resilience to herbicide exposure. Harmful Algae 75, 57–74.

Flood, S.L., Burkholder, J.M., Cope, W.G., 2018. Assessment of atrazine toxicity to the estuarine phytoplankter, *Dunaliella tertiolecta* (Chlorophyta) under varying nutrient conditions. Environ. Sci. Pollut. Res. 25, 11409–11423.

Flood, S.L., 2017. Ecotoxicology of estuarine phytoplankton growth and toxicity in response to atrazine exposures. Dissertation. North Carolina State University, Raleigh, NC.

Flynn, K.J., Mitra, A., Stoecker, D.K., Raven, J.A., Granéli, E., Glibert, P.M., Hansen, P.J., Burkholder, J.M., 2012. An ocean of mixotrophs – a new paradigm for marine ecology. J. Plankton Res. 35, 3–11.

Fu, M., Koulman, A., van Rijssel, M., Lützen, A., de Boer, M.K., Tyl, M.R., Liebezeit, G., 2004. Chemical characterisation of three haemolytic compounds from the microalgal species *Fibrocapsa japonica* (Raphidophyceae). Toxicon 43, 355–363.

Gascón, J., Oubiña, A., Barceló, D., 1997. Detection of endocrine-disrupting pesticides by enzyme-linked immunosorbent assay (ELISA): application to atrazine. Trends Anal. Chem. 16, 554–562.

Gilliom, R.J., Barbash, J.E., Crawford, C.G., Hamilton, P.A., Martin, J.D., Nakagaki, N., Nowell, L.H., Scott, J.C., Stackelberg, P.E., Thelin, G.P., 2006. Pesticides in the

Nation's Streams and Ground Water, 1992–2001. U.S. Geological Survey, Reston, VA.
Glibert, P.M., Anderson, D.M., Gentien, P., Graneli, E., Sellner, K.G., 2005a. The global, complex phenomena of harmful algal blooms. Oceanography 18, 136–147.
Glibert, P.M., Seitzinger, S., Heil, C.A., Burkholder, J.M., Parrow, M.W., Codispoti, L.A., Kelly, V., 2005b. Eutrophication – new perspectives on its role in the global proliferation of harmful algal blooms. Oceanography 18, 198–209.
Graham, L.E., Graham, J.M., Wilcox, L.W., Cook, M.E., 2016. Algae, 3rd edition. LJLM Press, LLC, Madison, WI.
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, 135–145.
Granéli, E., Weberg, M., Salomon, P.S., 2008. Harmful algal blooms of allelopathic microalgal species: the role of eutrophication. Harmful Algae 8, 94–102.
Granéli, E., Edvardsen, B., Roelke, D.L., Hagström, J.A., 2012. The ecophysiology and bloom dynamics of *Prymnesium* spp. Harmful Algae 14, 260–270.
Graymore, M., Stagnitti, F., Allinson, G., 2001. Impacts of atrazine in aquatic ecosystems. Environ. Int. 26, 483–495.
Guasch, H., Muñoz, I., Rosés, N., Sabater, S., 1997. Changes in atrazine toxicity throughout succession of stream periphyton communities. J. Appl. Phycol. 9, 137–146.
Guasch, H., Lehmann, V., Van Beusekom, B., Sabater, S., Admiraal, W., 2007. Influence of phosphate on the response of periphyton to atrazine exposure. Arch. Environ. Contam. Toxicol. 52, 32–37.
Guillard, R.R., Hargraves, P., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32, 234–236.
Guillard, R.R., 1973. Division rates. In: In: Stein, J.R. (Ed.), Handbook of Phycological Methods – Culture Methods and Growth Measurements Vol. 1. Cambridge University Press, New York, NY, pp. 290–311.
Gustavson, K., Wängberg, S.-Å., 1995. Tolerance induction and succession in microalgae communities exposed to copper and atrazine. Aquat. Toxicol. 32, 283–302.
Hallegraeff, G., Hara, Y., 2003. Taxonomy of harmful marine raphidophytes. In: 2nd edition. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae Vol. 11. International Oceanographic Commission of the United Nations Educational, Scientific, and Cultural Organization (IOC-UNESCO), Paris, France, pp. 511–522.
Hallegraeff, G., Munday, B., Baden, D., Whitney, P., 1998. *Chattonella marina* raphidophyte bloom associated with mortality of cultured bluefin tuna (*Thunnus maccoyii*) in South Australia. In: Reguera, B., Blanco, J., Fernández, M., Wyatt, T. (Eds.), Harmful Algae, VIII Conference on Harmful Algae. Vigo, Spain, June 25–29, 1997. IOC-UNESCO, Paris, France. pp. 93–96.
Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.
Hamala, J.A., Kollig, H.P., 1985. The effects of atrazine on periphyton communities in controlled laboratory ecosystems. Chemosphere 14, 1391–1408.
Hamilton, P.B., Jackson, G.S., Kaushik, N.K., Solomon, K.R., 1987. The impact of atrazine on lake periphyton communities, including carbon uptake dynamics using track autoradiography. Environ. Pollut. 46, 83–103.
Hamilton, J., Zangerl, A., DeLucia, E., Berenbaum, M., 2001. The carbon–nutrient balance hypothesis: its rise and fall. Ecol. Lett. 4, 86–95.
Hapeman, C.J., Dionigi, C.P., Zimba, P.V., McConnell, L.L., 2002. Agrochemical and nutrient impacts on estuaries and other aquatic systems. J. Agric. Food Chem. 50, 4382–4384.
Hardison, D.R., Sunda, W.G., Litaker, R.W., Shea, D., Tester, P.A., 2012. Nitrogen limitation increases brevetoxins in *Karenia brevis* (Dinophyceae): implications for bloom toxicity. J. Phycol. 48, 844–858.
Hardison, D.R., Sunda, W.G., Shea, D., Litaker, R.W., 2013. Increased toxicity of *Karenia brevis* during phosphate limited growth: ecological and evolutionary implications. PLoS One 8 (3), e58545. http://dx.doi.org/10.1371/journal.pone.0058545.
Harris, G.P., 1986. Phytoplankton Ecology: Structure, Function and Fluctuation. Chapman and Hall, London, United Kingdom.
Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., 2008. Eutrophication and harmful algal blooms: a scientific consensus. Harmful Algae 8, 3–13.
Ianora, A., Boersma, M., Casotti, R., Fontana, A., Harder, J., Hoffmann, F., Pavia, H., Potin, P., Poulet, S., Toth, G., 2006. New trends in marine chemical ecology. Estuar. Coasts 29, 531–551.
Ianora, A., Bentley, M.G., Caldwell, G.S., Casotti, R., Cembella, A.D., Engström-Öst, J., Halsband, C., Sonnenschein, E., Legrand, C., Llewellyn, C.A., 2011. The relevance of marine chemical ecology to plankton and ecosystem function: an emerging field. Mar. Drugs 9, 1625–1648.
Imai, I., Yamaguchi, M., 2012. Life cycle, physiology, ecology and red tide occurrences of the fish-killing raphidophyte *Chattonella*. Harmful Algae 14, 46–70.
Imai, I., Itakura, S., Itoh, K., 1991. Life cycle strategies of the red tide causing flagellates *Chattonella* (Raphidophyceae) in the Seto Inland Sea. Mar. Pollut. Bull. 23, 165–170.
Imai, I., Yamaguchi, M., Watanabe, M., 1998. Ecophysiology, life cycle, and bloom dynamics of *Chattonella* in the Seto Inland Sea, Japan. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin, Germany, pp. 95–112.
Ishimatsu, A., Oda, T., Yoshida, M., Ozaki, M., 1996a. Oxygen radicals are probably involved in the mortality of yellowtail by *Chattonella marina*. Fish. Sci. 62, 836–837.
Ishimatsu, A., Sameshima, M., Tamura, A., Oda, T., 1996b. Histological analysis of the mechanisms of *Chattonella*-induced hypoxemia in yellowtail. Fish. Sci. 62, 50–58.
Jeong, H.J., Seong, K.A., Kang, N.S., Yoo, Y.D., Nam, S.W., Park, J.Y., Shin, W., Glibert, P.M., John, D., 2010. Feeding by raphidophytes on the cyanobacterium *Synechococcus* sp. Aquat. Microb. Ecol. 58, 181–195.
Jeong, H.J., 2011. Mixotrophy in red tide algae raphidophytes. J. Eukaryot. Microbiol. 58, 215–222.
Johansson, N., Granéli, E., 1999a. Cell density, chemical composition and toxicity of *Chrysochromulina polylepis* (Haptophyta) in relation to different N:P supply ratios. Mar. Biol. 135, 209–217.
Johansson, N., Granéli, E., 1999b. 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, 243–258.
Kasai, F., Takamura, N., Hatakeyama, S., 1993. Effects of simetryne on growth of various freshwater algal taxa. Environ. Pollut. 79, 77–83.
Kasai, F., 1999. Shifts in herbicide tolerance in paddy field periphyton following herbicide application. Chemosphere 38, 919–931.
Katano, T., Yoshida, M., Lee, J., Han, M.-S., Hayami, Y., 2009. Fixation of *Chattonella antiqua* and *C. marina* (Raphidophyceae) using Hepes-buffered paraformaldehyde and glutaraldehyde for flow cytometry and light microscopy. Phycologia 48, 473–479.
Khan, S., Ahmed, M., Arakawa, O., Onoue, Y., 1995. Properties of neurotoxins separated from a harmful red tide organism *Chattonella marina*. Isr. J. Aquacult.-Bamid. 47, 137–141.
Khan, S., Arakawa, O., Onoue, Y., 1996. A toxicological study of the marine phytoflagellate, *Chattonella antiqua* (Raphidophyceae). Phycologia 35, 239–244.
Kim, D., Watanabe, M., Nakayasu, Y., Kohata, K., 2004. Production of superoxide anion and hydrogen peroxide associated with cell growth of *Chattonella antiqua*. Aquat. Microb. Ecol. 35, 57–64.
Kim, D., Watanabe, M., Nakayasu, Y., Kohata, K., 2005. Changes in O_2–and H_2O_2 production by *Chattonella antiqua* during diel vertical migration under nutrient stratification. Aquat. Microb. Ecol. 39, 183–191.
Kim, D., Yamaguchi, K., Oda, T., 2006. Nitric oxide synthase-like enzyme mediated nitric oxide generation by harmful red tide phytoplankton, *Chattonella marina*. J. Plankton Res. 28, 613–620.
Kim, D., Nakashima, T., Matsuyama, Y., Niwano, Y., Tamaguchi, K., Oda, T., 2007. Presence of the distinct systems responsible for superoxide anion and hydrogen peroxide generation in red tide phytoplankton *Chattonella marina* and *Chattonella ovata*. J. Plankton Res. 29, 241–247.
Kuroda, A., Nakashima, T., Yamaguchi, K., Oda, T., 2005. Isolation and characterization of light-dependent hemolytic cytotoxin from harmful red tide phytoplankton *Chattonella marina*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 297–305.
Lambers, H., Chapin III, F.S., Pons, T.L., 2008. Plant Physiological Ecology. Springer Science+Business Media, LLC, New York, NY, pp. 445–477.
Legrand, C., 2001. Phagotrophy and toxicity variation in the mixotrophic *Prymnesium patelliferum* (Haptophyceae). Limnol. Oceanogr. 46, 1208–1214.
Lehotay, S.J., Harman-Fetcho, J.A., McConnell, L.L., 1998. Agricultural pesticide residues in oysters and water from two Chesapeake Bay tributaries. Mar. Pollut. Bull. 37, 32–44.
Lewitus, A.J., Holland, A.F., 2003. Initial results from a multi-institutional collaboration to monitor harmful algal blooms in South Carolina. Environ. Monit. Assess. 81, 361–371.
Lewitus, A.J., Schmidt, L.B., Mason, L.J., Kempton, J.W., Wilde, S.B., Wolny, J.L., Williams, B.J., Hayes, K.C., Hymel, S.N., Keppler, C.J., 2003. Harmful algal blooms in South Carolina residential and golf course ponds. Popul. Environ. 24, 387–413.
Lewitus, A.J., Brock, L.M., Burke, M.K., DeMattio, K.A., Wilde, S.B., 2008. Lagoonal stormwater detention ponds as promoters of harmful algal blooms and eutrophication along the South Carolina coast. Harmful Algae 8, 60–65.
Lewitus, A.J., Horner, R.A., Caron, D.A., Garcia-Mendoza, E., Hickey, B.M., Hunter, M., Huppert, D.D., Kudela, R.M., Langlois, G.W., Largier, J.L., 2012. Harmful algal blooms along the North American west coast region: history, trends, causes, and impacts. Harmful Algae 19, 133–159.
Liu, W., Au, D.W., Anderson, D.M., Lam, P.K., Wu, R.S., 2007. Effects of nutrients, salinity, pH and light: dark cycle on the production of reactive oxygen species in the alga *Chattonella marina*. J. Exper. Mar. Biol. Ecol. 346, 76–86.
Lorente, C., Causape, J., Glud, R.N., Hancke, K., Merchan, D., Muniz, S., Val, J., Navarro, E., 2015. Impacts of agricultural irrigation on nearby freshwater ecosystems: the seasonal influence of triazine herbicides in benthic algal communities. Sci. Total Environ. 503, 151–158.
Lytle, J.S., Lytle, T.F., 2001. Use of plants for toxicity assessment of estuarine ecosystems. Environ. Toxicol. Chem. 20, 68–83.
Marshall, J.-A., Nichols, P.D., Hallegraeff, G.M., 2002. Chemotaxonomic survey of sterols and fatty acids in six marine raphidophyte algae. J. Appl. Phycol. 14, 255–265.
Marshall, J.-A., Nichols, P.D., Hamilton, B., Lewis, R.J., Hallegraeff, G.M., 2003. Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to damselfish (*Acanthochromis polycanthus*): the synergistic role of reactive oxygen species and free fatty acids. Harmful Algae 2, 273–281.
Marshall, J.-A., Salas, M., Oda, T., Hallegraeff, G., 2005a. Superoxide production by marine microalgae I. Mar. Biol. 147, 541–549.
Marshall, J.-A., Ross, T., Pyecroft, S., Hallegraeff, G., 2005b. Superoxide production by marine microalgae II. Mar. Biol. 147, 541–549.
Matsusato, T., Kobayashi, H., 1974. Studies on death of fish caused by red tide. Bull. Nansei Reg. Fish. Res. Lab. 7, 43–67.
Matsuyama, Y., Uchida, T., Kotani, Y., 2000. Effect of culture filtrate of raphidophytes *Heterosigma akashiwo* and *Chattonella antiqua* on the growth of diatom *Skeletonema costatum*. Bull. Environ. Inland Sea 2, 57–66.
Mitra, A., Flynn, K.J., Tillmann, U., Raven, J.A., Caron, D., Stoecker, D.K., Not, F., Hansen, P.J., Hallegraeff, G., Sanders, R., Wilken, S., McManus, G., Johnson, M., Pitt, P., Våge, S., Berge, T., Calbet, A., Thingstad, F., Jeong, H.J., Burkholder, J.M., Glibert, P.M., Granéli, E., Lundgren, V., 2016. Defining planktonic protist functional groups on mechanisms for energy and nutrient acquisition: incorporation of diverse mixotrophic strategies. Protist 167, 106–120.
Norberg-King, T.J., 1993. A linear interpolation method for sublethal toxicity: the

inhibition concentration (ICp) approach. National Effluent Toxicity Assessment Center Technical Report 39. pp. 3–93.
Nyström, B., Paulsson, M., Almgren, K., Blank, H., 2000. Evaluation of the capacity for development of atrazine tolerance in periphyton from a Swedish freshwater site as determined by inhibition of photosynthesis and sulfolipid synthesis. Environ. Toxicol. Chem. 19, 1324–1331.
Oda, T., Ishimatsu, A., Shimada, M., Takeshita, S., Muramatsu, T., 1992. Oxygen-radical-mediated toxic effects of the red tide flagellate *Chattonella marina* on *Vibrio alginolyticus*. Mar. Biol. 112, 505–509.
Oda, T., Nakamura, A., Shikayama, M., Kawano, I., Ishimatsu, A., Muramatsu, T., 1997. Generation of reactive oxygen species by raphidophycean phytoplankton. Biosci. Biotechnol. Biochem. 61, 1658–1662.
Pelley, J., 1998. What is causing toxic algal blooms? Environ. Sci. Technol. 32, 26A–30A.
Pennington, P.L., Scott, G.I., 2001. Toxicity of atrazine to the estuarine phytoplankter *Pavlova* sp. (Prymnesiophyceae): increased sensitivity after long-term, low-level population exposure. Environ. Toxicol. Chem. 20, 2237–2242.
Pennington, P.L., Daugomah, J.W., Colbert, A.C., Fulton, M.H., Key, P.B., Thompson, B.C., Strozier, E.D., Scott, G.I., 2001. Analysis of pesticide runoff from mid-Texas estuaries and risk assessment implications for marine phytoplankton. J. Environ. Sci. Health B 36, 1–14.
Peters, N.E., Meybeck, M., Chapman, D.V., 2005. Effects of human activities on water quality, Chapter 93. In: Anderson, M.G. (Ed.), Encyclopedia of Hydrological Sciences. John Wiley & Sons, Ltd., New York, NY 17 pp.
Pinckney, J.L., Örnólfsdóttir, E.B., Lumsden, S.E., 2002. Estuarine phytoplankton group-specific responses to sublethal concentrations of the agricultural herbicide, atrazine. Mar. Pollut. Bull. 44, 1109–1116.
Pistocchi, R., Guerrini, F., Pezzolesi, L., Riccardi, M., Vanucci, S., Ciminiello, P., Dell'Aversano, C., Forino, M., Fattorusso, E., Tartaglione, L., Milandri, A., Pompei, M., Cangini, M., Pigozzi, S., Riccardi, E., 2012. Toxin levels and profiles in microalgae from the north-western Adriatic Sea – 15 years of studies on cultured species. Mar. Drugs 10, 140–162.
Redfield, A.C., 1934. On the proportions of organic derivatives in sea water and their relation to the composition of plankton. In: Daniel, R.J. (Ed.), James Johnstone Memorial Volume. University Press of Liverpool, Liverpool, United Kingdom, pp. 177–192.
Redfield, A.C., 1958. The biological control of chemical factors in the environment. Am. Sci. 46, 205–221.
Ryberg, K.R., Vecchia, A.V., Martin, J.D., Gilliom, R.J., 2010. Trends in Pesticide Concentrations in Urban Streams in the United States, 1992–2008. United States Geological Survey, Reston, VA.
Scott, G.I., Holland, A.F., Sandifer, P.A., 2006. Afterword—managing coastal urbanization and development in the twenty-first century: the need for a new paradigm. In: Kleppel, G.S., DeVoe, M.R., Rawson, M.V. (Eds.), Changing Land Use Patterns in the Coastal Zone. Springer Science + Business Media, New York, New York, pp. 285–299.
Shen, M., Xu, J., Tsang, T.Y., Au, D.W., 2010. Toxicity comparison between *Chattonella marina* and *Karenia brevis* using marine medaka (*Oryzias melastigma*): evidence against the suspected ichthyotoxins of *Chattonella marina*. Chemosphere 80, 585–591.
Shimada, M., Murakami, T.H., Imahayashi, T., Ozaki, H.S., Toyoshima, T., Okaichi, T., 1983. Effects of sea bloom, *Chattonella antiqua*, on gill primary lamellae of the young yellowtail, *Seriola quinqueradiata*. Acta Histochem. Cytochem. 16, 232–244.
Smayda, T.J., 2002. Adaptive ecology, growth strategies and the global bloom expansion of dinoflagellates. J. Oceanogr. 58, 281–294.
Sorokin, C., 1973. Dry weight, packed cell volume and optical density. In: Stein, J.R. (Ed.), Handbook of Phycological Methods – Culture Methods and Growth Measurement. Cambridge University Press, Cambridge, United Kingdom, pp. 321–343.
Starr, A.V., Bargu, S., Maiti, K., DeLaune, R.D., 2017. The effect of atrazine on Louisiana Gulf Coast estuarine phytoplankton. Arch. Environ. Contam. Toxicol. 72, 1–11.
Strong, L.C., Rosendahl, C., Johnson, G., Sadowsky, M.J., Wackett, L.P., 2002. *Arthrobacter aurescens* TC1 metabolizes diverse *s*-triazine ring compounds. Appl. Environ. Microbiol. 68, 5973–5980.
Sunda, W.G., Granéli, E., Gobler, C.J., 2006. Positive feedback and the development and persistence of ecosystem disruptive algal blooms1. J. Phycol. 42, 963–974.
Thursby, G.B., Anderson, B.S., Walsh, G.E., Steele, R.L., 1993. A review of the current status of marine algal toxicity testing in the United States. In: First Symposium on Environmental Toxicology and Risk Assessment: Aquatic, Plant and Terrestrial. ASTM Philadelphia, PA. pp. 362–377.
United States Environmental Protection Agency (U.S. EPA), 2016a. National Coastal Condition Report IV. Report EPA-842-R-10-003. U.S. EPA, Washington, DC.
United States Environmental Protection Agency (U.S. EPA), 2016b. National Lakes Assessment 2012: A Collaborative Survey of Lakes in the United States. Report EPA 841-R-16-113. U.S. EPA, Washington, DC.
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. Mar. Ecol. Prog. Ser. 299, 137–148.
Van Dolah, F.M., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 108, 133–141.
Varkitzi, I., Pagou, K., Graneli, E., Hatzianestis, I., Pyrgaki, C., Pavlidou, A., Montesanto, B., Economou-Amilli, A., 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. Harmful Algae 9, 304–311.
Vencill, W.K., 2002. Herbicide Handbook, 10th Edition. Weed Science Society of America, Lawrence, KS.
Weiner, J.A., DeLorenzo, M.E., Fulton, M.H., 2007. Atrazine induced species-specific alterations in the subcellular content of microalgal cells. Pest. Biochem. Physiol. 87, 47–53.
Yamaguchi, H., Sakamoto, S., Yamaguchi, M., 2008. Nutrition and growth kinetics in nitrogen-and phosphorus-limited cultures of the novel red tide flagellate *Chattonella ovata* (Raphidophyceae). Harmful Algae 7, 26–32.
Zar, J.H., 1999. Biostatistical Analysis, 4th edition. Prentice Hall, Upper Saddle River, NJ, pp. 389–394.
Zhang, Y., Fu, F.-X., Whereat, E., Coyne, K.J., Hutchins, D.A., 2006. Bottom-up controls on a mixed-species HAB assemblage: a comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) isolates from the Delaware Inland Bays, USA. Harmful Algae 5, 310–320.