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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.