Harmful Algae 39 (2014) 334–341

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Effects of nutrient-limiting supply ratios on toxin content of *Karenia brevis* grown in continuous culture

Alina A. Corcoran*, Bill Richardson, Leanne J. Flewelling

Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 8th Avenue SE, St. Petersburg, FL 33701, USA

ARTICLE INFO

Article history:
Received 19 November 2013
Received in revised form 29 August 2014
Accepted 29 August 2014
Available online 1 October 2014

Keywords:
Harmful algal blooms
Brevetoxin
Chemostat
Nitrogen
Phosphorus
Growth rate

ABSTRACT

Toxins produced as secondary metabolites can play important roles in phytoplankton communities and contribute to the ecological success of harmful algal bloom (HAB) taxa. Toxin composition and content in phytoplankton are affected by a suite of environmental factors, including nutrient availability. Changes in nutrient availability can increase or decrease toxin content and alter toxin composition, depending on toxin stoichiometry and the mechanisms by which nutrient limitation affects toxin production. The studies that have assessed the effects of nutrient availability on brevetoxin content of the HAB species *Karenia brevis* have reported contradictory results, although there is growing support that nutrient limitation increases brevetoxin content. In this study, we assessed the effects of decreased nitrogen (N) and phosphorus (P) availability on brevetoxin content and composition of *K. brevis* grown in chemostats at steady state by altering the nutrient supply ratios of incoming media from the Redfield Ratio. Overall, brevetoxin content was greatest in cultures grown at the lowest rate, regardless of the nutrient supply ratio (i.e., under both Redfield and N-limiting supply ratios). Compared to cultures grown at 0.2 d^{-1}, cultures grown at 0.1 d^{-1} exhibited 5-fold increases in intracellular toxin content. In contrast, at constant growth rates, N-limiting supply ratios decreased intracellular brevetoxin content by approximately one-third, although this result was significant only in cultures growing at the fastest rate of 0.23 d^{-1}. P-limiting supply ratios had no effect on brevetoxin content or composition. In addition, when cultures grown at rates of 0.2 d^{-1} were supplied with balanced/Redfield N:P supply ratios, but different absolute nutrient concentrations, toxin content was greater under greater nutrient concentrations. These findings suggest that when growth rate is not nutrient limited, there is a positive relationship between nutrient availability and brevetoxin content. This work contributes to previous studies by demonstrating strong growth rates effects on brevetoxin content and that growth rate and nutrient availability can independently or together affect toxin content of *K. brevis*. Moreover, our work underscores the value of the chemostat as a tool to elucidate the mechanisms by which nutrient availability and growth rate affect toxin production and content of HAB species.

1. Introduction

Primary producers ranging from unicellular algae to terrestrial plants often produce toxins as secondary metabolites (Hay and Fenical, 1988; Bennett and Wallsgrove, 1994; Cembella, 2003). Toxins play critical roles in phytoplankton communities and may, in fact, contribute to the success of bloom-forming organisms (Ianora et al., 2006). Many toxins produced by harmful algal bloom (HAB) taxa can deter predators, reduce grazing rates, and lessen competition by other microalgae (Paul et al., 2007; Poulson et al., 2009). Environmental factors, including nitrogen (N) and phosphorus (P) availability, influence the amount and composition of toxins produced by HAB species (Usup et al., 1994; Pan et al., 1998; Adolf et al., 2009; Van de Waal et al., 2009). However, the mechanisms by which nutrients affect toxin production and content vary with toxin stoichiometry and species' physiological constraints. Nutrient limitation may directly influence toxin production if the toxin is rich in the limiting nutrient. For example, N limitation reduces the production of N-rich saxitoxin (Anderson et al., 1990) and shifts the production of N-rich microcystins to less N-rich microcystin variants (Van de Waal et al., 2009). More often, nutrient limitation affects toxin production and content indirectly.

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* Corresponding author. Tel.: +1 727 892 4156; fax: +1 727 550 4222.
E-mail address: alina.corcoran@myfwc.com (A.A. Corcoran).

http://dx.doi.org/10.1016/j.hal.2014.08.009

Although indirect effects have been described as a general stress response (Pan et al., 1996; Johansson and Granéli, 1999b), specific mechanisms by which nutrient limitation affects toxin production and content have been documented. For example, changes in growth rate due to nutrient limitation affect cell size and toxin accumulation within cells, effectively increasing cellular toxin quotas (Varkitzi et al., 2010). It has also been proposed that plants accumulate carbon (C)-based secondary metabolites during nutrient-limited growth and reduced photosynthetic rates (Bryant et al., 1983, since termed the carbon-nutrient balance hypothesis). Despite equivocal support of this hypothesis (Koricheva et al., 1998; Hamilton et al., 2001), it has merit for some phytoplankton species, including the HAB taxon *Karenia brevis* (Van de Waal et al., 2009; Hardison et al., 2012, 2013).

Karenia brevis is a marine dinoflagellate that blooms nearly annually along the southwest Florida shelf in the eastern Gulf of Mexico and causes fish kills, marine mammal mortalities, and human illnesses (Flewelling et al., 2005; Landsberg et al., 2009; Fleming et al., 2011). This species constitutively produces a suite of C-rich cyclic polyether neurotoxins called brevetoxins. Although more than nine brevetoxin congeners have been isolated from *K. brevis* and characterized (Baden et al., 2005), the parent toxins PbTx-1 and PbTx-2 dominate total brevetoxin content within *K. brevis* cells. In the field, brevetoxin content can vary considerably, even during the same bloom (Pierce et al., 2008). Clones of *K. brevis* also exhibit variability in toxin content ranging from ~1 to 20 pg cell^{-1} (Baden and Tomas, 1988; Errera et al., 2010; Lekan and Tomas, 2010). Batch and semi-continuous culture studies have revealed variable effects of nutrient limitation on brevetoxin content and composition of *K. brevis* clones, although there is growing support that nutrient limitation increases toxin content (Roszell et al., 1990; Greene et al., 2000; Monroe et al., 2005; Lekan and Tomas, 2010; Hardison et al., 2012, 2013). To effectively manage *K. brevis* blooms, it is important to understand the factors, including nutrient availability, that affect bloom toxicity.

In this study, we quantified the short-term effects of changes in N and P availability on brevetoxin content and composition of a *Karenia brevis* clone. We used continuous culture systems, specifically chemostats, to separate the effects of nutrient availability from the effects of growth rate, since both can affect toxin content. We know of no published studies describing the physiology of *K. brevis* cultures grown in chemostats. We grew *K. brevis* at constant growth rates under nutrient-limiting and non-limiting supply ratios to assess the effects of N and P availability on toxin content. We hypothesized that, at steady state, N- and P-limiting supplies will minimally or not affect intracellular brevetoxin content or composition because brevetoxin is a C-rich compound.

2. Material and methods

2.1. Growth conditions

To separate the direct effects of nutrient availability from the indirect effects of nutrient-induced changes in growth rate on toxin content and composition, we conducted our experiments in chemostats – homogenous, open systems in which the influx of sterile media is matched by the efflux of used media, living cells, and cellular debris. At steady state, cultures exhibit a constant growth rate equal to the dilution rate of the cultures set by the experimenter, given that the dilution rate is less than the intrinsic growth rate of the study organism (Smith and Waltman, 1995). The nutrient concentrations in the media do not affect the growth rate of the culture, but do determine the concentration of steady-state biomass (Goldman, 1977). Nutrient limitation can be imposed by manipulating incoming nutrient supply ratios from a “balanced ratio” (i.e., the Redfield Ratio of 16:1) to an unbalanced ratio where a single nutrient is in short supply. Under balanced growth, the absolute nutrient concentrations will determine the biomass of the culture, whereas under nutrient-limited or unbalanced growth, the absolute concentration of only the limiting nutrient will determine the biomass of the culture. In sum, the dilution rate sets the growth rate of a culture and the nutrient concentrations set its biomass.

Our chemostat system consisted of an array of culture vessels, which were mechanically stirred, rather than bubbled, and fed with media by a single peristaltic pump (Fig. 1). Each of 12 cylindrical one-liter polycarbonate wide-mouth bottles (17 cm × 9.5 cm), contained a Teflon® stir paddle (10 cm × 8 cm) cm) and Delrin® shaft (1.3 cm diameter) to mix cultures, an inflow tube to deliver media, and an outflow bulkhead fitting to efflux used media and cells. All stir paddles were mechanically driven by sprockets connected to a single steel chain and DC gear motor (Dayton model #1LPW4), resulting in a synchronous rotation rate of ~8 rpm. The chain, sprockets, and motor were enclosed in a Plexiglas® box mounted above the culture vessels. Sterile medium from reservoirs was delivered to vessels via tubing (Masterflex Pharmed BPT L/S 13 pump tubing and 1/16″-ID PTFE tubing) with a peristaltic pump (Masterflex L/S Variable-Speed Digital Drive with two L/S eight-channel pump heads).

The chemostat array was kept in an environmental chamber at an average temperature of 25 °C. Light was supplied on a 12 h L/D cycle from one side by a bank of 40 W fluorescent bulbs (GE Cool White, GE Chroma 50 Full Spectrum, and GE Daylight) covered with polycarbonate tube guards (Diversified Lighting Inc., Farmingdale, NY) to shield UV radiation. The average (±SD) intensity of photosynthetically active radiation reaching the center of each vessel was 209 ± 24 μmol photons m^{-2} s^{-1}. For all experiments, treatment replicates were haphazardly distributed along the light banks.

2.2. Experimental approach and setup

We conducted three separate experiments to quantify the effects of decreases in nutrient availability on intracellular toxin content and composition (Table 1). In two experiments, we quantified the effects of changes in N availability, and in one experiment we quantified the effects of changes in both N and P availability. Therefore, the number of treatments per experiment

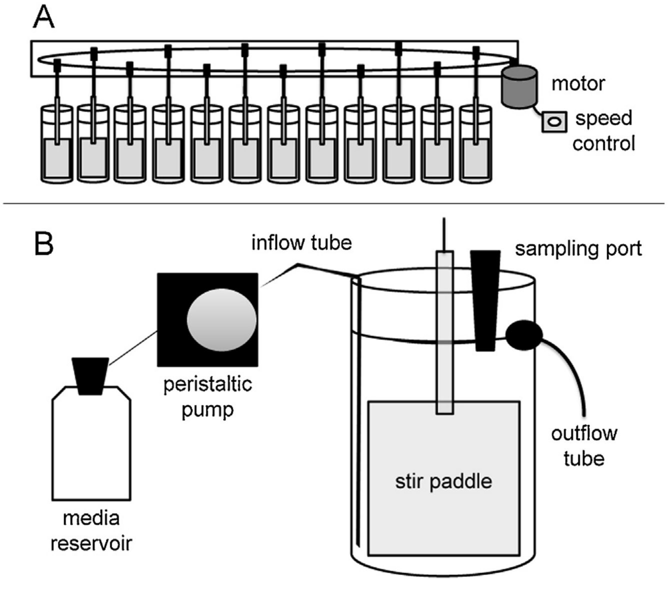

Fig. 1. Schematic of the 12-chemostat array (A) showing a single motor- and chain-driven stirring mechanism and an individual chemostat (B). All chemostats were fed by a single peristaltic pump.