Toxin production by *Crocosphaera watsonii:*
structure and influence on nutrient cycling in the upper ocean
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Abstract

Cyanobacteria are abundant components of the biosphere and play a crucial role in carbon sequestration and oxygen supply to the atmosphere. In addition to their photosynthetic capabilities, some cyanobacteria contribute to global ocean productivity by fixing inert atmospheric nitrogen gas (N_2) into a bioavailable form. Many of these nitrogen-fixing bacteria (diazotrophs) also produce toxic metabolites, whose physiological and ecological roles are poorly understood. *Crocosphaera watsonii* has recently been shown to possess non-ribosomal peptide synthetases and polyketide synthetases, which have been implicated in toxin production. Cell extracts of *C. watsonii* exhibited toxicity to human cell lines. We propose to purify and structurally characterize the toxin. In addition, experiments will be conducted to determine the conditions for optimal toxin production, shedding light on the significance of the toxin to the producing organism. As cyanobacterial toxins are capable of inhibiting the photosynthetic and nitrogen-fixing capabilities of other cyanobacteria, we will perform algicide assays to determine the potential effects of the toxin on the planktonic community. Nitrogen-fixing bacteria (diazotrophs) are thought to significantly contribute to global ocean productivity and carbon export to the deep sea, yet nitrogen-fixation rates and their control mechanisms are poorly understood. To improve our understanding of the controls on nitrogen fixation, we must understand the physiological role of toxins in diazotrophs. In addition, if we are to correctly gage the contribution of nitrogen-fixation to marine productivity, we must have a thorough understanding of the interactions between diazotrophs, competing photoautotrophs, and grazer populations in the open ocean.

Introduction

Cyanobacterial natural products. Cyanobacteria are potent producers of structurally and functionally diverse natural compounds that exhibit a wide range of biological activities (Figure 1). Such natural products have been the target of extensive pharmaceutical screening efforts, which have lead to the discovery of antibiotic, antifungal, anti-inflammatory, anti-HIV, and tumor suppressing structures (Burja *et al*.). The diversity and complexity of these structures often exceed the synthetic capabilities and creative design of organic chemists, and nearly half of all current cancer drug discovery efforts are directed at marine organisms (Clardy & Walsh, Burja *et al*.). The cyanobacterial influence on human health and economics extends beyond medicinal applications, as many metabolites are extremely toxic to higher organisms, causing massive fish kills, mammalian paralysis, and fatality (Hitzfeld *et al*.). Natural products have also exhibited anti-algal and grazer-deterring activity, indicating that they may play a role in nutrient balances, species succession, and ecosystem dynamics (Legrand *et al*., Wiegand and Pflugmacher).

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Figure 1. Structually diverse cyanobacterial natural products. Reproduced from Smith & Doan, Wiegand & Pflugmacher.

The pharmacology of natural products has been extensively evaluated, yet their ecological role is not well understood. This is largely an artifact of the analytical technique used to isolate novel bioactive compounds; crude extracts are screened against primarily vertebrate bioassays, such as human, mouse, or porcine cell lines. While this is effective for identifying the therapeutic potential of a compound, it leaves us with little understanding of the physiological role of the compound in the producing organism. Indeed, natural products are often referred to as secondary metabolites, as they are believed to be non-essential for growth, development, or reproduction. In a given genera, there are perfectly functional strains that do not produce secondary metabolites, while

other strains of the same genera actively produce the structurally complex compounds (Singh *et al.*, Wiegand *et al*).

Natural product biosynthesis. Two large (200-2000 kDa), polyfunctional enzymes have been implicated in natural product synthesis; polyketide synthetases (PKSs) and nonribosomal polypeptide synthetases (NRPSs). Both enzymes are organized into functional modules, independently folded protein domains, each of which is responsible for specific group modification at sequential stages of ketide (PKSs) or peptide (NRPSs) chain elongation (Cane & Walsh). NRPS and PKS genes are similar in their operation and organization, but diverse in their specific enzymatic mechanisms (Cane *et al.*). The genes encoding these megasynthetases seem to be well distributed amongst cyanobacterial species, indicating that they are ancestral to the cyanobacteria (Christiansen *et al.*). Their absence from *Gleobacter* PCC7421, which is thought to represent a common predecessor of modern cyanobacteria, and other strains that typically contain NRPS and PKS genes supports the notion of gene loss through time and indicates that PKS and NRPS products are not essential for survival (Ehrenreich *et al.*, Turner.). The retention of metabolically expensive, complex synthesis machinery for the production of nonessential compounds seems like a poor strategy for survival in a competitive, dynamic ecosystem. While secondary metabolites appear to be expendable, the conservation and expression of their genetic precursors indicates that natural products serve some physiological or ecological role promoting the survival and proliferation of the producing organism in certain environments.

Ecological role of toxins. Of the more than 850 identified cyanobacterial natural products, only 15 have been studied in terms of their effect on planktonic communities (Burja *et al.*, Legrand *et al.*). Only six of the 15 compounds have elucidated structures, so it is difficult to make generalizations relating compound structure and allopathic effect. Typically, the metabolite hinders the success of target organisms either through inhibition of photosynthetic machinery or degradation of the cell wall (Figure 2) (Mason *et al.*, Wiegand and Pflugmacher, Legrand *et al.*, Smith *et al.*, von Elert and Jüttner). Additionally, extracellular release of an unidentified allelochemical was shown to increase under phosphorus-limited conditions (von Elert and Jüttner). These results suggest that cyanobacteria may attempt to regulate the nutrient availability of their environment using secondary metabolites to limit the growth of competing microalgae. It has also been hypothesized that natural products can function as feeding deterrents; grazing zooplankton have been shown to either completely avoid or selectively limit consumption of allelopath-producing cyanobacteria (Wolfe, Wiegand and Pflugmacher).

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Figure 2. Effect of *Scytonema hofmanni* cell-free extract on structure of *Synechococcus* sp. Reproduced from Mason *et al.* (a) An untreated *Synechococcus* cell at 30,000 magnification. (b) A section of the cell wall at 100,000-fold magnification. The cell wall has the multilayered structure typical of cyanobacteria. Two parallel arrays of thylakoid membranes are clearly visible in the cytoplasm at the periphery of the cell. (c) Morphology of *Synechococcus* cell after 2-day treatment with 1.6 mg mL^{-1} *Scytonema* extract. Clear areas in the cytoplasm indicate a loss of cellular components, presumably due to increased permeability of the cell wall and membrane. (d) The cell wall, although still multilayered, is grossly distorted. No thylakoid membranes are evident in the treated cells.

Toxin-producing organisms have been the subject of extensive study, but the ecological role of toxins is very poorly understood. Toxins target vertebrate neuroskeletal systems, hepatic systems, dermal and gill tissue, and have resulted in human, livestock, and commercially important fish fatalities. As a result, research efforts have focused on the mechanisms of toxicity on vertebrate systems and environmental conditions preceding toxic algal blooms. A large body of knowledge exists describing the toxic effects on vertebrates, but there is no conclusive evidence regarding why cyanobacteria would produce the structurally complex molecules. There is no clear or direct competitive advantage for a cyanobacterium to limit the livelihood of terrestrial livestock, and some other selective pressure must have led to the development, retention, and expression of the intricate toxin-synthesis machinery.

The first identified biochemical mechanism of cyanotoxin activity was the blockage of voltage-gated sodium channels in nerve cells by saxitoxin (Strichartz *et al.*). Thus, it was believed that vertabrate toxins would not affect microalgae, grazers, or other members of the planktonic community that do not possess nervous systems. However, it has come to light in recent years that the modes of action of the toxins are as diverse as their structures. Toxins have been identified that inhibit ATP synthesis, disassemble DNA strands, and inactivate protein phosphatases (Wiegand and Pflugmacher). These structures are important to almost all living oraganisms, and so it is possible that cyanotoxins are targetting other microalgae, zooplankton, and bacteria.

Evidence for the allelopathic role of toxins. To date, more than eighty-two vertebrate cyanotoxins have been identified, but only one has been investigated for its allelopathic potential (Burja *et al.*, Legrand *et al.*, Wiegand and Plugmacher). Microcystin-LR (MC-LR) is a lethal hepatotoxin that induces cytoskeletal disorganization, lipid peroxidation, loss of membrane integrity, DNA damage, apoptosis, necrosis, and intrahepatic bleeding, which ultimately leads to death by hemorrhage shock in terrestrial mammals. Numerous

fish kills associated with cyanobacterial blooms have also been attributed to microcystin-induced liver damage. The toxicity of MC-LR arises from its ability to inhibit protein phosphatase, a vital enzyme for protein synthesis in vertebrates and microalgae alike. When assayed for algicidal activity, MC-LR paralyzed the motile green algae *Chlamydomonas reinhardtii*, causing the organism to settle out of the water column (Kearns and Hunter, 2001). A microcystin isolated from *M. aeruginosa* reduced the photosynthetic capabilities of the cyanobacteria *Nostoc muscorum, Anabaena*, and the dinoflagellate *Peridinium gatunense* (Sukenik *et al*, Singh *et al.*). In addition, nitrogenase activity of *N. muscorum* and *Anabaena* decreased after the toxin was administered, and cell lysis increased after 6 days of exposure (Singh *et al.*). These results indicate that toxins may be produced to limit the success of competing organisms, and Engelke *et al.* showed that the presence of a competing, non-toxic organism (*Planktothrix agardhii*) or its spent medium stimulated microcystin production of *M. aeruginosa*. The protein phosphatases of *M. aeruginosa* share less than 20% identity with the protein phosphatases of eukaryotic, archaeal, or other bacterial organisms, rendering them insensitive to microcystin-LR and okadaic acid (another protein phosphatase inhibitor) (Wiegand and Pflugmacher). The necessity of this adaptation suggests that algae are indeed susceptible to cyanotoxins. Thus, the argument that algal toxins do not affect planktonic organisms is falling out of favor with the marine natural product community.

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Figure 3a. Growth response of *Anabaena* BT1 (A) and *N. muscorum* (B) to *M. aeruginosa* toxin (microcystin). Reproduced from Singh *et al*. Results for the control □, 25 µg ml^{-1} Δ, and 50 µg ml^{-1} ◯ treatments are shown. Arrow indicates the time of toxin addition. Each point represents mean ± standard deviation.

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Figure 3b. Effect of microcystin on O₂ evolution activity. Reproduced from Singh *et al*. Results for the *N. muscorum* ○ and *Anabaena* BT1 □ controls, and *N. muscorum* Δ and *Anabaena* BT1◊ treated with 25 μg toxin ml^{-1}. Arrow indicates the time of toxin addition. Each point represents mean ± standard deviation.

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Figure 3c. Effect of microcystin on nitrogenase activity in *Anabaena* BT1 (A) and *N. muscorum* (B). Reproduced from Singh *et al*. Results for the control □, 25 μg ml^{-1} Δ, and 50 μg ml^{-1} ○ treatments are shown. Each point represents mean ± standard deviation.

Physiological role of toxins. In spite of these findings, it is still unclear as to whether the primary function of toxins is to limit competitors and grazers or to perform some physiologic function. The majority of secondary metabolites are lipophilic and would require active secretion or very high intracellular concentrations in order to impact aquatic target organisms. Such excretion has not been observed and, when investigated, intracellular quantities of toxins are almost always higher. Toxins seem to be released to the water column exclusively through cell lysis, which is often augmented by wave action (Hitzfeld *et al.*, Singh *et al.*, Keating). The retention of toxins by the producing organisms suggests that the molecules serve some physiologic function and that they are not simply "secondary metabolites." We have a vested interest in determining the significance of these toxins to the producing organisms, as it may enable us to forecast toxin outbreaks, minimize human impact, and understand the role of the toxins in the productivity of the global ocean.

Toxin production in a globally important cyanobacterium. The open-ocean, unicellular diazotroph, *Crocosphaera watsonii* WH8501, has recently been shown to contain numerous PKS and NRPS gene fragments (Ehrenreich *et al.*). Cell-free extracts inhibited the growth of human embryonic kidney cells (adherent HEK-293), indicating that *C. watsonii* is producing a toxic metabolite (Webb and Ehrenreich, personal communication). As a nitrogen-fixing, photosynthetic organism, *C. watsonii* plays an important role in the CO_2 balance of the modern ocean and atmosphere. Recent studies have shown that unicellular cyanobacteria may contribute to global nitrogen fixation as much or more than *Trichodesmium*, which was previously assumed to be the predominant oceanic N_2-fixer (Zehr *et al.*, Montoya *et al.*). Unicellular diazotrophs have a broader distribution throughout the euphotic zone of oligotrophic tropical waters, and *C. watsonii* can achieve cell densities up to 1000 cells/mL (Montoya *et al.*, Joint Genome Institute). The relatively high abundance and rapid N_2-fixation rates (520 ± 160 µmol N m^{-2} d^{-1} in North Pacific Subtropical Gyre, Montoya *et al.*) indicate that *C. watsonii* may significantly influence carbon and nitrogen cycles in tropical oceans, and production of toxins will almost certainly affect *C.watsonii's* role in the biogeochemical cycles and ecology of the microbial community.

The goal of this study is to determine the physiological and ecological role of the toxin produced by *C. watsonii* by determining the optimal conditions for toxin production. We will isolate and identify the toxic compound(s) using bioassay-guided separations and a structure-dependant combination of robust analytical techniques (^{13}C-NMR, ^{1}H-NMR, mass spectrometry, x-ray crystallography). If the toxin is integral to the growth, development, or reproduction of the organism, we expect to see a relationship between the growth phase of the culture and toxin levels in the cells. If the primary function of the toxin is to act as an allelochemical or a feeding deterant, we expect to see increased toxin concentrations when *C. watsonii* is cultured with competing microalgae or grazing zooplankton. Montoya *et al.* noted that N_2-fixation rates of *"Synechocystis* [*Crocosphaera*]-like" unicellular diazotrophs "could supply new nitrogen to the upper water column at a rate comparable to that of the equatorial upwelling system, although without any accompanying PO_4^{3-} to support the balanced growth of plankton." *C. watsonii* could compensate for the lack of PO_4^{3-} by hindering the growth or weakening

the cell walls of competing microalgae. This may manifest itself as increased toxin production and release under P-limited conditions.

Recent studies have noted that there seems to be a relationship between the ability to fix nitrogen and the maintenance and expression of NRPS gene fragments in the cyanobacteria (Ehrenreich *et al.*, Smith and Doan). Nitrogen-fixing cyanobacteria may require toxins for some essential intracellular process or may be using toxins to sequester other nutrients from the upper ocean, influencing the rapidly cycled nutrient inventories in oligotrophic waters. Such activity would alter our present understanding of nutrient cycling and dynamics in the upper ocean and reshape our view of the role of nitrogen-fixing organisms in oceanic carbon sequestration. Climate models rely on accurate estimates of global ocean productivity. Unicellular diazotrophs may represent a significant source of new nitrogen to the N-limited oceans, and without knowledge of the physiologic significance and ecological impact of their toxins, we cannot accurately describe of the contribution to primary productivity and influence on biogeochemical cycles.

Objectives

1. Determine optimal conditions for production of C. watsonii *toxin*. Ehrenreich *et al.* determined that *C. watsonii* produces a toxic compound, but the optimal production conditions were not determined. Prior to describing the structure of the compound, we will rely on increased bioassay activity to indicate increased toxin production, as toxicity and concentration are typically directly (although not linearly) related. Optimizing the production conditions will facilitate isolation and identification of the compound. Additionally, we may be able to gain a first-order understanding of the physiological significance of the compound by understanding the factors controlling its expression.

2. Purify and identify of the biologically active compound. Using standard organic extraction, bioassay-guided separations and chromatographic techniques, we will isolate the toxic compound from *C. watsonii*. Structural analysis will be preformed using primarily ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry (MS), and, if necessary, x-ray crystallography. Structural determination will allow more accurate compound quantification, calculation of production rates, and an improved understanding of the transport, partitioning, and cycling of the compound in the natural environment.

3. Determine allelopathic activity. We will assess the activity of the toxin against fast-growing cyanobacterial tester strains. The growth and cell-integrity of the target organisms will be assayed to determine the effect of the compound on the planktonic community and assess the potential effects of the toxin on nutrient cycling in the upper ocean.

4. Isolate the toxin from open-ocean Crocosphaera. In order to confirm toxin production by *C. watsonii* in open ocean conditions, we will isolate the compound during a transect of the North Pacific Gyre, where *C. watsonii* is abundant and activity fixing nitrogen.

We will perform shipboard incubations of *C. watsonii*, monitoring the change in toxin production with changing nutrient availabilities. In addition, we will administer purified toxin to incubations of the natural microalgal community and monitor the effects on nitrogen fixation, growth, and cell integrity. We acknowledge that this is an ambitious objective whose completion and exact protocol are contingent on preceding laboratory results.

Proposed Research

Optimizing growth conditions for maximum toxin production. The Waterbury & Webb Lab at the Woods Hole Oceanographic Institution has successfully cultured five axenic strains of *Crocosphaera,* and they have agreed to assist in the culturing of cyanobacteria. (See attached letter of support). *C. watsonii* has been grown as described in Webb *et al.* and maintained as described in Waterbury *et al.* Purity of the cultures will be monitored by microscopic observation of any sign of bacterial contamination. Expression of the synthetic genes for toxins might be related to growth phase (lag, log, or stationary) or the nutritional status of the medium. Thus, we will perform a series of experiments using large volume cultures, tracking the activity of the compound throughout the different growth phases of the culture. These experiments will include phosphorous, nitrogen, temperature, and light stress and will be compared to cultures grown in ideal, nutrient replete conditions (Webb *et al*., Waterbury *et al*.).

Prior to identifying the structure of the compound, we will rely on bioassay activity to indicate the production of the compound. The toxin exhibits activity against human embryonic kidney cells (adherant HEK-293), an epithelial cell line that has commonly been used to study toxicity (Webb and Ehrenreich, personal communication). HEK cells will be grown in a dilution series (0.5, 1, 2%) of extract concentrations and a 2% methanol-only (no extract) control. Following a 24-hr exposure period and a two-hour reagent incubation period, the absorbance will be monitored at 485 nm (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay), giving a quantitative measure of cellular viability. Data will be corrected for growth medium blanks and then normalized to the methanol control. Once we have identified the compound (Objective 2), we will perform a more rigorous quantification of the compound using standard analytical techniques (such as high-performance liquid chromatography or mass spectrometry).

We are interested in the physiologic significance of the vertebrate toxins to the producing microorganisms. Identifying the culture phase of maximal toxin production will suggest weather the toxin is important to growth, reproduction, or simply cell maintenance. If the toxin has a role in nitrogen fixation, we expect to see maximal concentrations under N-limited conditions or when N_2-fixation rates are maximal. If the primary function of the compound is to influence extracellular phosphorus availability, we expect to see maximal production under P-limited conditions.

We are also interested in the potential allelopathic activity of the vertebrate toxin, and while we acknowledge that activity against HEK-293 does not imply allelopathic activity, the use of HEK-293 will aid in the isolation of the vertebrate toxin as HEK-293 is a

sensitive indicator that has known susceptibility to the *C. watsonii* toxin (CW-1). Following structure identification, we will assay these vertebrate toxins against cyanobacterial tester strains.

Cell-free and growth media extract generation. To determine the relative extracellular/ intracellular distribution of CW-1, we will centrifuge 250 mL of the axenic cultures to separate the cells and the growth medium (Figure 4). After decanting the supernatant, the pellet will be transferred to 2-mL screw cap tubes. Samples will be sonicated for six minutes with alternating 30-second pulses (Ehrenreich *et al.*). Icewater will be pumped through a microcup horn using a peristaltic pump to prevent overheating. The cell pellets will be treated with chloroform and water, homogenized by vortexing, and subsequently centrifuged to remove cell debris. The organic extract will transferred to tapered glass ampoules and allowed to evaporate to dryness in a fume hood. The aqueous extract will first be concentrated by rotary evaporation and subsequently evaporated to dryness a fume hood. Dried samples will be resuspended in a small volume (250 μL) of methanol. To assess the extracellular release of the toxin, the growth medium (supernatant from initial centrifugation) will be partitioned against chloroform. The aqueous and organic extracts will be evaporated to dryness and resuspended in 250 μL of methanol. This procedure will be used to generate extracts to estimate the optimal conditions for production of biological active compounds.

Isolation and identification of the toxic compound. Each extract (organic and aqueous fractions from the growth media and cell extractions) will be checked for activity against the HEK-293 cell line. The bioactive extract will be charged onto a silica gel column and eluted with solvents of increasing polarity from hexane/chloroform to chloroform/methanol. Each fraction will be assayed for biological activity, and active fractions will be pooled and subjected to preparative thin-layer chromatography or high-pressure liquid chromatography (HPLC) using a C_{18} or C_8 reverse phase column. Additional separations will be preformed until a pure compound is isolated.

The exact protocol for structural determination will depend on the complexity of the toxin. High resolution MS provides an accurate mass and elemental composition, and advanced MS/MS can be used to identify how molecular fragments are attached in the toxin. Using one- and two-dimensional ^{1}H, ^{15}N, and ^{13}C- NMR spectroscopy, we will be able to determine the placement of atoms in CW-1. Complex toxin structures (e.g. the dinoflagelate-produced polyether brevetoxin) can be investigated using x-ray crystallography, provided that the toxin can be crystallized. Catherine Drennan at the Massachusetts Institute of Technology is a leader in the field of natural product and natural product synthesis protein identification using crystallography, and she has agreed to perform these analyses if necessary, as we do not have the instrumental capability at WHOI. (See attached letter of support). Once the compound has been successfully identified, we will be able to quantify its production with improved precision and accuracy.
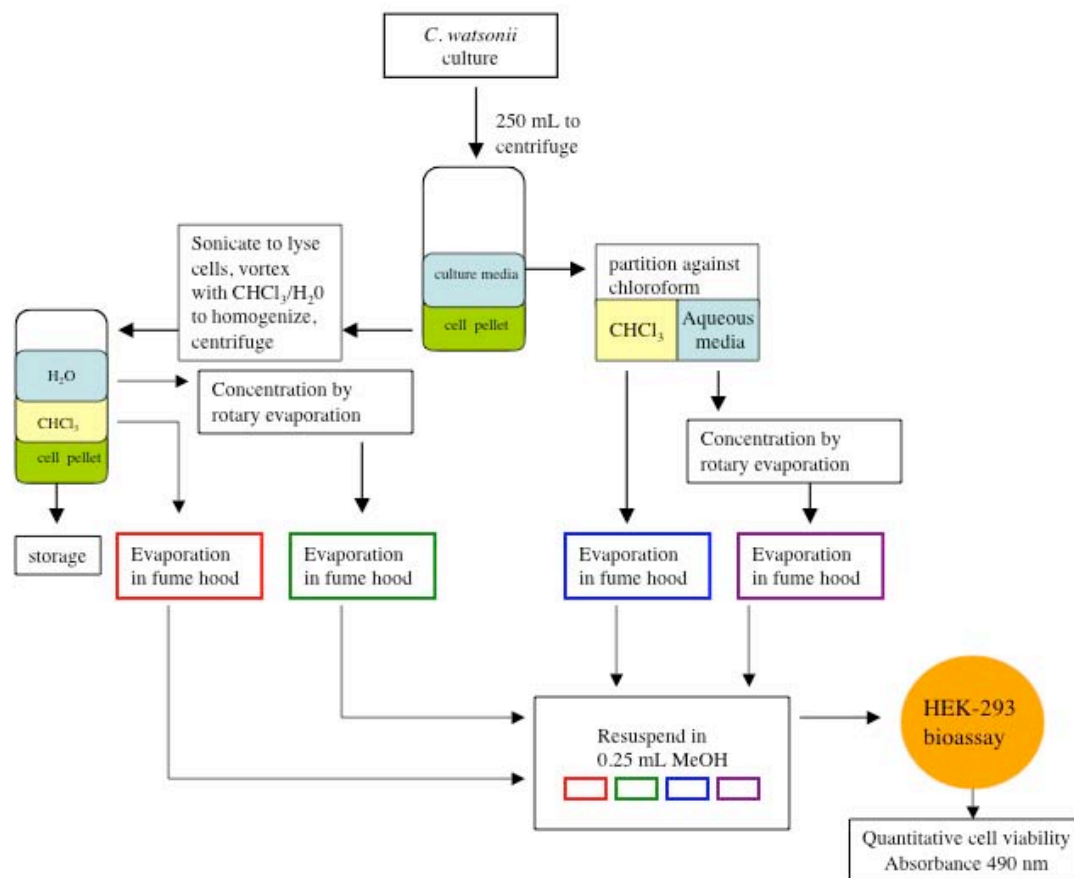
Figure 4. Schematic of protocol for toxin extraction from *Crocosphaera watsonii* laboratory cultures.

Determining allelopathic activity. To date, only one of the more than eighty-two identified cyanobacterial toxins has been evaluated in terms of its allelopathic effects. While we are uncertain that CW-1 will exhibit anti-algal activity, we believe that the experiments must be preformed in order to fully describe the role of the toxin in the physiology of *C. watsonii* and the ecology of planktonic communities. The algicide assays are facile and relatively rapid and can yield useful information regarding the mode of action of the toxin.

We will employ *Synechococcus* PCC7002 and *Synechocystis* PCC6803 as test organisms, as they have relatively short doubling times of four and twelve-hours, respectively. Other marine cyanobacteria have doubling times on the order of days, which would significantly prolong the experiment. Presently, the general make up of algal communities found in close association with *C. watsonii* has not been described (Montoya *et al.*). Once more information is available, we will assay the effects of CW-1 against species that typically coexist with *C. watsonii*. In the time being, *Synechococcus*

PCC7002 and *Synechocystis* PCC6803 will provide an efficient means of evaluating the algicidal activity of CW-1.

Anti-algal activity will be monitored using the method described by Ehrenreich *et al.*. The tests will be conducted in a 96-well plate format, with total culture volumes of 100 µL and 0, 2.5, and 5 µg of CW-1. Each well will then be inoculated with 10 µL of the cyanobacterial test strains (in stationary phase) and monitored visually for growth relative to CW-1 free controls over a five-day period. At the end of the experiment, the chlorophyll a absorbance maximum (670 nm) will be measured using a Versamax Tunable Microplate Reader to assess the inhibition of growth relative to the control.

If algicidal activity is observed, we will proceed with tests to determine the mode of action of CW-1. The cellular integrity of the test strains will be examined microscopically to determine if cell lysis or cell wall degradation occurred. As photosystem inhibition is a common mode of allelopathic interference, we may conduct additional experiments monitoring the evolution of oxygen in cultures treated with and without CW-1 (after Singh *et al.*). Nitrogenase activity may also be reduced in the presence of the toxin, and we can monitor these effects using the acetylene reduction procedure and a diazotrophic cyanobacterium test organism, such as *Anabaena* BT1 (Capone and Montoya, Singh *et al.*). We can confirm the uptake of the toxin to the target organism using radiocarbon-labeled CW-1, which can be generated by culturing *C.watsonii* on $NaH^{14}CO_3$-spiked growth medium (Singh *et al.*).

We may observe no algicidal activity, suggesting that either: 1) *Synechococcus* PCC7002 or *Synechocystis* PCC6803 are not susceptible to the effects of CW-1. This may suggest that CW-1 plays a role in the physiology of *C. watsonii* and does not directly affect the community structure of the upper ocean. Assaying the effects of CW-1 against other cyanobacterial test strains would help confirm that resistance to CW-1 is not unique to PCC7002 or PCC6803. 2) The use of HEK-293 in the isolation protocol precludes the purification of an allelochemical. As mentioned earlier, activity against HEK-293 does not imply anti-algal activity, and while *C. watsonii* could be producing an allelochemical, we may fail to track that chemical and instead isolate a vertebrate toxin with no allelopathic activity. To ensure that we track potential allelochemicals, we can perform the isolation using the algicide assay in place of the HEK-293 cell viability assay. We do not employ the algicide assay in the isolation protocol *a priori* because HEK-293 has previously established sensitivity to *C. watsonii* extracts and gives quantitative cell-viability estimates (Ehrenreich *et al.*). We are confident that we will be able to isolate the bioactive compound, elucidate its structure, and determine its optimal production conditions. These results are of interest, as they will lay the foundation for investigating the significance of CW-1 to *C. watsonii*, an ecologically important diazotroph (Zehr *et al.*, Montoya *et al.*). Additionally, as human contact with *C. watsonii* increases, structural information on the vertebrate toxin will be useful to facilitate treatment and understanding the production conditions may help limit human contact with the toxin.

Open ocean sampling. To assess the *in situ* production of CW-1 by *C. watsonii*, we will sample the North Pacific Gyre (transect from 32°N, 120°W to 28°N, 160 ^{O}W), as *C.*

watsonii has been observed in abundance in those, as well as other, warm oligotrophic, tropical waters. Additionally, Montoya *et al.* showed that unicellular diazotrophs are well distributed throughout the euphotic zone and actively fixing nitrogen in this region. *C. watsonii*-like organisms fix nitrogen at night, while other unicellular diazotrophs fix nitrogen during the day (Zehr *et al.*). To isolate the compound from seawater, we will filter 500-1000 L of water from 25 m depth once during the day and once at night. (There are few estimates on water column toxin levels in the literature. Fastener *et al.* measured toxin concentrations from a planktonic *Nodularian* ranging from 0.01-0.35 mg/L. We will obtain an improved estimate of the necessary volume of water based on laboratory toxin concentrations/ cell and *in situ* cell counts at sea). Seawater will be passed through a 10-μM Nitex filter and a pre-combusted 1.0-μM glass fiber filter (GF/F). The GF/Fs will be extracted with ethyl acetate, chloroform, or methanol, depending on the toxin's structural characteristics (as determined by Objective # 1). Concentrated filter extracts will be dried, resuspended in methanol, and assayed against HEK-293. Further bioassay-guided and standard analytical (e.g. HPLC) separations will be performed until a pure compound is obtained. We will have purified samples of CW-1 on hand so that we may perform a rapid, first-order confirmation that the toxin isolated from the field is CW-1 by HPLC peak or thin-layer chromatography (TLC) spot alignment (if CW-1 is amenable to those methods). The exact protocol for isolation, *in situ* sampling, and shipboard incubations will be influenced by the results of the preceding objectives. Here, we propose potential pathways for investigation.

Shipboard incubations will be used to determine 1) the effect of nutrient availability on toxin production and 2) the effect of the toxin on target cyanobacteria. Samples will be collected from varying depths in the euphotic zone for shipboard incubations at night and during the day. They will be passed through a 10 μM Nitex filter to exclude *Trichodesmium* and large diatoms that might contain diazotrophic symbionts. We will determine the relative abundance of *C. watsonii* by direct cell counts, as they are easily detectable under microscope due to their 2-5 μM size and orange color. The toxin will be isolated from cultures using the organic extraction procedures and HEK-293 cell viability assay as outline above (Figure 4).

We will assess the production of CW-1 by natural populations using a series of shipboard incubations with varying levels of nutrient availability. Cell-normalized toxin production will be measured *in situ* during the night and day, in a control incubation with no added nutrients, in a phosphate-spiked, and in a nitrogen-spiked incubation. A decrease in toxin production relative to the control after phosphate addition will suggest that the toxin plays a role in extracellular nutrient availability or sequestration. The addition of nitrate will inhibit *nifH* (nitrogenase gene) expression (Zehr *et al.*), and a decrease in toxin production following the nitrogen spike would suggest a relationship between the expression of nitrogenase and natural product synthesis genes. An increase in toxin production following nutrient addition would imply that the compound is related to growth, development, or reproduction.

To investigate the allelopathic effects of CW-1, we will administer the purified toxin to shipboard incubations of the natural microalgal populations. Zehr *et al.* identified two

groups of nitrogen-fixing cyanobacteria using reverse transcriptase polymerase chain reaction (RT-PCR). "Group A" actively fixed nitrogen during the day and was isolated from around 100m depth. "Group B," which contains *C. watsonii*, was isolated from 25 m depth and fixed nitrogen at night. Montoya *et al.* recovered RNA from both types throughout the water column and measured fairly constant nitrogen fixation throughout the day and night, but did not attribute the fixation to a specific group. It is possible that both Group A and Group B type cyanobacteria coexist throughout the water column and fix nitrogen with alternating circadian cycles. We will collect unicellular diazotrophs at day, when Group A is actively fixing nitrogen, and monitor the expression of *nifH* using RT-PCR prior to and following addition of CW-1 to the incubation. Diminished expression of *nifH*$_{GroupA}$ following CW-1 addition would suggest a mechanism for community shifts and identify a limitation on the nitrogen fixing capacity of the system. We will also monitor growth, cell integrity, and extracellular nutrient levels (HPO_4^{2-}, NO_3^-) during these incubations to assess the affect of the toxin on the viability of target organisms and nutrient availability in the upper water column.

Summary

Toxins pose a substantial threat to human health and the health of vertebrate marine communities of economical importance. In addition, genomic evidence suggests that there is a relationship between toxin production and the ability to fix nitrogen in the cyanobacteria. As the unicellular diazotrophs, *C. watsonii* in particular, account for a significant fraction of marine global nitrogen fixation, it is important that we understand the physiological significance of the toxins. Knowing when and why toxins are produced, their role in nitrogen fixation, as well as the effects on other primary producers, will further our understanding of nutrient dynamics and biogeochemical cycles in the modern ocean.

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