# SAXITOXINS: ROLE OF PROKARYOTES

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A

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# ABSTRACT

Saxitoxins, the toxins associated with paralytic shellfish poisoning (PSP), are synthesized by dinoflagellates, cyanobacteria, and possibly bacteria. The specific objectives of this study were to determine growth conditions that promote high and low levels of toxin accumulation in *Aphanizomenon flos-aquae* (cyanobacterium) and *Pseudomonas stutzeri* (bacterium). Putative saxitoxins of *P. stutzeri* identified by HPLC-FLD in this study, and previously by other laboratories, were determined to be "imposters" based on their chemical and physical properties, suggesting that this bacterium may not synthesize PSP toxins. In the cyanobacterium, toxin production was enhanced under higher light intensities and temperatures. Toxin accumulation reached maximal levels when cellular nitrogen was from either ($NO_3^-$ + $NH_4^+$)-N or $N_2$-N, while urea-N drastically reduced toxin levels. These data will be used in future studies aimed at identifying the genes involved in saxitoxin synthesis via molecular technologies that rely upon expression of the "saxitoxin genes" under different growth conditions.

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**Introduction**

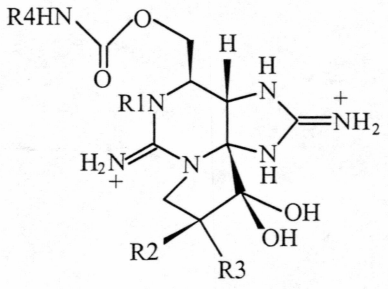
The base of the pelagic food chain in the world's ocean is made up of microscopic planktonic algae. These organisms are critical to the survival of filter-feeding bivalves and the larval stages of finfish and crustaceans. When these algae experience favorable conditions they can go through a sudden increase in numbers or bloom. These blooms can be beneficial to the surrounding biota, but in other cases they can have negative effects on the environment and upper trophic levels, in which case they are referred to as Harmful Algal Blooms (HABs). HABs can be caused by a number of organisms and their presence can sometimes be identified as a discoloring of the water (e.g., red tides) or from noticeable impacts on other organisms. Negative impacts can be due to the algae clogging gills, creating anoxic conditions, and/or producing toxins (Anderson 1994). HABs are usually dominated by a single species, are not very predictable, and are categorized by their effects, whether it is physical factors, over-abundance, or toxin production (Table 1-1).

There are certain variables that have been found to enhance or diminish blooms and/or toxin production. Water column physics, life cycles of the organisms, nutrient availability, temperature, community structure, growth, and grazing pressure all combine to regulate an algal bloom. HABs can be caused by a number of different organisms, including: dinoflagellates, diatoms, raphidophytes, prymnesiophytes, cyanobacteria, silicoflagellates, and seaweeds. Bacteria may also play a role in HABs (Kodoma 1988a, 1990; Doucette 1995; Franca et al. 1996). Of the 5000 extant marine phytoplankton species, 300 are known to be organisms that proliferate and cause a bloom while only about 40 species can produce potentially lethal toxins (Hallegraeff 1993). These toxins are transferred through the food chain following the ingestion of toxic algae by zooplankton and filter feeders, such as shellfish (e.g. clams, mussels, scallops, oysters).

Table 1-1. The events, species, location, and description of the toxins involved in some marine HAB events. The data are incomplete, but cover major trends.

| TOXIC EVENT | CAUSATIVE SPECIES | LOCATION | TOXIN (TYPE COMPOUND) | MODE OF ACTION |
|---|---|---|---|---|
| **Diarrhetic Shellfish Poisoning (DSP)** | *Dinophysis spp.* *Prorocentrum lima* | Cold/temperate Atlantic and Pacific | Okadaic acid dinophysistoxins pectenotoxins yessotoxins (polyethers) | Inhibits protein phosphorylase phosphatase 1 and 2a |
| **Paralytic Shellfish Poisoning (PSP)** | *Alexandrium spp.* *Gymnodinium catenatum,* *Pyrodinium bahamense,* Cyanobacteria, Red algae? Bacteria? | World-wide in cold and warm waters | Saxitoxins (alkaloids) | Blocks $Na^+$ influx at sodium channels |
| **Neurotoxic Shellfish Poisoning (NSP)** | *Gymnodinium breve* | Southeast US; Campeche, Mexico | Brevetoxins (polyethers) | Induces $Na^+$ infux at sodium channels |
| **Amnesic Shellfish Poisoning (ASP)** | *Pseudo-nitzschia spp.; Nitzschia actydrophila;* *Amphora coffaeiformis;* Red algae? | NE and SW Canada; NE, Gulf, and W coast of US; Australia; Europe | Domoic acid (excitatory amino acid) | Receptor-induced depolarization and excitation |
| **Ciguatera Fish Poisoning** | *Gambierdiscus toxicus* *Prorocentrum spp.* *Ostreopsis spp.* *Coolia monotis* *Amphidinium spp.* | World-wide in tropics | Ciguatoxin maitotoxin scaritoxin gambiertoxin (polyethers) | Opens $Na^+$ channels and prevents their inactivation (ciguatoxin); $Ca^{++}$ influx (maitotoxin) |
| **Ambush Predator** | *Pfiesteria piscicida* | Southeast US | Unknown | Respiratory irritation, problems with concentration, memory and learning |

The HAB toxic syndromes have been described as paralytic, amnesic, diarrhetic, neurotoxic and ciguatera fish poisoning (Smayda 1990). Toxic algae generally cause neurological or gastrointestinal symptoms including nausea, tingling, and vomiting, but other symptoms are specific to the species and toxin that causes the illness. Paralytic shellfish poisoning (PSP) is caused mostly by dinoflagellates and has been described worldwide in warm and cold waters. The toxins that cause PSP are the saxitoxins (Figure 1-1), which have been found to be fifty times more lethal than strychnine, and 1000 times more toxic than cyanide (RaLonde 1996); and a pinhead sized amount (500 μg) can be fatal to humans (Hallegraeff 1993). These compounds block the flux through sodium channels and cause symptoms ranging from tingling and numbness to respiratory paralysis within 2-4 hours.




| Name | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Saxitoxin | H | H | H | H |
| Neosaxitoxin | OH | H | H | H |
| $GTX_1$ | OH | $OSO_3^-$ | H | H |
| $GTX_2$ | H | $OSO_3^-$ | H | H |
| $GTX_3$ | H | H | $OSO_3^-$ | H |
| $GTX_4$ | OH | H | $OSO_3^-$ | H |
| $C_1$ | H | $OSO_3^-$ | H | $SO_3^-$ |
| $C_2$ | H | H | $OSO_3^-$ | $SO_3^-$ |
| $C_3$ | OH | $OSO_3^-$ | H | $SO_3^-$ |
| $C_4$ | OH | H | $OSO_3^-$ | $SO_3^-$ |

**Figure 1-1.** Structures of PSP toxins.

**The Role of Dinoflagellates in the Production of Saxitoxins**

American Native Indian tribes were perhaps the first to realize the connection between toxic shellfish and dinoflagellates. These astute observers of nature realized that shellfish toxicity and water bioluminescence were interrelated (Halstead 1965). Subsequent studies revealed that dinoflagellates, obviously the source of bioluminescence observed by Indians, were the toxic agent responsible for PSP events.

Several distinct PSP-toxin producing gonyaulacoid dinoflagellates of comparable morphology have been identified (Steidinger et al. 1980). These dinoflagellates were originally classified as *Gonyaulax* (Steidinger 1980) but were subsequently reclassified as *Protogonyaulax* (Taylor 1979), *Gesnerium* (Loeblich and Loeblich 1979), and most recently *Alexandrium* (Balech 1985). The latter has been generally accepted as a justifiable assignment and is presently being applied. Other dinoflagellate species confirmed as PSP toxin producers include *Pyrodinium bahamense* (Steidinger et al. 1980) and *Gymnodinium catenatum* (Morey-Gaines 1982). *Pyrodinium bahamense* , a thecate dinoflagellate with detailed plates, has caused toxic outbreaks in New Guinea (Maclean 1977), Guatemala (Rosales-Loessener et al. 1989), and the West Pacific (Harada et al. 1982). *Gymnodinium catenatum*, a catenate dinoflagellate without a theca, has been associated with PSP episodes in Mazatlan (Morey-Gaines 1982), Spain (Fraga and Sanchez 1985), Portugal (Franca and Almeida 1989), Tasmania (Hallegraeff et al. 1989), and Japan (Ikeda 1989). Over 350 human mortalities from paralytic shellfish poisoning have been recorded. (Lo Cicero 1975; Taylor and Selinger 1979; Anderson et al. 1985). PSP caused by dinoflagellates has been documented in the United States, Mexico, Canada, Europe, Australia, South America, South Africa, Scandinavia and Southeast Asia.

Growth conditions are known to impact the accumulation of toxins in dinoflagellates. For instance, saxitoxin accumulation and growth rates of dinoflagellates vary with growth

temperature (Hall 1982; Ogata et al.1987; Anderson et al. 1990b), salinity (White 1978; Parkhill and Cembella 1999), nutrient limitation (Hall 1982; Boyer et al. 1987; Anderson et al. 1990b; Flynn et al. 1995), and light intensity (Ogata et al. 1987). Growth conditions affect not only toxin accumulation but also toxin composition (Anderson et al. 1990a). Since individual saxitoxin derivatives have different toxicity potency values (reported in terms of mouse units/μmol toxin), growth conditions could, in theory, cause considerable changes in toxicity, without a corresponding effect on the total amount of toxin produced.

**Cyanobacteria and Saxitoxin Production**

As the history of PSP and the saxitoxins unfolded in the last 200 years, one of the most startling revelations was the demonstration that freshwater cyanobacteria also make saxitoxins. Certain cyanobacteria have long been known to make a number of toxins. For example, liver toxins termed microcystins and nodularins have been reported to have been produced by organisms in the genera *Microcystis*, *Anabaena*, *Hapalosiphon*, *Nodularia*, *Nostoc*, and *Oscillatoria* (Carmichael 1997). It was first suggested in the late 1800's that cyanobacteria could produce toxic substances (Francis 1878). Water from the Murray River, Australia, rich in blue-green filaments (*Nodularia spumigena*) caused unconsciousness and rapid death in animals that relied on the river as a water source. The first reports of PSP toxins in cyanobacteria were from lakes in New Hampshire in 1968 and 1980 (Sawyer et al. 1968). These blooms were dominated by a single species, *Aphanizomenon flos-aquae,* and the toxins from this cyanobacterium were shown to resemble PSP toxins in that they halted membrane excitability without affecting the transmembrane resting potential. Researchers continued to compare the toxins from these lakes, referred to as aphantoxins, to the saxitioxins produced by dinoflagellates. The toxicity of these compounds was tested on mice, fish and waterfleas (Jakim and Gentile 1968). It was established

that blockage of the sodium channels by aphantoxins was reversible and had no effect on potassium conductance, transmembrane potential, or membrane resistance, which are properties of saxitoxin (Adelman et al. 1982). Alam et al. (1973, 1978) collected natural bloom material from these New Hampshire lakes and purified three toxic components, one of which appeared to be a guanidine derivative. HPLC analysis of the toxins suggested the presence of saxitoxin as well as three other similar compounds. Izawa et al. (1982) and Mahood and Carmichael (1986) cultured strains of the putatively toxic *Aphanizomenon flos-aquae* from these lakes and demonstrated that unialgal cultures could produce compounds with a high degree of similarity to neosaxitoxin and saxitoxin. One of the cyanobacterial isolates, *Aphanizomenon flos-aquae* NH-1, played an instrumental role in the first study to conclusively demonstrate the "unexpected" pathway of saxitoxin biosynthesis involving arginine and acetate (i.e., saxitoxin had previously been considered a purine derivative based on the similarity in structure of purines and saxitoxins; Shimizu 1984). The identification of these substates has permitted hypotheses of the biosynthetic pathway and the variables involved in toxin production (Figure 1-2). The studies of Shimizu involved supplying cultures with isotopically labeled substrates and subsequent analysis of the products by NMR. These NMR studies confirmed that the toxic compounds produced by this cyanobacterium were chemically identical to saxitoxins. The *A. flos-aquae* NH-1 strain used by Shimuzu (1984) was also used in this study.

In 1991, a second cyanobacterium that produced PSP toxins was discovered in Australia, *Anabeana circinalis* (Humpage et al. 1993). Detailed analyses indicated that *A. circinalis* accumulates saxitoxin, the gonyautoxins, and some of the C toxins (Humpage et al. 1994). Other saxitoxin-synthesizing cyanobacteria have been found in the Tennessee River system (Carmichael et al., 1997) and in the Montargil reservoir, Portugal (Pereira et al. 2000)

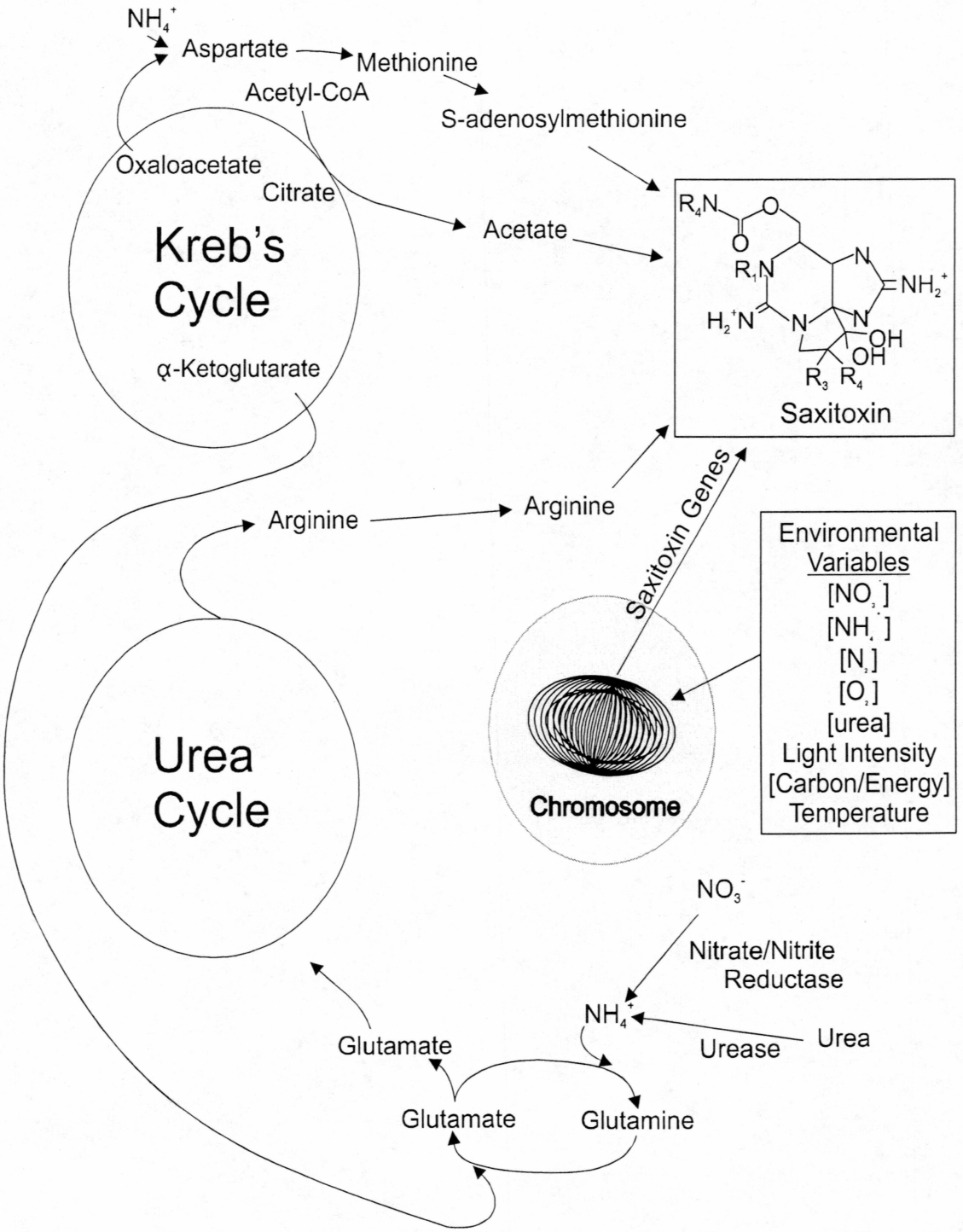



**Figure 1-2:** Hypothetical biochemical pathway of saxitoxin synthesis. Modified from Plumley (1997).

suggesting that these toxic cyanobacteria are widespread. The chemical structures of several cyanobacterial toxins have been confirmed by NMR and found to include previously unknown saxitoxin derivatives (Ondodera et al. 1997).

**The Bacterial Role in PSP Toxin Events**

With the realization that cyanobacteria also synthesize saxitoxins, an earlier hypothesis of de Silva (1962) suggesting that bacteria are involved in toxin production, was re-examined by Kodama and colleagues (Kodama et al. 1988a, 1988b, 1990a; Ogata et al. 1990). This work was initiated with a bacterial strain of *Moraxella* sp. isolated from a putatively axenic culture of the toxic dinoflagellate, *Alexandrium tamarense*. The bacterium had escaped detection until cells of the dinoflagellate were broken, releasing the intracellular bacterium. In culture, *Moraxella* was shown to produce compounds that were indistinguishable from saxitoxins based on numerous chemical properties as well as toxicity to mice. Doucette and Trick (1995) were subsequently able to confirm the production of PSP toxins from one of Kodama's cultures of *Moraxella* PTB-1. These later studies (Doucette and Trick 1995) also indicated that *Moraxella* PTB-1 should be reclassified as *Altermonas/Pseudomonas* PTB-1. The *Alteromonas/Pseudomonas* PTB-1 strain was one of two bacterial strains used in studies reported in this thesis.

The work of Kodama and colleagues, confirmed by Doucette and Trick, served as a catalyst to ignite numerous studies of bacteria capable of autonomous production of PSP-toxins. Many studies focused on the intracellular bacteria associated with toxic dinoflagellates and revealed putatively toxic bacteria from *Alexandrium lusitanicum* (Franca et al. 1995, 1996), *A. tamarense* and *A. affine* (Gallacher et al. 1997), and *Gymnodinium catenatum* (Franca et al. 1996). One of the bacterial strains isolated by Franca et al. (1995, 1996) from *A. lusitanicum*

was studied in this thesis. Other putatively toxic bacterial strains have been isolated directly from seawater (Levasseur et al. 1996) suggesting they may live entirely as free-living forms. Bacterial strains reported to produce PSP toxins have been isolated from diverse habitats and geographical locations. For instance, bacteria strains have been isolated from both open ocean locations and sheltered lagoons. Putatively toxic bacteria have been isolated from warm, temperate regions such as Portugal (Franca et al. 1995), Japan (Kodama et al. 1988a), Spain (Gallacher et al. 1997) and from colder regions including the St. Lawrence River (Levasseur et al. 1996) and the United Kingdom (Gallacher et al. 1997).

Since the first isolation and detection of PSP toxins in these bacteria, other investigators have reported toxin production using several different detection methods. Kodoma and colleagues (Kodama et al. 1988a, 1988b, 1990a; Ogata et al. 1990) reported positive results utilizing the mouse bioassay, TLC, and HPLC. PSP toxins have since been identified by HPLC coupled with a post-column derivitization protocol and fluorometric detection (Doucette 1995, Gallacher et al. 1997, Ogata et al. 1990, Levasseur et al. 1996), capillary electrophoresis-MS (Gallacher et al. 1997), and by cell-based viability assays that utilize ouabane-veratradine as tests for sodium channel blocking compounds (Gallacher et al. 1997). Anti-saxitoxin antibodies have also been used to demonstrate bacterial production of saxitoxin (Gallacher et al. 1997) and a receptor binding assay that detects displacement of $^{3}H$ saxitoxin from rat brain synaptosome (Doucette et al. 1997) yielded positive results when challenged with extracts from putatively toxic bacteria.

**The Alaska PSP Story**

The PSP problem is prominent in Alaska due to the vast coastline, the abundance of shellfish, and the apparently large populations of toxic dinoflagellates. In a recent 20 year study,

Alaska averaged seven reported illnesses a year. One of the first reported episodes of PSP took place in Alaska and much of the initial research was done in Alaska and on Alaskan species. The first outbreak that is attributed to toxic shellfish took place in Alaska just before the nineteenth century. Cases of PSP continued throughout the following years and eventually led to the demise of a potentially lucrative shellfish industry.

In 1799, near Sitka, Alaska, 100 Aleut hunters died a few hours after eating mussels (Halstead 1965). Natives continued to warn travelers when they saw the discoloration of the water and bioluminescence that they associated with the illness. During World War II the demand for shellfish increased and razor and butter clam harvests grew in southeast and southcentral Alaska. In 1946, the U.S. Food and Drug Administration (FDA) detected high levels of toxins in a number of butter clams and shut down a growing commercial market (Magnussen and Carlson 1951). In 1954 the US Public Health Service withdrew Alaska from the National Shellfish Sanitation Program (NSSP) because it could not meet the requirements to assure a safe product. This stopped interstate transport of Alaskan shellfish. Soon thereafter (1962) the cockle harvest ended due to PSP restrictions. An immense earthquake destroyed 43% of the razor clam population in Cordova and caused widespread destruction of shellfish habitat throughout southcentral and southeast Alaska in 1964. In 1970 the Department of Health and Social Services restricted harvesting in Alaska to Cordova, Cook Inlet and Shelikof Strait. Alaska regained access to the interstate human consumption market in 1975 when it was readmitted into the NSSP. Later that same year, harvesting of razor clams was closed due to toxicity values greater than 80 μg toxin/100 gm shellfish meat in 3-5% of the clams tested. A lot sampling system for PSP was initiated to allow for harvests of shellfish other than razor clams. A three year study (1985-1987) conducted by the Department of Fish and Game and the Department of Environmental Conservation showed that butter clams were toxic year round and

that bay mussels, littlenecks and cockles were seasonal. Harvests have increased in the past few years due to increased availability of testing facilities, but the commercial harvesting of shellfish is still practically nonexistent in Alaska. Shellfish growers also constantly battle the PSP problem (RaLonde 1996).

There have been few studies conducted on toxic dinoflagellates in Alaska. One study demonstrated a correlation between dinoflagellate abundance and mussel toxicity at some, but not all, test sites near Ketchikan, Alaska (Neal 1965). The most thorough study of *Alexandrium* spp. in Alaska was conducted by Hall (1982), who reported detailed studies on the distribution of dinoflagellate cysts and the effects of nutrients on toxin accumulation in *Alexandrium* spp. Hall also conducted detailed studies on the chemical properties of PSP toxins.

**Overall Thesis Objectives**

The research reported in this thesis is part of a larger project funded by the Alaska Sea Grant College Program. The goals of the research project are to identify the genes involved in the synthesis of saxitoxin (i.e., the "saxitoxin genes"). Although many dinoflagellates are known to synthesize saxitoxins, these algae can be difficult to grow, cannot be plated on agar plates, have very large genomes, and are not amenable to study using many techniques that are routinely used in molecular genetic studies (reviewed in Plumley 1997). In contrast, bacteria and cyanobacteria are easier to grow, can be plated on agar plates, have small genomes, and are amenable to molecular studies including reverse genetics. For these reasons, I undertook a project to ascertain the role of prokaryotes in the synthesis of saxitoxins. My initial focus was on defining growth conditions that maximized toxin production in bacteria. The overall goals of the project included defining conditions for enhanced toxin production, because another study being conducted with these bacteria relied on Tn5 mutagenesis and subsequent detection of mutants

unable to synthesize toxin (Plumley et al., 1999). Preliminary studies had shown that under most growth conditions, the bacteria produced such low levels of toxin that they could not be reproducibly assayed. We reasoned that enhanced toxin production, achieved by growing cells under "optimal" growth conditions, would minimize selection of "false negatives" (i.e., bacterial mutants that were still able to produce saxitoxins). My work in this area revealed an unpleasant surprise. At least one, and possibly all, of the putative saxitoxin compounds that had been identified from the bacterial strains under study were not authentic saxitoxins, but were "imposters." The second chapter of this thesis reports on the physical and chemical properties of five different imposter compounds that have chemical, physical, and toxicity properties similar to one saxitoxin derivative, $GTX_4$.

The third chapter of this thesis is a brief report on a different prokaryote, the photosynthetic cyanobacterium, *Aphanizomenon flos-aquae* NH-1. As outlined above, many studies have conclusively demonstrated that this prokaryote produces saxitoxins. Similar to the work with bacteria, one goal of the lab was to identify growth conditions that enhanced toxin production. The work reported in Chapter 3 clearly demonstrates that toxin production is enhanced under higher light intensities and at higher temperatures. Growth with different nitrogen sources was also found to affect toxin accumulation. Surprisingly, growth with $N_2$-N resulted in high levels of toxin, suggesting that nitrogen fixation and toxin production may not be competing pathways. Another unexpected result was observed when urea was included in the growth medium; toxin production was drastically reduced under all light/temperature regimes tested. I had predicted that urea, a precursor of arginine and the source of all the nitrogen atoms in saxitoxin, would enhance toxin production. Obviously, much remains to be learned about the cellular and biochemical pathways of saxitoxin synthesis and the effects of environmental growth conditions on toxin production.

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# Characterization of Fluorescent Compounds from *Pseudomonas stutzeri* SF/PS and *Pseudomonas/Alteromonas* PTB-1, Bacteria Associated with PSP Toxin-Producing *Alexandrium* spp.

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**Abstract**

Saxitoxins, the etiologic agent of paralytic shellfish poisoning, are synthesized by dinoflagellates and cyanobacteria. Several reports indicate that bacteria are also capable of saxitoxin synthesis. Two bacterial strains, isolated from saxitoxin-producing dinoflagellates, *Alexandrium tamarense* and *A. lusitanicum* (=*A. minutum*), were grown under a variety of culture conditions including those previously reported to induce saxitoxin synthesis. Five fluorescent compounds were identified by HPLC-FLD with retention times similar to a reference standard of $GTX_4$, one of the saxitoxin congeners. However, we were unable to detect $GTX_1$, the epimeric partner of $GTX_4$, in the bacterial samples. The $GTX_4$ standard was labile to treatment with NaOH but four of the bacterial compounds were stable. Unlike $GTX_4$, none of the five bacterial compounds were detectable by HPLC-FLD following electrochemical oxidation. The fluorescence emission spectrum of each of the five bacterial compounds was unique and readily discernable from the spectrum of $GTX_4$. None of the samples containing the putative $GTX_4$ toxin yielded consistently positive results when analyzed by a $^3$H-saxitoxin receptor-binding assay for saxitoxin-like activity. We cannot rule out the possibility that these bacteria produce saxitoxins; however, our data clearly demonstrate that they accumulate at least five different fluorescent compounds that could be easily mistaken for $GTX_4$. We conclude that these five fluorescent compounds are $GTX_4$ imposters.

*Keywords:* saxitoxin, paralytic shellfish poisoning, *Alexandrium*, bacteria

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## 1. Introduction

Paralytic shellfish poisoning (PSP) imposes serious health problems and economic losses on many coastal communities around the world. PSP results from human consumption of shellfish that have fed on toxic algae containing saxitoxins. With an $LD_{50}$ as low as 9 μg per kg body weight (in pigeons), saxitoxin is 1000 times more lethal than cyanide (Shimizu, 1978), and a pinhead-sized amount (500 μg) could be fatal to humans (Hallegraeff, 1993). Saxitoxins are potent, but reversible, blockers of voltage-gated sodium channels that provide the basis for nerve and muscle excitability. The positively charged saxitoxins are thought to bind to negatively charged groups near the mouth of the sodium channel thereby preventing normal operation (Terlau et al., 1991). Respiratory paralysis is usually the cause of death in serious cases of PSP toxin ingestion; lesser symptoms involve numbness of the lips and/or fingers. Ironically, saxitoxin's mode of action has made this compound quite useful in medical research, especially in the characterization of $Na^+$ channels and nerve transmission (reviewed in Terlau et al., 1991).

Saxitoxins are alkaloids having the general structure of purines (Fig. 1). The parent toxin molecule, presumably saxitoxin, is modified by addition/removal of hydroxyl, carbamyl, and/or sulfate moieties. These substitutions yield different congeners whose potencies vary more than three orders of magnitude, and include some forms that are essentially non-toxic (Onodera et al., 1997). To date, more than 20 derivatives of saxitoxin have been characterized (Hall et al., 1990; Oshima et al., 1993; Onodera et al., 1997). The large number of saxitoxin congeners has made it difficult to easily identify specific members of this toxin family. However, several HPLC methods that rely upon post-column oxidation reactions to produce fluorescent derivatives have been successfully employed for this purpose. The most commonly used post-column oxidation method relies upon chemical oxidation (Oshima, 1995) though recent data indicate that electrochemical oxidation may be preferred for some derivatives (Boyer et al., 1997; Boyer and Goddard, 1999).

Outbreaks of PSP are routinely associated with blooms of dinoflagellates, known colloquially as red tides (Hallegraeff, 1993; Anderson, 1994). Marine dinoflagellates were once considered to be solely responsible for saxitoxin production, but it is now known that certain freshwater cyanobacteria also synthesize saxitoxins (Onodera et al., 1996, 1997). Bacteria also accumulate compounds that have been referred to as PSP toxins (reviewed in Doucette, 1995;

Doucette et al., 1998). In culture, the accumulation of these bacterial PSP toxins is highly dependent upon growth conditions. For instance, growth with reduced phosphorus or with a single carbon/energy (C/E) source enhances toxin production relative to growth in P-replete medium or with multiple C/E sources such as those present in yeast extract (Kodama et al., 1990; Doucette and Trick, 1995).

Although many bacterial compounds have been referred to as PSP toxins, crucial evidence (e.g., NMR or MS/MS data) that would confirm the identity of these compounds as authentic saxitoxins has been difficult to obtain. At least one neosaxitoxin "imposter" has been reported (Sato and Shimuzu, 1998) from a bacterial strain that was thought to be capable of autonomous saxitoxin synthesis. In this report, we demonstrate that fluorescent compounds isolated from bacterial strains associated with toxic dinoflagellates (Kodama et al., 1988; Franca et al., 1996) that share many physical and chemical properties with $GTX_4$ are PSP toxin imposters.

## 2. Materials and methods

### *2.1 Bacterial strains and culture conditions*

#### *2.1.1 Bacterial strains*

*Pseudomonas stutzeri* SF/PS was isolated from a culture of the toxic dinoflagellate, *Alexandrium lusitanicum* (=*Alexandrium minutum*), isolated from Obidos Lagoon, Portugal (Franca et al., 1995). *Alteromonas/Pseudomonas* PTB-1 was isolated from a culture of toxic *Alexandrium tamarense* from Ofunata Bay, Japan (Ogata et al., 1990); this bacterial strain was originally referred to as *Moraxella* sp.

#### *2.1.2 Growth media*

Cultures were maintained in artificial seawater (ASW; Doucette and Trick, 1995) consisting of 15.5 g NaCl, 0.75 g KCl, 12.35 g $MgSO_4 \cdot 7H_2O$, 2.9 g $CaCl_2 \cdot H_2O$, 1 g $NH_4Cl$, 0.065 g $Na_2HPO_4$ per liter deionized distilled water ($ddH_2O$) with 10 mL of 200 mM $NaHCO_3$ and 10

mL of ferric sequestrene added after autoclaving. Different organic compounds, listed below, were used as the carbon/energy (C/E) sources.

Cultures were also grown in seawater complete medium (SWC; Haygood and Nealson, 1985) consisting of 5 g Bacto Tryptone, 3 g yeast extract, 6 ml 50% glycerol per L of seawater. Freshwater complete medium (FWC) was made as SWC using $ddH_2O$. Cultures were also grown with the different C/E sources, listed below, in modified JM (Thompson et al., 1988), a freshwater medium containing 20 mg $Ca(NO_3)_2$-$4H_2O$, 6.2 mg $KH_2PO_4$, 50 mg $MgSO_4$-$7H_2O$, 7.3 mg EDTA, 2.4 mg $FeCl_3$-$6H_20$, 2.4 mg $H_3BO_3$, 1.3 mg $MnCl_2$-$4H_2O$, 1.0 mg $(NH_4)_6Mo_7O_{24}$-$4H_2O$, 40 µg cyanocobalamine ($B_{12}$), 40 µg thiamine HCl ($B_1$), 40 µg biotin, 80 mg $NaNO_3$, 14.2 mg $Na_2HPO_4$ per liter $ddH_2O$. After autoclaving, 61 mg $HCO_3$ were added to achieve a pH 7.4. We refer to this modified medium as MJM.

### *2.1.3 Carbon/energy sources*

Bacteria were grown in ASW or MJM utilizing one of the following C/E sources: 25.0 mM arginine, 25.0 mM citrate, or 37.5 mM succinate. (Table 1).

### *2.1.4 Standard culture conditions*

An overnight culture grown in 3 ml of SWC was used to inoculate 300 ml of minimal medium contained in a 500 ml Erlenmeyer flask and incubated on a rotary shaker (40 rpm) at 20-22°C for 20 – 25 days under ambient laboratory illumination.

### *2.1.5 Modified culture conditions*

The effects of reduced nutrient and salt concentrations were tested by diluting ASW 1:2, 1:4, and 1:10 with $ddH_2O$. Temperature effects were investigated by growing bacteria as above but at 37, 25, 20, 18, or 15°C. All incubations were carried out on a rotary shaker at 40 rpm. To test the effects of microaerobic conditions, 70 ml of ASW in 75 ml test tubes were heated at 100°C for 15 min, inoculated with 3 ml culture, and tightly capped to minimize exposure to

oxygen. Other than the culture containers and volume of medium, incubations were carried as described in 2.1.4.

Bacteria were also grown under conditions identical to those previously reported to induce accumulation of saxitoxins by these two bacterial strains (Franca et al., 1996; Levasseur et al., 1996). Briefly, an overnight culture of *Pseudomonas stutzeri* (SF/PS) was used to inoculate ASW containing 50 mM HEPES pH 7.4 and 37.5 mM succinate. Cultures were grown in 500 ml flasks containing 250 ml of ASW and incubated at 20°C in reduced light with shaking (40 rpm), until the culture reached an $OD_{660}$ of 1.2-1.5 (Franca et al., 1996). An overnight culture of *Alteromonas/Pseudomonas* PTB-1 was added to 30 ml of ASW media with 37.5 mM succinate and incubated at 20°C with shaking (40 rpm) in the dark for three days (Levasseur et al., 1996).

## *2.2 Characterization of putatively toxic compounds*

### *2.2.1 Extraction*

Bacteria were harvested by centrifugation (16,000 *g* for 15 min). Both the supernatant (i.e., the spent culture medium) and the pellet were analyzed. The pellets were resuspended in 0.5N HOAc , whereas concentrated acetic acid was added to the supernatant to give a final concentration of 0.1N HOAc. Both supernatants and pellets were homogenized with a microtip sonic probe (10 min, 0°C, 50 W; Sonicator™ Ultrasonic Processor, Heat Systems, Ultrasonic, Inc.) and centrifuged at 16,000g, then the supernatant was passed through either a C18-10 SPE column (J.T. Baker Chemical Co.) or an Ultracent-10 filtration cartridge (BioRad); comparable results were obtained with both clean-up devices.

Toxin extraction protocols for experiments designed to specifically reproduce the results of Franca et al. (1996) and Levasseur et al. (1996) were carried out as above, with minor modifications. *Pseudomonas stutzeri* SF/PS was harvested by centrifugation at 9,000 *g* for 20 min and the homogenized cell pellet centrifuged at 2,000 *g* for 10 min before passage through Ultracent-10 filters (Franca et al., 1996). *Alteromonas/Pseudomonas* PTB-1 cells were harvested by centrifugation at 12,000 *g* for 20 min. The supernatants were adjusted to 0.1N HOAc, while the pellets were disrupted by sonication as described above. All cell pellet samples were adjusted to 0.1N HCl and heated at 100°C for 15 min before passage through Ultracent-10 filters.

The stability of the toxins and the interconversion of various saxitoxin congeners were tested after addition of 0.1 N HCl and heating at 100 °C for 10 min or by addition of 0.2 N NaOH and heating at 37°C for 3 hours (Boyer et al., 1986).

### *2.2.2 HPLC*

Supernatants and cell pellet extracts were resolved by post-column oxidation HPLC-FLD protocols of Oshima (1995). Briefly, we employed a C8-bonded silica gel column (Supelco LC-8 column; 25 cm x 4.6 mm, 5 μm), three isocratic mobile phases, and post column periodic acid oxidation. Fluorescent compounds were detected with either a SLM Aminco SPF-500C spectrofluorometer or an Agilent Technologies 1100 series FLD detector. Excitation for both detectors was at 330 nm, and emission was recorded at 390 nm, using slit widths of 20 nm. The fluorescence emission spectrum of each HPLC peak was recorded in separate runs with the Agilent Technologies FLD detector with excitation at 320 nm to minimize overlap with the scanned emission wavelengths. For electrochemical oxidation, the separation used an Inertsil (15 cm X 4.6 mm, 5 μm) column connected to a coulometic cell powered by an ESA Coulochem II detector operated at 900 mV. Peak signals were detected with a Shimadzu RF535 fluorescence detector at the same excitation and emission wavelengths as above. The putative PSP toxins were also monitored using direct electrochemical detection by measuring the output of the ESA analytical cell (Boyer et al., 1997; Boyer and Goddard, 1999). All standards for saxitoxin congeners were obtained from NRC-CNRC (Halifax, N.S., Canada) except for saxitoxin which was from RBI (Natick, MA).

### *2.2.3 $^{3}$H-Saxitoxin receptor binding assay*

$^{3}$H-Saxitoxin competitive binding assays were performed using rat brain synaptosomes as the source of toxin receptors. The bacterial supernatants and cell extracts served as the unlabeled competitor. Details of the receptor assay protocol are given by Doucette et al. (1997) and by Powell and Doucette (1999).

# 3. Results

## *3.1 HPLC-FLD*

The $GTX_{1,4}$ standard clearly showed the two epimers, $GTX_1$ and $GTX_4$, in the HPLC-FLD traces. The peak area of $GTX_1$ was approximately 3.5 times larger than that of the earlier eluting $GTX_4$ (Fig. 2 A, B). Treatment with NaOH and heat resulted in loss of HPLC-FLD signals from the $GTX_{1,4}$ standard (Fig. 2 C).

When *P. stutzeri* SF/PS was grown in either MJM or ASW with citrate as the C/E source or in SWC under microaerobic conditions, the spent medium yielded a peak during HPLC-FLD that eluted at a time similar to $GTX_4$. In Fig. 2, these HPLC peaks are labeled as $X_1$, $X_2$, and $X_3$, respectively (Fig. 2 D, H, L). Upon treatment with 0.1N HCl, the $X_1$ peak increased in size, there was no effect on $X_2$, and $X_3$ decreased slightly (Fig. 2 E, I, M, respectively). The compound eluting as $X_1$ was unaffected by NaOH/heat treatment (Fig. 2 F), whereas $X_2$ was no longer detectable (Fig. 2 J) and the signal from $X_3$ was decreased (Fig. 2 N) by the same treatment. Co-injection of these bacterial samples with the $GTX_{1,4}$ standard indicated that $X_1$ eluted just before $GTX_4$ (Fig. 2 G) and both $X_2$ and $X_3$ co-eluted with $GTX_4$ (Fig. 2 K and O). No peaks corresponding to $GTX_1$ were observed in these bacterial samples (Fig. 2 E, I, M).

Cell extracts of *P. stutzeri* SF/PS grown under standard culture conditions (2.1.4) yielded HPLC-FLD results that were generally consistent with those obtained from the spent culture medium shown in Fig. 2. The cell extracts generally had considerably larger HPLC-FLD peaks in the void volume and frequently had additional peaks eluting late in the run (data not shown). In no cases were we able to correlate any of these peaks to $GTX_4$ or any other saxitoxin for which we had reference standards available.

*P. stutzeri* SF/PS and *Alteromonas/Pseudomonas* PTB-1 were grown and toxin extracts prepared as previously described (Franca et al., 1996; Levasseur et al., 1996). Both extracts yielded a HPLC-FLD peak that was acid/heat stable and that eluted at the same time as $GTX_4$ (Figs. 3 D and G). The HPLC signal from these fluorescent compounds either increased or decreased slightly following NaOH/heat treatment ($X_4$ and $X_5$ in Fig. 3 E and H, respectively). The peaks were also observed without the post column oxidation reaction, though $X_4$ was reduced in size (Figs. 3 F and I). The $GTX_{1,4}$ standard also had two HPLC-FLD peaks without

post column oxidation, but the $GTX_4$ peak was considerably larger without oxidation (i.e., compare Fig. 3 C and Fig. 3 A). No peaks corresponding to $GTX_1$ were detected in the bacterial samples (Fig. 3 D - I).

The fluorescence emission spectra were recorded for the $GTX_{1,4}$ standard and the five bacterial fluorescent compounds, $X_1$-$X_5$ (Fig. 4). The emission spectrum of $GTX_4$ peaked at 396 nm, a wavelength similar to other saxitoxins (Lawrence et al., 1991). The emission spectra of $X_1$- $X_5$ were maximal at 418, 426, 416, 445, and 421 nm, respectively (Fig. 4).

### *3.2 HPLC-EC/FLD*

Bacterial samples were also analyzed by HPLC employing an electrochemical cell for post-column oxidation (HPLC-EC/FLD). Compounds that co-eluted at the same time as $GTX_4$ were not identified in any of the bacterial samples analyzed (Table 1), though several of the resulting HPLC traces had fluorescent peaks that eluted earlier than $GTX_4$. A peak that could be due to saxitoxin was found in one sample, while a possible $GTX_3$ signal, but no $GTX_2$ signal, was observed in two samples (Table 1).

### *3.3 Receptor binding assays*

The $^3$H-saxitoxin receptor binding assay provided evidence for saxitoxins in three bacterial samples (Table 1). However, when bacteria were grown under identical conditions and tested a second time, the signals were below the limits of detection and thus inconclusive.

## 4. Discussion

Several laboratories have provided evidence in support of the idea that bacteria accumulate PSP toxins. In this study, we found evidence for PSP toxins when bacteria were grown under specific growth conditions using the $^3$H-saxitoxin receptor binding assay. However, we were unable to correlate these positive results with HPLC chromatographic signals of known saxitoxin derivatives. Five different growth conditions induced bacteria to accumulate fluorescent compounds with characteristics similar to $GTX_4$. As explained below, each of the five bacterial

compounds differed from the others as well as from $GTX_4$. We concluded that each of the bacterial compounds was a different $GTX_4$ imposter. Our results are generally consistent with those of Sato and Shimizu (1998) who reported that a different fluorescent bacterial compound was an imposter that could be confused with neosaxitoxin, another derivative of saxitoxin.

The $GTX_{1,4}$ standard produced two HPLC-FLD peaks that were readily cleaved under basic conditions. These toxins were stable to mild acid hydrolysis, i.e. conditions that would cleave any N21-sulfo groups but would not cleave the 11-hydroxy sulfate linkage, and formed an epimeric mixture. The $GTX_4$ peak was approximately one fourth the size of the $GTX_1$ peak when post-column chemical oxidation was employed (Fig. 2 A, B) and the $GTX_4$ peak was substantially larger when the oxidation step was omitted (Fig. 3 C). These results are consistent with those previously reported by Onodera et al. (1996). This stability of the α-isomer ($GTX_1$) over the β-isomer ($GTX_4$) is in agreement with what has been observed by others (Laycock et al., 1997, Onodera et al., 1996, Boyer et al., 1978). This equilibrium ratio of $GTX_1$ and $GTX_4$ can be easily established by heating the toxins under acidic conditions. In contrast, when the putative $GTX_4$ peak, $X_1$, obtained from extracts of *P. stutzeri* SF/PS grown in MJM plus citrate, was heated in acid, it increased in size but no formation of the more stable $GTX_1$ was observed (Fig. 2 E). The increase in peak height could, in theory, be easily explained if the bacterial extract also contained $C_4$, one of the saxitoxin congeners (Fig. 1). Acid treatment of $C_4$ would result in the transformation of $C_4$ to $GTX_4$ by hydrolysis of the N21-sulfate, its corresponding carbamate form (Boyer et al., 1986). However, inspection of the data indicated that the sample did not contain either $C_3$ or $C_4$ when the HPLC-FLD was run using the C-toxin conditions of Oshima (1995; data not shown). The absence of a $GTX_1$ peak in the acid treated sample (Fig. 2 E) and the absence of $C_4$ in the native sample provided evidence that $X_1$ was not $GTX_4$. Further evidence that $X_1$ was not $GTX_4$ included: 1) $X_1$ was NaOH/heat stable but $GTX_4$ is heat labile (Fig. 2 F); 2) $X_1$ did not co-elute with $GTX_4$ upon co-injection with the standard (Fig. 2 G); 3) the fluorescence emission spectrum of $X_1$ differed from that of $GTX_4$ (Fig. 4); and 4) $X_1$ was not detectable by HPLC-ECOS (Table 1). Furthermore, samples containing $X_1$ did not consistently exhibit the presence of a compound(s) able to compete against $^3$H-saxitoxin in the receptor binding assay (Table 1). Taken together, these results, summarized in Table 2, allow us to conclude that $X_1$ was a $GTX_4$ imposter.

We also concluded that $X_2$, $X_3$, $X_4$, and $X_5$ were $GTX_4$ imposters based on similar evidence. Although each of these four bacterial compounds shared many properties with $GTX_4$ (Table 2), none of them had the expected epimeric partner, $GTX_1$, and the fluorescence emission spectrum of each was unique and different from $GTX_4$. The compound identified as $X_2$ was the only one of these four that was not stable under NaOH/heat treatment, a property shared with $GTX_4$ (Table 2).

Based on the various properties of $X_1 - X_5$ (Table 2), it is clear that each of these five fluorescent compounds is unique. We have no firm chemical data on the identity of any of the five compounds. In our early work, we suspected that iron-scavenging siderophores were present in these samples, especially when citrate, a known iron chelator, was the C/E source. However, subsequent experiments (Baker, 2001) with different levels of iron, iron chelators, and iron:chelator ratios convinced us that these fluorescent compounds were not siderophores.

In a larger, parallel study (Baker, 2001), *Pseudomonas stutzeri* SF/PS was grown under 76 different growth conditions and tested for toxin production. Most of the growth conditions used C/E sources involved in the urea and/or Kreb cycles. These compounds were tested because saxitoxin is synthesized from arginine and acetate (Shimizu et al., 1984) and we reasoned that these compounds should stimulate saxitoxin synthesis. Other growth conditions tested included combined C/E/N sources, again focusing on compounds such as arginine that might stimulate toxin production. Temperature and light intensity were also tested, as were the effects of growth under a variety of different nutrient-limited conditions (e.g., P-, N-, etc.). Of these 76 different growth conditions tested, only 15-20 conditions induced *P. stutzeri* SF/PS to synthesize a compound that co-eluted with $GTX_4$. However, in all cases except one, the putative $GTX_4$ peak was not accompanied by its expected epimeric partner, $GTX_1$. Growth of *P. stutzeri* SF/PS on ASW + succinate induced the accumulation of two compounds that could be mistaken for the $GTX_{1,4}$ epimeric pair. However, the fluorescence emission spectrum of the putative $GTX_4$ peaked at 450 nm, clearly distinct from $GTX_4$ (Table 2), while the putative $GTX_1$ compound peaked at 405 nm, somewhat red shifted from the $GTX_1$ signal at 396 nm (data not shown). In addition to the putative $GTX_{1,4}$ peaks, a number of different possible saxitoxin derivatives were identified by HPLC-FLD in samples taken from one or more of the 76 growth conditions tested (Baker, 2001). However, all of the compounds failed the relatively simple tests shown in Figs. 2 and 3, indicating they were also saxitoxin imposters.

Importantly, despite our inability to detect saxitoxin derivatives in this study, it is clear that under certain growth conditions, *Pseudomonas stutzeri* and *Pseudomonas/Alteromonas* PTB-1 accumulate compounds that could be associated with PSP events (Table 1; Lavesseur et al., 1996; Kodama et al., 1990; Doucette and Trick, 1995; Gallacher et al., 1997). These PSP toxins may, or may not, be members of the saxitoxin family. New saxitoxin congeners are being reported with regularity (e.g., Onodera et al., 1997) and it is possible that these bacteria primarily accumulate saxitoxin derivatives that are currently unknown. Alternatively, we may have failed to find the very specific combination of temperature, nutrients, pH, etc. that induces production of saxitoxin in *P. stutzeri* SF/PS and *Pseudomonas/Alteromonas* PTB-1. For instance, we noted that different C/E sources produced significantly different growth rates in our studies. Medium made with multiple C/E sources (e.g., SWC) supported rapid growth rates. Only when growth was slowed under microaerobic conditions in SWC (Fig. 2 L) was it possible to detect fluorescent compounds that could be confused with $GTX_4$ present in this medium. Given the variety of $GTX_4$ imposters identified in this study (Table 2), it is clear that other, as yet undetermined conditions, could induce the synthesis of authentic $GTX_4$ and/or other saxitoxin congeners.

We observed that each of the five bacterial compounds ($X_1 - X_5$) were present in the culture medium as well as inside the bacterial cells. Previous reports of bacterial PSP toxins also noted a dual localization, both extra and intracellular (Gallacher et al., 1997). We also note that authentic saxitoxins associated with Gram negative cyanobacteria were present both in the culture medium as well as inside the cell (Onodera et al., 1996, Baker, 2001).

There appears to be a significant increase in the global frequency and severity of harmful algal blooms (Smayda, 1992), including blooms of algae that produce saxitoxins, the causative agent of PSP. The role of bacteria in HAB events is exceedingly complex (Doucette, 1995; Doucette et al., 1998). The research reported here addresses only the issue of whether bacteria are capable of synthesizing of one of the PSP toxins, $GTX_4$. We cannot rule out the possibility that *P. stutzeri* SF/PS is capable of $GTX_4$ synthesis under specific growth conditions not tested herein (Franca et al., 1996); however, we do show that many compounds that sharing physical and chemical properties with $GTX_4$ are, in fact, imposters. Our results, along with those of Sato and Shimizu (1998), indicate that considerable care must be taken when describing toxin compounds based primarily, if not solely, on their HPLC-FLD characteristics.

**Acknowledgments**

The *P. stutzeri* SF/PS and *Alteromonas/Pseudomonas* PTB-1 cultures were kindly provided by Dr. S. Franca and M. Kodama, respectively. This publication is the result of research sponsored by Alaska Sea Grant with funds from the National Oceanic and Atmospheric Administration Office of Sea Grant, Department of Commerce, under grant number NA 86RG0050-R/95-01, and from the University of Alaska with funds appropriated by the state. We also acknowledge support from NOAA/National Ocean Service operational funds.

**Table 2-1.** Summary of toxin results obtained with *P. stutzeri* SF/PS and *Alteromonas/ Pseudomonas* PTB-1 cultured under different growth conditions. Toxins were analyzed by HPLC employing two different post-column oxidation methodologies and by the $^{3}$H-saxitoxin receptor binding assay.

| Bacterial Strain | Growth Condition | Medium | C/E Source | Sample Type | HPLC-FLD[a] | HPLC-EC[b] | RBA[c] |
|---|---|---|---|---|---|---|---|
| *Pseudomonas stutzeri* SF/PS | standard | ASW | citrate | SM[d] | ⊗[e] ($X_2$[f]) | ⊗ | 45[g] ⊗[h] |
| | | ASW | citrate | CE[i] | ⊗ | $GTX_3$?[j] | ND[k] |
| | | MJM | citrate | SM | ⊗ ($X_1$) | ND | ?[l] |
| | | ASW | succinate | CE | ⊗ | $GTX_3$? | ND |
| | | ASW | arginine | SM | ⊗ | ⊗ | ⊗ |
| | | SWC | yeast ext., peptone | SM | ⊗ ($X_3$) | ND | 28 ⊗ |
| | | FWC | yeast ext., peptone | SM | ⊗ | ND | 5 ⊗ |
| | modified, medium 1:10 | ASW/10 | citrate | SM | ⊗ | ND | ⊗ |
| | modified, 37°C | ASW | citrate | SM | ⊗ | ⊗ | ⊗ |
| | Franca et al. 1996 | ASW | succinate | CE | ⊗ ($X_4$) | STX? ⊗ | ND |
| Altermonas/Pseudmonas PTB-1 | Levasseur et al. 1996 | ASW | succinate | CE | ⊗ ($X_5$) | ⊗ | ND |

[a] toxins identified include $GTX_{1-4}$, $C_{1-4}$, Neo and STX
[b] toxins identified include $GTX_{1-4}$, Neo and STX ($C_{1-4}$ converted to GTX before assay)
[c] receptor binding assay; sensitivity is based on ability of toxins to displace $^{3}$H-saxitoxin
[d] SM, spent medium
[e] ⊗, no toxin detected
[f] $X_1$-$X_5$ refer to compounds in Figures 2 and 3
[g] results reported as nM saxitoxin equivalents per liter
[h] split column provides assay results from two independent analyses
[i] CE, cell extract
[j] ?, results inconclusive
[k] ND, not determined

**Table 2-2**. Summary of physical and chemical properties of $GTX_4$ and the five fluorescent bacterial compounds identified in Figures 1 and 2.

| Compound | Treatment/ Characteristics | | | | | |
|---|---|---|---|---|---|---|
| | Acid/heat stable | NaOH/heat labile | Elution with $GTX_4$ standard | HPLC-FLD signal enhanced without post column oxidation | Detection of epimeric pair after acid/heat | Fluorescence emission maximium (nm) |
| $GTX_4$ | √[a] | √ | √ | √ | √ | 396 |
| $X_1$[b] | √ | ⊗[c] | ⊗ | ND[d] | ⊗ | 418 |
| $X_2$ | √ | √ | √ | ⊗ | ⊗ | 426 |
| $X_3$ | √ | ⊗ | √ | ND | ⊗ | 416 |
| $X_4$ | √ | ⊗ | √ | √ | ⊗ | 445 |
| $X_5$ | √ | ⊗ | √ | ⊗ | ⊗ | 421 |

[a] √-compound exhibited properties described
[b] $X_1$-$X_5$ refer to compounds in Figures 2 and 3
[c] ⊗, no toxin detected
[d] ND, not determined

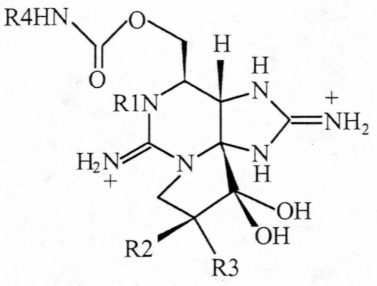
Figure Legends

**Figure 2-1.** Structures of saxitoxin derivatives.

**Figure 2-2.** HPLC-FCD chromatograms of the $GTX_{1,4}$ standard (A-C) and the spent culture medium of *P. stutzeri* SF/PS grown in MJM + citrate (D-G), SWC + citrate (H-K), or SWC under microaerobic conditions (L-O). Samples were processed as described in Materials and Methods and injected without further treatment (A, D, H, L), or after treatment with 0.1 N HCl/heat (B, E, I, M), 0.2 N NaOH/heat (C, F, J, N). Bacterial samples were also co-injected with the $GTX_{1,4}$ standard (G, K, O).

**Figure 2-3.** HPLC-FCD chromatograms of the $GTX_{1,4}$ standard (A-C) and the cell extracts of *P. stutzeri* SF/PS (D - F) and *Alteromonas/Pseudomonas* PTB-1 (G-I) grown as previously described (Franca, et al., 1996; Levasseur et al., 1996, respectively). Samples were processed as described in Materials and Methods and injected after treatment with 0.1 N HCl/heat (A, D, G) or 0.2 N NaOH/heat (B, E, H). Samples were also analyzed without post-column oxidation (C, F, I).

**Figure 2-4.** Fluorescence emission spectra of $GTX_4$ and the five bacterial compounds, $X_1 - X_5$, identified in Figures 2 and 3. Each spectrum is normalized to full scale at its emission maximum.




| Name | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Saxitoxin | H | H | H | H |
| Neosaxitoxin | OH | H | H | H |
| $GTX_1$ | OH | $OSO_3^-$ | H | H |
| $GTX_2$ | H | $OSO_3^-$ | H | H |
| $GTX_3$ | H | H | $OSO_3^-$ | H |
| $GTX_4$ | OH | H | $OSO_3^-$ | H |
| $C_1$ | H | $OSO_3^-$ | H | $SO_3^-$ |
| $C_2$ | H | H | $OSO_3^-$ | $SO_3^-$ |
| $C_3$ | OH | $OSO_3^-$ | H | $SO_3^-$ |
| $C_4$ | OH | H | $OSO_3^-$ | $SO_3^-$ |

**Figure 2-1**

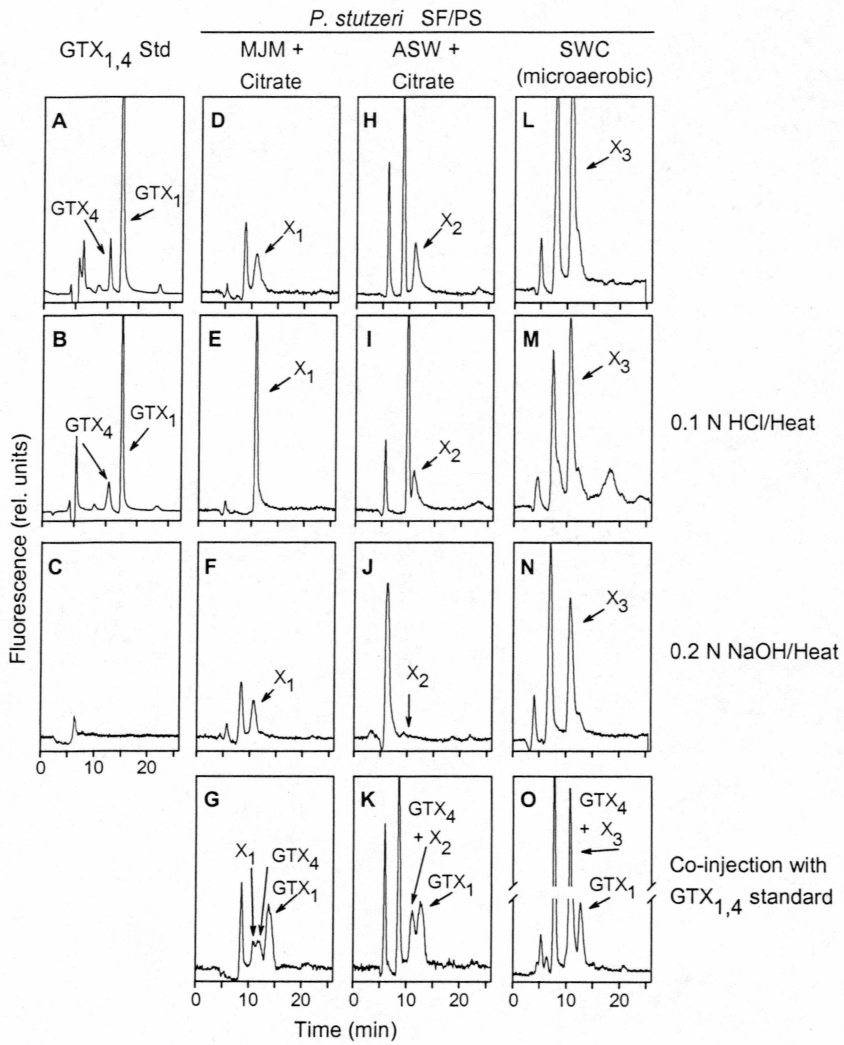

P. stutzeri SF/PS
GTX1,4 Std
MJM + Citrate
ASW + Citrate
SWC (microaerobic)
A
D
H
L
B
E
I
M
C
F
J
N
G
K
O
GTX4
GTX1
X1
X2
X3
+ X2
+ X3
0.1 N HCl/Heat
0.2 N NaOH/Heat
Co-injection with GTX1,4 standard
Fluorescence (rel. units)
Time (min)
0
10
20

**Figure 2-2**

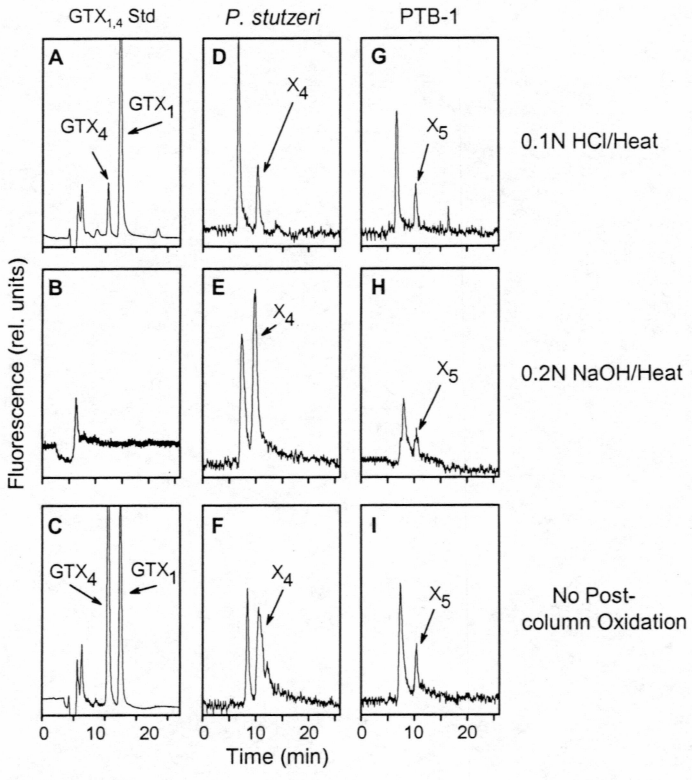

GTX1,4 Std
P. stutzeri
PTB-1
A
D
G
GTX4
GTX1
X4
X5
0.1N HCl/Heat
B
E
H
X4
X5
0.2N NaOH/Heat
C
F
I
GTX4
GTX1
X4
X5
No Post-column Oxidation
Fluorescence (rel. units)
0
10
20
Time (min)

**Figure 2-3**

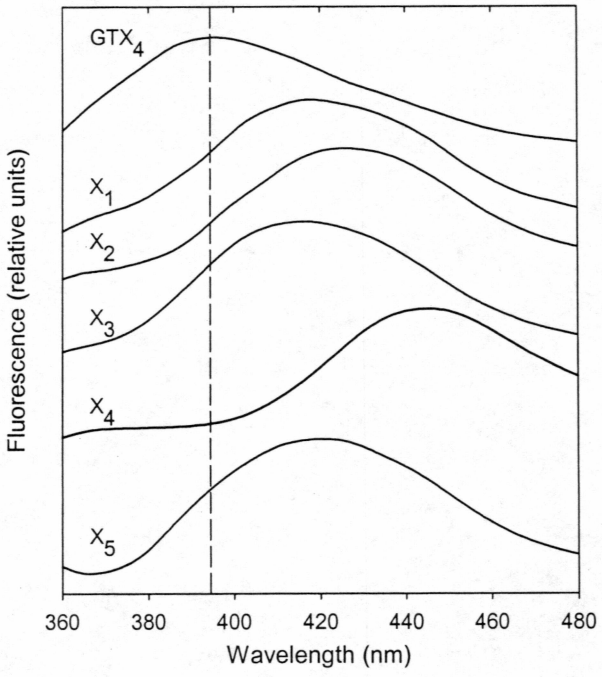

GTX4
X1
X2
X3
X4
X5
Fluorescence (relative units)
360
380
400
420
440
460
480
Wavelength (nm)

**Figure 2-4**

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# Saxitoxin Accumulation in *Aphanizomenon flos-aquae* as Affected by Nitrogen Source, Temperature, and Light Intensity

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Abstract

The effects of growth conditions on saxitoxin accumulation were examined in the cyanobacterium, *Aphanizomenon flos-aquae*. Maximum toxin levels (per ml) were observed when cultures were grown under a combination of high light (120 μ Ein/$m^2$/sec) and high temperature (26°C). Reduced light intensity (to 73 μ Ein/$m^2$/sec) *or* temperature (to 20°C) caused toxin levels to decline to the same low levels caused by reduced light *and* temperature. Toxin levels were higher when either $N_2$-N or ($NH_4^+$ + $NO_3^-$)-N were the sources of cellular nitrogen and decreased dramatically when urea-N was added to the growth medium. The molar ratio of saxitoxin to neosaxitoxin was also affected by light intensity, temperature, and nitrogen source. Neosaxitoxin was always more abundant than saxitoxin, but saxitoxin ranged from 10-35% of the total toxin under different growth conditions. Significant amounts of toxin were present in the extracellular toxin pool under virtually all conditions tested, suggesting that release of toxin from the cells may provide a competitive advantage to this microorganism in its natural environment. The results obtained in this study are similar to those obtained with other saxitoxin-producing cyanobacteria, but significant differences were observed, suggesting that there may be considerable variation in the physiological ecology of different toxic cyanobacteria. These results also indicate little competition between the two high-energy pathways of $N_2$ fixation and saxitoxin synthesis. Urea, a potential substrate in the saxitoxin biosynthetic pathway, unexpectedly inhibits toxin accumulation.

Note: This manuscript will be submitted for publication as part of the symposium proceedings of the next International Conference on Toxic Marine Phytoplankton.

**Introduction**

Over the last century, harmful algal blooms (HABs) have become an increasingly persistent problem in coastal regions as well as in fresh water lakes and rivers around the world (Anderson 1989; Hallegraeff 1993; Carmichael 1997). Algal blooms have had deleterious effects on human health and the success of fisheries. Toxin production is associated with many, but not all, types of HAB events. One of the most widely distributed toxic HAB syndromes is caused by saxitoxin, the etiological agent of paralytic shellfish poisoning (PSP). Saxitoxins are potent neurotoxins that bind to sodium channels, stopping nerve and muscle cell impulses and causing numbness, paralysis, disorientation and death in humans (Mosher et al. 1964).

Saxitoxins are produced by both marine dinoflagellates and freshwater cyanobacteria. The first reports of PSP toxins in cyanobacteria were from ponds in New Hampshire (Sawyer 1968). The PSP toxins in these ponds were produced by a cyanobacterium, *Aphanizomenon flos-aquae*, and resembled saxitoxins in their ability to inhibit membrane excitability without affecting the transmembrane resting potential. The toxins were subsequently purified and confirmed to be saxitoxins by testing them on other organisms (Jakim and Gentile 1968), by examining transmembrane potentials and membrane resistance (Adelman et al. 1982), by HPLC (Alam et al. 1978) and, most conclusively, by NMR (Shimizu et al. 1984).

The competitive advantage(s) and biochemical mechanisms of algal synthesis of saxitoxins are not well understood. Most workers have approached these problems by examining the physiological ecology of toxic algae and ascertaining the effects of environmental growth conditions on toxin production. For instance, saxitoxin accumulation and growth rates of dinoflagellates vary with environmental factors including temperature (Hall 1982; Ogata et al. 1987; Anderson et al. 1990), salinity (White 1978; Parkhill et al. 1999), nutrient limitation (Hall 1982; Boyer et al. 1987; Anderson et al. 1990; Flynn et al. 1995), and light intensity (Ogata et al. 1987). In contrast to the numerous studies conducted on the physiological ecology of saxitoxin-producing dinoflagellates, few studies have examined the role of environmental variables on the accumulation of saxitoxins in cyanobacteria. Saxitoxin accumulation in *A. flos-aquae* NH-5 was shown to be inversely related to growth rates, when growth was decreased as a consequence of sub-optimal low temperature, low light, and either P or N limitation (Ibrahim 1990). Another PSP toxin producing cyanobacteriun, *Lyngbya wollei*, exhibited reduced toxin accumulation when the temperature increased or decreased from optimal (26°C; Yin et al. 1997). High toxicity

and biomass were observed when *L. wollei* was grown with low phosphorus and nitrate and high calcium levels (Yin et al. 1997). In this study, we examine the effects of different N sources at two light intensities and two temperatures on the accumulation of saxitoxin, for the first time in the NH-1 strain of *Aphanizomenon flos-aquae.*

**Materials and Methods**

Cyanobacterial strain: *Aphanizomenon flos-aquae* NH-1 was obtained from Dr. Y. Shimizu (Univ. Rhode Island) and maintained in MJM.

Growth medium: Cultures were grown in Jaworski medium (JM) modified in our lab to contain 20 mg $Ca(NO_3)_2$-$4H_2O$, 6.2 mg $KH_2PO_4$, 50 mg $MgSO_4$-$7H_2O$, 7.3 mg EDTA, 2.4 mg $FeCl_3$-$6H_20$, 2.4 mg $H_3BO_3$, 1.3 mg $MnCl_2$-$4H_2O$, 1.0 mg $(NH_4)_6Mo_7O_{24}$-$4H_2O$, 40 µg cyanocobalamine ($B_{12}$), 40 µg thiamine HCl ($B_1$), 40 µg biotin, 80 mg $NaNO_3$, 14.2 mg $Na_2HPO_4$ per liter deionized distilled water ($ddH_2O$). After autoclaving, 61 mg $HCO_3$ was added to reach pH 7.4. We refer to this modified version of JM as MJM.

Culture conditions: *Aphanizomenon flos-aquae* was grown in MJM either without combined N in the medium (refered as $N_2$ -N and assumed to be N fixing conditions) or with N supplied at: 0.5 mM $NO_3^-$ + 0.5 mM $NH_4^+$; 1.0mM $NO_3^-$; 1.0 mM $NH_4^+$; or 1.0 mM urea. Replicate cultures were grown under conditions of high or low light intensities (HL= 120 µ Ein/$m^2$/sec; LL = 73 µEin/$m^2$/sec) at either a high or low temperature (HT = 26°C; LT = 20°C).

Starter cultures were grown in MJM to mid log phase. Experimental cultures were initiated by addition of 3 ml of starter culture to 300 ml of MJM (with different N sources) in 500 ml flasks. Cultures were grown without aeration or shaking. Various time points were sampled throughout the growth period. Only samples from day 68 are shown. The experiment was repeated two times, with only the results from the second experiment shown. Results between the two experiments demonstrated the same general trends in toxin accumulation.

Toxin Extractions: Five milliliters of culture were centrifuged (32,000 g, 10 min.). The supernatant (i.e., spent culture medium) was brought to pH 4 with HOAc. The cell pellet was resuspended in 1.0 ml of 0.1N HOAc and toxins were released by three cycles of freezing (-20°C) and thawing (22°C). Cell debris was removed by centrifugation (14,000 rpm for 10 min).

HPLC Analyses: The spent culture medium, containing extracellular toxins, and the cell extracts, containing the intracellular toxins, were analyzed by HPLC employing post-column oxidation methods with fluorescence detection (Oshima 1995). A C8-bonded Silicagel column (Supelco LC-8 column; 25 cm x 4.6 mm, 5 μm) was employed with three isocratic mobile phases, and post column periodic acid oxidation. Fluorescent compounds were detected with an Agilent Technologies 1100 series FLD detector. Excitation was at 330 nm, emission was recorded at 390 nm, slit widths were 20 nm. The neosaxitoxin standard was from NRC-CNRC (Halifax, N.S., Canada). Saxitoxin levels were calculated based on the neosaxitoxin standard, assuming a differential sensitivity of 1.1:4 (Lawrence et al. 1991), a ratio consistent with our preliminary analysis of a saxitoxin sample from RBI (Natick, MA).

**Results**

Intracellular levels of saxitoxin and neosaxitoxin accumulation were maximal in this study when *A. flos-aquae* was grown under a HL/HT regime on a combination of N sources, ($NH_4^+ + NO_3^-$)-N (Figure 1A). Growth under the same HL/HT regime, but with $N_2$-N, resulted in reduced levels of intracellular toxins. The presence of urea in the growth medium further decreased toxin levels (Figure 1A), especially under the LL/HT conditions (Figure 1B). Growth under either LL or LT conditions resulted in decreased intracellular toxin levels relative to HL/HT regardless of the N source (i.e., compare Figure 1B and C, with Figure 1A). Toxin levels following growth under LL/LT conditions (Figure 1D) did not reduce toxin levels more than growth under conditions where only light (Figure 1B) or temperature (Figure 1C) was reduced. Extracellular levels of toxins (Figure 2) followed the same general trend as intracellular toxins (Figure 1) in relation to light intensity, temperature, and N source. The extracellular toxin pool was generally larger than the intracellular toxin pool regardless of growth conditions or N source (compare Figure 1 A, B, C and D with same panel in Figure 2). The stability of extracellular toxins would be expected to vary as a function of pH and temperature. However, within treatments, temperature was held constant and should not differentially affect extracellular toxin stability. Similarly, a pH of 8.3 to 8.5 was observed in the culture medium of all growth conditions tested after the first few days of growth, indicating that pH effects on toxin stability were minimal.

Neosaxitoxin was the most abundant toxin accumulated regardless of light intensity, temperature, or N source. There were, however, substantial variations in the saxitoxin:neosaxitoxin ratio under different growth conditions. For instance, cultures grown under HL/HT with $N_2$-N accumulated approximately 25% and 35% of their intracellular (Figure 1A) and extracellular (Figure 2A) toxin as saxitoxin, respectively. In contrast, only about 10-15% of the toxin accumulated under HL/LT with $N_2$-N was in the form of saxitoxin in both the intracellular (Figure 1B) and extracellular (Figure 2B) pools. No saxitoxin derivatives other than saxitoxin and neosaxitoxin were detectable under any of the growth conditions examined. The total toxin pools, the combination of the intracellular and extracellular pools, were greater under HT/HL conditions with ($NH_4^+ + NO_3^-$)-N (Figure 3A). Growth on $N_2$-N reduced toxin accumulation by approximately 33% whereas urea-N decreased total toxin levels by > 90% (Figure 3A). Growth under LT, LL, or a combination of LT/LL reduced total toxin levels relative to HL/HT, regardless of the N source (Figure 3 B, C, D). Growth in the presence of urea resulted in the lowest levels of total toxin accumulation, regardless of light intensity or temperature.

**Discussion**

Growth of *Aphanizomenon flos-aquae* with different N sources substantially affected accumulation of saxitoxin, both in the intracellular and the extracellular toxin pools. Nitrogen fixation did not result in substantial reductions in toxin accumulation under most growth conditions relative to growth on ($NH_4 + NO_3$)-N. In contrast, addition of urea-N resulted in reduced toxin pools. Optimal toxin production was observed under higher light intensities and higher temperatures. Reduction of either the light intensity or the temperature reduced toxin levels to the same extent as reductions in both light and temperature. Together, these results indicate that this cyanobacterium is well adapted to N fixation as a mode of obtaining its N quota and that there is little, if any, competition for high-energy anabolic metabolites between the two high-energy pathways of N fixation and saxitoxin synthesis. The cellular mechanism(s) underlying reduced toxin levels when cells are grown on urea-N remain(s) enigmatic. It should also be noted that N fixation in *A. flos-aquae* occurs at low oxygen concentrations, which could also be affecting saxitoxin production.

Maximal toxin production was recorded in this study at 120 μEin/$m^2$/sec and 26°C. This temperature optimum, 26°C, is roughly comparable to that observed in two other saxitoxin-producing cyanobacteria, the NH-5 strain of *Aphanizomenon flos-aquae* (Ibrahim 1990) and *Lyngbya wollei* (Yin et al. 1997). The light intensity of maximal toxin production in this study, 120 μEin/$m^2$/sec, is considerably higher than that reported for the other two toxic cyanobacteria. *Lyngbya wollei* accumulated saxitoxins optimally at either 11 or 22 μEin/$m^2$/sec (Yin et al. 1997) while the NH-5 strain of *Aphanizomenon flos-aquae* accumulated toxin maximally at 20 μEin/$m^2$/sec (Ibrahim 1990). Mahmood and Carmichael (1986) commented about the slow growth of NH-1 relative to NH-5 but it is unlikely that high light intensities were tested in this earlier study because cyanobacteria generally grow optimally under very low light intensities. Our results are consistent with those of Mahmood and Carmichael (1986) and indicate that different ecotypes of *Aphanizomenon flos-aquae* may be present in nature. We suggest that these different ecotypes flourish under different environmental growth conditions and/or at different stages of a bloom. Similar "bloom stage-specific ecotypes" have been reported previously in diatoms (Gallagher and Alberte 1985).

In this report, toxin levels were presented for *Aphanizomenon flos-aquae* after 68 days of growth under 12 different growth conditions. Cultures were also analyzed for toxins at 15, 28 and 52 days; toxins were always present at much lower levels. These results clearly indicate that toxin levels continued to increase with time, long after the cultures stopped growing (at approximately three weeks). The production of toxin during the stationary phase of growth suggests these compounds are not required for growth, but that they more likely play a role as anti-predatory compounds. It seems unlikely that saxitoxins play a role as N storage compounds, though this is possible. For instance, N fixation and photosynthetic $CO_2$ reduction could both be high during stationary phase, especially under HL/HT conditions. Toxin synthesis could represent a "safety valve" allowing for dissipation of light energy when other factors (e.g., P or Fe) limit growth.

Bacteria likely affected toxin levels in these experiments. The NH-1 culture of *Aphanizomenon flos-aquae* is not axenic. Repeated attempts to make it axenic have not been successful. Axenic strains of other saxitoxin-producing cyanobacteria "cannot" be obtained (pers. comm. J. Boyer, W. Carmichael, B. Neiland). When the NH-1 strain was plated on nutrient-rich agar plates (YTG; yeast extract, tryptone, glucose), 13 unique bacterial colony types

were observed (note, different colony types were defined as those with unique pigmentation and/or growth characteristics). Contaminating bacteria can be reduced by growth under different conditions and cultures have been obtained with only two detectable types of bacteria. For the experiments shown, there were approximately 10 different types of bacteria present in the starter culture. Importantly, the bacterial populations (in culturable cells/ml) were approximately the same under the 12 different culture conditions, but the abundance of different bacterial types varied by more than three orders of magnitude under the different growth conditions. For instance, small bacteria colonies that were white, pink and yellow tend to predominate in cultures grown with urea-N while large and small colonies that were white, orange, red, and beige predominate with $N_2$-N as the N source (data not shown). The effects of bacteria, both direct and indirect, on toxin production are well documented (Doucette 1995) and likely were an important variable in this study.

At the 68 day time point, all cultures had a similar outward appearance, as judged by pigmentation and cell densities. However, during the first few weeks of this experiment, different culture conditions resulted in distinctly different pigmentation patterns in this cyanobacterium. Cells grown on urea-N were distinctly blue, indicative of high levels of phycocyanin and/or allophycocyanin, the two blue pigments responsible for the "blue" coloration of blue-green algae. These two pigments have been proposed as N storage depots in cyanobacteria, suggesting that urea-N stimulates N storage. After a few weeks of growth on urea-N, the blue pigmentation diminished and cultures grown on urea-N were indistinguishable from those grown on other N sources. Toxin levels in cultures grown with urea-N were low at the early stage of the experiment (when cultures were blue) and remained low, even after the putative N storage compounds (the phycocyanins) were diminished. We are intrigued by the possible interplay between phycocyanin and toxin accumulation as affected by urea-N. However, too little is known about the pathway of saxitoxin synthesis to advance concrete hypotheses about the molecular effects of urea on toxin production. More studies are needed to identify the "saxitoxin genes" and subsequent studies are needed that make use of the saxitoxin gene probes to ascertain how environmental variables, including exogenous N sources, affect the flow of carbon and nitrogen into the saxitoxin biosynthetic pathway.

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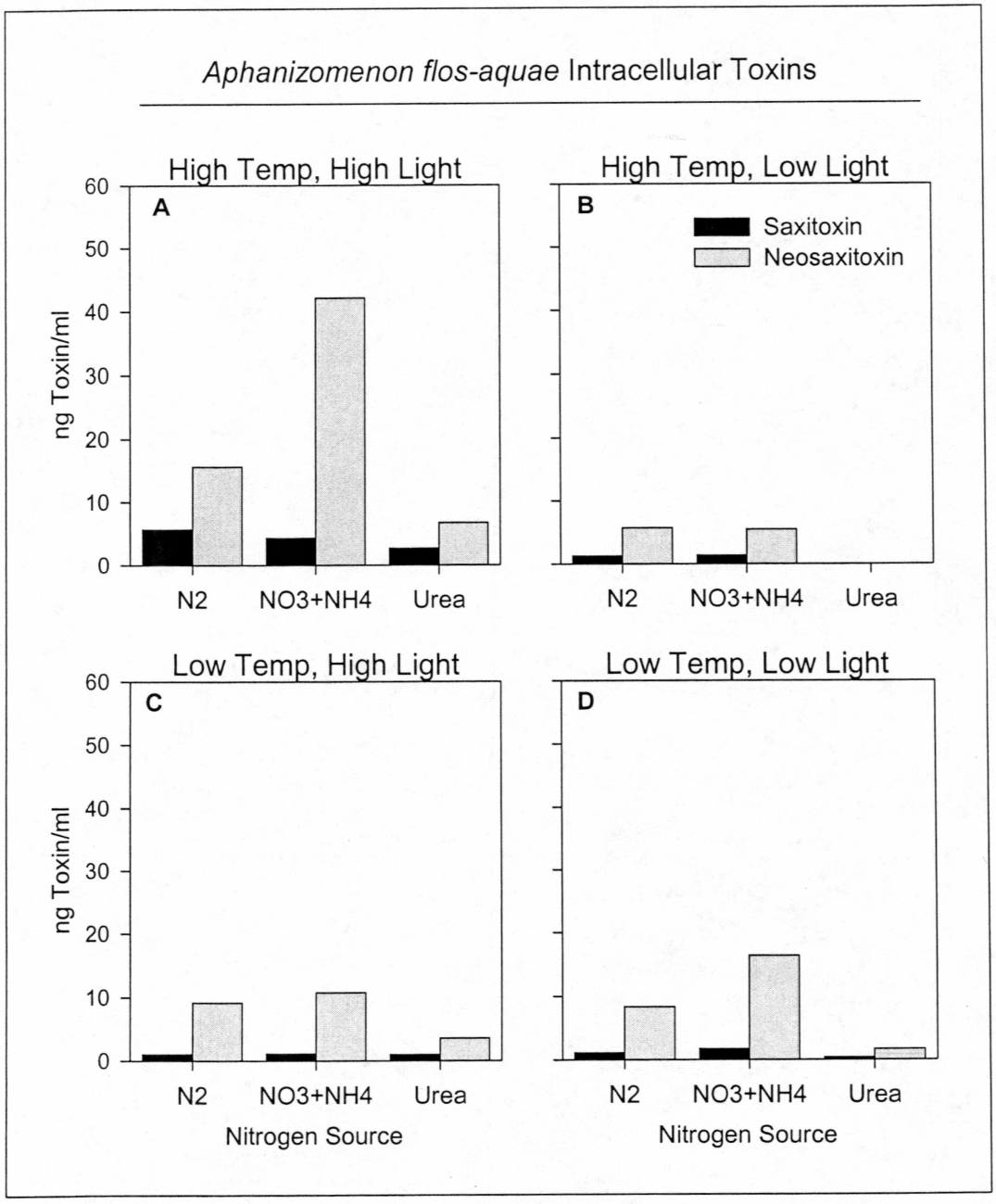


Figure 3-1. Effects of nitrogen source on intracellular toxin accumulation in *Aphanizomenon flos-aquae* grown under high and low light intensities and at high and low temperature. The nitrogen sources were: nitrogen fixation (N2), nitrate + ammonium (NO3+NH4), and urea. Light and temperature conditions are described above each panel, A-D.

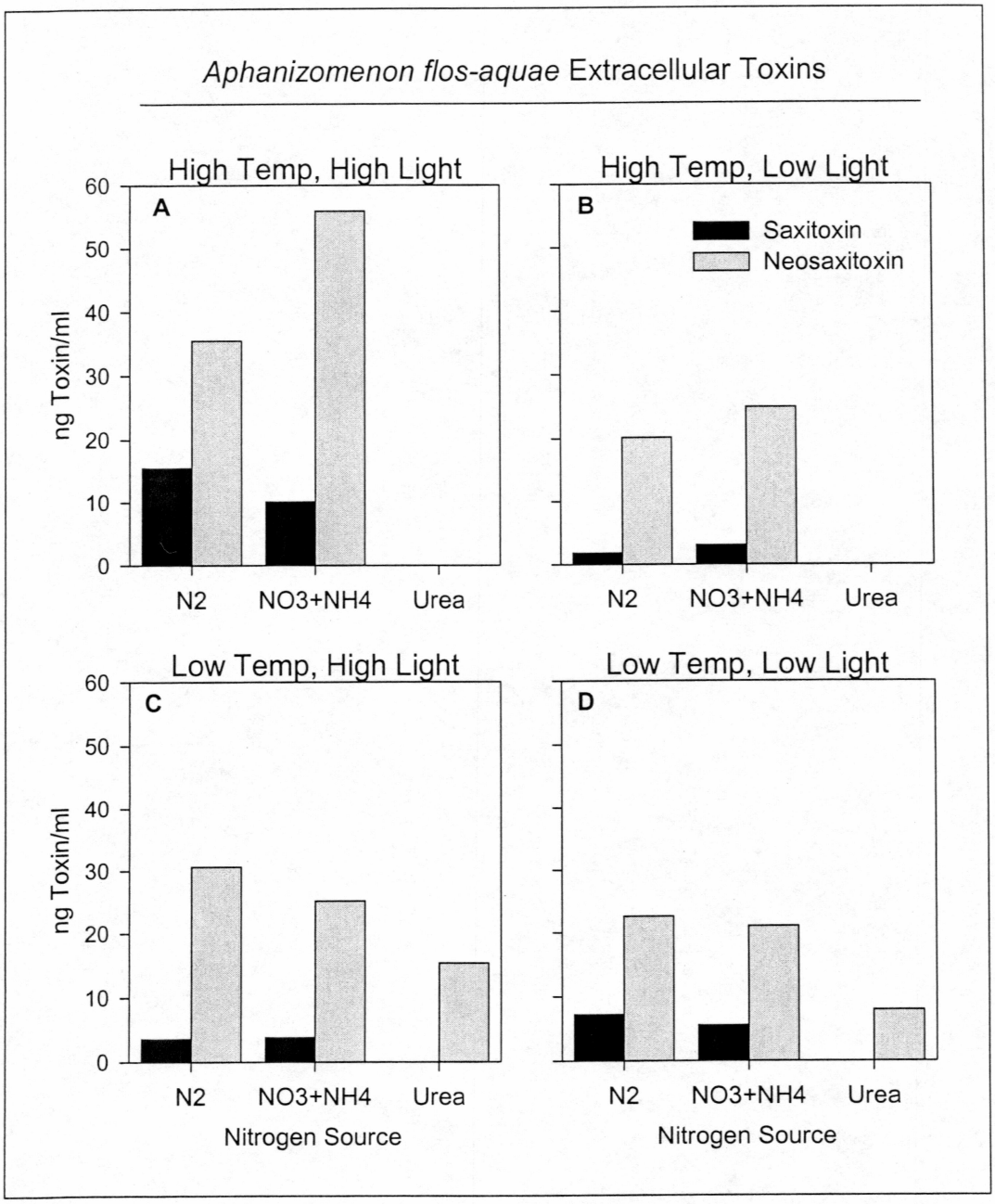


Figure 3-2. Effects of nitrogen source on extracellular toxin accumulation in *Aphanizomenon flos-aquae* grown under high and low light intensities and at high and low temperature. Labels are as in Figure 1.

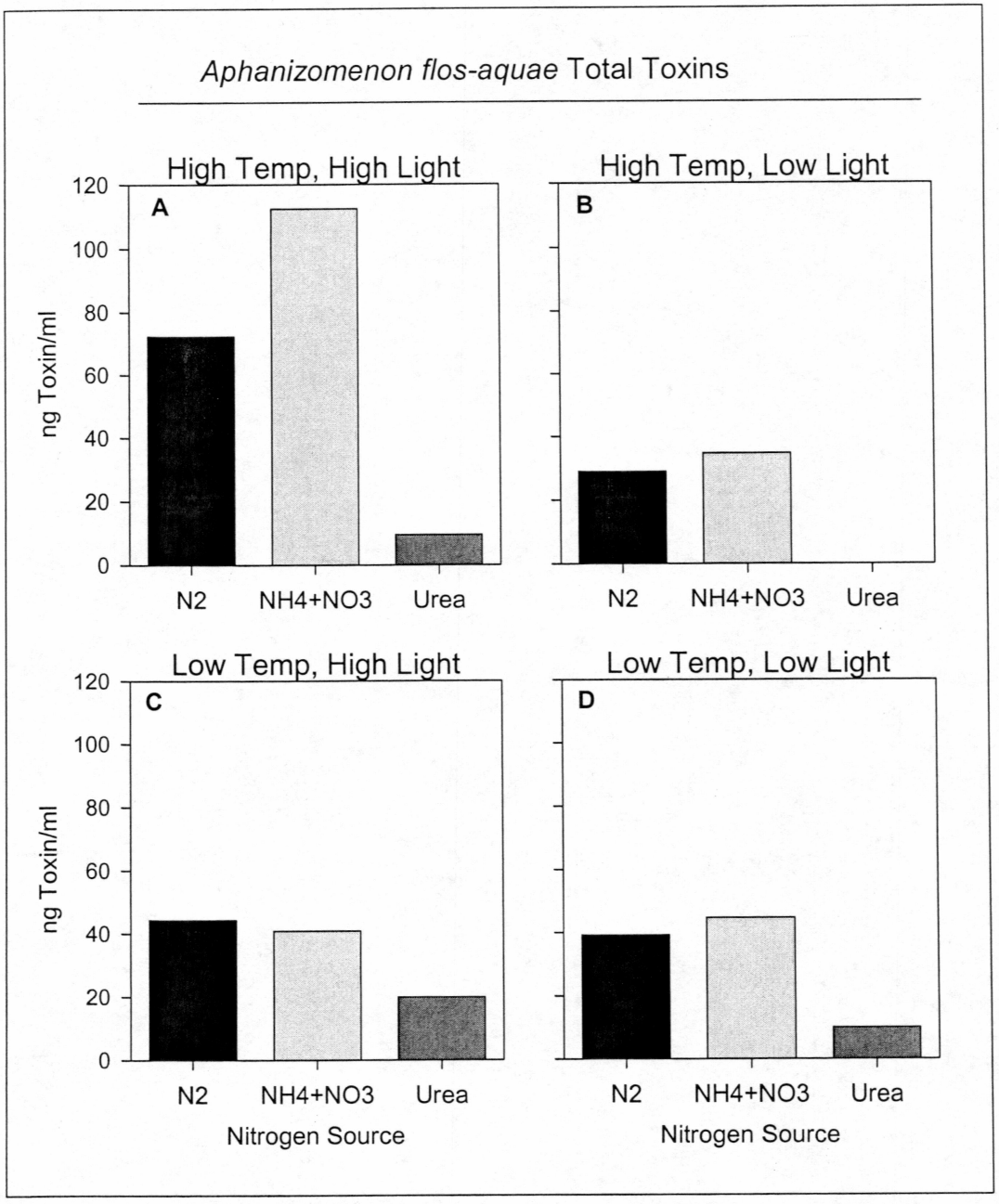


Figure 3-3. Effects of nitrogen source on total toxin accumulation in *Aphanizomenon flos-aquae* grown under high and low light intensities and at high and low temperature. Total toxin is defined as the sum of saxitoxin and neosaxitoxin in the intracellular and extracellular pools. Labels are as in Figure 1.

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