

Development of Microfluidic Technologies for the Detection and Quantification of Marine Biotoxins

A Final Report Submitted to

**The NOAA/UNH Cooperative Institute for Coastal and Estuarine
Environmental Technology (CICEET)**

Submitted by

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1. Expanded Executive Summary and Key Findings

(Maximum three pages) Please be brief and concise in addressing the following questions.

- a) What is the coastal resource issue the project sought to address?
This project addresses: Marine biotoxins resulting from harmful algal blooms
- b) What is the tool? *Automated Microfluidic Platform for Harmful Algal Bloom Toxin Detection which employs electrophoretic separations combined with laser induced fluorescence detection for the identification and quantification of marine toxins from sea water or phytoplankton. The detection platform will ultimately be combined with automated sample preparation to produce a stand alone detection platform for monitoring toxins in seawater or phytoplankton samples.*
- c) How does it address the problem? *By looking for the toxin in the algal mass or the associated seawater itself, rather than waiting for it to bioaccumulate in the shellfish. Rapid and sensitive detection of marine toxins in seawater or phytoplankton will allow much earlier identification of harmful algal bloom generation. In addition to aiding shellfish and marine mammal safety, earlier or more sensitive detection of the onset of toxin production may ultimately shed light on some of the environmental triggers of HAB development.*
- d) How is it an improvement over existing tools? *This approach has the potential to be much more rapid and sensitive than traditional methodology, replacing the standard mouse bioassay and the attendant issues with animal testing. It also eliminates the time lag associated with the bioaccumulation of various toxins in the shellfish, enabling* Moreover, *in conjunction with systems engineering and microfluidic capabilities of Sandia, it has potential for miniaturization and unattended operation, eliminating the need for skilled users and enabling the field deployment at remote or dockside/shipboard or coastal locations.*
- e) What is the current stage of development (bench, lab, field, prototype)? *While the specific methods development associated with the marine toxins is at the preliminary bench stage/proof of concept, requiring further validation (especially in real sample matrices) and protocol optimization, the bulk of the enabling technology is at a field prototype level. This includes the various microfluidic and control components associated with the sample preparation and toxin detection.*
- f) Describe any regulatory barriers to application? *N/A*
- g) Who are the end user groups for this tool? *State and local fisheries managers and HAB researchers*

Key Findings

The purpose of this section is to directly compare your tool to existing methods. This section was developed with feedback from coastal managers. Please format in a bulleted list.

Compare the performance of the tool to existing methods in terms of the following:

- a) *Cost. The primary costs associated with the technology will be in initial outlay for the platform itself. The platform, which is currently envisioned as a stand alone, portable, automated or semi-automated (depending on the needs of the specific end-users) will include all the hardware needed to take and prepare a sample from seawater or a suspension of algal mass and detect the marine toxins present. No expensive or specialty reagents are needed (eg. monoclonal antibodies). The only consumables, beyond standard electrical power (running off batteries is possible as well) will be a few cents worth of buffers and reagents per assay.*
- b) *Maintenance requirements. Since the system will be automated, little routine maintenance is anticipated, though replenishment of fluids will be needed from time to time. This interval will depend on the frequency of measurements but analogous systems built for the water utilities are able to carry enough fluid on board to easily run for a month of testing at 30 minute intervals. Given the very small consumption for individual analyses (few microliters), fluid reservoirs for up to 6 months operation should also be possible).*
- c) *Accuracy. TBD*
- d) *Speed. It is anticipated that cycle time (sampling- to-analysis) should be achieved in 45 minutes or less.*
- e) *Ease of use. The associated automation should make the system extremely easy to use, “pre-loaded methods in a computer/laptop/PDA controlled device.*
- f) *End user capacity requirements (supplies, skills, hardware). We anticipate essentially no specialized skills or equipment will be required. Depending on the ultimate application space, the user might be required to simply supply a sample of seawater or a suspension of algal mass to the instrument, either poured into a reservoir or possibly introduced into a port through a simple syringe.*
- g) *If the tool is knowledge, describe the advancement of science over current level.*

2. Project Development

a) Abstract

(Maximum one page) Summarize the project. Include a problem statement, project rationale, objectives, methods and results. Include a brief statement on the management implications of the project results.

Global increase in occurrence of harmful algal blooms (HABs) and their associated marine biotoxins has led to a higher frequency of seafood-borne illnesses and, ultimately, the implementation or expansion of coastal monitoring programs. Because of greater demand in recent years, several reports have articulated the need for rapid and automated methods of detection and measurement of marine biotoxins for

research and monitoring purposes. In response, the goals of this project are to develop microfluidic analysis techniques for rapid (minutes), sensitive (pg/injection) and high-resolution separation and quantification of algal toxins. These techniques can ultimately be incorporated into field portable and automated microfluidic devices, with follow-on funding.

Under this preliminary one year effort, work centered on the development of methods for the measurement of domoic acid and saxitoxins, but could eventually be extended to the analysis of other toxins such as brevetoxins and okadaic acid. The approach consisted of capillary- & microchip-based electrokinetic separations. Capillary zone electrophoresis (CZE) was used to separate analytes based on the charge/mass ratio. Methods were also developed to label the toxins for fluorescent detection and lyse the cells and extract toxins in a fashion compatible with subsequent labeling and electrophoretic separations.

b) Introduction

This technology will significantly reduce the time of analysis as compared to conventional assays. This would allow researchers to take multiple rapid field measurements resulting in a finer mapping of toxin concentrations on both temporal and spatial scales. High sensitivity will allow early warning of the presence of toxin in the lower trophic levels of the food chain and before high levels are reached in shellfish and effects are manifest in the human and animal populations. This project will benefit the marine and estuarine scientists, environmental and wildlife protection agencies, and, potentially, government public health agencies

c) Objectives

The objectives for this one year proof-of-concept effort were four-fold:

- 1. Optimize sample preparation techniques; steps to include dewatering, lysis, solid phase extraction and fluorescent labeling schemes*
- 2. Develop CZE separation methods in capillaries and/or microfluidic chips for biotoxin analysis using UV-absorbance and/or laser-induced fluorescence detection*
- 3. Obtain cultured, natural, and spiked samples of toxigenic phytoplankton (Dr. Anderson) or toxin spiked seawater samples and analyze for marine biotoxins in capillaries and/or microchips*
- 4. Compare detection limits, speed of assay, and multianalyte detection capability with conventional HPLC-based methods (carried out in Dr. Anderson's lab)*

d) Methods

The methods development work related to electrophoretic separations used pure samples of the toxins, saxitoxin, neosaxitoxin and domoic acid diluted in clean buffers. A variety of electrophoretic conditions were evaluated to yield optimal separation of the toxins under investigation. The range of conditions were first investigated in capillary using an Agilent Capillary Electrophoresis instrument in the absorbance detection mode.

These included:

- *CZE*
 - *at pH 5, MES buffer,*
 - *pH 7.4, phosphate buffer*
 - *pH 8.4, Tris Borate buffer, and*
 - *pH 9.5, Borate buffer*
- *MEKC (micellar electrikinetic chromatography, a special class of CZE using borate buffer with anionic detergent SDS)*
- *Buffer additives were also evaluated to modify electroosmotic flow (the bulk flow of fluid resulting from charges on the surface of the capillary or microchannel,*
 - *Normal and reverse polarity separations (detector at the cathode or the anode, respectively)*
 - *Non Viscous additives*
 - *Acetonitrile*
 - *Isopropanol*
 - *Brij 35 non-ionic detergent*
 - *Viscosity additives (Polymers)*
 - *0-1% hydroxyethyl cellulose*
 - *0-0.2% polyethylene oxide.*
- *Pseudo-Isotachopheresis (a separation method used to enhance sensitivity and resolution employing large sample injections containing high concentrations of both salt and acetonitrile)*

A variety of both fluorogenic and non-fluorogenic labeling reagents were evaluated for the fluorescent labeling of the saxitoxins and domoic acid. These were evaluated for labeled toxins peaks in absorbance mode prior to transition to the microchip platform (μ ChemLabTM) for laser induced fluorescence detection.

These included:

- *Fluorescamine*
- *4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F)*
- *AlexaFluor 488 & AlexaFluor 647 hydrazides combined with carbodiimide chemistry*

Methods development related to sample preparation protocols began with monocultures of both diatoms and dinoflagellates. A small number of lysis and extraction protocols were tried that were compatible with the subsequent labeling and electrophoretic separations.

e) Results

Fluorescent Labeling

Domoic Acid: We demonstrated successful labeling of domoic acid with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), which is another fast-reacting fluorogenic dye that is capable of reacting with both primary and secondary amines (AlDirbashi et al., 1998; Wallenborg et al., 2000; James et al., 2000). The detection of NBD-F-labeled domoic acid was observed in μ ChemLab™ and was resolved from free NBD-F dye using reverse polarity as shown in **Figure 1**. The detection limit was initially estimated at ~5 nM (corresponding to ~1 fg in the analysis volume) without any appreciable optimization; this is expected to improve by using pre-concentration strategies, such as pseudo-isotachopheresis or voltage stacking (Albin et al., 1993). Moreover, this use of NBD-F labeling can only deliver one dye molecule per toxin molecule (single nitrogen labeling site).

Labeling via the carboxy moiety would allow the possibility to couple as many as three dye molecules per toxin molecules due to the presence of three carboxy groups. We also successfully coupled Alexa Fluor dyes to domoic acid via the carboxylic acid moiety and standard carbodiimide chemistry. This approach is not as rapid as NBD-F labeling (e.g., ~10 minute versus 1 minute), but it uses the Alexa Fluor dyes, which are among the brightest commercially available dyes (Alexa Fluor 647 extinction coefficient >235,000; Invitrogen) and are significantly brighter than NBD-F. This factor, combined with the larger number of available labeling sites, should have a strong positive impact on sensitivity and detection limit.

Saxitoxins: Labeling of saxitoxins using NBD-F was evaluated with the hopes of developing a “universal” labeling strategy; it was hoped that the unusually low pKa values of the guanidinium nitrogens available in the saxitoxins would allow reaction with the amine specific NBD-F described above for domoic acid labeling. However, NBD-F appeared to be a poor choice for both the saxitoxin and the neosaxitoxin and little if any fluorescence/visible absorbance was observed that could be attributed to a labeled toxins species. Likewise fluorescamine, another fluorogenic, fast reacting dye proved to be non-reactive for the amine groups in the saxitoxins under any of the conditions tested.

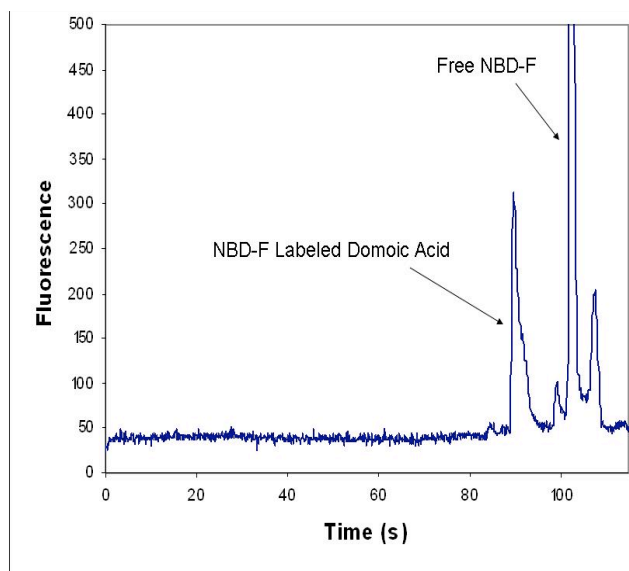


Figure 1. Microchip separation and detection of domoic acid labeled with NBD-F.

In light of these observations, we identified three alternative approaches. The first approach, involves oxidizing saxitoxins to fluorescent products using either periodate or hydrogen peroxide. This method has been used successfully for the detection of

high performance liquid chromatographic (HPLC)-separated toxins (Cianca et al., 2007; He et al., 2005); however, it is a less attractive option initially because the optical properties of the oxidized products (330 nm excitation and 390 nm emission) would require the development of an alternate detector—still employing common hardware but requiring different excitation and collection optics and significantly increasing cost of the ultimate platform. In addition, it appears that the fluorescent intensity of the oxidized products is relatively poor, based on fluorescence measurements made in-house. The intensity may be adequate for HPLC application where the path length in the larger HPLC column allows for sufficient intensity but will be problematic in the very small microchip and capillary.

The second approach, using indirect fluorescence detection, potentially represents a universal detection strategy. In this strategy, dye is added to the running buffer. When a molecule travels through the channel, it typically takes up molecular space otherwise occupied by dye molecules, and the detection peaks in the presence of the target molecule tend to be negative, representing a drop in fluorescence (Dolnik and Liu, 2005; Bishop et al., 2007). This approach is attractive because it eliminates the need for a separate labeling step, and it does not require new detection hardware. However, indirect fluorescence detection tends to be somewhat less sensitive than using conventional LIF.

A third approach is to use the Alexa Fluor dyes to label the saxitoxins, after oxidation to a carboxylic acid (Bhakuni and Rawat, 2006). This method also has the advantage of being almost a universal labeling strategy because it can be used for all of the toxins being studied here as they all possess carboxy groups: domoic acid, saxitoxin and neosaxitoxin, after an oxidation step. The drawback here is the potential for longer analysis times, resulting from the longer labeling process. We began evaluation of this in the late stages of the program and the preliminary results are favorable, suggesting successful post-oxidation labeling. Figure 2 shows the labeling of neosaxitoxin with AlexaFluor 488 hydrazide after prior oxidation using periodic acid. The apparent detection limit in the absorbance mode is estimated at ~150 nM. LIF detection can be expected to drop this detection limit at least one, if not two, orders of magnitude.

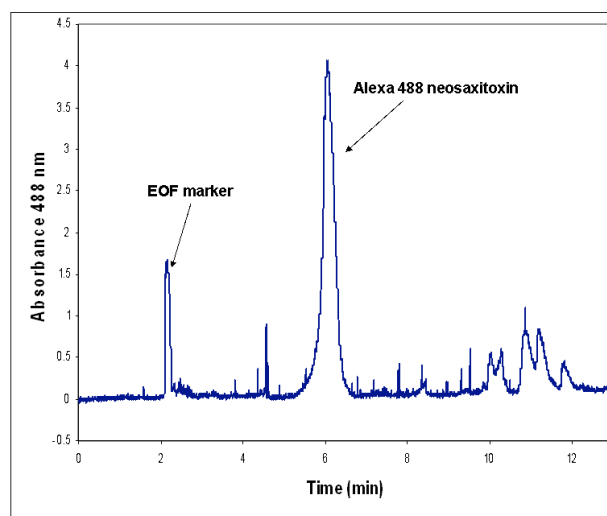


Figure 2: Alexa Fluor 488 labeled neosaxitoxin, after oxidization by periodic acid

Separation Methods in Capillaries and/or Microfluidic Chips

Literature about the chemistry of the saxitoxins suggested that at physiological pH, there is a distinct difference in charge between structurally similar saxitoxin and neosaxitoxin because of differences in the relative pKa values of the guanidinium groups (Bhakuni and Rawat, 2006). This was experimentally verified in capillary using with separations at pH 7.4 in a pseudo-isotachphoresis mode (p-ITP) which fully resolved the saxitoxin and neosaxitoxin (R value »2). This approach yielded sufficient sensitivity to observe the saxitoxins in absorbance mode; without the p-ITP amplification the underivatized toxins are not detectable by absorbance using the Agilent system at the concentrations available (commercial stock solutions are 65 µM).

Using pH 7.4 buffer with the polymer hydroxyethyl cellulose added to reduce electroosmotic flow, domoic acid was also fully resolved from both saxitoxin and neosaxitoxin, migrating in the opposite voltage polarity (anode near the detector) to both saxitoxin and neosaxitoxin (cathode near the detector). This is expected due to the charge differences between the toxins, at pH 7.4, the saxitoxins are positively charged while the domoic acid is negatively charged.

Sample Preparation

Given the time and budget constraints and that the labeling and detection of the saxitoxins proved more difficult than originally expected, the bulk of the effort in this one year effort was focused on labeling and separations experiments.

Preliminary work was carried out to develop the lysis and extraction protocols that are compatible with the downstream labeling and microfluidic separations. These have focused on lysis protocols and harvesting of domoic acid spiked diatom samples. Further development work will progress should follow-on funding become available.

Extracts of toxigenic dinflagellates from our collaborator Don Anderson at WHOI have been collected and received at SNL but there was insufficient time to analyze them using our fluorescent labeling strategy and separation conditions. Samples will be archived and tested as resources or follow-on funding becomes available.

f) Discussion

Summarize the project results. How do the results compare to existing methods/technologies currently used to address the management issue or problem? *Though time and budget constraints in this preliminary one-year project prevented the full demonstration of labeling, separation, sample preparation protocol optimization and natural sample validation, we feel that there has been excellent progress and that the fundamental proof of principle has been achieved. We have successfully labeled toxins for fluorescent detection, demonstrated separation conditions for the toxins by capillary electrophoresis and begun preliminary development of lysis and sample preparation protocols from phytoplankton that*

would be compatible with the labeling and separations. In addition, there were no fundamental hurdles identified that might prevent successful development of this technology for marine toxin applications. The necessity to use somewhat slower chemistries to fluorescently label the saxitoxins will limit the speed of the ultimate assay, requiring possibly an additional 10 to 20 minutes reaction time than was previously anticipated. In spite of this, the proposed approach and analysis platform can still be expected to have a cycle time, including sample preparation and analysis, of less than an hour. This still represents a spectacular improvement over the existing techniques (especially bioassay). In addition, the potential for miniaturization and field deployment of the platform remains a profound advancement.

3. Utilization

a) End User Application

N/A

b) Intellectual Property and Partnerships

As a result of the CICEET funded Market Assessment carried out by RTI, we have built a partnership with RTI in this area. We have jointly submitted a proposal to the Office of Naval Research to further this effort; the proposal is currently in review. As part of that process, we have also built relationships with additional researchers in the field, Dr. Chris Scholin at MBARI, Dr. Greg Doucette at NOAA and Dr. Julie Dyble at NOAA.

c) Knowledge Exchange

What methods were used to disseminate information about the project?

Please list all of the following that apply:

- Conference presentations. *The work will be presented in an upcoming conference in April (PacRim Shellfish Conference, San Diego, CA Apr 22-24, 2008).*
- Manuscripts (with citations) published or submitted to refereed journals. *One manuscript in preparation on the chip based fluorescent detection of domoic acid in diatoms.*
- Students that worked on the project. Please identify the student's level (undergrad, grad) and if applicable, the degree awarded and dissertation titles. *Undergraduate student, Deanna Curtis, worked part time on the project, and is continuing with her studies.*
- Other. *We met with End User Advisor Langlois and Dr. Kusum Perera, an analytical chemist at the California Department of Health Services and an expert in the detection and quantification of marine biotoxins. At this we discussed the needs of the Marine Biotoxin Monitoring Program and various concepts of operation for the device. Because of an unusual domoic acid event off of coastal California there was significant media interest in our project. We were featured with our advisor Gregg Langlois on the local news broadcast (KTVU news station 04/25/07) and on a pod cast produced by the American Society for Microbiology: MicrobeWorld which aired 6/26/2007 (Early Detection of Harmful Algal Blooms; <http://www.flpradio.com/microbeworld/audio/070611-070706/070626.mp3>)*

4. Next Steps to Application

Please describe next steps for the application of this technology.

- Is the tool readily accessible to coastal resource managers now? *The technology is not yet ready for coastal resource managers. If so, how?*
- What is the next stage of development? *What was achieved under this effort is preliminary proof-of-concept. The next stage of development, in the event of follow on funding, is to further optimize the methods, expanding the target analyte to include a larger number of marine toxins, and build the first integrated prototype. The prototype development will be very straightforward as it will be based heavily on existing platforms developed under other programs at Sandia. Once the prototype has been built it can be tested by a variety of researchers and coastal resource managers.*
- What are the current obstacles to application? Please consider technical as well as non-technical (regulatory, political, social, economic) obstacles. *We see no specific obstacles to development, either technical or non-technical, other than funding. The suite of tools or components already have been developed and all that remains is to select the appropriate components for this application and build the first unit. Automation of the unit should facilitate the utilization of the platform by non-skilled and users*
- What steps could be taken to overcome those obstacles? *N/A*
- Please identify the parties that you feel should be involved in developing an application plan for this technology. *State health officials and or fisheries managers.*
- Roughly estimate the time and cost of bringing the technology to the next stage of development. *Approximately \$1M in a 1 year period should allow the final optimization of methods, building of a prototype and testing both in the field and by a small number of end user participants. Further development to iterate the design to incorporate any changes suggested by initial end users (eg. for enhanced ruggedness, improved maintainability) to bring the prototype platform to a point where commercialization partners could be engaged, could be carried out for ~\$0.5 M over a 6-9 month period. If potential funding were more limited, such as \$250 K for each of two years, a smaller scope could be envisioned in which we would optimize the methods and build a breadboard system, suitable for testing by end users in a laboratory setting (not fully automated or fieldable) .*

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