Florida Harmful Algal Bloom Mitigation Year Five Neutralizing Brevetoxin Using the Amino Acid Derivative, Cysteine Methyl Ester

Collin McAliley
Melbourne Beach, Florida
inventor@cfl.rr.com

ABSTRACT

Harmful algal blooms (HABs) occur worldwide in bodies of water that range from ponds to oceans. A few species of algae produce and release lethal toxins that under certain conditions are sufficient to damage human health and marine life and severely impact coastal economies. HABs of *Karenia brevis* ("red tides") can develop over hundreds of square miles of open water, such as the Gulf of Mexico, and their control can be challenging. To succeed, control methods for HABs must be based on sound theory, must address the toxic effects of the bloom, and must be applicable on a large scale.

This project investigated a chemical approach for mitigating the effects of Florida Harmful Algal Blooms (FHABs) using the amino acid derivative, cysteine methyl ester (CME). *Karenia brevis*, the alga causing FHABs, releases powerful neurotoxic brevetoxins. CME was evaluated because it can chemically neutralize brevetoxins by molecular binding (Naar, et al, 2007). The specific objective of this research was to determine *in vitro* the length of time that a single CME treatment could neutralize brevetoxins at the typical Gulf of Mexico summer and winter temperatures of 29°C and 18°C. Sonicated *Karenia brevis* was combined with artificial seawater, treated with CME, and incubated. The incubated samples were processed using Solid Phase Extraction (SPE) to collect the resulting toxins and derivatives. SPE eluted products were analyzed for brevetoxin content using High Performance Liquid Chromatography–Mass Spectrometry. CME was found to be very effective in neutralizing brevetoxins. A single CME treatment remained 100% effective for 37 days and partially effective for at least an additional seven days. Temperature in the range 18°-29°C did not have a significant influence on how long a treatment was effective (P=0.0978).

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It was hypothesized that cysteine methyl ester (CME) would be equally effective in the *in vitro* neutralization of brevetoxin at the typical Gulf of Mexico summer and winter temperatures of 29°C and 18°C. It was further hypothesized that the effectiveness of a single CME treatment would decline with time.

KEYWORDS: cysteine methyl ester, harmful algal blooms (HABs), *Karenia brevis*, brevetoxins, molecular binding, cysteine, ester, red tides.

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1. INTRODUCTION

Over the last several decades, there has been evidence that the threat from the HABs has increased (Alcock, 2007). Conditions favorable to the development of HABs may occur more frequently in the future due to global warming and increased pollution by nutrient runoff into near-coastal waters (Alcock, 2007). There is a critical need for new methods and approaches for mitigating the harmful effects of Florida HABs. This study examined a chemical method for mitigation that targets the toxins themselves rather than the organism(s) producing them.

Why evaluate cysteine methyl ester?

CME is a readily manufactured amino acid derivative that is known to rapidly neutralize brevetoxins by molecular binding (Naar et al., 2007; Yang & Naar, 2007). It is possible that the CME chemical structure is less soluble in water than unmodified cysteine, perhaps resulting in a longer effective period and making it a good candidate for further study. Naar et al. (2007) found that CME reacted quickly with brevetoxin at low concentrations, making it a good candidate for further research (Naar et al., 2007) because it could neutralize brevetoxin before it could cause damage to other viable organisms (Jerome Naar). Brevetoxins damage both human and marine animals by binding to Site 5 voltage sensitive sodium ion channels in the cell membranes and preventing them from closing. This causes nerve damage from repetitive firing (Sperelakis et al., 2001). In humans, brain, heart, and skeletal muscle cells are affected (Baden et al., 2005). The repetitive nerve firing results in sensory abnormalities, nerve dysfunction, and sometimes, respiratory failure (Baden et al., 2005). CME is able to

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chemically react to available bonding sites on brevetoxins, essentially preventing further bonding by the brevetoxins to the sodium ion channels and avoiding the ensuing harmful effects (Jerome Naar).

2. MATERIALS AND METHODS

Experimental Design

Low concentrations of an aging CME solution were added to sonicated *Karenia brevis* then incubated *in vitro* at the typical Gulf of Mexico summer and winter temperatures of 29°C and 18°C respectively. Following incubation, the samples were then concentrated by Solid Phase Extraction (SPE). The SPE was eluted with methanol and the eluates were analyzed for toxin concentrations using High Performance Liquid Chromatography–Mass Spectrometry (HPLC/MS). The following three types of control samples were run:

- Original CME solution: Two liters of an 0.2 mg CME/mL solution in artificial seawater (ASW) was and divided into two 1-L portions and these were stored throughout the experimental period at 29°C and 18°C respectively. Samples taken from these solutions were tested every Friday and Sunday during the 44-day experimental period.
- Fresh CME solution: Fresh CME solutions with the same composition as the Original CME solution were prepared using fresh, CME treated seawater. This control allowed direct comparison each week between the effectiveness of the Original CME and the Fresh CME solutions.
  - Positive Control: On each testing day, positive control samples (50/50 untreated seawater and sonicated *Karenia brevis* culture) were prepared (positive control sample).
  - Negative Control: On each testing day, negative control samples (100% untreated seawater) were prepared (negative control sample).

Project Duration and Testing Frequency

The experimental period of 44 days allowed time for sufficient test replication and for determination of the effectiveness of CME in neutralizing brevetoxins over an extended period. The project started on a Friday and sampling and analysis were performed every Friday and Sunday.

Equipment

The following equipment was constructed for the project:

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- Solid Phase Extraction (SPE) Processor: SPE processing was performed using 3 mL Strata X SPE cartridges, 20 mL syringes, and Luer adapter caps. Without access to a vacuum manifold, the SPE process is a slow, multi-step process. To insure an appropriate flow rate and to reduce processing time an SPE processor was designed and built for a cost of approximately $15. This equipment reduced processing time by at least six-fold by allowing six samples to be processed simultaneously and provided a mechanical control for syringe depression that ensured reproducible flow rates.

SPE Processor

- Incubators: Two identical incubators were fabricated from small, commercial, off-the-shelf wine coolers. One was maintained at 18°C ±0.5^{o}C and the other at 29°C±0.5^{o}C. The pre-existing cooling element was satisfactory for the lower temperature incubator. To provide a stable heat source for the higher temperature incubator, three 50-watt aquarium heaters were suspended in a sealed glass flask of deionized water. The heaters were then calibrated to 29°C±0.5^{o}C. To monitor temperature the incubators were fitted with digital thermometers equipped with remote sensors.

Incubator

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Test Sample Preparation

Triplicate solutions were prepared as indicated in Table 1.

Table 1. ***Test Sample Solutions***

	Friday	Sunday
1	10 mL Original 18°C CME treated ASW and 10 mL18°C *Karenia brevis*	10 mL Original 18°C CME treated ASW and 10 mL 18°C *Karenia brevis*
2	10 mL Original 29°C CME treated ASW and 10 mL 29°C *Karenia brevis*	10 mL Original 29°C CME treated ASW and 10 mL 29°C *Karenia brevis*
3	Positive Control – 10 mL untreated 18°C ASW and 10 mL 18°C *Karenia brevis*	Positive Control – 10 mL untreated 18°C ASW and 10 mL 18°C *Karenia brevis*
4	Negative Control – 20 mL untreated 18°C ASW	Negative Control – 20 mL untreated 18°C ASW
5	n/a	Additional Control -10 mL Fresh 18°C CME treated ASW and 10 mL 18°C *Karenia brevis*
6	n/a	Additional Control - 10 mL Fresh 29°C CME treated ASW and 10 mL 29°C *Karenia brevis*

Prepared samples were stored in their respective incubators for two hours to allow the CME to react with the *Karenia brevis.* Untreated controls were also stored in their respective incubators during this interval.

Solid Phase Extraction (SPE) Processing

Immediately following the two hour incubation period, toxins and derivatives in the samples were isolated using SPE. Samples were processed in groups described in Table 1, using the SPE processing apparatus. Three separate replicates of each group were processed for each testing day. SPE processing steps were as follows:

1. Setup for each sample included one 20 mL syringe connected to a 3 mL Strata-X SPE cartridge using an adapter cap.
2. SPE cartridges were conditioned with 10 mL (additional 5 mL for second and third replicates for cleaning purposes) methanol 100% each at a 10 mL/minute flow rate. They were not allowed to run dry.

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3. SPE cartridges were then equilibrated with 10 mL deionized water (each) at a 10 mL/minute flow rate and were not allowed to run dry.
4. One set of 20 mL samples (Table 1) were loaded by pipetter into the 20 mL syringes and filtered at a 5 mL/minute flow rate and were not allowed to run dry.
5. SPE cartridges were washed with 5 mL deionized water (each) at a 10 mL/minute flow rate and were allowed to run dry.
6. SPE cartridges were eluted with 2 mL methanol (100%) each. Eluted products were directly collected in pre-labeled 7 mL glass vials and tightly capped.
7. Eluted products were immediately placed in a freezer and stored at –23°C.
8. Waste was disposed of according to laboratory safety procedures.

Toxin analysis

The frozen eluted products were packed in ice and shipped overnight to the Center for Marine Science, University of North Carolina Wilmington (UNCW). This laboratory used HPLC/MS to determine residual brevetoxin concentrations. The UCNW results were provided in the form of peak areas.

Statistical analysis

Statistical analyses were performed using Excel® and Minitab®.

- Normality: Standard deviations were calculated to test data distribution and variability. Results were cross-checked with normal probability plots using the maximum likelihood estimation method (Murray, 1972) at a 95% confidence interval.
- Statistical differences: The impact of the manipulated variables, time and temperature, were analyzed using Student's t-tests, interaction plots, and main effect plots. Student's t-tests were performed comparing each of the test groups to each of the controls and to each other.

3. RESULTS

HPLC/MS Results

CME was effective in neutralizing brevetoxins at both summer and winter temperatures. A single treatment remained 100% effective for 37 days and partially effective for at least seven additional

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days. Temperature did not have a statistically significant influence on the time that a treatment was effective (P=0.0978). No brevetoxins were detected in any of the Original CME treated samples until 42 days from the treatment date (Figure 1) (Note that no brevetoxin analysis was conducted between Day 37 and Day 41. On testing Days 42 and 44, brevetoxins were detected at 52% (mean) of the positive control levels at the 18°C and at 17% (mean) of the positive control levels in the 29°C CME treated samples. Brevetoxins were detected in all positive control samples. No brevetoxins were detected any of the negative control samples. No brevetoxins were detected in any of the fresh CME treated samples (Figure 1. and Table 2)

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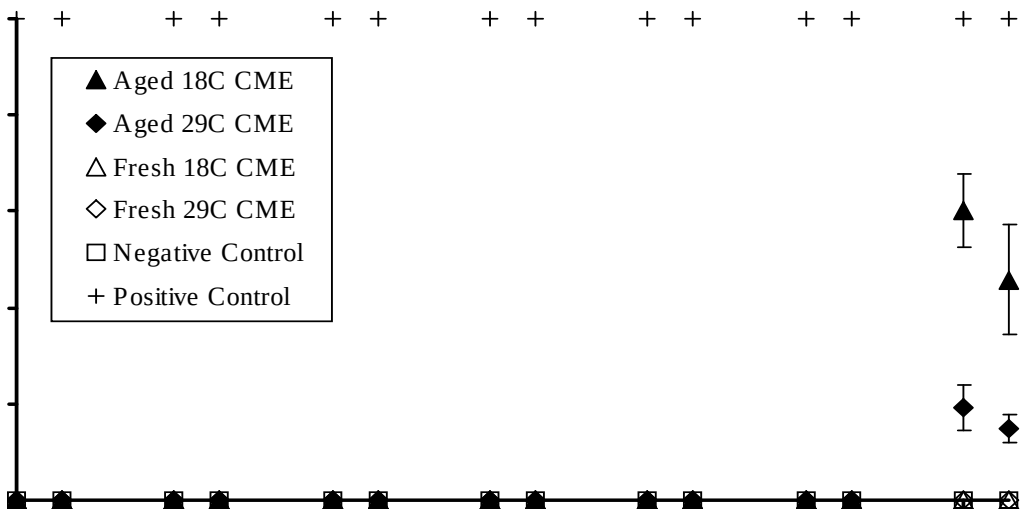

Fig 1. Brevetoxin concentration detected through HPLC/MS analysis. Values are means +/- one standard deviation.

Table 2. Mean concentration (%) of detected brevetoxin with standard deviations

	Brevetoxin concentration						s		
Project Day	Negative Control	Fresh 18C CME	Fresh 29C CME	Aged 18C CME	Aged 29C CME	Positive Control	Aged 18C CME	Aged 29C CME	Positive Control
0	0%	0%	0%	0%	0%	100%	-	-	0.12
2	0%	0%	0%	0%	0%	100%	-	-	0.08
7	0%	0%	0%	0%	0%	100%	-	-	0.16
9	0%	0%	0%	0%	0%	100%	-	-	0.11
14	0%	0%	0%	0%	0%	100%	-	-	0.16
16	0%	0%	0%	0%	0%	100%	-	-	0.32
21	0%	0%	0%	0%	0%	100%	-	-	0.12
23	0%	0%	0%	0%	0%	100%	-	-	0.21
28	0%	0%	0%	0%	0%	100%	-	-	0.25
30	0%	0%	0%	0%	0%	100%	-	-	0.16
35	0%	0%	0%	0%	0%	100%	-	-	0.16
37	0%	0%	0%	0%	0%	100%	-	-	0.09
42	0%	0%	0%	60%	19%	100%	0.07	0.05	0.05
44	0%	0%	0%	46%	15%	100%	0.11	0.03	0.12

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There was a statistically significant difference between the negative control samples and the Original CME samples at both 18°C and 29°C (Student's t-test; p=0.0209 and p=0.0118). Statistical analysis also indicated that over the experimental period there was a statistically significant difference between the positive control samples and the Original CME samples at both 18°C and 29°C (Student's t-test; p=0.000 and p=0.000). Statistical analysis showed that results at both temperatures for both Fresh and Original CME treated samples were normally distributed (Figures 2 and 3).

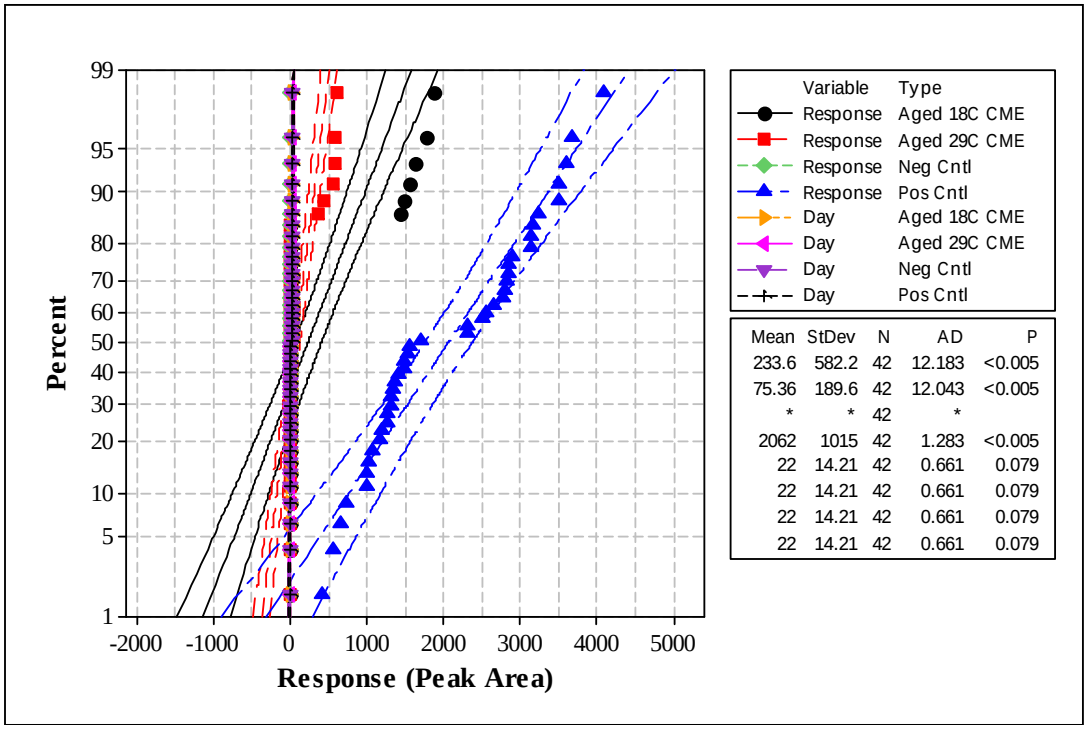

Figure 2. Original CME treated samples vs. Controls. Probability Plot of HPLC/MS response (for presence of Brevetoxins) by day. Normal - 95% confidence interval.

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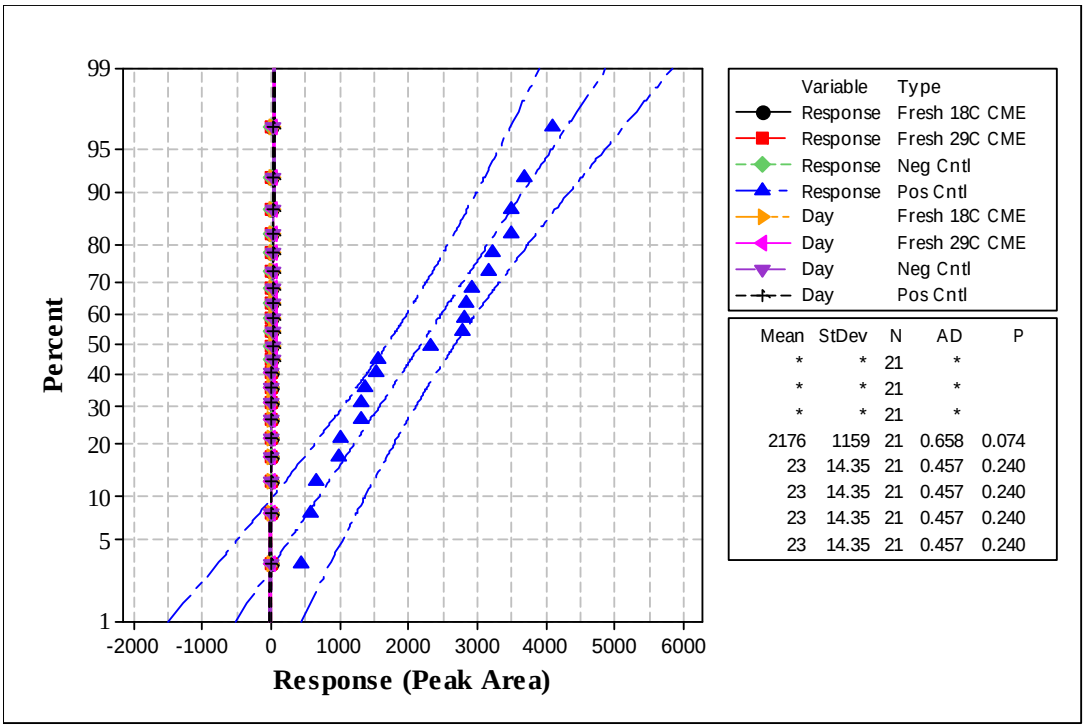

Figure 3. Fresh CME treated samples vs. Controls. Probability Plot of HPLC/MS response (for presence of Brevetoxins) by day. Normal - 95% confidence interval.

Impact of Temperature

Temperature (at 18°C and 29°C) had no influence on CME effectiveness. Brevetoxin concentrations in the Original CME treated samples at 18°C and 29°C did not differ significantly (Student's t-test; p=0.0978) for the experimental period. Fresh CME treatments were consistently completely effective in neutralizing the brevetoxins at both 18°C and 29°C over the 44 days experimental period. Forty-two days after treatment with the Original CME, brevetoxins were detected at both 18°C and 29°C. The observed brevetoxin concentrations at 18°C were approximately triple those at 29°C in both the Day 42 and the Day 44 samples.

Degradation of Cysteine Methyl Ester Over Time

As indicated above, Brevetoxins were detected on Day 44 indicating that CME had become less effective in neutralizing them. On Days 42 and 44 (the last two days) an increasing sulfur odor was observed suggesting that CME degradation was occurring.

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Effectiveness of Cysteine Methyl Ester on Different Forms of Brevetoxin

At least nine forms of brevetoxin have been identified (PbTx-1 through PbTx-9) (Baden, et al., 2005). In this study HPLC/MS detected the presence of either PbTx-2 or PbTx-3 (Table 2). Neither of these brevetoxin forms was detected in CME-treated samples through Day 37. Thus CME treatment was effective in neutralizing PbTx-3 as well as PbTx-2. Previous findings on the effectiveness of CME treatment were limited to PbTx-2 (Naar, et al., 2007; Yang & Naar, 2007; Xingye Yang).

4. DISCUSSION

The long-range purpose of this research is to find an effective method of mitigating the harmful effects of Florida HABs. Current HAB mitigation efforts largely consist of identifying the presence and position of a HAB and issuing warnings to interested parties that a HAB event may occur. As a result of the HAB warning, commercial fisheries may be closed, but there is no existing method of protecting shellfish harvesting or coastal communities from damage by a HAB.

Naar et al (2007) found that a CME concentration of 0.2 mg CME/mL ASW was effective in neutralizing brevetoxins *in vitro*- but did not determine how long a CME addition to *Karenia brevis* would remain effective. This research showed that a CME treatment remained effective for over five weeks at typical Gulf of Mexico winter and summer temperatures. Thereafter CME effectiveness degraded. Further work on the mechanism and products of CME degradation is needed prior to its introduction into the environment.

On Days 42 and 44 the brevetoxin concentration was significantly higher at 18°C than at 29°C suggesting that treatment may last longer at summer temperatures when *Karenia brevis* may become more toxic (McAliley, 2008). This needs to be confirmed by further study because this effect was observed during the final two testing days of the experiments.

For CME treatment to become practical the chemical lifecycle of CME in a marine environment must be determined. This would include evaluation of environmental risks from CME as well as the effect of the marine environment on the efficacy of CME. CME treatment could be used in two ways: protection of an ecosystem or an aquaculture system; and, treatment of individual animals (e.g. a manatee that has been exposed to brevetoxins). In the United Kingdom, CME is used as a prescription medication (Visclair) for respiratory illness at a recommended daily dose of 800 mg. (The CME

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concentration in this study was 200 mg/L per liter of water. CME is also an effective and economical material for removing toxic heavy metal ions from drinking water (Wildgoose et al., 2005).

Although CME binds to brevetoxins and neutralizes them, it may not be the best substance for this purpose. Further study of the available binding sites on brevetoxins, analysis of possible improved binding agents, and consideration of a binding agent delivery system would be necessary to produce the most efficient molecular binding approach to HAB mitigation.

5. CONCLUSIONS, ASSUMPTIONS AND LIMITATIONS

Conclusions:

1. A single CME treatment for neutralizing brevetoxins was completely effective for 37 dyas and partially effective for at least seven additional days.
2. Temperature in the range 18°C and 29°C did not significantly influence the duration over which a treatment was effective.
3. CME neutralized both the PbTx-2 and PbTx-3 forms of brevetoxin.

Assumptions:

- The absence of detectable brevetoxin, after treatment with CME, results from a chemical reaction between the CME and the brevetoxins. This reaction forms a new nontoxic derivative (Yang & Naar, 2007).
- This new brevetoxin derivative does not bind to the sodium ion channel (Yang & Naar, 2007).
- All containers of *Karenia brevis* provided by the research laboratory were of a consistent concentration.
- No contaminants were introduced into any of the samples.

Limitations:

- Data collected was limited to *in vitro*.
- Data was within a 95% confidence interval.

6. ABREVIATIONS AND ACRONYMS

ASW: Artificial seawater

CME: Cysteine methyl ester

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HAB: Harmful algal bloom

HPLC/MS: High performance liquid chromatography / mass spectrometry

SPE: Solid phase extraction

7. ACKNOWLEDGEMENTS

Credits

I would like to thank Dr. Jerome Naar and Dr. Xingye Yang, from the Center for Marine Science University of North Carolina Wilmington and the Red Tide Control and Mitigation Program for their assistance on this project. They provided *Karenia brevis* cultures, supplies, advice, and performed the HPLC/MS analysis. I would also like to thank Ms. Yvette Managan and the City of Cocoa Beach, Fla., Water Reclamation Laboratory for providing the laboratory facility where this project was conducted.

Author

I am finishing the tenth grade at Cocoa Beach High School, Cocoa Beach, Fla., in the International Baccalaureate Programme. I live in Melbourne Beach, Fla., on a narrow barrier island between the Atlantic Ocean and the Indian River Lagoon, surrounded by a unique marine environment. I play piano and enjoy music composition and computer gaming. I have entered the Brevard County, Fla. Science Fairs with projects involving indigenous algae for the past six years. I was a Discovery Channel Young Scientist Challenge National Finalist (participated in Washington, D.C.) in 2006 and 2007, and won the first Planet Green Prize, awarded in 2007 by the Planet Green and Discovery Channels. At the 2009 Intel International Science and Engineering Fair in Reno, Nev., I was awarded the NOAA "Pulse of the Planet" Award. This award allows me to participate in a summer internship at a NOAA research facility or on a research vessel. In addition, I was awarded a scholarship from the Office of Naval Research, and a 4th place Grand Award in Biochemistry. I am currently formulating next year's science project, which will continue my research on HAB mitigation.

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