

Evaluating the Potential Oyster Contamination from
Cyanobacterial Toxins in the Breton Sound Estuary

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Introduction:

Toxin producing cyanobacteria blooms occur in estuarine environments throughout the world causing negative impacts on the environmental health and creating a human health risk through the shellfish consumption. In the northern Gulf of Mexico (GOM), marine harmful algal bloom (HAB) toxins have been the subject of regional interest for many decades, while the presence of toxic cyanobacteria in estuaries have recently received increased interest. Louisiana's (LA) coastline on the northern coast of the GOM has large numbers of both private and state oyster beds that may be adversely affected by the presence of HAB toxins. One important oyster seed ground, Breton Sound, LA, which is influenced by nutrient-rich freshwater coming from the Mississippi River (via Caernarvon diversion) has over 10 % bottom coverage by oyster beds and contains a great potential to be contaminated by both marine and freshwater toxins. It is now known that when the water flow is high in the upper part of the estuaries due to river inflow, toxic cyanobacteria populations can reach to coastal part of the estuaries, where high toxin intake into the filter-feeding oysters can occur (Czubakowski 2010).

The main goals of this research were to determine whether toxic cyanobacteria can grow successfully in more saline coastal stations of Louisiana estuaries, and whether they have the potential to contaminate oysters in that area. To address these goals, following experiments were run: (A) Laboratory based toxic cyanobacteria growth experiments at varying salinities to see whether fresh water adapted toxic cyanobacteria can survive in more saline areas of the Breton Sound Estuary; (B). Laboratory based oyster-grazing

experiments with toxic cyanobacteria diet to see whether oysters can contain toxic cyanobacteria as part of their diet and whether they can accumulate toxins in their tissue.

Materials and Methods:

Cyanobacteria cultures and maintenance:

The freshwater cyanobacterium used in these experiments was a toxic strain of *Anabaena* that was isolated from Fargo, North Dakota, and was obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP 2066). Freshwater was collected during a non-bloom period from the Breton Sound estuary. Natural phytoplankton were removed using 0.2 μm glass fiber filters (GF/F) and nutrient rich stock media was diluted in it to prepare the experimental media. As a final step, sterilization of the media was accomplished by autoclaving at 121°C, 15 lb/in² for 15 minutes or longer depending on the volume involved. The semi-continuous stock cultures of toxic cyanobacteria were maintained in 250 mL Erlenmeyer flasks containing approximately 150 mL of freshwater media and kept in a light and temperature controlled incubator at ~25 C° on a 12-h light/12-h dark cycle.

A. Toxic Cyanobacteria Growth Experiments at Varying Salinities

For the first set of experiment, 250-ml cultures of toxic *Anabaena* at 0ppt, 5ppt, 10ppt, 15ppt, and 20ppt salinities (n = 3 for each) were maintained in controlled incubator (25 C° on a 12-h light/12-h dark cycle) for 10 days. Every 24 hours, starting from t = 0, 10 ml subsample was removed from each flask for chlorophyll a (Chl a) measurements.

Chl a levels were determined as the indicator of phytoplankton biomass. Subsamples were filtered onto 25 mm GF/F filters and the filters were placed in 15-ml centrifuge tubes, covered with aluminum foil and kept frozen until the analyses. Filters were then extracted for 24 h in 90% aqueous acetone at -20°C , and fluorescence was measured before and after acidification with hydrochloric acid using a Turner fluorometer (Model 10-AU) (Parsons et al. 1984). Chl a values were then converted to cell numbers using laboratory determined values of Chl a/cell measured in cultures.

For the second set of experiments, the first experiment was repeated at narrower salinity range 5-9 ppt to be able to define the tolerance threshold better. The highest salinity used was 9 ppt since the previous experiment showed that *Anabaena* did not grow in any culture with salinities above 9 ppt. Sampling and processing were the same as the first set of experiments, except at the end of this experiment, subsamples were also taken for the cyanotoxin, microcystin, and measurements.

ELISA (Enzyme-Linked Immunosorbant Assay) was used to measure microcystin following the protocol in Garcia et al. 2010. ELISA is a competitive binding assay that is highly sensitive for MCs with a detection limit of $0.10\ \mu\text{g l}^{-1}$ based on the most common variant, MC-LR, and its congeners. All the processed samples were temporarily stored at -20°C until needed for analysis.

3-h time course experiment with eastern oysters (Crassostrea virginica) grazing on a low toxicity strain of Anabaena sp.

Oyster collection and maintenance:

Eastern oysters were collected from Breton Sound, LA. At the same time water samples were taken from a transect in the Breton Sound Estuary area that covered both freshwater dominated upper estuarine waters and more saline waters of lower estuarine waters, where the oysters were collected. Chl a and toxin analyses were done with the collected field samples (water and oysters), to obtain information of the water conditions at the time of the oyster collection for the grazing experiments. Chl a and toxin presence were measured with the remaining water samples. Both Chl a and toxin analyses were conducted the same way as described above.

The oysters were brought back to the lab within five hours of collection, and then were scrubbed and placed in 10 gal aquariums. Oysters were held in 10 gal aquariums with continuous flow of ambient water and bubbled air for 24 hours to acclimate.

Grazing Experiment:

After the 24 hours of acclimation the grazing experiment began immediately. This was done to decrease the amount of time that the oysters were out of their natural habitat. The 24 hours of acclimation also served as a starvation period.

The cyanobacteria *Anabaena* was added to each clear polycarbonate containers (Tundra Specialties) (n = 14) with 2 l of sterilized media at 10 ppt. Each container held one oyster. The containers were air bubbled to ensure even distribution of *Anabaena* culture. Animals were allowed to feed on *Anabeana* with an initial concentration of 10^{-7} cells l^{-1} for 3 h. 10-ml water aliquots were withdrawn from each container at the beginning and at every 20 min, and were filtered onto 25 mm GF/F filters. Filters were then extracted for 24 h in 90% aqueous acetone and analyzed for Chl a. Each oyster was removed individually at the end of 3 h and frozen for further stomach content and toxin analyses. Water samples were measured for toxin levels at time zero and at the end of 3 h.

Both ingestion and clearance rates were calculated from changes in Chl a concentrations in the feeding containers relative to changes in Chl a concentrations in control bottles using Frost (1972) equations. Chl a values were then converted to cell numbers using laboratory determined values of Chl a/cell measured in cultures obtained immediately prior to the feeding experiments. The measurements of the filtration and clearance rates are ongoing.

Three sets of controls were used in the oyster grazing experiment. The first control (n= 3) was with oysters that were scrubbed with a 10% bleach solution to kill any possible epibionts. The shells were then rinsed in fresh water and placed in a container with the same set-up as the experimental containers. The second control (n=3) was with algae and a bleached oyster shell that had been shucked. This was done to account for any possible grazing by unknown fauna on the empty shell and changes in the accumulation of the

culture due to the shell being in the water. The third control (n=2) was with only algae to correct for potential phytoplankton growth. It was air bubbled like the rest of the experimental containers. Subsamples were similarly taken every 20 min to measure Chl α for phytoplankton biomass for 3h.

Toxin Analysis:

Microcystin (MC) equivalents in water subsamples and oyster tissues were determined using a commercially available Enzyme-Linked Immunosorbant Assay (ELISA). ELISA is a competitive binding assay that is highly sensitive for MCs with a detection limit of 0.10 $\mu\text{g l}^{-1}$ based on the most common variant, MC-LR, and its congeners. Water samples were collected at the end of the salinity experiment and the grazing experiment by filtering the water through GF/F filters until clogged. All the processed samples were temporarily stored at -20°C until needed for analysis.

Particulate samples were extracted using the methods developed by Garcia et al. (2010). First filters were allowed to come to room temperature and placed in glass test tubes, and then 5mL of 50% methanol plus 1% acetic acid was added to each tube. Each sample was vortexed for 1 minute, sonicated (6.0 setting) for 2 minutes and then centrifuged at 3000 rpm for 10 minutes. The supernatant was then extracted placed in a 7 ml scintillation vial before the extraction process was repeated. The pooled supernatant was then passed directly through a 0.20 μm syringe filter with a SFCA membrane filter

and collected in a new 7 ml scintillation vial. These samples were stored in 4C until analyzed with the ELISA assay.

The viscera toxin was extracted by homogenizing the viscera in a plastic beaker until it was smooth using a Biohomogenizer with a 1.4 cm generator (Biospec). Four grams (or less when samples did not contain 4 g) of the homogenate was weighed on a Mettler balance, as the Biosense ELISA protocol specifies. Homogenate was then placed in a clean 50mL centrifuge tube and 50% MeOH was added in a 1:4 weight to volume ratio, 1 part viscera weight to 4 parts MeOH. After this step, the homogenate was vortexed, sonicated, filtered and stored in the same manner as the water samples.

Results:

The growth experiments at varying salinities showed that *Anabaena* can grow within a range of 0 ppt to 8 ppt salinity (Figure 1). Results showed there was a significant growth from 0 ppt to 7 ppt, with a die off starting at 8 ppt. 5 ppt showed the greatest optimal growth over a 10-day span with concentrations reaching up to Chl a concentration of 448.815 (ug/L) and there was a continuing decrease in biomass at higher salinities. Low toxin levels were detected at all ranges of salinity; however the levels were variable with no significant trend to follow.

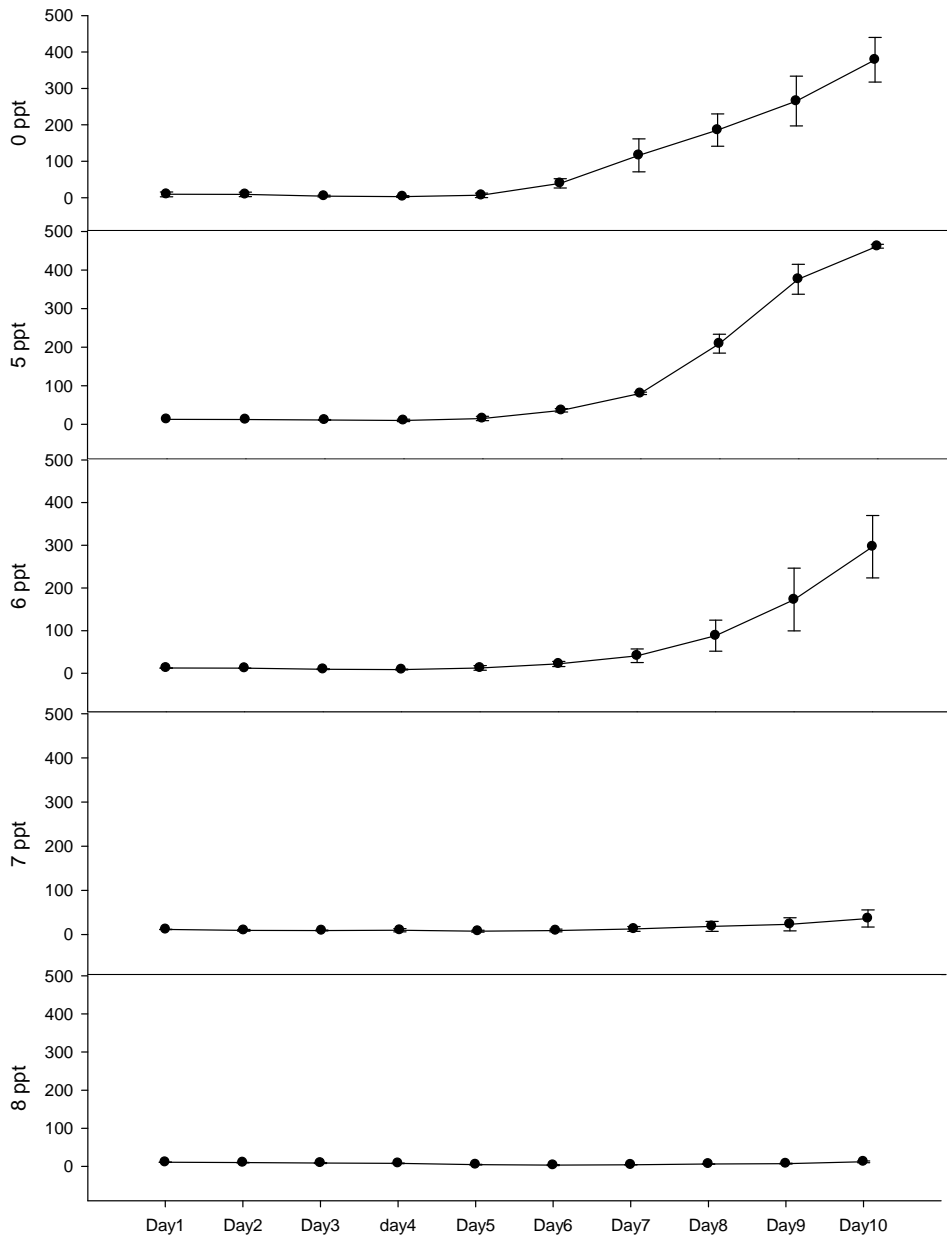


Figure 1. Chl a concentrations at 0-8 ppt salinity.

In the 3-h grazing experiment, Chl a in grazing containers decreased significantly over time, while the Chl a levels remained the same in the controls (Figure 2), indicating oyster grazing on the toxic *Anabaena* cells.

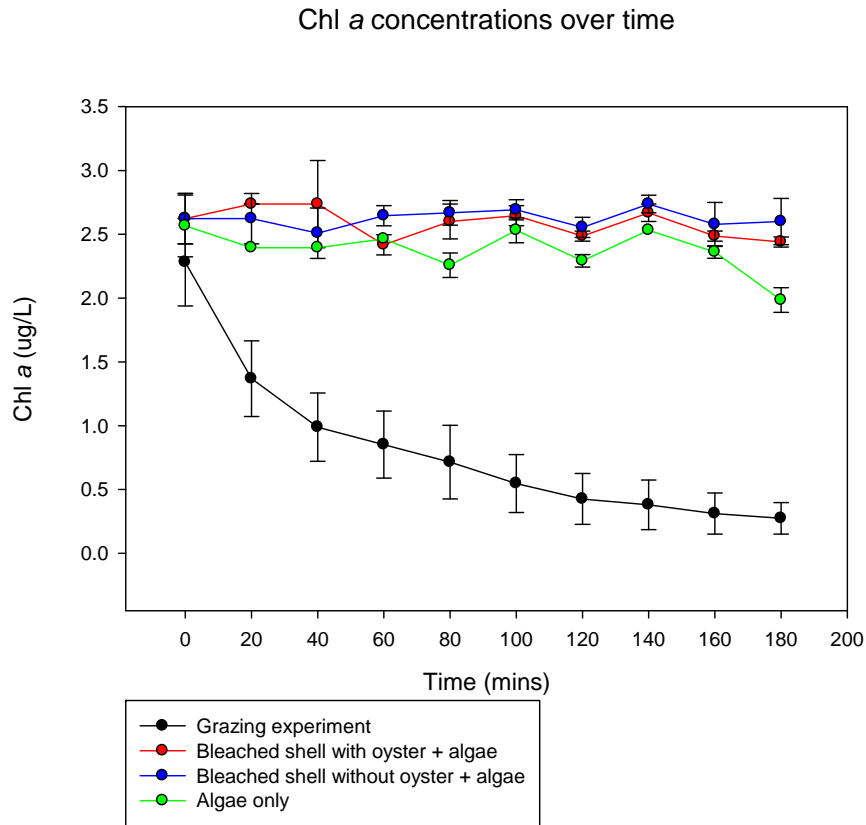


Figure 2. Chl a concentrations over time for grazing experiment.

Initial toxin measurements showed that the native population did have toxins present in their viscera. The toxin samples taken from $t=0$, after the acclimation, had a lower MC concentration, showing that the oysters are capable of depurating the toxin. The viscera that was measured for MC after 3 hours of exposure showed an increase in the concentration from the initial samples, indicating uptake of toxin during the filtration. At $t=0$ the water had low levels of toxins present and then had a decrease over time (Table 1).

**Table 1. Toxin concentrations in water (pg/mL) and oysters (ug/kg).
Time is measured in minutes.**

Experiment	Time	pg/mL	ug/kg
Water in the container	0	0.004	
Oyster Replicate #1	180	BD	.87
Oyster Replicate #5	180	BD	2.61
Oyster Replicate #12	180	0.006	BD
Oyster Replicate # 13	180	0.007	16.78
Oyster Replicate # 14	180	BD	2.29
Oyster 1a*	180	BD	BD
Oyster 1b*	180	0.0003	
Oyster 1c*	180	BD	
Oyster 2a*	180	BD	
Oyster 2b*	180	BD	
Oyster 2c*	180	BD	
Oyster 3a*	180	0.005	
Oyster 3b*	180	BD	
Oyster 4a*	180	0.002	
Oyster 4b*	180	BD	
Oyster 4c*	0	BD	
Oyster 4c*	180	0.02	

*=Controls of experiment. 1=Bleached shell with oyster. 2=Oyster no food.
3= Bleached shell with no oyster. 4=No oyster just algae

Discussion:

The experiments' results show that it is likely that oysters can be affected by toxic cyanobacteria. If a bloom of toxic cyanobacteria were to occur, oysters have a chance of ingesting the cyanobacteria cells and containing the toxin in their tissue.

The salinity experiments proved that *Anabaena* could survive and grow not only in freshwater but also in more saline waters with 8 ppt being the highest tolerance for growth. This shows that if water from the Mississippi River, through the diversions, pushes the fresh water of the upper estuary to the oyster beds of the lower estuary, *Anabaena* can survive and grow potentially initiating a bloom.

Toxin data also showed that the oysters could take in the toxic *Anabaena* and contain the toxin within their viscera. Although the concentrations were low, over a longer period of time, the concentrations could amplify in the oysters. Because humans consume oysters as a food source, the toxins can cause negative effects on human health.

References:

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Personal Gains through this Project And Acknowledgements

First I would like to thank Sea Grant for allowing me to experience and be part of, what I would have to say, is the experience of a life time. It has been a learning experience and also great preparation for what I would like to do in the future. It has given me the experience necessary to enter the work force, or to apply to a graduate program. I learned the fundamentals of doing a project and all that it entails. This project taught me skills that I may have never used or even known I had. All in all, this project created the greatest experience for me and I look forward to becoming a research scientist, hopefully in the near future.

I'd like to now take the time to think all who have given their time and knowledge in helping me along the way, because without them I may still be trying to figure out how to use a pipette. First to Dr. Bargu who has been a huge source of knowledge and great help. She made the project enjoyable while also making sure I understood what was going on. She made sure I knew the reason behind whatever I was doing. She is a great inspiration and help even for the things that was not involving my project.

I would like to also thank Emily Smith. She has helped on many occasions and without her I feel I may never have gotten pass day one. Her knowledge on the matter made it very easy to understand what was going on, no matter what. She was always making sure every thing was going right. Great help when the grazing experiment was going on and samples were being taken every 20 minutes. I joined her at a conference where she presented, and that was an added bonus experience. It was a great time and a

side of science I have never seen. I would like to thank her for allowing me to go to the conference with her and being part of that.

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I would now like to thank my fiancée Catherine Melancon. She made sure I always kept my head up in times I thought I was overwhelmed. On days when I'd have class from 8:30 am – 12:00 p.m. and then work from 1:00 p.m. – 9:00 p.m., she'd make sure I was still up to filter my samples. Without her, my papers would be total chaos. She's my proofreader and grammar queen. She is my inspiration on what I do and why I always try my hardest.

After this project is complete, I do not intend to leave science. It did not scare me away. I would like to continue working in a lab environment, possibly in different areas of science just to add to my experience. I would like to also submit an abstract for a conference and present my findings. I plan on attending State of the Coast Conference in New Orleans in June 2012.

Once again I would like to thank all that were involved with this project and allowing me to have the experience of a lifetime.

Thank You,

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