An Analysis of Bacterial Content in *Cladophora* at Presque Isle State Park

Figure One- *Cladophora* present along the shoreline of Presque Isle State Park

Picture taken in the summer of 2007 by Nicole Ruffo, former undergraduate student at Mercyhurst College

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Introduction

Cladophora is a nuisance algae that is present in the beach waters and shoreline of Presque Isle State Park (Figure 1). The presence of this algae not only detracts from the beauty of the beachfront, but also emits a foul odor, which likely has a negative impact on the attraction of the millions of visitors who use Presque Isle State Park beaches for recreational purposes. In addition to the unpleasant look and smell of this algae, it has recently been found that *Cladophora* can contain high levels of bacteria residing on its surface, presenting potential concern on human health in *Cladophora* laden waters (Whitman et. al, Appl. Environ. Microbiol., 2003, Olapade et. al., Appl. Environ. Microbiol., 2006, Ishii et. al., Appl. Environ. Microbiol., 2006, Byappanahalli et. al., Water Res., 2007, Englebert et. al., Sci Total Environ., 2008).

Our initial studies in the summer of 2007, funded by Pennsylvania Sea Grant, confirmed the presence of high levels of bacteria obtained from *Cladophora* samples floating in the beach waters of Presque Isle State Park. To examine this in greater detail, this study, also funded by Pennsylvania Sea Grant, examines the spatial and temporal variability in bacterial content present in *Cladophora* samples obtained from different beach waters of Presque Isle State Park. Our results demonstrate that the abundance and distribution of bacteria in *Cladophora* is dependent on the location and day that a sample was obtained. While showing spatial and temporal disparity, bacterial abundance in *Cladophora* remained closely correlated with bacterial abundance in the water, demonstrating a link between bacterial contamination in beach water and *Cladophora*.

Methods

Sample collection- Water sampling occurred as described in Standard Methods for the Examination of Water and Wastewater. Water was taken in duplicate from each site depicted in Figure 2. Duplicate *Cladophora* samples were obtained from pieces of free floating algae in the beach water with the exception of the last two sample dates. On these dates, free floating *Cladophora* could not be observed, and samples were taken from the surf zone instead. Each sample was placed in a sterile bottle and placed on ice until processing.

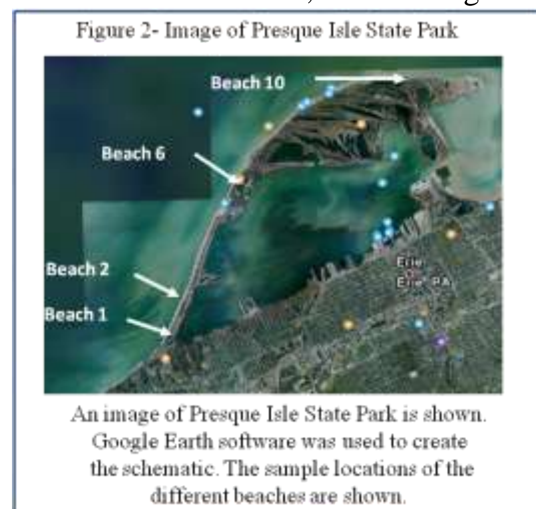


Figure 2- Image of Presque Isle State Park

An image of Presque Isle State Park is shown. Google Earth software was used to create the schematic. The sample locations of the different beaches are shown.

DNA isolation- Water samples were filtered onto 0.45 micron mixed cellulose filters as described in Standard Methods for the Examination of Water and Wastewater. Bacterial DNA was released and isolated from the filter following a procedure outlined in the Appendix of this report. *Cladophora* samples were isolated in a similar

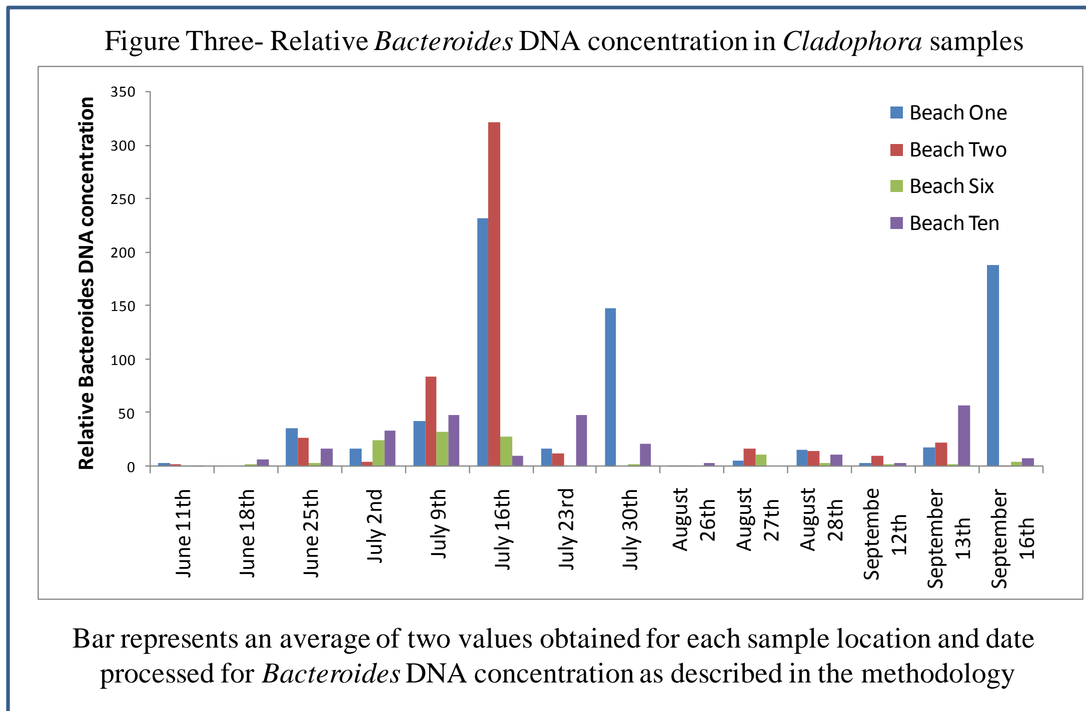
way, with the exception that bacteria was first released in a buffered saline solution from the *Cladophora* by agitation.

Quantitative PCR- The isolated DNA was analyzed for *Bacteroides* content using quantitative PCR using an Applied Biosystems machine and software using the procedure and conditions reported by Layton et. al. in Applied and Environmental Microbiology, 2006. All samples were normalized to a concentration of DNA obtained from a standard curve of positive controls. To test for human contribution to bacterial pollution, *Bacteroides* DNA primers that amplified bacterial DNA from human sources only were represented as a percent to amplified *Bacteroides* DNA that amplified from the same sample that originated from all possible sources.

Results

Bacterial content in Cladophora- We utilized *Bacteroides* DNA content to estimate bacterial abundance in *Cladophora* obtained from beaches 1,2,6,and 10 on days in June, July, August, and September of 2008. The results are displayed in Figure 3 and demonstrate a great deal of spatial and temporal variability in bacterial content in these samples. For example, beaches 1 and 2 had very high level of *Bacteroides* DNA present in *Cladophora* samples on July 16th compared to beaches 6 and 10, demonstrating that the separation of *Cladophora* samples by just a few miles can play a major role in the amount of bacteria that is harbored in a sample. Interestingly, beaches 1 and 2 did not always contain higher levels of bacteria compared to beaches 6 and 10, as evidenced by similar amounts of *Bacteroides* DNA present in *Cladophora* samples in June and August. Together, this data demonstrates that bacterial content in *Cladophora* is dynamic, and depends on the site and date a sample was analyzed.

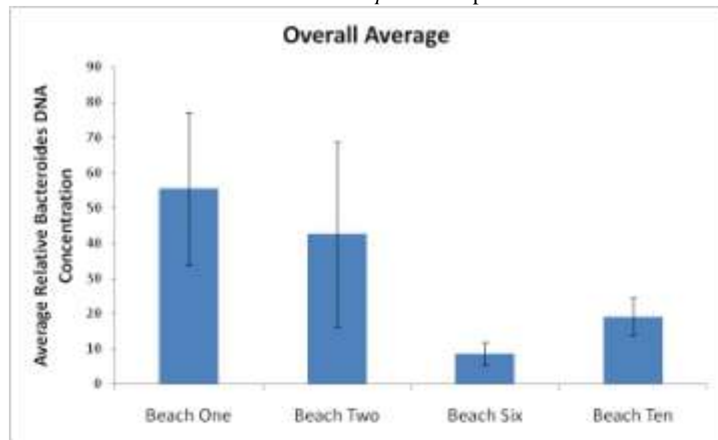
Figure Three- Relative *Bacteroides* DNA concentration in *Cladophora* samples



Bar represents an average of two values obtained for each sample location and date processed for *Bacteroides* DNA concentration as described in the methodology

While bacterial content in *Cladophora* exhibited a high degree of spatial and temporal variability, there was a trend of higher overall bacterial content present in *Cladophora* samples in beaches 1 and 2 compared to beaches 6 and 10. This is evident by comparing the averages of all days *Bacteroides* DNA content was analyzed in *Cladophora* samples (Figure 4). This suggests that beaches 1 and 2 are more heavily impacted by bacterial content compared to beaches 6 and 10.

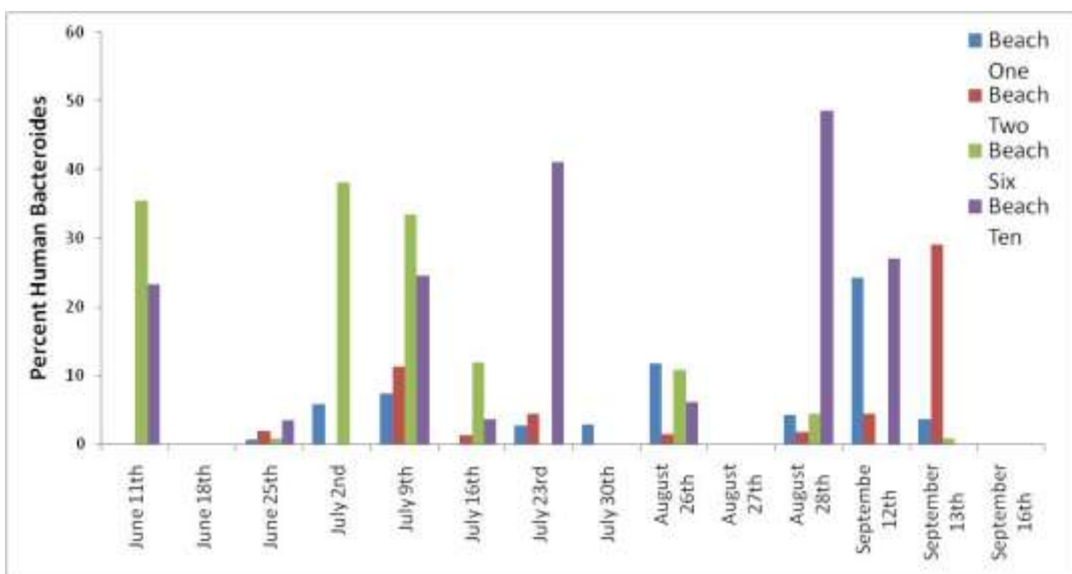
Figure Four- A comparison of the average relative *Bacteroides* DNA concentraiton in *Cladophora* samples



Value obtained on each beach by averaging together values obtained on all Sample days for that beach. Standard error bars are shown

Estimation of human dependent bacterial contamination in Cladophora- One of the advantages of utilizing *Bacteroides* DNA as a bacterial indicator is that it allows for an estimation of the *Bacteroides* DNA that arose from humans compared to *Bacteroides* DNA that have arisen from other sources. Using this approach, it then becomes possible to estimate the human contribution to bacterial pollution in a sample. We compared the percent human *Bacteroides* content in bacterial DNA isolated from the *Cladophora* samples we obtained (Figure 5).

Figure Five- Percent Human *Bacteroides* in bacterial samples isolated from *Cladophora*



Sample dates are provided for each of the beach locations where *Cladophora* samples were obtained. Percent human *Bacteroides* was determined as explained in the methods section.

On most days sampled, the estimation of human contribution to bacterial pollution present in *Cladophora* samples is low for all sample locations. For example, all samples analyzed on June 18th, June 25th, July 30th, August 28th, and September 16th exhibited a human *Bacteroides* DNA content below 10% of total *Bacteroides* DNA content. However, some beaches did contain higher percentages of *Bacteroides* arising from human sources. Interestingly, the sample locations that indicated the highest levels of human dependent bacterial contamination were beaches 6 and 10, as indicated in Figure 5 and the overall averages of percentage of *Bacteroides* arising from human sources depicted in Figure 6. Together, this data suggests that human dependent bacterial deposition has a greater impact on bacterial abundance in beaches 6 and 10 compared to beaches 1 and 2.

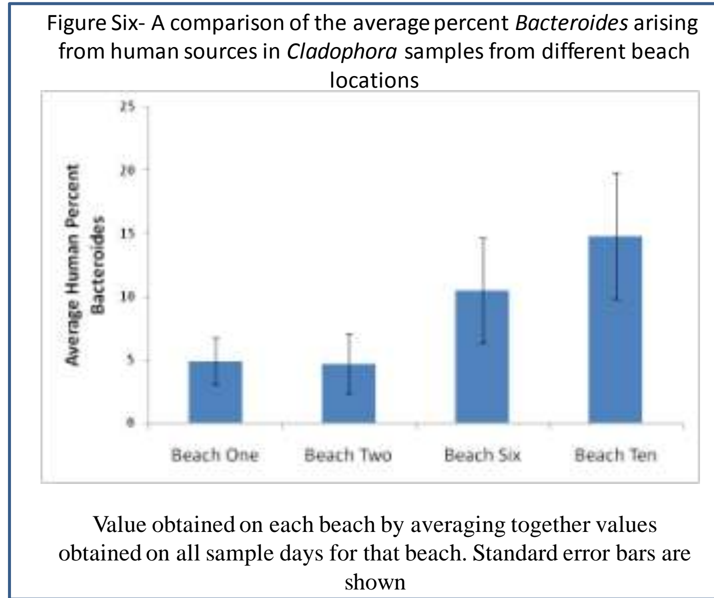


Figure Six- A comparison of the average percent *Bacteroides* arising from human sources in *Cladophora* samples from different beach locations

Value obtained on each beach by averaging together values obtained on all sample days for that beach. Standard error bars are shown

Association of bacterial abundance and content with beach water- To better understand the association that bacterial content has in *Cladophora* in comparison to the beach water in which a sample was taken, we directly compared *Bacteroides* DNA content in *Cladophora* samples to the beach water that sample was obtained from. The results are depicted in Figure 7 and indicate a close association with bacterial abundance in *Cladophora* to the bacterial abundance present in the water. This is also true for the percent of human *Bacteroides* DNA present in a sample (not shown), demonstrating that bacterial identity is also similar between *Cladophora* and the water sources it was obtained from.

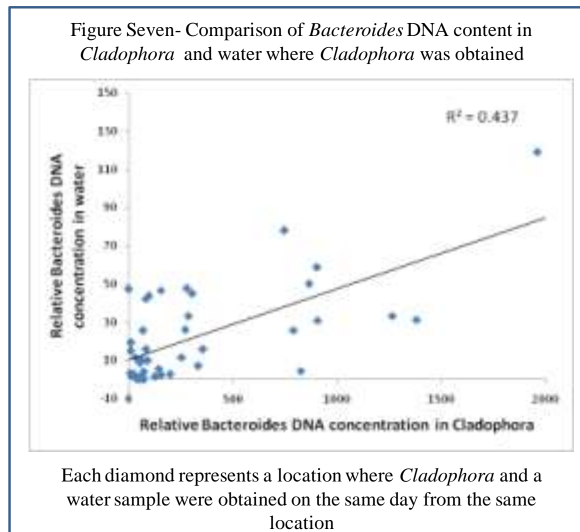


Figure Seven- Comparison of *Bacteroides* DNA content in *Cladophora* and water where *Cladophora* was obtained

Each diamond represents a location where *Cladophora* and a water sample were obtained on the same day from the same location

Discussion

The results of this study provides important insights into bacterial content in *Cladophora* samples present in the beach waters of Presque Isle State Park. Similar to studies done elsewhere (Whitman et. al, Appl. Environ. Microbiol., 2003, Olapade et. al., Appl. Environ. Microbiol., 2006, Ishii et. al., Appl. Environ. Microbiol., 2006, Byappanahalli et. al., Water Res., 2007, Englebert et. al., Sci Total Environ., 2008), we have established that *Cladophora* consistently harbors bacteria at Presque Isle State Park. Additionally, we have also shown that bacterial abundance in *Cladophora* samples varies according to their site and date located, with a bias towards higher abundances in samples obtained from the beaches on the west side of Presque Isle State Park. This finding may be of critical importance when considering that these beaches are traditionally those that are most heavily impacted by high levels of bacteria. The close association with bacterial abundance and content in *Cladophora* in comparison to beach water that a *Cladophora* sample was obtained further supports the idea that *Cladophora* is playing some role in bacterial presence at Presque Isle State Park beaches.

What remains unclear from this data is whether *Cladophora* is serving as a source, sink, or is not playing a role at all in the bacterial presence in the beach waters of Presque Isle State Park. A preliminary laboratory analysis that we have done on *Cladophora* samples indicates that this algae can either donate or accept bacteria from water depending on whether levels of bacteria in the water source is high or low. Thus, *Cladophora* appears to be in an equilibrium state with the water source in which it resides, donating bacteria to the water if bacterial levels in *Cladophora* are high, or accepting bacteria from the water if bacterial levels in *Cladophora* are low. The general movement of *Cladophora* from near the mouths of tributaries high in bacterial content west of Presque Isle to the beaches would suggest that *Cladophora* is acting as a source of bacterial content to the beach waters, depositing the high bacterial content it has accumulated as it traveled from the mouths of tributaries to the beach waters. We have been unsuccessful in thoroughly testing this idea, since we have not found it possible to monitor bacterial content in a single piece of *Cladophora* from its origin to Presque Isle State Park. However, we are in the process of performing DNA sequence comparisons of bacteria in *Cladophora* samples, tributary water, and beach water, which will allow for a bacterial fingerprinting approach to unravel the relatedness of bacteria present and consequently the interplay between these different sources of this aquatic ecosystem that are capable of harboring bacteria.

Appendix

DNA Isolation from water-Standard Operating Procedure

The day before

- Autoclave everything you will need, including collection bottles and filter units.
- Label all of your eppindorf tubes and place 0.3 grams of glass beads in each tube that will be used.

The day of

- Make plates if necessary
- Fill ice bucket and place all collection bottles so they are half-way submerged in the ice bucket and travel to sample site.
- To collect the sample, walk out into the area up to your waist, dip the bottle down to your knee, open the bottle, and collect water sample. For those of you sampling streams, go out to a deeper part and collect the sample half-way between the top of the water and bottom of the water.
- Place sample in ice chest so it is half way submerged in ice. Note the conditions and fill out the sheet for collection of that sample. Repeat this until all of the samples have been collected. All samples have to be back at the lab for processing in 3.5 hours!
- Once back in the lab, place a filter (gridded side up) in each of the filter units. Each time you touch a new filter, make sure you wash your forceps using an ethanol wash jar! Once all the filters have been placed, shake your sample vigorously and place 100mls of water in each of the filter units. Once all the samples have been placed, turn the vacuum on, and then slowly open the apparatus to allow the water to pass through.
- Once the water has passed through all of the samples, close the apparatus and then turn off the vacuum. It must be done in this order!
- Remove the filter and fold into quarters (gridded side out) and lightly place in the glass beads that you have filled with 600uL of TE. Don’t forget to wash the forceps with ethanol between each sample!
- If using the filters again, wash around the edges with approximately 20mls of distilled water and filter that through. Then replace filters on the filter units and repeat previous steps until all the samples have been finished.
- Once all the samples have been finished, flick each tube to equally disperse the TE/glass bead mixture across your filter. Vortex at full speed for 7 minutes.
- Remove the filter by scraping against the side of the tube. Spin the remaining solution at 5,000rpm for 1 minute in the centrifuge.
- Transfer 200ul of the solution to a new tube. If you don’t get 200uL, write this in your notebook.
- At this point, you can freeze your samples and continue to isolate another day.
- To continue, add 200uL of phenol:chloroform, shake to mix, and spin full speed in the centrifuge for 5 minutes.
- Transfer 125uL of sample to a new tube.
- To this sample, add 1uL glycoblue, 12.5uL 3M NaAcetate, and 425uL Ethanol that is ice cold.
- Spin for 20 minutes at full speed in the refrigerated microcentrifuge.
- Decant supernatant and resuspend in 50uL 10mM Tris. Take the absorbance of one or two of your samples to make sure you successfully isolated DNA.
- Place your tubes in a labeled box for later analysis with real-time PCR.
- Clean up your mess! Make sure all bottled are placed in the washing machine and washed.

Real time PCR (Standard Operating Procedure)

1. Fill out the 96 well or 16 well sheet so you can identify your samples. You will want to reserve 12 sites (6 sites done in duplicate) for controls. Each sample should also be run in duplicate with a dilution scheme (see number 4 below).

2. Make 2X Taq Buffer

   940uL of Taq Buffer

   20uL 25mM DNTP

   40uL Taq

- The amount above will be enough for a 96 well plate reaction (100 samples). If you do not plan on using all 96 wells, you should scale down the amount of Taq Buffer you make accordingly.
- Taq is a viscous solution. When you pipette, don't plunge your tip all the way down in the tube, or retract your plunger too rapidly. Each person will have their own tube of Taq. Taq should only be taken out of the freezer immediately before dispensing into tube and should be put back immediately.
- After DNTPs thaw, they must always be kept on ice!
- Mix all the contents by gently pipetting up and down in the tube a number of times. Try very hard not to get too many air bubbles!

3. Make master mix

   2X Taq Buffer= 10uL

   5uM Forward= 1.32uL

   5uM Reverse= 1.32uL

   10mM Tris= 0.96uL

   5uM fluorescent probe= 1.0uL

- When creating a master mix, add all of the above in a single tube and multiply by the total number of tubes you will be doing, but make sure you overshoot the total number for the master mix you are creating to compensate for volume loss during pipette transfer.

- Add the fluorescent probe to the master mix last. If you are using different primers and probes in the same reaction, you can just add them all together. If you are using different primers and probes for different samples, you have to create a master mix for that.
- Pipette up and down when finished, again being careful not to get air bubbles!

4. Distribute samples and master mix in tubes

- Place 15uL of master mix in each well according to the well sheet you filled out. As long as you are using the same master mix, you do not have to change tips each time.

- Place 5uL or 5uL of a 1:5 dilution (for the duplicate sample) of sample in each well that you are using according to the well sheet you filled out. When you place this in, pipette gently up and down a few times to mix the two solutions.

- After all samples are in their respective wells, cover with adhesive film. Start at one end and try to work your way across the plate without getting folds or bends in the film. When it is attached, seal all edges with a 15mL canonical tube bottom. If you are not going to do a full plate, you can use the caps instead.

- Balance the plate in the centrifuge and spin for a few seconds. To do this, turn the machine on, let it get up to 2200rpm, and then hit stop. That is sufficient to get the material to the bottom of the tube.

- Place the plate in the machine, and set up the run according to the protocol you plan on using (I will give instructions for this).

- Fill out a real-time run sheet, staple it to the well sheet, and place it in the real-time PCR folder.

- Run the real-time reaction according to the instruction manual to determine a CT value. The CT value that is equivalent to 235 *E.coli* colonies/100mls of water sampled is currently being determined.