Jamaica Bay Long-Term Control Plan
Ribbed Mussel (*Geukensia demissa*) Project

Phase I Experimental Set-up Final Report

October 2018

Hazen

Introduction

The New York City Department of Environmental Protection (NYCDEP) proposes to mitigate the influence of pathogens derived from combined sewer overflow (CSO) events in marine surface waters. The goal is to cultivate and deploy ribbed mussels that will provide bacterial filtration of the waterbody. Evidence in the literature shows that ribbed mussel (*Geukensia demissa*) are capable of filtering out particles as small as bacteria (< 1 micron at varying levels of efficiency) from the water column. It is hypothesized that ribbed mussel can filter *E. coli* and other fecal coliform bacteria commonly found in the water near wastewater treatment plants (WTPs) during and after CSO events.

The NYCDEP is addressing research priorities that are focused on quantifying the potential for ribbed mussel to filter coliform bacteria associated with CSO events. This project has been broken up into several phases beginning with laboratory-based trials (Phase 1) that will eventually scale up to mesocosm simulations (Phase II) and then field trials (Phase III). Each phase will target specific goals, and build upon the previous phases utilizing the information and data obtained. This report will summarize the first phase of this project which included the following four tasks: (1) a literature review, (2) preliminary cultivation of ribbed mussel and testing of gonadal maturation during the fall, (3) initial testing of deployment structures for ribbed mussel and monitoring the growth of fouling organisms and (4) experiments to determine and identify base-line information regarding the ribbed mussel filtration capabilities of *E. coli*.

Literature Review

A literature review was conducted to identify any research that has been previously conducted on ribbed mussel (*Geukensia demissa*) filtration of bacteria. This review can be found in Appendix A and is also the source from which all papers cited in this report are listed. Its primary purpose was to collect information regarding the ability of the ribbed mussel to effectively filter bacteria out of the water column, specifically species associated with WTPs. This review was expanded to include papers regarding: 1) the general biology and ecology of ribbed mussel, 2) aquaculture techniques of mussel species and other non-commercially cultivated species, 3) papers that focused on ribbed mussel filtration of bacterioplankton and other small particles and 4) papers that used specific experimental methodology for measuring filtration of bacteria and other small particles by various bivalves. Each section provided a brief summary of the most relevant facts from the papers regarding their significance to the overall goal of the NYC DEP. Tables were provided for each section listing the authors, title and journal from which each paper came. Overall, a total of 60 papers were compiled. The General Biology and Ecology section contained 20 papers, the Aquaculture section contained 12 papers, the Filtration section which focused on ribbed mussel filtration of bacteria contained 7 papers; further exhibiting the small amount of research that exists in this area, and the Experimental Methodology section contained 10 papers. There was also another section containing 11 papers that were thought to contain relevant information prior, but were ultimately deemed less pertinent. These were also included in the literature review, however, no summary about them was provided. While the literature search showed various studies that demonstrated the ability of ribbed mussel to filter bacteria size particles, only one specifically used *E. coli* bacteria (Bernard 1989). This study looked at the filtration of *E. coli* by four species of shellfish and provided some useful baseline data that our study can be compared too. Although ribbed mussel was not

one of the species used, the study did use blue mussel (*Mytilus edulis*), which is closely related and shares similar physiological traits including filtration capabilities (Wright *et al* 1982). It did not, however, address the more complex questions that the NYC DEP is interested in, rather just determined the elimination of *E. coli* by the shellfish species' tested nor did it use flow cytometry which provides a more accurate detection of bacteria cells.

Aquaculture of Ribbed Mussel

There is very little known regarding the conditioning, spawning and cultivation of larval and juvenile ribbed mussel. The only two studies found in the primary literature regarding ribbed mussel cultivation were developed at the Rutgers University Aquaculture Innovation Center (AIC), the Bin-Silo Method (Landau 2014) and a poster presented at a conference (Jones *et. al* 2013). There has been minimal interest in the cultivation of this species primarily because it had no commercial value. However, with the recent interest in utilizing ribbed mussel for living shoreline, bio-extraction, and wetland restoration applications, there is an increased need for supply; in particular to protect the removal of ribbed mussel from healthy wetlands that would negatively impact the marsh. Developing basic and reliable culturing methods is critical to providing a supply of ribbed mussels to projects.. We have begun to explore some of these in Phase 1 of this project.

Broodstock ribbed mussel were collected (n = 100) the last week of July 2018. Animals were inspected for gonadal maturation and they appeared to have minimal development, indicating that they had already spawned at this point in the season (*i.e.* natural spawn in the wild). Since, we were unable to use these un-ripened adults to spawn in the hatchery at this time, we attempted to re-condition them and test if it is possible to successfully produce a secondary spawn. The animals were separated into groups of 8 to 10 and placed inside plastic planter pots (Figure 1) where they would remain throughout the conditioning and spawning periods. This allows them to be handled and transported with minimal disturbance or destruction of their byssal threads. Two batches of 30 animals (broodstock) were placed inside a 100 gallon conditioning tank with water maintained at a constant temperature of 20°C. One broodstock batch was placed inside the conditioning tank two weeks after the first. This would allow for the first batch to be spawned, raised in the conicals and moved to downwellers in time for the second batch to be spawned. The broodstock was fed a steady diet of cultivated algae (*Tetraselmis* spp., *Isochrysis galbana* and *Pavlova lutherii*) for 6 - 8 weeks to condition them and allow their gonads to mature. The broodstock tank was drained, cleaned and refilled with filtered (1µm) seawater at 20°C every other day.

Figure 1. Ribbed mussel maintained in plastic planter pots during conditioning and spawning periods. This reduces the effects of handling related to byssal thread disturbance.

Under typical hatchery settings, this conditioning period takes place during the winter months usually starting in January or February for other species such as clam (*Mercenaria mercenaria*) and oyster (*Crassostrea virginica*). This time period is preferred because it allows for a longer growing season for the cultured species, therefore increasing the chances of survival once the animal has been released into the field. Our conditioning period for this study, however, started in the first week of August. After 6 weeks of conditioning, the first batch of broodstock was inspected for gonadal maturation. Visual inspection on September 14th 2018 revealed significant maturation of gonads (Figures 2 and 3) and the animals were prepared for spawning the following week. This was actually a key piece of information that was yielded from this study, as it was uncertain whether ribbed mussel could be re-conditioned later in the season following their natural spawning cycle. It will allow for spawning of ribbed mussels to be done throughout the year, which will assist with future cultivation practices. Other species such as clam and oyster have been known to be re-conditioned after undergoing their natural spawn but when they are re-conditioned, it is typical for the animals to not produce as large a spawn as they may have earlier in the season (G. Rivara, Cornell Cooperative Extension, pers. comm.). This would likely be similar for the ribbed mussel as well.

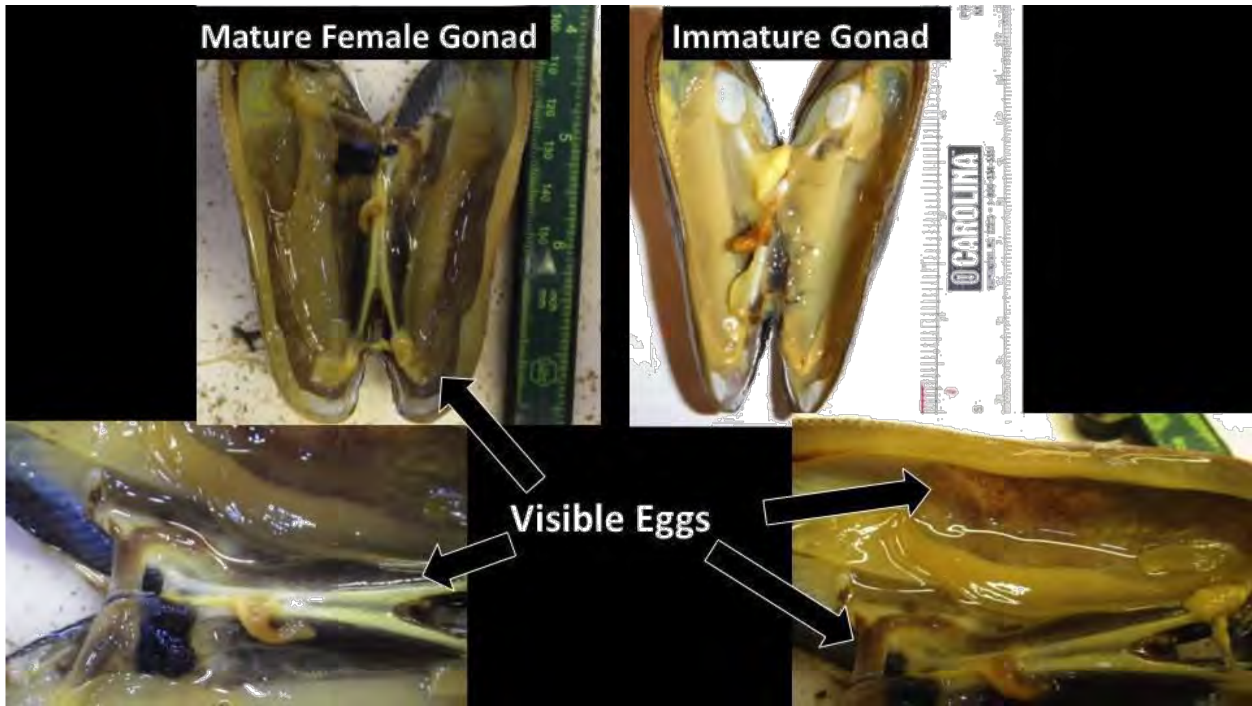


Figure 2. Photos of female ribbed mussel with eggs visible within the tissue compared to an animal with immature gonad tissue.

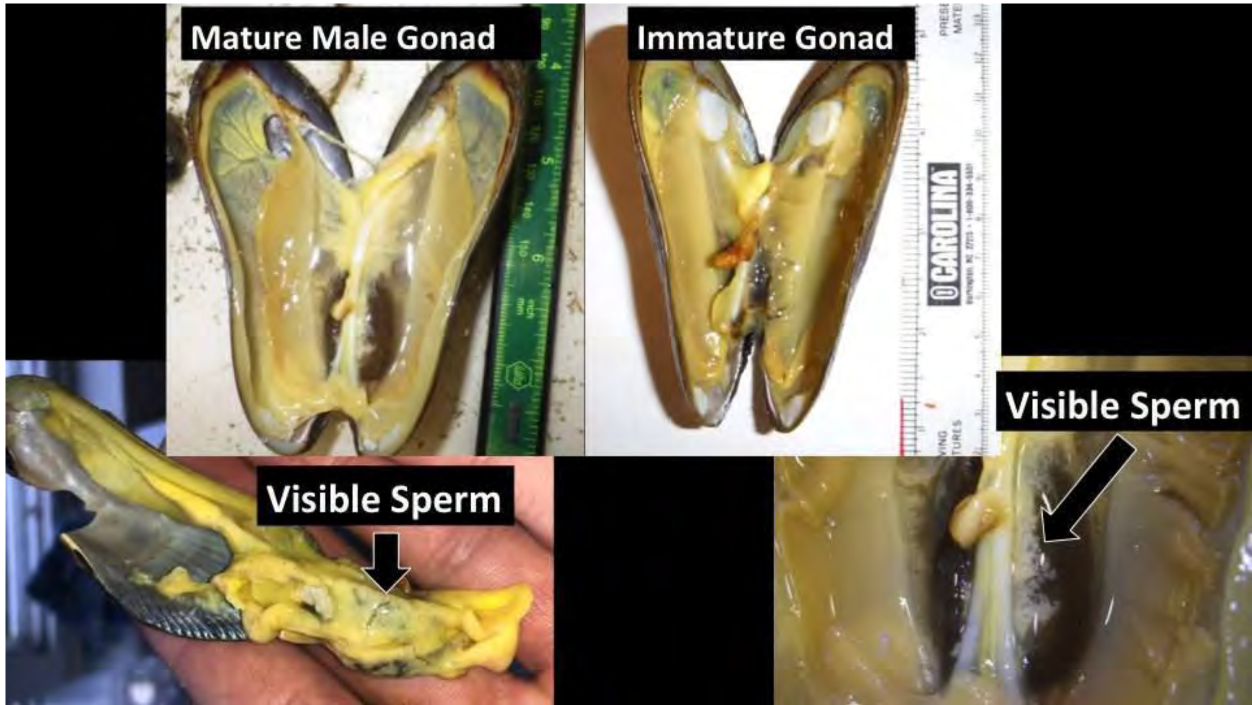


Figure 3. Pictures of mature male ribbed mussel with visible sperm observed within its tissue compared to an animal with immature gonad tissue.

Previous cultivation attempts by CCE and others (*e.g.* Rutgers University Aquaculture Innovation Center (AIC) and Martha's Vineyard Shellfish Group) have not had success spawning ribbed mussel using traditional methods (*i.e.* thermal cycling) that are used for clam and oyster. Loosanoff and Davis (1963) had the same issue, but found that the ribbed mussel would spawn without artificial stimulation in their holding tank overnight. The Bin-Silo Method, a passive system designed for spawning and larval collection, was developed by Landau (2014) at Rutgers AIC and has been used as a successful technique for spawning ribbed mussel. Figure 4 shows an image from the Landua (2014) study as well as a cross cut image of this set up that CCE used to spawn the animals. The broodstock in our study were placed inside a Bin-Silo containing filtered (1µm) seawater, and held overnight at 30°C. There were two successful spawns that yielded approximate total of 900,000 D-stage larvae (Figure 5) and were transferred after each spawn from the Bin-Silo into conicals.

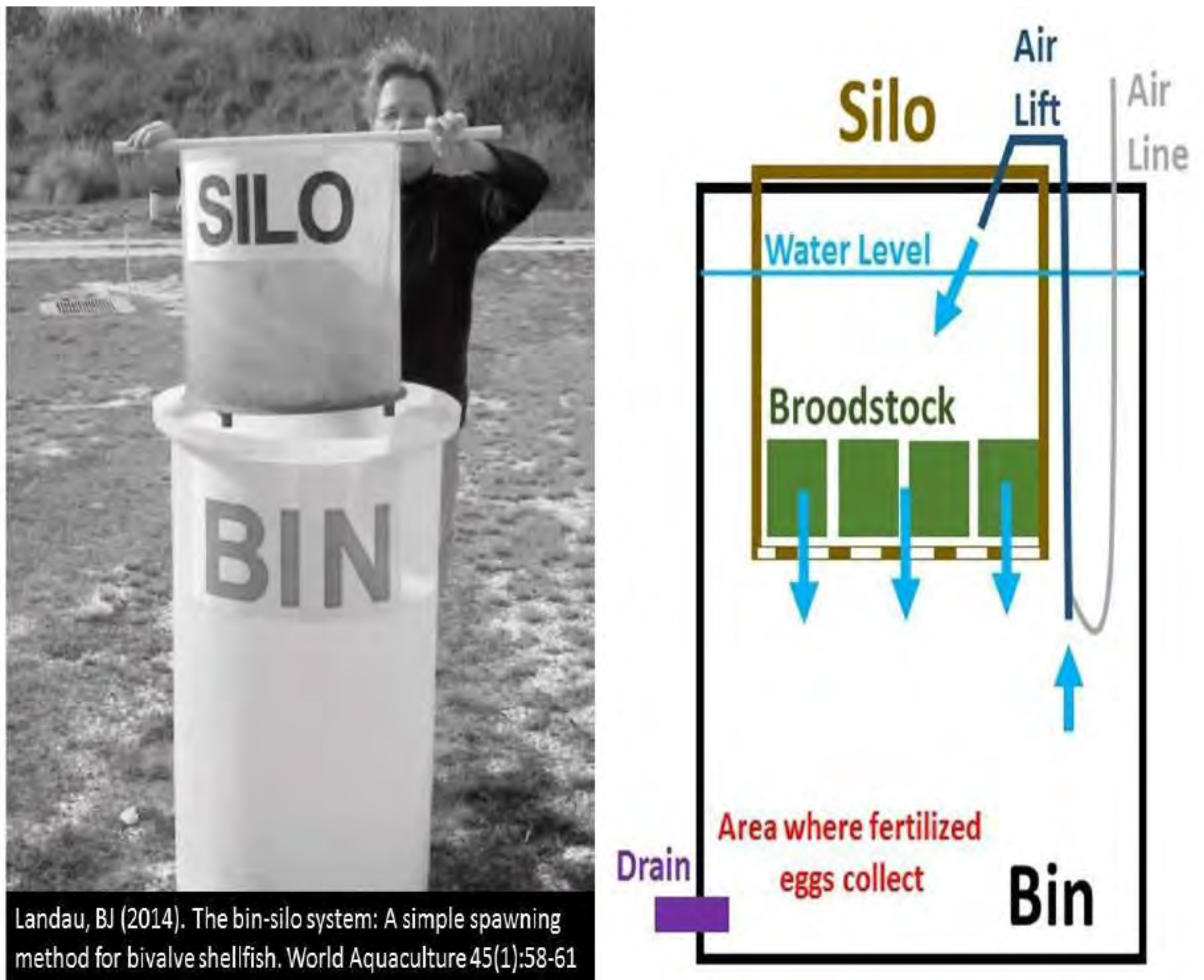


Figure 4. Photo from Landau (2014) and cross cut view of the Bin-Silo used by CCE. The air lift provides a flow of water that forces the sperm, eggs and ultimately fertilized eggs to gather in the lower area of the "Bin". This reduces the chances of eggs being inadvertently consumed by the broodstock animals undergoing their natural filtration of the water.

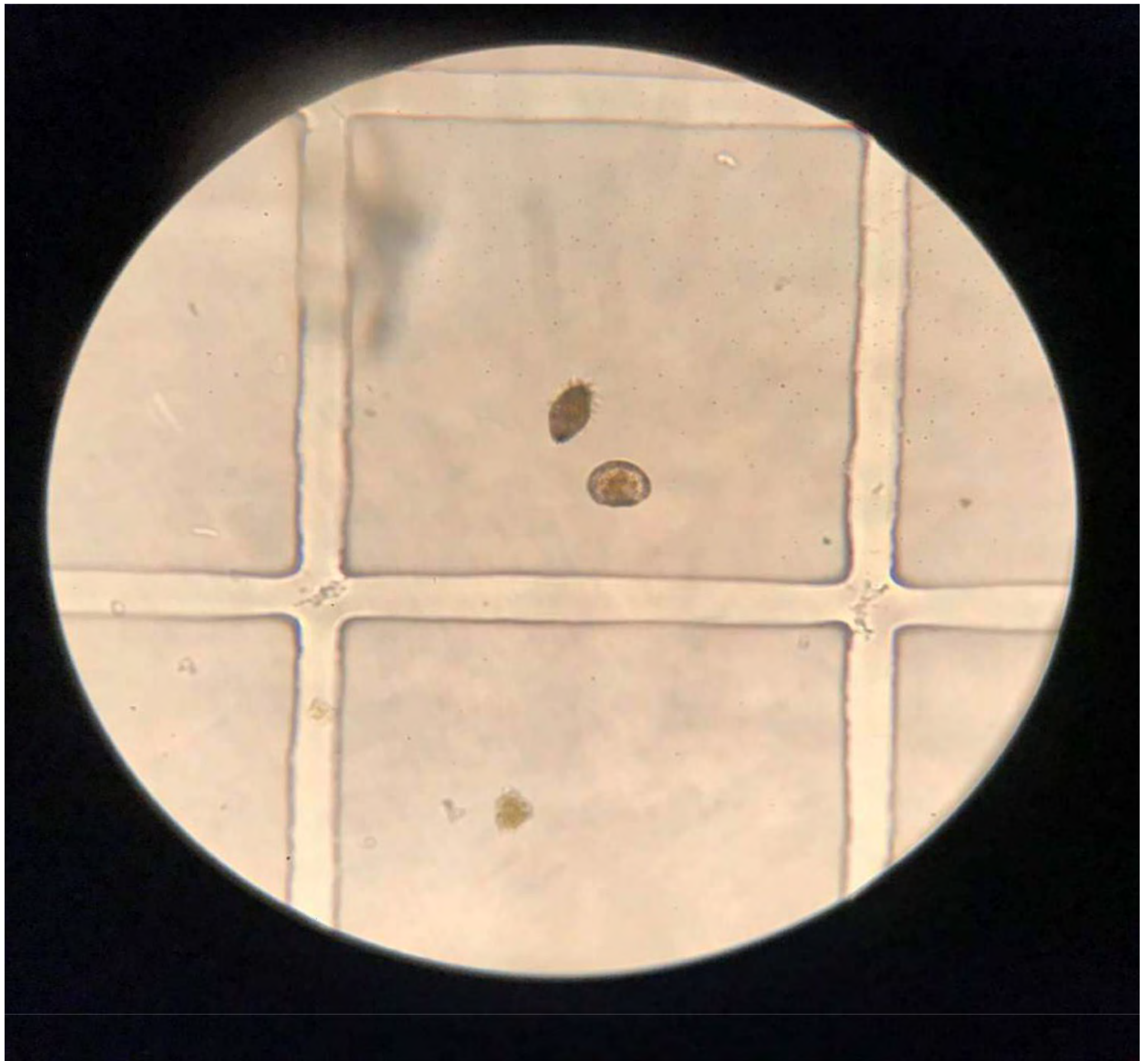


Figure 5. Photo of D-Stage ribbed mussel larvae (74-90μm in size) cultivated in this study at magnification of 400x.

The larvae were transferred to 100-gallon conical shaped rearing tanks where they were held for 2 weeks. These conicals are static tanks (Figure 6) that contain filtered (1μm) seawater heated to 22-24°C. While in the conical stage, the tanks were drained, cleaned and refilled on the same schedule as in the conditioning period. The larvae were fed a steady diet of cultivated algae (*Isochrysis galbana* and *Pavlova lutherii*) ad libidum for about 2 weeks until they developed an eye-spot and metamorphosed. This is referred to as the "setting stage".

Once the animals reached the setting stage, they were moved to downwellers. This stage involves PVC rings used as silos with a small mesh on the bottom containing the animals, but allowing water to pass through. As the animals grow in size, they were moved up to larger mesh sizes (range of mesh sizes used: 115 ∝m – 275 ∝m, see Figure 5). The downwellers were held in a static 150 gallon tank and receive the same drain down and cleaning routine as in the conditioning and conical stages. Another challenge in the cultivation of ribbed mussel is that there is a consistent and dramatic loss of larvae during the conical stage, causing reduced numbers of larvae to ultimately reach the setting stage. Approximately 21,500 animals reached this stage (>115 ∝m), which was approximately 97.6% mortality over 1 month. We typically see 75-90% mortality at a similar stage of development in oyster larvae. Further research is needed to determine if there are better methods to reduce mortality rates at this stage of culturing. The surviving cohort will remain on downwellers as long as possible until they can be moved to ambient water where they will remain for the winter period in flow through tanks.

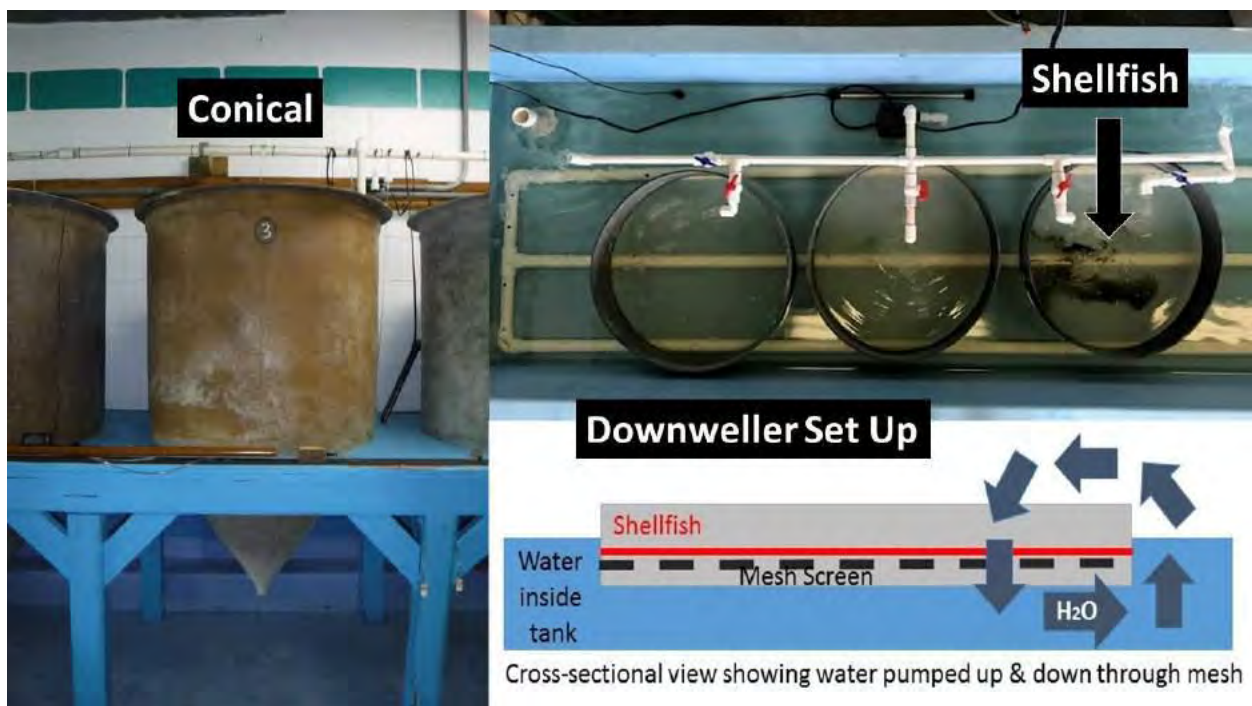


Figure 6. 100 gallon conical shaped tank (left) used for hosting the shellfish larvae where they will remain (approximately 2 weeks) until they metamorphose and reach the “setting stage” when they are transferred to downwellers (right). Shellfish can remain on downwellers for 2-6 weeks or until they are able to transition to ambient water temperatures.

Structure Testing

To accomplish to goal of the NYC DEP, the plan is to deploy ribbed mussel near a CSO outfall.. This will require the ribbed mussel to be installed in, or attached to some sort of structure that can be placed in the desired location. Several methods have been considered including concrete structures such as oyster reef castles, gabions and oyster cages, shown in Figure 7.

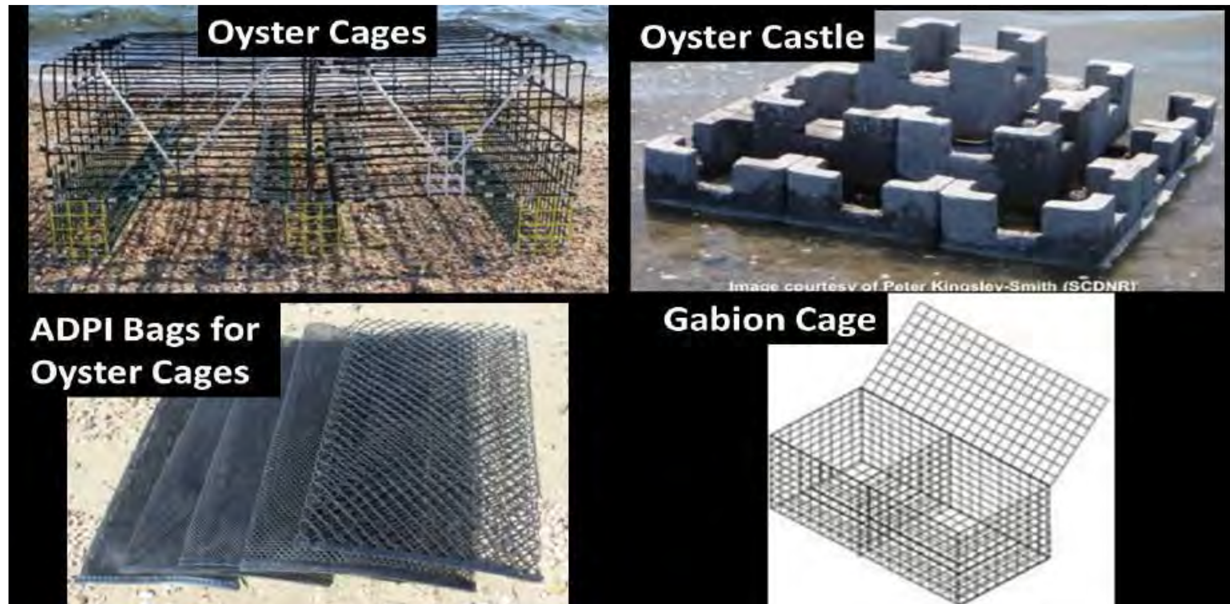


Figure 7. Potential structures to be used for ribbed mussel deployment included: oyster cages, oyster castles, and gabion cages. ADPI bags would be inserted into the oyster cages and lay flat, each cage receiving at least 3 bags.

The oyster castle method has had success in other areas, but primarily because they are designed specifically for oyster, which cement themselves to hardened structures and will remain there for life. The ribbed mussel will be cultivated in a hatchery and grown until they reach a size of at least 15 mm (3/8 in) before they will be deployed using the selected structure. Using a structure such as the oyster castle or concrete blocks will pose logistical problems since the structures should be deployed with the mussel already attached. To have the mussel attach to concrete blocks, the blocks would need to be submerged into seawater tanks and then the ribbed mussel added to the tank and then allowed time to attach themselves to the blocks via their byssal threads. The blocks would then need to be removed from the tanks and transported to a project site. Due to the inability to control whether or not the animals would attach to the concrete blocks and the complexity that would exist to remove these blocks from the site if needed, it is recommended to use something more portable such as the oyster or gabion cages.

Oyster and gabion cages were ordered (n = 3 of each) to test deployment ability and also to monitor the growth of fouling organisms. The gabion cages that were ordered and recommended by the supplier turned out to be insufficient for our needs. The material was a flexible plastic and designed to be filled with rock or other material to maintain its shape and therefore could not be used for these trials. The correct style needed could not be obtained within the timeframe of this phase of the project and will therefore be examined more closely in phase II of the project.

Only the oyster cages were used for the monitoring of fouling organisms in this phase. The oyster cage frame is made of heavy-duty 8-gauge marine grade vinyl coated wire (similar to lobster traps). The openings on the cage are 4.5 in and have feet made of the same material but with a smaller opening size (1.5”) for greater strength. These are commonly used in the aquaculture industry and have been observed to last for over 6 years in the water with no sign of deterioration. Cages have 3 tiers that can each hold at least 1 grow bag or ADPI bag. These are made from rigid polyethylene plastic, are UV resistant and will last for great lengths of time without breaking down.

As an initial pilot test of this concept, CCE deployed a cage during the last week of July 2018, prior to the start of the project. Ribbed mussel were collected from a nearby marsh, placed inside ADPI bags and put into the cage that was chained to a dock piling. This location, at CCE’s shellfish grow-out facility in Huntington, NY is representative of the many city wetland habitats. It is located on the north shore of Long Island and experiences large semi-diurnal tidal fluctuations with amplitudes of greater than 6 ft. The oyster cage was placed in a location where it would be under at least 2 ft of water at high tide and completely exposed at low tide. It was also placed within the shade of a dock to reduce the effect the sun would have on the fouling organisms to potentially promote as much growth as possible. Weekly photographs were taken of the cage to visually document growth of the fouling organisms. These pictures are contained in Appendix B. Figure 8 shows the condition of the cages at the start and at week 15 in November 2018. Figure 9 shows the same cage with photos taken at various weekly intervals. The oyster cages that were ordered were not received until the third week of September 2018. At that point, it was quite apparent there was minimal (if any) growth of the fouling organisms so only one more cage with ribbed mussel was deployed rather than using all three. The animals contained in the oyster cages were checked closely from time to time to ensure that they were still alive, and this was confirmed. Based on these observations, it is likely that there will be minimal fouling of cages deployed at a project site. The caveat to this aspect of the project was that these cages were not exposed to a full year of growth including the spring and early summer, so it is possible that more fouling organisms may grow at those time periods than was observed here. It is likely, however, that given the nature of the tidal fluctuation at a project site, being exposed at low tide for approximately half the day, and being in direct sunlight will greatly reduce the growth of fouling organisms on whatever form of cage that is used. These cages will be deployed again in the spring of 2019 to continue to track the growth of fouling organisms throughout the entire course of the growing season.

A total of 2.5 million ribbed mussel are estimated to be necessary for deployment. Each oyster cage can hold at least 3 ADPI bags and be stocked with approximately 1,000 animals (3,000 per cage). This will require 834 oyster cages to be deployed at the site to reach the filtration goals of NYC DEP’s proposed project. The size of the mussel at the time of deployment will affect the quantity that can fit inside each bag and also whether or not more than 3 bags can be placed inside each cage. Due to the issue with the gabion cages, it is still uncertain which of these two units may be the preferable structure for field deployment. Once the gabion cages are received in early 2019, a comparison will be done. Both structures are very similar and the concept for both is that the animals would be placed inside ADPI bags and then inside one of the cages. The degree of fouling should not change between the structures; the question is more about what unit holds a greater amount of ribbed mussel and which structure will be the most durable over time.

Figure 8. Oyster cage on left represents time zero and the same cage on the right is at 15 weeks later. Evidence of fouling organisms is minimal and animals inside the cages remained alive during this time period.

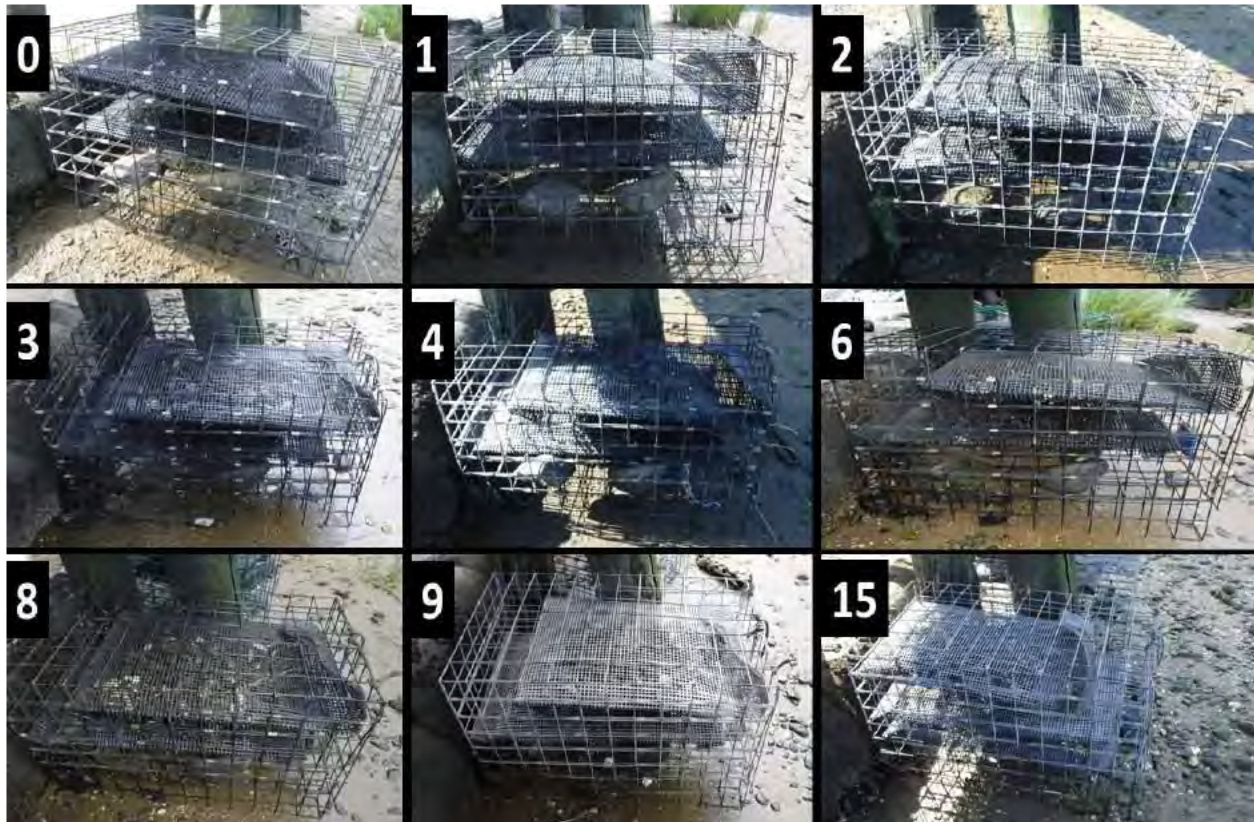


Figure 9. Photos of the same cage and viewpoint from start of deployment (0) though 15 weeks.

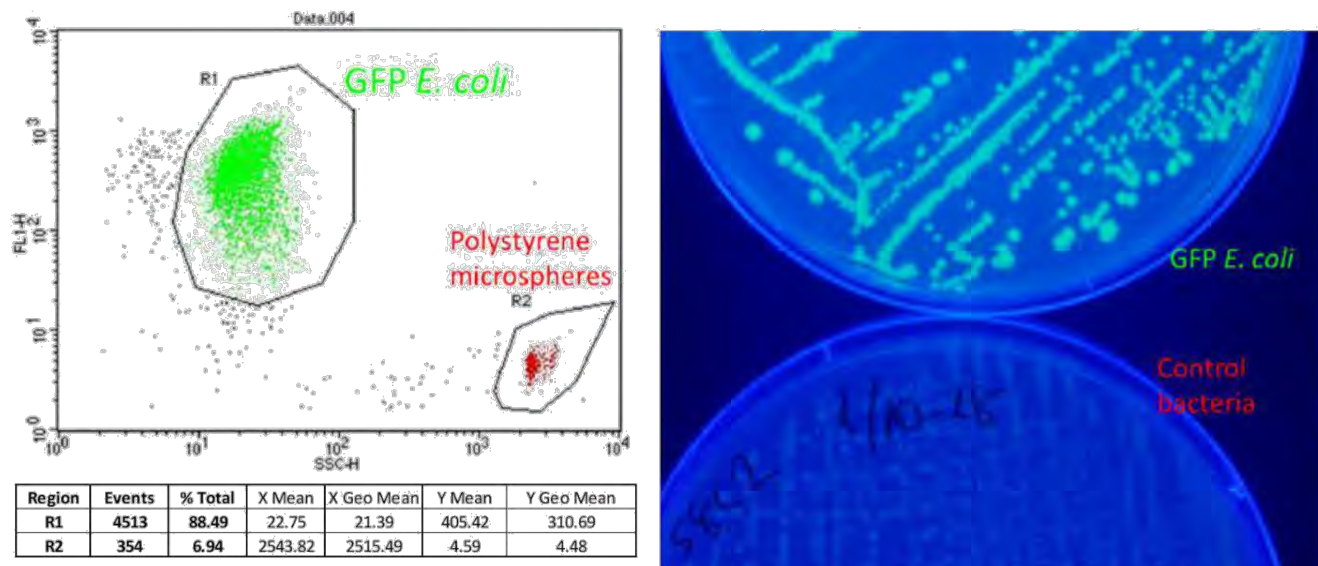
Laboratory Experiments

Handling of Ribbed Mussel

The Phase 1 experiments focused on determining the filtration of *E. coli* bacteria by the ribbed mussel using small-scale laboratory experiments to identify base-line levels of clearance rates. Ribbed mussel were collected from a salt marsh in Huntington, NY which is located on the north shore of Long Island and represents a similar habitat to that found in many city wetlands. At the end of July, the animals were placed in ADPI bags inside a metal cage that was kept within the intertidal zone. Prior to each experiment, animals were removed from the ADPI bags, measured (size range: 65.2 – 73.8 mm) and scrubbed clean with a brush to remove any debris or fouling organisms (*e.g.* barnacles). The animals were then transported to the lab at Stony Brook University and held inside a small aquarium tank with seawater filtered to 0.2 µm and sterilized via UV bulb for 24-48 hours without being fed to purge any residual contents within their digestive system. Prior to placement in the acclimation tank, the ribbed mussel had a piece of Velcro (<1 sq. inch) superglued to its shell so they could be attached to a stationary piece of plexiglass that would maintain the mussel above the bottom of the experimental tanks. Animals were removed from the acclimation tank, wrapped in a wet paper towel and left out overnight (12-16 hours) prior to the next morning's experiment. The purpose of this was to increase the chances of the ribbed mussel to open up and attempt to feed once placed into the experimental tanks; and is a technique that is commonly used in experiments of this nature.

Bacteria Culture

Bacterial clearance was assessed using *E. coli* (ATCC 25922GFP) containing a plasmid that codes for a green fluorescent protein (GFP). There are several major advantages for using this bacteria. The GFP tag allows for an easy detection of the bacteria using both flow cytometry and culture methods (Figure 10). The GFP plasmid also codes for ampicillin resistance making this strain grow on a culture media that contains ampicillin, which inhibits the growth of other natural bacteria, further facilitating quantification. All bacterial cultures were made on Luria-Bertani (also known as lysogeny broth) agar plates (VWR 25384-342), supplemented with 100 mg/L ampicillin (sodium salt) (VWR 101174-208, CAS 69-52-3), and incubated at 37ºC for 24-48 hr. Prior to the start of each experiment, bacterial colonies were scraped from the plates and suspended in sterile-filtered seawater. A spectrophotometer was used to measure the optical density of the suspension. Based on the optical density, the number of colony-forming units (CFU) per milliliter was estimated based on a standard curve established earlier using known bacterial counts. The volume of the bacterial suspension to be added to each experimental tank was then calculated for the different experiments. Overall, we targeted approximately 25,000 CFU per mL in each experimental tank at the beginning of each experiment, and bacteria were added subsequently by pulses (Experiment 1 and 3) or by continuous drips (Experiments 2 and 3) to each experimental beaker (see details below).



Region	Events	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	4513	88.49	22.75	21.39	405.42	310.69
R2	354	6.94	2543.82	2515.49	4.59	4.48

Figure 10. Flow cytometry (left) and culture-based (right) identification of GFP E. coli is made easy by the fluorescence signal emitted by this bacteria. In the left panel, the x-axis represents side light scatter (indicative of particle size and complexity) and the y-axis represents green fluorescence signal. The flow cytometry allows instantaneous determination of the counts and optical characteristics of different particles in a given sample (table, bottom left).

General Experimental Design

We used twelve, 2 L replicate beakers (experimental tank) containing 1600 mL of seawater that was filtered to 0.2 µm and sterilized via UV bulb. Flow cytometry indicated that the water was well filtered as no particles were observed in the bacterial size range. The beakers were placed on a magnetic stir plate and each contained a magnetic stir bar that would allow the water to move and keep the contents of the water suspended. The mussels were given 15-30 min to acclimate in each beaker prior to the addition of bacteria to allow them to open up before the start of the experiment. Cultured GFP *E. coli* and polystyrene microspheres, 5 to 6µm in size (size range that ensures 100% capture efficiency by the gills; used here to assess mussel filtration rate), would be added at known concentrations to the desired beakers during the experiments. New stock solutions of the cultured bacteria were made each day

The laboratory trials had three treatments each with four replicates: (1) Experimental - live ribbed mussel with cultured GFP *E. coli* and polystyrene microspheres, (2) Control A - live ribbed mussel with no bacteria (only microspheres), and (3) Control B - empty ribbed mussel shell with cultured GFP *E. coli* and polystyrene microspheres. The empty ribbed mussel shells were of similar size to the live animals used and were super-glued shut so as to replicate the displacement and location of the living mussels. The purpose of Control A was to provide a reference for filtration capabilities of the mussels which would have been filtering just the polystyrene microspheres and to show whether or not natural levels of bacteria may have been introduced by the ribbed mussel themselves. The purpose of Control B was to identify whether or not the bacterial loads and microcapsule concentration in experimental containers change over time as a result of particle settling and bacteria natural multiplication or decay, if any. The treatments were randomly assigned to a beaker each day (trial) throughout the experiments. This experimental design was replicated 3 times for a total of 12 replicates for each treatment. However, we elected to modify this design after the first experiment to optimize the number of

treatment replicates. Specifically, Controls A and B were reduced to 2 replicates each (per day), and Experimental increased to 8, which enabled the Trials to run for two days instead of three (with similar statistical power for replication). All mussels, both living and empty shells were attached via Velcro to a 25 mm wide x 80 mm long piece of plexiglass that was held to the rim of the beaker via a clothespin. This kept all the mussels a fixed distance (approximately 40 mm) above the bottom of the beaker and completely submerged during the experiment (Figure 11).

The clearance of the *E. coli* and the microspheres was calculated by measuring the exponential reduction of the particles in the beakers with a known volume of water (V, in liters) using the formula: $F = V/t \times \ln C_0/C_t$, where t is the time in hours and C_0 and C_t are the particle concentrations at time 0 and t. This method is commonly used in experiments of this nature and was used by Riisgard (1988) and Wright *et al* (1982) as well as other studies Dr. Allam and Dr. Espinosa have conducted. For the continuous and continuous + pulse experiments (2 and 3) we accounted for the different initial concentrations at each time interval and the change in volume of the beaker.

Figure 11. Experimental layout, with 12 beakers on magnetic stir plates each containing 1 mussel (either live or empty shell) attached via Velcro to a plexiglass strip held to beaker with a clothespin.

Each beaker contained 1 mussel (live or empty shell) and the experiment would run for 6 hours from time zero (T0), which was when bacteria and beads were added. At T0, a 100 μL sample was taken from each beaker and plated on an LB agar plate supplemented with ampicillin and allowed to incubate for 24 hr. at 37°C before counting. This was also done at the end of the experiment to provide a comparison between the flow cytometer measurements of bacterial counts and CFU measurements. During the first experiment, it became apparent that there was fluctuation of mussel opening and closing, so we began documenting this as well. At the end of the experiments, the tissue was removed from the living ribbed mussel and placed in a drying oven for at least 48 hr. to determine the clearance rates per gram of tissue. The temperature of the water was not controlled during these experiments and was affected by the ambient air temperature within the laboratory. This was monitored however, since it did fluctuate during the course of each experiment, starting off cooler and then warming up. A total of 3 experiments were performed during this first phase: (1) pulses of bacteria, (2) continuous flow of bacteria and (3) continuous flow of bacteria plus additional timed pulses.

Experiment 1 – Pulse

This experiment was conducted over the course of 3 days, October 11th, 12th and the 15th (1 experimental trial per day). Table 1 shows the breakdown for each trial detailing the amount of bacteria and beads added, the temperature fluctuation and the number of mussels open at designated times. Each trial followed the same sampling protocol. Mussels in the Experimental beakers (n = 4) and Control B beakers (n = 4) received 40 x 10^6 CFU *E. coli*/mL (equating 25 x 10^3 CFU/mL) and all beakers (total of 12) received 4 x 10^6 microspheres/mL (equating 2.5 x 10^3 microspheres/mL). A 100 μL sample was taken at T0 from the Experimental and Control B beakers and plated for CFU counts, as well as a 500 μL sample from all 12 beakers for the flow cytometer. Samples (500 μL) were subsequently taken every 15 min from each beaker and measured using the flow cytometer. At hour 1, a sample was taken for the flow cytometer, then another pulse of bacteria (40 x 10^6 CFU) was added to each of the 8 Experimental and Control B beakers and then another sample was immediately taken for the flow cytometer. Sampling continued every 15 min until hour 2 where the procedure from hour 1 was repeated along with the addition of polystyrene microspheres. Sampling continued every 15 min until hour 3. At that time, no additional pulses of bacteria or beads were added and sampling was reduced to every 30 min after hour 3. This was done so that the decay curve of the bacteria loads could be measured. At hour 6, one last sample was taken for the flow cytometer and a 100 μL sample was taken and plated again for CFU counts.

After the first two days of experiments, the data regarding the controls was quickly analyzed to determine if they were behaving as expected. Since there were no flow cytometry signals from beakers not added with GFP *E. coli*, and no significant settling or decay from controls with empty shells, we reduced the two controls (A and B) to 2 replicates each. This allowed for more experimental mussels to be used and additional experiments to be reduced from 3 days to 2 days, yet still achieving the desired number of experimental replicates. This was also done to compensate for reduced number of mussels open during the experimental trials. This changes the number of potentially open experimental mussels from a maximum of 4 to 8.

Table 1. The amount of *E. coli* (CFU) and polystyrene microspheres added during the experiment, temperature, number of ribbed mussels that were open and the number of samples taken for the flow cytometer during the corresponding hour for Pulse Treatment.

Day	Time	*E. coli*	Beads	Temperature (°C)	Mussels Open	Samples Taken
1	0	40×10^6	4×10^6	23.2	5	4 (every 15 min)
1	1 hour	40×10^6	0	24.3	8	4 (every 15 min)
1	2 hour	40×10^6	4×10^6	25.2	8	4 (every 15 min)
1	3 hour	0	0	25.4	8	2 (every 30 min)
1	4 hour	0	0	25.7	8	2 (every 30 min)
1	5 hour	0	0	25.9	8	2 (every 30 min)
1	6 hour	0	0	26.0	8	1 last sample
2	0	40×10^6	4×10^6	23.3	6	4 (every 15 min)
2	1 hour	40×10^6	0	23.6	6	4 (every 15 min)
2	2 hour	40×10^6	4×10^6	24.5	Not recorded	4 (every 15 min)
2	3 hour	0	0	24.9	Not recorded	2 (every 30 min)
2	4 hour	0	0	25.2	Not recorded	2 (every 30 min)
2	5 hour	0	0	25.4	Not recorded	2 (every 30 min)
2	6 hour	0	0	25.7	Not recorded	1 last sample
3	0	40×10^6	4×10^6	17.2	6	4 (every 15 min)
3	1 hour	40×10^6	0	18.0	6	4 (every 15 min)
3	2 hour	40×10^6	4×10^6	19.4	Not recorded	4 (every 15 min)
3	3 hour	0	0	20.4	Not recorded	2 (every 30 min)
3	4 hour	0	0	Not taken	Not recorded	2 (every 30 min)
3	5 hour	0	0	21.5	5	2 (every 30 min)
3	6 hour	0	0	22.2	Not recorded	1 last sample

Results – Experiment 1: Pulse

In this experiment, the difference in bacterial levels is clearly discernible between experimental and control beakers (Figure 12). The levels measured in Control B (empty shell) show a steady and continuous level of *E. coli* due to zero filtration (after the increase from each pulse), and the Control A beakers (no bacteria) show zero levels. The experimental beakers demonstrate a strong decrease (approximately 50%) in the *E. coli* within the first 60 min after each pulse. At the conclusion of the experiment, the Control B levels reached an average of approximately 13,000 bacteria cells per 60 µL, while the Experimental beakers had an average of approximately 1,550 bacteria cells per 60 µL, which is a difference of approximately 838%.

Appendix C contains the graphs showing the results from each day this experiment was conducted. These graphs also show the interindividual variability seen each day. Variations can be attributed to size of the ribbed mussel and whether or not the animal was opened or closed at the various time intervals.

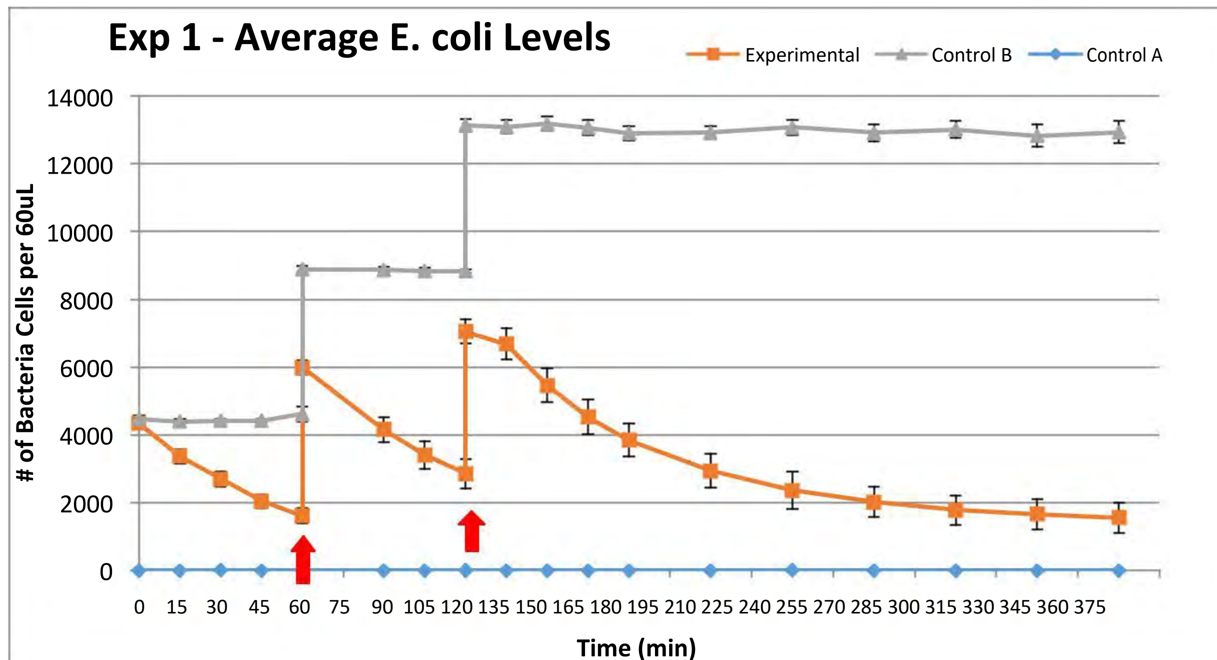


Figure 12. Average (+SEM) of bacterial counts measured by a flow cytometer for the experimental and control beakers in the Pulse experiment. Samples were collected every 15 or 30 min excluding for samples surrounding each pulse (red arrows) where samples were taken immediately before and after the pulses were given.

Experiment 2 – Continuous

This experiment was conducted over the course of 2 days (1 trial per day), October 25th and 26th. Overall, this experiment was laid out in the same manner as the Pulse Experiment although continuing with the reduced number of controls. Therefore, a total of 8 Experimental beakers were used each day, along with 2 for Control A and 2 for Control B. At T0, each Experimental and Control B beakers received a pulse of *E. coli* (40×10^6 CFU) and polystyrene microspheres (9.6×10^6). The concentration of the polystyrene microspheres was increased as compared to the Pulse Experiment (above) to allow beads to remain in experimental beakers throughout the experiment. The same quantity of microspheres was added again three hours after the beginning of the experiment. Peristaltic pumps were used to continuously drip a mixture of seawater with *E. coli* (500 µL containing ~3.3×10^5 CFU per min from a bacteria head tank) into the 10 corresponding beakers (Experimental and Control B) throughout the course of the experiment. Figure 13 shows the experimental layout. The bacteria head tank was placed on a stir plate with a stir bar to keep the bacteria from settling during the course of the experiment. The concentration of the bacteria added over time was the same as in the Pulse Treatment (40×10^6 CFU introduced to each beaker every hour). The 2 beakers used for Control A (live mussel with no bacteria) received seawater from a separate head tank (Figure 13). Samples (500 µL) were taken for the flow cytometer every 15 min until T3 where they transitioned to every 30 min. At T0 and at T6, a 100 µL sample was taken from the Experimental and Control B beakers and plated to determine CFU counts as in the Pulse Treatment Experiment.

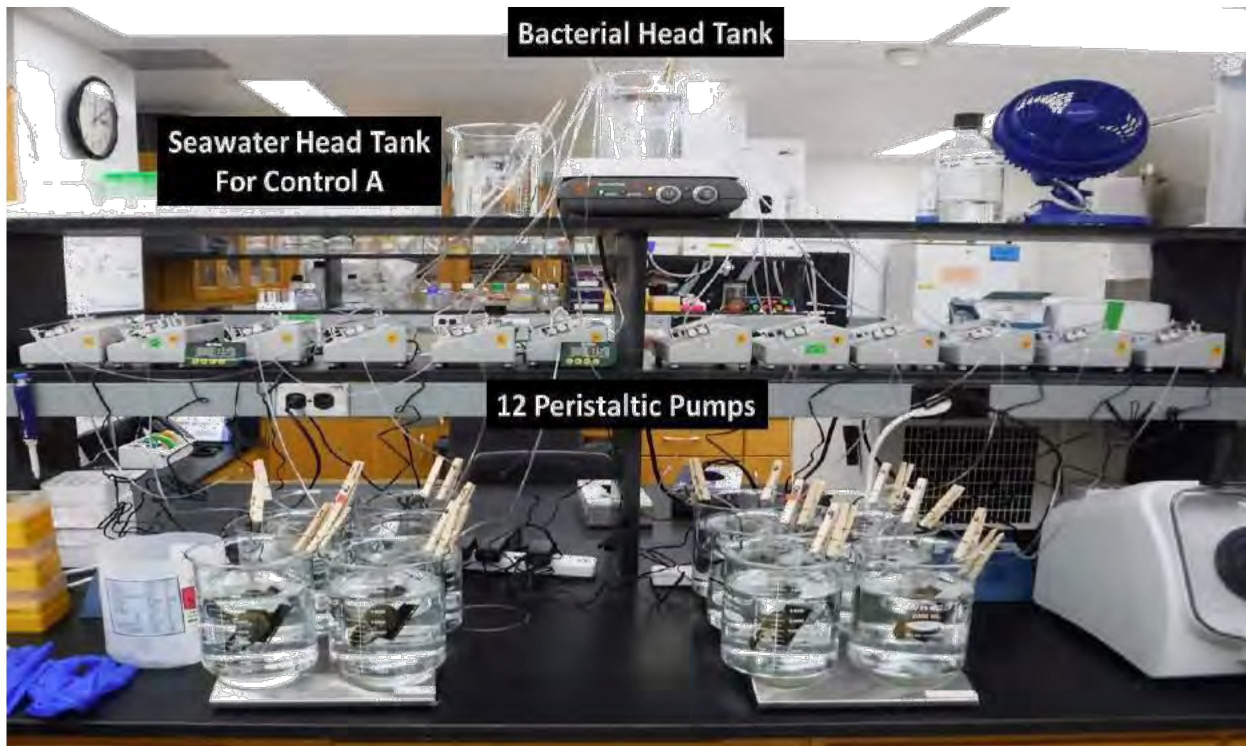


Figure 13. Experimental layout is the same as in Pulse experiment (Figure 8). Peristaltic pumps (n = 12) on shelf above experimental beakers and the bacterial head tank on top shelf was used to supply a continual input of *E. coli* into the experimental tanks. The bacterial head tank was placed on a stir plate, keeping bacteria in suspension, and the seawater head tank (Control A) sat adjacent.

Table 2. The amount of *E. coli* (CFU) and polystyrene microspheres added during the experiment, temperature, number of ribbed mussels that were open and the number of samples taken for the flow cytometer during the corresponding hour for continuous treatment. The continuous drip added 40 x 10^6 CFU to the beakers every hour.

Day	Time	*E. coli*	Beads	Temperature (°C)	Mussels Open	Samples Taken
1	0	40 x 10^6	9.6 x 10^6	18.2	9	4 (every 15 min)
1	1 hour	Continuous drip	0	Not recorded	9	4 (every 15 min)
1	2 hour	Continuous drip	0	20.2	9	4 (every 15 min)
1	3 hour	Continuous drip	9.6 x 10^6	Not recorded	10	4 (every 15 min)
1	4 hour	Continuous drip	0	Not recorded	9	2 (every 30 min)
1	5 hour	Continuous drip	0	Not recorded	10	2 (every 30 min)
1	6 hour	End	0	22.0	8	1 last sample
2	0	40 x 10^6	9.6 x 10^6	16.7	3	4 (every 15 min)
2	1 hour	Continuous drip	0	17.5	5	4 (every 15 min)
2	2 hour	Continuous drip	0	18	7	4 (every 15 min)
2	3 hour	Continuous drip	9.6 x 10^6	18.6	7	4 (every 15 min)
2	4 hour	Continuous drip	0	18.9	7	2 (every 30 min)
2	5 hour	Continuous drip	0	19.8	8	2 (every 30 min)
2	6 hour	End	0	20.2	8	1 last sample

Results – Experiment 2: Continuous

In this experiment, the bacteria measured in Control B (empty shell) show a steady and continuously increasing level of *E. coli* due to no filtration occurring, and the Control A beakers (no bacteria) show zero levels (Figure 14). The experimental beakers show an immediate difference within the first 15 min sampling period when compared to Control B levels and this trend continued as the slope only increased slightly as opposed to the slope of the levels from Control B (Figure 14). At the conclusion of the experiment, the Control B levels reached an average of approximately 24,000 bacteria cells per 60 µL while Experimental beakers had an average of approximately 9,200 bacteria cells per 60 µL, which is a difference of approximately 260%.

Appendix C contains the graphs showing the results from each day this experiment was conducted and the variation between ribbed mussels in each beaker. Variations can be attributed to size of the ribbed mussel and whether or not the animal was opened or closed.

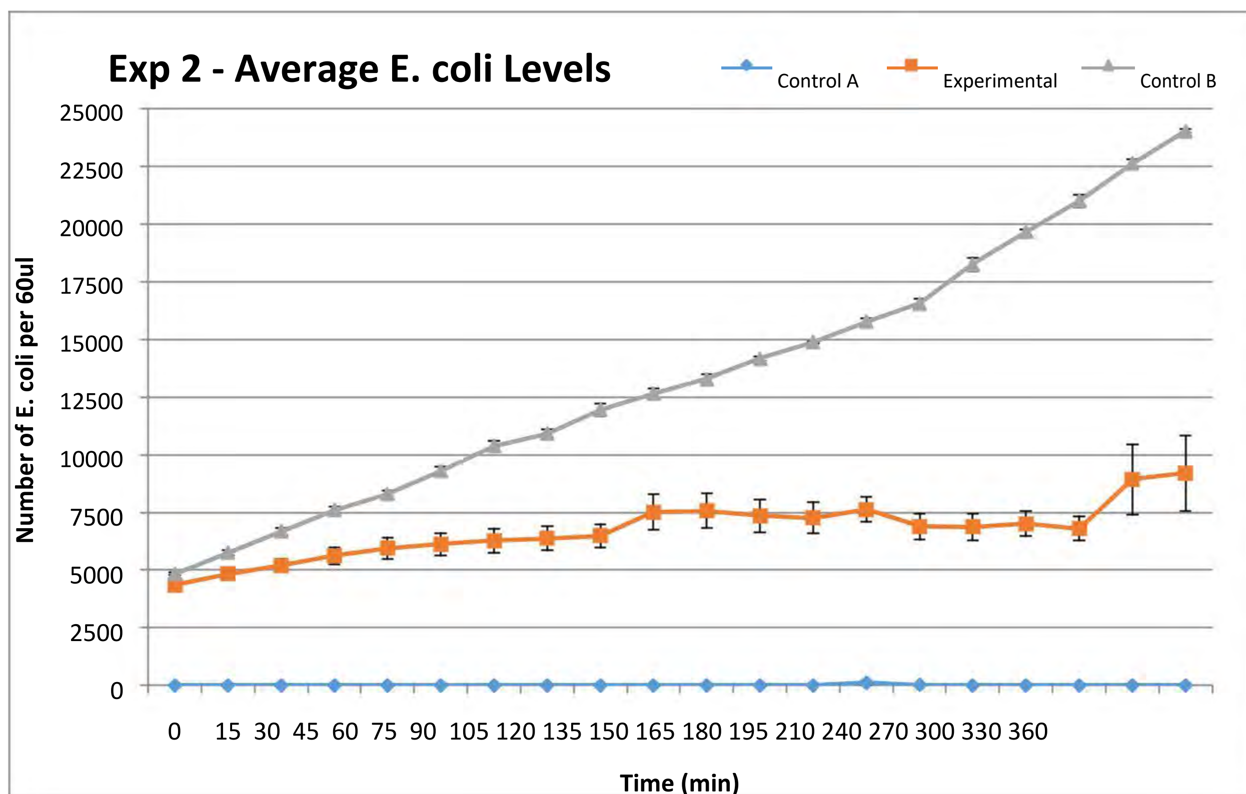


Figure 14. Average (+SEM) of bacterial counts measured by a flow cytometer for the experimental and control beakers with a continual input of bacteria over time.

Experiment 3 – Continuous + Pulse

This experiment was conducted over the course of 2 days, October 30th and 31st and was a combination of the first 2 experiments using the same layout as Experiment 2, but with additional pulses of bacteria added. There were 8 Experimental beakers, 2 Control A and 2 Control B beakers. At T0, each Experimental (8) and Control B (2) beakers received a pulse of *E. coli* (40×10^6 CFU) and polystyrene microspheres (9.6×10^6). The peristaltic pumps delivered a continuous drip of bacteria (500 µL containing ~3.3×10^5 CFU per min) in seawater to the Experimental and Control A beakers from the bacteria head tank (Figure 13, Exp. 2). Additionally, pulses of bacteria (40×10^6 CFU) were added during the course of the experiment at hour 1.5 and hour 3. The continuous flow of bacteria never ceased during the experiment even when the pulse was added. Beakers for Control B received filtered seawater without bacteria via the peristaltic pumps from the seawater head tank. Microspheres were added again 2 hours after T0. Samples were taken with pipettes at the beginning and the end of the experiment for plating on agar and every 15 min for the flow cytometer until T4 when it transitioned to every 30 min.

Table 3. The amount of *E. coli* (CFU) and polystyrene microspheres added during the experiment, temperature, number of ribbed mussels that were open, and the number of samples taken for the flow cytometer during the corresponding hour for Continuous + Pulse Treatment. The continuous drip added 40×10^6 CFU to the beakers every hour and continued even when a pulse was added.

Day	Time	*E. coli*	Beads	Temperature (°C)	Mussels Open	Samples Taken
1	0	40×10^6	9.6×10^6	19.8	6	4 (every 15 min)
1	1 hour	Continuous drip	0	20.2	7	4 (every 15 min)
1	1.5 hour	40×10^6	0	Not recorded	7	n/a
1	2 hour	Continuous drip	9.6×10^6	20.7	7	4 (every 15 min)
1	3 hour	40×10^6	0	21.5	9	4 (every 15 min)
1	4 hour	Continuous drip	0	21.6	9	2 (every 30 min)
1	5 hour	Continuous drip	0	21.8	10	2 (every 30 min)
1	6 hour	End	0	22.0	10	1 last sample
2	0	40×10^6	9.6×10^6	20.2	4	4 (every 15 min)
2	1 hour	Continuous drip	0	21.1	6	4 (every 15 min)
2	1.5 hour	40×10^6	0	Not recorded	6	n/a
2	2 hour	Continuous drip	9.6×10^6	21.6	6	4 (every 15 min)
2	3 hour	Continuous drip	0	22.1	9	4 (every 15 min)
2	4 hour	Continuous drip	0	22.8	9	2 (every 30 min)
2	5 hour	Continuous drip	0	23.2	9	2 (every 30 min)
2	6 hour	End	0	23.5	9	1 last sample

Results - Experiment 3: Continuous + Pulse

In this experiment the levels of bacteria measured in Control B (empty shell) show a steady and continuously increasing level of *E. coli* due to no filtration occurring, and the Control A beakers (no bacteria) show zero levels (Figure 15). Prior to the first pulse in this experiment, the experimental beakers show a similar trend to what was observed in Experiment 2 regarding the bacteria levels in Control B versus the experimental beakers (*i.e.* step function at pulsed times, but declining in Experimental tanks and increasing in Control B). At the conclusion of the experiment, the Control B levels reached an average of approximately 31,000 bacteria cells per 60 µL and the Experimental beakers had an average of approximately 12,000 bacteria cells per 60 µL. That is a difference of approximately 258%. It is also interesting that the final level of bacteria in this experiment only reached a slightly higher level than that observed in Experiment 2 (9,200 vs. 12,000) even though in this experiment a greater overall concentration of bacteria was introduced. For Control B, the final level of bacteria had a difference of approximately 7,000 bacteria cells per 60 µL yet the experimental beakers kept that difference to approximately 1,000 bacteria cells per 60 µL.

Appendix C contains the graphs showing the results from each day this experiment was conducted and the variation between each beaker. Variations can be attributed to size of the ribbed mussel and whether or not the animal was opened or closed.

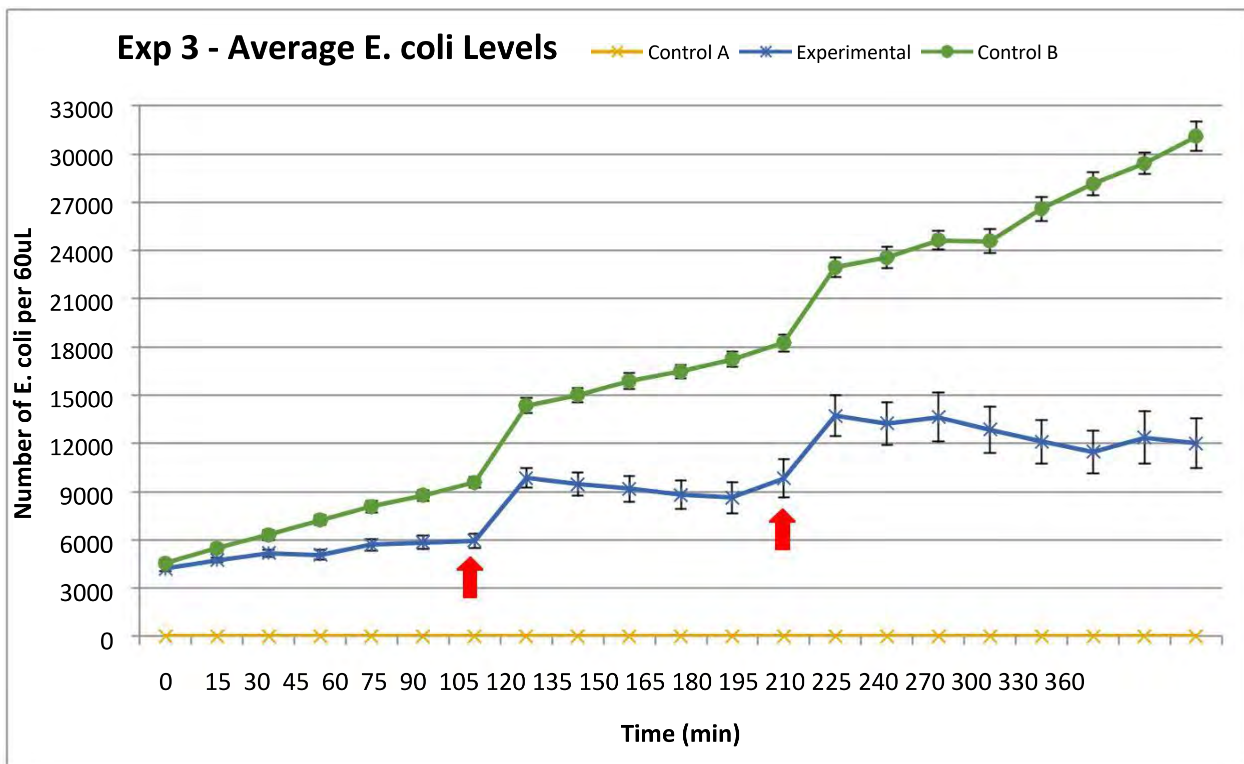


Figure 15. Average (+SEM) of bacterial counts measured by a flow cytometer for the experimental and control beakers treated with a continuous input and additional pulses of bacteria. Initial concentrations of bacteria at time 0 was followed by subsequent pulses of bacteria at time 90 and 180 (red arrows) with samples taken prior to the pulses and 15 min after.

Discussion

Various aspects of ribbed mussel (*Geukensia demissa*) were explored in this Phase 1 study including, their ability to filter *E. coli* bacteria, evaluate basic culture methods in the hatchery and monitor the growth of fouling organisms on field deployed cages. These studies will provide useful information to NYC DEP regarding the potential for ribbed mussel to reduce bacteria levels found in the water column following CSO events. It will also allow for improvements to the cultivation techniques of *G. demissa* to be developed and to identify gaps in knowledge for additional investigation needed before larger scale implementation.

The experimental work done with culture of ribbed mussel in the hatchery indicated that it is possible to back-condition previously spawned individuals. This is beneficial in that it will enable a greater time period when the production of larvae in a hatchery setting can be accomplished. Given the high rates of mortality that were also observed in this study at the larval stages, it is quite valuable to have more opportunity throughout the year to spawn the animals. More work is needed to further improve the hatchery rearing process and to maximize survival at the larval stages.

The preliminary field trials performed on different structures to place ribbed mussels in after they have been grown out, suggests that oyster or gabion cages may be useful for deployment. They will serve as a lightweight unit that will experience minimal sinking when placed on a soft substrate and also provide for removal from the area if the need were to arise. Placing juvenile or adult ribbed mussels in ADPI bags that are stacked inside the cages may be a suitable method for use in the field. In addition, the fouling that was observed on the cages over the 15 weeks indicated that it would not be an issue in the inter-tidal areas. However, a more robust test of this is warranted in the early spring through the summer months when fouling organisms may be more prevalent.

The results of these preliminary laboratory investigations indicate that ribbed mussels are effective at filtering *E. coli*. Flow cytometry provided an efficient and accurate real-time means of quantifying bacteria and is a preferred method to use for future experiments, but CFU counts were also performed to provide counts similar to those used to measure bacteria in the field such as during CSO events. Overall, the CFU counts correlated well with the flow cytometer measurements with R^2 values as high as 0.84 although some variability in CFU counts were noted (possibly as a result of change in the culturability of bacteria held in seawater for extended periods). Specifically, the studies reveal that ribbed mussel, when exposed to levels of *E. coli* bacteria similar to CSO events, can filter them quite effectively over short periods of time (62% reduction in Experiment 1 within 1 hour) after an initial pulse (Figure 12 and 16). Similarly, mussels were still effectively able to clear *E. coli* even when a continuous flow of bacteria was applied as seen in Experiment 2 (62% reduction as compared to controls over the 6-hour length of the trial) and Experiment 3 (61% reduction). In fact, the calculated clearance rates of bacteria were higher in Experiments 2 and 3 (Table 4) as compared to Experiment 1 despite an apparent steeper downward trend observed in the latter (Figure 12 vs. 14 and 15; Figure 16). This is an artifact resulting from the principal difference in how the bacteria were introduced to the system (pulse vs. flow). Put another way, a greater decrease is observed at a pulse in a short time span but this slope will begin to decrease over time. An interesting result was that at the end of the 6 hours, the experimental beakers in Experiments 2 and 3 only differed by approximately 3,000

bacterial cells per 60 µL (Figure 16) despite those beakers in Experiment 3 receiving over 9,000 additional *E. coli* cells (per 60 µL) in 2 pulses over the course of the treatment. Overall, mussel clearance rates measured here were fairly consistent between all three experiments with greatest filtration rates of microspheres observed in Experiment 1 (Table 4).

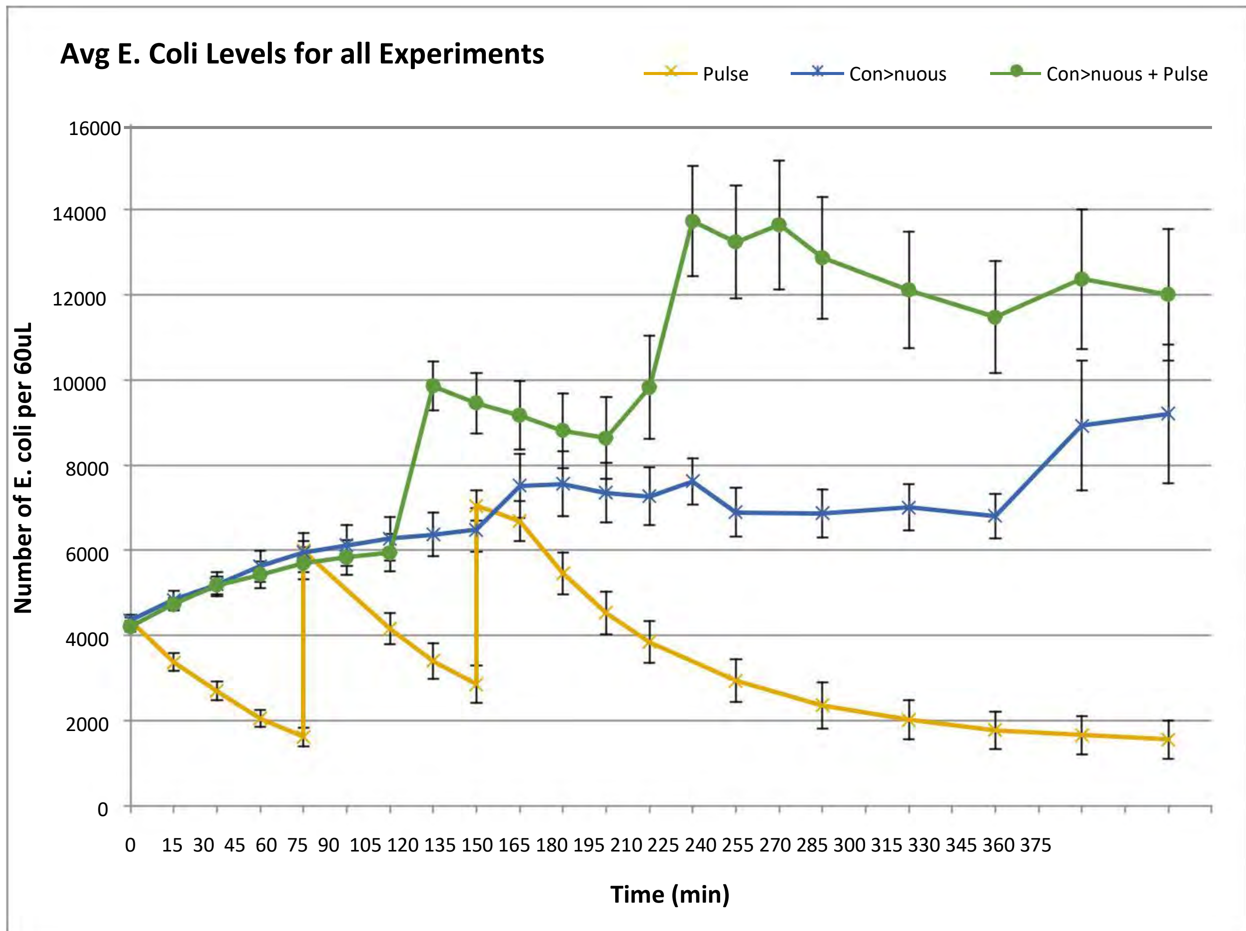


Figure 16: Average (+SEM) of bacterial counts measured by a flow cytometer for all three experimental trials (Pulse, Continuous and Continuous with additional pulses of bacteria). Initial concentrations of bacteria at time 0 was followed by subsequent pulses of bacteria at 60 min for the Pulse Experiment with a sample taken prior and after the pulse given and repeated at 120 min. The Continuous plus pulse experiment received a pulse at 90 and 180 min with samples taken prior to the pulses and 15 min after.

Clearance rates calculated based on polystyrene microspheres (1.195 to 1.629 L g^{-1} h^{-1}) uptake were slightly higher than those calculated from the E. coli data (0.991 to 1.152 L g^{-1} h^{-1}). This is expected since all microspheres used in our experiments (5 to 6µm in diameter) were above the 4µm threshold considered by Riisgard (1988) to trigger 100% particle retention. Nevertheless, the slim differences between both particle types (clearance rates based on *E. coli* represented 61-93% of those calculated based on microspheres uptake) indicate a very high retention of *E. coli* by ribbed mussel. The clearance rates for bacteria were higher than those observed by Wright *et al* (1982) who reported 0.46 ± 0.03 L g^{-1} h^{-1}. These differences could be related to the techniques used for bacteria quantification (flow cytometry in our study vs. microscopic counts by Wright *et al* (1982) and the qualitative characteristics of mussel (size, physiological status) and bacteria (*e.g.* size) used.

Table 4. Clearance rates for *E. coli* and microspheres per trial in L g^{-1} h^{-1}. Standard errors are given for all parameters (shown between parentheses for weight and length).

Experiment	n	Dry Weight (g)	Shell Length (mm)	*E. coli*	Microspheres
Pulse	24	0.405-1.589 (0.03)	68.7-72.9 (0.12)	0.991 ± 0.12	1.629 ± 0.16
Continuous	18	0.729-1.259 (0.03)	66.0-73.8 (0.56)	1.109 ± 0.149	1.195 ± 0.14
Continuous + Pulse	20	0.739-1.124 (0.03)	65.2-67.7 (0.17)	1.152 ± 0.12	1.375 ± 0.09

While these initial trials show the potential for *G. demissa* to filter fecal coliform bacteria associated with CSO events, there are still many areas of investigation needed to further quantify their efficiency to achieve the overall goal of NYC DEP. Additional experiments will investigate the ribbed mussel ability to filter *E. coli* under different environmental conditions (*e.g.* temperature, salinity) as well as with the presence of other size particles including phytoplankton. Evaluating how the size of the mussel affects its filtration capabilities and demands under different parameters also needs to be conducted. After experimenting with these other parameters in the lab, the next step would be to conduct larger scale experiments in mesocosms that will allow for a simulated tidal exchange that will better represent the actual creek system with water flowing past the ribbed mussel. These will all be examined in subsequent phases.

Appendix A – Literature Review

Introduction

The New York City Department of Environmental Protection (NYCDEP) would like to mitigate the influence of CSO dischargesusing ribbed mussels. The goal is to cultivate and deploy ribbed mussels in the surrounding area of the WTP for bacterial filtration. Evidence in the literature shows that ribbed mussel (*Geukensia demissa*) are capable of filtering out particles as small as bacteria from the water column but no work has been conducted specifically looking at the filtration of E. coli or other fecal coliform bacteria from the water column. It is hypothesized that the ribbed mussel is able to filter E. coli and other fecal coliform bacteria commonly found in the water near WTPs, that it will reduce the levels; especially during and after combined sewer overflow (CSO) events. The primary focus of this review was to compile information from the literature on the ability of ribbed mussel to filter bacteria out of the water column and assist with the design of laboratory testing and future field experiments. Along with that though, there is pertinent information found in the general biology and ecology, and aquaculture categories that is relevant to the ultimate goal of the NYCDEP.

This literature review will categorize papers related to ribbed mussels into five categories of information: general biology and ecology, aquaculture, filtration studies that focused on bacteria uptake, and an experimental methodology category which includes papers that conducted similar laboratory experiments that Cornell Cooperative Extension (CCE) and Stony Brook University (SBU) will be performing for this project. A final category contains additional papers that were reviewed but were found to have minimal (if any) relevance to the overall project, but included as part of the literature search.

General Biology and Ecology Related Papers

An extensive amount of literature exists focusing on the ecological role the ribbed mussel plays in the ecosystem. Ribbed mussels have been shown to play an important role in the salt marsh community where it commonly exists. This includes filtration of the water (Jordan and Valiela 1982, Kuenzler 1961), providing nutrient laden feces and pseudofeces to the cordgrass, *Spartina alterniflora* (Bertness 1980 and 1984), and stabilization of the marsh sediments as well as contributing to sediment accretion which combats sea level rise (Bertness 1984, Kreeger *et al.* 2001, 2015). There are many other ecosystem functions that are provided by ribbed mussel that are beyond the scope of this search, but we have included a wide variety of literature in Table 1 that include them. A few papers of relevance to NYDEP were conducted by Franz (1993, 1997, and 2001) who examined ribbed mussel populations in Jamaica Bay, NY and provided estimates on the population densities found in the waterbodies of interest. Galimany *et al.* (2013a and b, 2017) conducted a study in the Bronx River Estuary at Hunts Point, NY. This location was selected because the area is closed to shellfish harvest due to bacterial contamination likely related to its proximity to the Hunts Point WTP. To the best of our knowledge, this project was the first of its kind and found that ribbed mussel were capable of filtering just as effectively when submerged 100% of the time despite naturally being found in the intertidal zone. This indicated that this species would be suitable for bioextraction purposes in highly eutrophic urban areas. This study also demonstrated the difficulty of collecting and recruiting natural ribbed mussel from the wild and reinforces the need for hatchery cultivated ribbed mussel stock to use for projects.

#	Author	Paper Title	Year	Journal
Table 1. Literature related to general background information of ribbed mussel ecology.				
1	Bertness	Growth and mortality in the ribbed mussel *Geukensia demissa*	1980	Veliger 23 (1):62-69
2	Bertness	Ribbed mussels and *Spartina alterniflora* in a New England salt marsh	1984	Ecology 65 (6):1794-1807
3	Bertness & Grosholz	Population Dynamics of the ribbed mussel, *Geukensia demissa:* The cost and benefits of an aggregated distribution	1985	Oecologia 67 (2):192-204
4	Borreo	Tidal height and gametogenesis: reproductive variation among populations of *Geukensia demissa*	1987	Biological Bulletin 173:160-168
5	Charles & Newell	Digestive physiology of the ribbed mussel *Geukensia demissa* (Dillwyn) held at different tidal heights	1997	Experimental Marine Biology & Ecology 209:201-213
6	Franz	Allometry of shell and boy weight in relation to shore level in the intertidal bivalve *Geukensia demissa* (Bivalvia: Mytilidae)	1993	Experimental Marine Biology & Ecology 174:193-207
7	Franz	Recruitment, survivorship, and age structure of a NY ribbed mussel population *(Geukensia demissa*) in relation to shore level: a nine year study	2001	Estuaries 24 (3):319-327
8	Franz	Resource allocation in the intertidal salt-marsh mussel *Geukensia demissa* in relation to shore level	1997	Estuaries 20 (1):134-148
9	Galimany *et al.*	Quantifying feeding behavior of ribbed mussels (*Geukensia demissa*) in two urban sites (Long Island Sound, USA) with different seston characteristics	2013	Estuaries & Coasts 36 (6):1265-1273
10	Galimany *et al.*	Short communication: adaptability of the feeding behavior of intertidal ribbed mussels (*Geukensia demissa*) to constant submersion	2013	Aquaculture International 21:1009-1015
11	Galimany *et al.*	Cultivation of the Ribbed Mussel (*Geukensia demissa)* for nutrient bioextraction in an urban estuary	2017	Environmental Science & Technology 51 (22):13311-13318
11	Jordan & Valiela	A nitrogen budget of the ribbed mussel, *Geukensia demiss,* and its significance in a nitrogen flow in a New England salt marsh	1982	Limnology & Oceanography 27 (1):75-90
12	Kreeger *et al.*	Marine bivalve shellfish restoration priorities for the Delaware Estuary	2011	Partnership for the Delaware Estuary, Wilmington, DE. PDE Report No. 11-03. 54 p.
13	Kreeger *et al.*	Geospatial and seasonal variation in the capture, flux and fate of seston and associated nitrogen by ribbed mussels (*Geukensia demissa*) in representative mid-Atlantic salt marshes	2015	Partnership for the Delaware Estuary, Wilmington, DE. PDE Report No. 15-09. 135 p.
14	Kuenzler	Phosphorus budget of a mussel population	1961	Limnology & Oceanography 6 (4):400-415
15	Kuenzler	Structure and energy flow of a mussel population in a GA salt marsh	1961	Limnology & Oceanography 6 191-204
16	Lent	Adaptations of the ribbed mussel, *Modiolus demissa* (Dillwyn), to the intertidal habitat	1969	Am. Zoologist 9:283-292
17	Lin	Influence of location in a salt marsh on survivorship of ribbed mussels	1989	Marine Ecology Progress Series 56:105-110
18	Nielsen & Franz	The influence of adult conspecifics and shore level on recruitment of the ribbed mussel *Geukensia demissa* (Dillwyn)	1995	Experimental Marine Biology &Ecology 188:89-98
19	Stiven & Gardner	Population processes in the ribbed mussel *Geukensia demissa* (Dillwyn) in a NC salt marsh tidal gradient: spatial pattern, predation, growth and mortality	1992	Experimental Marine Biology & Ecology 160:81-102
20	Whalen *et al.*	Practitioner's Guide: Shellfish-based living shorelines for salt marsh erosion control and environmental enhancement in the Mid-Atlantic	2011	Partnership for the Delaware Estuary, Wilmington, DE. PDE Report No. 11-04. 48 p.

Aquaculture Related Papers

Although there are numerous articles related to shellfish aquaculture, there are few specifically related to ribbed mussel cultivation. This is primarily due to the lack of commercial interest in the species since they are considered a non-edible species. Cornell Cooperative Extension of Suffolk County (CCE), the New Jersey Aquaculture Innovation Center at Rutgers University (NJAIC) and the Martha's Vineyard Shellfish Group and are the only hatcheries that have had any success cultivating ribbed mussel; although on a small scale. Efforts to cultivate ribbed mussel have shown that they do not respond to traditional methods used for clam and oyster (Loosanoff and Davis 1963). As a result, other methods for spawning various species of mussels that may also apply to ribbed mussel, have been investigated in Table 2. Landua (2014) has developed a method known as the Bin-Silo system and this has proven successful by NJAIC (Jones et al. 2013) and was successfully adopted by CCE with a few adaptations. Rather than cycling the adult broodstock through temperature changes as done with clam or oyster, the mussels are actually placed in a warm water bath (30°C) and allowed to cool overnight and thus spawn during the night.

Table 2. Literature articles related to shellfish and mussel species aquaculture.

#	Author	Paper Title	Year	Journal
1	Brousseau	Gametogenesis and spawning in a population of *Geukensia demissa* (Pelecypoda: Mytilidae) from Westport, CT	1981	Veliger 24:247-251
2	Farfan *et al.*	Seed production and growth of *Modiolus capax* in laboratory conditions	2007	Shellfish Research 26 (4):1075-1080
3	Iglesias *et al.*	Feeding, particle selection and absorption in cockles *Cerastoderma edule* (L.) exposed to variable conditions of food concentration and quality	1992	ExperimentalMarine Biology & Ecology 162:177-198
4	Jones *et al.*	Commercial-Scale Production of the Ribbed Mussel *Geukensia demissa*	2013	Poster presented at: DE EstuaryScience& Environmental Summit Cape May, NJ
5	Landau	The Bin-silo system: A simple spawning method for bivalve shellfsih	2014	World Aquaculture 45 (1):58-61
6	Loosanoff & Davis	Rearing of bivalve mollusks	1963	Advances in Marine Biology 1:1-136
7	Ompi	Settlement behavior and size of mussel larvae from the family Mytilidae	2010	Coastal Development 13 (3):215-227
8	Pronker *et al.*	Hatchery cultivation of the common cockle (*Cerastoderma edule* L.): from conditioning to grow-out	2015	Aquaculture Research 46:302-312
9	Schwaebe *et al.*	Comparative efficacy of *Dreissena rostriformis bugensis* (Bivalvia: Dreissenidae) spawning techniques	2013	Aquatic Invasions 8 (1):45-52
10	Stoeckel *et al.*	Laboratory culture of *Dreissena polymorpha* larvae: spawning success, adult fecundity and larval mortality patterns	2004	Canadian Journal of Zoology 82:1436-1443
11	Utting & Millican	Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability	1997	Aquaculture 155:45-54
12	Wong & Arshad	Induced spawning and early development of *Modiolus philippinarum*	2013	Asian Journal of Animal & Veterinary Advances 8(1)100-107

Filtration Papers

There are only a few studies that have been conducted looking at the filtration of bacteria by ribbed mussel (Table 3). Based on those studies (Kemp *et al.* 1990, Langdon and Newell 1990, Newell and Krambeck 1995, Riisgard 1988, and Wright *et al.* 1982) it has become accepted that ribbed mussel are capable of filtering out smaller particles (< 2µm) and with greater efficiency than other species such as oyster and clam. Wright *et al.* (1982) was the first study to look at this and also analyzed the gill structure of ribbed mussel to compare with other bivalve species (e.g. oyster, clam, and blue mussel). Wright *et al.* (1982) documented the unique structure of their gills and inferred the differences from the other species is what enables the ribbed mussel to filter out particles as small as 0.2µm in size. Other studies (Kemp *et al.* 1990, Langdon and Newell 1990, Newell and Krambeck 1995, and Riisgard 1988) also corroborated the findings of Wright *et al.* (1982) and examined how the bacteria were being assimilated by the ribbed mussel. Kemp *et al.* (1990) and Langdon and Newell (1990) identified that ribbed mussels can uptake nutrients from the bacterioplankton at times when they are nutrient limited. Newell and Krambeck (1995) used lab and field tests in their study and found that during the winter months when temperatures are lowest, there was no significant evidence observed for bacterioplankton filtration. They inferred that this could be due to lower nutritional needs of the ribbed mussel during this time of year. This is relevant to the NYC DEP's project since there is a possibility that the ribbed mussel deployed may not reduce bacteria loads from CSO events as effectively during the winter months. However, CSO events may be less frequent in winter months. The temperature ranges where filtration is significantly reduced should be identified. Kemp *et al.* (1990) looked at various sizes of mussel classes (40, 26 and 16 mm) and how their filtration rates changed in response to different particle size. The small mussels showed a higher clearance rate of all particle sizes than the medium and large size classes. The plan for deployment would involve utilizing hatchery cultivated ribbed mussel, which would be in the smaller size class for the first few years of deployment.

The study conducted by Bernard (1989) is very similar to the lab experiments that CCE and SBU will be conducting where *E. coli* was tested on various bivalve species to determine their ability to filter this particular species of bacteria. This study was conducted in the Pacific Northwest and used bivalves native to that area, including the blue mussel, *Mytilus edulis*. Bernard (1989) experimented at 3 different temperatures (7, 12, and 17°C) to determine if there was an effect on filtration. Prior to the filtration experiments, the *E. coli* was suspended in seawater at these varying temperatures to determine how its concentrations would change over 24 hours. After inoculation, the concentration quickly decreased over the first 6 hours and then stabilized for about 6 hours before they began to decline again for final 12 hours. The experiments using the bivalves were conducted for a period of 10 hours and showed that the *E. coli* levels were almost zero in the blue mussel treatment tanks, with the greatest decreases observed at the warmest temperature. Bernard (1989) also indicated that the initial reduction of *E. coli* is very rapid but then declined after 3-4 hours and inferred that a plateau phase may be reached. In the study conducted by Wright *et al.* (1982)*,* the gill structure of ribbed mussel and blue mussels were examined. It was documented that the ribbed mussel gill structure had a greater density of gill filaments than the blue mussels had. It is likely that the ribbed mussel will be able to filter out the *E. coli* better than the blue mussel.

Govorin (2000) provides a summary of numerous studies that investigated bivalves and their ability to filter bacteria, including *E. coli*. This summary supports much of what has been documented here already including Bernard (1989), Kemp *et al.* (1990) and Wright *et al.* (1982)

studies. Although there were more species covered than ribbed mussel, there was discussion regarding the plateau phase observed by Bernard (1989), with the initial filtration rates being highest in the first 2-6 hours of exposure. The fate of the bacteria filtered was also discussed although without a definitive conclusion whether or not the bacteria was digested in the gut of the bivalve or potentially eliminated from the organism in the form of pseudofeces. All of these aspects pertaining to bacterial fate and effect of long-term exposure to *E. coli* on bacterial clearance by *G. demissa* will be explored in the proposed new phases of this project.

Table 3. Literature related to bacteria filtration by shellfish, primarily ribbed mussel (RM).

#	Author	Paper Title	Year	Journal	Key Info
1	Bernard	Uptake and elimination of coliform bacteria by four marine bivalve mollusks	1989	Canadian Journal of Fisheries Aquatic Science 46:1592-1599	Blue mussel, *Mytilus edulis,* filtered and eliminated coliforms at higher rate than other species
2	Govorin	Role of bivalves in the depuration of seawaters contaminated by bacteria	2000	Russian Journal of Marine Biology 26 (2):81-88	Summary paper
3	Kemp *et al*	Effects of filter feeding by the ribbed mussel *Geukensia demissa* on the water column microbiota of a *Spartina alterniflora* saltmarsh	1990	Marine Ecology Progress Series 59:119-131	RM have ability to select among particle size and alter preference over short time span. Similar filtration efficiency to Riisgard and Wright
4	Langdon & Newell	Utilization of detritus and bacteria as food sources by two bivalve suspension feeders, the oyster *Crassostrea virginica* and the ribbed mussel *Geukensia demissa*	1990	Marine Ecology Progress Series 58:299-310	Retained free bacteria with 15.8% efficiency
5	Newell & Krambeck	Responses of bacterioplankton to tidal inundations of a saltmarsh in a flume and adjacent mussel enclosures	1995	Experimental Marine Biology & Ecology 190:79-95	Net removal of about 30-35% bacterioplankton cells. Bacterial filtration may be seasonal and not significant in winter
6	Riisgard	Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves	1988	Marine Ecology Progress Series 45:217-223	Geukensia retains particles 2 microns in size with 70% efficiency
7	Wright *et al*	Field and laboratory measurements of bivalve filtration of natural marine bacterioplankton	1982	Limnology and Oceanography 27 (1):91-98	Retains bacteria particles from 0.2-0.4µm and 0.4-0.6µm with 30% and 86% efficiency, respectively

Experimental Methodologies Focused on Bacteria

An important component of this project involves laboratory testing to measure the filtration of E. coli by ribbed mussel. While there was some literature that investigated the filtration of bacteria by a bivalve species, we did not find any studies that specifically tested *E. coli* bacterial filtration in ribbed mussel. Bernard's (1989) study using blue mussel (Table 3) was the closest. However, the general methodologies used in all of the studies was similar with respect to counting and measuring various particles for shellfish filtration (Table 4). CCE is working with Dr. Bassem Allam of Stony Brook University to conduct these laboratory tests, and using flow cytometry methods observed in some of the existing literature. Dr. Allam is also a co-author of Espinosa *et al*. 2008 (Table 4) study of particle size selection in ribbed mussels. Many of these papers used various methods that will be utilized by our lab testing as well as some methodology from Table 3 (Bernard 1989, Langdon and Newell 1990, Wright *et al.* 1982).

Table 4. Literature related to experimental methodology that will be utilized during lab testing				
#	Author	Paper Title	Year	Journal
1	Cucci *et al*	Flow cytometry: a new method for characterization of differential ingestion, digestion and egestion by suspension feeders	1985	Marine Ecology Progress Series 24:210-204
2	Espinosa *et al*	Particle selection in the ribbed mussel *Geukensia demisssa* and the Eastern Oyster *Crassostrea virginic:* Effect of microalgae growth stage	2008	Estuarine, Coastal and Shelf Science 79:1-6
3	Iglesias *et al*	Variability of feeding processes in the cockles Cerastoderma edule (L.) in response to changes in seston concentration and composition	1996	Experimental Marine Biology and Ecology 197:121-143
4	Iglesias *et al*	Measuring feeding and absorption in suspension-feeding bivalves: an appraisal of the biodeposition method	1998	Experimental Marine Biology and Ecology 219:71-86
5	Kiorboe & Mohlenberg	Particle selection in suspension feeding bivalves	1981	Marine Ecology Progress Series 5:291-296
6	Ma *et al*	The biofilteration ability of oysters (*Crassostrea gigas*) to reduce *Aeromonas salmonicida* in salmon culture	2017	Applied Microbial Biotechnology 101:5869-5880
7	Macdonald & Ward	Feeding activity of scallops and mussels measured simultaneously in the field: Repeated measures sampling and implications for modelling	2009	Experimental Marine Biology and Ecology 371:42-50
8	Riisgard	On measurement of filtration rates in bivalves - the stony road to reliable data: review and interpretation	2001	Marine Ecology Progress Series 211:275-291
9	Silverman *et al*	Clearance of laboratory-cultured bacteria by freshwater bivalves: differences between lentic and lotic unionids	1997	Canadian Journal of Zoology 75:1857-1866
10	Velasco & Navarro	Feeding physiology of two bivalves under laboratory and field conditions in response to variable food concentrations	2005	Marine Ecology Progress Series 291:115-124

Other Papers of Minimal Relevance

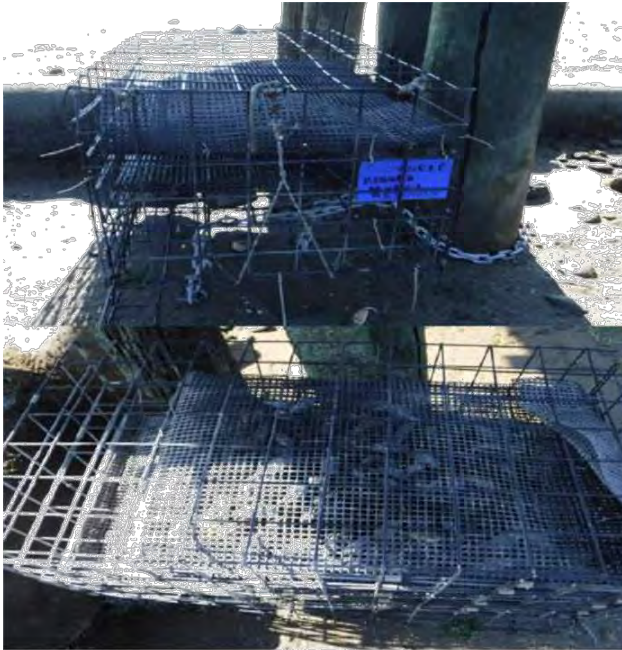
The final category shown in Table 5, contains various studies that were reviewed because the title or abstract of the paper appeared to be relevant. However, upon reviewing them, they were determined to either provide minimal or no information that would be relevant to the overall goals of NYC DEP's project.

Table 5. Literature that was reviewed but deemed non-relevant to the literature review.				
#	Author	Paper Title	Year	Journal
1	Cognie *et al*	Selective feeding of the Oyster *Crassostrea gigas* fed on a natural microphytobenthos assemblage	2001	Estuaries 24 (1):126-131
2	Dabrowski *et al*	Numerical modelling of blue mussel (*Mytilus edulis*) bacterial contamination	2014	Sea Research 89:52-63
3	Evgenidou & Valiela	Response of growth and density of a population to land-derived Nitrogen loading in Waquoit Bay, MA	2002	Estuarine, Coastal & Shelf Science 55:125-138
4	Kreeger *et al*	Utilization of refractory cellulosic carbon derived from *Spartina alterniflora* by the ribbed mussel *Geukensia demissa*	1988	Marine Ecology Progress Series 42:171-179
5	Kreeger *et al*	Effect of tidal exposure on utilization of dietary lignocellulose by the ribbed mussel *Geukensia demissa*	1990	Experimental Marine Biology & Ecology 144:85-100
6	Kreeger & Newell	Seasonal utilization of different seston carbon sources by the ribbed mussel, *Geukensia demissai* in a mid-Atlantic salt marsh	2001	Experimental Marine Biology & Ecology 260:71-91
7	Kreeger & Newell	Ingestion and assimilation of carbon from cellulolytic bacteria and heterotrophic flagellates by the mussels *Geukensia demissa* and *Mytilus edulis*(Bivalvia, Mollusca)	1996	Aquatic Microbial Ecology 11:205-214
8	Lee *et al*	Sulfide-stimulation of oxygen consumption rate and cytochrome reduction in gills of the estuarine mussel Geukensia demissa	1996	Biological Bulletin 191(3):421-430
9	McKinney *et al*	Ribbed mussel nitrogen isotope signatures reflect nitrogen sources in coastal salt marshes	2001	Ecological Applications 11(1):203-214
10	Navarro & Winter	Ingestion rate, assimilation efficiency and energy balance in *Mytilus chilensis* in relation to body size and different algal concentrations	1982	Marine Biology 67:255-266
11	Strubbia *et al*	Geographic and temporal variation of *E. coli* and norovirus in mussels	2016	Marine Pollution Bulletin 107:66-70

Appendix B – Structure Testing Photos

2 Weeks
Aug 15 2018

3 Weeks
Aug 21 2018



4 Weeks
Aug 30 2018

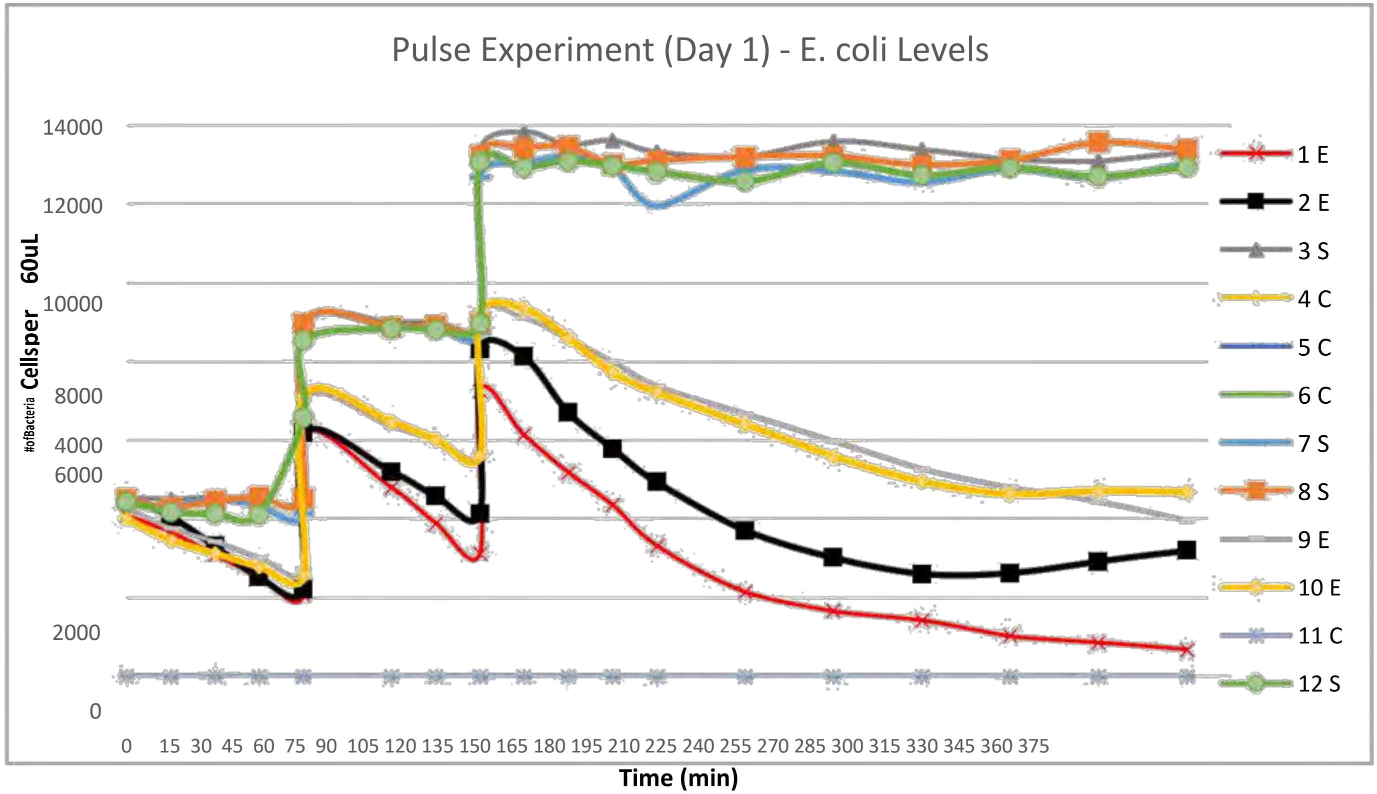
6 Weeks
Sept 13 2018

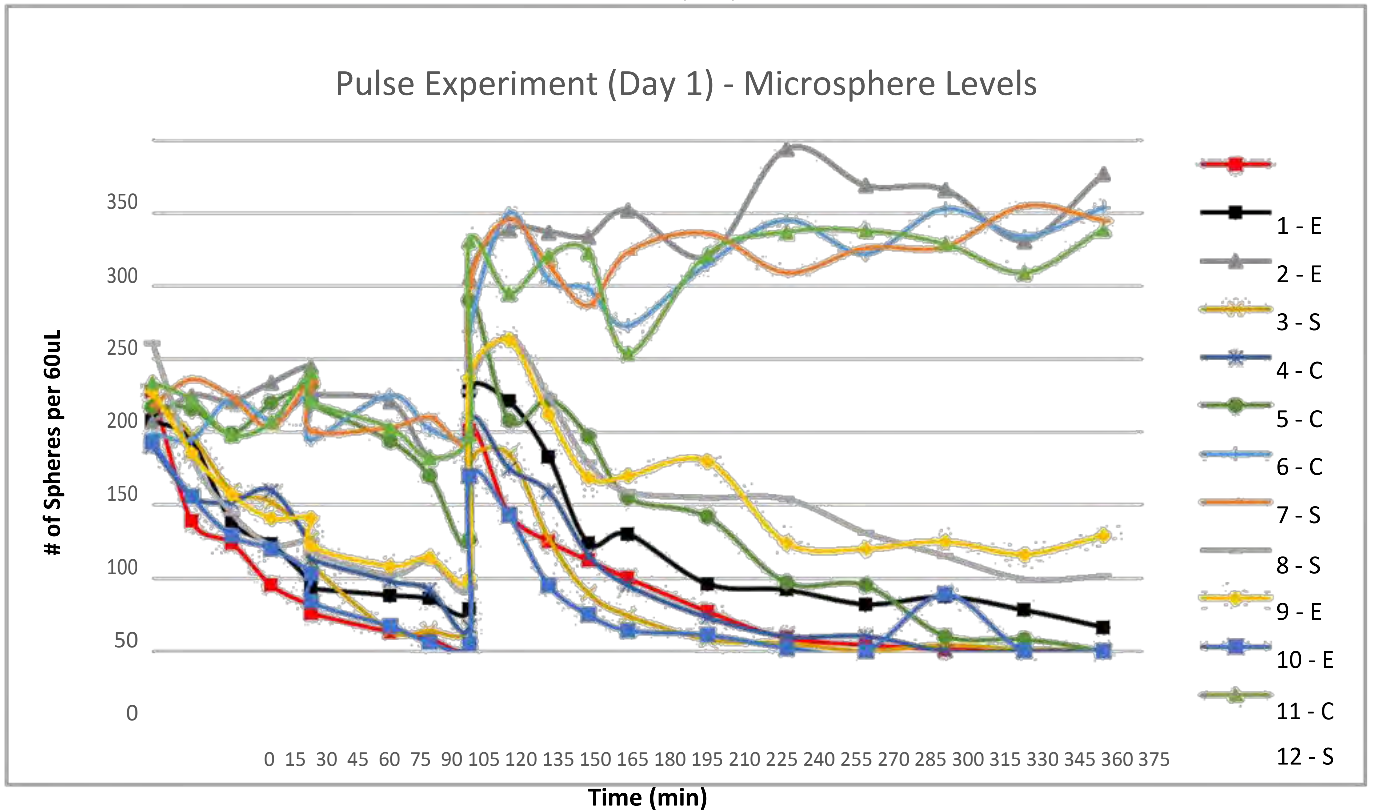
8 Weeks
Sept 25 2018

9 Weeks
Oct 5 2018

15 Weeks
Nov 12 2018

Appendix C – Lab Experiment Data





Pulse Exp (Day 2) - E. coli Levels

# of Spheres per 60uL

14000
12000
10000
8000
6000
4000
2000
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 - E
2 - E
3 - S
4 - C
5 - S
6 - S
7 - C
8 - C
9 - E
10 - S
11 - E
12 - C

Pulse Exp (Day 2) - Microsphere Levels

# of Spheres per 60uL

500
450
400
350
300
250
200
150
100
50
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 - E
2 - E
3 - S
4 - C
5 - S
6 - S
7 - C
8 - C
9 - E
10 - S
11 - E
12 - C

Pulse Exp (Day 3) - E. coli Levels

16000
14000
12000
10000
8000
6000
4000
2000
0

# of Spheres per 60uL

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 E
2 E
3 E
4 E
5 E
6 E
7 C
8 S
9 C
10 E
11 S
12 E

Pulse Exp (Day 3) - Microsphere Levels

350
300
250
200
150
100
50
0

# of Spheres per 60uL

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 E
2 E
3 E
4 E
5 E
6 E
7 C
8 S
9 C
10 E
11 S
12 E

Con>nuous Exp (Day 1) - E. coli Levels

# of Bacteria Cells per 60uL

25000
20000
15000
10000
5000
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 E
2 C
3 E
4 E
5 E
6 E
7 E
8 C
9 E
10 S
11 E
12 S

Con>nuous Exp (Day 1) - Microsphere Levels

# of Spheres per 60uL

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 E
2 C
3 E
4 E
5 E
6 E
7 E
8 C
9 E
10 S
11 E
12 S

Con>nuous Exp (Day 2) - E. coli Levels

# of Bacteria Cells per 60uL

27500
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 E
2 C
3 E
4 E
5 S
6 E
7 C
8 E
9 E
10 S
11 E
12 E

Con>nuous Exp (Day 2) - Microsphere Levels

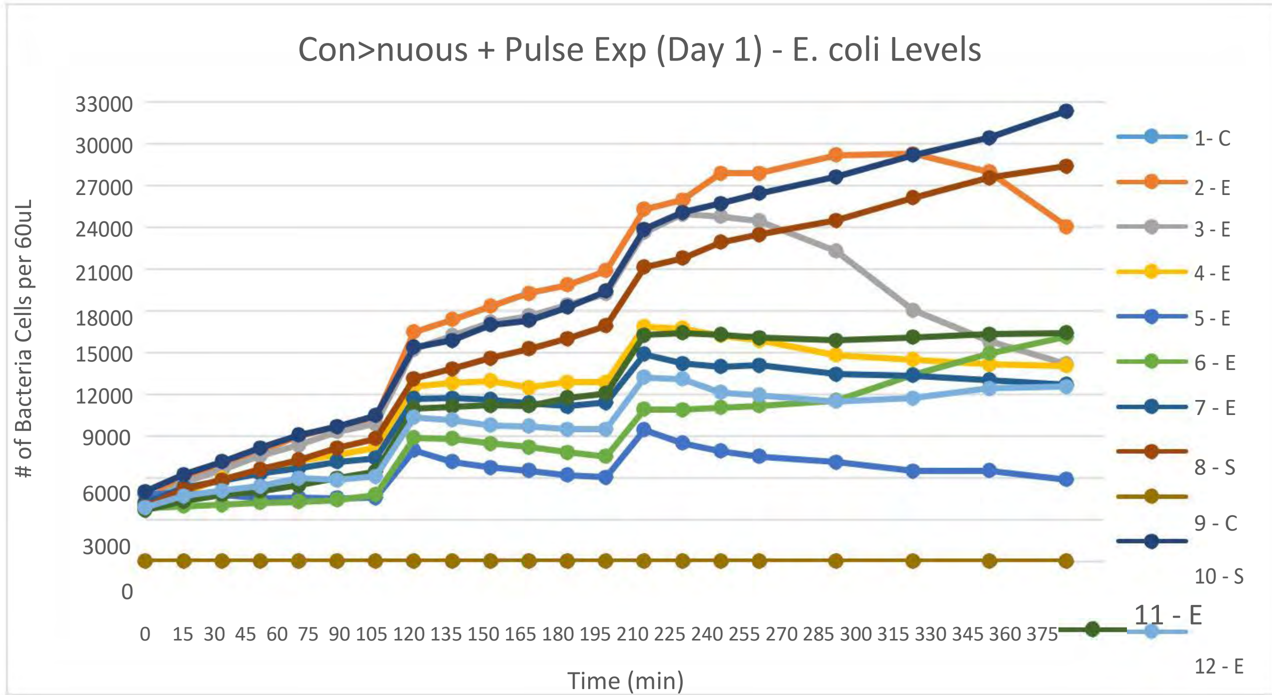
# of Spheres per 60uL

750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0

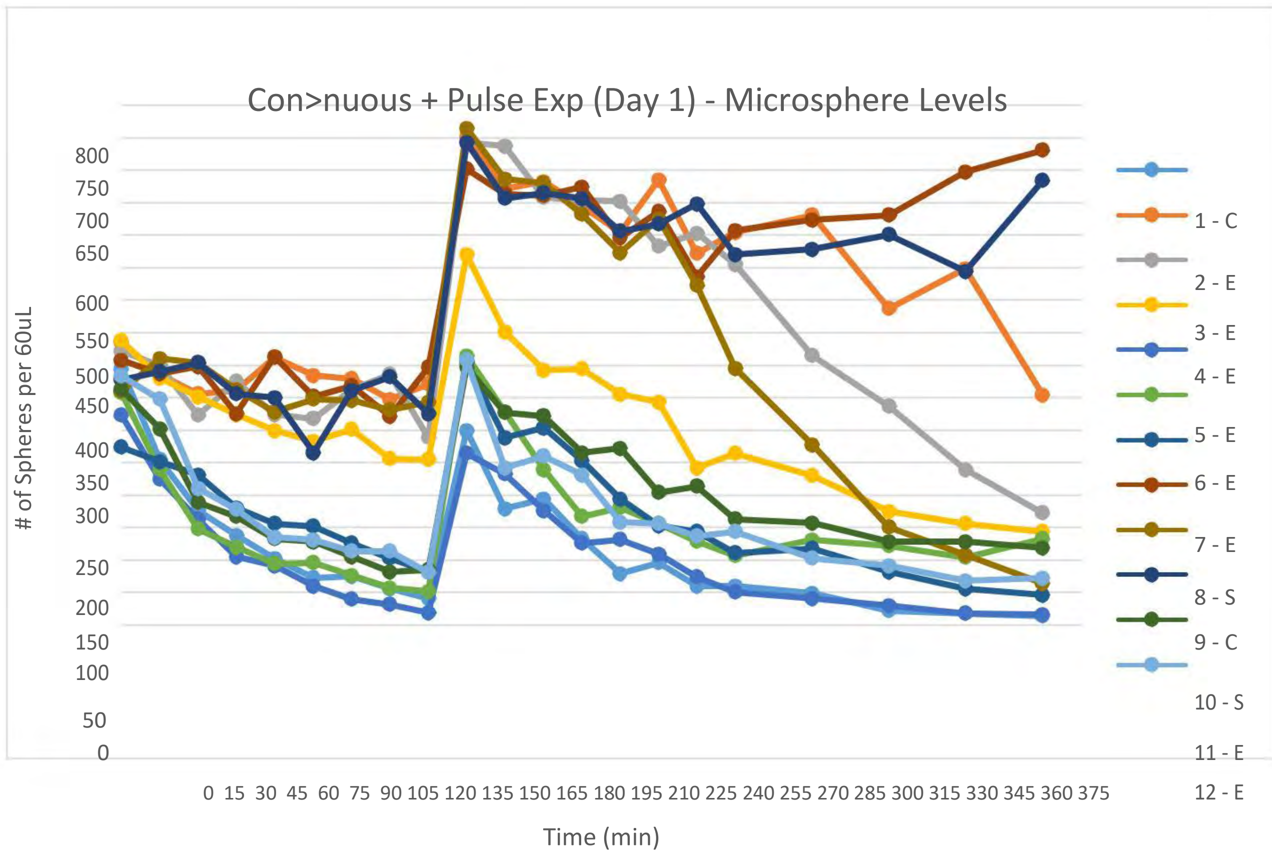
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375

Time (min)

1 E
2 C
3 E
4 E
5 S
6 E
7 C
8 E
9 E
10 S
11 E
12 E



Con>nuous + Pulse Exp (Day 1) - E. coli Levels
# of Bacteria Cells per 60uL
33000
30000
27000
24000
21000
18000
15000
12000
9000
6000
3000
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375
Time (min)
1- C
2 - E
3 - E
4 - E
5 - E
6 - E
7 - E
8 - S
9 - C
10 - S
11 - E
12 - E



Con>nuous + Pulse Exp (Day 1) - Microsphere Levels
# of Spheres per 60uL
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375
Time (min)
1 - C
2 - E
3 - E
4 - E
5 - E
6 - E
7 - E
8 - S
9 - C
10 - S
11 - E
12 - E

ConGnuous + Pulse Exp (Day 2) - E. coli Levels

of Bacteria Cells per 60uL

33000
30000
27000
24000
21000
18000
15000
12000
9000
6000
3000
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 270 300 330 360

Time (min)

1 E
2 E
3 C
4 S
5 E
6 E
7 C
8 E
9 S
10 E
11 E
12 E

ConGnuous + Pulse Exp (Day 2) - Microsphere Levels

of Spheres per 60uL

650
600
550
500
450
400
350
300
250
200
150
100
50
0

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 270 300 330 360

Time (min)

1 E
2 E
3 C
4 S
5 E
6 E
7 C
8 E
9 S
10 E
11 E